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Functional Analysis of Nuclear β-Adrenergic Receptors in the Myocardium

par

George Vaniotis

Département de Biochimie

Faculté de Médecine

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Functional Analysis of Nuclear β-Adrenergic Receptors in the Myocardium

présentée par:

George Vaniotis

a été évalué par un jury compose des personnes suivantes:

Dr. Nikolaus Heveker

President-rapporteur

Dr. Bruce Gordon Allen

Directeur de recherche

Dr. Terence E. Hébert

Co-directeur de recherché

Dr. Denis deBlois

Membre du jury

Dr. Timothy O'Connell

Examinateur externe

Dr. Nathalie Arbour

Représentant du doyen

ABSTRACT

Recently several G protein-coupled receptors (GPCRs) have been shown to localize to intracellular membranes, in particular the nuclear membrane. As such, we sought to determine if the β-adrenergic receptor (βAR) subtypes and their associated signalling machinery are functionally localized to nuclear membranes. We demonstrated the presence of β_1AR and the β_3AR, but not the β_2AR, in adult ventricular myocyte nuclei by western blotting, confocal microscopy and functional assays. Downstream signalling partners such as $G\alpha_S$, $G\alpha_i$ and adenylyl cyclase II and V/VI were also present. Nuclear-localized βARs were functional with respect to ligand binding and effector activation. In isolated nuclei, the non-selective βAR agonist isoproterenol (ISO) and the β_3AR-selective ligand BRL37344, but not the β_1AR-selective xamoterol, stimulated transcription initiation in a pertussis toxin (PTX)-sensitive manner. In contrast, stimulation of type B endothelin receptors (ETB), another GPCR family shown to be present on the nuclear membrane, decreased *de novo* RNA synthesis. To investigate the signalling pathway(s) involved in GPCR-mediated regulation of RNA synthesis, nuclei were isolated from intact adult rat hearts and treated with receptor agonists in the presence or absence of inhibitors of the PI3K/PKB and mitogen-activated protein kinase (MAPK) pathways. Components of p38, JNK, and ERK1/2 MAPK cascades as well as PKB were detected in nuclear preparations. Inhibition of PKB with triciribine converted the activation of the βAR from stimulatory to inhibitory with regards to transcription initiation. Analysis by qPCR indicated isoproterenol treatment increased 18S rRNA but decreased NFκB mRNA. In contrast, ET-1 had no effect on 18S rRNA expression. Further investigation using pathway-specific PCR arrays revealed that isoproterenol treatment also reduced the expression of several other genes involved in the activation of NFκB and that ERK1/2 and PKB inhibitors attenuated this effect. Subsequent genome-wide microarray analysis has revealed that nuclear βAR and ETB regulated a host of genes in an overlapping but distinct manner. Moreover, both ET-1 and ISO produced an L-NAME-sensitive increase in NO production in isolated cardiac nuclei. These observations were confirmed in intact cardiomyocytes using novel caged

analogues of ISO and ET-1 and the cell-permable NO-sensitive fluorescent dye, DAF-2 DA. Briefly, both ET-1 and isoproterenol increased NO production, and this increase was prevented upon preincubation with L-NAME. Moreover, the ability of isoproterenol to increase transcription initiation in isolated nuclei was blocked by L-NAME or the PKG inhibitor KT5823, indicating the NO-GC-PKG pathway is involved in the regulation of gene expression by nuclear βARs. Hence, we have shown that βARs and ETRs in the nuclear membrane activate distinct signalling pathways, resulting in different effects on gene transcription and thus represent potentially important targets for drug development.

Keywords: β-adrenergic receptors, endothelin receptors, G protein coupled receptors (GPCRs), heart, nuclear membrane, transcription, nitric oxide.

RÉSUMÉ

Récemment plusieurs récepteurs couplés aux protéines G (RCPGs) ont été caractérisés au niveau des membranes intracellulaires, dont la membrane nucléaire. Notre objectif était de déterminer si les sous-types de récepteurs β-adrénergiques (βAR) et leurs machineries de signalisation étaient fonctionnels et localisés à la membrane nucléaire des cardiomyocytes. Nous avons démontré la présence des β_1AR et β_3AR, mais pas du β_2AR à la membrane nucléaire de myocytes ventriculaires adultes par immunobuvardage, par microscopie confocale, et par des essais fonctionnels. De plus, certains partenaires de signalisation comme les protéines $G\alpha_S$, $G\alpha_i$, l'adénylate cyclase II, et V/VI y étaient également localisés. Les sous-types de βAR nucléaires étaient fonctionnels puisqu'ils pouvaient lier leurs ligands et activer leurs effecteurs. En utilisant des noyaux isolés, nous avons observé que l'agoniste non-sélectif isoprotérénol (ISO), et que le BRL37344, un ligand sélectif du β_3AR, stimulaient l'initiation de la synthèse de l'ARN, contrairement à l'agoniste sélectif du β_1AR, le xamotérol. Cette synthèse était abolie par la toxine pertussique (PTX). Cependant, la stimulation des récepteurs nucléaires de type B de l'endothéline (ETB) causaient une réduction de l'initiation de la synthèse d'ARN. Les voies de signalisations impliquées dans la régulation de la synthèse d'ARN par les RCPGs ont ensuite été étudiées en utilisant des noyaux isolés stimulés par des agonistes en présence ou absence de différents inhibiteurs des voies MAP Kinases (proteines kinases activées par mitogènes) et de la voie PI3K/PKB. Les protéines impliquées dans les voies de signalisation de p38, JNK, ERK MAP Kinase et PKB étaient présents dans les noyaux isolés. L'inhibition de PKB par la triciribine, inhibait la synthèse d'ARN. Nous avons ensuite pu mettre en évidence par qPCR que la stimulation par l'ISO entrainait une augmentation du niveau d'ARNr 18S ainsi qu'une diminution de l'expression d'ARNm de NFκB. En contraste, l'ET-1 n'avait aucun effet sur le niveau d'expression de l'ARNr 18S. Nous avons ensuite montré que la stimulation par l'ISO réduisait l'expression de plusieurs gènes impliqués dans l'activation de NFκB, tandis que l'inhibition de ERK1/2 et PKB renversait cet effet. Un microarray global nous a ensuite permis de démontrer que les βARs et les ETRs nucléaires régulaient un grand nombre de gènes distincts. Finalement, les βARs et ETRs nucléaires augmentaient

aussi une production de NO de noyaux isolés, ce qui pouvait être inhibée par le L-NAME. Ces résultats ont été confirmés dans des cardiomyocytes intacts en utilisant des analogues cagés et perméables d'ISO et de l'ET-1: l'augmentation de NO nucléaire détectée par DAF2-DA, causée par l'ET-1 et l'ISO, pouvait être prévenue par le L-NAME. Finalement, l'augmentation de l'initiation de la transcription induite par l'ISO était aussi bloquée par le L-NAME ou par un inbitheur de PKG, le KT5823, suggérant que la voie NO-GC-PKG est impliquée dans la régulation de la transcription par les βAR. En conclusion, les βARs et les ETRs nucléaires utilisent des voies de signalisation différentes et exercent ainsi des effets distincts sur l'expression des gènes cardiaques. Ils représentent donc une avenue intéressante pour le développement de drogues pharmacologiques.

Mots Clés: récepteur β-adrénergiques, récepteurs aux endothelin, récepteurs couplé aux protéines G (RCPG), coeur, membrane nucléaire, transcription, oxide nitrique.

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LIST OF ABBREVIATIONS

αAR	α-adrenergic receptor
βAR	β-adrenergic receptor
AC	adenylyl cyclase
AKAP	A kinase anchoring protein
AMP	adenosine monophosphate
ATF-2	activating transcription factor 2
ATP	adenosine triphosphate
BAD	Bcl-2 associated death promoter
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CRE	cAMP response elements
CREB	cAMP response element binding
DAG	diacylglycerol
DNA	deoxyribonucleic acid
ECE	endothelin converting enzyme
eNOS	endothelial NOS
EPAC	exchange proteins activated by cAMP
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase 1/2
ET-1	endothelin 1
ETA	endothelin receptor A
ETB	endothelin receptor B
ETR	endothelin receptor
GAP	GTPase activating protein
GC	guanylyl cyclase
GDP	guanosine diphosphate
GEF	guanosine nucleotide exchange factor
GIRK	G protein-coupled inwardly rectifying potassium channel

GMP	guanosine monophosphate
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GSK-3	glycogen synthase kinase-3
GTP	guanosine triphosphate
HAT	histone acetyltransferase
HDAC	histone deacetylase
IκB	inhibitor of κB
IKK	Iκ-B kinase
iNOS	inducible NOS
IP3	inositol trisphosphate
ISO	isoproterenol
JNK	c-Jun N-terminal kinases
MAPK	mitogen-activated protein kinase
mGLU	metabotropic glutamate
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NFκB	nuclear factor kappa-light-chain-enhancer of activated B-cells
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal NOS
PDE	phosphodiesterase
PDK1	phosphoinositide-dependent protein kinase 1
PI3K	phosphoinositide 3-kinase
PIP2	phosphotidylinositol 4, 5-bisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKG	protein kinase G
PLA	phospholipase A
PLC	phospholipase C

POL I	RNA polymerase I
POL II	RNA polymerase II
POL III	RNA polymerase III
PP2	protein phosphatase 2
PTX	pertussis toxin
qPCR	quantitative real-time polymerase chain reaction
RGS	regulator of G protein signalling
RNA	ribonucleic acid
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
rRNA	ribosomal RNA
SERCA	sarco/endoplasmic reticulum calcium ATPase
sGC	soluble guanylate cyclase
SR	sarcoplasmic reticulum

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CHAPTER 1: INTRODUCTION

I) The Cardiovascular System

The heart is responsible for pumping blood throughout the body to ensure adequate delivery of oxygen and nutrients as wells as removal of metabolic waste products. The myocardium is composed primarily of cardiac muscle cells, endothelial cells and fibroblasts. The mammalian heart is divided into four chambers, consisting of a right and left ventricle and atrium (Figure 1). The right atrium and ventricle are responsible for pumping blood to the respiratory system, while the left atrium and ventricle pump blood to the remainder of the body. The heart acts as a dual pump with the atria contracting first followed in quick succession by the ventricles. The contraction of the atria ensures the proper filling of the ventricle, termed the preload. This contraction is achieved by a change in the membrane potential from its resting negative state to a positive one. This depolarization causes the contraction of the cardiomyocyte and is the basis for the electrical activity that controls the heart. The original depolarization, which controls the basic automaticity of the heart occurs in the sinoatrial node, located in the right atrium, and quickly spreads throughout the atria and into the ventricles. The discharge rate of the sinoatrial node determines the heart rate. Depolarization is a result of the opening of voltage-gated sodium channels and generates an action potential in the plasma membrane of muscle cells. This in turn increases the cell's cytosolic calcium concentration, causing a contraction. The cardiac action potential consists of five phases (Figure 2). The primary phase is the resting membrane potential of the cardiomyocyte, prior to stimulation. Upon stimulation, the action potential enters the rapid depolarization phase, due to the opening of the fast sodium channels. The following phase involves the inactivation of the sodium channels, followed by the plateau phase, resulting from a sustained balance between inward movement of calcium through L-type calcium channels and outward movement of potassium through slow delayed rectifier potassium channels. The final phase is the rapid repolarization phase, where the calcium channels close, and other potassium channels open, mainly the rapid delayed rectifier potassium channels. This final phase allows for a return to the resting membrane potential. During the depolarization of the

cardiomyocyte, voltage-gated calcium channels located in the T tubules open, allowing calcium to enter the cell. This small increase in calcium facilitates calcium binding to ryanodine-sensitive calcium channels (RyR) on the sarcoplasmic reticulum (SR) and this leads to opening of the RyR allowing a large efflux of calcium from the SR into the cytosol. This release of calcium from the SR is termed calcium-induced calcium release and is responsible for cardiac contraction. Contraction occurs as a result of binding of calcium to the regulatory protein troponin, which initiates cross-bridge formation between actin and myosin. Following cardiomyocyte contraction is relaxation, which allows the cardiomyocytes to return to their basal state, where they can undergo another contraction/relaxation cycle. Cardiomyocyte relaxation involves the reuptake of calcium into the sarcoplasmic reticulum, and is mediated primarily by sarco/endoplasmic reticulum calcium ATPase (SERCA). Calsequestrin, a calcium-binding protein located within the lumen of the SR, facilitates SERCA function by lowering the free calcium concentration within the SR. The rate of calcium reuptake by SERCA is regulated by phospholamaban, which is in turn under the control of PKA phosphorylation. Phospholamban normally inhibits SERCA, though phosphorylation of phospholamban by PKA relieves this inhibition, resulting in an increase in the rate of calcium movement.

Cardiac output is the product of heart rate (HR) and stroke volume (SV), the blood volume ejected by the left ventricle with each beat. Changes in either of these two parameters can alter cardiac output, and increase or decrease the amount of blood pumped by each ventricle. HR and SV are primarily controlled by the sympathetic (increase) and parasympathetic (decrease) nervous systems. The main target of the sympathetic nervous system, our focus here, are the adrenergic receptors, which respond to the hormones epinephrine and norepinephrine. Under baseline conditions cardiac output should remain fairly stable, while it will increase in response to stress or strenuous activity.

Control of arterial pressure by the sympathetic system is normally acute, and subsides as soon as the stimulus passes, commonly referred to as the “fight-or-flight”

response. However, sustained changes in arterial pressure can also lead to alterations of cardiac output and eventually to the development of various pathological conditions. One such instance is the case of chronically increased arterial blood pressure, which leads to the development of hypertension. Many factors can contribute to the development of hypertension, though the main underlying cause remains unclear. Hypertension causes a variety of problems throughout the cardiovascular system, and is a primary cause of left ventricular hypertrophy. Hypertrophy is at first an adaptive increase in muscle mass that helps to compensate for the increased pressure. However, this adaptive response causes changes in the organization and function of the cells within the myocardium, and eventually leads to the development of maladaptive hypertrophy. This in turn can lead to the development of heart failure. The development of heart failure can lead to pulmonary edema, arrhythmias, myocardial infarction and ultimately, mortality.

Cardiovascular diseases are among the largest causes of death worldwide, and as such their treatment is of paramount importance. In fact, 77% of all Canadians over the age of 60 are either hypertensive or prehypertensive. Currently one of the most common treatment options for both hypertension and end-stage heart failure is administration of β-adrenergic receptor antagonists (β-blockers). However, while effective, these drugs are not without side effects, which have required the development of newer more selective and specific β-blockers, in terms of both receptor subtype (discussed below) and signalling pathways modulated. As such, developing a better understanding of the signalling pathways the β-adrenergic receptors target in the heart, which genes they might regulate, as well as how they are regulated, is of critical importance in the development of the next generation of β-blockers.

II) G Protein-Coupled Receptors

a) GPCR Classes

G protein-coupled receptors (GPCRs) comprise the largest family of receptors encoded by the human genome with roughly 900 members, and represent the largest class of proteins currently targeted by therapeutic agents (1-3). Drugs targeting GPCRs

are used to treat a wide range of human pathologies including neurodegenerative, immune, cardiovascular and renal diseases, and cancer (3). There are 5 recognized families of GPCRs, each consisting of several sub-families.

The class A, or rhodopsin-like receptors, is the largest class of GPCRs. This class contains 672 family members, divided into 19 separate sub-families, representing almost 75% of all GPCRs, making it the most diverse of all the classes (4). Almost half of these receptors are predicted to be olfactory receptors. Resulting from its large diversity, class A receptors have been shown to respond to a variety of different ligands, including hormones and neurotransmitters. This class contains the adrenergic, endothelin, angiotensin and chemokine receptors sub-families, to name a few. This class also comprises of a large number of receptors having no known physiologic ligand.

Class B GPCRs consist primarily of hormone receptors, including secretin, glucagon and parathyroid hormone (5). This receptor class contains three main sub-families, the B1 sub-family of secretin and glucagon receptors, the B2 sub-family receptors which are differentiated by large N-terminal extensions that mediate ligand binding (6) and the B3 sub-family which includes methuselah and methuselah-related *Drosophila* receptors (7). There are also several as yet unclassified receptors that make up this class.

Class C includes receptors that play a particular role in the central and peripheral nervous systems. This includes the metabotropic glutamate (mGlu) as well as the taste receptors and the GABA-B receptors (8, 9). The defining characteristic of the class C receptors is their large N-terminal domain, which is necessary for ligand binding (6).

The final two GPCR classes are the adhesion and frizzled/taste receptor families, which encompass the rest of the GPCRs (4). The frizzled/taste family is an important regulator of the Wnt signalling pathway, through the activation of the protein dishevelled.

b) Heterotrimeric G Proteins

The best known property of GPCRs is the way in which they activate intracellular signalling pathways through their association with and activation of heterotrimeric G protein complexes, consisting of an α, β and γ subunit. The classic GPCR signalling paradigm as written in textbooks holds that upon ligand binding, the receptor undergoes a conformational change leading to the activation of the G protein α (Gα). In their inactive state, Gα subunits are bound to guanosine diphosphate (GDP) (10). However, once activated by receptors, Gα exchanges GDP for guanosine triphosphate (GTP) and dissociates from the receptor, forming the Gα-GTP monomer as well as a Gβγ dimer, which are now free to modulate the activity of other intracellular proteins (11). Gα inactivation is mediated via its intrinsic GTPase activity which hydrolyses GTP to GDP (12). This process is facilitated by regulator of G protein signalling (RGS) proteins, a type of GTPase-activating protein (GAP) that have the ability to stimulate the GTPase activity of Gα. Following the activation of the effector, GTP is hydrolyzed, resulting in the inactivation of Gα, and its reassociation with Gβγ to re-form the G protein heterotrimer, which can again bind the GPCR. However, recent evidence indicates that in certain cases the heterotrimeric G protein complex will simply undergo a conformational change, rather than dissociating, as a result of ligand binding. This conformational change would then allow the G ptotein to interact with its effector either by revealing the binding site, or by bringing them into close proximity, this in turn leads to the activation of the effector (13). Furthermore, there is also recent evidence indicating that GPCRs can also signal independently of their G proteins, leading to the suggestion that they be renamed seven transmembrane receptors instead of GPCRs (4).

There are four sub-classes of Gα subunit which differ in their sensitivity to different bacterial toxins such as cholera toxin or pertussis toxin. The Gα that is associated with the receptor determines, in large measure, which downstream effectors are activated, leading to the production or release of different second messengers and, as a result, the activation of diverse signalling pathways (Figure 3). Certain GPCRs can bind more than one subclass of Gα subunit and can even switch between them

depending on how they are regulated. One of the main subclasses is $G\alpha_S$ which also includes G_{olf} and G_{gust}. $G\alpha_S$ activation primarily leads to the activation of adenylyl cyclase (AC) located at the plasma membrane. There are 10 known types of AC, nine of which are membrane bound and one which is soluble (sAC): AC5 and AC6 are the major isoforms present in myocytes, though all except AC8 are expressed in the heart (14). Once activated, AC catalyzes the conversion of its substrate ATP to 3',5'-cyclic AMP (cAMP) (14). The second messenger cAMP, then binds and activates a protein serine/threonine kinase, protein kinase A (PKA), also known as cAMP-dependent protein kinase. PKA is normally inactive, in the form of a tetrameric holoenzyme, consisting of two catalytic and two regulatory subunits (15). cAMP binds to the regulatory subunits, causing their dissociation from the catalytic subunits and thereby allowing the catalytic subunits to bind to and phosphorylate their specific substrates. cAMP has also been shown to activate exchange proteins activated by cAMP (EPAC) in a similar manner, leading to the activation of small Ras-like GTPase proteins (16). There are two isoforms of EPAC, and recent data has revealed that they can play critical roles in both physiologic and pathologic processes (16). cAMP is subsequently degraded to 5'-AMP by cyclic nucleotide phosphodiesterases (PDEs) (14). The cyclic nucleotide phosphodiesterases can either be specific for cAMP (PDE4, 7, 8), cGMP (PDE5, 6, 9) or can hydrolyze both (PDE1, 2, 3, 10, 11).

The effects of PKA actvation can vary greatly depending on the cell type. In the cardiomyocyte, PKA has been shown to regulate calcium uptake into the sarcoplasmic reticulum (SR) through the phosphorylation of phospholamban (17). Phospholamban normally inhibits the sarco/endoplasmic reticulum calcium ATPase (SERCA). This inhibition is relieved upon phosphorylation of phospholamban by PKA, thus increasing the rate of muscle relaxation as well as contractility (17). Furthermore, PKA can also regulate voltage-gated L-type calcium channels directly through phosphorylation (18). PKA is also able to directly activate cAMP response element binding (CREB), a transcription factor that is able to bind cAMP response elements on DNA and thus affect transcription (19).

The accessibility of PKA to its downstream substrates is also tightly regulated by the localization of PKA within the cell. This localization is in turn regulated by the scaffolding proteins A-kinase anchor proteins (AKAPs). AKAPs interact with the regulatory domains of PKA, and usually, also assemble other signalling elements into a complex, thus facilitating PKA function. AKAPs often bring together opposing regulatory molecules, thereby setting up localized temporal regulation of signal transduction pathways. There are over 50 different AKAPs with AKAP6, also termed mAKAP, being a predominant member in cardiac muscle, localized predominantly to the SR and the nucleus (20). In fact, mAKAP has been shown to determine the subcellular localization of several proteins that are thought to be involved in cardiac hypertrophy, including protein phosphatases and MAPKs (14).

$G\alpha_{i/o}$ subunits work in the exact opposite manner to $G\alpha_S$. $G\alpha_{i/o}$ inhibits AC function, and in so doing decreases cellular levels of cAMP and thus the activity of PKA. Hence the activity of AC is tightly controlled by these two Gα proteins, and the balance between the two in conjunction with cAMP-selective phosphodiesterases, determines the cytosolic levels and subcellular distribution of the second messenger cAMP.

Another sub-class of Gα protein is $G\alpha_{q/11}$ which activates phospholipase C (PLC). There are 6 PLC sub-families that differ in mode of activation, regulation and cellular localization (21). PLC-δ is considered the prototypical PLC isoform, and is the best understood, although PLC-β is the one most commonly associated with GPCR signalling. Nevertheless, all isoforms of PLC catalyze the same reaction, the hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and 1,2-diacylglycerol (DAG) (21). Unlike DAG, IP3 is soluble and can more freely diffuse through the cytoplasm, where it can bind IP3-sensitive calcium channels (IP3R) on the endoplasmic reticulum (ER), or SR in muscle, and cause the release of calcium from the ER into the cytoplasm. In the heart, the entry of calcium from the voltage-sensitive calcium channels can also lead to the activation of the RyR on the SR, triggering a further increase in cytosolic calcium. This process is termed calcium-induced calcium

release. On the other hand, DAG remains in the plasma membrane as a result of its hydrophobic nature. DAG is able to recruit the serine/threonine protein kinase C (PKC) to the plasma membrane, and thus facilitate its activation. There are three main sub-families of PKC, the conventional, the novel and the atypical. The conventional family of PKC requires DAG as well as calcium in order to be activated, while the novel PKC family requires DAG but not calcium. The atypical PKC family however requires neither DAG nor calcium for its activation (22). Similar to PKA, the effects of PKC vary greatly depending on the cell type in question. PKC has been implicated in a multitude of cellular functions including receptor desensitization, and regulating cell growth and transcription. In the heart, PKC is primarily involved in the regulation of ion channel-linked receptor, or ionotropic receptor, function, and has been implicated in the devolpment of heart failure (23). One method by which PKC alters contractility is by phosphorylating G protein receptor kinase 2 (GRK2), which is known to regulate the βARs (23).

The fourth and final type of Gα subunit is $G\alpha_{12/13}$. $G\alpha_{12/13}$ regulates cellular processes through the modulation of guanosine nucleotide exchange factors (GEF) known as RhoGEFs (12). When bound to $G\alpha_{12/13}$, these RhoGEFs can allosterically activate the small GTPase Rho, and are typically implicate in the regulation of the cellular cytoskeleton (12). The Rho family of GTPases is part of the Ras superfamily, and contains 20 known members. However, only three members have been seriously studied to date; cdc42, Rac1 and RhoA. Once Rho binds GTP it can activate a host of proteins, including Rho-associated protein kinase (ROCK), a serine/threonine kinase and a major downstream effector of RhoA (24). There are two ROCK isoforms, with ROCK2 found predominantly in the brain and heart, where the RhoA/ROCK pathway contributes to remodeling following ischemic injury or persistent hypertrophic stress (24).

In addition, the ability of Gβγ subunits to modulate signalling should not be overlooked. While Gβγ was long believed to primarily be important for the inhibition of Gα, subsequent work indicates that Gβγ can activate signalling pathways independently

of Gα. There are five Gβ isoforms and twelve Gγ isoforms, resulting in the formation of a host of different Gβγ dimers depending on the cell type. Gβγ has been shown to regulate ion channels, particularly the G protein-coupled inwardly rectifying potassium channel (GIRK or Kir3 channels), phospholipase A and C, and of particular interest to the work presented here, phosphoinositide 3-kinase (PI3K) (12).

The PI3K family is subdivided into three classes, with class I shown to be activated by GPCRs. Class I PI3K are composed of a regulatory and catalytic subunit, with at least seven known variants of the regulatory subunit, and three of the catalytic subunit (25). Upon activation, PI3K produces various 3-phosphorylated phosphoinositides including PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (25). These two phosphoinositides bind the pleckstrin homology domain of serine/threonine protein kinase B (PKB, also known as Akt), and hence activation of PI3K can result in the recruitment of PKB to the plasma membrane. The phosphoinositide-dependent protein kinase 1 (PDK1) is similarly recruited to the plasma membrane following the activation of PI3K. The colocalization of PKB with activated PDK1 allows PDK1 to phosphorylate PKB at threonine 308, leading to the partially activation of PKB (26). PKB is then phosphorylated at serine 473; this double phosphorylation fully activates the enzyme. The phosphorylation of PKB at serine 473 is mediated by either the PI3K related family mammalian target of rapamycin complex 2 (mTORC2) or DNA-PK, depending on the original stimulus as well as cellular context (26). There are three PKB family members, each the product of a different gene. PKB has a variety of different downstream targets, including but not limited to GSK3β, mTOR, Bcl-2-associated death promoter (BAD), Iκ-B kinase (IKK) and Pim-1, and is involved in cell proliferation, differentiation and survival (25). Glycogen synthase kinase 3 (GSK3β) and BAD are involved in the regulation of apoptosis, and both are inhibited by PKB phosphorylation. On the other hand, mTOR and nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) are activated by PKB, thus regulating protein synthesis as well as the pro-survival pathway (27). In the case of NFκB, PKB leads to its activation by phosphorylating IKK, causing its dissociation from NFκB, which allows it to freely migrate to the nucleus, where it can modulate transcription. Finally, Pim-1 expression

has been linked to many of the cardioprotective effects normally associated with PKB, as well as the regulation of c-Myc (28, 29). Like many of the other protein kinases discussed, PKB is deactivated by a protein phosphatase (PP2). This serine/threonine phosphatase has broad substrate specificity and is fairly ubiquitously expressed. PP2 consists of a dimeric core enzyme composed of the structural A and catalytic C subunits, and a regulatory B subunit.

More recent studies have begun to assign a role in cellular signalling to the Gβγ dimer, independent of its association to GPCRs. In fact, there appears to be a role for G proteins in other receptor-mediated signalling pathways, as well as modulation of other proteins, like GEFs and protein phosphatases. The Gβγ dimer has also been implicated in the direct regulation of transcription through potential regulation of transcription factors like the AP-1 complex, or even direct interaction with histone deacetylase 5 (HDAC5) (12, 30).

c) GPCR Ligands

Given the variety of GPCRs, the multiple G protein subtypes, and the wide array of downstream effectors, it comes as no surprise that there is a wide diversity of ligands for GPCRs. In fact GPCRs have been shown to be activated by everything from small amines, to peptides, and lipids (31). Ligands are usually classified as agonists, antagonists or inverse agonists. Agonists would thus result in the activation of a given signalling pathway. Inverse agonist however would have the opposing effect of the agonist, resulting in the inhibition of constitutive signalling in the absence of agonist. Antagonists block the effect of either agonists or inverse agonists. In the case where all three types of ligands are present, the affinity of the receptor for the individual ligands, termed receptor specificity, will determine which ligand binds and as a result the level of activation of the downstream signalling pathway. Full agonists or inverse agonists, bind the receptor and display full efficacy, producing a maximal functional response. This efficacy is determined relative to the endogenous ligand. Partial agonists on the other hand have only partial efficacy, and result in a reduced activation compared to the endogenous ligand.

In the case of the antagonists, there are both competitive and non-competitive antagonists. Competitive antagonists bind reversibly to the receptor, usually at the same binding site as the agonist, blocking agonist or inverse agonist binding. As such, these ligands compete for the same binding site, and the level of receptor activity will be determined by the receptor's relative affinity for each ligand as well as the concentration of each ligand. In this case, simply increasing the concentration of the agonist can overcome the effect of the antagonist and will result in a greater activation of the receptor. Non-competitive antagonists however bind the receptor irreversibly, or nearly so, and at either the active site or an allosteric site. Non-competitive antagonists that bind to an alternative alosteric site usually exert their function by preventing conformational changes in the receptor required for activation (32). In both cases, this results in a decrease in the magnitude of the maximal possible response, regardless of the amount of agonist present.

Complicating the issue of ligand diversity is the matter of ligand specificity versus selectivity. Recent evidence has begun to mount indicating that a single receptor binding one distinct ligand, may result in the differential activation of particular signalling pathways, or even the activation of completely distinct pathways, termed ligand-biased signalling (4). The standard ligand nomenclature has arisen as a way to define the action of each receptor on a specific signalling pathway following ligand binding. Adding yet another level of complexity to GPCR signalling dynamics is the fact that certain individual ligands can also bind more than just one receptor. As a result, a particular ligand can affect a specific signalling pathway in a completely different way depending on the receptor to which it has bound. This phenomenon, termed binding selectivity, results in ligands potentially acting as either agonists or inverse-agonists towards a single signalling pathway, depending on the receptor being activated (33). Hence, the classification of ligands is much more complicated than originally believed, and necessitates the precision of a receptor and signalling pathway of interest. This likely plays a factor in why many currently available drugs that target specific GPCRs usually have unwanted side effects.

d) GPCR Regulation

GPCR activation is also a tightly regulated process, as they become desensitized, as well as internalized, as a result of sustained activation. There are two types of desensitization; homologous, where the activated receptor type is desensitized upon chronic stimulation and heterologous, where repeated stimulation leads to general desensitization towards multiple agonists. One way in which GPCRs are desensitized is by PKA phosphorylation. As a sort of feedback mechanism, PKA which is activated by cAMP, can then in turn phosphorylate the receptor and desensitize it. In the case of the β_2AR, this phosphorylation can actually switch its coupling from $G\alpha_S$ to $G\alpha_i$ (34). There are also protein kinases which specifically regulate GPCRs. These proteins, termed G protein-coupled receptor kinases (GRKs) are serine/threonine protein kinases that phosphorylate only activated GPCRs. There are seven known GRKs with different types associated to the regulation of different GPCRs. Upon phosphorylation by GRKs, the GPCR is endocytosed, dephosphorylated within endosomal vesicles and then recycled to the plasma membrane (35). This allows for the resensitization of the receptor. Alternatively, the GPCR can also be degraded by being targeted to the lysozome. The phosphorylation of GPCRs by GRKs also has another effect, the recruitment of arrestin proteins. The phosphorylated receptor can recruit arrestin, which reduces the receptor's ability to productively activate G proteins.

There are 4 arrestin subtypes, termed simply arrestins 1-4. The subtype most commonly found in the heart is arrestin-2, also known as β-arrestin. Once β-arrestin has bound to the GPCR it undergoes a conformational change that allows it to act as a scaffolding protein for the adaptor complex AP-2, which in turn recruits clathrin (36). This complex, which also includes PI3K, facilitates internalization of the GPCR via clathrin-coated pits (37). PI3K also aids in endocytosis by phosphorylating non-muscle tropomyosin, thereby enhancing the endocytotic process (37). β-arrestin also has the ability to recruit other proteins, and has been shown to mediate post G protein signalling. β-arrestin can mediate these signalling cascades by selectively scaffolding certain components, including the small GTPases like Ras, and members of the mitogen-activated protein kinase (MAPK) cascade (36).

The MAPKs are serine/threonine protein kinases activated in response to extracellular stimuli, regulating signalling cascades involved in cellular proliferation, gene expression, cell survival and apoptosis. The MAPK family consists of three main groups, the classical extracellular signal-regulated kinases (ERK1/2), and the stress activated kinases which consist of c-Jun N-terminal kinases (JNKs) and p38. As with all MAPKs, ERK1/2 is activated by a cascade that involves a MAPK kinase (MAPKK) as well as a MAPK kinase kinase (MAPKKK). The activation of ERK1/2 involves Ras GTPases, leading to the activation of Raf-1, a MAPKKK. Raf-1 can directly bind the Ras family members, leading to its activation (38). Once activated, Raf-1 phosphorylates the MAPKKs 1/2 also known as MEK1/2. MEK1/2 will then in turn phosphorylate, and hence activate, ERK1/2. ERK1/2 regulates cell proliferation and differentiation, and has been shown to activate various transcription factors, like Elk-1 (39). Furthermore, ERK1/2 is implicated in cardiac hypertrophy, though it does not appear to be absolutely necessary, it does appear to provide critical protective effects (40). In addition, a role for ERK1/2 in the nucleus has also been shown, under the apparent control of casein kinase 2, as well as a novel auto-phosphorylation site that directs ERK1/2 to the nucleus leading to enhanced phosphorylation of nuclear targets (41, 42). ERK5 is also a member of this family, and is activated by MEK5. The MEK5/ERK5 pathway is less studied, although it has been shown to be implicated in survival, and anti-apoptotic signalling (43). In addition, the C-terminus of ERK5 is significantly larger than most MAPKs, and contains both a auto-inhibitory domain and nuclear-shuttling functions (43). There are also two atypical MAPKs, ERK3 and ERK4 (44). These MAPKs only require a single phosphorylation to be activated, unlike the classical MAPKs that require dual phosphorylation, and display major differences in their C-terminal tail. ERK3 is unstable, unlike ERK4, and both are primarily found in the cytoplasm (44). Otherwise, not much is known about their regulation, possible substrates and function in the cell.

There are three main JNK genes (JNK1, JNK2 and JNK3), each with multiple isoforms. Activation of JNK is mediated by MAPKK4 and MAPKK7, and requires dual phosphorylation of threonine and tyrosine residues within a thr-pro-tyr motif (45). As

previously stated, JNKs are activated by stress stimuli, such as ultraviolet radiation, cytokines, and/or heat or osmotic shock. Once activated, JNKs have been shown to be involved in the regulation of differentiation, survival and apoptosis, through the activation of various proteins, including c-Jun, ATF2 and p53 (45).

Four p38 MAPKs have been identified (α, β, γ and δ), with p38α and p38β more widely expressed, as compared to the other two isoforms (46). Similar to JNKs, the p38 MAPKs are activated by MAPKK3 and MAPKK6 by dual phosphorylation of threonine and tyrosine residues within a thr-gly-tyr motif. p38 MAPK is activated by similar stress stimuli as JNK, and also regulates transcription factors involved in survival and apoptosis, like ATF2 and MEF2 (47).

e) GPCR Oligomerization

GPCR oligomerization has begun to be studied in a wide range of different GPCR subfamilies, and appears to play a role predominantly in class C GPCRs. However, the notion of GPCR oligomerization remains controversial, and opinions tend to vary according to which class, and even subfamily is being discussed (6). While the question of oligomerization of class C receptors is now more widely accepted, whether this is also the case for class A remains debated. The study of stable class C receptor dimers has however produced a wealth of knowledge on the subject, which could then potentially be applied to the other classes of GPCRs. Recent evidence has indicated that while these receptors might form stable dimers, one receptor is sufficient for everything from ligand binding, to G protein activation and even β-arrestin binding (6). Although dimerization can alter which G protein associates with a given receptor (4). Oligomerization of GPCRs can also play a role in receptor trafficking, as is the case for the GABA receptors, where heterodimerization of $GABA_{B1}$ with $GABA_{B2}$ is necessary for proper trafficking to the plasma membrane (48). Moreover, the GABA-B receptors are not the only GPCRs that appear to be able to heterodimerize, as other GPCRs have recently also been shown to have the ability to heterodimerize, as is the case of the β-adrenergic receptors and the endothelin receptors (49, 50).

Moreover, the notion of oligomerization is further complicated by recent evidence that indicates that, contrary to the classical view of GPCR signalling, GPCRs might in fact form stable complexes with their heterotrimeric G proteins as well as downstream effectors and even regulatory proteins (51). These GPCR complexes would undergo a conformational change as a result of ligand binding, rather than the classical separation of the G protein heterotrimer (52). These complexes could potentially form as early as in the ER, where the β_2AR has been shown to interact with the G$\beta\gamma$ dimer, and would thus be exported to the plasma membrane as an intact complex (51). GPCRs have been shown to associate with AC as well as with Kir3.1 potassium channels, indicating that these two effectors might form stable complexes with the receptors and their G proteins (53).

f) Intracellular GPCRs

Recently, GPCRs have also been shown to localize to intracellular membranes, in particular the nuclear membrane (Table 1). GPCRs have also been shown to signal from other intracellular locations, with the angiotensin receptor localized to mitochondria (54). The discovery of nuclear GPCRs is particularly relevant for class A GPCRs, as many sub-families have been found to localize to the nucleus including but not limited to the endothelin receptor (ETR), angiotensin receptor (ATR) and adrenergic receptor families (55-57). Class B and C GPCRs have also been shown to localize to the nucleus, with the parathyroid receptor being discovered in the nucleus of osteoclast cells (58), and metabotropic glutamate receptors (mGluR) have been demonstrated on the nuclear membrane of neurons (9, 59). In addition, all four types of Gα proteins and various G$\beta\gamma$ subtypes have also been detected at the level of the nucleus (60-62). Furthermore, many of the downstream effectors associated to GPCRs have also been shown to localize to the plasma membrane, including PLA, PLC and AC (63-65), and several ion channels, including Ca^{2+} and K^{+} channels (66-68). Moreover, the various second messengers generated by these effectors have also been observed at the level of the nucleus, including cAMP (69), IP3 and DAG, as well as cGMP (70). Additionally, these nuclear GPCRs have been shown to interact with their downstream effectors. Nuclear bradykinin type 2, ETB, mGlu5 and AT1 receptors, to name a few, have been

demonstrated to be able to regulate nuclear calcium, with mGlu5 and AT1 receptors also shown to generate nuclear IP3 (61, 70-75). On the other hand, nuclear β_1AR appears to activate AC and leads to the increase of nuclear cAMP (57). Furthermore, several of the GPCR regulatory proteins are also located at the nucleus. This includes the RGS proteins (76), β-arrestin 1 (77), as well as the GRKs (78, 79). This implies that nuclear GPCRs are also under tight regulation, not unlike GPCRs located at the plasma membrane, and further supports the notion that these receptors are in fact functionally relevant. In addition, other downstream effectors, associated with both G protein-mediated and G protein-independent signalling have also been shown at the nucleus, and appear to be activatable (30, 80, 81). Further, some GPCRs, such as the GABA-B receptors, have also been shown to interact directly with certain transcription factors, as have certain G proteins (82-84), while others appear to exert their effects by way of histone acetylation, as is the case of the gonadotropin and bradykinin receptors (74, 85).

The unique structure of the nuclear envelope however does make the organization of nuclear GPCR signalling more complex than at the plasma membrane. The nuclear envelope consists of a dual membrane with an intermembrane space, the perinuclear space or lumen, which is contiguous with the ER. Additionally, at regular intervals the two membranes are joined together, forming nuclear pores. As such nuclear GPCRs can potentially be present on either the inner or outer nuclear membrane, or perhaps both (Figure 4). However, we believe that the N-terminal end of the receptor, containing the ligand binding sites, will never the less be situated in the lumen. This would mean that the ligand would have to be transported into the lumen of the nuclear membrane in order to bind its receptor and effect signalling. Furthermore, depending on its orientation nuclear GPCRs would either be directing signalling toward the nucleus or the cytosol. To date no study has conclusively shown the orientation of any nuclear-localized GPCR.

The presence of GPCRs at the level of the nuclear membrane means there must be a pool of intracrine ligands that can reach and bind to these nuclear receptors (Table 2). These ligands can be either synthesized within the cell, or internalized from the extracellular space. Intracrine ligands synthesized within the cell can be targeted to the

Golgi apparatus for secretion, though they may act intracellularly before or following secretion, by way of reuptake. Intracrine ligands that are internalized from the extracellular space may do so in a variety of ways. These ligands might be internalized in conjunction with their native receptor located at the plasma membrane, or can potentially enter the cell through passive diffusion. Internalized intracrine ligands can also be internalized by way of transmembrane transporters, or pass through channels or pores in the membrane.

III) Adrenergic Receptors

One GPCR sub-family of particular interest is the adrenergic receptor family. The adrenergic receptor family is part of the class A GPCRs, with the ability to bind catecholamines, especially epinephrine (adrenaline) as well as norepinephrine (noradrenaline) endogenously. Upon binding their ligand, these receptors will generally mediate sympathetic nervous system responses; this is normally referred to as the "fight-or-flight" response. Catecholamines are synthesized from DOPA, with dopamine being the first catecholamine synthesized, followed by norepinephrine and epinephrine. Norepinephrine and epinephrine are produced in certain neurons of the sympathetic nervous system, the latter more predominantly in the chromaffin cells of the adrenal medulla. Both catecholamines can act as either a hormone or a neurotransmitter, depending on their site of action, and have half-lives of only a few minutes in the circulation. Their release throughout the entire body is triggered by a variety of stresses, including a physical threat, excitement and loud noises. Catecholamines have been shown to directly increase heart and respiratory rate, trigger the release of glucose from energy stores, as well as lipolysis and increase blood flow and muscle contraction.

There are two primary types of adrenergic receptors, the α-adrenergic receptors (αAR) and the β-adrenergic receptors (βAR). These two groups can be further subdivide into 5 subtypes; the α_1AR and α_2AR, and the β_1AR, β_2AR and β_3AR. The α and βARs have differential cellular localization and usually play opposing roles in the tissues where they are both present. In fact, in the circulation, the αARs are less sensitive to

epinephrine than the βARs, however when activated they override the vasodilatory effects of the βARs. This will only occur at higher levels of circulating epinephrine.

There are three α_1AR subtypes, α(1A), α(1B) and α(1D), and all display a higher affinity for norepinephrine than epinephrine. All three subtypes generally activate $G\alpha_q$. The α_1AR is found predominantly in smooth muscle, where it mediates vasoconstriction. Activation of the receptor/G protein complex leads to the activation of PLC. PLC in turn causes an increase in DAG and IP3, triggering further effects, including calcium release and the activation of PKC (86). The α_1AR is also present in other tissues, regulating glycogen metabolism in adipose tissue and liver, sodium reabsorption in the kidney, and a variety of other effects in the nervous system. In the heart, α_1AR activation tends to have a positive inotropic effect, though to a lesser extent than the βARs, with α(1B) being the predominant isoform (86, 87). The physiologic function of the α_1AR in the heart is still not completely clear, though they have been shown to play a role in hypertrophy and stimulation of transcription (87). Under pathologic conditions, it is believed that the αAR may play a compensatory role to counterbalance the desensitization of the βAR. Furthermore, the α_1AR is believed to be important in developmental cardiac growth, as well as pathologic hypertrophy (86). The α_1AR also appears to produce pro-survival effects in the cardiomyocyte, while also protecting it from decompensated heart failure. Moreover, the α_1AR has been shown to localize to the nucleus in adult ventricular myocytes, where it is capable of activating the ERK1/2 pathway (88, 89).

Similarly to α_1AR, the α_2AR also has three subtypes, α(2A), α(2B) and α(2C). However, α_2AR displays higher affinity for epinephrine, and primarily activate $G\alpha_i$ (90). Upon receptor activation, $G\alpha_i$ is able to associate with AC, and thereby inhibit its activity. This in turn causes a reduction in the levels of cAMP and a decrease in the activity of PKA. The α_2AR is predominantly expressed in the nervous system and in smooth muscle. The α_2AR can cause either vasoconstriction or vasodilation, depending on the tissue of interest (91). In the nervous system, α_2AR can act as either an

autoreceptor, inhibiting the exocytosis of its own neurotransmitters, or as a heteroreceptor, inhibiting the release of many other neurotransmitters (90). The α_2AR has also been shown to inhibit insulin release from the pancreas, to inhibit the norepinephrine system in the brain and increase thrombocyte aggregation (92). In fact, during the development of hypertrophy, the α_2AR are desensitized by GRK2 in adrenergic neurons, causing an increase in circulating adrenaline levels, likely contributing to the eventually development of heart failure (90). In the heart, α_2AR appear to play a cardioprotective role, as α_2AR agonists are a common perioperative treatment, though this remains a contentious and controversial area of medicine.

The β_1AR displays equal affinity for both epinephrine and norepinephrine and signals mainly through $G\alpha_S$ (34). Upon activation of $G\alpha_S$, the G protein associates with AC, and promotes the conversion of ATP to cAMP, increasing its levels, and leading to the activation of PKA. This activation of PKA can be a fairly diffuse cellular response, leading to effects throughout the cell. The β_1AR is the predominant βAR expressed in the heart, where it represents roughly 70% of the βAR density (93). The β_1AR has also been shown to be expressed in adipose tissue and the cerebral cortex. In the heart, activation of the β_1AR leads to an increase in cardiac output, via increases in heart rate as well as contractility, and while the β_2AR can perform a similar role, knock-out studies have shown that the β_1AR is sufficient (93). β_1AR-mediated cAMP signalling has also been shown to regulate L-type calcium channels as well as phospholamban (93). During the development of hypertrophy, and eventually heart failure, there is an increase in circulating catecholamines, resulting in chronic stimulation of the β_1AR which is thought to play a role in the further development of pathologic hypertrophy, and heart failure. During the progression towards heart failure, several changes occur within the cardiomyocyte, which include alterations in β_1AR signalling. There is a downregulation of the β_1AR subtype by around 50%. Such changes are not seen in the other subtypes, although the β_2AR does undergo desensitization (94). Similar to other GPCRs, the βAR also undergo desensitization following phosphorylation by GRKs. The principal GRK responsible for desensitizing the βARs is GRK2, also termed β-

adrenergic receptor kinase 1 (βARK) (34). Moreover, there is an increase in the expression of GRK2, which is responsible for the pronounced desensitization of the βARs. This downregulation of βAR signalling, coupled with desensitization, is initially cardioprotective but eventually contributes to the onset of heart failure. This has lead to the use of βAR antagonists, or β-blockers to treat heart failure. However, the method by which they improve cardiac function is still somewhat contested. While some believe that β-blockers work by antagonizing the βARs, there is also evidence that they may work by resensitizing the failing myocardium to adrenergic stimulation. Another change in β_1AR signalling is the observation that persistent activation augments contractility through calcium/calmodulin-dependent kinase II (CAMKII) signalling, and independently of the cAMP/PKA signalling system (93). This was shown through the use of inhibitors and a dominant-negative mutant of CAMKII. In conditions of chronic β_1AR stimulation, inhibition of PKA is unable to block the sustained increases in myocyte contractility and calcium transients, while CAMKII inhibition does (95). Furthermore, studies have shown that following chronic β_1AR stimulation, along with a progressive activation of CAMKII, there is also a concomitant desensitization of the PKA pathway, indicating that in response to chronic stimulation there is a time dependent switch from PKA to CAMKII (95).

CAMKII is a serine/threonine protein kinase regulated by the calcium/calmodulin complex. CAMKII consists of four structural domains, a catalytic domain, an autoinhibitory domain, a variable segment and a self-association domain (96). The sensitivity of CAMKII to calcium and calmodulin is regulated by the variable and self-association domains. As greater amounts of calcium and calmodulin become present, CAMKII autophosphorylates, and together with a partially reversible oxidation of a pair of methionines in the CAMKII regulatory domain, leads to persistent activation for a short period of time (96). CAMKII has also been linked to the apoptotic effects of sustained β_1AR stimulation, as inhibition of CAMKII can block the apoptotic effects, and overexpression of CAMKII-δC, a cardiac CAMKII isoform, markedly aggravates this apoptotic effect (97). As such, the switch from PKA to CAMKII has also been linked to the catecholamine-induced hypertrophic response in cardiomyocytes (98). This

has been further demonstrated by treatment with a CAMKII peptide inhibitor. Overexpression of this inhibitor in transgenic mice prevents the maladaptive cardiac remodeling and contractile dysfunstion associated with chronic βAR stimulation and myocardial infarction (99). Also involved in this PKA independent hypertrophic response is EPAC, which has been shown to lead to the activation of CAMKII through the release of calcium by ryanodine receptors located on the SR (16). Given that EPAC plays a role as a GEF for small GTPases like Rap1 and Rap2, this would suggest that this switch to CAMKII activity involves the activation of small GTPases rather than cAMP production.

The β_2AR displays higher affinity for epinephrine over norepinephrine, and signals through both $G\alpha_S$ and $G\alpha_i$. Similarly to the β_1AR, upon activation the β_2AR leads to the activation of AC, catalyzing the formation of cAMP, and leading to the activation of PKA. However, unlike the β_1AR, this activation of PKA remains highly localized. The mechanism responsible for this localized response is likely its association with AKAPs, although coupling of the β_2AR to $G\alpha_i$ is suggested to also play a role (100). The β_2AR is primarily expressed in smooth muscle and the heart, where it represents roughly 30% of the βAR density (93). In the heart, the β_2AR has been linked to positive inotropic and chronotropic effects, and has been shown to regulate the class C L-type calcium channels. A key feature of the β_2AR is an ability to undergo spontaneous activation which has not been observed in the β_1AR (101). As such, the β_2AR can signal in the absence of its endogenous ligand, and this effect is dependent on receptor density. Moreover, in contrast to the β_1AR, chronic activation of the β_2AR is actually believed to play a cardioprotective effect (102). This cardioprotective effect is largely believed to be mediated through $G\alpha_i$, and involves $G\beta\gamma$, and the activation of the PI3K/PKB survival pathway. This pathway is thought to be responsible due to the fact that the β_1AR cannot interact with $G\alpha_i$, and that blocking any of these elements results in the loss of the protective effect (93). In addition, one of the main recognized changes in βAR signalling during the development of heart failure is the switch of β_2AR coupling from $G\alpha_S$ to $G\alpha_i$ in cardiomyocytes (34). This potential cardioprotective effect

of β_2AR signalling has lead, in part, to the development of a new generation of β-blockers that are β_1AR selective agents. Furthermore, the development of β-blockers that would antagonize the β_1AR while acting as partial agonists towards the β_2AR has also been explored. Furthermore, recent evidence indicates that the β_2AR is able to form a heterodimer with the β_1AR (103), this heterodimerization might be important in the proper localization of the receptors, as well as cause the heterodimer to have a unique ligand binding profile.

The β_3AR displays higher affinity for norepinephrine than epinephrine and, similarly to the β_2AR, signals through both $G\alpha_S$ and $G\alpha_i$. The β_3AR is found predominantly in adipose tissue, where it is involved in the regulation of lipolysis and thermogenesis (104). However, the β_3AR has also been localized to the brain, gut, liver and myocardium (105). A key distinguishing feature of the β_3AR is the relatively fewer serine and threonine sites in its C-terminal tail, and the lack of a consensus PKA phosphorylation site, of which there are two in the β_2AR and one in the β_1AR (105, 106). These differences affect its propensity to be desensitized, and in fact in remains to be determinded conclusively whether or not the β_3AR can desensitize. In adipose tissue, where the β_3AR can interact with both $G\alpha_S$ and $G\alpha_i$, it has been shown to activate two parallel signalling pathways, the cAMP/PKA pathway through $G\alpha_S$, and the ERK MAPK pathway through $G\alpha_i$ and recruitment of the tyrosine kinase c-Src (105). The activation of PKA activates the protein kinase cascade leading to p38 activation. This in turn activates a subset of transcription factors, including ATF2. The β_3AR is also present in the heart, though it represents a negligible percentage of the total βAR density in comparison to the other two βAR subtypes (104). As in adipose tissue, it has been linked to both $G\alpha_S$ and $G\alpha_i$, although the signalling effectors downstream of either G protein are less well understood than in the adipose tissue. Interestingly, stimulation of the β_3AR actually results in a decrease in cardiac contractility, which is in clear contrast to the other βARs. Also of note, in cardiac tissue, β_3AR activation of $G\alpha_i$ appears to lead to the production of nitric oxide (NO) by a constitutively expressed isoform of NO synthase (NOS), probably endothelial NOS (eNOS) (104). The production of NO is

likely responsible for some of the negative inotropic effects observed following β_3AR activation, through the activation of sGC (107). As in adipose tissue, in the heart, the β_3AR is believed to activate various MAPK cascades, which potentially contribute to the observed negative inotropic effects. Another unique feature of the β_3AR, is that its expression is upregulated in the failing heart, in contrast to the β_1AR and β_2AR (104). Given the negative inotropic effect of the β_3AR, this upregulation might prove beneficial at first, but likely contributes to the eventual development of heart failure. Interestingly, the β_3AR has also been linked to various cardioprotective effects, principally through its activation of the PI3K/PKB pathway, demonstrating the complexity of β_3AR signalling, and the necessity for further study (93).

IV) Endothelin Receptors

The endothelin receptors (ETR) also belong to the class A family of GPCRs. The ligands for ETR are endothelins, vasoconstricting peptides consisting of 21 amino acids, primarily produced in the endothelium, which play a role in vascular homeostasis. There are three isoforms of endothelin, derived from different genes, termed endothelin 1 (ET-1), ET-2 and ET-3. All three endothelins are synthesized as larger precursor proteins, pre-proETs, and subsequently cleaved to 37-41 amino acid proforms, termed big endothelins. Endothelin converting enzymes (ECEs) are responsible for converting big endothelins to their mature forms (108). In the myocardium, ET-1 is believed to play a paracrine/autocrine role, with ET-1 synthesized, stored and secreted by adult cardiomyocytes under basal conditions and in response to various stimuli (109, 110). Moreover, extracellular ET-1 has been shown to regulate ET-1 mRNA levels, thereby regulating its own expression (111). In addition, there are two receptor subtypes, ETA and ETB, each with its own expression and signalling profile. Another subtype, ETC, has been cloned in *Xenopus laevis*, although a mammalian homolog has yet to be discovered (112).

ETA is equally selective for ET-1 and ET-2, over ET-3, couples predominantly to $G\alpha_q$, although it has been shown to couple to $G\alpha_S$ as well, and is implicated in the regulation of intracellular calcium. ETA is located primarily in the smooth muscle of

blood vessels, though it has also been shown in the heart. On the other hand, ETB is non-selective with respect to the three endothelins, though it does appear to have higher affinity for ET-3. Similarly to ETA, ETB couples primarily to $G\alpha_q$, though it has also been shown to couple to $G\alpha_i$, and plays a role in calcium regulation. The ETR mainly regulate calcium through the activation of PLA, PLC and PLD, leading to the synthesis of the second messengers, IP3 and DAG, and the eventual activation of PKC (108). However, ETR have also been shown to regulate cGMP through NO and several MAPKs, in particular ERK1/2 (108). Both receptor subtypes have been found in the heart, where they mediate positive inotropic and chronotropic effects and can prolong the action potential (113, 114). ET-1 has also been implicated in the development of hypertrophy, myocardial infarction, and congestive heart failure (108). The hypertrophic effects of ET-1 have been linked to its activation of ERK1/2, which in turn is strongly implicated in transcriptional regulation (115) including the immediate early gene Atf3, which has been shown to be important in cardiomyocyte hypertrophy (116). In addition, the ETA and ETB may form homodimers and heterodimers, which can affect their ligand binding, as well as receptor activation and desensitization (50).

Furthermore, the ETR have also been found to localize to the nucleus in various tissues, including the liver, endothelial cells, the brain and the heart (117). These nuclear receptors have been shown to affect several pathways, including calcium regulation, gene transcription and can mediate the phosphorylation of various protein kinases (117). In the heart, ETB appears to localize predominantly to the nucleus, while ETA is primarily found on the plasma membrane (56). These nuclear ETR have been shown to regulate calcium independently of their plasma membrane counterparts, and likely accomplish this via a R-type calcium channel in human aortic smooth muscle cells (118). ET-1 has also been demonstrated to result in a significant increase in reactive oxygen species (ROS) in endocardial endothelial cells, and in vascular smooth muscle cells (119). The potential for nuclear ROS to also affect nuclear calcium levels clearly exists, with intracellular ROS having been shown to be linked to calcium signalling through NO production via NOS (120). Furthermore, nuclei isolated from rat liver, when treated with an NO donor show calcium mobilization, mediated through the

NO/cGMP/PKG pathway, which leads to the activation of NFκB (121). In addition, this nuclear localization of the ETR has lead to the development of a caged analogue of ET-1 that can freely cross the plasma membrane, and once uncaged by UV light can be used to directly activate internal ETR (122).

V) Nitric Oxide

Nitric oxide (NO) is an important signalling molecule involve in many physiological and pathological events, and acts primarily as a vasodilator, thanks to its freely diffusible nature, though it has a half-life of only a few seconds in the bloodstream. In particular, NO is an important mediator in the immune, nervous and cardiovascular systems. NO plays a key role in vascular smooth muscle relaxtion, via vasodilation and increased blood flow (123). In addition, NO has been shown to act as a neurotransmitter, and as such has been implicated in the development of various neurodegenerative diseases (124). Moreover, NO has been implicated in such pathologic conditions as hypertension, stroke and neurodegenerative diseases. NO is synthesized from the amino acid L-arginine, and this reaction is catalyzed by various NOS enzymes (125). There are three NOS subtypes, which differ primarily in their tissue localization. The three subtypes are, endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (125). The NOS subtype primarily found in endothelial cells, and responsible for the vasodilator effects of NO, is eNOS. This subtype is calcium-dependent, and mediates constitutive release of NO (125). eNOS is also involved in the regulation of cardiac function and angiogenesis, and is the primary controller of smooth muscle tone. Furthermore, NO has been shown to play an important negative feedback regulatory role on eNOS, and as a result on endothelial cell function (125). The neuronal isoform is also calcium-dependent, and is found primarily in the nervous system, being implicated in its development, and skeletal muscle. nNOS is also involved in the regulation of cardiac function, as well as sexual arousal. The inducible isoform, unlike the other two subtypes, has been described as calcium-insensitive (125). iNOS is primarily implicated in the immune system, and is synthesized in response to various cytokines. Similarly to eNOs and nNOS, iNOS is also expressed in the cardiovascular

system. Interestingly, iNOS appears to be modulated by cAMP, indicating that some cross-talk might occur between NO signalling and the cAMP/PKA pathway (126, 127).

The primary mode of action of NO is to cause the activation of the enzyme guanylyl cyclase (GC). GC, in response to calcium levels, catalyzes the synthesis of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). GC is usually activated by low levels of calcium, and inhibited by higher intracellular calcium levels. In addition, there are two types of GCs, a membrane bound type, and a soluble type (sGC), with NO generally described as activating the soluble form (128). cGMP is a second messenger, similar to cAMP, and is involved in the activation of intracellular protein kinases, is implicated in ion channel conductance, glycogenolysis and apoptosis. Also in similar fashion to cAMP, cGMP is degraded by cyclic nucleotide PDEs, mainly PDE5, PDE6 and PDE9. PDE5 is of particular interest as its inhibition, with sildenafil, has been shown to have some cardioprotective effects, as well as other uses. The main protein kinase activated by cGMP is the serine/threonine cGMP-dependent protein kinase G (PKG) (129). PKG is comprised of three domains, the first is an N-terminal domain that regulates interactions with other proteins, mainly substrates, and suppresses the kinase domain in the absence of cGMP. The second, the regulatory domain, contains two non-identical cGMP binding domains, and the kinase domain which is responsible for phosphorylating the target proteins. Binding of cGMP to the regulatory domain causes a conformational change which removes the inhibition of the kinase domain, and allows PKG to phosphorylate its substrates. Unlike PKA, the regulatory subunits do not dissociate from the complex. PKG has several biologically important targets and is known to regulate smooth muscle relaxation, neural plasticity, gene transcription and to play a role in cardiac protection.

All three NOS subtypes are present in the heart, though they appear to have different subcellular localization patterns, and as such differ in their signalling effects (130, 131). In fact, eNOS appears to localize to caveolae, while nNOS appears to localize to cardiac SR (132). As such, NO has been linked to a variety of physiologic effects in the heart, including cardiac contractility, apoptosis and calcium regulation

(125, 131). In the heart, NO inhibits L-type calcium channels, while stimulating SR calcium release (132). Furthermore, NO production in the heart is tightly regulated by both calcium and NO levels, and certain protein kinases, like PKB (133, 134). Additionally, NO has been linked to gene regulation, in particular through its regulation of the transcription factor NFκB (128, 135). Recently, evidence has also revealed a possible role for NO in regulating calcium homeostasis in the nucleus, potentially under the control of ET-1 (119). The potential role of NO signalling in nucleus is further supported by recent studies showing that the nuclear lysophosphatidic acid receptors (LPAR) (72), the nuclear prostaglandin E_2 receptor (EP_3) (61, 136), and the bradykinin B2 receptors (B2Rs) (74), all appear to regulate the expression of iNOS.

VI) Gene Transcription

Several of the pathways previously discussed have as their end target the regulation of gene expression. While some pathways alter the expression of a specific gene, or a particular set of genes, others may affect gene transcription as a whole. In addition, the mechanism(s) by which they accomplish this may also differ. Targets may include transcription factor activity, histone modification, or the RNA polymerase complex itself.

Gene transcription is the process of creating a complimentary RNA copy of the host DNA. For any gene to be transcribed, RNA polymerase must first bind to the promoter of the gene of interest. The promoter is a specific sequence of DNA nucleotides, located near the transcription start site of the gene, on the strand that is to be transcribed. Once bound, the RNA polymerase hydrolyses the nucleotide triphosphates, initiating the separation of the two strands of DNA. The polymerase then progresses along the DNA strand, reading the DNA sequence and adding the corresponding nucleotides to the RNA transcript as it goes. The RNA polymerase will eventually reach a “stop” signal, and release the newly formed RNA transcript. Upon its release, a series of 100 to 200 adenine nucleotides are added to the end of the transcript, forming a poly-A tail. This tail acts as a signal to allow RNA to be exported from the nucleus, and bind ribosomes in the cytoplasm. The primary RNA transcript will normally undergo splicing

by spliceosomes, to remove introns, stretches of noncoding RNA. The RNA transcription unit also contains regulatory sequences that direct and control its synthesis, usually located in its 5' untranslated region. Moreover, there are three types of RNA, all with different roles in the cell. One type of RNA is messenger RNA (mRNA), which can be translated into the various proteins they encode for. There are also ribosomal RNAs (rRNA) and transfer RNAs (tRNA). These two types mainly play a role in the translation of mRNA, with the various rRNA transcripts forming the ribosome, and tRNA mediating recognition of the codon and providing the necessary amino acid. However, rRNA accounts for the largest pool of RNA in the cell.

There are three principal RNA polymerases, though others have been discovered in plants. RNA polymerase I (POL I) is responsible for the transcription of rRNA. POL I transcribes one large transcript that itself encodes the 18S, the 5.8S and the 28S rRNA transcripts (137). These rRNA transcripts, along with the 5S rRNA come together to form the ribosome. Given that the rate of cell growth is intricately linked to protein synthesis, which itself is linked to ribosome synthesis, rRNA synthesis and POL I-mediated transcription are usually tightly regulated along with other components of protein translation. RNA polymerase II (POL II) is the polymerase responsible for the transcription of mRNA. Given that POl II is responsible for the transcription of the majority of genes, it is no wonder it is also the most studied and most tightly regulated polymerase. POL II, along with various general transcription factors and regulatory proteins, forms the RNA polymerase II holoenzyme, responsible for transcription of mRNA (138). Chromatin structure has also been shown to regulate POL II function, mainly through alterations in histone modification. The third polymerase, RNA polymerase III (POL III) is responsible for the transcription of the last rRNA transcript, 5S, as well as tRNAs and other small RNAs (137). The genes transcribed by POL III are generally considered housekeeping genes and as such POL III requires little regulation, although recent evidence has begun to show a possible role for the POL III transcription system in other nuclear processes, including direct effects on POL II transcription (139).

Transcription is also regulated by histones. Histones are a family of proteins found in the nucleus that are responsible for packaging and ordering the DNA into discrete structural units called nucleosomes. There are five main families of histones, with histones H2A, H2B, H3 and H4 known as the core histones, and H1 and H5 as the linker histones. The more tightly that the DNA is wound around the histones, or condensed, the less likely it is that a polymerase may bind and the DNA will be transcribed. As the DNA becomes less condensed, the more the genes become accessible and can be transcribed. As such, the regulation of histones is an important part of gene transcription. In evidence of this fact, there are no less than eight distinct types of modifications that histones can undergo, including but not limited to, methylation, phosphorylation, acetylation and ubiquitination. These various modifications affect not only gene expression, but also genome stability and cell mitosis. As a result there are numerous histone-modifying enzymes. The main enzymes regulating gene expression however, are the histone acetyltransferases (HATs) and histone deacetylases (HDACs). In general, histone acetylation is linked to transcriptional activation, while deacetylation has the opposite effect.

Another set of proteins important in regulating transcription are the transcription factors, with between 2000 and 3000 sequence-specific DNA-binding transcription factors found in humans (140). These proteins contain DNA binding domains that allow them to interact directly with specific sequences of DNA and can affect gene transcription by either acting as activators or repressors of RNA polymerase, catalyzing the acetylation or deacetylation of histone proteins, or by recruiting coactivator or corepressor proteins to the transcription factor DNA complex. One particular transcription factor of interest is the cAMP response element binding (CREB) protein. CREB binds to DNA at specific sequences termed cAMP response elements (CRE). CREB is the main transcriptional regulatory target of the cAMP/PKA pathway (19).

Another transcription factor of note is nuclear factor kappa-light chain enhancer of activated B cells (NFκB). NFκB is an important transcription factor as it belongs to the category of rapid acting primary transcription factors, that are already present in most

cells, and does not require protein synthesis to be activated. This allows NFκB to quickly respond to harmful external stimuli. Under basal conditions, NFκB is maintained in an inhibited state by a family of inhibitor proteins called “inhibitor of κB” (IκB), which sequester NFκB in the cytoplasm. Activation of NFκB requires the degradation of these inhibitor proteins. This is accomplished by the activation of IκB kinase (IKK). Once activated, IKK phosphorylates the regulatory domain of IκB, which causes its ubiquitination and eventual degradation by the proteasome. The degradation of IκB than allows the NFκB to translocate to the nucleus, where it can bind to specific DNA sequences, and affect DNA transcription. One particular gene activated by NFκB is its own repressor IκB, which can then reinhibit NFκB, thus forming an auto feedback loop and results in oscillations in NFκB signalling (141). NFκB activation has been implicated in the inflammatory and immune responses, as well as in cell survival and proliferation. The two main signalling pathways known to activate the NFκB pathway are the PI3K/PKB and the PLC/PKC pathways.

Figure 1: Cross section of the heart. Illustrated cross section of the heart. Taken from (http://www.clivir.com/lessons/show/diagram-of-the-human-heart-for-kids.html).

Figure 2: Cardiac action potential. Illustration of the cardiac action potential, detailing the five phases and the key ion channels involved.

Figure 3: Diversity of G protein-coupled receptor signalling. Various ligands use G protein-coupled receptors (GPCRs) to regulate membrane, cytoplasmic and nuclear targets. GPCRs interact with heterotrimeric G proteins composed of α, β and γ subunits that are GDP bound in the resting state. Agonist binding triggers a conformational change in the receptor, which catalyses dissociation of GDP from the α subunit followed by GTP-binding to Gα and the dissociation of Gα from Gβγ subunits. The α subunits of G proteins are divided into four subfamilies: $G\alpha_S$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$, and a single GPCR can couple to either one or more families of Gα proteins. Each G protein activates several downstream effectors. Typically $G\alpha_S$ stimulates adenylyl cyclase and increases levels of cyclic AMP (cAMP), whereas $G\alpha_i$ inhibits adenylyl cyclase and lowers cAMP levels and members of the $G\alpha_q$ family bind to and activate phospholipase C (PLC), which cleaves phosphatidylinositol bisphosphate (PIP2) into diacylglycerol and inositol triphosphate (IP3). The Gβ subunits and Gγ subunits function as a dimer to activate many signalling molecules, including phospholipases, ion channels and lipid kinases. Besides the regulation of these classical second-messenger generating systems, Gβγ subunits and Gα subunits such as $G\alpha_{12}$ and $G\alpha_q$ can also control the activity of key intracellular signal-transducing molecules, including small GTP-binding proteins of the Ras and Rho families and members of the mitogen-activated protein kinase (MAPK) family of serine-threonine kinases, including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), p38 and ERK5, through an intricate network of signalling events that has yet to be fully elucidated. Ultimately, the integration of the functional activity of the G protein-regulated signalling networks control many cellular functions. 5-HT, 5-hydroxytryptamine; ECM, extracellular matrix; GABA, gamma-aminobutyric acid; GEF, guanine nucleotide exchange factor; GRK, G protein receptor

kinase; LPA, lysophosphatidic acid; PI3K, phophatidylinositol 3-kninase; PKA and PKC, protein kinase A and C; S1P sphingosine-1-phosphate. Taken from (31).

Figure 4: Possible orientation of nuclear β-AR signalling complexes. Both the β_1-AR, coupled to Gs, and the β_3-AR, coupled to Gi, but not the β_2-AR, are resident on the nuclear membrane, at least in rat and mouse adult ventricular cardiomyocytes. How these receptors are trafficked to distinct endomembrane compartments is not well understood and could either be a result of receptor internalization from the cell surface or via de novo delivery from the biosynthetic pathway. The outer nuclear membrane is continuous with both the endoplasmic reticulum (ER) and the inner nuclear membrane, with which it is joined at the level of nuclear pore complex insertion. Ligands must be able to reach the space between the two nuclear membranes to activate these receptors, or receptors must be constitutively active. The possibility that there may be two distinct orientations for nuclear G protein-coupled receptors (GPCRs), i.e., either capable of delivering signals toward the cytosol or the nucleoplasm, is something that can only be explored in an intact cell context. Also, the role of the nuclear pore complex in determining the sidedness of nuclear GPCR signalling remains to be determined. C, COOH-terminus; N, NH2-terminus; AC, adenylyl cyclase; PI3K, phosphatidylinositol 3-kinase. Taken from (30).

Figure 5: Subtype-specific signalling pathways of cardiac βARs. The dual coupling of β_2AR to G_i proteins activates the G_i–Gβγ–PI3K–Akt pathway, which, in turn, leads to functional localization and inhibition of the G_s–AC–cAMP–PKA signalling and protects cardiomyocytes against apoptosis. In contrast, β_1AR couples exclusively to G_S, which activates the G_S–AC–cAMP–PKA pathway, resulting in positive inotropic and relaxant effects. The newly identified, PKA-independent activation of CaMKII is necessary and sufficient in mediating persistent β_1AR stimulation-induced myocyte apoptosis, perhaps cardiac hypertrophy as well. Emerging evidence suggests that cardiac β_3AR might also couple to G_i, in addition to G_S, resulting in the activation of the NOS–NO signalling pathway, which negatively regulates cardiac contractility (PTX, pertussis toxin). Taken from (93).

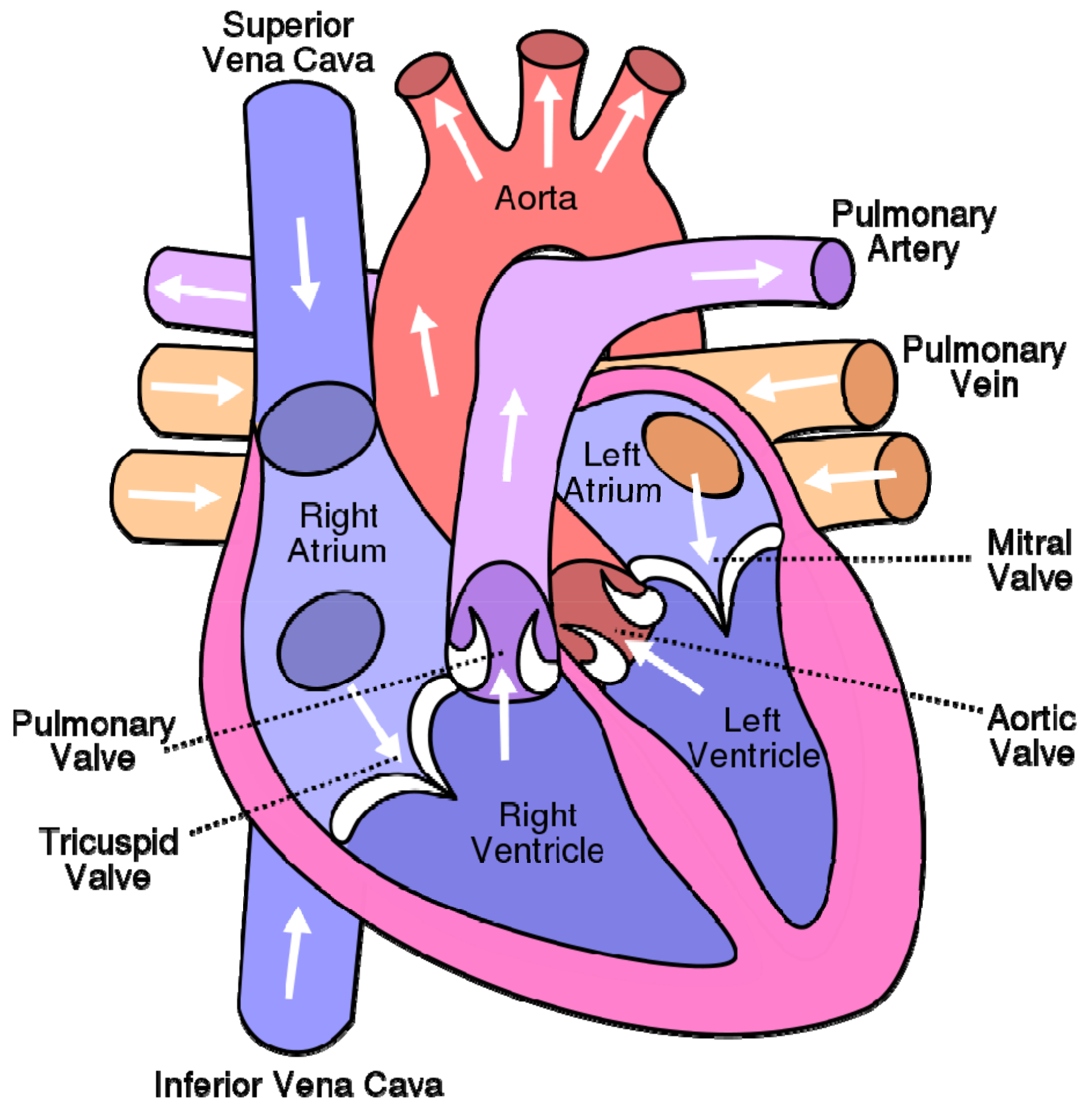


Figure 1: Cross section of the Heart

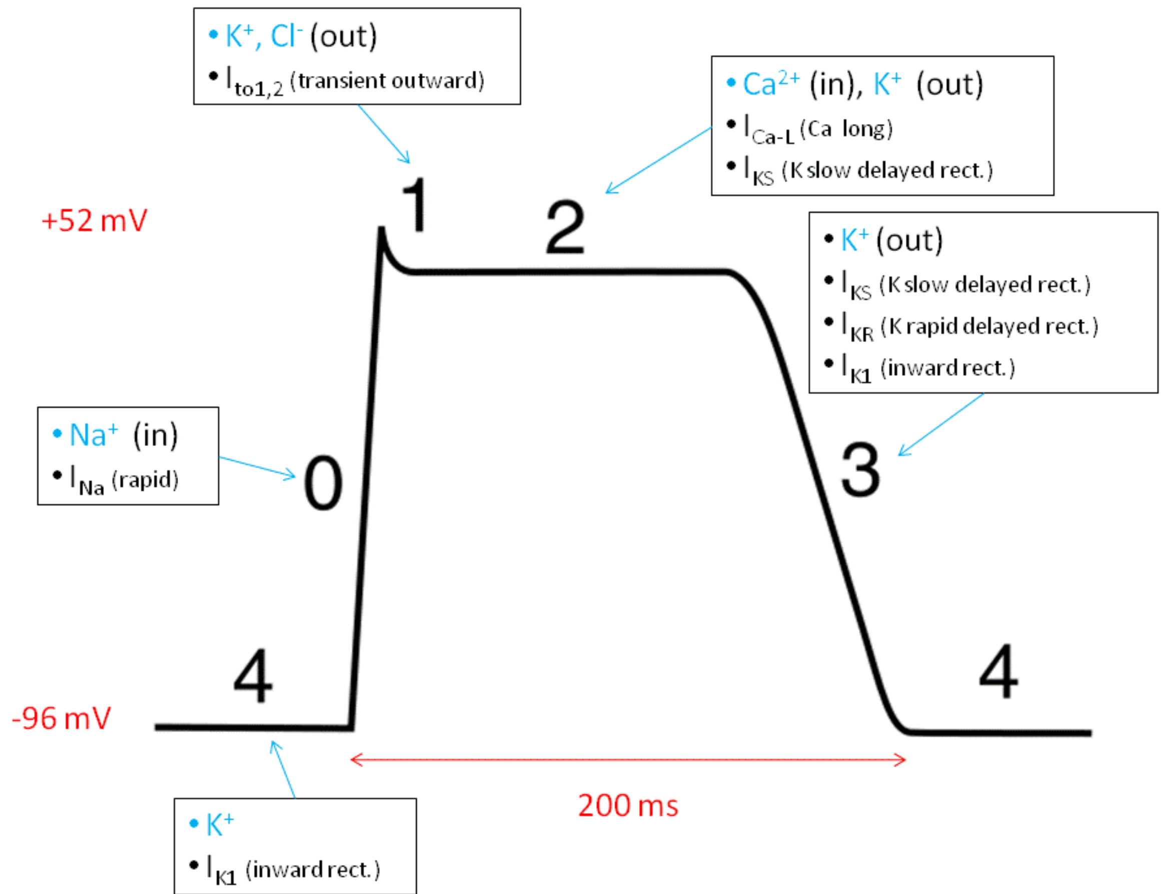
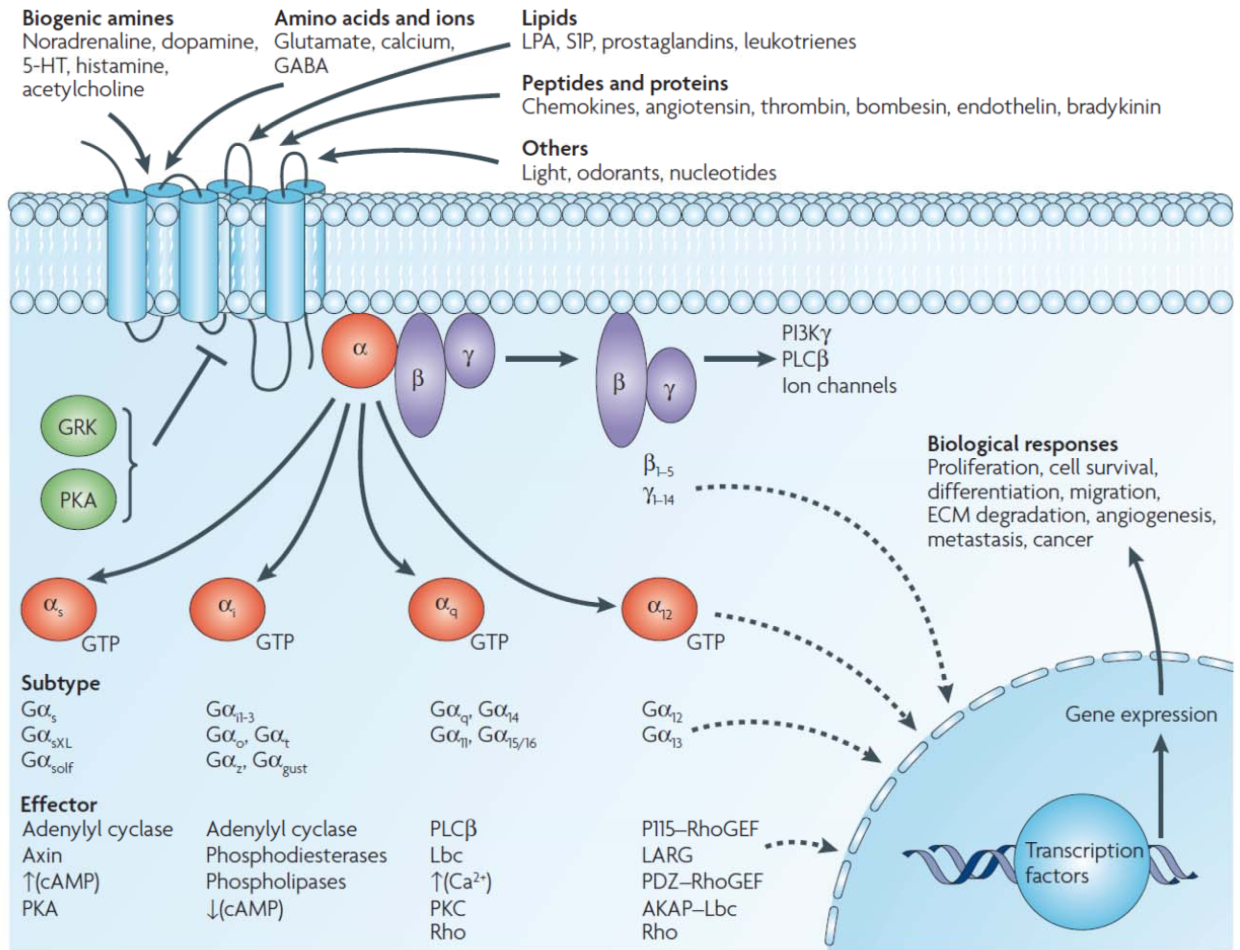


Figure 2: **Cardiac action potential.**



<u>Figure 3</u>: Diversity of G protein coupled receptor signalling.

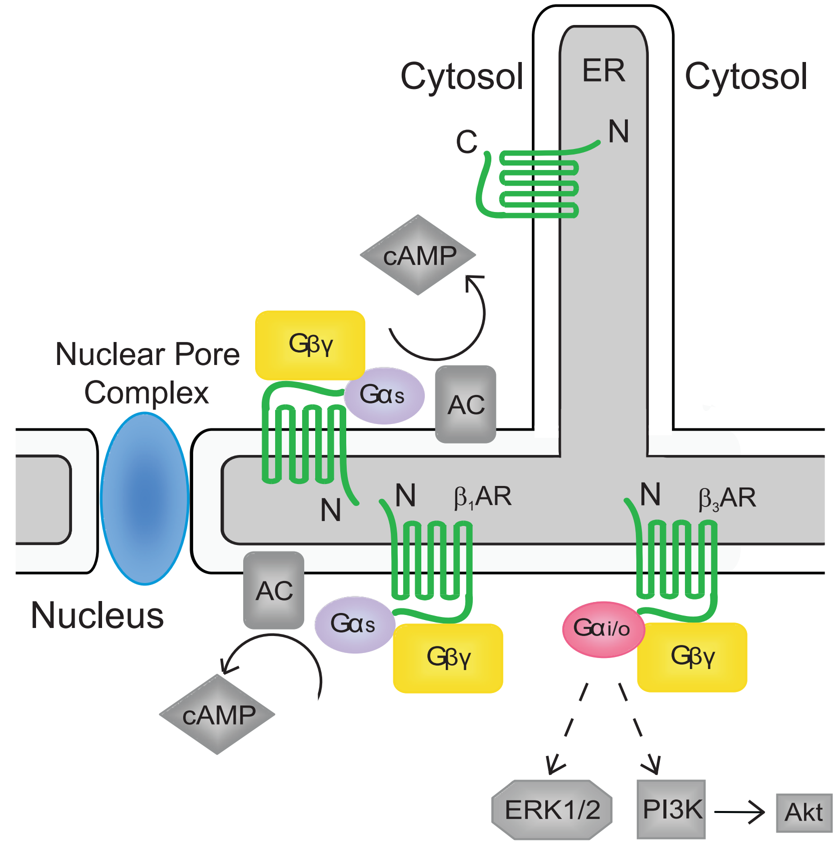


Figure 4: Possible orientation of nuclear βAR signalling complexes

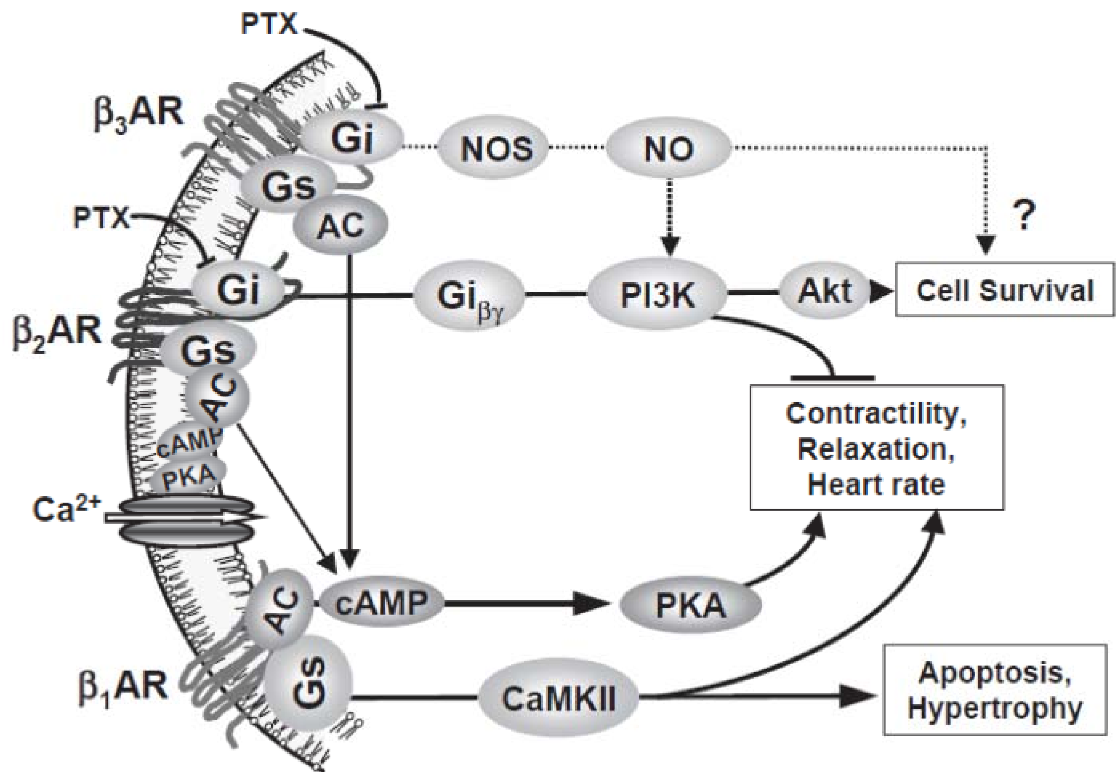


Figure 5: Subtype-specific signalling pathways of cardiac βARs.

Table 1. Nuclear GPCRs in various organ systems

GPCR subfamilies	Cell/Tissue	Species	Method of detection	Function	Reference
Family 1					
Muscarinic acetylcholine receptor (mAChR)	Cornea, corneal epithelial & endothelial cells	Rabbit	Autoradiographic ligand binding, ligand binding assay, functional assays	↑ DNA and RNA Pol II activity, ↑ nuclear cGMP	(Cavanagh & Colley, 1989) (Lind & Cavanagh, 1993, 1995)
Apelin receptor (APJ)	Cerebellum, hypothalamus, transfected cerebellum D283 Med cells	Human	IHC, GFP-CM, Western blot*	Unknown	(Lee *et al.*, 2004)
Alpha- adrenergic receptor (α-AR)	Neonatal ventricular cardiomyocytes, adult ventricular α_1ARKO cardiomyocytes	Rat, murine	Ligand binding assay, GFP-CM, subcellular ligand binding	α-AR; ERK activation, proliferation, survival	(Buu *et al.*, 1993) (Huang *et al.*, 2007)
Beta- adrenergic receptor (β-AR)	Adult & neonatal ventricular cardiomyocytes	Rat, murine	Ligand binding, Western blot,[+] ICC,[+] functional assays	↑ Nuclear cAMP, G protein coupling, transcription, PKB activation	(Buu *et al.*, 1993) (Boivin *et al.*, 2006) (Vaniotis *et al.*, 2011b)
Angiotensin receptor (ATR)	Vascular endothelial cells (ECs), VSMCs,adult ventricular cardiomyocytes, human adrenal tissue, neuronal cells, hepatocytes,HEK-293T cells, cerebellar cortex	Rat, hamster	Autoradiographic ligand binding, ligand binding assay, GFP-CM, ICC,[+] Western blot,[+] IHC,[+] functional assays	↑ Nuclear [Ca^{2+}], ERK & p38 activation, tyrosine phosphorylation, G protein coupling, transcription, proliferation	(Lee *et al.*, 2004) (Re *et al.*, 1984) (Robertson & Khairallah, 1971)(Haller *et al.*, 1999; Cook *et al.*, 2006) (Tadevosyan *et al.*, 2010)
Cysteinyl leukotriene receptor 1 ($CysLT_1$)	Colon tissue, colorectal carcinomas, Int 407 cells, CaCo-2 cells	Human	IHC,[+] ICC,[+] GFP-CM, Western blot,[+] functional assays	↑ Nuclear [Ca^{2+}], ERK activation, DNA replication, proliferation?	(Nielsen *et al.*, 2005)
C-X-C chemokine receptor type 4 (CXCR4)	Hepatoma, colorectal cancer HT-29 cells, adenocarcinoma, (NSCLS), HeLa	Human	IHC,[+] ICC,[+] flow cytometry	Tumorigenesis?	(Shibuta *et al.*, 2002) (Gobeil *et al.*, 2006a) (Spano *et al.*, 2004)

Prostaglandin receptor (EPR)	Brain, myometrium, liver, transfected HEK293 & Swiss 3T3, cerebralmicrovessel ECs	Murine, human, porcine, rat	Radioligand binding, ICC,+ IHC,+ Western blot,+ functional assays	↑ Nuclear [Ca^{2+}], ERK & PKB activation, ↑ nuclear cAMP & IP_3,↑ eNOS mRNA,G-protein coupling,transcription, pro-inflammatoryresponse?	(Bhattacharya *et al.*, 1998) (Bhattacharya *et al.*, 1999) (Gobeil *et al.*, 2002) (Provost *et al.*, 2010)
Endothelin receptor (ETR)	Liver, VSMCs, adult ventricular myocytes, heart, brain, endocardial ECs	Rat, human, canine, sheep	Ligand binding assay, ICC,+ Western blot,+ radioligand-binding-SDS-PAGE, functional assays	↑ Nuclear [Ca^{2+}], ↑ phosphorylation, transcription	(Hocher *et al.*, 1992) (Boivin *et al.*, 2006) (Jacques *et al.*, 2005) (Vaniotis *et al.*, 2011b)
Gonadotropin-releasing hormone Receptor (GnRHR)	Germline, intestine, HEK293HTR-8/SVneo	*C. elegans*, human, murine	IHC+	Acetylation & phosphorylation of Histone H3	(Vadakkadath Meethal *et al.*, 2006) (Re *et al.*, 2010)
Lysophosphatidic acid-1-receptor (LPA_1R)	Hepatocytes, cerebral microvessel ECs, transfected HTC4 cells	Rat, porcine	Western blot,+ ligand binding assay, IHC,ICC, functional assays	↑ Nuclear [Ca^{2+}], ERK & PKB activation, iNOS activation, transcription	(Gobeil *et al.*, 2003) (Gobeil *et al.*, 2006b) (Waters *et al.*, 2006)
Melanocortin type 2 receptor (MC2R)	Adrenal Y6 cells	Human	Functional assays	Unknown	(Doufexis *et al.*, 2007)
Melatonin receptor (MT2)	Placental choriocarcinoma cells	Human	ICC*	Unknown	(Lanoix *et al.*, 2006)
Natural killer receptor (NKR)	Dorsal root ganglia neural cells, HEK 293, Ventral tegmental area (VTA) neurons	Rat	ICC,+ IHC,+ electron microscopy*	Unknown	(Boer & Gontijo, 2006) (Lessard *et al.*, 2009)
Opioid peptide receptor (OPR 1/2)	NG 108-15 neurohybrid cells (OPR1), cardiomyopathic adult ventricular myocytes (OPR2)	Human, hamster	IHC,+ ICC,+ ligand binding assay, functional assays, transcription	G protein coupling, PKC activation	(Belcheva *et al.*, 1993) (Ventura *et al.*, 1998)
Platelet activating factor receptor (PAFR)	Liver, cerebral microvascular ECs, brain, transfected CHO, HEK293 & COS1cells, brain ECs	Rat, porcine	Flow cytometry, ICC,+ IHC,+ radio ligand binding assay, GFP-CM, Western blot,+ functional assays	↑ Nuclear DAG, ↑ nuclear [Ca^{2+}],↓ nuclear cAMP, ERK phosphorylation,G protein coupling, transcription, PLC activation. Pro-inflammatory response?	(Miguel *et al.*, 2001) (Marrache *et al.*, 2002) (Zhu *et al.*, 2006)

Prolactin receptor (PRLR)	Liver, splenocytes	Rat	Functional assays	Mitogenesis?	(Buckley *et al.*, 1988)
Vasoactive intestinal peptide receptor(VPAC)	HT29 cells, breast cancer cells	Human	Ligand binding assay, radioligand-binding-SDS-PAGE	cAMP production	(Omary & Kagnoff, 1987) (Valdehita *et al.*, 2010)
Neuropeptide Y1 receptor (Y1R)	Pituitary gland, pituitary cells, endocardial EC	Rat, human	ICC[+]	Unknown	(Chabot *et al.*, 1988) (Jacques *et al.*, 2003)
Family 2					
Parathyroid hormone 1 receptor (PTH_1R)	Kidney, liver, gut, uterus, ovary, MC3T3-E1, ROS17/2.8, UMR106 and SaOS-2 cells, osteoclast-like multinucleated cells, LLC-PK1 cells	Rat, murine, human, deer	IHC,[+] ICC,[+] Western blot,[+] far Western blot GFP-CM	DNA synthesis, mitosis	(Watson *et al.*, 2000a) (Watson *et al.*, 2000b) (Faucheux *et al.*, 2002) (Pickard *et al.*, 2006)
Family 3					
Metabotropic glutamate receptor (mGluR)	Neuronal cells, transfected HEK293cells, neonatal striatal neurons	Murine	IHC,[+] ICC,[+] Western blot,[+] functional assays	↑ Nuclear $[Ca^{2+}]$, transcription, CREB phosphorylation	(O'Malley *et al.*, 2003) (Jong *et al.*, 2005) (Jong *et al.*, 2007)

Abbreviations for detection methods: IHC, Immunohistochemistry; GFP-CM, green fluorescent protein-confocal microscopy; ICC, immunocytochemistry

Antibodies used for verification of nuclear localisation by Western blotting or immunochemistry were targeted to either [*]epitope tagged versions of the receptor, or (more commonly) [+]endogenous receptors. Taken from (112).

Table 2. Intracellular ligands involved in cardiac physiology

Intracrines	References
Angiogenin	(Moroianu & Riordan, 1994b)
Angiotensin II	(Re *et al.*, 1984; Erdmann *et al.*, 1996)
Angiotensin (1-7)	(Camargo de Andrade *et al.*, 2006)
Angiotensin converting Enzyme (ACE)	(Cristovam *et al.*, 2008)
Angiotensinogen	(Sherrod *et al.*, 2005)
Atrial natriuretic peptide (ANP)	(Morel *et al.*, 1988)
Brain-derived neurotrophic factor	(Marmigere *et al.*, 2001)
Dynorphin	(Ventura *et al.*, 1998)
Endothelial cell growth factor, platelet-derived (PD-ECGF)	(Fox *et al.*, 1995; Matsukawa *et al.*, 1996)
Endothelin Converting Enzyme	(Russell & Davenport, 1999)
Epidermal growth factor (EGF)	(Rakowicz-Szulczynska *et al.*, 1986; Wiley *et al.*, 1998)
Erythropoietin	(Mitjavila *et al.*, 1991)
Fibroblast growth factor (FGF; 1, 2, 3, 10)	(Bouche *et al.*, 1987)
Gonadotropin	(Millar *et al.*, 2004)
Growth hormone	(Ardail *et al.*, 2010)
Hepatoma-derived growth factor	(Kishima *et al.*, 2002)
Hepatopoietin	(Lu *et al.*, 2002)
Insulin	(Goldfine *et al.*, 1982)
Insulin-like growth factor binding Protein (IGFBP; 3, 5, 6)	(Schedlich *et al.*, 2000; Iosef *et al.*, 2008)
Interferon-γ (IFN-γ)	(Nagy *et al.*, 2000)
Interleukins	(Clevenger *et al.*, 1990; Curtis *et al.*, 1990)
Nerve growth factor (NGF)	(Rakowicz-Szulczynska *et al.*, 1986)
Neuropeptide Y	(Wirth *et al.*, 2005)
Oxytocin	(Kinsey *et al.*, 2007)
Parathyroid hormonerelated protein (PHTrP)	(Nguyen & Karaplis, 1998)
Phospholipase A2-I	(Fayard *et al.*, 1998)
Platelet-derived growth factor (PDGF)	(Rakowicz-Szulczynska *et al.*, 1986)
Renin/prorenin	(Lee-Kirsch *et al.*, 1999)
Thrombospondin-1	(Ashton *et al.*, 2004)
Thyroid-releasing hormone (TRH)	(Garcia *et al.*, 2000)
Transforming growth factor α (TGF-α)	(Grasl-Kraupp *et al.*, 2002)
Vascular endothelial growth factor (VEGF)	(Li & Keller, 2000)
WNT13	(Struewing *et al.*, 2006)

Taken from (117).

HYPOTHESIS & OBJECTIVES

Hypothesis

Recently, several GPCRS have been discovered at the level of the nuclear membrane, in a variety of different tissues and cell systems. The potential role of these nuclear-localized receptors still remains unclear. One such receptor subtype is the βARs, which we recently showed to be localized to the nucleus in adult ventricular cardiomyocytes. The guiding hypothesis of this study is that βARs do not act exclusively as cell surface receptors, but can also modulate intracellular signalling pathways when targeted to nuclear membranes in adult ventricular cardiomyocytes. To test this overarching hypothesis, I set out the following objectives.

1) Further characterize the distinct roles that nuclear βAR subtypes play in the nucleus of adult ventricular cardiomyocytes.
2) Determine the signalling pathways downstream of receptor activation in the nuclear membrane.
3) Determine the transcriptional targets regulated by nuclear βARs.
4) Confirm our findings obtained using freshly isolated cardiac nuclei within the context of the intact cell.

CHAPTER 2: MATERIALS AND METHODS

I) Materials

Anti-PKB, anti-phospho-PKB (threonine 308 and serine 473), anti-phospho-ERK p44/42 (threonine 202/tyrosine 204), anti-MEK1/2 and anti-phospho-MEK1/2, anti-p38 and anti-JNK2 antibodies were from Cell Signaling Technology. Anti-Raf1 and Lamin B antibodies were from Santa Cruz Biotechnology. Anti-NFκB antibody was from eBioscience. Horseradish peroxide (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence (ECL) reagent Renaissance Plus was from Perkin Elmer Life Sciences (Woodbridge, Ontario). Triton X-100 (TX-100), leupeptin, PMSF and DNase I were from Roche Applied Science (Laval, Quebec). SDS-polyacrylamide gel electrophoresis reagents and nitrocellulose (0.22μm) were from Bio-Rad Laboratories (Mississauga, Ontario). Isoproterenol, BRL 37344 and KT5823 were from Tocris Bioscience (Ellisville, MO). Endothelin-1 (ET-1) was from Peninsula Laboratories (Torrance, CA). Pertussis toxin (PTX), Tri Reagent, microcystin LR, CGP 20712A, xamoterol, forskolin, alprenolol, EEDQ, L-NAME, ICI 118,551 and α-amanitin were from Sigma (Mississauga, Ontario). PD98059, SB203580, SP600125, LY294002, wortmannin, triciribine, diaminofluorescein-2 (DAF-2), and diaminofluorescein-2 diacetate (DAF-2 DA) were from Calbiochem. U0126 was from Upstate Cell Signalling Solutions. RNaseOut, dNTP Mix, First Strand buffer and M-MLVRT were from Invitrogen. Primers, as well as SyBR Green and ROX were also from Invitrogen. Draq5 was from Enzo Life Sciences (Farmingdale, NY). RNA extraction kits were from Qiagen. RT^2 First strand kits, SABiosciences RT^2 qPCR Master Mix and rat NFκB signalling pathway RT^2 Profiler PCR arrays were from SABiosciences. Unless otherwise stated, all reagents were of analytical grade and were purchased from VWR Canlab (Ville Mont-Royal, Quebec) or Fisher Scientific (Mississauga, Ontario). [α^{32}P]UTP (specific activity 3000 Ci/mmol) and ^{125}I-CYP were from Perkin Elmer.

II) Methods

1) Nuclear isolation from rat heart

Two adult rat hearts were harvested and then pulverized under liquid nitrogen, resuspended in cold PBS (18 ml PBS, 0.5 mM DTT, 0.25 mM Na_3VO_4 and 0.25 mM PMSF) and homogenized (Polytron, 8000 rpm; 2 x 10 s, Fraction 1). All subsequent steps were carried out on ice or at 5°C. Homogenates were centrifuged for 15 min at 500 x g. The resulting supernatants, referred to as Fraction 2, were diluted 1:1 with buffer A (10 mM K-HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4), incubated 10 min on ice, and centrifuged for 15 min at 2000 x g. The resulting supernatant was discarded. The pellet, referred to as crude nuclei (Fraction 3) was resuspended in 5 ml buffer B (0.3 M K-HEPES pH 7.9, 1.5 M KCl, 0.03 M $MgCl_2$, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4), incubated on ice for 20 min, and centrifuged for 15 min at 2000 x g. The pellet, an enriched nuclear fraction (Fraction 4), was resuspended in buffer C (20 mM Na-HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4) or 1x transcription buffer (50 mM Tris pH 7.9, 0.15 M KCl, 1 mM $MnCl_2$, 6 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 1 U/μl RNase inhibitor) and either used fresh or aliquoted, snap-frozen using liquid nitrogen, and stored at -80°C.

2) Transcription initiation

10 μl of isolated nuclei (resuspended in 1 x transcription buffer) were incubated at 30°C for 30 min in the presence of 10 μl of the desired agonist/antagonist and 10 μCi [α^{32}P]UTP (3000 Ci/mmol). CTP and GTP were omitted to prevent chain elongation. Following termination of reactions by digestion with DNase I, nuclei were lysed with 30 μl of lysis buffer (10 mM Tris–HCl pH 8.0, 10 mM EDTA and 1% SDS). Duplicate 55 μl aliquots were transferred onto Whatman GF/C glass fiber filters, washed twice with 5% TCA containing 20 mM sodium pyrophosphate, and air-dried. ^{32}P incorporation was determined by liquid scintillation counting. DNA concentrations were determined spectrophotometrically and [α^{32}P]UTP incorporation expressed as cpm/ng DNA. Where

indicated, isolated nuclei were pre-treated with the appropriate pharmacological inhibitor.

3) Immunoblotting

SDS PAGE and immunoblotting were performed as previously described (56). Immunoreactive bands were quantified using a BioRad VersaDoc 4000 imaging system and Quantity One® software (Version 4.5.2 for Mac).

4) RNA isolation from isolated nuclei

Roughly 300 µl of isolated nuclei was resuspended in 1 ml of Tri Reagent, and then incubated for 5 min at room temperature (RT). 200 µl of chloroform was added, vortexed and incubated for 3 min at RT, followed by a centrifugation of 15 min at 14 000 rpm and 4°C. All subsequent steps were at RT. The upper aqueous layer was collected and an equal volume of 70% ethanol was added in order to precipitate the RNA. Added to Qiagen columns, then centrifuged 15 sec at 14 000 rpm. Remaining sample added to same column, recentrifuged for 15 sec at 14 000 rpm. Columns were washed twice with 350 µl RW1 buffer and centrifuged 15 sec at 14 000 rpm. The collection tubes was changed and then the columns were washed twice with 500 µl RPE buffer and centrifuged for 15 sec at 14 000 rpm. Columns were then centrifuged 1 min at 14 000 rpm to remove excess RPE buffer and the collection tubes were changed once again. To elute RNA, 25 µl of RNase-free water was added to the columns, and then incubated 1 min. Columns were then centrifuged 1 min at 14 000 rpm, and the eluate collected. The RNA content in the eluate was quantified by determining absorbance at 260 and 280 nm and only samples having an A260/A280 ratio greater than 1.8 were used in subsequent studies.

5) RNA isolation from isolated myocytes

Cardiac ventricular myocytes were isolated from adult male rats as described previously (142). Isolated myocytes were centrifuged for 5 min at 1 000 rpm and 4°C. The cell pellet was resuspended in 1 ml of Tri Reagent, homogenized by polytron for 20 sec, then incubated for 5 min at RT. 200 µl of chloroform was added, vortexed and

incubated for 3 min at RT, followed by a centrifugation of 15 min at 14 000 rpm and 4°C. All subsequent steps were at RT. The upper aqueous layer was collected and an equal volume of 70% ethanol was added in order to precipitate the RNA. RNA was then isolated as described above.

6) cDNA preparation

To prepare cDNA, 1 µg of RNA was mixed with 100 ng of random primers and 1 µl of 10 mM dNTP mix, diluted to a final volume of 12 µl, heated to 65°C for 5 min, and then immediately quick chilled on ice. Next, 4 µl of 5 x first strand buffer, 2 µl of 0.1 M DTT and 1 µl of RNase Out were added, and reactions were incubated for 2 min at 37°C. Following addition of 1 µl of M-MLV reverse transcriptase (200 units), reactions were mixed, centrifuged and then incubated for: 1) 10 min at 25°C, 2) 50 min at 37°C, and 3) 15 min at 70°C.

7) Quantitative real-time PCR

qPCR reactions contained 12.5 µl of SyBR Green PCR master mix, 0.03 µl ROX Dye (an internal fluorescence standard), 2.5 µl of primers (300 nM), and 10 µl of cDNA. The program entailed 1 cycle of 10 min at 95°C, then 40 cycles of (30 s at 95°C, followed by 1 min at 60°C and 1 min at 72°C), followed by 1 cycle of (1 min at 95°C, 30 s at 55°C and 30 s at 95°C) to obtain the dissociation curve, using a Stratagene Mx3000P system. Samples were assayed in triplicate and normalized to β-actin expression. Primers used for real-time qPCR are shown in Table 1. The specificity of each primer pair for the amplicon of interest was verified by dissociation curve analysis.

8) RT^2 Profiler PCR Arrays

RNA was isolated from purified nuclei using Qiagen RNA Extraction Kits. cDNA was prepared from 1 µg of RNA using the SABiosciences RT^2 First Strand Kit. cDNA (102 µl) was mixed with 1275 µl of 2 x SABiosciences RT^2 qPCR Master Mix plus 1173 µl of ddH_2O and 25 µl of the final mixture was transferred to each well of the RT^2 PCR array. Arrays were assayed for 1 cycle of 10 min at 95°C, then 40 cycles of (15 s at

95°C, followed by 1 min at 60°C) using a Stratagene Mx3000P system. All conditions were done in triplicate and normalized to the untreated control. Each 96-well plate also included primers for 5 housekeeping genes as well as positive and negative controls. Results were analyzed using web-based analysis software (SABiosciences). Changes were considered significant when $p < 0.05$.

9) Measurement of NO Production

Isolated nuclei were preincubated with the fluorescent dye DAF-2 (5 µg/ml) in a buffer containing 140 mM NaCl, 14 mM glucose, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.8 mM $MgSO_4$, 1.8 mM KH_2PO_4, and 0.1 mM L-arginine (final pH 7.4) for 30 min at 37 °C. Nuclei were washed twice in standard buffer to remove any unbound dye and then incubated in buffer containing the indicated inhibitor. Nuclei were then treated with the desired agonist or vehicle for 30 min at 37 °C. DAF fluorescence, indicative of NO production, was measured using a microplate reader at wavelengths of 485 nm (excitation) and 510 nm (emission).

10) Radioligand binding assays

Membranes were prepared from HEK 293 cells or isolated myocytes. Cells were washed twice with cold PBS and then, 10 ml of lysis buffer (5 mM Tris-HCl pH 7.4, 2 mM EDTA, with protease inhibitor cocktail; for HEK cells), (5 mM Tris-HCl pH 7.4, 5 mM EGTA, with protease inhibitor cocktail; for myocytes) was added to each flask. Cells were disrupted using a Polytron, 2x10 sec (HEK cells), or homogenized with 15 strokes (myocytes) on ice, and then centrifuged at 1000 rpm for 5 min at 4 °C. The lysate was then centrifuged at 16000 rpm for 20 min at 4 °C and the pellet resuspended in 1 ml of binding buffer (75 mM Tris-HCl pH 7.4, 2 mM EDTA, 12.5 mM $MgCl_2$). [^{125}I]CYP (50 µl, 400000 cpm) was added to 10 µl of membranes in a total volume of 0.5 ml, in triplicate, for each condition. Alprenolol (10 µl) was used to measure non-specific binding. Membranes were incubated at room temperature for 90 min and subsequently captured and washed using a Brandel cell harvester. [^{125}I]CYP binding was quantified using a β-counter.

11) EEDQ treatment

Calcium-tolerant cardiac ventricular myocytes were isolated from adult male Sprague-Dawley rats by langendorff perfusion as previously described (142). Freshly isolated myocytes were treated with either 100 μM EEDQ or vehicle (2 h, 37 °C) to irreversibly alkylate β-adrenergic receptors present at the cell surface, followed by treatment with either 1 μM isoproterenol or vehicle (30 min, 30 °C).

12) Measurement of NO production in live cells

Cardiac ventricular myocytes were isolated from adult male rats as described previously (142) and plated on laminin-coated glass-bottomed culture dish for 1 h at 37 °C (95% O_2, 5% CO_2) and 30 min at 4°C. ACVMs were then incubated with DAF-2 DA (10 μM) in 20 mM HEPES pH 7.4, 134 mM NaCl, 6 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1% BSA for 30 min at room temperature and in the absence or presence of a photolabile caged isoproterenol (ZCS-1-67, 30 μM) or a photolabile caged ET-1 analog ([Trp-ODMNB^{21}]ET-1, 1.5 μM) (122) and either the NO synthase inhibitor L-NAME (1 mM) or vehicle, as indicated. ACVMs were then washed three times and a cell-permeable fluorescent DNA dye, DRAQ5 (1 μM), was added and L-NAME was readded where indicated. Fluorescence imaging was accomplished using a Zeiss LSM 7 Duo microscope (combined LSM 710 and Zeiss Live systems) with a 63x/1.4 oil Plan-Apochromat objective. DAF-2 DA was excited with a 488 nm/100 mW diode (2-3% laser intensity) and fluorescence emitted between 495 nm and 550 nm was collected. Cells were scanned approximately every 10 s. Pixel size was set at 0.264 μm and the pinhole at 2 Airy units. To visualize the nucleus, DRAQ5 was excited with a 635 nm/50 mW diode and fluorescence emitted at >655 nm was collected. DAF-2 DA and DRAQ5 were excited and fluorescence collected simultaneously using 2 different Zeiss Live detectors. Images were acquired over a total period of 9 min (60 frames). After acquiring 13 frames (114 s) to establish a baseline, cET-1 or ZCS-1-67 was photolysed by administering a 4 s pulse of UV light using a 405 nm/30 mW diode (100% laser intensity). DRAQ5 emissions were used to focus the UV laser into a 60 μm^2 rectangular region overlapping the nucleus. The microscope stage (Zeiss Observer Z1) was equipped with a BC 405/561 dichroic mirror that allowed simultaneous photolysis of

ZCS-1-67 or caged ET-1 (LSM 710 405 nm laser) and image acquisition (Zeiss Live).

13) EPAC assays for cAMP detection

HEK 293 cells were plated onto 6-well plates at least 24 h prior to transfection (143). The cells were transfected with 3 μg of EPAC construct and 1 μg of FLAG-tagged β_2AR using 5 μl of Lipofectamine 2000. 72 h post transfection, the cells were washed twice with 1X PBS and resuspended in 500 μl of 1X PBS. Cell suspensions (80 μl) were distributed in 96-well Opti-plates and left to incubate for 2 h at room temperature. Fluorescence was then measured using a Synergy2 (Biotek) microplate reader. Immediately after reading the fluorescence, the cells were incubated with 10X coelenterazine h (final well concentration 50 μM) and total luminescence and BRET ratios were collected for 5 min. The average of these BRET ratios represents basal BRET of the cells. The cells were then treated with 10 μl of 10X isoproterenol prepared in 100 μM of ascorbic acid solution (final well concentration of isoproterenol 10 μM) or with 10 μl of 100 μM ascorbic acid (vehicle) and BRET ratios were read for 30 min. Upon completion of the assay, the final five BRET readings were averaged and taken to represent the final average BRET. The net BRET for agonist or vehicle treatment was calculated by subtracting basal BRET from the final average BRET. The ΔBRET for each transfection was then calculated by subtracting the net BRET of agonist from respective net BRET of vehicle.

14) Miscellaneous methods

Protein concentrations were determined by the method of Bradford using bovine γ-globulin standard.

CHAPTER 3

Boivin B, Lavoie C, Vaniotis G, Baragli A, Villeneuve LR, Ethier N, Trieu P, Allen BG, and Hébert TE. "Functional β-adrenergic receptor signalling on nuclear membranes in adult rat and mouse ventricular cardiomyocytes." Cardiovasc Res. 2006 Jul 1;71(1):69-78.

Contribution: Figure 7

Functional β-adrenergic receptor signalling on nuclear membranes in adult rat and mouse ventricular cardiomyocytes.

Benoit Boivin[1,2], Catherine Lavoie[1], George Vaniotis[1,2], Alessandra Baragli[3], Louis-Robert Villeneuve[1], Nathalie Ethier[3], Phan Trieu[3], Bruce G. Allen[1,2,4], and Terence E. Hébert[2,3†]

1) Centre de recherche, Institut de Cardiologie de Montréal
2) Département de biochimie et Groupe de recherche universitaire sur les medicaments, Université de Montréal
3) Department of Pharmacology and Therapeutics, McGill University
4) Département de médecine, Université de Montréal

Word count : 6,259

† Address correspondence to:

Terence E. Hébert

Department of Pharmacology and Therapeutics, Faculty of Medicine, 13th floor, Room 1303, McIntyre Medical Sciences Building, 3655 Promenade Sir William Osler, Montreal, QC, H3G 1Y6, Canada

Running title: Subcellular distribution of βAR signalling machinery.

Abstract

Objective: We sought to determine if different β-adrenergic receptor (βAR) subtypes, and their associated signalling machinery, are functionally localized to nuclear membranes.

Methods: Employing enriched nuclear preparations, we assayed the specific presence of βAR by measuring ^{125}I-cyanopindolol (CYP) binding, Western blotting, confocal microscopy and functional assays.

Results: Western blots of rat heart nuclear fractions and confocal immunofluorescent analysis of adult rat and mouse ventricular cardiomyocytes displayed the presence of β_1AR and β_3AR but, surprisingly, not the β_2AR on nuclear membranes. Nuclear localization of downstream signalling partners Gs, Gi and adenylyl cyclases II and V/VI was also demonstrated. The functional relevance of nuclear βAR was shown by receptor-mediated stimulation of adenylyl cyclase activity by isoproterenol but not the β_3AR-selective agonist CL 316243 in enriched nuclear preparations. We also examined the effect of subtype-selective ligands on the initiation of RNA synthesis in isolated nuclei. Both isoproterenol and another β_3AR-selective agonist, BRL 37344, increased RNA synthesis which was inhibited by pertussis toxin (PTX). Neither a β_1AR-selective agonist, xamoterol, or a β_2AR-selective agonist, procaterol, were able to stimulate transcription. However, both CGP 20712A and ICI 118,551 blocked isoproterenol-mediated effects to varying extents. PTX treatment also revealed that nuclear βAR may be coupled to other signalling pathways in addition to Gi, as stimulation under these conditions reduced initiation of transcription below basal levels.

Conclusion: These results highlight differential subcellular localization for βAR subtypes and indicate that βAR may have specific roles in regulating nuclear function in cardiomyocytes.

Introduction

An increasing number of GPCRs are targetted to the nuclear membrane, including lysophosphatidic acid receptors (1), metabotropic glutamate receptors (MGluR5, (2)), apelin receptors (3), platelet activating factor (PAF) receptors (4), bradykinin B2 receptors (3), angiotensin 2 type I receptors (3,5-7), prostaglandin receptors (8) and endothelin receptors (9). In addition, a large number of proteins, classically associated with receptor-mediated events at the cell surface, including heterotrimeric G proteins ((8,10,11); reviewed in (12)), adenylyl cyclase isoforms (13,14), phospholipase A_2 (15), phospholipase Cβ (16), phospholipase D (17), RGS proteins (reviewed in (18)), β-arrestin1 (19,20), G protein-coupled receptor kinases (21-23), A kinase anchoring proteins (AKAPs) and PKA (24), among others, have been demonstrated to be trafficked to the nucleus and/or nuclear membrane.

In mammalian cardiomyocytes, three βAR subtypes are found which differ in their signalling partners, subcellular localization and desensitization profiles. β_1ARs, localized to the sarcolemma and t-tubular network, plays a predominant role in regulating myocyte contractility. β_2ARs, similarly distributed, play a more modest role in regulating inotropic and lusitropic responses. Both receptors signal through Gs and adenylyl cyclase with similar efficacy although the signals are compartmentalized differently possibly due to localization in distinct membrane microdomains and/or dual coupling of the β_2AR to Gs and Gi. (reviewed in (25-27)). β_3AR are also found in cardiomyocytes although their function remains ill-defined. Hence, βAR subtypes may serve unique functions and play non-redundant roles within the cardiomyocyte.

In the process of studying receptor trafficking and heterodimerization in mouse ventricular cardiomyocytes using recombinant adenoviruses, we noted a perinuclear distribution of overexpressed β_1AR and β_2AR (28). We therefore wished to study receptor distribution in native myocytes and assess what function putative nuclear βAR might have.

Experimental Procedures

Materials

Polyclonal rabbit anti-β_1AR, β_2AR, Gαs, Gαi1-3, adenylyl cyclases II, V/VI and polyclonal goat anti-β_3AR were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-nucleoporin p62 (Nup-62) monoclonal antibodies (1:200) were from BD Transduction Laboratories. Appropriate TRITC-, CY5-, and HRP-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Chemiluminescence reagent Renaissance Plus was from Perkin-Elmer Life Sciences (Woodbridge, Ontario). Triton X-100, leupeptin, Protector RNAse inhibitor and PMSF were from Roche (Laval, Quebec). SDS-polyacrylamide gel electrophoresis reagents, nitrocellulose (0.22 μm) were from Bio-Rad (Mississauga, Ontario). Essential fatty acid-free BSA, CL 316243, PTX, isoproterenol, CGP 20712A and ICI 118,551 were from Sigma (Mississauga, Ontario). Xamoterol, procaterol and BRL 37344 were from Tocris (Ellisville, MO). Unless otherwise stated, all reagents were of analytical grade and were purchased from VWR Canlab (Ville Mont-Royal, Quebec) or Fisher Scientific (Mississauga, Ontario). [α^{32}P]-UTP (specific activity 3000 Ci/mmol), [^{125}I]-cyanopindolol (CYP) and [α^{32}P]-ATP were from Perkin-Elmer.

Isolation of Ventricular Myocytes

Calcium-tolerant cardiomyocytes were isolated from adult rats (29) or mice (30) as described. These preparations provided 8-10 million cells/heart with 70 to 85% viability, as assessed by the presence of quiescent cells with rod-shaped morphology. The experimental protocol conformed 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).

Isolation of nuclei

Rat cardiac nuclei were isolated as described with modifications (31). Briefly, rat hearts were pulverized under liquid nitrogen, resuspended in cold PBS and homogenized (Polytron, 8000 rpm; 2 x 10 s, Fr. 1). All subsequent steps were carried out on ice or at 5°C. Homogenates were centrifuged for 15 min at 500*g* and supernatants, referred to as Fr. 2, diluted 1:1 with buffer A (10 mM K-HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4), incubated 10 min on ice, and centrifuged for 15 min at 2000*g*. The resulting supernatant was discarded. The pellet, referred to as crude nuclei (Fr. 3) was resuspended in buffer B (0.3 M K-HEPES pH 7.9, 1.5 M KCl, 0.03 M $MgCl_2$, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4), incubated on ice for 10 min, and centrifuged for 15 min at 2000*g*. The pellet, an enriched nuclear fraction (Fr. 4), was resuspended in buffer C (20 mM Na-HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4) or 1X transcription buffer (see below) and either used fresh or aliquoted, snap-frozen using liquid nitrogen, and stored at -80°C.

Receptor quantification

Nuclei were prepared and washed as described above. Total βAR number was calculated using a saturating concentration (300 pM) of [^{125}I]-cyanopindolol (CYP; Perkin Elmer) as the radioligand as described (32).

Measurement of adenylyl cyclase activity

Adenylyl cyclase activity was assayed in nuclear preparations as described (33) using 100 μg protein in a total volume of 50 μl. Enzyme activities were determined following a 15 min incubation in the presence of various receptor ligands, 100 μM forskolin, 10 mM NaF or vehicle at 37°C. Data were calculated as pmol cAMP/min/mg protein, and analyzed by least squares regression using Prism 4.0cx (GraphPad Software).

Transcription initiation

Measurements of transcription initiation were as described (34). Briefly, 10 μl of isolated nuclei were incubated at 30°C for 30 min in 20 μl of 1X transcription buffer (50 mM Tris pH 7.9, 0.15 M KCl, 1 mM $MnCl_2$, 6 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 1U/μl RNAse inhibitor) and 10 μCi [α^{32}P]-UTP (3000 Ci/mmol) in the absence of CTP and GTP to prevent chain elongation. After termination of reactions by digestion with DNase I, nuclei were lysed with 10 mM Tris-HCl pH 8.0, 10 mM EDTA and 1% SDS. Duplicate 5 μl aliquots were dried onto Whatman GF/C glass fibre filters which were batch washed with 5% TCA, containing 20 mM sodium pyrophosphate and dried. ^{32}P incorporation was determined by scintillation counting. DNA concentration was determined by spectrophotometry and incorporation expressed as dpm/μg DNA. Ligands were used as for activation of adenylyl cyclase. Where indicated, nuclei were pretreated with PTX (5 μg/ml) for 2 hours.

Western blotting

Immunoblots were performed on the subcellular fractions described above. Protein concentrations were measured as described (35) using bovine γ-globulin as a standard (9). We first demonstrated using HEK 293 cells, transfected or not with the three βAR subtypes that individual antibodies recognized cognate receptors specifically in both Western and immunocytochemical applications (data not shown). These antibodies have been used in other studies on rat ventricular cardiomyocytes under similar conditions (36,37). Similarly, we performed control experiments for our other antibodies.

Confocal Microscopy

The intracellular localization of βAR subtypes and downstream signalling partners was studied using scanning confocal microscopy (LSM 510 Carl Zeiss). Freshly isolated adult ventricular myocytes were plated on laminin-coated coverslips for 1 h (37 °C, 95% O2/5% CO2), fixed for 20 min in PBS (pH 7.4) containing 2% (w/v) paraformaldehyde, then washed thrice in PBS and blocked 1 h at room temperature in PBS containing 5% normal donkey serum (NDS) plus 0.2% (v/v) TX-100. Coverslips were rinsed thrice in PBS

and incubated 1 hr at 4°C in PBS containing 10% NDS. Excess serum was removed and cells were incubated overnight at 4°C with primary antibody diluted 1:100 in PBS containing 2% NDS. Coverslips were rinsed gently three times with PBS, drained, and incubated for 1 hr at room temperature with a 1:500 (v/v) dilution of appropriate secondary antibodies (TRITC-conjugated anti-rabbit antibody, or ALEXA 488-conjugated anti-sheep antibody) in PBS containing 2% NDS. Coverslips were washed three times with PBS, drained, and mounted onto glass slides. For co-localization experiments, protocols were modified slightly. Freshly isolated mouse cardiomyocytes were plated on laminin-coated coverslips for 1 hr (37°C, 95% O_2/5% CO_2) and fixed for 20 min in 2% paraformaldehyde. Coverslips were rinsed three times in PBS and incubated 1 hr at 4°C in PBS containing 2% normal goat serum (NGS) and Triton 0.2% (v/v) when anti-β_1AR (1:100) and β_2AR (1:100) were used and 2% normal donkey serum (NDS) and Triton 0.2% (v/v) when β_3AR (1:100) was used. ALEXA 555-conjugated anti-mouse antibody (1:500 in NGS) was used when murine cardiomyocytes were stained for Nup62. Fluorescence was visualized as serial 0.5 μm-thick optical sections in the z-axis plane of each cell. For each secondary antibody, control experiments were performed in the absence of primary antibody.

Statistical Analysis

Data are presented as the mean ± the standard error of the mean (S.E.). The significance of differences between groups was estimated by one-way ANOVA followed by Tukey's multiple comparison test (Prism 4.0cx, GraphPad Software). Differences were considered significant when $p < 0.05$.

Results

Localization of β-adrenergic signalling machinery on nuclear membranes.

To determine whether βAR subtypes were trafficked to the nuclear membrane, we used a standard protocol for enrichment of nuclei. As shown in Figure 1, binding of the βAR-specific ligand [^{125}I]-CYP was detected in both crude (Fr. 3) and enriched (Fr. 4) nuclear preparations. The efficiency of fractionation is well characterized (9). Fr. 3 and Fr. 4 are significantly enriched for the nuclear membrane marker, Nup62 (38) and have clearly lost both annexin II (a cell surface marker (39)), BiP (an ER chaperone (40)) and GM 130 (a Golgi resident protein (41)). The nuclear fraction is never totally pure but in going from Fraction 3 to Fraction 4, there is a significant enrichment for Nup62 (which was undetectable in Fractions 1 and 2) with concomitant depletion of markers for the cell surface (annexin II is reduced significantly in Fraction 4), ER (BiP is almost undetectable in Fraction 4), and Golgi (GM 130 is reduced significantly in Fraction 4). Initial experiments to determine the exact subtypes present in enriched nuclear fractions were performed using competition experiments with various subtype-selective ligands. These experiments could not clearly distinguish between the different subtypes in isolated nuclei. However, as described below, we used subtype-selective agonists to elicit specific responses in isolated nuclei.

Similar results to those obtained in ligand binding studies were obtained using Western blots on the different crude and enriched nuclear fractions (Figure 2). However, whereas all three subtypes could be detected in crude lysates (Fr. 1), only the β_1AR and β_3AR were detected in the enriched nuclear fraction (Fr. 4). A lower molecular weight protein (around 45 kDa) was revealed as a faint band by β_2AR antiserum in Frs.1-3; however this band was unrelated to the β_2AR.

Next, confocal microscopy was used in order to further investigate the subcellular localization of βAR subtypes. Confocal image stacks were deconvolved to reduce the amount of out-of-focus haze from secondary antibodies (11). Immunofluorescence for all three receptors was detected as punctate signals localized on the cell surface and in a

striated pattern within myocytes (Figure 3A, C, E). Although the t-tubular system is formed of continuous invaginations of the plasma membrane, β_1AR and β_2AR immunofluorescence was more intense on t-tubular membranes than surface membranes. Most interestingly, cell images of β_1AR- or β_3AR-decorated myocytes revealed an intense intracellular signal on the periphery of the nucleus (Figure 3A) and in structures that appeared to extend outwards from its apex (Figure 3E), indicating that they were localized in nuclear or perinuclear membranes. In contrast, no β_2AR staining was detected on the nuclear membranes (Arrows, Figure 3A, C, E). No staining was observed in the absence of the primary antibody (Figure 3B, D, F). Co-localization studies were also performed using mouse ventricular cardiomyocytes where relevant secondary antibodies were available and these confirmed our results using isolated rat cardiomyocytes. Nup-62 colocalized with β_1AR and β_3AR but not with β_2AR (Figure 4). Colocalization of all three subtypes was detected with caveolin-3 but not with the Golgi marker GM 130 (data not shown) confirming our results using subcellular fractionation.

Functional effects of nuclear β-adrenergic receptors.

To assess potential function of the nuclear βAR receptors, immunofluorescence was employed to localize downstream effector molecules. Known downstream effectors, $G\alpha_i$- and $G\alpha_s$- subunits as well as adenylyl cyclases (AC) II and V/VI, were examined. $G\alpha_i$ immunofluorescence was apparent on t-tubules and surface membranes, including the intercalated disc region (Figure 5A). In addition, punctate $G\alpha_i$ immunofluorescence was observed on the nuclear periphery and in intranuclear structures (arrow, Figure 5A). Images from anti-$G\alpha_s$-labeled cardiomyocytes revealed punctate immunofluorescence on plasma membranes, t-tubules and nuclear membranes, where staining was most intense (arrow, Figure 5C). Thus, both G proteins known to couple to βAR subtypes in adult ventricular myocytes (25) were present on nuclear membranes and intranuclear structures. AC II antibodies decorated the cell surface, showed a striated intracellular pattern and labeled nuclei (Figure 5E). AC II localization to t-tubules was mainly peripheral. AC V/VI immunofluorescence was observed at the surface membranes, t-tubules and was most

intense in the intercalated disc regions and nuclei (Figure 5G). Hence, the relevant βAR signalling machinery is present on nuclear membranes in cardiomyocytes.

To more directly explore the functional consequences of nuclear localization of βAR, we first measured the ability of a non-selective agonist, isoproterenol, to stimulate adenylyl cyclase activity in isolated nuclei from rat hearts. We recognize that only 30-40% of the nuclei in the heart come from cardiomyocytes (42). However, the mechanical procedure we used for nuclear isolation is much more efficient on whole tissues than isolated cells. As can be seen in Figure 6A, there was a dose-dependent stimulation of adenylyl cyclase activity in enriched nuclear preparations demonstrating that the entire signalling system from receptor to Gs to effector is indeed functional. In the absence of ligand, basal enzyme activity was detected suggesting that the system may also be functional constitutively, i.e. even in the absence of the endogenous ligand (Figure 6B). Direct activation of G protein with NaF or adenylyl cyclase with forskolin also led to an increase in the production of cAMP in isolated nuclei confirming the functional presence of the signalling system downstream of the receptor (Figure 6B). Interestingly, a β_3AR-selective agonist CL 316243 had no effect on adenylyl cyclase activity, suggesting that the β_1AR is primarily responsible for activation of this effector in nuclei.

To determine if there were consequences downstream of βAR activation particular to nuclear function, purified nuclei were incubated with [α^{32}P]-UTP to measure initiation of RNA transcription in response to both non-selective and subtype-selective ligands. In addition to isoproterenol, subtype-selective ligands, xamoterol (β_1AR), procaterol (β_2AR) and BRL 37344 (β_3AR) were used to stimulate transcription. Isoproterenol and BRL 37344, but not xamoterol (Figure 7a) or procaterol (data not shown), could stimulate initiation of transcription. Since both Gs and Gi were detected on cardiomyocyte nuclear membrane, we next sought to determine the G protein coupling of each receptor. Nuclei were pretreated with PTX for 2 hours prior to agonist stimulation in the presence of [α^{32}P]-UTP. For both isoproterenol- and BRL 37344-stimulated nuclei, treatment with PTX abolished initiation of transcription demonstrating that the nuclear β_3AR coupling to Gi is responsible for this

activity. Further, basal activity was not affected by PTX treatment although the responses to both ligands became inhibitory. It is noteworthy to mention that stimulation of nuclear endothelin receptors induces a similar decrease in basal transcriptional activity (data not shown). This indicates that under basal conditions, a balance exists between Gi-mediated stimulatory signals and inhibitory signals mediated by other G proteins. This notion is also supported by the observation that forskolin, a direct activator of adenylyl cyclase, tended toward an inhibitory effect (7525 +/- 2394 pmol cAMP/mg/min, basal versus 5637.2 +/- 1600 pmol cAMP/mg/min, 100 μM forskolin, n=6).

To further define the subtype selectivity for the transcriptional response and to overcome difficulties with the use of weak partial agonists such as xamoterol, we used either CGP 20712A or ICI 118,551 to block β_1AR and β_2AR, respectively. Curiously, we noted that isoproterenol-stimulated transcription initiation was inhibited in both cases (Figure 7b). There was residual stimulation in the case of CGP 20712A. However, when we used these compounds, in the absence of agonist, CGP 20712A had no effect, consistent with its role as an antagonist. Surprisingly, ICI 118,551 had a strong inhibitory effect on the basal level of transcription initiation by itself. Further, we have shown that ICI 118,551 can compete for CYP binding (data not shown). These data suggest the possibility that levels of β_2AR undetectable by immunological methods may be found in the nuclei as well.

Taken together, our data suggest that two effector systems are stimulated by βAR in isolated rat heart nuclei, one coupled to Gs which leads to an activation of adenylyl cyclase and another acting through Gi which leads to an increase in RNA transcription initiation.

Discussion

Our results demonstrate functional βAR receptors on the nuclear membrane. By immunological, ligand-binding and functional criteria, these seem to be predominantly β_1AR and β_3AR. However, it is also possible that the β_2AR may be there but undetectable using available antibodies. These receptors were inhibited by ICI 118,551 even in the absence of agonist, i.e. ICI 118,551 is acting as an inverse agonist reducing constitutive receptor activity. CGP 20712 blocked the effects of isoproterenol as well. It has been demonstrated that CGP 20712 can antagonize β_3AR (43) and this may explain the effects we have seen here. We have so far considered the effects of stimulation on nuclear βAR as if the receptors were monomeric or homodimeric entities. Alternatively, we (44) and others (45,46) have demonstrated that receptor heterodimers may comprise unique receptors with altered pharmacological properties, i.e. subtype-selective ligands may not be informative. Our results may thus be complicated by the possibility that the β_1AR and β_3AR may form heterodimers. Both β_1AR and β_2AR (47,48) and β_2AR and β_3AR (49) form heterodimeric receptors and it remains to be seen (although likely) if β_1AR and β_3AR do as well. Other potentially confounding and cell- or even organelle-specific factors might affect the pharmacology, signalling and/or trafficking of homodimeric or heterodimeric GPCRs to different subcellular destinations. As βAR signalling changes during development (50,51), and during the progression to heart failure (52), it is possible that trafficking itineraries on an absolute or relative basis may be altered as well.

β_1AR and β_3AR also seem to serve different functions in the nuclear membrane, as they are coupled to distinct G protein partners. β_3AR stimulation, coupled to a PTX-sensitive G protein, likely Gi, which colocalizes with the receptor to the nuclear membrane, can initiate *de novo* transcription in isolated nuclei. The β_3AR-mediated transcriptional activity is independent of adenylyl cyclase as stimulation with forskolin does not mimic these results. Our studies, being performed in native tissues, confirm that these results are not simply artifacts due to overexpression in heterologous expression systems and suggest a novel role for the β_3AR in cardiac function. Also, we verified the existence of these receptors on the

nucleus using multiple techniques, not only relying on antibodies but also demonstrating the presence of receptors and functional signalling pathways using ligand binding and by measuring two distinct agonist-mediated events. Further, as has been demonstrated for a number of other GPCRs, not only are the receptors themselves trafficked to the nuclear membrane but in many cases entire signalling pathways are also present.

A recent study that deorphanized GPR30 has demonstrated that this receptor is retained in the ER where it serves as a receptor for estrogen (53). For other GPCRs such as the PAF or prostaglandin receptors, which are also activated by hydrophobic ligands which can cross the plasma membrane, it is quite possible to envision particular roles for these receptors in intracellular compartments. For receptors with peptidic ligands such as those for angiotensin, bradykinin or endothelin, it is intrinsically easier to imagine how these intracrine signalling pathways might be activated as well. This is because the ligands themselves may be released into intracellular compartments as part of their own independent trafficking itineraries following synthesis. In some cases, as for EGF receptors, a fragment of a cell surface receptor is trafficked to the nucleus after a proteolytic event (54,55). This is unlikely to be the case here as Western blots of enriched nuclei indicate molecular weights for the β_1AR and β_3AR consistent with published values for full-length receptors (36,37). Furthermore, no reduction in molecular weight was observed for the β_1AR and β_3AR in the nuclear fraction (Fr. 4) compared to total cell extract (Fr. 1). For adrenergic and other small ligand-activated receptors to have intracellular effects, one could consider an uptake mechanism. The classic reuptake system seen in neurons is unlikely to operate in cardiomyocytes. However, there are other non-selective uptake systems such as EMT (extraneuronal monoamine transporter, or OCT3) found in several tissues including heart ((56,57), reviewed in (58)) which could transport catecholamines to intracellular targets. One study using neonatal rat cardiomyocytes determined that extracellular [^{3}H]-norepinephrine is taken up into these cells and >60% is distributed to nuclear fractions (59). Further, these authors also demonstrate that nuclei isolated from these cells (purity confirmed by electron microscopy) possess approximately 60 fmol/mg βAR (roughly comparable to our values of 15-20 fmol/mg) as determined by ligand binding. It has also become clear in recent years that many GPCRs including βAR have constitutive, agonist-

independent activity (60,61). Depending on local expression levels in a given subcellular fraction, this constitutive activity could be quite important.

A growing number of GPCRs are trafficked to the nuclear or other endomembranes where they can activate signalling pathways that may be intrinsically different than those activated at the plasma membrane. The actual subcellular location of a given receptor may be critical for determining which signalling pathways get activated. Thus decisions about trafficking and formation of signalling complexes with distinct compositions may represent key events in determining the specificity of cellular communication.

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Figure Legends

Figure 1. *^{125}I-CYP binding in enriched nuclear fractions.* Specific βAR binding (total minus non-specific as measured using 10 μM alprenolol) in various fractions associated with the nuclear enrichment procedure. Fractions are as described in Materials and Methods. Data represent mean ± S.E. of at least three separate experiments performed in triplicate.

Figure 2. *Detection of βAR isoforms in crude and enriched nuclei using Western blotting.* Cell fractions were prepared as in Figure 1. Lanes contained 100 μg protein from Frs. 1-4. Blots were probed using anti-β_1AR (A), anti-β_2AR (B) or anti-β_3AR (C). Molecular weight markers are indicated on the left of the figure. Arrows indicate specific antibody signals previously described in the literature (see text for details). Data are representative of three independent experiments.

Figure 3. *Detection of βAR isoforms in isolated adult rat ventricular cardiomyocytes using immunocytochemistry.* Panels A, C and E are representative confocal images showing the distribution of β_1AR (A), β_2AR (C) or β_3AR (E), respectively. Controls were performed in the absence of primary antibody (B, D and F). Scale bar is 10 μm. Arrows indicate nuclei. Data are representative of three independent experiments.

Figure 4. *Colocalization of βAR isoforms in adult mouse ventricular cardiomyocytes with a marker of the nuclear membrane.* Panels A, B and C are representative confocal images showing the colocalization of β_1AR, β_2AR or β_3AR, respectively with Nup-62, a component of the nuclear pore complex. Controls were performed in the absence of primary antibody (D). Data are representative of at least three independent experiments.

Figure 5. *Detection of βAR signalling partners in isolated adult rat left ventricular cardiomyocytes using immunocytochemistry.* Panels A, C, E and G are representative confocal images showing the distribution of Giα (A), Gsα (C) ACII (E) or ACV/VI (G),

respectively. Controls were performed in the absence of primary antibody (B, D, F and H). Scale bar is 10 μm. Arrows indicate nuclei. Data are representative of three independent experiments.

Figure 6. *Functional coupling of β_1AR but not β_3AR to adenylyl cyclase stimulation in enriched nuclear preparations.* A) Membranes isolated from enriched nuclear fractions were stimulated with increasing concentrations of isoproterenol (1 nM to 100 μM) and the production of [^{32}P]-cAMP from [α^{32}P]-ATP was assessed. B) Stimulation of adenylyl cyclase activity in response to activation by 1 μM isoproterenol, the β_3AR-selective agonist CL 316243 (1 μM), direct activation of G protein with 10 mM NaF or adenylyl cyclase with 100 μM forskolin. Data represent mean ± S.E. of at least three separate experiments performed in triplicate.

Figure 7. *Functional coupling of β_3AR but not β_1AR to initiation of de novo transcription in enriched nuclear preparations.* A) Enriched nuclear fractions were stimulated with 1 μM isoproterenol, the β_1AR-selective agonist xamoterol (1 μM) or the β_3AR-selective agonist BRL 37344. [α^{32}P]-UTP incorporation was measured in membranes either untreated or pretreated with 5 μg/ml PTX for 2 hours to inhibit Gi-specific signalling. Significant differences (* for $p<0.05$ between control and ligand-stimulated) were determined by a paired *t* test for three or more experiments. B) Enriched nuclear fractions were stimulated with 1 μM isoproterenol, with and without the β_1AR-selective antagonist CGP 20712A (1 nM) or the β_2AR-selective antagonist ICI 118,551 (0.5 nM). [α^{32}P]-UTP incorporation was also measured in response to CGP 20712A or ICI 118,551 alone. Data represent mean ± S.E. of at least three separate experiments performed in triplicate and are normalized to control. Significant differences (** and * for $p<0.05$ between vehicle and PTX-treated and control and ligand-stimulated, respectively) were determined by a paired *t* test for three or more experiments.

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Figure 1

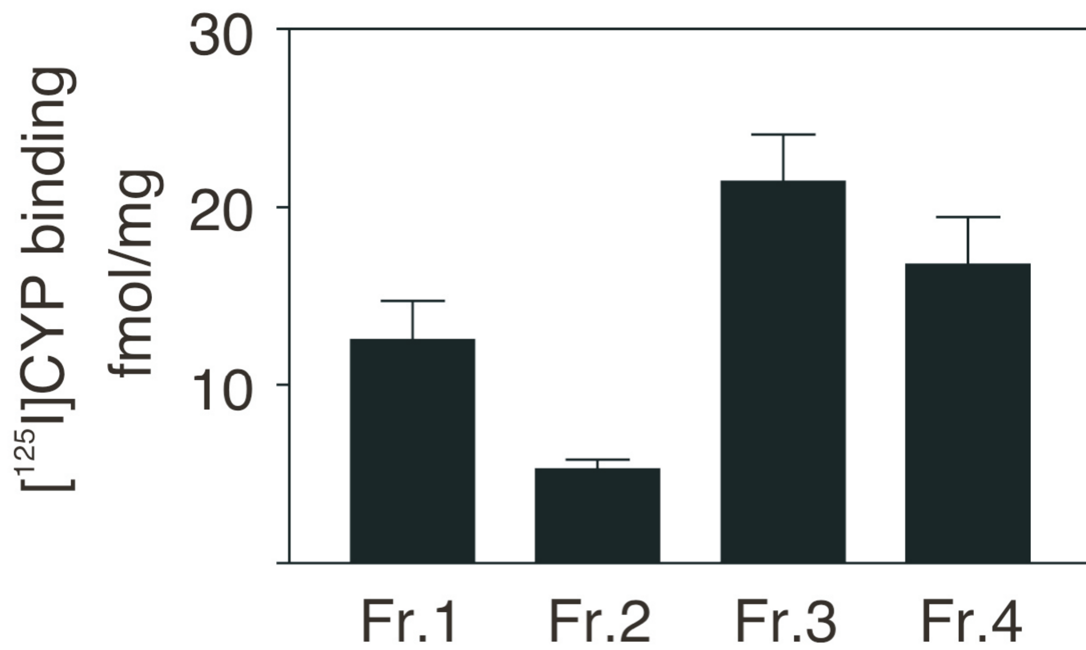

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Figure 2

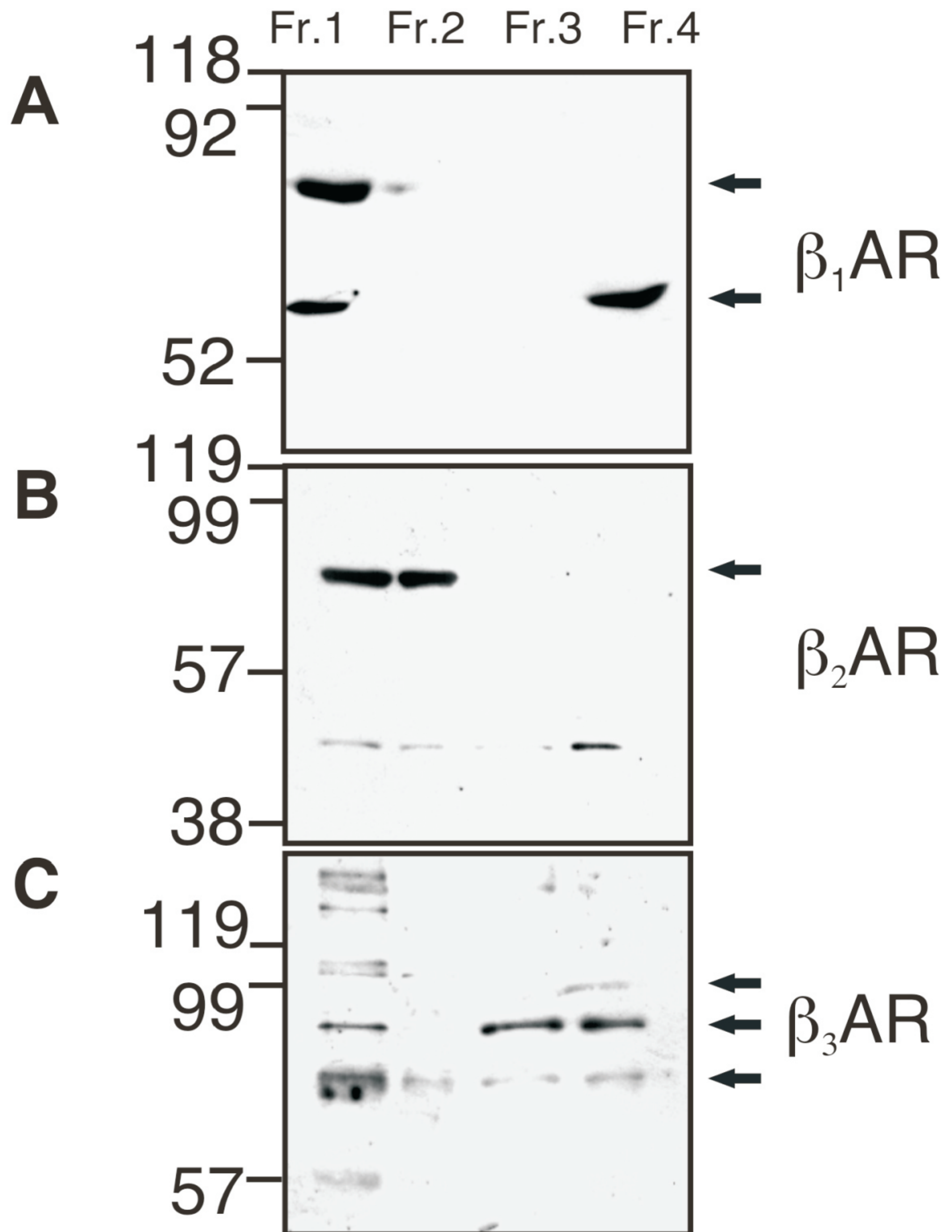

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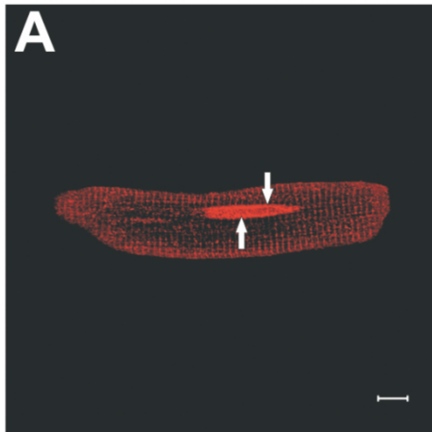
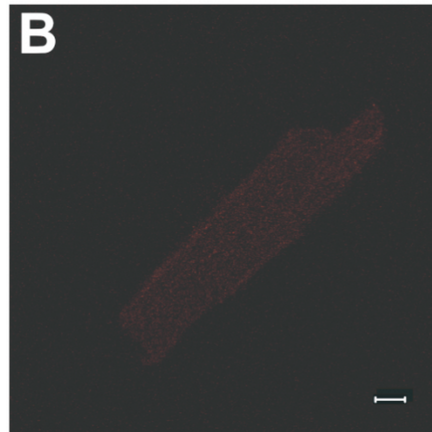
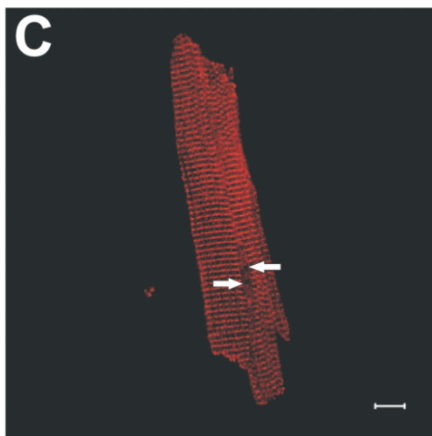
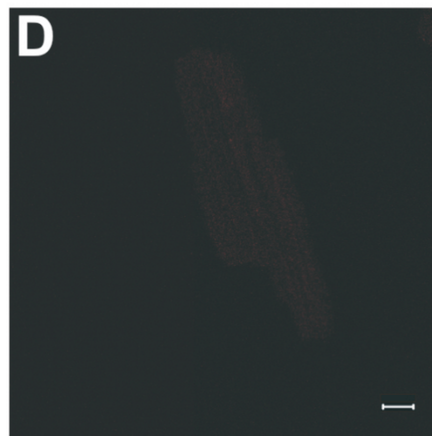
Figure 3





β_1AR





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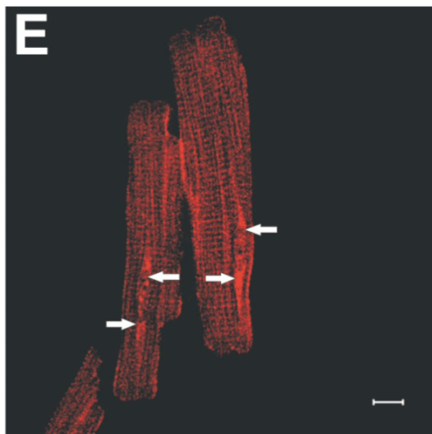

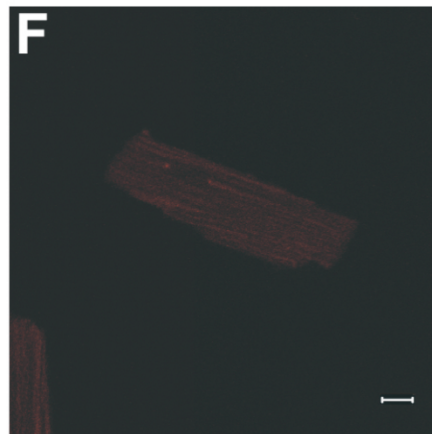

β_3AR

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Figure 4

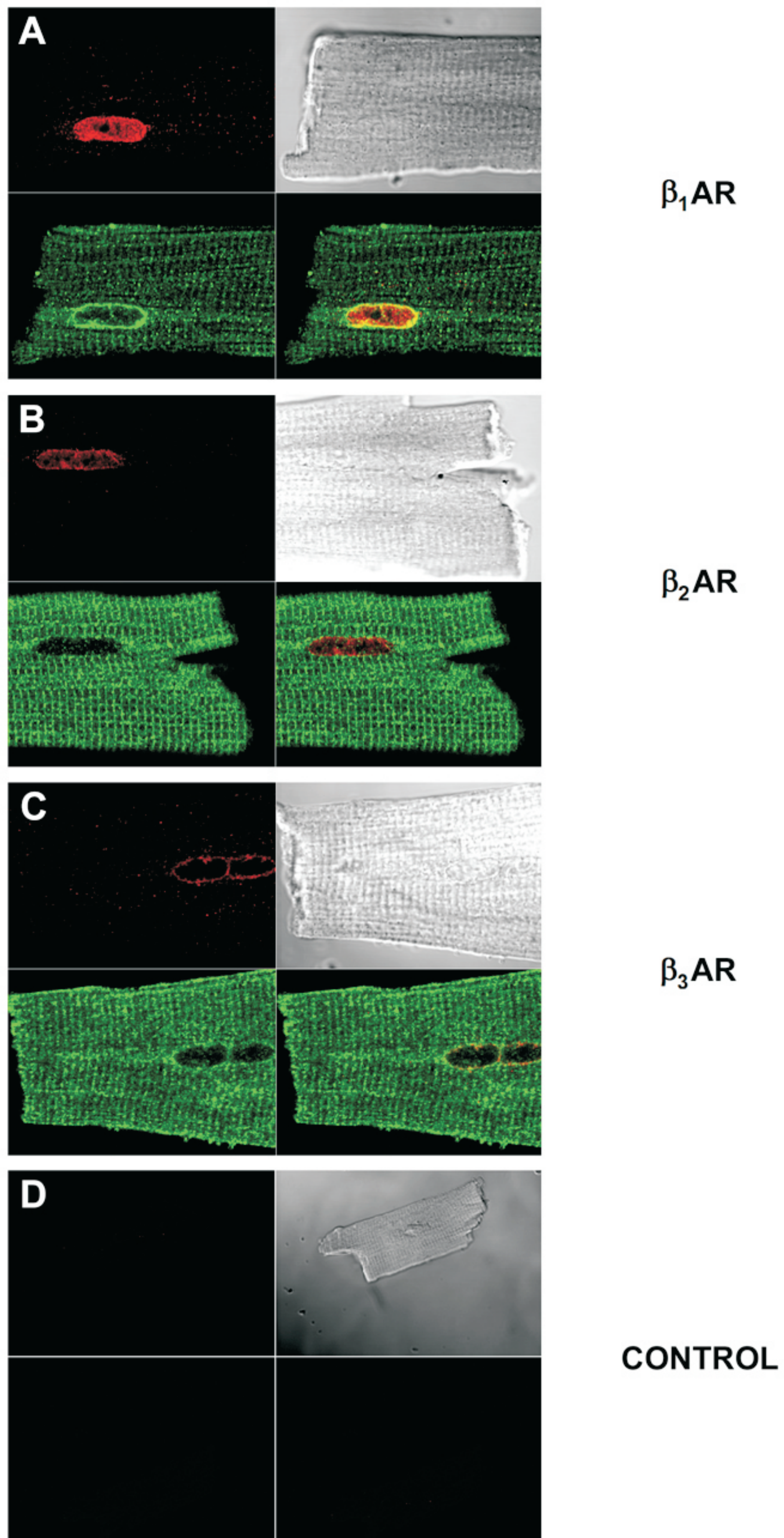

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Figure 5

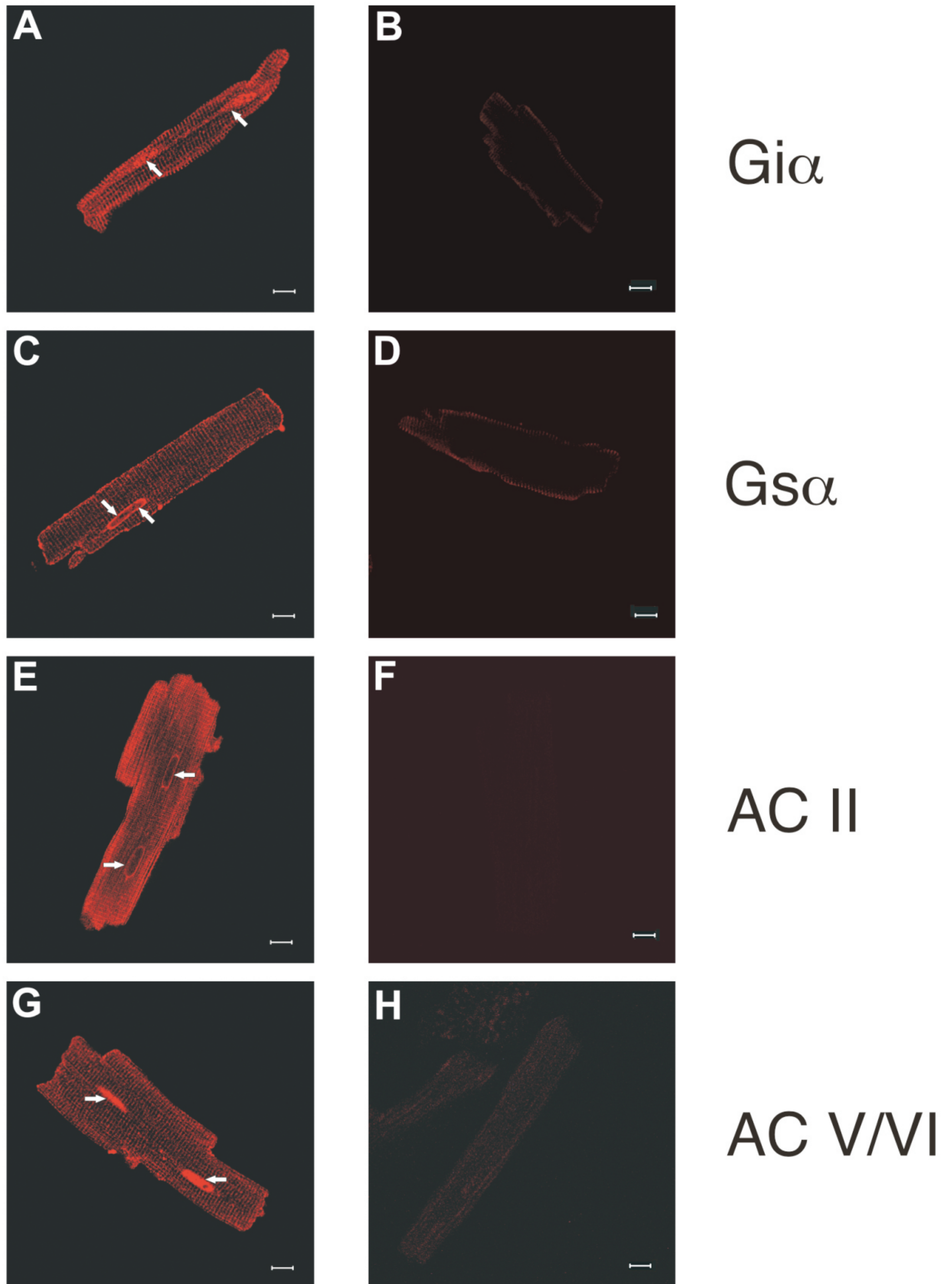

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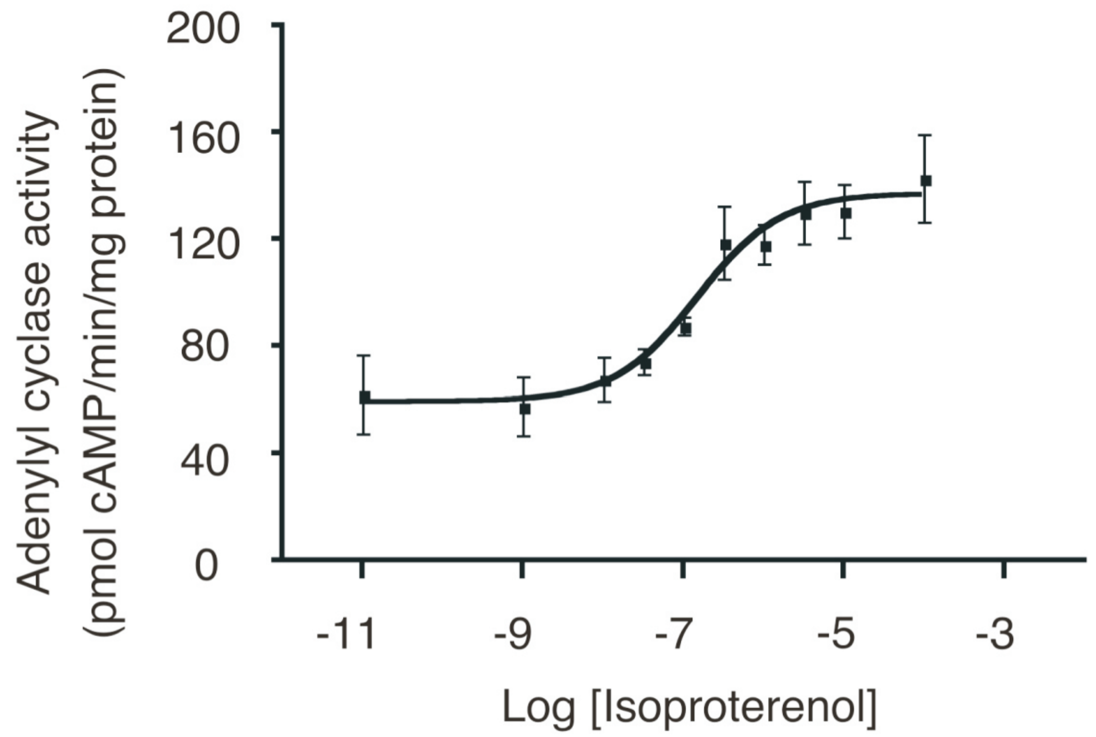
Figure 6



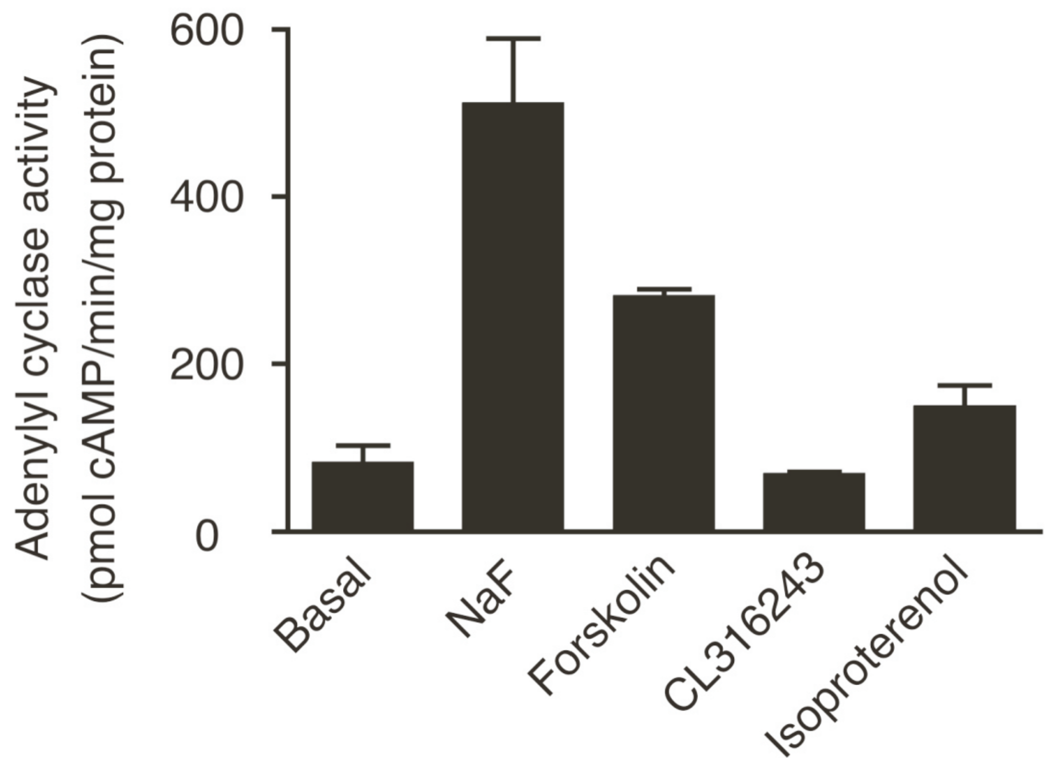


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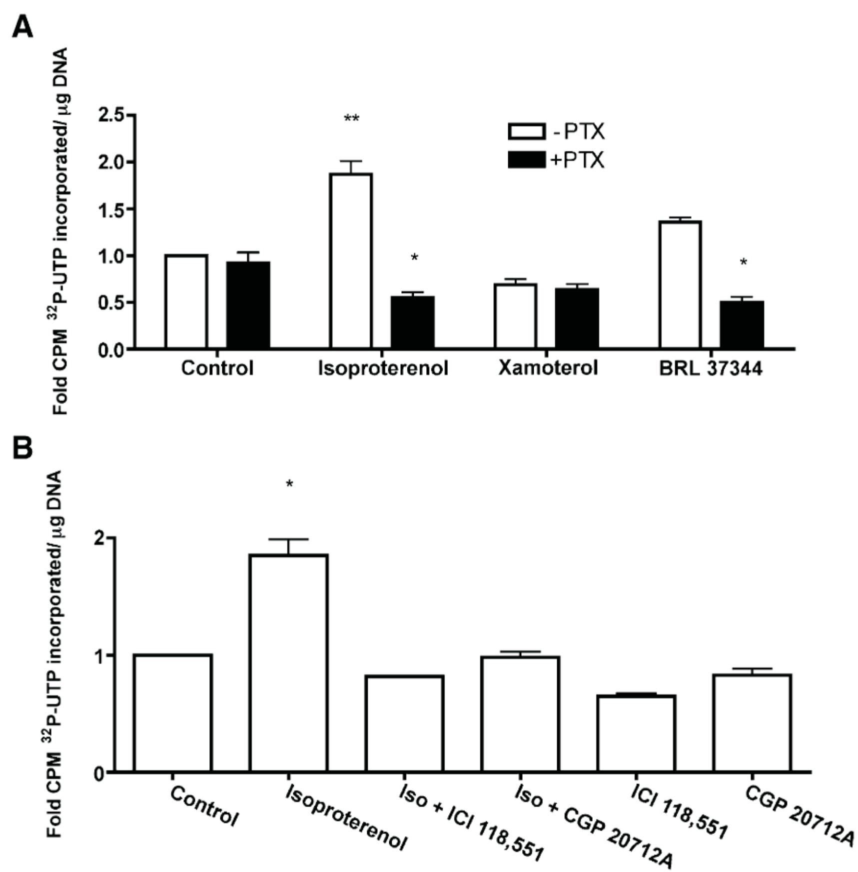
Figure 7



CHAPTER 4

Vaniotis G, Del Duca D, Trieu P, Rohlicek CV, Hébert TE, Allen BG. “Nuclear β-adrenergic receptors modulate gene expression in adult rat heart.” Cell Signal. 2011 Jan; 23(1):89-98.

Contribution: Figures 1-10

Nuclear β-adrenergic receptors modulate gene expression in adult rat heart

George Vaniotis[a,b], Danny Del Duca[c], Phan Trieu[d], Charles V. Rohlicek [e,f,g], Terence E. Hébert[d], and Bruce G. Allen[a,b,d,h]

Departments of Biochemistry[a] and Medicine[h], Montreal Heart Institute[b]; Université de Montréal; and
Divisions of Cardiovascular Surgery[c] and Cardiology[g],
Montréal Children's Hospital - McGill University Health Centre;
Departments of Pharmacology and Therapeutics[d], Physiology[e], and Pediatrics[f],
McGill University, Montréal, Canada

Corresponding authors:

Terence E. Hébert, PhD
Department of Pharmacology and Therapeutics
McGill University Faculty of Medicine
McIntyre Medical Sciences Building
3655 Promenade Sir William Osler, Room 1303
Montréal, Québec, Canada
H3G 1Y6

Bruce G. Allen, PhD
Department of Medicine
Montreal Heart Institute
Université de Montréal
5000, Belanger St
Montréal, Québec, Canada
H1T 1C8

Running Head: Transcriptional regulation by nuclear βARs

Abstract

Both β_1- and β_3-adrenergic receptors (β_1ARs and β_3ARs) are present on nuclear membranes in adult ventricular myocytes. These nuclear-localized receptors are functional with respect to ligand binding and effector activation. In isolated cardiac nuclei, the non-selective βAR agonist isoproterenol stimulated *de novo* RNA synthesis measured using assays of transcription initiation (Boivin et al, 2006 Cardiovasc Res. 71:69-78). In contrast, stimulation of endothelin receptors, another G protein-coupled receptor (GPCR) that localizes to the nuclear membrane, resulted in decreased RNA synthesis. To investigate the signalling pathway(s) involved in GPCR-mediated regulation of RNA synthesis, nuclei were isolated from intact adult rat hearts and treated with receptor agonists in the presence or absence of inhibitors of different mitogen-activated protein kinase (MAPK) and PI3K/PKB pathways. Components of p38, JNK, and ERK1/2 MAP kinase cascades as well as PKB were detected in nuclear preparations. Inhibition of PKB with triciribine, in the presence of isoproterenol, converted the activation of the βAR from stimulatory to inhibitory with regards to RNA synthesis, while ERK1/2, JNK and p38 inhibition reduced both basal and isoproterenol-stimulated activity. Analysis by qPCR indicated an increase in the expression of 18S rRNA following isoproterenol treatment and a decrease in NFκB mRNA. Further qPCR experiments revealed that isoproterenol treatment also reduced the expression of several other genes involved in the activation of NFκB, while ERK1/2 and PKB inhibition substantially reversed this effect. Our results suggest that GPCRs on the nuclear membrane regulate nuclear functions such as gene expression and this process is modulated by activation/inhibition of downstream protein kinases within the nucleus.

Keywords: beta-adrenergic receptor, endothelin receptor, nuclear membrane, G protein-coupled receptor (GPCR), transcription, signal transduction, protein kinase.

1. Introduction

β-adrenergic receptors (βARs) are part of the GPCR[1] superfamily that signal through heterotrimeric G proteins. GPCRs activate a wide range of downstream effectors and regulate diverse cellular functions in cardiomyocytes, including contractility, metabolism and gene expression. Additionally, the downstream signalling pathways activated following ligand binding can vary depending on the composition of heterotrimeric G proteins, and particularly which α subunits interact with the receptor.

In mammalian cardiomyocytes, all three known βAR subtypes have been detected. β_1ARs are the predominant subtype found in the heart, representing roughly 70% of the total βAR density (1). Primarily involved in the regulation of cardiomyocyte contractility, β_1ARs are known to signal through $G\alpha_s$ and adenylyl cyclase (AC). β_2ARs, representing roughly 30% of the total βAR density, are also involved in the regulation of contractility, but to a lesser extent (2). β_1ARs and β_2ARs signal through $G\alpha_s$ and AC with similar efficacy. However, the signals are compartmentalized differently in cardiomyocytes, possibly due to their localization in distinct membrane

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[1] Abbreviations: AC, adenylyl cyclase; ATP, adenosine triphosphate; βAR, β-adrenergic receptor; CAMKII, Ca^{2+}/calmodulin-dependent protein kinase II; DNA, deoxyribonucleic acid; DTT, dithiothreitol; ET-1, endothelin 1; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; HRP, horseradish peroxide; ISO, isoproterenol; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B-cells; PBS, phosphate buffered saline; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PMSF, phenylmethanesulphonylfluoride; PTX, pertussis toxin; qPCR, quantitative real-time polymerase chain reaction; RNA, ribonucleic acid; RNAP, RNA polymerase; TCA, trichloroacetic acid; TX-100, Triton X-100; UTP, uridine 5' triphosphate.

microdomains and/or dual coupling of the β_2AR to Gα_s and Gα_i (3). β_2ARs also have a high level of spontaneous activity not detected for β_1AR (4). During the development of heart failure, β_1ARs are internalized, their synthesis is reduced, and they begin to signal predominantly through a Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII)-dependent mechanism, while β_2ARs switch from Gα_s to Gα_i signalling, potential activating cardioprotective mechanisms (2). Additionally, expression and activity of GRK2 (βARK1), the primary GPCR kinase (GRK) in the heart, is increased during the development of heart failure, providing a molecular mechanism for βAR desensitization in heart failure (1). While β_3ARs are also expressed in healthy cardiomyocytes, their functions remain ill-defined, and they represent a negligible part of the total βAR density (1). In fact, as opposed to the β_1ARs and β_2ARs, β_3ARs appear to have negative inotropic effects and are actually up-regulated in response to heart failure (5). Hence, βAR subtypes likely play non-redundant roles within the cardiomyocyte.

Recently, evidence has accrued showing that functional GPCRs are not solely localized at the plasma membrane but can also signal from different endogenous membrane compartments, including the nuclear membrane (reviewed in (6)). Recent evidence also seems to indicate that these intracellular receptors may have the capacity to regulate signalling pathways that differ from those of their plasma membrane counterparts, as was recently demonstrated for the metabotropic glutamate receptor 5 (mGluR5) (7). In the case of the βARs, functional β_1AR and β_3AR, but not the β_2AR, have been detected at the level of the nuclear membrane in rat and mouse adult ventricular myocytes (8). Moreover, several studies have demonstrated that a number of their normal cell surface interactors, including Gα_s, Gα_i, Gα_q, adenylyl cyclase, and PKA, as well as other regulatory molecules known to interact with GPCRs, are also associated with the nucleus or the nuclear membrane (9, 10). In fact, the literature even seems to support the existence of nuclear-localized phosphoinositide signalling pathways that can regulate nuclear PKB/Akt signalling (11). Furthermore, βARs on the nuclear membrane have been shown to be functional with respect to both ligand binding and effector activation (8). Indeed, in isolated nuclei, β_1ARs activate AC following treatment with isoproterenol, while β_3ARs appear to stimulate *de novo* transcription,

through $G\alpha_i$ activation (8). However, the post-receptor signalling pathways that become activated following ligand binding and lead to changes in gene transcription remain to be identified.

Given the presence of GPCRs at the level of the nuclear membrane, we wished to assess what role they might play in adult cardiomyocytes, and to determine what pathways might be activated downstream of nuclear receptor activation and lead to modulation of gene transcription. To this end we used a pharmacological approach to investigate the involvement of different MAPKs and the PI3K/PKB pathway.

2 Material and Methods

2.1. Materials

Anti-PKB, anti-phospho-PKB (threonine 308 and serine 473), anti-phospho-ERK p44/42 (threonine 202/tyrosine 204), anti-MEK1/2 and anti-phospho-MEK1/2, anti-p38 and anti-JNK2 antibodies were from Cell Signaling Technology. Anti-Raf1 and Lamin B antibodies were from Santa Cruz Biotechnology. Anti-NFκB antibody was from eBioscience. Horseradish peroxide (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence (ECL) reagent Renaissance Plus was from Perkin Elmer Life Sciences (Woodbridge, Ontario). Triton X-100 (TX-100), leupeptin, PMSF and DNase I were from Roche Applied Science (Laval, Quebec). SDS-polyacrylamide gel electrophoresis reagents and nitrocellulose (0.22μm) were from Bio-Rad Laboratories (Mississauga, Ontario). Isoproterenol was from Tocris Bioscience (Ellisville, MO). Endothelin-1 (ET-1) was from Peninsula Laboratories (Torrance, CA). Pertussis toxin (PTX), microcystin LR, CGP 20712A, ICI118,551 and α-amanitin were from Sigma (Mississauga, Ontario). PD98059, SB203580, SP600125, LY294002, wortmannin and triciribine were from Calbiochem. U0126 was from Upstate Cell Signaling Solutions. RNaseOut, dNTP Mix, First Strand buffer and M-MLVRT were from Invitrogen. Primers, as well as SyBR Green and ROX were also from Invitrogen. RNA extraction kits were from Qiagen. RT^2 First strand kits, SABiosciences RT^2 qPCR Master Mix and rat NFκB signalling pathway RT^2 Profiler PCR arrays were from SABiosciences. Unless otherwise stated, all reagents were of analytical grade and were purchased from VWR Canlab (Ville Mont-Royal, Quebec) or Fisher Scientific (Mississauga, Ontario). [α^{32}P]UTP (specific activity 3000 Ci/mmol) was from Perkin Elmer.

2.2. Isolation of Nuclei

Rat cardiac nuclei were isolated as described previously (12). Briefly, rat hearts were pulverized under liquid nitrogen, resuspended in cold PBS, and homogenized

(Polytron, 8000 rpm; 2 x 10 s, Fraction 1). All subsequent steps were carried out on ice or at 5°C. Homogenates were centrifuged for 15 min at 500 x g. The resulting supernatants, referred to as Fraction 2, were diluted 1:1 with buffer A (10 mM K-HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4), incubated 10 min on ice, and centrifuged for 15 min at 2000 x g. The resulting supernatant was discarded. The pellet, referred to as crude nuclei (Fraction 3) was resuspended in buffer B (0.3 M K-HEPES pH 7.9, 1.5 M KCl, 0.03 M $MgCl_2$, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4), incubated on ice for 10 min, and centrifuged for 15 min at 2000 x g. The pellet, an enriched nuclear fraction (Fraction 4), was resuspended in buffer C (20 mM Na-HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4) or 1 x transcription buffer (50 mM Tris pH 7.9, 0.15 M KCl, 1 mM $MnCl_2$, 6 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 1 U/μl RNase inhibitor) and either used fresh or aliquoted, snap-frozen using liquid nitrogen, and stored at -80°C.

2.3. *Transcription Initiation*

Measurements of transcription initiation were performed as previously described (8). Briefly, 10 μl of isolated nuclei (resuspended in 1 x transcription buffer) were incubated at 30°C for 30 min in the presence of the indicated agonist/antagonist and 10 μCi [α^{32}P]UTP (3000 Ci/mmol). CTP and GTP were omitted to prevent chain elongation. Following termination of reactions by digestion with DNase I, nuclei were lysed with 10 mM Tris–HCl pH 8.0, 10 mM EDTA and 1% SDS. Duplicate 5 μl aliquots were transferred onto Whatman GF/C glass fiber filters, washed twice with 5% TCA containing 20 mM sodium pyrophosphate, and air-dried. ^{32}P incorporation was determined by liquid scintillation counting. DNA concentrations were determined spectrophotometrically and [^{32}P]UTP incorporation expressed as cpm/μg DNA. Where indicated, isolated nuclei were pre-treated with the appropriate pharmacological inhibitors. Nuclei were either pre-treated at room temperature with 10 μM PD98059 for 1 h, 20 μM U0126 for 30 min, 100 nM wortmannin for 30 min, 50 μM LY294002 for

30 min, 10 μM SB203580 for 1 h, 20 μM SP600125 for 1 h, 1 μM triciribine for 30 min, 3 μM α-amanitin (a potent inhibitor of RNA polymerase II and III) for 30 min, or vehicle (DMSO) for either 30 min or 1 h, according to which inhibitor was used.

2.4. Immunoblotting

SDS PAGE and immunoblotting were performed as previously described (12). Immunoreactive bands were quantified using a BioRad VersaDoc 4000 imaging system and Quantity One® software (Version 4.5.2 for Mac).

2.5. Quantitative real-time PCR

RNA was isolated from purified nuclei using Qiagen RNA extraction kits. To prepare cDNA, 1 μg of RNA was mixed with 100 ng of random primers and 1 μl of 10 mM dNTP mix, diluted to a final volume of 10 μl, heated to 65°C for 5 min, and then immediately quick chilled on ice. Next, 4 μl of 5 x first strand buffer, 2 μl of 0.1 M DTT and 1 μl of RNase Out were added, and reactions were incubated for 2 min at 37°C. Following addition of 1 μl of M-MLV reverse transcriptase (200 units), reactions were mixed, centrifuged and then incubated for 10 min at 25°C, 50 min at 37°C, and finally 15 min at 70°C. qPCR reactions, containing 12.5 μl of SyBR Green PCR master mix, 0.03 μl ROX Dye (an internal fluorescence standard), 2.5 μl of primers, and 10 μl of cDNA were 1 cycle of 10 min at 95°C, then 40 cycles of (30 s at 95°C, followed by 1 min at 60°C and 1 min at 72°C) using a Stratagene Mx3000P system. Samples were assayed in triplicate and normalized to β-actin expression. Primers used for real-time qPCR are shown in Table 1. The specificity of each primer pair for the amplicon of interest was verified by dissociation curve analysis.

2.6. RT^2 Profiler PCR Arrays

RNA was isolated from purified nuclei using Qiagen RNA Extraction Kits. cDNA

was prepared from 1 μg of RNA using the SABiosciences RT^2 First Strand Kit. cDNA (102 μl) was mixed with 1275 μl of 2 x SABiosciences RT^2 qPCR Master Mix plus 1173 μl of ddH_2O and 25 μl of the final mixture was transferred to each well of the RT^2 PCR array. Arrays were assayed for 1 cycle of 10 min at 95°C, then 40 cycles of (15 s at 95°C, followed by 1 min at 60°C) using a Stratagene Mx3000P system. All conditions were done in triplicate and normalized to the untreated control. Each 96-well plate also included primers for 5 housekeeping genes as well as positive and negative controls. Results were analyzed using web-based analysis software (SABiosciences). Changes were considered significant when $p < 0.05$.

2.7. Statistical Analysis

Data are presented as the mean ± the standard error of the mean (S.E.M.). The significance of differences between groups was determined using one-way ANOVA followed by Tukey's multiple comparison tests (Prism 4.0cx, GraphPad Software). Differences were considered significant when $p < 0.05$.

2.8. Miscellaneous Methods

Protein concentrations were determined by the method of Bradford using bovine γ-globulin standard.

3. Results

3.1. Regulation of β-adrenergic transcriptional responses in isolated nuclei by protein kinases.

We have shown previously that isoproterenol (ISO) increases *de novo* RNA synthesis in nuclei freshly isolated from rat heart (8). MAPK and PKB signalling are known to lead to the phosphorylation of a variety of transcription factors, including some that are regulated by multiple signalling pathways. ERK1/2 primarily activates activator protein-1 (AP-1) through its coordinated stimulation of c-fos, while JNK also activates AP-1, though through its control of c-jun expression (13). PKB on the other hand primarily activates the FoxO family of transcription factors as well as NFκB (14). Moreover, ATF-2 and p53 can be activated by JNK as well as p38 MAPK, while c-myc has been shown to be activated by ERK1/2, ERK5 and JNK (15-17). Hence, it is reasonable to propose that one or more of these pathways may be involved in regulating transcription in response to activation of βARs in the nuclear membrane. First, we set out to determine if the ERK1/2 MAPK pathway modulated the effects of βAR stimulation on transcriptional initiation. Freshly isolated nuclei were incubated with ISO (1 μM), in the absence or presence of an inhibitor of either MEK1/2 activation (PD98059) or activity (U0126), and [α^{32}P]UTP to measure initiation of RNA transcription. Endothelin type B receptors (ETB) have also been shown to be present on the nuclear envelope (12); hence, purified nuclei were also treated with endothelin 1 (ET-1, 10 nM). Interestingly, whereas ISO increased [^{32}P]UTP incorporation (Fig. 1A) treatment with ET-1 decreased RNA transcription below control levels. Treatment with PD98059 also resulted in a decrease in basal levels of *de novo* transcription whereas U0126 did not suggesting a difference between having activatable MEK1/2 or not. However, pre-treatment with either PD98059 or U0126 blocked the increase in RNA synthesis following ISO treatment. Neither compound altered the inhibition of RNA synthesis induced by ET-1, suggesting that the signals from βAR and ETB are compartmentalized differently. The presence of the activated, phosphorylated form of ERK1/2 in isolated nuclei was confirmed by immunoblot, where isolated nuclei were pretreated with either vehicle, ISO, microcystin LR, or ISO and microcystin LR and

then probed with anti-phosphoERK1/2 (p42/p44, Fig. 4A). The presence of other components of the ERK1/2 pathway was also assessed by immunoblotting, with the results demonstrating the presence of both Raf-1 and the phosphorylated form of MEK1/2 (Fig 4B, 5A). The ability of PD98059 to reduce the basal level of transcription, taken together with the ability of both PD98059 and U0126 to block the ISO-induced up regulation of RNA synthesis, and the presence of Raf-1, MEK1/2 and ERK1/2 immunoreactivity, demonstrate that all of the components of the ERK1/2 MAPK pathway are present in the nucleus and appear to already be in an activated form prior to βAR stimulation. Isoproterenol treatment did not activate ERK1/2 *per se*. A number of signals likely converge on ERK1/2 and other MAPKs suggesting that the net state of their activation may serve a modulatory role for βAR-mediated transcription.

Next, we examined the potential involvement of the p38 and JNK MAPK pathways. Pre-treatment with SB203580, an inhibitor of p38α and p38β activity, again resulted in the inhibition of basal RNA synthesis (Fig. 1B), while pre-treatment with SP600125 (which inhibits JNK) showed an inhibitory trend that did not reach statistical significance. Both SB203580 and SP600125 blocked the ISO-induced increase in transcription, but had no appreciable effect on the inhibition of transcription induced by ET-1, again highlighting differences in how the two signalling pathways are organized in nuclei. Both p38 and JNK were detected by immunoblot (Fig. 5B, 5C), however levels of phospho-p38 and phospho-JNK were too low to detect with the antibodies available (data not shown). These results indicate that both the p38 and JNK MAPK signalling cascades are present within the nucleus. The ability of p38 and JNK inhibitors to completely block ISO-induced up regulation of RNA synthesis, while not being directly activated by isoproterenol treatment, seems to indicate that these MAPK might also play important modulatory roles in this process. Thus the different MAPK systems all seem to mediate the ability of βAR to increase transcriptional activity. Systems which control MAPK activity or distribution in the nucleus would either be permissive or restrictive to transcriptional regulation by βAR.

We then turned our attention to the PI3K/PKB pathway. LY294002 and wortmannin (which inhibit PI3K) both inhibited *de novo* transcription in the absence of agonist stimulation (Fig. 2A), and blocked the increase in transcription in response to ISO. Again neither inhibitor had any effect on ET-1 induced inhibition. In line with these experiments, it was not surprising that pre-treatment with the PKB inhibitor triciribine alone resulted in a similar decrease in basal *de novo* transcription since PKB is downstream of PI3K (Fig. 2B). A striking finding was that triciribine not only blocked the increase in transcription produced by ISO, but actually converted the normal stimulatory effect of ISO to an inhibitory one, i.e. levels of RNA synthesis initiation were lower in the presence of triciribine and ISO than with triciribine alone. Thus, the ability of triciribine to turn ISO from stimulatory to inhibitory indicates that ISO, when PKB cannot be activated, becomes an inverse agonist for the transcriptional pathway. In addition, triciribine enhanced the inhibitory effect of ET-1, suggesting that ET-1 and triciribine inhibit *de novo* RNA synthesis by two distinct pathways.

3.2. Regulation of β-adrenergic transcriptional responses in isolated nuclei by protein serine/threonine phosphatase activity

Given that ERK1/2, p38, JNK and PKB are all protein serine/threonine kinases, we next examined the involvement of protein serine/threonine phosphatase activity in regulating [^{32}P]UTP incorporation. Microcystin LR, a potent inhibitor of type I and IIA protein serine/threonine phosphatases, produced 50% increases in transcription initiation with vehicle, ISO, and ET-1 (Fig. 3). The latter observation is interesting in the sense that it changes the direction of ET-1 signalling with respect to transcription initiation in an analogous way to how triciribine affected the response to ISO. Since PKB inhibition converted ISO from an activator into an inhibitor of transcriptional initiation, the effect of ISO and microcystin upon PKB phosphorylation was also examined. Isolated nuclei were pretreated with either vehicle, ISO, microcystin LR, or ISO and microcystin, and either phospho-PKB threonine 308 or serine 473 was quantified by immunoblotting. Treatment with microcystin increased phosphorylation of PKB at both threonine 308 and serine 473 (Fig. 6A, 6B) indicating the presence of, and dephosphorylation by, type I and/or IIA protein serine/threonine phosphatase activity in isolated nuclei.

Additionally, although ISO alone did not increase PKB phosphorylation at either threonine 308 or serine 473, treatment with ISO in the presence of microcystin increased the level of PKB phosphorylation at both sites, particularly in the case of serine 473, indicating that ISO is in fact able to activate PKB in the nucleus, but that PKB is subject to rapid dephosphorylation. The levels of both total ERK and total PKB immunoreactivity remained constant (Fig. 4A, 6A, 6B), confirming that the changes in phospho-PKB levels were due to activation of local PKB pools in isolated nuclei following receptor stimulation.

3.3. Targets of β-adrenergic transcriptional responses in isolated nuclei

We next wished to determine which type(s) of RNA were being altered upon stimulation of βAR on isolated nuclear membranes. Ribosomal RNA (rRNA) represents the majority of the RNA within the cell. Consequently, to identify if an increase in rRNA synthesis was responsible for the observed increase in *de novo* RNA synthesis, isolated nuclei were treated with ISO or vehicle for 30 minutes, 1, 6, 12 or 24 hours and the quantity of 18S rRNA determined by qPCR, and normalized against β-actin mRNA. Large variations in the abundance of 18S rRNA could be seen at the later time points, including a decrease at 12 hours and a significant increase at 24 hours (data not shown). However, a closer examination of the qPCR data revealed the variability observed from the 6 h time point forward was due to changes in both 18S rRNA and β-actin mRNA levels, which appeared to be degraded more rapidly in the ISO treated samples. For that reason we decided to focus solely on the 30 min time point. A significant increase, of close to 5 fold, in 18S rRNA was observed, indicating that an increase in rRNA is likely primarily responsible for the increase in global transcription observed upon βAR stimulation (Fig. 7A).

We also wanted to determine whether mRNA abundance was affected in response to activation of βAR in the nuclear membrane. Given that NFκB is a well characterized transcription factor downstream of PKB signalling, and its message levels have recently been shown to be regulated by another nuclear GPCR, the angiotensin receptor 1 (AT-1) (18), we reasoned that it might be an interesting candidate. Using primers directed

against NFκB1, we observed a decrease in NFκB mRNA levels after 30 min of stimulation with ISO (Fig. 7B). These results were further confirmed at the protein level as a small decrease in NFκB was observed following treatment with ISO (Fig. 9). Levels of NFκB protein were normalized using an anti-lamin B antibody. Hence, the abundance of NFκB mRNA appears to be down-regulated by nuclear βAR stimulation. The fact that inhibition of RNA polymerase II (RNAP II), with α-amanitin, substantially reduced the effects of ISO on transcription initiation (Fig. 8A) also suggests that some of the transcriptional events depend on this enzyme. ISO reduced NFκB mRNA levels and no added effect was observed in the presence of α-amanitin (Fig. 8B). Interestingly, we also noted an effect of α-amanitin on 18S rRNA levels, which depends on RNAP I (Fig. 8C).

To further explore the regulation exerted by nuclear βARs on the NFκB pathway, we used RT^2 profiler PCR arrays (purchased from SABiosciences) with RNA from isolated nuclei treated with ISO or vehicle for 30 minutes in the presence or absence of either ERK1/2 inhibition (U0126) or PKB inhibition (triciribine, Table 2). The abundance of several transcripts was significantly altered in each of the conditions. Consistent with our initial experiments with NFκB, treatment with ISO also resulted in the down-regulation of ATF-2, Il1r1 and Tnfrsf1b, along with the modest up-regulation of Ripk2. ATF-2, which is known to interact with c-jun, is a transcription factor primarily regulated by the stress activated MAPKs JNK and p38 and is known to play a role in the hypertrophic response (19), while Il1r1 is mainly involved in the immune and inflammatory response, through its interaction with Myd88, although it also plays a role in the activation of NFκB (20). Tnfrsf1b plays a role in the activation of NFκB, but only in the absence of Rip proteins like Ripk2 (21). Taken together these results support the idea that ISO stimulation leads to general inhibition of the NFκB pathway along with the suppression of the inflammatory response.

Further, isolated nuclei pretreated with U0126 prior to ISO stimulation showed an increase in Irak2, Myd88 and Ippk along with a decrease in Traf3. Both the protein kinase Irak2 and adaptor protein Myd88 play key roles in the activation of NFκB (22),

whereas Traf3 has been shown to inhibit NFκB activation (23). The role of the enzyme Ippk is still unclear, but it has been shown to play a role in the inhibition of apoptosis (24). In addition, c-jun is also up-regulated in comparison to the ISO stimulated nuclei. Our results indicate that inhibition of ERK1/2 activation by U0126 in the presence of ISO leads to the activation of NFκB and the suppression of apoptosis. Hence, U0126 completely reverses the inhibitory effect exerted on the NFκB pathway by ISO stimulation, indicating that ERK1/2 might be responsible for this inhibitory effect.

Isolated nuclei pretreated with triciribine prior to ISO stimulation showed an increase in Casp1 and Lpar1 mRNA, along with increases in Tnfsf14, Tlr6 and Tnfrsf1b that were not quite statistically significant. Casp1 is an enzyme involved in the up-regulation of inflammation and apoptosis (25), while Lpar1 is a GPCR involved in developmental, physiologic and pathophysiologic processes, and has also been shown to activate PKB as well as Rho pathways (26). In addition, Tnfsf14 and Tnfrsf1b both have been shown to play a role in the activation of NFκB and apoptosis (21), while Tlr6, through its interaction with Tlr2, is involved in the immune response (27). Hence, it appears that PKB inhibition leads to an increase in the abundance of mRNAs arising from genes important for induction of apoptosis, suggesting a role for PKB in the inhibition of apoptosis following nuclear βAR stimulation. The results of the RT^2 PCR arrays were in accordance with the qPCR data, where ISO stimulation leads to the reduction of NFκB mRNA, while also demonstrating a role for both ERK1/2 and PKB in modulating this pathway. Taken together, our data indicate that nuclear βAR modulate a number of transcriptional events through localized signalling pathways (i.e. PKB activation) and these are modulated by the tone of ERK, p38 and protein phosphatase signalling in the nucleus.

4. Discussion

We have demonstrated that GPCRs located on the nuclear membrane are not only functional with respect to ligand binding and effector activation, but also can play very distinct roles in regards to the regulation of RNA synthesis (summarized in Fig. 10). Whereas βAR activation has been shown previously to both activate AC via Gs and stimulate global RNA synthesis via Gi (8), we now show that ETR activation by ET-1 has the opposite effect, actually inhibiting *de novo* RNA synthesis, in a protein phosphatase-sensitive manner. That these two types of GPCRs exert opposing effects on RNA synthesis indicates that they either integrate signals relevant for target binding sites in the genome or may have distinct sets of targets. Additionally, βARs in nuclear membranes, similarly to the βARs located at the level of the plasma membrane, appear to be able to activate the PI3K/PKB pathway, a process which also appears to be highly regulated via serine/threonine phosphatases. MAPK signalling pathways, although not directly activated by the βAR in the nucleus, do in fact modulate the ability of βAR to mediate transcriptional events, again in a manner that is sensitive to serine/threonine phosphatase inhibition. Different levels of control are also suggested by the differences in basal transcriptional activity in the presence of an activatable MEK or not. Whereas PD98059 inhibits MEK1/2 activation, U0126 inhibits MEK1/2 activity. As a result, in the nuclei treated with U0126, MEK1/2 can still be activated and hence might play some other role unrelated to its kinase activity *per se*, perhaps as a scaffold.

Nuclear-targeted PKB overexpression has previously been shown to have several beneficial effects including enhanced contractility and protection from ischaemic injury (28), while sustained PKB activation can lead to hypertrophic cardiomyopathy primarily via phosphorylation of substrates localized to the cytoplasm (11, 29), indicating that PKB not only plays important roles in the nuclei of cardiomyocytes, but that these roles can differ depending on its localization.

Furthermore, we show that inhibition of PKB with triciribine causes ISO to behave as an inverse agonist, leading to the inhibition of RNA synthesis, as opposed to its

previously noted stimulatory effect. At the level of the plasma membrane, PI3K activation leads to the recruitment of phosphoinositide-dependent kinase-1 (PDK1) and PKB via their pleckstrin homology (PH) domains, where PDK1 is able to phosphorylate PKB at threonine 308 and thereby activate it (30). Once activated, PKB can accumulate both in the cytoplasm and the nucleus (11, 31, 32). However, inhibition of PI3K with either wortmannin or LY294002 did not produce the same effect, indicating that either PKB activation in this case might be mediated by another pathway, or that PKB is only one of multiple effectors activated downstream of PI3K in the nucleus. One such possibility would be that PKB is being activated by DNA-dependent protein kinase (DNA-PK) through the control of cAMP levels (33, 34), although in this case DNA-PK would not be exported, but would instead phosphorylate PKB within the nucleus. Moreover, it is unclear how inhibition of PKB with triciribine reverses the effect of ISO treatment on transcriptional activity. One possibility is that the conformational state of the receptor is altered such that ISO now acts as an inverse agonist for the transcriptional pathway. Recent evidence demonstrates that βAR inverse agonists for AC may act as agonists for activation of MAPKs via a β-arrestin-mediated pathway (35). In our case, similar pleiotropic effects of ISO may result from PKB-mediated alterations in the receptor or its signalling partners, leading to the inhibition of *de novo* RNA synthesis, when PKB is inhibited and activation when it remains activatible. Given the high level of basal RNA transcription seen, in the case where PKB is inhibited, ISO might simply be inhibiting the spontaneous activity of these nuclear localized receptors in a functionally selective manner. Further experiments to examine the involvement of β-arrestin in this pathway and the identification of the relevant PKB targets are required. At this point, it is still unclear if the nuclear localized receptors interact with β-arrestin or GRKs although both are present in the nucleus (36, 37). Pim-1, a protein kinase downstream of PKB, may also be involved as it plays a pivotal role in the inhibition of both apoptosis and hypertrophy in cardiomyocytes, and its expression affects both JNK and ERK pathways (38). This may in part explain why each of the MAPK pathways appear to play some role in the βAR stimulated increase in *de novo* RNA synthesis. Additionally, Pim-1 has been shown to phosphorylate and stabilize c-myc (39). Given the established role of c-myc as an activator of RNAP I (40), this may suggest a

pathway involving the receptor, PKB and RNAP I being important for rRNA transcription.

Although the bulk of the transcriptional response to βARs stimulation involved synthesis of rRNA, we did detect regulation of specific mRNA targets, such as NFκB, which was down-regulated in response to treatment with ISO, and our experiments with α-amanitin demonstrate a role for RNAP II in the receptor-mediated transcriptional responses. The effects on rRNA transcription in response to pretreatment with α-amanitin were somewhat surprising. A similar effect of α-amanitin on RNAP I- and RNAP III-transcribed genes has been reported previously (41, 42) and RNAP II was recently shown to bind near and regulate the transcription of genes transcribed by RNAP III (41). Alternatively, it may be that signalling via the βAR alters the activity and/or distribution of shared components of RNAP I and RNAP II but this is beyond the scope of the present study.

Genes for other modulators and targets of the NFκB pathway, such as ATF-2 and Tnfrsf1b were also down-regulated. Nuclear GPCRs may therefore be important regulators of both apoptosis and the inflammatory pathway in the heart. Moreover, the fact that both ERK1/2 and PKB inhibition seem to reverse the effects of ISO stimulation on these identified genes would seem to indicate that both pathways play a role in the regulation of gene expression by nuclear βARs. This may be through MAPK or PKB modulation of the signalling proteins themselves, modulation of the transcriptional machinery or via alterations in the occupancy of chromatin sites by ERK and other protein kinases (43, 44). Further, given the already identified role of MAPKs in regulating mRNA stability (45), further experiments need to be conducted to determine if the regulation that we see is actually due to a changes in the level of *de novo* mRNA synthesis or reduced mRNA stability. These results reveal that βARs located on the nuclear envelope can in fact affect specific genes, and pathways, though further large-scale analysis by microarray would be useful in developing a broader picture of genes regulated by these receptors.

5. Conclusions

We have shown that ETB and βARs in the nuclear membrane can effect RNA synthesis, and in opposing ways, implying that they have different roles to play in regulating this process. Additionally, using a pharmacological approach we have shown that MAPK and PKB modulate RNA synthesis observed following ISO treatment in isolated nuclei. Moreover, we show that not only is rRNA synthesis increased but specific mRNA targets, such as NFκB, also appear to be regulated. Together, these results begin to demonstrate that not only are these GPCRs present at the level of the nuclear membrane, functional with respect to ligand binding and effector activation, but also have a role to play in the regulation of gene transcription, in addition to other functions yet to be discovered.

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Figure Legends

Figure 1: **Effect of MAP kinase inhibition on transcription initiation.** A) Enriched nuclear fractions were stimulated with either 1 μM isoproterenol (ISO), or 100 nM ET-1. [α^{32}P]UTP incorporation was measured in either untreated nuclei or nuclei pretreated with either an inhibitor of MEK1/2 activation, PD98059 (10 μM, 1 h), or activity U0126 (20 μM, 30 min). B) Enriched nuclear fractions were stimulated with either 1 μM isoproterenol (ISO), or 100 nM ET-1. [α^{32}P]UTP incorporation was measured in either untreated nuclei or nuclei pretreated with p38 MAPK inhibitor SB203580 (50 μM, 30 min) or JNK MAPK inhibitor SP600125 (100 nM, 30 min). Data represent mean ± S.E. of at least three separate experiments performed in triplicate. Significant differences (* for $p<0.05$) were determined by one-way ANOVA for three or more experiments.

Figure 2: **Effect of PI3K/PKB inhibition on transcription initiation.**
A) Enriched nuclear fractions were stimulated with either 1 μM isoproterenol (ISO), or 100 nM ET-1. [α^{32}P]UTP incorporation was measured in nuclei either untreated or pretreated with PI3K inhibitors LY294002 (50 μM, 30 min) or wortmannin (100 nM, 30 min). B) Enriched nuclear fractions were stimulated with either 1 μM isoproterenol (ISO), or 100 nM ET-1. [α^{32}P]UTP incorporation was measured in nuclei either untreated or pretreated with PKB inhibitor triciribine (1 μM, 30 min). Data represent mean ± S.E. of at least three separate experiments performed in triplicate. Significant differences (* for $p<0.05$) were determined using one-way ANOVA for three or more experiments.

Figure 3: **Effect of microcystin on transcription initiation.**
Enriched nuclear fractions were stimulated with either 1 μM isoproterenol (ISO), or 100 nM ET-1. [α^{32}P]UTP incorporation was measured in nuclei either untreated or pretreated with 1 μM microcystin LR (an inhibitor of type I and IIA protein serine/threonine phosphatases) prior to the addition of agonist. Data represent mean ± S.E. of at least three separate experiments performed in triplicate. Significant differences (* for $p<0.05$) were determined using one-way ANOVA for three or more experiments.

Figure 4: **Phosphorylation of ERK1/2 and MEK1/2 in isolated nuclei following treatment with isoproterenol.**

A) Enriched nuclear fractions were either untreated (1), treated with 1 μM microcystin LR (an inhibitor of type I and IIA protein serine/threonine phosphatases) (2), stimulated with 1 μM isoproterenol (3), or with 1 μM isoproterenol plus 1 μM microcystin LR (4). All treatments were for 30 min at 30°C. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with an anti-phospho ERK1/2 (threonine 202/tyrosine 204) antibody. B) Enriched nuclear fractions were treated as described in A, separated on SDS-PAGE, transferred to nitrocellulose, and probed with an anti-phospho MEK1/2 antibody. Data represent mean ± S.E. of at least three separate experiments. Significant differences (* for $p<0.05$) were determined using one way ANOVA for three or more experiments.

Figure 5: **Detection of Raf1, p38 and JNK2 in isolated nuclei.**

Enriched nuclear fractions were treated as described in Figure 4A, separated by SDS-PAGE, transferred to nitrocellulose and probed with A) an anti-phospho Raf1 antibody, B) an anti-p38 antibody or C) an anti-JNK2 antibody. Images on the left of each panel show representative immunoblots and data on the right of each panel represent mean ± S.E. of at least three separate experiments. Significant differences (* for $p<0.05$) were determined by one-way ANOVA for three or more experiments.

Figure 6: **Phosphorylation of PKB following treatment with isoproterenol.** Enriched nuclear fractions were treated as described in Figure 4A, separated by SDS-PAGE, transferred to nitrocellulose and probed with A) an anti-phospho PKB (threonine 308) antibody or B) an anti-phospho PKB (serine 473) antibody. Images on the left of each panel show representative immunoblots and data on the right of each panel represent mean ± S.E. of at least three separate experiments. Significant differences (* for $p<0.05$) were determined by one-way ANOVA for three or more experiments.

Figure 7: **βAR stimulation alters the abundance of 18S rRNA and NFκB mRNA.**

A) Enriched nuclear fractions were stimulated with either 1 μM isoproterenol or vehicle for 30 min at 37°C. RNA was then extracted, cDNA prepared and 18S rRNA (A) or NFκB1 mRNA (B) quantified by qPCR. C_T values were normalized to those for β-actin mRNA. Data represent mean ± S.E. of at least three separate experiments. Significant differences (* for $p<0.05$) were determined by one way ANOVA for three or more experiments.

Figure 8: **Effects of α-amanitin on isoproterenol-mediated transcription initiation**.

A) Nuclear [α^{32}P]UTP incorporation was determined following α-amanitin (3 μM, 30 min) or vehicle pre-treatment and subsequent treatment with 1 μM isoproterenol. B) Levels of NFκB1 mRNA following α-amanitin or vehicle pre-treatment and subsequent treatment with 1 μM isoproterenol. Samples were treated as described above in Figure 5. C_T values were normalized to β-actin mRNA levels. C) Levels of 18S rRNA following α-amanitin or vehicle pre-treatment and subsequent treatment with 1 μM isoproterenol. Samples were treated as described above in Figure 5. C_T values were normalized to β-actin mRNA levels. Data represent mean ± S.E. of at least three separate experiments. Significant differences (* for $p<0.05$) were determined by one way ANOVA for three or more experiments.

Figure 9: **Detection of NFκB following treatment with isoproterenol.**

Enriched nuclear fractions were treated as described in Figure 4A, separated by SDS-PAGE, transferred to nitrocellulose and probed with an anti-NFκB antibody. Expression of NFκB was normalized to the levels of Lamin B protein. Images on the left of each panel show representative immunoblots and data on the right of each panel represent mean ± S.E. of at least three separate experiments. Significant differences (* for $p<0.05$) were determined by one-way ANOVA for three or more experiments.

Figure 10: **Signalling diagram of post-nuclear βAR and ETR activation**

Signalling diagram illustrating major findings of our study.

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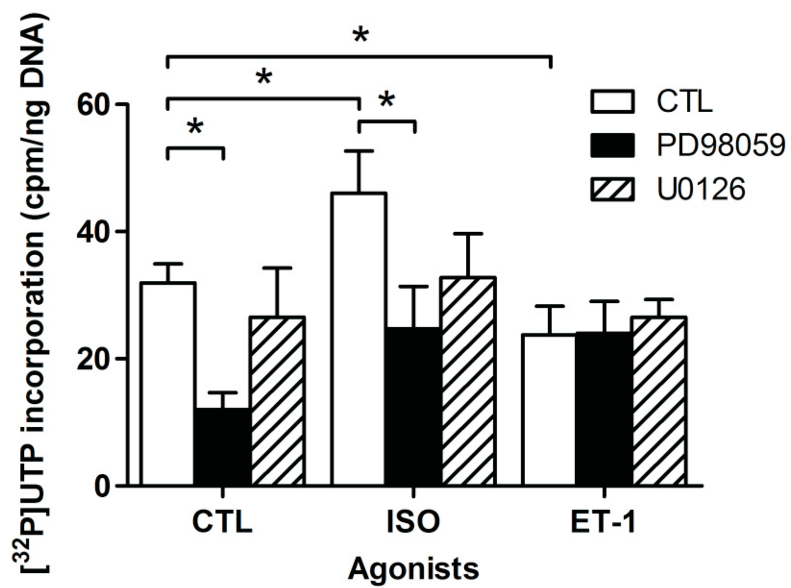
Figure 1



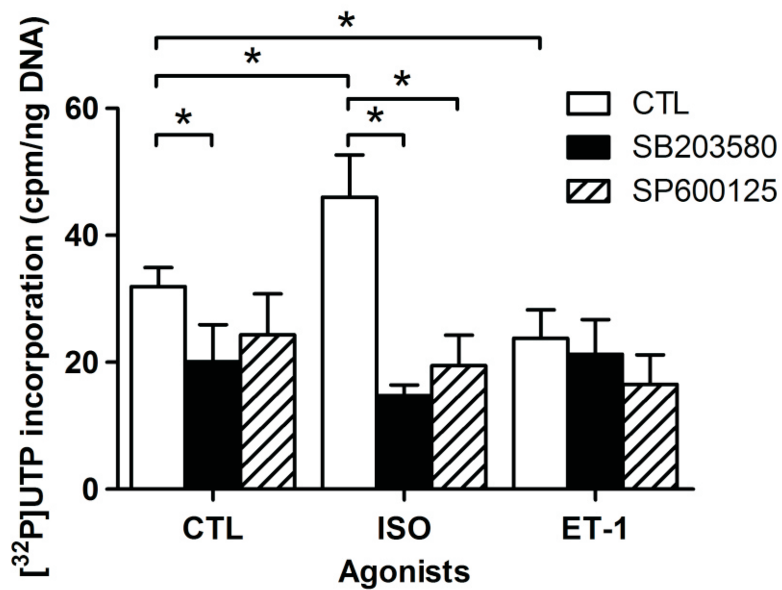


Figure 2

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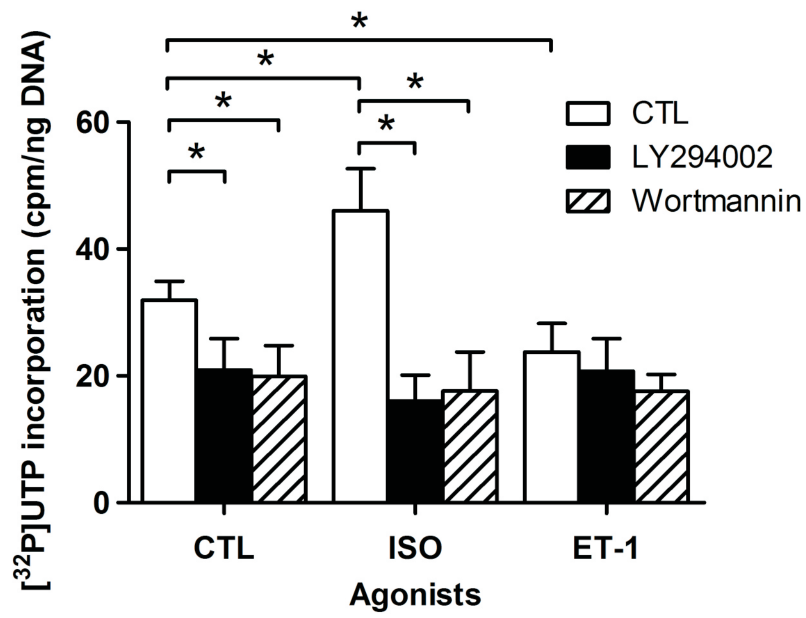

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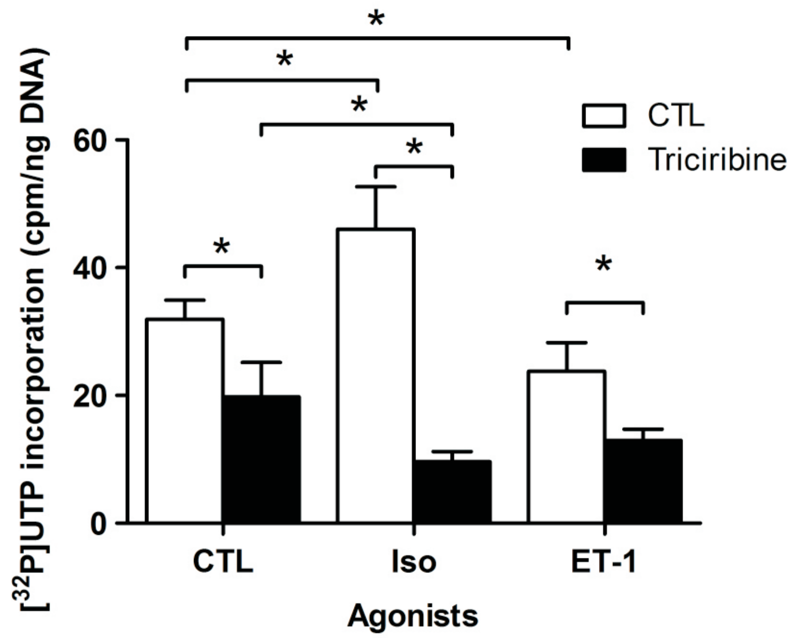


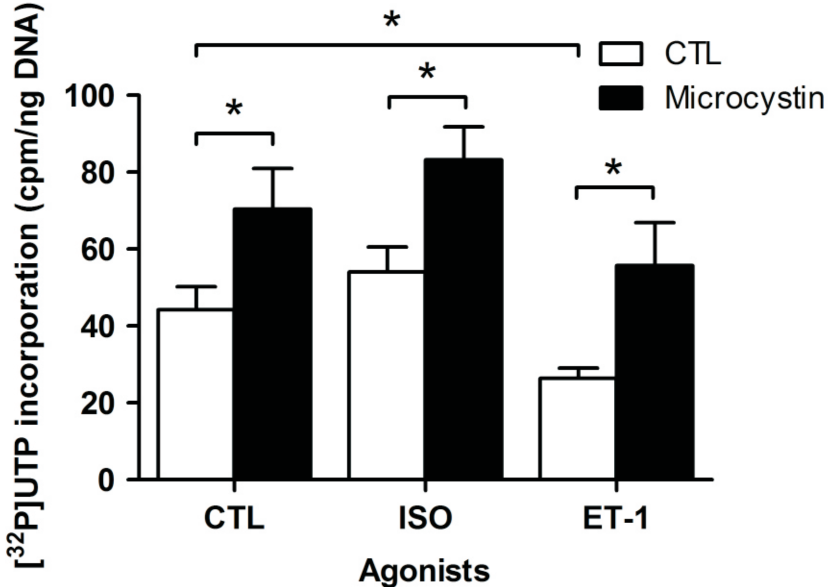
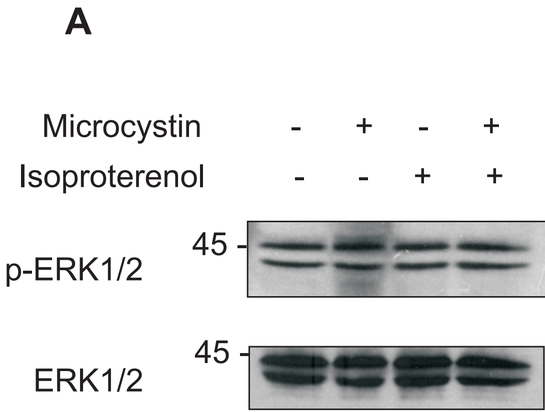
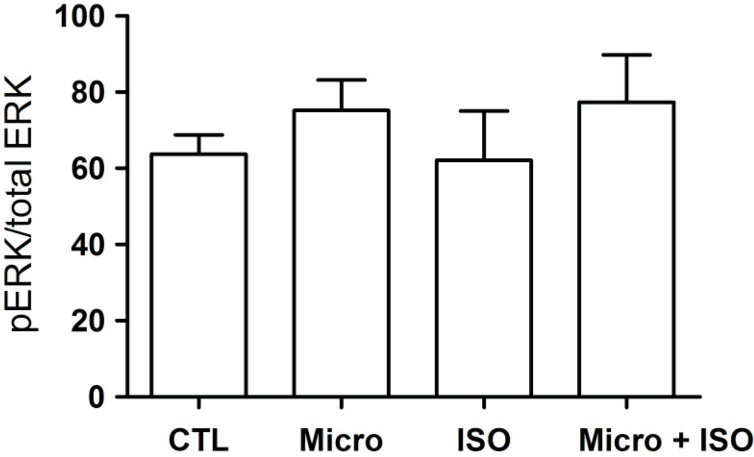

[32P]UTP incorporation (cpm/ng DNA)
100
80
60
40
20
0
CTL
Microcystin
CTL
ISO
ET-1
Agonists
*
*
*
*

Figure 4

A





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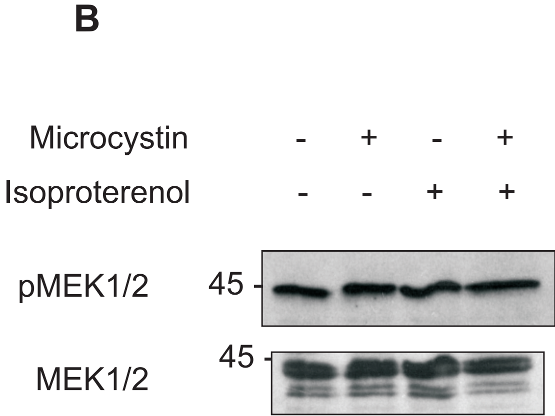


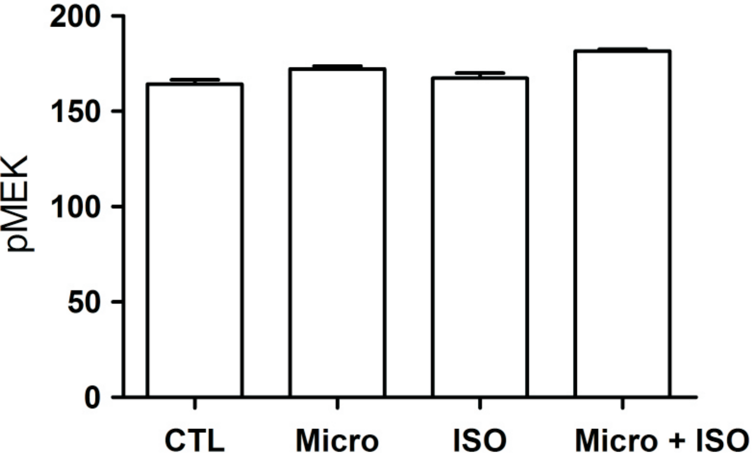
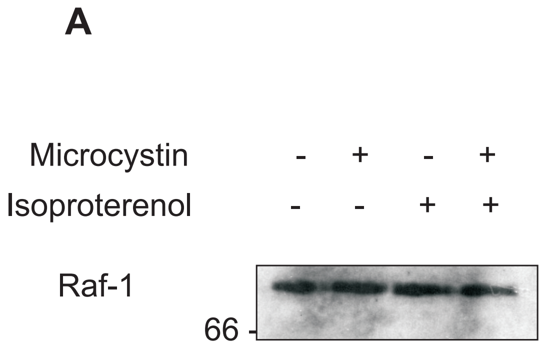
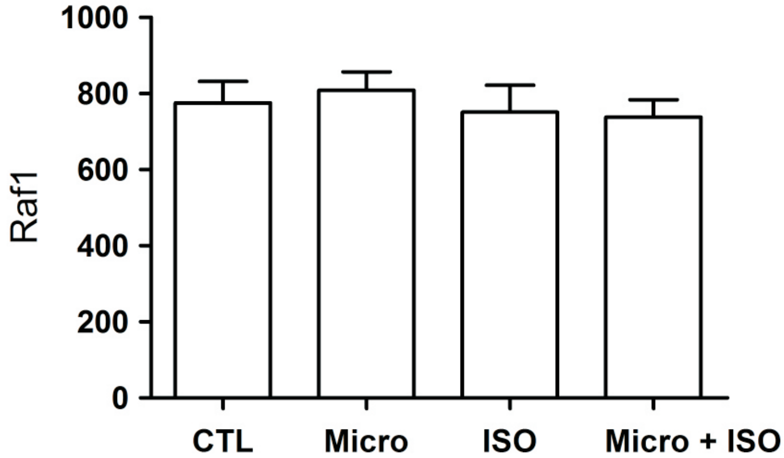


Figure 5

A





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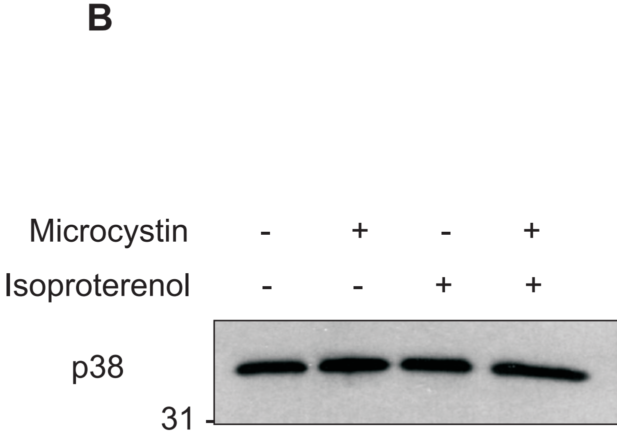

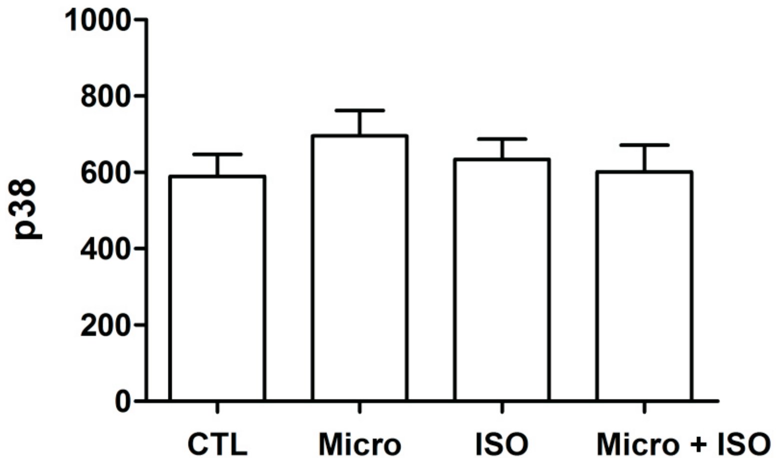

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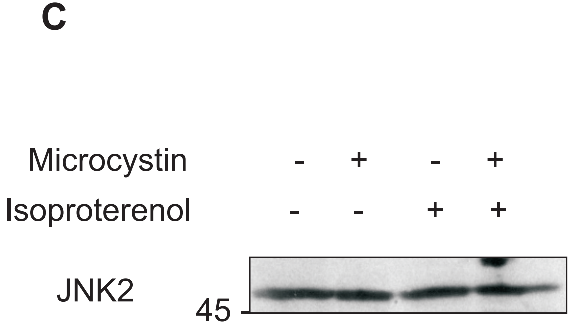


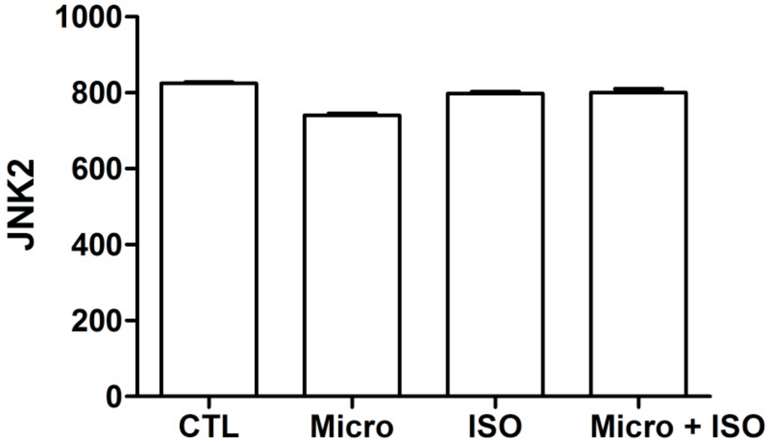
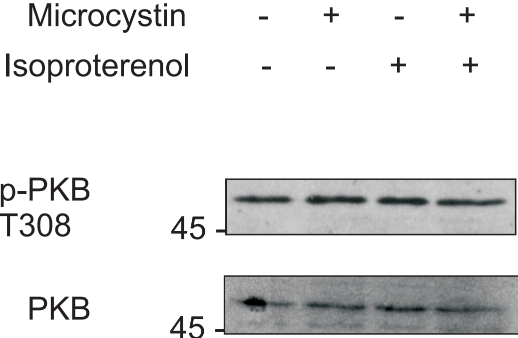
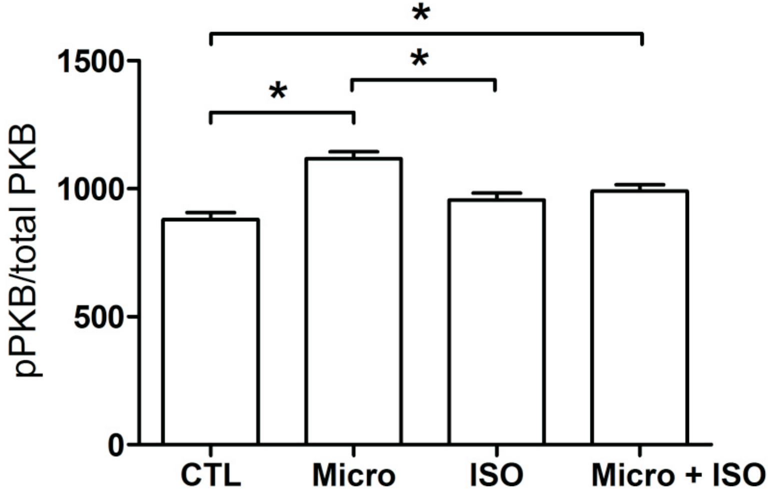


Figure 6

A





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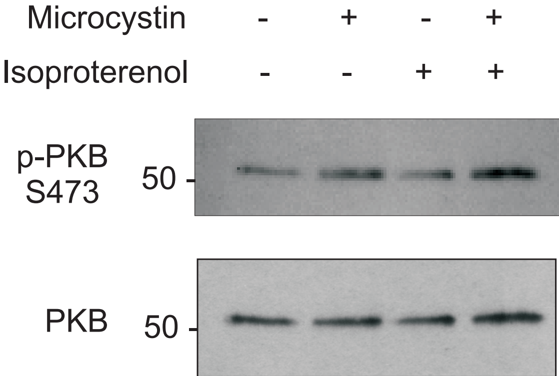


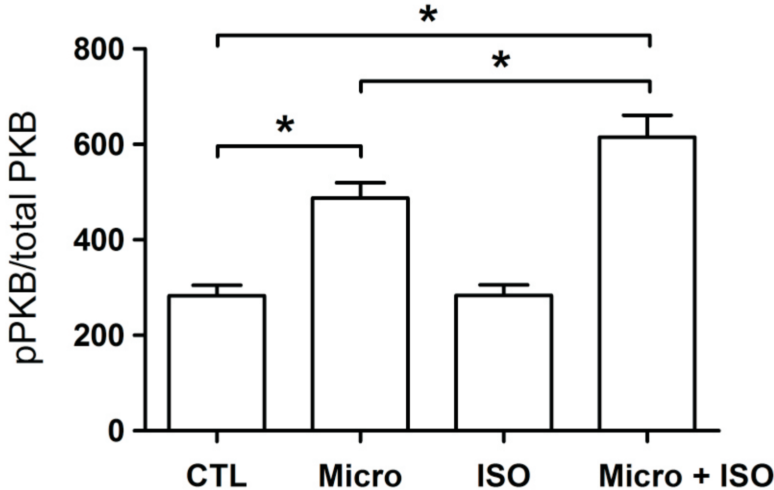
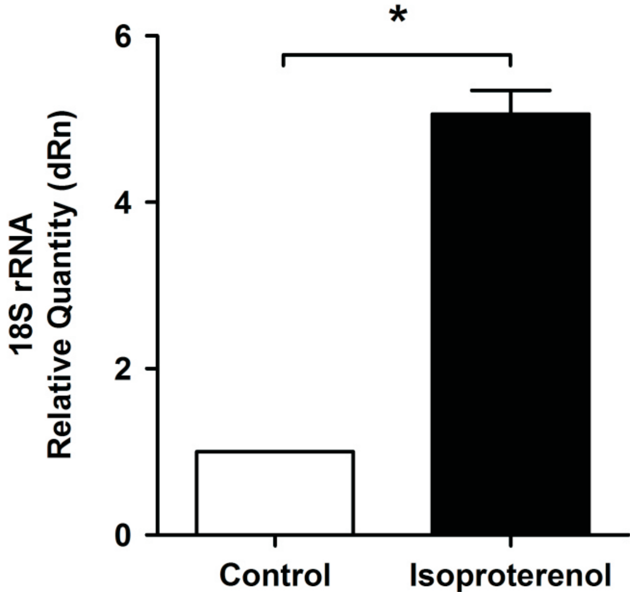


Figure 7



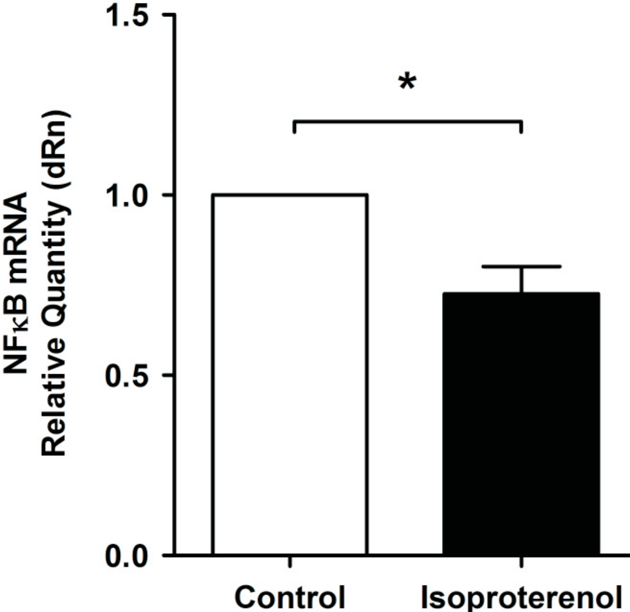
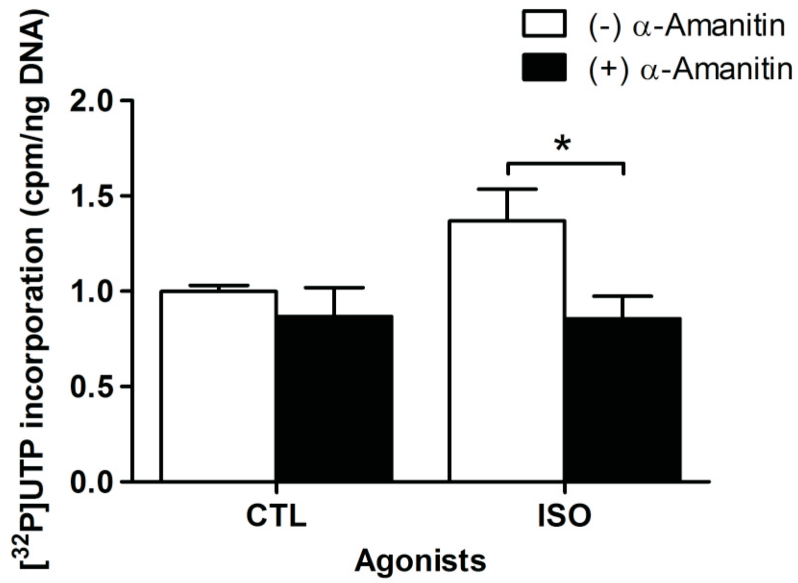
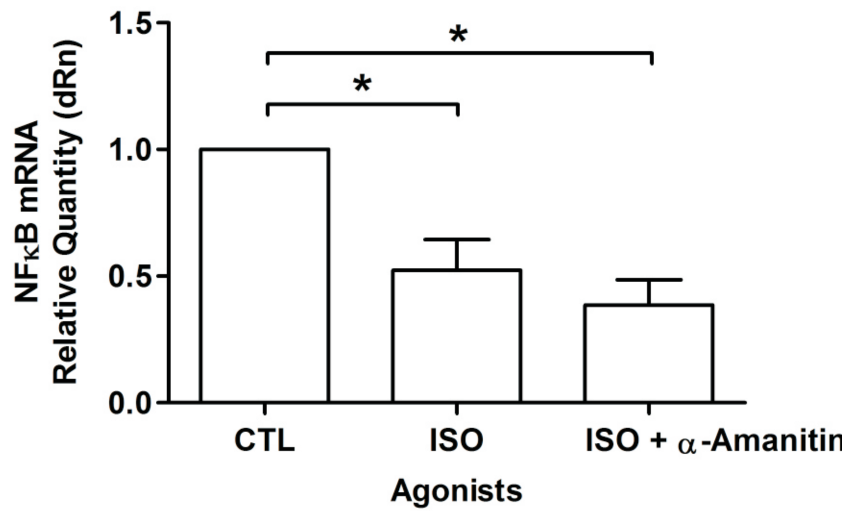


Figure 8





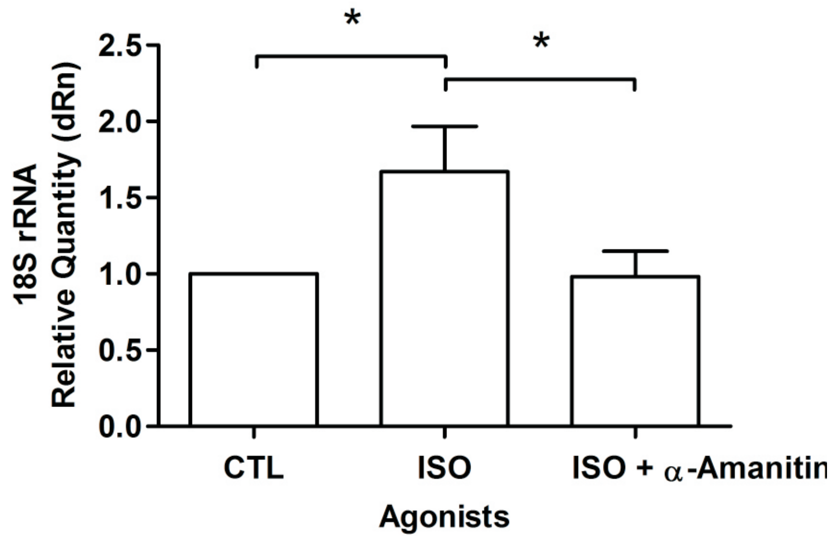
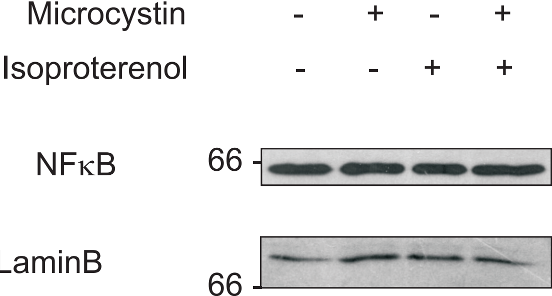


Figure 9



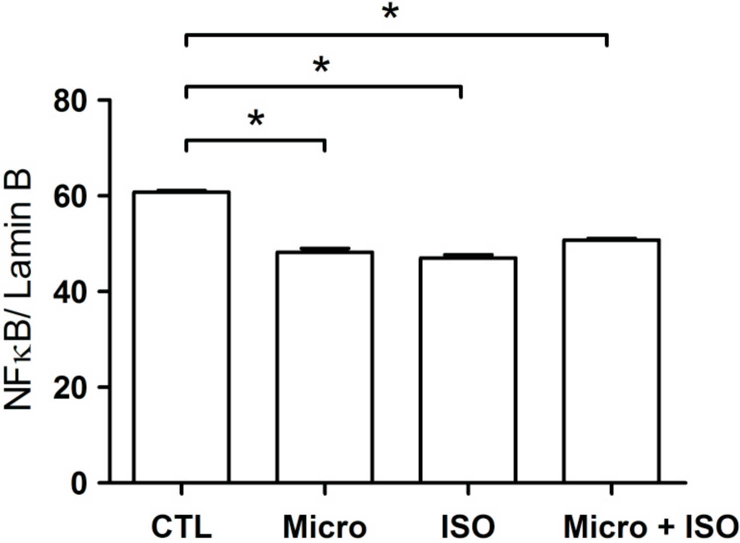


Figure 10

Nucleus

ETR

β3AR

Gi

Raf-1

PI3K

Wortmannin
LY294002

MEK3/6

MEK4/7

MEK1/2

U0126
PD98059

PDK1 ?

SB203580

SP600125

Triciribine

p38

JNK

ERK1/2

PKB

PP2A

Microcystin

Transcription Factors

POLI

POLII

α-Amanitin

↑18S

↓ NFκb
↓ ATF-2
↓ IL1r1
↑ Ripk2
↑ Tnfrsf1b

Stimulatory: →
Inhibitory: ⊣
Unknown: ⋯→

Table 1: Primers used for real-time qPCR

Target	Primers
18S Sense 18S Antisense	5'- ACG GAC CAG AGC GAA AGC AT -3' 5'- TGT CAA TCC TGT CCG TGT CC -3'
NFκB Sense NFκB Antisense	5'- CTG CGA TAC CTT AAT GAC AGC G -3' 5'- AAT TTT GGC TTC CTT TCT TGG CT -3'
β-Actin Sense β-Actin Antisense	5'- TTC AAT TCC ATC ATG AAG TGT G -3' 5'- CTG ATC CAC ATC TGC TGG AAG GTG -3'

Table 2

Gene		ISO	ISO + U0126	ISO + Triciribine
Symbol	**Name**			
ATF-2	Activating transcription factor 2	-1.89 (0.042)	1.32 (0.202)	1.18 (0.448)
Casp1	Caspase 1	-1.04 (0.906)	1.43 (0.093)	1.49 (0.045)
Lpar1	Lysophosphatidic acid receptor 1	1.15 (0.376)	1.51 (0.193)	1.28 (0.016)
Il1r1	Interleukin 1 receptor, type 1	-1.20 (0.017)	1.16 (0.405)	-1.16 (0.776)
Irak2	Interleukin-1 receptor-associated kinase 1	-1.21 (0.615)	2.12 (0.012)	1.25 (0.310)
Jun	Jun oncogene	-1.45 (0.176)	1.70 (0.086)	1.04 (0.975)
Tnfsf14	Tumor necrosis factor superfamily, member 14	-1.12 (0.648)	1.16 (0.416)	1.43 (0.069)
Myd88	Myeloid differentiation primary response gene 88	-1.22 (0.489)	1.42 (0.007)	1.22 (0.194)
Ippk	Inositol 1,3,4,5,6 pentakisphosphate 2-kinase	1.27 (0.585)	2.17 (0.003)	1.20 (0.305)
Ripk2	Receptor-interacting serine-threonine kinase 2	1.33 (0.023)	1.11 (0.562)	-1.10 (0.459)
Tlr6	Toll-like receptor 6	1.51 (0.228)	1.16 (0.418)	1.43 (0.068)
Tnfrsf1b	Tumor necrosis factor receptor superfamily 1b	-2.15 (0.049)	1.28 (0.147)	1.36 (0.066)
Tradd	Tnfrsf1a-associated via death domain	1.74 (0.071)	1.11 (0.544)	1.12 (0.523)
Traf3	TNF receptor-associated factor 3	1.10 (0.832)	-1.33 (0.008)	-1.32 (0.219)

Numeric values indicate calculated fold change in gene expression. *P* values are indicated in parentheses.

CHAPTER 5

Vaniotis G, Glazkova I, Merlen C, Smith C, Villeneuve , Chatenet D, Therien M, Fournier A, Tadevosyan A, Nattel S, Hébert TE and Allen BG. "Regulation of cardiac nitric oxide signalling by nuclear β-adrenergic and endothelin receptors."

Contribution: Figures 1-7, SF1-3

Caged compounds made by David Chatenet, Michel Therien and Alain Fournier

Figure 8 provided by Irina Glazkova and Carter Smith

Figure 9 provided by Clemence Merlen

Regulation of cardiac nitric oxide signaling by nuclear β-adrenergic and endothelin receptors*

George Vaniotis[a,c], Irina Glazkova[d,§], Clémence Merlen[a,c,§], Carter Smith[d], Louis R. Villeneuve[c], David Chatenet[e], Michel Therien[f], Alain Fournier[e], Artavazd Tadevosyan[b,c], Phan Trieu[d], Stanley Nattel[c,d], Terence E. Hébert[d] and Bruce G. Allen[a,b,c,d]

From the Departments of [a]Biochemistry and [b]Medicine, Université de Montréal, Montreal, Quebec H3T 1J4, Canada; [c]Montreal Heart Institute, Montréal, Québec, H1T 1C8, Canada; [d]Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, H3G 1Y6, Canada; [e]Laboratoire d'Études Moléculaires et Pharmacologiques des Peptides, Institut National de la Recherche Scientifique – Institut Armand-Frappier, Université du Québec, Laval, Quebec, H7V 1B7, Canada; and [f]Zamboni Chemistry Solutions, Department of Chemistry, McGill University, Montreal, Quebec, Canada

[§] Both authors contributed equally to this work

To whom correspondence should be addressed:

Terence E. Hébert, Department of Pharmacology and Therapeutics, McGill University Faculty of Medicine, McIntyre Medical Sciences Building, 3655 Promenade Sir William Osler, Room 1303, Montréal, Québec, H3G 1Y6, Canada.

Bruce G. Allen, Department of Medicine, Montreal Heart Institute, Université de Montréal, 5000 Belanger St., Montréal, Québec, H1T 1C8, Canada.

Running head: Nuclear GPCRs regulate NO in cardiomyocytes

Abstract

At the cell surface, βARs and endothelin receptors can regulate nitric oxide (NO) production. β-adrenergic receptors (βARs) and type B endothelin receptors (ETB) are present in cardiac nuclear membranes and regulate transcription. The present study investigated the role of the NO pathway in the regulation of gene transcription by these nuclear G protein-coupled receptors. Nitric oxide production and transcription initiation were measured in nuclei isolated from adult rat heart. The cell-permeable fluorescent dye 4,5-diaminofluorescein diacetate (DAF2 DA) was used to provide a direct assessment of nitric oxide release. Both isoproterenol and endothelin increased NO production in isolated nuclei. Furthermore, a β_3AR-selective agonist, BRL 37344, increased NO synthesis whereas the β_1AR-selective agonist xamoterol did not. Isoproterenol increased, whereas ET-1 reduced, *de novo* transcription. The NO synthase inhibitor L-NAME prevented isoproterenol from increasing either NO production or *de novo* transcription. L-NAME also blocked ET-1-induced NO-production but did not alter the suppression of transcription initiation by ET-1. Inhibition of the cGMP-dependent protein kinase (PKG) using KT5823 also blocked the ability of isoproterenol to increase transcription initiation. Furthermore, immunoblotting revealed eNOS, but not nNOS, in isolated nuclei. Finally, caged, cell-permeable isoproterenol and endothelin-1 analogs were used to selectively activate intracellular β-adrenergic and endothelin receptors in intact adult cardiomyocytes. Intracellular release of caged ET-1 or isoproterenol analogs increased NO production in intact adult cardiomyocytes. Hence, activation of the NO synthase/guanylyl cyclase/PKG pathway is necessary for nuclear β_3ARs to increase *de novo* transcription. Furthermore, we have demonstrated the potential utility of caged receptor ligands in selectively modulating signaling via endogenous intracellular G protein-coupled receptors.

Keywords. β-adrenergic receptors, endothelin receptors, **nuclear** membranes, transcription, Nitric oxide, protein kinase G

1. Introduction

The G protein-coupled receptor (GPCR)[7] superfamily consists of a large group of seven transmembrane domain-containing receptors that signal via heterotrimeric G proteins. GPCRs modulate a wide range of downstream effectors and, as a result, regulate multiple cellular functions in cardiomyocytes. β-adrenergic receptors (βARs) and endothelin receptors (ETRs) are two such members of the GPCR superfamily, both of which are expressed in the myocardium. Recently, potential roles for these receptors when localized to intracellular compartments such as the nuclear membrane have been identified under both physiological and pathological conditions (reviewed in [1, 2]). We have shown that ETB, β_1AR and β_3AR are present on the nuclear membrane in adult cardiomyocytes [3, 4]. In addition, several of their downstream effectors have also been identified at the level of the nucleus or nuclear membrane [5, 6]. These receptors have been shown to bind ligand, couple to effectors, and regulate gene expression in isolated nuclei, with the βARs having a stimulatory effect on transcription initiation, whereas ETB activation is inhibitory [7]. The precise signaling pathways involved in regulation of transcriptional initiation by GPCRs in the nuclear membrane have not been clearly defined and require further study.

Nitric oxide (NO) is an important signaling molecule involved in many physiological processes. NO is produced from the amino acid L-arginine via the action of NO synthases (NOS, [8]. There are three NOS subtypes: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) [8]. All three NOS subtypes are expressed in cardiomyocytes and play roles in cardiac physiology and pathology [9, 10]. In fact, NO has been linked to a wide variety of effects in both the heart and vasculature, from regulation of vascular tone and myocardial contractility to calcium handling and apoptosis [8, 11]. Regulation of NOS activity is complex, involving several mechanisms

mediated by calcium, protein kinases, and NO levels [12, 13]. The exact effect exerted by NO however, appears to depend on the NOS isoform being activated, as well as its subcellular localization and mode of action [11, 14]. NO exerts its effects via modulation of guanylyl cyclase activity leading to increases in cyclic guanosine 3',5'-monophosphate (cGMP) levels and the subsequent activation of protein kinase G (PKG), but has also been shown to signal in a guanylyl cyclase-independent manner [15]. NO has also been implicated in the regulation of gene expression through the transcriptional regulator nuclear factor κB (NF-κB) [15, 16]. In addition, recent findings have also demonstrated that the PI3K/PKB pathway, which is activated by nuclear βARs [7], is capable of activating both eNOS and iNOS, leading to the stimulation of NO production [17, 18]. Moreover, iNOS upregulation in the nucleus appears to be linked to $G\alpha_i$, the protein kinase ERK1/2, and potentially eNOS as well [19-21]. Furthermore, a link has also been established between NO production and both ETA and ETB [22] as well as the βARs [10, 23] localized at the cell surface. Additionally recent evidence has also shown a role for NO in the nucleus, where it appears to modulate calcium homeostasis and is also potentially regulated by ET-1 [20, 24].

Given the involvement of NO in the regulation of cardiac function, and its established link with both the ETRs and βARs, we wished to ascertain whether the NO pathway was involved in the regulation of gene expression observed following stimulation of nuclear ETRs and βARs, and to identify which components of NO signaling might be implicated. Toward this end, we used a pharmacologic approach to study NO production in both isolated nuclei and intact cardiomyocytes following treatment with various agonists and inhibitors. Further, we demonstrate the potential utility of caged receptor ligands in selectively modulating nuclear signaling via GPCRs.

2. Materials and Methods

2.1. Materials

Triton X-100 (TX-100), leupeptin, PMSF and DNase I were from Roche Applied Science (Laval, Quebec). Isoproterenol, BRL 37344, CGP20712, ICI118551, SR59230A, 8-bromo-cGMP, Rp-8-Br-PET-cGMPS (Rp-8-Br) and KT5823 were from Tocris (Ellisville, MO). Endothelin-1 (ET-1) was from Peninsula Labs (Torrance, CA). Pertussis toxin (PTX), xamoterol, forskolin, alprenolol, EEDQ and L-NAME, were from Sigma-Aldrich (Mississauga, Ontario). Triciribine, diaminofluorescein-2 (DAF-2), and diaminofluorescein-2 diacetate (DAF-2 DA) were from Calbiochem. RNaseOut, dNTP Mix, First Strand buffer, and M-MLVRT were from Invitrogen. Primers, as well as SYBR Green and ROX were also from Invitrogen. RNA extraction kits were from Qiagen. DRAQ5 was from Enzo Life Sciences (Farmingdale, NY). nNOS- and eNOS-selective antibodies were from Cell Signaling Technology and BD Transduction Laboratories, respectively. Horseradish peroxide (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence (ECL) reagent Renaissance Plus was from Perkin Elmer Life Sciences (Woodbridge, Ontario). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and nitrocellulose (0.22 μm) were from Bio-Rad Laboratories (Mississauga, Ontario). Bovine aortic endothelial cells (BAEC) were a generous gift from Dr. Martin Sirois (Montréal Heart Institute). Unless otherwise stated, all reagents were of analytical grade and were purchased from VWR Canlab (Ville Mont-Royal, Quebec) or Fisher Scientific (Mississauga, Ontario). [α^{32}P]UTP (specific activity 3000 Ci/mmol) and [^{125}I]CYP were from Perkin Elmer (Montreal, Quebec).

2.2. Synthesis of caged isoproterenol

Previous studies have shown that it is possible to create caged versions of isoproterenol [25]. We wanted to engineer a more hydrophobic version that would be more readily absorbed by cells before uncaging with UV light. The caged compounds were prepared by treatment of the amine starting material, isoproterenol, with the corresponding 2-nitrobenzyl bromide in dimethylsulfoxide and potassium carbonate as shown in Figure 1. After purification by silica gel column chromatography, the desired amines were treated with HCl in dioxane to obtain the hydrochloride salt. To (-)-isoproterenol hydrochloride, (150 mg) in DMSO (1.8 mL), was added 2-bromomethyl-4-methoxy-1-nitrobenzene (149 mg) and potassium carbonate (169 mg). The mixture was stirred overnight, then diluted with dichloromethane, washed with water (2X), brine and dried over Na_2SO_4, filtered, and the solvent evaporated. Purification by silica gel chromatography yielded 81 mg (R)-4-[1-hydroxy-2-[isopropyl-(5-methoxy-2-nitrobenzyl)-amino]-ethyl]-benzene-1,2-diol which was dissolved in diethyl ether and treated with 4 M HCl in dioxane (0.16 mL) to form a precipitate which was stirred for 1 h, then filtered and air dried, affording the desired (R)-4-[1-hydroxy-2-[isopropyl-(5-methoxy-2-nitrobenzyl)-amino]-ethyl]-benzene-1,2-diol hydrochloride. This compound is referred to herein as ZCS-1-67.

2.3. Isolation of nuclei

Rat cardiac nuclei were isolated and the purity of the nuclear preperation was tested as described previously [3, 26]. Briefly, rat hearts were pulverized under liquid nitrogen, resuspended in cold PBS, and homogenized (Polytron, 8000 rpm; 2 × 10 s). All subsequent steps were carried out on ice or at 5 °C. Homogenates were centrifuged for 15 min at 500 ×g. The supernatants were diluted 1:1 with buffer A (10 mM K-

HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 25 μg/mL leupeptin, 0.2 mM Na_3VO_4), incubated 10 min on ice, and centrifuged for 15 min at 2000 ×g. The resulting supernatant was then discarded. The pellet was resuspended in buffer B (0.3 M K-HEPES pH 7.9, 1.5 M KCl, 0.03 M $MgCl_2$, 25 μg/mL leupeptin, 0.2 mM Na_3VO_4), incubated on ice for 20 min, and centrifuged for 15 min at 2000 ×g. The pellet, an enriched nuclear fraction, was resuspended in buffer C (20 mM Na-HEPES pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT, 25 μg/mL leupeptin, 0.2 mM Na_3VO_4) or 1× transcription buffer (50 mM Tris pH 7.9, 0.15 M KCl, 1 mM $MnCl_2$, 6 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 1 U/μL RNAse inhibitor) and used fresh.

2.4. Measurement of NO production

Isolated nuclei were preincubated with the fluorescent dye DAF-2 (5 μg/mL) in a buffer containing 140 mM NaCl, 14 mM glucose, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.8 mM $MgSO_4$, 1.8 mM KH_2PO_4, and 0.1 mM L-arginine (final pH 7.4) for 30 min at 37 °C. Nuclei were washed twice in standard buffer to remove any unbound dye and then resuspended in our nuclear isolation buffer C. The inhibitors used were L-NAME (1 mM, 30 min at 37 °C), PTX (5 μg/mL, 2 h at 37 °C), KT5823 (1 μM, 30 min at 37 °C), Rp-8-Br-PET-cGMPS (1 μM, 1 h at 37 °C) and triciribine (1 μM, 30 min at 37 °C). Pre-treatment with subtype-selective βAR antagonists CGP20712 (5 nM, 30 min at 37 °C), ICI 118,551 (10 nM, 30 min at 37 °C) and SR59230A (1 μM, 30 min at 37 °C) was also performed in certain experiments. Nuclei were then treated with either isoproterenol (1 μM), ET-1 (10 nM), BRL 37344 (1 μM), xamoterol (1 μM), forskolin (100 nM) or vehicle for 30 min at 37 °C. DAF fluorescence, indicating NO production, was measured using a microplate reader at wavelengths of 485 nm (excitation) and 510 nm (emission) and expressed as arbitrary fluorescence units (AU).

2.5. Transcription initiation

Measurements of transcription initiation were performed as previously described [4]. Briefly, 10 μL of freshly isolated nuclei (resuspended in 1× transcription buffer) were incubated at 30 °C for 30 min in a final volume of 20 μL containing agonist/antagonist and 10 μCi [α^{32}P]UTP (3000 Ci/mmol) in the absence of CTP and GTP to prevent chain elongation. Following termination of reactions by digestion with DNase I, nuclei were lysed with 10 mM Tris-HCl pH 8.0, 10 mM EDTA and 1% SDS. Duplicate 5 μL aliquots were transferred onto Whatman GF/C glass fibre filters, washed twice with 5% TCA containing 20 mM sodium pyrophosphate, and air-dried. Incorporation of [^{32}P] was determined by liquid scintillation counting. DNA concentrations were determined by spectrophotometry. [^{32}P] incorporation was expressed as dpm/ng DNA. Where indicated, isolated nuclei were pre-treated at 37 °C with 1 mM L-NAME, 1 μM KT5823, 1 μM Rp-8-Br, 100 μM cGMP or vehicle for 30 min.

2.6. Radioligand binding assays

Membranes prepared from naive HEK 293 cells and from a stable HEK 293 cell line expressing the β_2AR and preincubated with 100 μM EEDQ or vehicle for 2 h at 37 °C. Briefly, cells were washed twice with cold PBS and then 10 mL of lysis buffer (5 mM Tris-HCl pH 7.4, 2 mM EDTA, with protease inhibitor cocktail) was added to each flask. Cells were disrupted using a Polytron (2 × 10 s) on ice, and then centrifuged for 5 min at 1000 rpm and 4 °C. The lysate was centrifuged at 16000 rpm for 20 min at 4 °C and the pellet resuspended in 1 mL of binding buffer (75 mM Tris-HCl pH 7.4, 2 mM EDTA, 12.5 mM $MgCl_2$). Membranes from whole rat heart were prepared as previously described [27]. Briefly, hearts were pulverized under liquid nitrogen then homogenized

by polytron in 15 mL of ice cold lysis buffer (5 mM Tris-HCl, pH 7.4, 2 mM EDTA, plus protease inhibitor cocktail). The resulting homogenate was centrifuged at 500 × g for 15 min at 4 °C. The supernatant was centrifuged at 45000 ×g for 15 min, and the pellet resuspended in binding buffer. [^{125}I]CYP (50 μL, 400,000 cpm) was added to 10 μL of membranes in a total volume of 0.5 mL, in triplicate, for each condition. Alprenolol (10 μM) was used to measure non-specific binding. In some cases, membranes were treated with ISO (1 μM), caged or uncaged ZCS-1-67 (1 and 5 μM) were used to compete with specific CYP binding. Membranes were incubated at room temperature for 90 min and subsequently captured and washed using a Brandel cell harvester. [^{125}I]CYP binding was quantified using a γ-counter.

2.7. EEDQ treatment

Calcium-tolerant cardiac ventricular cardiomyocytes were isolated from adult male Sprague-Dawley rats by Langendorff perfusion as previously described [28]. Freshly isolated cardiomyocytes were treated with either 100 μM EEDQ or vehicle (2 h, 37 °C) to irreversibly alkylate β-adrenergic receptors present at the cell surface, followed by treatment with either 1 μM isoproterenol or vehicle (30 min, 30 °C).

2.8. Quantitative real-time PCR

RNA was isolated from either purified nuclei or isolated myocytes using the Qiagen RNA extraction kit. To prepare cDNA, 1 μg of RNA was mixed with 100 ng of random primers and 1 μL of 10 mM dNTPs in a final volume of 10 μL, heated to 65 °C for 5 min, and then immediately quick chilled on ice. Next, 4 μL of 5× first strand buffer, 2 μL of 0.1 M DTT and 1 μL of RNAse Out were added and the reactions were then incubated for 2 min at 37 °C. Following addition of 1 μL of M-MLVRT (200 units), reactions were mixed, centrifuged and then incubated for 10 min at 25 °C, then at

37 °C for 50 min, and finally at 70 °C for 15 min. qPCR reactions, containing 12.5 µL of SyBR Green PCR master mix, 0.03 µL ROX dye (an internal fluorescence standard), 2.5 µL of primers, and 10 µL of cDNA were for 1 cycle of 10 min at 95 °C, then 40 cycles of (30 s at 95 °C, followed by 1 min at 60 °C and 1 min at 72 °C) using a Stratagene Mx3000P system. Samples were assayed in duplicate and normalized to β-actin expression. Primers used for real-time qPCR are shown in Table 1. The selectivity of the primers for a single product was validated by dissociation curve analysis.

2.9. Measurement of NO production in intact cells

Left ventricular cardiomyocytes were isolated from adult male rats as described previously [28] and plated on laminin-coated glass-bottomed culture dishes for 1 h at 37 °C (95% O_2, 5% CO_2) and 30 min at 4 °C. Cardiomyocytes were then incubated with DAF-2 DA (10 µM) in 20 mM HEPES pH 7.4, 134 mM NaCl, 6 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1% BSA (buffer D) for 30 min at room temperature and in the absence or presence of a photolabile caged isoproterenol (ZCS-1-67, 30 µM) or a photolabile caged ET-1 analog ([Trp-ODMNB^{21}]-ET-1, 1.5 µM) [29] and either the NO synthase inhibitor L-NAME (1 mM) or vehicle, as indicated. Cardiomyocytes were then washed three times with buffer D and a cell-permeable fluorescent DNA dye, DRAQ5 (1 µM), was added and L-NAME was re-added where indicated. Fluorescence imaging was performed using a Zeiss LSM 7 Duo microscope (combining LSM 710 and Zeiss Live systems) with a 63x/1.4 oil Plan-Apochromat objective. DAF-2 DA was excited with a 488 nm/100 mW diode (2-3% laser intensity) and fluorescence emitted between 495 nm and 550 nm was collected. Cells were scanned approximately every 10 s. Pixel size was set at 0.264 µm and the pinhole at 2 Airy units. To visualize the nucleus, DRAQ5 was excited with a 635 nm/50 mW diode and fluorescence emitted at >655 nm was collected. DAF-2 DA and DRAQ5 were

excited and fluorescence collected simultaneously using 2 different Zeiss Live detectors. Images were acquired over a total period of 9 min (60 frames). After acquiring 13 frames (114 s) to establish a baseline, cET-1 or ZCS-1-67 was photolysed by administering a 4 s pulse of UV light using a 405 nm/30 mW diode (100% laser intensity). DRAQ5 emissions were used to focus the UV laser into a 60 μm^2 rectangular region overlapping the nucleus. The microscope stage (Zeiss Observer Z1) was equipped with a BC 405/561 dichroic mirror that allowed simultaneous photolysis of ZCS-1-67 or caged ET-1 (LSM 710 405 nm laser) and image acquisition (Zeiss Live). Cardiomyocytes were maintained at 35-36 °C, using a stage incubator and objective heater, for the duration of image acquisition.

2.10. EPAC Assays for cAMP detection

HEK 293 cells were plated onto 6-well plates at least 24 h prior to transfection. The cells were transfected with 3 μg of EPAC construct [30] and 1 μg of FLAG-tagged β_2AR using 5 μL of Lipofectamine 2000. 72 h post transfection, the cells were washed twice with PBS and resuspended in 500 μl of PBS. Cell suspensions (80 μL) were distributed in 96-well Opti-plates and left to incubate for 2 h at room temperature. Fluorescence was then measured using a Synergy2 (Biotek) microplate reader. Immediately after reading the fluorescence, the cells were incubated with coelenterazine h (final well concentration 50 μM) and total luminescence and BRET ratios were collected for 5 min. The average of these BRET ratios represents basal BRET of the cells. ZCS-1-67 was exposed to 15 minutes of UV light (black-ray long wave, model B100AP lamp, Thermo-Fisher) 3.0 cm above the plate to uncage compounds prior to being added to the assay plates. The cells were then stimulated by the addition of 10 μL of 100 μM isoproterenol prepared in 100 μM of ascorbic acid (final isoproterenol concentration 10 μM) or with 10 μl of 100 μM ascorbic acid (vehicle) and BRET ratios

were read for 30 min. Upon completion of the assay, the final five BRET readings were averaged and taken to represent the final average BRET. The net BRET for agonist or vehicle treatment was calculated by subtracting basal BRET from the final average BRET. The ΔBRET for each transfection was then calculated by subtracting the net BRET of agonist from respective net BRET of vehicle.

2.11. Immunoblotting

SDS-PAGE and immunoblotting were performed as previously described [31]; however, nitrocellulose membranes were employed in the present studies.

2.12. Statistical analysis

Data are presented as the mean ± the standard error of the mean (S.E.M.). The significance of differences between groups was determined using one-way ANOVA followed by Tukey's multiple comparison tests (Prism 4.0cx, GraphPad Software). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Measurement of NO production

The link between plasma membrane GPCR signaling and nitric oxide (NO) production has been well characterized for both the ETB [24, 32] and the βARs, including the β_3AR [8, 10]. Hence, given the presence of these receptors on the nuclear membrane, the recapitulation of cell surface signaling pathways in the nucleus (reviewed in [1, 2]) and the demonstrated effects of certain nuclear prostaglandin E2, bradykinin, lysophosphatidic acid type-1 receptors on iNOS and eNOS expression in non-cardiac cells [18-20, 33, 34], we sought to determine if either βARs or ETB also regulated NO production in cardiac nuclei. We first ascertained whether treatment of isolated rat heart nuclei with either isoproterenol or ET-1 resulted in a change in NO levels. Isolated rat heart nuclei were preincubated with the fluorescent dye DAF-2 and then treated with isoproterenol (ISO, 1 μM), ET-1 (10 nM) or vehicle, for 5, 10, 15, and 30 min. An increase in NO production could be detected as early as 5 min after treatment with either agonist, with a maximal response detectable at 30 min (Supplemental Figure 1). Hence, treatment was for 30 min in all subsequent experiments. Given that we observed a time-dependent increase in NO production following agonist treatment, we next wanted to determine if we could block this increase with the non-selective NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME). Toward this end, isolated nuclei were again preincubated with the fluorescent dye DAF-2 and then incubated for 30 min with or without L-NAME (1 mM) before being treated with either ISO (1 μM, 30 min) or ET-1 (10 nM, 30 min, Figure 2A). Again, NO release increased following agonist treatment; however, pre-treatment of nuclei with L-NAME blocked this increase in both ISO and ET-1 treated nuclei. Immunoblot experiments using antibodies against the two constitutive NOS isoforms revealed that it is likely that

eNOS is responsible for this effect, as eNOS immunoreactivity was detected in enriched nuclear preparations whereas nNOS was not (Supplemental Figure 2). Pre-treatment of isolated nuclei with two inhibitors of PKG, KT5823 or Rp-8-Br, did not alter the ability of ET-1 or ISO to increase NO release (Supplemental Figure 3). However, treatment with either inhibitor alone resulted in an increase in NO production, indicating that PKG may inhibit NOS activity. Negative-feedback regulation of NOS activity by PKG has been observed previously in intact vascular endothelial cells [35, 36]. These results clearly demonstrate that both ETB and βARs can increase NOS activity in isolated cardiac nuclei, likely through activation of eNOS.

Given that two βAR subtypes, β_1AR and the β_3AR, were shown to be present at the level of the nuclear membrane [4], we next sought to determine which of these two βAR subtypes was responsible for the increase in NO production following ISO treatment. Thus, isolated nuclei preincubated with DAF-2 were treated with a β_1AR specific agonist, xamoterol, a β_3AR specific agonist, BRL37344, or forskolin, which can directly activate adenylyl cyclase (AC), bypassing the receptor (Figure 2B). Interestingly, the β_3AR-specific agonist had an effect similar to ISO, whereas xamoterol and forskolin had no appreciable effect on DAF-2 fluorescence. This was further confirmed with specific antagonists against all three βAR subtypes (Figure 2C). As expected, the β_3AR antagonist SR59230A was able to inhibit the increase in NO production produced by ISO while the β_1AR antagonist CGP20712 had no significant effect. Interestingly, the specific β_2AR antagonist ICI 118,551 also inhibited the ISO stimulated increase indicating that there may be differences in receptor selectivity between the nucleus and plasma membrane. We previously showed that ligand selectivity was altered when multiple βAR subtypes were co-expressed in the same cells [37]. Further study would be required to more carefully examine pharmacological differences between receptors on the nucleus versus the plasma membrane. Taken

together, our data indicates that ISO regulates NOS activity via β_3AR whereas the β_1AR-mediated AC/cAMP pathway is not directly involved in isolated cardiac nuclei.

We next wished to determine which heterotrimeric G protein mediated the effects of β_3AR stimulation upon NOS activity in the nuclear membrane. Given that the AC/cAMP pathway did not seem to play a role, and that both the nuclear β_3AR [4] and ETB [38] can activate Gαi, we used pertussis toxin (PTX) to block Gαi activation. Of note is the fact that we have already shown that PTX inhibits ISO-stimulated increases in transcription initiation by β_3AR, demonstrating the interaction between the receptor and Gαi [4]. Following preincubation with DAF-2, we pre-treated isolated nuclei with PTX (5 μg/mL, 2 h) and then treated with either ISO or ET-1. Interestingly, PTX treatment alone was able to increase the basal level of NO production in isolated nuclei, and to such an extent that further treatment with either agonist had no further appreciable effect (Figure 3A). This suggests that, in the absence of receptor stimulation, basal Gαi tone integrating inputs from several nuclear Gi-coupled GPCRs may play a role in modulating NOS activation and maintaining the basal levels of NO release. PTX prevents Gαi from functionally coupling with these different receptors might explain why no further increase is seen in the agonist treated samples, as both stimulatory and inhibitory inputs would both be lost. Identifying other putative Gαi-coupled receptors at the level of the nucleus leading to additional regulation of NO production would be an interesting avenue for future investigation. Alternatively, interactions with other G proteins, potentially Gαq in the case of ETB, might also be involved in regulating NOS activity.

Given the established link between PKB and ETRs [39] and nuclear βARs [7], as well as the well-characterized regulation of NOS activation by PKB [17, 21], we next examined the effect of PKB inhibition on NO production in isolated cardiac nuclei. Toward this end, isolated nuclei were loaded with DAF-2, then pre-treated with the PKB

inhibitor triciribine (1 μM, 30 min), and finally treated with either ISO or ET-1. While treatment with either agonist lead to an increase in NO production, concomitant treatment with triciribine actually potentiated this effect, for both agonists, although to a lesser extent for ET-1 (Figure 3B). Treatment with triciribine alone, however, had no effect on basal NO production. Hence, PKB inhibition can lead to an increase in NO production, but only when the receptors are activated: basal activity was unaffected. The exact mechanism responsible for the potentiation remains to be elucidated. However, this might be explained in part by the mode of action of triciribine, which inhibits PKB activation, but whose exact molecular target is unknown. This would also leave the upstream effectors of PKB active; hence if they are activating other kinases as well they may be compensating for the lack of PKB activity. In addition while PKB is known to activate NO production in the cytoplasm, it is entirely possible that a different signaling mechanism exists at the level of the nucleus. We have previously shown that the βARs activate the PI3K/PKB signaling pathway in isolated nuclei and that treatment with triciribine profoundly alters the modulation of transcription initiation, switching ISO from an agonist to an inverse agonist [7].

3.2. Transcription initiation

We next determined if NOS activation played a role in the previously described effects of ISO and ET-1 on transcription initiation in isolated cardiac nuclei [4, 7]. Thus, isolated nuclei were treated with either ISO (1 μM) or ET-1 (10 nM) in the presence or absence of L-NAME (1 mM). Transcription was assessed by measuring [α^{32}P]UTP incorporation. As previously reported, ISO increased *de novo* transcription (Figure 4A). L-NAME inhibited both the basal and ISO-induced increase in *de novo* transcription. ET-1, in the absence or presence of L-NAME, reduced [^{32}P] UTP incorporation to a level comparable to that induced by L-NAME alone. Hence, although ISO and ET-1

both increased NO release in isolated nuclei, they induced opposite effects on the initiation of global transcription, indicating there exists additional complexity in the organization of the two pathways.

NO exerts its effects by activation of soluble guanylyl cyclase and, possibly, by S-nitrosylation of cysteine residues in target proteins (see [11]). Hence cGMP-mediated activation of PKG may represent a means whereby activation of either β_3AR or ETB is transduced into changes in promoter activity. To further investigate this possibility, we examined the effect of PKG inhibition on transcription. Isolated nuclei were treated with either ISO (1 μM), ET-1 (10 nM) or cGMP (100 μM) in the presence or absence of 1 μM KT5823 or 1 μM Rp-8-Br, PKG inhibitors, and transcription initiation was assessed as [α^{32}P]UTP incorporation (Figure 4B). As with L-NAME, KT5823 exhibited an inhibitory effect on *de novo* transcription in the absence of agonist, and was also able to block the stimulatory effect of ISO. KT5823 alone reduced the basal level of [^{32}P]UTP incorporation to levels comparable to those induced by ET-1. While inhibition of PKG resulted in an increase in NO production, it is perhaps not unexpected to see a decrease in transcription as PKG is likely involved in a feedback inhibition loop (discussed above), and thus the increased NO production is likely an attempt to compensate for the inhibition of PKG activity. Unlike KT5823, Rp-8-Br had no significant effect on the basal level of transcription likely due to the fact that it acts as a competitive inhibitor of cGMP and hence has no effect on the pool of already activated PKG. Rp-8-Br did however still inhibit the ISO stimulated increase. Furthermore, in the presence of ET-1 neither of these agents had any additional significant inhibitory effect upon transcription initiation. The inability of ET-1 to increase *de novo* transcription may reflect the fact that ISO increases the abundance of 18S ribosomal RNA (rRNA) [7] whereas ET-1 does not (Figure 4C) and rRNA accounts for approximately 90% of the total RNA pool. Microarray analysis has revealed that ET-1 and ISO regulate transcription of distinct,

but overlapping, populations of genes in isolated cardiac nuclei (GV, TEH and BGA, in preparation). Treatment with cGMP had no significant effect on the basal level of transcription, though a slight inhibition was observed. Taken together, these results raise the possibility that PKG is involved in mediating the effects of nuclear βAR activation upon transcription, though further study is required to determine its exact role. ETB activation, although increasing NO production, may act to suppress the stimulatory effects of the NOS-GC-PKG pathway upon 18S RNA expression through the activation of additional signaling pathways. Alternatively, β_3ARs may activate one or more additional signaling pathways required for the initiation of 18S RNA transcription.

3.3. Nuclear GPCRs in intact cardiomyocytes

We next extended our findings into the context of the intact cardiomyocyte as isolated cardiac nuclei are derived from a heterogenous cell population and, in addition, would lack regulatory elements normally recruited from the cytosol. We have previously shown that the treatment of isolated nuclei with ISO increases the levels of 18S rRNA [7]. Hence, we first ascertained that we could reproduce the changes in 18S rRNA following ISO treatment in the intact cardiomyocyte. To ensure that the changes in 18S rRNA were in fact due to the specific action of ISO upon nuclear βARs, we first treated cardiomyocytes with EEDQ, an impermeable alkylating agent that irreversibly binds and inactivates surface dopamine and adrenergic receptors [40]. In our hands, EEDQ totally ablated binding of the hydrophobic non-selective βAR ligand [^{125}I]CYP to HEK 293 cells transfected with the β_2AR (Supplemental Figure 4) but a small fraction of binding was preserved in cardiomyocytes suggesting an internal pool of βAR is protected (Figure 5A). Following alkylation, cardiomyocytes were incubated with ISO (Figure 5B). As seen previously in isolated nuclei, ISO produced an increase in 18S rRNA and this increase was not completely blocked by EEDQ. This supports the notion

that nuclear βARs are indeed functional in the intact cardiomyocyte independent of the activation status of surface receptors. This notion was further confirmed by measuring levels of Pim-1 mRNA (Figure 5C). Pim-1 is a protein kinase that has been linked to the pro-survival effects of PKB, and has been shown to play a role in apoptosis and transcriptional activation [41, 42]. Pim-1 mRNA was also increased following ISO treatment, and again EEDQ treatment did not completely block this increase. Thus, although ISO is highly hydrophilic, it is able to enter cardiomyocytes. This has already been shown for norepinephrine [43], and it is believed that an active transporter, norepinephrine-uptake-2, is responsible for the uptake of catecholamines into the cardiomyocyte [44]. However, we cannot exclude the possibility that some surface receptors were spared in the treatment with EEDQ and thus we sought another, more direct way to show that internal receptors could be activated in the intact cell context.

To selectively target the nuclear receptors, we used caged agonists for both ETRs (caged ET-1; cET-1, [29]) and βARs (caged isoproterenol; ZCS-1-67). The "cages" are protecting groups that allow these compounds to freely cross the plasma membrane but can be removed by exposure to UV light, resulting in release of the parent compound with no residual structural modification (e.g., ET-1 or ISO). To first test the caged βAR ligand, we performed direct uncaging experiments in solution by exposing the compounds to UV light before incubating them with HEK 293 cells transfected with the β_2AR. As can be seen in Figures 6A and 6B, at concentrations as high as 10 μM, the caged ISO analog, ZCS-1-67, was not able to significantly activate βAR signaling until uncaged by exposure to UV light, as measured by stimulation of cAMP production in an EPAC-based BRET assay. Caged ET-1 has been characterized previously [29]. Similarily, radioligand binding assays in membranes prepared from whole rat heart revealed that at concentrations of 1 and 5 μM, caged ZCS-01-67 produced a non-significant reduction in [^{125}I]CYP binding (Figure 6C). Upon photolysis of 1 μM ZCS-

01-67, no further reduction was observed, whereas photolysis of 5 μM ZCS-01-67 reduced [^{125}I]CYP binding to a similar extent to that observed with 1 μM ISO. These data indicate that, like alprenolol (a non-selective βAR antagonist) and ISO (a non-selective βAR agonist) uncaged ZCS-01-67 competes with [^{125}I]CYP for binding to βAR. Caged ZCS-01-67 may also compete with [^{125}I]CYP, but very weakly in comparison with ISO, alprenolol, or uncaged ZCS-01-67. Taken together, these data indicate that, at best, ZCS-01-67 may act as a very weak partial agonist (which may still compete for binding to surface receptors) for βARs, having both reduced affinity and efficacy.

We next investigated whether we could still detect increases in NO production following ISO treatment in the intact cardiomyocyte. Measurements of intracellular NO release were performed using live-cell confocal fluorescence microscopy. Cardiomyocytes were loaded with either cET-1 or ZCS-1-67. Higher concentrations of the caged ligands were used to compensate for the reduced UV intensity used to uncage them (to preserve cell viability). Once loaded with agonist, cardiomyocytes were washed to remove excess ligand, and subsequently exposed to a 4 s pulse of UV light (405 nm/30 mW diode, 100% laser intensity) in order to photolyse the caged ligands. Cardiomyocytes were also loaded with a cell-permeable analog of DAF-2, DAF-2 DA, which is hydrolysed to DAF-2 by intracellular esterases, and the fluorescent DNA dye, DRAQ5. Changes in intracellular NO were assessed by monitoring DAF-2 fluorescence using a Zeiss LSM 710 DUO confocal microscope: DRAQ5 fluorescence was measured to delineate the nucleus and quantify changes in nuclear DAF-2 fluorescence (Figure 7A). DAF-2 and DRAQ5 fluorescence was acquired simultaneously. Both cET-1 and ZCS-1-67 evoked increases in NO upon photolysis and this increase was blocked by pre-treatment with L-NAME (Figure 7B). Including ETA (BQ610, 1 μM) and ETB (BQ788, 1 μM) antagonists in the extracellular medium did not prevent the effects of

cET-1, indicating that photolysed ET-1 was not being secreted by the cells and acting upon ETRs at the cell surface (CM and BGA, manuscript in preparation), These results reinforce our findings using EEDQ and support the conclusion that nuclear ETB and β_3AR regulate NO production locally in the nucleus, independently of cell surface receptors. The whole cell images reveal that 5 min after photolysis, the increase in DAF-2 fluorescence induced by cET-1 and ZCS-1-67 differed in terms of compartmentalization. cET-1 increased DAF-2 fluorescence within the nucleus to a level similar to that in the surrounding cytosol (Figure 7C, panel 2). This increase was not observed in the vehicle control (also exposed to UV) or in the presence of cET-1 plus L-NAME (Figure 7C, panels 1 and 3, respectively). In contrast, although ZCS-1-67 produced a significant increase in nuclear NO levels (Figure 7A,B), its effects were far more pronounced in extra-nuclear compartments (Figure 7C, panel 4). In addition, ZCS-1-67 induced a greater overall increase in whole-cell DAF-2 fluorescence than observed with cET-1 (data not shown). The increase in DAF-2 fluorescence induced by ZCS-1-67 was also inhibited by L-NAME (Figure 7C, bottom panel).

4. Discussion

We have demonstrated that the ETB and βAR in the nuclear membrane regulate NO production in isolated cardiac nuclei and more importantly, in intact cardiomyocytes. Using a NO-sensitive fluorescent dye, DAF-2, we observed an increase in NO production following treatment with both ISO and ET-1. In addition, pre-treatment with the NOS inhibitor L-NAME blocked the increase in DAF 2 fluorescence, clearly indicating that these two agonists enhance NOS activity in the nucleus. These results implicate NO in the regulatory effects mediated by these two nuclear GPCRs, including calcium handling for the ETRs and regulation of gene expression for the βARs [7, 45].

Nuclear lysophosphatidic acid receptors (LPAR) have been shown to upregulate iNOS expression in porcine cerebral microvascular endothelial cells through the activation of the PI3K/PKB pathway, and in a PTX-sensitive manner [34]. Moreover, in isolated hepatocyte nuclei, this upregulation is dependent on nuclear eNOS activation [20]. Furthermore the nuclear prostaglandin E_2 receptor (EP_3) has been shown to lead to an increase in both iNOS and eNOS expression in microvessel endothelial cells, through the activation of the ERK1/2 pathway, also in a PTX-sensitive manner [19, 33]. In addition, the nuclear bradykinin B2 receptors (B2Rs) also appear to regulate iNOS expression in isolated hepatocyte nuclei, again through the PI3K/PKB pathway [18]. This is the first study examining the effects of nuclear GPCRs on the NO pathway in adult cardiomyocytes and exact NOS subtype(s) being activated remains to be determined. However, since iNOS expression is not detected in isolated adult cardiac myocytes and iNOS mRNA is only detected after 6 h of stimulation with cytokines [46], it is unlikely that iNOS is responsible for the NO release induced by ISO or ET-1 in isolated cardiac nuclei (30 min) or cardiomyocytes (5 min). In contrast, both eNOS and

nNOS are constitutively expressed in heart and appear to have overlapping subcellular distribution and function [47]. In general, eNOS is more abundant at the sarcolemma whereas nNOS is more predominant at the sarcoplasmic reticulum. However, in response to external stimuli nNOS may relocate to the cell periphery whereas eNOS shifts to intracellular sites, including the nucleus (see [11]). In contrast, in HUVECs, eNOS localizes to the perinuclear region, cytosol, and cell periphery but then translocates to the cell periphery during hypoxia [48]. Although we demonstrated the presence of eNOS immunoreactivity in isolated cardiac nuclei, further study is required to determine the identity and localization of the NOS subtypes activate by ET-1 and isoproterenol in cardiac nuclear membranes.

Given the presence of both β_1AR and β_3AR in cardiac nuclear membranes [4], as well as the propensity of these receptors to interact with multiple G proteins [38, 49], we also investigated which receptor and G protein subtypes were specifically involved in regulating NOS activity. We established that β_3AR was likely responsible for the observed increase in NO production, as the β_3AR selective agonist, BRL 37344, reproduced the stimulatory effect seen with ISO, whereas the β_1AR selective agonist, xamoterol, had no effect. In addition, treatment with forskolin, which directly activates adenylyl cyclase (AC), was also without effect. Taken together, and given the already established link between the nuclear β_1AR and AC/cAMP activation [4], it would appear that the β_1AR-AC/cAMP pathway does not regulate NO production at the level of the nucleus, while the β_3AR does. This is not entirely surprising as it has already been demonstrated that the β_3AR can activate all three NOS subtypes in the heart, and appears to be an important mediator of NO signaling [10]. We also demonstrated that Gαi played a role in this regulation, as its inhibition with PTX resulted in an increase in NO production. Further treatment with either agonist did not potentiate this effect. The mechanism by which Gαi inhibits NO production under basal conditions remains

unclear, although the relationship between Gαi and NOS regulation is well established [50, 51], including in regards to nuclear GPCRs [33, 34]. While the nuclear β_3AR has already been shown to signal through $G\alpha_i$ [4], no such link has yet been established for the nuclear ETB. While the β_3AR is only expressed at low levels in cardiomyocytes under basal conditions, its expression does increase during the development of hypertrophy and heart failure [52]. As such, the pathways activated by nuclear β_3AR might play a role in the development of cardiac pathology. Moreover, given the lack of the β_2AR subtype at the level of the nucleus, the β_3AR may represent a greater proportion of the nuclear βARs as compared to total heart βAR density.

We have also demonstrated that the PI3K/PKB signaling pathway is involved in the regulation of NO production by the βARs, as treatment with the PKB inhibitor triciribine potentiated the effect of ISO. Triciribine however had no effect on basal NO production. The PI3K/PKB pathway is a known regulator of NO production [17, 21, 50, 53], and has been previously linked to nuclear βAR activation [7], and to the upregulation of iNOS expression [18, 34]. The fact that PKB inhibition potentiated the effect of ISO indicates that multiple pathways might be activated following ISO treatment, with both stimulatory and inhibitory pathways being activated. The ability of NO to attenuate signaling of serine/threonine protein kinases may also play a factor in this complex cascade [22]. The possibility of differential effects depending on the NOS subtype being targeted also merits consideration.

We have now demonstrated that NO is involved in the previously characterized regulation of global transcription by nuclear βARs. When examining *de novo* gene transcription in isolated nuclei, we noted that treatment with the NOS inhibitor L-NAME decreased the basal level of *de novo* gene transcription, while also blocking the observed increase following ISO treatment. No appreciable effect was seen in the ET-1 treated samples, indicating that NO signaling is differentially compartmentalized for the

two receptor subtypes (as shown in Figure 7C). This likely indicates that NO is indeed involved to some degree in βAR-mediated regulation of gene transcription. This isn't all that surprising given the pre-established role of NO in the regulation of gene transcription through modulation of nuclear NF-κB[16], and that the nuclear β_3AR also appears to regulate the NF-κB pathway [7]. Moreover, we have also shown that PKG is involved in this pathway as well. Similarly to the treatment with L-NAME, treatment with KT5823 or Rp-8-R, potent and selective PKG inhibitors, reduced the basal level of *de novo* gene transcription, and was also able to block the observed increase following ISO treatment. That PKG is a well-established downstream mediator of NO, and has also been shown to regulate PKB and calcium signaling [9], only reinforces these findings.

We also wanted to show that the regulation of NO production observed in isolated nuclei could be observed in the intact cardiomyocyte. Toward this end, we have first shown that nuclear βARs in isolated cardiomyocytes can in fact respond to extracellular ISO, as cells pre-treated with EEDQ, a potent irreversible alkylating agent [40], to inactivate cell surface receptors, still showed an increase in 18S rRNA by qPCR following ISO treatment, as previously observed in isolated nuclei [7]. An increase in levels of Pim-1 mRNA, a protein kinase downstream of PKB [41], was also observed, indicating that mRNA is also regulated by nuclear βARs in intact myocytes. These results do not discount the possibility that nuclear βARs are directly trafficked to the nucleus following their biosynthesis, and not after internalization, as internalized receptors would still have EEDQ bound and thus would remain unable to respond to agonist treatment. The recent identification of nuclear localization sequences in the α_1ARs [54], reveals the need for further study of the βAR in this regard.

Finally, we showed that both the βARs and ETRs can in fact regulate NO production in the intact cardiomyocyte, as treatment with a caged ISO analog (ZCS-1-

67) as well as with caged ET-1 (cET-1) resulted in increased NO production as visualized by an increase in DAF-2 fluorescence by live cell confocal microscopy. These caged compounds can freely enter cardiomyocytes allowing us to specifically target intracellular receptors and demonstrate conclusively that internal receptors are functional in the intact cardiomyocyte. These results are further supported by the fact that pre-treatment with L-NAME was able to block this observed increase. These results confirm our previous findings in regard to NO regulation and lend further credence to the notion that nuclear GPCRs do in fact play a role *in vivo*.

5. Conclusions

We have shown that the nuclear β_3AR and ETB regulate NO production in the cardiomyocyte, and that Gαi is implicated in this regulation. Increased NO production was required for the ISO-mediated increase in *de novo* transcription. Furthermore, both PKB and PKG are involved in this pathway. Moreover, we have demonstrated that nuclear receptors can regulate both rRNA and mRNA targets even in the context of the intact cell. Taken together, these results demonstrate for the first time that nuclear GPCRs directly upregulate NO production, and imply that the NO-GC-PKG pathway is involved in the regulation of gene transcription by nuclear GPCRs, as well as that this pathway is active in cardiomyocytes. Furthermore, the spectrum of effects of βAR ligands may be strongly influenced both by their receptor subtype selectivity and their ability to cross the plasma membrane and modulate signaling in βARs located on the nuclear membranes.

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Abbreviations

*The abbreviations used are: βAR, β-adrenergic receptor; αAR, α-adrenergic receptor; cET-1, caged ET-1; ISO, isoproterenol; ET-1, endothelin 1; ETB, endothelin type B receptor; ETR, endothelin receptor; GC, guanylyl cyclase; GPCR, G protein-coupled receptor; NO, nitric oxide; NOS nitric oxide synthase; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKG, protein kinase G; cGMP, cyclic guanosine 3',5'-monophosphate; AC, adenylyl cyclase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B-cells; PTX, pertussis toxin; DAF-2, diaminofluorescein-2; PMSF, phenylmethanesulphonylfluoride; DTT, dithiothreitol; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; PBS, phosphate buffered saline; TCA, trichloroacetic acid; ATP, adenosine triphosphate; UTP, uridine 5' triphosphate; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; qPCR, quantitative real-time polymerase chain reaction; TX-100, Triton X-100.

Figure legends

Figure 1: Synthesis of a caged isoproterenol analog. N-(2-nitrobenzyl)-L-isoproterenol (ZCS-1-67) was synthesized as described in *Methods*.

Figure 2: Effect of various agonists on NO production. Enriched nuclear fractions were preincubated with the fluorescent dye DAF-2 (5 μg/mL), then stimulated with either A) 1 μM isoproterenol or 100 nM ET-1, in the absence or presence of the NOS inhibitor L-NAME (1 mM). B) NO production in response to 1 μM isoproterenol, 100 nM forskolin, 1 μM xamoterol, or 1 μM BRL 37344. C) NO production in response to 1 μM isoproterenol in the presence or absence of 5 nM CGP20712, 10 nM ICI 118,551 or 1 μM SR59230A. NO production was determined as a measure of DAF-2 fluorescence at wavelengths of 485 nm (excitation) and 510 nm (emission). Data represents mean ± S.E. of at least three separate experiments performed in duplicate and are normalized to control. Significant differences (*, $p<0.05$) were determined by one-way ANOVA for three or more experiments.

Figure 3: Effect of pertussis toxin and triciribine on NO production. Enriched nuclear fractions were preincubated with the fluorescent dye DAF-2 (5 μg/mL) and then incubated with either A) the Gαi inhibitor pertussis toxin (PTX, 5 μg/mL), or B) the PKB inhibitor triciribine (1 μM) and then stimulated with 1 μM isoproterenol or 100 nM ET-1. NO production was determined as a measure of DAF-2 fluorescence at wavelengths of 485 nm (excitation) and 510 nm (emission). Data represents mean ± S.E. of at least three separate experiments performed in duplicate and are normalized to control. Significant differences (*, $p<0.05$) were determined by one-way ANOVA for three or more experiments.

<u>Figure 4</u>: Effect of NOS pathway inhibition on isoproterenol and ET-1 induced transcription initiation. A) Enriched nuclear fractions were stimulated with either 1 μM isoproterenol or 100 nM ET-1. [α^{32}P]UTP incorporation was measured in nuclei pre-treated with either vehicle alone (DMSO) or an inhibitor of NOS, L-NAME (1 mM). B) Enriched nuclear fractions were stimulated with either 1 μM isoproterenol, 100 nM ET-1 or 100 μM cGMP. [α^{32}P]-UTP incorporation was measured in nuclei pre-treated with either vehicle alone or inhibitors of PKG, KT5823 (1 μM) or Rp-8-Br (1 μM). C) Enriched nuclear fractions were stimulated with either 100 nM ET-1 or vehicle. RNA was extracted, cDNA prepared, and 18S rRNA quantified by qPCR. C_T values for 18S rRNA were normalized to those for β-actin mRNA. Data represents mean ± S.E. of at least three separate experiments performed in triplicate and are normalized to control. Significant differences (*, p<0.05) were determined by one-way ANOVA for three or more experiments.

<u>Figure 5</u>: βARs regulate rRNA and mRNA transcription in intact cardiomyocytes. A) Membranes prepared from freshly isolated myocytes that were preincubated with 100 μM EEDQ or vehicle for 2 h at 37 °C and then lysed. [^{125}I]-CYP (50 μL, 400,000 cpm) was added to 10 μL of membranes in a total volume of 0.5 mL, in triplicate, for each condition. Alprenolol (10 μM) was used to measure non-specific binding. Membranes were incubated at room temperature for 90 min and subsequently captured and washed using a Brandel cell harvester. [^{125}I]-CYP binding was quantified using a γ-counter. B) Enriched nuclear fractions were preincubated with 100 μM EEDQ or vehicle, then stimulated with either 1 μM isoproterenol. RNA was extracted, cDNA prepared and 18S rRNA quantified by qPCR. C_T values for 18S rRNA were normalized to those for β-actin mRNA. C) Enriched nuclear fractions were preincubated with 100 μM EEDQ or vehicle, then stimulated with either 1 μM isoproterenol. RNA was

extracted, cDNA prepared and Pim-1 mRNA quantified by qPCR. C_T values for Pim-1 mRNA were normalized to those for β-actin mRNA. Data represents mean ± S.E. of at least three separate experiments. Significant differences (*, $p<0.05$) were determined by one-way ANOVA for three or more experiments.

<u>Figure 6</u>: **Activation of βAR signaling by a caged isoproterenol analog, ZCS-1-67, in HEK 293 cells.** A) To assess cAMP/PKA pathway activation of endogenous β-adrenergic receptors, an EPAC assay was used. HEK 293 cells were transfected with 1.5 μg of EPAC, a cAMP-sensitive BRET-based biosensor. 48 h later, real-time BRET ratios were measured with 10 μM isoproterenol, vehicle or caged/uncaged ZCS-1-67. The caged compounds were exposed to 15 min of UV light (black-ray long wave, model B100AP lamp) 3.0 cm above the plate to uncage compounds prior to the assay. To calculate net BRET, variations in BRET ratio (ΔBRET) were calculated for each treatment and subtracted from ΔBRET associated to vehicle treated cells. Average of net BRET values representative of three independent experiments. B) Dose-responses for isoproterenol, caged and un-caged isoproterenol analog, ZCS-1-67, were assessed by EPAC assay. The assay was performed as described above and average net BRET responses of three independent experiments were calculated for various concentrations of the compounds. C) βAR ligand binding in membranes prepared from adult rat hearts. [^{125}I]CYP (50 μL, 400,000 cpm) was added to 10 μL of membranes in a total volume of 0.5 mL, 5 replicates were used for each condition. Alprenolol (10 μM), ISO (1 μM), caged or uncaged ZCS-1-67 (1 and 5 μM) were used to compete with specific CYP binding. Membranes were incubated at room temperature for 90 min and subsequently filtered and washed using a Brandel Cell h\Harvester. [^{125}I]CYP binding was quantified using a γ-counter. Data represents mean ± S.E. of at least three separate experiments.

Significant differences (*, p<0.05) were determined by one-way ANOVA for three or more experiments.

__Figure 7__: **Measurement of NO production in live cardiomyocytes by confocal microscopy.** A) Ventricular cardiomyocytes were isolated from adult male rats, allowed to rest for 1 h, then loaded with DAF-2 DA (5 μg/mL) and DRAQ5. Cardiomyocytes were then treated with either ZCS-1-67 (30 μM), cET-1 (1.5 μM), a caged analog of ET-1 or vehicle, as described in *Methods*. Ligand photolysis and fluorescence imaging were accomplished using a Zeiss LSM 7 Duo microscope. Nucleoplasmic DAF-2 fluorescence was recorded before (F_0), during, and after photolysis (F). DRAQ5 fluorescence was used to delineate the nucleus. Representative traces are shown. B) Nucleoplasmic DAF-2 fluorescence before (F_0) and after (t=500 s: F) photolysis. Signals are presented as background-subtracted normalized fluorescence (%F/F_0), where F is the fluorescence intensity and F_0 is the basal level of DAF-2 fluorescence recorded 1 s prior to photolysis. Data represents mean ± S.E. of at least three separate experiments. Significant differences (*, p<0.05) were determined by one-way ANOVA. C) Images showing whole cell DAF-2 and DRAQ5 fluorescence acquired immediately before photolysis (time = 0 s) and 5 min after photolysis of cET-1 or ZCS1-67. Arrows indicate the position of the nuclei in each cell.

Supplemental Figure Legends

__Supplemental Figure 1__: **Regulation of NO production.**

Enriched nuclear fractions were preincubated with the fluorescent dye DAF-2 (5 μg/ml), then stimulated with either 1 μM isoproterenol or 100 nM ET-1 for either 5, 10, 15 or 30 min. NO production was determined as a measure of DAF-2 fluorescence at

wavelengths of 485 nm (excitation) and 510 nm (emission). Data represents mean ± S.E. of at least three separate experiments performed in duplicate and are normalized to control. Significant differences (*, $p<0.05$) were determined by one-way ANOVA for three or more experiments.

Supplemental Figure 2: Identification of NOS isoforms in isolated nuclei.

A) Bovine aortic endothelial cell lysates (EC; lane 1; 5 μg) and enriched nuclear fractions from three separate preparations (nuclei; lanes 2-4; 100 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with an eNOS-specific antibody. B) Rat brain cytosol (RB; lane 1; 100 μg) and enriched nuclear fractions from three separate preparations (nuclei; lanes 2-4; 100 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with an nNOS-specific antibody. Membranes were stripped and reprobed using a nucleoporin62-specific antibody (Nup62). The immunoblots shown are representative of 3 independent experiments.

Supplemental Figure 3: Effect of KT5823 on NO production.

Enriched nuclear fractions were preincubated with the fluorescent dye DAF-2 (5 μg/ml) and then stimulated with either 1 μM isoproterenol or 100 nM ET-1, as well as the PKG inhibitors KT5823 (1 μM) or Rp-8-Br (1 μM). NO production was determined as a measure of DAF-2 fluorescence at wavelengths of 485 nm (excitation) and 510 nm (emission). Data represents mean ± S.E. of two separate experiments performed in duplicate and are normalized to control. Significant differences (*, $p<0.05$) were determined by one-way ANOVA for three or more experiments.

Supplemental Figure 4: Effect of EEDQ on βAR binding in HEK 293 cells.

Membranes prepared from native HEK 293 cells and from a stable HEK 293 cell line

expressing the β_2AR were preincubated with 100 μM EEDQ or vehicle for 2 h at 37 °C and then lysed. [^{125}I]CYP (50 μl, 400000 cpm) was added to 10 μl of membranes in a total volume of 0.5 ml, in triplicate, for each condition. Alprenolol (10 μM) was used to measure non-specific binding. Membranes were incubated at room temperature for 90 min and subsequently captured and washed using a Brandel cell harvester. [^{125}I]-CYP binding was quantified using a γ-counter.

Disclosure:

The authors declare no conflicts of interest.

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Figure 1

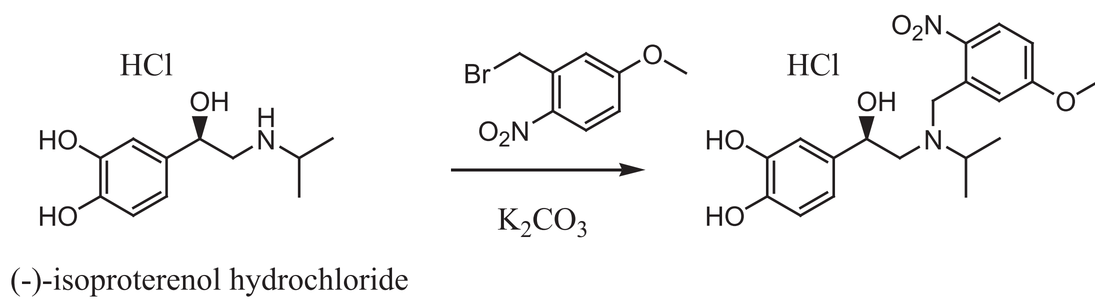


Figure 2

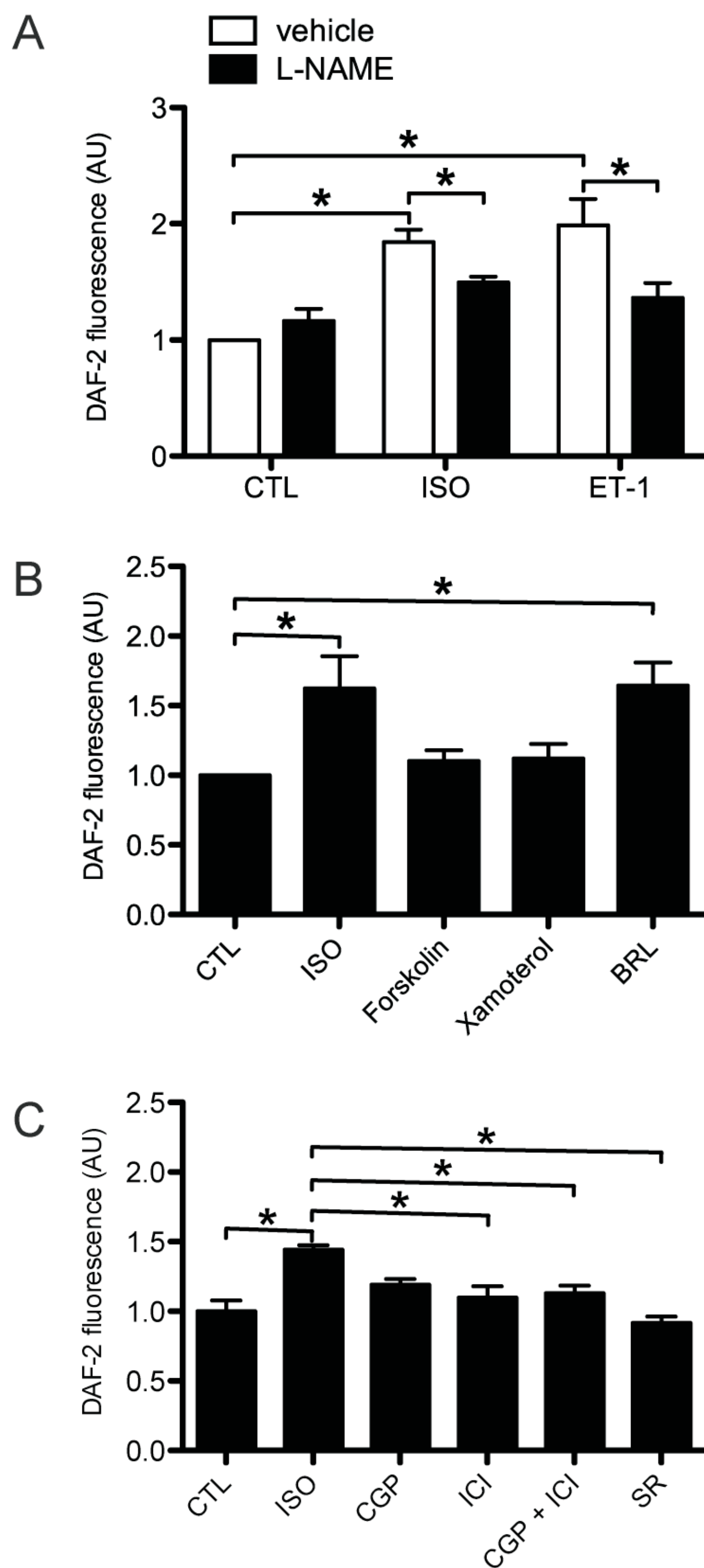


Figure 3

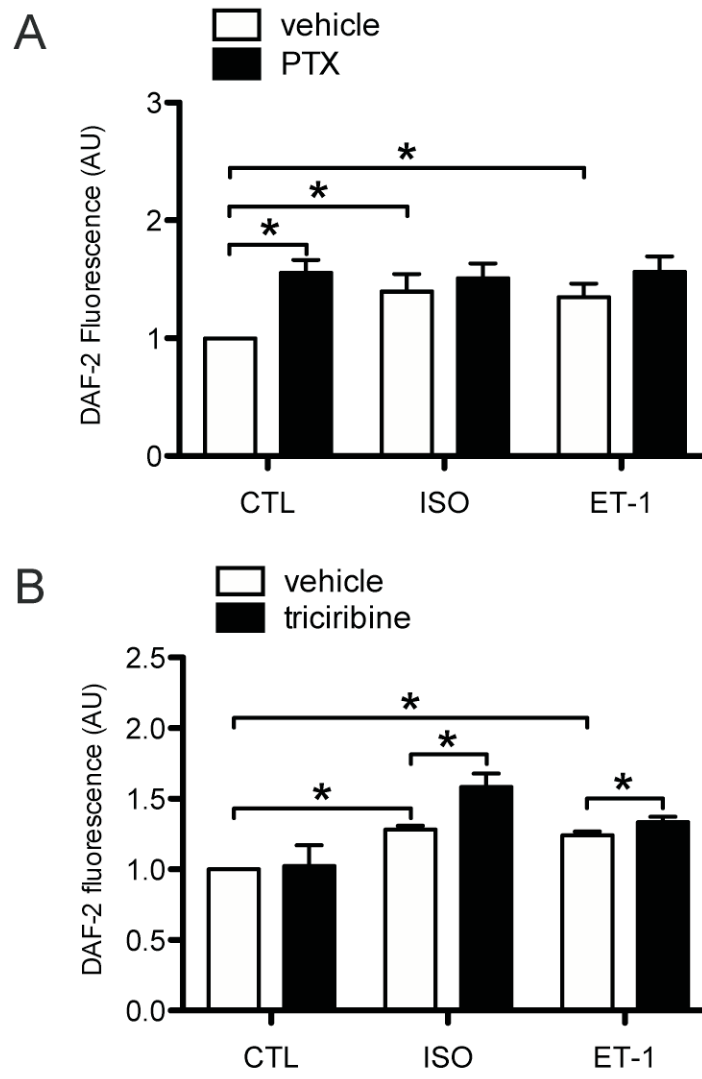
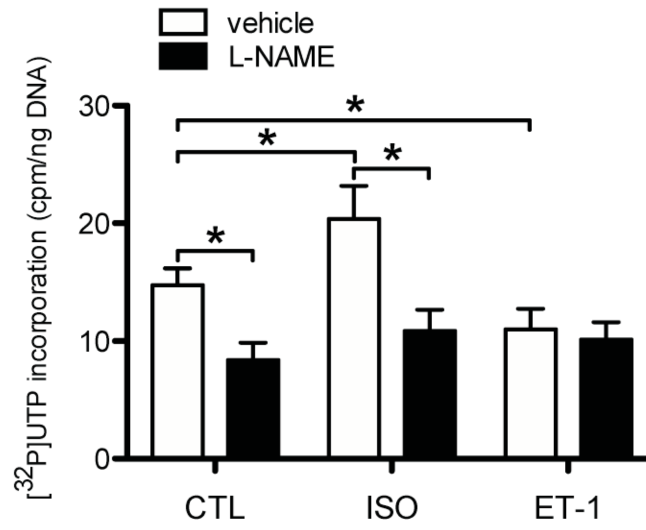


Figure 4

A



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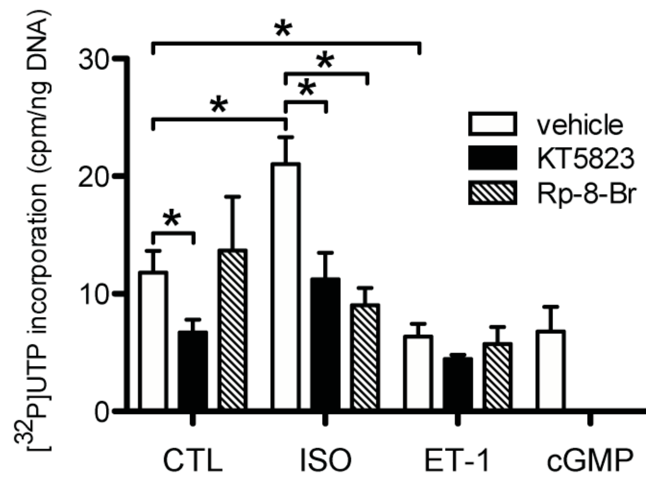

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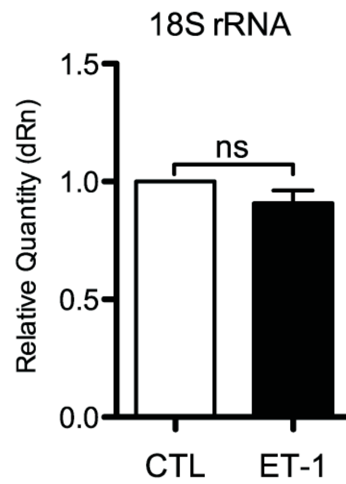
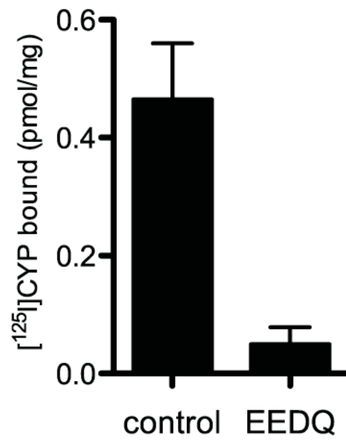


Figure 5

A



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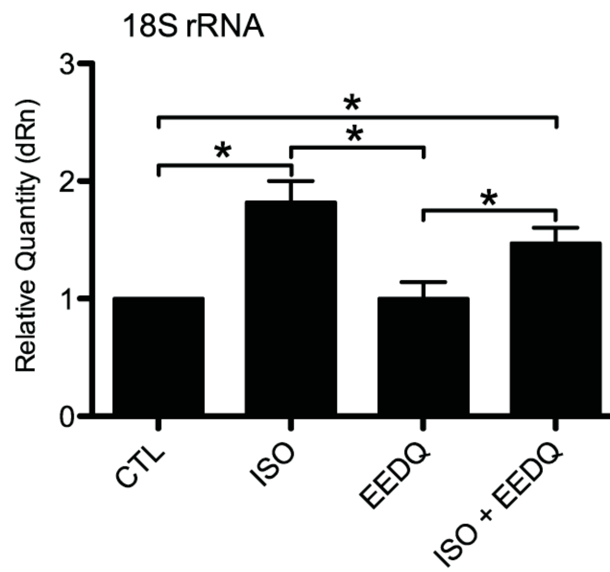

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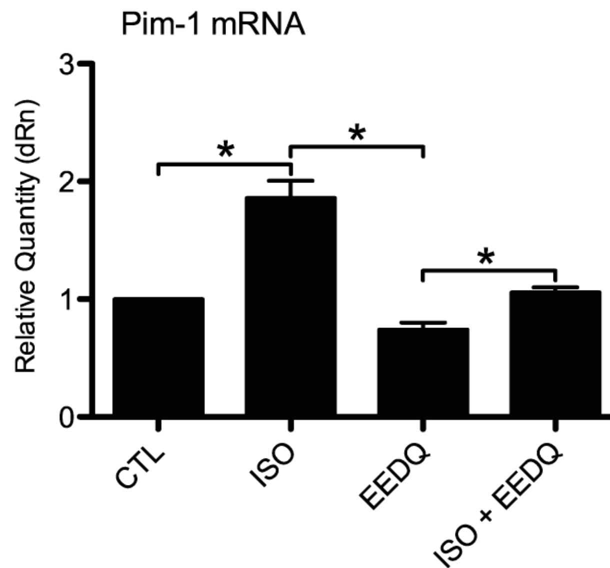


Figure 6

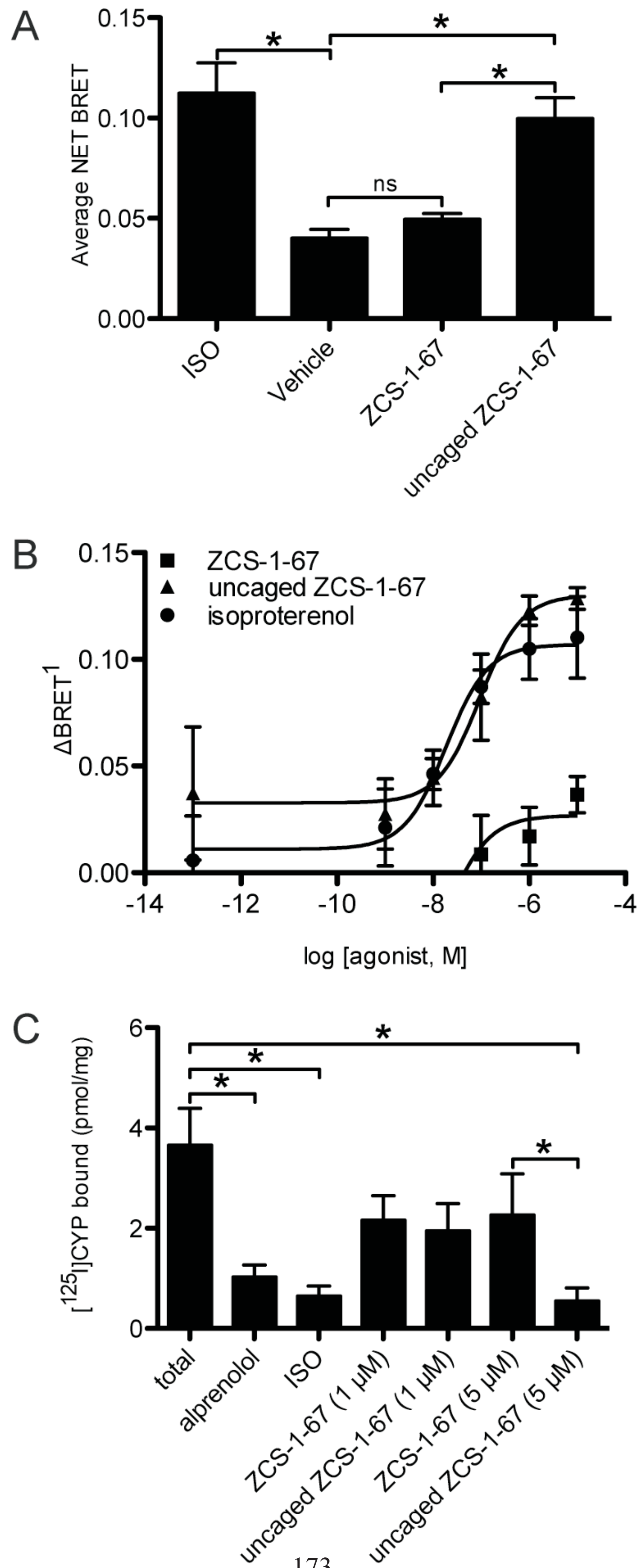
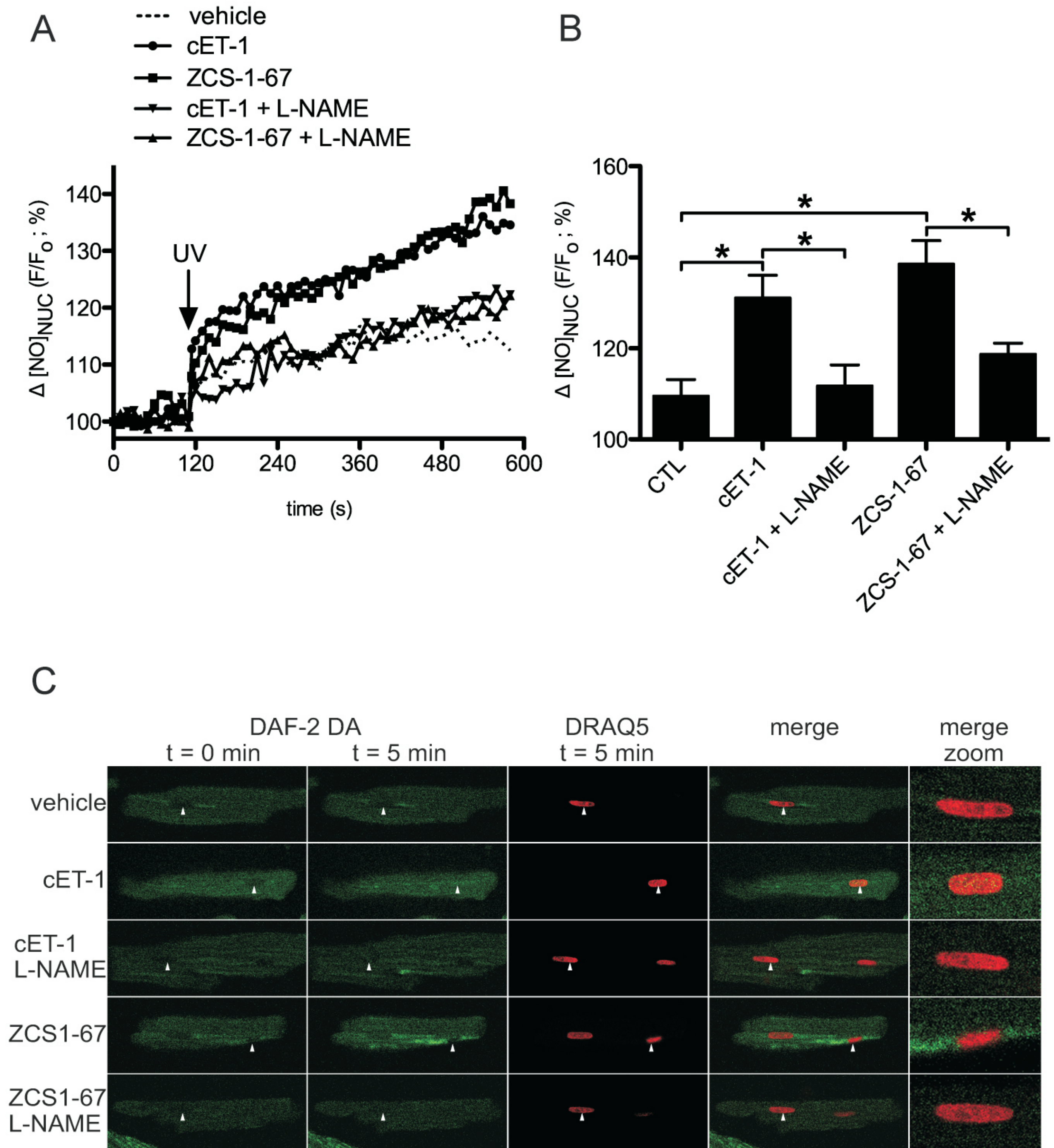
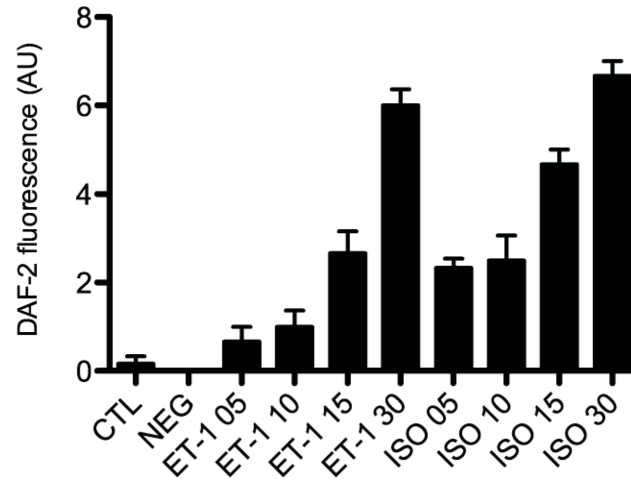


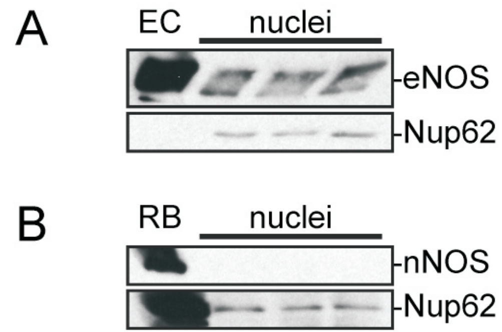
Figure 7



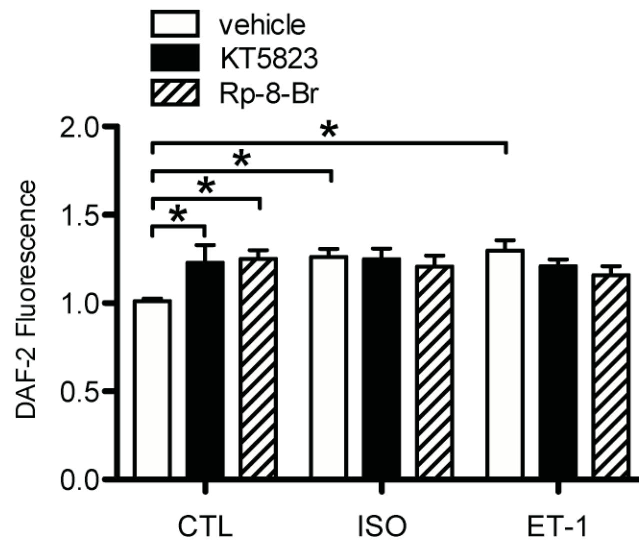
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

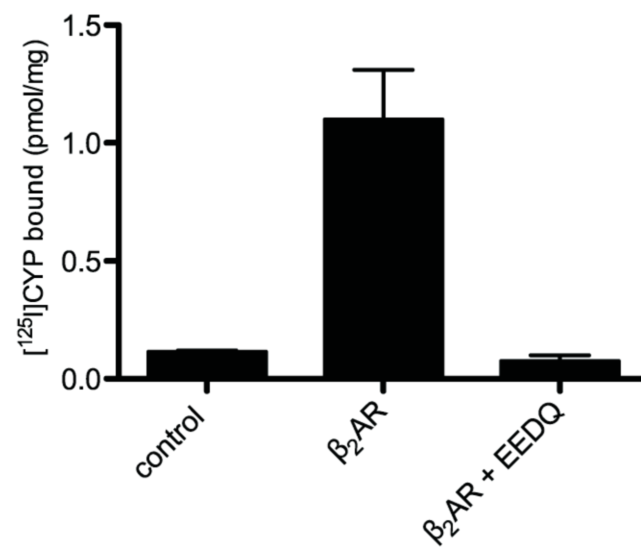


Table 1: Primers used for real-time qPCR

Target	Primers
18S Sense 18S Antisense	5’- ACG GAC CAG AGC GAA AGC AT -3’ 5’- TGT CAA TCC TGT CCG TGT CC -3’
Pim-1 Sense Pim-1 Antisense	5’- TTC GGC TCG GTC TAC TCT GG -3’ 5’- CAC GTG CTT AAT GGC CAC C -3’
β-Actin Sense β-Actin Antisense	5’- TTC AAT TCC ATC ATG AAG TGT G -3’ 5’- CTG ATC CAC ATC TGC TGG AAG GTG -3’

CHAPTER 7: GENERAL DISCUSSION

GPCRs have long been known to affect signalling pathways at the cell surface. However, as I discuss in this thesis, there is mounting evidence that GPCRs can also affect signalling cascades while located on intracellular membranes, such as the nucleus. Through the use of immunoblotting and immunocytochemistry, our labs were the first to show the presence of the β_1AR and the β_3AR, but not the β_2AR at the level of the nuclear membrane in adult ventricular cardiomyocytes. This is in contrast to the cell surface where the β_1AR and β_2AR are the dominant subtypes, representing roughly 70% and 30% of the βAR density respectively (93). In addition, we were able to show the presence of both $G\alpha_S$ and $G\alpha_i$, and AC, and that the β_1AR was able to activate AC in isolated nuclei. These results demonstrate that the βARs localize to the nuclear membrane in adult ventricular cardiomyocytes, and that they are functional in respect to ligand binding and effector activation. We then set out to determine which signalling pathways were activated downstream of receptor activation, and what effect this would have on gene transcription.

We first showed by way of a transcription initiation assay, that stimulation of nuclear βAR with ISO can in fact lead to an increase in *de novo* transcription, and that we could block this with βAR antagonists, indicating that this effect is in fact mediated by the βAR. In addition, the β_3AR-specific agonist BRL37344 was able to reproduce this effect, while the β_1AR specific agonist xamoterol had no effect, indicating that the β_3AR is responsible for the observed increase in transcription initiation. Furthermore, preincubation with the $G\alpha_i$ inhibitor PTX also blocked the stimulatory effects of ISO, demonstrating that the β_3AR is likely signalling through $G\alpha_i$.. The nuclear βAR are not however the first nuclear GPCR to have been shown to regulate gene transcription. The nuclear metabotropic glutamate receptor 5 (mGluR5) was previously demonstrated to activate transcription (144). In addition, the nuclear prostaglandin receptors (EP) have been shown to specifically regulate eNOS mRNA expression through NFκB (61), and the nuclear muscarinic acetylcholine receptor (mAChR) have been shown to activate

POL II (145). There is already evidence indicating that the β_3AR can couple to $G\alpha_i$ at the cell surface (93). In addition we also showed that activation of ETRs by ET-1 exerts a net inhibitory effect on transcription initiation, in contrast to the βAR effect. This opposing effect of nuclear GPCR signalling further reinforces the hypothesis that they are in fact playing important yet distinct regulatory roles in the cell.

We next turned our attention towards the downstream signalling pathways activated by these receptors in the nuclear membrane. Using a pharmacological approach, in combination with immunoblotting, we were able to show that the PI3K/PKB pathway was activated by nuclear βAR stimulation and that the other MAPK pathways present in the nucleus played a modulatory role in βAR regulation of gene transcription. This activation of PKB was also sensitive to the phosphatase inhibitor microcystin, indicating it to be under the control of type 2A protein phosphatases. These results are consistent with previous studies that have shown that surface β_3AR can signal via the activation of the PI3K/PKB pathway, while also having the ability to regulate ERK and p38 (93, 105). Furthermore, ERK1/2 as well as the other MAPKs have long been recognized to play a role in gene transcription, with ERK2 even shown to bind DNA and act as a transcriptional regulator independently of its kinase activity (146). As such, it is not surprising to associate a role in the regulation of gene transcription by nuclear GPCRs to these protein kinases. In fact, several nuclear GPCRs have previously been shown to activate the various MAPK cascades, with nuclear αAR shown to activate ERK (89), nuclear angiotensin receptors (ATR) demonstrated to activate both ERK and p38 (147), while nuclear lysophosphatidic acid-1 receptor (LPA_1R) has been shown to activate PKB (72). In addition, nuclear GPCRs have been observed to signal through modulation of several second messengers, with the nuclear vasoactive intestinal peptide receptor (VPAC) and the nuclear platelet-activating factor receptor (PAFR) both shown to regulate cAMP levels (80, 148), and the nuclear mAChR demonstrated to regulate cGMP levels (145). Several nuclear GPCRs have been shown to regulate calcium levels via stimulation of DAG and IP3 production (9, 149). Moreover, nuclear GPCRs have also been shown to activate other protein kinases, such as PKC (150),

likely a consequence of their ability to activate PLC (149). Taken together, these results suggest that there is a complex signalling network present at the level of the nucleus in various cells, and lend further support to the identification of a βAR signalling complex that regulates gene transcription through the activation of PKB and MAPKs. Overexpression of nuclear PKB has been previously shown to be cardioprotective, leading to enhanced contractility and conferring protection from ischemic injury (151). However, sustained activation of PKB has been linked to hypertrophic cardiomyopathy (152). Moreover, inhibition of PKB with triciribine actually altered the effect of ISO treatment upon transcription initiation from being stimulatory to inhibitory. Interestingly, while PI3K is primarily responsible for the activation of PKB, inhibition of PI3K activity did not produce the same effect as observed with triciribine. This would seem to indicate that PI3K leads to the activation of multiple downstream signalling pathways, one of which is PKB-dependent, and the other having a potential inhibitory effect on gene transcription. One possibility is that βAR stimulation also leads to the activation of the Ras/MAPK pathway, which is known to have multiple levels of cross-talk with the PI3K/PKB pathway, including between Ras and PI3K (153). Additionally, PKB might play a role in regulating the conformational state of the βAR, and hence its inhibition leads to a change in downstream signalling.

A limitation of the transcription initiation assay is that it reports on net changes in *de novo* transcription. In theory, an agonist could induce a large change in the transcriptome, comprising activation and inhibition of numerous genes, without causing a net change in transcription initiation. Thus, we investigated what type of RNA was being transcribed following nuclear βAR stimulation. Given that rRNA represents the most abundant species of RNA within the cell we begun by looking at rRNA. Looking at the expression level of 18S rRNA by qPCR as representative of total rRNA we observed a significant increase in rRNA following ISO treatment. This indicates that the bulk of the increase seen following ISO treatment in the transcription assay was likely due to an increase in rRNA. In contrast, ET-1 which resulted in a net decrease in global transcription was shown to have no effect on 18S rRNA levels. Given that increased

transcription of rRNA is usually tied to increased protein synthesis and various survival pathways, the observed increase in 18S rRNA induce by ISO indicates that the nuclear βAR might play a pro-survival, pro-hypertrophic, role in cardiomyocytes. This would be consistent with previous results showing that βAR stimulation leads to the activation of PKB, as the PI3K/PKB has long been implicated in the regulation of protein synthesis and the pro-survival pathway (25, 27).

We also wanted to investigate whether nuclear βAR stimulation leads to changes in mRNA levels. To this end we first used a pharmacologic inhibitor of POL II, the RNA polymerase responsible for the transcription of mRNA, to see if POL II was implicated in the observed pathway. α-amanitin, an inhibitor of POL II and POL III, blocked the increase observed in the transcription initiation assay, as well as the increase in 18S rRNA seen by qPCR. The ability of α-amanitin to inhibit 18S rRNA transcription is surprising given that 18S rRNA is transcribed by POL I, though similar results on POL I and POL III transcribed genes have been previously reported (154, 155). As such, while POL II does appear to be involved, further experiments were needed to fully ascertain its role. Therefore, we next looked at the expression of NFκB, a transcription factor known to be downstream of PKB, by qPCR. Interestingly, we observed a decrease in NFκB mRNA expression. However, it is currently unclear if this decrease was due to a reduction in NFκB transcription or an alteration in the stability of NFκB mRNA. Given the ability of NFκB to activate its own auto-feedback loop through the increased transcription of its own repressor IκB (141), this observed decrease does not necessarily mean the NFκB pathway is inhibited by nuclear βAR stimulation, but more likely indicates its downregulation subsequent to its prior activation by PKB. To further investigate the changes in mRNA transcription we also performed an RT profiler PCR array analysis using a microarray that specifically targets the NFκB pathway. The results of the PCR array were consistent with our previous qPCR data, showing a general reduction in the abundance of mRNAs of the genes involved in the NFκB pathway. The genes inhibited by ISO treatment were those implicated in the activation of NFκB, including the inteuleukin receptor Il1r1 and the tumor necrosis factor (TNF)

receptor Tnfrsf1b, which can lead to the activation of NFκB, however only in the absence of Ripk2, one of the few genes actually upregulated in response to ISO treatment (156, 157). In addition, the mRNA levels of some other transcription factors were reduced, in particular ATF-2, which is known to be regulated by the stress activated MAPKs, and has been shown to interact with c-jun as well as with CREB (158). These results support the hypothesis that βAR stimulation leads to the downregulation of the NFκB pathway. Furthermore, pretreatment with an inhibitor of either the ERK MAPK pathway or PKB was able to reverse the effects of ISO treatment. Under these conditions we observed an increase in the levels of protein kinase Irak2 and the adaptor protein Myd88, both of which play a role in the activation of NFκB (156, 159). A decrease in the protein TRAF3, a member of the TNF receptor superfamily known to play a role in the inhibition of NFκB, was also observed (160). Moreover, upregulation of the transcription factor c-jun, the receptor Tnfrsf1b and the TNF ligand superfamily member Tnfsf14 were also observed. These results further support our previous findings and indicate that the inhibition of the NFκB pathway can be alleviated by the repression of the activation of PKB, and possibly also ERK1/2. These results also further support the notion of substantial cross-talk between the ERK MAPK pathway and the PI3K/PKB pathway previously discussed. Taken together with our previous transcription initiation and qPCR results, we can see that nuclear βAR can in fact modulate a number of transcriptional events, mediated by both RNA POL I and POL II, through the activation of distinct signalling pathways, including PKB, and this cascade is further modulated by the tone of ERK phosphorylation as well as by protein phosphatase activity.

We have performed a preliminary microarray analysis on isolated nuclei treated with one of three agonists, ISO, ET-1, AngII or vehicle. Results from the analysis, while still somewhat preliminary, further support our findings. We have previously shown the presence of three subtypes of GPCRs at the level of the nucleus in adult ventricular myocytes. These three GPCRs include the βAR, ETR and the angiotensin receptors (ATR) (56, 57, 75). All three have also been shown to be functional in respect to ligand

binding and effector activation. In addition, all three GPCRs have been shown to have some effect on gene transcription, whether at the global level, or on specific transcripts (75, 161). Their regulation of gene transcription indicates that these nuclear GPCRs likely play some role in the intact myocyte. Furthermore, there appears to be some overlap between the downstream signalling pathways activated by these individual GPCRs, as NFκB seems to be activated by both the ATR and βAR, while the ATR and the ETR both appear to regulate calcium. As such we wanted to examine the transcriptome as a whole in order to get a better picture of what genes are regulated by each individual GPCR, but also to see how these three GPCRs differ in their regulation of said transcriptome. To this end we performed gene expression profiling on isolated nuclei from adult male rats. This analysis revealed that the three GPCRs regulate a substantial number of genes, and while there is some overlap in certain pathways, there is also a variety of genes that are differentially regulate by the individual receptors.

Treatment with ISO resulted in changes in the abundance of mRNAs involved in various molecular and cellular functions (Table 1). Based on these changes, our analysis predicted that the nuclear βAR are likely involved in the regulation of cell death, cellular development and molecular transport, among other pathways. The regulation of these pathways was predicted as a result of the observed increases in such genes as Fas, eNOS and iNOS and the downregulation of JNK1/2 and IGF-1. Moreover, the software also predicted that the nuclear βAR are involved in the regulation of cardiovascular development and function as well as tissue development and morphology. The regulation of cardiovascular development and function was predicted based on the changes in a specific set of genes, such as the upregulation of β_1AR, ETB, eNOS and iNOS as well as the downregulation of PKC. Furthermore, analysis also predicted several possible transcription factors that might be responsible for the observed changes in gene transcription (Table 2). These transcription factors include POL II, CEBPB and Cbp, the NFκB complex is also one of the listed potential upstream regulators, with 31 of its target genes showing changes in expression. In addition, several pathologic conditions were also predicted to be regulated by ISO treatment, including neurological

and cardiovascular disease (Table 3). This prediction is not surprising, given that the nervous and cardiovascular systems are two primary sites of βAR action. Two neurological diseases predicted to be associated with nuclear βAR stimulation were neuromuscular disease and neurodegenerative disorders. In regards to cardiac disease, ISO treatment was predicted to mainly play a role in the regulation of cardiac hypertrophy and heart failure. Some of the altered genes used to predict a role in regulation of hypertrophy include the β_1AR, HDAC5 and PLC which were all upregulated and IGF-1 and PKC which were downregulated. In regards to heart failure, several of the same genes were used in the prediction, but also included the upregulation of eNOS, iNOS and phosphodiesterase 5A (PDE5A).

ET-1 treatment while also having a substantial effect on the transcriptome, similar to ISO treatment also differed in certain respects (Table 1). ET-1 was also predicted to play a role in cellular development and cell death similarly to ISO, though it appears to regulate a greater subset of genes involved in these pathways, and also appears to be involved in the regulation of cellular movement and small molecule biochemistry. While ET-1 treatment is also predicted to be involved in cell death and cellular devopment, we noted the upregulation of a slightly different subset of genes, including α_{1b}AR, PKB, and CAMKIIB, certain other genes were altered similarly to ISO treatment including Fas, eNOS and IGF-1. ET-1 treatment was also predicted to be involved in the development and function of similar systems as ISO, including tissue development and morphology as well as cardiovascular development and function, though again a larger set of genes appeared to be involved. Some of the same genes were also used to make this prediction, including ETB, PKC and eNOS which were all altered similarly to ISO, though there were also some that were not altered by ISO including α_{1b}AR and PKB which were upregulated and ET-1 which was downregulated. The most likely transcription factors implicated in the observed transcription changes from ET-1 treatment include CEBPA and CEBPB, as well as PPARG (Table 2). Also similarly to the βAR, ET-1 might be predicted to be involved in both neurological and cardiovascular disease, though it also appears to have a role in developmental disorder

and inflammatory responses (Table 3). ET-1 is however linked to different areas of these diseases, including movement disorder and disorder of basal ganglia in the case of neurological disease. The nuclear ETR were also predicted to be involved in cardiac hypertrophy, necrosis/cell death, infarction and heart failure in particular. In regards to cardiac infarction, the main upregulated genes were α_{1b}AR, renin and eNOS. The predicted involvement of ET-1 in hypertrophy and heart failure used many of the same altered genes as ISO, with a few differences. For example, in the case of ET-1, α_{1b}AR was upregulated in the place of β_1AR in hypertrophy, and PDE4A was upregulated in heart failure in place of PDE5A. Interestingly, whereas ET-1 has been shown previously to induce hypertrophy, activation of nuclear ETB in isolated nuclei induces a change in gene expression that was predicted to be antihypertrophic. These findings further support the hypothesis that these two nuclear GPCRs play different roles in the cardiomyocyte, though might also have some overlapping physiologic effects, while exerting their effects via different signalling pathways and by altering different physiologic processes.

AngII treatment appears to have had the greatest overall effect on the transcriptome compared to the other two treatments, and primarily altered genes were involved in cellular development and cell death (Table 1). This would seem to imply that nuclear receptors as a whole might be particularily involved in these biological functions. Ang II does however appear to have altered a much larger subset of genes in both of these pathways, almost double that seen with ISO treatment. In terms of system development and function, Ang II seems to have altered a variety of genes in multiple processes, in particular in tissue morphology and organismal development. Some of the altered genes used to make these predictions include Fas, PKC and eNOS and iNOS which were upregulated and TNF, ET-1 and Bcl-XL which were downregulated. PKC is of particular interest as it is one gene that was downregulated by both ISO and ET-1, and downregulated by AT-2. The software also predicted that the transcription factors Sp1, HDAC1 and MTPN are responsible for some of the observed changes in the transcriptome (Table 2). The NFκB complex is also one of the top suggested upstream

regulators, with 60 altered genes linked to NFκB. In terms of pathology, Ang II treatment is primarily predicted to be involved in cardiovascular and neurological disease, similarly to both ISO and ET-1 and in inflammatory response and disease (Table 3). In addition, similarly to ISO and ET-1, AT-2 also is predicted to be involved in cardiac hypertrophy, necrosis/cell death and heart failure, though it also seems to have a more potent role in cardiac fibrosis. The predicted role of Ang II in fibrosis was made based in part on the upregulation of angiotensinogen, AT2R and eNOS and the downregulation of $AT1_BR$.

The gene array results show that these three nuclear GPCRs all have a substantial role in the regulation of the transcriptome, resulting in either the upregulation or downregulation of multiple genes. These three GPCRs regulate several genes in common, but also have a variety of differrences, indicating that they likely play redundant roles in certain pathways, while also having their own distinct effects on other pathways. Isoproterenol treatment appears to result in the regulation of fewest genes, compared to the other two treatments, and similarly to ETR and ATR, βAR appears to be involved in the regulation of cell development and death. The βAR also seem to be primarily involved in the regulation of genes important in the nervous and cardiovascular systems, both in physiologic conditions and in pathology. These findings are not surprising as the βAR are primarily known to signal in the nervous and cardiovascular systems, and have been previously linked to the regulation of apoptosis in cardiomyocytes (162). In the cardiac pathologic condition, ISO treatment alters genes primarily in hypertrophy and heart failure, both conditions known to be affected by chronic βAR stimulation (17, 34). These results begin to paint a picture of the effect exerted by nuclear βAR, though further analysis is still required. A further study using specific agonists for either the β_1AR or the β_3AR would be of particular interest, as both have been shown to localize to the nucleus in adult ventricular cardiomyocytes. Given that ISO is non-specific, the results we observe are from the stimulation of both receptors, which raises the possibility that some genes may appear to have not changed due to opposing effects of the two receptor subtypes. A further analysis would be useful

in the elucidation of the role of each βAR subtype. Hence, caged ligands appear to be a viable way to target nuclear versus cell surface receptors in the intact cells. This could also lead to the development of drugs that target intracellular receptors specifically, though a different method of un-caging would be necessary, potentially using esterases.

ET-1 treatment appears to regulate a larger subset of genes, though is also predicted to be involved in some of the same developmental pathways as ISO as well as the development and function of the cardiovascular system. ET-1 is also predicted to be particularily involved in the inflammatory response, which has already been shown in coronary arteries (163). While ET-1 treatment is predicted to be involved in hypertrophy and heart failure (108), similarly to ISO, it is also predicted to play a role in cardiac infarction. Interestingly ET-1 is actually predicted to be anti-hypertrophic which is in contrast to the pro-hypertrophic effect associated with ETR at the cell surface. The predicted involvement in cardiac infarction is also consistent with the known litterature, as ET-1 has been shown to be increased following cardiac infarction (164, 165). In the case of ET-1, ETB is the only nuclear endothelin receptor found in cardiomyocytes, and as such further analysis with specific agonists is unnecessary, though further analysis of the results is still required.

AngII treatment resulted in the alteration of an even larger set of genes, and was also predicted to be involved in development and cell death, similarly to the other two treatments. The nuclear ATR were also predicted to be involved in the development and function of the nervous and cardiovascular systems, which has been shown previously for plasma membrane receptors (166). Furthermore, similarly to ET-1 and ISO, AngII treatment is also predicted to be involved in the regulation of hypertrophy and heart failure, which has been shown previously as well (167). In addition, nuclear ATR are predicted to be involved in cardiac fibrosis, a process also already associated with the ATR (168). Moreover, similarly to the βAR, the ATR also have been shown to have two subtypes present at the level of the nucleus, the AT1R and the AT2R. As such, a follow

up study with specific agonsists for each receptor would again be of interest, in order to obtain a more representative look at the effects of each receptor subtype.

Taken together these results begin to give us an idea of the possible roles these nuclear receptors might play in the cardiomyocyte in both physiologic and pathologic conditions, and how they differ between themselves. The analysis done to date however remains preliminary, with a more detailed analysis required in order to get a proper grasp of the data. Once properly analyzed, this data should allow us to make educated hypothesis upon the function of these receptors as well as give us clues as to what genes and pathways to investigate going forward. We can then further study and validate these predictions using qPCR and various functional studies, including assessing changes in transcription factor activity by immunoblotting. Further gene arrays with specific agonsists as well as with antagonsists, and phamacologic inhibitors could be used to further clarify the above picture, and reinforce our previous results. Finally, this study must be performed in the context of intact cells, as signalling molecules or transcriptional regulators normally recruited from the cytosol would have been absent in the isolated nuclei. Given the number of genes altered by these three nuclear GPCRs, and the predicted link to several cardiotoxic effects, the potential to use caged compounds to treat cardiovascular disease cannot be overlooked. Caged compounds can be made to target specific intracellular receptor subtypes, which might help alleviate some of the associated side-effects of currently used β-blockers and ACE inhibitors.

Table Legend

Nuclei, isolated from four adult rat hearts, as previously described (161), were treated with one of three agonists, ISO (1 μM), ET-1 (10 nM), AngII (10 nM) or vehicle, for 30 min at 37°C. RNA was then isolated, using the Qiagen RNA isolation kit, as previously described (161), from each sample and sent for gene CHIP analysis. RNA from the three experiments was than pooled. Gene expression analysis was performed using Agilent Rat Whole Genome microarrays. Data was analyzed using Ingenuity Systems software and presented as a ratio of expression, versus vehicle control. Total rat RNA was used to normalize the results. The selected results from this analysis are shown in the tables below, centered on the predicted biological functions, toxicity and upstream regulators for the three agonists.

Table 1: Genes regulated by ETR or β_2AR classified according to biological function

Molecular & Cellular	ISO/CTL	ET-1/CTL	AT-2/CTL
Lipid Metabolism	92	143	156
Carbohydrate Metabolism	71	108	134
Small Molecule Biochemistry	121	190	242
Cellular Development	157	264	324
Molecular Transport	132	171	224
Cellular Movement	110	192	207
Cell Death	164	283	326

Values represent number of genes altered.

System Development & Functions	ISO/CTL	ET-1/CTL	AT-2/CTL
Cardiovascular (Dev & Fns)	93	157	177
Endocrine (Dev & Fns)	46	83	94
Nervous (Dev & Fns)	96	140	216
Reproductive (Dev & Fns)	39	109	103
Tissue Development	139	187	241
Tissue Morphology	115	233	275
Organismal Development	154	217	287
Organismal Survival	90	164	187

Values represent number of genes altered.

Table 2: Predicted Upstream Regulators

Isoproterenol/Control

Symbol	Name	General Function
POL II	RNA polymerase II	mRNA transcription
NCOR2	Nuclear receptor co-repressor 2	HDAC recruitment
TCF7	T cell factor 7	General transcription factor
CEBPB	CCAAT/enhancer binding protein beta	Trans-activating transcription factor
Cbp	CREB binding Protein	Transcriptional co-activator

Endothelin-1/Control

Symbol	Name	General Function
CEBPB	CCAAT/enhancer binding protein beta	Trans-activating transcription factor
CEBPA	CCAAT/enhancer binding protein alpha	Trans-activating transcription factor
NR0B1	Nuclear receptor subfamily 0, group B, member 1	Dominant-negative transcriptional regulator
PPARG	Peroxisome proliferator-activated receptor gamma	Regulation of fatty acid storage and glucose metabolism
Sp1	Specificity protein 1	Transcription factor involved in early organism development

Angiotensin-2/Control

Symbol	Name	General Function
Sp1	Specificity protein 1	Transcription factor involved in early organism development
HDAC1	Histone deacetylase 1	Histone deacetylation
CTNNB1	β-catenin gene	Transcriptional co-factor
MTPN	Myotrophin gene	Co-factor of NFκB
SMAD3	Mothers against decapentaplegic homology 3	Transcription factor & member of the TGFβ superfamily of modulators

Table 3: Pathology

Diseases & Disorders	ISO/CTL	ET-1/CTL	AT-2/CTL
Gastrointestinal Disease	39	49	55
Renal & Urological Disease	38	24	78
Nutritional Disease	44	55	70
Neurological Disease	115	128	198
Cardiovascular Disease	72	126	142
Inflammatory Disease	42	63	157
Inflammatory Response	65	111	158
Developmental Disorder	54	128	163
Organismal Injury, Abnormalities	67	119	100

Values represent number of genes altered.

Cardiotoxicity	ISO/CTL	ET-1/CTL	AT-2/CTL
Cardiac Hypertrophy	28	40	42
Cardiac Proliferation	12	13	18
Cardiac Dilation	6	15	10
Cardiac Fibrosis	15	18	21
Cardiac Dysfunction	6	15	8
Cardiac Infarction	15	22	15
Cardiac Necrosis/Cell Death	12	24	27
Heart Failure	22	25	28

Values represent number of genes altered.

Given the already well established link between the β_3AR and NO (107, 169) and that NO has also been shown to be activated by PKB (134, 170), not to mention regulated by ETB (171), we sought to determine if NO production was implicated in the previously described pathway. Through the use of the fluorescent dye DAF-2 we were able to show that both the nuclear ISO and ET-1 increased NO in isolated nuclei. This increase was blocked by the non-selective NOS inhibitor, L-NAME. This shows that the NO pathway is in fact regulated by these two GPCRs at the level of the nucleus. In fact, regulation of NO has already been demonstrated for other nuclear GPCR in different tissues: nuclear LPA_1R has been shown to increase iNOS expression in nuclei isolated from the liver (72), while nuclear EPR has been shown to regulate eNOS expression in cerebral microvessel endothelial cells (61), and the nuclear mAChR can increase nuclear cGMP levels in corneal epithelial cells (145). However, there are likely cell type-specific differences in expression of nuclear GPCRs, as well as differences in their downstream signalling that require further study. Furthermore, inhibition of PKG, the protein kinase downstream of NO, did not block the observed increase following either ISO or ET-1 treatment, further indicating that this is a real effect. However, inhibition of PKG did result in an increase in NO production in the basal state, indicating the potential presence of a negative feedback loop involving PKG regulating NOS activity. Moreover, using specific agonists for the β_1AR and the β_3AR we demonstrated that the β_3AR, but not the β_1AR increase NO production. This result is particularily revealing, as the β_3AR has been shown to regulate all three NOS isoforms in the heart and appears to play a critical role in the regulation of NO signalling (169). Furthermore, using the pharmacologic inhibitor PTX we were able to show that $G\alpha_i$ was likely involved in this process, and our results would also suggest that under basal conditions $G\alpha_i$ is actively inhibiting NO production and βAR activation likely works by lifting this inhibition. The method by which $G\alpha_i$ leads to the inhibition of NO production remains to be elucidated, although $G\alpha_i$ has been previously linked to the NO pathway (172, 173). Inhibition of PKB with triciribine also would seem to indicate that PKB is involved, though its exact involvement remains unclear, and likely depends on the NOS subtype being activated.

These results support the general idea that the NO pathway is in fact involved in the regulation of transcription by nuclear βAR, through $G\alpha_i$ and PKB.

To further investigate the possible role of NO in regulating transcription initiation in response to βAR activation we performed the transcription initiation assay looking at whether L-NAME could block the previously described increase in global transcription following ISO treatment. L-NAME was in fact able to decrease the basal level of *de novo* gene transcription, as well as block the increase produced by ISO. L-NAME, however, had no effect on the inhibitory effect of ET-1. This further indicates that the NO pathway is in fact involved in the β_3AR mediated regulation of gene transcription. What role NO might play downstream of the ETR remains unclear. However, the lack of any effect of L-NAME in the transcription assay is not surprising as we have shown that ET-1 has no effect on 18S rRNA expression and, as such, has little effect on global transcriptional levels which are primarily represented by rRNA levels. Additionally, inhibition of PKG, a known kinase downstream of NO, with KT5823, had an effect similar to that of L-NAME, indicating that PKG is part of this pathway. Moreover, PKG has been shown to regulate PKB, indicating the possible involvement of a feedback loop (129). These results are consistent with our previous findings indicating that NFκB is likely responsible for some of the transcriptional effects we have seen, as nitric oxide has been shown to play a role in gene transcription through the modulation of nuclear NFκB (128, 135). Taken together these results begin to paint a clearer picture of the downstream signalling pathways activated by nuclear βAR, in particular the β_3AR, and their role on gene transcription.

We also wanted to determine if the previously described results obtained using isolated cardiac nuclei can also be recapitulated in intact cardiomyocytes. The reason for this was to further validate our findings and show that the signalling pathways we describe are functional in the intact cell setting. To accomplish this we first investigated whether ISO stimulation could still produce the same effect on 18S RNA transcription as seen in isolated nuclei. In order to ensure that we were in fact are looking at a nuclear

effect, we blocked the plasma membrane βAR with EEDQ, an alkylating agent, which we have shown in both HEK cells and isolated myocytes can block the surface βAR. We observed that ISO does increase 18S rRNA in intact myocytes, and EEDQ treatment was unable to block this effect. This finding confirms that the nuclear βAR are functional in the intact myocyte and appear to play a similar role to that described in isolated nuclei. These results also confirm the presence of an intrinsic pathway in the cardiomyocyte that enable the uptake of catecholamines, and their entry into the nuclear cisternae. This is not the first study to suggest such a mechanism. Norepinephrine has already been shown to localize to the nucleus of neonatal ventricular myocytes (174), and it has been suggested that the norepinephrine-uptake-2 transporter is responsible for this internalization (88). Studies using either hydrophobic or hydrophilic agonists against the mAChR have demonstrated that surface receptors versus intracellular receptors can affect different signalling pathways and, hence, have differential effects (175). Our findings further emphasize the importance of studying these nuclear receptors and their associated functions. In addition, in the case of the mACHR, the use of hydrophobic ligands also reveals another method by which to potentially target these intracellular receptors without activating their cell surface counterparts. Moreover we were able to show that ISO stimulation as results in an increase in Pim-1 mRNA expression. Pim-1 is a protein kinase downstream of PKB that has been linked to many of the pro-survival effects of PKB. In addition, Pim-1 has been shown to stabilize c-myc, a known activator of RNA POL I, potentially linking it to the previously described increase in 18S rRNA (29, 176). The increase in Pim-1 expression implies that PKB is also activated by nuclear βAR in the intact cardiomyocyte. Moreover, this further demonstrates that nuclear βAR are functional, and can also regulate mRNA expression in the intact myocyte.

In order to further investigate the role of these nuclear GPCRs in intact cardiomyocytes, we employed caged analogues of both ET-1 and ISO. Caged ET-1 has been described previously (122), however for these studies a caged analogue of ISO was developed. These analogues are able to freely enter the myocyte, where we could then

expose the cells to a brief pulse of UV light in order to uncage the ligands. Once the cage was removed, the compounds could then work similarly to their endogenous counterparts, as the cage moeity leaves no residual modification. An EPAC assay was used to verify the functionality of the caged ISO analogue in HEK 293 cells. The assay tested the ability of both the caged and uncaged analogue to stimulate cAMP formation in comparison to parent compound ISO, with the caged analogue having no effect, while the uncaged ISO has a similar effect to ISO. We investigated whether the uncaged analogues could result in a similar increase in NO production as seen in isolated nuclei. Photolysis of caged ISO and ET-1 did increase NO production in intact cardiomyocytes, and we could block this increase with the non-selective NOS inhibitor L-NAME. These results further demonstrate that these nuclear GPCR are functional and also supports the hypothesis that they play a physiological role in the intact myocyte.

Conclusions

We have shown that the βAR and ETR located in the nuclear membrane of adult ventricular cardiomyocytes are functional and play a role in the regulation of gene transcription. In the case of the βAR, transcriptional regulation is mediated through its interaction with $G\alpha_i$, and the activation of the PI3K/PKB pathway. We have also shown the presence of phosphatases within the nucleus that are able to dephosphorylate, and inactivate PKB, thus terminating its effect on transcription. Once activated, PKB likely regulates gene transcription by activation several different pathways including the nitric oxide pathway leading to PKG, the NFκB pathway, which then causes its own downregulation and potentially Pim-1 as well. The various MAPKs all play a modulatory role on this pathway. These pathways interact to cause the observed increase in 18S rRNA, likely indicating an increase in protein synthesis and hence the activation of a pro-survival pathway. ET-1 on the other hand inhibits global transcription, but has a positive effect on a variety of genes, as seen in the gene chip data. Similarly to the βAR, ET-1 also increases NO production, although its role in the cardiomyocyte remains to be elucidated. In addition, we have shown that these GPCRs can regulate NO production in the intact myocyte, and that nuclear βAR are capable of regulating 18S rRNA expression in the intact myocyte as well. Taken together these results clearly demonstrate a role for the nuclear βAR in the regulation of gene transcription and potentially reveal a mechanism by which chronic adrenergic stimulation might affect the gene expression profile of the cardiomyocyte. Additionally, it is possible that these changes are involved in the development of hypertrophy and, possibly, heart failure. It should not be overlooked that the receptor responsible for these effects is the β_3AR. The physiologic role for the β_3AR remains to be defined, and the β_3AR is the sole βAR that is actually upregulated rather than being downregulated or desensitized as a result of chronic adrenergic stimulation. Moreover, even the role of nuclear β_1AR activation remains to be determined.

Going forward, experiments looking at the differences between cell surface and nuclear signaling events driven by GPCRs would help further define the role of these nuclear GPCRs. This can be accomplished in a number of different ways, one of which we have already begun to use, the caged ligand analogues. Intracellular receptors can be targeted with the caged compounds while the cell surface receptors can be activated using the endogenous ligand. However, given that we have shown that even isoproterenol which is highly hydrophilic can potential enter the cell this approach alone probably would not suffice. Nonetheless, the βARs have a vast library of available ligands with diverse hydrophilic/hydrophobic profiles that we can use to target either cell surface or internal receptors respectively, though an extensive study of these compounds would likely be necessecary to fully characterize their selectivity. Another way to approach this problem is to block the cell surface receptors with EEDQ, which we have already shown to be possible. Additionally, we could also use patch clamp and microinjection systems to directy inject endogenous ligand into cardiomyocytes.

Furthermore, additional mircroarray analyses with follow-up qPCR experiments to validate some of the targets would be necessary. Moreover, chromatin immunoprecipitation experiments looking at the activity of the predicted transcription factors would also help further build the pathway. A follow-up microarray experiment using specific agonists against either the β_1AR or the β_3AR could also be useful, in order to better understand the effect exerted by each subtype. Additional gene arrays with agonist in the presence of different inhibitors could also prove beneficial. In regards to the intact cardiomyocyte study, the development of a caged βAR antagonist could help further validate our previous findings. In addition, given the number of nuclear GPCRs that have been shown to regulate calcium, it would be interesting to investigate if the βAR also play some role in the regulation of this pathway, what effect this might have on transcription and eventually if the addition of different inhibitors, like an inhibitor of IP3, could block this effect. This could be done similarly to the NO production experiment using the caged compounds, with Fluo-4 AM in place of DAF-2. In addition, it would also be of interest to repeat some of these studies in a disease

model, to see how that affects the observed signalling pathways. For this we could use an aortic constriction model in rats to induce hypertrophy. Further study of the different signalling pathways activated by the nuclear versus surface βAR, and as such their differential effects in cardiomyocyte function could lead to a better understanding of adrenergic signalling and the development of more specific β-blockers. These results point to a need to study the hydrophobicity of the currently available β-blockers to clarify exactly which βAR are actually being targeted by these drugs.

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