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Régulation des hémicanaux de connexine 43: Implication dans la cardioprotection contre les lésions ischémiques

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Cette thèse intitulée :

Régulation des hémicanaux de connexine 43: Implication dans la cardioprotection contre les lésions ischémiques

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a été évaluée par un jury composé des personnes suivantes :

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Résumé

La connexine 43 (Cx43) est l'unité protéique de base dans la formation des canaux des jonctions gap (JG) responsables des échanges intercellulaires. Toutefois, elle forme aussi des canaux non-jonctionnels à large conductance, nommés hémicanaux (Hc), qui fournissent un accès entre l'intérieur des cellules et le milieu extracellulaire. Bien qu'ils soient beaucoup moins étudiés que les JG, on estime que les Hc restent normalement à l'état fermé, et ce, grâce à la phosphorylation des connexines qui les forment. Suite à un stress ischémique, les Cx43 se déphosphorylent et entraînent ainsi l'ouverture des Hc de Cx43 (HcCx43), un effet qui compromet la survie des cellules.

La protéine kinase C (PKC) est l'enzyme de phosphorylation qui possède le plus grand nombre de sites de phosphorylation sur la Cx43 en comparaison avec les autres kinases. Ses fonctions dépendent de la mise en jeu d'un répertoire d'au moins 12 isoformes distinctes. Dans les cardiomyocytes, les isoformes de PKC participent au développement des réponses adaptées ou mésadaptées au stress ischémique. Malgré que la régulation des canaux de Cx43 par la PKC lors d'une ischémie soit bien documentée, il n'existe pas à l'heure actuelle de connaissances sur les effets fonctionnels spécifiques qu'exercent des différentes isoformes de PKC sur les HcCx43, ni sur la valeur thérapeutique de la modulation de ces derniers. Dans ce contexte, nous avons proposé que les HcCx43 sont régulés sélectivement et différentiellement par les différentes isoformes de PKC et que l'inhibition spécifique de ces hémicanaux peut protéger le

cœur lors d'un événement ischémique. Le présent travail comporte trois études qui ont été entreprises spécialement dans le but de valider ces hypothèses.

Dans la première étude, nous avons profité de l'expertise du laboratoire du Dr Baroudi dans la dissection des isoformes de PKC pour étudier le rôle fonctionnel de chacune d'elles dans la régulation des HcCx43 en utilisant une gamme unique de peptides synthétiques inhibiteurs et activateurs spécifiques des isoformes de PKC, en combinaison avec la technique du patch-clamp. Nous avons démontré, entre autre, que les HcCx43 sont particulièrement inhibés par l'isoforme PKC epsilon, connue pour son effet cardioprotecteur contre les dommages ischémiques lors d'un préconditionnement ischémique.

Dans la deuxième étude, nous avons caractérisé l'effet d'un peptide synthétique mimétique structural de la Cx43 sur la fonction des HcCx43. En plus d'avoir élucidé ses effets sur les propriétés fonctionnelles du canal, nous avons démontré d'une manière directe et indéniable que le peptide Gap26 inhibe spécifiquement les HcCx43 et que son administration *in vitro* (cardiomyocytes isolés) et *ex vivo* (cœur intact) confère à ces modèles expérimentaux une résistance importante contre le stress ischémique.

Dans la troisième étude, nous avons investigué pour la première fois *in vivo* le potentiel de deux peptides uniques mimétiques structuraux de la Cx43, Gap26 et Gap27, dans la cardioprotection contre les lésions ischémiques lorsqu'ils sont administrés à basse dose sous forme d'un bolus intraveineux unique. Nous avons démontré que l'injection de ces peptides avant ou après la survenue de l'ischémie réduit significativement la taille de l'infarctus qui en résulte.

En conclusion, l'ensemble de ces résultats révèlent le rôle bénéfique de l'inhibition des HcCx43 lors d'une ischémie et dévoilent un potentiel thérapeutique prometteur des mimétiques structuraux de Cx43 dans la prévention et le traitement de l'infarctus du myocarde.

Mots-clés : Cœur, Ischémie, Connexine 43, Protéine kinase C, Peptides synthétiques.

Abstract

Connexin 43 (Cx43) is the basic unit in the composition of Gap junction channels but also of the non-junctional unapposed hemichannels (Hc). Gap junction channels play key roles in cardiac function by allowing conduction of electrical impulses and exchange of biologically important molecules between cells. The unapposed Hc, however, perform functions different from those achieved by Gap junction channels mainly by providing pathways between the cytosol and the extracellular space allowing movement of ions and other small metabolites. Although they are much less studied than Gap junction channels, Hc are believed to remain normally in a closed state and that phosphorylation is an important factor promoting their closure. Under ischemic stress, the amount of non-phosphorylated Cx43 increases resulting in increasing hemichannels opening, an effect that can lead to irreversible tissue injury and cell death.

Protein kinase C (PKC) possesses the largest number of phosphorylation sites on Cx43 and exerts significant control on Cx43 channels. Its function depends on the involvement of at least 12 distinct isoformes. Various PKC isoforms exert specific cellular and cardiovascular functions, nonetheless the functional role of PKC isoforms in the modulation of the unapposed Cx43 hemichannels has never been assessed, neither has the therapeutic potential of Cx43Hc modulation in the protection of ischemic heart. In this context, three studies have been performed, they form the body of this thesis.

In the first study, a unique set of synthetic PKC isoform-selective activator and inhibitor peptides was utilised. In combination with the patch-clamp technique, we have

demonstrated that Cx43Hc conductance is strongly inhibited by, among many isoforms, epsilon PKC isoforme, known for its cardioprotective effect against ischemic injury.

In the second study, we characterized the effect of a synthetic structural mimetic peptide of Cx43. Using patch clamp technique, we have demonstrated that the peptide Gap26 inhibits directly and specifically Cx43Hc, we also showed that Gap26 can confer resistance to cardiomyocytes (*in vitro*) and intact heart (*ex vivo*) against ischemia.

In the third study, we investigated for the first time *in vivo* the capability of a unique pair of structural Cx43 mimetic peptides, Gap26 and Gap27, to protect heart from ischemic injury when administered in single low-dose intravenous boluses. We demonstrated that administration of either one or both peptides, before or after the onset of ischemia renders heart more resistant to ischemia and reduces significantly the size of myocardial infarct.

Altogether, our results revealed salvatory effect of Cx43Hc inhibition during ischemia and uncovered therapeutic potential of the synthetic structural mimetic peptides of Cx43 in ischemic heart disease.

Keywords: Heart, Ischemia, Connexin 43, Protein kinase C, Synthetic peptides.

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Liste des abréviations et symboles

4αPDD	: 4 α phorbol-12,13 didecanoate
AMPc	: adénosine monophosphate cyclique
BMPF	: débit myocardique basal (*basal myocardial perfusate flow*)
C	: cystéine
CD8-a	: antigène de surface CD8-a
Ca^{2+}	: ion calcium
$CaCl_2$	: chlorure de calcium
COOH	: groupement carboxy-terminal
Cx	: connexine
Cx40	: connexine 40
Cx43	: connexine 43
Cx45	: connexine 45
EGTA	: acide éthylèneglycol tétraacétique
FBS	: sérum du fœtus de bœuf
Hc	: hémicanal
HEK293	: cellules rénales embryonnaires humaines 293
HEPES	: acide 4-(2-hydroxyéthyl)-1-pipérazine éthane sulfonique
HcCx43	: hémicanaux de connexine 43
GF109203X	: bisindolylmaleinide
JG	: jonction gap
K^+	: ion potassium
KCl	: chlorure de potassium
KD	: kilodalton
$MgCl_2$	: chlorure de magnésium
MPTP	: les pores de transition de perméabilité mitochondriale
Ms	: millisecondes

mV	: millivolts
MPF	: débit myocardique du perfusat (*myocardial perfusate flow*)
MΩ	: mégaohms
Na^+	: ion sodium
NaCl	: chlorure de sodium
NAV	: nœud auriculo-ventriculaire
NH_2	: groupement amino-terminal
NS	: nœud sinusal
pHi	: pH intracellulaire
PKC	: protéine kinase C
pS	: picosiemens
αPKC	: l'isoforme PKC alpha
βIPKC	: l'isoforme PKC beta I
βIIPKC	: l'isoforme PKC beta II
δPKC	: l'isoforme PKC delta
εPKC	: l'isoforme PKC epsilon
μPKC	: l'isoforme PKC mu
PMA	: phorbol 12-myristate 13-acétate
SV40	: virus simien 40
tsA201	: cellules rénales embryonnaires humaines dérivées des cellules HEK 293
αC_{2-4}	: inhibiteur de l'isoforme αPKC
βIV_{5-3}	: inhibiteur de l'isoforme βIPKC
$\beta II\ V_{5-3}$	: inhibiteur de l'isoforme βIIPKC
δV_{1-7}	: inhibiteur de l'isoforme δPKC
εV_{1-2}	: inhibiteur de l'isoforme εPKC
εV_{1-7}	: activateur de l'isoforme εPKC

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À mes bijoux…. Ghawsi, Aladdin et Adam

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Chapitre I : Introduction

I. Le cœur : structure et physiologie

Le cœur est le principal organe du système cardiovasculaire. Grâce à ses contractions coordonnées, il assure la circulation du sang dans les vaisseaux afin de transporter les ressources vers les systèmes de l'organisme et de collecter leurs déchets métaboliques.

Le cœur comprend deux oreillettes et deux ventricules (Figure 1). Parmi ceux-là, le ventricule gauche est le plus développé. Il est responsable de pomper le sang vers tout l'organisme. Il est aussi le site le plus vastement touché par les maladies coronariennes.

Du point de vue de l'histologie, la paroi du cœur est composée de trois couches tissulaires superposées. Le péricarde, composé de cellules épithéliales et de tissu conjonctif tapissant la surface externe du cœur. Le myocarde, constitué majoritairement de cellules contractiles. Et, l'endocarde, formé de cellules épithéliales et de tissu conjonctif tapissant la surface interne du cœur.

Le muscle cardiaque est nourri par trois artères coronaires principales : l'artère coronaire droite, l'artère coronaire gauche et l'artère circonflexe. L'obstruction d'une ou de plusieurs artères coronaires entraine une ischémie. Cette dernière est à la base de la formation de l'infarctus du myocarde.

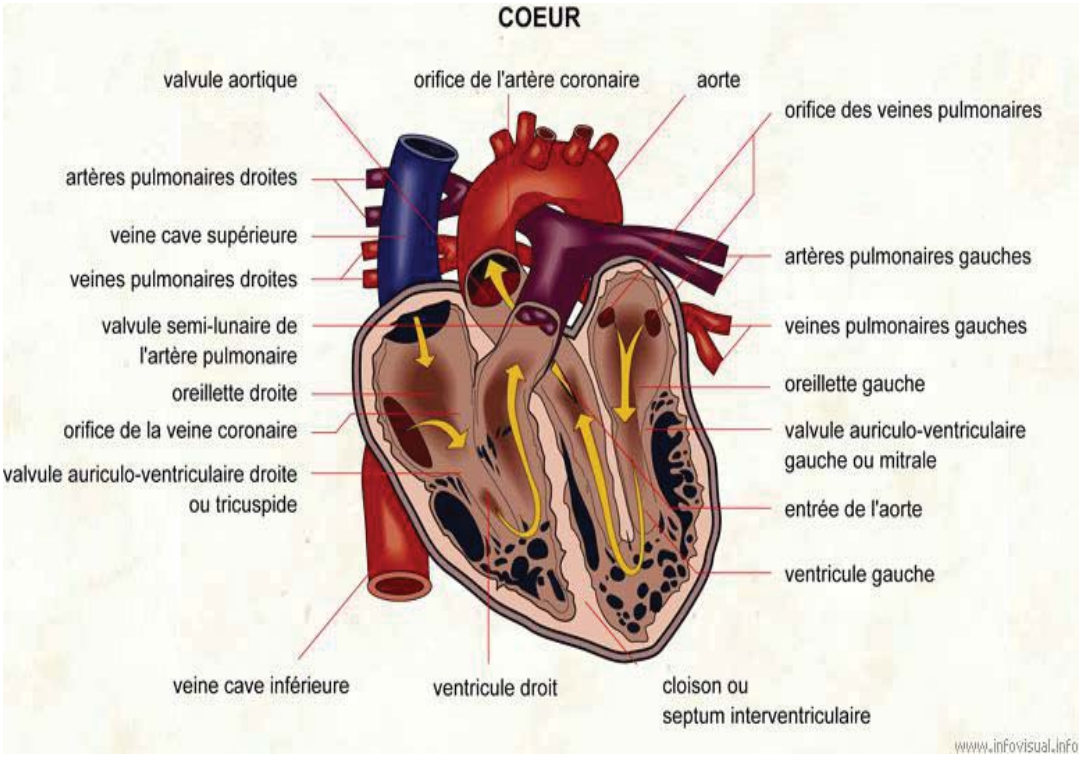

Figure 1. Coupe latérale du cœur humain.

Source de Patrick J. Lynch (avec permission)

I.1. L'infarctus du myocarde

L'infarctus du myocarde est une cause majeure de mortalité et de morbidité dans le monde. Chaque année, 1,5 million de nouvelles personnes d'âges variés développent un infarctus du myocarde et le taux de mortalité se situe à près de 30 % des personnes affectées (Rosamond *et al.*, 2008). Au Canada, les maladies cardiaques constituent la deuxième cause de mort après le cancer. En particulier, la cardiopathie ischémique est responsable de prés de 16 % de toutes les mortalités.

Chapitre I : Introduction

L'infarctus du myocarde se caractérise par des lésions tissulaires irréversibles à la suite d'une ischémie. Cette dernière survient lors d'une réduction anormale de l'apport sanguin dans une région du cœur à cause de l'obstruction d'une ou de plusieurs artères coronaires. En clinique, l'infarctus du myocarde provient, le plus souvent, d'une coronaropathie, mais aussi de spasmes artériels ou d'une réponse inadéquate à un besoin accru en oxygène (un effort physique, par exemple). Dans la plupart des cas, l'ischémie est régionale et n'affecte que la zone située en aval de l'obstruction coronaire.

Les lésions ischémiques surviennent généralement en deux phases (Jennings *et al.*, 1983). La phase précoce correspond à une période durant laquelle les lésions sont encore réversibles si le myocarde est reperfusé (Jennings *et al.*, 1960). Pendant cette phase, le ralentissement du métabolisme oxydatif entraine une diminution marquée de la production mitochondriale d'ATP. Alternativement, ces derniers seront alors produits par la glycolyse anaérobique, mais en quantité insuffisante, qui ne compense que partiellement la carence en énergie et ne permet pas de couvrir les besoins du myocarde. De plus, l'accumulation des produits du catabolisme de l'ATP (adénosine et phosphate inorganique) a pour conséquence l'augmentation de l'acidose. Cette dernière déclenche l'activation des échangeurs Na^+/H^+ et l'accumulation intracellulaire du sodium qui est à l'origine d'un déséquilibre osmotique et à l'apparition d'un œdème intracellulaire (Benhabbouche *et al.*, 2011). L'activation des échangeurs Na^+/Ca^{2+} amène l'accumulation de calcium intracellulaire qui conduit à un état de contraction permanente et au dysfonctionnement diastolique au cours de l'ischémie (van Echteld *et al.*, 1991).

La phase tardive survient lorsque l'ischémie persiste et se caractérise par des lésions irréversibles (Reimer *et al.*, 1977). Elle mène, au bout d'une vingtaine de minutes, à une nécrose progressive (Nayler, 1981; Koretsune and Marban, 1990; Steenbergen *et al.*, 1990). Des altérations biochimiques fonctionnelles et structurelles surviendront. La production de l'ATP est complètement arrêtée ce qui conduit à l'épuisement de la réserve d'énergie et la diminution d'élimination des métabolites toxiques (Neely, 1989; Koretsune and Marban, 1990; Steenbergen *et al.*, 1990). L'amplification de la surcharge calcique entraine l'activation des enzymes telles que les protéases et les phospholipases. La structure des myocytes se désorganise à la suite de la rupture de la membrane plasmique et la fuite de nombreuses composantes cellulaires vers le compartiment extracellulaire (Feuvray, 1981;Opie, 1991). Par conséquent, les cellules meurent et un infarctus se forme.

I.1.1. Les médiateurs de l'infarctus du myocarde

Pendant une ischémie, de nombreux changements métaboliques surviennent et contribuent aux dégâts structuraux et aux altérations de la performance contractile du cœur (Allen *et al.*, 1993). Certains médiateurs de ces changements sont largement étudiés. Parmi ceux-ci, il existe les canaux de connexine 43 (Cx43), les échangeurs Na^+/H^+ et les pores de transition de perméabilité mitochondriale (MPTP).

I.1.1.1. Les canaux de Cx43

Les jonctions Gap

Les jonctions Gap (JG) sont situées principalement sur les disques intercalaires (Figure 2) et relient les cytoplasmes des cellules voisines (Sosinsky and Perkins, 2000; Harris, 2000). Leur implication dans les dommages causés par ischémie a été mise en évidence par plusieurs groupes. Toutefois, deux théories opposées ont été émises quant à leur fonction exacte pendant cette condition.

La première, énoncée par McCallister en 1979 et appuyée par d'autres, suggère que les JG se ferment pendant l'ischémie et que cette fermeture est à la base des arythmies et de la mort cellulaire (McCallister *et al.*, 1979; Kleber *et al.*, 1987; Dekker *et al.*, 1996; Beardslee *et al.*, 2000; Matsushita *et al.*, 2006). Tandis que la deuxième théorie suggère que la conductance à travers les JG persiste pendant l'ischémie et entraine la propagation du calcium entre les cellules adjacentes causant ainsi la contraction permanente et la mort des cellules (Garcia-Dorado *et al.*, 1989; Cinca *et al.*, 1997; Ruiz-Meana *et al.*, 1999; Ruiz-Meana *et al.*, 2001; Rodriguez-Sinovas *et al.*, 2003).

Les hémicanaux de Cx43

En contraste avec les JG, les hémicanaux de Cx43 (HcCx43) se localisent principalement sur les membranes latérales des cardiomyocytes (Figure 2) et fournissent un accès entre l'intérieur des cellules et le milieu extracellulaire.

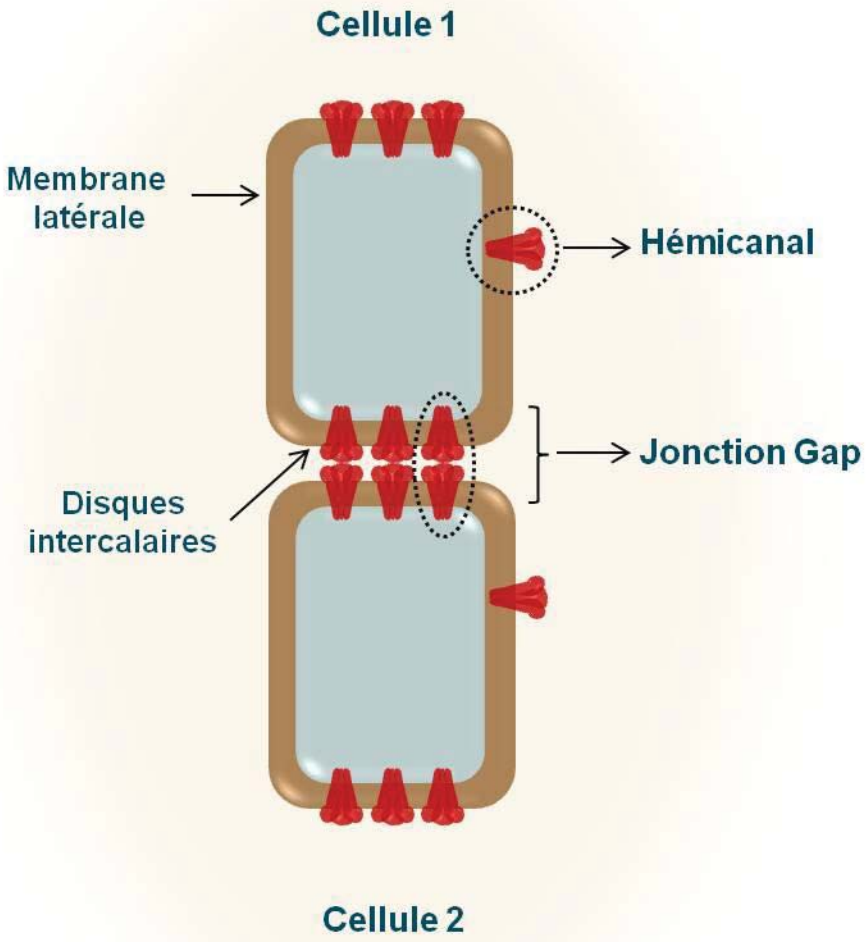

Figure 2. Distribution des canaux de Cx43 sur les membranes des cardiomyocytes. Les jonctions Gap prédominent dans les disques intercalaires alors que les hémicanaux se localisent principalement sur les membranes latérales.

Dans des conditions physiologiques normales, les HcCx43 restent fermés (Saez *et al.*, 2005). Cet état est surtout favorisé par la phosphorylation des Cx43 par des protéines kinases (Saez *et al.*, 2005; Bao *et al.*, 2007). Lors d'un stress ischémique, les Cx43 se déphosphorylent (Beardslee *et al.*, 2000; Schulz *et al.*, 2003; Jain *et al.*, 2003; Miura *et al.*, 2004). Par conséquent, les HcCx43 s'ouvrent et entrainent un déséquilibre dans le gradient ionique régnant de part et d'autre de la membrane cellulaire et la perte de métabolites vitaux (John *et al.*, 1999; Bruzzone *et al.*, 2001; Bennett *et al.*, 2003; Braet *et al.*, 2003; Goodenough and Paul, 2003). La mort cellulaire à la suite de l'ouverture des Hc a été mise en évidence dans plusieurs modèles cellulaires incluant les cardiomyocytes néonataux (Shintani-Ishida *et al.*, 2007) et adultes (John *et al.*, 1999).

La présente thèse est fondée sur l'idée que l'inhibition spécifique des HcCx43 devrait conférer au cœur une résistance contre les dommages induits par l'ischémie et le protéger contre l'infarctus du myocarde.

Les Cx43 mitochondriales

La Cx43 est encore présente sur les membranes internes des mitochondries (Boengler *et al.*, 2005 ; Boengler *et al.*, 2009). L'implication des Cx43 mitochondriales dans la cardioprotection contre l'ischémie est démontrée par plusieurs (Heinzel *et al.*, 2005; Schulz *et al.*, 2007). Toutefois, l'inhibition de la translocation de ces structures vers les mitochondries n'a pas empêché la protection du cœur par préconditionnement

ischémique (Rodriguez-Sinovas *et al.*, 2006). Ceci laisse suggérer l'existence d'autres voies de protection indépendantes des Cx43 mitochondriales.

I.1.1.2. Autres médiateurs

Plusieurs autres médiateurs sont identifiés à ce jour. Nous citons ici deux des plus connus : les échangeurs Na^+/H^+ et les pores MPTP.

Les échangeurs Na^+/H^+

L'augmentation de l'acidité dans les cardiomyocytes ventriculaires pendant l'ischémie entraine l'activation des échangeurs Na^+/H^+ (Pierce and Philipson, 1985; Seiler *et al.*, 1985) suivie de celle des échangeurs Na^+/Ca^{2+} qui s'opposent à l'entrée massive des ions Na^+ (Tani and Neely, 1989; Meng and Pierce, 1991; du Toit and Opie, 1992). Il est accepté que ce mécanisme contribue à la surcharge cytosolique en calcium responsable de la contraction cellulaire et les lésions tissulaires irréversibles.

Les pores de transition de perméabilité mitochondriale

Lors d'un stress oxydatif, les MPTP situés sur la membrane interne des mitochondries s'ouvrent et provoquent l'augmentation de la pression osmotique à l'intérieur des mitochondries, le gonflement de ces dernières et la rupture de leurs membranes (Crompton, 1999; Halestrap, 1999; Halestrap *et al.*, 2002). Ceci entraine le

découplage de la chaine respiratoire (Argaud and Ovize, 2004) et le déclenchement de l'apoptose à la suite de la libération des molécules pro-apoptotiques telles que le cytochrome c, les caspases 2, 3 et 9 ainsi que l'Apoptose Inducing Factor (AIF) dans le cytosol (Crompton, 1999; Halestrap, 1999).

I.2. Ischémie et arythmies

Le rythme cardiaque est assuré par des impulsions électriques initiées dans le nœud sinusal et propagées tout au long de la masse contractile du myocarde.

Pendant une ischémie, l'altération dans la propagation des impulsions dans le myocarde contribue au développement des arythmies (Janse and Wit, 1989). Les courants Na^+, qui sont à l'origine de l'initiation et de la propagation du potentiel d'action à travers le myocarde et qui jouent un rôle central dans l'excitabilité des cellules et la conduction des pulses électriques dans le cœur, sont perturbés (Nguyen-Thi *et al.*, 1981; Myerburg *et al.*, 1982). L'entrée massive d'ions Na^+ à la suite de l'ouverture des HcCx43 (John *et al.*, 1999; Kondo *et al.*, 2000; Shintani-Ishida *et al.*, 2007), l'arrêt de l'expulsion de Na^+ par chute d'activité des pompes $Na^+/K^+_{ATPases}$ (Benhabbouche *et al.*, 2011) et l'augmentation de l'entrée lente d'ions Na^+ par l'intermédiaire des échangeurs Na^+/H^+ (Chakrabarti *et al.*, 1997), entrainent un excès de sodium intracellulaire. Cette augmentation dans la teneur sodique intracellulaire provoque à son tour un influx cellulaire d'ions Ca^{2+} par l'intermédiaire des échangeurs Na^+/Ca^{2+} (Tani and N eely, 1989). L'accumulation du Ca^{2+} intracellulaire produit des effets inotropes positifs, comme déjà mentionné, mais peut aussi conduire à des anomalies de genèse du potentiel d'action (des déclenchements spontanés) se traduisant par des post-dépolarisations tardives qui sont à la base de la tachycardie ventriculaire (Pogwizd *et al.*, 2001).

D'autre part, ces perturbations des propriétés électrophysiologiques des cardiomyocytes entrainent une diminution du potentiel membranaire de repos (de -90 mV jusqu'à -60 mV) (Downar *et al.*, 1977; Kleber *et al.*, 1978; Kleber, 1983) et une

réduction de l'amplitude du potentiel d'action et de la vitesse de dépolarisation (Kleber *et al.*, 1978). Ceci est à la base de la réduction de la vitesse de conduction dans le tissu affecté (Bhandari *et al.*, 1987). Il est important de remarquer que ces modifications ne sont pas homogènes dans tout le myocarde, mais sont restreintes au tissu situé à proximité de la zone ischémique ce qui cause une hétérogénéité électrique à travers le muscle cardiaque (Hill and Gettes, 1980; Coronel *et al.*, 1988). Cette hétérogénéité favorise la formation d'une région de bloc unidirectionnel (Jozwiak and Dhein, 2008) qui représente un substrat principal pour la fibrillation ventriculaire par réentrée (Smith *et al.*, 1995; Duffy and Wit, 2008).

Finalement, il est à noter que les arythmies qui surviennent lors d'une ischémie ont largement été associées à la réduction du couplage électrique cardiomyocytaire, qui lui est causé par la réduction de la conductance à travers les JG sur les disques intercalaires des cardiomyocytes (Kleber *et al.*, 1987; Smith *et al.*, 1991; Beardslee *et al.*, 2000; Ruiz-Meana *et al.*, 2001; Miura *et al.*, 2004; Dhein, 2006; Miura *et al.*, 2007). Cette réduction dans la conductance des canaux des JG résulte de plusieurs facteurs dont le changement du niveau d'expression des connexines (Peters *et al.*, 1993 Huang *et al.*, 1999), la relocalisation des canaux de JG à partir des disques intercalaires vers les côtés latéraux des cardiomyocytes (Beardslee *et al.*, 2000) et l'altération de leur état de phosphorylation (Beardslee *et al.*, 2000).

Sur le tracé de l'ECG, les troubles du rythme cardiaque reliés à l'ischémie peuvent se manifester de diverses façons incluant la dépression du segment S-T, la modification dans la forme du complexe QRS ou la prolongation de l'intervalle QJ (Hanna *et al.*,

2001) (Figure 3). Toutes ces manifestations sont causées par la perturbation de la conduction intraventriculaire (Gettes and Maruyama, 1992;Hatala *et al.*, 1995).

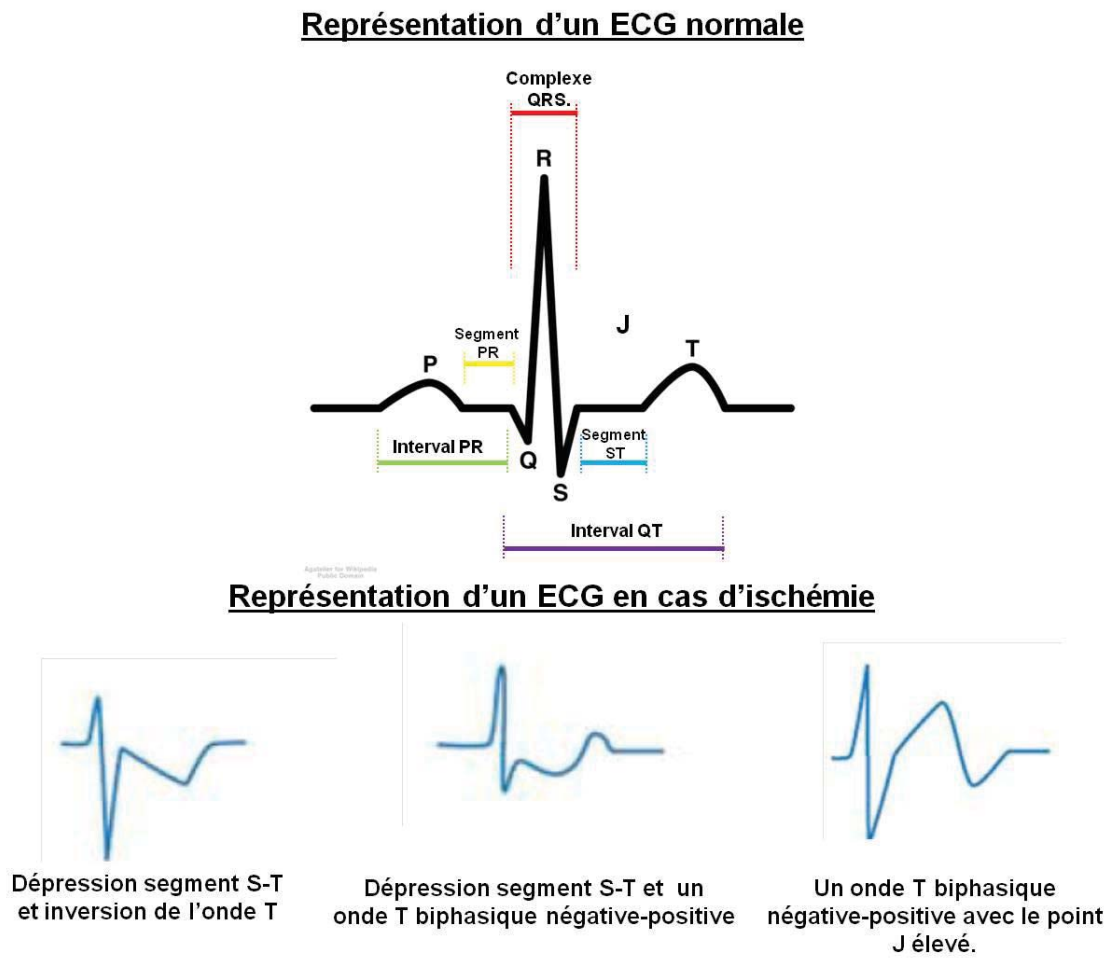

Figure 3. Représentation d'un ECG cardiaque. Variations dans la longueur et la forme des ondes et des intervalles dans un cœur ischémique (Hanna and Glancy, 2011).

Au point de vue du traitement, les anti-arythmiques de classe II (les β–bloquants) tel que le Métoprolol sont souvent utilisés pour ralentir le rythme cardiaque rapide en cas de tachycardie et prévenir les arythmies. De plus, le Métoprolol a été démontré stabiliser les plaques des JG dans les disques intercalaires et empêcher la dégradation de la Cx43 ce qui contribue aussi à la conduction normale du potentiel d'action (Salameh *et al.*, 2009; Salameh *et al.*, 2010). Les anti-arythmiques de classe IV (les inhibiteurs calciques) comme le Diltiazem et le Vérapamil sont également employés pour contrôler la fréquence cardiaque et prévenir les arythmies à la suite d'une ischémie. Les inhibiteurs des anti-arythmiques de classe III (les canaux potassiques), tels que l'Amiodarone et le Sotalol, servent à traiter les fibrillations causées par réentrées et prévenir leurs récidives.

I.3. Le préconditionnement ischémique

En 1986, Murry et collaborateurs (Murry *et al.*, 1986) a démontré que l'application de courtes périodes d'ischémie au cœur, entrecoupées d'une reperfusion brève, le rend plus résistant pendant une ischémie subséquente plus prolongée suivie d'une reperfusion longue. Ce phénomène a été appelé le préconditionnement ischémique (PI). La cardioprotection induite par PI a été mise en évidence chez plusieurs espèces animales (Baines *et al.*, 1998; Schulz *et al.*, 2003;Lochner *et al.*, 2009). De plus, le PI a été décrit dans des organes autres que le cœur, comme le cerveau, le foie ou le poumon (Tauskela *et al.*, 1999; Xue *et al.*, 2012; Waszak *et al.*, 2012).

Kuzuya et collaborateurs en 1993 ont montré que la protection induite par PI est biphasique: une phase immédiate et une phase tardive survenant après une période de vingt-quatre heures du PI et qui est responsable de la limitation de la taille de l'infarctus (Kuzuya *et al.*, 1993). Le PI entraine une réduction dans la consommation d'oxygène, une diminution des arythmies, le retard dans l'épuisement de l'ATP (Murry *et al.*, 1986) ainsi que dans l'accumulation du lactate et des ions H^+ (Kida *et al.*, 1991) résultant en une réduction de la taille de l'infarctus.

Le mécanisme d'action du PI est complexe et incomplètement élucidé à ce jour. Toutefois, on distingue les inducteurs qui déclenchent les modifications intracellulaires; les médiateurs, qui transmettent la signalisation et aboutissent à des cibles pharmacologiques, ou les effecteurs, qui induisent eux-mêmes la cardioprotection. De nombreuses études ont été réalisées pour caractériser les médiateurs et les effecteurs qui

peuvent être impliqués dans les voies de signalisation du PI. Plusieurs médiateurs du PI sont identifiés à ce jour, dont la Cx43.

La libération de médiateurs endogènes (comme l'acétylcholine et l'adénosine) au cours de l'induction du PI ou bien l'administration d'agonistes pharmacologiques pour mimer le PI, stimulent des récepteurs membranaires des myocytes couplés aux protéines G (des récepteurs d'adénosine, de noradrénaline, d'acétylcholine et de bradykinine) et des récepteurs à activité tyrosine kinase (Benhabbouche *et al.*, 2011). La stimulation de ces récepteurs conduit à l'activation intracellulaire des protéines kinases dont la protéine kinase C (PKC). Par la suite, de nombreuses protéines, dont les effecteurs finaux (tel que la Cx43), vont être phosphorylées (Benhabbouche *et al.*, 2011). L'importance et l'implication de la PKC dans le phénomène du PI ont été démontrées dans plusieurs études (Gray *et al.*, 1997; Schulz *et al.*, 2003; Budas *et al.*, 2007).

I.3.1. Les médiateurs du préconditionnement ischémique

I.3.1.1. La connexine 43

La Cx43 est certainement un médiateur important du PI (Schulz *et al.*, 2003; Jain *et al.*, 2003). En effet, les cœurs déficients en Cx43 ne peuvent pas être préconditionnés (Schwanke *et al.*, 2002; Heinzel *et al.*, 2005). Bien qu'à l'origine l'effet cardioprotecteur du PI ait été attribué à la fermeture des canaux des JG (Garcia-Dorado *et al.*, 1997; Schwanke *et al.*, 2002; Li *et al.*, 2002; Schwanke *et al.*, 2003; Garcia-Dorado *et al.*, 2004), la résistance acquise par les cardiomyocytes isolés (donc n'ayant pas de canaux

de JG) contre les dommages ischémiques lorsqu'ils sont préconditionnés suggère qu'un préconditionnement efficace ne requiert pas nécessairement la présence des canaux de JG (Li *et al.*, 2004). De même, des enregistrements faits sur le cœur de cochon *in vivo* ont montré que l'impédance du tissu cardiaque chez les animaux qui ont subi un PI ne diffère pas de celle des cœurs qui n'ont pas été préconditionnés, ce qui suggère davantage que l'effet cardioprotecteur du PI n'implique pas les JG en particulier (Padilla *et al.*, 2003). Et puisque l'inhibition de voie de translocation des Cx43 vers la membrane interne de mitochondrie n'empêche pas la protection par PI (Rodriguez-Sinovas *et al.*, 2006), ces données nous ont incités à croire que le rôle de la Cx43 dans la cardioprotection par PI est très probablement médié par les hémicanaux non-apposés présents sur le sarcolemme. Précisément, nous avons pensé que l'inhibition de ces hémicanaux anormalement activés par l'ischémie est un des mécanismes importants à la base de la cardioprotection par PI.

Il est suggéré que l'augmentation du Ca^{2+} au moment de l'ischémie active les phosphatases et accélère de ce fait la déphosphorylation des Cx43 (Beardslee *et al.*, 2000; Contreras *et al.*, 2002; Retamal *et al.*, 2006). Par conséquent, les HcCx43 s'ouvrent (John *et al.*, 1999; Kondo *et al.*, 2000; Li *et al.*, 2001; Contreras *et al.*, 2002). Parallèlement, il a été noté aussi que dans un cœur préconditionné, les Cx43 restent hautement phosphorylées pendant une ischémie prolongée subséquente (Schulz *et al.*, 2003; Jain *et al.*, 2003; Miura *et al.*, 2004). Cet effet a été attribué principalement à l'activation de la PKC (Schulz et al., 2003). En particulier, l'isoforme PKCε exerce un

effet cardioprotecteur contre les lésions ischémiques (Gray *et al.*, 1997; Liu *et al.*, 1999; Cross *et al.*, 2002).

En nous basant sur ce qui précède, nous avons formulé notre première hypothèse spécifique stipulant que la phosphorylation des Cx43 par l'isoforme PKCε exerce un effet inhibiteur sur les HcCx43. Par la suite, cet effet pourra alors représenter, au moins partiellement, le mécanisme cellulaire à la base de la résistance myocardique conférée par le PI contre le stress ischémique.

I.3.1.2. Autres médiateurs potentiels

Plusieurs autres médiateurs du PI sont connus. Nous citons pour mémoire quelques-uns des plus caractérisés, dont l'implication est modulée par la PKC.

La protéine kinase C

L'isoforme PKCε est l'un des médiateurs les plus importants et les plus connus de la cardioprotection contre l'ischémie (Yoshida *et al.*, 1997; Gray *et al.*, 1997; Liu *et al.*, 2001; Ping *et al.*, 2002; Inagaki and Mochly-Rosen, 2005; Kim *et al.*, 2006; Sivaraman *et al.*, 2009). L'inhibition spécifique de cette isoforme abolit l'effet protecteur du PI (Budas *et al.*, 2007). Plusieurs effets sont reliés à l'activation de cette enzyme dont l'activation des canaux potassiques ATP-dépendants (K_{ATP}) mitochondriaux (Jaburek *et al.*, 2006a) et l'inhibition des MPTP (Baines *et al.*, 2003).

Les canaux potassiques ATP-dépendants

Les canaux K_{ATP} contribuent aussi à la protection par PI. En fait, l'inhibition sélective des canaux K_{ATP} mitochondriaux abolit tout effet protecteur du PI (Mubagwa and Flameng, 2001). De plus, les souris knockouts pour le gène K_{ATP} du sarcoplasme ayant subi un PI développent un infarctus significativement plus grand que celui des souris normales préconditionnées (Suzuki *et al.*, 2002). On croit que cet effet survient grâce à la capacité de ce canal à antagoniser la surcharge calcique mitochondriale (Nakaya *et al.*, 2003). Plusieurs études suggèrent que l'isoforme PKCε est impliquée dans l'activation des canaux K_{ATP} mitochondriaux (Andrukhiv *et al.*, 2006; Jaburek *et al.*, 2006a).

Les protéines MAP kinases

Le p38MAPK est un membre de la famille des protéines kinases activées par les mitogènes (MAPK) et joue le rôle de second messager pendant le PI. Son activité augmente au cours de ce phénomène (Weinbrenner *et al.*, 1997) et son inhibition abolit la protection par PI (Loubani and Galinanes, 2002). Cette enzyme activée par PKC phosphoryle une variété d'autres protéines impliquées dans le PI. (Loubani and Galinanes, 2002).

II. Les connexines

Les connexines sont des protéines transmembranaires et les unités de base dans la structure des JG. Vingt isoformes différentes de connexines sont identifiées chez l'humain (Willecke *et al.*, 2002). Elles sont souvent désignées par le préfixe Cx suivi d'un chiffre indiquant la masse moléculaire de la connexine en Kilodalton (KD) (Beyer *et al.*, 1990). Les connexines sont sélectivement et différentiellement exprimées dans les divers tissus de l'organisme. La Cx43 est la connexine la plus répandue dans le corps humain (Severs, 1990; Dermietzel *et al.*, 1991; Dermietzel and Spray, 1993; Rozental *et al.*, 2000).

II.1. Structure générale des connexines

Toutes les connexines possèdent la même structure générale (Figure 3). Elles sont principalement formées par quatre segments transmembranaires en forme d'hélices α reliés entre eux par deux boucles extracellulaires et une boucle intracellulaire. Les deux extrémités amino- et carboxy- terminales de la protéine sont situées du côté cytosolique de la membrane plasmique (Hertzberg *et al.*, 1988; Milks *et al.*, 1988; Quist *et al.*, 2000).

Parmi ces composants, les quatre segments transmembranaires, les deux boucles extracellulaires et la région amino-terminale sont hautement conservés à travers les différentes isoformes de connexines. Au contraire, la longueur et la composition en

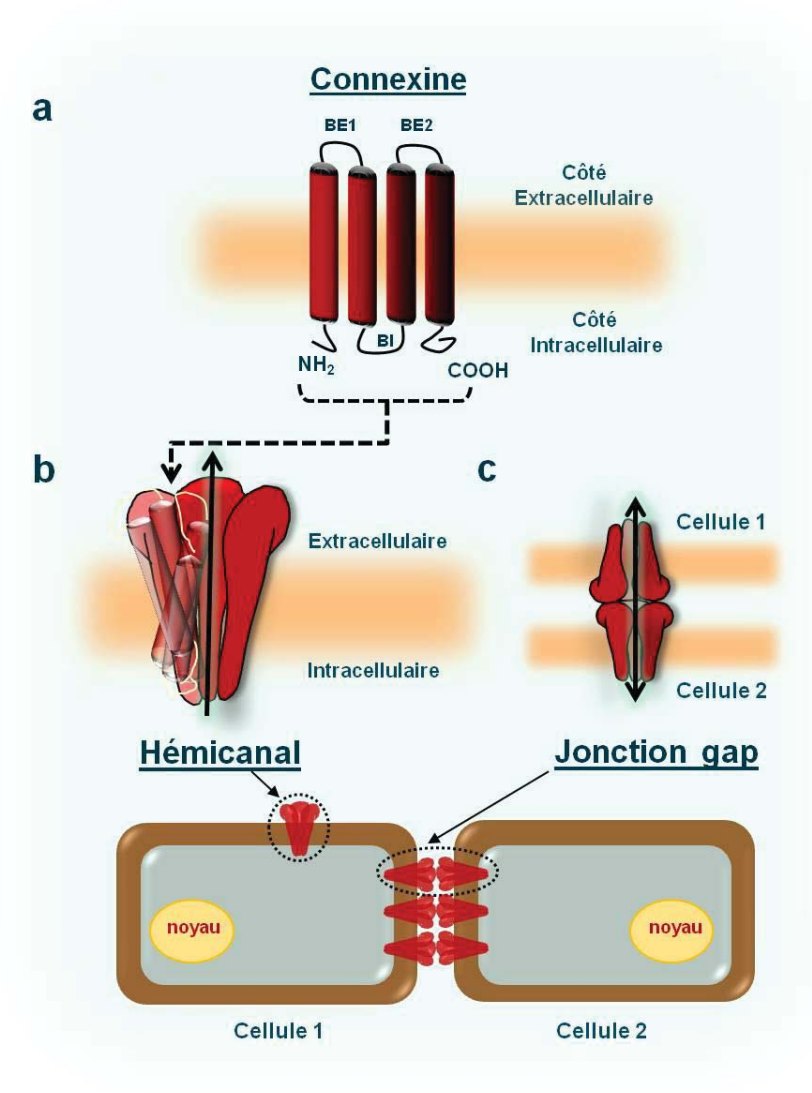

Figure 4. (a) Structure secondaire des connexines, laquelle consiste en quatre segments transmembranaires en forme d'hélices α reliés entre eux par deux boucles extracellulaires et une boucle intracellulaire. (b) Représentation schématique d'un hémicanal non-apposé formé par l'association de six connexines. (c) Une JG se forme lorsque deux hémicanaux provenant de deux cellules adjacentes s'apposent ensemble. BE1: première boucle extracellulaire; BE2 : deuxième boucle extracellulaire; BI: boucle intracellulaire; S: segment transmembranaire.

acides aminés de la boucle cytoplasmique et de l'extrémité carboxy-terminale varient considérablement entre les connexines et sont généralement uniques à chaque isoforme (Bennett *et al.*, 1991).

L'organisation spatiale de six exemplaires de connexines dans la membrane plasmique donne lieu à un pore central transmembranaire de 1 à 1,5 nm de diamètre, perméable aux ions et aux petites molécules dont la masse moléculaire est inférieure à 1KD (Goldberg *et al.*, 2004). Cette structure est connue sous le nom *connexon* ou *hémicanal*. L'apposition de deux hémicanaux forme un canal complet d'une JG (Bennett *et al.*, 1991). Ainsi, un canal d'une JG traverse les deux membranes plasmiques et met en communication directe les cytoplasmes des deux cellules voisines (Figure 4). La liaison entre ces deux hémicanaux se fait grâce à des ponts disulfures que se forment entre des cystéines conservées sur les boucles extracellulaires des connexines formant les Hc apposés et est maintenue par des interactions hydrophobes (Musil and Goodenough, 1991).

Différents types de connexines peuvent s'associer ensembles dans de nombreuses combinaisons. Les hémicanaux peuvent alors contenir un ou plusieurs types de connexines, ils formeront alors respectivement des hémicanaux homomériques ou hétéromériques. De même, un canal de JG peut se former par l'apposition de deux hémicanaux identiques (canal homotypique) ou non identiques (canal hétérotypique). La composition du canal en connexines influence significativement ses propriétés biophysiques, telle que la perméabilité, la conductance et la manière dont ils sont régulés par des molécules régulatrices (Kumar and Gilula, 1996).

II.2. Expression des connexines dans le cœur

Trois isoformes de connexines sont exprimées dans le cœur. La Cx43 représente la forme la plus abondante. Elle prédomine surtout dans les ventricules droit et gauche (Gourdie *et al.*, 1993). Au contraire, la Cx40 se trouve principalement dans les oreillettes, le faisceau de His et les fibres du Purkinje du myocarde adulte (Gourdie *et al.*, 1993; Firouzi *et al.*, 2004; Gollob, 2006). Finalement, la Cx45 est la moins abondante dans le cœur adulte et est localisée surtout dans les nœuds sinusal et auriculo-ventriculaire.

Bien que la majorité des Cx43 dans les cellules soient localisées sur la membrane plasmique, la protéine est également présente sur d'autres structures à l'intérieur des cellules, telles que le noyau (Dang *et al.*, 2003), le réticulum endoplasmique (Musil and Goodenough, 1991) et la membrane interne de la mitochondrie (Boengler *et al.*, 2005). La présente thèse est consacrée à l'étude des Cx43 membranaires dans leur forme d'hémicanaux non-apposés.

II.2.1. Expression des connexines dans les vaisseaux

Au point de vue histologique, un vaisseau sanguin présente trois couches cellulaires distinctes. La couche externe de la paroi artérielle, ou l'adventice, est composée de tissu conjonctif et de fibres élastiques. La couche moyenne, ou la média, est constituée de fibres de collagène et d'élastine, mais aussi des fibres musculaires lisses responsables de la vasomotricité des vaisseaux de moyen et petit calibres. Et la

couche interne, ou l'intima, est formée de fines fibres de tissu conjonctif et de cellules endothéliales.

Pour qu'un réseau vasculaire se contracte et se dilate d'une façon coordonnée afin de contrôler le débit sanguin dans les tissus, les cellules doivent communiquer les unes avec les autres. Ceci se fait par le biais des JG (Segal and Jacobs, 2001) qui permettent de coupler les cellules vasculaires en coordonnant à la fois la transmission nerveuse et les réponses cellulaires (Christ *et al.*, 1996; Haddock *et al.*, 2006; Tran and Welsh, 2009; Hawat *et al.*, 2010).

En permettant la diffusion des ions et des seconds messagers entre les cellules adjacentes, les JG sont aussi impliquées dans plusieurs autres fonctions vasculaires vitales telles que dans l'angiogenèse, le développement et la différenciation cellulaire, et la signalisation intracellulaire (Sandow and Hill, 2000; Gairhe *et al.*, 2011).

Dans les vaisseaux, quatre principaux types de connexines sont identifiés. Les Cx37, Cx40 et Cx43 sont exprimées dans les cellules musculaires lisses et les cellules endothéliales. Une quatrième, la Cx45, n'a été identifiée que dans les cellules musculaires lisses des vaisseaux (Yeh *et al.*, 1998; Hill *et al.*, 2001; Hill *et al.*, 2002; Kwak *et al.*, 2002).

La régulation des Cx a été associée avec diverses pathologiques vasculaires telles que l'hypertension (Haefliger *et al.*, 2004) et la formation de plaques d'athérosclérose (Kwak *et al.*, 2003). Une surexpression de la Cx43 a aussi été aperçue après une angioplastie coronarienne (Yeh *et al.*, 1997).

Pendant une ischémie, la mort cellulaire endothéliale est attribuée à l'ouverture des hémicanaux de la Cx43, et une inhibition spécifique de cette connexine prévient la mort cellulaire pendant une ischémie (O'Carroll *et al.*, 2008).

II.3. Fonctions des connexines

Les connexines sont impliquées dans des fonctions cellulaires très diversifiées allant de la prolifération jusqu'à la différenciation des cellules, en passant par la signalisation, la régulation de l'expression des gènes et le maintien de l'homéostasie cellulaire (Pearson *et al.*, 2005). Cette diversité de fonctions a été réalisée surtout après l'identification de nombreux troubles pathologiques causés par des mutations sur les gènes codant pour des connexines (Omori *et al.*, 1996; Kelsell *et al.*, 2001), incluant l'épilepsie et d'autres neuropathies (Seeman *et al.*, 2001; Delgado-Escueta, 2007), la surdité (Kelley *et al.*, 1999), les cataractes (Maestrini *et al.*, 1999), les maladies de la peau (Richard *et al.*, 1998; Richard, 2000) mais aussi l'arythmie et les malformations cardiaques (Britz-Cunningham *et al.*, 1995; Reaume *et al.*, 1995; Simon and Goodenough, 1998). Les expériences de ciblage génétique ont permis l'élucidation de plusieurs de leurs rôles dans les fonctions physiologiques (Gutstein *et al.*, 2001; Willecke *et al.*, 2002).

Dans les sections suivantes, nous abordons les fonctions fondamentales des formes membranaires de Cx43: les jonctions Gap et les hémicanaux non-apposés.

II.3.1. Les fonctions des jonctions Gap

Du fait qu'ils traversent les membranes des cellules adjacentes et relient leurs cytoplasmes, les canaux de JG permettent le couplage intercellulaire. Deux modes de couplage sont connus, le mode électrique et le mode métabolique.

II.3.1.1. Le couplage électrique

Dans le muscle cardiaque, le couplage électrique a été mis en évidence la première fois en 1932 (Wilson *et al.*, 1933). Le potentiel d'action, généré dans le nœud sinusal, se propage rapidement sous forme d'une vague d'excitation tout le long du muscle cardiaque. La transmission de cette onde de dépolarisation à travers le muscle cardiaque et la synchronisation des contractions sont assurées grâce à la présence des JG (Manjunath and Page, 1986).

En contraste avec les autres canaux ioniques, les canaux de JG ne sont pas sélectifs pour les ions. Ils laissent passer presque tous les types d'ions par diffusion passive selon le gradient électrochimique qui règne de part et d'autre de la membrane plasmique (Bennett *et al.*, 1991). L'altération du couplage électrique peut entrainer des arythmies létales (Dillon *et al.*, 1988; Smith *et al.*, 1991; Luke and Saffitz, 1991).

II.3.1.2. Couplage métabolique

En plus des ions, les JG permettent également le passage des molécules biologiques ayant un poids moléculaire inférieur à 1 KD et un diamètre plus petit que

1,5 nm (Simpson *et al.*, 1977; Steinberg *et al.*, 1994; Elfgang *et al.*, 1995; Bevans *et al.*, 1998; Nicholson *et al.*, 2000). Parmi ces molécules, on connait des seconds messagers et des métabolites naturels (Bevans *et al.*, 1998; Goldberg *et al.*, 1999) tels que l'inositol trisphosphate (Saez *et al.*, 1989; Sanderson, 1995), l'AMPc (Lawrence *et al.*, 1978), l'ADP, l'ATP, le glutamate, le glutathion et l'adénosine (Goldberg *et al.*, 1999; Goldberg *et al.*, 2002) ainsi que des acides aminés (Johnson and Sheridan, 1971), des petits peptides (Simpson *et al.*, 1977), des cofacteurs vitaminiques, et des sucres (Saez *et al.*, 1989). Comme dans le cas du couplage électrique, le transfert métabolique se fait rapidement et par diffusion passive selon le gradient électrochimique (KANNO and LOEWENSTEIN, 1964; KANNO and Loewenstein, 1966; Stewart and Page, 1978).

Le couplage métabolique est surtout important pour la croissance et la différenciation cellulaires (Lo *et al.*, 1996; Kanczuga-Koda, 2004).

II.3.2. Les fonctions des hémicanaux non-apposés

À ce jour, les HcCx43 sont connus surtout pour leur implication dans la régulation du volume cellulaire (Quist *et al.*, 2000) et la signalisation (Plotkin *et al.*, 2002; Plotkin *et al.*, 2006; Plotkin *et al.*, 2008; Lima *et al.*, 2009).

Toutefois, contrairement aux canaux des JG, les fonctions des Hc non-apposés sont relativement moins étudiées. En fait, ce n'est qu'en 1991 que des Hc fonctionnels ont été détectés pour la première fois, et ce, dans des ovocytes de Xénope transfectées avec la Cx46 (Paul *et al.*, 1991). Depuis, des Hc de différents types de connexines ont été

caractérisés dans plusieurs types cellulaires y compris les cardiomyocytes (John *et al.*, 1999; Kondo *et al.*, 2000; Contreras *et al.*, 2002). Les données fonctionnelles disponibles sur les Hc montrent que certaines propriétés de ces derniers diffèrent de celles des canaux de JG.

En effet, sous des conditions physiologiques normales, les Hc non-apposés restent à l'état fermé (Saez *et al.*, 2005). Leur ouverture n'est observée que dans des conditions particulières telles qu'à basses concentrations de calcium extracellulaire (Pfahnl and Dahl, 1999; Quist *et al*., 2000) ou de H^+ cytoplasmique (Trexler *et al.*, 1999). Elle survient aussi dans des conditions hypo-osmotiques (Quist *et al.*, 2000), par des dépolarisations à des voltages élevés (Li *et al.*, 1996; Contreras *et al.*, 2003) ou par stimulation mécanique (Bao *et al.*, 2004).

Le stress ischémique dans ses différentes formes, incluant l'hypoxie, l'inhibition métabolique, la privation d'oxygène et de glucose et l'ischémie, favorise aussi l'ouverture des Hc (John *et al.*, 1999; Kondo *et al.*, 2000; Contreras *et al.*, 2002; Vergara *et al.*, 2003; Ramachandran *et al.*, 2007; Retamal *et al.*, 2007). Lorsqu'ils sont activés, les HcCx43 engendrent de gros courants ioniques qui reflètent leur forte conductance.

Tout comme les canaux de JG, ils sont perméables aux petits métabolites (≤1 KD) et ne sont pas sélectifs vis-à-vis les ions. De ce fait, leur activation peut perturber sévèrement la composition ionique du milieu cytosolique. L'accumulation intracellulaire excessive d'ions Na^+ et Ca^{2+} (Barry and Bridge, 1993; Silverman and Stern, 1994) et la libération de NAD^+ (Bruzzone *et al.*, 2001) et d'ATP (Zhao *et al.*, 2005) à la suite de l'ouverture

des HcCx43 compromet la survie des cellules et peut entrainer leur mort (Pierce GN. *et al*., 1995). L'ouverture des HcCx43 à la suite d'un stress ischémique a été rapportée dans plusieurs types cellulaires (Contreras *et al.*, 2002; Contreras *et al.*, 2004; Thompson *et al.*, 2006), incluant les cardiomyocytes (Kondo *et al.*, 2000; Schulz and Heusch, 2004; Shintani-Ishida *et al.*, 2007; Clarke *et al.*, 2009). Dans ces derniers, cet effet entraine la mort cellulaire (John *et al*., 1999; Kondo *et al*., 2000).

II.4. La régulation des canaux de connexines

Les connaissances dont nous disposons aujourd'hui montrent que les canaux de connexines sont hautement régulés depuis leur formation jusqu'à leur dégradation. Entre ces deux étapes, la régulation fonctionnelle de ces canaux reste active et implique des mécanismes bien variés.

La concentration des ions Na^+, Ca^{2+} ou Mg^{2+} intracellulaires, les variations du pH ou la perte d'ATP peuvent tous moduler ces canaux (De Mello, 1975; De Mello, 1976; Maurer and Weingart, 1987; Noma and Tsuboi, 1987; Sugiura *et al.*, 1990; Ek *et al.*, 1994). Les Hc non-apposés en particulier peuvent être régulés par la dépolarisation de la membrane cellulaire, les conditions hypo-osmotiques, la stimulation mécanique et le stress oxydatif (Li *et al.*, 1996; John *et al.*, 1999; Quist *et al.*, 2000; Kondo *et al.*, 2000; Contreras *et al.*, 2002; Vergara *et al.*, 2003; Contreras *et al.*, 2003; Bao *et al.*, 2004; Ramachandran *et al.*, 2007; Retamal *et al.*, 2007). Aujourd'hui, la phosphorylation des connexines par les protéines kinases reste l'un des mécanismes de régulation les plus

dominants et les mieux étudiés (Warn-Cramer *et al.*, 1998; Duncan and Fletcher, 2002). Elle représente le mécanisme de régulation par excellence pendant les conditions physiologiques et pathophysiologiques (Pierce and Czubryt, 1995).

II.4.1 Régulation par phosphorylation

La plupart des connexines, incluant la Cx43, sont des phosphoprotéines (Traub *et al.*, 1994). La biosynthèse, l'assemblage des connexines en Hc, l'insertion des Hc dans la membrane, le fonctionnement de ces canaux, leur internalisation et ensuite leur dégradation dépendent tous de l'état de phosphorylation des connexines (Musil and Goodenough, 1991; Elvira *et al.*, 1993; Lampe, 1994; Hertlein *et al.*, 1998; Berthoud *et al.*, 2000).

La Cx43 contient un grand nombre de sites de phosphorylation où interviennent plusieurs protéines kinases dont la protéine kinase A (PKA), la protéine kinase G (PKG), les tyrosines kinases, les MAP kinases, la caséine kinase (Lampe and Lau, 2000) mais surtout la protéine kinase C (PKC) qui intervient, à elle seule, sur le plus grand nombre de sites de phosphorylation putatifs de la Cx43, soit une vingtaine de sites (Blom *et al.*, 2004). Pour comparer, la Cx43 ne contient que trois sites de phosphorylation pour la PKA, trois pour la MAPK et quatre pour la PKG (Kemp and Pearson, 1990; van Veen *et al.*, 2001) (Figure 5).

Le rôle de la PKC dans le cycle de vie de la Cx43 semble être complexe et n'est pas entièrement élucidé. Toutefois, il est établi que l'activation de la PKC augmente la

phosphorylation de la Cx43 et possède des effets sur l'assemblage des canaux de JG, sur leur fermeture et ouverture ainsi que sur la demi-vie de la Cx43 sur la membrane (Lampe, 1994; Ek-Vitorin *et al.*, 2006; Moreno and Lau, 2007).

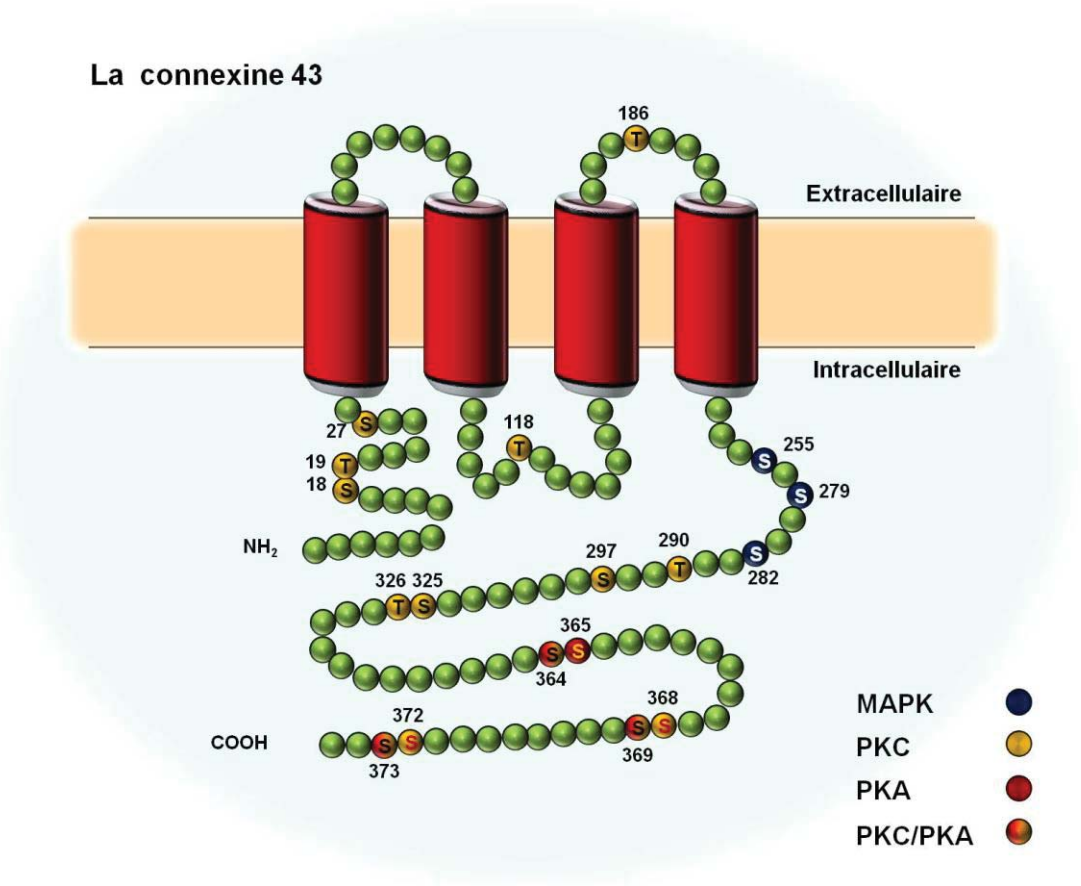

Figure 5. Sites de phosphorylation putatifs sur la Cx43. S et T représentent respectivement les résidus sérine et thréonine. Les chiffres correspondent à la position du résidu dans la séquence des acides aminés qui forment la protéine.

Précisément, l'activation de la PKC diminue d'une manière spectaculaire l'assemblage des JG en déstabilisant la formation de nouveaux JG sans affecter la structure des plus anciens (Pitts and Burk, 1987; Lampe, 1994). Elle réduit également la perméabilité des HcCx43 aux grosses molécules sans provoquer la fermeture totale du canal (Lampe and Lau, 2000; Bao *et al.*, 2007); (Kwak and Jongsma, 1996). Les amplitudes des courants engendrés par les canaux unitaires des JG régressent aussi (Pitts and Burk, 1987; Lampe and Lau, 2000; Ek-Vitorin *et al.*, 2006). Toutefois, la probabilité de leur ouverture augmente (Kwak and Jongsma, 1996), ce qui laisse peu affecté le couplage électrique entre les cellules.

II.4.2. Régulation des canaux de Cx43 pendant une ischémie

À la suite d'une ischémie, l'activité des protéines kinases et phosphatases est altérée. La régulation de l'état de phosphorylation de Cx43 détermine la conductance et la perméabilité des Hc et des canaux des JG.

II.4.2.1. Régulation des canaux des jonctions Gap

Pendant une ischémie, le couplage cellulaire est altéré. Toutefois, la nature exacte de cette altération est encore débattue. Certains proposent que la communication à travers les JG baisse considérablement (Green and Severs, 1993; Matsushita *et al.*, 1999; Lampe *et al.*, 2006). L'augmentation du calcium cytosolique, la réduction de l'ATP, l'augmentation de l'acidité ainsi que le changement de l'état de phosphorylation

pendant l'ischémie sont tous des facteurs qui favorisent la fermeture des JG (Noma and Tsuboi, 1987; Sugiura *et al.*, 1990). Elle atténue de ce fait la propagation des métabolites toxiques de la zone lésée vers les régions saines et réduit la perte de métabolites cellulaires vitaux tels que l'ATP et le glucose (Dhein, 1998). Il est largement accepté que ce découplage résulte de la déphosphorylation rapide des Cx43 et la migration des JG à partir des disques intercalaires vers les côtés latéraux des cardiomyocytes (Green and Severs, 1993; Matsushita *et al.*, 1999; Lampe *et al.*, 2006). La densité des JG entre les cardiomyocytes ventriculaires est alors réduite (Peters *et al.*, 1993).

Au contraire, d'autres chercheurs suggèrent que l'ischémie favorise la phosphorylation des Cx43 (Shintani-Ishida *et al.*, 2009). Les auteurs de cette théorie pensent que cet effet accélère la translocation des Cx43 vers les JG dans les disques intercalaires et que l'accumulation des canaux phosphorylés dans ces dernières augmente la communication à travers les JG, l'échappement d'un grand nombre de molécules vitales et l'entrée massive d'ions calcium. Par conséquent, les cellules meurent et la nécrose survient (Shintani-Ishida *et al.*, 2009).

Entre ces deux théories, plusieurs groupes avaient démontré qu'au début de l'ischémie, l'activité des protéines kinases A et C augmente (Lochner *et al.*, 1999; Albert and Ford, 1999), les JG restent phosphorylées et actives (Schulz *et al*. 2003). Leur ouverture contribue alors à la transmission des facteurs déclenchant l'hypercontraction (Schulz R *et al*., 2003). Au fur et à mesure que l'ischémie se prolonge, l'activité des kinases régresse. Les Cx43 commencent alors à se déphosphoryler (Jeyaraman *et al.*, 2003; Jain *et al.*, 2003) et le découplage se produit (Schulz and Heusch, 2004).

II.4.2.2. Régulation des hémicanaux

Pendant les conditions physiologiques normales, les Cx43 dans le myocarde sont majoritairement phosphorylées (van Veen *et al.*, 2001) et les HcCx43 demeurent à l'état fermé (Saez *et al.*, 2005). Lorsqu'un stress ischémique survient, les HcCx43 s'ouvrent (John *et al.*, 1999; Kondo *et al.*, 2000; Contreras *et al.*, 2002; Shintani-Ishida *et al.*, 2007; Ramachandran *et al.*, 2007; Retamal *et al.*, 2007). Bien que le mécanisme exact à la base de l'ouverture de ces Hc pendant une ischémie n'ait pas été complètement élucidé, plusieurs relient cet effet principalement à la réduction du calcium extracellulaire et au changement de l'état de phosphorylation des Cx43 (Beardslee *et al.*, 2000; Li and Nagy, 2000; Contreras *et al.*, 2002; Contreras *et al.*, 2004; Retamal *et al.*, 2006). Ceci nous a incités à croire que l'ouverture des HcCx43, pendant une ischémie, soit un facteur important dans la formation de l'infarctus du myocarde et que son inhibition devrait protéger le myocarde contre ces lésions en rendant les cellules plus résistantes à l'ischémie.

II.5. Les inhibiteurs des canaux de connexines

Des bloqueurs de canaux de connexine sont utilisés dans le traitement de plusieurs conditions pathologiques (Tsai *et al.*, 1996; Laird *et al.*, 1999). À ce jour, on connait deux classes d'inhibiteurs de canaux de connexine, les inhibiteurs classiques et les inhibiteurs synthétiques mimétiques structuraux des connexines.

II.5.1. Les inhibiteurs classiques

Les inhibiteurs classiques (ou conventionnels) agissent simultanément sur les JG et les Hc. De plus, ils présentent une faible spécificité envers les divers types de connexines qui forment ces canaux. Leurs effets sur les canaux de connexines sont indirects et leurs mécanismes d'action sont complexes (Spray *et al.*, 2002; Evans *et al.*, 2006). On connait plusieurs inhibiteurs dans cette classe :

Les agents lipophiliques

Ce groupe comprend des produits chimiques comme l'octanol, l'heptanol et les anesthésiques halothanes. Ces produits exercent des effets réversibles et agissent en dissolvant les lipides membranaires modifiant ainsi la fluidité de la membrane. Ils affectent la probabilité d'ouverture ainsi que la conductance unitaire du canal (Burt and Spray, 1989; Bastiaanse *et al.*, 1993). D'autres canaux ioniques et récepteurs membranaires sont également affectés par ces produits (Rozental *et al.*, 2000).

L'acide 18 α-glycyrrhétinique

L'acide 18 α–glycyrrhétinique agit indirectement sur les connexines en activant des protéines kinases et des protéines G (Evans and Boitano, 2001). Il induit un changement dans la conformation des connexines et affecte leur assemblage et la distribution des canaux dans les plaques de JG (Goldberg *et al.*, 1996). Il modifie aussi les propriétés biophysiques des canaux et altère la communication intercellulaire (Goodenough *et al.*, 1996).

Les acides gras

Les acides gras, en particulier, les acides oléiques et arachidoniques altèrent la biosynthèse des lipides et les voies de signalisation qui sous-tendent le fonctionnement normal des JG (Burt *et al.*, 1991; Lavado *et al.*, 1997; Schmilinsky-Fluri *et al.*, 1997). L'oléamide, un amide de l'acide oléique, peut affecter de nombreuses fonctions physiologiques, y compris le sommeil, la thermorégulation et la sensibilité à la douleur (Boger *et al.*, 1998; Carrero *et al.*, 2005; Michaud and Denlinger, 2006).

II.5.2. Les peptides synthétiques mimétiques structuraux des connexines

Dans le but de concevoir des inhibiteurs spécifiques aux JG, des chercheurs ont développé de courts peptides synthétiques qui miment l'une ou l'autre des deux boucles extracellulaires des connexines normalement impliquées dans l'interaction Hc-Hc lors

de leur apposition pour former un canal de JG (Dahl *et al.*, 1994; Warner *et al.*, 1995; Evans *et al.*, 2006). Par leur liaison sur les séquences homologues sur la connexine, ces peptides feront un obstacle à l'apposition des hémicanaux et inhiberont ainsi la formation et la fonction des canaux de JG (Kwak and Jongsma, 1999; Evans and Boitano, 2001).

À la surprise de leurs propres inventeurs, ces peptides ont exercé peu ou pas d'effets sur les JG (Braet *et al.*, 2003). Toutefois, leurs effets fonctionnels apparents ont incité d'autres auteurs à présumer que ces peptides inhibent plutôt les Hc non-apposés (Braet *et al.*, 2003; Leybaert *et al.*, 2003; Gomes *et al.*, 2005; Pearson *et al.*, 2005; Romanov *et al.*, 2007).

Étant donné que les structures visées sur les boucles extracellulaires possèdent des séquences en acide aminés spécifiques à chaque type de connexine, il est présumé que les peptides mimétiques interagissent uniquement et spécifiquement avec le type de connexine duquel ils dérivent sans affecter les autres connexines. À ce jour, on connait au moins une douzaine de peptides mimétiques de plusieurs variantes de connexines (Tableau I) (Chaytor *et al.*, 1997; Chaytor *et al.*, 1998; Kwak and Jongsma, 1999; Evans and Boitano, 2001).

Nom	Séquence	Domaine	Propriétés
Gap26	43, 32, 26 VCYDKSFPISHVR	BE1	Inhibe les HcCx (Chaytor et al.,1997; Chaytor et al., 1999)
Gap27/Cx43	43, 37, 32, 26 SRPTEKTIFII	BE2	Inhibe les HcCx (Chaytor et al., 1997; Chaytor et al., 1999; Dora et al., 1999; Bitano et al., 2000; Isakson et al., 2001; Ovieto-Orta et al., 2004; Isakson et al., 2005; Martin et al., 2005)
Gap27/Cx40	40 SRPTEKNVFIV	BE2	Inhibe les JG de la Cx40 (Evans et Boitano . 2001; Chaytor et al., 1999)
Gap24	32 GHGDPLHLEEVKC	BI	Inhibe les HcCx32 et non pas les JG (De Vuyst et al., 2006)
L2	43 DGVNVEMHLKQIEIK KFKYGIEEHGK	BI	Influence les JG (Seki et al., 2004)
Peptide 1848	43 CNTQQPCCENVCY	BE1	Inhibe les JG dans les cellules dendritiques
P 180-195	43 SLSAVYTCKRDPCPHE	BE2	Inhibe les JG de la Cx43 (Kwak et Jongsma. 1999)
P177-192	40 FLDTLHVCRRSPCPHP	BE2	Inhibe les JG de la Cx40 (Kwak et Jongsma. 1999)
Sans nom	32 ICNTLQPGCNSV	BE1	Inhibe les JG de la Cx32 dans les hépatocytes (Eugenin et al., 1998)

Tableau I. Les peptides synthétiques mimétiques de connexines. BE1 : première boucle extracellulaire; BE2 : deuxième boucle extracellulaire; BI : boucle intracellulaire.

Les peptides Gap26 (VCYDKSFPISHVR) et Gap27 (SRPTEKTIFII) miment respectivement la première et la deuxième boucle extracellulaires de la Cx43 (Figure 6) (Chaytor *et al.*, 1999;Figueroa *et al.*, 2006).

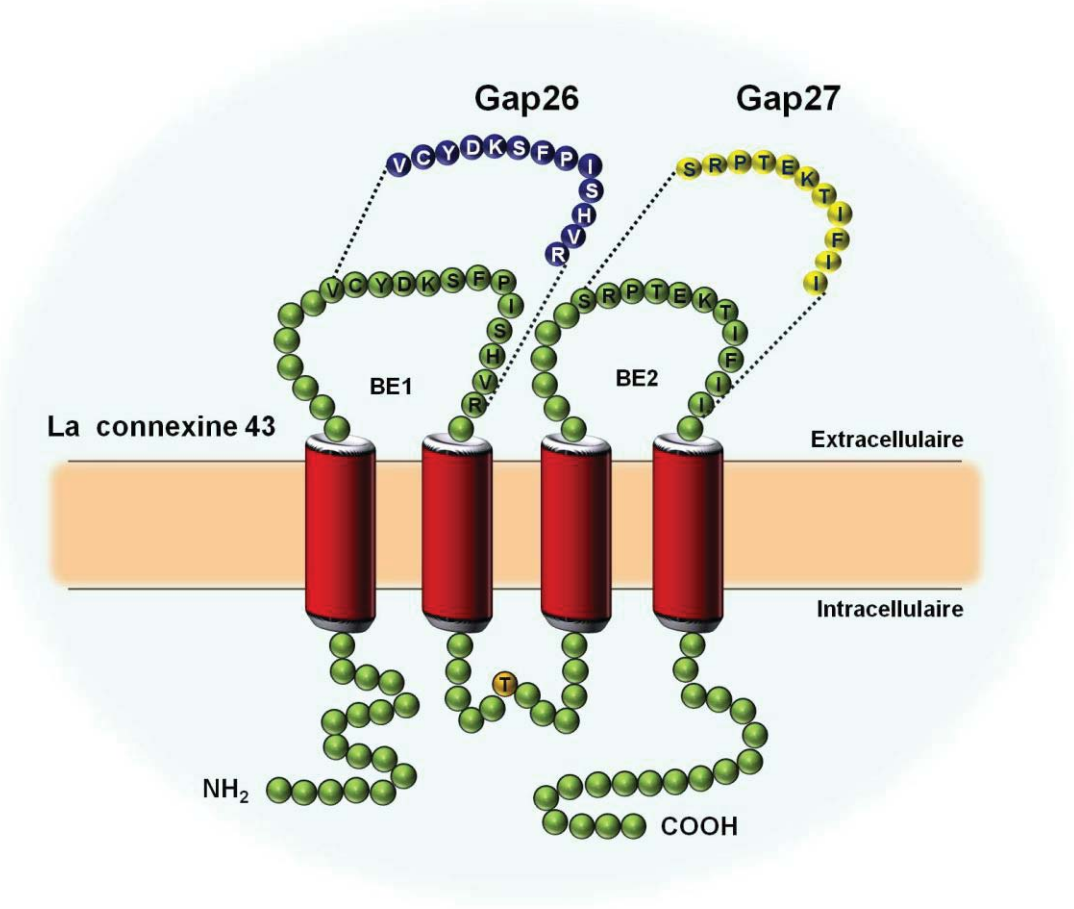

Figure 6. Les peptides synthétiques Gap26 et Gap 27 se lient respectivement sur la première et la deuxième boucle extracellulaires de la connexine 43.

Les motifs qu'ils contiennent ne sont pas présents dans aucune autre protéine cellulaire (Dahl *et al.*, 1992; Warner *et al.*, 1995) ce qui suggère que ces deux peptides interagissent uniquement avec la Cx43 sans interférer avec d'autres connexines ou protéines de surface (Warner *et al.*, 1995; Braet *et al.*, 2003). L'application du peptide Gap26 aux préparations cellulaires a bloqué sélectivement la libération d'ATP, l'absorption du colorant et l'entrée du calcium dans les cellules soumises à une ischémie (Braet *et al.*, 2003; Shintani-Ishida *et al.*, 2007), et ce, sans affecter la communication à travers les JG. Néanmoins, l'effet inhibiteur du peptide Gap26 directement sur de vrais HcCx43 n'a jamais été démontré (Dahl, 2007).

Dans ce projet, nous avons fourni pour la première fois une preuve directe et indéniable de l'effet inhibiteur des peptides Gap26 et Gap27 sur des HcCx43 exogènes exprimés dans les cellules tsA201. Nous avons alors étudié l'effet de ces deux peptides dans la protection du cœur contre les dommages ischémiques.

III. La famille des protéines kinases C

III.1. Généralités

Les protéines kinases C forment une superfamille de kinases regroupant au moins une douzaine d'isoformes différentes (Nishizuka, 1988; Osada *et al.*, 1992). Ces dernières phosphorylent de nombreux substrats sur des résidus sérine ou thréonine et sont impliquées dans la régulation de beaucoup de phénomènes cellulaires, tels que la croissance et la différenciation (Nishizuka, 1988; Hug and Sarre, 1993; Nishizuka, 1995; Sugden and Bogoyevitch, 1995; Steinberg *et al.*, 1995) et des mécanismes physiologiques tels que la fréquence cardiaque et la contraction du myocarde (Croop *et al.*, 1980; Leatherman *et al.*, 1987). Il en résulte qu'un dérangement dans l'activité de ces enzymes pourra entrainer des conséquences pathologiques bien graves (Boneh, 1995). La PKC est associée à plusieurs pathologies incluant la cardiomyopathie, rétinopathie, néphropathie, neuropathie, dysfonction endothéliale (Geraldes and King, 2010), différentes tumeurs (Basu, 1993; Baron-Delage and Cherqui, 1997) et des maladies neurodégénératives (Chen *et al.*, 2003).

Bien qu'on retrouve les isoformes de PKC presque partout dans notre organisme, ces enzymes sont sélectivement et différentiellement exprimées dans les tissus et à l'intérieur des cellules (Knopf *et al.*, 1986; Coussens *et al.*, 1987; Ohno *et al.*, 1988). Elles possèdent des mécanismes d'activation variés suggérant que chacune de ces

isoformes accomplit un rôle spécifique et déterminé (Banan *et al.*, 2003; Banan *et al.*, 2004) d'où leur importance physiologique.

III.2. Classification des différentes isoformes de PKC

Du point de vue structurel, chaque PKC possède une région catalytique du côté C-terminal contenant les domaines C3 et C4, sites de fixation de l'ATP et des substrats, respectivement; et une autre région régulatrice du côté N-terminal qui contient un domaine pseudosubstrat auto-inhibiteur et deux domaines C1 et C2 sensibles au diacylglycérol (DG)/phorbol ester (PE) et au calcium, respectivement (Chambers *et al.*, 1993; Mellor and Parker, 1998; Toker, 1998) (Figure 6). La présence et le positionnement de ces domaines dans la structure de l'enzyme déterminent la classe de cette dernière. En se basant sur ces critères, les douze isoformes de PKC se divisent en trois classes principales (Figure 7).

La classe des **PKC classiques**, ou cPKC, regroupe les isoformes α, βI, βII et γ. Elles sont sensibles au calcium, aux phospholipides, et au DG (Nishizuka, 1988). Les isoformes de cette classe possèdent les deux sites C1 et C2 dans leur domaine régulateur. Le site C1 contient deux doigts de zinc riches en cystéine, appelés C1a et C1b (Hurley and Grobler, 1997). Ce dernier est impliqué dans la liaison du DG et des PE (Takai *et al.*, 1979; Castagna *et al.*, 1982; Zhang *et al.*, 1995). Le domaine C2 lie les phospholipides d'une manière dépendante du calcium (Stahelin and Cho, 2001).

La classe des **PKC nouvelles**, ou nPKC, comprend les isoformes δ, ε, η et θ. Elles sont indépendantes du calcium, mais sensibles aux phospholipides et au DG. Comme les cPKC, les nPKC possèdent les deux domaines C1 et C2 (le C2 précède le domaine C1) dans leur région régulatrice.

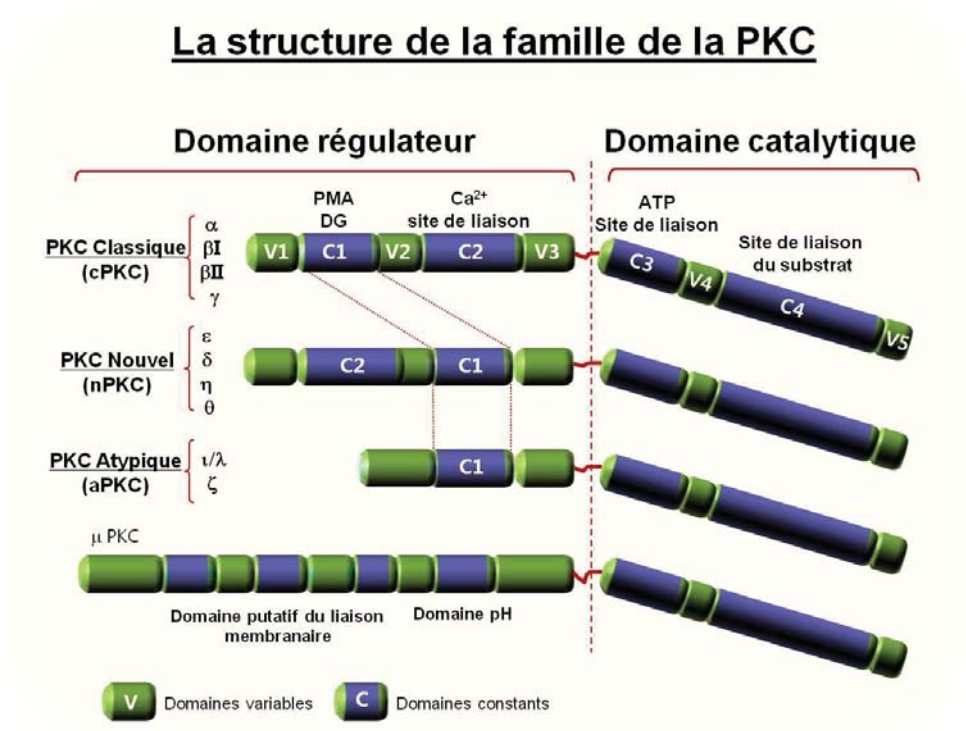

Figure 7. Structure générale des PKC avec les domaines régulateurs et catalytiques et leurs emplacements. La présence et l'emplacement de ces domaines sites déterminent le classement de la PKC.

Toutefois, la séquence du domaine C2 manque de chaines latérales liant le calcium d'où le fait que les isoformes des nPKC s'activent par le DG et les PE sans nécessiter la présence du calcium (Ono *et al.*, 1988; Pappa *et al.*, 1998).

La troisième grande classe est la classe des **PKC atypiques**, ou aPKC. Elle comprend les isoformes ζ et λ / ι insensibles au calcium, aux phorbols esters et au DG. Les isoformes aPKC ne possèdent pas le site C2 sensible au calcium. De même, le site C1 de ces isoformes ne contient qu'un seul doigt de zinc riche en cystéine qui ne lie ni le DG ni le PMA (Ono *et al.*, 1988;Sakane *et al.*, 1996). Par conséquent, les isoformes aPKC sont indépendantes du calcium, du DG et du PE.

Finalement, il existe une isoforme individuelle unique, nommée μPKC, dont la structure ne ressemble à aucune des isoformes appartenant aux trois classes décrites ci-haut. Elle forme une classe par elle-même et est sensible aux phospholipides, aux PE et au DG, mais insensible au calcium (Johannes *et al.*, 1994).

La régulation des HcCx43 par les différentes isoformes de PKC n'est pas encore élucidée. Dans cette étude, nous avons étudié le rôle fonctionnel des isoformes de PKC dans la régulation des HcCx43. Nous avons mis en évidence l'effet inhibiteur de l'isoforme PKCε, connu pour son rôle cardioprotecteur contre les dommages myocardiques ischémiques sur les HcCx43. Ce travail fait l'objet du chapitre II de la présente thèse.

III.3. Expression et mécanismes d'activation et d'inactivation

Dans le cœur, plusieurs isoformes de PKC sont exprimées (Bowling *et al.*, 1999; Shin *et al.*, 2000; Simonis *et al.*, 2007). Parmi celles-ci, les isoformes PKCα, PKCβI et βII prédominent dans les ventricules. Au contraire, les oreillettes sont surtout dominées par les isoformes PKCδ et ζ (Simonis *et al.*, 2007). Les isoformes PKCε and λ sont réparties presque également dans les oreillettes et les ventricules (Simonis *et al.*, 2007).

Isoformes de PKC	Inactive	Active
PKCα	Cytoplasme	Région périnucléaire (Disatnik MH. et al., 1994)
PKCβI	Cytoplasme Région périnucléaire	Intérieur du noyau (Disatnik MH. et al., 1994)
PKCβII	Structures fibrillaires	Région périnucléaire et périphérie de la cellule (Disatnik MH. et al., 1994)
PKCδ	Noyau	Stries des cellules et région périnucléaire (Disatnik MH. et al., 1994; Johnson JA. et al., 1996)
PKCε	Noyau	Stries des cellules et disques intercalaires (Disatnik MH. et al., 1994; Johnson JA. et al., 1996)
PKCζ	Cytoplasme	Région périnucléaire (Disatnik MH. et al., 1994)
PKC*η*	Cytoplamse	Cytoplasme (Johnson JA. et al., 1996)

Tableau II. Localisation des isoformes de PKC dans les cardiomyocytes avant et après leur activation.

À l'intérieur d'une cellule, les isoformes de PKC sont réparties sélectivement dans les divers compartiments. Suite à l'activation d'une isoforme particulière, cette dernière se déplace d'un compartiment cellulaire à un autre tout en changeant son état physique, soit de l'état dissous à l'état granulé ou vice versa (Tanaka *et al.*, 1991). Ce mouvement est appelé "translocation".

La localisation des différentes isoformes dans des sites subcellulaires distincts, avant et après leur activation (Tableau II), suggère que chaque isoforme exerce des fonctions uniques.

Du point de vue structurel, une isoforme inactive de PKC se trouve normalement repliée sur elle-même (Parker and Murray-Rust, 2004). Le domaine pseudosubstrat est alors fixé sur le domaine de fixation du substrat (Figure 8a) empêchant ainsi toute reconnaissance d'une cible potentielle. Sous cette conformation, la PKC ne peut pas phosphoryler ses substrats.

Pour induire son activation, le DG ou le calcium par exemple se fixent sur les domaines régulateurs C1 et/ou C2 (Edwards *et al.*, 1999). Cette fixation écarte le pseudosubstrat du domaine de fixation du substrat, déplie la molécule et expose le domaine de fixation du substrat (Figure 8b) (Nishizuka, 1995; Griner and Kazanietz, 2007). L'activation de l'isoforme de PKC s'accompagne par sa translocation vers un site cellulaire précis où elle sera attachée à des protéines spécifiques, appelées RACKS (*Receptor for Activated C kinase*) afin d'interagir ensuite avec son substrat (Mochly-Rosen, 1995) (Figure 8).

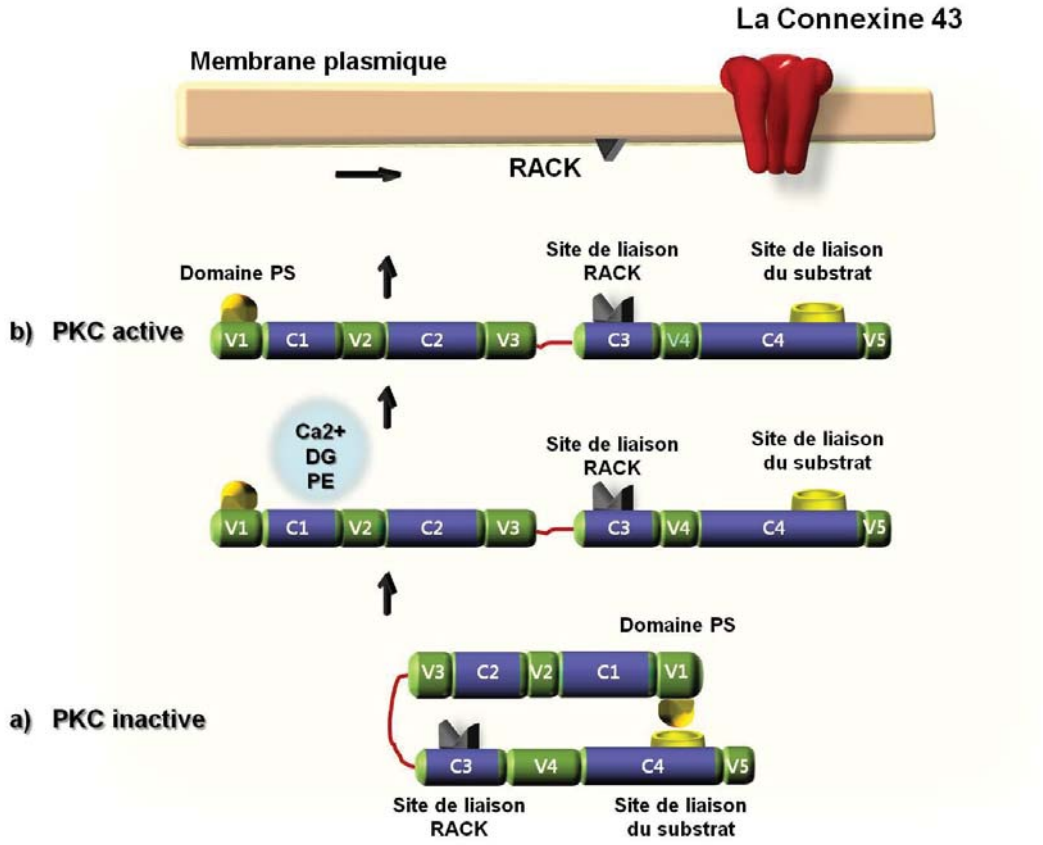

Figure 8. Activation d'une isoforme de PKC. (a) L'isoforme inactive est repliée sur elle-même de façon à ce que le domaine pseudosubstrat se lie sur le site de liaison du substrat. (b) Dans sa forme active, le DG, le calcium ou le PE se lient sur les domaines C1 et C2, induisant l'ouverture de la PKC et exposant les sites de liaison du substrat.

III.4. Les peptides synthétiques modulateurs spécifiques des isoformes de PKC

Jusqu'à la fin des années 90, les modulateurs spécifiques des isoformes de PKC n'existaient pas. C'est vers l'année 1998 que le groupe du professeur Daria Mochly-Rosen à Stanford University en Californie a réussi à moduler individuellement la fonction des isoformes de PKC en utilisant de courts peptides synthétiques (Ron *et al.*, 1995;Souroujon and Mochly-Rosen, 1998) (Tableau III). En mimant le site de liaison d'une isoforme particulière de PKC sur son propre récepteur RACK, le peptide se lie sur ce dernier et empêche sa liaison sur l'isoforme PKC. Par conséquent, ceci empêche le transport de la PKC vers son substrat et inhibe alors la phosphorylation de ce dernier.

Des peptides activateurs ont aussi été conçus. En se liant à l'isoforme de PKC correspondante, le peptide activateur provoque un changement dans la conformation de l'isoforme de manière à exposer son site catalytique et faciliter la fixation de la PKC sur son RACK. Ceci permettra ensuite sa liaison sur son substrat pour le phosphoryler (Mochly-Rosen, 1995).

Aujourd'hui, ces peptides sont le seul outil connu pour disséquer les rôles fonctionnels individuels des différentes isoformes de PKC. En combinaison avec la technique du patch-clamp, nous avons utilisé ces peptides pour déterminer le rôle que jouent les différentes isoformes de PKC dans la régulation des HcCx43 (chapitre II).

Peptide	Effets	Séquence
aC2-4	Inhibiteur de PKCα	S-L-N-P-Q-W-N-E-T
bIV5-3	Inhibiteur de PKCβI	K-L-F-I-M-N
bIIV5-3	Inhibiteur de PKCβII	Q-E-V-I-R-N
bc2-2	Activateur de PKCβ	S-V-E-I-W
dV1-1	Inhibiteur de PKCδ	S-F-N-S-Y-E-L-G-S-L
eV1-2	Inhibiteur de PKCε	E-A-V-S-L-K-P-T
eV1-7	Activateur de PKCε	H-D-A-P-I-G-Y-D
hV1-2	Inhibiteur de PKCη	E-A-V-G-L-Q-P-T

Tableau III. Les peptides modulateurs spécifiques des isoformes de PKC utilisés dans cette étude, leurs effets et leurs séquences.

III.5. Les isoformes de PKC et la cardioprotection contre l'ischémie

Le développement de modulateurs spécifiques pour les isoformes de PKC a permis d'élucider le rôle de ces derniers dans la physiopathologie de l'infarctus du myocarde (Chen *et al.*, 2001; Budas *et al.*, 2007; Churchill and Mochly-Rosen, 2007). À cet égard, l'administration de l'activateur spécifique de la PKCε ou de l'inhibiteur de la PKCδ s'est avérée cardioprotectrice contre les lésions ischémiques dans plusieurs modèles animaux et a réduit la taille de l'infarctus (Hu *et al.*, 2000; Chen *et al.*, 2001; Di-Capua *et al.*, 2003; Inagaki *et al.*, 2003a). Au contraire, l'activation de la PKCδ augmente significativement les lésions tissulaires lors d'une ischémie (Dorn *et al.*, 1999;

Chen *et al.*, 2001; Inagaki *et al.*, 2003b; Tanaka *et al.*, 2004; Inagaki and Mochly-Rosen, 2005). Ces observations ont souligné le rôle bénéfique de l'isoforme PKCε et défavorable de l'isoforme PKCδ sur le cœur pendant l'ischémie.

Le mécanisme exact à la base de la cardioprotection par la modulation des isoformes de PKC n'est pas encore clairement élucidé. Toutefois, plusieurs médiateurs ont été proposés. Parmi ceux-ci, les canaux K_{ATP} du sarcolemme, ceux des mitochondries (Aizawa *et al.*, 2004) ainsi que les MPTP (Pravdic *et al*. 2009).

Dans une étude publiée en 2004, Aizawa et collaborateurs démontre que l'activation spécifique de l'isoforme PKCε entraîne une augmentation du niveau de phosphorylation des canaux K_{ATP} dans le sarcolemme et les mitochondries et stimule de ce fait leur ouverture (Aizawa *et al.*, 2004). Ce mécanisme a été également attribué à la cardioprotection par PI (Liang, 1998; Andrukhiv *et al.*, 2006; Jaburek *et al.*, 2006b). Au contraire, la phosphorylation des pores MPTP par PKCε induit leur fermeture (Korge *et al.*, 2002; Hausenloy *et al.*, 2004). On pense que cet effet réduit l'entrée du calcium et prévient le gonflement cellulaire pendant l'ischémie (Baines *et al.*, 2003; Pravdic *et al.*, 2009). D'autres médiateurs pourraient potentiellement être impliqués, parmi ceux-là les canaux ioniques. En fait, la PKC est impliquée dans la régulation de plusieurs canaux ioniques (Lacerda *et al.*, 1988; Dosemeci *et al.*, 1988; Walsh and Kass, 1988; Tseng and Boyden, 1991; Puceat *et al.*, 1994; Xiao *et al.*, 2001; Baroudi *et al.*, 2006). Par exemple, l'activation de l'isoforme PKCε entraine l'inhibition des canaux sodiques (Xiao *et al.*, 2001) et des canaux calciques de type L (Baroudi *et al.*, 2006). Ces effets pourront

réduire l'entrée des ions sodium et calcium survenant lors de l'ischémie (Miyawaki *et al.*, 1996) et jouer par conséquent un rôle bénéfique dans la cardioprotection lors d'une ischémie.

Dans la présente étude, nous proposons que les HcCx43, dont la conductance, la perméabilité et la sélectivité dépassent de loin celles des autres canaux et récepteurs membranaires, soient l'un des médiateurs les plus importants de la cardioprotection contre l'ischémie. Les Hc de connexines sont régulés par plusieurs facteurs incluant la phosphorylation par PKC (Bao *et al.*, 2004). L'implication de l'isoforme PKCε en particulier dans la phosphorylation des Cx43 a déjà été démontrée chez le rat (Doble *et al.*, 2000) et l'humain (Bowling *et al.*, 2001).

Au repos, lorsque les Cx43 sont phosphorylées, la conductance des HcCx43 est réduite (Saez *et al.*, 2005). À la suite d'une ischémie, les sous-unités de Cx43 se déphosphorylent et la perméabilité des HcCx43 augmente (Beardslee *et al.*, 2000; Schulz *et al.*, 2003; Jain *et al.*, 2003; Miura *et al.*, 2004). L'ouverture des HcCx entraîne la mort des cellules dans plusieurs modèles expérimentaux (Shintani-Ishida *et al.*, 2007).

Ces observations sont à la base des hypothèses sous-jacentes au projet et à l'origine des objectifs déterminés pour les diverses études qui les composent.

IV. Hypothèses et objectifs

En récapitulant ce qui précède dans ce premier chapitre, nous remarquons particulièrement les principaux faits suivants. Les HcCx43 restent fermés dans les conditions physiologiques normales (Saez *et al.*, 2005). Cet état est favorisé par plusieurs facteurs dont la phosphorylation des Cx43 par les protéines kinases, surtout par la PKC (Bao *et al.*, 2004). Or, plusieurs isoformes de PKC sont présentes dans le cœur. Le rôle fonctionnel des isoformes de PKC dans la modulation des HcCx43 n'est pas encore connu. En nous basant sur ces faits, nous avons postulé en premier que les HcCx43 sont régulés sélectivement et différentiellement par les isoformes de PKC.

De plus, à la suite d'un stress ischémique, les Cx43 se déphosphorylent (Beardslee *et al.*, 2000; Schulz *et al.*, 2003; Jain *et al.*, 2003; Miura *et al.*, 2004) et les HcCx43 s'ouvrent (Contreras *et al*. 2002). L'ouverture des HcCx43 entraine la mort cellulaire et des lésions tissulaires irréversibles (John *et al.*, 1999; Bruzzone *et al.*, 2001; Bennett *et al.*, 2003; Braet *et al.*, 2003; Goodenough and Paul, 2003). Après un PI, le niveau de phosphorylation des Cx43 dans le cœur reste préservé. L'isoforme PKCε en particulier joue un rôle primordial dans la protection par PI (Yoshida *et al.*, 1997; Gray *et al.*, 1997; Liu *et al.*, 2001; Ping *et al.*, 2002; Inagaki and Mochly-Rosen, 2005; Kim *et al.*, 2006; Sivaraman *et al.*, 2009). Pour cette raison, nous estimons dans une deuxième hypothèse que l'isoforme cardioprotectrice PKC ε joue le rôle d'inhibiteur des HcCx43.

À l'heure actuelle, les moyens mis en œuvre pour traiter un infarctus du myocarde pendant sa phase aiguë se concentrent à limiter la durée de l'ischémie en rétablissant la

circulation coronarienne normale. Aucun traitement n'est présentement disponible pour prévenir les dommages cardiaques qui surviennent après ces interventions. Dans une deuxième partie de notre étude, nous proposons que l'inhibition spécifique des HcCx43 rende les cellules myocardiques plus résistantes au stress ischémique et protège le cœur contre l'infarctus du myocarde. Tous les inhibiteurs classiques des canaux de connexine connus à ce jour ne sont pas spécifiques aux HcCx43. Ils affectent tous les JG de même que les Hc, et ne sont pas sélectifs envers les différents types de connexines qui forment ces canaux. Des peptides mimétiques structuraux de la Cx43 ont récemment émergé. Hypothétiquement, nous pensons que ces peptides inhibent sélectivement les HcCx43 en exerçant peu ou pas d'effets immédiats sur les canaux des JG ou sur les Hc faits par les autres connexines.

Ici, nous avons postulé que l'administration de ces peptides pourra exercer un effet protecteur contre les dommages causés par une ischémie. Si confirmée, cette hypothèse ouvrira sans doute un nouvel horizon très prometteur dans le traitement de cette pathologie mortelle.

Dans le but de valider, ou pas, les hypothèses qui ont découlé, nous avons proposé les objectifs spécifiques suivants:

1) Déterminer le rôle des différentes isoformes de PKC dans la régulation fonctionnelle des HcCx43 exprimés dans un système d'expression cellulaire privé de tout canal ionique, les cellules tsA201. En combinaison avec la technique du patch-clamp, l'effet des peptides modulateurs spécifiques des isoformes de PKC livrés dans le cytosol des cellules sur la modulation des HcCx43 sera évalué.

2) Étudier l'effet protecteur potentiel que l'administration des peptides mimétiques structuraux de Cx43, Gap26 et Gap27, peut conférer au cœur de rat adulte contre une ischémie locale induite par occlusion de l'artère coronaire antérieure descendante gauche (ADG), en comparant la taille de la zone de l'infarctus dans un cœur perfusé avec les peptides versus des cœurs témoins non traités.

Spécifiquement, nous nous sommes engagés à :

i. Démontrer les effets inhibiteurs des peptides Gap26 et Gap27 directement sur les HcCx43 exprimés dans les cellules tsA201 par patch-clamp.

ii. Démontrer la spécificité de ces peptides vis-à-vis les HcCx43 en testant leurs effets sur des Hc composés par d'autres connexines cardiaques, soit les HcCx40 et HcCx45.

iii. Étudier l'effet cardioprotecteur de ces peptides sur les cardiomyocytes isolés auxquels nous avons imposé une ischémie simulée par privation d'oxygène et de glucose.

iv. Étudier l'effet protecteur de la perfusion continue du cœur isolé intact par le peptide Gap26 contre une ischémie locale induite par occlusion de l'artère coronaire ADG.

v. Élucider et comparer *in vivo* dans un modèle de rat d'infarctus du myocarde l'effet cardioprotecteur des peptides Gap26 et Gap27 contre les lésions ischémiques lorsqu'ils sont injectés à basse dose sous forme d'un bolus unique avant la reperfusion.

V. Reference List

1. Aizawa,K., Turner,L.A., Weihrauch,D., Bosnjak,Z.J., Kwok,W.M., 2004. Protein kinase C-epsilon primes the cardiac sarcolemmal adenosine triphosphate-sensitive potassium channel to modulation by isoflurane. Anesthesiology 101**,** 381-389.

2. Albert,C.J., Ford,D.A., 1999. Protein kinase C translocation and PKC-dependent protein phosphorylation during myocardial ischemia. Am. J. Physiol 276**,** H642-H650.

3. Allen,D.G., Cairns,S.P., Turvey,S.E., Lee,J.A., 1993. Intracellular calcium and myocardial function during ischemia. Adv. Exp. Med. Biol. 346**,** 19-29.

4. Andrukhiv,A., Costa,A.D., West,I.C., Garlid,K.D., 2006. Opening mitoKATP increases superoxide generation from complex I of the electron transport chain. Am. J. Physiol Heart Circ. Physiol 291**,** H2067-H2074.

5. Argaud,L., Ovize,M., 2004. [How to use the paradigm of ischemic preconditioning to protect the heart?]. Med. Sci. (Paris) 20**,** 521-525.

6. Baines,C.P., Song,C.X., Zheng,Y.T., Wang,G.W., Zhang,J., Wang,O.L., Guo,Y., Bolli,R., Cardwell,E.M., Ping,P., 2003. Protein kinase Cepsilon interacts with and inhibits the permeability transition pore in cardiac mitochondria. Circ. Res. 92**,** 873-880.

7. Baines,C.P., Wang,L., Cohen,M.V., Downey,J.M., 1998. Protein tyrosine kinase is downstream of protein kinase C for ischemic preconditioning's anti-infarct effect in the rabbit heart. J. Mol. Cell Cardiol. 30**,** 383-392.

8. Banan,A., Farhadi,A., Fields,J.Z., Zhang,L.J., Shaikh,M., Keshavarzian,A., 2003. The delta-isoform of protein kinase C causes inducible nitric-oxide synthase and nitric oxide up-regulation: key mechanism for oxidant-induced carbonylation, nitration, and disassembly of the microtubule cytoskeleton and hyperpermeability of barrier of intestinal epithelia. J. Pharmacol. Exp. Ther. 305**,** 482-494.

9. Banan,A., Zhang,L.J., Farhadi,A., Fields,J.Z., Shaikh,M., Keshavarzian,A., 2004. PKC-beta1 isoform activation is required for EGF-induced NF-kappaB inactivation and IkappaBalpha stabilization and protection of F-actin assembly and barrier function in enterocyte monolayers. Am. J. Physiol Cell Physiol 286**,** C723-C738.

10. Bao,L., Sachs,F., Dahl,G., 2004. Connexins are mechanosensitive. Am. J. Physiol Cell Physiol 287**,** C1389-C1395.

11. Bao,X., Lee,S.C., Reuss,L., Altenberg,G.A., 2007. Change in permeant size selectivity by phosphorylation of connexin 43 gap-junctional hemichannels by PKC. Proc. Natl. Acad. Sci. U. S. A 104**,** 4919-4924.

12. Baron-Delage,S., Cherqui,G., 1997. [Protein kinase C and tumorigenic potential]. Bull. Cancer 84**,** 829-832.

13. Baroudi,G., Qu,Y., Ramadan,O., Chahine,M., Boutjdir,M., 2006. Protein kinase C activation inhibits Cav1.3 calcium channel at NH2-terminal serine 81 phosphorylation site. Am. J. Physiol Heart Circ. Physiol 291**,** H1614-H1622.

14. Barry,W.H., Bridge,J.H., 1993. Intracellular calcium homeostasis in cardiac myocytes. Circulation 87**,** 1806-1815.

15. Bastiaanse,E.M., Jongsma,H.J., van der Laarse,A., Takens-Kwak,B.R., 1993. Heptanol-induced decrease in cardiac gap junctional conductance is mediated by

a decrease in the fluidity of membranous cholesterol-rich domains. J. Membr. Biol. 136**,** 135-145.

16. Basu,A., 1993. The potential of protein kinase C as a target for anticancer treatment. Pharmacol. Ther. 59**,** 257-280.

17. Beardslee,M.A., Lerner,D.L., Tadros,P.N., Laing,J.G., Beyer,E.C., Yamada,K.A., Kleber,A.G., Schuessler,R.B., Saffitz,J.E., 2000. Dephosphorylation and intracellular redistribution of ventricular connexin43 during electrical uncoupling induced by ischemia. Circ. Res. 87**,** 656-662.

18. Benhabbouche,S., Crola da,S.C., Abrial,M., Ferrera,R., 2011. [The basis of ischemia-reperfusion and myocardial protection]. Ann. Fr. Anesth. Reanim. 30 Suppl 1**,** S2-16.

19. Bennett,M.V., Barrio,L.C., Bargiello,T.A., Spray,D.C., Hertzberg,E., Saez,J.C., 1991. Gap junctions: new tools, new answers, new questions. Neuron 6**,** 305-320.

20. Bennett,M.V., Contreras,J.E., Bukauskas,F.F., Saez,J.C., 2003. New roles for astrocytes: gap junction hemichannels have something to communicate. Trends Neurosci. 26**,** 610-617.

21. Berthoud,V.M., Tadros,P.N., Beyer,E.C., 2000. Connexin and gap junction degradation. Methods 20**,** 180-187.

22. Bevans,C.G., Kordel,M., Rhee,S.K., Harris,A.L., 1998. Isoform composition of connexin channels determines selectivity among second messengers and uncharged molecules. J. Biol. Chem. 273**,** 2808-2816.

23. Beyer,E.C., Paul,D.L., Goodenough,D.A., 1990. Connexin family of gap junction proteins. J. Membr. Biol. 116**,** 187-194.

24. Bhandari,A.K., Au,P.K., Rose,J.S., Kotlewski,A., Blue,S., Rahimtoola,S.H., 1987. Decline in inducibility of sustained ventricular tachycardia from two to twenty weeks after acute myocardial infarction. Am. J. Cardiol. 59**,** 284-290.

25. Blom,N., Sicheritz-Ponten,T., Gupta,R., Gammeltoft,S., Brunak,S., 2004. Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics. 4**,** 1633-1649.

26. Boengler,K., Dodoni,G., Rodriguez-Sinovas,A., Cabestrero,A., Ruiz-Meana,M., Gres,P., Konietzka,I., Lopez-Iglesias,C., Garcia-Dorado,D., Di,L.F., Heusch,G., Schulz,R., 2005. Connexin 43 in cardiomyocyte mitochondria and its increase by ischemic preconditioning. Cardiovasc. Res. 67**,** 234-244.

27. Boengler,K., Stahlhofen,S., van de Sand,A., Gres,P., Ruiz-Meana,M., Garcia-Dorado,D., Heusch,G., Schulz,R., 2009. Presence of connexin 43 in subsarcolemmal, but not in interfibrillar cardiomyocyte mitochondria. Basic Res. Cardiol. 104**,** 141-147.

28. Boger,D.L., Patterson,J.E., Guan,X., Cravatt,B.F., Lerner,R.A., Gilula,N.B., 1998. Chemical requirements for inhibition of gap junction communication by the biologically active lipid oleamide. Proc. Natl. Acad. Sci. U. S. A 95**,** 4810-4815.

29. Boneh,A., 1995. Possible role for protein kinase C in the pathogenesis of inborn errors of metabolism. J. Cell Biochem. 59**,** 27-32.

30. Bowling,N., Huang,X., Sandusky,G.E., Fouts,R.L., Mintze,K., Esterman,M., Allen,P.D., Maddi,R., McCall,E., Vlahos,C.J., 2001. Protein kinase C-alpha and -epsilon modulate connexin-43 phosphorylation in human heart. J. Mol. Cell Cardiol. 33**,** 789-798.

31. Bowling,N., Walsh,R.A., Song,G., Estridge,T., Sandusky,G.E., Fouts,R.L., Mintze,K., Pickard,T., Roden,R., Bristow,M.R., Sabbah,H.N., Mizrahi,J.L., Gromo,G., King,G.L., Vlahos,C.J., 1999. Increased protein kinase C activity and expression of Ca2+-sensitive isoforms in the failing human heart. Circulation 99**,** 384-391.

32. Braet,K., Vandamme,W., Martin,P.E., Evans,W.H., Leybaert,L., 2003. Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26. Cell Calcium 33**,** 37-48.

33. Britz-Cunningham,S.H., Shah,M.M., Zuppan,C.W., Fletcher,W.H., 1995. Mutations of the Connexin43 gap-junction gene in patients with heart malformations and defects of laterality. N. Engl. J. Med. 332**,** 1323-1329.

34. Bruzzone,S., Guida,L., Zocchi,E., Franco,L., De,F.A., 2001. Connexin 43 hemi channels mediate Ca2+-regulated transmembrane NAD+ fluxes in intact cells. FASEB J. 15**,** 10-12.

35. Budas,G.R., Churchill,E.N., Mochly-Rosen,D., 2007. Cardioprotective mechanisms of PKC isozyme-selective activators and inhibitors in the treatment of ischemia-reperfusion injury. Pharmacol. Res. 55**,** 523-536.

36. Burt,J.M., Massey,K.D., Minnich,B.N., 1991. Uncoupling of cardiac cells by fatty acids: structure-activity relationships. Am. J. Physiol 260**,** C439-C448.

37. Burt,J.M., Spray,D.C., 1989. Volatile anesthetics block intercellular communication between neonatal rat myocardial cells. Circ. Res. 65**,** 829-837.

38. Carrero,J.J., Lopez-Huertas,E., Salmeron,L.M., Baro,L., Ros,E., 2005. Daily supplementation with (n-3) PUFAs, oleic acid, folic acid, and vitamins B-6 and E increases pain-free walking distance and improves risk factors in men with peripheral vascular disease. J. Nutr. 135**,** 1393-1399.

39. Castagna,M., Takai,Y., Kaibuchi,K., Sano,K., Kikkawa,U., Nishizuka,Y., 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257**,** 7847-7851.

40. Chakrabarti,S., Hoque,A.N., Karmazyn,M., 1997. A rapid ischemia-induced apoptosis in isolated rat hearts and its attenuation by the sodium-hydrogen exchange inhibitor HOE 642 (cariporide). J. Mol. Cell Cardiol. 29**,** 3169-3174.

41. Chambers,T.C., Pohl,J., Raynor,R.L., Kuo,J.F., 1993. Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. J. Biol. Chem. 268**,** 4592-4595.

42. Chaytor,A.T., Evans,W.H., Griffith,T.M., 1997. Peptides homologous to extracellular loop motifs of connexin 43 reversibly abolish rhythmic contractile activity in rabbit arteries. J. Physiol 503 (Pt 1)**,** 99-110.

43. Chaytor,A.T., Evans,W.H., Griffith,T.M., 1998. Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. J. Physiol 508 (Pt 2)**,** 561-573.

44. Chaytor,A.T., Martin,P.E., Evans,W.H., Randall,M.D., Griffith,T.M., 1999. The endothelial component of cannabinoid-induced relaxation in rabbit mesenteric artery depends on gap junctional communication. J. Physiol 520 Pt 2**,** 539-550.

45. Chen,D.H., Brkanac,Z., Verlinde,C.L., Tan,X.J., Bylenok,L., Nochlin,D., Matsushita,M., Lipe,H., Wolff,J., Fernandez,M., Cimino,P.J., Bird,T.D., Raskind,W.H., 2003. Missense mutations in the regulatory domain of PKC gamma: a new mechanism for dominant nonepisodic cerebellar ataxia. Am. J. Hum. Genet. 72**,** 839-849.

46. Chen,L., Hahn,H., Wu,G., Chen,C.H., Liron,T., Schechtman,D., Cavallaro,G., Banci,L., Guo,Y., Bolli,R., Dorn,G.W., Mochly-Rosen,D., 2001. Opposing

cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. Proc. Natl. Acad. Sci. U. S. A 98**,** 11114-11119.

47. Christ,G.J., Spray,D.C., el-Sabban,M., Moore,L.K., Brink,P.R., 1996. Gap junctions in vascular tissues. Evaluating the role of intercellular communication in the modulation of vasomotor tone. Circ. Res. 79**,** 631-646.

48. Churchill,E.N., Mochly-Rosen,D., 2007. The roles of PKCdelta and epsilon isoenzymes in the regulation of myocardial ischaemia/reperfusion injury. Biochem. Soc. Trans. 35**,** 1040-1042.

49. Cinca,J., Blanch,P., Carreno,A., Mont,L., Garcia-Burillo,A., Soler-Soler,J., 1997. Acute ischemic ventricular arrhythmias in pigs with healed myocardial infarction: comparative effects of ischemia at a distance and ischemia at the infarct zone. Circulation 96**,** 653-658.

50. Clarke,T.C., Williams,O.J., Martin,P.E., Evans,W.H., 2009. ATP release by cardiac myocytes in a simulated ischaemia model: inhibition by a connexin mimetic and enhancement by an antiarrhythmic peptide. Eur. J. Pharmacol. 605**,** 9-14.

51. Contreras,J.E., Saez,J.C., Bukauskas,F.F., Bennett,M.V., 2003. Gating and regulation of connexin 43 (Cx43) hemichannels. Proc. Natl. Acad. Sci. U. S. A 100**,** 11388-11393.

52. Contreras,J.E., Sanchez,H.A., Eugenin,E.A., Speidel,D., Theis,M., Willecke,K., Bukauskas,F.F., Bennett,M.V., Saez,J.C., 2002. Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. Proc. Natl. Acad. Sci. U. S. A 99**,** 495-500.

53. Contreras,J.E., Sanchez,H.A., Veliz,L.P., Bukauskas,F.F., Bennett,M.V., Saez,J.C., 2004. Role of connexin-based gap junction channels and hemichannels in ischemia-induced cell death in nervous tissue. Brain Res. Brain Res. Rev. 47**,** 290-303.

54. Coronel,R., Fiolet,J.W., Wilms-Schopman,F.J., Schaapherder,A.F., Johnson,T.A., Gettes,L.S., Janse,M.J., 1988. Distribution of extracellular potassium and its relation to electrophysiologic changes during acute myocardial ischemia in the isolated perfused porcine heart. Circulation 77**,** 1125-1138.

55. Coussens,L., Rhee,L., Parker,P.J., Ullrich,A., 1987. Alternative splicing increases the diversity of the human protein kinase C family. DNA 6**,** 389-394.

56. Crompton,M., 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 341 (Pt 2)**,** 233-249.

57. Croop,J., Toyama,Y., Dlugosz,A.A., Holtzer,H., 1980. Selective effects of phorbol 12-myristate 13-acetate on myofibrils and 10-nm filaments. Proc. Natl. Acad. Sci. U. S. A 77**,** 5273-5277.

58. Cross,H.R., Murphy,E., Bolli,R., Ping,P., Steenbergen,C., 2002. Expression of activated PKC epsilon (PKC epsilon) protects the ischemic heart, without attenuating ischemic H(+) production. J. Mol. Cell Cardiol. 34**,** 361-367.

59. Dahl,G., 2007. Gap junction-mimetic peptides do work, but in unexpected ways. Cell Commun. Adhes. 14**,** 259-264.

60. Dahl,G., Nonner,W., Werner,R., 1994. Attempts to define functional domains of gap junction proteins with synthetic peptides. Biophys. J. 67**,** 1816-1822.

61. Dahl,G., Werner,R., Levine,E., Rabadan-Diehl,C., 1992. Mutational analysis of gap junction formation. Biophys. J. 62**,** 172-180.

62. Dang,X., Doble,B.W., Kardami,E., 2003. The carboxy-tail of connexin-43 localizes to the nucleus and inhibits cell growth. Mol. Cell Biochem. 242**,** 35-38.

63. De Mello,W.C., 1975. Effect of intracellular injection of calcium and strontium on cell communication in heart. J. Physiol 250**,** 231-245.

64. De Mello,W.C., 1976. Influence of the sodium pump on intercellular communication in heart fibres: effect of intracellular injection of sodium ion on electrical coupling. J. Physiol 263**,** 171-197.

65. Dekker,L.R., Fiolet,J.W., VanBavel,E., Coronel,R., Opthof,T., Spaan,J.A., Janse,M.J., 1996. Intracellular Ca2+, intercellular electrical coupling, and mechanical activity in ischemic rabbit papillary muscle. Effects of preconditioning and metabolic blockade. Circ. Res. 79**,** 237-246.

66. Delgado-Escueta,A.V., 2007. Advances in genetics of juvenile myoclonic epilepsies. Epilepsy Curr. 7**,** 61-67.

67. Dermietzel,R., Hertberg,E.L., Kessler,J.A., Spray,D.C., 1991. Gap junctions between cultured astrocytes: immunocytochemical, molecular, and electrophysiological analysis. J. Neurosci. 11**,** 1421-1432.

68. Dermietzel,R., Spray,D.C., 1993. Gap junctions in the brain: where, what type, how many and why?. Trends Neurosci. 16**,** 186-192.

69. Dhein,S., 1998. Gap junction channels in the cardiovascular system: pharmacological and physiological modulation. Trends Pharmacol. Sci. 19**,** 229-241.

70. Dhein,S., 2006. Cardiac ischemia and uncoupling: gap junctions in ischemia and infarction. Adv. Cardiol. 42**,** 198-212.

71. Di-Capua,N., Sperling,O., Zoref-Shani,E., 2003. Protein kinase C-epsilon is involved in the adenosine-activated signal transduction pathway conferring protection against ischemia-reperfusion injury in primary rat neuronal cultures. J. Neurochem. 84**,** 409-412.

72. Dillon,S.M., Allessie,M.A., Ursell,P.C., Wit,A.L., 1988. Influences of anisotropic tissue structure on reentrant circuits in the epicardial border zone of subacute canine infarcts. Circ. Res. 63**,** 182-206.

73. Doble,B.W., Ping,P., Kardami,E., 2000. The epsilon subtype of protein kinase C is required for cardiomyocyte connexin-43 phosphorylation. Circ. Res. 86**,** 293-301.

74. Dorn,G.W., Souroujon,M.C., Liron,T., Chen,C.H., Gray,M.O., Zhou,H.Z., Csukai,M., Wu,G., Lorenz,J.N., Mochly-Rosen,D., 1999. Sustained in vivo cardiac protection by a rationally designed peptide that causes epsilon protein kinase C translocation. Proc. Natl. Acad. Sci. U. S. A 96**,** 12798-12803.

75. Dosemeci,A., Dhallan,R.S., Cohen,N.M., Lederer,W.J., Rogers,T.B., 1988. Phorbol ester increases calcium current and simulates the effects of angiotensin II on cultured neonatal rat heart myocytes. Circ. Res. 62**,** 347-357.

76. Downar,E., Janse,M.J., Durrer,D., 1977. The effect of acute coronary artery occlusion on subepicardial transmembrane potentials in the intact porcine heart. Circulation 56**,** 217-224.

77. du Toit,E.F., Opie,L.H., 1992. Modulation of severity of reperfusion stunning in the isolated rat heart by agents altering calcium flux at onset of reperfusion. Circ. Res. 70**,** 960-967.

78. Duffy,H.S., Wit,A.L., 2008. Is there a role for remodeled connexins in AF? No simple answers. J. Mol. Cell Cardiol. 44**,** 4-13.

79. Duncan,J.C., Fletcher,W.H., 2002. alpha 1 Connexin (connexin43) gap junctions and activities of cAMP-dependent protein kinase and protein kinase C in developing mouse heart. Dev. Dyn. 223**,** 96-107.

80. Edwards,A.S., Faux,M.C., Scott,J.D., Newton,A.C., 1999. Carboxyl-terminal phosphorylation regulates the function and subcellular localization of protein kinase C betaII. J. Biol. Chem. 274**,** 6461-6468.

81. Ek,J.F., Delmar,M., Perzova,R., Taffet,S.M., 1994. Role of histidine 95 on pH gating of the cardiac gap junction protein connexin43. Circ. Res. 74**,** 1058-1064.

82. Ek-Vitorin,J.F., King,T.J., Heyman,N.S., Lampe,P.D., Burt,J.M., 2006. Selectivity of connexin 43 channels is regulated through protein kinase C-dependent phosphorylation. Circ. Res. 98**,** 1498-1505.

83. Elfgang,C., Eckert,R., Lichtenberg-Frate,H., Butterweck,A., Traub,O., Klein,R.A., Hulser,D.F., Willecke,K., 1995. Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. J. Cell Biol. 129**,** 805-817.

84. Elvira,M., Diez,J.A., Wang,K.K., Villalobo,A., 1993. Phosphorylation of connexin-32 by protein kinase C prevents its proteolysis by mu-calpain and m-calpain. J. Biol. Chem. 268**,** 14294-14300.

85. Evans,W.H., Boitano,S., 2001. Connexin mimetic peptides: specific inhibitors of gap-junctional intercellular communication. Biochem. Soc. Trans. 29**,** 606-612.

86. Evans,W.H., De,V.E., Leybaert,L., 2006. The gap junction cellular internet: connexin hemichannels enter the signalling limelight. Biochem. J. 397**,** 1-14.

87. Feuvray,D., 1981. Structural, functional, and metabolic correlates in ischemic hearts: effects of substrates. Am. J. Physiol 240**,** H391-H398.

88. Figueroa,X.F., Isakson,B.E., Duling,B.R., 2006. Vascular gap junctions in hypertension. Hypertension 48**,** 804-811.

89. Firouzi,M., Ramanna,H., Kok,B., Jongsma,H.J., Koeleman,B.P., Doevendans,P.A., Groenewegen,W.A., Hauer,R.N., 2004. Association of human connexin40 gene polymorphisms with atrial vulnerability as a risk factor for idiopathic atrial fibrillation. Circ. Res. 95**,** e29-e33.

90. Gairhe,S., Bauer,N.N., Gebb,S.A., McMurtry,I.F., 2011. Myoendothelial gap junctional signaling induces differentiation of pulmonary arterial smooth muscle cells. Am. J. Physiol Lung Cell Mol. Physiol 301**,** L527-L535.

91. Garcia-Dorado,D., Inserte,J., Ruiz-Meana,M., Gonzalez,M.A., Solares,J., Julia,M., Barrabes,J.A., Soler-Soler,J., 1997. Gap junction uncoupler heptanol prevents cell-to-cell progression of hypercontracture and limits necrosis during myocardial reperfusion. Circulation 96**,** 3579-3586.

92. Garcia-Dorado,D., Rodriguez-Sinovas,A., Ruiz-Meana,M., 2004. Gap junction-mediated spread of cell injury and death during myocardial ischemia-reperfusion. Cardiovasc. Res. 61**,** 386-401.

93. Garcia-Dorado,D., Theroux,P., Desco,M., Solares,J., Elizaga,J., Fernandez-Aviles,F., Alonso,J., Soriano,J., 1989. Cell-to-cell interaction: a mechanism to explain wave-front progression of myocardial necrosis. Am. J. Physiol 256**,** H1266-H1273.

94. Geraldes,P., King,G.L., 2010. Activation of protein kinase C isoforms and its impact on diabetic complications. Circ. Res. 106**,** 1319-1331.

95. Gettes,L.S., Maruyama,T., 1992. [Metabolic regulation of the cardiac gap junctional current flow--electrophysiological and magnetic resonance studies]. Jpn. Circ. J. 56 Suppl 5**,** 1334-1338.

96. Goldberg,G.S., Lampe,P.D., Nicholson,B.J., 1999. Selective transfer of endogenous metabolites through gap junctions composed of different connexins. Nat. Cell Biol. 1**,** 457-459.

97. Goldberg,G.S., Moreno,A.P., Bechberger,J.F., Hearn,S.S., Shivers,R.R., MacPhee,D.J., Zhang,Y.C., Naus,C.C., 1996. Evidence that disruption of connexon particle arrangements in gap junction plaques is associated with inhibition of gap junctional communication by a glycyrrhetinic acid derivative. Exp. Cell Res. 222**,** 48-53.

98. Goldberg,G.S., Moreno,A.P., Lampe,P.D., 2002. Gap junctions between cells expressing connexin 43 or 32 show inverse permselectivity to adenosine and ATP. J. Biol. Chem. 277**,** 36725-36730.

99. Goldberg,G.S., Valiunas,V., Brink,P.R., 2004. Selective permeability of gap junction channels. Biochim. Biophys. Acta 1662**,** 96-101.

100. Gollob,M.H., 2006. Cardiac connexins as candidate genes for idiopathic atrial fibrillation. Curr. Opin. Cardiol. 21**,** 155-158.

101. Gomes,P., Srinivas,S.P., Van,D.W., Vereecke,J., Himpens,B., 2005. ATP release through connexin hemichannels in corneal endothelial cells. Invest Ophthalmol. Vis. Sci. 46**,** 1208-1218.

102. Goodenough,D.A., Goliger,J.A., Paul,D.L., 1996. Connexins, connexons, and intercellular communication. Annu. Rev. Biochem. 65**,** 475-502.

103. Goodenough,D.A., Paul,D.L., 2003. Beyond the gap: functions of unpaired connexon channels. Nat. Rev. Mol. Cell Biol. 4**,** 285-294.

104. Gourdie,R.G., Severs,N.J., Green,C.R., Rothery,S., Germroth,P., Thompson,R.P., 1993. The spatial distribution and relative abundance of gap-junctional connexin40 and connexin43 correlate to functional properties of

components of the cardiac atrioventricular conduction system. J. Cell Sci. 105 (Pt 4), 985-991.

105. Gray,M.O., Karliner,J.S., Mochly-Rosen,D., 1997. A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. J. Biol. Chem. 272, 30945-30951.

106. Green,C.R., Severs,N.J., 1993. Robert Feulgen Prize Lecture. Distribution and role of gap junctions in normal myocardium and human ischaemic heart disease. Histochemistry 99, 105-120.

107. Griner,E.M., Kazanietz,M.G., 2007. Protein kinase C and other diacylglycerol effectors in cancer. Nat. Rev. Cancer 7, 281-294.

108. Gutstein,D.E., Morley,G.E., Tamaddon,H., Vaidya,D., Schneider,M.D., Chen,J., Chien,K.R., Stuhlmann,H., Fishman,G.I., 2001. Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. Circ. Res. 88, 333-339.

109. Haddock,R.E., Grayson,T.H., Brackenbury,T.D., Meaney,K.R., Neylon,C.B., Sandow,S.L., Hill,C.E., 2006. Endothelial coordination of cerebral vasomotion via myoendothelial gap junctions containing connexins 37 and 40. Am. J. Physiol Heart Circ. Physiol 291, H2047-H2056.

110. Haefliger,J.A., Nicod,P., Meda,P., 2004. Contribution of connexins to the function of the vascular wall. Cardiovasc. Res. 62, 345-356.

111. Halestrap,A.P., 1999. The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. Biochem. Soc. Symp. 66, 181-203.

112. Halestrap,A.P., McStay,G.P., Clarke,S.J., 2002. The permeability transition pore complex: another view. Biochimie 84, 153-166.

113. Hanna,E.B., Glancy,D.L., 2011. ST-segment depression and T-wave inversion: classification, differential diagnosis, and caveats. Cleve. Clin. J. Med. 78**,** 404-414.

114. Harris,A., 2000. Communication gaps. Br. Dent. J. 189**,** 582.

115. Hatala,R., Savard,P., Tremblay,G., Page,P., Cardinal,R., Molin,F., Kus,T., Nadeau,R., 1995. Three distinct patterns of ventricular activation in infarcted human hearts. An intraoperative cardiac mapping study during sinus rhythm. Circulation 91**,** 1480-1494.

116. Hausenloy,D., Wynne,A., Duchen,M., Yellon,D., 2004. Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. Circulation 109**,** 1714-1717.

117. Hawat,G., Benderdour,M., Rousseau,G., Baroudi,G., 2010. Connexin 43 mimetic peptide Gap26 confers protection to intact heart against myocardial ischemia injury. Pflugers Arch. 460**,** 583-592.

118. Heinzel,F.R., Luo,Y., Li,X., Boengler,K., Buechert,A., Garcia-Dorado,D., Di,L.F., Schulz,R., Heusch,G., 2005. Impairment of diazoxide-induced formation of reactive oxygen species and loss of cardioprotection in connexin 43 deficient mice. Circ. Res. 97**,** 583-586.

119. Hertlein,B., Butterweck,A., Haubrich,S., Willecke,K., Traub,O., 1998. Phosphorylated carboxy terminal serine residues stabilize the mouse gap junction protein connexin45 against degradation. J. Membr. Biol. 162**,** 247-257.

120. Hertzberg,E.L., Disher,R.M., Tiller,A.A., Zhou,Y., Cook,R.G., 1988. Topology of the Mr 27,000 liver gap junction protein. Cytoplasmic localization of amino- and carboxyl termini and a hydrophilic domain which is protease-hypersensitive. J. Biol. Chem. 263**,** 19105-19111.

121. Hill,C.E., Phillips,J.K., Sandow,S.L., 2001. Heterogeneous control of blood flow amongst different vascular beds. Med. Res. Rev. 21**,** 1-60.

122. Hill,C.E., Rummery,N., Hickey,H., Sandow,S.L., 2002. Heterogeneity in the distribution of vascular gap junctions and connexins: implications for function. Clin. Exp. Pharmacol. Physiol 29**,** 620-625.

123. Hill,J.L., Gettes,L.S., 1980. Effect of acute coronary artery occlusion on local myocardial extracellular K+ activity in swine. Circulation 61**,** 768-778.

124. Hu,K., Mochly-Rosen,D., Boutjdir,M., 2000. Evidence for functional role of epsilonPKC isozyme in the regulation of cardiac Ca(2+) channels. Am. J. Physiol Heart Circ. Physiol 279**,** H2658-H2664.

125. Huang,X.D., Sandusky,G.E., Zipes,D.P., 1999. Heterogeneous loss of connexin43 protein in ischemic dog hearts. J. Cardiovasc. Electrophysiol. 10**,** 79-91.

126. Hug,H., Sarre,T.F., 1993. Protein kinase C isoenzymes: divergence in signal transduction?. Biochem. J. 291 (Pt 2)**,** 329-343.

127. Hurley,J.H., Grobler,J.A., 1997. Protein kinase C and phospholipase C: bilayer interactions and regulation. Curr. Opin. Struct. Biol. 7**,** 557-565.

128. Inagaki,K., Chen,L., Ikeno,F., Lee,F.H., Imahashi,K., Bouley,D.M., Rezaee,M., Yock,P.G., Murphy,E., Mochly-Rosen,D., 2003a. Inhibition of delta-protein kinase C protects against reperfusion injury of the ischemic heart in vivo. Circulation 108**,** 2304-2307.

129. Inagaki,K., Hahn,H.S., Dorn,G.W., Mochly-Rosen,D., 2003b. Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. Circulation 108**,** 869-875.

130. Inagaki,K., Mochly-Rosen,D., 2005. DeltaPKC-mediated activation of epsilonPKC in ethanol-induced cardiac protection from ischemia. J. Mol. Cell Cardiol. 39**,** 203-211.

131. Jaburek,M., Costa,A.D., Burton,J.R., Costa,C.L., Garlid,K.D., 2006a. Mitochondrial PKC epsilon and mitochondrial ATP-sensitive K+ channel copurify and coreconstitute to form a functioning signaling module in proteoliposomes. Circ. Res. 99**,** 878-883.

132. Jaburek,M., Costa,A.D., Burton,J.R., Costa,C.L., Garlid,K.D., 2006b. Mitochondrial PKC epsilon and mitochondrial ATP-sensitive K+ channel copurify and coreconstitute to form a functioning signaling module in proteoliposomes. Circ. Res. 99**,** 878-883.

133. Jain,S.K., Schuessler,R.B., Saffitz,J.E., 2003. Mechanisms of delayed electrical uncoupling induced by ischemic preconditioning. Circ. Res. 92**,** 1138-1144.

134. Janse,M.J., Wit,A.L., 1989. Electrophysiological mechanisms of ventricular arrhythmias resulting from myocardial ischemia and infarction. Physiol Rev. 69**,** 1049-1169.

135. Jennings,R.B., Reimer,K.A., Jones,R.N., Peyton,R.B., 1983. High energy phosphates, anaerobic glycolysis and irreversibility in ischemia. Adv. Exp. Med. Biol. 161**,** 403-419.

136. Jennings,R.B., SOMMERS,H.M., SMYTH,G.A., FLACK,H.A., LINN,H., 1960. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. Arch. Pathol. 70**,** 68-78.

137. Jeyaraman,M., Tanguy,S., Fandrich,R.R., Lukas,A., Kardami,E., 2003. Ischemia-induced dephosphorylation of cardiomyocyte connexin-43 is reduced by okadaic acid and calyculin A but not fostriecin. Mol. Cell Biochem. 242**,** 129-134.

138. Johannes,F.J., Prestle,J., Eis,S., Oberhagemann,P., Pfizenmaier,K., 1994. PKCu is a novel, atypical member of the protein kinase C family. J. Biol. Chem. 269**,** 6140-6148.

139. John,S.A., Kondo,R., Wang,S.Y., Goldhaber,J.I., Weiss,J.N., 1999. Connexin-43 hemichannels opened by metabolic inhibition. J. Biol. Chem. 274**,** 236-240.

140. Johnson,R.G., Sheridan,J.D., 1971. Junctions between cancer cells in culture: ultrastructure and permeability. Science 174**,** 717-719.

141. Jozwiak,J., Dhein,S., 2008. Local effects and mechanisms of antiarrhythmic peptide AAP10 in acute regional myocardial ischemia: electrophysiological and molecular findings. Naunyn Schmiedebergs Arch. Pharmacol. 378**,** 459-470.

142. Kanczuga-Koda,L., 2004. [Gap junctions and their role in physiology and pathology of the digestive tract]. Postepy Hig. Med. Dosw. (Online.) 58**,** 158-165.

143. KANNO,Y., LOEWENSTEIN,W.R., 1964. INTERCELLULAR DIFFUSION. Science 143**,** 959-960.

144. KANNO,Y., Loewenstein,W.R., 1966. Cell-to-cell passage of large molecules. Nature 212**,** 629-630.

145. Kelley,P.M., Abe,S., Askew,J.W., Smith,S.D., Usami,S., Kimberling,W.J., 1999. Human connexin 30 (GJB6), a candidate gene for nonsyndromic hearing loss: molecular cloning, tissue-specific expression, and assignment to chromosome 13q12. Genomics 62**,** 172-176.

146. Kelsell,D.P., Di,W.L., Houseman,M.J., 2001. Connexin mutations in skin disease and hearing loss. Am. J. Hum. Genet. 68**,** 559-568.

147. Kemp,B.E., Pearson,R.B., 1990. Protein kinase recognition sequence motifs. Trends Biochem. Sci. 15**,** 342-346.

148. Kida,M., Fujiwara,H., Ishida,M., Kawai,C., Ohura,M., Miura,I., Yabuuchi,Y., 1991. Ischemic preconditioning preserves creatine phosphate and intracellular pH. Circulation 84**,** 2495-2503.

149. Kim,M.Y., Kim,M.J., Yoon,I.S., Ahn,J.H., Lee,S.H., Baik,E.J., Moon,C.H., Jung,Y.S., 2006. Diazoxide acts more as a PKC-epsilon activator, and indirectly activates the mitochondrial K(ATP) channel conferring cardioprotection against hypoxic injury. Br. J. Pharmacol. 149**,** 1059-1070.

150. Kleber,A.G., 1983. Resting membrane potential, extracellular potassium activity, and intracellular sodium activity during acute global ischemia in isolated perfused guinea pig hearts. Circ. Res. 52**,** 442-450.

151. Kleber,A.G., Janse,M.J., van Capelle,F.J., Durrer,D., 1978. Mechanism and time course of S-T and T-Q segment changes during acute regional myocardial ischemia in the pig heart determined by extracellular and intracellular recordings. Circ. Res. 42**,** 603-613.

152. Kleber,A.G., Riegger,C.B., Janse,M.J., 1987. Electrical uncoupling and increase of extracellular resistance after induction of ischemia in isolated, arterially perfused rabbit papillary muscle. Circ. Res. 61**,** 271-279.

153. Knopf,J.L., Lee,M.H., Sultzman,L.A., Kriz,R.W., Loomis,C.R., Hewick,R.M., Bell,R.M., 1986. Cloning and expression of multiple protein kinase C cDNAs. Cell 46**,** 491-502.

154. Kondo,R.P., Wang,S.Y., John,S.A., Weiss,J.N., Goldhaber,J.I., 2000. Metabolic inhibition activates a non-selective current through connexin hemichannels in isolated ventricular myocytes. J. Mol. Cell Cardiol. 32**,** 1859-1872.

155. Koretsune,Y., Marban,E., 1990. Mechanism of ischemic contracture in ferret hearts: relative roles of [Ca2+]i elevation and ATP depletion. Am. J. Physiol 258**,** H9-16.

156. Korge,P., Honda,H.M., Weiss,J.N., 2002. Protection of cardiac mitochondria by diazoxide and protein kinase C: implications for ischemic preconditioning. Proc. Natl. Acad. Sci. U. S. A 99**,** 3312-3317.

157. Kumar,N.M., Gilula,N.B., 1996. The gap junction communication channel. Cell 84**,** 381-388.

158. Kuzuya,T., Hoshida,S., Yamashita,N., Fuji,H., Oe,H., Hori,M., Kamada,T., Tada,M., 1993. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. Circ. Res. 72**,** 1293-1299.

159. Kwak,B.R., Jongsma,H.J., 1996. Regulation of cardiac gap junction channel permeability and conductance by several phosphorylating conditions. Mol. Cell Biochem. 157**,** 93-99.

160. Kwak,B.R., Jongsma,H.J., 1999. Selective inhibition of gap junction channel activity by synthetic peptides. J. Physiol 516 (Pt 3)**,** 679-685.

161. Kwak,B.R., Mulhaupt,F., Veillard,N., Gros,D.B., Mach,F., 2002. Altered pattern of vascular connexin expression in atherosclerotic plaques. Arterioscler. Thromb. Vasc. Biol. 22**,** 225-230.

162. Kwak,B.R., Veillard,N., Pelli,G., Mulhaupt,F., James,R.W., Chanson,M., Mach,F., 2003. Reduced connexin43 expression inhibits atherosclerotic lesion formation in low-density lipoprotein receptor-deficient mice. Circulation 107**,** 1033-1039.

163. Lacerda,A.E., Rampe,D., Brown,A.M., 1988. Effects of protein kinase C activators on cardiac Ca2+ channels. Nature 335**,** 249-251.

164. Laird,D.W., Fistouris,P., Batist,G., Alpert,L., Huynh,H.T., Carystinos,G.D., Alaoui-Jamali,M.A., 1999. Deficiency of connexin43 gap junctions is an independent marker for breast tumors. Cancer Res. 59**,** 4104-4110.

165. Lampe,P.D., 1994. Analyzing phorbol ester effects on gap junctional communication: a dramatic inhibition of assembly. J. Cell Biol. 127**,** 1895-1905.

166. Lampe,P.D., Cooper,C.D., King,T.J., Burt,J.M., 2006. Analysis of Connexin43 phosphorylated at S325, S328 and S330 in normoxic and ischemic heart. J. Cell Sci. 119**,** 3435-3442.

167. Lampe,P.D., Lau,A.F., 2000. Regulation of gap junctions by phosphorylation of connexins. Arch. Biochem. Biophys. 384**,** 205-215.

168. Lavado,E., Sanchez-Abarca,L.I., Tabernero,A., Bolanos,J.P., Medina,J.M., 1997. Oleic acid inhibits gap junction permeability and increases glucose uptake in cultured rat astrocytes. J. Neurochem. 69**,** 721-728.

169. Lawrence,T.S., Beers,W.H., Gilula,N.B., 1978. Transmission of hormonal stimulation by cell-to-cell communication. Nature 272**,** 501-506.

170. Leatherman,G.F., Kim,D., Smith,T.W., 1987. Effect of phorbol esters on contractile state and calcium flux in cultured chick heart cells. Am. J. Physiol 253**,** H205-H209.

171. Leybaert,L., Braet,K., Vandamme,W., Cabooter,L., Martin,P.E., Evans,W.H., 2003. Connexin channels, connexin mimetic peptides and ATP release. Cell Commun. Adhes. 10**,** 251-257.

172. Li,F., Sugishita,K., Su,Z., Ueda,I., Barry,W.H., 2001. Activation of connexin-43 hemichannels can elevate [Ca(2+)]i and [Na(+)]i in rabbit ventricular myocytes during metabolic inhibition. J. Mol. Cell Cardiol. 33**,** 2145-2155.

173. Li,G., Whittaker,P., Yao,M., Kloner,R.A., Przyklenk,K., 2002. The gap junction uncoupler heptanol abrogates infarct size reduction with preconditioning in mouse hearts. Cardiovasc. Pathol. 11**,** 158-165.

174. Li,H., Liu,T.F., Lazrak,A., Peracchia,C., Goldberg,G.S., Lampe,P.D., Johnson,R.G., 1996. Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells. J. Cell Biol. 134**,** 1019-1030.

175. Li,W.E., Nagy,J.I., 2000. Connexin43 phosphorylation state and intercellular communication in cultured astrocytes following hypoxia and protein phosphatase inhibition. Eur. J. Neurosci. 12**,** 2644-2650.

176. Li,X., Heinzel,F.R., Boengler,K., Schulz,R., Heusch,G., 2004. Role of connexin 43 in ischemic preconditioning does not involve intercellular communication through gap junctions. J. Mol. Cell Cardiol. 36**,** 161-163.

177. Liang,B.T., 1998. Protein kinase C-dependent activation of KATP channel enhances adenosine-induced cardioprotection. Biochem. J. 336 (Pt 2)**,** 337-343.

178. Lima,F., Niger,C., Hebert,C., Stains,J.P., 2009. Connexin43 potentiates osteoblast responsiveness to fibroblast growth factor 2 via a protein kinase C-delta/Runx2-dependent mechanism. Mol. Biol. Cell 20**,** 2697-2708.

179. Liu,G.S., Cohen,M.V., Mochly-Rosen,D., Downey,J.M., 1999. Protein kinase C-epsilon is responsible for the protection of preconditioning in rabbit cardiomyocytes. J. Mol. Cell Cardiol. 31**,** 1937-1948.

180. Liu,H., McPherson,B.C., Yao,Z., 2001. Preconditioning attenuates apoptosis and necrosis: role of protein kinase C epsilon and -delta isoforms. Am. J. Physiol Heart Circ. Physiol 281**,** H404-H410.

181. Lo,W.K., Shaw,A.P., Takemoto,L.J., Grossniklaus,H.E., Tigges,M., 1996. Gap junction structures and distribution patterns of immunoreactive connexins 46 and 50 in lens regrowths of Rhesus monkeys. Exp. Eye Res. 62**,** 171-180.

182. Lochner,A., Genade,S., Tromp,E., Podzuweit,T., Moolman,J.A., 1999. Ischemic preconditioning and the beta-adrenergic signal transduction pathway. Circulation 100**,** 958-966.

183. Lochner,A., Marais,E., Genade,S., Huisamen,B., du Toit,E.F., Moolman,J.A., 2009. Protection of the ischaemic heart: investigations into the phenomenon of ischaemic preconditioning. Cardiovasc. J. Afr. 20**,** 43-51.

184. Loubani,M., Galinanes,M., 2002. Long-term administration of nicorandil abolishes ischemic and pharmacologic preconditioning of the human myocardium: role of mitochondrial adenosine triphosphate-dependent potassium channels. J. Thorac. Cardiovasc. Surg. 124**,** 750-757.

185. Luke,R.A., Saffitz,J.E., 1991. Remodeling of ventricular conduction pathways in healed canine infarct border zones. J. Clin. Invest 87**,** 1594-1602.

186. Maestrini,E., Korge,B.P., Ocana-Sierra,J., Calzolari,E., Cambiaghi,S., Scudder,P.M., Hovnanian,A., Monaco,A.P., Munro,C.S., 1999. A missense mutation in connexin26, D66H, causes mutilating keratoderma with sensorineural deafness (Vohwinkel's syndrome) in three unrelated families. Hum. Mol. Genet. 8**,** 1237-1243.

187. Manjunath,C.K., Page,E., 1986. Rat heart gap junctions as disulfide-bonded connexon multimers: their depolymerization and solubilization in deoxycholate. J. Membr. Biol. 90**,** 43-57.

188. Matsushita,S., Kurihara,H., Watanabe,M., Okada,T., Sakai,T., Amano,A., 2006. Alterations of phosphorylation state of connexin 43 during hypoxia and

reoxygenation are associated with cardiac function. J. Histochem. Cytochem. 54**,** 343-353.

189. Matsushita,T., Oyamada,M., Fujimoto,K., Yasuda,Y., Masuda,S., Wada,Y., Oka,T., Takamatsu,T., 1999. Remodeling of cell-cell and cell-extracellular matrix interactions at the border zone of rat myocardial infarcts. Circ. Res. 85**,** 1046-1055.

190. Maurer,P., Weingart,R., 1987. Cell pairs isolated from adult guinea pig and rat hearts: effects of [Ca2+]i on nexal membrane resistance. Pflugers Arch. 409**,** 394-402.

191. McCallister,L.P., Trapukdi,S., Neely,J.R., 1979. Morphometric observations on the effects of ischemia in the isolated perfused rat heart. J. Mol. Cell Cardiol. 11**,** 619-630.

192. Mellor,H., Parker,P.J., 1998. The extended protein kinase C superfamily. Biochem. J. 332 (Pt 2)**,** 281-292.

193. Meng,H., Pierce,G.N., 1991. Involvement of sodium in the protective effect of 5-(N,N-dimethyl)-amiloride on ischemia-reperfusion injury in isolated rat ventricular wall. J. Pharmacol. Exp. Ther. 256**,** 1094-1100.

194. Michaud,M.R., Denlinger,D.L., 2006. Oleic acid is elevated in cell membranes during rapid cold-hardening and pupal diapause in the flesh fly, Sarcophaga crassipalpis J. Insect Physiol 52**,** 1073-1082.

195. Milks,L.C., Kumar,N.M., Houghten,R., Unwin,N., Gilula,N.B., 1988. Topology of the 32-kd liver gap junction protein determined by site-directed antibody localizations. EMBO J. 7**,** 2967-2975.

196. Miura,T., Ohnuma,Y., Kuno,A., Tanno,M., Ichikawa,Y., Nakamura,Y., Yano,T., Miki,T., Sakamoto,J., Shimamoto,K., 2004. Protective role of gap junctions in

preconditioning against myocardial infarction. Am. J. Physiol Heart Circ. Physiol 286**,** H214-H221.

197. Miura,T., Yano,T., Naitoh,K., Nishihara,M., Miki,T., Tanno,M., Shimamoto,K., 2007. Delta-opioid receptor activation before ischemia reduces gap junction permeability in ischemic myocardium by PKC-epsilon-mediated phosphorylation of connexin 43. Am. J. Physiol Heart Circ. Physiol 293**,** H1425-H1431.

198. Miyawaki,H., Zhou,X., Ashraf,M., 1996. Calcium preconditioning elicits strong protection against ischemic injury via protein kinase C signaling pathway. Circ. Res. 79**,** 137-146.

199. Mochly-Rosen,D., 1995. Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science 268**,** 247-251.

200. Moreno,A.P., Lau,A.F., 2007. Gap junction channel gating modulated through protein phosphorylation. Prog. Biophys. Mol. Biol. 94**,** 107-119.

201. Mubagwa,K., Flameng,W., 2001. Adenosine, adenosine receptors and myocardial protection: an updated overview. Cardiovasc. Res. 52**,** 25-39.

202. Murry,C.E., Jennings,R.B., Reimer,K.A., 1986. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74**,** 1124-1136.

203. Musil,L.S., Goodenough,D.A., 1991. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. J. Cell Biol. 115**,** 1357-1374.

204. Myerburg,R.J., Epstein,K., Gaide,M.S., Wong,S.S., Castellanos,A., Gelband,H., Bassett,A.L., 1982. Electrophysiologic consequences of experimental acute ischemia superimposed on healed myocardial infarction in cats. Am. J. Cardiol. 49**,** 323-330.

205. Nakaya,H., Miki,T., Seino,S., Yamada,K., Inagaki,N., Suzuki,M., Sato,T., Yamada,M., Matsushita,K., Kurachi,Y., Arita,M., 2003. [Molecular and functional diversity of ATP-sensitive K+ channels: the pathophysiological roles and potential drug targets]. Nippon Yakurigaku Zasshi 122**,** 243-250.

206. Nayler,W.G., 1981. The role of calcium in the ischemic myocardium. Am. J. Pathol. 102**,** 262-270.

207. Neely,J.R., 1989. Metabolic disturbances after coronary occlusion. Hosp. Pract. (Off Ed) 24**,** 81-86.

208. Nguyen-Thi,A., Ruiz-Ceretti,E., Schanne,O.F., 1981. Electrophysiologic effects and electrolyte changes in total myocardial ischemia. Can. J. Physiol Pharmacol. 59**,** 876-883.

209. Nicholson,B.J., Weber,P.A., Cao,F., Chang,H., Lampe,P., Goldberg,G., 2000. The molecular basis of selective permeability of connexins is complex and includes both size and charge. Braz. J. Med. Biol. Res. 33**,** 369-378.

210. Nishizuka,Y., 1988. The heterogeneity and differential expression of multiple species of the protein kinase C family. Biofactors 1**,** 17-20.

211. Nishizuka,Y., 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. 9**,** 484-496.

212. Noma,A., Tsuboi,N., 1987. Dependence of junctional conductance on proton, calcium and magnesium ions in cardiac paired cells of guinea-pig. J. Physiol 382**,** 193-211.

213. O'Carroll,S.J., Alkadhi,M., Nicholson,L.F., Green,C.R., 2008. Connexin 43 mimetic peptides reduce swelling, astrogliosis, and neuronal cell death after spinal cord injury. Cell Commun. Adhes. 15**,** 27-42.

214. Ohno,S., Akita,Y., Konno,Y., Imajoh,S., Suzuki,K., 1988. A novel phorbol ester receptor/protein kinase, nPKC, distantly related to the protein kinase C family. Cell 53**,** 731-741.

215. Omori,Y., Mesnil,M., Yamasaki,H., 1996. Connexin 32 mutations from X-linked Charcot-Marie-Tooth disease patients: functional defects and dominant negative effects. Mol. Biol. Cell 7**,** 907-916.

216. Ono,Y., Fujii,T., Ogita,K., Kikkawa,U., Igarashi,K., Nishizuka,Y., 1988. The structure, expression, and properties of additional members of the protein kinase C family. J. Biol. Chem. 263**,** 6927-6932.

217. Opie,L.H., 1991. Role of calcium and other ions in reperfusion injury. Cardiovasc. Drugs Ther. 5 Suppl 2**,** 237-247.

218. Osada,S., Mizuno,K., Saido,T.C., Suzuki,K., Kuroki,T., Ohno,S., 1992. A new member of the protein kinase C family, nPKC theta, predominantly expressed in skeletal muscle. Mol. Cell Biol. 12**,** 3930-3938.

219. Padilla,F., Garcia-Dorado,D., Rodriguez-Sinovas,A., Ruiz-Meana,M., Inserte,J., Soler-Soler,J., 2003. Protection afforded by ischemic preconditioning is not mediated by effects on cell-to-cell electrical coupling during myocardial ischemia-reperfusion. Am. J. Physiol Heart Circ. Physiol 285**,** H1909-H1916.

220. Pappa,H., Dekker,L.V., Parker,P.J., McDonald,N.Q., 1998. Preliminary X-ray analysis of a C2-like domain from protein kinase C-delta. Acta Crystallogr. D. Biol. Crystallogr. 54**,** 693-696.

221. Parker,P.J., Murray-Rust,J., 2004. PKC at a glance. J. Cell Sci. 117**,** 131-132.

222. Paul,D.L., Ebihara,L., Takemoto,L.J., Swenson,K.I., Goodenough,D.A., 1991. Connexin46, a novel lens gap junction protein, induces voltage-gated currents in

nonjunctional plasma membrane of Xenopus oocytes. J. Cell Biol. 115**,** 1077-1089.

223. Pearson,R.A., Dale,N., Llaudet,E., Mobbs,P., 2005. ATP released via gap junction hemichannels from the pigment epithelium regulates neural retinal progenitor proliferation. Neuron 46**,** 731-744.

224. Peters,N.S., Green,C.R., Poole-Wilson,P.A., Severs,N.J., 1993. Reduced content of connexin43 gap junctions in ventricular myocardium from hypertrophied and ischemic human hearts. Circulation 88**,** 864-875.

225. Pfahnl,A., Dahl,G., 1999. Gating of cx46 gap junction hemichannels by calcium and voltage. Pflugers Arch. 437**,** 345-353.

226. Pierce,G.N., Czubryt,M.P., 1995. The contribution of ionic imbalance to ischemia/reperfusion-induced injury. J. Mol. Cell Cardiol. 27**,** 53-63.

227. Pierce,G.N., Philipson,K.D., 1985. Na+-H+ exchange in cardiac sarcolemmal vesicles. Biochim. Biophys. Acta 818**,** 109-116.

228. Ping,P., Song,C., Zhang,J., Guo,Y., Cao,X., Li,R.C., Wu,W., Vondriska,T.M., Pass,J.M., Tang,X.L., Pierce,W.M., Bolli,R., 2002. Formation of protein kinase C(epsilon)-Lck signaling modules confers cardioprotection. J. Clin. Invest 109**,** 499-507.

229. Pitts,J.D., Burk,R.R., 1987. Mechanism of inhibition of junctional communication between animal cells by phorbol ester. Cell Tissue Kinet. 20**,** 145-151.

230. Plotkin,L.I., Lezcano,V., Thostenson,J., Weinstein,R.S., Manolagas,S.C., Bellido,T., 2008. Connexin 43 is required for the anti-apoptotic effect of bisphosphonates on osteocytes and osteoblasts in vivo. J. Bone Miner. Res. 23**,** 1712-1721.

231. Plotkin,L.I., Manolagas,S.C., Bellido,T., 2002. Transduction of cell survival signals by connexin-43 hemichannels. J. Biol. Chem. 277**,** 8648-8657.

232. Plotkin,L.I., Manolagas,S.C., Bellido,T., 2006. Dissociation of the pro-apoptotic effects of bisphosphonates on osteoclasts from their anti-apoptotic effects on osteoblasts/osteocytes with novel analogs. Bone 39**,** 443-452.

233. Pogwizd,S.M., Schlotthauer,K., Li,L., Yuan,W., Bers,D.M., 2001. Arrhythmogenesis and contractile dysfunction in heart failure: Roles of sodium-calcium exchange, inward rectifier potassium current, and residual beta-adrenergic responsiveness. Circ. Res. 88**,** 1159-1167.

234. Pravdic,D., Sedlic,F., Mio,Y., Vladic,N., Bienengraeber,M., Bosnjak,Z.J., 2009. Anesthetic-induced preconditioning delays opening of mitochondrial permeability transition pore via protein Kinase C-epsilon-mediated pathway. Anesthesiology 111**,** 267-274.

235. Puceat,M., Hilal-Dandan,R., Strulovici,B., Brunton,L.L., Brown,J.H., 1994. Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. J. Biol. Chem. 269**,** 16938-16944.

236. Quist,A.P., Rhee,S.K., Lin,H., Lal,R., 2000. Physiological role of gap-junctional hemichannels. Extracellular calcium-dependent isosmotic volume regulation. J. Cell Biol. 148**,** 1063-1074.

237. Ramachandran,S., Xie,L.H., John,S.A., Subramaniam,S., Lal,R., 2007. A novel role for connexin hemichannel in oxidative stress and smoking-induced cell injury. PLoS. One. 2**,** e712.

238. Reaume,A.G., de Sousa,P.A., Kulkarni,S., Langille,B.L., Zhu,D., Davies,T.C., Juneja,S.C., Kidder,G.M., Rossant,J., 1995. Cardiac malformation in neonatal mice lacking connexin43. Science 267**,** 1831-1834.

239. Reimer,K.A., Lowe,J.E., Rasmussen,M.M., Jennings,R.B., 1977. The wavefront phenomenon of ischemic cell death. 1. Myocardial infarct size vs duration of coronary occlusion in dogs. Circulation 56**,** 786-794.

240. Retamal,M.A., Cortes,C.J., Reuss,L., Bennett,M.V., Saez,J.C., 2006. S-nitrosylation and permeation through connexin 43 hemichannels in astrocytes: induction by oxidant stress and reversal by reducing agents. Proc. Natl. Acad. Sci. U. S. A 103**,** 4475-4480.

241. Retamal,M.A., Schalper,K.A., Shoji,K.F., Bennett,M.V., Saez,J.C., 2007. Opening of connexin 43 hemichannels is increased by lowering intracellular redox potential. Proc. Natl. Acad. Sci. U. S. A 104**,** 8322-8327.

242. Richard,G., 2000. Connexins: a connection with the skin. Exp. Dermatol. 9**,** 77-96.

243. Richard,G., White,T.W., Smith,L.E., Bailey,R.A., Compton,J.G., Paul,D.L., Bale,S.J., 1998. Functional defects of Cx26 resulting from a heterozygous missense mutation in a family with dominant deaf-mutism and palmoplantar keratoderma. Hum. Genet. 103**,** 393-399.

244. Rodriguez-Sinovas,A., Boengler,K., Cabestrero,A., Gres,P., Morente,M., Ruiz-Meana,M., Konietzka,I., Miro,E., Totzeck,A., Heusch,G., Schulz,R., Garcia-Dorado,D., 2006. Translocation of connexin 43 to the inner mitochondrial membrane of cardiomyocytes through the heat shock protein 90-dependent TOM pathway and its importance for cardioprotection. Circ. Res. 99**,** 93-101.

245. Rodriguez-Sinovas,A., Garcia-Dorado,D., Padilla,F., Inserte,J., Barrabes,J.A., Ruiz-Meana,M., Agullo,L., Soler-Soler,J., 2003. Pre-treatment with the Na+/H+ exchange inhibitor cariporide delays cell-to-cell electrical uncoupling during myocardial ischemia. Cardiovasc. Res. 58**,** 109-117.

246. Romanov,R.A., Rogachevskaja,O.A., Bystrova,M.F., Jiang,P., Margolskee,R.F., Kolesnikov,S.S., 2007. Afferent neurotransmission mediated by hemichannels in mammalian taste cells. EMBO J. 26**,** 657-667.

247. Ron,D., Luo,J., Mochly-Rosen,D., 1995. C2 region-derived peptides inhibit translocation and function of beta protein kinase C in vivo. J. Biol. Chem. 270**,** 24180-24187.

248. Rosamond,W., Flegal,K., Furie,K., Go,A., Greenlund,K., Haase,N., Hailpern,S.M., Ho,M., Howard,V., Kissela,B., Kittner,S., Lloyd-Jones,D., McDermott,M., Meigs,J., Moy,C., Nichol,G., O'Donnell,C., Roger,V., Sorlie,P., Steinberger,J., Thom,T., Wilson,M., Hong,Y., 2008. Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 117**,** e25-146.

249. Rozental,R., Carvalho,A.C., Spray,D.C., 2000. Gap junctions in the cardiovascular and immune systems. Braz. J. Med. Biol. Res. 33**,** 365-368.

250. Ruiz-Meana,M., Garcia-Dorado,D., Hofstaetter,B., Piper,H.M., Soler-Soler,J., 1999. Propagation of cardiomyocyte hypercontracture by passage of Na(+) through gap junctions. Circ. Res. 85**,** 280-287.

251. Ruiz-Meana,M., Garcia-Dorado,D., Lane,S., Pina,P., Inserte,J., Mirabet,M., Soler-Soler,J., 2001. Persistence of gap junction communication during myocardial ischemia. Am. J. Physiol Heart Circ. Physiol 280**,** H2563-H2571.

252. Saez,J.C., Connor,J.A., Spray,D.C., Bennett,M.V., 1989. Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions. Proc. Natl. Acad. Sci. U. S. A 86**,** 2708-2712.

253. Saez,J.C., Retamal,M.A., Basilio,D., Bukauskas,F.F., Bennett,M.V., 2005. Connexin-based gap junction hemichannels: gating mechanisms. Biochim. Biophys. Acta 1711**,** 215-224.

254. Sakane,F., Imai,S., Kai,M., Wada,I., Kanoh,H., 1996. Molecular cloning of a novel diacylglycerol kinase isozyme with a pleckstrin homology domain and a C-terminal tail similar to those of the EPH family of protein-tyrosine kinases. J. Biol. Chem. 271**,** 8394-8401.

255. Salameh,A., Blanke,K., Dhein,S., Janousek,J., 2010. Cardiac gap junction channels are upregulated by metoprolol: an unexpected effect of beta-blockers. Pharmacology 85**,** 203-210.

256. Salameh,A., Krautblatter,S., Karl,S., Blanke,K., Gomez,D.R., Dhein,S., Pfeiffer,D., Janousek,J., 2009. The signal transduction cascade regulating the expression of the gap junction protein connexin43 by beta-adrenoceptors. Br. J. Pharmacol. 158**,** 198-208.

257. Sanderson,M.J., 1995. Intercellular calcium waves mediated by inositol trisphosphate. Ciba Found. Symp. 188**,** 175-189.

258. Sandow,S.L., Hill,C.E., 2000. Incidence of myoendothelial gap junctions in the proximal and distal mesenteric arteries of the rat is suggestive of a role in endothelium-derived hyperpolarizing factor-mediated responses. Circ. Res. 86**,** 341-346.

259. Schmilinsky-Fluri,G., Valiunas,V., Willi,M., Weingart,R., 1997. Modulation of cardiac gap junctions: the mode of action of arachidonic acid. J. Mol. Cell Cardiol. 29**,** 1703-1713.

260. Schulz,R., Boengler,K., Totzeck,A., Luo,Y., Garcia-Dorado,D., Heusch,G., 2007. Connexin 43 in ischemic pre- and postconditioning. Heart Fail. Rev. 12**,** 261-266.

261. Schulz,R., Gres,P., Skyschally,A., Duschin,A., Belosjorow,S., Konietzka,I., Heusch,G., 2003. Ischemic preconditioning preserves connexin 43 phosphorylation during sustained ischemia in pig hearts in vivo. FASEB J. 17**,** 1355-1357.

262. Schulz,R., Heusch,G., 2004. Connexin 43 and ischemic preconditioning. Cardiovasc. Res. 62**,** 335-344.

263. Schwanke,U., Konietzka,I., Duschin,A., Li,X., Schulz,R., Heusch,G., 2002. No ischemic preconditioning in heterozygous connexin43-deficient mice. Am. J. Physiol Heart Circ. Physiol 283**,** H1740-H1742.

264. Schwanke,U., Li,X., Schulz,R., Heusch,G., 2003. No ischemic preconditioning in heterozygous connexin 43-deficient mice--a further in vivo study. Basic Res. Cardiol. 98**,** 181-182.

265. Seeman,P., Mazanec,R., Ctvrteckova,M., Smilkova,D., 2001. Charcot-Marie-Tooth type X: A novel mutation in the Cx32 gene with central conduction slowing. Int. J. Mol. Med. 8**,** 461-468.

266. Segal,S.S., Jacobs,T.L., 2001. Role for endothelial cell conduction in ascending vasodilatation and exercise hyperaemia in hamster skeletal muscle. J. Physiol 536**,** 937-946.

267. Seiler,S.M., Cragoe,E.J., Jr., Jones,L.R., 1985. Demonstration of a Na+/H+ exchange activity in purified canine cardiac sarcolemmal vesicles. J. Biol. Chem. 260**,** 4869-4876.

268. Severs,N.J., 1990. The cardiac gap junction and intercalated disc. Int. J. Cardiol. 26**,** 137-173.

269. Shin,H.G., Barnett,J.V., Chang,P., Reddy,S., Drinkwater,D.C., Pierson,R.N., Wiley,R.G., Murray,K.T., 2000. Molecular heterogeneity of protein kinase C expression in human ventricle. Cardiovasc. Res. 48**,** 285-299.

270. Shintani-Ishida,K., Uemura,K., Yoshida,K., 2007. Hemichannels in cardiomyocytes open transiently during ischemia and contribute to reperfusion injury following brief ischemia. Am. J. Physiol Heart Circ. Physiol 293**,** H1714-H1720.

271. Shintani-Ishida,K., Unuma,K., Yoshida,K., 2009. Ischemia enhances translocation of connexin43 and gap junction intercellular communication, thereby propagating contraction band necrosis after reperfusion. Circ. J. 73**,** 1661-1668.

272. Silverman,H.S., Stern,M.D., 1994. Ionic basis of ischaemic cardiac injury: insights from cellular studies. Cardiovasc. Res. 28**,** 581-597.

273. Simon,A.M., Goodenough,D.A., 1998. Diverse functions of vertebrate gap junctions. Trends Cell Biol. 8**,** 477-483.

274. Simonis,G., Briem,S.K., Schoen,S.P., Bock,M., Marquetant,R., Strasser,R.H., 2007. Protein kinase C in the human heart: differential regulation of the isoforms in aortic stenosis or dilated cardiomyopathy. Mol. Cell Biochem. 305**,** 103-111.

275. Simpson,I., Rose,B., Loewenstein,W.R., 1977. Size limit of molecules permeating the junctional membrane channels. Science 195**,** 294-296.

276. Sivaraman,V., Hausenloy,D.J., Kolvekar,S., Hayward,M., Yap,J., Lawrence,D., Di,S.C., Yellon,D.M., 2009. The divergent roles of protein kinase C epsilon and

delta in simulated ischaemia-reperfusion injury in human myocardium. J. Mol. Cell Cardiol. 46**,** 758-764.

277. Smith,J.H., Green,C.R., Peters,N.S., Rothery,S., Severs,N.J., 1991. Altered patterns of gap junction distribution in ischemic heart disease. An immunohistochemical study of human myocardium using laser scanning confocal microscopy. Am. J. Pathol. 139**,** 801-821.

278. Smith,W.T., Fleet,W.F., Johnson,T.A., Engle,C.L., Cascio,W.E., 1995. The Ib phase of ventricular arrhythmias in ischemic in situ porcine heart is related to changes in cell-to-cell electrical coupling. Experimental Cardiology Group, University of North Carolina. Circulation 92**,** 3051-3060.

279. Sosinsky,G.E., Perkins,G.A., 2000. Electron crystallographic methods for investigating gap junction structure. Methods 20**,** 140-155.

280. Souroujon,M.C., Mochly-Rosen,D., 1998. Peptide modulators of protein-protein interactions in intracellular signaling. Nat. Biotechnol. 16**,** 919-924.

281. Spray,D.C., Rozental,R., Srinivas,M., 2002. Prospects for rational development of pharmacological gap junction channel blockers. Curr. Drug Targets. 3**,** 455-464.

282. Stahelin,R.V., Cho,W., 2001. Roles of calcium ions in the membrane binding of C2 domains. Biochem. J. 359**,** 679-685.

283. Steenbergen,C., Murphy,E., Watts,J.A., London,R.E., 1990. Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat heart. Circ. Res. 66**,** 135-146.

284. Steinberg,S.F., Goldberg,M., Rybin,V.O., 1995. Protein kinase C isoform diversity in the heart. J. Mol. Cell Cardiol. 27**,** 141-153.

285. Steinberg,T.H., Civitelli,R., Geist,S.T., Robertson,A.J., Hick,E., Veenstra,R.D., Wang,H.Z., Warlow,P.M., Westphale,E.M., Laing,J.G., ., 1994. Connexin43 and connexin45 form gap junctions with different molecular permeabilities in osteoblastic cells. EMBO J. 13**,** 744-750.

286. Stewart,J.M., Page,E., 1978. Improved stereological techniques for studying myocardial cell growth: application to external sarcolemma, T system, and intercalated disks of rabbit and rat hearts. J. Ultrastruct. Res. 65**,** 119-134.

287. Sugden,P.H., Bogoyevitch,M.A., 1995. Intracellular signalling through protein kinases in the heart. Cardiovasc. Res. 30**,** 478-492.

288. Sugiura,H., Toyama,J., Tsuboi,N., Kamiya,K., Kodama,I., 1990. ATP directly affects junctional conductance between paired ventricular myocytes isolated from guinea pig heart. Circ. Res. 66**,** 1095-1102.

289. Suzuki,M., Sasaki,N., Miki,T., Sakamoto,N., Ohmoto-Sekine,Y., Tamagawa,M., Seino,S., Marban,E., Nakaya,H., 2002. Role of sarcolemmal K(ATP) channels in cardioprotection against ischemia/reperfusion injury in mice. J. Clin. Invest 109**,** 509-516.

290. Takai,Y., Kishimoto,A., Kikkawa,U., Mori,T., Nishizuka,Y., 1979. Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. Biochem. Biophys. Res. Commun. 91**,** 1218-1224.

291. Tanaka,M., Terry,R.D., Mokhtari,G.K., Inagaki,K., Koyanagi,T., Kofidis,T., Mochly-Rosen,D., Robbins,R.C., 2004. Suppression of graft coronary artery disease by a brief treatment with a selective epsilonPKC activator and a deltaPKC inhibitor in murine cardiac allografts. Circulation 110**,** II194-II199.

292. Tanaka,S., Tominaga,M., Yasuda,I., Kishimoto,A., Nishizuka,Y., 1991. Protein kinase C in rat brain synaptosomes. Beta II-subspecies as a major isoform associated with membrane-skeleton elements. FEBS Lett. 294**,** 267-270.

293. Tani,M., Neely,J.R., 1989. Role of intracellular Na+ in Ca2+ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H+-Na+ and Na+-Ca2+ exchange. Circ. Res. 65**,** 1045-1056.

294. Tauskela,J.S., chakravarthy,B.R., Murray,C.L., Wang,Y., Comas,T., Hogan,M., Hakim,A., Morley,P., 1999. Evidence from cultured rat cortical neurons of differences in the mechanism of ischemic preconditioning of brain and heart. Brain Res. 827**,** 143-151.

295. Thompson,R.J., Zhou,N., MacVicar,B.A., 2006. Ischemia opens neuronal gap junction hemichannels. Science 312**,** 924-927.

296. Toker,A., 1998. Signaling through protein kinase C. Front Biosci. 3**,** D1134-D1147.

297. Tran,C.H., Welsh,D.G., 2009. Current perspective on differential communication in small resistance arteries. Can. J. Physiol Pharmacol. 87**,** 21-28.

298. Traub,O., Eckert,R., Lichtenberg-Frate,H., Elfgang,C., Bastide,B., Scheidtmann,K.H., Hulser,D.F., Willecke,K., 1994. Immunochemical and electrophysiological characterization of murine connexin40 and -43 in mouse tissues and transfected human cells. Eur. J. Cell Biol. 64**,** 101-112.

299. Trexler,E.B., Bukauskas,F.F., Bennett,M.V., Bargiello,T.A., Verselis,V.K., 1999. Rapid and direct effects of pH on connexins revealed by the connexin46 hemichannel preparation. J. Gen. Physiol 113**,** 721-742.

300. Tsai,H., Werber,J., Davia,M.O., Edelman,M., Tanaka,K.E., Melman,A., Christ,G.J., Geliebter,J., 1996. Reduced connexin 43 expression in high grade, human prostatic adenocarcinoma cells. Biochem. Biophys. Res. Commun. 227**,** 64-69.

301. Tseng,G.N., Boyden,P.A., 1991. Different effects of intracellular Ca and protein kinase C on cardiac T and L Ca currents. Am. J. Physiol 261**,** H364-H379.

302. van Echteld,C.J., Kirkels,J.H., Eijgelshoven,M.H., van der Meer,P., Ruigrok,T.J., 1991. Intracellular sodium during ischemia and calcium-free perfusion: a 23Na NMR study. J. Mol. Cell Cardiol. 23**,** 297-307.

303. van Veen,A.A., van Rijen,H.V., Opthof,T., 2001. Cardiac gap junction channels: modulation of expression and channel properties. Cardiovasc. Res. 51**,** 217-229.

304. Vergara,L., Bao,X., Bello-Reuss,E., Reuss,L., 2003. Do connexin 43 gap-junctional hemichannels activate and cause cell damage during ATP depletion of renal-tubule cells?. Acta Physiol Scand. 179**,** 33-38.

305. Walsh,K.B., Kass,R.S., 1988. Regulation of a heart potassium channel by protein kinase A and C. Science 242**,** 67-69.

306. Warn-Cramer,B.J., Cottrell,G.T., Burt,J.M., Lau,A.F., 1998. Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. J. Biol. Chem. 273**,** 9188-9196.

307. Warner,A., Clements,D.K., Parikh,S., Evans,W.H., DeHaan,R.L., 1995. Specific motifs in the external loops of connexin proteins can determine gap junction formation between chick heart myocytes. J. Physiol 488 (Pt 3)**,** 721-728.

308. Waszak,P., Alphonse,R., Vadivel,A., Ionescu,L., Eaton,F., Thebaud,B., 2012. Preconditioning Enhances the Paracrine Effect of Mesenchymal Stem Cells in Preventing Oxygen-Induced Neonatal Lung Injury in Rats. Stem Cells Dev.

309. Weinbrenner,C., Liu,G.S., Cohen,M.V., Downey,J.M., 1997. Phosphorylation of tyrosine 182 of p38 mitogen-activated protein kinase correlates with the protection of preconditioning in the rabbit heart. J. Mol. Cell Cardiol. 29**,** 2383-2391.

310. Willecke,K., Eiberger,J., Degen,J., Eckardt,D., Romualdi,A., Guldenagel,M., Deutsch,U., Sohl,G., 2002. Structural and functional diversity of connexin genes in the mouse and human genome. Biol. Chem. 383**,** 725-737.

311. Wilson,F.N., Macleod,A.G., Barker,P.S., 1933. THE DISTRIBUTION OF THE ACTION CURRENTS PRODUCED BY HEART MUSCLE AND OTHER EXCITABLE TISSUES IMMERSED IN EXTENSIVE CONDUCTING MEDIA. J. Gen. Physiol 16**,** 423-456.

312. Xiao,G.Q., Qu,Y., Sun,Z.Q., Mochly-Rosen,D., Boutjdir,M., 2001. Evidence for functional role of epsilonPKC isozyme in the regulation of cardiac Na(+) channels. Am. J. Physiol Cell Physiol 281**,** C1477-C1486.

313. Xue,Q., Yuan,Z., Chen,Z., Hao,R., Liu,C., Tu,B., 2012. Protective role of nitric oxide induced by ischemic preconditioning on cold ischemic-reperfusion injury of rat liver graft. Transplant. Proc. 44**,** 948-951.

314. Yeh,H.I., Lupu,F., Dupont,E., Severs,N.J., 1997. Upregulation of connexin43 gap junctions between smooth muscle cells after balloon catheter injury in the rat carotid artery. Arterioscler. Thromb. Vasc. Biol. 17**,** 3174-3184.

315. Yeh,H.I., Rothery,S., Dupont,E., Coppen,S.R., Severs,N.J., 1998. Individual gap junction plaques contain multiple connexins in arterial endothelium. Circ. Res. 83**,** 1248-1263.

316. Yoshida,K., Kawamura,S., Mizukami,Y., Kitakaze,M., 1997. Implication of protein kinase C-alpha, delta, and epsilon isoforms in ischemic preconditioning in perfused rat hearts. J. Biochem. 122**,** 506-511.

317. Zhang,G., Kazanietz,M.G., Blumberg,P.M., Hurley,J.H., 1995. Crystal structure of the cys2 activator-binding domain of protein kinase C delta in complex with phorbol ester. Cell 81**,** 917-924.

318. Zhao,H.B., Yu,N., Fleming,C.R., 2005. Gap junctional hemichannel-mediated ATP release and hearing controls in the inner ear. Proc. Natl. Acad. Sci. U. S. A 102**,** 18724-18729.

Chapitre II : Méthodologie

Pour cette thèse, la méthodologie n'a pas été détaillée totalement dans le but de faciliter la lecture de l'introduction. Cependant, tous les détails techniques concernant la procédure chirurgicale, ainsi que la réalisation des tests électropphysiologiques, biochimiques de chacun des articles, sont présentés dans la partie « *methods* ».

Chapitre III:

Differential Modulation of Unapposed Connexin 43 Hemichannel Electrical Conductance by Protein Kinase C Isoforms

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Abstract

Opening of unapposed connexin 43 hemichannels (Cx43Hc) in the plasma membrane results in altered ionic homeostasis leading to cell damage. Although it is generally acknowledged that Cx43Hc function is regulated by protein kinase C (PKC), information regarding PKC functional role in the modulation of Cx43Hc electrical conductance is lacking. In this work, we used the patch clamp technique to study the effect of phorbol 12-myristate 13-acetate (PMA), a general PKC activator, on the electrical conductance of exogenous Cx43Hc expressed in tsA201 cells. Subsequently, a matrix of synthetic PKC isoform-specific inhibitor peptides was used to dissect the functional role of individual PKC isoforms in Cx43Hc regulation. Superfusion with 10nM PMA abolished Cx43Hc currents by 74%, an effect that was prevented by retreatment with a general PKC inhibitor, GF109203X. Interestingly, intracellular diffusion of εV1-2 (0.1μM), an εPKC-specific inhibitor peptide, completely antagonized PMA-induced current inhibition. Cell dialysis with either β_{II}- or δPKC inhibitor peptides partially decreased PMA effect. Neither α- nor β_IPKC inhibition altered PMA-induced current reduction. This study shows for the first time that Cx43Hc electrical conductance is inhibited following PKC activation. Moreover, this inhibition is predominantly mediated by the "novel" εPKC isoform, whereas partial inhibition may be provided by the "conventional" β_{II} as well as the "novel" δPKC isoforms.

Keywords: Patch clamp, Connexin 43, Hemichannels, PKC, Peptides, tsA201 cells.

Introduction

Connexin 43 hemichannels (Cx43Hc) consist of a hexameric association of Cx43 subunits. Whereas complete gap junctional channels result from head-to-head apposition of hemichannel pairs from adjacent cells creating direct communication pathways between the cytoplasmic compartments of neighboring cells (Harris, 2001), the unapposed Cx43Hc have recently been shown to be expressed independently and to exert functions different from those of gap junctional channels. Functional unapposed Cx43Hc have been identified in several tissues including heart muscle (Kondo *et al.*, 2000; John *et al.*, 2003), central nervous system (Hofer and Dermietzel, 1998; Contreras *et al.*, 2002; Saez *et al.*, 2003; Boucher and Bennett, 2003), renal tubules (Vergara *et al.*, 2003) and bone (Romanello and D'Andrea, 2001). They provide communication between the intracellular and extracellular compartments and are involved in Ca signaling (Stout *et al.*, 2002) and glutamate release (Ye *et al.*, 2003) in astrocytes, in transduction of cell survival signals in bone (Plotkin and Bellido, 2001; Plotkin *et al.*, 2002), as well as in promoting cell injury in response to metabolic inhibition in isolated ventricular myocytes (Kondo *et al.*, 2000; Shintani-Ishida *et al.*, 2007), astrocytes (Contreras *et al.*, 2002) and renal tubule cells (Vergara *et al.*, 2003). Opening of Cx43Hc can be induced by depolarization and/or low extracellular [Ca^{2+}] (Li *et al.*, 1996; Contreras *et al.*, 2003), metabolic inhibition (John *et al.*, 1999; Kondo *et al.*, 2000; Contreras *et al.*, 2002), mechanical stimulation (Stout *et al.*, 2002) and treatment with bisphosphonates (Plotkin and Bellido, 2001).

Phosphorylation of Cx43 subunits is known to be an important regulatory mechanism of Cx43 channels, i.e. unapposed hemichannels (Bao *et al.*, 2004; Saez *et al.*, 2005) as well as gap junctional channels (van Veen *et al.*, 2001). The relative proportions of Cx43 existing in either the phosphorylated or dephosphorylated states varies between physiological and pathophysiological conditions (Beardslee *et al.*, 2000; Schwanke *et al.*, 2003; Hatanaka *et al.*, 2004; Ek-Vitorin *et al.*, 2006). Indeed, Cx43 contains a significantly greater number of motifs for phosphorylation by PKC than it does for any other kinase (reviewed in (van Veen *et al.*, 2006)). However, information is lacking regarding the functional role of PKC in the modulation of Cx43Hc electrical conductance.

In this study, this issue was addressed using the patch-clamp technique by measuring whole-cell currents between cytoplasmic and extracellular compartments in individual cells following phorbol 12-myristate 13-acetate (PMA)-mediated PKC activation. Because studies on native Cx43Hc in cells are often complicated by interference from other membrane channels and other coexisting connexin isoforms, we studied the regulation of Cx43Hc in a Cx43-transfected tsA201 cell expression system, which is deprived of any native membrane ionic channel, but endogenously expresses most of the PKC isoforms (Leaney *et al.*, 2001). To dissect the role of the various endogenous PKC isoforms in Cx43Hc regulation, we employed a matrix of PKC isoform-specific modulator peptides targeting, individually, the α, β_I and β_{II} "conventional" isoforms (i.e. dependent on Ca, phospholipid and diacylglycerol) as well as the ε and δ "novel" isoforms (i.e. phospholipid and diacylglycerol-dependent but Ca-

independent). Five inhibitor peptides targeting α-, β_I-, β_{II}-, δ- and εPKC isoforms and one activator peptide targeting εPKC were diffused separately through the patch pipette. By structurally mimicking the binding site of the *receptor of activated C kinase* (RACK) on one particular PKC isoform, inhibitor peptides inhibit the function of that isoform and prevent its action. In contrast, activator peptide binds to the εPKC isoform, causes a conformational change in its structure and exposes its catalytic site therefore enhancing its activity (Souroujon and Mochly-Rosen, 1998). These peptides have successfully been used to dissect functional roles of individual PKC isoforms in the modulation of various on channels (Hu *et al.*, 2000; Xiao *et al.*, 2001; Xiao *et al.*, 2003; Baroudi *et al.*, 2006).

The principal objectives were therefore to assess the functional implication of PKC in regulating Cx43Hc electrical conductance and to unravel the specific PKC isoforms involved in such regulation during PMA-mediated PKC activation.

Materials and methods

Transfection of tsA201 cell line

Rat pBlue-Cx43 plasmid was kindly provided by Prof. Pascal Daleau (Université Laval, Sainte-Foy, Québec). The mammalian cell line tsA201 is derived from human embryonic kidney HEK293 cells by stable transfection with SV40 large-T antigen. Cells were grown in high glucose DMEM supplemented with 10% fetal bovine serum, L-glutamine (2mM), penicillin G (100 U/ml), and streptomycin (10mg/ml; Invitrogen). Cells were incubated in a 5% CO2 humidified atmosphere. The tsA201 cells were transfected using the calcium phosphate method with the following modification: to identify transfected cells, 7 μg of EBO/CD8 plasmid was cotransfected with 7 μg of pBlue-Cx43. For patch clamp experiments, cells 2-3 days posttransfection were incubated for 2 min in medium containing anti-CD8 coated beads (Invitrogen) (Jurman *et al.*, 1994). The unattached beads were removed by washing with bath solution. Beads were prepared according to the manufacturer's instruction. Cells expressing CD8-a, and therefore binding beads, were distinguished from nontransfected cells by light microscopy.

Solutions, drugs and peptides

For whole cell recordings, the pipette solution contained (in mM): 140 KCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES and 5 EGTA (estimated free Ca, 37 nM) adjusted to pH 7.4.

The bath solution contained (in mM): 140 NaCl, 1 $MgCl_2$, 5.4 KCl, 1.8 $CaCl_2$, 10 HEPES and 10 EGTA adjusted to pH 7.4. PKC isoform peptides were added in the pipette solution as previously described (Hu *et al.*, 2000; Xiao *et al.*, 2001; Xiao *et al.*, 2003). PKC isoform-specific modulator and scrambled negative control peptides used in this study include αC_{2-4} (inhibitor of αPKC), $\beta_I V_{5-3}$ (inhibitor of β_IPKC), $\beta_{II} V_{5-3}$ (inhibitor of β_{II}PKC), εV_{1-2} (inhibitor of εPKC), δV_{1-7} (inhibitor of δPKC), εV_{1-7} (activator of εPKC) and scrambled εV_{1-2}. To allow for peptides to access the cytoplasm, cells were dialyzed with peptide (0.1 μM) for at least 5 min after breaking the membrane seal prior to current recordings. All peptides had >90% purity and were custom-made at Sheldon Biotechnology Inc, McGill University, Montréal, QC. The sequence and properties of PKC isoform peptides were reported previously (Hu *et al.*, 2000; Xiao *et al.*, 2001; Xiao *et al.*, 2003). PMA, 4α-phorbol 12,13-didecanoate (4αPDD), and the GF109203X were obtained from Sigma-Aldrich (St. Louis, MO).

Electrophysiology

Cx43 hemichannels-mediated currents were recorded in whole-cell configuration of the patch clamp technique (Hamill *et al.*, 1981). Data were digitized at 5 kHz with an analog-to-digital converter (Digidata 1440A, Molecular Devices, CA). The recordings were filtered with a low pass corner frequency of 2 kHz. Glass electrodes (Corning model 8161, outer diameter 1.5 mm) with an electrode tip resistance of 0.8-1.0 MΩ were connected to a patch-clamp amplifier (Axon model 200B). A voltage error of 4 mV,

attributable to liquid junction potential, was corrected. Data were analyzed using pClamp 10.1 (Molecular Devices). Cells were maintained at a holding potential of 0 mV. To generate Cx43Hc current-voltage relationship traces, cells were depolarized by test pulses ranging from 0 to 130 mV in a 10 mV increment for 5 s. The effect of PMA and other tested agents were studied by depolarizing cells in 30 mV steps for pulse durations of 5s.

Statistical analysis

Data are expressed as means ± SE. Percent inhibition was calculated as the difference between the basal current amplitude and the intervention. Paired t-test was used to compare measurements made under different conditions (e.g. basal versus PMA) in individual cells. Measurements of PMA effects made in the presence of the different PKC isoform modulator peptides were compared to the measurements of the effect of PMA alone using unpaired Dunnett's t-test. Differences were deemed significant at a P value < 0.05.

Results

PMA inhibits Cx43Hc-induced current

The representative outward current traces shown in (Fig. 1a) illustrate the voltage-dependent Cx43Hc-mediated current amplitudes in response to membrane depolarization under basal conditions. Application of the general PKC activator PMA (10nM) in the bath consistently inhibited Cx43Hc currents (Fig. 1b). The time course for PMA-induced inhibition of Cx43Hc currents elicited at 30 mV is shown in (Fig. 1c). Steady state inhibition was reached in all cells studied. For easy comparison, reduction in Cx43Hc current amplitudes is represented throughout the text as a percentage with reference to the basal value. Averaged data show that PMA resulted in Cx43Hc current reduction by 74.0 ± 4.3% (n=6, $P<0.05$). To exclude the possibility that the current decay observed with PMA is due to rundown or other nonspecific effects, a set of control experiments was conducted. Cx43Hc currents recorded in the absence of PKC modulators did not show significant current reduction over a 15 min time frame (1.1 ± 5.1%, n=3, *P*=NS) (Fig. 2a). Superfusion of tsA201 cells with 10nM 4αPDD, a phorbol ester analog which does not activate PKC, did not significantly affect Cx43Hc current amplitude (increase of 2.0 ± 1.5%, n=5, *P*=NS) (Fig. 2b), indicating that the PMA effect reported above is mediated via PKC activation. To further substantiate the role of PKC in the PMA-induced inhibition, we tested the ability of a general PKC inhibitor, bisindolylmaleimide (GF109203X), to antagonize the effect of PMA. As shown in (Fig. 2c), preincubation of Cx43-expressing cells with 15μM GF109203X for 10-15 min

before superfusion with PMA resulted in a current increase to 133.0 ± 9.3%, n=3, $P<0.05$ (compared to 100% current at basal conditions), suggesting the presence of basal PKC activity in tsA201 cells. According to expectation, application of PMA could not significantly alter Cx43Hc current amplitudes (144.6 ± 1.4%, n=3, *P*=NS) (Fig. 2c).

PMA inhibition of Cx43Hc current is primarily mediated through εPKC isoform

To dissect the role of individual PKC isoforms in the regulation of Cx43Hc, we tested the ability of five PKC isoform-specific inhibitor peptides (Souroujon and Mochly-Rosen, 1998) to antagonize the effect of PMA, i.e. αC_{2-4}, $\beta_I V_{5-3}$, $\beta_{II} V_{5-3}$, δV_{1-2} and εV_{1-2} targeting α-, β_I-, β_{II}-, δ- and ε-PKC isoforms, respectively. Each of the tested peptides targets a corresponding native PKC isoform. Cell dialysis with αC_{2-4} or $\beta I V_{5-3}$ (0.1 μM) did not prevent Cx43Hc inhibition by PMA (Fig. 3, a and b). In comparison with the 74.0 ± 4.3% (n=6) inhibition when cells were superfused with PMA alone, PMA-induced inhibition occurred at statistically comparable levels of 74.9 ± 5.1% (n= 7, *P*=NS) and 80.5 ± 1.8% (n=5, *P*=NS) in cells dialyzed with αC_{2-4} and $\beta_I V_{5-3}$, respectively. These results suggest that α- and β_I- isoforms are not involved in PKC-dependent regulation of Cx43Hc conductance. In contrast, slight but statistically significant reductions of the PMA-induced current inhibition to 50.2 ± 5.3% (n=7, $P<0.05$) and to 34.2 ± 6.1% (n=5, $P<0.05$) were observed upon inhibition of β_{II}- and δPKC isoforms, respectively (Fig. 3, c and d).

Interestingly, when cells were dialysed with the εPKC inhibitor peptide, εV_{1-2} (0.1μM), the PMA-induced current inhibition was completely abolished (118.5 ± 15.8%, n=6, significantly different from the reference PMA effect) (Fig. 4a). To substantiate the specificity of the εV_{1-2} inhibitor peptide effect, we performed additional experiments using a scrambled sequence of the εV_{1-2} amino acids as a negative control (Fig. 4b). As expected, in the presence of scrambled εV_{1-2} (0.1 μM) the inhibitory effect of PMA on Cx43Hc current was not significantly different compared with the PMA effect alone (69.4 ± 4.7%, n=3, P=NS). On the other hand, using an εPKC activator peptide εV_{1-7} (0.1μM) alone was able to cause a 75.7 ± 2.6% reduction in the basal Cx43Hc current (n=3, $P<0.05$) (Fig. 4c), thus confirming the involvement of εPKC in Cx43Hc inhibition.

The histogram shown in Fig. 5 summarizes our main findings that Cx43Hc conductance was significantly inhibited by PMA and that, moreover, such inhibition was partially reversed by the β_{II}- and δPKC isoform peptide antagonists (since the conductance remained smaller than basal) but completely abolished by εPKC antagonism.

Discussion

Inhibition of Cx43Hc conductance by PMA

In the present study, we demonstrate that Cx43Hc electrical conductance is modulated by PKC and that such modulation is selectively and differentially regulated by PKC isoforms. Using tsA201 cells derived from HEK293 cells, known to endogenously express most of the phorbol ester-sensitive PKC isoforms including α, β_I, β_{II}, δ and ε (Leaney *et al.*, 2001), we have shown that whole-cell Cx43Hc conductance is inhibited by 74% during superfusion of Cx43-expressing cells with a general PKC activator, PMA. This inhibitory effect can be, with confidence, attributed to the activation of PKC since a similar effect could not been reproduced using 4αPDD, a biologically inactive phorbol ester analog that does not activate PKC. In addition, inhibition of Cx43Hc current by PMA was prevented when cells were dialyzed with a general PKC inhibitor, GF109203X. A time-dependent Cx43Hc current rundown effect (Weng *et al.*, 2002) can be ruled out since no variation in Cx43Hc current amplitude was observed in control experiments in which patch clamp measurements were maintained in the same cell for over 15 min.

Inhibition of Cx43Hc electrical conductance following its phosphorylation by PKC is possibly mediated via conformational change in the hemichannel protein therefore restricting ions passage through the pore structure. In principle, whole-cell current amplitude could also be affected by modification of the number of hemichannels expressed on the cell surface. However, since the half life of Cx43 is known to be in the

range of 1 to 2 hours (Laird *et al.*, 1991;Gaietta *et al.*, 2002;Berthoud *et al.*, 2004), it is unlikely that variations in hemichannel expression at the plasma membrane could have contributed to the inhibition of electrical conductance which occurs within a few minutes of exposure to PMA.

Bao et al. (Bao *et al.*, 2007) recently reported an altered Cx43Hc size selectivity upon PKC activation which caused a reduction in purified hemichannels permeability to large solutes without affecting permeation to ions or hemichannels conductance. This is in contrast with observations reported herein, as well as with previously reported observations based on single channel conductance measurements (Moreno *et al.*, 1992;Kwak *et al.*, 1995), indicating that PKC activation drastically reduces the Cx43Hc electrical conductance. One possible explanation for the discrepancy could be the fact that the biophysical properties of hemichannels purified from solubilized Cx43 subunits, as used by Bao et al. (Bao *et al.*, 2007), differ from those of hemichannels studied in cellular systems that provide a physiological environment and cellular metabolism. In another study conducted on gap junction channels, PKC activation induced an increase in intercellular electrical conductance in cell pairs expressing exogenous Cx43 (Kwak and Jongsma, 1996). It is possible that dissimilarities between the functional properties of Cx43Hc and Cx43 gap junctional channels result from interactions between Cx43 and their many binding partner proteins during Cx43Hc assembly into full gap junction channels and their processing toward their final position (Barker *et al.*, 2001;Giepmans, 2004) or simply result from conformational changes during docking with an apposed hemichannel in the adjacent cell (Thimm *et al.*, 2005).

PKC isoform-selective modulation of Cx43Hc

Another novel finding is the unravelling of selective and differential implication of PKC isoforms in the modulation of Cx43Hc conductance. We dissected the role of individual PKC isoforms in the regulation of Cx43Hc using a matrix of well-established PKC isoform-specific inhibitor peptides (Souroujon and Mochly-Rosen, 1998). These peptides have successfully been used to study functional roles of individual PKC isoforms in the modulation of various ion channels (Hu *et al.*, 2000;Xiao *et al.*, 2001;Xiao *et al.*, 2003;Baroudi *et al.*, 2006). In the present work, εPKC was found to be the isoform predominantly involved in the modulation of Cx43Hc conductance following PMA superfusion. In fact, after diffusing the εPKC-specific inhibitor peptide, the PMA effect was not only prevented but there was actually a moderate Cx43Hc current increase to 118.5% when compared to basal values (100%). Although not significant, this slight current increase may be interpreted as an inhibition, by inhibitor $\varepsilon V_{1\text{-}2}$, of endogenous PKC basal activity. This interpretation is consistent with the increase in conductance induced by the general PKC inhibitor GF109203X (Fig. 2c). Smaller but significant antagonism of the PMA effect was identified with the β_{II}PKC and δPKC isoform inhibitors (peptides $\beta_{IIV5\text{-}3}$ and $\delta V_{1\text{-}2}$, respectively), whereas the PMA effect was not significantly altered following application of the α or β_I inhibitor peptides. Assuming a perfect selectivity profile of the inhibitory peptides (Souroujon and Mochly-Rosen, 1998), this suggests that the β_{II} and δ PKC isoforms may also contribute to the PMA effect on conductance, together with a markedly stronger εPKC

contribution. Thus, the electrical conductance of Cx43Hc appears to be differentially regulated by PKC in an isoform-specific manner.

Activation of isoforms belonging to the conventional PKC subfamily (i.e., α, β_I and β_{II}) is known to require intracellular calcium (Nishizuka, 1988). On the other hand, Cx43 hemichannel opening is inhibited at intracellular Ca concentrations >1μM (Shintani-Ishida *et al.*, 2007). Therefore, a balance was achieved whereby moderate Ca concentrations were maintained in the intracellular and bath solutions with the use of EGTA in order to enhance hemichannels opening without compromising conventional PKC activity. Indeed, the estimated intracellular free calcium concentration in our experiments is sufficient to activate the Ca-sensitive PKC isoforms. Moreover, the affinity of Ca-sensitive PKC isoforms to the cytosolic Ca is further increased in the presence of phorbol esters (Nishizuka, 1986).

Perspective

Cx43Hc dephosphorylation (Beardslee *et al.*, 2000;Schulz *et al.*, 2003;Jain *et al.*, 2003;Jeyaraman *et al.*, 2003;Miura *et al.*, 2004) and opening (John *et al.*, 1999;Kondo *et al.*, 2000;Shintani-Ishida *et al.*, 2007) have been proposed as a key mechanism underlying cell injury in response to simulated ischemia in cardiomyocytes. Preservation of Cx43 phosphorylation by selective activation of the εPKC isoform occurs in response to ischemic pre- (Ytrehus *et al.*, 1994;Liu *et al.*, 1999;Chou and Messing, 2005) and post-conditioning (Philipp *et al.*, 2006;Zatta *et al.*, 2006;Penna *et al.*, 2006), in which brief episodes of ischemia reduce the adverse effects of subsequent or preceding myocardial ischemia, respectively. PKC isoform-selective modulator drugs are currently under development for the treatment of a variety of diseases (Serova *et al.*, 2006;Bar-Am *et al.*, 2007;Casellini *et al.*, 2007). As the function of a multiple proteins may be affected by such drugs, delineation of the isoform-specific regulation of downstream targets, e.g. Cx43Hc, is important to improve our understanding of the pathogenesis of ischemic heart disease and to identify new opportunities for drug development.

In conclusion, our results establish that electrical conductance of Cx43Hc is regulated by PKC-dependent phosphorylation. Furthermore, the results points mainly to ε, but also βII and δPKC isoforms as the key players in this modulation. Our results suggest that εPKC, β_{II}PKC and δPKC may constitute suitable candidates for the development of targeted therapeutic for cardiac pathophysiology in which Cx43Hc is involved.

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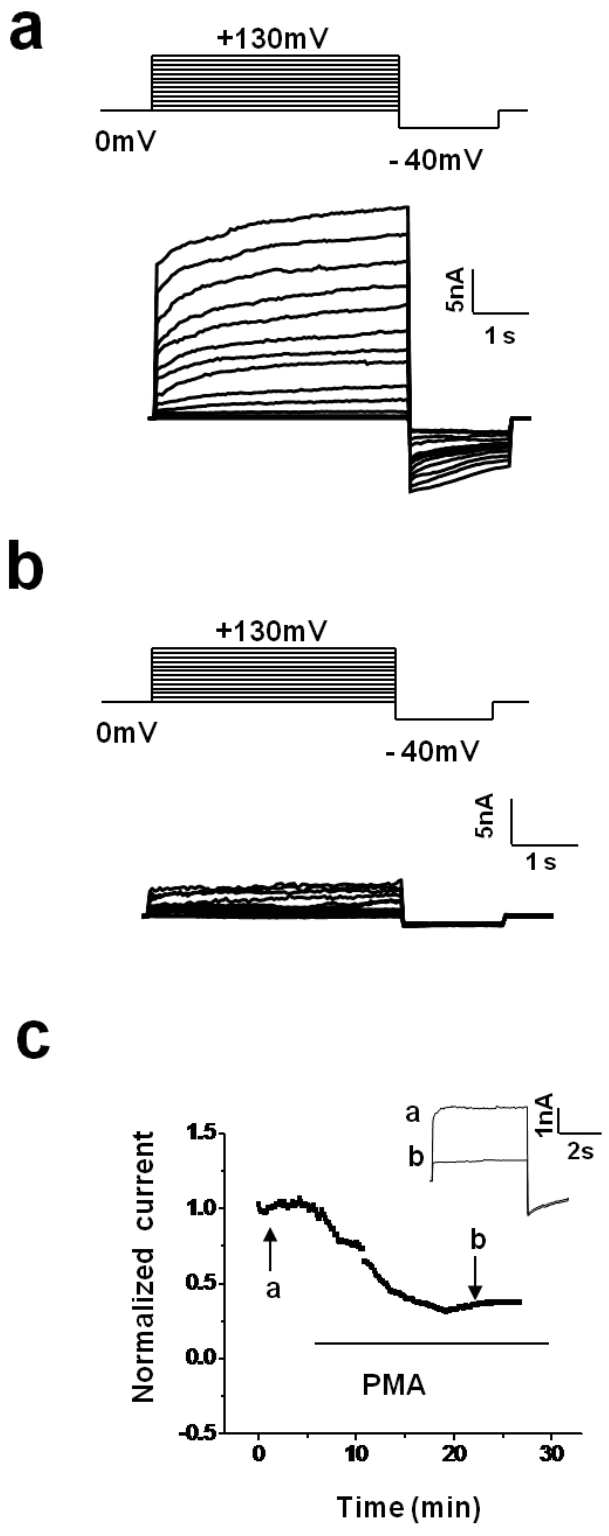
a
+130mV
0mV
-40mV
5nA
1 s
b
+130mV
0mV
-40mV
5nA
1 s
c
Normalized current
1.5
1.0
0.5
0.0
-0.5
0
10
20
30
Time (min)
a
b
PMA
a
b
1nA
2s

Figure 1.

Figure 1. Whole-cell Cx43Hc mediated currents recorded from tsA201 cells. a Starting from 0 mV, cells were depolarized using test pulses ranging from 0 to 130 mV in 10-mV increments during 5 s and then hyperpolarized to −40 mV for 2 s. b Zero-current traces recorded from a non transfected tsA201 cells, using same protocol as in a. c Cx43Hc current traces recorded from transfectedtsA201 cell superfused with the general PKC activator, PMA(10 nM). d Time course recording plotting Cx43Hc current amplitudes in the presence of PMA against time. The inset illustrates Cx43Hc current traces obtained at time points indicatedby arrows a and b.

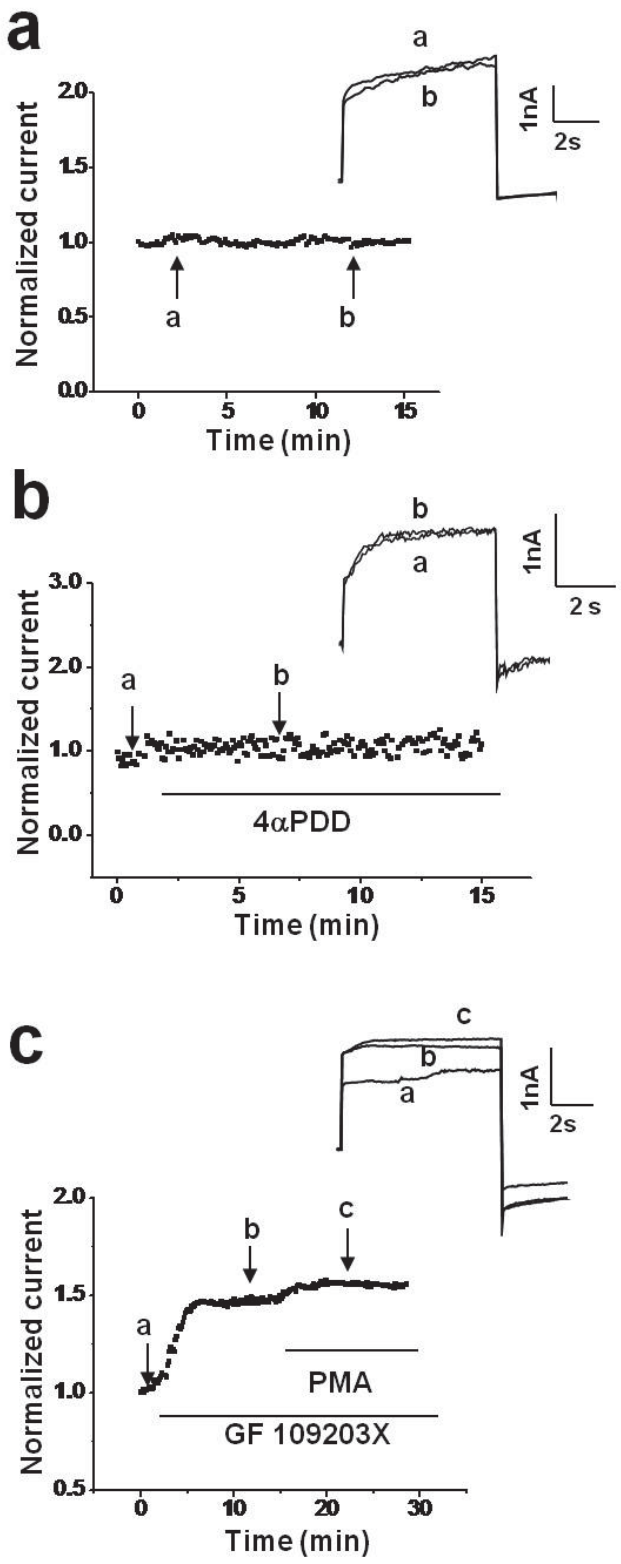
a
a
b
1nA
2s
Normalized current
2.0
1.5
1.0
0.5
0.0
a
b
0
5
10
15
Time (min)
b
b
a
1nA
2 s
3.0
2.0
1.0
0.0
a
b
4αPDD
0
5
10
15
Time (min)
c
c
b
a
1nA
2s
2.0
1.5
1.0
0.5
b
c
a
PMA
GF 109203X
0
10
20
30
Time (min)

Figure 2.

Figure 2. Time course recording of Cx43Hc currents. a Rundown test, the cell is depolarized using a 30-mV test pulse every 7 s for a total duration of 15 min. b The cell is superfused with 10 nM 4αPDD. c The cell is superfused with general PKC inhibitor GF109203X during 15 min before addition of 10 nM PMA. d Time course showing Cx43Hc current inhibition after PMA superfusion when normal Ca concentration is used in the patch pipette and the bath solutions. Insets illustrate Cx43Hc current traces recorded at time points indicated by arrows a and b.

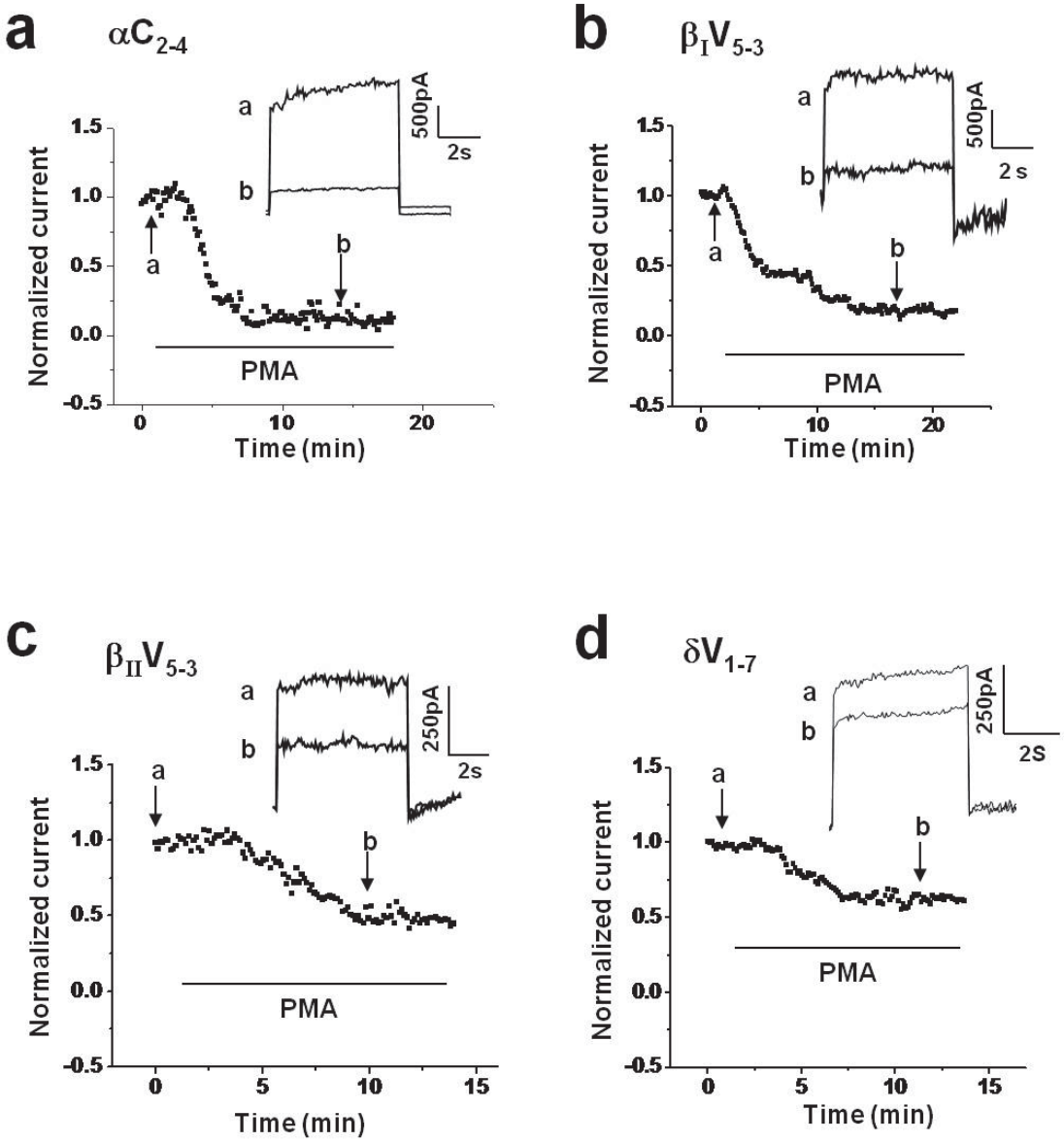
a
αC2-4
Normalized current
Time (min)
PMA
500pA
2s
b
βIV5-3
2 s
c
βIIV5-3
250pA
d
δV1-7

Figure 3.

Figure 3. Time course recordings from cells dialyzed with various PKC isoform-specific inhibitor peptides. The effect of PMA is observed on Cx43Hc currents in the presence of a αC2–4, b βIV5–3, c βIIV5–3, and d δV1–7. Insets illustrate current traces recorded at time points indicated by arrows a and b.

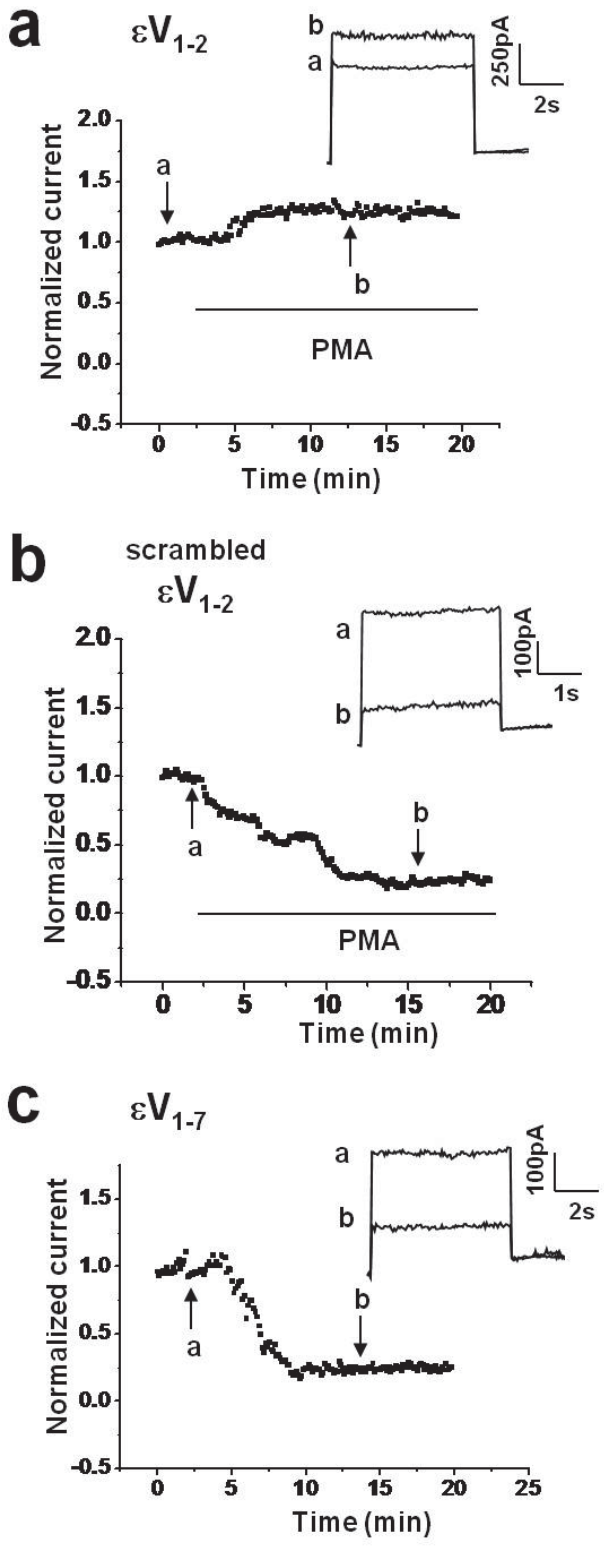
a
εV1-2
b
a
250pA
2s
Normalized current
2.0
1.5
1.0
0.5
0.0
-0.5
a
b
PMA
0
5
10
15
20
Time (min)
b
scrambled
εV1-2
a
b
100pA
1s
Normalized current
2.0
1.5
1.0
0.5
0.0
-0.5
a
b
PMA
0
5
10
15
20
Time (min)
c
εV1-7
a
b
b
100pA
2s
Normalized current
1.5
1.0
0.5
0.0
-0.5
a
0
5
10
15
20
25
Time (min)

Figure 4.

Figure 4. Cx43Hc current regulation by εPKC modulator peptide. The effect of PMA (10 nM) on Cx43Hc current amplitudes is shown during time course recording from cell dialyzed with a εPKC inhibitor peptide and εV1–2 and b scrambled εPKC inhibitor, scrambled εV1–2. c Time course recording shows effect of εPKC activator peptide alone, εV1–7. Insets illustrate current traces recorded at time points indicated by arrows a and b.

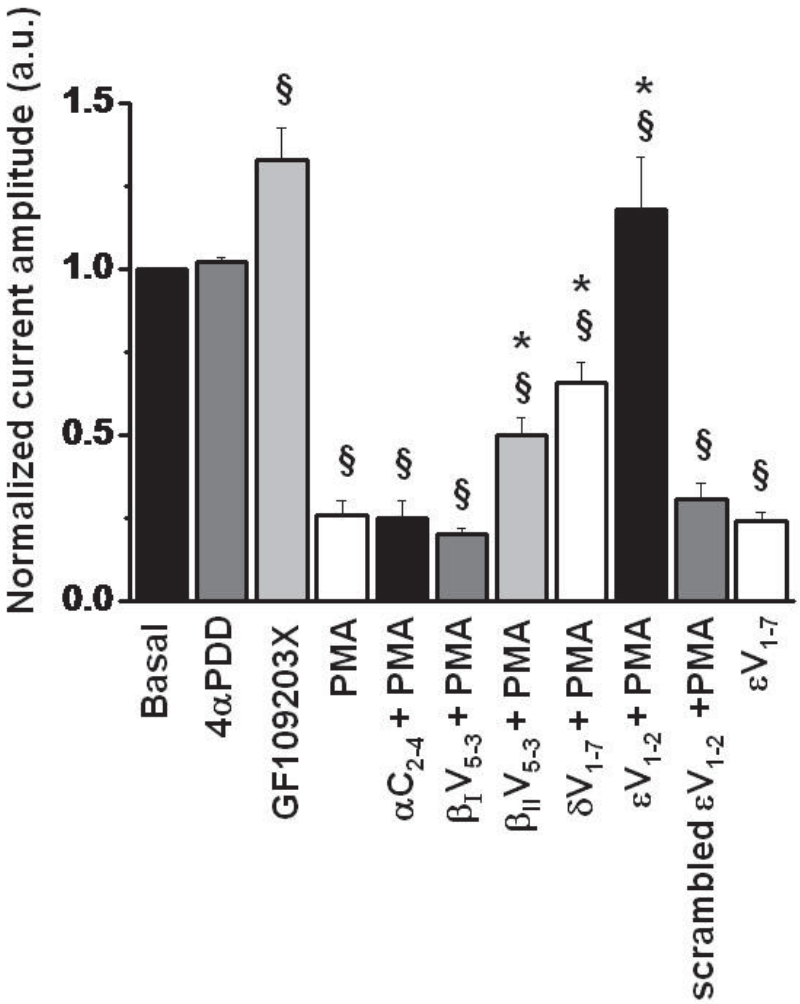
Normalized current amplitude (a.u.)
1.5
1.0
0.5
0.0
Basal
4αPDD
GF109203X
PMA
αC2-4 + PMA
βIV5-3 + PMA
βIIV5-3 + PMA
δV1-7 + PMA
εV1-2 + PMA
scrambled εV1-2 +PMA
εV1-7
§
*

Figure 5.

Figure 5. Data summary. The histogram illustrates percentage of Cx43Hc currents in presence of 4αPDD, GF109203X, PMA, PMA with various PKC isoform-specific peptides, εPKC activator, and scrambled εPKC inhibitor. Current amplitudes are normalized against the control and are represented in arbitrary units (a.u.). Section symbol, $P<0.05$, significantly different from basal. Asterisk, $P<0.05$,significantly different from PMA alone.

Reference List

1. Bao,X., Altenberg,G.A., Reuss,L., 2004. Mechanism of regulation of the gap junction protein connexin 43 by protein kinase C-mediated phosphorylation. Am. J. Physiol Cell Physiol 286**,** C647-C654.

2. Bao,X., Lee,S.C., Reuss,L., Altenberg,G.A., 2007. Change in permeant size selectivity by phosphorylation of connexin 43 gap-junctional hemichannels by PKC. Proc. Natl. Acad. Sci. U. S. A 104**,** 4919-4924.

3. Bar-Am,O., Amit,T., Youdim,M.B., 2007. Aminoindan and hydroxyaminoindan, metabolites of rasagiline and ladostigil, respectively, exert neuroprotective properties in vitro. J. Neurochem. 103**,** 500-508.

4. Barker,R.J., Price,R.L., Gourdie,R.G., 2001. Increased co-localization of connexin43 and ZO-1 in dissociated adult myocytes. Cell Commun. Adhes. 8**,** 205-208.

5. Baroudi,G., Qu,Y., Ramadan,O., Chahine,M., Boutjdir,M., 2006. Protein kinase C activation inhibits Cav1.3 calcium channel at NH2-terminal serine 81 phosphorylation site. Am. J. Physiol Heart Circ. Physiol 291**,** H1614-H1622.

6. Beardslee,M.A., Lerner,D.L., Tadros,P.N., Laing,J.G., Beyer,E.C., Yamada,K.A., Kleber,A.G., Schuessler,R.B., Saffitz,J.E., 2000.

Dephosphorylation and intracellular redistribution of ventricular connexin43 during electrical uncoupling induced by ischemia. Circ. Res. 87**,** 656-662.

7. Berthoud,V.M., Minogue,P.J., Laing,J.G., Beyer,E.C., 2004. Pathways for degradation of connexins and gap junctions. Cardiovasc. Res. 62**,** 256-267.

8. Boucher,S., Bennett,S.A., 2003. Differential connexin expression, gap junction intercellular coupling, and hemichannel formation in NT2/D1 human neural progenitors and terminally differentiated hNT neurons. J. Neurosci. Res. 72**,** 393-404.

9. Casellini,C.M., Barlow,P.M., Rice,A.L., Casey,M., Simmons,K., Pittenger,G., Bastyr,E.J., III, Wolka,A.M., Vinik,A.I., 2007. A 6-month, randomized, double-masked, placebo-controlled study evaluating the effects of the protein kinase C-beta inhibitor ruboxistaurin on skin microvascular blood flow and other measures of diabetic peripheral neuropathy. Diabetes Care 30**,** 896-902.

10. Chou,W.H., Messing,R.O., 2005. Protein kinase C isozymes in stroke. Trends Cardiovasc. Med. 15**,** 47-51.

11. Contreras,J.E., Saez,J.C., Bukauskas,F.F., Bennett,M.V., 2003. Gating and regulation of connexin 43 (Cx43) hemichannels. Proc. Natl. Acad. Sci. U. S. A 100**,** 11388-11393.

12. Contreras,J.E., Sanchez,H.A., Eugenin,E.A., Speidel,D., Theis,M., Willecke,K., Bukauskas,F.F., Bennett,M.V., Saez,J.C., 2002. Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. Proc. Natl. Acad. Sci. U. S. A 99**,** 495-500.

13. Ek-Vitorin,J.F., King,T.J., Heyman,N.S., Lampe,P.D., Burt,J.M., 2006. Selectivity of connexin 43 channels is regulated through protein kinase C-dependent phosphorylation. Circ. Res. 98**,** 1498-1505.

14. Gaietta,G., Deerinck,T.J., Adams,S.R., Bouwer,J., Tour,O., Laird,D.W., Sosinsky,G.E., Tsien,R.Y., Ellisman,M.H., 2002. Multicolor and electron microscopic imaging of connexin trafficking. Science 296**,** 503-507.

15. Giepmans,B.N., 2004. Gap junctions and connexin-interacting proteins. Cardiovasc. Res. 62**,** 233-245.

16. Hamill,O.P., Marty,A., Neher,E., Sakmann,B., Sigworth,F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch. 391**,** 85-100.

17. Harris,A.L., 2001. Emerging issues of connexin channels: biophysics fills the gap. Q. Rev. Biophys. 34**,** 325-472.

18. Hatanaka,K., Kawata,H., Toyofuku,T., Yoshida,K., 2004. Down-regulation of connexin43 in early myocardial ischemia and protective effect by ischemic preconditioning in rat hearts in vivo. Jpn. Heart J. 45**,** 1007-1019.

19. Hofer,A., Dermietzel,R., 1998. Visualization and functional blocking of gap junction hemichannels (connexons) with antibodies against external loop domains in astrocytes. Glia 24**,** 141-154.

20. Hu,K., Mochly-Rosen,D., Boutjdir,M., 2000. Evidence for functional role of epsilonPKC isozyme in the regulation of cardiac Ca(2+) channels. Am. J. Physiol Heart Circ. Physiol 279**,** H2658-H2664.

21. Jain,S.K., Schuessler,R.B., Saffitz,J.E., 2003. Mechanisms of delayed electrical uncoupling induced by ischemic preconditioning. Circ. Res. 92**,** 1138-1144.

22. Jeyaraman,M., Tanguy,S., Fandrich,R.R., Lukas,A., Kardami,E., 2003. Ischemia-induced dephosphorylation of cardiomyocyte connexin-43 is reduced by okadaic acid and calyculin A but not fostriecin. Mol. Cell Biochem. 242**,** 129-134.

23. John,S., Cesario,D., Weiss,J.N., 2003. Gap junctional hemichannels in the heart. Acta Physiol Scand. 179**,** 23-31.

24. John,S.A., Kondo,R., Wang,S.Y., Goldhaber,J.I., Weiss,J.N., 1999. Connexin-43 hemichannels opened by metabolic inhibition. J. Biol. Chem. 274**,** 236-240.

25. Jurman,M.E., Boland,L.M., Liu,Y., Yellen,G., 1994. Visual identification of individual transfected cells for electrophysiology using antibody-coated beads. Biotechniques 17**,** 876-881.

26. Kondo,R.P., Wang,S.Y., John,S.A., Weiss,J.N., Goldhaber,J.I., 2000. Metabolic inhibition activates a non-selective current through connexin hemichannels in isolated ventricular myocytes. J. Mol. Cell Cardiol. 32**,** 1859-1872.

27. Kwak,B.R., Hermans,M.M., De Jonge,H.R., Lohmann,S.M., Jongsma,H.J., Chanson,M., 1995. Differential regulation of distinct types of gap junction channels by similar phosphorylating conditions. Mol. Biol. Cell 6**,** 1707-1719.

28. Kwak,B.R., Jongsma,H.J., 1996. Regulation of cardiac gap junction channel permeability and conductance by several phosphorylating conditions. Mol. Cell Biochem. 157**,** 93-99.

29. Laird,D.W., Puranam,K.L., Revel,J.P., 1991. Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. Biochem. J. 273(Pt 1)**,** 67-72.

30. Leaney,J.L., Dekker,L.V., Tinker,A., 2001. Regulation of a G protein-gated inwardly rectifying K+ channel by a Ca(2+)-independent protein kinase C. J. Physiol 534**,** 367-379.

31. Li,H., Liu,T.F., Lazrak,A., Peracchia,C., Goldberg,G.S., Lampe,P.D., Johnson,R.G., 1996. Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells. J. Cell Biol. 134**,** 1019-1030.

32. Liu,G.S., Cohen,M.V., Mochly-Rosen,D., Downey,J.M., 1999. Protein kinase C-epsilon is responsible for the protection of preconditioning in rabbit cardiomyocytes. J. Mol. Cell Cardiol. 31**,** 1937-1948.

33. Miura,T., Ohnuma,Y., Kuno,A., Tanno,M., Ichikawa,Y., Nakamura,Y., Yano,T., Miki,T., Sakamoto,J., Shimamoto,K., 2004. Protective role of gap junctions in preconditioning against myocardial infarction. Am. J. Physiol Heart Circ. Physiol 286**,** H214-H221.

34. Moreno,A.P., Fishman,G.I., Spray,D.C., 1992. Phosphorylation shifts unitary conductance and modifies voltage dependent kinetics of human connexin43 gap junction channels. Biophys. J. 62**,** 51-53.

35. Nishizuka,Y., 1986. Studies and perspectives of protein kinase C. Science 233**,** 305-312.

36. Nishizuka,Y., 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334**,** 661-665.

37. Penna,C., Rastaldo,R., Mancardi,D., Raimondo,S., Cappello,S., Gattullo,D., Losano,G., Pagliaro,P., 2006. Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-

sensitive K+ channel and protein kinase C activation. Basic Res. Cardiol. 101**,** 180-189.

38. Philipp,S., Yang,X.M., Cui,L., Davis,A.M., Downey,J.M., Cohen,M.V., 2006. Postconditioning protects rabbit hearts through a protein kinase C-adenosine A2b receptor cascade. Cardiovasc. Res. 70**,** 308-314.

39. Plotkin,L.I., Bellido,T., 2001. Bisphosphonate-induced, hemichannel-mediated, anti-apoptosis through the Src/ERK pathway: a gap junction-independent action of connexin43. Cell Commun. Adhes. 8**,** 377-382.

40. Plotkin,L.I., Manolagas,S.C., Bellido,T., 2002. Transduction of cell survival signals by connexin-43 hemichannels. J. Biol. Chem. 277**,** 8648-8657.

41. Romanello,M., D'Andrea,P., 2001. Dual mechanism of intercellular communication in HOBIT osteoblastic cells: a role for gap-junctional hemichannels. J. Bone Miner. Res. 16**,** 1465-1476.

42. Saez,J.C., Contreras,J.E., Bukauskas,F.F., Retamal,M.A., Bennett,M.V., 2003. Gap junction hemichannels in astrocytes of the CNS. Acta Physiol Scand. 179**,** 9-22.

43. Saez,J.C., Retamal,M.A., Basilio,D., Bukauskas,F.F., Bennett,M.V., 2005. Connexin-based gap junction hemichannels: gating mechanisms. Biochim. Biophys. Acta 1711**,** 215-224.

44. Schulz,R., Gres,P., Skyschally,A., Duschin,A., Belosjorow,S., Konietzka,I., Heusch,G., 2003. Ischemic preconditioning preserves connexin 43 phosphorylation during sustained ischemia in pig hearts in vivo. FASEB J. 17**,** 1355-1357.

45. Schwanke,U., Li,X., Schulz,R., Heusch,G., 2003. No ischemic preconditioning in heterozygous connexin 43-deficient mice--a further in vivo study. Basic Res. Cardiol. 98**,** 181-182.

46. Serova,M., Ghoul,A., Benhadji,K.A., Cvitkovic,E., Faivre,S., Calvo,F., Lokiec,F., Raymond,E., 2006. Preclinical and clinical development of novel agents that target the protein kinase C family. Semin. Oncol. 33**,** 466-478.

47. Shintani-Ishida,K., Uemura,K., Yoshida,K., 2007. Hemichannels in cardiomyocytes open transiently during ischemia and contribute to reperfusion injury following brief ischemia. Am. J. Physiol Heart Circ. Physiol 293**,** H1714-H1720.

48. Souroujon,M.C., Mochly-Rosen,D., 1998. Peptide modulators of protein-protein interactions in intracellular signaling. Nat. Biotechnol. 16**,** 919-924.

49. Stout,C.E., Costantin,J.L., Naus,C.C., Charles,A.C., 2002. Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. J. Biol. Chem. 277**,** 10482-10488.

50. Thimm,J., Mechler,A., Lin,H., Rhee,S., Lal,R., 2005. Calcium-dependent open/closed conformations and interfacial energy maps of reconstituted hemichannels. J. Biol. Chem. 280**,** 10646-10654.

51. van Veen,A.A., van Rijen,H.V., Opthof,T., 2001. Cardiac gap junction channels: modulation of expression and channel properties. Cardiovasc. Res. 51**,** 217-229.

52. van Veen,T.A., van Rijen,H.V., Jongsma,H.J., 2006. Physiology of cardiovascular gap junctions. Adv. Cardiol. 42**,** 18-40.

53. Vergara,L., Bao,X., Cooper,M., Bello-Reuss,E., Reuss,L., 2003. Gap-junctional hemichannels are activated by ATP depletion in human renal proximal tubule cells. J. Membr. Biol. 196**,** 173-184.

54. Weng,S., Lauven,M., Schaefer,T., Polontchouk,L., Grover,R., Dhein,S., 2002. Pharmacological modification of gap junction coupling by an antiarrhythmic peptide via protein kinase C activation. FASEB J. 16**,** 1114-1116.

55. Xiao,G.Q., Mochly-Rosen,D., Boutjdir,M., 2003. PKC isozyme selective regulation of cloned human cardiac delayed slow rectifier K current. Biochem. Biophys. Res. Commun. 306**,** 1019-1025.

56. Xiao,G.Q., Qu,Y., Sun,Z.Q., Mochly-Rosen,D., Boutjdir,M., 2001. Evidence for functional role of epsilonPKC isozyme in the regulation of cardiac Na(+) channels. Am. J. Physiol Cell Physiol 281**,** C1477-C1486.

57. Ye,Z.C., Wyeth,M.S., Baltan-Tekkok,S., Ransom,B.R., 2003. Functional hemichannels in astrocytes: a novel mechanism of glutamate release. J. Neurosci. 23**,** 3588-3596.

58. Ytrehus,K., Liu,Y., Downey,J.M., 1994. Preconditioning protects ischemic rabbit heart by protein kinase C activation. Am. J. Physiol 266**,** H1145-H1152.

59. Zatta,A.J., Kin,H., Lee,G., Wang,N., Jiang,R., Lust,R., Reeves,J.G., Mykytenko,J., Guyton,R.A., Zhao,Z.Q., Vinten-Johansen,J., 2006. Infarct-sparing effect of myocardial postconditioning is dependent on protein kinase C signalling. Cardiovasc. Res. 70**,** 315-324.

Chapitre IV:

Connexin 43 Mimetic Peptide Gap26 Confers Protection to Intact Heart Against Myocardial Ischemia Injury.

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Abstract

Unapposed connexin 43 hemichannels (Cx43Hc) are present on sarcolemma of cardiomyocytes. Whereas Cx43Hc remain closed during physiological conditions, their opening under ischemic stress contributes to irreversible tissue injury and cell death. To date, conventional blockers of connexin channels act unselectively on both gap junction channels and unapposed hemichannels. Here, we test the hypothesis that Gap26, a synthetic structural-mimetic peptide deriving from the first extracellular loop of Cx43 and a presumed selective blocker of Cx43Hc, confers resistance to intact rat heart against ischemia injury. Langendorff-perfused intact rat hearts were utilized. Regional ischemia was induced by 40 min occlusion of the left anterior descendent coronary and followed by 180 min of reperfusion. Gap26 was applied either 10 min before or 30 min after the initiation of ischemia. Interestingly, myocardial infarct size was reduced by 48% and 55% in hearts treated with Gap26 before or during ischemia, respectively, compared to untreated hearts. Additionally, myocardial perfusate flow was increased in both groups during reperfusion by 37% and 32%, respectively. Application of Gap26 increased survival of isolated cardiomyocytes after simulated ischemia-reperfusion by nearly 2-fold compared to untreated cells. On the other hand, superfusion of tsA201 cells transiently expressing Cx43 with Gap26 caused 61% inhibition of Cx43Hc-mediated currents recorded using the patch clamp technique. In summary, we demonstrate for the first time that Cx43-mimetic peptide Gap26 confers protection to intact heart against ischemia-reperfusion injury whether administered before or after the occurrence of

ischemia. In addition, we provide unequivocal evidence for Gap26 inhibitory effect on genuine Cx43Hc.

Keywords: Myocardial Infarction, Connexin 43, Connexin Hemichannels, Peptides, Prevention, Treatment.

Introduction

Connexin 43 hemichannels (Cx43Hc) are hydrophilic transmembrane pathways formed by hexameric associations of Cx43 subunits. While most Cx43Hc are engaged in gap junction channels (GJC), some of them remain unapposed on the sarcolemma (Verselis *et al.*, 2000). Unapposed Cx43Hc perform functions different from those achieved by GJC mainly by providing a pathway between cytosol and extracellular space to ions and other small metabolites (Goodenough and Paul, 2003; Bennett *et al.*, 2003).

During physiological conditions, unapposed Cx43Hc remain predominantly closed (Contreras *et al.*, 2003;Saez *et al.*, 2005). However, their opening is enhanced under non-physiological conditions like ischemia (John *et al.*, 1999; Kondo *et al.*, 2000; Contreras *et al.*, 2002; Vergara *et al.*, 2003). The increased activity of Cx43Hc is thought to disturb cellular homeostasis by inducing abnormal elevation of intracellular Na^+ and Ca^{2+} loads (Li *et al.*, 2001), release of ATP(Braet *et al.*, 2003a;Braet *et al.*, 2003b) and osmotic imbalance (Tranum-Jensen *et al.*, 1981; Steenbergen *et al.*, 1985; Jennings *et al.*, 1986; Quist *et al.*, 2000). Consequently, cell swelling (Steenbergen *et al.*, 1985) and death (Wilde and Aksnes, 1995; Shintani-Ishida *et al.*, 2007) occur. The purpose of this study is to investigate whether specific inhibition of unapposed Cx43Hc renders intact hearts more resistant to ischemia and reduces the resulting infarct size.

Conventional blockers of connexin channels act unselectively on both GJC and unapposed hemichannels, independently of the connexin isoforms forming these channels (Scemes *et al.*, 2009). Interestingly, synthetic connexin-deriving structural-mimetic peptides (CxMPs) emerged as powerful and unique tools capable to block

unapposed hemichannels with little or no immediate effects on GJC. While initially developed with the intention to modulate the function of GJC (Dahl, 1996; Chaytor *et al.*, 1997; Chaytor *et al.*, 1998; Kwak and Jongsma, 1999; Evans and Boitano, 2001), subsequent studies unravelled their preferential action on unapposed hemichannels (Leybaert *et al.*, 2003; Braet *et al.*, 2003a; Braet *et al.*, 2003b; Gomes *et al.*, 2005; Evans *et al.*, 2006). By mimicking short amino acid sequences on connexins extracellular loops, CxMPs bind to these structures and cause inhibition of unapposed hemichannels by a yet undetermined mechanism (see review by (Dahl, 2007). Because each CxMP contains a conserved motif that is not consistently found in other connexins or cell surface proteins, it is generally admitted that CxMPs interact specifically with connexins in an isoform-selective manner. Intriguingly, direct evidence for the CxMP-mediated inhibitory effect on genuine and unequivocally identified connexin hemichannels has never been reported.

Here, we utilized *for the first time* Gap26, a structural CxMP of the Cx43 first extracellular loop and a presumed selective blocker of Cx43Hc, to assess its potential role in the protection of intact heart against regional ischemia and to substantiate its inhibitory effect on Cx43Hc. We demonstrate that acute exposure to Gap26 whether administered before or during ischemia provides protection to heart against ischemia injury. We also utilized the ion channels-deficient tsA201 cells to assess the functional effect of Gap26 on exogenous Cx43Hc. The results provide the most direct evidence so far for the specific inhibitory effect of Gap26 on genuine Cx43Hc-mediated currents using a remarkably smaller concentration than previously reported (Romanov *et al.*,

2007).

Materials and methods

Peptides

Amino acid sequences for Gap26 peptide and its biological inactive scrambled version (sGap26) are VCYDKSFPISHVR and YSIVCKPHVFDRS, respectively. Both peptides were synthesized with ≥85% purity by Sheldon Biotechnology Inc, Québec, Canada. In all experiments, peptides were utilized at a final concentration of 0.5 μM.

Intact heart study

Male Sprague-Dawley rats weighing 350 to 450 g were handled in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the Comité d'Éthique de l'Expérimentation Animale at our research center. Anaesthesia was induced with ketamine/xylazine, 50 mg/kg and 5 mg/kg, respectively. After thoracotomy, the heart was quickly excised, canulated via the aorta and perfused on a non-recirculating Langendorff perfusion apparatus using Krebs-Henseleit solution containing (in mM): 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.8 $CaCl_2$ and 5 glucose (pH 7.4) at 37ºC. The perfusate was oxygenated by continuous bubbling with 95% O_2/ 5% CO_2. Ten min were allowed for each heart to stabilize. Regional ischemia was then induced by ligation of the left anterior descendent (LAD) coronary artery using a 4.0 surgical thread. After 40 min ischemia, reperfusion was

induced by removing ligation for 180 min. Ischemia was confirmed by myocardial surface cyanosis and reduction in myocardial perfusate flow (MPF).

The MPF was determined by measuring the volume of perfusate recovered from Langendorff-perfused heart during one minute at 10 min after LAD occlusion or at 10 min after reperfusion and compared to the basal MPF (BMPF). The BMPF corresponds to the volume of perfusate recovered from the Langendorff-perfused heart during one minute at 11 min before the LAD occlusion.

When it applied, peptides were introduced in the perfusion solution starting at 10 min before or 30 min after the LAD occlusion (i.e. 10 min before reperfusion) depending on the experimental group as outlined in Figure 1.

To determine the area at risk, the LAD was re-occluded and the heart was infused with 2 mL of 0.5% Evans Blue (Aldrich, USA) via the aorta. To determine infarct size, the heart was frozen at -80^{o}C for 40 min and transversely sectioned. Heart slices were next incubated in 1.5% triphenyltetrazolium dye for 10 min at 37^{o}C. After both stages, slices were scanned into computer. The perfused area (stained in dark blue), the infarct area (whitish zone) and the area at risk (the red zone including whitish infarct areas) were measured using a homemade image analysis software.

Isolated cardiomyocytes study

Langendorff-perfused hearts were washed from blood with oxygenated Tyrode solution containing (in mM): 128 NaCl, 0.47 NaH_2PO_4, 1.18 $MgSO_4 \cdot 7H_2O$, 20.1 $NaHCO_3$, 11.1 dextrose, 4.69 KCl and 2.23 $CaCl_2 2 \cdot 2H_2O$ for 5 min at 37^{o}C (Bendukidze *et al.*, 1985).

Next, hearts were stabilized for 5 min with calcium free solution containing (in mM): 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 5 $MgSO_4 \cdot 7H_2O$, 20 glucose, 50 Taurine, 10 MOPS, pH 7.4. After stabilization, hearts were digested during 5 min by perfusing with the same calcium-free solution to which 13.3 U/mL collagenase type I (Roche Diagnostics, Laval, Québec) was added. Ventricular tissues were chopped with surgical scissors and cardiomyocytes were dispersed gently using a wide-tipped pipette. Cells were pelleted by low speed centrifugation for 1 min and washed with Krebs-Henseleit buffer.

To simulate ischemia, cells were washed twice with degassed glucose-free Krebs-Henseleit buffer then gassed with N_2 for 15 min and pelleted as before. After removing 90% of the supernatant, mineral oil was layered over the remaining buffer to create an air-tight environment for 40 min as previously described (Armstrong *et al.*, 1994;Armstrong *et al.*, 1997). To simulate reperfusion, mineral oil was removed, cells were then washed with oxygenated Krebs-Henseleit buffer and incubated in the same buffer for 3 hours at 37^{o}C. Five groups of isolated myocytes were prepared for the study (Fig. 1): a control group using Gap26-free solutions, two groups in which Gap26 was introduced in Krebs-Henseleit solution either 10 min before or 30 min after the onset of ischemia, and two groups using the inactive sGap26 introduced either 10 min before or 30 min after the onset of ischemia. Gap26 concentration was kept constant until the end of experiments.

Cell viability was assessed using Trypan blue exclusion and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays. In the former test, viable cells were counted in a hemocytometer using 0.4% Trypan blue. Dead cells with

damaged membranes absorb the blue dye and were therefore distinguishable from viable unstained cells under light microscope. In MTT assay (Mosmann, 1983) viability was deduced from the ability of cells to reduce the tetrazolium salt, MTT, and to form a blue formazan product by the mitochondrial enzyme succinate dehydrogenase . To this end, cells were incubated in 0.5 mg/mL MTT for 4 hours at 37^{o}C. Then, a 100 µL of solubilisation solution (0.04 M HCl-isopropanol) was added. The amount of MTT formazan product was quantified by measuring the optical density at 570 nm. The mitochondrial activity in each experimental group was presented as a percentage of the mitochondrial activity measured in a control group of cardiomyocytes maintained at normoxic conditions during a similar time frame, i.e. oxygenated Krebs-Henseleit buffer at 37^{o}C during 4 hours.

Electrophysiological study

Hemichannel-mediated currents were studied in tsA201 cells transiently transfected with connexin-enconding plasmids as previously described (Hawat and Baroudi, 2008). The tsA201 cells are ion channels-deficient mammalian cell line derived from the human embryonic kidney HEK293 cells by stable transfection with SV40 large T antigen (Margolskee *et al.*, 1993). Briefly, cells were grown in high-glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin G (100 U/mL), and streptomycin (10 mg/mL; Invitrogen Canada Inc, Burlington, ON) in 5% CO_2-humidified atmosphere.

Using the calcium phosphate method, cells were transfected with 7 μg of rat pBlue-Cx40, 43 or 45 and 7 μg of EBO plasmid, encoding for the CD8 surface antigen. All plasmids were a generous gift from Prof. Pascal Daleau (Université Laval, QC). Two to three days post-transfection, cells were incubated for 2 min prior to patch clamp experiments in medium containing anti-CD8-coated beads (Invitrogen Canada, Inc). Transfected cells expressing CD8 and therefore binding beads were distinguished from non-transfected cells by light microscopy.

Cx43Hc currents were recorded in whole cell configuration of the patch clamp technique using Axopatch200B amplifier (Molecular Devices, CA) as previously described (Hawat and Baroudi, 2008). For whole-cell recordings, the pipette solution contained (in mM): 140 KCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 ethylene glycol tetraacetic acid (EGTA) adjusted to pH 7.2. The bath solution contained (in mM): 140 NaCl, 1 $MgCl_2$, 5.4 KCl, 1.8 $CaCl_2$, 10 HEPES, and 10 EGTA adjusted to pH 7.2. Data were digitized with a Digidata1440A analog-to-digital convertor and analyzed using pClamp 10.1 software (Molecular Devices, CA).

Statistical analysis

Data are expressed as mean ± SE. Analysis of variance (ANOVA) adapted to factorial design (groups and time) with orthogonalization according to Winer (Winer, 1978) was used. Differences were deemed significant when $P<0.05$.

Results

Study on intact hearts

Administration of Gap26 before ischemia significantly decreased infarct size from 36.3 ± 2.1 of area at risk in untreated hearts (n=7) to 18.8 ± 1.9% (n=6, $P<0.05$) (Fig 2a). In order to assess the peptide capability to counteract an existing ischemia, a separate group of hearts was studied in which Gap26 was introduced 30 min after the LAD coronary occlusion. Similarly, the infarct size was significantly reduced to 16.2 ± 0.8% (n=7, $P<0.05$) of area at risk. These results correspond to infarct size reductions by 48.2% and 55.4% when Gap26 was introduced before or during ischemia, respectively. The size of area at risk to total ventricles did not change significantly in both experimental groups (41.3 ± 4.5%, n=6, $P>0.05$ and 40.6 ± 3.2%, n=7, $P>0.05$, respectively) compared to the group of untreated hearts (44.8 ± 2.0%, n=7) (Fig. 2b). As negative control, sGap26 was also tested. Administration of sGap26 did not statistically affect the size of infarct area to area at risk when introduced before ischemia (41.1 ± 3.0%, n=3, $P>0.05$) or during ischemia (39.2 ± 2.0%, n=4, $P>0.05$) in comparison to untreated hearts (36.3 ± 2.1%, n=7). The global size of total ventricles was comparable between groups of untreated hearts and hearts treated with Gap26 or sGap26 (data not shown). Percentage values for different heart areas obtained from various experimental groups are listed in Table 1. Transversal slices obtained from hearts representing the groups of untreated hearts, hearts treated with Gap26 before ischemia and hearts treated with Gap26 during ischemia are shown in Fig. 2c, 2d and 2e.

In order to investigate the effect of Gap26 on the function of Langendorff-perfused hearts, we compared the MPF measured 10 min after LAD occlusion and 10 min after reperfusion to the BMPF. The BMPF is determined for each heart during normal conditions, i.e. before LAD occlusion in the absence of Gap26. Application of Gap26 to non-ischemic hearts during 1 hour did not cause significant changes to MPF (data not shown). Importantly, addition of Gap26 in the perfusion solution increased MPF during reperfusion of ischemic hearts from 65.7 ± 5.1% (n=7) of BMPF in untreated hearts to 89.8 ± 4.9% (n=7, $P<0.05$) and 86.7 ± 4.8% (n=4, $P<0.05$) in hearts treated with Gap26 before or during ischemia, respectively (Fig. 3). As negative control, sGap26 did not affect MPF when introduced before or during ischemia. Values for MPF variations in different experimental groups are listed in Table 1.

Study on isolated cardiomyocytes

In order to elucidate the cellular basis for the Gap26-mediated cardioprotection observed in intact hearts, we investigated the effect of peptide on isolated cardiomyocytes from adult rat hearts subjected to simulated ischemia-reperfusion. Application of Gap26 in the bath solution either 10 min before or 30 min after the initiation of simulated ischemia increased the number of surviving cardiomyocytes after 180 min reperfusion from 28.4 ± 6.5% of total cells (n=8) to 53.1 ± 6.8% (n=8, $P<0.05$) and 55.0 ± 6.5% (n=8, $P<0.05$), respectively (Fig. 4a). The percentage of surviving cells did not differ statistically between groups treated with Gap26 before or during simulated ischemia. As negative control, the percentage of surviving cells following application of sGap26 before

ischemia (22.9 ± 6.9%, n=8) or during ischemia (26.0 ± 6.6%, n=8) did not differ significantly in comparison to untreated cells (28.4 ± 6.5%, n=8, $P>0.05$). In order to substantiate these findings, we assessed mitochondrial activity in each experimental group of cardiomyocytes using the colorimetric MTT assay. To alleviate comparison, results are expressed as percentages of the mitochondrial activity measured in a control group of cardiomyocytes maintained at normoxic conditions during a similar time frame. Simulated ischemia-reperfusion reduced mitochondrial activity in untreated cardiomyocytes to 49.7 ± 1.2%, n=5, $P<0.05$ in comparison to the control cardiomyocytes 100.0 ± 1.2%, n=5. Treatment of cardiomyocytes with Gap26 prevented partially but significantly this mitochondrial dysfunction when introduced before or during ischemia (83.0 ± 1.1%, n=5, $P<0.05$ and 82.1 ± 1.1%, n=5, $P<0.05$, respectively). Application of sGap26 did not prevent the mitochondrial dysfunction when applied before ischemia (50.3 ± 1.0%, n=5, $P>0.05$) or during ischemia (51.8 ± 0.9%, n=5, $P>0.05$) in comparison to untreated cells. Results obtained from the different experimental groups are illustrated in the histogram (Fig. 4b).

Gap26 inhibits Cx43Hc currents

Because intracellular ionic imbalance is a key factor in the mechanism of cell death during ischemic stress, we investigated the functional effect of Gap26 on Cx43Hc-mediated currents in individual tsA201 cells transiently expressing Cx43. Figure 5a shows a family traces of macroscopic Cx43Hc-mediated currents elicited by

depolarizing the cell from a resting potential of +0 mV to test pulses ranging from +0 mV to +130 mV in a low calcium bath solution. To substantiate the identity of currents recorded from Cx43-transfected cells, patch clamp experiments were performed on non-transfected tsA201 cells. No currents could be recorded from these cells (Fig. 5b) therefore confirming the absence of contaminating endogenous currents. In Figure 5c, a representative time course recording shows rapid Cx43Hc currents reduction when Gap26 was introduced in bath solution. A steady state inhibition was reached in all experiments. Averaged data indicate that Gap26 caused 60.1 ± 4.6% (n=5, $P<0.05$) current reduction. In presence of sGap26 peptide, the amplitude of Cx43Hc currents did not vary over a similar time frame (99.0 ± 1.1%, n=5, $P>0.05$) (Fig. 5d). The histogram (Fig. 5e) summarizes effects of both peptides on normalized Cx43Hc currents. Curves representing the current-voltage (I-V) relationship for Cx43Hc in absence and in presence of Gap26 are illustrated in Fig.5f. I-V data were elicited by depolarizing cells with voltage steps ranging from +0 mV to +90 mV.

Because connexin 40 (Cx40) and connexin 45 (Cx45) may also form hemichannels in the heart, we examined whether Gap26 has effects on currents recorded from tsA201 cells transfected with either connexin isoforms. Importantly, application of Gap26 did not cause significant reduction in currents mediated by Cx40Hc (0.7 ± 1.6%, n=4, $P>0.05$) or Cx45Hc (0.2 ± 0.7%, n=4, $P>0.05$) over a time frame similar to that used in Cx43Hc experiments (Fig. 6).

Discussion

The most important finding in this study is the protection that Cx43-mimetic peptide Gap26 conferred to intact rat heart against regional ischemia induced by LAD coronary occlusion. We showed that administration of Gap26 prior to LAD occlusion resulted in more than 48% reduction of infarct size compared to untreated hearts. Similarly, Gap26 reduced infarct size by 55% when administered during ischemia. We also showed that the salutary effect of Gap26 extends to heart function by increasing the MPF during reperfusion by nearly 37% and 32% when the peptide was administered before or during LAD occlusion, respectively, in comparison to untreated hearts. These results indicate that whereas Gap26 can confer resistance to 'normal' hearts against subsequent ischemia, it also has capability to salvage injured hearts when administered after the occurrence of ischemia. Hypothetically, we ascribe these effects to the presumable Gap26-mediated inhibition of cardiomyocytic Cx43Hc opened by the ischemic stress. Indeed, Gap26 is also known to inhibit Cx43 GJCs when used at relatively high concentrations and/or following prolonged exposure (Evans *et al.*, 2006). Nonetheless, all experiments in this study were performed using low concentration of Gap26 (0.5μM) previously shown to selectively inhibit hemichannels without directly affecting GJCs (Verma *et al.*, 2009; Clarke *et al.*, 2009). Therefore, we consider that neither the peptide concentration nor the duration of exposure to Gap26 (which varied depending on protocols between ~3 and 4 hours) were sufficient to modulate GJCs in the studied hearts.

In concordance with the fact that death of cardiomyocytes, and therefore myocardial injury, principally occurs during reperfusion (Gottlieb *et al.*, 1994; Zhao *et al.*, 2000; Shintani-Ishida *et al.*, 2007), both structural and functional improvements in intact hearts did not differ significantly whether Gap26 was introduced before occlusion or before reperfusion. To investigate the cellular basis of the protection of intact hearts, we assessed the effect of Gap26 on isolated cardiomyocytes subjected to simulated ischemia-reperfusion. We found a nearly 2-fold increase in the number of surviving cells when Gap26 was administered either before or during the simulated ischemia. A similar increase was previously noted in isolated neonatal rat cardiomyocytes treated with Gap26 prior to simulated ischemia (Shintani-Ishida *et al.*, 2007). Clearly, both observations underscore the capability of Gap26 to confer protection to cardiomyocytes in absence of direct intercellular communication, precisely through GJC, and therefore point to the inhibition of unapposed hemichannels as the underlying mechanism of cardioprotection.

Another important finding in this study is the rapid depression of Cx43Hc electrical conductance in response to the application of Gap26. To date, the involvement of unequivocally identified unapposed hemichannels in CxMPs-mediated phenomena, such as regulation of ATP release and calcium propagation, is still debated (Dahl, 2007). Here, we showed for the first time that superfusion of the ion channels-deficient tsA201 cells, transiently expressing Cx43, with Gap26 readily inhibits Cx43Hc-mediated currents recorded from individual cells. This observation complements with previous studies reporting on inhibitory effect of Gap26 on the *permeability* of Cx43Hc (Leybaert

et al., 2003; Braet *et al.*, 2003a; Gomes *et al.*, 2005; Pearson *et al.*, 2005), the second major functional characteristic of connexin channels beside conductance. Although not surprisingly, this result represents the most direct evidence reported so far for the Gap26 inhibitory effect on Cx43Hc. Curiously, this inhibition occurred using a peptide concentration that is 1000 times lower than what has previously been utilized to block hemichannel currents (0.5 μM in this study *versus* 500 μM in (Romanov *et al.*, 2007). Occasionally, the specificity of CxMPs toward hemichannels has been challenged (Wang *et al.*, 2007). Therefore, we tested the effect of Gap26 on hemichannels of Cx40 and Cx45 which are also present in the heart. Importantly, Gap26 did not affect currents from Cx40Hc or Cx45Hc.

Altogether, these results strongly suggest that the observed cardioprotection conferred by Gap26 is most likely mediated by the specific inhibition of Cx43Hc opened by the ischemic stress.

Scientific context and perspectives

The non-junctional Cx43 have previously been associated with ischemic preconditioning-mediated cardioprotection (Padilla *et al.*, 2003; Li *et al.*, 2004), a phenomenon whereby brief episodes of ischemia reduce adverse effects of subsequent prolonged ischemia (Murry *et al.*, 1986; Shiki and Hearse, 1987; Cohen *et al.*, 1991). This effect has been related to increase in Cx43 phosphorylation (Schulz *et al.*, 2003; Hatanaka *et al.*, 2004; Miura *et al.*, 2004; Hund *et al.*, 2007). Interestingly, different studies pointed to the isozyme epsilon of protein kinase C (εPKC) as principal mediator of ischemic preconditioning-mediated cardioprotection (Gray *et al.*, 1997; Ping *et al.*, 1997; Liu *et al.*, 1999; Saurin *et al.*, 2002; Inagaki *et al.*, 2003; Hund *et al.*, 2007). More recently, we demonstrated using a unique set of PKC isozyme-specific modulator peptides that the εPKC, among other PKC isozymes, selectively inhibits the conductance of Cx43Hc (Hawat and Baroudi, 2008). These data prompt us to believe that inhibition of Cx43Hc is also a fundamental basis for the cardioprotection conferred by ischemic preconditioning. In the light of our current findings and given the ubiquitous expression of PKC and the abundance of its substrates within cells and throughout tissues, we put forth that the selective inhibition of the abnormally open Cx43Hc, which we believe are solely localized in the ischemic region of the heart, would be more suitable to mimic pharmacologically the cardioprotection conferred by ischemic preconditioning than to modulate PKC isozymes as previously proposed (Chen *et al.*, 2001a; Chen *et al.*, 2001b). The accessibility of CxMP binding sites from extracellular space, which

circumvents the need of conjugating the peptides with transmembrane carriers and the limitations deriving from their use, is indeed another therapeutic advantage over use of intracellular modulators. Certainly, studies using more elaborated experimental models are needed to substantiate the therapeutic potentials of Gap26.

In conclusion, we provide the most direct evidence so far for the inhibitory effect of Gap26 on Cx43Hc-specific currents. We demonstrate for the first time that administration of Gap26 prior to ischemia prevents injury by making intact heart more resistant to ischemic stress. Moreover, usage of Gap26 as a treatment following occurrence of ischemia reduces cardiac tissue damage and improves intact heart function. The discovery of new agents capable to make heart more resistant to ischemia and/or to improve its recovery after injury caused by ischemia will certainly be promising tools to fight ischemic heart disease.

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Conflict of Interest

None declared.

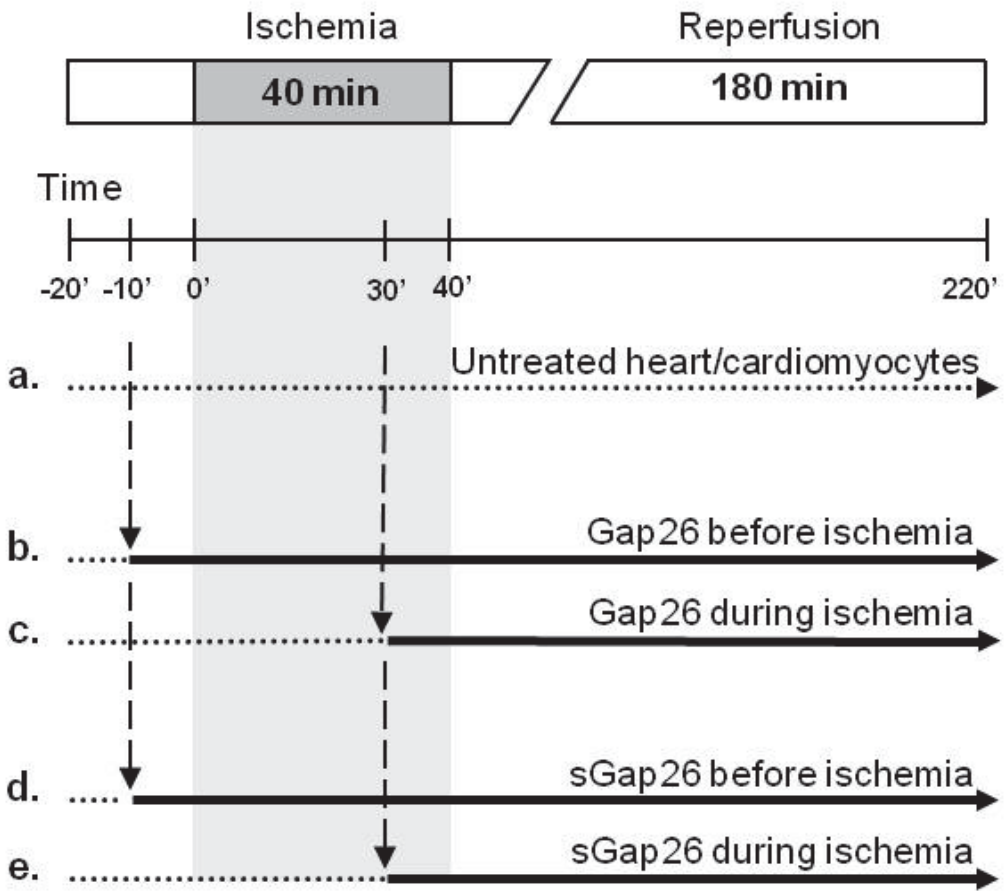
Ischemia
Reperfusion
40 min
180 min
Time
-20'
-10'
0'
30'
40'
220'
a.
Untreated heart/cardiomyocytes
b.
Gap26 before ischemia
c.
Gap26 during ischemia
d.
sGap26 before ischemia
e.
sGap26 during ischemia

Figure 1.

Figure 1. A schematic representation of different experimental groups utilized to assess the effect of Gap26 during ischemia-reperfusion, in intact heart and isolated cardiomyocytes. The gray zone covers the ischemic period. The dashed arrows indicate the time when peptide is introduced. (a) Untreated hearts/cardiomyocytes; (b) hearts/cardiomyocytes treated with Gap26 starting 10 min before ischemia; (c) hearts/cardiomyocytes treated with Gap26 starting 30 min after the onset of ischemia; (d) hearts/cardiomyocytes treated with sGap26 starting 10 min before ischemia; and, (e) hearts/cardiomyocytes treated with sGap26 starting 30 min after the onset of ischemia.

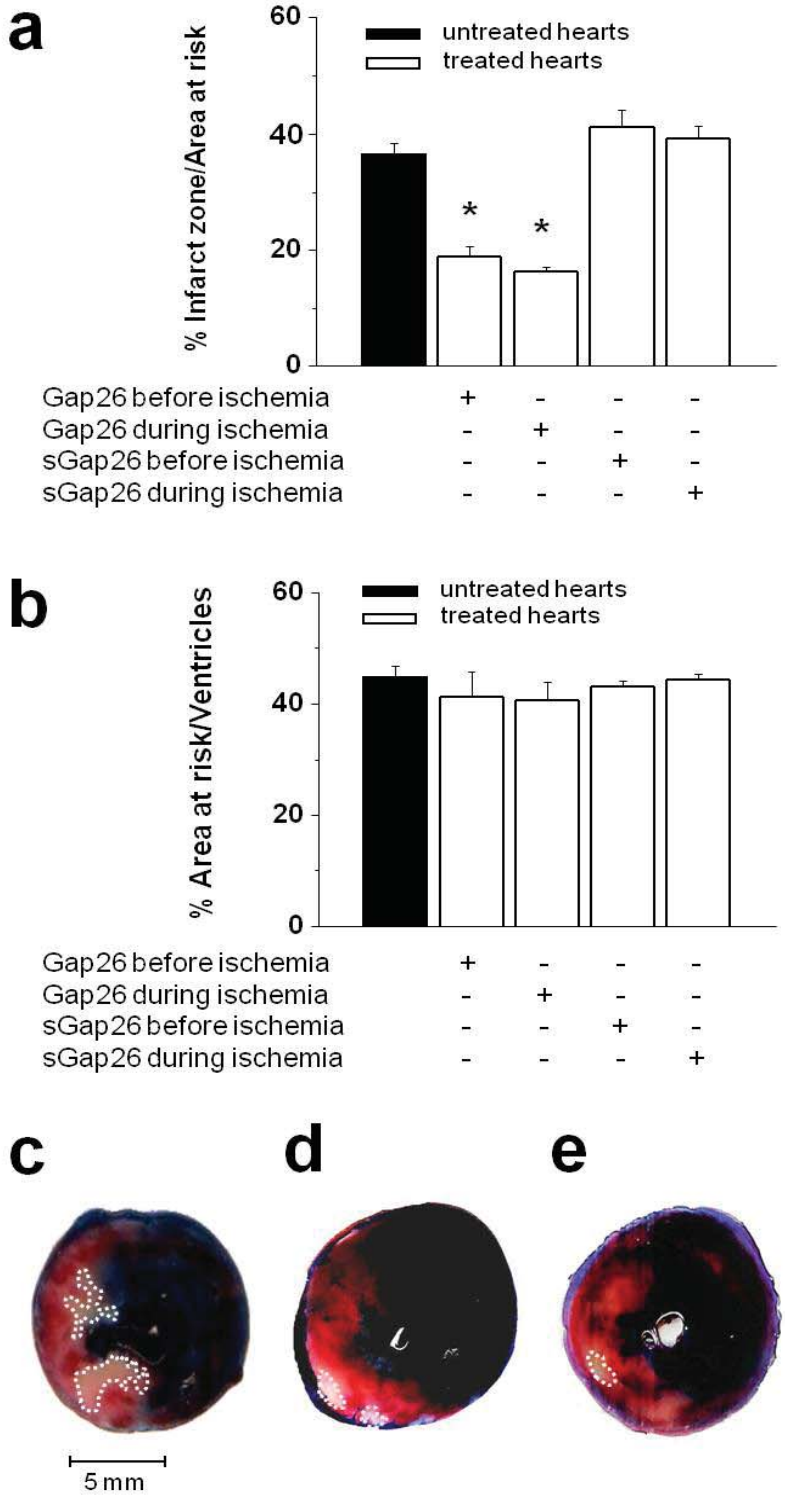
a
% Infarct zone/Area at risk
60
40
20
0
untreated hearts
treated hearts
*
*
Gap26 before ischemia + - - -
Gap26 during ischemia - + - -
sGap26 before ischemia - - + -
sGap26 during ischemia - - - +
b
% Area at risk/Ventricles
60
40
20
0
untreated hearts
treated hearts
Gap26 before ischemia + - - -
Gap26 during ischemia - + - -
sGap26 before ischemia - - + -
sGap26 during ischemia - - - +
c
d
e
5 mm

Figure 2.

Figure 2. Effect of synthetic peptides on intact hearts subjected to regional ischemia by LAD ligation. (a) Histogram representing the proportion of infarct zone to the area at risk, (b) Histogram representing the proportion of area at risk to total ventricles. Values obtained from ischemic hearts in presence of Gap26 or sGap26 added either 10 min before (n=6 and n=3, respectively) or 30 min after (n=7 and n=4, respectively) the occlusion of LAD are reported. * indicates statistically significant difference in comparison to untreated hearts (n=7), $P<0.05$. Photographs of TTC-stained heart sections after 40 min ischemia and 180 min reperfusion representing (c) untreated heart; (d) heart treated with Gap26 introduced 10 min before LAD occlusion; and (e) Gap26 introduced 30 min after LAD occlusion. In panels c-e, the infarct zone is the whitish area delimited with white dashed lines; the area at risk corresponds to the red region including the infarct zone; and the perfused area is in dark blue.

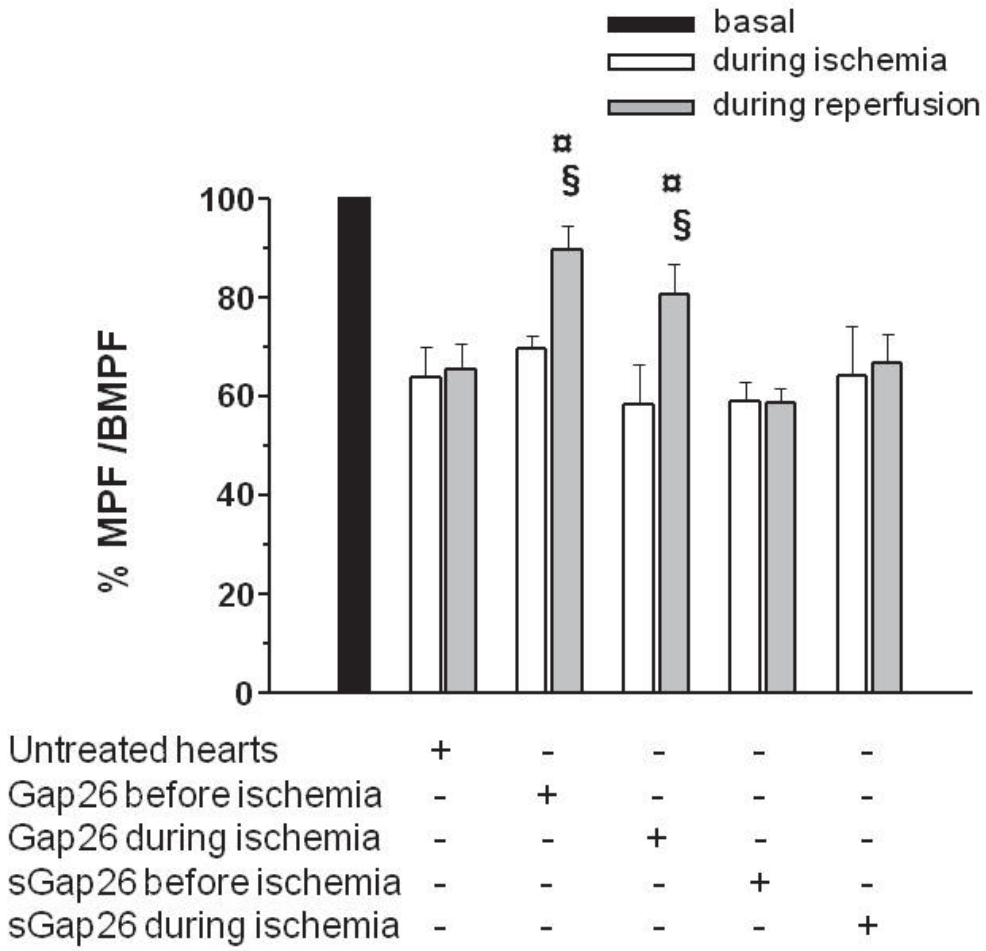
basal
during ischemia
during reperfusion
% MPF /BMPF
100
80
60
40
20
0
¤
§
¤
§
Untreated hearts + - - - -
Gap26 before ischemia - + - - -
Gap26 during ischemia - - + - -
sGap26 before ischemia - - - + -
sGap26 during ischemia - - - - +

Figure 3.

Figure 3. Percentages of MPF measured at 10 min after LAD occlusion (open columns) or at 10 min after reperfusion (filled columns) to BMPF. The groups shown correspond to the untreated hearts (n=7), hearts treated with Gap26 before ischemia (n=7), hearts treated with Gap26 during ischemia (n=4), hearts treated with the inactive sGap26 before ischemia (n=3), and heart treated with sGap26 during ischemia (n=4). ¤ indicates data significantly different from untreated hearts, $P<0.05$. § indicates data significantly different from MPF during ischemia, $P<0.05$.

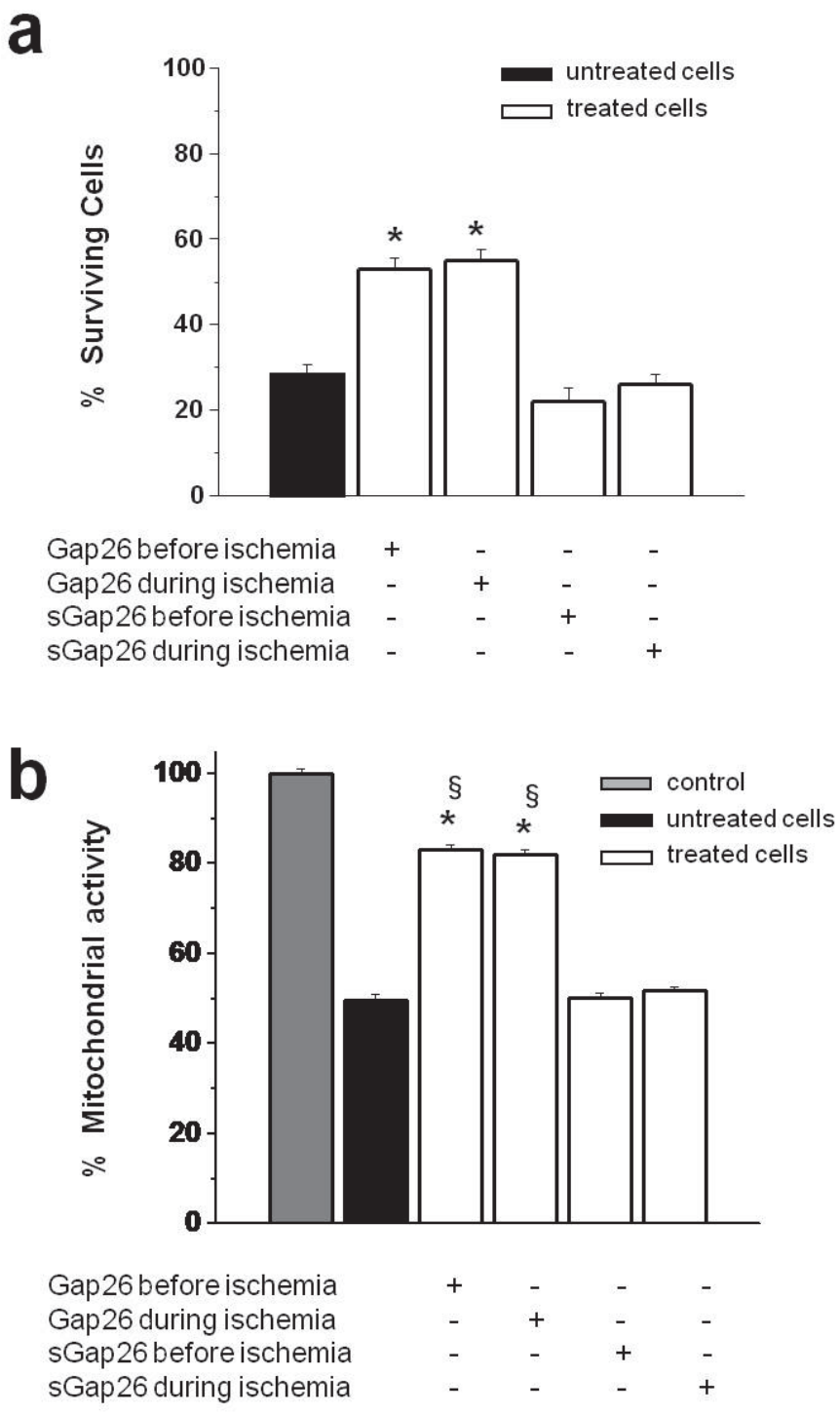
a
% Surviving Cells
100
80
60
40
20
0
untreated cells
treated cells
*
*
Gap26 before ischemia + - - -
Gap26 during ischemia - + - -
sGap26 before ischemia - - + -
sGap26 during ischemia - - - +
b
% Mitochondrial activity
100
80
60
40
20
0
control
untreated cells
treated cells
§
*
§
*
Gap26 before ischemia + - - -
Gap26 during ischemia - + - -
sGap26 before ischemia - - + -
sGap26 during ischemia - - - +

Figure 4.

Figure 4. Effect of Gap26 on viability of isolated adult rat cardiomyocytes during simulated ischemia-reperfusion. (a) The percentage of surviving cells to total cells after reperfusion was determined in absence or presence of Gap26 or sGap26 added to the bath solution before or during ischemia (n=8 in each group). Data are expressed as mean +/- SEM of eight experiments using myocytes from 3 different animals; (b) Mitochondrial dysfunction was determined with the MTT test (n=5 in each group, obtained from a single rat heart). * indicates significant difference in comparison to untreated cells, $P<0.05$. § indicates significant difference in comparison to control, $P<0.05$.

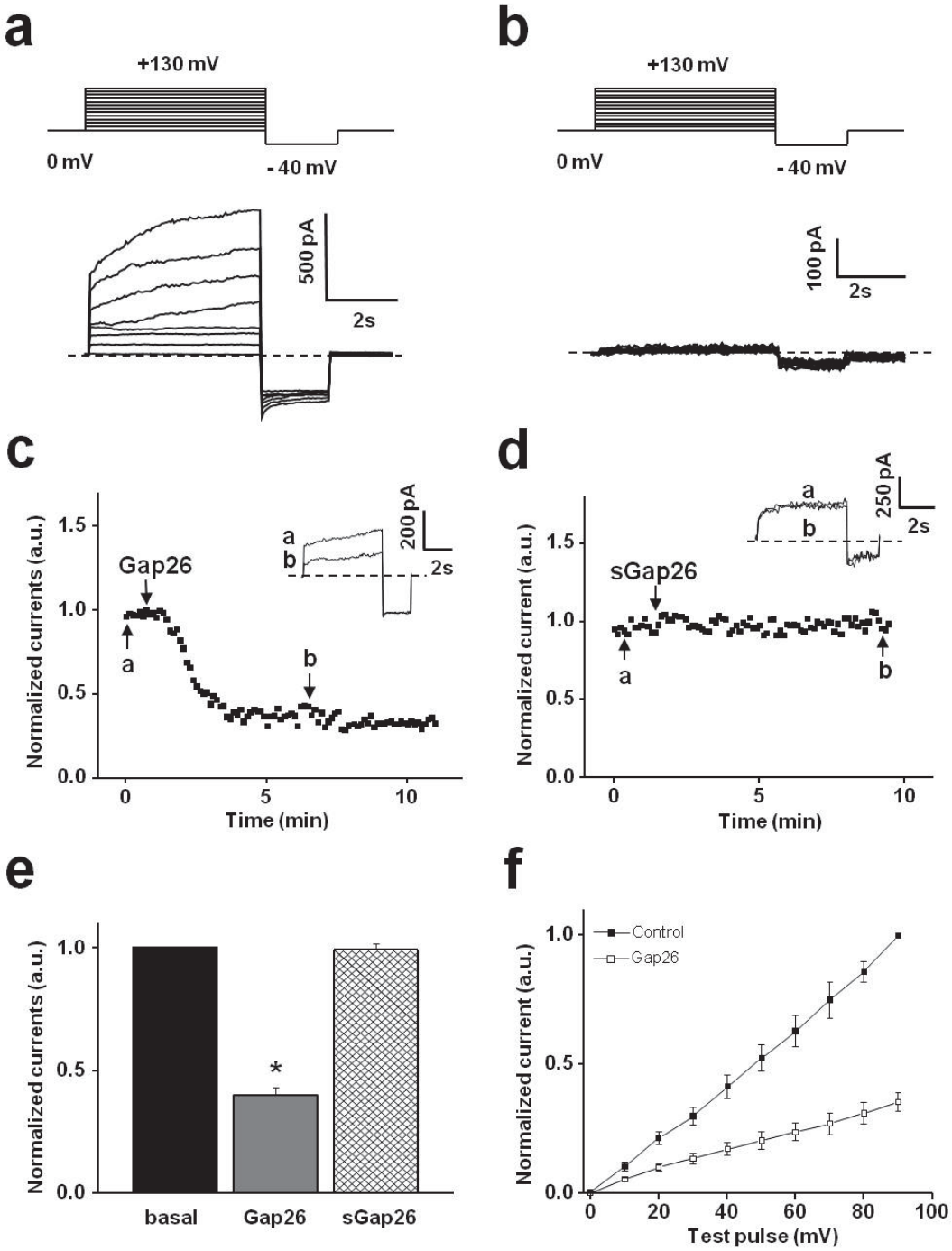
a
+130 mV
0 mV
- 40 mV
500 pA
2s
b
+130 mV
0 mV
- 40 mV
100 pA
2s
c
Normalized currents (a.u.)
Gap26
a
b
200 pA
2s
Time (min)
d
Normalized current (a.u.)
sGap26
a
b
250 pA
2s
Time (min)
e
Normalized currents (a.u.)
basal
Gap26
sGap26
f
Normalized current (a.u.)
Control
Gap26
Test pulse (mV)

Figure 5.

Figure 5. Cx43Hc-mediated whole-cell current inhibition by Gap26 recorded from individual tsA201 cells expressing exogenous Cx43. (a) A family traces of Cx43Hc-mediated currents recorded from a typical cell by depolarizing from a holding potential of 0 mV to test pulses ranging from 0 mV to +130 mV. (b) non detectable currents could be recorded from a non transfected tsA201 cell using same protocol as in (a). The dashed line represents the zero current. Time course recorded from typical cells dialysed with Gap26 (c) and sGap26 (d). Insets illustrate Cx43Hc current traces in response to membrane depolarisation recorded at time points indicated by arrows a and b. (e) Histogram showing percentage of Cx43Hc currents inhibition in response to Gap26 (n=5) and sGap26 (n=6). Current amplitudes are normalized against control and represented in arbitrary units (a.u.). * $P<0.05$ in comparison to basal currents. (f) I–V curves obtained from Cx43-expressing tsA201 cells in the absence (n=5) and in the presence (n=5) of Gap26.

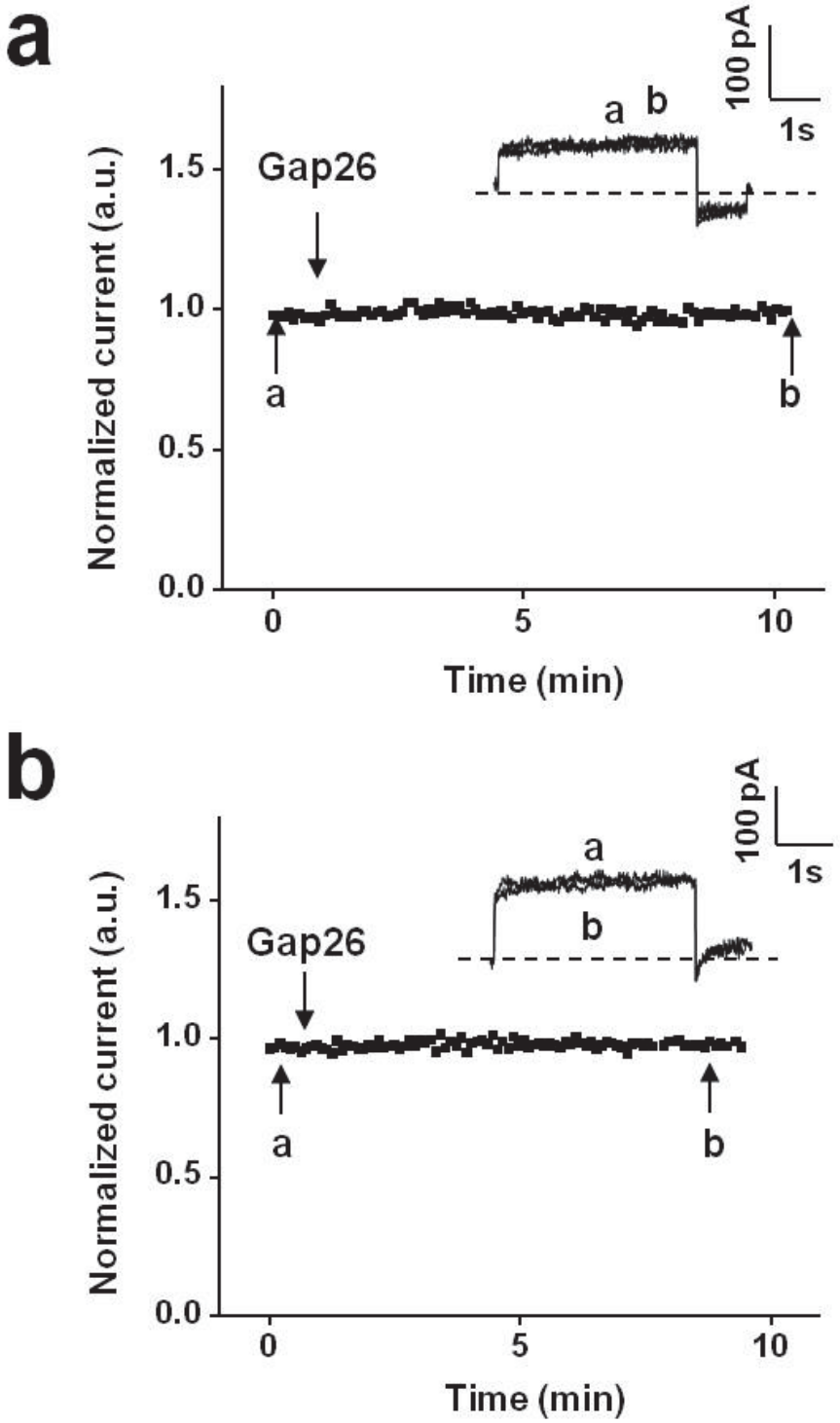
a
100 pA
1s
a b
Gap26
Normalized current (a.u.)
1.5
1.0
0.5
0.0
a
b
0
5
10
Time (min)
b
100 pA
1s
a
b
Gap26
Normalized current (a.u.)
1.5
1.0
0.5
0.0
a
b
0
5
10
Time (min)

Figure 6.

Figure 6. Effect of Gap26 on Cx40Hc and Cx45Hc. Time course recording from a typical individual tsA201 cell expressing Cx40 (a) or Cx45 (b) dialysed with Gap26. Insets illustrate the current traces recorded at time points indicated by arrows a and b.

Table 1: Percentage values for heart areas and myocardial perfusate flows

Groups	IZ/AAR (%)	AAR/V (%)	n	MPFI/BMPF (%)	MPFR/BMPF (%)	n
Untreated hearts	36.3 ± 2.1	44.8 ± 2.0	7	63.79 ± 6.2	65.7 ± 5.1	7
Gap26 before ischemia	18.8 ± 1.9 §	41.3 ± 4.5	6	69.9 ± 2.3	89.8 ± 4.9 § ¤	7
Gap26 during ischemia	16.2 ± 0.8 §	40.6 ± 3.2	7	58.50 ± 7.4	86.7 ± 4.8 § ¤	4
sGap26 before ischemia	41.1 ± 3.0	43.1 ± 0.9	3	59.07 ± 3.7	57.1 ± 3.1	3
sGap26 during ischemia	39.2 ± 2.0	44.4 ± 1.0	4	64.23 ± 9.9	66.7 ± 5.9	4

Table 1.

Table 1. IZ infarct zone, AAR area at risk, V ventricles, MPFI myocardial perfusate flow measured during ischemia, MPFR myocardial perfusate flow measured during reperfusion, BMPF basal myocardial flow. § Statistically different in comparison to untreated group. ¤ Statistically different in comparison to MPF during ischemia.

Reference List

1. Armstrong,S., Downey,J.M., Ganote,C.E., 1994. Preconditioning of isolated rabbit cardiomyocytes: induction by metabolic stress and blockade by the adenosine antagonist SPT and calphostin C, a protein kinase C inhibitor. Cardiovasc. Res. 28**,** 72-77.

2. Armstrong,S.C., Kao,R., Gao,W., Shivell,L.C., Downey,J.M., Honkanen,R.E., Ganote,C.E., 1997. Comparison of in vitro preconditioning responses of isolated pig and rabbit cardiomyocytes: effects of a protein phosphatase inhibitor, fostriecin. J. Mol. Cell Cardiol. 29**,** 3009-3024.

3. Bendukidze,Z., Isenberg,G., Klockner,U., 1985. Ca-tolerant guinea-pig ventricular myocytes as isolated by pronase in the presence of 250 microM free calcium. Basic Res. Cardiol. 80 Suppl 1**,** 13-17.

4. Bennett,M.V., Contreras,J.E., Bukauskas,F.F., Saez,J.C., 2003. New roles for astrocytes: gap junction hemichannels have something to communicate3. Trends Neurosci. 26**,** 610-617.

5. Braet,K., Aspeslagh,S., Vandamme,W., Willecke,K., Martin,P.E., Evans,W.H., Leybaert,L., 2003a. Pharmacological sensitivity of ATP release triggered by photoliberation of inositol-1,4,5-trisphosphate and zero extracellular calcium in brain endothelial cells. J. Cell Physiol 197**,** 205-213.

6. Braet,K., Vandamme,W., Martin,P.E., Evans,W.H., Leybaert,L., 2003b. Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26. Cell Calcium 33**,** 37-48.

7. Chaytor,A.T., Evans,W.H., Griffith,T.M., 1997. Peptides homologous to extracellular loop motifs of connexin 43 reversibly abolish rhythmic contractile activity in rabbit arteries1. J. Physiol 503 (Pt 1)**,** 99-110.

8. Chaytor,A.T., Evans,W.H., Griffith,T.M., 1998. Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries2. J. Physiol 508 (Pt 2)**,** 561-573.

9. Chen,L., Hahn,H., Wu,G., Chen,C.H., Liron,T., Schechtman,D., Cavallaro,G., Banci,L., Guo,Y., Bolli,R., Dorn,G.W., Mochly-Rosen,D., 2001a. Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC1. Proc. Natl. Acad. Sci. U. S. A 98**,** 11114-11119.

10. Chen,L., Wright,L.R., Chen,C.H., Oliver,S.F., Wender,P.A., Mochly-Rosen,D., 2001b. Molecular transporters for peptides: delivery of a cardioprotective epsilonPKC agonist peptide into cells and intact ischemic heart using a transport system, R(7). Chem. Biol. 8**,** 1123-1129.

11. Clarke,T.C., Williams,O.J., Martin,P.E., Evans,W.H., 2009. ATP release by cardiac myocytes in a simulated ischaemia model: inhibition by a connexin

mimetic and enhancement by an antiarrhythmic peptide1. Eur. J. Pharmacol. 605**,** 9-14.

12. Cohen,M.V., Liu,G.S., Downey,J.M., 1991. Preconditioning causes improved wall motion as well as smaller infarcts after transient coronary occlusion in rabbits. Circulation 84**,** 341-349.

13. Contreras,J.E., Saez,J.C., Bukauskas,F.F., Bennett,M.V., 2003. Gating and regulation of connexin 43 (Cx43) hemichannels8. Proc. Natl. Acad. Sci. U. S. A 100**,** 11388-11393.

14. Contreras,J.E., Sanchez,H.A., Eugenin,E.A., Speidel,D., Theis,M., Willecke,K., Bukauskas,F.F., Bennett,M.V., Saez,J.C., 2002. Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. Proc. Natl. Acad. Sci. U. S. A 99**,** 495-500.

15. Dahl,G., 1996. Where are the gates in gap junction channels? Clin. Exp. Pharmacol. Physiol 23**,** 1047-1052.

16. Dahl,G., 2007. Gap junction-mimetic peptides do work, but in unexpected ways2. Cell Commun. Adhes. 14**,** 259-264.

17. Evans,W.H., Boitano,S., 2001. Connexin mimetic peptides: specific inhibitors of gap-junctional intercellular communication1. Biochem. Soc. Trans. 29**,** 606-612.

18. Evans,W.H., De,V.E., Leybaert,L., 2006. The gap junction cellular internet: connexin hemichannels enter the signalling limelight2. Biochem. J. 397**,** 1-14.

19. Gomes,P., Srinivas,S.P., Van,D.W., Vereecke,J., Himpens,B., 2005. ATP release through connexin hemichannels in corneal endothelial cells10. Invest Ophthalmol. Vis. Sci. 46**,** 1208-1218.

20. Goodenough,D.A., Paul,D.L., 2003. Beyond the gap: functions of unpaired connexon channels. Nat. Rev. Mol. Cell Biol. 4**,** 285-294.

21. Gottlieb,R.A., Burleson,K.O., Kloner,R.A., Babior,B.M., Engler,R.L., 1994. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J. Clin. Invest 94**,** 1621-1628.

22. Gray,M.O., Karliner,J.S., Mochly-Rosen,D., 1997. A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death5. J. Biol. Chem. 272**,** 30945-30951.

23. Hatanaka,K., Kawata,H., Toyofuku,T., Yoshida,K., 2004. Down-regulation of connexin43 in early myocardial ischemia and protective effect by ischemic preconditioning in rat hearts in vivo. Jpn. Heart J. 45**,** 1007-1019.

24. Hawat,G., Baroudi,G., 2008. Differential modulation of unapposed connexin 43 hemichannel electrical conductance by protein kinase C isoforms. Pflugers Arch. 456**,** 519-527.

25. Hund,T.J., Lerner,D.L., Yamada,K.A., Schuessler,R.B., Saffitz,J.E., 2007. Protein kinase Cepsilon mediates salutary effects on electrical coupling induced by ischemic preconditioning. Heart Rhythm. 4**,** 1183-1193.

26. Inagaki,K., Hahn,H.S., Dorn,G.W., Mochly-Rosen,D., 2003. Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. Circulation 108**,** 869-875.

27. Jennings,R.B., Reimer,K.A., Steenbergen,C., 1986. Myocardial ischemia revisited. The osmolar load, membrane damage, and reperfusion. J. Mol. Cell Cardiol. 18**,** 769-780.

28. John,S.A., Kondo,R., Wang,S.Y., Goldhaber,J.I., Weiss,J.N., 1999. Connexin-43 hemichannels opened by metabolic inhibition. J. Biol. Chem. 274**,** 236-240.

29. Kondo,R.P., Wang,S.Y., John,S.A., Weiss,J.N., Goldhaber,J.I., 2000. Metabolic inhibition activates a non-selective current through connexin hemichannels in isolated ventricular myocytes. J. Mol. Cell Cardiol. 32**,** 1859-1872.

30. Kwak,B.R., Jongsma,H.J., 1999. Selective inhibition of gap junction channel activity by synthetic peptides. J. Physiol 516 (Pt 3)**,** 679-685.

31. Leybaert,L., Braet,K., Vandamme,W., Cabooter,L., Martin,P.E., Evans,W.H., 2003. Connexin channels, connexin mimetic peptides and ATP release. Cell Commun. Adhes. 10**,** 251-257.

32. Li,F., Sugishita,K., Su,Z., Ueda,I., Barry,W.H., 2001. Activation of connexin-43 hemichannels can elevate [Ca(2+)]i and [Na(+)]i in rabbit ventricular myocytes during metabolic inhibition. J. Mol. Cell Cardiol. 33**,** 2145-2155.

33. Li,X., Heinzel,F.R., Boengler,K., Schulz,R., Heusch,G., 2004. Role of connexin 43 in ischemic preconditioning does not involve intercellular communication through gap junctions. J. Mol. Cell Cardiol. 36**,** 161-163.

34. Liu,G.S., Cohen,M.V., Mochly-Rosen,D., Downey,J.M., 1999. Protein kinase C-epsilon is responsible for the protection of preconditioning in rabbit cardiomyocytes. J. Mol. Cell Cardiol. 31**,** 1937-1948.

35. Margolskee,R.F., McHendry-Rinde,B., Horn,R., 1993. Panning transfected cells for electrophysiological studies. Biotechniques 15**,** 906-911.

36. Miura,T., Ohnuma,Y., Kuno,A., Tanno,M., Ichikawa,Y., Nakamura,Y., Yano,T., Miki,T., Sakamoto,J., Shimamoto,K., 2004. Protective role of gap junctions in preconditioning against myocardial infarction. Am. J. Physiol Heart Circ. Physiol 286**,** H214-H221.

37. Mosmann,T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65**,** 55-63.

38. Murry,C.E., Jennings,R.B., Reimer,K.A., 1986. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74**,** 1124-1136.

39. Padilla,F., Garcia-Dorado,D., Rodriguez-Sinovas,A., Ruiz-Meana,M., Inserte,J., Soler-Soler,J., 2003. Protection afforded by ischemic preconditioning is not mediated by effects on cell-to-cell electrical coupling during myocardial ischemia-reperfusion. Am. J. Physiol Heart Circ. Physiol 285**,** H1909-H1916.

40. Pearson,R.A., Dale,N., Llaudet,E., Mobbs,P., 2005. ATP released via gap junction hemichannels from the pigment epithelium regulates neural retinal progenitor proliferation. Neuron 46**,** 731-744.

41. Ping,P., Zhang,J., Qiu,Y., Tang,X.L., Manchikalapudi,S., Cao,X., Bolli,R., 1997. Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. Circ. Res. 81**,** 404-414.

42. Quist,A.P., Rhee,S.K., Lin,H., Lal,R., 2000. Physiological role of gap-junctional hemichannels. Extracellular calcium-dependent isosmotic volume regulation. J. Cell Biol. 148**,** 1063-1074.

43. Romanov,R.A., Rogachevskaja,O.A., Bystrova,M.F., Jiang,P., Margolskee,R.F., Kolesnikov,S.S., 2007. Afferent neurotransmission mediated by hemichannels in mammalian taste cells. EMBO J. 26**,** 657-667.

44. Saez,J.C., Retamal,M.A., Basilio,D., Bukauskas,F.F., Bennett,M.V., 2005. Connexin-based gap junction hemichannels: gating mechanisms. Biochim. Biophys. Acta 1711**,** 215-224.

45. Saurin,A.T., Pennington,D.J., Raat,N.J., Latchman,D.S., Owen,M.J., Marber,M.S., 2002. Targeted disruption of the protein kinase C epsilon gene abolishes the infarct size reduction that follows ischaemic preconditioning of isolated buffer-perfused mouse hearts. Cardiovasc. Res. 55**,** 672-680.

46. Scemes,E., Spray,D.C., Meda,P., 2009. Connexins, pannexins, innexins: novel roles of "hemi-channels". Pflugers Arch. 457**,** 1207-1226.

47. Schulz,R., Gres,P., Skyschally,A., Duschin,A., Belosjorow,S., Konietzka,I., Heusch,G., 2003. Ischemic preconditioning preserves connexin 43 phosphorylation during sustained ischemia in pig hearts in vivo. FASEB J. 17**,** 1355-1357.

48. Shiki,K., Hearse,D.J., 1987. Preconditioning of ischemic myocardium: reperfusion-induced arrhythmias. Am. J. Physiol 253**,** H1470-H1476.

49. Shintani-Ishida,K., Uemura,K., Yoshida,K., 2007. Hemichannels in cardiomyocytes open transiently during ischemia and contribute to reperfusion injury following brief ischemia. Am. J. Physiol Heart Circ. Physiol 293**,** H1714-H1720.

50. Steenbergen,C., Hill,M.L., Jennings,R.B., 1985. Volume regulation and plasma membrane injury in aerobic, anaerobic, and ischemic myocardium in vitro. Effects of osmotic cell swelling on plasma membrane integrity. Circ. Res. 57**,** 864-875.

51. Tranum-Jensen,J., Janse,M.J., Fiolet,W.T., Krieger,W.J., D'Alnoncourt,C.N., Durrer,D., 1981. Tissue osmolality, cell swelling, and reperfusion in acute regional myocardial ischemia in the isolated porcine heart. Circ. Res. 49**,** 364-381.

52. Vergara,L., Bao,X., Cooper,M., Bello-Reuss,E., Reuss,L., 2003. Gap-junctional hemichannels are activated by ATP depletion in human renal proximal tubule cells. J. Membr. Biol. 196**,** 173-184.

53. Verma,V., Hallett,M.B., Leybaert,L., Martin,P.E., Evans,W.H., 2009. Perturbing plasma membrane hemichannels attenuates calcium signalling in cardiac cells and HeLa cells expressing connexins. Eur. J. Cell Biol. 88**,** 79-90.

54. Verselis,V.K., Trexler,E.B., Bukauskas,F.F., 2000. Connexin hemichannels and cell-cell channels: comparison of properties. Braz. J. Med. Biol. Res. 33**,** 379-389.

55. Wang,J., Ma,M., Locovei,S., Keane,R.W., Dahl,G., 2007. Modulation of membrane channel currents by gap junction protein mimetic peptides: size matters. Am. J. Physiol Cell Physiol 293**,** C1112-C1119.

56. Wilde,A.A., Aksnes,G., 1995. Myocardial potassium loss and cell depolarisation in ischaemia and hypoxia. Cardiovasc. Res. 29**,** 1-15.

57. Winer,B.J., 1978. Statistics and data analysis: trading bias for reduced mean squared error. Annu. Rev. Psychol. 29**,** 647-681.

58. Zhao,Z.Q., Nakamura,M., Wang,N.P., Wilcox,J.N., Shearer,S., Ronson,R.S., Guyton,R.A., Vinten-Johansen,J., 2000. Reperfusion induces myocardial apoptotic cell death. Cardiovasc. Res. 45**,** 651-660.

Chapitre V:

Single Intravenous Low-Dose Injections of Connexin 43 Mimetic Peptides Protect Ischemic Heart In Vivo Against Myocardial Infarction

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Abstract

The opening of unapposed connexin 43 hemichannels (Cx43Hc) under ischemic stress leads to cell death and irreversible tissue injury. Here, we investigate for the first time *in vivo* the cardioprotective potentials of two unique Cx43 structural-mimetic peptides (Cx43MPs) presumed specific blockers of Cx43Hc, Gap26 and Gap27, when injected intravenously using a rat model of myocardial infarction.

Sprague Dawley rats were utilized. Myocardial infarction was induced by occluding the left anterior descendent coronary during 40 min followed by 2 days of reperfusion. Interestingly, single bolus injections of Gap26 or Gap27 (1μg/Kg) into jugular vein caused infarct size reductions by up to 61% with reference to control rats injected with saline at similar timings. Infarct reductions did not vary significantly whether peptides were administered before or after the onset of ischemia. Although the two peptides allegedly interact with distinct structures of Cx43, co-administration of Gap26/Gap27 in equal doses did not confer additive protection to hearts (maximum infarct reduction by 64%). Using patch clamp technique, we provide unique and direct evidence for the inhibitory effect of Cx43MPs on genuine human Cx43Hc transiently expressed in the ion channel-deficient tsA201 cells. In concordance with the cardioprotective effect observed *in vivo,* co-application of both peptides did not cause cumulative current inhibition. A safety profile of Cx43MPs was also addressed.

Our results reveal great therapeutic potential of Cx43MPs in treatment of myocardial infarction. Their practical way and timing of administration and their apparent safe profile make them promising tools to fight ischemic heart disease.

Keywords: Myocardial infarction; Connexin 43; Hemichannels; Peptides; Prevention; Treatment.

Abbreviations

Cx: Connexin

Cx43: Connexin 43

Hc: hemichannels

MPs: Connexin-deriving structural mimetic peptides

LAD: Left anterior descendant coronary

sGap26: scrambled Gap26

sGap27: scrambled Gap27

1. Introduction

In cardiac myocytes, most sarcolemmal connexin 43 hemichannels (Cx43Hc) appose with counterparts from adjacent cells to form gap junction channels. Until apposition occurs, the unapposed Cx43Hc remain predominantly closed [1]. Abnormally, these Hc can open under ischemic stress [2-4]. The increased permeability of Hc leads to disturbances in cellular homeostasis [5, 6] and uncontrolled release of ATP [7, 8], which consequently causes cell death and irreversible tissue injury [9-11].

Initially, the role of Cx43 in myocardial ischemia injury was suggested by Garcia-Dorado and collaborators by pharmacological inhibition with heptanol, a nonspecific inhibitor of Cx channels that blocks both gap junction channels and unapposed Hc [12]. This was later confirmed by others [13] and extended to other nonspecific blockers [14]. Similar results have also been described in transgenic mice models [15, 16]. Remarkably, whereas all these studies addressed the role of connexins forming gap junctions, none of them exclude the possibility that these effects were due to actions on unapposed Hc. In fact, several other studies - including ours - support the hypothesis that unapposed CxHc are key mediators of ischemia injury [7, 11, 17-20].

In contrast with the classical inhibitors of Cx channels that are neither specific (Hc *versus* Gap junction channels) nor selective (vis-à-vis various Cx isoforms), synthetic Cx structural-mimetic peptides (CxMPs) have emerged more recently as unique and powerful specific blockers of unapposed Hc that exert little or no effect on gap junction channels [21, 22]. CxMPs are also believed to interact selectively with various Cx isoforms. Until recently, the inhibitory effect of CxMPs has never been demonstrated

directly and specifically on identified Hc but rather deduced indirectly [23]. Exceptionally, we recently demonstrated using the ion channel-deficient tsA201 cells transiently expressing the rat variant of Cx43 that Gap26, a Cx43MP mimicking the first extracellular loop of Cx43, readily inhibits macroscopic Cx43Hc-mediated currents. In support to its alleged selectivity, we also demonstrated that Gap26 does not inhibit currents from other cardiac CxHc (i.e., Cx40Hc and Cx45Hc) [20].

Interestingly, treatment with Gap26 of isolated cardiomyocytes *in vitro* and of excised rat hearts *ex vivo* made these experimental models significantly resistant to ischemia injury [20]. In concordance with these findings, we hypothesized here that Cx43MPs, if administered in a more complex setting (i.e. systemically *in vivo*), will preserve - at least partially - similar protective potentials against ischemia injury. Therefore, we assess here for the first time *in vivo* the cardioprotective potentials of the only two known Cx43MPs, Gap26 and Gap27 mimicking the first and the second extracellular loops of Cx43 respectively, when administered intravenously in low-dose single bolus injections before or after the onset of ischemia. We also investigate the functional effect of both Cx43MPs directly on human Cx43Hc (hCx43Hc) exogenously expressed in a human cellular expression system. To our knowledge, this work represents the foremost attempt yet to assess CxMPs therapeutic applicability in a complete organism for these increasingly promising tools in fighting ischemic diseases

2. Methods

2.1 Peptides

Amino acid sequences for Cx43MPs are VCYDKSFPISHVR for Gap26 and SRPTEKTIFII for Gap27, their biological inactive scrambled versions are YSIVCKPHVFDRS for sGap26 and SETKIRPITFI for sGap27. The sequence of a flag-tagged Gap26 peptide utilized for confocal microscopy imaging consisted of the following 21-amino acid sequence, DYKDDDDKVCYDKSFPISHVR. All peptides were synthesized with ≥85% purity by Canpeptide Inc, Montréal, Canada. For intravenous injections, peptides were diluted in saline solution and administered at a 1 μg/Kg dose when injected individually or 1 μg/Kg for each peptide when injected in combination. For patch clamp experiments, peptides were diluted in bath solution as previously described [20] to a final concentration of 0.5 μmol/L when peptides are tested individually or 0.5 μmol/L for each peptide when peptides are combined.

2.2 Immunostaining

Four to six-micron sections were cut from the heart of a rat injected with the flag-tagged Gap26. Tissues were then fixed using a 1:3 acetone/methanol solution for 20 min before they were permeabilized using 0.1% Triton into 1 mmol/L PBS-0.5% BSA solution and incubated with antibodies as previously described.[24] The mouse anti-flag primary antibody utilized in 1:200 dilution against the flag-tagged Gap26 peptide was purchased from ProtTech, Inc (Fairview Village, PA). The secondary antibody, a conjugated

DyLight594 goat anti-mouse, was purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA) and diluted at 1:400. To detect Cx43, we utilized a rabbit anti-Cx43 primary antibody targeted against the C-terminus diluted at 1:500 from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The corresponding secondary antibody (Conjugated DyLight488 anti-rabbit) used in 1:400 dilution was also from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA).

2.3 Confocal microscopy

Fluorescent probe-labelled myocardial tissues were viewed by a Bio-Rad MRC-1024 confocal imaging system equipped with a krypton-argon laser beam and mounted on a Zeiss microscope. A 60X oil objective with a 1.4 numerical aperture was used. Confocal settings were as follows: 1-mw laser power, 1.2 zoom, 1 second per scan, Kalman filter, and 4 frames per image. The photomultiplier gain was set to maximum, and the confocal aperture was adjusted for maximum resolution.

2.4 In vivo study

Male Sprague-Dawley rats weighing 350 to 450 g were handled in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). All procedures were approved by the *Comité d'Éthique de l'Expérimentation Animale* at our research center. Anesthesia was induced with ketamine/xylazine, 50 and 5 mg/kg intramuscular, respectively, after which the rats were intubated and placed on an artificial respirator and

anesthesia was maintained with isoflurane (1.5%) ventilation. Absence of response to painful stimuli was used as index to adequate anesthesia. A left thoracotomy was performed at the fifth intercostal space, and the left coronary artery was occluded with a silk suture. A bolus of saline with or without peptides was injected into jugular vein 10 min before or 30 min after the LAD occlusion (i.e., 10 min before reperfusion) (Fig. 2A). The occlusion was removed after 40 min, the chest was closed, and the animal was returned to its cage. Forty-eight hours later, a blood sample was drawn for testing and animals were then euthanized by excising heart under anesthesia by intramuscular injection of ketamine/xylazine (50 and 5 mg/kg, respectively). The heart, brain, lungs, liver, and kidneys were rapidly removed for histopathology study. To determine the area at risk in excised heart, the LAD was re-occluded and heart was infused with 2 mL of 0.5% Evans Blue (Aldrich, USA) via the aorta. To determine infarct size, the heart was frozen at −80°C for 40 min and transversely sectioned. Heart slices were next incubated in 1.5% triphenyltetrazolium dye for 10 min at 37°C. After both stages, slices were scanned into computer. The perfused area (stained in dark blue), the infarct area (whitish zone), and the area at risk (the red zone including whitish infarct areas) were measured using a homemade image analysis software following the method previously described by Reimer and Jennings [25].

2.5 Cell electrophysiology

Cx43Hc-mediated currents were studied in tsA201 cells transiently transfected with hCx43 gene as previously described [26]. Briefly, tsA201 cells were grown in high-

glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), penicillin G (100 U/ml), and streptomycin (10 mg/ml; Invitrogen Canada Inc, Burlington, ON) in 5% CO_2-humidified atmosphere. Cells were transfected using calcium phosphate method with 7 μg of human pcDNA3.1-Cx43 and 7 μg of EBO plasmid, encoding for the CD8 surface antigen, simultaneously. Two to three days post-transfection, cells were incubated for 2 min prior to patch clamp experiments in medium containing anti-CD8-coated beads (Invitrogen Canada, Inc). Transfected cells expressing CD8 and therefore binding beads are distinguished from nontransfected cells by light microscopy. Cx43Hc currents were recorded in whole cell configuration of the patch clamp technique using Axopatch200B amplifier (Molecular Devices, CA) as previously described [26]. For whole-cell recordings, the pipette solution contained (in mM): 140 KCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 ethylene glycol tetraacetic acid (EGTA) adjusted to pH 7.2. The bath solution contained (in mM): 140 NaCl, 1 $MgCl_2$, 5.4 KCl, 1.8 $CaCl_2$, 10 HEPES and 10 EGTA adjusted to pH 7.2. Data were digitized with Digidata 1440A analog-to-digital convertor and analyzed using pClamp 10.1 software both purchased from Molecular Devices, CA.

2.6 Western blot

Proteins were extracted from left ventricle tissues obtained from rats euthanized 40 min after reperfusion using a lysis buffer containing in mmol/L: 50 Tris-HCl (pH 7.5), 20 β-glycerophosphate, 20 NaF, 5 EDTA, 10 EGTA, 1 Na_3VO_4, supplemented with 0.2

mol/L PMSF, 1 mol/L DTT, 10% TritonX-100, 10 mg/mL leupeptin and 1 mmol/L microcystin. Samples were centrifuged at 10,000g for 30 min. Supernatants were then submitted to protein quantification. Equal amounts of protein lysates (50 μg) were boiled for 5 min and loaded on 12.5% polyacrylamide SDS-gel and transferred to a nitrocellulose membrane. The membranes were blocked with 10% non-fat dried milk in Tris-buffered saline containing 1% Tween (TBS-T) for 1 h at room temperature, washed with TBS-T and incubated overnight with anti-Cx43 primary antibody (Santa Cruz Biotechnology, CA) diluted 1:200 in TBS-T buffer. After sequential washes in TBS-T, membranes were incubated for 1 h with the corresponding horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted at 1:10,000 in TBS-T buffer then rinsed again in TBS-T and revealed with the SuperSignal West chemiluminescent substrate kit, according to the manufacturer's instructions (Thermo Scientific, IL). The blots were stripped with stripping solution containing 100 mmol/L Glycine, 1% sodium dodecyl sulphate (SDS), pH 2 and then reprobed with mouse anti-β-actin antibody (Jackson Immunoresearch laboratories Inc, PA) diluted at 1:1000 in TBS-T buffer to confirm equal loading of proteins in each lane. Comparison of bands intensity was performed using the online software ImageJ as described, (http://rsb.info.nih.gov/ij/index.html).

2.7 Histopathology study

Specimens were fixed in 10% buffered formaldehyde solution immediately after surgical excision and embedded in paraffin at (40-60°C) following standard procedures. Four to

six 2-μm sections were cut from each specimen. Before examination, tissue sections were sequentially deparaffinised and rehydrated through an alcohol gradient. Then, sections were stained with haematoxylin and eosin.

2.8 Blood count and chemistry

Three groups of Sprague-Dawley rats were injected with saline, Gap26 (1 μg/Kg) or Gap27 (1 μg/Kg). Blood samples were drawn 10 min before and 3 h after injection for testing. Blood count and biochemistry tests were carried out at the biology medical laboratory facilities at the Hôpital du Sacré-Cœur de Montréal, Montréal, Canada.

2.9 Statistical analysis

All data are expressed as mean ± SE. Analysis of variance (ANOVA) adapted to factorial design (groups and time) with orthogonalization according to Winer [27] was used. Differences were deemed significant when $P<0.05$.

3. Results

3.1 Localization of injected flag-tagged Gap26

Because peptides will be administered intravenously throughout the study, we first assessed using confocal microscopy whether the flag-tagged Gap26 can attain cardiomyocytes in the ventricular myocardium after being injected into a jugular vein of an adult rat. Interestingly, confocal microscopy experiments indicate that the peptide administered at a dose of 1 μg/Kg successfully reaches ventricular tissues (Fig. 1). Images from ventricular sections obtained 60 min after peptide injection illustrates the flag-tagged Gap26 peptide (in *red*) co-localizing with Cx43 (in *green*) on the lateral membranes of cardiac myocytes (in *orange*) where unapposed Hc preferentially exist, but not in the intercalated disks where gap junctions predominate (Fig. 1A-D). Noticeably, the superimposition of both colors in panels 1*C* and 1*D* also illustrates an intracellular co-localization pattern (in *orange*). This pattern was not observed in a control experiment where ventricular tissues from normal heart of a rat injected with saline alone was immunostained using the same procedure (Fig. 1E-F). Additionally, Cx43 was absent from lateral membranes of ventricular cardiomyocytes obtained from a normal heart thus ruling out the possibility that lateral membranes staining is caused by fixation artifact (Fig. 1E).

3.2 Effects of Cx43MPs on ischemic hearts

Three doses of Gap26 (0.5, 1 and 10 μg/Kg) were initially tested. All of them caused decrease in the myocardial infarct size when administered before ischemia (Fig. 2B) (Supplementary material, Table I). Because infarct size reductions did not vary significantly (P>0.05) when using 1 and 10 μg/Kg, we utilized 1 μg/Kg-dose in the rest of the study. For alleviation, infarct size is represented in the text as percentage of area at risk. Importantly, bolus injection of Gap26 before ischemia significantly decreased infarct size from 32.9 ± 1.5% (n=6) in control rats injected with saline at similar timing to 13.9 ± 1.3% (n=6, P<0.05) (Fig. 2C). In order to investigate the capability of Gap26 to counteract an existing ischemia under an emergency-like setting, a separate group of rats was studied where peptide was injected 30 min after the onset of ischemia - thus 10 min before reperfusion. Similarly, infarct size was reduced from 33.1 ± 1.7% (n=6) in rats treated with saline at similar timing to 12.9 ± 0.5% (n=6, P<0.05). These results correspond to infarct size reductions by 57.7 ± 2.8% and 61.0 ± 2.2% when Gap26 was injected before or after the onset of ischemia, respectively. The size of area at risk to total ventricles did not vary significantly in groups treated with Gap26 before or during ischemia (43.7 ± 1.3%, n=6, P>0.05 and 43.2 ± 1.5%, n=6, P>0.05, respectively) in comparison to control groups treated with saline at similar timings (45.1±1.0%, n=6; and 45.8 ± 1.0%, n=6, respectively) (Fig. 2D). These data confirm that changes in the infarct size among groups are not related to the size of area at risk that depends on the occlusion by our surgical technique. As negative control, a scrambled version of Gap26 (sGap26) was also tested. Administration of sGap26 did not affect infarct size when introduced

before ischemia (32.5 ± 1.0%, n=3, $P>0.05$) or during ischemia (31.6 ± 1.2%, n=4, $P>0.05$) in comparison to corresponding saline groups.

To assess cardiac cytolysis, troponin T blood levels were measured 48 h after ischemia. Although areas at risk were comparable between groups, Gap26-treatment before or during ischemia resulted in ~83% and 77% reduction in troponin T levels, respectively, with reference to the corresponding saline-controls (Fig. 2E). These results provide additional evidence for the cardioprotective effect exerted by Gap26 on ischemic hearts. Importantly, Cx43 protein levels did not differ in ventricles of rats treated with saline or Gap26 before ischemia as indicated by Western blot analysis (Fig. 2F). The ratio of Cx43 over β-actin is comparable between saline-control (0.99 ± 0.1, n=4) and Gap26-treated (0.96 ± 0.2, n=4; $P>0.05$) groups therefore ruling out the possibility that Gap26-treatment causes impairment of the overall Cx43 expression.

We next investigated the effect of Gap27, the only known structural-mimetic peptide of Cx43 besides Gap26. Although the infarct size was also reduced when rats were treated with Gap27 before ischemia (reduction by 37.7 ± 2.0%, n=6, $P<0.05$) or during ischemia (reduction by 40.5 ± 3.2%, n=6, $P<0.05$), the extent of reductions was significantly smaller than that observed using Gap26 (Fig. 2C).

Because each of the tested Cx43MPs allegedly interacts specifically with distinct structures of Cx43, we assessed whether co-injection of both Gap26 and Gap27 in equal doses could confer additive protection to ischemic hearts. Analysis of heart sections from rats injected with single bolus of combined Gap26/Gap27 peptides before or after the onset of ischemia did not cause statistically different infarct size reductions

(reduction by 63.5 ± 3.3%, n=6, $P<0.05$; and 64.3 ± 2.7%, n=6, $P<0.05$, respectively) compared to groups treated with Gap26 alone. Transversal sections from hearts representing groups treated with saline, Gap26, Gap27, and Gap26/Gap27 before ischemia are illustrated in Fig. 2G-J. Sizes of various heart areas obtained from different experimental groups are provided in supplementary material, Table I.

3.3 Effects of Cx43MPs on hCx43Hc currents

In order to elucidate the mechanism underlying the differential cardioprotection conferred by Cx43MPs on the cell level and to assess whether the reported salutary effects exerted in rat can be extrapolated to human, we assessed the effects of Gap26 and Gap27 - when administered individually or combined - directly on hCx43Hc-mediated currents. Figure 3A shows family traces of macroscopic hCx43Hc currents elicited by depolarizing a typical cell from a 0 mV resting potential to test-pulses ranging from 0 to +80 mV. To substantiate the identity of currents recorded from hCx43-transfected cells, patch clamp experiments were performed on non-transfected tsA201 cells. No current could be recorded from these cells (Fig. 3B) therefore confirming the absence of any contaminating endogenous currents. In Figure 3C and D, representative time course recordings show rapid currents reduction when Gap26 and Gap27 were introduced in bath solution, respectively. Steady state inhibition was reached in all experiments. Averaged data indicate current reductions by 55.6 ± 1.1 % (n=8, $P<0.05$) and 43.9± 2.3% (n=7, $P<0.05$) in presence of Gap26 or Gap27 (0.5 μM), respectively. Curiously, co-application of both peptides in equal concentrations did not cause additive inhibition

of the Cx43Hc-mediated currents (Fig. 3E). An average current inhibition by 60.1 ± 0.8 % (n=8) was statistically (P<0.05) but not significantly different from the inhibition when using Gap26 alone. Negative control peptides, sGap26 and sGap27, did not affect current amplitudes significantly over similar time frames (reduction by 1.8 ± 0.8 %, n=3, P>0.05 for sGap26 and 2.0±0.4%, n=3, P>0.05 for sGap27). The histogram in Fig. 3F summarizes effects of Cx43MPs on normalized hCx43Hc currents.

3.4 Safety remarks

Indeed, safety is a crucial issue in any potential therapeutic application involving Cx43MPs. Therefore, we document some observations that we think are relevant to this concern. First, although in some experimental groups heart rates during 1 to 2 h following peptide treatment were statistically different from controls (Supplementary material, Table I), these variations were not abnormal and remained within the normal range of the adult rat heart rate. On the other hand, while downregulation of Cx43 channels in general is known to be arrhythmogenic [28-30], we noticed only sporadic ventricular fibrillations occurring during the ischemic period while animals were still under surgery. A total of 5 rats died during ischemia following a ventricular fibrillation (Supplementary material, Table II). No arrhythmic events were noted during 1-2h of reperfusion while ECGs were still recorded. It should be noted, however, that mortality from undetermined reasons has also been remarked after the ECG recording period but remained comparable between groups (Supplementary material, Table III). A total of 9 rats died within 48 h of reperfusion. Besides heart, Cx43 is also expressed in many other

organs which raises the possibility that administration of Cx43MPs may exert adverse side effects on these organs. In this regard, examination of histological sections from brain, lung, kidney and liver failed to show apparent structural differences between experimental groups (data not shown). Similarly, peptide-treatment did not cause perceptible abnormal alterations in blood cell counts or serum chemistry parameters in blood samples drawn before and after treatment with Cx43MPs (Supplementary material, Table IV). Rats did not develop any apparent abnormal signs or behaviour during the 2 days after injection with Cx43MPs.

4. DISCUSSION

The main significance of this study resides essentially in the experimental setting where Cx43MPs are administered for the first time systemically *in vivo* through intravenous injections which represents an ideal physiological context to assess the therapeutic potentials and applicability of treatment with these molecules. We demonstrate that single bolus injections of Cx43MPs can successfully confer protection to adult rat hearts against ischemia injury and reduce the resulting infarct size significantly. The first important observation arose from the confocal microscopy study which unravelled the Gap26 capability to leave vasculature, attain ventricular myocardial tissues and colocalize with Cx43 on cell surfaces when injected through a jugular vein. Strikingly, while the peptide successfully reached lateral membranes of cardiomyocytes, it remained noticeably absent from intercalated discs where gap junction channels essentially form. Although the exact reason for this selective localization remains to be elucidated, we expect this pattern to result from the inaccessibility of Gap26 binding sites on Cx43 first extracellular loops due to their engagement in the apposition of Hc forming the gap junction channels. Regardless the reason behind this observation, the latter represents indeed a unique insight to explain the mechanism underlying the specificity of Gap26 toward the unapposed Cx43Hc. In addition to its peripheral localization on lateral membranes, staining for Gap26 was also noticed in intracellular spaces. Indeed, Cx43 are continuously formed and trafficked through the cytoplasm to the plasma membrane and then internalized and degraded [31]. Hence, the protein is

naturally present in various organelles involved in these processes including the ER, the Golgi apparatus, lysosomes and proteosomes [31] but also in other subcellular structures such as mitochondria [32]. Therefore, the intracellular staining for Cx43 and thus the flag-tagged Gap26 as observed by confocal microscopy was not unexpected.

While decrease of infarct size was previously reported *ex vivo* when Gap26 [20] and Gap27 [33] were directly infused into hearts via inert perfusate buffers, our results here underscore the capability of these peptides to preserve their cardioprotective potentials even when injected in the systemic circulation at a relatively remote site from injury (see Opanasopit et al. [34] for factors affecting drug delivery). Effectively, the injection of any or both Cx43MPs using a substantially low dose of 1 μg/Kg caused infarct size reduction by up to ~65% compared to animals treated with saline only. Curiously, the extent of the infarct size reductions caused by single doses of Gap26 as reported here exceeded those we have previously observed using the *ex vivo* model of myocardial infarction where isolated rat hearts were constantly perfused with a nonrecirculating Gap26-containing buffer (concentration of Gap26 maintained stable at 0.5μM) during ischemia and for three hours following reperfusion [20]. Although we cannot assert the exact reason behind this dissimilarity between the two experimental settings, we believe this may be caused at least partially by the enhanced oxygenation of myocardium when (re)perfused with whole blood compared to perfusate buffer [35].

Theoretically, we ascribe the Cx43MPs cardioprotective effect to the specific inhibition of sarcolemmal unapposed Cx43Hc. Indeed, when high concentrations are utilized,

Cx43MPs have also been shown to inhibit Cx43 gap junction channels [36]. Whereas the exact concentration of peptides diffused in heart tissues could not be determined in our model, the final concentration of Cx43MPs in total blood volume (estimated 16-30 mL) would certainly not exceed 0.5 μM, a concentration previously shown to selectively inhibit Hc without affecting gap junction channels [7, 37]. Relevant to this concern, the treatment of isolated cardiomyocytes - thus lacking any gap junction channels - with Gap26 (0.5 μM) has also been shown to be protective as it was noted by the significant increase in the number of surviving cells following simulated ischemia-reperfusion [11, 20]. Together with results from the current confocal miscroscopy study, these observations incite us to believe that the involvement of gap junction channels in the Cx43MPs-mediated cardioprotection is not likely. Similarly, we exclude the implication of the other cardiac isoforms of CxHc (i.e., Cx40Hc and Cx45Hc) given the lack of Gap26 effect on these Hc as we previously demonstrated [20]. It is important to remark that upregulation of mitochondrial Cx43 has also been associated with ischemic preconditioning-mediated cardioprotection [38-41]. In this regard, we showed earlier that superfusion of cardiomyocytes with Gap26 preserves mitochondrial activity following ischemic stress [20]. Arguably, this may indicate that Cx43MPs-mediated cardioprotection involves the mitochondrial Cx43 modulation by a yet unknown mechanism. Nonetheless, none of these assumptions can be substantiated under our current experimental conditions. Indeed, the permeability of mitochondrial membranes to Cx43MPs has yet to be demonstrated.

It is noteworthy that infarct size reduction did not differ statistically whether peptides were administered before or after the onset of ischemia. Whereas this observation could result from the fact that death of cardiomyocytes and consequent myocardial injury essentially occur during reperfusion [42, 43], it is also possible that the injected peptides do not reach adequately the areas at risk during LAD occlusion when coronary circulation is interrupted. Therefore, it is conceivable that peptides exert their protective effect only during reperfusion, regardless the timing of administration.

Intriguingly, while both Cx43MPs successfully conferred resistance to hearts against ischemia injury, the extent of the infarct size reduction was significantly smaller when rats were treated with Gap27 (~40% reduction with Gap27 *versus* ~60% with Gap26). Because of the resemblance in biophysical properties between both peptides (i.e., relative length, amino acid composition, size, polarity and hydrophobicity of amino acids that compose these peptides), we do not ascribe the noted disparity to differences in the delivery process of peptides to their substrate but rather to potentially distinct functional effects that these peptides exert on Cx43Hc once they attain their targets. This assumption was subsequently assessed in the electrophysiological study. In a related result, co-injection of both Gap26 and Gap27 in equal doses did not confer additive protection to ischemic hearts. We believe these results point to the existence of a saturating level of Cx43MPs-mediated protection that was reached, beyond which no further protection can be added.

Interestingly, both peptides readily inhibited the exogenous hCx43Hc-mediated currents recorded from the ion channel-deficient tsA201 cells. Given the concordance with previous reported effects of Gap26 on rat Cx43Hc and in cardioprotection [20], the electrophysiological data reported in the present study could represent the initial functional evidence supporting the therapeutic potential of Cx43MPs if applied to human ischemic heart. Indeed, the inhibitory effect of Gap27 was noticeably less pronounced than that of Gap26, and co-application of both peptides did not cause additive current inhibition but rather was comparable to the effect exerted by Gap26 alone. These results correlate with those obtained from the *in vivo* study and could be at the basis of the differential protective effect conferred by Cx43MPs.

Finally, it worth to note that assuming Cx43MPs inhibit specifically the unapposed Cx43Hc that open abnormally under ischemia as it is widely believed, it becomes then plausible to believe that the functional/therapeutic effects induced by the systemic administration of Cx43MPs are mainly restricted to the ischemic area of the heart with little or no effects on other non-ischemic tissues in the rest of the organism. This assumption could partly be supported by the preliminary safety remarks that we reported here. Indeed, more extensive toxicity studies are necessary to further confirm safety of Cx43MPs.

5. Conclusion and relevance

We show here for the first time *in vivo* that single intravenous low-dose bolus injections of Cx43MPs can confer significant protection to the heart against ischemia injury whether injected before or after the onset of ischemia. These practical advantages give Cx43MPs great potential for future therapeutic applications especially in emergency and out-of-hospital settings. We believe that the efficacy of Cx43MPs and their apparent safety make them promising tools to fight ischemic heart disease

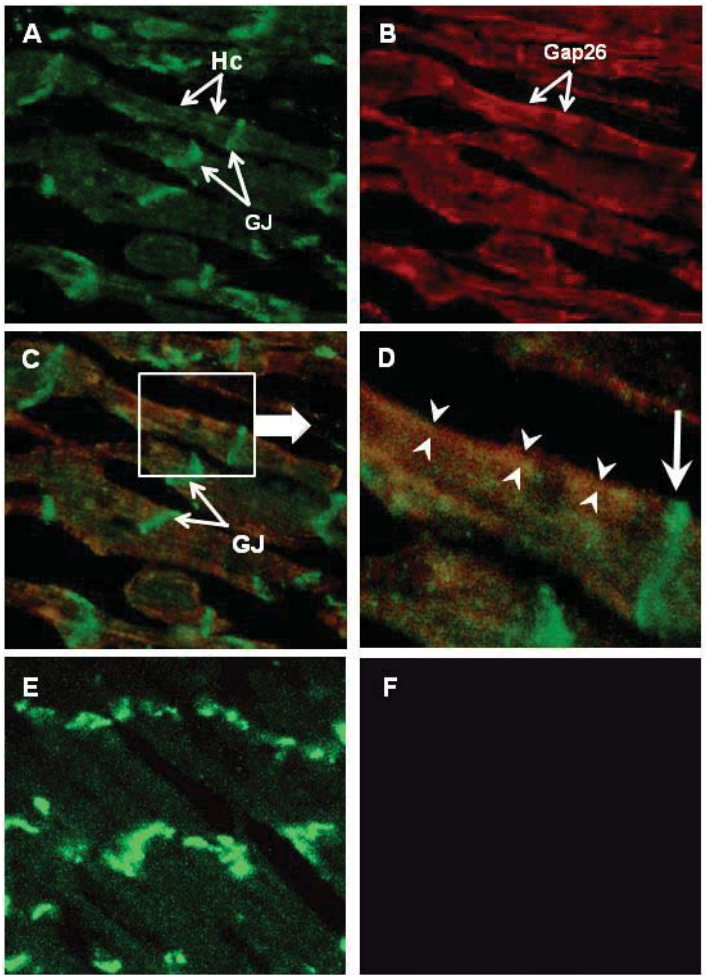

Figure 1.

Figure 1. Confocal images showing immunostaining of a ventricular section obtained from heart of rat injected with flag-tagged Gap26 peptide. A, staining of Cx43 from ischemic myocardium shows both peripheral and sarcoplasmic localization of the protein in green. The arrows point to lateral membranes and intercalated discs where Hc and gap junction channels dominate, respectively. B, same ventricular section is shown where flag-tagged Gap26 peptide is stained in red. Gap26 essentially localizes on the lateral membrane and in sarcoplasm but is remarkably absent in the intercalated discs. C, superimposition of the green and the red (orange color) shows colocalization of the flag-tagged Gap26 and Cx43 on lateral membranes and inside cardiomyocytes but not in the intercalated discs (green). The frame in white indicates a selected section enlarged in panel D. D, a magnified view of the selected section from panel C. Arrowheads point to lateral membrane where flag-tagged Gap26 and Cx43 colocalize. E, staining of Cx43 in normal myocardium of a rat injected with saline only shows sarcoplasmic and polar localization of Cx43 in green, but not on lateral membranes. F, same ventricular section as in E after immunoreaction with anti-flag and the corresponding secondary antibody.

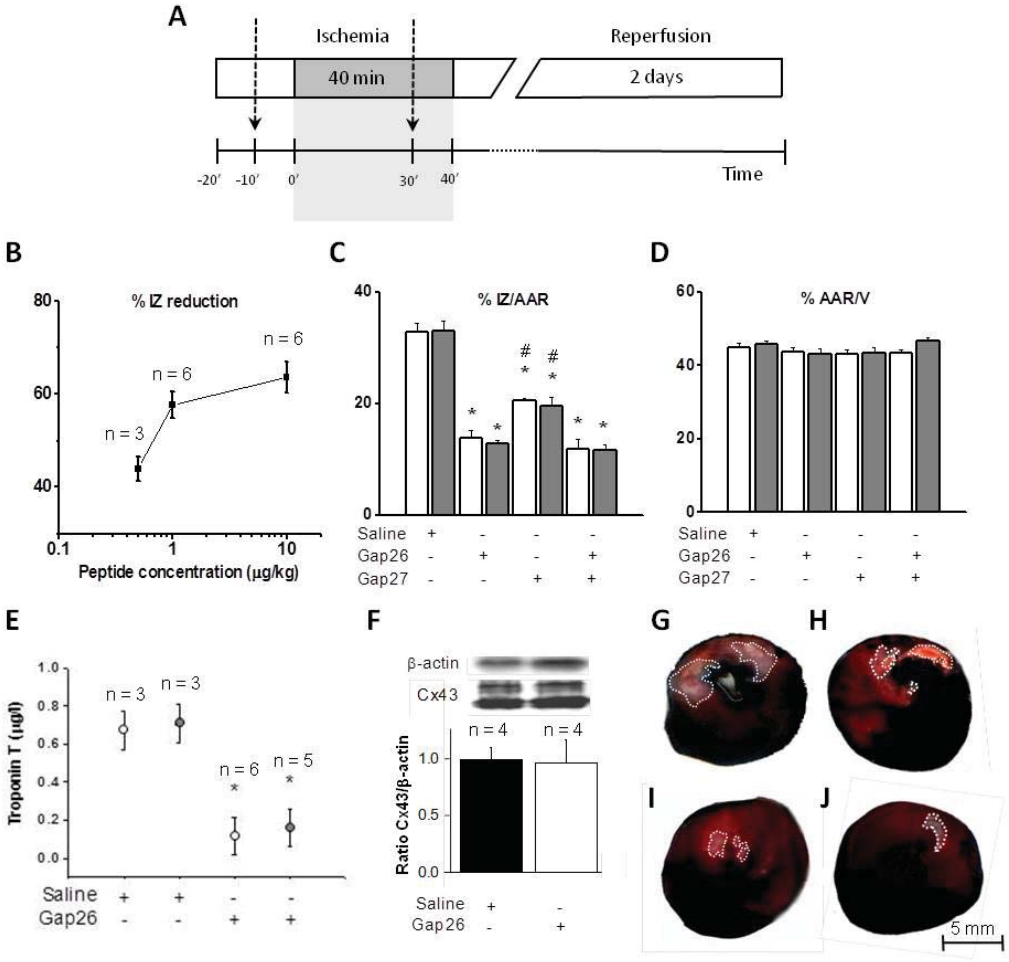
A
Ischemia
Reperfusion
40 min
2 days
-20'
-10'
0'
30'
40'
Time
B
% IZ reduction
n = 3
n = 6
n = 6
Peptide concentration (µg/kg)
C
% IZ/AAR
Saline
Gap26
Gap27
D
% AAR/V
Saline
Gap26
Gap27
E
Troponin T (µg/l)
n = 3
n = 3
n = 6
n = 5
Saline
Gap26
F
β-actin
Cx43
n = 4
n = 4
Ratio Cx43/β-actin
Saline
Gap26
G
H
I
J
5 mm

Figure 2.

Figure 2. A, a schematic representation of the experimental protocol used to assess the effect of Cx43MPs during ischemia-reperfusion *in vivo*. The gray zone covers the ischemic period. The dashed arrows indicate the time at which peptides are injected, either 10 min before or 30 after the initiation of ischemia. B, dose-response curves showing infarct size reduction in function of three different doses of Gap26. C, a histogram representing the proportion of infarct zone to area at risk in various groups. Open and gray columns represent groups treated before and during ischemia, respectively. * indicates statistically different compared to saline-controls at similar timings, $P<0.05$. # indicates statistically different compared to Gap26-treated groups at similar timings, $P<0.05$. D, a histogram showing the proportion of areas at risk to total ventricles. E, analysis of troponin T levels in blood samples. Open and gray dots represent groups treated before and during ischemia, respectively. * indicates statistically different compared to saline-controls at similar timings, $P<0.05$. F, a Western blot analysis of Cx43 protein levels in 10,000g supernatants from ventricular tissues obtained 40 min after reperfusion from rats treated (black column) or not (open column) with Gap26. The histogram displays averaged data from four experiments. Photographs of TTC-stained heart sections obtained 2 days after reperfusion from rats injected with (G) saline; (H) Gap26; (I) Gap27; and (J) Gap26/Gap27 before LAD occlusion. Infarct zones are whitish areas delimited with dashed lines; areas at risk correspond to the red regions including infarct zones; and perfused areas are in dark blue.

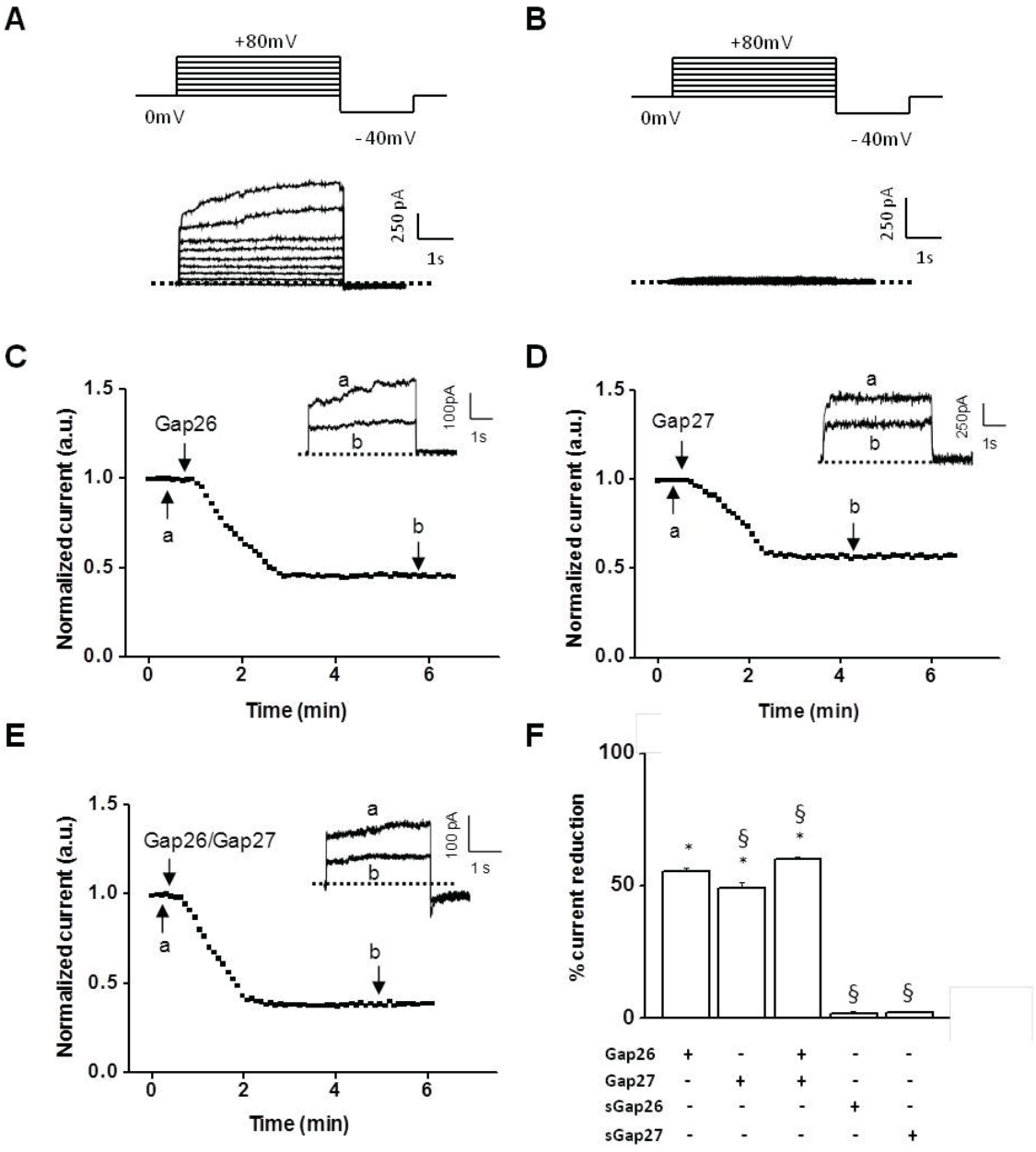
A
+80mV
0mV
-40mV
250 pA
1s
B
+80mV
0mV
-40mV
250 pA
1s
C
Gap26
a
b
100pA
1s
Normalized current (a.u.)
Time (min)
D
Gap27
a
b
250pA
1s
Normalized current (a.u.)
Time (min)
E
Gap26/Gap27
a
b
100 pA
1 s
Normalized current (a.u.)
Time (min)
F
% current reduction
Gap26 + - + - -
Gap27 - + + - -
sGap26 - - - + -
sGap27 - - - - +

Figure 3.

Figure 3. Cx43Hc current inhibition by Cx43MPs. A, a family traces of Cx43Hc-mediated currents recorded from a typical cell depolarized from a holding potential of 0 mV to pulses ranging from 0 to +80 mV. B, traces showing no detectable currents from non-transfected tsA201 cell. Dashed lines represent the zero current. Time course recorded using 30 mV depolarizing pulses from typical cells dialysed with (C) Gap26, (D) Gap27 or (E) Gap26/Gap27. In panels C-E, current amplitudes were normalized to the maximal current recorded. Insets illustrate Cx43Hc current traces in response to the depolarizing pulse recorded at time-points indicated by arrows a and b. (F) Histogram showing percentages of Cx43Hc current inhibition in response to Gap26 (n=8), Gap27 (n=7), Gap26/Gap27 (n=8), sGap26 (n=3) and sGap27 (n=3). Current amplitudes are normalized against control and represented in arbitrary units (a.u.). * $P<0.05$ in comparison to current reduction in non-treated cells at similar timing. § $P<0.05$ in comparison to current reduction in cells treated with Gap26 at similar timing.

Table I. Values obtained from various experimental groups.

Groups	Dose of peptide (µg/Kg)	IZ/AAR (%)	AAR/V (%)	n	HR before ischemia	HR after ischemia	n
Saline before ischemia	0	32.9 ± 1.5	45.1 ±1.0	6	162.8 ± 5.8	174.7 ± 6	4
Saline during ischemia	0	33.1 ± 1.7	45.8 ± 1.0	6	177.8 ± 6.3	179.7 ± 4.5	4
Gap26 before ischemia	0.5	18.0 ± 0.5[a]	46.7 ± 2.1	3	-	-	
Gap26 before ischemia	1	13.9 ± 1.3[a]	43.7 ± 1.3	6	184.1 ± 3.5	181.4 ± 3	4
Gap26 before ischemia	10	11.9 ± 1.9[a]	46.3 ± 1.4	6	-	-	
Gap26 during ischemia	1	12.9 ± 0.5[b]	43.2 ± 1.5	6	189.2 ± 3.7	183.3 ± 7	4
Gap27 before ischemia	1	20.5 ± 0.5[a]	43.3 ± 1.0	6	157.74 ± 12.5	158.37 ± 13	4
Gap27 during ischemia	1	19.7 ± 1.5[b]	43.4 ± 1.5	6	148.7 ± 9.5	155.65 ± 10	4
Gap26+Gap27 before ischemia	1	12.0 ± 1.8[a,c]	43.3 ± 1.0	6	171 ± 8	182 ± 7.5	4
Gap26+Gap27during ischemia	1	11.8 ± 1.0[b,d]	46.7 ± 1.0	6	169.3 ± 4.8	174.6 ± 8.5	4
sGap26 before ischemia	1	32.5 ± 1.0	44.8 ± 1.5	3	142.2 ± 5.7	145.5 ± 5	3
sGap26 during ischemia	1	31.6 ± 1.2	46.7 ± 1.2	4	149.3 ± 5	151.3 ± 5.9	3
sGap27 before ischemia	1	30.3 ± 1.0	46.0 ± 0.5	3	147.4 ± 6.5	153.1 ± 13	3
sGap27 during ischemia	1	32.8 ± 1.0	45.4 ± 1.6	3	151.6 ± 3.5	156.2 ± 4.6	3

IZ infarct zone, AAR area at risk, V ventricles, HR heart rate

[a] Statistically different in comparison to group treated with saline before ischemia

[b] Statistically different in comparison to group treated with saline during ischemia

[c] Statistically different in comparison to group treated with Gap27 before ischemia

[d] Statistically different in comparison to group treated with Gap27 during of ischemia

Table I

Table II. Mortality following ventricular fibrillation during ischemia.

Group	n	Timing of administration	Dose of peptide (µg/Kg)
Saline	1	10 min before ischemia	--
	1	30 min after ischemia	--
Gap26	1	10 min before ischemia	10
Gap26/Gap27	1	10 min before ischemia	1
sGap26	1	30 min after ischemia	1

Table II

Table III. Mortality of undetermined reasons during reperfusion.

Group	n	Time of administration	Dose of peptide (µg/Kg)	Approx. timing of death after reperfusion (hours)
Saline	1	10 min before ischemia	--	24
	1	30 min after ischemia		48
Gap26	1	30 min after ischemia	1	24
Gap27	1	10 min before ischemia	1	4
Gap26/Gap27	2	10 min before ischemia	1/1	24
sGap26	1	10 min before ischemia	1	1
sGap27	1	10 min before ischemia	1	48
	1	30 min after ischemia	1	48

Table III

Table IV. Complete blood count and blood chemistry parameters.

Groups	Untreated n=7	Saline n=3	Gap26 n=4	Gap27 n=4
Complete blood counts				
Red cells (10^{12}/L)	8.34 ± 0.41	9.60 ± 0.42	9.28 ± 0.37	9.33 ± 0.43
White cells (10^{9}/L)	7.93 ± 0.72	6.06 ± 0.81	4.22 ± 0.31	5.67 ± 0.40
Hematocrit (L/L)	0.42 ± 0.03	0.37 ± 0.05	0.39 ± 0.01	0.42 ± 0.02
Platlets (10^{9}/L)	902.00 ± 54.70	819.33 ± 30.25	797.33 ± 63.10	814.66 ± 113.15
Hemoglobin (g/L)	154.5 ± 6.68	167 ± 6.77	152.75 ± 5.21	153.25 ± 2.72
Neutrophil (%)	21.33 ± 3.19	23 ± 0.95	25.5 ± 5.39	19.66 ± 1.99
Monocyte (%)	2.5 ± 0.53	0	0.5 ± 0.43	2.75 ± 0.61
Lymphocyte (%)	85.5 ± 3.93	80.0 ± 3.81	98.25 ± 23.8	83.5 ± 1.75
Eosinophil (%)	1.00 ± 0.37	0	0.75 ± 0.41	1.25 ± 0.41
Basophil (%)	0	0	0	0
Mean cell volume (fl)	50.09 ± 2.91	42.69 ± 0.22	42.81 ± 0.86	49.52 ± 1.71
Mean cell hemoglobine concentration (g/L)	372.86 ± 16.64	375 ± 17.85	379.94 ± 7.05	362.75 ± 20.49
Red cell distribution width (%)	14.25 ± 0.34	14.2 ± 0.71	13.87 ± 0.62	13.75 ± 0.32
Platlet volume concentration (%)	53.60 ± 12.95	54.20 ± 40	46.97 ± 6.88	46.80 ± 8.13
Blood chemistry				
Glucose (mmol/L)	20.95 ± 1.79	20.23 ± 3.73	27.45 ± 1.81	28.73 ± 3.20
Urea nitrogen (mmol/L)	7.21 ± 0.28	7.5 ± 1.85	8.7 ± 0.71	8.36 ±0.51
Creatinin (µmol/L)	57.57 ± 3.67	52 ± 3.92	57 ± 3.74	60.5 ± 2.68
Aspartate aminotransferase (U/L)	55.71 ± 3.43	68 ± 1.50	65.5 ± 6.67	55 ± 2.54
Alkaline phosphatase (U/L)	214.14 ± 57.75	199.5 ± 45.25	195.75 ± 18.74	162 ± 9.51
Proteins (g/L)	55.85 ± 0.68	58.8 ± 1.24	57.05 ± 1.53	55.55 ± 1.34
Albumin (g/L)	36.15 ± 0.39	34.46 ± 0.35	34.05 ± 0.56	32.2 ± 0.46
Globulin (g/L)	23.59 ± 0.98	24.33 ± 1.55	22.97 ± 1.89	23.55 ± 0.88
Ratio albumin/globulin	1.85 ± 0.08	1.43 ± 0.11	1.53 ± 0.13	1.37 ± 0.03

Table IV

Reference List

[1] Contreras JE, Saez JC, Bukauskas FF, Bennett MV. Gating and regulation of connexin 43 (Cx43) hemichannels. Proc Natl Acad Sci U S A 2003;100:11388-93.

[2] Contreras JE, Sanchez HA, Eugenin EA, Speidel D, Theis M, Willecke K et al. Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. Proc Natl Acad Sci U S A 2002;99:495-500.

[3] John SA, Kondo R, Wang SY, Goldhaber JI, Weiss JN. Connexin-43 hemichannels opened by metabolic inhibition. J Biol Chem 1999;274:236-40.

[4] Kondo RP, Wang SY, John SA, Weiss JN, Goldhaber JI. Metabolic inhibition activates a non-selective current through connexin hemichannels in isolated ventricular myocytes. J Mol Cell Cardiol 2000;32:1859-72.

[5] Li F, Sugishita K, Su Z, Ueda I, Barry WH. Activation of connexin-43 hemichannels can elevate [Ca(2+)]i and [Na(+)]i in rabbit ventricular myocytes during metabolic inhibition. J Mol Cell Cardiol 2001;33:2145-55.

[6] Quist AP, Rhee SK, Lin H, Lal R. Physiological role of gap-junctional hemichannels. Extracellular calcium-dependent isosmotic volume regulation. J Cell Biol 2000;148:1063-74.

[7] Clarke TC, Williams OJ, Martin PE, Evans WH. ATP release by cardiac myocytes in a simulated ischaemia model: inhibition by a connexin mimetic and enhancement by an antiarrhythmic peptide. Eur J Pharmacol 2009;605:9-14.

[8] Eltzschig HK, Eckle T, Mager A, Kuper N, Karcher C, Weissmuller T et al. ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function. Circ Res 2006;99:1100-8.

[9] Wilde AA, Aksnes G. Myocardial potassium loss and cell depolarisation in ischaemia and hypoxia. Cardiovasc Res 1995;29:1-15.

[10] Steenbergen C, Hill ML, Jennings RB. Volume regulation and plasma membrane injury in aerobic, anaerobic, and ischemic myocardium in vitro. Effects of osmotic cell swelling on plasma membrane integrity. Circ Res 1985;57:864-75.

[11] Shintani-Ishida K, Uemura K, Yoshida K. Hemichannels in cardiomyocytes open transiently during ischemia and contribute to reperfusion injury following brief ischemia. Am J Physiol Heart Circ Physiol 2007;293:H1714-H1720.

[12] Garcia-Dorado D, Inserte J, Ruiz-Meana M, Gonzalez MA, Solares J, Julia M et al. Gap junction uncoupler heptanol prevents cell-to-cell progression of

hypercontracture and limits necrosis during myocardial reperfusion. Circulation 1997;96:3579-86.

[13] Saltman AE, Aksehirli TO, Valiunas V, Gaudette GR, Matsuyama N, Brink P et al. Gap junction uncoupling protects the heart against ischemia. J Thorac Cardiovasc Surg 2002;124:371-6.

[14] Garcia-Dorado D, Rodriguez-Sinovas A, Ruiz-Meana M. Gap junction-mediated spread of cell injury and death during myocardial ischemia-reperfusion. Cardiovasc Res 2004;61:386-401.

[15] Kanno S, Kovacs A, Yamada KA, Saffitz JE. Connexin43 as a determinant of myocardial infarct size following coronary occlusion in mice. J Am Coll Cardiol 2003;41:681-6.

[16] Rodriguez-Sinovas A, Sanchez JA, Gonzalez-Loyola A, Barba I, Morente M, Aguilar R et al. Effects of substitution of Cx43 by Cx32 on myocardial energy metabolism, tolerance to ischaemia and preconditioning protection. J Physiol 2010;588:1139-51.

[17] Bargiotas P, Monyer H, Schwaninger M. Hemichannels in cerebral ischemia. Curr Mol Med 2009;9:186-94.

[18] Saez JC, Schalper KA, Retamal MA, Orellana JA, Shoji KF, Bennett MV. Cell membrane permeabilization via connexin hemichannels in living and dying cells. Exp Cell Res 2010;316:2377-89.

[19] Orellana JA, Hernandez DE, Ezan P, Velarde V, Bennett MV, Giaume C et al. Hypoxia in high glucose followed by reoxygenation in normal glucose reduces the viability of cortical astrocytes through increased permeability of connexin 43 hemichannels. Glia 2010;58:329-43.

[20] Hawat G, Benderdour M, Rousseau G, Baroudi G. Connexin 43 mimetic peptide Gap26 confers protection to intact heart against myocardial ischemia injury. Pflugers Arch 2010;460:583-92.

[21] Braet K, Vandamme W, Martin PE, Evans WH, Leybaert L. Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26. Cell Calcium 2003;33:37-48.

[22] Leybaert L, Braet K, Vandamme W, Cabooter L, Martin PE, Evans WH. Connexin channels, connexin mimetic peptides and ATP release. Cell Commun Adhes 2003;10:251-7.

[23] Dahl G. Gap junction-mimetic peptides do work, but in unexpected ways. Cell Commun Adhes 2007;14:259-64.

[24] Baroudi G, Pouliot V, Denjoy I, Guicheney P, Shrier A, Chahine M. Novel mechanism for Brugada syndrome: defective surface localization of an SCN5A mutant (R1432G). Circ Res 2001;88:E78-E83.

[25] Reimer KA, Jennings RB. Myocardial Ischemia, Hypoxia and Infarction. In: Fozzard HA, Haber.E., Jennings RB, Katz AM, Morgan HE, editors. The Heart and Cardiovscular System Scientific Foundations: Raven Press; 1991; p. 1947.

[26] Hawat G, Baroudi G. Differential modulation of unapposed connexin 43 hemichannel electrical conductance by protein kinase C isoforms. Pflugers Arch 2008;456:519-27.

[27] Winer BJ. Statistical principles in experimental design. New York: McGraw-Hill; 1971.

[28] Mayama T, Matsumura K, Lin H, Ogawa K, Imanaga I. Remodelling of cardiac gap junction connexin 43 and arrhythmogenesis. Exp Clin Cardiol 2007;12:67-76.

[29] Waghabi MC, Coutinho-Silva R, Feige JJ, Higuchi ML, Becker D, Burnstock G et al. Gap junction reduction in cardiomyocytes following transforming growth factor-beta treatment and Trypanosoma cruzi infection. Mem Inst Oswaldo Cruz 2009;104:1083-90.

[30] Imanaga I. Pathological remodeling of cardiac gap junction connexin 43-With special reference to arrhythmogenesis. Pathophysiology 2010;17:73-81.

[31] Salameh A. Life cycle of connexins: regulation of connexin synthesis and degradation. Adv Cardiol 2006;42:57-70.

[32] Boengler K, Stahlhofen S, van de SA, Gres P, Ruiz-Meana M, Garcia-Dorado D et al. Presence of connexin 43 in subsarcolemmal, but not in interfibrillar cardiomyocyte mitochondria. Basic Res Cardiol 2009;104:141-7.

[33] Przyklenk K, Maynard M, Darling CE, Whittaker P. Pretreatment with D-myo-inositol trisphosphate reduces infarct size in rabbit hearts: role of inositol trisphosphate receptors and gap junctions in triggering protection. J Pharmacol Exp Ther 2005;314:1386-92.

[34] Opanasopit P, Nishikawa M, Hashida M. Factors affecting drug and gene delivery: effects of interaction with blood components. Crit Rev Ther Drug Carrier Syst 2002;19:191-233.

[35] Schenkman KA, Beard DA, Ciesielski WA, Feigl EO. Comparison of buffer and red blood cell perfusion of guinea pig heart oxygenation. Am J Physiol Heart Circ Physiol 2003;285:H1819-H1825.

[36] Evans WH, De Vuyst E, Leybaert L. The gap junction cellular internet: connexin hemichannels enter the signalling limelight. Biochem J 2006;397:1-14.

[37] Verma V, Hallett MB, Leybaert L, Martin PE, Evans WH. Perturbing plasma membrane hemichannels attenuates calcium signalling in cardiac cells and HeLa cells expressing connexins. Eur J Cell Biol 2009;88:79-90.

[38] Boengler K, Dodoni G, Rodriguez-Sinovas A, Cabestrero A, Ruiz-Meana M, Gres P et al. Connexin 43 in cardiomyocyte mitochondria and its increase by ischemic preconditioning. Cardiovasc Res 2005;67:234-44.

[39] Schulz R, Boengler K, Totzeck A, Luo Y, Garcia-Dorado D, Heusch G. Connexin 43 in ischemic pre- and postconditioning. Heart Fail Rev 2007;12:261-6.

[40] Dhein S. New, emerging roles for cardiac connexins. Mitochondrial Cx43 raises new questions. Cardiovasc Res 2005;67:179-81.

[41] Ruiz-Meana M, Rodriguez-Sinovas A, Cabestrero A, Boengler K, Heusch G, Garcia-Dorado D. Mitochondrial connexin43 as a new player in the pathophysiology of myocardial ischaemia-reperfusion injury. Cardiovasc Res 2008;77:325-33.

[42] Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 1994;94:1621-8.

[43] Zhao ZQ, Nakamura M, Wang NP, Wilcox JN, Shearer S, Ronson RS et al. Reperfusion induces myocardial apoptotic cell death. Cardiovasc Res 2000;45:651-60.

Chapitre VI : Discussion et conclusions

La cardiopathie ischémique est la première cause de mortalité dans le monde (près de 13 % des décès), soit 7,3 millions de décès en 2008 dont 6,7 millions dans les pays à revenu intermédiaire et élevé[1]. Au Canada, plus de 16 000 personnes succombent chaque année à la suite de cette pathologie[2]. À l'heure actuelle, les interventions thérapeutiques utilisées lors d'une ischémie cardiaque se limitent principalement à empêcher cette dernière de s'aggraver (par thrombolyse, fibrinolyse ou angioplastie) et de prévenir ses récidives (par l'administration d'anti-aggrégants plaquettaires, de bêta-bloquants, de statines, etc.). Toutefois, aucun traitement n'est actuellement disponible pour contrer les dommages myocardiques irréversibles qui se produisent après la survenue de l'ischémie. Cette problématique est au cœur du présent travail.

Dans ce cadre, la première étude (chapitre 3) a été consacrée à élucider les propriétés des HcCx43 – médiateurs potentiels de l'infarctus du myocarde – ainsi que leur régulation fonctionnelle par les différentes isoformes de PKC présentes dans le cœur, y compris l'isoforme PKCε connue pour ses effets cardioprotecteurs lors du PI. Parce que l'utilisation des cardiomyocytes pour caractériser les HcCx43 implique une limitation naturelle non négligeable représentée par la possibilité de contamination des courants par ceux engendrés par d'autres canaux ioniques endogènes tels que les canaux

[1] Organisation mondiale de la santé

[2] Fondation des maladies du cœur du Canada

calciques, sodiques ou les hémicanaux formés par les autres connexines cardiaques, nous avons eu recours à une lignée cellulaire privée de tout canal ionique endogène, les cellules tsA201. Nous avons démontré pour la première fois dans ces cellules transfectées avec la Cx43 que les HcCx43 sont modulés sélectivement et différentiellement par les diverses isoformes de PKC, et ce, en ayant recours à une méthode utilisant une matrice unique de peptides synthétiques modulateurs spécifiques des isoformes de PKC, dont le laboratoire du Dr Baroudi est pionnier au Canada. Au cours de l'étude, l'isoforme cardioprotectrice PKCε s'est avérée la plus impliquée dans la modulation fonctionnelle des HcCx43, inhibant plus de 60 % des courants engendrés par ces derniers. Les isoformes PKCβII et PKCδ ont eu également des effets inhibiteurs sur les HcCx43; toutefois, ceux-ci ont été significativement moins importants comparés à l'effet exercé par l'isoforme PKCε. Au contraire, les isoformes PKCα et PKCβI ont été sans aucun effet fonctionnel apparent. Ces résultats nous incitent alors à croire que la fermeture des HcCx43 sous les conditions physiologiques normales est assurée, au moins partiellement, par la phosphorylation des Cx43 spécifiquement par l'isoforme PKCε et que, par conséquent, la baisse de l'activité de cette isoforme à la suite d'une ischémie contribue à l'ouverture anormale des HcCx43 et conduit à la nécrose cardiomyocytaire et aux lésions myocardiques irréversibles. Cette hypothèse nous a menés ensuite à postuler que l'identification d'un agent pharmacologique capable de mimer spécifiquement l'effet inhibiteur de l'isoforme PKCε sur les HcCx43, lorsque ces derniers sont déphosphorylés à cause d'ischémie, pourra servir d'outil pharmacologique unique pour la prévention de la nécrose cellulaire, des dommages tissulaires irréversibles

et la protection du cœur contre l'infarctus du myocarde, et ceci sans affecter les nombreux autres substrats de PKCε présents à travers le cœur et le reste de l'organisme.

Cette hypothèse a fait le sujet de la deuxième étude (chapitre 4) où nous avons démontré pour la première fois la capacité de Gap26, le peptide synthétique mimétique structural de la première boucle extracellulaire des Cx43, d'inhiber directement et sélectivement les HcCx43 sans affecter les hémicanaux faits par d'autres connexines myocardiques. Outre l'élucidation de son effet inhibiteur direct et spécifique sur les courants engendrés par les HcCx43, l'observation la plus intéressante qui a découlé de l'étude sur le peptide Gap26 a été sa capacité de conférer une résistance significative contre le stress ischémique, non seulement au niveau des cellules isolées, mais aussi au niveau du cœur intact du rat adulte. Le nombre des cardiomyocytes qui ont survécu après un stress ischémique *in vitro* a plus que doublé lorsque le peptide Gap26 a été introduit avant ou même après l'initiation de ce stress. De même, il a été également intéressant de constater que dans le cœur intact, non seulement la taille de l'infarctus a diminué significativement, mais aussi la circulation coronarienne s'est grandement améliorée. Il est important de noter aussi qu'au cours de cette étude la concentration utilisée du peptide Gap26 a été 1000 fois plus petite que la plus basse concentration utilisée dans des études antérieures, un fait qui souligne la puissance inhibitrice préalablement sous-estimée de ce peptide.

Remarquablement, les améliorations observées à la suite de l'administration du peptide Gap26 ne différèrent pas significativement lorsque le peptide a été introduit avant ou

après l'initiation de l'ischémie. Ces observations nous laissent croire que l'effet protecteur du peptide Gap26 est potentiellement exercé au début de la reperfusion lorsque la circulation coronarienne est reprise, et cela, quel que soit le moment de son administration. Le peptide montre donc une capacité non seulement à conférer une résistance au cœur "sain" contre une ischémie subséquente, mais aussi de sauver un cœur déjà ischémique s'il est administré avant la reperfusion. Aussi prometteurs que ces résultats soient, le modèle du cœur intact *ex vivo* ainsi que la technique de perfusion utilisée impliquent surement plusieurs limitations. Une limitation majeure résulte du fait que les résultats obtenus au cours de l'étude reflètent uniquement les effets du peptide sur le muscle cardiaque isolé de son environnement physiologique naturel et privé des ses interactions existant normalement avec les différents systèmes de l'organisme (hormonal, nerveux, sanguin, immunitaire, température, etc.). La validation du potentiel thérapeutique du peptide dans un contexte physiologique plus complet, précisément *in vivo*, s'est avérée alors primordiale pour élucider si le peptide Gap26 pourrait conférer au cœur ischémique une protection similaire ou, au moins, significative, lorsqu'il est administré systémiquement. Avec cet objectif déterminé, une troisième étude a été entreprise.

En effet, dans la troisième étude (chapitre 5), nous avons étudié chez le rat *in vivo* le potentiel thérapeutique de Gap26, mais aussi d'un deuxième peptide mimétique de la deuxième boucle extracellulaire de Cx43, nommé Gap27. Aujourd'hui, ces deux peptides sont considérés les deux seuls inhibiteurs spécifiques connus d'HcCx43. Au cours de

l'étude, nous avons tenté d'établir les réponses à plusieurs questions fondamentales. Par exemple, ces peptides, ont-ils la capacité de quitter la circulation et d'atteindre leurs cibles (les Cx43) sur la surface des cardiomyocytes ventriculaires s'ils sont administrés par la voie intraveineuse? Quels seront alors les effets sur le cœur et les autres organes? Qu'en est-il de leur profil de sécurité?

La première découverte a été élucidée par imagerie confocale lorsque nous avons observé que le peptide Gap26, injecté dans la veine jugulaire de l'animal, a été capable d'atteindre les tissus ventriculaires du myocarde et de colocaliser avec la Cx43 sur les membranes latérales des cardiomyocytes, lieu principal des HcCx43, mais pas dans les disques intercalaires où se localisent majoritairement les canaux de JG. Cette observation a représenté à ce jour la preuve la plus directe de la spécificité du peptide Gap26 envers les HcCx43. Malgré que la raison exacte de cette localisation sélective ne soit pas encore déterminée, nous pensons que les boucles extracellulaires des Cx43 formant les canaux de JG sont inaccessibles aux peptides mimétiques diffus dans les milieux interstitiels à cause de l'engagement préalable de ces boucles dans l'apposition des Hc provenant des cellules voisines.

Le résultat le plus important a été cependant la mise en évidence qu'une simple et faible dose de Gap26 (1μg/Kg) administrée sous forme d'un bolus intraveineux est capable de protéger le cœur du rat *in vivo* significativement contre les dommages tissulaires causés par l'ischémie. Ceci étant manifesté par la réduction de la taille de l'infarctus de plus de 60 % en comparaison avec les cœurs des animaux non traités. D'une manière surprenante, l'effet protecteur du peptide Gap27 a été toutefois moins important que

celui de Gap26. L'administration d'une dose similaire à celle de Gap26 et sous des conditions expérimentales semblables n'a réduit la taille de l'infarctus que de 40 % par rapport aux cœurs des animaux non traités. Il est fort probable que ces effets différentiels des deux peptides résultent, tel que le suggère les résultats de l'étude fonctionnelle du patch clamp, du pouvoir inhibiteur distinct qu'exerce chacun des deux peptides sur les HcCx43 au niveau cellulaire.

Curieusement, la co-administration des deux peptides, Gap26 et Gap27, en doses égales n'a pas conféré une protection cumulative au cœur. Des résultats comparables ont été aussi obtenus lors de l'étude fonctionnelle sur l'inhibition des courants des HcCx43. Bien que la cause à la base de ces observations reste toujours à déterminer, nous pensons que l'inhibition des HcCx43 confère un niveau de protection maximale au-delà duquel aucune protection supplémentaire ne pourra être ajoutée par ces peptides.

En conclusion, ce travail souligne l'importance des HcCx43 comme médiateurs importants de la formation de l'infarctus du myocarde et démontre qu'ils peuvent représenter une cible thérapeutique de choix pour les maladies ischémiques. La caractérisation des effets protecteurs des mimétiques synthétiques de la Cx43 que nous avons effectuée dans plusieurs modèles expérimentaux est sans doute indispensable pour tout prochain travail visant l'utilisation de ces peptides dans un contexte thérapeutique. L'identification de nouveaux agents capables de rendre le cœur plus résistant à l'ischémie et d'améliorer son rétablissement après une crise cardiaque fournira sans

doute des outils prometteurs longuement attendus pour la lutte contre la maladie la plus fatale au Canada et dans le monde.

Limitations et perspectives

Ce travail comporte certaines limites. Bien que nous ayons découvert l'effet bénéfique du peptide Gap26 sur la réduction de la taille de l'infarctus myocardique chez le modèle du rat *in vivo,* nous n'avons pas pu déterminer la demi-vie de ce peptide. Une recherche pour savoir la demi-vie de Gap26 sera nécessaire afin de connaitre la période du temps pour laquelle la quantité du peptide Gap26 continue à avoir un effet après son injection par voie intraveineuse.

Une autre limitation présente dans cette étude consiste en un manque d'évaluation de la stabilité du Gap26 dans le sang pendant et après la chirurgie. Il aurait été approprié de confirmer la stabilité du Gap26 à différents moments du protocole, afin de s'assurer que l'administration du médicament se fasse dans des conditions optimales, et ainsi obtenir des résultats fiables.

Une étude histopathologique pour la plupart des organes a été faite, elle démontre que Gap26 est sans effet secondaire, mais il faut mettre en considération que ce peptide a été donné sous forme d'un bolus à faible dose, donc une étude plus approfondie à long terme sera nécessaire afin d'évaluer ses effets secondaires. Il aurait été également intéressant d'étudier les propriétés pharmacocinétiques de ce peptide, permettant de choisir la voie d'administration la plus appropriée et comprendre sa forme de distribution et d'élimination à la suite de son injection. Nous sommes parvenus à étudier l'effet du Gap26 sur l'amélioration du débit du perfusat myocardique chez le modèle du

rat *ex vivo* durant l'ischémie, mais il sera intéressant de regarder son effet sur les propriétés fonctionnelles du cœur du rat *in vivo*.

Nos résultats démontrent que Gap26 constitue certainement un outil prometteur pour combattre les maladies cardiaques ischémiques, mais de toute évidence des études plus élaborées utilisant des modèles expérimentaux de plus grandes tailles seront nécessaires pour soutenir le potentiel thérapeutique de Gap26 à long terme. En même temps, il sera intéressant de regarder l'effet de ce peptide s'il est introduit après la reperfusion afin de déterminer son rôle chez les patients qui ont déjà subi une crise cardiaque.

Annexe I

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ION CHANNELS

Differential modulation of unapposed connexin 43 hemichannel electrical conductance by protein kinase C isoforms

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Abstract Opening of unapposed connexin 43 hemichannels (Cx43Hc) in the plasma membrane results in altered ionic homeostasis leading to cell damage. Although it is generally acknowledged that Cx43Hc function is regulated by protein kinase C (PKC), information regarding the functional role of PKC in the modulation of Cx43Hc electrical conductance is lacking. In this work, we used the patch-clamp technique to study the effect of phorbol 12-myristate 13-acetate (PMA), a general PKC activator, on the electrical conductance of exogenous Cx43Hc expressed in tsA201 cells. Subsequently, a matrix of synthetic PKC isoform-specific inhibitor peptides was used to dissect the functional role of individual PKC isoforms in Cx43Hc regulation. Superfusion with 10 nM PMA abolished Cx43Hc currents by 74%, an effect that was prevented by pretreatment with a general PKC inhibitor, GF109203X. It is interesting to note that intracellular diffusion of εV_{1-2} (0.1 μM), an εPKC-specific inhibitor peptide, completely antagonized PMA-induced current inhibition. Cell dialysis with either β_{II}- or δPKC inhibitor peptides partially decreased PMA effect. Neither α- nor β_IPKC inhibition altered PMA-induced current reduction. This study shows for the first time that Cx43Hc electrical conductance is inhibited after PKC activation. Moreover, this inhibition is predominantly mediated by the "novel" εPKC isoform, whereas partial inhibition may be provided by the "conventional" β_{II}PKC as well as the "novel" δPKC isoforms.

Keywords Patch clamp · Connexin 43 · Hemichannels · PKC · Peptides · tsA201 cells

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Introduction

Connexin 43 hemichannels (Cx43Hc) consist of a hexameric association of Cx43 subunits. Whereas complete gap junctional channels result from head-to-head apposition of hemichannel pairs from adjacent cells creating direct communication pathways between the cytoplasmic compartments of neighboring cells [19], the unapposed Cx43Hc have recently been shown to be expressed independently and to exert functions different from those of gap junctional channels. Functional unapposed Cx43Hc have been identified in several tissues including heart muscle [25, 26, 28], central nervous system [8, 13, 21, 44], renal tubules [55], and bone [43]. They provide communication between the intracellular and extracellular compartments and are involved in Ca signaling [51] and glutamate release [59] in astrocytes, in transduction of cell survival signals in bone [41, 42], and in promoting cell injury in response to metabolic inhibition in isolated ventricular myocytes [28, 49], astrocytes [13], and renal tubule cells [55]. Currents from Cx43Hc have been characterized in various native cell

types and expression systems [9, 12, 14, 28, 51]. They are relatively large and nonselective currents reflecting the high conductance and permeability (molecules ≤1 kDa) characteristics of connexin hemichannels [26, 33]. The opening of Cx43Hc is significantly facilitated by depolarization and/or low extracellular [Ca^{2+}] [12, 33], metabolic inhibition [13, 26, 28], mechanical stimulation [51], and treatment with bisphosphonates [41].

Phosphorylation of Cx43 subunits is known to be an important regulatory mechanism of Cx43 channels, i.e., unapposed hemichannels [1, 45] as well as gap junctional channels [53]. The relative proportions of Cx43 existing in either the phosphorylated or dephosphorylated states varies between physiological and pathophysiological conditions [6, 15, 20, 47]. Indeed, Cx43 contains a significantly greater number of motifs for phosphorylation by protein kinase C (PKC) than it does for any other kinase (reviewed in van Veen et al. [54]). However, information is lacking regarding the functional role of PKC in the modulation of Cx43Hc electrical conductance.

In this study, this issue was addressed using the patch-clamp technique by measuring whole-cell currents between cytoplasmic and extracellular compartments in individual cells after phorbol 12-myristate 13-acetate (PMA)-mediated PKC activation. Because studies on native Cx43Hc in cells are often complicated by interference from other membrane channels and other coexisting connexin isoforms, we studied the regulation of Cx43Hc in a Cx43-transfected tsA201 cell expression system, which is deprived of any native membrane ionic channel, but endogenously expresses most of the PKC isoforms [32]. To dissect the role of the various endogenous PKC isoforms in Cx43Hc regulation, we employed a matrix of PKC isoform-specific modulator peptides targeting, individually, the α, β_I, and β_{II} "conventional" isoforms (i.e., dependent on Ca, phospholipids, and diacylglycerol) as well as the ε and δ "novel" isoforms (i.e., phospholipid- and diacylglycerol-dependent but Ca-independent). Five inhibitor peptides targeting α-, β_I-, β_{II}-, δ-, and εPKC isoforms and one activator peptide targeting εPKC were diffused separately through the patch pipette. By structurally mimicking the binding site of the *receptor of activated C kinase* on one particular PKC isoform, inhibitor peptides inhibit the function of that isoform and prevent its action. In contrast, activator peptide binds to the εPKC isoform, causes a conformational change in its structure, and exposes its catalytic site therefore enhancing its activity [50]. These peptides have successfully been used to dissect functional roles of individual PKC isoforms in the modulation of various ion channels [5, 22, 57, 58].

The principal objectives were therefore to assess the functional implication of PKC in regulating Cx43Hc electrical conductance and to unravel the specific PKC isoforms involved in such regulation during PMA-mediated PKC activation.

Materials and methods

Transfection of tsA201 cell line

Rat pBlue-Cx43 plasmid was kindly provided by Prof. Pascal Daleau (Université Laval, Sainte-Foy, Québec). The mammalian cell line tsA201 is derived from human embryonic kidney HEK293 cells by stable transfection with SV40 large-T antigen. Cells were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (10 mg/ml; Invitrogen). Cells were incubated in a 5% CO_2-humidified atmosphere. The tsA201 cells were transfected using the calcium phosphate method with the following modification: To identify transfected cells, 7 μg of EBO/CD8 plasmid was cotransfected with 7 μg of pBlue-Cx43. For patch-clamp experiments, cells 2–3 days post-transfection were incubated for 2 min in a medium containing anti-CD8-coated beads (Invitrogen) [27]. The unattached beads were removed by washing with bath solution. Beads were prepared according to the manufacturer's instruction. Cells expressing CD8-a, and therefore binding beads were distinguished from nontransfected cells by light microscopy.

Solutions, drugs, and peptides

For whole-cell recordings, pipette solution contained (in mM): 140 KCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 ethylene glycol tetraacetic acid (EGTA; estimated free Ca, 37 nM) adjusted to pH 7.2. The bath solution contained (in mM): 140 NaCl, 1 $MgCl_2$, 5.4 KCl, 1.8 $CaCl_2$, 10 HEPES, and 10 EGTA adjusted to pH 7.2. PKC isoform peptides were added in the pipette solution as previously described [22, 57, 58]. PKC isoform-specific modulator and scrambled negative control peptides used in this study include αC_{2-4} (inhibitor of αPKC), $\beta_I V_{5-3}$ (inhibitor of β_IPKC), $\beta_{II} V_{5-3}$ (inhibitor of β_{II}PKC), εV_{1-2} (inhibitor of εPKC), δV_{1-7} (inhibitor of δPKC), εV_{1-7} (activator of εPKC), and scrambled εV_{1-2}. To allow for peptides to access the cytoplasm, cells were dialyzed with peptide (0.1 μM) for at least 5 min after breaking the membrane seal before current recordings. All peptides had greater than 90% purity and were custom made at Sheldon Biotechnology, McGill University, Montréal, QC. The sequence and properties of PKC isoform peptides were reported previously [22, 57, 58]. PMA, 4α-phorbol 12,13-didecanoate (4αPDD), and the GF109203X were obtained from Sigma-Aldrich (St. Louis, MO).

Electrophysiology

Cx43 hemichannel-mediated currents were recorded in whole-cell configuration of the patch-clamp technique [18]. Data were digitized at 5 kHz with an analog-to-digital converter (Digidata 1440A, Molecular Devices, California, USA). The recordings were filtered with a low-pass corner frequency of 2 kHz. Glass electrodes (Corning model 8161, outer diameter 1.5 mm) with an electrode tip resistance of 0.8–1.0 MΩ were connected to a patch-clamp amplifier (Axon model 200B). A voltage error of 4 mV, attributable to a liquid junction potential, was corrected. Data were analyzed using pClamp 10.1 (Molecular Devices). Cells were maintained at a holding potential of 0 mV. To generate Cx43Hc current–voltage relationship traces, cells were depolarized by test pulses ranging from 0 to 130 mV in a 10-mV increment for 5 s. The effect of PMA and other tested agents were studied by depolarizing cells in 30-mV steps for pulse durations of 5 s.

Statistical analysis

Data are expressed as means±SE. Percent inhibition was calculated as the difference between the basal current amplitude and the intervention. Paired *t* test was used to compare measurements made under different conditions (e.g., basal vs PMA) in individual cells. Measurements of PMA effects made in the presence of the different PKC isoform modulator peptides were compared to the measurements of the effect of PMA alone using unpaired Dunnett's *t* test. Differences were deemed significant at a *P* value less than 0.05.

Results

PMA inhibits Cx43Hc-induced current

The representative outward current traces shown in (Fig. 1a) illustrate the voltage-dependent Cx43Hc-mediated current amplitudes in response to membrane depolarization under basal conditions. No current could be recorded from nontransfected tsA201 cells (Fig. 1b). The application of the general PKC activator PMA (10 nM) in the bath consistently inhibited Cx43Hc currents (Fig. 1c). The time course for PMA-induced inhibition of Cx43Hc currents elicited at 30 mV is shown in (Fig. 1d). Steady-state inhibition was reached in all cells studied. For easy comparison, reduction in Cx43Hc current amplitudes is represented throughout the text as a percentage with reference to the basal value. Averaged data show that PMA resulted in Cx43Hc current reduction by 74.0±4.3% (n=6, P<0.05). To exclude the possibility that the current decay observed with PMA is due to rundown or other nonspecific effects, a set of control experiments was conducted. Cx43Hc currents recorded in the absence of PKC modulators did not show significant current reduction over a 15-min time frame (1.1±5.1%, n=3, P=not

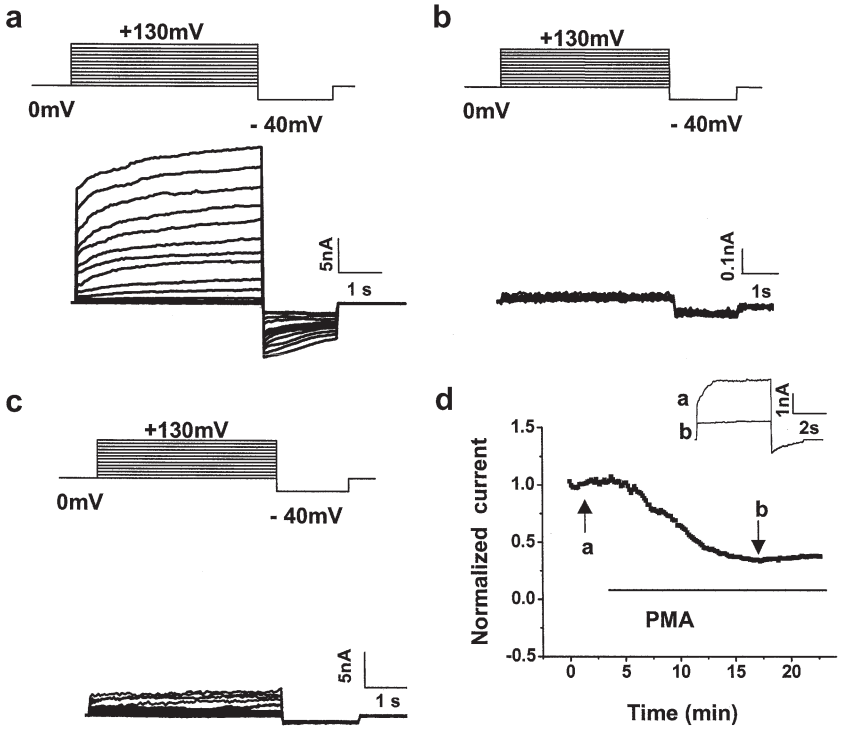

Fig. 1 Whole-cell Cx43Hc-mediated currents recorded from tsA201 cells. **a** Starting from 0 mV, cells were depolarized using test pulses ranging from 0 to 130 mV in 10-mV increments during 5 s and then hyperpolarized to −40 mV for 2 s. **b** Zero-current traces recorded from a nontransfected tsA201 cells, using same protocol as in **a**. **c** Cx43Hc current traces recorded from transfected tsA201 cell superfused with the general PKC activator, PMA (10 nM). **d** Time course recording plotting Cx43Hc current amplitudes in the presence of PMA against time. The *inset* illustrates Cx43Hc current traces obtained at time points indicated by *arrows a* and *b*

significant [NS]; Fig. 2a). Superfusion of tsA201 cells with 10 nM 4αPDD, a phorbol ester analog that does not activate PKC, did not significantly affect Cx43Hc current amplitude (increase of 2.0±1.5%, n=5, P=NS; Fig. 2b), indicating that the PMA effect reported above is mediated via PKC activation. To further substantiate the role of PKC in the PMA-induced inhibition, we tested the ability of a general PKC inhibitor, bisindolylmaleimide (GF109203X), to antagonize the effect of PMA. As shown in Fig. 2c, preincubation of Cx43-expressing cells with 15 μM GF109203X for 10–15 min before superfusion with PMA resulted in a current increase to 133.0±9.3%, n=3, P<0.05 (compared to 100% current at basal conditions), suggesting the presence of basal PKC activity in tsA201 cells. According to expectation, application of PMA could not significantly alter Cx43Hc current amplitudes (144.6± 1.4%, n=3, P=NS; Fig. 2c). Because conventional PKC isoforms depend on Ca, we examined PMA effect under physiological Ca concentrations using lower concentrations of the Ca chelator EGTA in pipette (3 mM) and bath (0 mM) solutions (Fig. 2d). To induce Cx43Hc opening, currents were elicited by depolarizing cells to higher voltage (50 mV). The results show that the PMA-induced inhibitory effect on Cx43Hc current was not significantly affected (72.2±2.6%, n=4, P=NS).

PMA inhibition of Cx43Hc current is primarily mediated through the εPKC isoform

To dissect the role of individual PKC isoforms in the regulation of Cx43Hc, we tested the ability of five PKC isoform-specific inhibitor peptides [50] to antagonize the effect of PMA, i.e., αC_{2-4}, $\beta_I V_{5-3}$, $\beta_{II} V_{5-3}$, δV_{1-7}, and εV_{1-2} targeting α-, β_I-, β_{II}-, δ-, and ε-PKC isoforms, respectively. Each of the tested peptides targets a corresponding native PKC isoform. Cell dialysis with αC_{2-4} or $\beta_I V_{5-3}$ (0.1 μM) did not prevent Cx43Hc inhibition by PMA (Fig. 3a and b). In comparison with the 74.0±4.3% (n=6) inhibition when cells were superfused with PMA alone, PMA-induced inhibition occurred at statistically comparable levels of 74.9±5.1% (n=7, P=NS) and 80.5±1.8% (n=5, P=NS) in cells dialyzed with αC_{2-4} and $\beta_I V_{5-3}$, respectively. These results suggest that the α- and β_I-isoforms are not involved in PKC-dependent regulation of Cx43Hc conductance. In contrast, slight but statistically significant reductions in the PMA-induced

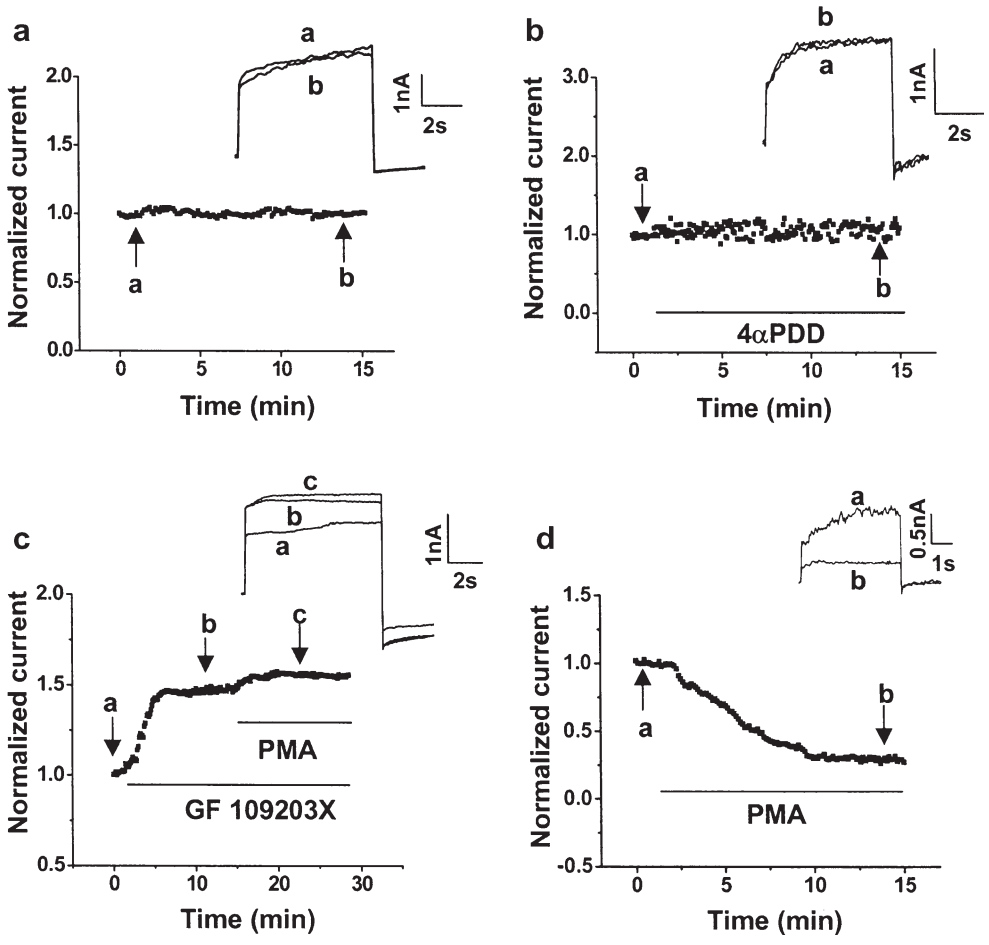

Fig. 2 Time course recording of Cx43Hc currents. **a** Rundown test, the cell is depolarized using a 30-mV test pulse every 7 s for a total duration of 15 min. **b** The cell is superfused with 10 nM 4αPDD. **c** The cell is superfused with general PKC inhibitor GF109203X during 15 min before addition of 10 nM PMA. **d** Time course showing Cx43Hc current inhibition after PMA superfusion when normal Ca concentration is used in the patch pipette and the bath solutions. *Insets* illustrate Cx43Hc current traces recorded at time points indicated by *arrows a* and *b*

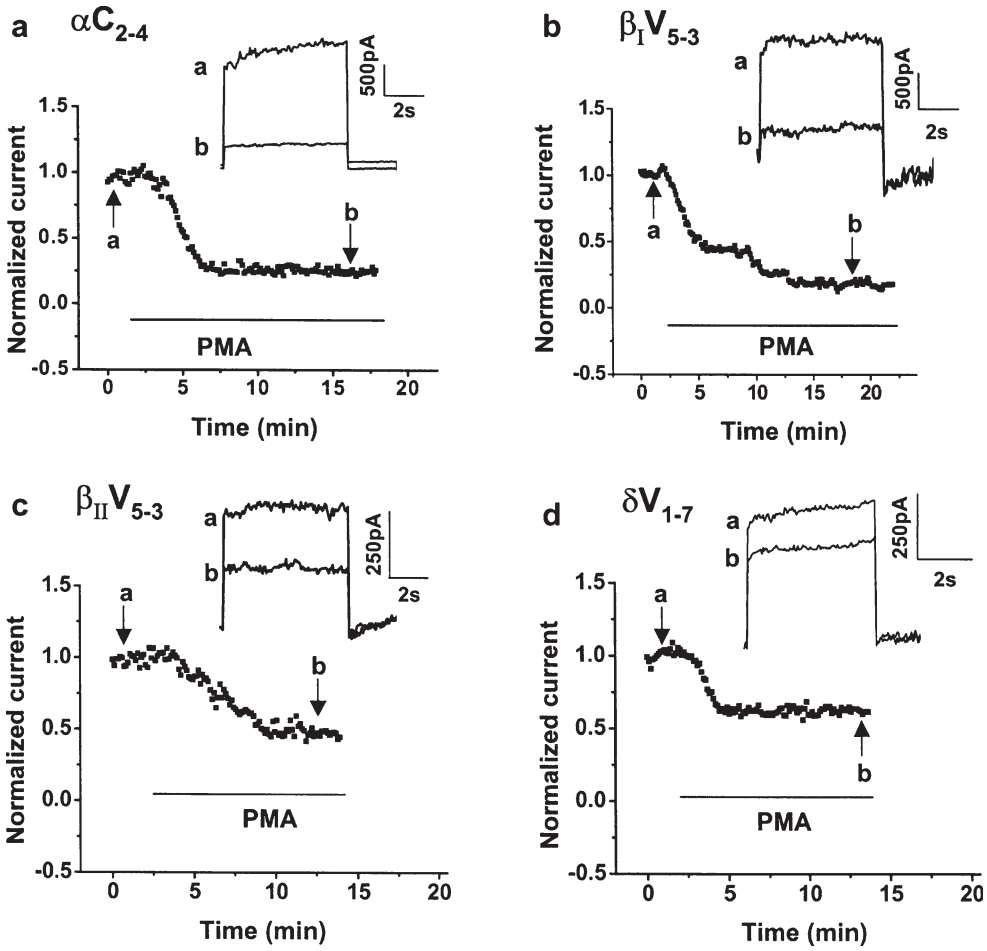

Fig. 3 Time course recordings from cells dialyzed with various PKC isoform-specific inhibitor peptides. The effect of PMA is observed on Cx43Hc currents in the presence of **a** αC_{2-4}, **b** $\beta_I V_{5-3}$, **c** $\beta_{II} V_{5-3}$, and **d** δV_{1-7}. *Insets* illustrate current traces recorded at time points indicated by *arrows a* and *b*

current inhibition to 50.2±5.3 (n=7, P<0.05) and to 34.2±6.1% (n=5, P<0.05) were observed upon inhibition of β_{II}- and δPKC isoforms, respectively (Fig. 3c and d).

It is interesting to note that when cells were dialysed with the εPKC inhibitor peptide, εV_{1-2} (0.1 μM), the PMA-induced current inhibition was completely abolished (118.5±15.8%, n=6, significantly different from the reference PMA effect; Fig. 4a). To substantiate the specificity of the εV_{1-2} inhibitor peptide effect, we performed additional experiments using a scrambled sequence of the εV_{1-2} amino acids as a negative control (Fig. 4b). As expected, in the presence of scrambled εV_{1-2} (0.1 μM), the inhibitory effect of PMA on the Cx43Hc current was not significantly different compared with the PMA effect alone (69.4±4.7%, n=3, P=NS). On the other hand, using an εPKC activator peptide εV_{1-7} (0.1 μM) alone was able to cause a 75.7±2.6% reduction in the basal Cx43Hc current (n=3, P<0.05; Fig. 4c), thus confirming the involvement of εPKC in Cx43Hc inhibition.

The histogram shown in Fig. 5 summarizes our main findings that Cx43Hc conductance was significantly inhibited by PMA and that, moreover, such inhibition was partially reversed by the β_{II}-and δPKC isoform peptide antagonists (because the conductances remained smaller than basal) but completely abolished by εPKC antagonism.

Discussion

Inhibition of Cx43Hc conductance by PMA

In the present study, we demonstrate that Cx43Hc electrical conductance is modulated by PKC and that such modulation is selectively and differentially regulated by PKC isoforms. Using tsA201 cells derived from HEK293 cells, known to endogenously express most of the phorbol ester-sensitive PKC isoforms including α, β_I, β_{II}, δ, and ε [32], we have shown that whole-cell Cx43Hc conductance is inhibited by 74% during superfusion of Cx43-expressing cells with a general PKC activator, PMA. This inhibitory effect can be, with confidence, attributed to the activation of PKC because a similar effect could not been reproduced using 4αPDD, a biologically inactive phorbol ester analog that does not activate PKC. In addition, inhibition of

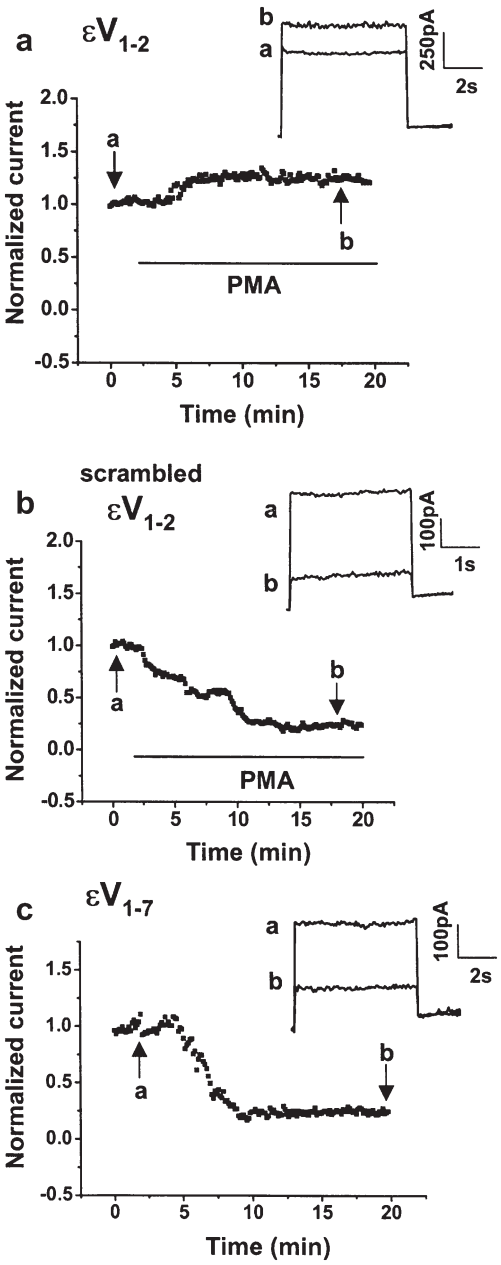

Fig. 4 Cx43Hc current regulation by ε PKC modulator peptides. The effect of PMA (10 nM) on Cx43Hc current amplitudes is shown during time course recording from cell dialyzed with **a** εPKC inhibitor peptide and εV_{1-2} and **b** scrambled εPKC inhibitor, scrambled εV_{1-2}. **c** Time course recording shows effect of εPKC activator peptide alone, εV_{1-7}. *Insets* illustrate current traces recorded at time points indicated by *arrows a* and *b*

Cx43Hc current by PMA was prevented when cells were dialyzed with a general PKC inhibitor, GF109203X. A time-dependent Cx43Hc current rundown effect [56] can be ruled out because no variation in Cx43Hc current amplitude was observed in control experiments in which patch-clamp measurements were maintained in the same cell for over 15 min. Although not significant, a slight increase in Cx43Hc current was observed in some cells upon application of 4αPDD or PMA in the presence of PKC inhibitors. We think that this is possibly due to a PKC-independent effect of phorbol esters that is unmasked when PKC activity is reduced.

Inhibition of Cx43Hc electrical conductance following its phosphorylation by PKC is possibly mediated via conformational change in the hemichannel protein therefore restricting ions passage through the pore structure. In principle, whole-cell current amplitude could also be affected by modification of the number of hemichannels expressed on the cell surface. However, since the half life of Cx43 is known to be in the range of 1 to 2 hours [7, 16, 31], it is unlikely that variations in hemichannel expression at the plasma membrane could have contributed to the inhibition of electrical conductance which occurs within a few minutes of exposure to PMA.

Bao et al. [2] recently reported an altered Cx43Hc size selectivity upon PKC activation, which caused a reduction in purified hemichannels permeability to large solutes without affecting permeation to ions or hemichannels conductance. This is in contrast with observations reported herein, as well as with previously reported observations based on single-channel conductance measurements [29, 36], indicating that PKC activation drastically reduces the Cx43Hc electrical conductance. One possible explanation for the discrepancy could be the fact that the biophysical properties of hemichannels purified from solubilized Cx43

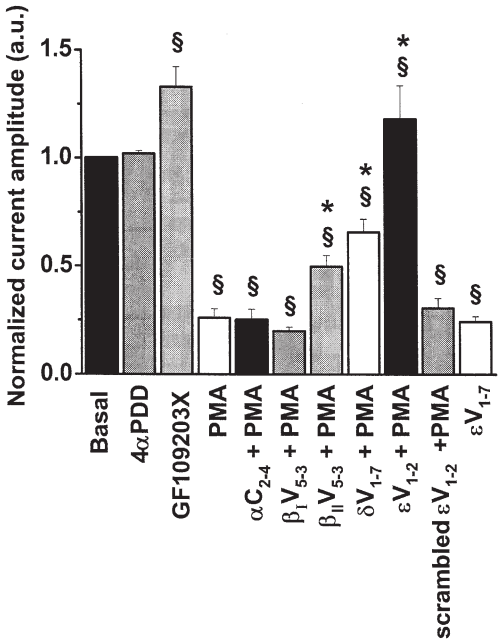

Fig. 5 Data summary. The histogram illustrates percentage of Cx43Hc currents in presence of 4αPDD, GF109203X, PMA, PMA with various PKC isoform-specific peptides, εPKC activator, and scrambled εPKC inhibitor. Current amplitudes are normalized against the control and are represented in arbitrary units (*a.u.*). *Section symbol*, $P<0.05$, significantly different from basal. *Asterisk*, $P<0.05$, significantly different from PMA alone

subunits, as used by Bao et al. [2], differ from those of hemichannels studied in cellular systems that provide a physiological environment and cellular metabolism. In another study conducted on gap junction channels, PKC activation induced an increase in intercellular electrical conductance in cell pairs expressing exogenous Cx43 [30]. It is possible that dissimilarities between the functional properties of Cx43Hc and Cx43 gap junctional channels result from interactions between Cx43 and their many binding partner proteins during Cx43Hc assembly into full gap junction channels and their processing toward their final position [4, 17] or simply result from conformational changes during docking with an apposed hemichannel in the adjacent cell [52].

PKC isoform-selective modulation of Cx43Hc

Another novel finding is the unraveling of selective and differential implication of PKC isoforms in the modulation of Cx43Hc conductance. We dissected the role of individual PKC isoforms in the regulation of Cx43Hc using a matrix of well-established PKC isoform-specific inhibitor peptides [50]. These peptides have successfully been used to study functional roles of individual PKC isoforms in the modulation of various ion channels [5, 22, 57, 58]. In the present work, εPKC was found to be the isoform predominantly involved in the modulation of Cx43Hc conductance after PMA superfusion. In fact, after diffusing the εPKC-specific inhibitor peptide, the PMA effect was not only prevented, but there was actually a moderate Cx43Hc current increase to 118.5% when compared to basal values (100%). Although not significant, this slight current increase may be interpreted as an inhibition, by inhibitor εV_{1-2}, of endogenous PKC basal activity. This interpretation is consistent with the increase in conductance induced by the general PKC inhibitor GF109203X (Fig. 2c). Smaller but significant antagonism of the PMA effect was identified with the β_{II}PKC and δPKC isoform inhibitors (peptides $\beta_{II}V_{5-3}$ and δV_{1-7}, respectively), whereas the PMA effect was not significantly altered after application of the α or β_I inhibitor peptides. Assuming a perfect selectivity profile of the inhibitory peptides [50], this suggests that the β_{II} and δPKC isoforms may also contribute to the PMA effect on conductance, together with a markedly stronger εPKC contribution. Thus, the electrical conductance of Cx43Hc appears to be differentially regulated by PKC in an isoform-specific manner.

Activation of isoforms belonging to the conventional PKC subfamily (i.e., α, β_I, and β_{II}) is known to require intracellular calcium [38]. On the other hand, the Cx43 hemichannel opening is inhibited at intracellular Ca concentrations greater that 1 μM [49]. Therefore, a balance was achieved throughout this study whereby moderate Ca concentrations were maintained in the intracellular and bath solutions with the use of EGTA to enhance hemichannels opening without compromising conventional PKC activity. Indeed, the affinity of Ca-sensitive PKC isoforms to the cytosolic Ca is further increased in the presence of phorbol esters [37]. Therefore, we believe that the estimated intracellular free calcium concentration in our experiments is sufficient to activate the Ca-sensitive PKC isoforms. This is further supported by the fact that at normal Ca concentrations, PKC modulation of Cx43Hc currents elicited at high-voltage depolarization was not significantly affected (Fig. 2d).

Perspective

Cx43Hc dephosphorylation [6, 23, 24, 35, 46] and opening [26, 28, 49] have been proposed as a key mechanism underlying cell injury in response to simulated ischemia in cardiomyocytes. Preservation of Cx43 phosphorylation by selective activation of the εPKC isoform occurs in response to ischemic pre- [11, 34, 60] and postconditioning [39, 40, 61], in which brief episodes of ischemia reduce the adverse effects of subsequent or preceding myocardial ischemia, respectively. PKC isoform-selective modulator drugs are currently under development for the treatment of a variety of diseases [3, 10, 48]. As the function of a multiple proteins may be affected by such drugs, delineation of the isoform-specific regulation of downstream targets, e.g., Cx43Hc, is important to improve our understanding of the pathogenesis of ischemic heart disease and to identify new opportunities for drug development.

In conclusion, our results establish that electrical conductance of Cx43Hc is regulated by PKC-dependent phosphorylation. Furthermore, the results points mainly to ε but also βII and δPKC isoforms as the key players in this modulation. Our results suggest that εPKC, β_{II}PKC, and δPKC may constitute suitable candidates for the development of targeted therapeutic for cardiac pathophysiology in which Cx43Hc is involved.

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References

1. Bao X, Altenberg GA, Reuss L (2004) Mechanism of regulation of the gap junction protein connexin 43 by protein kinase C-mediated phosphorylation. Am J Physiol Cell Physiol 286:C647–C654

2. Bao X, Lee SC, Reuss L, Altenberg GA (2007) Change in permeant size selectivity by phosphorylation of connexin 43 gap-junctional hemichannels by PKC. Proc Natl Acad Sci USA 104:4919–4924
3. Bar-Am O, Amit T, Youdim MB (2007) Aminoindan and hydroxyaminoindan, metabolites of rasagiline and ladostigil, respectively, exert neuroprotective properties in vitro. J Neurochem 103:500–508
4. Barker RJ, Price RL, Gourdie RG (2001) Increased co-localization of connexin43 and ZO-1 in dissociated adult myocytes. Cell Adhes Commun 8:205–208
5. Baroudi G, Qu Y, Ramadan O, Chahine M, Boutjdir M (2006) Protein kinase C activation inhibits Cav1.3 calcium channel at NH2-terminal serine 81 phosphorylation site. Am J Physiol Heart Circ Physiol 291:H1614–H1622
6. Beardslee MA, Lerner DL, Tadros PN, Laing JG, Beyer EC, Yamada KA, Kleber AG, Schuessler RB, Saffitz JE (2000) Dephosphorylation and intracellular redistribution of ventricular connexin43 during electrical uncoupling induced by ischemia. Circ Res 87:656–662
7. Berthoud VM, Minogue PJ, Laing JG, Beyer EC (2004) Pathways for degradation of connexins and gap junctions. Cardiovasc Res 62:256–267
8. Boucher S, Bennett SA (2003) Differential connexin expression, gap junction intercellular coupling, and hemichannel formation in NT2/D1 human neural progenitors and terminally differentiated hNT neurons. J Neurosci Res 72:393–404
9. Bukauskas FF, Verselis VK (2004) Gap junction channel gating. Biochim Biophys Acta 1662:42–60
10. Casellini CM, Barlow PM, Rice AL, Casey M, Simmons K, Pittenger G, Bastyr EJ III, Wolka AM, Vinik AI (2007) A 6-month, randomized, double-masked, placebo-controlled study evaluating the effects of the protein kinase C-beta inhibitor ruboxistaurin on skin microvascular blood flow and other measures of diabetic peripheral neuropathy. Diabetes Care 30:896–902
11. Chou WH, Messing RO (2005) Protein kinase C isozymes in stroke. Trends Cardiovasc Med 15:47–51
12. Contreras JE, Saez JC, Bukauskas FF, Bennett MV (2003) Gating and regulation of connexin 43 (Cx43) hemichannels. Proc Natl Acad Sci USA 100:11388–11393
13. Contreras JE, Sanchez HA, Eugenin EA, Speidel D, Theis M, Willecke K, Bukauskas FF, Bennett MV, Saez JC (2002) Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. Proc Natl Acad Sci USA 99:495–500
14. Ebihara L, Steiner E (1993) Properties of a nonjunctional current expressed from a rat connexin46 cDNA in Xenopus oocytes. J Gen Physiol 102:59–74
15. Ek-Vitorin JF, King TJ, Heyman NS, Lampe PD, Burt JM (2006) Selectivity of connexin 43 channels is regulated through protein kinase C-dependent phosphorylation. Circ Res 98:1498–1505
16. Gaietta G, Deerinck TJ, Adams SR, Bouwer J, Tour O, Laird DW, Sosinsky GE, Tsien RY, Ellisman MH (2002) Multicolor and electron microscopic imaging of connexin trafficking. Science 296:503–507
17. Giepmans BN (2004) Gap junctions and connexin-interacting proteins. Cardiovasc Res 62:233–245
18. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391:85–100
19. Harris AL (2001) Emerging issues of connexin channels: biophysics fills the gap. Q Rev Biophys 34:325–472
20. Hatanaka K, Kawata H, Toyofuku T, Yoshida K (2004) Down-regulation of connexin43 in early myocardial ischemia and protective effect by ischemic preconditioning in rat hearts in vivo. Jpn Heart J 45:1007–1019
21. Hofer A, Dermietzel R (1998) Visualization and functional blocking of gap junction hemichannels (connexons) with antibodies against external loop domains in astrocytes. Glia 24: 141–154
22. Hu K, Mochly-Rosen D, Boutjdir M (2000) Evidence for functional role of epsilonPKC isozyme in the regulation of cardiac Ca(2+) channels. Am J Physiol Heart Circ Physiol 279: H2658–H2664
23. Jain SK, Schuessler RB, Saffitz JE (2003) Mechanisms of delayed electrical uncoupling induced by ischemic preconditioning. Circ Res 92:1138–1144
24. Jeyaraman M, Tanguy S, Fandrich RR, Lukas A, Kardami E (2003) Ischemia-induced dephosphorylation of cardiomyocyte connexin-43 is reduced by okadaic acid and calyculin A but not fostriecin. Mol Cell Biochem 242:129–134
25. John S, Cesario D, Weiss JN (2003) Gap junctional hemichannels in the heart. Acta Physiol Scand 179:23–31
26. John SA, Kondo R, Wang SY, Goldhaber JI, Weiss JN (1999) Connexin-43 hemichannels opened by metabolic inhibition. J Biol Chem 274:236–240
27. Jurman ME, Boland LM, Liu Y, Yellen G (1994) Visual identification of individual transfected cells for electrophysiology using antibody-coated beads. Biotechniques 17:876–881
28. Kondo RP, Wang SY, John SA, Weiss JN, Goldhaber JI (2000) Metabolic inhibition activates a non-selective current through connexin hemichannels in isolated ventricular myocytes. J Mol Cell Cardiol 32:1859–1872
29. Kwak BR, Hermans MM, De Jonge HR, Lohmann SM, Jongsma HJ, Chanson M (1995) Differential regulation of distinct types of gap junction channels by similar phosphorylating conditions. Mol Biol Cell 6:1707–1719
30. Kwak BR, Jongsma HJ (1996) Regulation of cardiac gap junction channel permeability and conductance by several phosphorylating conditions. Mol Cell Biochem 157:93–99
31. Laird DW, Puranam KL, Revel JP (1991) Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. Biochem J 273(Pt 1):67–72
32. Leaney JL, Dekker LV, Tinker A (2001) Regulation of a G protein-gated inwardly rectifying K+ channel by a Ca(2+)-independent protein kinase C. J Physiol 534:367–379
33. Li H, Liu TF, Lazrak A, Peracchia C, Goldberg GS, Lampe PD, Johnson RG (1996) Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells. J Cell Biol 134:1019–1030
34. Liu GS, Cohen MV, Mochly-Rosen D, Downey JM (1999) Protein kinase C-epsilon is responsible for the protection of preconditioning in rabbit cardiomyocytes. J Mol Cell Cardiol 31:1937–1948
35. Miura T, Ohnuma Y, Kuno A, Tanno M, Ichikawa Y, Nakamura Y, Yano T, Miki T, Sakamoto J, Shimamoto K (2004) Protective role of gap junctions in preconditioning against myocardial infarction. Am J Physiol Heart Circ Physiol 286:H214–H221
36. Moreno AP, Fishman GI, Spray DC (1992) Phosphorylation shifts unitary conductance and modifies voltage dependent kinetics of human connexin43 gap junction channels. Biophys J 62:51–53
37. Nishizuka Y (1986) Studies and perspectives of protein kinase C. Science 233:305–312
38. Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665
39. Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D, Losano G, Pagliaro P (2006) Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K + channel and protein kinase C activation. Basic Res Cardiol 101:180–189

40. Philipp S, Yang XM, Cui L, Davis AM, Downey JM, Cohen MV (2006) Postconditioning protects rabbit hearts through a protein kinase C-adenosine A2b receptor cascade. Cardiovasc Res 70:308–314
41. Plotkin LI, Bellido T (2001) Bisphosphonate-induced, hemichannel-mediated, anti-apoptosis through the Src/ERK pathway: a gap junction-independent action of connexin43. Cell Adhes Commun 8:377–382
42. Plotkin LI, Manolagas SC, Bellido T (2002) Transduction of cell survival signals by connexin-43 hemichannels. J Biol Chem 277:8648–8657
43. Romanello M, D'Andrea P (2001) Dual mechanism of intercellular communication in HOBIT osteoblastic cells: a role for gap-junctional hemichannels. J Bone Miner Res 16:1465–1476
44. Saez JC, Contreras JE, Bukauskas FF, Retamal MA, Bennett MV (2003) Gap junction hemichannels in astrocytes of the CNS. Acta Physiol Scand 179:9–22
45. Saez JC, Retamal MA, Basilio D, Bukauskas FF, Bennett MV (2005) Connexin-based gap junction hemichannels: gating mechanisms. Biochim Biophys Acta 1711:215–224
46. Schulz R, Gres P, Skyschally A, Duschin A, Belosjorow S, Konietzka I, Heusch G (2003) Ischemic preconditioning preserves connexin 43 phosphorylation during sustained ischemia in pig hearts in vivo. FASEB J 17:1355–1357
47. Schwanke U, Li X, Schulz R, Heusch G (2003) No ischemic preconditioning in heterozygous connexin 43-deficient mice–a further in vivo study. Basic Res Cardiol 98:181–182
48. Serova M, Ghoul A, Benhadji KA, Cvitkovic E, Faivre S, Calvo F, Lokiec F, Raymond E (2006) Preclinical and clinical development of novel agents that target the protein kinase C family. Semin Oncol 33:466–478
49. Shintani-Ishida K, Uemura K, Yoshida KI (2007) Hemichannels in cardiomyocytes open transiently during ischemia and contribute to reperfusion injury following brief ischemia. Am J Physiol Heart Circ Physiol 293:H1714–H1720
50. Souroujon MC, Mochly-Rosen D (1998) Peptide modulators of protein–protein interactions in intracellular signaling. Nat Biotechnol 16:919–924
51. Stout CE, Costantin JL, Naus CC, Charles AC (2002) Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. J Biol Chem 277:10482–10488
52. Thimm J, Mechler A, Lin H, Rhee S, Lal R (2005) Calcium-dependent open/closed conformations and interfacial energy maps of reconstituted hemichannels. J Biol Chem 280: 10646–10654
53. van Veen AA, van Rijen HV, Opthof T (2001) Cardiac gap junction channels: modulation of expression and channel properties. Cardiovasc Res 51:217–229
54. van Veen TA, van Rijen HV, Jongsma HJ (2006) Physiology of cardiovascular gap junctions. Adv Cardiol 42:18–40
55. Vergara L, Bao X, Cooper M, Bello-Reuss E, Reuss L (2003) Gap-junctional hemichannels are activated by ATP depletion in human renal proximal tubule cells. J Membr Biol 196:173–184
56. Weng S, Lauven M, Schaefer T, Polontchouk L, Grover R, Dhein S (2002) Pharmacological modification of gap junction coupling by an antiarrhythmic peptide via protein kinase C activation. FASEB J 16:1114–1116
57. Xiao GQ, Mochly-Rosen D, Boutjdir M (2003) PKC isozyme selective regulation of cloned human cardiac delayed slow rectifier K current. Biochem Biophys Res Commun 306: 1019–1025
58. Xiao GQ, Qu Y, Sun ZQ, Mochly-Rosen D, Boutjdir M (2001) Evidence for functional role of epsilonPKC isozyme in the regulation of cardiac Na(+) channels. Am J Physiol Cell Physiol 281:C1477–C1486
59. Ye ZC, Wyeth MS, Baltan-Tekkok S, Ransom BR (2003) Functional hemichannels in astrocytes: a novel mechanism of glutamate release. J Neurosci 23:3588–3596
60. Ytrehus K, Liu Y, Downey JM (1994) Preconditioning protects ischemic rabbit heart by protein kinase C activation. Am J Physiol 266:H1145–H1152
61. Zatta AJ, Kin H, Lee G, Wang N, Jiang R, Lust R, Reeves JG, Mykytenko J, Guyton RA, Zhao ZQ, Vinten-Johansen J (2006) Infarct-sparing effect of myocardial postconditioning is dependent on protein kinase C signalling. Cardiovasc Res 70:315–324

Annexe II

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CARDIOVASCULAR PHYSIOLOGY

Connexin 43 mimetic peptide Gap26 confers protection to intact heart against myocardial ischemia injury

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Abstract Unapposed connexin 43 hemichannels (Cx43Hc) are present on sarcolemma of cardiomyocytes. Whereas Cx43Hc remain closed during physiological conditions, their opening under ischemic stress contributes to irreversible tissue injury and cell death. To date, conventional blockers of connexin channels act unselectively on both gap junction channels and unapposed hemichannels. Here, we test the hypothesis that Gap26, a synthetic structural mimetic peptide deriving from the first extracellular loop of Cx43 and a presumed selective blocker of Cx43Hc, confers resistance to intact rat heart against ischemia injury. Langendorff-perfused intact rat hearts were utilized. Regional ischemia was induced by 40-min occlusion of the left anterior descendent coronary and followed by 180 min of reperfusion. Gap26 was applied either 10 min before or 30 min after the initiation of ischemia. Interestingly, myocardial infarct size was reduced by 48% and 55% in hearts treated with Gap26 before or during ischemia, respectively, compared to untreated hearts. Additionally, myocardial perfusate flow was increased in both groups during reperfusion by 37% and 32%, respectively. Application of Gap26 increased survival of isolated cardiomyocytes after simulated ischemia–reperfusion by nearly twofold compared to untreated cells. On the other hand, superfusion of tsA201 cells transiently expressing Cx43 with Gap26 caused 61% inhibition of Cx43Hc-mediated currents recorded using the patch clamp technique. In summary, we demonstrate for the first time that Cx43 mimetic peptide Gap26 confers protection to intact heart against ischemia–reperfusion injury whether administered before or after the occurrence of ischemia. In addition, we provide unequivocal evidence for the inhibitory effect of Gap26 on genuine Cx43Hc.

Keywords Myocardial infarction · Connexin 43 · Connexin hemichannels · Peptides · Prevention · Treatment

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Introduction

Connexin 43 hemichannels (Cx43Hc) are hydrophilic transmembrane pathways formed by hexameric associations of Cx43 subunits. While most Cx43Hc are engaged in gap junction channels (GJC), some of them remain unapposed on the sarcolemma [54]. Unapposed Cx43Hc perform functions different from those achieved by GJC mainly by providing a pathway between cytosol and extracellular space to ions and other small metabolites [4, 20].

During physiological conditions, unapposed Cx43Hc remain predominantly closed [13, 44]. However, their opening is enhanced under non-physiological conditions like ischemia [14, 28, 29, 52]. The increased activity of

Cx43Hc is thought to disturb cellular homeostasis by inducing abnormal elevation of intracellular Na^+ and Ca^{2+} loads [32], release of ATP [5, 6], and osmotic imbalance [27, 42, 50, 51]. Consequently, cell swelling [50] and death [49, 56] occur. The purpose of this study is to investigate whether specific inhibition of unapposed Cx43Hc renders intact hearts more resistant to ischemia and reduces the resulting infarct size.

Conventional blockers of connexin channels act unselectively on both GJC and unapposed hemichannels, independent of the connexin isoforms forming these channels [46]. Interestingly, synthetic connexin-deriving structural mimetic peptides (CxMPs) emerged as powerful and unique tools capable of blocking unapposed hemichannels with little or no immediate effects on GJC. While initially developed with the intention to modulate the function of GJC [7, 8, 16, 17, 30], subsequent studies unravelled their preferential action on unapposed hemichannels [5, 6, 18, 19, 31]. By mimicking short amino acid sequences on connexins extracellular loops, CxMPs bind to these structures and cause inhibition of unapposed hemichannels by a yet undetermined mechanism (see review by Dahl [15]). Because each CxMP contains a conserved motif that is not consistently found in other connexins or cell surface proteins, it is generally admitted that CxMPs interact specifically with connexins in an isoform-selective manner. Intriguingly, direct evidence for the CxMP-mediated inhibitory effect on genuine and unequivocally identified connexin hemichannels has never been reported.

Here, we utilized for the first time Gap26, a structural CxMP of the Cx43 first extracellular loop and a presumed selective blocker of Cx43Hc, to assess its potential role in the protection of intact heart against regional ischemia and to substantiate its inhibitory effect on Cx43Hc. We demonstrate that acute exposure to Gap26 whether administered before or during ischemia provides protection to the heart against ischemia injury. We also utilized the ion channel-deficient tsA201 cells to assess the functional effect of Gap26 on exogenous Cx43Hc. The results provide the most direct evidence so far for the specific inhibitory effect of Gap26 on genuine Cx43Hc-mediated currents using a remarkably smaller concentration than previously reported [43].

Materials and methods

Peptides

Amino acid sequences for Gap26 peptide and its biological inactive scrambled version (sGap26) are VCYDKSF-PISHVR and YSIVCKPHVFDRS, respectively. Both peptides were synthesized with ≥85% purity by Sheldon Biotechnology Inc, Québec, Canada. In all experiments, peptides were utilized at a final concentration of 0.5 μM.

Intact heart study

Male Sprague–Dawley rats weighing 350 to 450 g were handled in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the Comité d'Éthique de l'Expérimentation Animale at our research center. Anesthesia was induced with ketamine/xylazine, 50 and 5 mg/kg, respectively. After thoracotomy, the heart was quickly excised, cannulated via the aorta, and perfused on a non-recirculating Langendorff perfusion apparatus using Krebs-Henseleit solution containing (in mM): 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.8 $CaCl_2$, and 5 glucose (pH7.4) at 37°C. The perfusate was oxygenated by continuous bubbling with 95% O_2/5% CO_2. Ten minutes was allowed for each heart to stabilize. Regional ischemia was then induced by ligation of the left anterior descendent (LAD) coronary artery using a 4.0 surgical thread. After 40-min ischemia, reperfusion was induced by removing ligation for 180 min. Ischemia was confirmed by myocardial surface cyanosis and reduction in myocardial perfusate flow (MPF).

The MPF was determined by measuring the volume of perfusate recovered from Langendorff-perfused heart during 1 min at 10 min after LAD occlusion or at 10 min after reperfusion and compared to the basal MPF (BMPF). The BMPF corresponds to the volume of perfusate recovered from the Langendorff-perfused heart during 1 min at 11 min before the LAD occlusion.

When applied, peptides were introduced in the perfusion solution starting at 10 min before or 30 min after the LAD occlusion (i.e., 10 min before reperfusion) depending on the experimental group as outlined in Fig. 1.

To determine the area at risk, the LAD was re-occluded and the heart was infused with 2 mL of 0.5% Evans Blue (Aldrich, USA) via the aorta. To determine infarct size, the heart was frozen at −80°C for 40 min and transversely sectioned. Heart slices were next incubated in 1.5% triphenyltetrazolium dye for 10 min at 37°C. After both stages, slices were scanned into a computer. The perfused area (stained in dark blue), the infarct area (whitish zone), and the area at risk (the red zone including whitish infarct areas) were measured using a homemade image analysis software.

Isolated cardiomyocytes study

Langendorff-perfused hearts were washed from blood with oxygenated Tyrode solution containing (in mM): 128 NaCl,

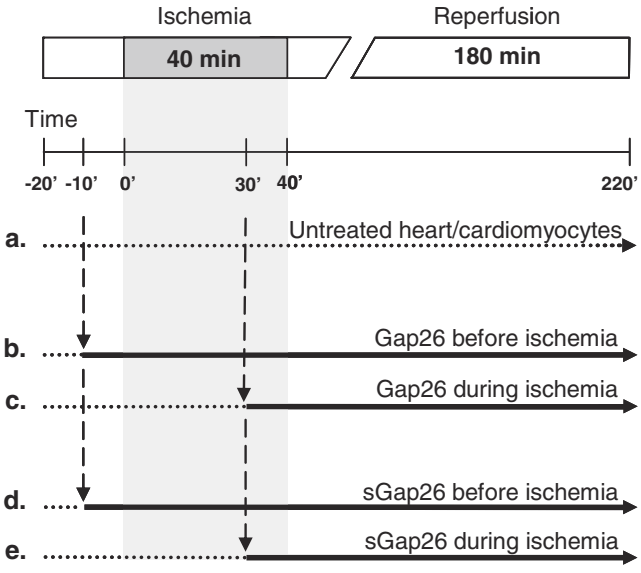

Fig. 1 A schematic representation of different experimental groups utilized to assess the effect of Gap26 during ischemia–reperfusion, in intact heart and isolated cardiomyocytes. The *gray zone* covers the ischemic period. The *dashed arrows* indicate the time when peptide is introduced. *a* Untreated hearts/cardiomyocytes; *b* hearts/cardiomyocytes treated with Gap26 starting 10 min before ischemia; *c* hearts/cardiomyocytes treated with Gap26 starting 30 min after the onset of ischemia; *d* hearts/cardiomyocytes treated with sGap26 starting 10 min before ischemia; and *e* hearts/cardiomyocytes treated with sGap26 starting 30 min after the onset of ischemia

0.47 NaH_2PO_4, 1.18 $MgSO_4{\cdot}7H_2O$, 20.1 $NaHCO_3$, 11.1 dextrose, 4.69 KCl, and 2.23 $CaCl_22{\cdot}2H_2O$ for 5 min at 37°C [3]. Next, hearts were stabilized for 5 min with calcium-free solution containing (in mM): 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 5 $MgSO_4{\cdot}7H_2O$, 20 glucose, 50 Taurine, 10 MOPS, pH7.4. After stabilization, hearts were digested during 5 min by perfusing with the same calcium-free solution to which 13.3 U/mL collagenase type I (Roche Diagnostics, Laval, Québec) was added. Ventricular tissues were chopped with surgical scissors and cardiomyocytes were dispersed gently using a wide-tipped pipette. Cells were pelleted by low speed centrifugation for 1 min and washed with Krebs-Henseleit buffer.

To simulate ischemia, cells were washed twice with degassed glucose-free Krebs-Henseleit buffer then gassed with N_2 for 15 min and pelleted as before. After removing 90% of the supernatant, mineral oil was layered over the remaining buffer to create an air-tight environment for 40 min as previously described [1, 2]. To simulate reperfusion, mineral oil was removed and cells were then washed with oxygenated Krebs-Henseleit buffer and incubated in the same buffer for 3 h at 37°C. Five groups of isolated myocytes were prepared for the study (Fig. 1): a control group using Gap26-free solutions, two groups in which Gap26 was introduced in Krebs-Henseleit solution either 10 min before or 30 min after the onset of ischemia, and two groups using the inactive sGap26 introduced either 10 min before or 30 min after the onset of ischemia. Gap26 concentration was kept constant until the end of experiments.

Cell viability was assessed using Trypan blue exclusion and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays. In the former test, viable cells were counted in a hemocytometer using 0.4% Trypan blue. Dead cells with damaged membranes absorb the blue dye and were therefore distinguishable from viable unstained cells under light microscope. In MTT assay [37], cell viability was deduced from the ability of cells to reduce the tetrazolium salt, MTT, and to form a blue formazan product by the mitochondrial enzyme succinate dehydrogenase. To this end, cells were incubated in 0.5 mg/mL MTT for 4 h at 37°C. Then, 100 μL of solubilization solution (0.04 M HCl-isopropanol) was added. The amount of MTT formazan product was quantified by measuring the optical density at 570 nm. The mitochondrial activity in each experimental group was presented as a percentage of the mitochondrial activity measured in a control group of cardiomyocytes maintained at normoxic conditions during a similar time frame, i.e., oxygenated Krebs-Henseleit buffer at 37°C during 4 h.

Electrophysiological study

Hemichannel-mediated currents were studied in tsA201 cells transiently transfected with connexin-encoding plasmids as previously described [24]. The tsA201 cells are ion channel-deficient mammalian cell line derived from the human embryonic kidney HEK293 cells by stable transfection with SV40 large T antigen [35]. Briefly, cells were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin G (100 U/mL), and streptomycin (10 mg/mL; Invitrogen Canada Inc, Burlington, ON) in 5% CO_2-humidified atmosphere.

Using the calcium phosphate method, cells were transfected with 7 μg of rat pBlue-Cx40, 43 or 45 and 7 μg of EBO plasmid, encoding for the CD8 surface antigen. All plasmids were a generous gift from Prof. Pascal Daleau (Université Laval, QC). Two to 3 days post transfection, cells were incubated for 2 min prior to patch clamp experiments in medium containing anti-CD8-coated beads (Invitrogen Canada, Inc). Transfected cells expressing CD8 and therefore binding beads were distinguished from non-transfected cells by light microscopy.

Cx43Hc currents were recorded in whole-cell configuration of the patch clamp technique using Axopatch200B amplifier (Molecular Devices, CA, USA) as previously described [24]. For whole-cell recordings, the pipette solution contained (in mM): 140 KCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid (HEPES), and 5 ethylene glycol tetraacetic acid (EGTA) adjusted to pH7.2. The bath solution contained (in mM): 140 NaCl, 1 $MgCl_2$, 5.4 KCl, 1.8 $CaCl_2$, 10 HEPES, and 10 EGTA adjusted to pH7.2. Data were digitized with a Digidata1440A analog-to-digital convertor and analyzed using pClamp 10.1 software (Molecular Devices, CA).

Statistical analysis

Data are expressed as mean ± SE. Analysis of variance adapted to factorial design (groups and time) with orthogonalization according to Winer [57] was used. Differences were deemed significant when $P<0.05$.

Results

Study on intact hearts

Administration of Gap26 before ischemia significantly decreased infarct size from 36.3±2.1 of area at risk in untreated hearts ($n=7$) to 18.8±1.9% ($n=6$, $P<0.05$) (Fig. 2a). In order to assess the peptide capability to counteract an existing ischemia, a separate group of hearts was studied in which Gap26 was introduced 30 min after the LAD coronary occlusion. Similarly, the infarct size was significantly reduced to 16.2±0.8% ($n=7$, $P<0.05$) of area at risk. These results correspond to infarct size reductions by 48.2% and 55.4% when Gap26 was introduced before or during ischemia, respectively. The size of area at risk to total ventricles did not change significantly in both experimental groups (41.3±4.5%, $n=6$, $P>0.05$ and 40.6±3.2%, $n=7$, $P>0.05$, respectively) compared to the group of untreated hearts (44.8±2.0%, $n=7$) (Fig. 2b). As negative control, sGap26 was also tested. Administration of sGap26 did not statistically affect the size of infarct area to area at risk when introduced before ischemia (41.1±3.0%, $n=3$, $P>0.05$) or during ischemia (39.2±2.0%, $n=4$, $P>0.05$) in comparison to untreated hearts (36.3±2.1%, $n=7$). The global size of total ventricles was comparable between groups of untreated hearts and hearts treated with Gap26 or sGap26 (data not shown). Percentage values for different heart areas obtained from various experimental groups are listed in Table 1. Transversal slices obtained from hearts representing the groups of untreated hearts, hearts treated with Gap26 before ischemia, and hearts treated with Gap26 during ischemia are shown in Fig. 2c–e.

In order to investigate the effect of Gap26 on the function of Langendorff-perfused hearts, we compared the MPF measured 10 min after LAD occlusion and 10 min after reperfusion to the BMPF. The BMPF is determined for

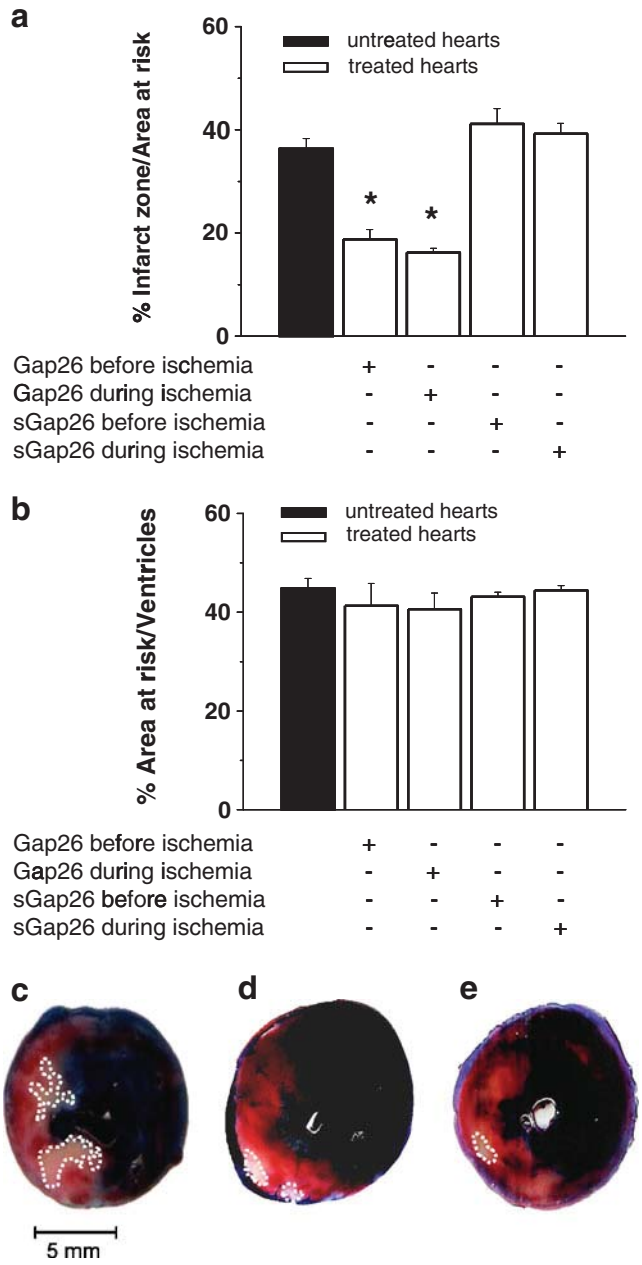

Fig. 2 Effect of synthetic peptides on intact hearts subjected to regional ischemia by LAD ligation. **a** Histogram representing the proportion of infarct zone to the area at risk, **b** histogram representing the proportion of area at risk to total ventricles. Values obtained from ischemic hearts in the presence of Gap26 or sGap26 added either 10 min before ($n=6$ and $n=3$, respectively) or 30 min after ($n=7$ and $n=4$, respectively) the occlusion of LAD are reported. *$P<0.05$ indicates statistically significant difference in comparison to untreated hearts ($n=7$). Photographs of TTC-stained heart sections after 40 min ischemia and 180 min reperfusion representing **c** untreated heart, **d** heart treated with Gap26 introduced 10 min before LAD occlusion, and **e** Gap26 introduced 30 min after LAD occlusion. **c**–**e** The infarct zone is the whitish area delimited with *white dashed lines*; the area at risk corresponds to the *red region* including the infarct zone; and the perfused area is in *dark blue*

Table 1 Percentage values for heart areas and myocardial perfusate flows

Groups	IZ/AAR (%)	AAR/V (%)	Number	MPFI/BMPF (%)	MPFR/BMPF (%)	Number
Untreated hearts	36.3±2.1	44.8±2.0	7	63.79±6.2	65.7±5.1	7
Gap26 before ischemia	18.8±1.9[a]	41.3±4.5	6	69.9±2.3	89.8±4.9[a, b]	7
Gap26 during ischemia	16.2±0.8[a]	40.6±3.2	7	58.50±7.4	86.7±4.8[a, b]	4
sGap26 before ischemia	41.1±3.0	43.1±0.9	3	59.07±3.7	57.1±3.1	3
sGap26 during ischemia	39.2±2.0	44.4±1.0	4	64.23±9.9	66.7±5.9	4

IZ infarct zone, *AAR* area at risk, *V* ventricles, *MPFI* myocardial perfusate flow measured during ischemia, *MPFR* myocardial perfusate flow measured during reperfusion, *BMPF* basal myocardial flow

[a] Statistically different in comparison to untreated group

[b] Statistically different in comparison to MPF during ischemia

each heart during normal conditions, i.e., before LAD occlusion in the absence of Gap26. Application of Gap26 to non-ischemic hearts during 1 h did not cause significant changes to MPF (data not shown). Importantly, addition of Gap26 in the perfusion solution increased MPF during reperfusion of ischemic hearts from 65.7±5.1% (n=7) of BMPF in untreated hearts to 89.8±4.9% (n=7, P<0.05) and 86.7±4.8% (n=4, P<0.05) in hearts treated with Gap26 before or during ischemia, respectively (Fig. 3). As negative control, sGap26 did not affect MPF when introduced before or during ischemia. Values for MPF variations in different experimental groups are listed in Table 1.

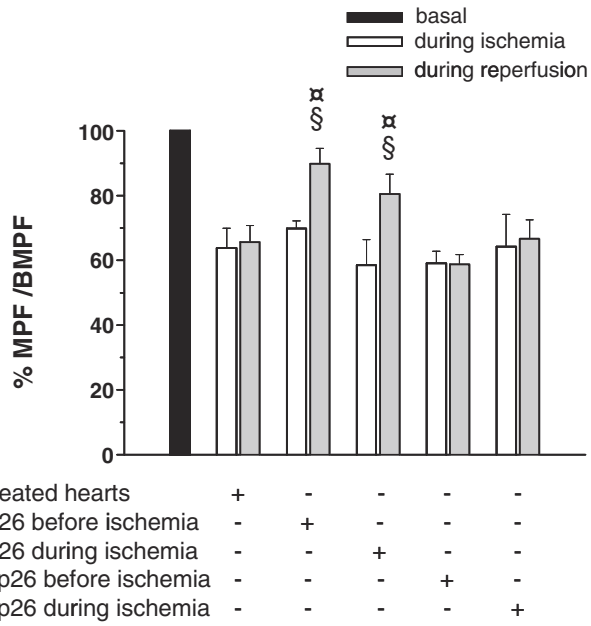

Fig. 3 Percentages of MPF measured at 10 min after LAD occlusion (*open columns*) or at 10 min after reperfusion (*filled columns*) to BMPF. The groups shown correspond to the untreated hearts (n=7), hearts treated with Gap26 before ischemia (n=7), hearts treated with Gap26 during ischemia (n=4), hearts treated with the inactive sGap26 before ischemia (n=3), and heart treated with sGap26 during ischemia (n=4). ¤P<0.05 indicates data significantly different from untreated hearts. §P<0.05 indicates data significantly different from MPF during ischemia

Study on isolated cardiomyocytes

In order to elucidate the cellular basis for the Gap26-mediated cardioprotection observed in intact hearts, we investigated the effect of the peptide on isolated cardiomyocytes from adult rat hearts subjected to simulated ischemia–reperfusion. Application of Gap26 in the bath solution either 10 min before or 30 min after the initiation of simulated ischemia increased the number of surviving cardiomyocytes after 180 min reperfusion from 28.4±6.5% of total cells (n=8) to 53.1±6.8% (n=8, P<0.05) and 55.0±6.5% (n=8, P<0.05), respectively (Fig. 4a). The percentage of surviving cells did not differ statistically between groups treated with Gap26 before or during simulated ischemia. As negative control, the percentage of surviving cells following application of sGap26 before ischemia (22.9±6.9%, n=8) or during ischemia (26.0±6.6%, n=8) did not differ significantly in comparison to untreated cells (28.4±6.5%, n=8, P>0.05). In order to substantiate these findings, we assessed mitochondrial activity in each experimental group of cardiomyocytes using the colorimetric MTT assay. To alleviate comparison, results are expressed as percentages of the mitochondrial activity measured in a control group of cardiomyocytes maintained at normoxic conditions during a similar time frame. Simulated ischemia–reperfusion reduced mitochondrial activity in untreated cardiomyocytes to 49.7±1.2%, n=5, P<0.05 in comparison to the control cardiomyocytes 100.0±1.2%, n=5. Treatment of cardiomyocytes with Gap26 prevented partially but significantly this mitochondrial dysfunction when introduced before or during ischemia (83.0±1.1%, n=5, P<0.05 and 82.1±1.1%, n=5, P<0.05, respectively). Application of sGap26 did not prevent the mitochondrial dysfunction when applied before ischemia (50.3±1.0%, n=5, P>0.05) or during ischemia (51.8±0.9%, n=5, P>0.05) in comparison to untreated cells.

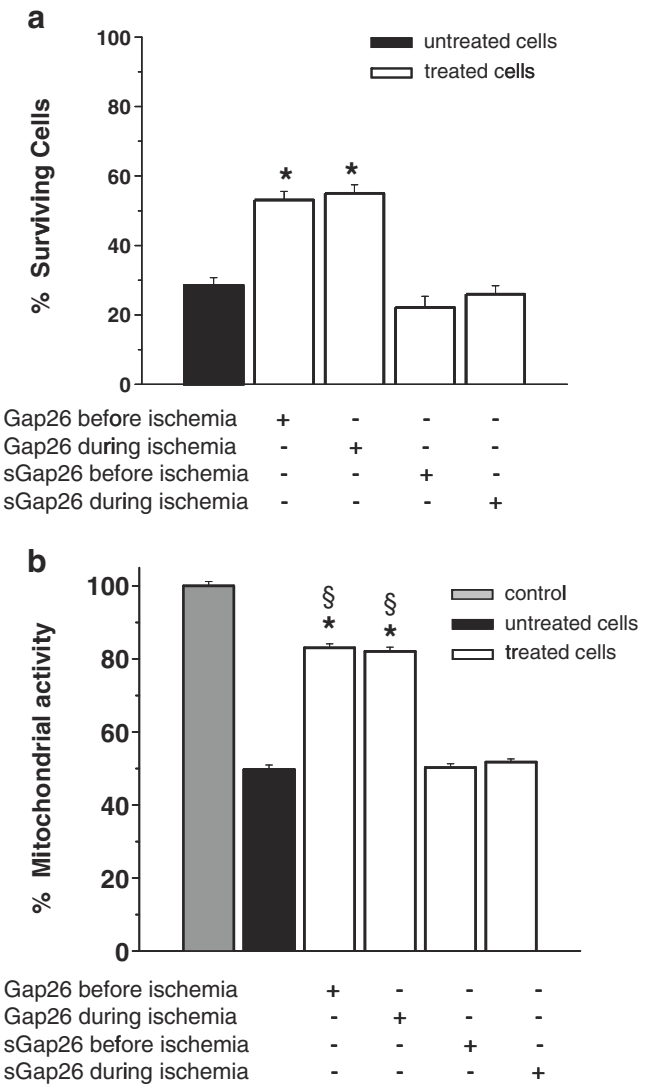

Fig. 4 Effect of Gap26 on viability of isolated adult rat cardiomyocytes during simulated ischemia–reperfusion. **a** The percentage of surviving cells to total cells after reperfusion was determined in the absence or presence of Gap26 or sGap26 added to the bath solution before or during ischemia (n=8 in each group). Data are expressed as mean ± SEM of eight experiments using myocytes from three different animals. **b** Mitochondrial dysfunction was determined with the MTT test (n=5 in each group, obtained from a single rat heart). *P<0.05 indicates significant difference in comparison to untreated cells. §P< 0.05 indicates significant difference in comparison to control

Results obtained from the different experimental groups are illustrated in the histogram (Fig. 4b).

Gap26 inhibits Cx43Hc currents

Because intracellular ionic imbalance is a key factor in the mechanism of cell death during ischemic stress, we investigated the functional effect of Gap26 on Cx43Hc-mediated currents in individual tsA201 cells transiently expressing Cx43. Figure 5a shows family traces of macroscopic Cx43Hc-mediated currents elicited by depolarizing the cell from a resting potential of +0 mV to test pulses ranging from +0 to +130 mV in a low calcium bath solution. To substantiate the identity of currents recorded from Cx43-transfected cells, patch clamp experiments were performed on non-transfected tsA201 cells. No currents could be recorded from these cells (Fig. 5b), therefore confirming the absence of contaminating endogenous currents. In Fig. 5c, a representative time course recording shows rapid Cx43Hc currents reduction when Gap26 was introduced in the bath solution. A steady-state inhibition was reached in all experiments. Averaged data indicate that Gap26 caused 60.1±4.6% (n=5, P<0.05) current reduction. In the presence of sGap26 peptide, the amplitude of Cx43Hc currents did not vary over a similar time frame (99.0±1.1%, n=5, P>0.05) (Fig. 5d). The histogram (Fig. 5e) summarizes the effects of both peptides on normalized Cx43Hc currents. Curves representing the current–voltage (I–V) relationship for Cx43Hc in the absence and in the presence of Gap26 are illustrated in Fig. 5f. I–V data were elicited by depolarizing cells with voltage steps ranging from +0 to +90 mV.

Because connexin 40 (Cx40) and connexin 45 (Cx45) may also form hemichannels in the heart, we examined whether Gap26 has effects on currents recorded from tsA201 cells transfected with either connexin isoforms. Importantly, the application of Gap26 did not cause a significant reduction in currents mediated by Cx40Hc (0.7±1.6%, n=4, P>0.05) or Cx45Hc (0.2±0.7%, n=4, P>0.05) over a time frame similar to that used in Cx43Hc experiments (Fig. 6).

Discussion

The most important finding in this study is the protection that Cx43 mimetic peptide Gap26 conferred to intact rat heart against regional ischemia induced by LAD coronary occlusion. We showed that administration of Gap26 prior to LAD occlusion resulted in more than 48% reduction of infarct size compared to untreated hearts. Similarly, Gap26 reduced infarct size by 55% when administered during ischemia. We also showed that the salutary effect of Gap26 extends to heart function by increasing the MPF during reperfusion by nearly 37% and 32% when the peptide was administered before or during LAD occlusion, respectively, in comparison to untreated hearts. These results indicate that whereas Gap26 can confer resistance to "normal" hearts against subsequent ischemia, it also has the capability to salvage injured hearts when administered after the occurrence of ischemia. Hypothetically, we ascribe these effects to the presumable Gap26-mediated inhibition of cardiomyocytic Cx43Hc opened by ischemic stress. Indeed, Gap26 is also known to inhibit Cx43 GJCs when used at

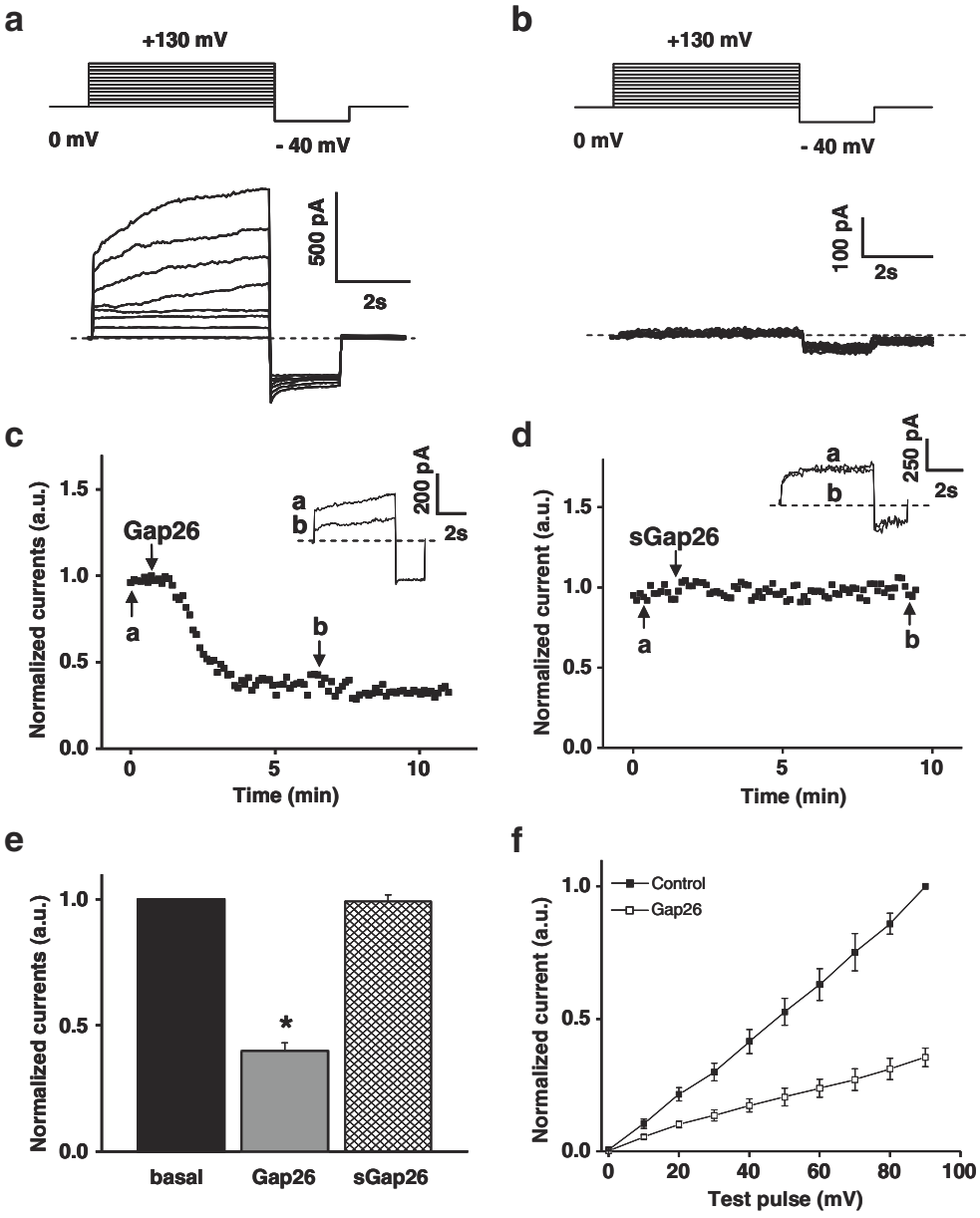

Fig. 5 Cx43Hc-mediated whole-cell current inhibition by Gap26 recorded from individual tsA201 cells expressing exogenous Cx43. **a** Family traces of Cx43Hc-mediated currents recorded from a typical cell by depolarization from a holding potential of 0 mV to test pulses ranging from 0 to +130 mV. **b** No detectable currents could be recorded from a non-transfected tsA201 cell using same protocol as in **a**. The *dashed line* represents the zero current. Time course recorded from a typical cell dialysed with Gap26 (**c**) or sGap26 (**d**). *Insets* illustrate Cx43Hc current traces in response to membrane depolarisation recorded at time points indicated by *arrows a* and *b*. **e** Histogram showing percentage of Cx43Hc currents inhibition in response to Gap26 (n=5) and sGap26 (n=5). Current amplitudes are normalized against control and represented in arbitrary units (a.u.). *P<0.05 in comparison to basal currents. **f** *I–V* curves obtained from Cx43-expressing tsA201 cells in the absence (n=5) and in the presence (n=5) of Gap26

relatively high concentrations and/or following prolonged exposure [18]. Nonetheless, all experiments in this study were performed using low concentration of Gap26 (0.5 μM) previously shown to selectively inhibit hemichannels without directly affecting GJCs [11, 53]. Therefore, we consider that neither the peptide concentration nor the duration of exposure to Gap26 (which varied depending on protocols between ~3 and 4 h) was sufficient to modulate GJCs in the studied hearts.

In concordance with the fact that death of cardiomyocytes, and therefore myocardial injury, principally occurs during reperfusion [21, 49, 58], both structural and functional improvements in intact hearts did not differ significantly whether Gap26 was introduced before occlusion or before reperfusion. To investigate the cellular basis of the protection of intact hearts, we assessed the effect of Gap26 on isolated cardiomyocytes subjected to simulated ischemia–reperfusion. We found a nearly twofold increase in the number of surviving cells when Gap26 was administered either before or during the simulated ischemia. A similar increase was previously noted in isolated neonatal rat cardiomyocytes treated with Gap26 prior to simulated ischemia [49]. Clearly, both observations underscore the capability of Gap26 to confer protection to cardiomyocytes in the absence of direct intercellular communication, precisely through GJC, and therefore point to the inhibition of unapposed hemichannels as the underlying mechanism of cardioprotection.

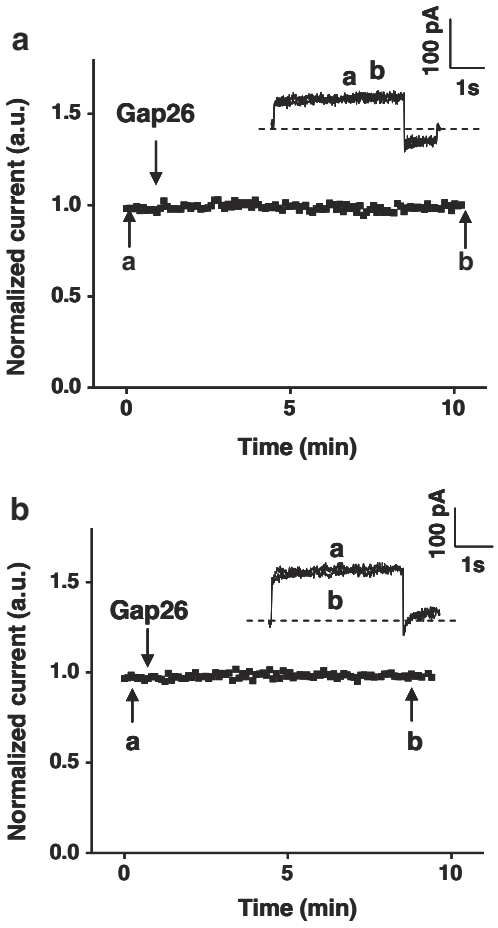

Fig. 6 Effect of Gap26 on Cx40Hc and Cx45Hc. Time course recording from a typical individual tsA201 cell expressing Cx40 (**a**) or Cx45 (**b**) dialysed with Gap26. *Insets* illustrate the current traces recorded at time points indicated by *arrows a* and *b*

Another important finding in this study is the rapid depression of Cx43Hc electrical conductance in response to the application of Gap26. To date, the involvement of unequivocally identified unapposed hemichannels in CxMPs-mediated phenomena, such as regulation of ATP release and calcium propagation, is still debated [15]. Here, we showed for the first time that superfusion of the ion channel-deficient tsA201 cells, transiently expressing Cx43, with Gap26 readily inhibits Cx43Hc-mediated currents recorded from individual cells. This observation complements with previous studies reporting on the inhibitory effect of Gap26 on the *permeability* of Cx43Hc [6, 19, 31, 40], the second major functional characteristic of connexin channels besides conductance. Although not surprisingly, this result represents the most direct evidence reported so far for the Gap26 inhibitory effect on Cx43Hc. Curiously, this inhibition occurred using a peptide concentration that is 1,000 times lower than what has previously been utilized to block hemichannel currents (0.5 μM in this study *versus* 500 μM in [43]). Occasionally, the specificity of CxMPs toward hemichannels has been challenged [55]. Therefore, we tested the effect of Gap26 on hemichannels of Cx40 and Cx45 which are also present in the heart. Importantly, Gap26 did not affect currents from Cx40Hc or Cx45Hc.

Altogether, these results strongly suggest that the observed cardioprotection conferred by Gap26 is most likely mediated by the specific inhibition of Cx43Hc opened by the ischemic stress.

Scientific context and perspectives

The non-junctional Cx43 have previously been associated with ischemic preconditioning-mediated cardioprotection [33, 39], a phenomenon whereby brief episodes of ischemia reduce the adverse effects of subsequent prolonged ischemia [12, 38, 48]. This effect has been related to increase in Cx43 phosphorylation [23, 25, 36, 47]. Interestingly, different studies pointed to the isozyme epsilon of protein kinase C (εPKC) as a principal mediator of ischemic preconditioning-mediated cardioprotection [22, 25, 26, 34, 41, 45]. More recently, we demonstrated using a unique set of PKC isozyme-specific modulator peptides that εPKC, among other PKC isozymes, selectively inhibits the conductance of Cx43Hc [24]. These data prompt us to believe that inhibition of Cx43Hc is also a fundamental basis for the cardioprotection conferred by ischemic preconditioning. In the light of our current findings and given the ubiquitous expression of PKC and the abundance of its substrates within cells and throughout tissues, we put forth that the selective inhibition of the abnormally open Cx43Hc, which we believe are solely localized in the ischemic region of the heart, would be more suitable to mimic pharmacologically the cardioprotection conferred by ischemic preconditioning than to modulate PKC isozymes as previously proposed [9, 10]. The accessibility of CxMP binding sites from extracellular space, which circumvents the need of conjugating the peptides with transmembrane carriers and the limitations deriving from their use, is indeed another therapeutic advantage over the use of intracellular modulators. Certainly, studies using more elaborated experimental models are needed to substantiate the therapeutic potentials of Gap26.

In conclusion, we provide the most direct evidence so far for the inhibitory effect of Gap26 on Cx43Hc-specific currents. We demonstrate for the first time that administration of Gap26 prior to ischemia prevents injury by making intact heart more resistant to ischemic stress. Moreover, usage of Gap26 as a treatment following occurrence of ischemia reduces cardiac tissue damage and improves intact heart function. The discovery of new agents capable to make heart more resistant to ischemia and/or to improve its recovery after injury caused by ischemia will certainly be promising tools to fight ischemic heart disease.

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Conflict of interest None declared.

References

1. Armstrong S, Downey JM, Ganote CE (1994) Preconditioning of isolated rabbit cardiomyocytes: induction by metabolic stress and blockade by the adenosine antagonist SPT and calphostin C, a protein kinase C inhibitor. Cardiovasc Res 28:72–77
2. Armstrong SC, Kao R, Gao W, Shivell LC, Downey JM, Honkanen RE, Ganote CE (1997) Comparison of in vitro preconditioning responses of isolated pig and rabbit cardiomyocytes: effects of a protein phosphatase inhibitor, fostriecin. J Mol Cell Cardiol 29:3009–3024
3. Bendukidze Z, Isenberg G, Klockner U (1985) Ca-tolerant guinea-pig ventricular myocytes as isolated by pronase in the presence of 250 microM free calcium. Basic Res Cardiol 80 (Suppl 1):13–17
4. Bennett MV, Contreras JE, Bukauskas FF, Saez JC (2003) New roles for astrocytes: gap junction hemichannels have something to communicate. Trends Neurosci 26:610–617
5. Braet K, Aspeslagh S, Vandamme W, Willecke K, Martin PE, Evans WH, Leybaert L (2003) Pharmacological sensitivity of ATP release triggered by photoliberation of inositol-1, 4, 5-trisphosphate and zero extracellular calcium in brain endothelial cells. J Cell Physiol 197:205–213
6. Braet K, Vandamme W, Martin PE, Evans WH, Leybaert L (2003) Photoliberating inositol-1, 4, 5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26. Cell Calcium 33:37–48
7. Chaytor AT, Evans WH, Griffith TM (1997) Peptides homologous to extracellular loop motifs of connexin 43 reversibly abolish rhythmic contractile activity in rabbit arteries. J Physiol 503(Pt 1):99–110
8. Chaytor AT, Evans WH, Griffith TM (1998) Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. J Physiol 508(Pt 2):561–573
9. Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GW, Mochly-Rosen D (2001) Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. Proc Natl Acad Sci U S A 98:11114–11119
10. Chen L, Wright LR, Chen CH, Oliver SF, Wender PA, Mochly-Rosen D (2001) Molecular transporters for peptides: delivery of a cardioprotective epsilonPKC agonist peptide into cells and intact ischemic heart using a transport system, R(7). Chem Biol 8:1123–1129
11. Clarke TC, Williams OJ, Martin PE, Evans WH (2009) ATP release by cardiac myocytes in a simulated ischaemia model: inhibition by a connexin mimetic and enhancement by an antiarrhythmic peptide. Eur J Pharmacol 605:9–14
12. Cohen MV, Liu GS, Downey JM (1991) Preconditioning causes improved wall motion as well as smaller infarcts after transient-coronary occlusion in rabbits. Circulation 84:341–349
13. Contreras JE, Saez JC, Bukauskas FF, Bennett MV (2003) Gating and regulation of connexin 43 (Cx43) hemichannels. Proc Natl Acad Sci U S A 100:11388–11393
14. Contreras JE, Sanchez HA, Eugenin EA, Speidel D, Theis M, Willecke K, Bukauskas FF, Bennett MV, Saez JC (2002) Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. Proc Natl Acad Sci U S A 99:495–500
15. Dahl G (2007) Gap junction-mimetic peptides do work, but in unexpected ways. Cell Commun Adhes 14:259–264
16. Dahl G (1996) Where are the gates in gap junction channels? Clin Exp Pharmacol Physiol 23:1047–1052
17. Evans WH, Boitano S (2001) Connexin mimetic peptides: specific inhibitors of gap-junctional intercellular communication. Biochem Soc Trans 29:606–612
18. Evans WH, De Vuyst E, Leybaert L (2006) The gap junction cellular internet: connexin hemichannels enter the signalling limelight. Biochem J 397:1–14
19. Gomes P, Srinivas SP, Van Driessche W, Vereecke J, Himpens B (2005) ATP release through connexin hemichannels in corneal endothelial cells. Invest Ophthalmol Vis Sci 46:1208–1218
20. Goodenough DA, Paul DL (2003) Beyond the gap: functions of unpaired connexon channels. Nat Rev Mol Cell Biol 4:285–294
21. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 94:1621–1628
22. Gray MO, Karliner JS, Mochly-Rosen D (1997) A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. J Biol Chem 272:30945–30951
23. Hatanaka K, Kawata H, Toyofuku T, Yoshida K (2004) Down-regulation of connexin43 in early myocardial ischemia and protective effect by ischemic preconditioning in rat hearts in vivo. Jpn Heart J 45:1007–1019
24. Hawat G, Baroudi G (2008) Differential modulation of unapposed connexin 43 hemichannel electrical conductance by protein kinase C isoforms. Pflugers Arch 456:519–527
25. Hund TJ, Lerner DL, Yamada KA, Schuessler RB, Saffitz JE (2007) Protein kinase C epsilon mediates salutary effects on electrical coupling induced by ischemic preconditioning. Heart Rhythm 4:1183–1193
26. Inagaki K, Hahn HS, Dorn GW, Mochly-Rosen D (2003) Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. Circulation 108:869–875
27. Jennings RB, Reimer KA, Steenbergen C (1986) Myocardial ischemia revisited. The osmolar load, membrane damage, and reperfusion. J Mol Cell Cardiol 18:769–780
28. John SA, Kondo R, Wang SY, Goldhaber JI, Weiss JN (1999) Connexin-43 hemichannels opened by metabolic inhibition. J Biol Chem 274:236–240
29. Kondo RP, Wang SY, John SA, Weiss JN, Goldhaber JI (2000) Metabolic inhibition activates a non-selective current through connexin hemichannels in isolated ventricular myocytes. J Mol Cell Cardiol 32:1859–1872
30. Kwak BR, Jongsma HJ (1999) Selective inhibition of gap junction channel activity by synthetic peptides. J Physiol 516(Pt 3):679–685
31. Leybaert L, Braet K, Vandamme W, Cabooter L, Martin PE, Evans WH (2003) Connexin channels, connexin mimetic peptides and ATP release. Cell Commun Adhes 10:251–257
32. Li F, Sugishita K, Su Z, Ueda I, Barry WH (2001) Activation of connexin-43 hemichannels can elevate [Ca(2+)]i and [Na(+)]i in rabbit ventricular myocytes during metabolic inhibition. J Mol Cell Cardiol 33:2145–2155
33. Li X, Heinzel FR, Boengler K, Schulz R, Heusch G (2004) Role of connexin 43 in ischemic preconditioning does not involve

intercellular communication through gap junctions. J Mol Cell Cardiol 36:161–163
34. Liu GS, Cohen MV, Mochly-Rosen D, Downey JM (1999) Protein kinase C-epsilon is responsible for the protection of preconditioning in rabbit cardiomyocytes. J Mol Cell Cardiol 31:1937–1948
35. Margolskee RF, Hendry-Rinde B, Horn R (1993) Panning transfected cells for electrophysiological studies. Biotechniques 15:906–911
36. Miura T, Ohnuma Y, Kuno A, Tanno M, Ichikawa Y, Nakamura Y, Yano T, Miki T, Sakamoto J, Shimamoto K (2004) Protective role of gap junctions in preconditioning against myocardial infarction. Am J Physiol Heart Circ Physiol 286:H214–H221
37. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 65:55–63
38. Murry CE, Jennings RB, Reimer KA (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74:1124–1136
39. Padilla F, Garcia-Dorado D, Rodriguez-Sinovas A, Ruiz-Meana M, Inserte J, Soler-Soler J (2003) Protection afforded by ischemic preconditioning is not mediated by effects on cell-to-cell electrical coupling during myocardial ischemia–reperfusion. Am J Physiol Heart Circ Physiol 285:H1909–H1916
40. Pearson RA, Dale N, Llaudet E, Mobbs P (2005) ATP released via gap junction hemichannels from the pigment epithelium regulates neural retinal progenitor proliferation. Neuron 46:731–744
41. Ping P, Zhang J, Qiu Y, Tang XL, Manchikalapudi S, Cao X, Bolli R (1997) Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. Circ Res 81:404–414
42. Quist AP, Rhee SK, Lin H, Lal R (2000) Physiological role of gap-junctional hemichannels. Extracellular calcium-dependent isosmotic volume regulation. J Cell Biol 148:1063–1074
43. Romanov RA, Rogachevskaja OA, Bystrova MF, Jiang P, Margolskee RF, Kolesnikov SS (2007) Afferent neurotransmission mediated by hemichannels in mammalian taste cells. EMBO J 26:657–667
44. Saez JC, Retamal MA, Basilio D, Bukauskas FF, Bennett MV (2005) Connexin-based gap junction hemichannels: gating mechanisms. Biochim Biophys Acta 1711:215–224
45. Saurin AT, Pennington DJ, Raat NJ, Latchman DS, Owen MJ, Marber MS (2002) Targeted disruption of the protein kinase C epsilon gene abolishes the infarct size reduction that follows ischaemic preconditioning of isolated buffer-perfused mouse hearts. Cardiovasc Res 55:672–680
46. Scemes E, Spray DC, Meda P (2009) Connexins, pannexins, innexins: novel roles of "hemi-channels". Pflugers Arch 457:1207–1226
47. Schulz R, Gres P, Skyschally A, Duschin A, Belosjorow S, Konietzka I, Heusch G (2003) Ischemic preconditioning preserves connexin 43 phosphorylation during sustained ischemia in pig hearts in vivo. FASEB J 17:1355–1357
48. Shiki K, Hearse DJ (1987) Preconditioning of ischemic myocardium: reperfusioninduced arrhythmias. Am J Physiol 253:H1470–H1476
49. Shintani-Ishida K, Uemura K, Yoshida K (2007) Hemichannels in cardiomyocytes open transiently during ischemia and contribute to reperfusion injury following brief ischemia. Am J Physiol Heart Circ Physiol 293:H1714–H1720
50. Steenbergen C, Hill ML, Jennings RB (1985) Volume regulation and plasma membrane injury in aerobic, anaerobic, and ischemic myocardium in vitro. Effects of osmotic cell swelling on plasma membrane integrity. Circ Res 57:864–875
51. Tranum-Jensen J, Janse MJ, Fiolet WT, Krieger WJ, D'Alnoncourt CN, Durrer D (1981) Tissue osmolality, cell swelling, and reperfusion in acute regional myocardial ischemia in the isolated porcine heart. Circ Res 49:364–381
52. Vergara L, Bao X, Cooper M, Bello-Reuss E, Reuss L (2003) Gap-junctional hemichannels are activated by ATP depletion in human renal proximal tubule cells. J Membr Biol 196:173–184
53. Verma V, Hallett MB, Leybaert L, Martin PE, Evans WH (2009) Perturbing plasma membrane hemichannels attenuates calcium signalling in cardiac cells and HeLa cells expressing connexins. Eur J Cell Biol 88:79–90
54. Verselis VK, Trexler EB, Bukauskas FF (2000) Connexin hemichannels and cell–cell channels: comparison of properties. Braz J Med Biol Res 33:379–389
55. Wang J, Ma M, Locovei S, Keane RW, Dahl G (2007) Modulation of membrane channel currents by gap junction protein mimetic peptides: size matters. Am J Physiol Cell Physiol 293:C1112–C1119
56. Wilde AA, Aksnes G (1995) Myocardial potassium loss and cell depolarisation in ischaemia and hypoxia. Cardiovasc Res 29:1–15
57. Winer BJ (1971) Statistical principles in experimental design, 2nd edn. McGraw-Hill, New York
58. Zhao ZQ, Nakamura M, Wang NP, Wilcox JN, Shearer S, Ronson RS, Guyton RA, Vinten-Johansen J (2000) Reperfusion induces myocardial apoptotic cell death. Cardiovasc Res 45:651–660

Annexe III

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Original Article

Single intravenous low-dose injections of connexin 43 mimetic peptides protect ischemic heart in vivo against myocardial infarction

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ABSTRACT

The opening of unapposed connexin 43 hemichannels (Cx43Hc) under ischemic stress leads to cell death and irreversible tissue injury. Here, we investigate for the first time in vivo the cardioprotective potentials of two unique Cx43 structural-mimetic peptides (Cx43MPs) presumed specific blockers of Cx43Hc, Gap26 and Gap27, when injected intravenously using a rat model of myocardial infarction.Sprague Dawley rats were utilized. Myocardial infarction was induced by occluding the left anterior descending coronary for 40 min followed by 2 days of reperfusion. Interestingly, single bolus injections of Gap26 or Gap27 (1 μg/kg) into the jugular vein caused infarct size reductions by up to 61% with reference to control rats injected with saline at similar timings. Infarct reductions did not vary significantly whether peptides were administered before or after the onset of ischemia. Although the two peptides allegedly interact with distinct structures of Cx43, co-administration of Gap26/Gap27 in equal doses did not confer additive protection to hearts (maximum infarct reduction by 64%). Using patch clamp technique, we provide unique and direct evidence for the inhibitory effect of Cx43MPs on genuine human Cx43Hc transiently expressed in the ion channel-deficient tsA201 cells. In concordance with the cardioprotective effect observed in vivo, co-application of both peptides did not cause cumulative current inhibition. A safety profile of Cx43MPs was also addressed.Our results reveal great therapeutic potential of Cx43MPs in treatment of myocardial infarction. Their practical way and timing of administration and their apparent safe profile make them promising tools to fight ischemic heart disease.

1. Introduction

In cardiac myocytes, most sarcolemmal connexin 43 hemichannels (Cx43Hc) appose with counterparts from adjacent cells to form gap junction channels. Until apposition occurs, the unapposed Cx43Hc remain predominantly closed [1]. Abnormally, these Hc can open under ischemic stress [2–4]. The increased permeability of Hc leads to disturbances in cellular homeostasis [5,6] and uncontrolled release of ATP [7,8], which consequently cause cell death and irreversible tissue injury [9–11].

Initially, the role of Cx43 in myocardial ischemia injury was suggested by Garcia-Dorado and collaborators by pharmacological inhibition with heptanol, a nonspecific inhibitor of Cx channels that blocks both gap junction channels and unapposed Hc [12]. This was later confirmed by others [13] and extended to other nonspecific blockers [14]. Similar results have also been described in transgenic mice models [15,16]. Remarkably, whereas all these studies addressed the role of connexins forming gap junctions, none of them excludes the possibility that these effects were due to actions on unapposed Hc. In fact, several other studies – including ours – support the hypothesis that unapposed CxHc are key mediators of ischemic injury [7,11,17–20]. In concordance with this theory, studies conducted to elucidate the role of Cx43 in cardioprotection mediated by ischemic preconditionning clearly point to the fact that the implication of Cx43 in this phenomenon is not exerted through gap junction channels [21,22].

All these observations led us to believe that the specific inhibition of unapposed Cx43Hc confers protection to ischemic heart against injury.

In contrast with the classical inhibitors of Cx channels that are neither specific (Hc versus Gap junction channels) nor selective (vis-à-vis various Cx isoforms), synthetic Cx structural-mimetic peptides (CxMPs) have emerged more recently as unique and powerful specific blockers of unapposed Hc that exert little or no effect on gap junction channels [23,24]. CxMPs are also believed to interact selectively with various Cx isoforms. Until recently, the inhibitory effect of CxMPs has never been

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demonstrated directly and specifically on identified Hc but rather deduced indirectly [25]. Exceptionally, we recently demonstrated that using the ion channel-deficient tsA201 cells transiently expressing the rat variant of Cx43 that Gap26, a Cx43MP mimicking the first extracellular loop of Cx43, readily inhibits macroscopic Cx43Hc-mediated currents. In support of its alleged selectivity, we also demonstrated that Gap26 does not inhibit currents from other cardiac CxHc (i.e., Cx40Hc and Cx45Hc) [20].

Interestingly, treatment with Gap26 of isolated cardiomyocytes in vitro and of excised rat hearts ex vivo made these experimental models significantly resistant to ischemic injury [20]. In agreement with these findings, we hypothesized that Cx43MPs, if administered systemically in vivo, exert similar cardioprotective effects against ischemic injury. Therefore, we assess here for the first time in vivo the cardioprotective potentials of the only two known Cx43MPs, Gap26 and Gap27, mimicking the first and the second extracellular loops of Cx43 respectively, when administered intravenously in low-dose single bolus injections before or after the onset of ischemia. We also investigate the functional effect of both Cx43MPs directly on human Cx43Hc (hCx43Hc) exogenously expressed in a human cellular expression system. To our knowledge, this work represents the foremost attempt yet to assess CxMPs therapeutic applicability in a complete organism for these increasingly promising tools in fighting ischemic diseases.

2. Methods

2.1. Peptides

Amino acid sequences for Cx43MPs are VCYDKSFPISHVR for Gap26 and SRPTEKTIFII for Gap27, their biological inactive scrambled versions are YSIVCKPHVFDRS for sGap26 and SETKIRPITFI for sGap27. The sequence of a flag-tagged Gap26 peptide utilized for confocal microscopy imaging consisted of the following 21-amino acid sequence, DYKDDDDKVCYDKSFPISHVR. All peptides were synthesized with ≥85% purity by Canpeptide Inc., Montréal, Canada. For intravenous injections, peptides were diluted in saline solution and administered at 1 μg/kg dose when injected individually or 1 μg/kg for each peptide when injected in combination. For patch clamp experiments, peptides were diluted in bath solution as previously described [20] to a final concentration of 0.5 μmol/L when peptides are tested individually or 0.5 μmol/L for each peptide when peptides are combined.

2.2. Immunostaining

Four to six-micron sections were cut from the heart of a rat injected with the flag-tagged Gap26. Tissues were then fixed using a 1:3 acetone/methanol solution for 20 min before they were permeabilized using 0.1% Triton into 1 mmol/L PBS-0.5% BSA solution and incubated with antibodies as previously described [26]. The mouse anti-flag primary antibody utilized in 1:200 dilution against the flag-tagged Gap26 peptide was purchased from ProtTech, Inc. (Fairview Village, PA). The secondary antibody, a conjugated DyLight594 goat anti-mouse, was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and diluted at 1:400. To detect Cx43, we utilized a rabbit anti-Cx43 primary antibody targeted against the C-terminus diluted at 1:500 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The corresponding secondary antibody (Conjugated DyLight488 anti-rabbit) used in 1:400 dilution was also from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

2.3. Confocal microscopy

Fluorescent probe-labeled myocardial tissues were viewed by a Bio-Rad MRC-1024 confocal imaging system equipped with a krypton–argon laser beam and mounted on a Zeiss microscope. A 60× oil objective with a 1.4 numerical aperture was used. Confocal settings were as follows: 1-mw laser power, 1.2 zoom, 1 s per scan, Kalman filter, and 4 frames per image. The photomultiplier gain was set to maximum, and the confocal aperture was adjusted for maximum resolution.

2.4. In vivo study

Male Sprague–Dawley rats weighing 350 to 450 g were handled in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the *Comité d'Éthique de l'Expérimentation Animale* at our research center. Anesthesia was induced with ketamine/xylazine, 50 and 5 mg/kg intramuscularly, respectively, after which the rats were intubated and placed on an artificial respirator and anesthesia was maintained with isoflurane (1.5%) ventilation. Absence of response to painful stimuli was used as index to adequate anesthesia. A left thoracotomy was performed at the fifth intercostal space, and the left coronary artery was occluded with a silk suture. A bolus of saline with or without peptides was injected into the jugular vein 10 min before or 30 min after the LAD occlusion (i.e., 10 min before reperfusion) (Fig. 2A). The occlusion was removed after 40 min, the chest was closed, and the animal was returned to its cage. Forty-eight hours later, a blood sample was drawn for testing and the animals were then euthanized by excising the heart under anesthesia by intramuscular injection of ketamine/xylazine (50 and 5 mg/kg, respectively). The heart, brain, lungs, liver, and kidneys were rapidly removed for histopathology study. To determine the area at risk in the excised heart, the LAD was re-occluded and heart was infused with 2 mL of 0.5% Evans Blue (Aldrich, USA) via the aorta. To determine infarct size, the heart was frozen at −80 °C for 40 min and transversely sectioned. Heart slices were next incubated in 1.5% triphenyltetrazolium dye for 10 min at 37 °C. After both stages, slices were scanned into the computer. The perfused area (stained in dark blue), the infarct area (whitish zone), and the area at risk (the red zone including whitish infarct areas) were measured using a homemade image analysis software following the method previously described by Reimer and Jennings [27].

2.5. Cell electrophysiology

Cx43Hc-mediated currents were studied in tsA201 cells transiently transfected with hCx43 gene as previously described [28]. Briefly, tsA201 cells were grown in high-glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), penicillin G (100 U/ml), and streptomycin (10 mg/ml; Invitrogen Canada Inc., Burlington, ON) in 5% CO_2-humidified atmosphere. Cells were transfected using calcium phosphate method with 7 μg of human pcDNA3.1-Cx43 and 7 μg of EBO plasmid, encoding for the CD8 surface antigen, simultaneously. Two to 3 days post-transfection, cells were incubated for 2 min prior to patch clamp experiments in medium containing anti-CD8-coated beads (Invitrogen Canada, Inc.). Transfected cells expressing CD8 and therefore binding beads are distinguished from nontransfected cells by light microscopy. Cx43Hc currents were recorded in whole cell configuration of the patch clamp technique using Axopatch200B amplifier (Molecular Devices, CA) as previously described [28]. For whole-cell recordings, the pipette solution contained (in mmol/L): 140 KCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 ethylene glycol tetraacetic acid (EGTA) adjusted to pH 7.2. The bath solution contained (in mmol/L): 140 NaCl, 1 $MgCl_2$, 5.4 KCl, 1.8 $CaCl_2$, 10 HEPES and 10 EGTA adjusted to pH 7.2. Data were digitized with Digidata 1440A analog-to-digital convertor and analyzed using pClamp 10.1 software both purchased from Molecular Devices, CA.

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2.6. Western blot

Proteins were extracted from left ventricle tissues obtained from rats euthanized 40 min after reperfusion using a lysis buffer containing in mmol/L: 50 Tris–HCl (pH 7.5), 20 β-glycerophosphate, 20 NaF, 5 EDTA, 10 EGTA, 1 Na_3VO_4, supplemented with 0.2 mol/L PMSF, 1 mol/L DTT, 10% TritonX-100, 10 mg/mL leupeptin and 1 mmol/L microcystin. Samples were centrifuged at 10,000 *g* for 30 min. Supernatants were then submitted to protein quantification. Equal amounts of protein lysates (50 μg) were boiled for 5 min and loaded on 12.5% polyacrylamide SDS-gel and transferred to a nitrocellulose membrane. The membranes were blocked with 10% non-fat dried milk in Tris-buffered saline containing 1% Tween (TBS-T) for 1 h at room temperature, washed with TBS-T and incubated overnight with anti-Cx43 primary antibody (Santa Cruz Biotechnology, CA) diluted 1:200 in TBS-T buffer. After sequential washes in TBS-T, membranes were incubated for 1 h with the corresponding horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted at 1:10,000 in TBS-T buffer then rinsed again in TBS-T and revealed with the SuperSignal West chemiluminescent substrate kit (Thermo Scientific, IL), according to the manufacturer's instructions. The blots were stripped with stripping solution containing 100 mmol/L glycine, 1% sodium dodecyl sulfate (SDS), pH 2 and then reprobed with mouse anti-β-actin antibody (Jackson Immunoresearch Laboratories Inc., PA) diluted at 1:1000 in TBS-T buffer to confirm equal loading of proteins in each lane. Comparison of bands intensity was performed using the online software ImageJ as described, (http://rsb.info.nih.gov/ij/index.html).

2.7. Histopathology study

Specimens were fixed in 10% buffered formaldehyde solution immediately after surgical excision and embedded in paraffin at (4–60 °C) following standard procedures. Four to six 2-μm sections were cut from each specimen. Before examination, tissue sections were sequentially deparaffinized and rehydrated through an alcohol gradient. Then, sections were stained with hematoxylin and eosin.

2.8. Blood count and chemistry

Three groups of Sprague–Dawley rats were injected with saline, Gap26 (1 μg/Kg) or Gap27 (1 μg/kg). Blood samples were drawn 10 min before and 3 h after injection for testing. Blood count and biochemistry tests were carried out at the biology medical laboratory facilities at the Hôpital du Sacré-Cœur de Montréal, Montréal, Canada.

2.9. Statistical analysis

All data are expressed as mean ± SE. Analysis of variance (ANOVA) adapted to factorial design (groups and time) with orthogonalization according to Winer [29] was used. Differences were deemed significant when $P<0.05$.

3. Results

3.1. Localization of injected flag-tagged Gap26

Because peptides will be administered intravenously throughout the study, we first assessed using confocal microscopy whether the flag-tagged Gap26 can attain cardiomyocytes in the ventricular myocardium after being injected into a jugular vein of an adult rat. Interestingly, confocal microscopy experiments indicate that the peptide administered at a dose of 1 μg/kg successfully reaches ventricular tissues (Fig. 1). Images from ventricular sections obtained 60 min after peptide injection illustrates the flag-tagged Gap26 peptide (in *red*) co-localizing with Cx43 (in *green*) on the lateral membranes of cardiac myocytes (in *orange*) where unapposed Hc preferentially exist, but not in the intercalated disks where gap junctions predominate (Figs. 1A–D). Noticeably, the superimposition of both colors in panels 1C and 1D also illustrates an intracellular co-localization pattern (in *orange*). This pattern was not observed in a control experiment where ventricular tissues from normal heart of a rat injected with saline alone was immunostained using the same procedure (Figs. 1E–F). Additionally, Cx43 was absent from lateral membranes of ventricular cardiomyocytes obtained from a normal heart thus ruling out the possibility that lateral membrane staining is caused by fixation artifact (Fig. 1E).

3.2. Effects of Cx43MPs on ischemic hearts

Three doses of Gap26 (0.5, 1 and 10 μg/kg) were initially tested. All of them caused decrease in the myocardial infarct size when administered before ischemia (Fig. 2B) (Supplementary material, Table 1). Because infarct size reductions did not vary significantly ($P>0.05$) when using 1 and 10 μg/kg, we utilized 1 μg/kg-dose in the rest of the study. For alleviation, infarct size is represented in the text as percentage of area at risk. Importantly, bolus injection of Gap26 before ischemia significantly decreased infarct size from 32.9 ± 1.5% (n = 6) in control rats injected with saline at similar timing to 13.9 ± 1.3% (n = 6, $P<0.05$) (Fig. 2C). In order to investigate the capability of Gap26 to counteract an existing ischemia under an emergency-like setting, a separate group of rats was studied where the peptide was injected 30 min after the onset of ischemia — thus 10 min before reperfusion. Similarly, infarct size was reduced from 33.1 ± 1.7% (n = 6) in rats treated with saline at similar timing to 12.9 ± 0.5% (n = 6, $P<0.05$). These results correspond to infarct size reductions by 57.7 ± 2.8% and 61.0 ± 2.2% when Gap26 was injected before or after the onset of ischemia, respectively. The size of area at risk to total ventricles did not vary significantly in groups treated with Gap26 before or during ischemia (43.7 ± 1.3%, n = 6, $P>0.05$ and 43.2 ± 1.5%, n = 6, $P>0.05$, respectively) in comparison with control groups treated with saline at similar timings (45.1 ± 1.0%, n = 6; and 45.8 ± 1.0%, n = 6, respectively) (Fig. 2D). These data confirm that changes in the infarct size among groups are not related to the size of area at risk that depends on the occlusion by our surgical technique. As negative control, a scrambled version of Gap26 (sGap26) was also tested. Administration of sGap26 did not affect infarct size when introduced before ischemia (32.5 ± 1.0%, n = 3, $P>0.05$) or during ischemia (31.6 ± 1.2%, n = 4, $P>0.05$) in comparison with corresponding saline groups.

To assess cardiac cytolysis, troponin T blood levels were measured 48 h after ischemia. Although areas at risk were comparable between groups, Gap26-treatment before or during ischemia resulted in ~83% and 77% reduction in troponin T levels, respectively, with reference to the corresponding saline-controls (Fig. 2E). These results provide additional evidence for the cardioprotective effect exerted by Gap26 on ischemic hearts. Importantly, Cx43 protein levels did not differ in ventricles of rats treated with saline or Gap26 before ischemia as indicated by Western blot analysis (Fig. 2F). The ratio of Cx43 over β-actin is comparable between saline-control (0.99 ± 0.1, n = 4) and Gap26-treated (0.96 ± 0.2, n = 4; $P>0.05$) groups therefore ruling out the possibility that Gap26-treatment causes impairment of the overall Cx43 expression.

We next investigated the effect of Gap27, the only known structural-mimetic peptide of Cx43 besides Gap26. Although the infarct size was also reduced when rats were treated with Gap27 before ischemia (reduction by 37.7 ± 2.0%, n = 6, $P<0.05$) or during ischemia (reduction by 40.5 ± 3.2%, n = 6, $P<0.05$), the extent of reductions was significantly smaller than that observed using Gap26 (Fig. 2C).

Because each of the tested Cx43MPs allegedly interacts specifically with distinct structures of Cx43, we assessed whether co-injection of both Gap26 and Gap27 in equal doses could confer additive protection to ischemic hearts. Analysis of heart sections from rats injected with single bolus of combined Gap26/Gap27 peptides before or after the onset

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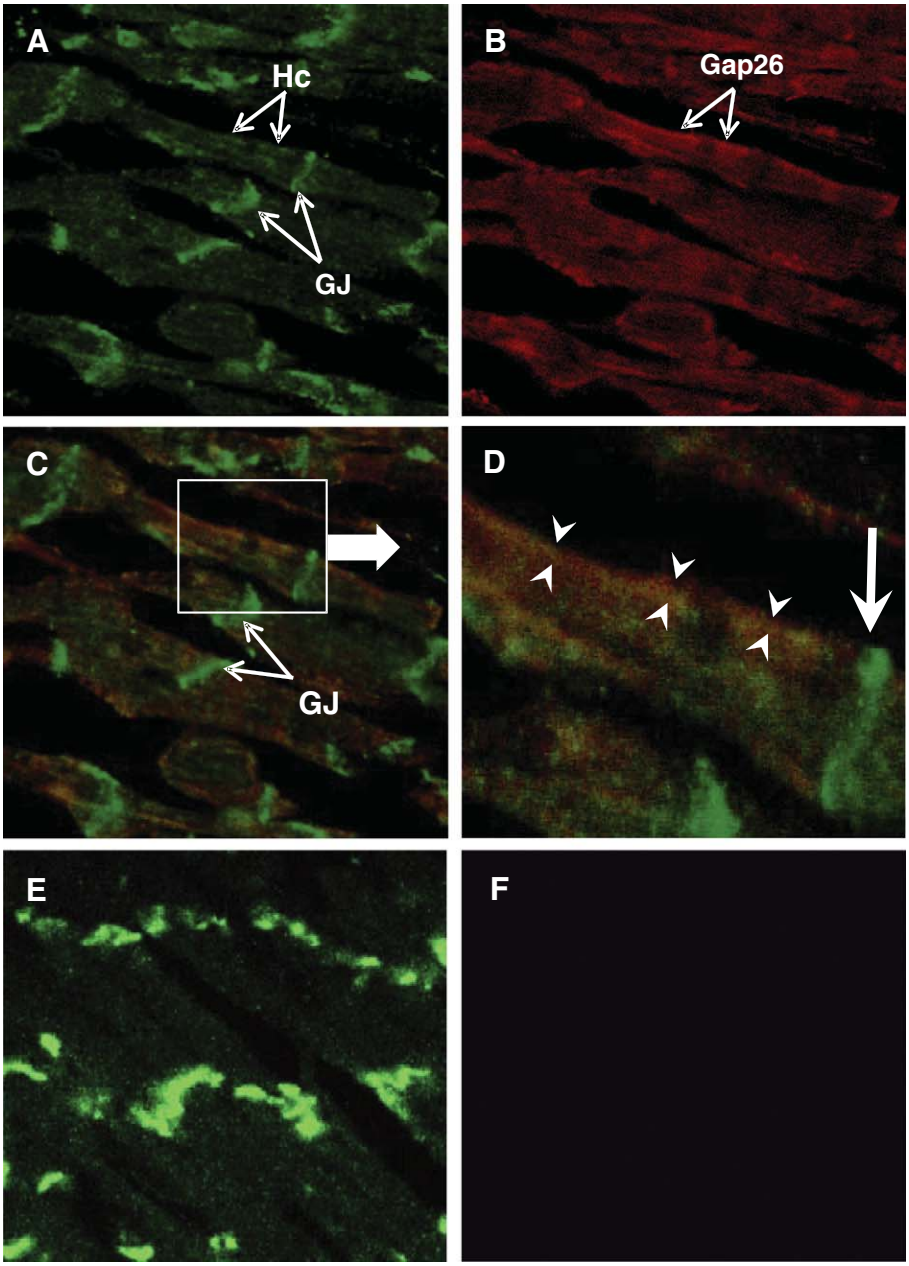

Fig. 1. Confocal images showing immunostaining of a ventricular section obtained from heart of rat injected with flag-tagged Gap26 peptide. A, staining of Cx43 from ischemic myocardium shows both peripheral and sarcoplasmic localization of the protein in green. The arrows point to lateral membranes and intercalated disks where Hc and gap junction channels dominate, respectively. B, same ventricular section is shown where flag-tagged Gap26 peptide is stained in red. Gap26 essentially localizes on the lateral membrane and in sarcoplasm but is remarkably absent in the intercalated disks. C, superimposition of the green and the red (orange color) shows colocalization of the flag-tagged Gap26 and Cx43 on lateral membranes and inside cardiomyocytes but not in the intercalated disks (green). The frame in white indicates a selected section enlarged in panel D. D, a magnified view of the selected section from panel C. Arrowheads point to lateral membrane where flag-tagged Gap26 and Cx43 colocalize. E, staining of Cx43 in normal myocardium of a rat injected with saline only shows sarcoplasmic and polar localization of Cx43 in green, but not on lateral membranes. F, same ventricular section as in E after immunoreaction with anti-flag and the corresponding secondary antibody.

of ischemia did not cause statistically different infarct size reductions (reduction by $63.5 \pm 3.3\%$, $n = 6$, $P < 0.05$; and $64.3 \pm 2.7\%$, $n = 6$, $P < 0.05$, respectively) compared to groups treated with Gap26 alone. Transversal sections from hearts representing groups treated with saline, Gap26, Gap27, and Gap26/Gap27 before ischemia are illustrated in Figs. 2G–J. Sizes of various heart areas obtained from different experimental groups are provided in Supplementary material, Table 1.

3.3. Effects of Cx43MPs on hCx43Hc currents

In order to elucidate the mechanism underlying the differential cardioprotection conferred by Cx43MPs on the cell level and to assess whether the reported salutary effects exerted in rats can be extrapolated to humans, we assessed the effects of Gap26 and Gap27 – when administered individually or combined – directly on hCx43Hc-mediated currents. Fig. 3A shows family traces of macroscopic hCx43Hc currents elicited by depolarizing a typical cell from a 0 mV resting potential to test-pulses ranging from 0 to +80 mV. To substantiate the identity of currents recorded from hCx43-transfected cells, patch clamp experiments were performed on non-transfected tsA201 cells. No current could be recorded from these cells (Fig. 3B) therefore confirming the absence of any contaminating endogenous currents. In Figs. 3C and D, representative time course recordings show rapid current reduction when Gap26 and Gap27 were introduced in bath solution, respectively.

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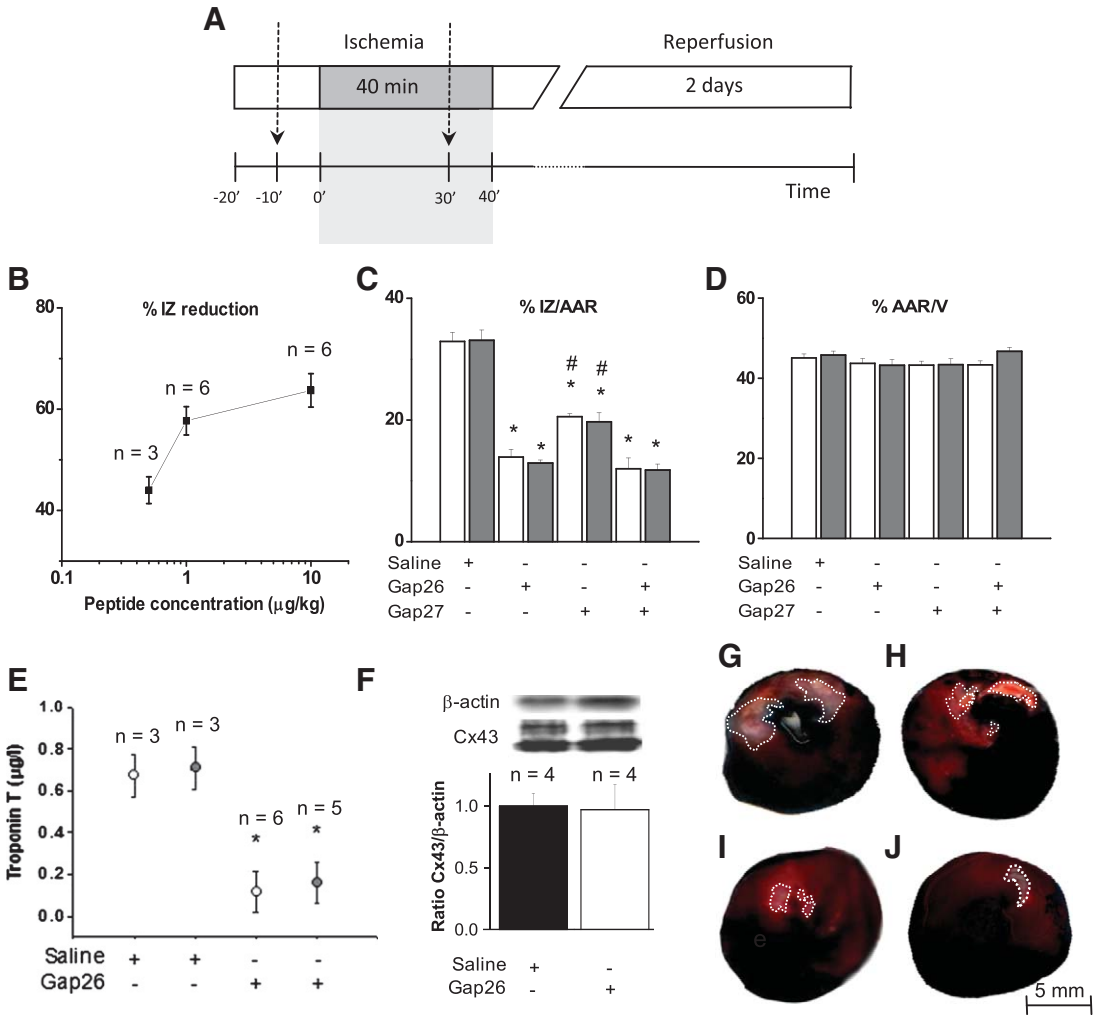

Fig. 2. A, a schematic representation of the experimental protocol used to assess the effect of Cx43MPs during ischemia–reperfusion in vivo. The gray zone covers the ischemic period. The dashed arrows indicate the time at which peptides are injected, either 10 min before or 30 after the initiation of ischemia. B, dose–response curves showing infarct size reduction in function of three different doses of Gap26. C, a histogram representing the proportion of infarct zone to area at risk in various groups. Open and gray columns represent groups treated before and during ischemia, respectively. * Indicates statistically different compared to saline-controls at similar timings, $P<0.05$. # Indicates statistically different compared to Gap26-treated groups at similar timings, $P<0.05$. D, a histogram showing the proportion of areas at risk to total ventricles. E, analysis of troponin T levels in blood samples. Open and gray dots represent groups treated before and during ischemia, respectively. * indicates statistically different compared to saline-controls at similar timings, $P<0.05$. F, a Western blot analysis of Cx43 protein levels in 10,000 g supernatants from ventricular tissues obtained 40 min after reperfusion from rats treated (black column) or not (open column) with Gap26. The histogram displays averaged data from four experiments. Photographs of TTC-stained heart sections obtained 2 days after reperfusion from rats injected with (G) saline; (H) Gap26; (I) Gap27; and (J) Gap26/Gap27 before LAD occlusion. Infarct zones are whitish areas delimited with dashed lines; areas at risk correspond to the red regions including infarct zones; and perfused areas are in dark blue.

Steady state inhibition was reached in all experiments. Averaged data indicate current reductions by 55.6 ± 1.1% (n = 8, $P<0.05$) and 43.9 ± 2.3% (n = 7, $P<0.05$) in presence of Gap26 or Gap27 (0.5 μM), respectively. Curiously, co-application of both peptides in equal concentrations did not cause additive inhibition of the Cx43Hc-mediated currents (Fig. 3E). An average current inhibition by 60.1 ± 0.8% (n = 8) was statistically ($P<0.05$) but not significantly different from the inhibition when using Gap26 alone. Negative control peptides, sGap26 and sGap27, did not affect current amplitudes significantly over similar time frames (reduction by 1.8 ± 0.8%, n = 3, $P>0.05$ for sGap26 and 2.0 ± 0.4%, n = 3, $P>0.05$ for sGap27). The histogram in Fig. 3F summarizes effects of Cx43MPs on normalized hCx43Hc currents.

3.4. Safety remarks

Indeed, safety is a crucial issue in any potential therapeutic application involving Cx43MPs. Therefore, we document some observations that we think are relevant to this concern. First, although in some experimental groups heart rates during 1 to 2 h following peptide treatment were statistically different from controls (Supplementary material, Table 1), these variations were not abnormal and remained within the normal range of the adult rat heart rate. On the other hand, while downregulation of Cx43 channels in general is known to be arrhythmogenic [30–32], we noticed only sporadic ventricular fibrillations occurring during the ischemic period while the animals were still under surgery. A total of 5 rats died during ischemia following a ventricular fibrillation (Supplementary material, Table 2). No arrhythmic events were noted during 1–2 h of reperfusion while ECGs were still recorded. It should be noted, however, that mortality from undetermined reasons has also been remarked after the ECG recording period but remained comparable between groups (Supplementary material, Table 3). A total of 9 rats died within 48 h of reperfusion. Besides in the heart, Cx43 is also expressed in many other organs which raises the possibility that administration of Cx43MPs may exert adverse side effects on these organs. In this regard, examination of histological sections from brain, lung, kidney and liver failed to show apparent structural differences

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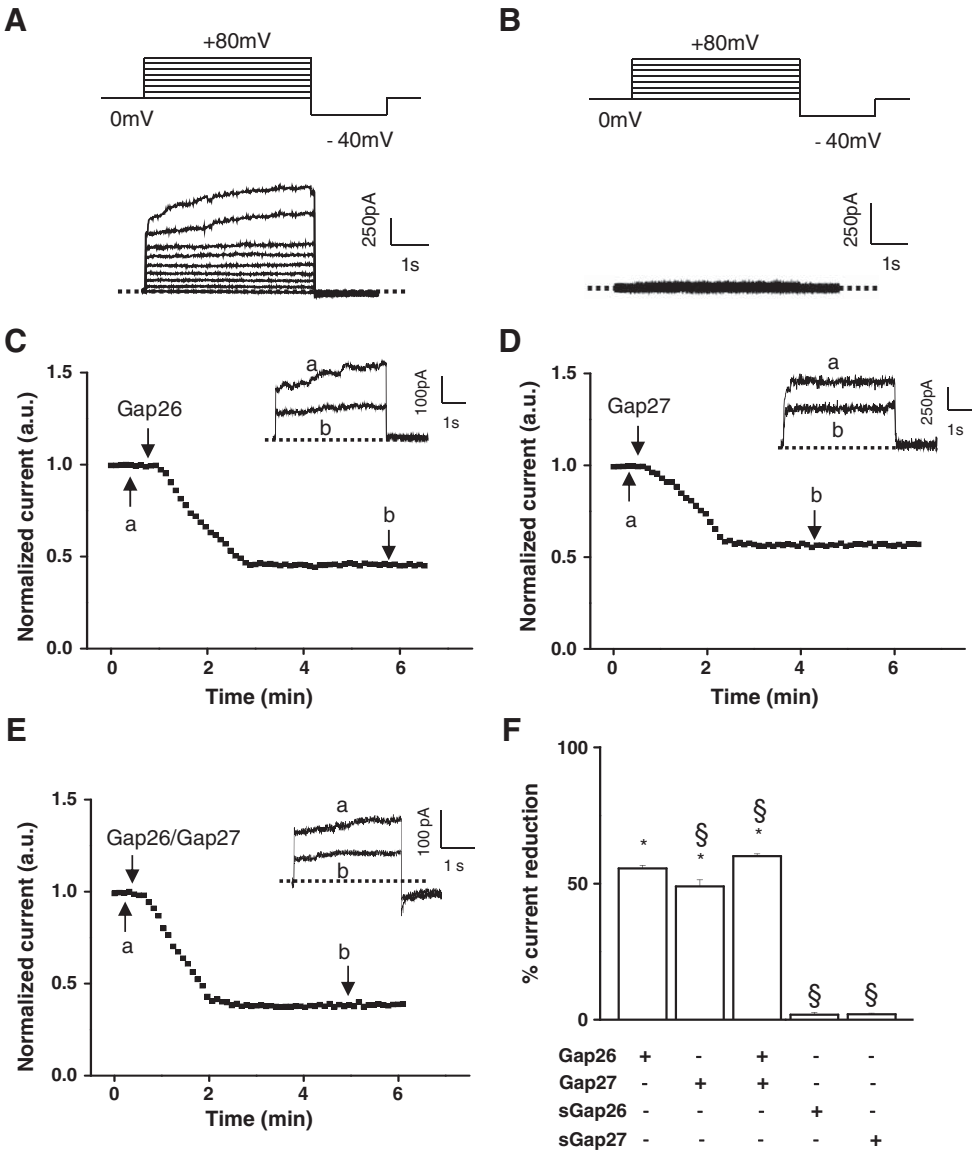

Fig. 3. Cx43Hc current inhibition by Cx43MPs. A, family traces of Cx43Hc-mediated currents recorded from a typical cell depolarized from a holding potential of 0 mV to pulses ranging from 0 to +80 mV. B, traces showing no detectable currents from non-transfected tsA201 cell. Dashed lines represent the zero current. Time course recorded using 30 mV depolarizing pulses from typical cells dialysed with (C) Gap26, (D) Gap27 or (E) Gap26/Gap27. In panels C–E, current amplitudes were normalized to the maximal current recorded. Insets illustrate Cx43Hc current traces in response to the depolarizing pulse recorded at time-points indicated by arrows a and b. (F) Histogram showing percentages of Cx43Hc current inhibition in response to Gap26 (n=8), Gap27 (n=7), Gap26/Gap27 (n=8), sGap26 (n=3) and sGap27 (n=3). Current amplitudes are normalized against control and represented in arbitrary units (a.u.). * $P<0.05$ in comparison to current reduction in non-treated cells at similar timing. § $P<0.05$ in comparison to current reduction in cells treated with Gap26 at similar timing.

between experimental groups (data not shown). Similarly, peptide-treatment did not cause perceptible abnormal alterations in blood cell counts or serum chemistry parameters in blood samples drawn before and after treatment with Cx43MPs (Supplementary material, Table 4). Rats did not develop any apparent abnormal signs or behavior during the 2 days after injection with Cx43MPs.

4. Discussion

The main significance of this study resides essentially in the experimental setting where Cx43MPs are administered for the first time systemically in vivo through intravenous injections which represents an ideal physiological context to assess the therapeutic potentials and applicability of treatment with these molecules. We demonstrate that single bolus injections of Cx43MPs can successfully confer protection to adult rat hearts against ischemic injury and reduce the resulting infarct size significantly. The first important observation arose from the confocal microscopy study which unraveled the Gap26 capability to leave vasculature, attain ventricular myocardial tissues and colocalize with Cx43 on cell surfaces when injected through a jugular vein. Strikingly, while the peptide successfully reached lateral membranes of cardiomyocytes, it remained noticeably absent from intercalated disks where gap junction channels essentially form. Although the exact reason for this selective localization remains to be elucidated, we expect this pattern to result from the inaccessibility of Gap26 binding sites on Cx43 first extracellular loops due to their engagement in the apposition of Hc forming the gap junction channels. Regardless of the reason behind this observation, the latter represents indeed a unique insight to explain the mechanism underlying the specificity of Gap26 toward the unapposed Cx43Hc. In addition to its peripheral localization on lateral membranes, staining for Gap26 was also noticed in intracellular spaces. Indeed, Cx43 are continuously formed and trafficked through the cytoplasm to the plasma membrane and then internalized and degraded [33]. Hence, the protein

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is naturally present in various organelles involved in these processes including the ER, the Golgi apparatus, lysosomes and proteosomes [33] but also in other subcellular structures such as mitochondria [34]. Therefore, the intracellular staining for Cx43 and thus the flag-tagged Gap26 as observed by confocal microscopy was not unexpected.

While reduction of infarct size was previously reported ex vivo when Gap26 [20] and Gap27 [35] were infused directly into hearts via an inert perfusate buffer, our results here underscore the capability of these peptides to preserve their cardioprotective potentials even when injected into the systemic circulation at a relatively remote site from the injury (see Opanasopit et al. [36] for factors affecting drug delivery). Effectively, the injection of any or both Cx43MPs using a substantially low dose of 1 μg/kg caused infarct size reduction by up to ~65% compared to animals treated with saline only. Curiously, the extent of the infarct size reductions caused by single doses of Gap26 as reported here exceeded those we have previously observed using the ex vivo model of myocardial infarction where isolated rat hearts were constantly perfused with a nonrecirculating Gap26-containing buffer (the concentration of Gap26 was maintained stable at 0.5 μM) during ischemia and for 3 h following reperfusion [20]. Although we cannot assert the exact reason behind this dissimilarity between the two experimental settings, we believe this may be caused at least partially by the enhanced oxygenation of the myocardium when (re)perfused with whole blood compared to perfusate buffer [37].

Theoretically, we ascribe the Cx43MPs′ cardioprotective effect to the specific inhibition of sarcolemmal unapposed Cx43Hc. Indeed, when high concentrations are utilized, Cx43MPs have also been shown to inhibit Cx43 gap junction channels [38]. Whereas the exact concentration of peptides diffused in heart tissues could not be determined in our model, the final concentration of Cx43MPs in total blood volume (estimated 16–30 mL) would certainly not exceed 0.5 μM, a concentration previously shown to selectively inhibit Hc without affecting gap junction channels [7,39]. Relevant to this concern, the treatment of isolated cardiomyocytes – thus lacking any gap junction channels – with Gap26 (0.5 μM) has also been shown to be protective as it was noted by the significant increase in the number of surviving cells following simulated ischemia–reperfusion [11,20]. Together with results from the current confocal miscroscopy study, these observations incite us to believe that the involvement of gap junction channels in the Cx43MPs-mediated cardioprotection is not likely. Similarly, we exclude the implication of the other cardiac isoforms of CxHc (i.e., Cx40Hc and Cx45Hc) given the lack of Gap26 effect on these Hc as we previously demonstrated [20]. It is important to remark that upregulation of mitochondrial Cx43 has also been associated with ischemic preconditioning-mediated cardioprotection [40–43]. In this regard, we showed earlier that superfusion of cardiomyocytes with Gap26 preserves mitochondrial activity following ischemic stress [20]. Arguably, this may indicate that Cx43MPs-mediated cardioprotection involves the mitochondrial Cx43 modulation by a yet unknown mechanism since, so far, the only established effect of Gap26 on Cx43Hc is rather inhibitory. Nonetheless, none of these assumptions can be substantiated under our current experimental conditions. Indeed, the permeability of mitochondrial membranes to Cx43MPs has yet to be demonstrated.

It is noteworthy that infarct size reduction did not differ statistically whether peptides were administered before or after the onset of ischemia. Whereas this observation could result from the fact that death of cardiomyocytes and consequent myocardial injury essentially occur during reperfusion [44,45], it is also possible that the injected peptides do not reach adequately the areas at risk during LAD occlusion when coronary circulation is interrupted. Therefore, it is conceivable that peptides are exerting their protective effect only during reperfusion, regardless of the timing of administration. Arguably, it may also be speculated that Cx43MPs exert their protective effect – up to a certain extent – through the inhibition of gap junction channels that connect the area at risk to the surrounding perfused myocardium. This is particularly likely to occur when the treatment is applied after the onset of ischemia when access to the area at risk is restrained. Importantly, the size of areas at risk under our experimental conditions remained comparable among the treated groups and in comparison with saline controls. This observation indicates that the protective effects of Cx43MPs in groups treated with Cx43MPs after the onset of ischemia are unlikely to be exerted through the gap junction channels.

Intriguingly, while both Cx43MPs successfully conferred resistance to hearts against ischemic injury, the extent of the infarct size reduction was significantly smaller when rats were treated with Gap27 (~40% reduction with Gap27 versus ~60% with Gap26). Because of the resemblance in biophysical properties between both peptides (i.e., relative length, amino acid composition, size, polarity and hydrophobicity of amino acids that compose these peptides), we do not ascribe the noted disparity to differences in the delivery process of peptides to their substrate but rather to potentially distinct functional effects that these peptides exert on Cx43Hc once they attain their targets. This assumption was subsequently assessed in the electrophysiological study. In a related result, co-injection of both Gap26 and Gap27 in equal doses did not confer additive protection to ischemic hearts. We believe these results point to the existence of a saturating level of Cx43MP-mediated protection that was reached, beyond which no further protection can be added.

Interestingly, both peptides readily inhibited exogenous hCx43Hc-mediated currents recorded from ion channel-deficient tsA201 cells. Given the concordance with previous reported effects of Gap26 on rat Cx43Hc and in cardioprotection [20], the electrophysiological data reported in the present study could represent the initial functional evidence supporting the therapeutic potential of Cx43MPs if applied to humans in the context of ischemic heart disease. Indeed, the inhibitory effect of Gap27 was noticeably less pronounced compared to Gap26, and co-application of both peptides did not cause additive current inhibition but rather was comparable to the effect exerted by Gap26 alone. These results correlate with those obtained from the in vivo study and could be the basis of the differential cardioprotection conferred by Cx43MPs.

In a related matter, it was proposed that the opening of only a few Cx43Hc to their highest conductance is sufficient to cause volume overload and cell death during simulated ischemia [3,4]. Therefore, the percentage of inhibition of Cx43Hc conductance by Cx43MPs as seen in the present study (40–60%) may arguably appear insufficient to protect cardiac myocytes against ischemic injury. However, it should be emphasized that the electrophysiological data from patch clamp experiments do not reflect quantitatively the exact inhibition level that normally occurs in cardiac myocytes of a beating heart in vivo given the differences between the two experimental settings. In fact, the activation of whole-cell Cx43Hc currents as recorded in vitro was enhanced insistently by using low calcium solutions and high depolarizing pulses in order to allow macroscopic current acquisition and adequate data analyses. Therefore, it becomes expectable that Cx43MPs-mediated inhibition is significantly more important under physiological conditions in vivo. Nonetheless, this remains to be substantiated.

Finally, it is worth noting that if Cx43MPs inhibit specifically the unapposed Cx43Hc that open abnormally under ischemia as it is widely believed, it then becomes plausible to believe that the functional/therapeutic effects induced by the systemic administration of Cx43MPs are mainly restricted to the ischemic area of the heart with little or no effects on other non-ischemic tissues in the rest of the organism. This assumption could be supported in part by the preliminary safety remarks that we reported here. Indeed, more extensive toxicity studies are necessary to further confirm the safety of Cx43MPs.

5. Conclusion and relevance

We show here for the first time in vivo that single intravenous low-dose bolus injections of Cx43MPs can confer significant protection

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to the heart against ischemic injury whether injected before or after the onset of ischemia. These practical advantages give Cx43MPs great potential for future therapeutic applications especially in emergency and out-of-hospital settings. We believe that the efficacy of Cx43MPs and their apparent safety make them promising tools to fight ischemic heart disease.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2012.07.008.

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Disclosure statement

None declared.

References

[1] Contreras JE, Saez JC, Bukauskas FF, Bennett MV. Gating and regulation of connexin 43 (Cx43) hemichannels. Proc Natl Acad Sci USA 2003;100:11388-93.
[2] Contreras JE, Sanchez HA, Eugenin EA, Speidel D, Theis M, Willecke K, et al. Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. Proc Natl Acad Sci U S A 2002;99:495-500.
[3] John SA, Kondo R, Wang SY, Goldhaber JI, Weiss JN. Connexin-43 hemichannels opened by metabolic inhibition. J Biol Chem 1999;274:236-40.
[4] Kondo RP, Wang SY, John SA, Weiss JN, Goldhaber JI. Metabolic inhibition activates a non-selective current through connexin hemichannels in isolated ventricular myocytes. J Mol Cell Cardiol 2000;32:1859-72.
[5] Li F, Sugishita K, Su Z, Ueda I, Barry WH. Activation of connexin-43 hemichannels can elevate [Ca(2+)]i and [Na(+)]i in rabbit ventricular myocytes during metabolic inhibition. J Mol Cell Cardiol 2001;33:2145-55.
[6] Quist AP, Rhee SK, Lin H, Lal R. Physiological role of gap-junctional hemichannels. Extracellular calcium-dependent isosmotic volume regulation. J Cell Biol 2000;148:1063-74.
[7] Clarke TC, Williams OJ, Martin PE, Evans WH. ATP release by cardiac myocytes in a simulated ischaemia model: inhibition by a connexin mimetic and enhancement by an antiarrhythmic peptide. Eur J Pharmacol 2009;605:9–14.
[8] Eltzschig HK, Eckle T, Mager A, Kuper N, Karcher C, Weissmuller T, et al. ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function. Circ Res 2006;99:1100-8.
[9] Wilde AA, Aksnes G. Myocardial potassium loss and cell depolarisation in ischaemia and hypoxia. Cardiovasc Res 1995;29:1–15.
[10] Steenbergen C, Hill ML, Jennings RB. Volume regulation and plasma membrane injury in aerobic, anaerobic, and ischemic myocardium in vitro. Effects of osmotic cell swelling on plasma membrane integrity. Circ Res 1985;57:864-75.
[11] Shintani-Ishida K, Uemura K, Yoshida K. Hemichannels in cardiomyocytes open transiently during ischemia and contribute to reperfusion injury following brief ischemia. Am J Physiol Heart Circ Physiol 2007;293:H1714-20.
[12] Garcia-Dorado D, Inserte J, Ruiz-Meana M, Gonzalez MA, Solares J, Julia M, et al. Gap junction uncoupler heptanol prevents cell-to-cell progression of hypercontracture and limits necrosis during myocardial reperfusion. Circulation 1997;96:3579-86.
[13] Saltman AE, Aksehirli TO, Valiunas V, Gaudette GR, Matsuyama N, Brink P, et al. Gap junction uncoupling protects the heart against ischemia. J Thorac Cardiovasc Surg 2002;124:371-6.
[14] Garcia-Dorado D, Rodriguez-Sinovas A, Ruiz-Meana M. Gap junction-mediated spread of cell injury and death during myocardial ischemia–reperfusion. Cardiovasc Res 2004;61:386-401.
[15] Kanno S, Kovacs A, Yamada KA, Saffitz JE. Connexin43 as a determinant of myocardial infarct size following coronary occlusion in mice. J Am Coll Cardiol 2003;41:681-6.
[16] Rodriguez-Sinovas A, Sanchez JA, Gonzalez-Loyola A, Barba I, Morente M, Aguilar R, et al. Effects of substitution of Cx43 by Cx32 on myocardial energy metabolism, tolerance to ischaemia and preconditioning protection. J Physiol 2010;588:1139-51.
[17] Bargiotas P, Monyer H, Schwaninger M. Hemichannels in cerebral ischemia. Curr Mol Med 2009;9:186-94.
[18] Saez JC, Schalper KA, Retamal MA, Orellana JA, Shoji KF, Bennett MV. Cell membrane permeabilization via connexin hemichannels in living and dying cells. Exp Cell Res 2010;316:2377-89.
[19] Orellana JA, Hernandez DE, Ezan P, Velarde V, Bennett MV, Giaume C, et al. Hypoxia in high glucose followed by reoxygenation in normal glucose reduces the viability of cortical astrocytes through increased permeability of connexin 43 hemichannels. Glia 2010;58:329-43.
[20] Hawat G, Benderdour M, Rousseau G, Baroudi G. Connexin 43 mimetic peptide Gap26 confers protection to intact heart against myocardial ischemia injury. Pflugers Arch 2010;460:583-92.
[21] Li X, Heinzel FR, Boengler K, Schulz R, Heusch G. Role of connexin 43 in ischemic preconditioning does not involve intercellular communication through gap junctions. J Mol Cell Cardiol 2004;36:161-3.
[22] Padilla F, Garcia-Dorado D, Rodriguez-Sinovas A, Ruiz-Meana M, Inserte J, Soler-Soler J. Protection afforded by ischemic preconditioning is not mediated by effects on cell-to-cell electrical coupling during myocardial ischemia–reperfusion. Am J Physiol Heart Circ Physiol 2003;285:H1909-16.
[23] Braet K, Vandamme W, Martin PE, Evans WH, Leybaert L. Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26. Cell Calcium 2003;33:37-48.
[24] Leybaert L, Braet K, Vandamme W, Cabooter L, Martin PE, Evans WH. Connexin channels, connexin mimetic peptides and ATP release. Cell Commun Adhes 2003;10:251-7.
[25] Dahl G. Gap junction-mimetic peptides do work, but in unexpected ways. Cell Commun Adhes 2007;14:259-64.
[26] Baroudi G, Pouliot V, Denjoy I, Guicheney P, Shrier A, Chahine M. Novel mechanism for Brugada syndrome: defective surface localization of an SCN5A mutant (R1432G). Circ Res 2001;88:E78-83.
[27] Reimer KA, Jennings RB. Myocardial ischemia, hypoxia and infarction. In: Fozzard HA, Haber E, Jennings RB, Katz AM, Morgan HE, editors. The heart and cardiovascular system scientific foundations. Raven Press; 1991. p. 1947.
[28] Hawat G, Baroudi G. Differential modulation of unapposed connexin 43 hemichannel electrical conductance by protein kinase C isoforms. Pflugers Arch 2008;456:519-27.
[29] Winer BJ. Statistical principles in experimental design. New York: McGraw-Hill; 1971.
[30] Mayama T, Matsumura K, Lin H, Ogawa K, Imanaga I. Remodelling of cardiac gap junction connexin 43 and arrhythmogenesis. Exp Clin Cardiol 2007;12:67-76.
[31] Waghabi MC, Coutinho-Silva R, Feige JJ, Higuchi ML, Becker D, Burnstock G, et al. Gap junction reduction in cardiomyocytes following transforming growth factor-beta treatment and *Trypanosoma cruzi* infection. Mem Inst Oswaldo Cruz 2009;104:1083-90.
[32] Imanaga I. Pathological remodeling of cardiac gap junction connexin 43—with special reference to arrhythmogenesis. Pathophysiol 2010;17:73-81.
[33] Salameh A. Life cycle of connexins: regulation of connexin synthesis and degradation. Adv Cardiol 2006;42:57-70.
[34] Boengler K, Stahlhofen S, van de SA, Gres P, Ruiz-Meana M, Garcia-Dorado D, et al. Presence of connexin 43 in subsarcolemmal, but not in interfibrillar cardiomyocyte mitochondria. Basic Res Cardiol 2009;104:141-7.
[35] Przyklenk K, Maynard M, Darling CE, Whittaker P. Pretreatment with D-myo-inositol trisphosphate reduces infarct size in rabbit hearts: role of inositol trisphosphate receptors and gap junctions in triggering protection. J Pharmacol Exp Ther 2005;314:1386-92.
[36] Opanasopit P, Nishikawa M, Hashida M. Factors affecting drug and gene delivery: effects of interaction with blood components. Crit Rev Ther Drug Carrier Syst 2002;19:191-233.
[37] Schenkman KA, Beard DA, Ciesielski WA, Feigl EO. Comparison of buffer and red blood cell perfusion of guinea pig heart oxygenation. Am J Physiol Heart Circ Physiol 2003;285:H1819-25.
[38] Evans WH, De Vuyst E, Leybaert L. The gap junction cellular internet: connexin hemichannels enter the signalling limelight. Biochem J 2006;397:1–14.
[39] Verma V, Hallett MB, Leybaert L, Martin PE, Evans WH. Perturbing plasma membrane hemichannels attenuates calcium signalling in cardiac cells and HeLa cells expressing connexins. Eur J Cell Biol 2009;88:79-90.
[40] Boengler K, Dodoni G, Rodriguez-Sinovas A, Cabestrero A, Ruiz-Meana M, Gres P, et al. Connexin 43 in cardiomyocyte mitochondria and its increase by ischemic preconditioning. Cardiovasc Res 2005;67:234-44.
[41] Schulz R, Boengler K, Totzeck A, Luo Y, Garcia-Dorado D, Heusch G. Connexin 43 in ischemic pre- and postconditioning. Heart Fail Rev 2007;12:261-6.
[42] Dhein S. New emerging roles for cardiac connexins. Mitochondrial Cx43 raises new questions. Cardiovasc Res 2005;67:179-81.
[43] Ruiz-Meana M, Rodriguez-Sinovas A, Cabestrero A, Boengler K, Heusch G, Garcia-Dorado D. Mitochondrial connexin43 as a new player in the pathophysiology of myocardial ischaemia–reperfusion injury. Cardiovasc Res 2008;77:325-33.
[44] Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 1994;94:1621-8.
[45] Zhao ZQ, Nakamura M, Wang NP, Wilcox JN, Shearer S, Ronson RS, et al. Reperfusion induces myocardial apoptotic cell death. Cardiovasc Res 2000;45:651-60.