Université de Montréal

Transporteurs d'ions dans des cellules d'épithélium rénal : rôle des purinocepteurs-P_2, de la protéine kinase C et des protéines de choc thermique

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Transporteurs d'ions dans des cellules d'épithélium rénal : rôle des purinocepteurs-P_2, de la protéine kinase C et des protéines de choc thermique

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SOMMAIRE

Des anomalies de l'activité des transporteurs d'ions et de l'expression des protéines de choc thermique (HSPs) ont été décrites dans les reins d'animaux hypertendus. Nous nous sommes particulièrement intéressés aux co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i. Les isoformes NKCC1 et NKCC2 du co-transporteur Na^+, K^+, Cl^- sont inhibés par des diurétiques tels que le furosémide et le bumétanide et sont impliqués dans la sécrétion et la réabsorption transépithéliale du sodium et de l'eau. L'implication de l'isoforme NKCC1 a aussi été démontrée dans la régulation du volume cellulaire ainsi que dans la progression du cycle cellulaire et dans la prolifération. Le co-transporteur Na^+, P_i de type II a un rôle majeur dans la réabsorption du phosphate par le tubule proximal ainsi que dans la régulation des concentrations plasmatiques du phosphate inorganique et du Ca^{2+}. La régulation de ces transporteurs est spécifique à chaque tissu et à chaque segment des tubules rénaux.

Puisque l'on savait que les HSPs sont impliquées dans la protection de certaines fonctions du transport transépithelial dans les tubules rénaux de poisson, notre hypothèse était que les HSPs modulent la régulation des transporteurs rénaux d'ions connus pour leur activité anormale dans l'hypertension artérielle. Toutefois, aucune étude n'avait évalué l'implication des HSPs dans la régulation des transporteurs d'ions dans des cellules d'épithélium rénal de mammifère. De plus, les HSPs peuvent jouer un rôle non seulement dans la modulation de l'activité basale des transporteurs d'ions mais aussi dans leur régulation. Encore une fois, il n'existait aucune donnée sur ce sujet. Pour cette thèse de doctorat, nous avons donc choisi d'étudier un modèle physiologique d'épithélium rénal avant d'entreprendre des études sur un modèle pathologique tel que l'hypertension artérielle. Le but de nos travaux de recherche était

d'examiner les voies de signalisation impliquées dans la régulation de différents transporteurs d'ions dans des cellules d'épithélium rénal de mammifère ainsi que la modulation de ces transporteurs par le stress thermique et les HSPs.

Nous avons choisi comme modèle d'épithélium rénal les cellules «Madin-Darby canine kidney» (MDCK) puisque cette lignée cellulaire a conservé, *in vitro*, plusieurs propriétés de l'épithélium rénal *in vivo* dont le transport vectoriel et la régulation hormonale. De plus, des protéines de choc thermique HSPs peuvent être induites dans cette lignée. L'activité des transporteurs d'ions a été évaluée à l'aide d'isotopes radioactifs. L'effet des stress thermiques modéré et sévère ainsi que du stress hyperosmotique a été évalué. L'accumulation de l'ARN messager et l'expression des protéines HSP70 et HSP27 ont été étudiées par buvardages de type northern et western. Dans des études complémentaires, nous avons aussi mesuré les concentrations intracellulaires de Na^+, de K^+, et de Cl^- à l'aide d'isotopes radioactifs; les concentrations intracellulaires de Ca^{2+} à l'aide du fluo-3; la production d'inositol triphosphate par la mesure du composé marqué myo-[2-^{3}H]-inositol et la phosphorylation de la protéine kinase activée par les mitogènes (MAPK) par immuno-buvardage de lysats cellulaires avec les anticorps anti-phospho ERK.

Nous avons démontré que, dans les cellules MDCK, la régulation du co-transporteur Na^+, K^+, Cl^- s'effectue principalement via des voies de signalisation impliquant les isoformes de la protéine kinase C sensibles au 4β-phorbol 12-myristate 13-acetate (PMA) et via l'activation des purinocepteurs-P_{2x} et/ou P_{2y}. Nous avons aussi démontré qu'aucun mécanisme connu de la signalisation induite par l'ATP n'est impliquée dans l'inhibition du co-transporteur Na^+, K^+, Cl^- par les purinocepteurs- P_2. Nos résultats suggèrent la présence d'une nouvelle voie de signalisation induite par les

purinocepteurs-P_2. Le co-transporteur Na^+, P_i est aussi régulé par le PMA et par l'ATP. L'activation des purinocepteurs-P_2 par l'ATP ainsi que l'activation de la protéine kinase C par le PMA diminuent de façon importante l'activité des co-transporteurs Na^+,K^+,Cl^- et Na^+, P_i. L'activité basale des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i est indépendante de la production des HSPs induites par le stress thermique. Nous avons observé que le stress thermique sévère augmente l'activité basale des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i et abolit complètement la régulation de ces transporteurs par le PMA, sans modifier significativement celle obtenue par l'activation des purinocepteurs-P_2. La production de HSP70 induite par le stress thermique modéré n'empêche pas l'activation des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i à la suite d'un stress thermique sévère ou leur inhibition par les purinocepteurs-P_2.

En conclusion, dans les cellules MDCK, les purinocepteurs-P_2 et les isoformes de la protéine kinase C sensibles au PMA sont impliqués dans la régulation de l'activité des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i par des mécanismes différents, l'un insensible et l'autre sensible au stress thermique, respectivement. Mais cette régulation est indépendante de l'induction des HSP70 et HSP27. De plus, la régulation du co-transporteur Na^+, K^+, Cl^- par les purinocepteurs-P_2 ne se fait pas par les mécanismes connus de la signalisation induite par l'ATP. Compte tenu de l'importance relative des membranes apicale et basolatérale pour la fonction de plusieurs transporteurs rénaux d'ions, dont les co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i, des travaux sur le rôle des HSPs dans la régulation de ces transporteurs dans des cellules d'épithélium rénal cultivées sur un support perméable méritent d'être entrepris.

TABLE DES MATIÈRES

CHAPITRE 2 : RÉGULATION DE LA POMPE Na^+, K^+ ET DES TRANSPORTEURS D'IONS COUPLÉS AU SODIUM DANS L'ÉPITHÉLIUM RÉNAL DE MAMMIFÈRES: RÔLE DE LA PROTÉINE KINASE C ET DES PURINOCEPTEURS-P_2

DEUXIÈME PARTIE : PRÉSENTATION DES MANUSCRITS

CHAPITRE 3 : INHIBITION COMPLÈTE DE L'ACTIVITÉ DU CO-TRANSPORTEUR Na^+, K^+, Cl^- DANS LES CELLULES MDCK PAR LES ISOFORMES DE LA PROTÉINE KINASE C SENSIBLES AU PMA

CHAPITRE 4 : INHIBITION DU CO-TRANSPORTEUR Na^+, K^+, Cl^- INDUITE PAR L'ATP DANS LES CELLULES MDCK : ABSENCE D'IMPLICATION DES VOIES CONNUES DE SIGNALISATION COUPLÉES AUX PURINOCEPTEURS

ANNEXE I

LISTE DES TABLEAUX

Chapitre 1

Chapitre 3

Chapitre 4

Chapitre 5

LISTE DES FIGURES

Chapitre 4

LISTE DES SIGLES ET ABRÉVIATIONS

$AACOF_3$: cétone arachidonyltrifluorométhyl

ATP : adénosine triphosphate

BAPTA-AM : ester 1,2-bis(2-aminophénoxy)-éthane-N,N,N',N'-tétra-acide acétique tetrakis acetoxymethyl

EGF : facteur de croissance de l'épiderme

EIPA : éthylisopropylamiloride

F : fluorescence

H-8 : N-[2-(méthylamino)éthyl]-5-iso-quinolinesulfonamide

H-89 : N-[2-((-bromocinnamyl)amino)ethyl(-5-isoisoquinolinesulfonamide)]

***hsp(s)* :** gène(s) codant pour la (les) protéine (s) de choc thermique

HSP(s) et hsp : protéine(s) de choc thermique et transcripts des gènes correspondants

$InsP_3$: inositol-1,4,5-triphosphate

LDH : lactate déhydrogénase

MAPK : protéine kinase activée par les mitogènes (ERK)

MAP kinases : protéines kinases activées par les mitogènes (incluant Raf, MEK et ERK)

MDCK : cellules canines de rein Madin-Darby

MHS : lignée de rats hypertendus de Milan

MNS : lignée de rats normotendus de Milan

NCCT : gène codant le co-transporteur Na^+, Cl^-

NDGA : acide nordihydroguaïarétique

NKCC : gène codant le co-transporteur Na^+, K^+,Cl^-

NPPB : acide 5-nitro-2-(3-phénylpropylamino)benzoïque

OSP :	protéine de stress osmotique
PKA :	protéine kinase dépendante de l'AMP-cyclique
PKC :	protéine kinase C
PKG :	protéine kinase dépendante du GMP-cyclique
PLA_2 :	phospholipase A_2
PLC :	phospholipase C
PMA :	4β-phorbol 12-myristate 13-acétate
PP_1 :	sérine-thréonine phosphatases de type 1
PP_{2A} :	sérine-thréonine phosphatases de type 2A
RFLP :	polymorphisme de longueur des fragments de restriction
SHM :	souris spontanément hypertendues
SHR :	rats spontanément hypertendus
U-73122 :	1-[6((17β-3-méthoxyestra-1,3,5(10)-trien-17-yl-amino)hexyl]-1H-pyrole-2.5-dione
VSMC :	cellules de muscles lisses vasculaires
WKY :	rats Wistar Kyoto

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À Vittorio,

À nos fils,

Fabiano et Roberto

À mes parents,

Janine et Paul-Émile

«Je veux être tout ce que je suis capable de devenir.»

KATHERINE MANSFIELD

PREMIÈRE PARTIE

REVUE DE LA LITTÉRATURE

CHAPITRE PREMIER

INTRODUCTION

ET

PERTINENCE DES TRAVAUX DE RECHERCHE

INTRODUCTION

Notre intérêt pour l'implication des transporteurs membranaires d'ions dans l'expression de l'hypertension essentielle découle d'expériences démontrant une activité anormale de ceux-ci dans les cellules des muscles lisses vasculaires (Jones 1973) et les érythrocytes (Postnov et al. 1976) de rats spontanément hypertendus ainsi que dans les érythrocytes de patients atteints d'hypertension essentielle (Postnov et al. 1977). Nous sommes particulièrement intéressés au co-transporteur Na^+, K^+, Cl^- (tableau I) dont l'activité est augmentée dans les érythrocytes (Bianchi et al. 1985, Orlov et al. 1988) et dans l'épithélium rénal (Orlov et al. 1991, Ferrandi et al. 1990) de rats génétiquement hypertendus ainsi que dans les erythrocytes d'un sous-groupe de patients hypertendus (Cacciafesta et al. 1994, Cusi et al. 1991). La régulation et les mécanismes moléculaires des anomalies de l'activité du co-transporteur Na^+, K^+, Cl^- décrites dans l'hypertension génétique demeurent inconnus. Puisque des anomalies de l'activité de ce transporteur ont été décrites, non seulement dans des cellules d'épithélium rénal mais aussi dans différents types cellulaires, l'implication de l'isoforme ubiquitaire NKCC1 dans l'expression de l'hypertension génétique doit être considérée. L'isoforme rénal NKCC2 semble aussi être impliqué dans l'expression de l'hypertension, du moins chez les rats Dahl sensibles au sel (Alvarez-Guerra et al. 1998).

En plus des anomalies de l'activité des transporteurs d'ions décrites dans les reins d'animaux hypertendus, des anomalies dans l'expression rénale des protéines de choc thermique (HSPs) ont aussi été documentées dans l'hypertension génétique (Hamet et al. 1990). Parce que les HSPs sont impliquées dans la protection de certaines fonctions du transport transépithélial dans les tubules rénaux de poissons

(Brown et al. 1992), notre hypothèse était que les HSPs modulent la régulation des transporteurs rénaux d'ions dont l'activité anormale est connue dans l'hypertension génétique. Toutefois, aucune étude n'avait évalué l'implication des HSPs dans la régulation du co-transporteur Na^+, K^+, Cl^- dans les cellules d'épithélium rénal. De plus, le co-transporteur Na^+, P_i est abondant sur la membrane apicale des cellules de l'épithélium rénal (tableau II). Ce co-transporteur est impliqué dans la réabsorption du phosphate inorganique et dans la régulation des concentrations de P_i et de Ca^{2+} dans les liquides extracellulaires. Puisque les concentrations plasmatiques de Ca^{2+} ionisé sont augmentées dans l'hypertension génétique (McCarron et al. 1980, McCarron 1982, Wright et al. 1980), nous avons aussi tenu compte du rôle potentiel des HSPs dans la modulation de ce transporteur.

Les HSPs peuvent, non seulement être impliquées dans la modulation de l'activité basale des transporteurs d'ions, mais aussi dans leur régulation. Jusqu'à présent, aucune étude n'a examiné la régulation hormonale de l'activité des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i dans des cellules d'épithélium rénal de mammifère en tenant compte du rôle des HSPs. Le but de nos travaux était donc d'aborder le sujet en étudiant un modèle cellulaire physiologique d'épithélium rénal avant d'entreprendre des études dans des cellules d'épithélium rénal d'animaux hypertendus.

Nous avons choisi les cellules MDCK comme modèle d'épithélium rénal. L'induction des HSPs par le stress thermique et par le stress osmotique a été démontré dans ce modèle cellulaire (Burg 1992, 1995, Cohen et al. 1991, Sheikh-Hamad et al. 1994). Les cellules MDCK commercialement disponibles sont dérivées de cellules d'épithélium rénal de chien mises en culture il y a près d'une quarantaine d'années. Cette lignée cellulaire est l'une des mieux caractérisées quant à l'étude des fonctions de

transport épithélial. Une caractéristique particulière des cellules MDCK est la formation de dômes lorsque les cellules sont ensemencées sur un support imperméable confirmant ainsi que ce type de cellules possède un système de transport vectoriel (Herzlinger el al. 1982, Kennedy et Lever 1984, Oberleithner et al. 1990). De plus, lorsqu'ensemencées sur un support perméable, ces cellules ont la capacité de développer une polarité morphologique et fonctionnelle (Cereijido et al. 1978). En fait, les premières données portant sur la régulation hormonale du mouvement transépithélial du sel et de l'eau, dont la régulation purinergique, furent obtenues dans des monocouches de cellules MDCK (Simmons 1981). Toutefois, les cellules MDCK commercialement disponibles sont hétérogènes et semblent constituées de deux principales populations, soit une à résistance électrique transépithéliale élevée et une autre à faible résistance (Gerkle et al. 1994, Valentich 1981). *In vivo*, l'épithélium du tubule collecteur comporte deux types de cellules, les cellules intercalaires et les cellules principales. La résistance transépithéliale mesurée dans les cellules MDCK utilisées pour nos expériences est d'environ 200 Ω/cm^2, ce qui correspond aux valeurs de résistance documentées dans les cellules intercalaires de tubule collecteur (Gerkle et al. 1994). Il semble donc que les cellules MDCK utilisées pour les expériences effectuées dans le cadre de cette thèse de doctorat étaient composées d'une population majoritaire de cellules intercalaires du tubule collecteur, ce qui n'exclut pas la présence de cellules principales à résistance élevée.

Parmi les systèmes de transport membranaire d'ions dont on a démontré la présence dans les cellules MDCK, il y a les co-transpoteurs Na^+, K^+, Cl^- et Na^+, P_i, auxquels nous sommes particulièrement intéressés, la pompe Na^+, K^+ (tableau III) et l'échangeur Na^+/H^+(tableau IV). Bien que les cellules MDCK soient dépourvues de flux ionique associé à l'isoforme NKCC2 (Isenring et al. 1998), un isoforme spécifique

au rein, il s'agit d'un excellent modèle pour l'étude de la régulation du co-transporteur Na^+, K^+, Cl^- puisque l'isoforme NKCC1 est abondant dans ces cellules. L'expression des ARNm des isoformes de NaP_i-1 (type I) et NaP_i-2 (type II) du co-transporteur Na^+, P_i n'a jamais été démontrée dans cette lignée cellulaire dans des conditions basales bien que l'activité du co-transporteur Na^+, P_i ait été mesurée par influx (Escoubet et al. 1989). Puisqu'il a été démontré que l'activité de ce transporteur dans les cellules MDCK est sous l'influence d'une régulation hormonale, on peut penser qu'il s'agit d'un isoforme du type II encore non identifié ou bien d'un type de transporteur de phosphate distinct des types I et II. Récemment, un troisième type de co-transporteur de phosphate a été identifié par Western blot dans des cellules d'épithélium rénal dérivées de l'opossum (Boyer 1998). La présence de cet isoforme du co-transporteur Na^+, P_i n'a pas été évaluée dans les cellules MDCK. Le complexe α_1-β_1 de la pompe Na^+, K^+ joue un rôle majeur dans la réabsorption du Na^+ dans l'épithélium rénal. La présence des ARNm des isoformes α_1 et β_1 a été démontrée dans les cellules MDCK. L'activité de l'isoforme NHE1 de l'échangeur Na^+/H^+ a aussi été décrite dans ces cellules. Bien que NHE3 soit l'isoforme spécifique au tissu rénal, son expression n'a pas été démontrée dans les cellules MDCK.

Dans cette thèse de doctorat, nous démontrons l'implication des récepteurs-P_2 dans la régulation du co-transporteur Na^+, K^+, Cl^- dans des cellules d'épithélium rénal de mammifère. Par la suite, nous avons tenté de caractériser cette voie de signalisation purinergique en utilisant différents inhibiteurs ou analogues spécifiques. L'approche utilisée est résumée à la figure 1 de ce chapitre. Brièvement, les protéines G_s et G_i, les phospholipases C et A_2, la $[Ca^{2+}]_i$, les protéines kinases A et G, les protéines phosphatases sérine-thréonine de types I et IIA, et la cascade des MAP-kinases ne semblent pas impliquées dans la régulation purinergique du co-transporteur

Na^+, K^+, Cl^-. L'ensemble de nos résultats suggère que l'inhibition du co-transporteur Na^+, K^+, Cl^- induite par l'ATP est activée par une voie de signalisation encore non décrite dans les cellules MDCK.

La régulation de fonctions cellulaires par la protéine kinase C est un phénomène répandu. Nos travaux ont démontré qu'un activateur de la protéine kinase C, le PMA, réduit de façon significative l'activité des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i dans les cellules MDCK. Dans des études additionnelles non présentées dans cette thèse de doctorat, nous avons observé l'effet inhibiteur du PMA sur l'activité du co-transporteur Na^+, K^+, Cl^- dans des cultures primaires d'épithélium rénal de lapin (Gagnon et al.1998). L'inhibition observée dans ces cultures primaires provenant de différents tubules rénaux de lapins était moindre que celle observée dans les cellules MDCK. Toutefois, ces résultats confirment qu'un tel phénomène de régulation est ubiquitaire bien qu'il y ait une différence entre le potentiel inhibiteur observé dans les cellules MDCK, une lignée cellulaire d'épithélium rénal d'origine canine bien établie, et celui de cultures primaires de lapin.

Finalement, nous avons étudié l'implication potentielle des HSPs dans la régulation des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i dans des cellules d'épithélium rénal de mammifère. Nous avons démontré que la régulation de l'activité des transporteurs d'ions étudiés est indépendante de l'induction de la HSP70 et de la HSP27 par un stress thermique modéré. De tel résultats suggèrent que les HSPs ne jouent pas de rôle dans l'augmentation de l'activité rénale du co-transporteur Na^+, K^+, Cl^- décrite dans l'hypertension génétique. Toutefois, cette conclusion devra être confirmée en comparant l'effet de l'induction des HSPs sur l'activité des transporteurs

d'ions dans des cellules d'épithélium rénal de rats génétiquement hypertendus par comparaison à celle des témoins normotendus.

TABLEAU I

Caractéristiques des différents isoformes des protéines de transport d'ions monovalents dépendantes du Cl^-

CARACTÉRISTIQUES	NKCC1 (*BSC2, CCC1*)	NKCC2 (*BSC1, CCC2*)	NCC (*TSC, CCC3*)	KCC1	KCC2
Prototype	co-transporteur Na^+, K^+, Cl^- de la glande rectale de requin	co-transporteur Na^+, K^+, Cl^- de reins de rat et de lapin	co-transporteur Na^+, K^+, Cl^- de reins de rat et de la vessie urinaire de la plie	co-transporteur K^+, Cl^- de plusieurs tissus dont le rein, le cerveau et les érythrocytes	co-transporteur K^+, Cl^- du cerveau de rat
Distribution tissulaire	ubiquitaire	reins	reins et ostéoblastes	ubiquitaire	neurones
Distribution rénale	plus abondant dans l'appareil juxtaglomérulaire et les cellules intercalaires du tubule collecteur	segment large ascendant de l'anse de Henlé du tubule distal et macula densa	tubule distal	ubiquitaire	indéterminée

Localisation membranaire dans les cellules épithéliales	basolatérale dans les cellules épithéliales sécrétrices; apicale dans le plexus choroïde	apicale	apicale	indéterminée	–
Localisation des gènes	humain : 5q23.3 rat : indéterminée	humain : 15q15-q21.1 rat : 3	humain : 16q13 Rat : indéterminée	humain : 16q22.1 rat : indéterminée	indéterminée
Stœchiométrie	1Na:1K:2Cl *	1Na:1K:2Cl *	1Na:1Cl	1K:1Cl	Probablement 1K:1Cl
Inhibiteurs	bumétanide > furosémide ; résistant aux thiazides	bumétanide > furosémide ; résistant aux thiazides	thiazides ; résistant à la bumétanide	DIOA > furosémide > bumétanide ; résistant aux thiazides	indéterminée
Longueur de l'ARNm	7,0-7,5 kb	4,6 –5,2 kb	3,0–4,4 kb	3,8 kb	5,6 kb
Poids moléculaire théorique de la protéine	130 kDa	120-130 kDa	~ 110 kDa	~ 120 kDa	~ 124 kDa

Homologie	identité des acides aminés : ~ 60 % avec NKCC2 ; ~ 45 % avec NCC ; ~ 25 % avec KCC1 et KCC2	identité des acides aminés : ~ 60 % avec NKCC1; ~ 45 % avec NCC ; ~ 25 % avec KCC1 et KCC2	identité des acides aminés : ~ 45 % avec NKCC1 ; ~ 45 % avec NCC2; ~ 25 % avec KCC1 et KCC2	identité des acides aminés : ~ 25 % avec NKCC1, NCC2, et NCC ; 67 % avec et KCC2	identité des acides aminés : ~ 25 % avec NKCC1, NCC2, et NCC ; 67 % avec et KCC1
Sites potentiels de phosphorylation	PKA et PKC	PKA et PKC	PKA et PKC	–	–
Effet du rétrécissement du volume cellulaire	activation	activation	indéterminé	inhibition	indéterminé
Effet de l'expansion du volume cellulaire	inhibition	inhibition	indéterminé	activation	indéterminé

Fonctions	• régulation des concentrations intracellulaires de Na^+, de K^+ et de Cl^- et du volume cellulaire • activité mitogénique • sécrétion transépithéliale du sel et de l'eau	• réabsorption transépithéliale du sel et de l'eau	• réabsorption transépithéliale du sel et de l'eau	• régulation du volume cellulaire	• indéterminées

Réf. Baekgaard et Bindsler 1998, Delpire et al. 1994, Gamba et al. 1994, Gillen et al. 1996, Guo et O'Brien 1996, Haas 1994, Haas et Forbush 1998, Kaplan et al. 1996, Mashimo K. et al. 1998, Orlov et al. 1998, Payne et al. 1994, 1995

* La stœchiométrie peut ausi être 1Na:2K:3Cl; K^+/K^+; Na^+/Na^+

TABLEAU II

Caractéristiques des différents isoformes du co-transporteur Na^+, P_i

CARACTÉRISTIQUES	TYPE I	TYPE II	TYPE III
Isoformes	**NaP_i-1**	**NaP_i-2, NaP_i-3 à 7**	**Glvr-1, Ram-1**
Distribution tissulaire	rein > foie	rein > poumon	cerveau, muscles squelettiques, cœur, testicules, reins (probablement ubiquitaire)
Distribution rénale	tubule proximal	tubules proximal et distal	indéterminée
Localisation membranaire dans les cellules épithéliales	apicale	apicale (sauf dans les cellules MDCK transfectées avec NaPi-2: apicale et basolatérale)	indéterminée
Localisation des gènes	humain: 6p22 souris: 13	humain: 5q35	indéterminée
Stœchiométrie	Na^+:P_i	$3Na^+$:$1P_i$	indéterminée

Poids moléculaire théorique de la protéine	55 kDa	68 kDa	70-85 kDa
Sites de phosphorylation	PKC	PKC	indéterminés
Effet de la PTH (hormone parathyroïdienne)	aucun	diminution de l'activité du co-transporteur et son expression (ARNm)	aucun effet sur l'expression de la protéine
Effet de la diète pauvre en phosphate	aucun	augmentation de l'activité du co-transporteur et de son expression (ARNm)	diminution de l'activité du co-transporteur sans changer le contenu membranaire de la protéine
Fonctions	• implication probable dans la régulation des concentrations intracellulaires de phosphate	• réabsorption du phosphate par le tubule proximal	• implication probable dans l'absorption du phosphate, provenant du liquide interstitiel, utilisé pour des fonctions cellulaires physiologiques dans différents tissus et chez différentes espèces

Réf. Biber 1989, Biber J. et al. 1996, Boyer et al. 1998, Busch et al. 1996, Cole et al. 1987, Escoubet et al. 1989, 1991, Hayes et al. 1995, Malmstroem 1988, Murer et al. 1996, 1997, Quabius et al. 1995, Werner et al. 1991

TABLEAU III

Caractéristiques des différents isoformes de la sous-unité α de la pompe Na^+, K^{+*}

CARACTÉRISTIQUES	α_1	α_2	α_3
Distribution tissulaire la plus abondante	reins >> cœur, muscles squelettiques, muscles lisses vasculaires	muscles squelettiques, cœur	cerveau >> muscles lisses vasculaires
Localisation membranaire dans les cellules épithéliales	basolatérale	basolatérale	basolatérale
Localisation des gènes	humain: 1p13-p11 rat: 2	humain: 1q21-q23 rat: indéterminée	humain: 19q12-q13.2 rat: indéterminée
IC_{50} pour la ouabaïne, μM	> 10	0,02-0,05	< 0,01
IC_{50} pour le Ca^{2+}_i, μM	10	1	1

$K_{0.5}$ pour le Na^+_i, mM	12	24	36
$K_{0,5}$ pour le K^+_o, mM	2,4	indéterminé	1,4
Poids moléculaire théorique de la protéine	~100 kDa	~100 kDa	~100 kDa
Sites de phosphorylation	PKC et PKA	PKC et PKA	PKC et PKA

Réf. Geering 1997, Herrera et Ruiz-Opazo 1990, Orlov et al. 1998, Schull et al. 1986

* Le rôle des isoformes de la sous-unité β de la pompe Na^+, K^+ (β_1-β_4) est encore peu connu mais on suggère un rôle dans la modulation du transport de la pompe à la surface cellulaire

TABLEAU IV

Caractéristiques des différents isoformes de l'échangeur Na^+/H^+

CARACTÉRISTIQUES	NHE1	NHE2	NHE3	NHE4 [4)]
Distribution tissulaire	ubiquitaire	rein, intestin, estomac, glande surrénale >> muscle squelettique, foie, poumon	rein, intestin et colon	estomac, intestin >> rein
Distribution rénale	ubiquitaire	tubules proximal et collecteur	membrane en brosse du tubule proximal >> segment large ascendant de l'anse de Henlé du tubule distal	tubule collecteur
Localisation membranaire dans les cellules épithéliales	surtout basolatérale mais aussi apicale	basolatérale et apicale	apicale	probablement basolatérale
Localisation des gènes	humain: 1p36.1-p35 rat: 5	humain: 2q11.2 rat: 9	humain: 5p15.3 rat: indéterminée	indéterminée

IC_{50}, amiloride [1)]	3 µM	3 µM	150 µM	100 µM
IC_{50}, dérivé de l'amiloride MPA [1)]	0,05 µM	0,5 µM	10 µM	< 20 µM
Autres inhibiteurs	HOE 694, EIPA, DMA	EIPA	EIPA, DMA	_
$K_{0,5}$ pour H^+_i (pH) [2)]	6,75	6,90	6,45	indéterminé
$K_{0,5}$ pour Na^+_o [2),3)]	10 mM	50 mM	5 mM	indéterminé
Poids moléculaire théorique de la protéine	~ 90-91 kDa	~ 79-91 kDa	~ 93 kDa	~ 81 kDa
Homologie (comparativement au NHE1 humain)	93-97 %	42-50 %	40-41 %	40 %
Effet du rétrécissement du volume cellulaire	activation	résultats controversés	inhibition	activation

Fonctions	• régulation du pH_i • régulation du volume cellulaire • implication dans la prolifération cellulaire	• régulation du volume cellulaire	• mouvement transépithélial du Na^+ • «membrane trafficking» • réabsorption du bicarbonate • probablement régulation du pH_i	• régulation du volume cellulaire

Réf. Bookstein et al. 1996, 1997, Chambrey et al. 1997a) b), Kapus et al. 1994, Noël et al. 1995, Orlov et al. 1998, Orlowski 1993, Sun et al. 1998, Tse et al. 1993, Wakabayashi et al. 1997

1) Données obtenues dans un milieu sans Na^+

2) Données obtenues chez le rat

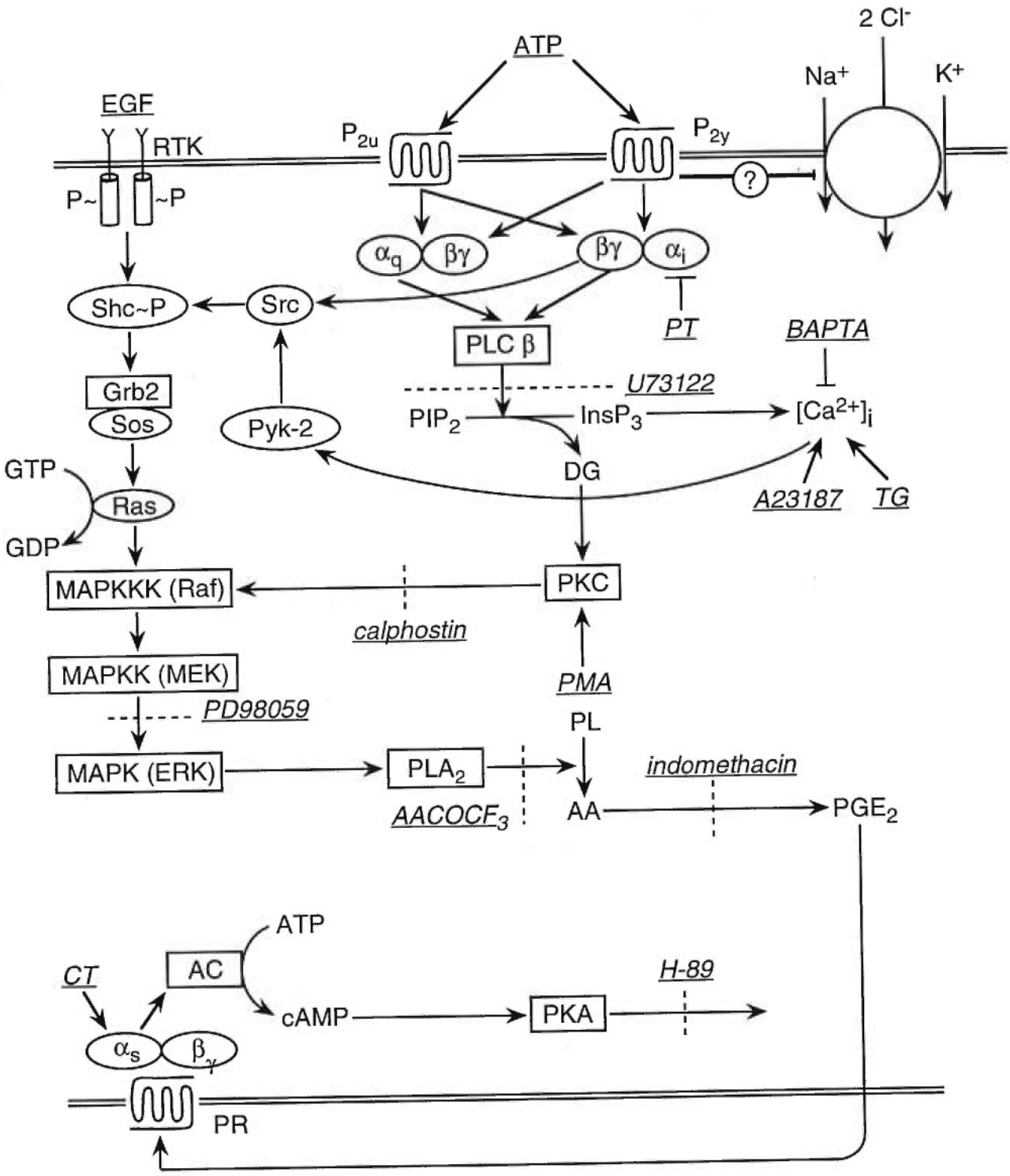
3) Pas de différence significative, pour ce paramètre, entre les isoformes NHE1-NHE3 chez l'humain ($K_{0,5}$ pour Na^+_o ~ 15-18 mM)

4) Ce transporteur est inactif dans des conditions iso-osmotiques, les données ont donc été obtenues dans des conditions de rétrécissement hyperosmotique

N.B. Les caractéristiques énumérées dans ce tableau ne sont pas disponibles pour l'isoforme NHE5 à l'exception de la localisation chromosomique du gène humain (16q22.1). Les isoformes NHE6 et NHE-β spécifiquement exprimés dans les mitochondries et les érythrocytes de poisson, respectivement.

FIGURE 1

Signalisation purinergique dans les cellules MDCK. **AC** – adénylate cyclase; **CT** – toxine du choléra ; **DG** – diacylglycerol; **PL** – phospholipides; **PR** – récepteur du PGE_2 ; **PT** – toxine de pertussis; **RTK** – récepteur pour les tyrosines kinases ; **TG** – thapsigargine ; **?** – indéterminé. → – activation ; ——| ou - - - - - – inhibition. Les agents modulateurs utilisés dans notre étude sont en italique.


ATP
2 Cl-
Na+
K+
EGF
RTK
P~
~P
P2u
P2y
?
αq
βγ
βγ
αi
PT
Shc~P
Src
PLC β
BAPTA
U73122
Grb2
Sos
Pyk-2
PIP2
InsP3
[Ca2+]i
GTP
GDP
Ras
DG
A23187
TG
MAPKKK (Raf)
PKC
calphostin
MAPKK (MEK)
PMA
PD98059
PL
MAPK (ERK)
PLA2
indomethacin
AACOCF3
AA
PGE2
ATP
CT
AC
H-89
cAMP
PKA
αs
βγ
PR

PERTINENCE DES TRAVAUX DE RECHERCHE

TRANSPORTEURS RÉNAUX D'IONS, PROTÉINES DE CHOC THERMIQUE ET HYPERTENSION ARTÉRIELLE

L'hypertension artérielle est un facteur de risque cardiovasculaire polygénique et multifactoriel. Environ 70% de l'hypertension artérielle est explicable par l'environnement alors que 30% l'est par la génétique. Toutefois, étant une maladie polygénique, c'est-à-dire que plusieurs gènes sont simultanément impliqués dans le développement de la pathologie, l'hypertension ne s'explique pas par une simple et unique distribution mendélienne (revues de la littérature, Hamet et al. 1994 et 1998). L'identification d'un gène causal par l'approche phénotypique classique devient alors difficile. Une des façons d'aborder l'étude de l'hypertension artérielle consiste à disséquer les différentes composantes de la maladie sur des bases physio-pathologiques par l'identification de phénotypes intermédiaires potentiellement impliqués dans différentes composantes de l'hypertension artérielle (Hamet et al. 1994 et 1998, Lander et Schork 1994). L'identification de certains phénotypes intermédiaires pourrait être déterminant dans le processus d'identification de gènes responsables de l'élévation de la tension artérielle.

L'hypertension artérielle n'étant pas une manifestation homogène, plusieurs sous-groupes d'hypertendus ont été identifiés. Parmi ces sous-groupes, il y a les individus dit «non-modulateurs», qui sont, par exemple, caractérisés par des anomalies du transport rénal du sodium et incluant les sujets sensibles au sel (Williams et al. 1992). Vers la fin des années 1970, les premières études suggérant une association entre des transporteurs d'ions et l'hypertension artérielle ont été publiées. Jones, ainsi

que Postnov et ses collaborateurs, furent les premiers à apporter une preuve directe de l'augmentation de la perméabilité membranaire aux flux d'ions dans les aortes (Jones 1973) et les érythrocytes de rats spontanément hypertendus ainsi que chez des individus hypertendus (Postnov et al. 1977). Au cours des vingt dernières années, plusieurs études portant sur l'hypertension artérielle ont confirmé la présence d'anomalies de l'activité de certains transporteurs d'ions couplés au sodium, dont le co-transporteur Na^+, K^+, Cl^-, l'échangeur Na^+/H^+ et la pompe Na^+, K^+ (revue de littérature, Hamet et al. 1995).

L'activité de plusieurs de ces transporteurs d'ions s'est avérée être un phénotype intermédiaire de l'hypertension artérielle. Les anomalies de l'activité des transporteurs d'ions pourraient être responsables, du moins en partie, de l'expression de l'hypertension chez les individus sensibles au sel. Les sujets sensibles au sel peuvent diminuer leur tension artérielle par une diète hyposodique et/ou par la prise de diurétiques, ce qui suggère une origine néphrogénique de l'hypertension artérielle. Toutefois, les mécanismes cellulaires et génétiques impliqués dans les anomalies de l'activité des transporteurs d'ions demeurent peu connus. Les gènes *hsp27* et *hsp70* sont des gènes candidats à une implication dans l'expression de certains phénotypes intermédiaires associés à l'hypertension artérielle. Des anomalies de l'expression des HSPs ont été décrites dans différents tissus d'animaux hypertendus, incluant les reins, ce qui nous suggère l'implication potentielle de ces gènes dans la régulation de transporteurs rénaux d'ions.

Transporteurs rénaux d'ions et hypertension artérielle

L'importance du rein dans la pathogenèse de l'hypertension a clairement été démontrée, il y a une vingtaine d'années, par Bianchi (1973), suite à une transplantation croisée de reins entre animaux hypertendus et normotendus. Ces expériences ont démontré que la transplantation d'un rein provenant d'un rat génétiquement hypertendu induit l'hypertension lorsque transféré à un rat normotendu (Bianchi 1973). De plus, le rein a un rôle important dans le maintien à long terme de l'élévation de la tension artérielle (Guyton 1991). Il a été proposé que l'adaptation du rein à l'hypertension est secondaire à une modification des systèmes de transport d'ions contrôlant le mouvement transcellulaire du sodium et de l'eau à travers l'épithélium rénal (Postnov and Orlov 1985, Hamet et al. 1995, Cusi 1997). Plus récemment, cette hypothèse a été appuyée par la démonstration, *in vivo*, d'une augmentation de l'activité de transporteurs rénaux d'ions (Salvati et al. 1990) et, *in vitro*, dans des cultures primaires et des fractions membranaires de cellules provenant d'épithélium rénal de rats génétiquement hypertendus (Orlov et al. 1991, Ferrandi et al. 1990).

L'implication des transporteurs rénaux d'ions dans la génétique de la régulation à long terme de la pression artérielle a été confirmée par les travaux de Lifton et de son équipe. Ils ont découvert, du moins en partie, l'origine du syndrome de Bartter par l'identification de mutations du gène NKCC2, qui code le cotransporteur Na^+, K^+, Cl^- (Simon et al. 1996a). Ces mutations sont associées à la diminution de l'activité de ce transporteur et le syndrome se caractérise, entre autres, par une perte urinaire excessive de sodium et de calcium, une diminution de la clairance de l'acide urique, une hypokaliémie et une diminution de la tension artérielle. Cette équipe a aussi identifié des mutations du gène NCCT codant le co-transporteur Na^+, Cl^-, qui est associé au syndrome de Gitelman. Le syndrome de Gitelman est caractérisé par de

l'hypotension et de l'hypokaliémie accompagné d'hypocalciurie (Simon et al. 1996b). D'autres systèmes de transport rénaux d'ions ont aussi été associés à la génétique de la régulation à la hausse de la tension artérielle. Par exemple, dans le syndrome de Liddle, des mutations des sous-unités β et γ des canaux sodiques sensibles à l'amiloride ont été identifiées dans différents groupes ethniques (Shimkets et al. 1994, Hansson et al. 1995). Ces mutations mènent à l'activation de ce transporteur, augmentant ainsi la réabsorption du sel et de l'eau dans le tubule collecteur. Les individus porteurs de ces mutations développent une hypertension sévère et une alcalose hypokaliémique (Schild et al. 1995). Le syndrome de Gordon est un autre exemple d'hypertension à transmission mendélienne associée à une anomalie du transport rénal d'ions (Gordon et al. 1995). Toutefois, pour cette pathologie, le gène causal reste à identifier (Hamet et al. 1998).

Pompe Na^+, K^+

Une augmentation de l'activité rénale de la pompe Na^+, K^+ est observée dans différentes souches de rats génétiquement hypertendus (Parenti et al. 1991, Husted et al. 1997). Certains auteurs suggèrent que cette augmentation de l'activité de la pompe Na^+, K^+ est associée à une sensibilité accrue au sel (Husted et al. 1997). Chez des animaux génétiquement sensibles au sel, la pompe Na^+, K^+ opère avec une stœchiométrie de $3Na^+$:$1K^+$, alors que chez les animaux résistants au sel, la stœchiométrie est de $2Na^+$:$1K^+$ (Canessa et al. 1993, Kunezsov et al. 1996). Une telle modification de la stœchiométrie est associée à une réabsorption accrue du sodium et de l'eau dans le tubule proximal, qui mène à une augmentation du volume extracellulaire et à l'hypertension (Orosz et Hopfer 1996). Parmi les gènes candidats dans la susceptibilté au sel, il y a le gène de la sous-unité α de la pompe Na^+, K^+. La substitution d'une leucine pour un acide glutamique en position 276 de l'unité α de ce

transporteur est rapportée chez les rats Dahl sensibles au sel (Herrera & Ruiz-Opazo 1990). Il est à noter toutefois que la différence de séquence décrite par Herrara et Ruiz-Opazo (1990) ne fait pas l'unanimité (Simonet et al. 1991). C'est donc avec réserve que cette mutation est associée à l'hypertension artérielle. De plus, il n'y a pas de ségrégation du gène codant pour l'unité α de la pompe Na^+, K^+ avec l'hypertension dans des rétrocroisements de rats Dalh sensibles et résistants au sel (Rapp et Dene 1990).

Bianchi et son équipe (1996), quant à eux, suggèrent l'implication d'une mutation au niveau du gène codant l'unité α de la protéine adducine dans certains aspects de la régulation de la tension artérielle tels que l'activité rénale de la pompe Na^+, K^+. L'adducine est impliquée dans la polymérisation de l'actine du cytosquelette et pourrait agir dans la redistribution et la modulation des molécules de la pompe Na^+, K^+ (Ferrandi et al. 1996). L'allèle mutant du gène de l'adducine est aussi associé à une diminution de la clairance de l'acide urique (Manunta et al. 1998).

Co-transporteur Na^+, K^+, Cl^-

Des équipes, dont celle du docteur Orlov, ont démontré que l'activité du co-transporteur Na^+, K^+, Cl^- est augmentée dans des cultures primaires (Orlov et al. 1991) et dans les fractions membranaires (Ferrandi et al. 1990) de cellules d'épithélium rénal de rats génétiquement hypertendus. Cette démonstration a aussi été faite *in vivo* dans les reins isolés de rats hypertendus (Salvati et al.1990). Toutefois, il est important de noter que les différences *in vitro* entre normotendus et hypertendus n'ont été observées qu'entre les souches MHS (*Milan Hypertensive Strain*) et MNS (*Milan Normortensive Strain*) alors qu'aucune différence n'a été observée entre les souches SHR (*Spontaneously Hypertensive Rats*) et WKY (*Wistar Kyoto*) (Orlov et al. 1991). L'une

des explications de cette apparente contradiction pourrait être l'utilisation de la souche WKY comme témoin dans les expériences avec la souche SHR. En effet, la souche WKY n'a pas été établi en parallèle avec la souche SHR (Rapp et al. 1987). La régulation et les mécanismes moléculaires de l'activité rénale du co-transporteur Na^+, K^+, Cl^- dans l'hypertension génétique demeurent encore inconnus. Toutefois, Alvarez et al. (1998) suggèrent que le défaut primaire de l'activité du co-transporteur Na^+, K^+, Cl^-, observée chez les rats Dahl-sensibles au sel, découle d'une anomalie de l'isoforme NKCC2, et/ou, d'un défaut dans la transduction de la signalisation membranaire impliquée dans la régulation de ce transporteur d'ions.

Échangeur Na^+/H^+

Plusieurs laboratoires ont décrit une activité et une expression accrues de l'échangeur Na^+/H^+ dans des cultures primaires de cellules provenant d'épithélium rénal ainsi que dans les tubules proximaux isolés de rats spontanément hypertendus (Orlov et al. 1991, Kelly et al. 1997, Hayashi et al. 1997). L'augmentation de l'activité de l'échangeur Na^+/H^+ dans les tubules proximaux isolés de rats SHR précède l'apparition de la tension artérielle élevée (Dagher et al. 1992), ce qui suggère un rôle primaire de ce transporteur dans la pathogenèse de l'hypertension. L'augmentation de l'activité rénale de l'échangeur Na^+/H^+ chez les rats SHR est associée à une transmission anormale du signal provenant des récepteurs dopaminergiques D1 situés dans le tubule proximal (Albrecht et al. 1996). Cette anomalie diminue l'effet inhibiteur de la dopamine sur l'échangeur Na^+/H^+. Albrecht et son équipe (1996) ont démontré une co-ségrégation de la diminution de la réponse de l'échangeur Na^+/H^+ aux agonistes D1 et de l'hypertension.

Co-transporteur Na^+, P_i

En plus des anomalies du transport d'ions monovalents, il est connu que les concentrations plasmatiques de Ca^{2+} ionisé sont augmentées chez les rats spontanément hypertendus et dans l'hypertension essentielle (McCarron et al. 1980, McCarron 1982, Wright et al. 1980). Ces modifications des concentrations plasmatiques de Ca^{2+} ionisé sont accompagnées d'une diminution des concentrations de phosphate sérique. Ces données suggèrent l'implication du co-transporteur Na^+, P_i dans la pathogenèse de l'hypertension artérielle puisque certains isoformes de ce transporteur contribuent à la régulation plasmatique de l'équilibre Ca^{2+}/P_i (Murer et al. 1991).

Protéines de choc thermique et hypertension artérielle

Présentation et fonctions générales des HSPs

L'exposition des cellules à un stress thermique s'accompagne de modifications morphologiques de la membrane, d'une diminution générale de la synthèse des protéines et de l'inhibition de plusieurs activités enzymatiques (Stevensen et al. 1986, Schlesinger et al. 1986, Lepock et al. 1983, Burdon et Cutmore 1982). Ces changements amorcent une réponse au stress qui se caractérise par la transcription de gènes spécifiques codant les protéines de stress HSPs (Schlesinger et al. 1986) et qui a pour but de restaurer et de protéger les fonctions cellulaires de base. Présents chez tous les organismes, ces gènes ont été nommés gènes de choc thermique parce que leur transcription a d'abord été observée dans les cellules de drosophiles soumises à un stress thermique. Depuis, l'activation de la transcription des HSPs a été observée dans différentes conditions aussi bien physiologiques que pathologiques (tableau V), *in vivo* et *in vitro*, d'où leur importance reconnue pour la survie des cellules.

Les différentes HSPs sont classées selon leur poids moléculaire sur gel d'électrophorèse SDS-PAGE en deux grandes familles, soit la famille des HSPs de haut poids moléculaire incluant HSP60-65, HSP70, HS90, et celle des HSPs de faible poids moléculaire. Pour le travail décrit ici, nous nous sommes peu intéressés aux familles HSP60-65 et HSP90 puisque respectivement celles-ci ne sont présentes que dans les mitochondries et sont peu abondantes dans le rein (revue de la littérature, Lovis 1994). La famille HSP70 est la plus abondante et la mieux conservée au cours de l'évolution. Le poids moléculaire des protéines de cette famille est d'environ 70 kDa. La principale protéine induite par le stress est la HSP72. Puisque dans la littérature, cette famille de protéines est communément appelée HSP70, c'est la nomenclature que nous utiliserons ici. C'est aussi à la grande famille des HSPs de haut poids moléculaire qu'appartiennent la OSP94, une protéine induite spécifiquement par le stress osmotique (Kojima et al. 1996) et la PBP74 (*peptide binding protein*) qui elle, n'est pas induite par le stress thermique. Les HSPs de faible poids moléculaire sont généralement exprimées de façon constitutive mais aussi à la suite d'un stress. Toutefois, elles sont beaucoup moins conservées dans l'évolution que la famille des HSP70. La famille des HSPs de faible poids moléculaire comprend les protéines de stress de poids moléculaires qui varient de 15 kDa à 30 kDa. Dans ce travail, nous nous sommes particulièrement intéressés à la protéine HSP27. Jusqu'à présent, une dizaine de gènes codant pour les HSP70 et un seul pour les HSP27 ont été localisés dans le génome humain.

Les HSPs sont induites en réponse à différents stress environnementaux dont la chaleur (Hamet et al. 1990, Sheikh-Hamad et al. 1994, Moseley et al. 1994) et l'hyperosmolarité (Sheikh-Hamad et al. 1994, Burg 1995, Cohen et al. 1991, Dasgupta et al. 1992, Kojima et al. 1996, Rauchman et al. 1997). Leurs nombreuses fonctions

sont résumées au tableau VI (revue de la littérature, Jacquier-Sarlin et Polla 1994). Une des principales fonctions des HSPs est celle de chaperoner, jouant un rôle dans l'assemblage et le transport des protéines nouvellement synthétisées et dans l'évacuation des protéines dénaturées (Craig et al. 1994, Gething et al. 1992). Ce processus a pour objet de prévenir l'aggrégation des protéines altérées suite au stress. L'induction de la synthèse et l'activation des HSPs par un stress modéré peut conférer une résistance accrue aux cellules qui subissent un stress subséquent plus sévère (Beck et De Maio 1994, Marber et al. 1993, Brown et al. 1992). Ce phénomène se nomme thermotolérance (Beck et Maio 1994) ou thermoprotection (Brown et al. 1992). Certaines HSPs ont aussi un rôle dans la signalisation cellulaire (Huot et al. 1996).

TABLEAU V

Classification des conditions inductrices des HSPs

Stress physiologiques	Stress pathophysiologiques	Stress environnementaux
Cycle cellulaire	Hypertrophie	Stress osmotique
Développement embryonnaire	Ischémie-reperfusion	Choc thermique
Différenciation cellulaire	Fièvre	Inhibition du métabolisme énergétique
Oncogenèse et proto-oncogenèse	Inflammation	Exercice physique
Stimulation hormonale	Infections	

TABLEAU VI

Fonctions des HSPs

- **Chaperon moléculaire**
  - Assemblage des protéines
  - Translocation des protéines
  - Maintien de la solubilité des protéines nucléaires et cytosoliques
  - *Clathrin uncoating ATPase*
  - Ciblage des protéines vers les lysosomes
- **Association aux récepteurs des stéroïdes**
- **Protéine de liaison du collagène**
- **Constitution du protéasome**
- **Dégradation protéique**

TABLEAU VII

Localisation des HSP27, HSP70 et HSP90 dans le rein

Localisation	HSP27[réf.1]	HSP70		HSP90[réf.4]
		Constitutive[réf.2]	Inductible[réf.3]	
Tubule proximal	+	++	++	±
Anse de Henlé	?	++	?	±
Tubule distal	+	++	?	+
Tubule collecteur cortical	++	?	?	+
Tubule collecteur médullaire	++	++	?	+

réf.1 Khan et al. 1996
réf.2 Komatsuda et al. 1992
réf.3 Van Why et al. 1991
réf.4 Matsubara et al. 1990
±, expression faible
+, expression modérée
++, expression abondante
?, non déterminé

HSPs et épithélium rénal

Un sommaire de la localisation des HSP27, HSP70 et HSP90 dans les différents tubules rénaux est présenté dans le tableau VII. De façon constitutive, la HSP70 est exprimée dans le noyau ainsi que dans le cytoplasme des cellules provenant des différents segments du néphron (Komatsuda et al. 1992). Par contre, la forme inductible de la HSP70, c'est-à-dire la HSP72, est absente dans le rein non-stressé et est induite par le stress thermique ou l'ischémie (Emani et al. 1991). La HSP90 est principalement exprimée dans le tubule distal (Matsubara et al. 1990). La HSP27 est exprimée surtout dans les cellules épithéliales du glomérule (Smoyer et al. 1996) et du tubule collecteur (Khan et al. 1996). Une expression préférentielle des HSPs a été observée dans la médullaire rénale par rapport au cortex rénal (Joannidis et al. 1995, Khan et al. 1996).

Peu d'études ont examiné la modulation des HSPs dans le rein (Joannidis et al. 1995, Brown et al. 1992, Hamet et al. 1990). Encore moins de travaux ont été publiés sur leur rôle dans cet organe (Brown et al. 1992, Joannidis et al. 1995, Emani et al. 1991). La plupart des données obtenues sur la modulation des HSPs dans le rein provient d'études utilisant les cellules MDCK, lignée cellulaire dérivée d'un épithélium rénal de chien (Cohen et al. 1991, Burg 1992, 1995, Sheikh-Hamad et al. 1994, Cowley et al. 1995, Moseley et al. 1994, Lovis et al. 1994). Quelques-unes ont étudié le rein en entier (Joannidis et al. 1995, Hamet et al. 1990). Les travaux démontrent, en résumé, que le stress thermique provoque une induction de l'ARNm spécifique à l'HSP70 de l'ordre de 8 à 20 fois la valeur observée dans les cellules MDCK non-stressées (Sheikh-Hamad et al. 1994, Burg 1995). Chez les souris génétiquement hypertendues, SHM, le stress thermique augmente de 13 fois le contenu rénal en

ARNm spécifique à l'HSP70 alors que l'augmentation est de 6 fois pour les animaux normotendus (Hamet et al. 1990).

Afin de conserver l'homéostasie corporelle en sel et en eau, le rein doit s'ajuster constamment aux variations environnementales telles l'hydratation de l'individu et sa consommation alimentaire en sel. Les cellules de l'épithélium rénal sont donc régulièrement soumises à des stress hyperosmotiques. Dans les cellules MDCK, le stress hyperosmotique induit une augmentation de 5 à 8 fois du contenu en ARNm spécifique à l'HSP70 (Cohen et al. 1991, Sheikh-Hamad et al. 1994, Burg 1995).

Quelques études *in vitro* ont démontré un phénomène de thermoprotection associé à l'induction des HSPs dans des cellules d'épithélium rénal de mammifères (Kulhmann et al. 1997, Moseley et al. 1994, Borkan et al. 1993). Toutefois, ces études ont généralement mesuré le phénomène de la thermoprotection par le maintien de l'intégrité cellulaire. *In vivo*, Perdrizet et al. (1993) ont démontré, dans un modèle porcin, qu'une augmentation modérée de la température corporelle protège des greffes allogéniques des lésions induites par l'ischémie inhérente aux techniques de transplantation. En effet, dix jours après la procédure chirurgicale, la survie des animaux du groupe ayant reçu un stress thermique était de 100% comparativement à 50-66% pour ceux des groupes témoins. Cette survie était accompagnée d'une récupération plus rapide de la fonction rénale. Parallèlement à une telle protection de la fonction rénale, les auteurs ont observé une induction de la HSP70. Par contre, Joannidis et al. (1995) n'ont pu démontrer l'acquisition de thermotolérance dans les reins de rats soumis à deux différents modèles d'ischémie-reperfusion, et ce, malgré l'induction des HSPs.

Implications des HSPs dans l'hypertension artérielle

Le docteur Hamet et son équipe ont démontré une réponse exagérée au stress chez des rongeurs spontanément hypertendus, et la ségrégation d'un tel trait avec l'élévation de la tension artérielle (Malo et al. 1989). Cette réponse est accompagnée d'une augmentation accrue de la production d'une protéine de choc thermique, HSP70 (Hamet et al. 1990). Cette équipe a suggéré que des modifications de la fonction du gène *hsp70* et celle d'autres membres des familles des HSPs sont impliquées dans la réponse exagérée au stress environnemental observée dans l'hypertension primaire (Hamet et al. 1994 et 1998). Cette hypothèse est basée sur la mise en évidence, par différentes équipes, d'une accumulation de l'ARNm du gène *hsp70* à la suite d'un stress thermique aigu dans différents tissus de rats (SHR) et de souris (SHM) spontanément hypertendus, aussi bien au niveau d'un stress subi par l'animal, ou un organe isolé, ou par des cellules en culture (Hamet et al. 1990, 1991, Gaia et al. 1995, Bongrazio et al. 1994, Huang et al. 1994). Notons que des résultats similaires ont été obtenus, chez l'humain, à partir de lymphocytes de sujets hypertendus (Kunes et al. 1992). La modification de la production des HSPs décrite précédemment a aussi été observée dans les reins d'animaux hypertendus soumis à un stress thermique (Hamet et al. 1990). De plus, l'équipe du docteur Hamet a mis en évidence la présence d'un polymorphisme par RFLP (Polymorphisme de longueur des fragments de restriction), du gène *hsp70* localisé sur le chromosome 20 chez des rats de souches congéniques (dans le complexe RT1 des lignées BN.1k et SHR.1N) et consanguines recombinantes (RIS-*Recombinants Inbred Strains*). Ce polymorphisme est associé à une différence de la tension artérielle de 15 mmHg entre les souches congéniques (Hamet et al. 1992). Ils ont également démontré la présence d'un polymorphisme, chez le rat, du gène *hsp27* (PCR-SSCP-Polymorphisme de conformation du simple brin), associé à l'hypertrophie ventriculaire, un prédicteur de complications de l'hypertension artérielle (Hamet et al. 1996). De plus, Gaia et ses collaborateurs (1995) ont observé une forte induction de la

HSP72 à la suite d'un stress thermique, non seulement dans le cœur, qui est un organe cible de l'hypertension artérielle, mais aussi dans le muscle squelettique. L'ensemble de ces résultats mettent donc en évidence l'implication des HSPs dans la pathogenèse de l'hypertension primaire. Les modifications de l'expression des HSPs ne seraient pas une conséquence de l'augmentation chronique de la tension artérielle.

Les HSPs semblent aussi impliquées dans l'hypertension aiguë. En effet, Udelman et ses collaborateurs ont démontré que des agents vasoconstricteurs, connus pour leur potentiel d'induction de l'hypertension via différentes voies de signalisation, augmentent le contenu en ARNm du gène *hsp70* dans les aortes de rats (Xu et al. 1995). De plus, le stress thermique qui induit la production des HSP70 et HSP27 protège de la nécrose dans les cellules du muscle lisse vasculaire (Champagne et al. 1998).

CONCLUSION

La revue de la littérature présentée dans cette section suggère que les anomalies du transport rénal d'ions observées dans l'hypertension génétique pourraient être reliées aux anomalies des HSPs décrites dans l'hypertension.

CHAPITRE 2

RÉGULATION DE LA POMPE Na^+, K^+ ET DES TRANSPORTEURS D'IONS COUPLÉS AU SODIUM DANS L'ÉPITHÉLIUM RÉNAL DE MAMMIFÈRES : RÔLE DE LA PROTÉINE KINASE C ET DES PURINOCEPTEURS-P_2

Na^+, K^+-pump and sodium-coupled ion carriers in mammalian kidney epithelia : regulation by protein kinase C and P_2-purinoceptors

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SUMMARY

This review up-dates our current knowledge on the regulation of Na^+/H^+ exchanger, Na^+,K^+,Cl^- cotransporter, Na^+,P_i cotransporter and Na^+,K^+ pump in renal epithelial cells by protein kinase C (PKC) and P_2-purinoceptors. In epithelial cells derived from different tubule segments, an activator of PKC, 4β-phorbol 12-myristate 13-acetate (PMA), inhibits apical Na^+/H^+ exchanger (NHE3) and Na^+,P_i cotransport as well as basolateral Na^+,K^+ cotransport (NKCC1) but augments the basolateral Na^+/H^+ exchanger (NHE1) and Na^+,K^+ pump. In PMA-treated proximal tubules, activation of Na^+,K^+ pump probably plays a major role in increased reabsorbtion of salt and osmotically-obliged water. In Madin-Darby canine kidney (MDCK) cells, which are highly abundant with intercalated cells from the collecting duct, PMA completely blocks Na^+,K^+,Cl^- cotransport and decreases the activity of Na^+,P_i cotransport by 30-40%. In these cells, agonists of P_2-purinoceptors inhibit Na^+,K^+,Cl^- and Na^+,P_i cotransport by 50-70% via a PKC-independent pathway. In contrast to MDCK, in epithelial cells derived from proximal and distal tubules of the rabbit kidney, Na^+,K^+,Cl^- cotransport is inhibited by PMA but is insensitive to P_2-receptor activation. In proximal tubules, PKC-induced inhibition of NHE3 and Na^+,P_i cotransporter can be triggered by parathyroid hormone. Both PKC and cAMP signaling contribute to dopaminergic inhibition of NHE3 and Na^+,K^+ pump. The receptors triggering PKC-mediated activation of Na^+,K^+ pump remain unknown. Recent data suggest that the PKC signaling system is involved in abnormalities of dopaminergic regulation of renal ion transport in hypertension and in the development of diabetic complications. The role of P_2-purinoceptors in the modulation of renal ion transporters under pathophysiological conditions has not yet been examined.

Key words: Na^+/H^+ exchanger, Na^+,K^+,Cl^- and Na^+,P_i cotransporters, Na^+,K^+ pump, protein kinase C, P_2-purinoceptors

INTRODUCTION

Na^+-coupled carriers localized on apical membrane of renal epithelial cells of proximal tubules (PT), thick ascending limb of Henle's loop (THAL) and distal tubules (DT), i.e. Na^+-coupled transporters of amino acids, glucose and inorganic phosphate, Na^+/H^+ exchange, Na^+,K^+,Cl^- and Na^+,Cl^- cotransport, contribute to reabsorption of ~80-90% of salt and osmotically-obliged water along nephron. Na^+,K^+,Cl^- cotransport localized on basolateral membrane along with apical K^+ channels are major pathways for K^+ secretion in collecting ducts (CD) and final adjustment of potassium homeostasis (Fig. 1). This transcellular ion movement machinery is under the complex control of a set of circulating and locally-released hormones and neurotransmitters, whose efficiency is varied along the nephron and in different species.

Apart from the release of cathecolamines, sympathetic stimulation in the kidney is accompanied by massive egress of ATP as co-transmitter (Rump et al., 1996). Using cDNA probes, mRNA species encoding several types of P_{2Y}, P_{2U} and P_{2X}-purinoceptors triggered by ATP and other nucleotides have been found in kidney epithelium (Rice et al., 1995; Tokuyama et al., 1995; Takeda et al., 1998; Webb et al., 1996). In several types of renal epithelial cells, including CD-derived Madin-Darby canine kidney (MDCK) cells, P_{2Y}- and P_{2U}- purinoceptors were characterized pharmacologically by analysis of the effects of P_2-agonists and antagonists on transcellular Cl^- transport, intracellular free Ca^{2+} concentration, the activity of Ca^{2+}-operated K^+ channels, phospholipase A_2, C, D (PLA_2, PLC, PLD) and MAP kinase (Simmons, 1981; Paulmichl et al., 1991; Zegarra-Moran et al., 1995; Firestein et al., 1996; Xing et al., 1997; Gagnon et al., 1998a). Recently, we reported that in MDCK cells, addition of ATP and other P_2-purinergic receptor agonists is accompanied by

~2-fold inhibition of Na^+,K^+,Cl^- cotransport, whereas activation of protein kinase C (PKC) completely blocks this carrier (Gagnon et al., 1998d). In these cells, activation of PKC and P_2-purinoceptors also leads to partial inhibition of Na^+,P_i-cotransport (Gagnon et al., 1998b). Keeping in mind these data and the fields of our expertise, the present review is focused on the regulation by PKC and P_2-purinoceptors of Cl^--dependent monovalent ion cotransporters, Na^+/H^+ exchange and Na^+,P_i cotransport. Data on the regulation of renal Na^+-coupled amino acid transporters, Na^+/Ca^{2+} exchanger and monovalent ion channels were summarized recently in other publications (Breyer, Ando, 1994; Douglas, Hopfer, 1994; Friedman, Gesek, 1995; Lang, Paulmichl, 1995; Aronson, Giebisch, 1997; Wright et al., 1997; Giebisch, 1998). Regulation of renal ion transporters by cAMP and cGMP signaling was also considered in these reviews.

Na^+,P_i cotransport and Na^+/H^+ exchange are highly sensitive to alteration of the transmembrane Na^+ gradient that is mainly under the control of Na^+,K^+ pump. Na^+_i as well as Cl^-_i can also inhibit Na^+,K^+,Cl^- cotransport via binding to regulatory sites of the carrier (Russell, 1983; Breitwieser et al., 1996). Considering this, the regulation of Na^+,K^+ pump by PKC is also a topic of the present review. In addition, the osmolality of lumen fluid is elevated in THAL due to a countercurrent multiplication mechanism and is varied in CD from 50-to1500 mosm, depending on the open probability of water channels in apical membrane of DT and CD controlled by antidiuretic hormone (Fig. 1). Keeping this in mind, the possible role of cell volume in regulation of the above-mentioned ion transporters by PKC and P_2-purinoceptors under normal and pathophysiological conditions was briefly analyzed.

It should be underlined that this review is concentrated on the regulation of renal ion transporters by diacylglycerol-sensitive isoforms of PKC whose activity can

be modulated selectively by derivatives of 4β-phorbol esters, such as 4β-phorbol 12-myristate 13-acetate (PMA). However, apart from classic (PMA/diacylglycerol+Ca^{2+})-sensitive cPKC (PKC-α, βI, βII and γ) and novel PMA/diacylglycerol-sensitive, Ca^{2+}-independent nPKC (PKC-δ, ε, η and ξ), atypical (Ca^{2+}+ PMA/diacylglycerol)-insensitive aPKC (PKC-ζ, ι, λ and μ) have been characterized (Kikawa et al., 1989; Akimoto et al., 1994; Selbie et al., 1993). Treatment of cells with antisense oligodeoxynucleotides and transfection with dominantly-expressed mutated forms of PKC are probably the best tools to dissect the relative contribution of different PKC isoforms in the regulation of renal function. Thus, using antisense oligodeoxynucleotides, it was demonstrated that PKC-ε is mainly involved in the regulation of epithelial Na^+ channels by vasopressin in primary cultured cells from rabbit CD (DeCoy et al., 1995). However, this approach has not been used yet to study the relative contribution of PKC isoforms in the regulation of renal transporters covered by this review. Finally, this review is limited by consideration of the acute effects of PKC and purinoceptors on ion transporters which are not mediated by *de novo* synthesis or catabolism of ion transporters and/or their regulatory proteins.

Na^+/H^+ exchanger

Na^+/H^+ exchanger providing electroneutral countertransport of Na^+ and H^+ was firstly cloned from the human genomic DNA library using a so-called H^+ suicide strategy in Na^+/H^+ exchange-deficient fibroblasts (Sardet et al., 1989). With mRNA probes, it was shown that this carrier is expressed in all mammalian cells studied so far, including the basolateral membrane of renal epithelial cells, and is referred to as ubiquitous Na^+/H^+ exchanger type 1 (NHE1). Later on, the tissue-specific forms of this carrier, including NHE3 which is highly expressed in brush-border membranes of PT, small intestine and colon, were revealed by low-stringency screening of cDNA

libraries (for more details, see (Wakabayashi et al., 1997; Levine et al., 1995; Tse et al., 1993; Aronson, 1996).

By comparing the properties of Na^+/H^+ exchanger isoforms in transfected cells, it was established that in contrast to NHE1, apical NHE3 is highly resistant to amiloride and its derivatives (EC_{50} for amiloride 3 and 150 μM, respectively) (Orlowski, 1993; Tse et al., 1993). Another striking feature of NHE3 is its regulation by cell volume. It was shown that shrinkage activates NHE1 in all types of cells studied so far (Orlov, Novikov, 1996; Lang et al., 1998) with the exception of human erythrocytes (Orlov et al., 1989). NHE4, which seems to be selectively expressed in the basolateral membrane of CD, is quenched under basal conditions and can be measured only under cell shrinkage (Chambrey et al., 1997a, b). In contrast to NHE1 and NHE4, cell shrinkage inhibits NHE3 in transfected cells (Nath et al., 1996) as well as in epithelial cells from PT of the opossum kidney (OK) and in LLC-PK_1 cell line derived from PT of the pig kidney (Soleimani et al., 1994).

The third peculiarity of NHE3 is its regulation by PKC. It is well documented that activation of PKC augments the activity of NHE1 due to an increase of its affinity for H^+_i (for review, see (Noel, Pouyssegur, 1995; Wakabayashi et al., 1997). The same results were obtained recently for NHE2 and NHE4 (Chambrey et al., 1997a, b; Kandasamy et al., 1995). In contrast, PMA treatment inhibits NHE3 in transfected cells (Orlowski et al., 1992; Kandasamy et al., 1995; Brant et al., 1995; Yip et al., 1997), OK (Helmle-Kolb et al., 1990), LLC-PK_1 (Singh et al., 1996), MCT cells derived from murine PT (Mrkic et al., 1992) and in stationary microperfused convoluted segments of rat renal PT (Reboucas, Malnic, 1996). Similarly to volume-dependent regulation, NHE3 inhibition by PKC is caused by a 25-35% decrease of V_{max} of the carrier and does not modulate its affinity for H^+_i. Several lines of evidence

indicate that NHE1 activation by growth factors is at least mediated in part by PKC-dependent elevation of the affinity of the carrier for H^+_i. (Wakabayashi et al., 1997). In contrast to NHE1, both serum and fibroblast growth factor (FGF) activate NHE3 in transfected PS120 cells via increment of V_{max} of the carrier (Brant et al., 1995), suggesting that PKC is not involved in the regulation of NHE3 by growth factors.

Both NHE1 and NHE3 exhibit several potential phosphorylation sites for PKA and PKC (Orlowski et al., 1992). However, neither FGF nor PMA affected phosphorylation of NHE3 in transfected cells (Yip et al., 1997), showing that this regulatory pathway is mediated by intermediate associated regulatory protein(s). Taking into account the abundance of intracellular vesicles in PT cells with NHE3 (Biemesderfer et al., 1997), it may also be assumed that NHE3 inhibition by PMA is caused by modification of the cytoskeleton and proteins controlling intracellular trafficking of the carrier. Recently, this hypothesis was confirmed by confocal morphometric analysis of the brush border/subapical distribution of NHE3 in PMA-treated Caco-2 cells derived from human colon adrenocarcinoma (Janecki et al., 1998).

In contrast to numerous data on the regulation of NHE1 and NHE3 by PKC, the involvement of P_2-receptors in the regulation of Na^+/H^+ exchanger in the renal epithelium has not yet been explored. To the best of our knowledge, data on the regulation of NHE1 in non-epithelial cells are limited to the report of De Souza with co-workers (1995) on its transient activation in PC12 cells treated with ATP or UTP.

Cl^--dependent cotransporters

Extensive studies performed during the last few years have led to cloning of several members of the superfamily of carriers providing Cl^--dependent cotransport (uniport) of monovalent cations. Two of them (NKCC1 and NKCC2) operate under the simultaneous presence of Na^+, K^+ and Cl^- (Na^+,K^+,Cl^- cotransport) and can be completely inhibited by a p-sulfamoylbenzoic acid derivative, furosemide, and its more potent and selective analogues, bumetanide and piretanide. NKCC1 is the ubiquitous isoform of the carrier, whereas NKCC2 is expressed exclusively on apical membrane of THAL. The other member of the superfamily, NCC, encodes K^+-independent, furosemide-resistant, thiazide-sensitive Na^+,Cl^- cotransport and is expressed in apical membrane of DT. Both ubiquitous and nerve tissue-specific isoforms of Na^+-independent K^+,Cl^- cotransporter (KCC1 and KCC2, respectively) are resistant to thiazide derivatives and much less sensitive to furosemide compared to NKCC1 and NKCC2. The role of KCC1 in renal function remains unclear (for more details, see (Haas, 1994; Payne, Forbush, 1994; Payne et al., 1995; Gillen et al., 1996; Payne et al., 1996; Kaplan et al., 1996; Ecelbarger et al., 1996; Barry et al., 1997).

One potential cAMP-dependent protein kinase (PKA) and 8 PKC phosphorylation sites have been found in the deduced amino acid sequence of mouse NKCC1 (Delpire et al., 1994). Several PKA and PKC phosphorylation sites have also benn found in winter flounder NCC and mammalian NKCC2 (Gamba et al., 1994; Igarashi et al., 1995). However, the regulation of NKCC1, NKCC2 and NCC in transfected cells by PKC has not yet been studied. This item becomes especially important when analysizing the tissue-specific action of PKC modulators on NKCC1 activity. Thus, in rat erythrocytes and vascular smooth muscle cells, PMA did not affect Na^+,K^+,Cl^- cotransport (Orlov et al., 1988; 1992), whereas in the rabbit tracheal

epithelium (Liedtke, Thomas, 1996) and NIH-3T3 fibroblasts (Hichami et al., 1996), it led to activation of this ion carrier. MDCK cells are highly abundant with basolateral NKCC1 (Delpire et al., 1994). In these cells, PKC activation results in complete inhibition of Na^+,K^+,Cl^- cotransport (Fig. 2a). In epithelial cells from PT, DT and CD of the rabbit kidney, PMA inhibited Na^+,K^+,Cl^- cotransport by 30, 45 and 20%, respectively (Gagnon et al., 1998c).

Fig. 2a shows that activation of P_2-receptors with ATP leads to ~50% inhibition of Na^+,K^+,Cl^- cotransport in MDCK cells. In contrast to MDCK cells, extracellular ATP increased Na^+,K^+,Cl^- cotransport by 2.5-fold in A6 cells derived from the *X.laevis* kidney (Middleton et al., 1993). However, the mechanism of NKCC1 regulation by P_2-purinoceptors in MDCK and amphibian kidney seems to be different. Thus, Cl^- channel blockers diminished the regulation of Na^+,K^+,Cl^- cotransport by ATP in A6 cells (Middleton et al., 1993) but did not affect it MDCK cells (Gagnon et al., 1998b).

Similarly to MDCK cells, ATP caused a transient increment of $[Ca^{2+}]_i$ in epithelial cells from PT of the rabbit kidney (Cejka et al., 1993; Gagnon et al., 1998c) and in DT-derived A6 cells (Nilius et al., 1995), suggesting that these cells also express P_2-receptors. However, we did not observe modulation of Na^+,K^+,Cl^- cotransport by ATP in epithelial cells derived from PT and DT of rabbit kidney (Gagnon et al., 1998). This observation indicates that purinergic inhibition of Na^+,K^+,Cl^- cotransport revealed in MDCK cells is limited to canine kidney or is caused by selection of a special cell phenotype of epithelial cells from CD under numerous passages.

The regulatory properties of basolateral NKCC1 and apical Na^+,K^+,Cl^- cotransporter that is expressed exclusively in THAL seem to be essentially different. Thus, in contrast to an obligatory requirement of K^+ for operation of NKCC1 (Orlov et al., 1996), furosemide-sensitive Na^+ uptake in epithelial cells from rabbit THAL was independent of the presence of K^+_o (Alvo et al., 1985). Kaji (1993) reported that alteration of osmolality in the range from 200 to 400 mosm leads to 3-fold activation of bumetanide-sensitive K^+ influx in mouse THAL. In contrast, the activity of NKCC1 expressed on the basolateral membrane of PT and in MDCK cells was increased by ~10-fold at the same range of osmolality (Raat et al., 1994; Gagnon et al., 1998a). Considering this striking difference, it may be assumed that potentiation of bumetanide-sensitive K^+ uptake by cell shrinkage observed in THAL by Kaji was partly mediated by basolateral NKCC1. Indeed, modulation of cell volume in anisotonic media has negligible effect on bumetanide-sensitive ion fluxes in NKCC2-transfected *X. laevis* oocytes (Gamba et al., 1994) and in NKCC2-transfected HEK-293 cells (Isenring et al., 1998). Moreover, it was reported that in contrast to basolateral NKCC1, cell swelling activates apical Na^+,K^+,Cl^- cotransport in THAL (Reeves et al., 1988). Interestingly, the same unusual properties of volume-dependent regulation were revealed in a study of Na^+,K^+,Cl^- cotransporter in primary cultured astrocytes from the rat brain (Mongin et al., 1994), C6 glioma cell line (Mongin et a.l., 1996) and in HEK-293 cells transfected with human NKCC1 (Isenring et al., 1998), again underlining the tissue-specific character of the regulation of Cl^--dependent cotransporters. Amlal and co-workers (1996) reported that in suspensions of medullary THAL of the rat kidney, NKCC2 measured as the bumetanide-inhibited component of HN_4^+ fluxes is slightly activated by PMA and inhibited by arachidonic acid and the Ca^{2+}-ionophore ionomycin. No information is currently available to indicate whether P_2-purinoceptors affect the activity of NKCC2 and NCC in renal epithelial cells.

Na^+,P_i cotransport

In the glomerular ultrafiltrate, P_i concentration is about 2 mM, whereas in luminal fluid leaving the proximal tubules it can be as low as 0.2 mM. Using monolayers of epithelial cells, it was shown that the activity of apical Na^+,P_i cotransport is a rate-limited step of intensive P_i reabsorption in this tubule segment (Biber, 1989). Recent electrophysiological studies demonstrated that despite different Hill coefficients (1 and 2 for P_i and Na^+, $K_{m(Na+)}$ ~50 mM, $K_{m(Pi)}$ ~0.15 mM), this carrier operates in electrogenic mode (for review, see (Biber et al., 1996)). Molecular biology experiments led to the identification of two subfamilies of Na^+,P_i cotransport (type I or NaPi-1 and type II or NaPi-2 related) sharing weak overall homology (20% identity) and possessing a distinct membrane architecture. The NaPi-1 isoform was originally cloned from the rabbit kidney cortex and identified by expression in *X.laevis* oocytes (Werner et al., 1991). Later on, several isoforms from the NaPi-2-related subfamily (NaPi-2 – NaPi-7) were cloned from human, rabbit, mouse, bovine and rat kidney cDNA libraries. Both type I and type II mRNA are expressed in PT. Type II mRNA and immunoreactive protein were also detected in distal nephron segments. Based on regulation by parathyroid hormone (PTH, see Section 6) and dietary P_i intake, type II isoforms of Na^+,P_i cotransporter mainly contribute to P_i reabsorption in PT. The functional significance of NaPi-1 remains unclear (for more details, see (Busch et al., 1996; Biber et al., 1996)).

Using OK cells, it was shown that addition of PMA is accompanied by up to 40% time-dependent inhibition of Na^+,P_i cotransport (Cole et al., 1987; Malmstroem et al., 1988). In MDCK cells, 30 min of preincubation with PMA leads to 30% inhibition of Na^+,P_i cotransport (Fig. 2b). Considering the lack of NaPi-1 mRNA expression in these cells (Quabius et al., 1995), it may be assumed that similarly to

OK cells, this effect of PMA is mediated by inhibition of the type II isoform of the carrier. Indeed, in oocytes injected with Na,Pi-II cDNA, PMA inhibited Na^+-dependent P_i uptake to the same extent (Hayes et al., 1995). Several consensus sites for PKC were found within the amino acid sequence of type II Na^+,P_i cotransporter. However, site-directed mutagenesis of serine-5, 91, 462, 625 and threonine-508 did not prevent PMA-induced inhibition of Na^+,P_i cotransport (Hayes et al., 1995). Thus, the precise mechanism of inhibition of this carrier by PKC remains unknown.

In MDCK cells, Na^+,P_i cotransport is also inhibited by 60-70% after addition of extracellular ATP (Fig. 2b). The type of purinoceptors involved in the inhibition of Na^+,P_i cotransport in MDCK cells has not yet been identified. Lederer and McLeish (1995) reported that in OK cells, addition of the agonist of P_{2X}–receptor, β–methylene-ATP (AMP-CPP), attenuates the inhibitory effect of PTH on the activity of Na^+,P_i cotransport, whereas an agonist of P_{2Y}-receptors, 2-methyl-thio-ATP, does not affect this regulatory pathway. However, the role of GTP-binding protein-independent P_{2X}-receptor possessing properties of intrinsic ion channels in the purinergic modulation of Na^+,P_i cotransport seems unlikely because of the lack of effect of AMP-CPP in pertussis toxin-treated OK cells (Lederer, McLeish, 1995).

Na^+,K^+ pump

Under basal conditions, Na^+,K^+ pump provides ouabain-sensitive hydrolysis of ATP coupled to electrogenic inward movement of $2K^+$ and outward movement of $3Na^+$. The functional unit of Na^+,K^+ pump is composed of α-and β-subunits in a 1:1 ratio. Three isoforms of α- and β- subunits have been cloned up to now. All α-subunits contain intracellular ATP- and Na^+- and extracellular ouabain- and K^+-binding sites, exhibit ouabain-sensitive, $(Na^+ + K^+)$-stimulated, Mg^{2+}-dependent ATP hydrolysis and are able to provide movement of Na^+ and K^+ against their

electrochemical gradient in the absence of the β-subunit that is probably involved in regulation of the affinity of enzyme for K^+_o and reassembling of pump in the plasma membrane. The major differences between the α-subunits are their tissue distribution and sensitivity to ouabain and Na^+_i. Thus, half-maximal activation by Na^+_i occurres at 12, 24 and 36 mM for α1, α2 and α3, respectively. α2- and α3-subunits are extremely sensitive to ouabain (EC_{50} 0.01-0.05 μM), whereas half-maximal inhibition of α1 is observed at 10 μM of ouabain. α1 and β1 are major subunits expressed in basolateral membranes of renal epithelia, and the low sensitivity of α1 to ouabain contributes to the side-effects of endogenous circulating ouabain-like factors involved in the pathogenesis of hypertension (for review, see (Hamet et al., 1995; DeWardener, 1996; Hamlyn et al., 1996)). Recently, a third γ-subunit codistributed with Na^+,K^+ pump activity along the nephron was identified. However, its functional significance remains unclear. For more details, see (McDonough, Farley, 1993; Ewart, Klip, 1995; Geering, 1997; Ueno et al., 1997)

It is well-documented that PKC activation with PMA enhances solute and water reabsorption in microperfused proximal tubules (Liu, Cogan, 1990; Wang, Chan, 1990), indicating the activation of major apical or/and basolateral pathways involved in reabsorption of salt and osmotically-obliged water in this nephron segment. As shown in Sections 1 and 3 both apical both Na^+/H^+ exchanger and Na^+,P_i cotransporter in PT are inhibited rather than activated by PMA, suggesting that potentiation of transcellular salt movement by PMA is mediated via activation of basolateral Na^+,K^+ pump. However, data on regulation of the enzymatic activity of renal Na^+,K^+ pump by PMA-sensitive PKC are rather contradictory. Thus, Satoh and Ominato with co-workers reported that PMA does not modulate the activity of Na^+,K^+ pump in homogenates from THAL and CD of the rat kidney but inhibits it by 40% in PT (Satoh et al., 1993; Ominato et al., 1996). In contrast, Pedemonte and co-workers

reported that in OK cells in the first 10 sec after addition of PMA, $[Na^+]_i$ is decreased from 20 to 6 mM without any activation of Na^+ influx, indicating ~100-fold transient elevation of Na^+,K^+ pump activity in these cells (Pedemonte et al., 1997). This discrepancy may be caused by the presence of species-specific regulatory pathways as well as by some uncontrolled steps in the experimental procedure. Such steps may be related to the level of tissue oxygenation. Indeed, it was demonstrated that PMA activates Na^+,K^+ pump in well oxygenated PT but inhibits it under conditions with uncontrolled oxygen supply (Feraille et al., 1995; Carranza et al., 1996).

We observed that 30-min treatment of MDCK cells with PMA increases the rate of ouabain-sensitive ^{86}Rb influx by 25-35% (Fig. 2c). This result is consistent with data obtained for OK (Pedemonte et al., 1997), but contradicts findings of 40-50% inhibition of Na^+,K^+ ATPase in MDCK cells reported by Shahedi and co-workers (1992). It should be underlined, however, that different approaches were used to estimate the regulation of Na^+,K^+ pump by PMA in these studies. Indeed, ouabain-sensitive ^{86}Rb uptake and $[Na^+]_i$ were measured in intact cells by our group and by Pedemonte and co-workers, respectively (Gagnon et al., 1998d; Pedemonte et al., 1997), whereas the maximal rate of $(Na^+{+}K^+)$-dependent ATP hydrolysis was measured in broken cells by Shahedi and co-workers (1992). It was shown that in oxygen-supplied PT, PMA did not affect the V_{max} of Na^+,K^+ pump but increased its apparent affinity for Na^+_i (Feraille et al., 1995; Carranza et al., 1996). This regulatory mechanism can not be observed by measurement of maximal activity of $(Na^+{+}K^+)$-dependent ATP hydrolysis in the presence of saturated Na^+ concentration. In addition to these methodological features, the preparation of cell lysates for the measurement of $(Na^+{+}K^+)$-dependent ATPase can lead to redistribution of PKC and proteins involved in PKC-mediated signal transduction that complicates interpretation of the results obtained by this method.

The contribution of direct phosphorylation of the α1-subunit and/or its regulators to modulation of the activity of Na^+,K^+ pump by PKC is still an unresolved issue. In oxygenated rat kidney PT, increment of the activity of Na^+,K^+ pump was positively correlated with the level of phosphorylation of the α1-subunit (Carranza et al., 1996). In Cos-7 and OK cells transfected with α1-Na^+,K^+ pump, modulation of the activity of the enzyme by PMA was abolished by deletion or mutation of the N-terminal containing PKC phosphorylation sites (Belusa et al., 1997; Pedemonte et al., 1997). However, this manipulation of the N-terminus can also lead to modification of the site that interacts with protein regulator(s) subjected to phosphorylation by PKC. Indeed, using transfected cells, it was shown that PMA-induced phosphorylation is abolished by mutation of PKA- but not PKC-phosphorylation sites (Beguin et al., 1996). In contrast to freshly-isolated PT (Feraille et al., 1995; Carranza et al., 1996), OK (Pedemonte et al., 1997) and MDCK cells (Gagnon et al., 1998d), PKC inhibits purified renal Na^+,K^+ pump by 40-50% which is accompanied by incorporation of 2 mol of phosphate per mole of the enzyme (Bertorello et al., 1991). Viewed collectively, these results strongly suggest that the activation of Na^+,K^+ pump by PMA, observed in intact cells and well-oxygenated tubules, is mediated by non-identified regulator(s).

In MDCK cells, activation of purinergic signaling with ATP augments Na^+,K^+ pump by 25-30% (Fig. 2c). To the best of our knowledge, there are no other data on regulation of the activity of renal Na^+,K^+ pump by P_2-purinoceptors.

Purinergic inhibition of Na^+,K^+,Cl^- and Na^+,P_i cotransporters: evidence of a PKC-independent mechanism

Data presented in the first four sections show that activation of purinergic signaling in MDCK cells leads to inhibition of Na^+,K^+,Cl^- and Na^+,P_i cotransporters and slightly increases ion fluxes mediated by Na^+,K^+ pump (Fig. 2). The rank-order potency of agonists of purinoceptors as inhibitors of Na^+,K^+,Cl^- cotransport in MDCK cells (ATP~ADP>UTP>>AMP) (Gagnon et al., 1998d) suggests that their effect is mediated by P_{2X}-, P_{2U}- or P_{2Y}-receptors. Indeed, the P_{2Z} subtype is completely insensitive to ADP and AMP, whereas P_{2T} is activated by ADP and antagonized by ATP and AMP and seems to be expressed exclusively in thrombocytes (Watson, Girdlestone, 1994). During the last few years, several isoforms of 7 membrane-spanning P2Y/2U-purinoceptors coupled to Gp/PLCβ-mediated breakdown of polyphosphoinositides and intracellular Ca^{2+}-release as well as P2X-receptors containing 2 transmembrane segments and possessing properties of intrinsic ion channel have been cloned (for review, see (Evans, 1996, Rassendren et al., 1997, Maier et al., 1997). The inhibitory effect of ATP on MDCK Na^+,K^+,Cl^- cotransporter was preserved under modulation of volume and intracellular monovalent ion content (Gagnon et al., 1998a). These results rule out the possible involvement of P_{2X}-receptors in volume- or Na^+_i/Cl^-_i-dependent modification of the activity of this carrier.

It is well-documented that activation of P_{2Y}- and P_{2U}-receptors in MDCK cell leads to transient elevation of $[Ca^{2+}]_i$ via release from intracellular stores and influx through Ca^{2+}-release activated channels (Paulmichl et al., 1991; Delles et al., 1995). The same receptors also cause activation of PLA_2, PKC, PKA and the MAP kinase signaling cascade (Paulmichl et al., 1991; Firestein et al., 1996; Xing et al., 1997). As mentioned in Sections 2 and 3, along with agonists of P_2-purinoceptors, both Na^+,P_i and Na^+,K^+,Cl^- cotransporters in MDCK cells are also inhibited by PMA, suggesting

that the effect of purinoceptors can be mediated by activation of diacylglycerol-sensitive isoforms of PKC. However, neither downregulation of PKC by prolonged incubation of MDCK cells with PMA nor an inhibitor of PKC, calphostin C, abolished the regulation of Na^+,K^+,Cl^- cotransporter by ATP (Gagnon et al., 1998a, d). In addition, ATP-induced inhibition of Na^+,P_i and Na^+,K^+,Cl^- cotransporters was insensitive to acute heat stress (46°C, 15 min), whereas regulation by PMA was completely abolished by this treatment (Gagnon et al., 1998b). These observations strongly suggest that diacylglycerol-sensitive isoforms of PKC are not involved in purinoceptor-induced inhibition of the activity of these carriers. We also did not see any effect of modulators of PLA_2, PKA, $Ca^{2+}{}_i$ and MAPK-signal transduction pathways on ATP-induced inhibition of Na^+,K^+,Cl^- cotransporter. Neither cholera nor pertussis toxin affects purinergic-induced inhibition of this carrier (Gagnon et al., 1998a). Consequently, we hypothesized that a novel membrane-delimited signaling pathway or/and a novel subtype of P_2-receptor coupled to GTP-binding proteins distinct from G_s and G_i is involved in the inhibition of Na^+,K^+,Cl^- cotransporter by ATP. This hypothesis is currently being examined in our laboratory.

Hormones and neuromediators involved in regulation of ion transporters by the PKC-dependent pathway

Several peptide hormones and neuromediators, including PTH, endothelin, dopamine and cathecholamine, are able to activate the PLCβ/PKC signaling cascade and modulate reabsorption of salt and osmotically-obliged water in different tubule segments (Vander, 1991; Breyer, Ando, 1994; Aronson, Giebisch, 1997; Wright et al., 1997; Giebisch, 1998). However, as mentioned in the previous section, activation of PLCβ/PKC by P_2-purinceptors is not involved in the inhibition of PKC-sensitive basolateral Na^+,K^+,Cl^- cotransporter by ATP. In this section, we analyze data on the

role of PKC in the regulation by hormones and neuromediators of other ion transporters covered by this review.

PTH

PTH and parathyroid hormone-related peptide interact with common GTP-binding protein-coupled receptors localized on the basolateral membrane of PT and stimulate the activity of adenylate cyclase and PLCβ. The latter enzyme mediates polyphosphoinositide hydrolysis and activates the diacyl glycerol-sensitive isoform of PKC (for review, see (Biber, 1989)). It is well-documented that PTH decreases NaCl reabsorption in PT (Vander, 1991). Data presented below show that both apical Na^+/H^+ exchange and Na^+,P_i cotransport contribute to this regulatory pathway.

The inhibition of NHE3 in rabbit PT by PTH was initially observed by Kahn and co-workers (1985). Data obtained with synthetic PTH fragments triggering selective activation of GTP-binding proteins coupled to adenylate cyclase and PLC suggest that both PKC and PKA contribute to 30-50% inhibition of NHE3 in OK cells, which is in accordance with the inhibition of this carrier by PMA and forskolin (Azarini et al., 1995). Despite the triggering of a transient $[Ca^{2+}]_i$ rise, inhibition of NHE3 in OK cells by PTH was insensitive to the modulation of $[Ca^{2+}]_i$ with ionomycin and BAPTA (Helmle-Kolb et al., 1990), showing that PKC isoforms with low sensitivity to Ca^{2+} are involved in suppression of the carrier's activity. In contrast to OK, in MCT cells, PTH does not augment cAMP content, and PTH-induced inhibition of Na^+/H^+ exchanger is probably mediated exclusively by PKC (Mrkic et al., 1992).

The regulation of Na^+,P_i cotransport by PTH was studied in more detail in OK cells. LLC-PK_1 cells lack PTH receptors, whereas in JTC-12 cells derived from the

monkey kidney, this transporter is resistant to PTH despite the presence of its receptors (Malmstroem, Murer, 1986). In OK cells, PTH leads to time-dependent ($\tau \sim 1.5$ hrs) inhibition of this carrier (Caverzasio et al., 1986). Using antibodies directed against NaPi-2 it has been demonstrated that PTH provokes a decrease of immunoreactive protein content in apical membranes (Kempson et al., 1995), suggesting that slow kinetics of the inhibition of Na^+,P_i cotransport in PT by PTH is caused by internalization of the carrier. As mentioned in Section 3, PKC activation in these cells leads to 40-60% inhibition of Na^+,P_i cotransport. The same level of inhibition was triggered by PKA activation with forskolin or 8-Br-cAMP (Caverzasio et al., 1986). However, the evidence listed below shows that PKC-coupled signaling pathways rather than PKA activation are dominan in the inhibition of Na^+,P_i cotransport by PTH in OK cells.

1) Half-maximal inhibition of Na^+,P_i cotransport was revealed at 10^{-11} M PTH, whereas half-maximal activation of cAMP production was observed at $5x10^{-9}$ M of this peptide (Cole et al., 1987).
2) PKC down-regulation by long-term treatment with PMA completely abolished the regulation of Na^+,P_i cotransport in OK cells by PTH (Siegfried et al., 1995).
3) In JTC-12 cells, PTH activates cAMP production but does not affect the activity of Na^+,P_i cotransport (Malmstroem, Murer, 1986).

These results contradict the direct involvement of PKA in PTH-induced inhibition of Na^+,P_i cotransport in PT. However, additional regulatory cAMP-mediated pathways can not be excluded. Indeed, using OK cells with a dominant mutated regulatory subunit of PKA, it was shown that Na^+,P_i cotransport is insensitive to PTH but still inhibited by PMA (Segal, Pollock, 1990). It may be assumed that this additional step is mediated by cAMP release and its degradation to adenosine by ecto-phosphodiesterase and 5'-nucleotidase. Indeed, inhibition of Na^+,P_i

cotransport by activation of adenosine receptors was confirmed recently (Friedlander et al., 1996). Interestingly, prolonged incubation with PTH or PMA increased ecto-5'-nucleotidase, indicating the presence of a feedback mechanism controlling the efficiency of regulation of the carrier by adenosine receptors (Siegfried et al., 1995). The relative contribution of this paracrine/autocrine pathway in the regulation of NHE3 by PTH-induced activation of cAMP signaling has not yet been explored.

Dopamine

Acute application of dopamine supresses salt and water reabsorption in rat and rabbit renal PT and THAL (Davis et al., 1968; Bello-Reuss et al., 1982; Lee, 1982; McGrath et al., 1980). In PT, this diuretic effect of dopamine is probably caused mainly by the inhibition of basolateral Na^+,K^+ pump and apical Na^+/H^+ exchanger. It has been shown that both dopamine-induced natriuresis and NHE3 supression are abolished by treatment with antagonists of D_1 receptors coupled to the cAMP signaling cascade (Moe et al., 1991). These results suggest that despite NHE3 inhibition by direct activators of PKC (see Section 1), stimulation of PLCβ/PKC-coupled D_2 receptors is not involved in dopaminergic supression of this carrier. However, to confirm this negative conclusion, additional experiments using downregulation PKC by longterm treatment with PMA and PKC antisense oligodeoxynucleotides should be carried out.

In contrast to NHE3, both D_1 and D_2 receptors contribute to inhibition of Na^+,K^+ pump revealed by measurement of (Na^++K^+)-dependent ATP hydrolysis in dopamine-treated permeabilized tubules and cell lysates (Bertorelo, Aperia, 1990; Pinto-de-O et al., 1997). When analyzing the role of Na^+,K^+ pump, it should be underlined that activation of D_1-receptors leads to ~2-fold activation of natriuresis (Nishi et al., 1993), whereas maximal inhibition of Na^+,K^+-ATPase by combined

excitation of D_1- and D_2-recepotrs does not exceed 20-30% (Aperia et al., 1987; Seri et al., 1988; Takemoto et al., 1992; Nishi et al., 1993). Moreover, Ibarra and co-workers reported that in the absence of the α-adrenergic agonist oxymetazoline, inhibition of Na^+,K^+-ATPase in permeabilized rat PT does not exceed 5-10% (Ibarra et al., 1993). It should also be underlined that the above-listed data on dopaminergic regulation of renal Na^+,K^+-ATPase were obtained by measurement of the maximal activity of this enzyme in broken cells. The limitations of this approach for data interpretation are considered in Section 4. We did not observe any modulation of the activity of Na^+,K^+ pump measured in intact MDCK cells as the rate of ouabain-sensitive ^{86}Rb influx after 30-min incubation with dopamine (Gagnon et al., 1998c). Thus, additional experiments should be conducted to draw a final conclusion on the role of Na^+,K^+ pump and apical ion carriers in dopaminergic-induced natriuresis and on the relative contribution of PKA and PKC to this phenomenon.

Endothelin and vasopressin

Chu and co-workers reported that in OK cells transfected with endothelin type B receptors, endothelin-1 increases NHE3 activity. However, this signaling pathway was insensitive to PKC inhibitors (Chu et al., 1996). Recently, it was shown that co-transfection of HEK293 cells with mutationally-activated Gα12 subunit decreases NHE1 activity but augmentes Na^+/H^+ exchange mediated by NHE3 (Lin et al., 1996). The role of this GTP-binding protein subfamily in the modulation of ion transport in renal epithelial cells by hormones and neuromediators has not yet been studied.

It was shown that vasopressin stimulates NaCl absorption in perfused mouse, rat and rabbit THAL (Hall, Varhey, 1980; Hebert et al., 1981; Sasaki, Imai 1980) probably by activating Na^+,K^+,Cl^- cotransport (Sun et al., 1991). However, in this nephron segment, vasopressin activates the cAMP signaling pathway triggered by V_2-

receptors and does not modify PKC which is under the control of PLCβ-coupled V_1 receptors (Hebert et al., 1987; Molony et al., 1989). The key role of cAMP signaling systems in the activation of NKCC2 in THAL was confirmed by observation of the activatory effect of 8-Br-cAMP and forskolin on this carrier in cells derived from mouse THAL (Vuillemin et al., 1992; Wu et al., 1994) and in freshly-isolated thick ascending limbs from the rat kidney (Amlal et al., 1996).

Pathophysiological implications

Hypertension

An unaltered level of salt excretion despite chronic hypertension termed as "kidney resetting" is one of the major determinants of longterm maintenance of elevated blood pressure (Guyton, 1991). By analyzing monogenous or Mendelian forms of hypertension and hypotension such as Liddle's syndrome, pseudohyperaldosteronism type 1, Gitelman's syndrome, Bartter's syndrome, Lifton and co-workers demonstrated that these forms of secondary hypertension are caused by single point mutations in genes encoding renal epithelial amiloride-sensitive Na^+ channels, NKCC2 and NCC (Lifton, 1995; 1996). Several lines of evidence suggest that abnormal activity of NHE3, NKCC2 and α1-Na^+,K^+ pump contributes to kidney resetting and the pathogenesis of primary (essential) hypertension (for review, see (Postnov, Orlov, 1985; Hamet et al., 1995; Cusi, 1997; Orlov et al., 1998). However, in contrast to symptomatic hypertension, the molecular determinants of altered activity of renal transporters in essential hypertension are poorly understood. It may be assumed that side by side with intrinsic determinants caused by mutations of ion transporters per se or their intracellular regulators, kidney resetting in primary hypertension is caused by peculiarities in the hormonal regulation of reabsorption of salt and osmotically-obliged water in the renal epithelium.

The most consistent results supporting this hypothesis were obtained by analysis of the dopaminergic regulation of transcellular salt transport in PT from spontaneously hypertensive rats (SHR). Using this experimental model of human essential hypertension, it was shown that dopamine and D_1-agonists or antagonists have little influence on sodium excretion, which can be partly attributed to the decreased ability of dopamine to inhibit NHE3 and Na^+,K^+-ATPase (Felder et al., 1990; Kaneko et al., 1990; Gesek, Schoolwerth, 1991; Chen et al., 1992; Nishi et al., 1993). As mentioned in Section 6, both PKA and PKC contribute to dopaminergic inhibition of Na^+,K^+ pump in PT. It has been shown that PLC (Chen et al., 1992), PKC (Kansra et al., 1995) and cAMP signaling (Felder et al., 1993) present an attenuated response to dopamine in PT from SHR compared with normotensive rats. This blunted response to dopamine in SHR seems to be limited to PT as it was not detected in CD from the SHR kidney (Ohbu, Felder, 1993).

A high-salt diet provokes increased dopamine production in PT (Moe et al., 1991), providing feedback control of blood pressure by extracellular fluid volume. Considering this, it is important to mention that in salt-resistant rats, dopamine infusion led to a ~2-fold increment of fractional sodium secretion but did not alter renal sodium handling in salt-sensitive rats which are extremely sensitive to the blood pressure-elevating effect of high-salt diet (Nishi et al., 1993). The relative contribution of altered coupling of dopamine receptors with PKA- and PKC-mediated signal transduction in the abnormal regulation of renal NHE3 and Na^+,K^+ ATPase in this experimental model of salt-sensitive essential hypertension remains unclear.

Diabetic complications

Diabetes mellitus is accompanied by vascular complications which are responsible for most of the morbidity and mortality associated with this disease. These complications are manifested as coronary and cerebrovascular diseases, nephropathy, hypertension and accelerated atherosclerosis. Clinical trials have concluded that normalization of glucose level prevents the further development of diabetic complications, indicating that chronic hyperglycemia rather than abnormal insulin level plays a key role in vascular damage (The Diabetic Control Research Group, 1993). It is suggested that diabetic complications triggered by hyperglycemia are mediated via intracellular accumulation of sorbitol under catabolism of glucose by aldose reductase and imbalance of cell volume regulation. Initially, this hypothesis was based on the rapid development of vascular complications in animals fed galactose, which is known to be the more preferable substrate for aldose reductase compared to glucose, and on prevention of complications caused by high glucose/galactose diet by aldose reductase inhibitors (Burg, Kador, 1988; McManus et al., 1995). Later on, it was demonstrated that vascular smooth cells growing on high glucose medium possess enhanced cell volume, DNA and protein synthesis, and inhibitors of aldose reductase partly abolish these differences (Yasunari et al., 1995). For our review, it is important to underline that side by side with abnormalities of cell volume regulation, vascular smooth muscle cells under hyperglycemic conditions exhibit enhanced PKC activity (Natarajan et al., 1992; Yasunari et al., 1996). Activation of this enzyme is probably caused by augmented diacylglycerol content as a consequence of increased intracellular hexoses (Xia et al., 1994) or/and abnormal activity of volume-dependent *myo*-inositol transporter (Burg, 1995). Among the various PKC isoforms, PKC-β and PKC-δ appear to be activated preferentially, with the key role of PKC-β being confirmed by analysis of cardiovascular complications in transgenic mice overexpressing this enzyme (for more details, see (Koya, King,

1998). Apart from enhanced proliferation, the elevated activity of PKC in vascular smooth muscle and nervous tissue cells is accompanied by inhibition of Na^+,K^+ pump (Greene et al., 1987; Koya, King, 1998).

Are diabetic complications in the kidney triggered by damage of the vasculature or do they also directly affect tubular epithelium cells via impairment of cell volume regulation and altered activity of PKC-dependent renal ion transporters? The transport of organic osmolites, such as betaine and myo-inositol, and modulation of aldose reductase activity (Burg, 1995) as well as NHE4 (Bookstein et al., 1994) seem have a major impact on sustained and acute cell volume regulation in CD. It has been suggested that side by side with normalization of cell volume caused by feedback regulation of transport and synthesis of organic osmolites, enhanced expression of heat shock proteins (HSP) contributes to protection of epithelial cells against chronic elevation of the osmolality of tubular fluid (Burg, 1995). Indeed, it was shown that sustained elevation of osmolality leads to heightened HSP70 mRNA expression in MDCK cells (Cohen et al., 1991; Sheikh-Hamad et al., 1994). However, despite enhanced HSP70 RNA content, western blot analysis did not reveal any increment of immunoreactive HSP70 and HSP27 protein in anisotonically-shrunken MDCK cells (Gagnon et al., 1998b), which may be attributed to nonspecific inhibition of protein synthesis in shrunken cells (Burg, Garcia-Perez, 1992). We also did not observe any modulation of Na^+,K^+,Cl^- and Na^+,P_i cotransporters under HSP70 and HSP27 induction by mild heat stress (Gagnon et al., 1998b). The role of a recently-discovered member of the HSP70/BiP superfamily, osmotic stress protein Osp94 (Kojima et al., 1996), in the protection of function of shrunken renal epithelial cells has not yet been explored.

Using three proximal tubule-like cell lines (JCT12, LLC-PK1 and OK cells) and primary cultures of human PT epithelia it was shown that hyperglycemia increases the activity of Na^+-coupled *myo*-inositol transporter and, in contrast to vascular smooth muscle and nervous tissue, enhances Na^+,K^+ pump activity. Similarly to vascular smooth muscle, the activity of (Ca^{2+}+phospholipid)-dependent PKC was augmented in JCT12 and human PT epithelia but was not altered in LLC-PK1 and OK cells (Cole et al., 1995). Under topics considered in the present review, it is important to underline that kidney ischemia and injury are accompanied by massive release of ATP and other purinoceptor-active nucleotides (Motte et al., 1995). Forthcoming experiments should further clarify the role of PKC and P_2-purinoceptors in the alteration of ion transporters during development of renal complications in diabetes mellitus and hypertension.

CONCLUSION AND UNRESOLVED ISSUES

The data summarized in Sections 1-4 show that in the mammalian kidney, PKC inhibits apical NHE3 and Na^+,P_i cotransport as well as basolateral NKCC1 and augments Na^+,K^+ pump (Fig. 3). In PT, PKC activation leads to enhanced salt reabsorption (Liu, Cogan, 1990; Wang, Chan, 1990), suggesting that activation of Na^+,K^+ pump prevails over PKC-induced suppression of apical carriers. PTH causes PKC activation but in contrast to direct activators of this enzyme, it decreases NaCl reabsorption in PT via inhibition of apical NHE3 and Na^+,P_i cotransporter without any indication of activation of basolateral Na^+,K^+ pump (Fig. 3). Dopamine, another activator of PKC, also decreases salt and water reabsorbtion, but in contrast to PMA, dopaminergic activation does not affect ouabain-sensitive ion fluxes in intact renal epithelial cells and slightly reduces the activity Na^+,K^+-ATPase in cell lysates. In MDCK cells, both Na^+,P_i and Na^+,K^+ cotransporters are inhibited by PMA but they are completely insensitive to activation of the PLCβ/PKA signaling cascade by angiotensin II, bradykinin, vasopressin, dopamine and methacholine (Gagnon et al., 1998d). Several explanations may be proposed by analysis of the different effects of direct and receptor-mediated activation of PKC on ion transport in the renal epithelium.

1) The overall action of excitation of PKC-coupled receptors on transcellular transport in the renal epithelium is under the control of cross-talk with other intracellular signaling systems triggered by these receptors, including PKA and PMA-insensitive isoforms of PKC. This cross-talk was considered in Section 6 under analysis of the regulation of NHE3, Na^+,P_i cotransport and Na^+,K^+ pump by PTH and dopamine.

2) Activation of PKC-coupled receptors is accompanied by release of biologically-active compounds, such as adenosine, metabolites of the arachidonic

acid cascade, etc. These paracrine/autocrine pathways complicate the comparison of data obtained in situ, isolated tubule segments, cultured cells and isolated membrane fractions.

3) Epithelial cells localized in proximal segments of THAL and in CD are subject to alteration of the osmolality of tubular fluid (Fig. 1). Side by side with anisotonic cell volume modulation, numerous pathways, including hormones and neuromediators, affect cell volume under isosmotic conditions (Hoffmann, Simonsen, 1989; Orlov, Novikov, 1996; Lang et al., 1998). As mentioned in Sections 1 and 2, cell shrinkage activates NHE1 and NKCC1 and inhibits NHE3. In OK cells, cell volume perturbations alter the activity of Na^+,P_i cotransport (Loghman-Adham, Motock, 1993). In electrically-excitable cells, including giant snail neurons (Suleimanian, et al., 1984), embryonic skeletal muscle-derived L6 myoblasts (Sen et al., 1995), frog skeletal muscle (Venosa, 1991), primary cultured astrocytes (Mongin et al., 1994) and rat brain synaptosomes (Aksentsev et al., 1994), hyperosmotic swelling leads to 2-fold activation of Na^+,K^+ pump independently of the modulation of activity of the other Na^+ transport pathways involved in the regulation of $[Na^+]_i$. The same results have been obtained with microdissected mouse cortical CD (Coutry et al., 1994). It should be underlined that, apart from the direct effect on the activity of ion transporters, cell volume affects their sensitivity to modulators of intracellular signaling (Orlov et al., 1992). Considering this, the possible modulation of cell volume should be taken into account when analyzing the regulation of ion transporters by activators of PKC and PKC-coupled receptors in the isolated kidney and *in vivo*.

In addition to modulation by PKC, in MDCK cells, agonists of P_2-receptors inhibit Na^+,K^+,Cl^- and Na^+,P_i cotransport by a PKC-independent mechanism (Fig. 3). In contrast to MDCK cells deived form CD, ATP-induced inhibition of Na^+,K^+,Cl^-

and Na^+,P_i cotransporters is not observed in epithelial cells derived from PT and DT of the rabbit kidney (Gagnon et al., 1998c). Two subtypes of cells possessing properties of principal and intercalated cells were isolated by Gekle and co-workers (1994) using the parental MDCK line. Based on the low value of transepithelial electrical resistance (~150 Ω/cm^2, Gagnon et al., 1998d), the MDCK cells used in our study are highly abundant with intercalated cells. Thus, it may be assumed that P_2-purinoceptor-induced inhibition of Na^+,K^+,Cl^- cotransport is limited to intercalated cells from CD only. This hypothesis is currently being examined in our laboratory. Purinergic modulation of ion transporters may be especially important in massive ATP release under local ischemia and kidney injury so that the pathophysiological implication of this signaling pathway deserve further investigation.

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ABBREVIATIONS

CD:	Collecting duct
DT:	Distal tubule
MDCK:	Madin-Darby canine kidney
NaiP1-NaiP7:	Isoforms of Na^+,P_i cotransporters
NCC:	Na^+,Cl^- cotransporter
NHE1-NHE4:	Isoforms of Na^+/H^+ exchangers
NKCC1 and **NKCC2**:	Isoforms of Na^+,K^+,Cl^- cotransporters
OK:	Opossum kidney
PKA:	cAMP-dependent protein kinase
PKC:	Protein kinase C
PLC:	Phospholipase C
PMA:	4β-phorbol 12-myristate 13-acetate
PT:	Proximal tubule
PTH:	Parathyroid hormone
THAL:	Thick ascending limb of Henle loop

FIGURE 1

Volume and osmolality of tubular fluid (**A**) and major transporters involved in reabsorption of salt and osmotically obliged water along nephron (**B**). **A**. – cell volume and osmolality of tubular fluid in proximal (**I**) and distal (**II**) parts of proximal tubule (**PT**), thick ascending limb of Henle loop (**THAL**), distal tubule (**DT**) and collecting duct (**CD**). Effect of antidiuretic hormone (**ADH**) on volume and osmolality in collecting duct is shown. The volume of tubular fluid passing through glomerular was taken as 100%. **B** – Schematic representation of major transporters involved in transcellular salt movement. **1** – Na^+-coupled cotransporters of inorganic phosphate, amino acid and glucose; **2** – apical Na^+/H^+ exchanger (NHE3 isoform); **3** – apical Na^+,K^+,Cl^- cotransport (NKCC2 isoform); **4** – Na^+,Cl^- cotransport; **5, 6** – Na^+ and K^+ channels; **7** - basolateral Na^+,K^+,Cl^- cotransport (NKCC1 isoform); **8** – Na^+,K^+ pump. **a.m**. and **b.m.** – apical and basolateral membrane, respectively. Inhibitory effect of intracellular Na^+ and Cl^- on the activity of ion transporters are shown by broken lines. Restricted permeability of apical membrane for water in DT and CD are shown as ----!. Adapted from (Vander, 1991; Aronson, 1996; Breyer, Ando, 1994).

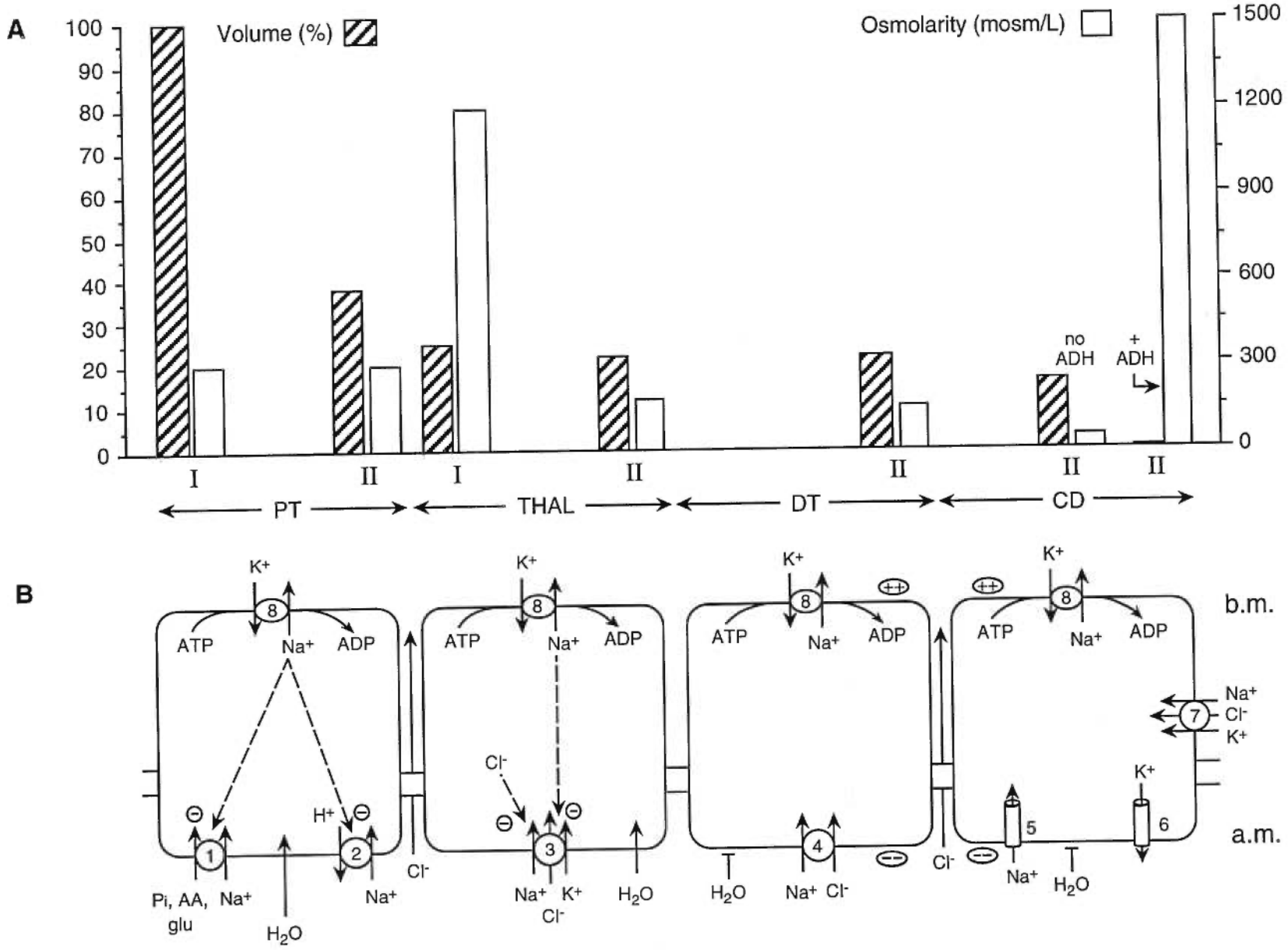

A
Volume (%)
Osmolarity (mosm/L)
100
90
80
70
60
50
40
30
20
10
0
1500
1200
900
600
300
0
no ADH
+ ADH
I
II
I
II
II
II
II
PT
THAL
DT
CD
B
K+
ATP
Na+
ADP
8
b.m.
a.m.
H+
Cl-
1
2
3
4
5
6
7
Pi, AA, glu
Na+
H2O
K+
Cl-
++
--

FIGURE 2

Effect of PMA and ATP on the activity of Na^+,K^+,Cl^- cotransport (**A**), Na^+,Pi cotransport (**B**) and Na^+,K^+ pump (**C**) in MDCK cells. Before the measurement of the activity of ion transporters cell were preincubated during 30 min in control medium or in the presence of 0.1 μM PMA or 100 μM ATP (Gagnon et al., 1998a,b).

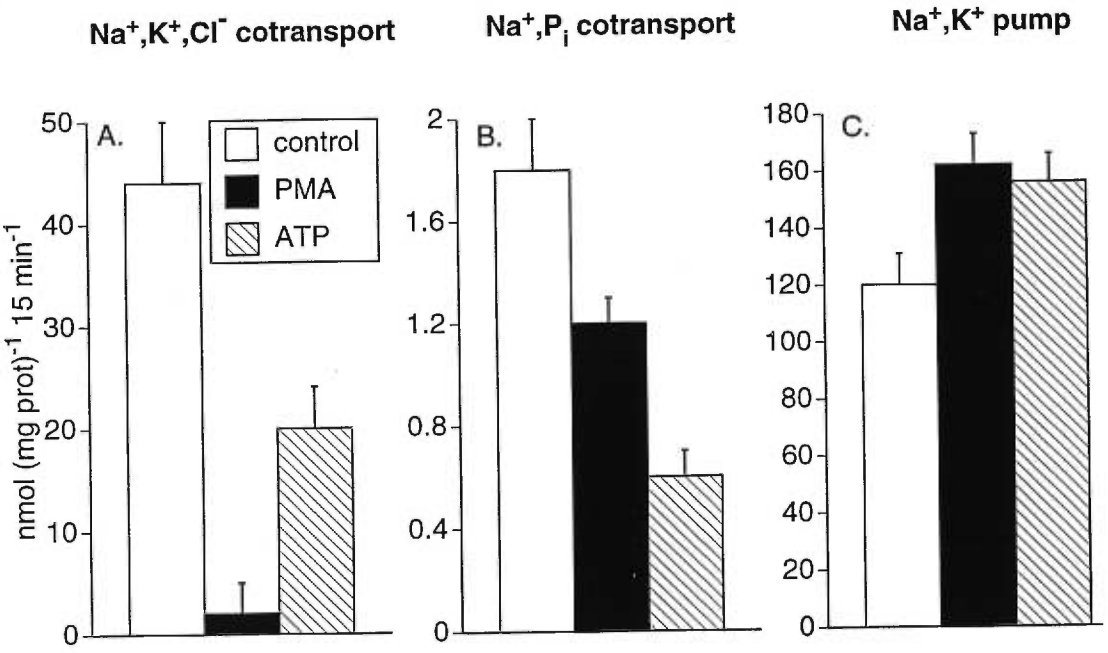
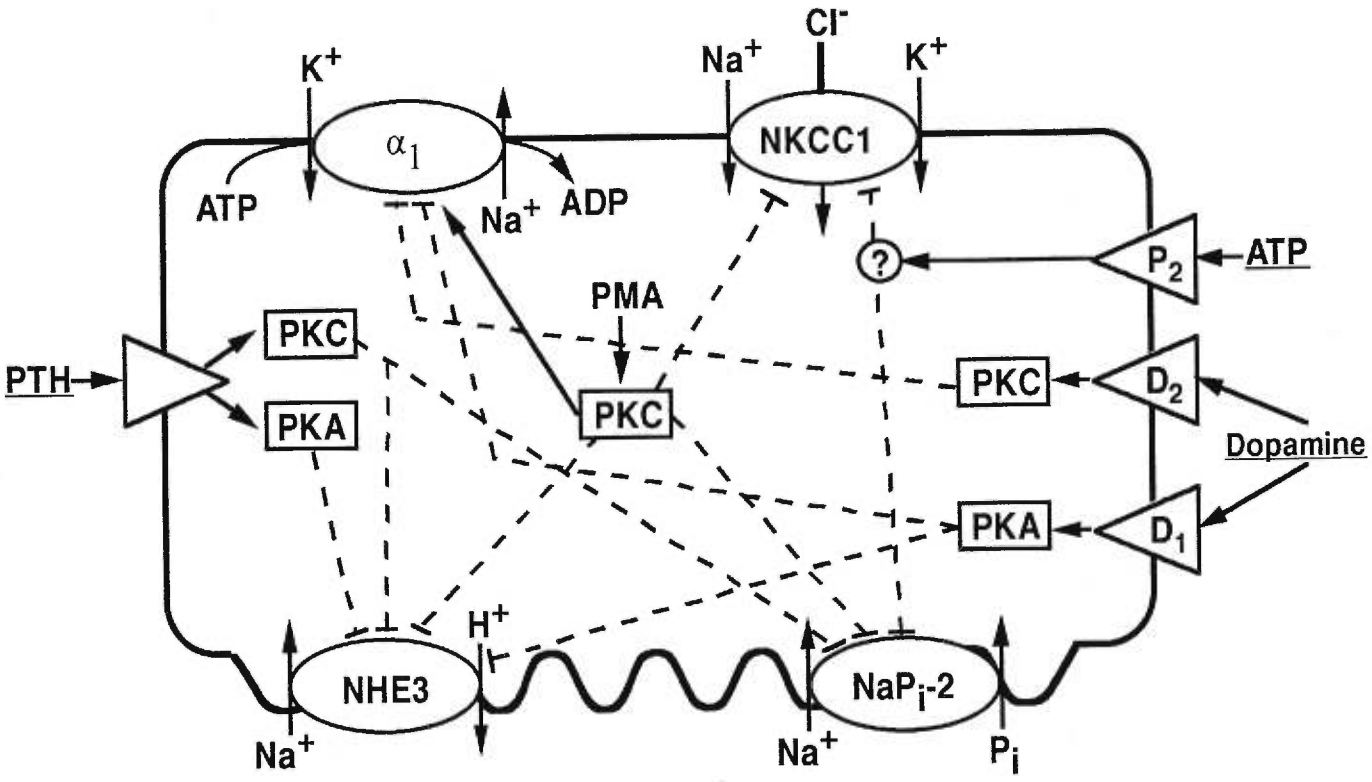

Na+,K+,Cl- cotransport
Na+,Pi cotransport
Na+,K+ pump
A.
B.
C.
control
PMA
ATP
nmol (mg prot)-1 15 min-1
50
40
30
20
10
0
2
1.6
1.2
0.8
0.4
0
180
160
140
120
100
80
60
40
20
0

FIGURE 3

The major pathways of the regulation of the activity of Na^+,K^+ pump ($\alpha 1$) and Na^+-coupled ion carriers (NHE3, NKCC1, NaPi-2) in renal epithelial cells by 4β-phorbol 12-myristate 13-acetate (PMA) –sensitive protein kinase C (PKC) and PKC and protein kinase A (PKA) coupled to PTH, dopaminergic (D_1, D_2) and P_2-purinergic receptors. --→ - activation; ------! – inhibition; ? – unidentified steps. For more details see text.


K+
Na+
Cl-
K+
α1
NKCC1
ATP
Na+
ADP
?
P2
ATP
PMA
PKC
PTH
PKA
PKC
PKC
D2
Dopamine
PKA
D1
H+
NHE3
NaPi-2
Na+
Na+
Pi

DEUXIÈME PARTIE

PRÉSENTATION DES MANUSCRITS

CHAPITRE 3

INHIBITION COMPLÈTE DE L'ACTIVITÉ DU CO-TRANSPORTEUR Na^+, K^+, Cl^- DANS LES CELLULES MDCK PAR LES ISOFORMES DE LA PROTÉINE KINASE C SENSIBLES AU PMA

Complete inhibition of Na^+, K^+, Cl^- cotransport in Madin-Darby canine kidney cells by PMA-sensitive protein kinase

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SUMMARY

This study examines the involvement of hormones and neuromediators in the regulation of Na^+,K^+,Cl^- cotransport in renal epithelial cells using Madin-Darby canine kidney cells with low transepithelial electrical resistance (194 ± 47 Ω/cm^2). In this cell line, Na^+,K^+,Cl^- cotransport measured as bumetanide-sensitive ^{86}Rb influx was inhibited up to 50-60% with agonists of P_2-purinoceptors (ATP≈ADP>UTP> AMP), slightly (15-30%) increased by activators of cAMP signaling (forskolin, 8-Br-cAMP) and was insensitive to activators of cGMP signaling (8-Br-cGMP, nitroprusside), EGF, angiotensin II, bradykinin, methacholine, propranolol, vasopressin, adenosine, dopamine and histamine. Thirty min preincubation of MDCK cells with 0.1 μM PMA completely blocked the activity of Na^+,K^+,Cl^- cotransport whereas down-regulation of this enzyme by 24 hr preincubation with 1 μ M PMA activated Na^+,K^+,Cl^- cotransport by 60% and abolished the effect of short-term treatment with PMA. Regulation of Na^+,K^+,Cl^- cotransport by ATP was insensitive to down-regulation of PMA-sensitive isoforms of protein kinase C. In addition, an inhibitor of protein kinase activity, staurosporine, abolished the effect of 0.1 μM PMA but did not change inhibition of this carrier by ATP. Thus, these results show for the first time that P_2-purinoceptors and PMA-sensitive isoforms of protein kinase C play a key role in the regulation of Na^+,K^+,Cl^- cotransport in MDCK cells. These results also show that neither PMA- nor staurosporine-sensitive forms of protein kinase are involved in the inhibition of Na^+,K^+,Cl^- cotransport by activators of P_2-purinoceptors.

Key words: Na^+,K^+,Cl^- cotransport, P_2-purinoceptors, intracellular signaling, MDCK cells

INTRODUCTION

Twenty years ago, using microperfused tubule preparations, it was established that the diuretic effect of sulfamoylbenzoic acid derivates (SAD) is caused by inhibition of transcellular movement of salt and osmotically obliged water in the thick ascending limb of Henle's loop (THAL) [1,2]. Later on, it was shown that SAD-sensitive ion transporter is operated as electroneutral Na^+,K^+,Cl^- cotransport with variable stoichiometry and topology in different nephron segments (for recent data, see [3,4]). The control of tubular reabsorption of salt and osmotically obliged water is also variable along tubular segments. Thus, in the collecting duct, cAMP directly increases the number of functioning water channels in apical membranes, the density of apical Na^+ channels and the open probability of 35 pS K^+ channels, whereas activation of protein kinase C-coupled signaling decreases the open probability of Na^+ channels and 35 pS ATP-sensitive K^+ channels (for review see [5]). Little is known about the regulation of Na^+,K^+,Cl^- cotransport in mammalians kidney. It has been shown that reabsorption of NaCl in the THAL is stimulated by cAMP signaling [6]. However, the relative contribution of Na^+,K^+,Cl^- cotransport and ion channels in this phenomenon is still a matter of controversy [7-9]. The present study examines the regulation of inward K^+ fluxes in epithelial cells derived from canine kidney (MDCK). Our results indicate that in these cells, Na^+,K^+,Cl^- cotransport is completely inhibited by activation of protein kinase C with 4ß-phorbol 12-myristate 13-acetate (PMA), and partly by activation of P_2-purinoceptors. We also present evidence that inhibition of Na^+,K^+,Cl^- cotransport by P_2-purinoceptor agonists is not mediated by PMA-sensitive forms of protein kinase C.

MATERIALS AND METHODS

Cell culture

MDCK cells from the American Type Culture Collection (ATCC No. CCL 34) were used in this study. MDCK cells seeded on permeable support have a transepithelial resistance of 194 ± 47 Ω/cm^2. These results suggest that MDCK cells from our stock have the properties of MDCK-strain II described by Simmons et al. [10] and clone C11 described by Gekle and co-workers [11]. In accordance with data presented in the last paper, this substrain of MDCK cells resembles intercalated cells of collecting duct. MDCK cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and grown in MEM (Gibco Laboratories, Burlington, Canada), supplemented with sodium bicarbonate 2.5 g/L, HEPES 2 g/L, penicillin 100 U/ml, streptomycin 100 μg/ml and 10% fetal bovine serum (Wisent, WI). The medium was changed 2-3 times per week. The cells were passaged at subconfluent density by treatment with 0.05% trypsin (Gibco Laboratories) in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline and scraped from the flasks with a rubber policeman. Cell were counted by Coulter Counter. Dishes were inoculated at 1.25 x 10^3 cell/cm^2. Both stock cultures and cultures for experiments were grown for 6 to 8 days to attain subconfluency, in 80 cm^2 culture flasks and 24-well plates, respectively.

Measurements of K^+ (^{86}Rb) influx

To study K^+ (^{86}Rb) influx, MDCK cells were washed twice with 2 ml of medium A containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES-tris buffer (pH 7.4, room temperature) and for 30 min at 37°C in 1 ml of medium B with and without agents indicated in the tables and figures. Medium B contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, D-glucose 5, HEPES-tris 20. Then, the preincubation medium was replaced with 0.25 ml of the same medium

B with or without 1 mM ouabain and 20 μM bumetanide. The cells were incubated at 37°C for 5 min, and thereafter 0.25 ml of medium B containing 1-2 μCi/ml ^{86}RbCl was added. ^{86}Rb uptake was terminated by the addition of 2 ml of ice-cold medium C containing 100 mM $MgCl_2$ and 10 mM HEPES-tris buffer (pH 7.4). The cells were then washed 4 times with 2 ml of ice-cold medium C and lysed with 1 ml of 1% SDS/4 mM EDTA mixture. The radioactivity of the cell lysate was measured with a liquid scintillation analyzer. ^{86}Rb (K^+) influx was calculated as $V=A/amt$ where A is the radioactivity in the sample (cpm), a is the specific radioactivity of ^{86}Rb (K^+) (cpm/nmol) in the incubation medium, m is the protein content (mg) and t is the incubation time (min). Protein content was measured by modified Lowry's method [12]. In the absence of ion transport inhibitors, the kinetics of ^{86}Rb uptake were linear up to at least 20 min (Fig.1A). Based on these finding, an incubation time of 15 min was used to determine the initial rate of K^+ influx.

Chemicals

PMA, 4α-PMA, bradykinin, norepinephrine, isoproterenol, ATP, AMP, 8-Br-cAMP, forskolin, arginine vasopressin, 8-Br-cGMP, ouabain, bumetanide, furosemide - Sigma (St. Louis, MO); dopamine, methacholine, phenylephrine, UTP, ADP, histamine - Research Biochemicals International (Natick, MA); angiotensin II - Armand-Frappier Institute (Laval, Canada); epidermal growth factor (EGF) - Gibco RBL (Gaithersburg, MD); ^{86}RbCl - Dupont (Boston, MA); salts, D-glucose and buffers were from Sigma and Anachemia (Montréal, Canada).

Statistical analysis

Experimental manipulations were performed in tri- or quadruplicate within any single experiment. The results are given as means ± standard errors. When appropriate, statistical significance was assessed by Student' s t-test for unpaired data. Differences of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The Na^+,K^+ pump and Na^+,K^+,Cl^- cotransport, identified as ouabain-sensitive and ouabain-resistant bumetanide-sensitive ^{86}Rb influx, respectively, mediated ~ 65% and 30% of total K^+ influx in MDCK cells (Fig. 1B). Both the absolute values and relative activity of the Na^+,K^+ pump and Na^+,K^+,Cl^- cotransport were in accordance with previously-reported data [13-17]. In the presence of 10 μM bumetanide, 1 mM furosemide did not affect ^{86}Rb influx (data not shown). It is known that at this concentration, furosemide inhibits Na^+-independent K^+,Cl^- cotransport [18]. Thus, it may be concluded that (ouabain + bumetanide)-resistant K^+ influx, accounting for about 5% of ^{86}Rb influx (Fig. 1B), represents ion transport along its electrochemical gradient via ion channels and leakage of the MDCK membrane. We use the term "passive permeability" to combine these minor ion transport pathways.

Table 1 shows that an activator of protein kinase C, PMA, completely blocked Na^+,K^+,Cl^- cotransport in MDCK cells, whereas ATP inhibited this carrier by 50-60% ($p<0.001$). Activation of cAMP signaling with 8-Br-cAMP, forskolin and cholera toxin increased the rate of Na^+,K^+,Cl^- cotransport by ~20%, 30% and 60%, respectively (Table 1), whereas activation of cGMP signaling with 8-Br-cGMP (1 mM) or nitroprusside (1 mM) did not affect Na^+,K^+,Cl^- cotransport (data not shown). Both PMA and ATP activated the Na^+,K^+ pump by 20-40% ($p<0.02$ and 0.05, respectively) (Table 1). We did not observe any significant effect of angiotensin II (1 μM), bradykinin (0.1 μM), vasopressin (0.1 μM), epidermal growth factor (1 μM), adenosine (100 μM), dopamine (100 μM), histamine (10 μM) and methacholine (100 μM) on K^+ influx in MDCK cells (data not shown). Previously, it was reported that in MDCK cells, PMA and dopamine inhibit Na^+,K^+-ATPase activity by 15-20% and 30-40% respectively [15,19]. It should be emphasized, however, that crude

membrane fractions of MDCK cells were used to estimate the activity of this enzyme in the above-cited studies.

Figure 2 displays the dose-dependency of inhibition of Na^+,K^+,Cl^- cotransport by ATP, ADP, AMP and UTP. The rank-order of potency revealed in this study (ATP~ADP>UTP>>AMP) suggests that inhibition of Na^+,K^+,Cl^- cotransport by these compounds is mediated by P_{2X}- or P_{2Y}-purinoceptors [20].

The data listed below suggest that inhibition of Na^+,K^+,Cl^- cotransport by PMA is mediated via activation of protein kinase C. Indeed, as seen in Figure 3, half-maximal inhibition of Na^+,K^+,Cl^- cotransport was observed at 10 nM of PMA, which is in accordance with data on the activation of protein kinase C by this compound in intact cells [21]. The inactive analogue of PMA, 4α-PMA, did not affect Na^+,K^+,Cl^- cotransport (Table 1). It was shown that 24 hr preincubation of epithelial cells with 1 μM of PMA [22] or 3 μM of an analogue of PMA (PDBu) [23] causes down-regulation of protein kinase C. In our study, treatment of MDCK cells with 1 μM PMA for 24 hr increased Na^+,K^+,Cl^- cotransport by 50-70% (Fig. 4). This treatment completely abolished the inhibition of this carrier by 0.1 μM PMA (Fig. 4) which suggest the down-regulation of PMA-receptors. Neither the basal activity of Na^+,K^+,Cl^- cotransport nor its regulation by 0.1 μM PMA was affected by long-term treatment with 4α-PMA. A non selective inhibitor of protein kinase activity, staurosporine ($K_{0.5}$ 7, 9 and 0.7 nM for purified protein kinase A, G and C, respectively [24]), decreased the activity of Na^+,K^+,Cl^- cotransport by 33% (Fig. 5). In staurosporine-treated cells, PMA did not significantly affected the activity of this carrier. In contrast to the regulation by PMA, neither staurosporine nor down-regulation of protein kinase C did affect the regulation of Na^+,K^+,Cl^- cotransport by ATP (Fig. 4, 5).

Thus, data obtained in the present study demonstrate for the first time that, in epithelial cells of the mammalian kidney, Na^+,K^+,Cl^- cotransport could be completely inhibited by PMA-sensitive forms of protein kinase C and partly by agonists of P_2-purinoceptors. The regulatory properties of mammalian Na^+,K^+,Cl^- cotransport are extremely tissue specific. Thus, cAMP signaling activates Na^+,K^+,Cl^- cotransport in the human intestinal secretory epithelium [25], the shark rectal gland [26], SV-40 transformed cells derived from rabbit THAL [9], and fetal human non-pigmental ciliary epithelial cells [27]. In contrast, in the rat vascular smooth muscle [28,29], monkey retinal pigment epithelium [30] and human lymphocytes [31], cAMP inhibits this carrier. In rat erythrocytes [32] and bovine tracheal epithelial cells [33], SAD-sensitive ion fluxes are not affected by cAMP. It has been reported that cGMP activates Na^+,K^+,Cl^- cotransport in neuroblastoma NB-OK-1 cells [34], inhibits it in bovine endothelial cells [35,36] and parathyroid cells [37], and does not affect this carrier in rat erythrocytes and vascular smooth muscle cells [28,32]. We did not observe any effect of PMA on Na^+,K^+,Cl^- cotransport in rat erythrocytes and vascular smooth muscle cells [28,32]. In the rabbit tracheal epithelium, PMA led to 10-fold activation of SAD-sensitive Na^+ and Cl^- effluxes [38]. In bovine lens epithelial cells, Na^+,K^+,Cl^- cotransport was inhibited by PMA up to 50% [39]. However, in that study, cells were treated with 10 μM PMA for 2 hr, whereas 1 hr of treatment with 1 μM did not significantly modify the activity of the carrier. Thus, it is not clear whether the effect of 10 μM PMA was caused by activation of protein kinase C, inactivation of the enzyme due to down-regulation of its expression, or by a nonspecific action of the high PMA concentration.

To our knowledge, there are no data on the inhibition of Na^+,K^+,Cl^- cotransport by agonists of P_2-purinoceptors. Middleton et al. [40] previously reported 2-fold stimulation of Na^+,K^+,Cl^- cotransport in ATP-treated A6 cells. These results are opposed to our data with a 2-3-fold decrease of Na^+,K^+,Cl^- cotransport in ATP-treated MDCK cells (Fig. 2). However, we have to keep in mind that the A6 cell line is derived from the amphibian kidney, where renal anatomy and physiology are quite different from that of the mammalian nephron. Data obtained in the present study (Fig. 4) show that PMA-sensitive isoforms of protein kinase C are not involved in ATP-induced inhibition of Na^+,K^+,Cl^- cotransport. The role of GTP-binding proteins, cell volume, intracellular Ca^{2+} and monovalent ions in the regulation of Na^+,K^+,Cl^- cotransport by purinoceptors is currently studied in our laboratory.

In terminals of the central and peripheral nervous systems, ATP and other P_2 agonists are colocalized with more specific neurotransmitters, such as catecholamines and acetylcholine. Local hypoxia and injury are another source of ATP release leading to purinergic activation [41]. We propose that P_2 receptor-mediated inhibition of Na^+,K^+,Cl^- cotransport, revealed for the first time in mammalian cells in this study, may be involved in the regulation of renal function under normal and pathophysiological conditions.

Screening the cDNA library obtained from different organs and species permitted cloning of 2 forms of Na^+,K^+,Cl^- cotransporters: NKCC1 and NKCC2. NKCC1 was revealed in all types of cells studied so far, whereas NKCC2 was selectively expressed in apical membranes of renal epithelial cells (for recent comparative analysis, see [42]). The side-specific effect of SAD on K^+ transport across the basolateral surface of the MDCK monolayer [13,43] indicates that the housekeeping NKCC1 is the major isoform expressed in these cells. Thus, it may be

suggested that the lack of drastic inhibition of NKCC1 in other non-epithelial and epithelial cells by PMA and ATP compared with MDCK cells is caused by tubular segment-specific post-translation modification of NKCC1 or by a specific set of regulators which are involved in transduction of purinoceptor-induced signals to the transporter.

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ABBREVIATIONS

MDCK:	Madin-Darby canine kidney
PMA:	4β-phorbol 12-myristate 13-acetate
4α-PMA:	4α-phorbol 12-myristate 13-acetate
SAD:	Sulfamoylbenzoic acid derivates
THAL:	Thick ascending limb of Henle's loop

TABLE 1

Regulation of K^+ influx in MDCK cells by activators of cAMP signaling, protein kinase C and ATP

Addition	**Na^+,K^+,Cl^- cotransport, %**	**Na^+,K^+ pump, %**	**Passive permeability for K^+, %**
None (control)	100	100	100
Forskolin, 10 μM	129 ± 13 (9)	95 ± 11(4)	93 ± 7 (9)
8-Br-cAMP, 1 mM	116 ± 9 (3)	119 ± 14 (1)	95 ± 6 (3)
Cholera toxin, 0.5 μg/ml	159 ± 11 (1)*	N.D.	98 ± 9 (1)
PMA, 0.1 μM	4 ± 7 (17)****	135 ± 9 (13)**	109 ± 6 (17)
4α-PMA, 0.1 μM	120 ± 8 (1)	N.D.	99 ± 4 (1)
ATP, 100 μM	43 ± 8 (11)***	130 ± 8 (5)*	103 ± 6 (11)

Cells were preincubated for 30 min with forskolin, 8-Br-cAMP, PMA, 4α-PMA, ATP or for 3 hr with cholera toxin before the measurement of ion fluxes. Values of Na^+,K^+,Cl^- cotransport, Na^+,K^+ pump and passive permeability for K^+ in the absence of any addition (control) were taken as 100%. Means ± S.E. obtained in (n) experiments performed in quadruplicate are given.

*, **, ***, **** for $p<0.05$, 0.02, 0.001 and 0.0001, respectively.

FIGURE 1

Kinetics of K^+ (^{86}Rb) influx (A) and activity of Na^+,K^+ pump (ouabain-sensitive ^{86}Rb influx), Na^+,K^+,Cl^- cotransport (ouabain-resistant, bumetanide-sensitive ^{86}Rb influx) and passive permeability for K^+ (ouabain+bumetanide-resistant ^{86}Rb influx) (B) in MDCK cells. Means ± S.E. obtained in 2 (A) and 20 (B) experiments performed in quadruplicate or triplicate are given.

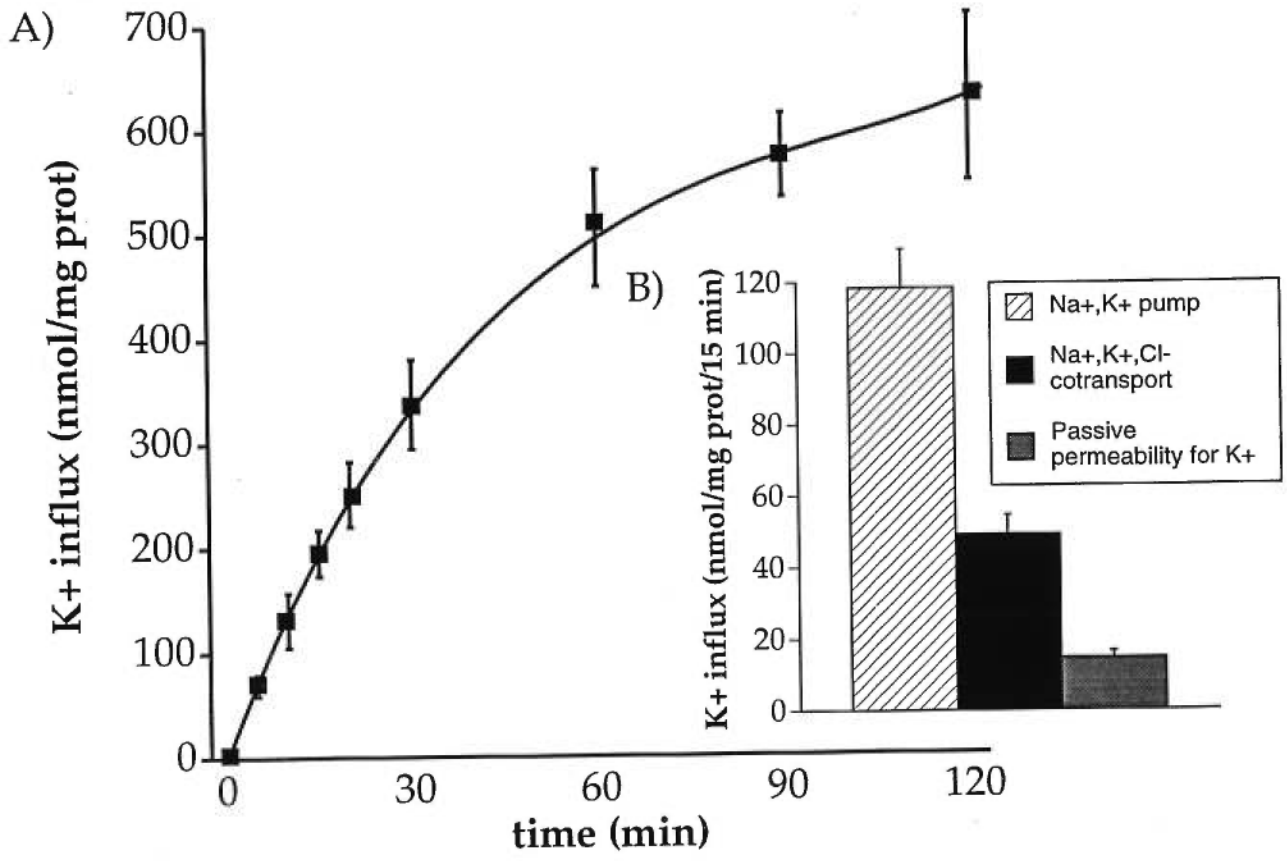

A)
700
600
500
400
300
200
100
0
K+ influx (nmol/mg prot)
0
30
60
90
120
time (min)
B)
120
100
80
60
40
20
0
K+ influx (nmol/mg prot/15 min)
Na+,K+ pump
Na+,K+,Cl- cotransport
Passive permeability for K+

FIGURE 2

Dose-dependence of Na^+,K^+,Cl^- cotransport on nucleotide concentrations. Cells were preincubated with nucleotide for 30 min before the measurement of ion fluxes. Values of Na^+,K^+,Cl^- cotransport in the absence of nucleotide were taken as 100%. Means ± S.E. obtained in experiments performed in quadruplicate are given.

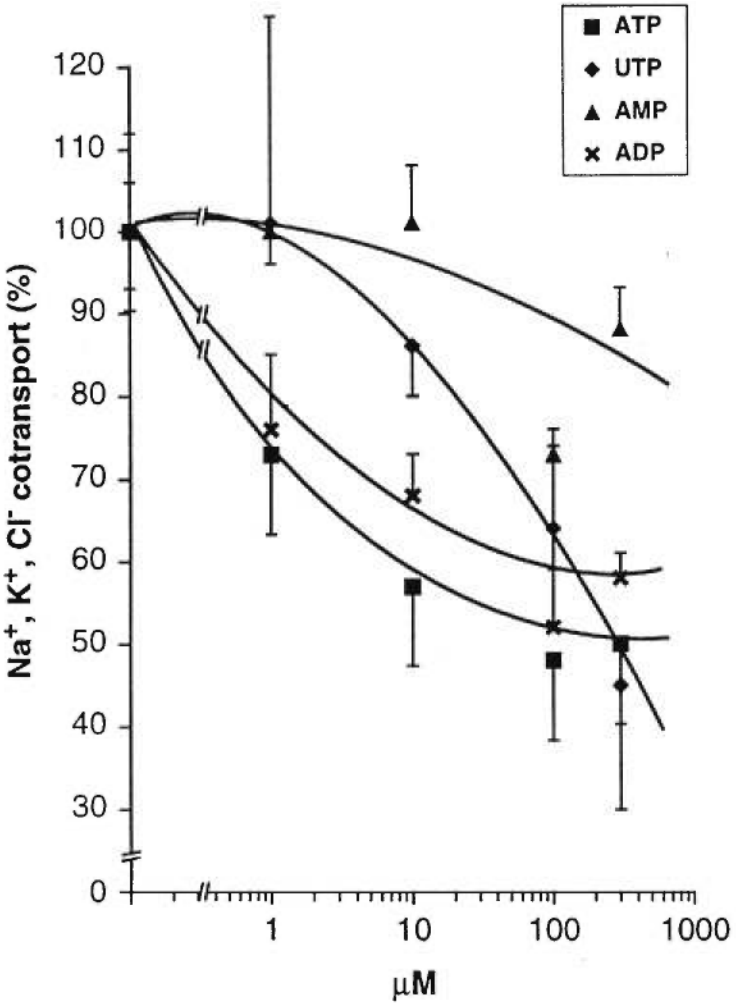

ATP
UTP
AMP
ADP
120
110
100
90
80
70
60
50
40
30
0
1
10
100
1000
μM
Na+, K+, Cl- cotransport (%)

FIGURE 3

Dose-dependence of Na^+,K^+,Cl^- cotransport on PMA. Cells were preincubated with PMA for 30 min before the measurement of ion fluxes. The value of Na^+,K^+,Cl^- cotransport in the absence of PMA was taken as 100%. Means ± S.E. obtained in experiments performed in quadruplicate are given.

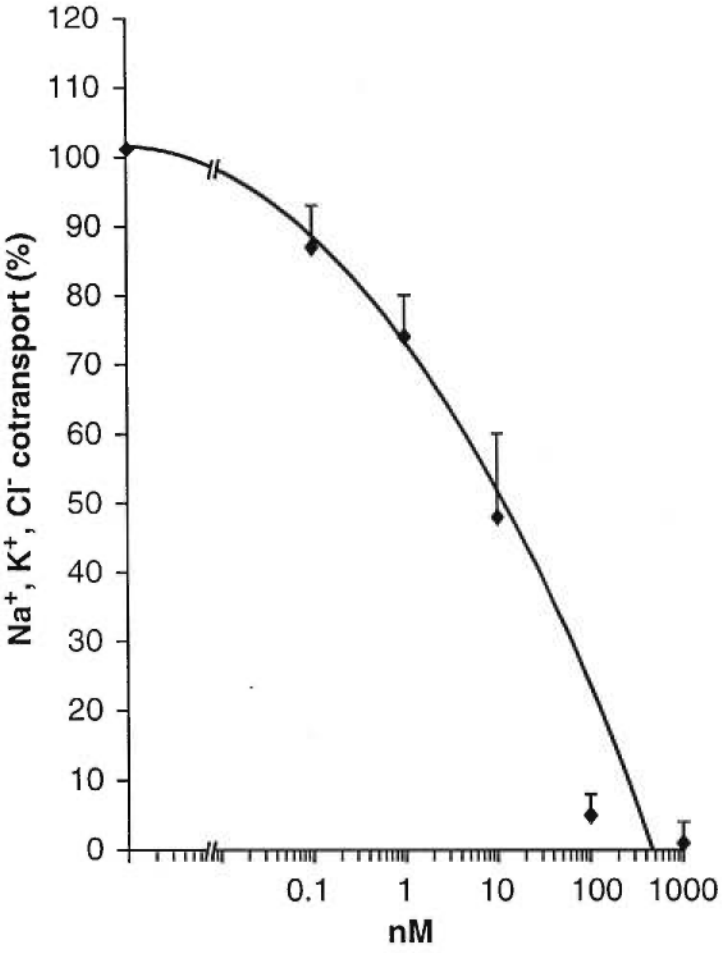

120
110
100
90
80
70
60
50
40
30
20
10
0
Na+, K+, Cl- cotransport (%)
0.1
1
10
100
1000
nM

FIGURE 4

Effect of down-regulation of protein kinase C activity on the regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP. Cells were treated for 24 hr with 1 μM of PMA or 4α-PMA in MEM containing 10% fetal calf serum. This medium was aspirated, cells were washed twice with medium A and incubated for 30 min in medium B with or without 0.1 μM PMA or 100 μM ATP. The value of Na^+,K^+,Cl^- cotransport in control cells without PMA or ATP (basal) was taken as 100%. Means ± S.E. obtained in experiments performed in quadruplicate are given.

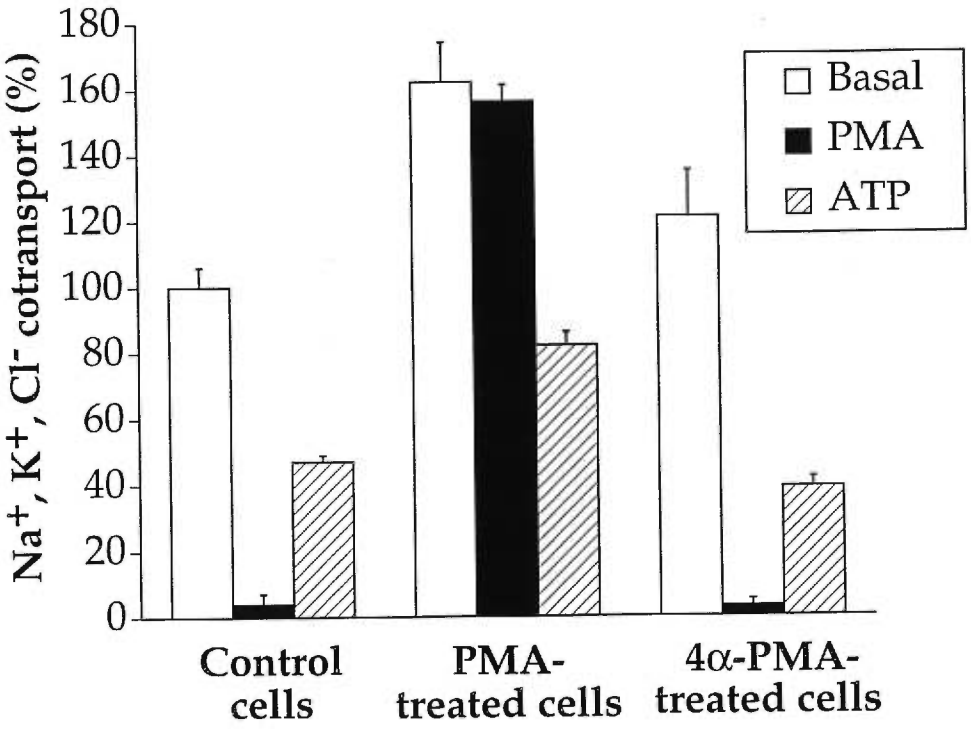
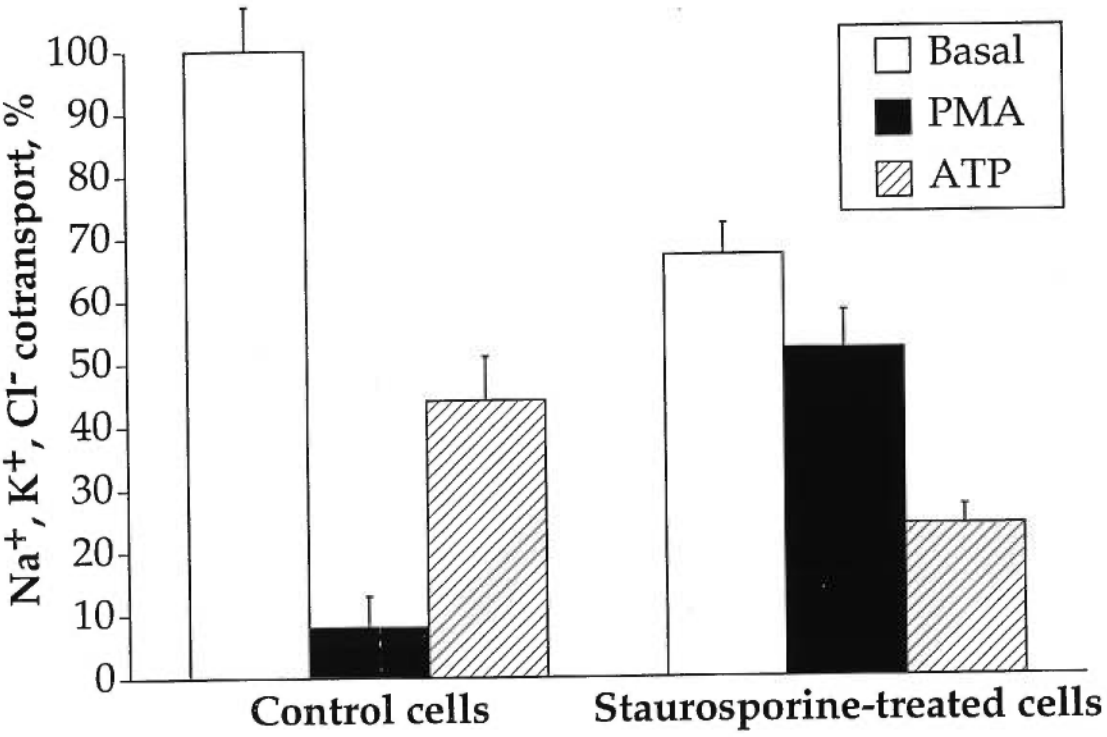

180
160
140
120
100
80
60
40
20
0
Na+, K+, Cl- cotransport (%)
Basal
PMA
ATP
Control cells
PMA-treated cells
4α-PMA-treated cells

FIGURE 5

Effect of staurosporine on the regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP. Cells were washed twice with medium A and incubated for 30 min in medium B. This medium was then aspirated and cells were incubated for 10 min in 0.25 ml of medium B with or without 0.5 μM of staurosporine followed by the addition of 0.25 ml of medium B with or without 0.1 μM PMA or 100 μM ATP. Then, cells were incubated for 30 min before the measurement of ion fluxes. The value of Na^+,K^+,Cl^- cotransport in control cells without PMA, ATP or staurosporine (basal) was taken as 100%. Means ± S.E. obtained in experiments performed in triplicate are given.


Basal
PMA
ATP
Na^+, K^+, Cl^- cotransport, %
100
90
80
70
60
50
40
30
20
10
0
Control cells
Staurosporine-treated cells

CHAPITRE 4

INHIBITION DU CO-TRANSPORTEUR Na^+, K^+, Cl^- INDUITE PAR L'ATP DANS LES CELLULES MDCK : ABSENCE D'IMPLICATION DES VOIES CONNUES DE SIGNALISATION COUPLÉES AUX PURINOCEPTEURS

ATP-induced inhibition of Na^+, K^+, Cl^- cotransport in Madin-Darby canine kidney cells: lack of involvement of known purinoceptor-coupled signaling pathways

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ABSTRACT

$P_{2U/2Y}$-receptors elicit multiple signaling in Madin-Darby canine kidney (MDCK) cells, including a transient increase of $[Ca^{2+}]_i$, activation of phospholipases C (PLC) and A_2 (PLA_2), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK). This study examines the involvement of these signaling pathways in the inhibition of Na^+,K^+,Cl^- cotransport in MDCK cells by ATP. The level of ATP-induced inhibition of this carrier (~50% of control values) was insensitive to cholera and pertussis toxins, to the PKC inhibitor calphostin C, to the cyclic nucleotide-dependent protein kinase inhibitors, H-89 and H-8 as well as to the inhibitor of serine-threonine type 1 and 2A phosphoprotein phosphatases okadaic acid. ATP led to a transient increase of $[Ca^{2+}]_i$ that was abolished by a chelator of $Ca^{2+}{}_i$, BAPTA. However, neither BAPTA nor the Ca^{2+} ionophore A231287, or an inhibitor of endoplasmic reticulum Ca^{2+}-pump, thapsigargin, modified ATP-induced inhibition of Na^+,K^+,Cl^- cotransport. An inhibitor of PLC, U73122, and an inhibitor of MAPK kinase (MEK), PD98059, blocked ATP-induced inositol-1,4,5-triphosphate production and MAPK phosphorylation, respectively. However, these compounds did not modify the effect of ATP on Na^+,K^+,Cl^- cotransport activity. Inhibitors of PLA_2 ($AACOCF_3$), cycloxygenase (indomethacin) and lypoxygenase (NDGA) as well as exogenous arachidonic acid also did not affect ATP-induced inhibition of Na^+,K^+,Cl^- cotransport. Inhibition of the carrier by ATP persisted in the presence of inhibitors of epithelial Na^+ channels (amiloride), Cl^- channels (NPPB) and Na^+/H^+ exchanger (EIPA) and was insensitive to cell volume modulation in anisosmotic media and to depletion of cells with monovalent ions, thus ruling out the role of other ion transporters in purinoceptor-induced inhibition of Na^+,K^+,Cl^- cotransport. Our data demonstrate that none of the known purinoceptor-stimulated signaling pathways

mediate ATP-induced inhibition of Na^+,K^+,Cl^- cotransport and suggest the presence of a novel P_2-receptor-coupled signaling mechanism.

Key words: Na^+,K^+,Cl^- cotransport, P_2-purinoceptors, intracellular signaling, MDCK cells

INTRODUCTION

The regulatory properties of the ubiquitous isoform of Na^+,K^+,Cl^- cotransport (NKCC1) are extremely tissue-specific. Thus, cyclic AMP activates Na^+,K^+,Cl^- cotransport in the human intestinal secretory epithelium (Matthews et al., 1992), shark rectal gland (Lytle, Forbush, 1992), and fetal human non-pigmented ciliary epithelial cells (Crook, Polansky, 1994). By contrast, cAMP inhibits this carrier in rat vascular smooth muscle cells (Smith, Smith, 1987; Orlov et al., 1992), monkey retinal pigment epithelium (Kennedy, 1992) and human lymphocytes (Feldman, 1992), whereas it has no effect on Na^+,K^+,Cl^- cotransport in rat erythrocytes (Orlov et al., 1988) and bovine tracheal epithelial cells (Musch, Field, 1989). It has been reported that cGMP activates Na^+,K^+,Cl^- cotransport in neuroblastoma NB-OK-1 cells (Delporte et al., 1993), inhibits it in bovine endothelium (O'Donnell, 1989), HeLa cells (Kort, Koch, 1990) and parathyroid cells (DeFeo et al., 1991), and does not affect the carrier in rat erythrocytes and vascular smooth muscle (Orlov et al., 1988; 1992). In rat erythrocytes and vascular smooth muscle cells, an activator of protein kinase C (PKC) 4β-phorbol 12-myristate 13-acetate (PMA), does not modulate Na^+,K^+,Cl^- cotransport (Orlov et al., 1988; 1992), whereas in the rabbit tracheal epithelium (Liedtke, Thomas, 1996) and NIH-3T3 fibroblasts (Hichami et al., 1996), PMA activates this ion carrier.

Madin-Darby canine kidney (MDCK) cells expressing NKCC1 (Delpire et al., 1994) are commonly used as a model of barrier epithelium (Lavelle et al., 1997). Recently, we have reported that in these cells, Na^+,K^+,Cl^- cotransport is insensitive to α-adrenergic, cholinergic and dopaminergic agonists as well as to vasopressin, bradykinin, angiotensin II and 8-Br-cGMP, but is completely blocked by activation of PKC with PMA and is partly inhibited by agonists of P_2-purinergic receptors (Gagnon

et al., 1998). It is well documented that in monolayers of MDCK cells, activation of P_2-receptors is accompanied by augmented Cl^- secretion (Simmons, 1981) linked to rise of $[Ca^{2+}]_i$ (Paulmichl et al., 1991) and activation of phospholipase C (PLC) and A_2 (PLA_2) (Paulmichl et al., 1991; Firestein et al., 1992; Yang et al., 1997). Recently, it has been reported that in MDCK cells, P_2-receptor agonists also stimulate mitogen-activated protein kinase (MAPK) (Xing et al., 1997). In the present study, we examined the involvement of these signaling pathways in ATP-induced inhibition of Na^+,K^+,Cl^- cotransporter. We demonstrated that inhibition of Na^+,K^+,Cl^- cotransport by P_2-receptors in MDCK cells is not mediated by Gs/Gi GTP-binding proteins and is not related to elevation of $[Ca^{2+}]_i$ as well as to the activation of MAPK, PLA_2, polyphosphoinositide breakdown and staurosporine/calphostin-sensitive PKC.

METHODS

Cell culture

MDCK cells from the American Type Culture Collection (ATCC No. CCL 34, Rockville, ML) were used in these study. They were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in MEM supplemented with 2.5 g/L sodium bicarbonate, 2 g/L HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Gibco Laboratories, Burlington, Canada). The cells were passaged upon reaching subconfluent density by treatment with 0.05% trypsin in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (Gibco Laboratories) and scarped from the flasks with a rubber policeman. Dispersed cells were counted and inoculated at $1.25x10^3$ cell/cm^2. Both stock cultures and cultures for experiments were grown for 6-8 days to attain subconfluency, in 80 cm^2 culture flasks and 6- or 24-well plates, respectively. In part of experiments (Fig.3-5), cells were serum starved for 2 days in DMEM containing 0.1% BSA.

Measurement of K^+ (^{86}Rb) influx

MDCK cells growing in 24-well plates were washed twice with 2 ml of medium A containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES-tris buffer (pH 7.4, room temperature) and incubated for 30 min at 37°C in 1 ml of medium B with or without tested compounds. Medium B contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, D-glucose 5, HEPES-tris 20 (pH 7.4). The preincubation medium was then replaced with 0.25 ml of the same medium containing 1 mM ouabain with or without 20 μM bumetanide. The cells were incubated at 37°C for 5 min, and thereafter 0.25 ml of medium B containing 1-2 μCi/ml $^{86}RbCl$ was added. ^{86}Rb uptake was terminated by the addition of 2 ml of ice-cold medium C containing 100 mM $MgCl_2$ and 10 mM HEPES-tris buffer (pH

7.4). The cells were then transferred on ice, washed 4 times with 2 ml of ice-cold medium C and lysed with 1 ml of 1% SDS/4 mM EDTA mixture. The radioactivity of the cell lysate was measured with a liquid scintillation analyzer. ^{86}Rb (K^+) influx was calculated as $V=A/amt$ where A is the radioactivity in the sample (cpm), a is the specific radioactivity of ^{86}Rb (K^+) (cpm/nmol) in the incubation medium, m is the protein content in the sample (mg) and t is the incubation time (min). Protein content was measured by modified Lowry's method (Hartee, 1972). As shown previously, the kinetics of ^{86}Rb uptake by MDCK cells were linear up to at least 20 min (Gagnon et al., 1998). Unless otherwise indicated, an incubation time of 15 min was used to determined the initial rate of K^+ influx. Na^+,K^+,Cl^- cotransport was estimated as the rate of ouabain-resistant bumetanide-sensitive ^{86}Rb influx.

Intracellular monovalent ion content

The intracellular content of Na^+, K^+ and Cl^- was determined as the distribution of isotopes between cells and extracellular medium under steady-state conditions (Orlov et al., 1996a). To adjust isotope equilibrium, MDCK cells were incubated for 2 hr in MEM containing 1 μCi/ml ^{86}Rb, ^{36}Cl or ^{22}Na, and for an additional 1 hr in medium B containing isotopes with the same specific radioactivity, with or without ouabain, PMA and ATP. To decrease the intracellular content of monovalent ions, cells were treated for 1 hr in a non-radioactive monovalent ion-depleted medium containing 300 mM sucrose, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose and 20 mM HEPES-tris buffer (pH 7.4, 37°C) (for more details see Fig.7). Aliquots of the incubation medium were then transferred into scintillation vials and the cells were washed 5 times with 2 ml of ice-cold medium C and lysed with SDS/EDTA mixture, as described above. Intracellular ion content (nmol/mg protein) was calculated as $V=A/am$ where A is the radioactivity of cell lysate (cpm), m is the

protein content (mg) and *a* is the specific radioactivity of the incubation medium (cpm/nmol).

Intracellular free calcium concentration

Cells growing in 80 cm^2 flasks were lifted by trypsin treatment as described above, and washed twice in DMEM containing 10% calf serum, followed by 2 washes in medium B. Cells resuspended in 3 ml of medium B were incubated for 1 hr at 37°C in the presence of 5 μM fluo 3AM and 0.02% pluronic F-127 under permanent stirring. In a part of the samples, this medium also contained 50 μM BAPTA-AM (BAPTA-loaded cells). The cells were centrifuged (800xg, 3 min), washed twice with medium B containing 1% BSA and 2.5 mM probenecid and then kept in 3 ml of the same medium at room temperature for no more than 3 hr. Before the measurement of fluorescence (F), 0.5 ml of cell suspension was centrifuged and the cells were washed with the medium B containing 1 mM probenecid, then resuspended in 2.5 ml of the same medium. F was measured at λ_{ex} = 483 nm and λ_{em} = 523 nm (slits 1 and 9 nm, respectively) using a SPEX FluoroMax spectrofluorimeter (Edison, NJ). Free intracellular Ca^{2+} concentration was measured as $[Ca^{2+}]_i = K_d (F - F_{min}) (F_{max} - F)^{-1}$, where F_{max} and F_{min} are maximal and minimal values of F measured in the presence of 0.5% triton X-100 and 2 mM $CaCl_2$ or 10 mM EGTA (pH 8.9), respectively; and K_d is the dissociation constant of the Ca-fluo 3 complex (864 nM at 37°C (Merritt et al., 1990)).

Inositol triphosphate production

Cells seeded on 24-well plates were prelabeled overnight with 3 μCi/ml *myo*-[2-^{3}H]-inositol. Prior to the experiment, radioactive medium was aspirated, and cells were washed 3 times with 1 ml of medium A, followed by 30 min preincubation in medium D containing 130 mM NaCl, 15 mM LiCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM

$CaCl_2$, 5 mM glucose and 20 mM HEPES-tris (pH 7.4; 37°C). Thereafter, this medium was replaced with 0.25 ml of medium D for an additional 30 min at 37°C, followed by the addition of 0.25 ml of medium B containing 200 μM ATP for 5 min. Incubation was terminated by the addition of SDS/EDTA mixture. Cell lysates were applied to column containing 0.5 g DOWEX-AG 1-X8 (formate form), and inositol-3-phosphate was resolved as described previously (Orlov et al., 1992).

MAPK phosphorylation

Serum-starved cells grown in 6-well plates were stimulated with desired agonists for 10 min, washed twice with ice-cold phosphate-buffered saline and lysed in 150 ml of the buffer containing 25 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% triton X-100, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml leupetin, 1 μg/ml aprotinin, 200 μM Na-orthovanadate and 1 mM NaF. The lysed cells were scarped and centrifuged at 14,000 rpm for 20 min in a microcentrifuge. An equal volume of clear lysates containing 20 μg of protein was applied on 10% polyacrylamide gel, followed by electrophoresis and transfer to Immobilon-P membrane (Millipore Corp, Bedford, MA). Phosphorylation of p42/p44 MAPK was determined by western blot analysis with antibodies against phospho-ERK following the manufacturer's instructions and by documenting the electrophoretic mobility shift of phosphorylated MAPK, using anti-p42 ERK antibodies.

Chemicals

PMA, ATP, ouabain, bumetanide, amiloride, probenecid, indomethacin, NDGA (nordihydroguaiaretic acid), arachidonic acid, DOWEX-AG 1-X8 – Sigma (St.Louis, MO); EIPA (ethylisopropylamiloride), okadaic acid, NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid – Research Biochemicals International (Natick,

MA); cholera toxin, pertussis toxin, thapsigargin, A23187, BAPTA-AM, staurosporine, calphostin C, H-8, H-89, $AACOCF_3$, U-73122 - Calbiochem (La Jolla, CA); fluo 3AM, pluronic F-127 – Molecular Probes (Eugene, OR); PD98059 and phospho-ERK antibodies – New England Biolab Inc. (Beverly, MA); $^{86}RbCl$, $^{22}NaCl$, $H^{36}Cl$, *myo*-[2-^{3}H]-inositol – Dupont (Boston, MA); salts, D-glucose and buffers – Sigma and Anachemia (Montreal, Canada). Anti-p42 ERK antibodies were kindly provided by Dr. Michael J.Dunn (Medical College of Wisconsin, Milwaukee, WI).

RESULTS

Role of cholera and pertussis toxin-sensitive GTP-binding proteins

To evaluate the role of G_p in purinergic-induced inhibition of Na^+,K^+,Cl^- cotransport, we examined the effect of exogenous ATP on this carrier in the presence of cholera and pertussis toxins. Previously, it has been shown that ADP ribosylation of G_i by pertussis toxin blocks ATP-induced inositol 1,4,5-triphosphate ($InsP_3$) production in MDCK cells (Paulmichl et al., 1991; Yang et al., 1997), whereas cholera toxin-sensitive Gs are involved in cAMP production triggered by ATP-induced PGE_2 release (Post et al., 1996). As shown in Table 1, treatment of MDCK cells with cholera toxin augmented Na^+,K^+,Cl^- cotransport by ~50%, which is consistent with a moderate elevation of its activity in MDCK cells treated with a direct activator of adenylate cyclase, forskolin (Gagnon et al., 1998). In contrast to cholera toxin, pertussis toxin did not affect basal Na^+,K^+,Cl^- cotransport activity. Neither cholera toxin nor pertussis toxin modified the level of inhibition of Na^+,K^+,Cl^- cotransport by ATP (40-50%) and the complete inhibition of the carrier by PMA (Table 1).

Role of polyphosphoinositide breakdown

Incubation of MDCK cells with ATP resulted in a 4-5-fold increase of $InsP_3$ production (Fig.1A), which is in accordance with previously reported data (Paulmichl et al., 1991). Preincubation of MDCK cells with an inhibitor of PLC, U73122, attenuated ATP-induced $InsP_3$ release by 90%. However, the same treatment with U73122 failed to alter the effect of ATP on Na^+,K^+,Cl^- cotransport activity (Fig.1B).

Role of $[Ca^{2+}]_i$

It is well documented that activation of P_2-receptors increases $[Ca^{2+}]_i$ in MDCK cells (Paulmichl et al., 1991; Delles et al., 1995). To evaluate the role of $[Ca^{2+}]_i$ in the regulation of Na^+,K^+,Cl^- cotransport, the effect of ATP and PMA on the carrier activity was measured in the presence of Ca^{2+}-modulating agents: an intracellular Ca^{2+} chelator, BAPTA, an inhibitor of endoplasmic reticulum Ca^{2+} pump, thapsigargin, and the Ca^{2+}-ionophore, A23187. As shown in Fig.2, thapsigargin, A23187 and ATP led to rise of $[Ca^{2+}]_i$ up-to 500-700 nM over control values. Preincubation of MDCK cells with BAPTA-AM decreased the basal $[Ca^{2+}]_i$ and dramatically inhibited the ATP-induced $[Ca^{2+}]_i$ response (Fig.2). However, none of the $[Ca^{2+}]_i$–modulating compounds had any significant effect on basal Na^+,K^+,Cl^- cotransport and on its inhibition by ATP and PMA (Table 2).

Role of serine-threonine protein kinases and phosphoprotein phosphatases

To investigate the role of serine-threonine phosphorylation in the regulation of Na^+,K^+,Cl^- cotransport by ATP, we first applied different inhibitors of serine-threonine protein kinases and phosphatases. Table 3 shows that the non specific inhibitor of protein kinases, staurosporine (Hidaka et al., 1984), decreased basal Na^+,K^+,Cl^- cotransport by ~25%, drastically attenuated the effect of PKC activator, PMA, and had somewhat of an impact on ATP-induced inhibition of Na^+,K^+,Cl^- cotransport. The selective inhibitor of PKC calphostin C (Kobayashi et al., 1989) enhanced the activity of the carrier by 40% and completely abolished the effect of PMA. However, calphostin C had no significant influence on the level of ATP-induced inhibition of Na^+,K^+,Cl^- cotransport (Table 3). The selective inhibitors of cAMP- and cGMP-dependent protein kinases (PKA and PKG), H-89 and H-8, respectively (Chijiwa et al., 1990) did not influence basal Na^+,K^+,Cl^- cotransport activity and its modulation by PMA and ATP. Interestingly, the inhibitor of serine-

threonine phosphatases type 1 and 2A (PP1/PP2A) okadaic acid (Cohen et al., 1990) enhanced Na^+,K^+,Cl^- cotransport by more than 2-fold, but did not alter regulation of the carrier by PMA and ATP (Table 3). These data suggest that despite the ability of PKC and PP1/PP2A to regulate Na^+,K^+,Cl^- cotransport, these enzymes as well as PKA and PKG are not involved in the inhibition of the carrier by ATP.

Role of MAP kinase

In MDCK cells, both ATP and PMA induced MAPK phosphorylation, as determined by immunoblotting of cell lysate with anti-phospho-ERK antibodies (Fig.3B) and by documenting the electrophoretic mobility shift of the p42 isoform of ERK (Fig.3A). It should be mentioned that, in contrast to vascular smooth muscle cells, calf serum only slightly potentiated MAPK phosphorylation in MDCK cells (Fig.3A and B). This peculiarity of MDCK cells is probably caused by their relatively low abundance of receptors to growth factor and lysophosphatidic acid, the major components involved in the activation of MAPK in vascular smooth muscle (Force, Bonventre, 1998). In contrast to ATP and PMA, calf serum did not inhibit Na^+,K^+,Cl^- cotransport in MDCK cells (Fig.3C). Fig.4A shows that phosphorylation of p42/p44 ERK by ATP was abolished in the presence of an inhibitor of MAPK kinase (MEK), PD98059. However, this compound did not alter the basal Na^+,K^+,Cl^- cotransport or its inhibition by ATP (Fig.4B).

As mentioned above, modulation of Ca^{2+}_i homeostasis with BAPTA and thapsigargin does not affect inhibition of Na^+,K^+,Cl^- cotransport by ATP (Table 2). Recently, we reported that purinoceptor-induced inhibition of this carrier is also insensitive to PKC downregulation under longterm treatment with PMA (Gagnon et al., 1998). To further examine the role of MAPK in ATP-dependent regulation of Na^+,K^+,Cl^- cotransport, we compared the effect of BAPTA and longterm exposure to

PMA on ATP-induced p42/p44 ERK phosphorylation, using PMA-, thapsigargin- and epidermal growth factor (EGF)-treated cells as controls. Fig.5 shows that, in contrast to regulation of Na^+,K^+,Cl^- cotransport, both BAPTA and PKC downregulation decreased ATP-induced MAPK phosphorylation indicating that this signaling pathway is mediated by Ca^{2+}- and diacyl glycerol-sensitive isoforms of PKC. BAPTA partially reduced PMA-induced p42/p44 phosphorylation, whereas PKC downregulation completely abolished the effect of acute exposure to PMA. Both BAPTA and PKC downregulation completely abolished thapsigargin-induced p42/p44 phosphorylation. In contrast to ATP, PMA and thapsigargin, the slight increment of MAPK phosphorylation induced by EGF was insensitive to downregulation of PKC and $Ca^{2+}{}_i$-chelation with BAPTA (Fig.5). The last observation is consistent with data on the $Ca^{2+}{}_i$- and PKC-independent mechanism of ERK phosphorylation by agonists of receptor tyrosine kinases (Force, Bonventre, 1988).

Role PLA_2-derived products

It has been documented that PMA as well as P_{2Y} and P_{2U} receptor agonists stimulate PLA_2 in MDCK cells, resulting in the production of arachidonic acid followed by PGE_2 release (Parker et al., 1987; Firestein et al., 1996). In monolayers of MDCK cells, PGE_2 mimicked the effect of ATP on short current and Cl^- secretion (Simmons, 1991; Steidl et al., 1991), whereas an active analogue of arachidonic acid, $AACOCF_3$ blocked the release of arachidonic acid from ATP-treated cells (Firestein et al., 1996). Therefore, we investigated a possible role of arachidonic acid and/or its metabolites in the regulation of Na^+,K^+,Cl^- cotransport by ATP. As shown in Table 4, neither the addition of exogenous arachidonic acid nor the inhibition of PLA_2 by $AACOCF_3$, of prostaglandin synthase by indomethacin and of cycloxygenase by NDGA affected basal activity of Na^+,K^+,Cl^- cotransport as well the regulation of this

carrier by PMA and ATP. This suggests that PLA_2 does not mediate the effect of ATP on Na^+,K^+,Cl^- cotransport.

Role of intracellular monovalent ions and cell volume

At the basolateral membrane of MDCK cells, Na^+,K^+,Cl^- cotransport operates in parallel to the Na^+,K^+ pump, Na^+/H^+ exchange and intermediate conductance K^+ (26 pS) and Cl^- (46 pS) channels. Both intermediate conductance and Ca^{2+}-activated maxi-K^+ channels (220 pS) are expressed in the apical membrane of MDCK cells. The apical membrane is also highly abundant with maxi-Cl^- channels (460 pS), whereas the content of Na^+ channels is rather low (for review see (Lang, Paulmichl, 1995)). It is well documented that intracellular Cl^- negatively regulates Na^+,K^+,Cl^- cotransport (Haas, 1994). In addition, Breitwiezer with co-workers (1996) reported that in squid axon this carrier is also inhibited by intracellular Na^+. Apart from direct regulation of Na^+,K^+,Cl^- cotransport, modulation of the content of intracellular monovalent ions can alter MDCK cell volume, thus also affecting volum-sensitive Na^+,K^+,Cl^- cotransporter (Haas, 1994). Considering this, it may be suggested that the regulation of bumetanide-sensitive K^+ influx by ATP is mediated by modulation of the activity of monovalent ion transporters distinct from Na^+,K^+,Cl^- cotransport. To test this hypothesis, we studied the effect of inhibitors of the above-mentioned ion transporters on the regulation of Na^+,K^+,Cl^- cotransport and modulation of the content of intracellular monovalent ions by ATP and PMA.

Table 5 shows that the addition of a potent inhibitor of epithelial Na^+ channels, amiloride, a putative Cl^- channel blocker, NPPB, and an inhibitor of Na^+/H^+ exchanger, EIPA, did not modify the regulation of Na^+,K^+,Cl^- cotransport by ATP and PMA. It is well documented that at a concentration of 3-5 mM Ba^{2+} completely blocks ion transport across different type of K^+ channels, including Ca^{2+}-activated

maxi-K^+ channels in MDCK cells (Tauc et al., 1993). Suprisingly, we observed that $BaCl_2$ inhibited basal activity of Na^+,K^+,Cl^- cotransport with an IC_{50} of ~1 mM (Fig.6, curve 1). In contrast to Na^+,K^+,Cl^- cotransport, (ouabain+bumetanide)-resistant ^{86}Rb influx termed here as a passive permeability for K^+ was not significantly affected by Ba^{2+} (Fig.6, curve 2), indicating that the contribution of Ba^{2+}-sensitive K^+ channels to K^+ influx in MDCK cells under basal condition is negligible.

Table 6 shows that both ATP and PMA slightly increase K^+_i content and decreased Na^+_i content, which is probably due to ~30% activation of Na^+,K^+ pump in PMA- and ATP-treated MDCK cells (Gagnon et al., 1998). Neither PMA nor ATP affected Cl^-_i content (Table 6). To further examine the role of intracellular monovalent ions in purinergic regulation of Na^+,K^+,Cl^- cotransport, we pretreated cells with an inhibitor of Na^+,K^+ pump, ouabain, and with monovalent ion depleted medium. Fig.7 shows that 1 hr treatment of MDCK cells with ouabain led to 8-fold increase of Na^+_i content and decreased K^+_i by 3-fold (column B). However, neither basal activity of Na^+,K^+,Cl^- cotransport nor its regulation by PMA and ATP was affected in ouabain-treated cells. Pretreatment of MDCK cells with monovalent ion-depleted medium decreased intracellular Na^+, K^+ and Cl^- content by 3-, 2- and 7-fold respectively (Fig.7, column C). In these cells, basal Na^+,K^+,Cl^- cotransport was increased by 2-3-fold, which is in accordance with the data on the feedback regulation of this carrier by Cl^-_i (Haas, 1994). Under these conditions, PMA inhibited Na^+,K^+,Cl^- cotransport by 50% only, whereas the level of inhibition of the carrier by ATP was unchanged.

The relatively low permeability of MDCK cells for water and [^{14}C]-urea (Lavelle et al.,1997), commonly used as a marker of intracellular water space, complicates the study of the effect of PMA and ATP on cell volume. However, the

lack of a significant effect of these compounds on the total content of intracellular ions (Table 6), i.e. of major intracellular osmolites, suggests that they did not lead to essential cell volume perturbation. To examine whether or not cell volume alteration can modify the sensitivity of Na^+,K^+,Cl^- cotransport to PMA and ATP, we measured the activity of this carrier in unisosmotic media. The shrinkage of MDCK cells under elevation of osmolality of the incubation medium from 200 to 480 mosm led to ~10-fold activation of Na^+,K^+,Cl^- cotransport (Fig.8), which is in accordance with data obtained for the majority of cells expressing NKCC1 isoform of the carrier (Haas, 1994). However, independently of cell volume, PMA still completely inhibited this ion transporter whereas ATP decreased its activity by 50-60%.

DISCUSSION

During the last decade, it was shown that P_2-purinoceptors affect the function of MDCK cells utilizing multiple signaling pathways, including activation of Gi/Gq proteins followed by elevation of the activity of PLC, PLA_2, adenylate cyclase, PKC and MAPK (Fig.9). Data obtained in the present study indicate that none of the above-mentioned signaling pathways are involved in recently discovered purinoceptor-induced inhibition of Na^+,K^+,Cl^- cotransport (Gagnon et al., 1998). The rank-order of potency of agonists of purinoceptors as inhibitors of Na^+,K^+,Cl^- cotransport in MDCK cells (ATP~ADP>UTP>>AMP) (Gagnon et al., 1998) suggest that their effect is mediated by P_{2X}- or P_{2Y}-receptors[1]. Indeed, in contrast to P_{2X} and P_{2Y}, the P_{2U} subtype exhibits the highest sensitivity to UTP, whereas the P_{2Z} subtype is completely insensitive to ADP and AMP. The P_{2T} subtype is activated by ADP and antagonized by ATP and AMP and appears to be exclusively expressed in platelets (Dubyak, El-Moatas, 1993; Fredholm et al., 1994; Watson, Gildelstone, 1994). The role of P_{2X}-purinoceptors possessing properties of intrinsic ion channels (Evans, 1996) in ATP-induced inhibition of Na^+,K^+,Cl^- cotransport seems to be unlikely because of the electroneutral mode of operation of this carrier (Haas, 1994) and the lack of significant effect of ATP on intracellular content of monovalent ions (Table 6). This conclusion is also supported by data on the same level of inhibition of Na^+,K^+,Cl^- cotransport by ATP in ouabain-treated and monovalent ion-depleted MDCK cells (Fig.7) as well as in shrunken and swollen cells (Fig.8).

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[1] The apparent affinity of P_2-receptors for ATP can be affected by the partial hydrolysis of this compound by ecto-ATPases. This hypothesis is currently examined in our laboratory.

Both P_{2Y} and P_{2U} receptors coupled to heterotrimeric GTP-binding proteins (Fredholm et al., 1994) have been characterized pharmacologically in MDCK cells, but their relative contribution in the regulation of cellular function is still a matter of controversy (Firestein et al., 1996; Insel et al., 1996). It has been shown that inhibition of Gi in MDCK cells with pertussis toxin prevents ATP-induced $InsP_3$ production (Paulmichl et al., 1991; Yang et al., 1997). In contrast to this observation, we did not discern any effect of pertussis toxin on the regulation of Na^+,K^+,Cl^- cotransport by ATP (Table 1). In the majority of cells studied so far, both α-subunit of Gq and βγ-dimer derived from Gi activate PLCβ (van Biesen et al., 1996; Rocca et al., 1997). Our results demonstrate that an inhibitor of PLC, U73122 blocked ATP-induced $InsP_3$ production but did not affect Na^+,K^+,Cl^- cotransport (Fig.1). Several laboratories have reported that ATP-triggers an elevation of $[Ca^{2+}]_i$ in MDCK cells due to $InsP_3$-induced Ca^{2+} release from intracellular store and influx via Ca^{2+}-release activated channels (CRAC) (Paulmichl et al., 1991; Delles et al., 1995). The present study confirms this observation (Fig.2). However, neither basal Na^+,K^+,Cl^- cotransport nor its inhibition by ATP was affected under modulation of $[Ca^{2+}]_i$ homeostasis with thapsigargin, BAPTA and A23187 (Table 2). These results show that PLC activation triggered by P_{2Y}/P_{2U}-purinoceptors and coupled to G_q/G_i-mediated polyphosphoinositide hydrolysis and elevation of $[Ca^{2+}]_i$ (Fig.9) is not involved in the inhibition of Na^+,K^+,Cl^- cotransport by ATP.

Recently we reported that side by side with purinergic inhibition of Na^+,K^+,Cl^- cotransport, an activation of PKC with PMA leads to complete inhibition of this carrier (Gagnon et al., 1998). The present study confirms this observation. To investigate whether or not PKC is involved in purinergic-induced inhibition of Na^+,K^+,Cl^- cotransport, we examined the effect of a selective inhibitor of PKC, calphostin C, on the regulation of the carrier by ATP using PMA-treated cells as a

positive control. This compound as well as the non-selective inhibitor of PKC, staurosporine, abolished the inhibitory effect of PMA but did not modify the regulation of Na^+,K^+,Cl^- cotransport by ATP (Table 3). These results are in accordance with our previous data on ATP-induced inhibition of this carrier revealed in MDCK cells subjected to downregulation of PKC under longterm treatment with PMA (Gagnon et al., 1998). Depletion of MDCK cells with monovalent ions drastically diminished the inhibitory effect of PMA but did not alter the regulation of Na^+,K^+,Cl^- cotransport by ATP (Fig.7). From this observation it seems that P_2-receptors are involved in regulating the number of functioning carrier or its turnover number, whereas PMA also increases the sensitivity of Na^+,K^+,Cl^- cotransport to inhibition by Cl^-_i or/and Na^+_i. Taken together, these results strongly suggest that the mechanism of the inhibition of Na^+,K^+,Cl^- cotransporter by PMA and ATP is different, and PMA/calphostin C-sensitive isoforms of PKC are not involved in the regulation of this carrier by ATP-coupled receptors. It should be mentioned, however, that apart from classic (PMA/diacylglycerol + Ca^{2+})-sensitive cPKC (PKC-α, βI, βII and γ) and novel PMA/diacylglycerol-sensitive Ca^{2+}-independent nPKC (PKC-δ, ε, η and ξ), the atypical (PMA/diacylglycerol + Ca^{2+})-insensitive aPKC (PKC-ζ, ι , λ and μ), which are more resistant to staurosporine and other available PKC inhibitors, have been characterized (Kikawa et al., 1989; Selbie et al., 1993; Akimoto et al., 1994). Considering this, the role of aPKC isoforms in inhibition of Na^+,K^+,Cl^- cotransport by ATP should be examined in additional experiments.

During the last few years, it has been documented that besides of receptor tyrosine kinases, G_p-coupled receptors, including receptors of lysophosphatidic acid, $PGF_{2\alpha}$, α-thrombin, A_1-adenosine, angiotensin II, α_{1B}- and α_{2A}-adrenergic and M_1-cholinergic receptors, are able to activate MAP kinase. Both Ras-dependent and Ras-independent pathways of MAPK activation triggered by α-subunits and $\beta\gamma$-dimer of

G_p have been identified in this signaling mechanism. The $p21^{ras}$-dependent pathway involves PLC-stimulated polyphosphoinositide hydrolysis, $InsP_3$-induced $Ca^{2+}{}_i$ release, activation of Pyk2 and Src tyrosine kinase followed by recruitment of the adapter protein Shc and Crb2-Soc complex. The $p21^{ras}$-independent pathway is mediated by activation of diacylglycerol-dependent isoforms of PKC (Fig.9) (for review see (van Biesen et al., 1996; Rocca et al., 1997; Force, Bonventre, 1998). Recently, it has been shown that purinergic activation leads to phosphorylation of MAPK in MDCK cells (Xing et al., 1997). Our results confirm this observation (Fig.4). MAPK phosphorylation was also observed under treatment of MDCK cells with PMA (Fig.3,5), suggesting that activation of the MAPK signaling cascade can be related to ATP- and PMA-induced inhibition of Na^+,K^+,Cl^- cotransporter. However, the data presented below contradict this hypothesis.

1. Inhibition of MAPK with PD98059 did not prevent the inhibition of Na^+,K^+,Cl^- cotransport by ATP (Fig.4).
2. Activation of MAPK with calf serum, EGF and thapsigargin (Fig.3A, 3B and 5) was not accompanied by inhibition of Na^+,K^+,Cl^- cotransport (Fig.3C, Table 2 and our recent data (Gagnon et al., 1998)).
3. Activation of MAPK signaling cascade by ATP was diminished after downregulation of PKC by longterm preincubation with PMA and after loading of MDCK cells with the $Ca^{2+}{}_i$-chelator, BAPTA (Fig.5). This suggests that both $p21^{ras}$-dependent and $p21^{ras}$-insensitive PKC-dependent pathways are involved in purinergic-induced activation of MAPK (Fig.9). On contrary, neither downregulation of PKC with PMA (Gagnon et al., 1998) nor modulation of $Ca^{2+}{}_i$ with BAPTA (Table 2) affected the purinergic regulation of Na^+,K^+,Cl^- cotransport.

Considering this, it may be concluded that activation of MAPK signaling is not involved in ATP-induced inhibition of Na^+,K^+,Cl^- cotransporter.

In MDCK cells, activation of P_2-receptors leads to increased PLA_2 activity via pertussis toxin-sensitive PKC- and MAPK-dependent signaling pathway. Indeed, it was shown that ATP-induced PLA_2 activation in these cells was accompanied by a massive release of arachidonic acid and PGE_2 as well as by cAMP production triggered by PGE_2 binding to G_s-coupled receptors (Insel et al., 1996; Post et al., 1996; Firestein et al., 1996). ATP-induced arachidonic acid release can be completely blocked by down regulation of PKC and by addition of MAPKK inhibitor, PD98059 (Xing et al., 1997). As mentioned above, we did not reveal any effect of PD98059 (Fig.4) and downregulation of PKC (Gagnon et al., 1998) on the regulation of Na^+,K^+,Cl^- cotransport by ATP. The inhibitory effect of ATP on this carrier also persisted after treatment of MDCK cells with cholera toxin (Table 1), exogenous arachidonic acid and inhibitors of PLA_2, cycloxygenase and lypoxygenase (Table 4). The role of PGE_2-induced cAMP production in the regulation of Na^+,K^+,Cl^- cotransport seems to be unlikely because of the slight augmentation rather than inhibition of its activity observed in forskolin- (Gagnon et al., 1998) and cholera-toxin-treated cells (Table 1) and because of the negative results obtained with an inhibitor of PKA, H-89 (Table 3). Taken together, these results do not support the involvement of PLA_2 in ATP-induced inhibition of Na^+,K^+,Cl^- cotransport.

In conclusion, our results show that purinergic-induced inhibition of Na^+,K^+,Cl^- cotransport in MDCK cells is not mediated by cholera and pertussis toxin-sensitive G_p. Inhibition of this carrier is independent of all known intracellular signaling pathways triggered by $P_{2U/2Y/2X}$-receptors, i.e. elevation of intracellular Ca^{2+} concentration, activation of PLC and PLA_2, diacylglycerol-sensitive isoforms of PKC, phosphorylation of MAPK, modulation of intracellular concentration of monovalent ions and cell volume. Taken together, these data suggest that ATP-induced inhibition of Na^+,K^+,Cl^- cotransport is triggered by a novel signal

transduction pathway, not yet described in MDCK cells. It is well documented that the activity of ion channels can be regulated by a membrane-delimited pathway via direct interaction of channels with G_p-derived subunits (Brown, Birnbaumer, 1990). This pathway has recently been shown to be also involved in the regulation of Na^+/H^+ exchanger by G_{12} and G_{13} (Voyno-Yasenetskaya et al., 1994; Lin et al., 1996). The role of G_p distinct from G_s and G_i in purinergic-induced inhibition of Na^+,K^+,Cl^- cotransport deserves further study.

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ABBREVIATIONS

MAPK:	mitogen-activated protein kinase
MDCK:	Madin-Darby canine kidney
PKA and **PKG**:	cAMP and cGMP-dependent protein kinases, respectively
PKC:	protein kinase C
PLA_2 and **PLC**:	phospholipase A_2 and C, respectively
PMA:	4β-phorbol 12-myristate 13-acetate

TABLE 1

Effect of cholera and pertussis toxins on the regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP in MDCK cells

Preincubation with toxins	Na^+,K^+,Cl^- cotransport, %		
	Control	PMA	ATP
None (control)	100 ± 6	4 ± 3 (96)*	57 ± 4 (43)*
Cholera toxin	155 ± 17#	8 ± 5 (95)*	78 ± 9 (50)*
Pertussis toxin	116 ± 11	6 ± 4 (95)*	61 ± 4 (47)*

Cells were preincubated with or without 0.5 μg/ml cholera or pertussis toxins for 3 hr, followed by stimulation with 0.1 μM PMA or 100 μM ATP for 30 min in medium B. This medium was then aspirated, and 0.25 ml of medium B containing 1 mM ouabain with or without 20 μM bumetanide, 0.1 μM PMA or 100 μM ATP was added. After 5 min, ^{86}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1-2 μCi/ml $^{86}RbCl$. The value of Na^+,K^+,Cl^- cotransport in toxin-untreated cells without PMA or ATP was taken as 100%. Means ± S.E. obtained in experiment performed in quadruplicate are given. The percentages of inhibition of Na^+,K^+,Cl^- cotransport by PMA and ATP are shown in parentheses. #$p<0.05$ as compared with toxin-untreated cells; *$p<0.005$ as compared with PMA- and ATP-untreated cells.

TABLE 2

Regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP in MDCK cells in the presence of Ca^{2+}-modulating compounds

Additions, μM	**Na^+,K^+,Cl^- cotransport, %**		
	Control	**PMA**	**ATP**
None (control)	100 ± 9	2 ± 4*	47 ± 6*
BAPTA-AM, 50	97 ± 11	3 ± 2*	39 ± 4*
Thapsigargin, 0.25	94 ± 7	4 ± 6*	45 ± 7*
A23187, 3	129 ± 17	3 ± 1*	36 ± 6*

Cells were preincubated in medium B with or without 0.2% BSA and BAPTA-AM for 30 min. Thereafter, the medium was aspirated and the cells were incubated for an additional 30 min with or without thapsigargin, A23187, 0.1 μM PMA and 100 μM ATP. This medium was then aspirated, and 0.25 ml of the same media containing 1 mM ouabain with or without 20 μM bumetanide was added. After 5 min, ^{86}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1-2 μCi/ml $^{86}RbCl$. The value of Na^+,K^+,Cl^- cotransport in the absence of any additions was taken as 100%. Means ± S.E. obtained in experiment performed in quadruplicate are given. *$p<0.005$ as compared with PMA- and ATP-untreated cells.

TABLE 3

Effect of staurosporine, calphostin C, H-89, H-8 and okadaic acid on the regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP in MDCK cells

Additions, μM	**Na^+,K^+,Cl^- cotransport, %**		
	Control	**PMA**	**ATP**
None (control)	100 ± 6	6 ± 4 (94)*	45 ± 7 (55)*
Staurosporine, 0.25	73 ± 6	54 ± 8 (22)	24 ± 3 (67)*
Calphostin C, 0.12	139 ± 11	135 ± 12 (3)	62 ± 6 (55)*
H-89, 20	104 ± 9	5 ± 2 (95)*	42 ± 7 (59)*
H-8, 20	96 ± 6	3 ± 2 (97)*	44 ± 8 (54)*
Okadaic acid, 1	229 ± 20#	6 ± 1 (97)*	57 ± 8 (75)*

Cells were preincubated in medium B with or without compounds listed in the left column, followed by 30 min of incubation in the same media with or without 0.1 μM PMA and 100 μM ATP. This medium was then aspirated, and 0.25 ml of the same media containing 1 mM ouabain with or without 20 μM bumetanide was added. After 5 min, ^{86}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1-2 μCi/ml $^{86}RbCl$. The value of Na^+,K^+,Cl^- cotransport in the absence of any additions was taken as 100%. Means ± S.E. obtained in 3 experiments performed in quadruplicate are given. The percentages of inhibition of Na^+,K^+,Cl^- cotransport by PMA and ATP are shown in parentheses. #$p<0.001$ as compared with control cells; *$p<0.001$ as compared with PMA- and ATP-untreated cells.

TABLE 4

Regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP in MDCK cells in the presence of inhibitors of PLA_2-mediated signaling pathways

Additions, μM	**Na^+,K^+,Cl^- cotransport, %**		
	Control	**PMA**	**ATP**
None (control)	100 ± 9	3 ± 4*	31 ± 7*
Arachidonic acid, 10	90 ± 6	5 ± 3*	35 ± 6
AACOCF3, 25	112 ± 10	6 ± 7*	35 ± 8*
NDGA, 10	93 ± 9	5 ± 3*	33 ± 6
Indomethacin, 10	99 ± 10	10 ± 6	36 ± 8

Cells were preincubated for 30 min in medium B with or without compounds listed in the left column, plus 0.1 μM PMA or 100 μM ATP. This medium was then aspirated, and 0.25 ml of the same media containing 1 mM ouabain with or without 20 μM bumetanide was added. After 5 min, ^{86}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1-2 μCi/ml $^{86}RbCl$. The value of Na^+,K^+,Cl^- cotransport in the absence of any additions was taken as 100%. Means ± S.E. obtained in experiment performed in quadruplicate are given. *$p<0.001$ as compared with PMA- and ATP-untreated cells.

TABLE 5

Effect of PMA and ATP on the activity of Na^+,K^+,Cl^- cotransport in presence of inhibitors of other ion transport pathways

Additions, μM	**Na^+,K^+,Cl^- cotransport, %**		
	Control	**PMA**	**ATP**
None (control)	100 ± 11	4 ± 6*	43 ± 6*
Amiloride, 20	96 ± 8	6 ± 7*	36 ± 5*
EIPA, 20	127 ± 17	6 ± 4*	41 ± 10*
NPPB, 100	93 ± 12	3 ± 2*	43 ± 6*

Cells were preincubated for 30 min in medium B with or without compounds listed in the left column, plus 0.1 μM PMA or 100 μM ATP. This medium was then aspirated, and 0.25 ml of the same media containing 1 mM ouabain with or without 20 μM bumetanide was added. After 5 min, ^{86}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1-2 μCi/ml $^{86}RbCl$. The value of Na^+,K^+,Cl^- cotransport in the absence of any additions was taken as 100%. Means ± S.E. obtained in experiment performed in quadruplicate are given. *$p<0.005$ as compared with PMA- and ATP-untreated cells.

TABLE 6

The content of monovalent ions in control and PMA- or ATP-treated MDCK cells

Additions, μM	**Intracellular ion content, nmol/mg prot**		
	K^+	**Na^+**	**Cl^-**
None (control)	719 ± 52	54 ± 5	225 ± 33
PMA, 0.1	825 ± 48	45 ± 6	236 ± 47
ATP, 100	788 ± 56	42 ± 4	240 ± 31

Cells were incubated for 2 hr in DMEM containing 1 μCi/ml ^{86}Rb, ^{22}Na or ^{36}Cl and for an additional 1 hr in medium B containing isotopes with the same specific radioactivity and with or without PMA or ATP. For more details see Methods. Means ± S.E. obtained in experiment performed in quadruplicate are given.

FIGURE 1

Effect of U73122 on ATP-induced inositol-1,4,5-triphosphate ($InsP_3$) production (**A**) and inhibition of Na^+,K^+,Cl^- cotransport (**B**) in MDCK cells. Cells were preincubated with or without 10 μM U73122 for 30 min, followed by stimulation with 100 μM ATP for 5 min (**A**) or 30 min (**B**). $InsP_3$ production and Na^+,K^+,Cl^- cotransport values in the absence of ATP were taken as 100%. Means ± S.E. obtained in experiments performed in quadruplicate are given. * $p<0.01$ as compared to ATP-untreated cells.

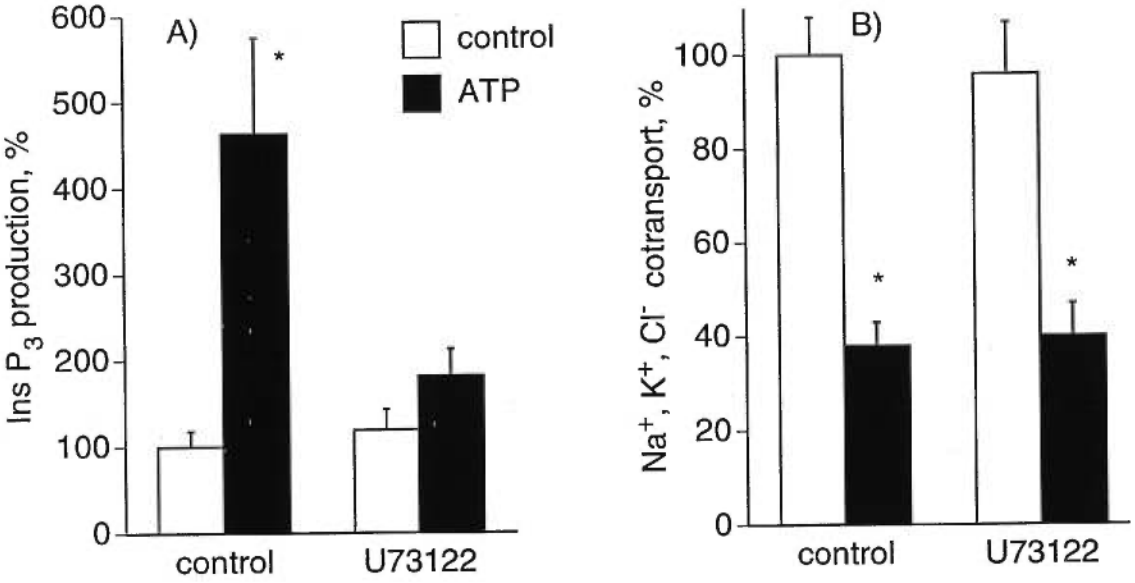

A)
control
ATP
Ins P_3 production, %
600
500
400
300
200
100
0
*
control
U73122
B)
Na^+, K^+, Cl^- cotransport, %
100
80
60
40
20
0
*
*
control
U73122

FIGURE 2

Effect of thapsigargin (TG), A23187 and ATP on intracellular free calcium concentration ($[Ca^{2+}]_i$) in control and BAPTA-loaded MDCK cells. $[Ca^{2+}]_i$) was measured as described in Methods after stimulation of intact MDCK cells (**a-c**) or BAPTA-loaded MDCK cells (**d**) with 0.25 μM thapsigargin (**a**), 3 μM A23187 (**b**), or 100 μM ATP (**c,d**).

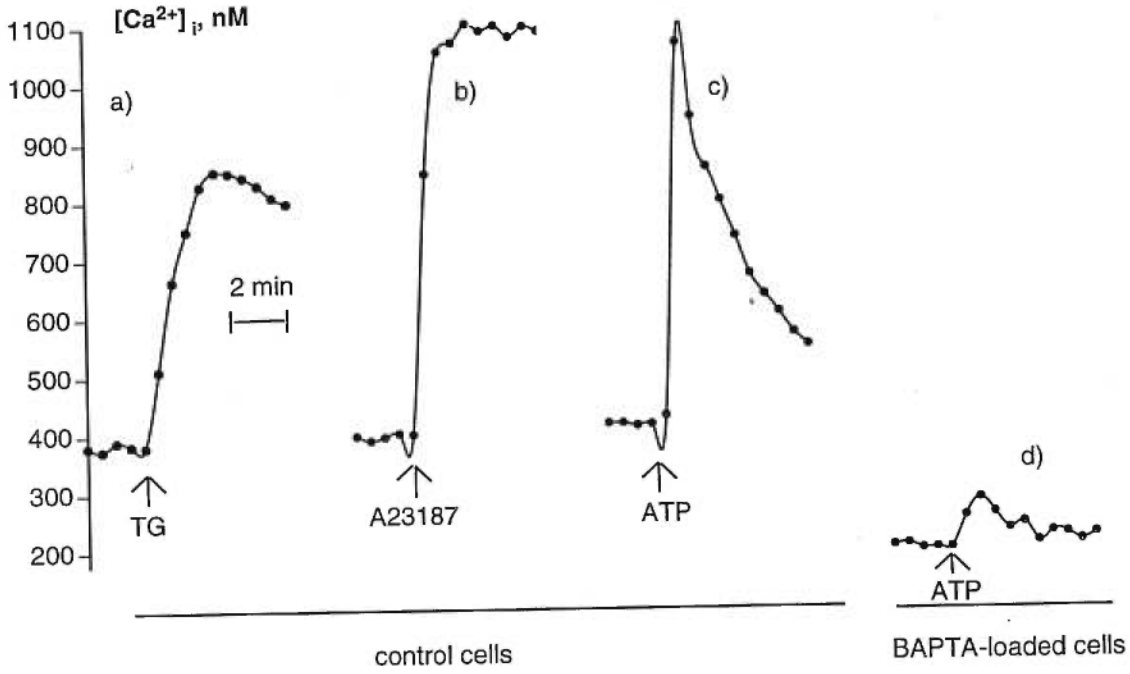

[Ca2+]i, nM
1100
1000
900
800
700
600
500
400
300
200
a)
2 min
TG
b)
A23187
c)
ATP
d)
ATP
control cells
BAPTA-loaded cells

FIGURE 3

Effect of ATP, PMA and calf serum on phosphorylation of MAP kinase (ERK) (**A,B**) and the activity of Na^+,K^+,Cl^- cotransport (**C**). MDCK cells or vascular smooth muscle cells (VSMC) were serum starved for 2 days in DMEM containing 0.1% BSA, followed by incubation in medium B (control – **1**) or in medium B with 100 μM ATP (**2**), 0.1 μM PMA (**3**) or 10% calf serum (**4**) for 10 min (**A,B**), or 30 min (**C**). Phosphorylation of MAPK was determined by gel retardation of p42 using anti-p42 ERK antibodies (**A**), or by immunoblotting with phospho-specific anti-p42/p44 ERK antibodies (**B**). VSMC were obtained from rat aorta as described previously in details (Orlov et al., 1996b) and were cultured similarly to MDCK cells. Means ± S.E. obtained in experiments performed in quadruplicate are given. * $p<0.001$ as compared with the controls.

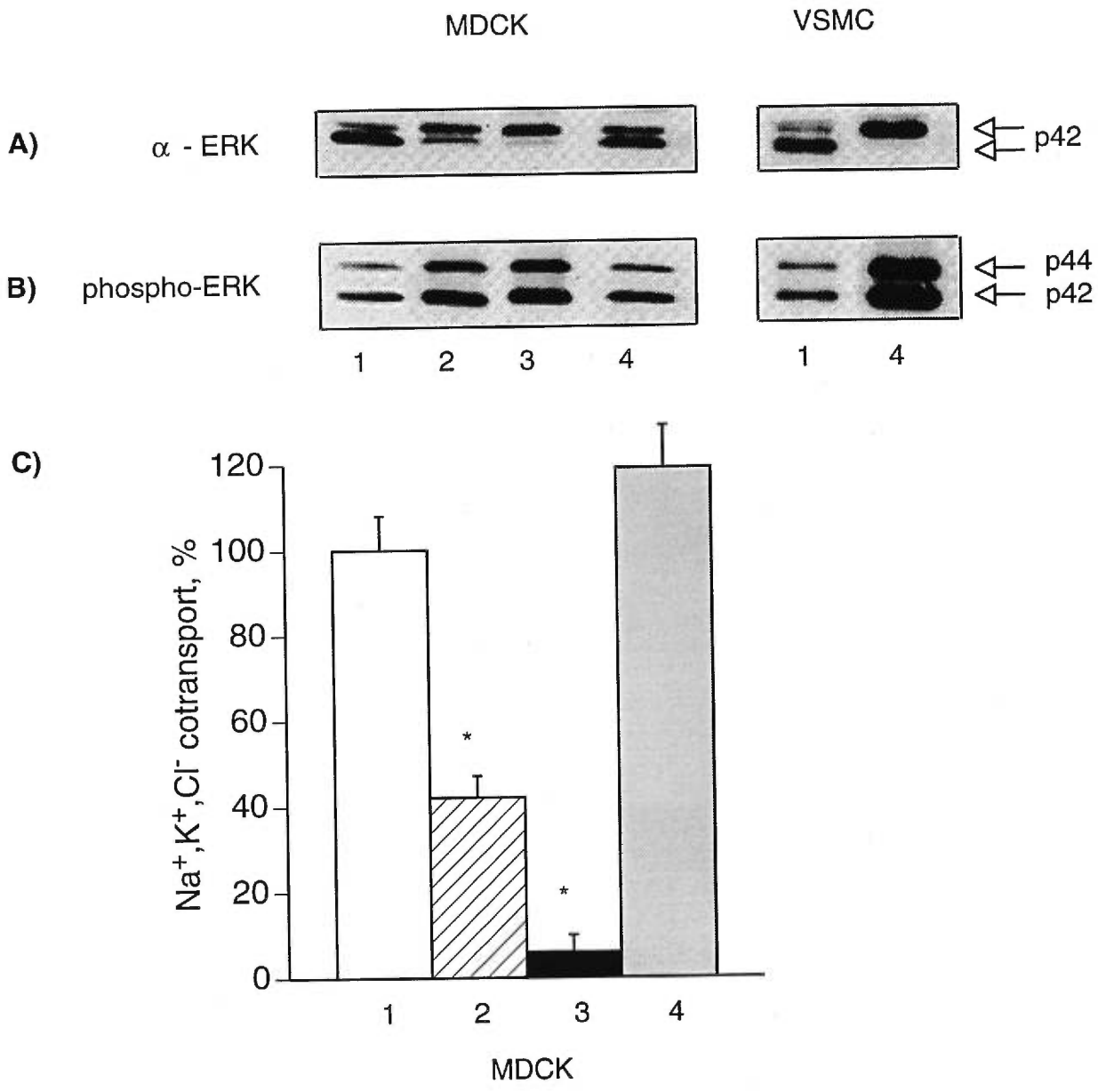

MDCK
VSMC
A)
α - ERK
p42
B)
phospho-ERK
p44
p42
1
2
3
4
1
4
C)
120
100
80
60
40
20
0
Na+,K+,Cl- cotransport, %
*
*
1
2
3
4
MDCK

FIGURE 4

Effect of PD98059 on ATP-induced MAPK phosphorylation (**A**) and Na^+,K^+,Cl^- cotransport inhibition (**B**). Serum-starved MDCK cells were preincubated in medium B with or without 50 μM PD98059 for 1 hr, followed by stimulation with 100 μM ATP for 10 min (**A**) or 30 min (**B**) as indicated. MAPK phosphorylation was determined by immunoblotting with phospho-specific anti-p42/p44 ERK antibodies. Means ± S.E. obtained in experiments performed in quadruplicate are given.

* $p<0.01$ as compared with the controls.

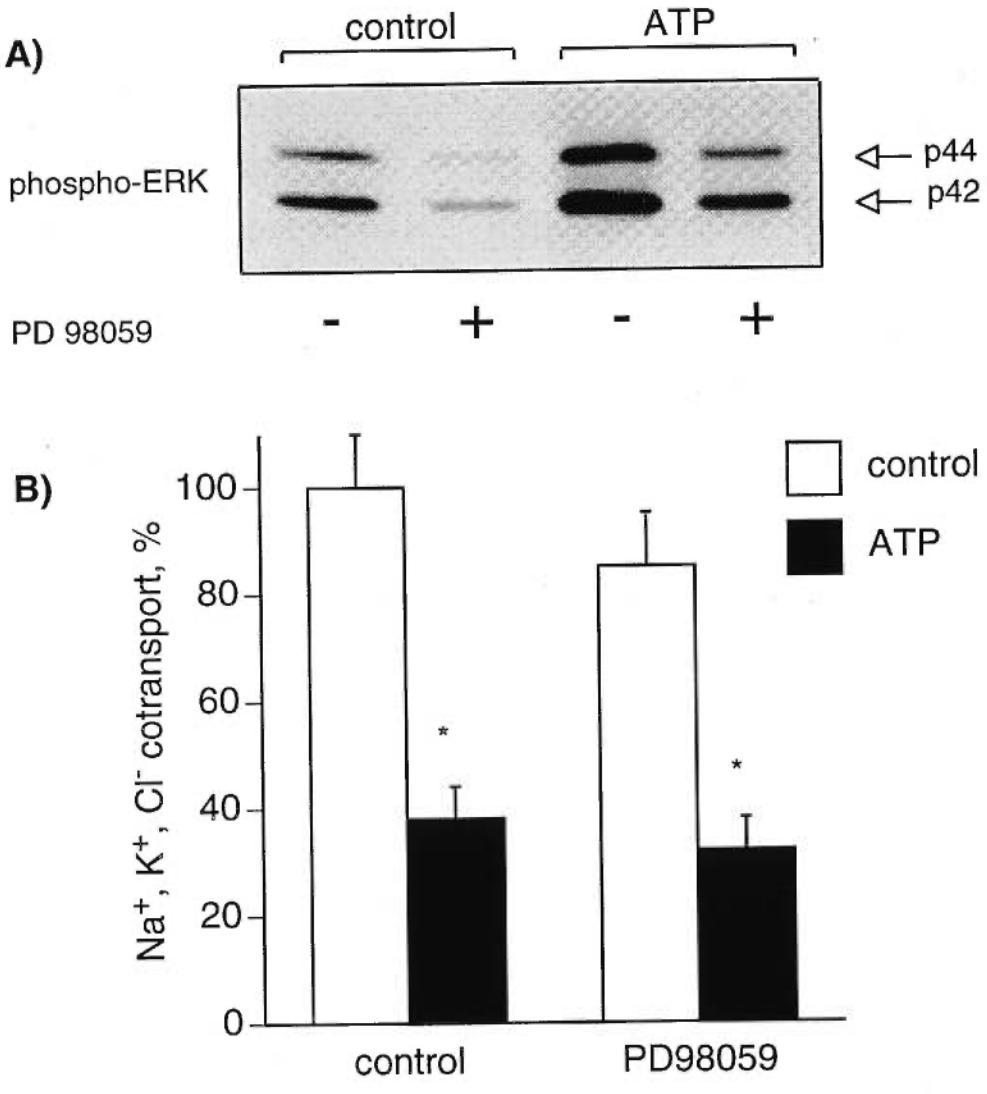

A)
control
ATP
phospho-ERK
p44
p42
PD 98059
-
+
-
+
B)
control
ATP
Na^+, K^+, Cl^- cotransport, %
100
80
60
40
20
0
*
*
control
PD98059

FIGURE 5

Effect of MAPK inhibition by PD98059, downregulation of PKC under longterm treatment with PMA and intracellular calcium depletion with BAPTA on MAPK phosphorylation induced by ATP, PMA, EGF or thapsigargin. Serum-starved cells were preincubated in DMEM containing 0.1% BSA with vehicle (**1**) or with 50 μM PD98059 for 1 hr (**2**), 1 μM PMA for 24 hr (**3**), or 50 μM BAPTA-AM for 1 hr (**4**), followed by stimulation with vehicle (control), 100 μM ATP, 0.1 μM PMA, 50 ng/ml EGF or 0.5 μM thapsigargin for 10 min. MAPK phosphorylation was determined by immunoblotting with phospho-specific anti-p42/p44 ERK antibodies.

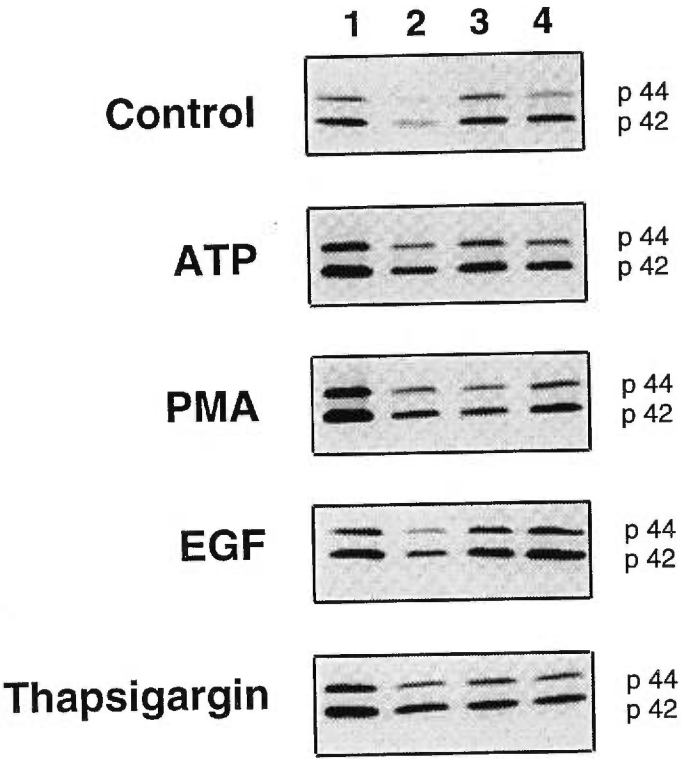

1 2 3 4
Control
p 44
p 42
ATP
p 44
p 42
PMA
p 44
p 42
EGF
p 44
p 42
Thapsigargin
p 44
p 42

FIGURE 6

Na^+,K^+,Cl^- cotransport activity and passive permeability for K^+ in MDCK cells preincubated with different $BaCl_2$ concentration for 30 min, followed by measurement of ouabain-resistant bumetanide-sensitive ^{86}Rb influx (Na^+,K^+,Cl^- cotransport activity – curve **1**), or (ouabain+bumetanide)-resistant ^{86}Rb influx (passive permeability for K^+ - curve **2**). Na^+,K^+,Cl^- cotransport values in the absence of $BaCl_2$ were taken as 100%. Means ± S.E. obtained in experiment performed in quadruplicate are given.

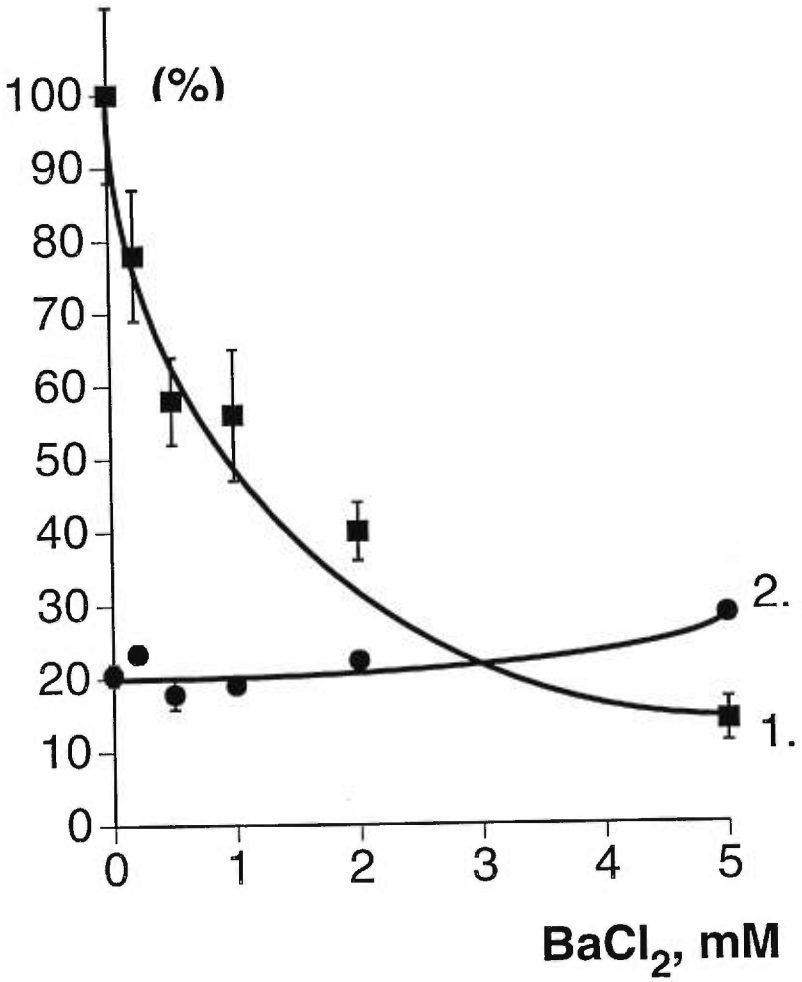
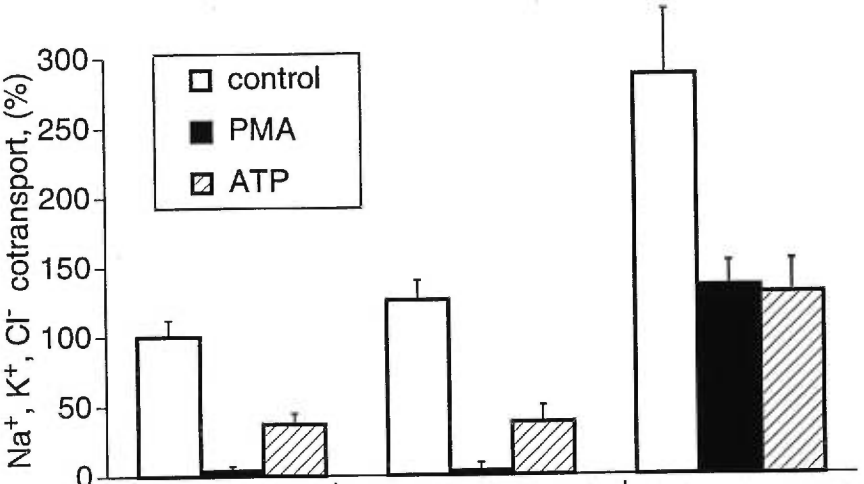

100
(%)
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
2.
1.
BaCl2, mM

FIGURE 7

Effect of intracellular monovalent ions on the regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP in MDCK cells preincubated in medium B (see Methods) for 30 min, followed by incubation with the same medium (**A** - control), medium B, containing 1 mM ouabain (**B** – ouabain-treated cells) or in monovalent ion-depleted medium (**C** – ion-depleted cells), containing 300 mM sucrose, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose, and 20 mM HEPES-tris (pH 7.4; 37°C). After 30 min, these media were replaced by the same media with or without 0.1 μM PMA or 100 μM ATP for an additional 30 min. The cells were then transferred onto ice, the media were aspirated, and 0.25 ml of medium B containing 1 mM ouabain with or without 20 μM bumetanide, 0.1 μM PMA or 100 μM ATP was added. ^{86}Rb uptake was triggered by the addition of 0.25 ml prewarmed medium B with 1 μCi/ml $^{86}RbCl$ and terminated after 5 min incubation at 37°C by the addition of ice-cold medium C (see Methods). The values of Na^+,K^+,Cl^- cotransport in control cells (**A**) without PMA and ATP were taken as 100%. The intracellular concentration of exchangeable Na^+, K^+ and Cl^- in control (**A**), ouabain-treated (**B**) and monovalent ion-depleted (**C**) cells was measured as described in Methods and is shown below the columns. Means ± S.E. obtained in 2 experiments performed in quadruplicate are given.



	A. control	B. ouabain-treated	C. ion-depleted
	intracellular ion content, nmol/mg prot		
$[Na^+]_i$	54 ± 5	423 ± 37	17 ± 4
$[K^+]_i$	719 ± 52	223 ± 18	398 ± 43
$[Cl^-]_i$	225 ± 33	201 ± 27	35 ± 8

FIGURE 8

Dependence of Na^+,K^+,Cl^- cotransport in MDCK cells on osmolality of the incubation medium. Cells were preincubated for 1 hr in medium B (see Methods), followed by incubation with 0.1 μM PMA or 100 μM ATP in the same medium. After 30 min of stimulation, this medium was aspirated, and 0.25 ml of medium B containing 1 mM ouabain with or without 20 μM bumetanide, 0.1 μM PMA or 100 μM ATP was added. After 5 min, ^{86}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1 μCi/ml $^{86}RbCl$. The final osmolality of the medium was decreased to 200 mosm and increased to 490 mosm by lowering NaCl concentration and by addition of sucrose to ^{86}Rb-containing medium, respectively. The final osmolality values was measured with a Knauer milliosmometer (Berlin, Germany). The values of Na^+,K^+,Cl^- cotransport in PMA- and ATP-untreated cells at 295 mosm were taken as 100%. Means ± S.E. obtained in 2 experiments performed in quadruplicate are given.

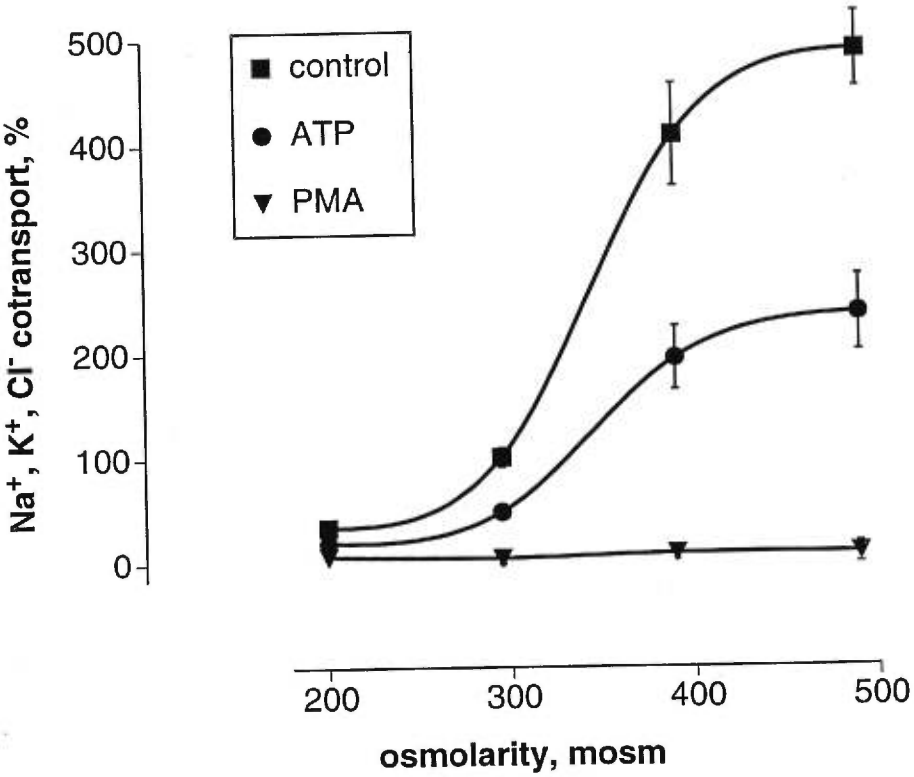
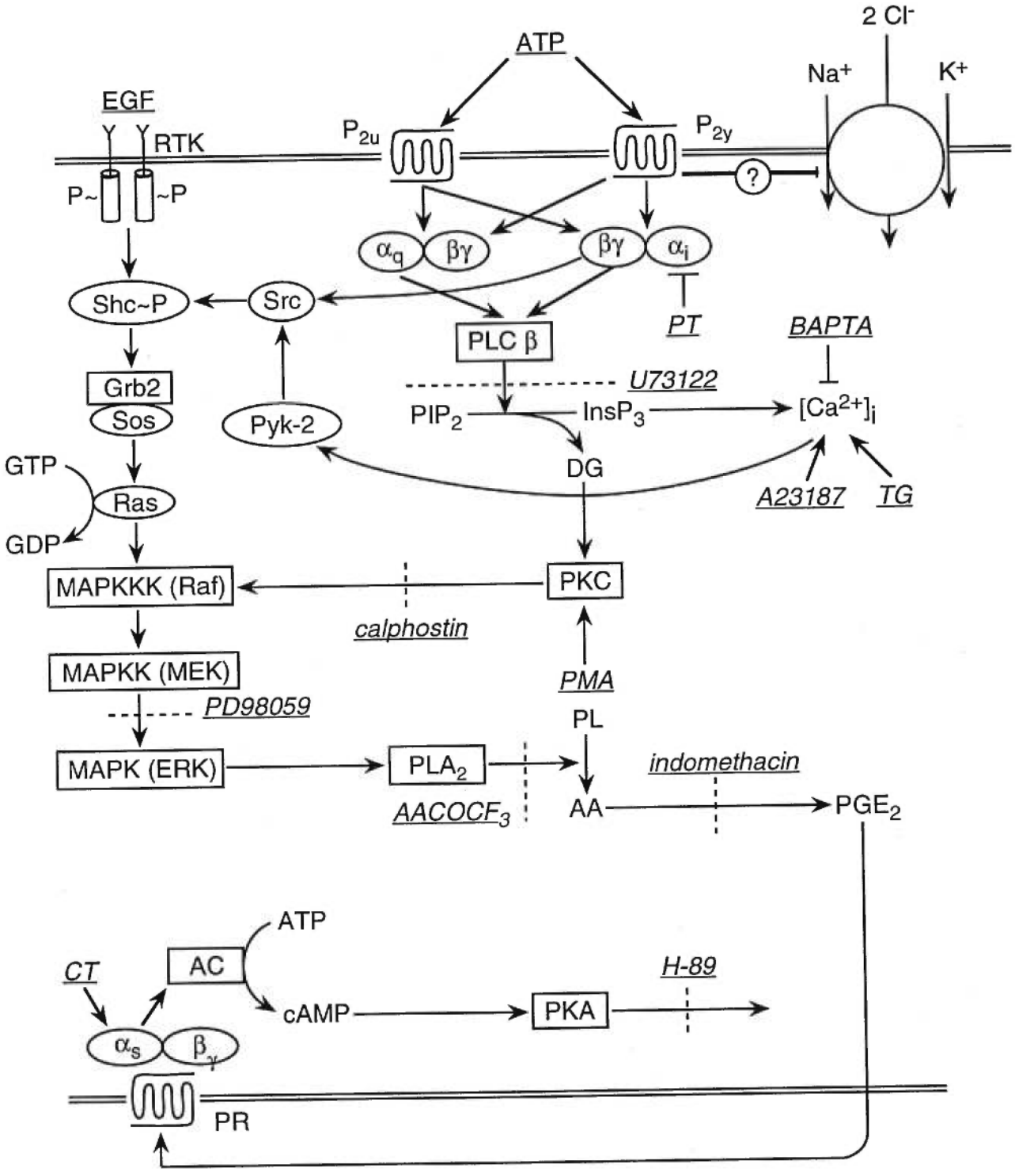

500
400
300
200
100
0
Na+, K+, Cl- cotransport, %
control
ATP
PMA
200
300
400
500
osmolarity, mosm

FIGURE 9

Purinergic signaling in MDCK cells. **AC** – adenylate cyclase; **CT** – cholera toxin; **DG** – diacylglycerol; **PL** – phospholipids; **PR** – PGE_2 receptors; **PT** – pertussis toxin; **RTK** – receptor tyrosine kinases; **TG** – thapsigargin; **?** – unidentified steps. → and ------ or - - - - - activatory and inhibitory stimulus, respectively. Compounds used in the present study to modulate the activity of signaling pathways are shown in *italics*. For more details see text.


2 Cl-
ATP
Na+
K+
EGF
RTK
P2u
P2y
P~
~P
?
αq
βγ
βγ
αi
Shc~P
Src
PT
BAPTA
PLC β
Grb2
Sos
U73122
PIP2
InsP3
[Ca2+]i
Pyk-2
GTP
Ras
DG
A23187
TG
GDP
MAPKKK (Raf)
PKC
calphostin
MAPKK (MEK)
PMA
PD98059
PL
MAPK (ERK)
PLA2
indomethacin
AACOCF3
AA
PGE2
ATP
CT
AC
H-89
cAMP
PKA
αs
βγ
PR

CHAPITRE 5

LE PRÉ-CONDITIONNEMENT PAR LE STRESS THERMIQUE NE PROTÈGE PAS LES CO-TRANSPORTEURS RÉNAUX Na^+, K^+, Cl^- ET Na^+, P_i DE LEUR MODULATION PAR LE STRESS THERMIQUE SÉVÈRE

Heat stress preconditionning does not protect renal epithelial Na^+, K^+, Cl^- and Na^+, P_i cotransporters from their modulation by severe heat stress

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ABSTRACT

This study compares the effects of heat and osmotic stress on heat stress protein (HSP) production while examining the putative protective action of HSPs on modulation of Na^+,K^+,Cl^- and Na^+,P_i cotransporters in Madin-Darby canine kidney (MDCK) epithelial cells by severe heat stress. Preconditioning heat stress (43°C, 20 min) followed by 4 hr recovery at 37°C led to a 35-fold increase of HSP70 mRNA expression measured by northern blot analysis. The protein content of HSP70 and HSP27, assessed by western blots, was augmented by 5- and 2- fold, respectively, after 6 hr of recovery. In contrast to preconditioning heat stress, hyperosmotic stress (500 vs 300 mosm) elevated HSP70 mRNA content only by 7-fold and did not significantly affect the protein content of HSP70 or HSP27. Neither cell survival, assessed as lactate dehydrogenase (LDH) release, nor the basal activities of the ion transporters and their modulation by protein kinase C, P_2-purinoceptor and cell volume were altered by preconditioning heat stress. Severe heat stress (46°C, 15 min) increased extracellular LDH content from 3 ± 2 to 23 ± 5% and enhanced Na^+,K^+,Cl^- and Na^+,P_i cotransport activity by 2-3-fold. The volume- and protein kinase C-dependent regulation of these carriers was abolished by severe heat stress while regulation by P_2-purinoceptors was preserved. Preconditioning heat stress diminished severe heat stress-induced LDH release to 11 ± 4% but did not protect Na^+,K^+,Cl^- and Na^+,P_i cotransporters from activation by severe heat stress and did not prevent severe heat stress-induced inactivation of protein kinase C- and volume-dependent signaling pathways. These results show that in mammalian kidney epithelial cells such as MDCK, preconditioning heat stress-induced HSPs are not involved in the regulation of Na^+,K^+,Cl^- and Na^+,P_i cotransporters and do not protect them from modulation by severe heat stress.

Key words: Heat stress proteins, thermotolerance, renal epithelium, Na^+,K^+,Cl^- cotransporter, Na^+, P_i cotransporter

INTRODUCTION

Heat stress proteins (HSPs) are encoded by a conserved gene family whose expression is increased in response to environmental stressors such as heat, hyperosmolarity, heavy metals, reactive oxygen species and chronically-applied excitatory stimuli (Schleisinger, 1986; Locke et al., 1990; Hamet et al., 1991; Salo et al., 1991; Udelsman et al., 1993; Burg, 1995; Kojima et al., 1996; Xu et al., 1997). Functioning as chaperons, HSPs play a role in both assembly and transport of newly-synthesized protein within cells as well as in the removal of denaturated proteins, thus preventing protein damage under environmental stress (Gething, Sambrook, 1992; Craig et al., 1994) and providing cellular resistance to subsequent stress that would otherwise be lethal (Brown et al., 1992; Beck, de Maio, 1994; Marber et al., 1993). This protection of cellular functions induced by sublethal heat stress against subsequent stress is termed thermotolerance or thermoprotection.

Special interest in acquired cytoprotection has arisen from experiments on ischemic preconditioning of cardiac tissue. These studies have demonstrated that preconditioning under transient ischemia leads to myocardial protection against subsequent ischemia and reperfusion injury (for recent review see (Millar et al., 1996; Schwarz et al., 1997; Meldrumd et al., 1998). Such research on preconditioning has spread to its putative application in the protection of other tissues including the kidney. Thus, it has been shown that mild heat stress or a brief period of ischemia-reperfusion in the rat kidney is associated with resistance to subsequent ischemic or oxidative stress (Emani et al., 1991; Perbrizet et al., 1993). In these studies, both mild preconditioning stress and brief ischemia were accompanied by induction of HSP70 production. However, the role of HSPs in kidney thermoprotection is still a matter of controversy (Bonventre, 1995; Joannidis et al., 1995).

Direct evidence for the role of HSPs in cell survival comes from experiments on cultured cells. Thus, it has been shown that transfection with HSP70 cDNA protects fibroblasts from severe heat stress, whereas microinjection of HSP70 antibody sensitizes these cells to heat (Li et al., 1991). It should be mentioned, however, that thermoprotection has been demonstrated mainly by estimation of cell survival, whereas data on thermotolerance of specific cellular functions are limited to a few observations. Moseley and co-workers reported that preconditioning heat stress elevates the threshold temperature required to disrupt transepithelial electrical resistance of Madin-Darby canine kidney (MDCK) cell monolayers (Moseley et al., 1994). Thermoprotection of Na^+-coupled transport functions has been documented in primary monolayer cultures from the flounder kidney (Brown et al., 1992). In both studies, thermoprotection of epithelial function was accompanied by HSP70 production. However, to the best of our knowledge, nothing is known about thermotolerance of ion transporters in the mammalian kidney and their regulation by HSPs.

Na^+,K^+,Cl^- and Na^+,P_i cotransporters, localized on the apical membrane of epithelial cells of the thick ascending Henle's loop and proximal tubule, respectively, are major contributors to adjustment of water and salt homeostasis and to regulation of calcium and inorganic phosphate content in extracellular fluids (Vander, 1991). This study examines the effect of preconditioning heat stress and osmotic heat stress on HSP70 and HSP27 production and compares these results with the action of preconditioning and severe heat stress on the basal activity of Na^+,K^+,Cl^- and Na^+,P_i cotransport in epithelial cells derived from MDCK and the regulation of these carriers by protein kinase C, P_2-purinoceptors and cell volume. Our results show that in this model of the mammalian kidney epithelium, severe heat stress increases the activity of both ion carriers and abolishes their regulation by cell volume and protein kinase

C. In contrast, we did not observe an effect of preconditioning heat stress on the modulation of these ion transport pathways by severe heat shock. The results of this study have been presented in part at Meetings of the International and European Societies of Hypertension (Gagnon et al., 1996; 1997).

MATERIALS AND METHODS

Cell culture

MDCK cells from the American Type Culture Collection (ATCC No. CCl 34) between passages 54 to 62 were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and grown in MEM, supplemented with sodium bicarbonate 2.5 g/L, HEPES 2 g/L, penicillin 100 U/ml, streptomycin 100 µg/ml and 10% fetal bovine serum (Gibco, Burlington, Canada). The medium was changed every 2-3 days. The cells were passaged at subconfluent density by treatment with 0.05% trypsin (Gibco) in Ca^{2+} - and Mg^{2+}-free Dulbecco's phosphate-buffered saline, scraped from the flasks with a rubber policeman and inoculated at 1.25 x 10^3 cell/cm². Both stock cultures and cultures for experiments were grown in 80 cm² culture flasks and 24-well plates, respectively, for 6 to 8 days to attain subconfluency.

Heat and osmotic stress

For preconditioning heat stress, cells were incubated for 20 min in a water bath at 43°C, then transfered to incubator at 37°C for up to 8 hr. To induce severe heat stress, they were incubated for 15 min in a water bath at 46°C. The medium was changed before heat stress was applied and replaced by prewarmed medium without fetal bovine serum. After heat stress, the medium was removed immediately and replaced by new medium prewarmed at 37°C, except in experiments for western blot analysis where it was not changed after heat stress. Hyperosmotic stress was induced by the addition of 100 mM NaCl or 200 mM sucrose (final osmolality 520 mosm) to the MEM containing fetal bovine serum for up to 24 h. To control the cytotoxicity of stress, lactate dehydrogenase (LDH) release and the relative content of attached cells were measured using an enzymatic assay kit (Sigma, St. Louis, MO) and the modified

Lowry method (Hartee et al., 1972), respectively. Table 1 shows that these parameters were not changed under preconditioning heat stress, whereas severe heat stress increased LDH release from 3 to 23% and decreased the content of attached cells by 28%. Both parameters were partly normalized when the cells were subjected to preconditioning heat stress. In the ion transport experiments, the effect of heat stress on cell survival was estimated by the measurement of protein content in attached cells.

Extraction of RNA and northern blot analysis

Total RNA from cells seeded in 80 cm^2 plates was isolated by the acid guanidium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi {1057} (34). Samples containing 10 μg of total RNA in a volume of 5 μl were denatured by heating at 65°C for 15 min in 20 μl of a buffer containing 20 mM MOPS, 50% formamide and 2.2 M formaldehyde, mixed with 5 μl of a loading buffer containing 20 mM MOPS, 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol. RNA was size-fractionated by electrophoresis on 1% agarose and 1.8% formaldehyde gel in 20 mM MOPS, 5 mM sodium acetate and 0.7 mM EDTA, pH 7.0, at 100 mA for 4 hr. The gels were stained with ethidium bromide, and RNA was transferred to Hybond nylon membranes following the manufacturer's instructions. To fix RNA, the membranes were treated for 5 min under UV light and stored at 4°C until prehybridization. The blots were prehybridized at 65°C for 2 to 3 hr in a buffer containing 1 M NaCl, 1% SDS and 10% dextran and hybridized overnight at 65°C in the prehybridization solution with the addition of 100 μg/ml of denatured salmon sperm DNA and 10^6 cpm/ml ^{32}P-labeled cDNA probes of rat HSP27 and human HSP70. The HSP27 probe was radiolabeled by polymerase chain reaction amplification method. The HSP70 probe was radiolabeled by the random priming technique. After hybridization, the membranes were washed 3 times, exposed on

phosphorus-sensitive cassettes for 24 hr, and HSP mRNA content was quantified by PhosphorImager densitometer (Molecular Dynamics, Sunnyvale, CA) and normalized for β-actin mRNA content.

Protein extracts and western blot analysis

Cells seeded in 80 cm^2 flasks were washed twice in ice-cold PBS, scraped and centrifuged for 10 min at 650 g at 4°C. Cell pellets were resuspended in 50 µl of hypotonic buffer (10 mM Tris, pH 7.4; 1 mM EDTA; 1 mM DTT; 1 mM PMSF; 50 µg/ml leupeptin), frozen immediately on dry ice and stored at -80°C. After 4 cycles of freezing-thawing, extracts were centrifuged at 15000 g for 45 min at 4°C and supernatants were kept for further analysis. The protein concentration of the supernatants was determined by the method of Bradford using bovine serum albumin as standard (Bradford, 1976) and 100 µg of cytoplasmic protein were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham, Mississauga, Canada) in 25 mM Tris and 192 mM glycine for 4 hr at 100 V. Nonspecific sites of the nitrocellulose membrane were blocked overnight at 4°C with phosphate buffered saline containing 5% non-fat dried milk (w/v) and 0.1% Tween 20 (v/v). HSP72, the inducible form of HSP70, and HSP27 were respectively detected with mouse Ab SPA-810 and rabbit polyclonal Ab SPA-801 (both from StressGen, Victoria, Canada) diluted 1:1000 and 1:2500 with phosphate buffered saline containing 1% non-fat dried milk (w/v). Immune complexes were revealed with specific ^{125}I-labeled second antibody at 0.25 µCi/ml. The membrane was exposed and analyzed with a PhosphorImager.

^{86}Rb and ^{32}P uptake studies

MDCK cells were washed twice with 2 ml of medium A containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES-tris buffer (pH 7.4, room temperature), then incubated for 30 min at 37°C in 1 ml of medium B with and without agents indicated in the figures. Medium B contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, D-glucose 5, 20 mM HEPES-tris (pH 7.4). For ^{86}Rb studies, the preincubation medium was replaced by 0.25 ml of the same medium B with 1 mM ouabain and with or without 20 μM bumetanide. For ^{32}P uptake studies, the cells were washed once with 1 ml of medium C containing (in mM): choline chloride 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, D-glucose 5, 20 mM HEPES-tris (pH7.4). Then, 0.25 ml of medium B or C, with and without the agents indicated in the figures, were added. The cells were incubated at 37°C for 5 min, and thereafter 0.25 ml of medium B containing 1-2 μCi/ml ^{86}RbCl, or medium B or C containing 1 μCi/ml [^{32}P]-orthophosphate and 0.2 mM K_2PO_4 was added. Radioisotope uptake was terminated by the addition of 2 ml of ice-cold medium W containing 100 mM $MgCl_2$ and 10 mM HEPES-tris buffer (pH7.4). The cells were then washed 4 times with 2 ml of ice-cold medium W and lysed with 1 ml of 1% SDS/4 mM EDTA mixture. The radioactivity of the cell lysate was measured with a liquid scintillation analyzer. ^{86}Rb (K^+) and ^{32}P uptake was calculated as $V=A/amt$ where A is the radioactivity in the sample (cpm), a is the specific radioactivity of ^{86}Rb (K^+) or ^{32}P (cpm/nmol) in the incubation medium, m is the protein content (mg) and t is the incubation time (min). The kinetics of ^{86}Rb (Gagnon et al., 1998) and ^{32}P uptake (data not shown) were linear up to 20 min. Based on these findings, an incubation time of 15 min was used to determine the initial rate of both K^+ and P_i influx. Figure 1 shows the effect of bumetanide (A) and omission of Na^+ (B) on the rate of ^{86}Rb (A) and $^{32}P_i$ (B) influx in MDCK cells. The absolute values of Na^+, K^+, Cl^- cotransport (49.1 ± 5.4 nmol/mg protein/15 min) and Na^+,P_i cotransport (1.80 ± 0.01 nmol/mg protein/15 min)

determined as the bumetanide-inhibited component of ^{86}Rb influx and as the Na^+-dependent component of ^{32}P influx, respectively, were in accordance with previously reported data (Rugg et al., 1986; Escoubet et al., 1989).

Chemicals

4β-phorbol 12-myristate 13-acetate (PMA), ATP, ouabain, bumetanide, sucrose- Sigma (St. Louis, MO); $^{86}RbCl$, ^{32}P-orthophosphate – Dupont (Boston, MA); ^{125}I – Amersham (Arlington, IL); salts, D-glucose and buffers were from Sigma and Anachemia (Montreal, Canada).

RESULTS

Effect of preconditioning heat stress and osmotic stress on HSP production

Figure 2A and B shows that preconditioning heat stress (43°C, 20 min) rapidly increased HSP70 mRNA content for a maximum 35-fold elevation after 4 hr recovery. In contrast to heat treatment, a maximal 8-fold increment of HSP70 mRNA was observed at 8 hr of osmotic stress. Prolongation of stress up to 24 hr did not significantly modify the HSP70 mRNA level (Fig. 2C and D). Therefore, the absolute values of the osmotic stress-induced increment were 4-5-fold less than with heat stress (Fig. 2E).

Figure 3 shows the effect of preconditioning heat stress (43°C for 20 min followed by 6 hr recovery at 37°C) and 8 hr osmotic stress on HSP70 and HSP27 protein content. Preconditioning heat stress induced 5- and 2-fold increases of HSP70 and HSP27 protein production, respectively. We did not observe any significant effect of osmotic stress on HSP70 and HSP27 protein content (Fig. 3). In view of the stronger effect of preconditioning heat stress on HSP production compared to hyperosmotic stress, heat stress was used as HSP inducer for subsequent experiments.

Effect of preconditioning and severe heat stress on the activity of ion transporter

Neither Na^+, K^+, Cl^- cotransport nor Na^+,P_i cotransport was significantly affected by preconditioning heat stress (Fig. 4). In contrast to preconditioning heat stress, severe heat stress augmented the rate of Na^+, K^+, Cl^- and Na^+,P_i cotransport by 2- and 2.5-fold, respectively. On the other hand, severe heat stress did not modify passive permeability for K^+ in MDCK cells ((ouabain + bumetanide)-resistant ^{86}Rb

influx) whereas the rate of Na^+_o-insensitive $^{32}P_i$-influx was increased by 70-80% (P<0.03, data not shown).

Effect of heat stress on the regulation of ion transporters by PMA and ATP

We demonstrated previously that an activator of protein kinase C, PMA, completely blocked Na^+, K^+, Cl^- cotransport in MDCK cells, whereas ATP inhibited this carrier by 50-60% (Gagnon et al., 1998) (35). Therefore, we also studied the effect of PMA and ATP on Na^+,P_i cotransport in MDCK cells. Figure 5 shows that PMA and ATP inhibited the activity of this carrier by 30 and 70%, respectively.

Preconditioning heat stress did not affect the regulation of Na^+, K^+, Cl^- cotransport and Na^+,P_i cotransport by PMA (Fig. 6) and by ATP (Fig. 7). Severe heat stress completely blocked the regulation of both cotransporters by PMA (Fig. 6) but did not significantly modify their sensitivity to regulation by ATP (Fig. 7). Preconditioning heat stress did not protect the PMA-sensitive signaling pathway from its inactivation by severe heat stress (Fig. 6).

Effect of heat stress on volume-dependent regulation of Na^+, K^+, Cl^- cotransport

It is known that Na^+, K^+, Cl^- cotransport is activated by hyperosmotic shrinkage in all types of cells studied so far (Haas, 1994) with the exception of human erythrocytes (Adragna, Tosteson, 1984; Orlov et al., 1989) (39). Figure 8 shows that cell shrinkage caused by increment of osmolality from 160 mosm to 470 mosm led to 10-fold activation of Na^+, K^+, Cl^- cotransport. Preconditioning heat stress did not significantly affect volume-dependent regulation of the activity of this carrier, whereas severe heat stress drastically diminished its efficiency. Preconditioning heat

stress did not protect the volume-dependent regulation pathway from its inactivation by severe heat stress.

DISCUSSION

The present study demonstrates that in MDCK cells severe heat stress drastically activates Na^+, K^+, Cl^- and Na^+,P_i cotransport, and abolishes their regulation by protein kinase C and cell volume. Mild preconditioning heat stress led to the increased production of HSP70 and HSP27 (Fig. 3) and protected MDCK cells from destruction by severe heat stress (Table 1). However, we did not observe a protective effect of preconditioning heat stress on the modulation of the activity of ion transporters by severe heat stress. This finding suggests that in this model of the mammalian renal epithelium neither Na^+, K^+, Cl^- nor Na^+,P_i is subjected to thermoprotection and regulation by mild heat stress-inducible HSPs.

To study the influence of HSPs on ion transport in MDCK cells, we compared the effects of preconditioning heat stress (43°C for 20 min, 4 h recovery) and hyperosmotic stress on HSP70 and HSP27 mRNA and protein production. We observed a 35-fold increase of HSP70 mRNA content following heat stress (Fig. 2) that is in accordance with previously-reported data (Sheikh-Hamad et al., 1994). It was demonstrated previously that after 6-10 hr of induction of hyperosmotic stress, HSP70 mRNA content in MDCK cells is elevated by 6-8-fold (Cohen et al., 1991; Sheikh-Hamad et al., 1994). These results concur with data obtained in our study (Fig. 2D). Using the rat DNA probe, we did not detect HSP27 mRNA in MDCK cells probably because of the lack of homology of this probe with canine HSP27 compared to the human HSP70 probe used for the detection of HSP70 in MDCK cells. To further examine the effect of preconditioning heat stress and osmotic stress on HSP production, we used western blot analysis. This study shows that preconditioning heat stress increased HSP70 and HSP27 protein content by 5- and 2-fold, respectively (Fig. 3). These results demonstrate that the mild heat stress-induced increment of

HSP70 protein content is much lower than the elevation of its mRNA level, which is consistent with data obtained for a neuroblastoma-derived cell line (Kiang et al., 1996). In contrast to heat stress, neither HSP70 nor HSP27 protein content in MDCK cells was significantly affected by hyperosmotic stress (Fig. 3). The lack of effect of osmotic stress on HSP70 protein content compared with the moderate induction of HSP70 mRNA is probably due to nonspecific inhibition of protein synthesis under hyperosmotic conditions (Burg, Garcia-Perez, 1992). Thus, based on data obtained by western blot analysis, the involvement of HSP27 and HSP70 in the protection of kidney epithelial cells under long-term increment of the osmolality of tubular fluid seems unlikely. This conclusion is in accordance with the negligible effect of dehydration of mice on HSP70 expression in the inner medulla as compared with a recently-discovered member of the HSP70/BiP superfamily, osmotic stress protein Osp94 (Kojima et al., 1996; Santos et al., 1998). In view of the stronger effect of heat stress on HSP production in comparison to hyperosmotic stress, preconditioning heat stress (43°C for 20 min followed by 4 h recovery) was used as HSP inducer in subsequent experiments.

We did not see any effect of preconditioning heat stress on the basal activity of Na^+, K^+, Cl^- and Na^+,P_i cotransporters and their regulation by the P_2-purinergic agonist ATP, protein kinase C and cell volume (Fig. 4, 6-8). These results suggest that the biochemical pathways involved in the above-listed cellular functions of mammalian kidney epithelial cells are resistant to modulation of the content of preconditioning heat stress-sensitive HSPs, including HSP70 and HSP27. In contrast to mild preconditioning heat stress, severe heat shock (46°C, 15 min) led to 2-3-fold activation of Na^+, K^+, Cl^- and Na^+,P_i cotransporters (Fig. 4). Under analysis of the possible mechanisms of this phenomenon it is important to underline that the activity of these carriers was sharply increased after elevation of the temperature of the

preincubation medium from 45°C to 46°C. Indeed, we did not observe any effect of 20-min preincubation at 43°C on the activity of Na^+, K^+, Cl^- and Na^+,P_i cotransporters (Fig. 4). An increase in temperature of the preincubation medium up to 45°C led to activation of these carriers by 20-30% only (data not shown) whereas preincubation at 46°C heightened their activity by 2-3-fold (Fig. 4). Further increment of temperature sharply decreased cell viability of cells as measured by LDH release (data not shown), that complicates ion transport studies. It seems unlikely that severe heat stress activates Na^+, K^+, Cl^- and Na^+,P_i cotransporters via a direct effect on the structural organization of these two non-homologous proteins. The most likely explanation is that this action is mediated by thermal modulation of a common element that is involved in the regulation of activity of these carriers. The role of protein kinase C in the activation of the carriers by severe heat stress should be probably excluded because of the slight (20-25%) inhibition of these enzyme observed after 1 hr exposure of cells at 45°C (Bagi, Hidvegi, 1990). Under analysis of these data it should be underlined that a complete inhibition of protein kinase C with calphostin C increased Na^+, K^+, Cl^- cotransport by 30-35% only and did not affect Na^+,P_i cotransporter in MDCK cells (data prepared for publication), whereas 15 min of exposure at 46°C increased the activity of Na^+,K^+,Cl^- and Na^+,P_i cotransporters by ~2- and 2.5-fold, respectively (Fig. 4). Considering this, we speculate that a thermosensitive regulator of these ion carriers is related to cytoskeleton network. Several lines of evidence support our assumption.

1. In several types of cells, including aortic smooth muscle cells and renal epithelial cells, modification of the cytoskeleton network drastically affects the activity of Na^+, K^+, Cl^- (Orlov et al., 1995; Wu et al., 1994) and Na^+,P_i (Friedlander et al., 1988; Hansch et al., 1993) cotransporters.
2. Treatment of aortic smooth muscle cells and renal epithelial cells with a disintegrator of microfilament bundles, cytocholasin B, or with a stabilizer of F-

actin, phalloidin, prevents the hormonal regulation of Na^+, K^+, Cl^- cotransporter (Orlov et al., 1995; Wu et al., 1994).

3. The interaction of protein kinase C with anchoring proteins of the cytoskeleton network plays a crucial role in the regulation of activity of the membrane-bound enzyme and transporters (Mochly-Rosen, 1995). Figure 6 shows that severe heat stress completely inhibited the regulation of both cotransporters in MDCK cells by the protein kinase C activator PMA.
4. In rat erythrocytes, disruption of the cytoskeleton network by annealing of the spectrin carcass under 10-min preincubation at 49°C completely abolishes the shrinkage-induced activation of Na^+, K^+, Cl^- cotransporter (Orlov et al., 1993) which is in accordance with data on the inhibition of volume-dependent regulation of Na^+, K^+, Cl^- cotransporter in MDCK cells subjected to severe heat stress (Fig. 8).

Brown and co-workers reported that preconditioning heat stress completely protects glucose and sulfate transport in the flounder tubular epithelium from its inactivation by severe heat stress (Brown et al., 1992). The involvement of members of the HSP family (HSP90, 70 and 28) in the protective effect of preconditioning heat stress on these renal transport functions was suggested since they were induced by the same heat stress protocol, and an inhibitor of protein synthesis, cycloheximide, prevented the thermoprotective action of preconditioning heat stress. The lack of effect of HSPs in protecting renal epithelial cell ion transport functions against heat stress in our study may have several explanations. First, the thermoprotection of ion transporters reported by Brown and co-workers may be a feature of epithelial cells from the fish kidney. Indeed, in the flounder epithelium, preconditioning heat stress led to a 15-20-fold increase of HSP content (Brown et al., 1992) that is 3-4-fold higher that in MDCK cells (Fig. 3). Second, thermoprotection may be limited to transport systems

studied by Brown and co-workers, i.e. carriers involved in the transepithelial movement of glucose and inorganic anions. Third, thermoprotection may be limited to a specific nephron segment. Indeed, the flounder kidney studied by Brown and co-workers is highly abundant in proximal tubules, whereas MDCK cells are derived from the distal tubule collecting duct. Fourth, non-asymmetrical cells seeded on plastic supports were used in our study whereas polarized cells on permeable supports were investigated by Brown and co-workers (Brown et al., 1992). It is well documented that polarization affects numerous cellular functions, including cytoskeleton organization (Cantiello, 1995).

In contrast to the complete inhibition of volume- and PMA-induced regulation, severe heat stress did not significantly modify the regulation of Na^+, K^+, Cl^- and Na^+,P_i cotransporter by ATP. These results suggest that the mechanisms of inhibition of these carriers by PMA and ATP are different. This conclusion is supported by data on the lack of effect of protein kinase C inhibitors and downregulation of protein kinase C under longterm treatment with PMA on the regulation of Na^+, K^+, Cl^- cotransporter by ATP (Gagnon et al., 1998).

In conclusion, in contrast to protection of the viability of severe heat stress-treated MDCK cells by mild preconditioning heat stress, we did not discern any thermoprotection of two major renal ion pathways, i.e. Na^+, K^+, Cl^- and Na^+,P_i cotransporters, by preconditioning heat stress. We also did not observe any involvement of preconditioning heat stress-inducable HSPs in the modulation of activity of these carriers. These data suggest that increased activity of the renal Na^+, K^+, Cl^- cotransporter in spontaneously hypertensive rats is not the consequence of an exaggerated HSP production caused by environmental stressors (for recent review see (Hamet et al., 1995; 1996; 1998); Righetti et al., 1995; Cusi 1997). However, taking

into account the extreme species-dependent character of renal ion transport mechanisms (Vander et al.,1991) to draw a final conclusion, additional experiments with monolayers of epithelial cells derived from rat nephron segments should be performed.

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ABBREVIATIONS

HSPs: Heat shock proteins

MDCK: Madin-Darby canine kidney

PMA: 4β-phorbol 12-myristate 13-acetate

TABLE 1

Effects of preconditioning and severe heat stress on the survival of MDCK cells

Cell treatment	LDH release, %	Content of attached cells, %
1. Control	3 ± 2	100 ± 5
2. Preconditioning heat stress (43°C - 20 min, 37°C – 4 hr)	7 ± 3	92 ± 8
3. Severe heat stress (46°C, 15 min)	23 ± 5	72 ± 7
4. Preconditioning heat stress + severe heat stress	11 ± 4	86 ± 8
$P_{1,3}$	<0.05	<0.01
$P_{1,4}$	NS	NS

MDCK cells grown in 24-well plates were washed twice with medium A and incubated for 4 hr in 1 ml of medium B at 37°C. In part of the experiment, cells were subjected to preconditioning and/or severe heat stress at the beginning and at the end of incubation, respectively. LDH release was estimated as the ratio of extracellular and total (extracellular + intracellular) enzyme activity. To determine intracellular LDH activity, the incubation medium was removed and the cells treated with 0.5% Triton X-100. To estimate the content of attached cells, the incubation medium was aspirated and cells washed 5 times with 2 ml of ice-cold medium C and treated with 0.5 N NaOH for protein measurement. Protein and total LDH content in control cells were taken as 100%. For the composition of medium A, B and C see the section on ion flux measurement in Methods. Means ± S.E obtained in 3 experiments performed in quadruplicate are given.

FIGURE 1

K^+ (^{86}Rb) (A) and P_i (B) influx in MDCK cells. A) K^+ influx was measured under control conditions (medium B + 1 mM ouabain) and in the presence of 20 μM bumetanide. Na^+,K^+,Cl^- cotransport represents the bumetanide-inhibited component of K^+ influx. B) P_i influx was measured in medium B containing 0.1 mM K_2HPO_4 (control) or under equimolar substitution of Na^+ with choline chloride (Na^+-free medium). Na^+,P_i cotransport represents the Na^+-sensitive component of $^{32}P_i$ influx. Means ± S.E. obtained in 20 (A) and 3 (B) experiments performed in quadruplicate or triplicate are given.

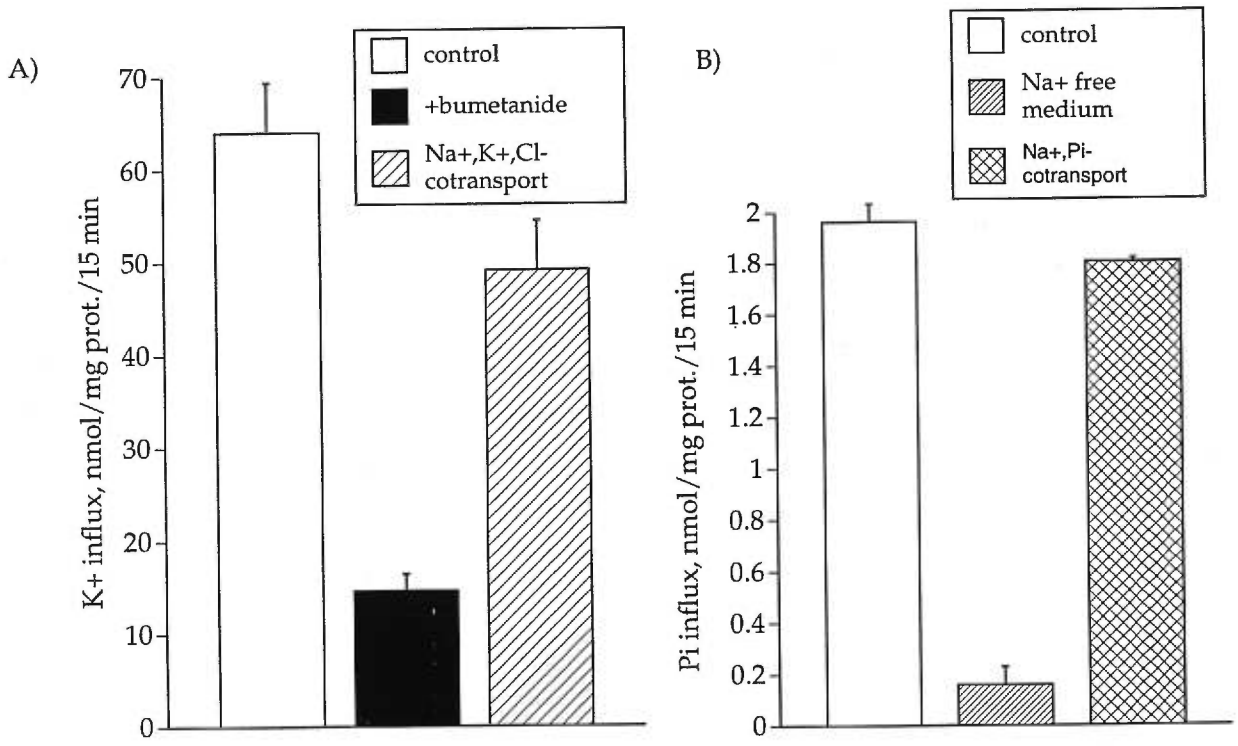
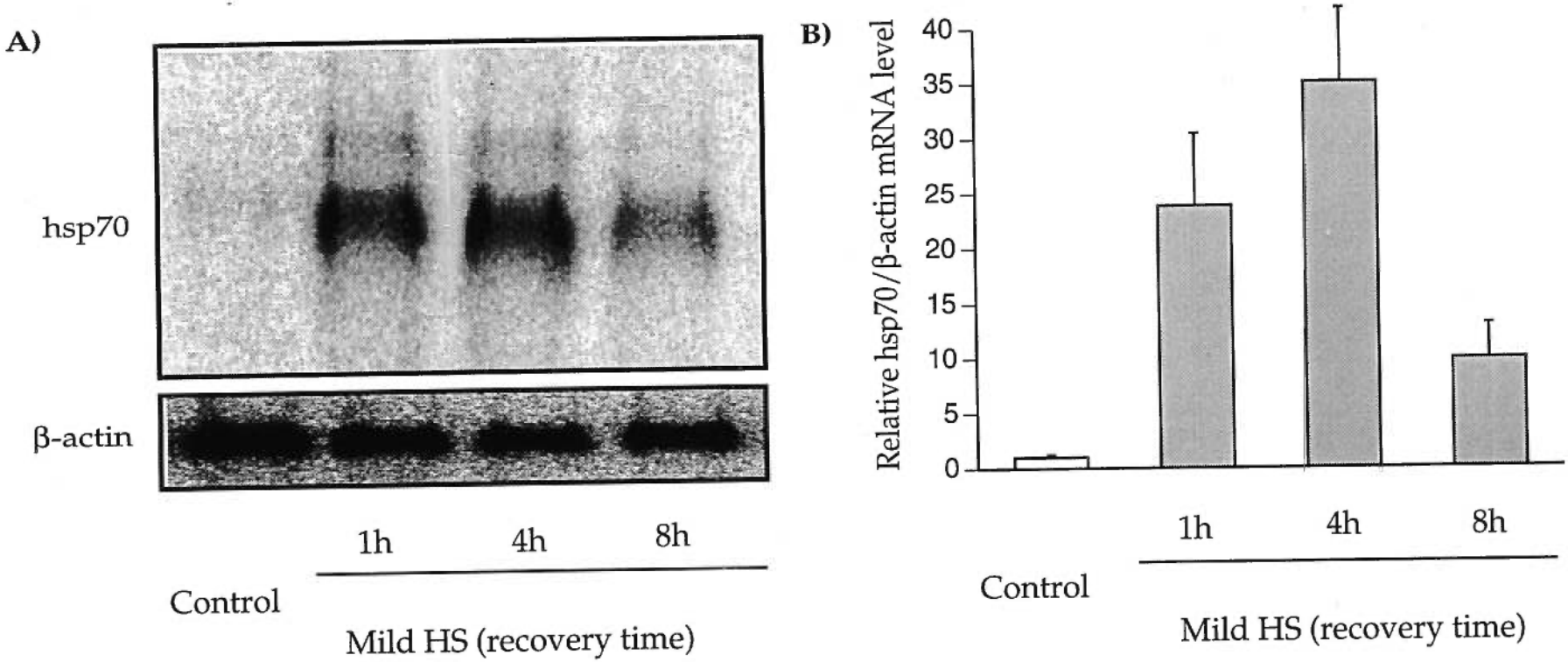
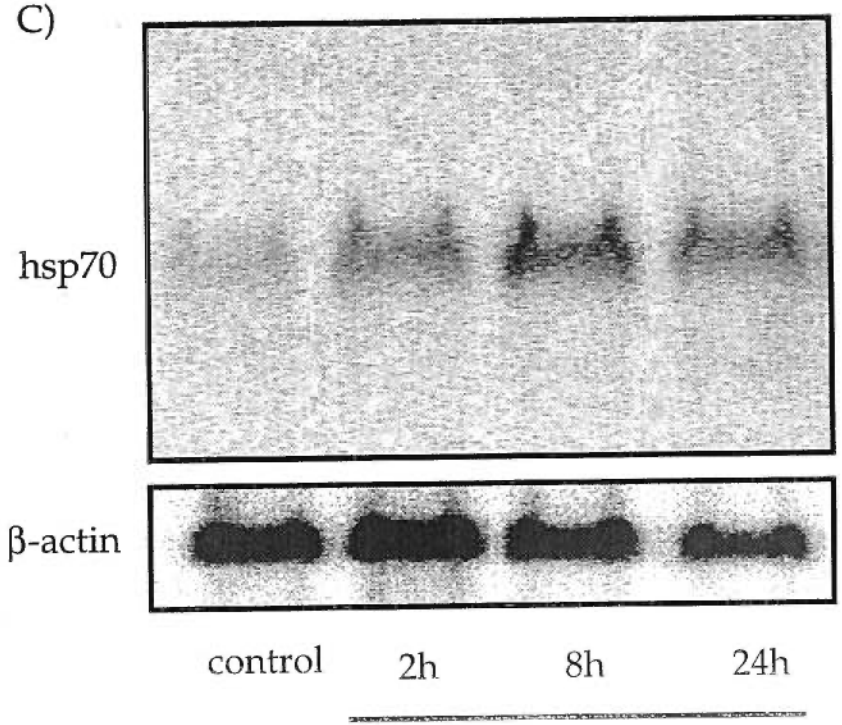

A)
control
+bumetanide
Na+,K+,Cl-
cotransport
K+ influx, nmol/mg prot./15 min
0
10
20
30
40
50
60
70
B)
control
Na+ free
medium
Na+,Pi-
cotransport
Pi influx, nmol/mg prot./15 min
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2

FIGURE 2

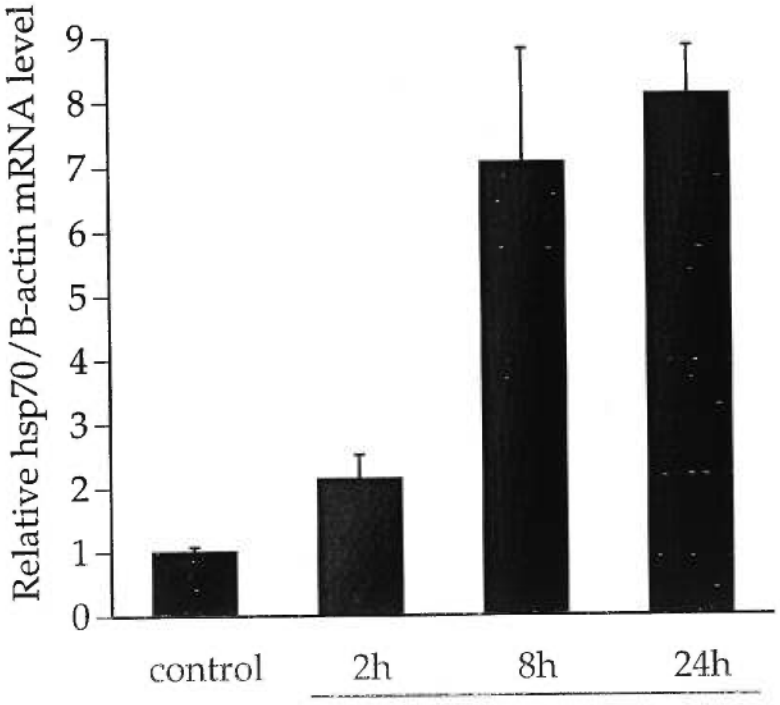
Northern blot (**A,C**) and HSP70/β-actin mRNA ratio (**B,D**) in MDCK cells 1, 4 and 8 hr after preconditioning heat stress (**HS**) (43°C for 20 min) (**A,B**) and 2, 8 and 24 hr after hyperosmotic stress (**HOS**) induced by the addition of 100 mM of NaCl to MEM (**C,D**). **E**) Northern blot comparing the effects of preconditioning heat stress (43°C, 4-hr recovery) and hyperosmotic stress induced by increased osmolality from 320 to 520 mosm by the addition of 100 mM of NaCl or 200 mM of sucrose to MEM for of 8 hr. The HSP70/β-actin mRNA ratio under control conditions was taken as 1.00. Means ± S.E. obtained in experiments performed in triplicate and duplicate are given.


A)
hsp70
β-actin
1h
4h
8h
Control
Mild HS (recovery time)
B)
Relative hsp70/β-actin mRNA level
40
35
30
25
20
15
10
5
0
1h
4h
8h
Control
Mild HS (recovery time)

C)


hsp70
β-actin
control
2h
8h
24h

D)


Relative hsp70/B-actin mRNA level
0
1
2
3
4
5
6
7
8
9
control
2h
8h
24h

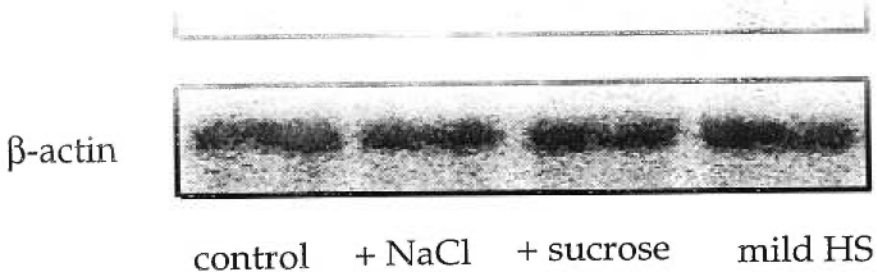
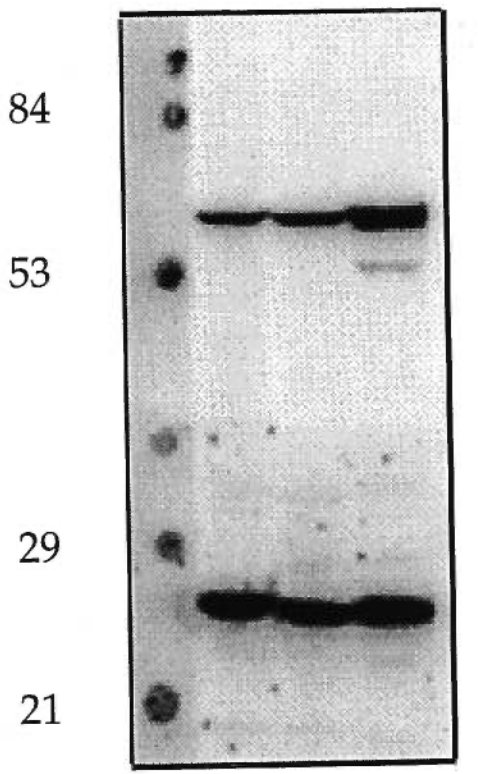

β-actin
control
+ NaCl
+ sucrose
mild HS

FIGURE 3

Western blot (**A**) and relative content (**B**) of HSP70 and HSP27 in MDCK cells subjected to hyperosmotic stress (**HOS**) induced by the addition of 200 mM of sucrose to MEM for 8 hr and by preconditioning heat stress (**HS**) (43°C, 20 min) followed by 6-hr recovery at 37°C. The level of HSPs in control conditions was taken as 1.00. Means ± S.E. from 2 experiments performed in duplicate are given.

A)


kDa
84
53
29
21
Control
HOS
Mild HS

B)

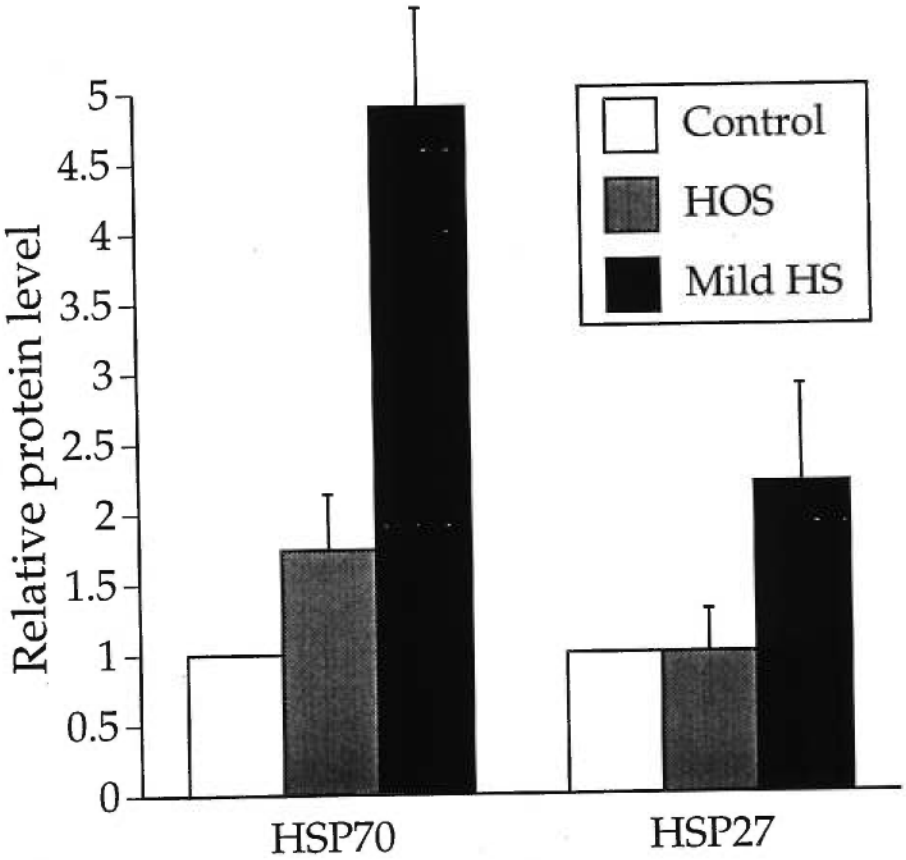
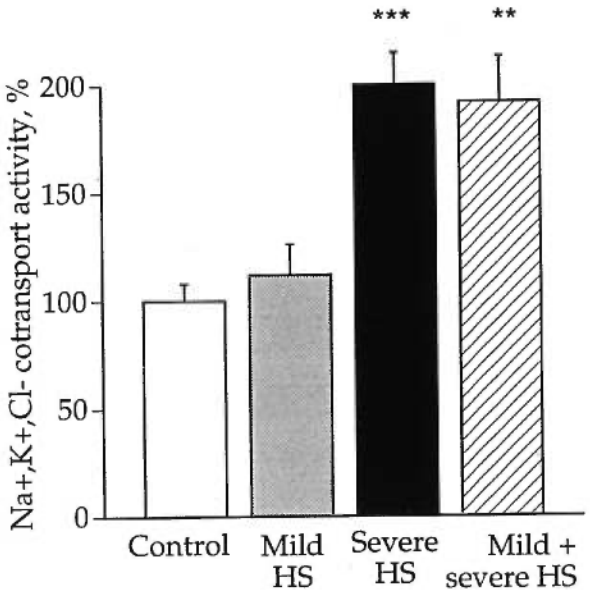

Relative protein level
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Control
HOS
Mild HS
HSP70
HSP27

FIGURE 4

Effects of preconditioning heat stress (HS) (43°C for 20 min, 4-hr recovery at 37°C) and severe heat stress (46°C for 15 min) on the activity of Na^+,K^+,Cl^- (**A**) and Na^+,P_i (**B**) cotransport. Values of the activity of these transporters under control conditions were taken as 100%. Means ± S.E. obtained in 7 (A) and 2 (B) experiments performed in quadruplicate or triplicate are given. *, **, *** for $p<0.02$, 0.002 and 0.0001, respectively, as compared to the controls.

A)


***
**
Na+,K+,Cl- cotransport activity, %
200
150
100
50
0
Control
Mild HS
Severe HS
Mild + severe HS

B)

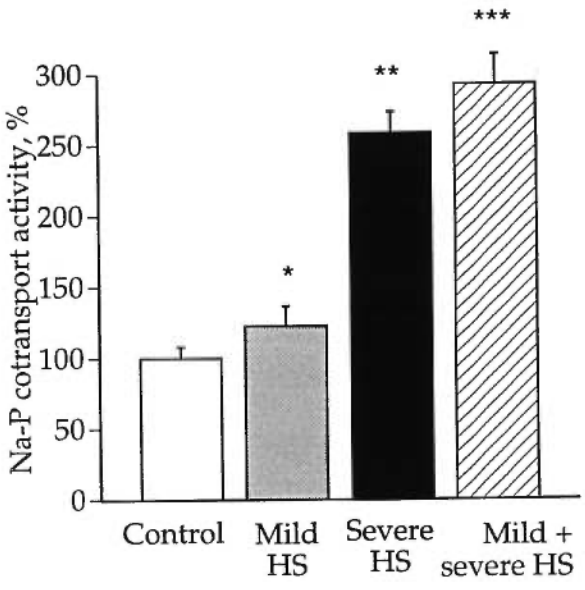

***
**
*
Na-P cotransport activity, %
300
250
200
150
100
50
0
Control
Mild HS
Severe HS
Mild + severe HS

FIGURE 5

Regulation of Na^+,P_i cotransport by PMA and ATP. Cells were incubated for 30 min in 1 ml of medium B with or without 0.1 μM PMA or 100 μM ATP. The medium was then removed and cells washed once with 1 ml of medium C. This medium was aspirated and the cells were incubated for 5 min in 0.25 ml of medium B or medium C containing 1 μCi/ml of ^{32}P and 0.2 mM K_2HPO_4. The value of Na^+,P_i cotransport in control cells without the addition of PMA or ATP was taken as 100%. Means ± S.E. obtained in 2 experiments performed in triplicate are given. *p<0.0002 as compared to the controls.

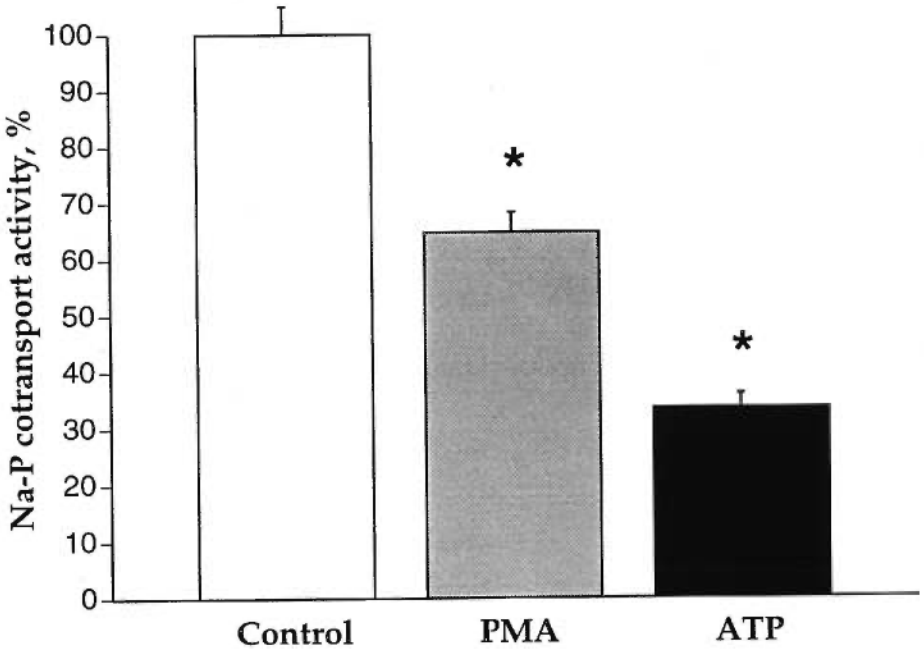
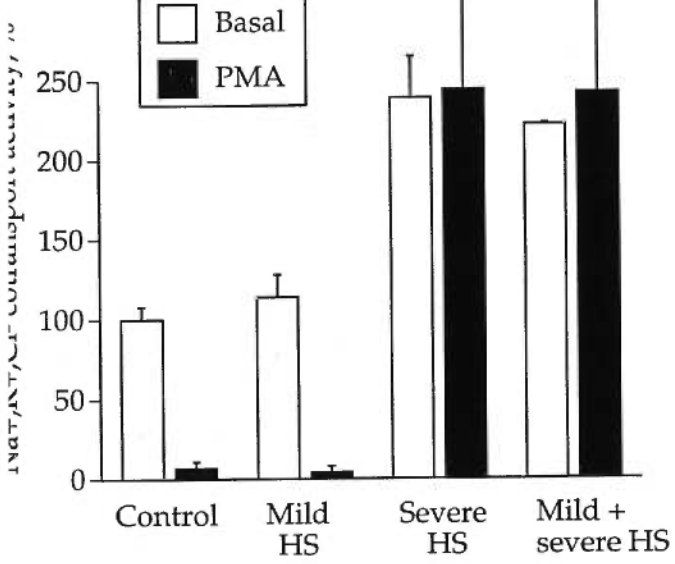

Na-P cotransport activity, %
100
90
80
70
60
50
40
30
20
10
0
*
*
Control
PMA
ATP

FIGURE 6

Effects of preconditioning heat stress (43°C for 20 min, 4-hr recovery at 37°C) and severe heat stress (46°C for 15 min) on the regulation of Na^+,K^+Cl^- cotransport (**A**) and Na^+,P_i cotransport (**B**) by PMA. Following the heat stress protocol, cells were preincubated for 30 min with or without 0.1 μM PMA in medium B or C before the measurement of ^{86}Rb or ^{32}P influx, respectively. The value of cotransporter activities in control PMA-untreated cells was taken as 100%. Means ± S.E. obtained in 3 (**A**) and 2 (**B**) experiments performed in quadruplicate and triplicate are given.


Basal
PMA
250
200
150
100
50
0
Control
Mild HS
Severe HS
Mild + severe HS

B)

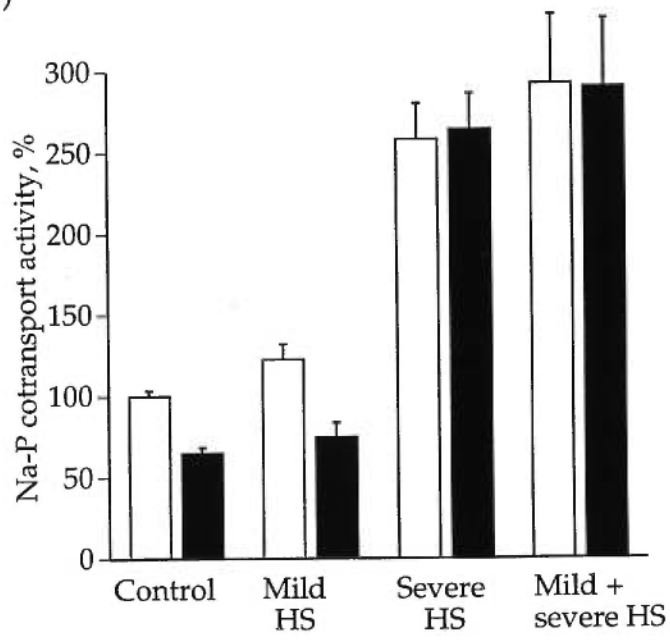

Na-P cotransport activity, %
300
250
200
150
100
50
0
Control
Mild HS
Severe HS
Mild + severe HS

FIGURE 7

Effects of preconditioning heat stress (43°C for 20 min, 4 hr recovery at 37°C) and severe heat stress (46°C for 15 min) on the regulation of Na^+,K^+,Cl^- cotransport (**A**) and Na^+,P_i cotransport (**B**) by ATP. Following the heat stress protocol, cells were preincubated for 30 min with or without 100 μM ATP in medium B or C before the measurement of ^{86}Rb or ^{32}P influx, respectively. The value of cotransporter activities in control ATP-untreated cells was taken as 100%. Means ± S.E. obtained in 3 (**A**) and 2 (**B**) experiments performed in quadruplicate and triplicate are given.

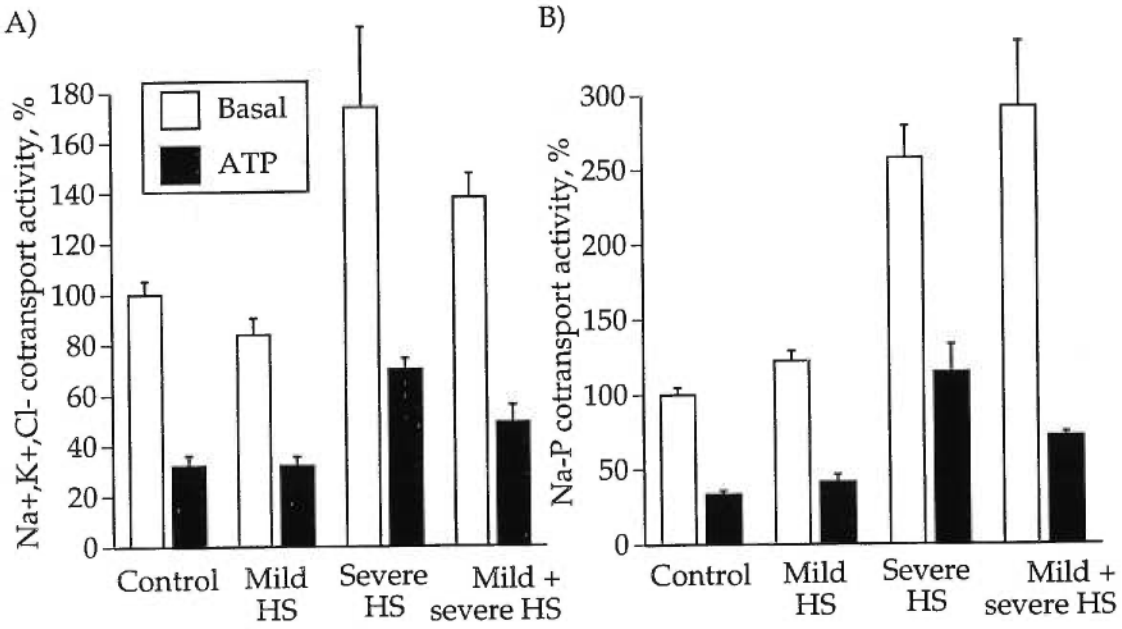
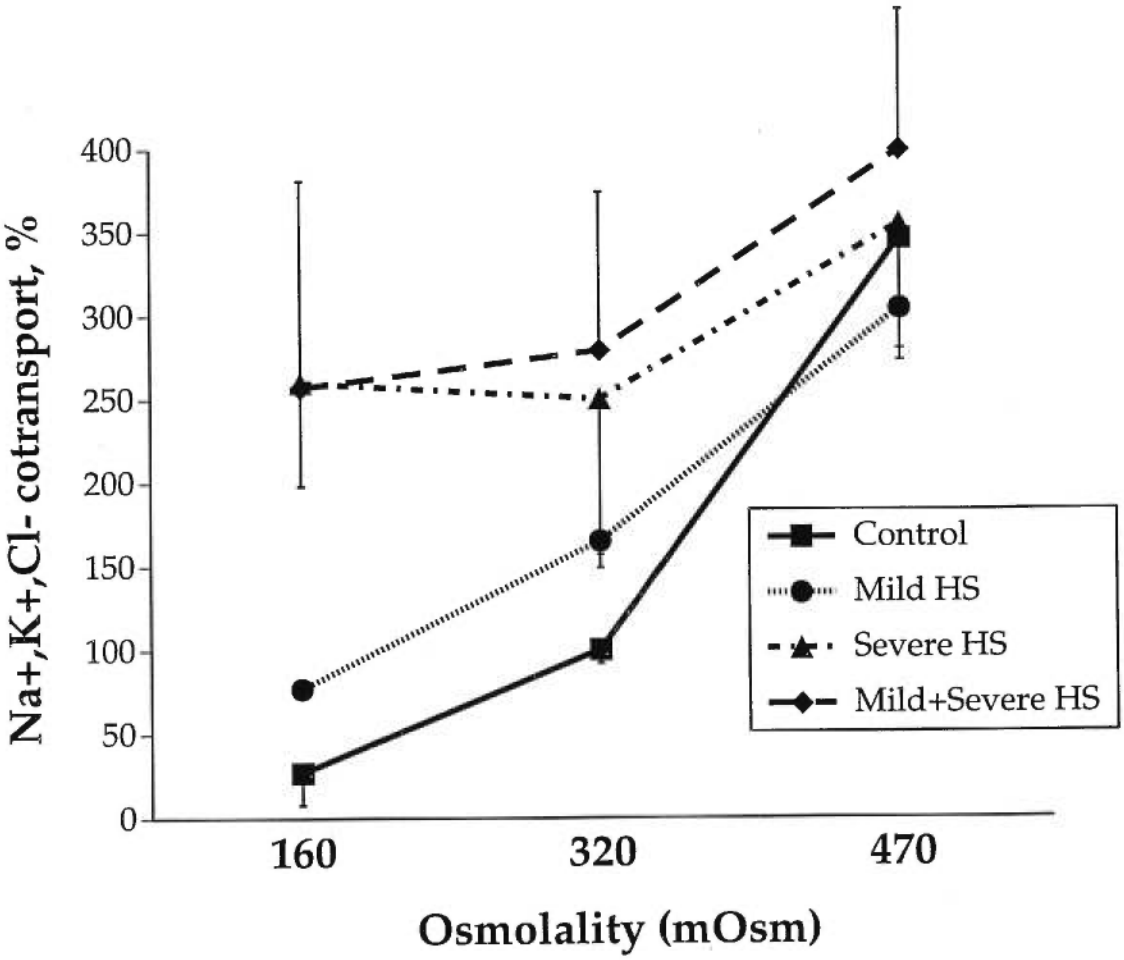

A)
Na+,K+,Cl- cotransport activity, %
180
160
140
120
100
80
60
40
20
0
Basal
ATP
Control
Mild HS
Severe HS
Mild + severe HS
B)
Na-P cotransport activity, %
300
250
200
150
100
50
0
Control
Mild HS
Severe HS
Mild + severe HS

FIGURE 8

Effects of preconditioning heat stress (42°C for 20 min, 4 hr recovery at 37°C) and severe heat stress (46°C for 15 min) on the volume-dependent regulation of Na^+,K^+,Cl^- cotransport. Following the heat stress protocol, cells were preincubated for 15 min in 0.25 ml of isosmotic medium B with 1 mM ouabain and with or without 20 μM bumetanide. Then, 0.25 ml of hyposmotic medium B containing 20 mM NaCl, isosmotic medium B and hyperosmotic medium B containing 300 mM sucrose was added to adjust the final osmolality of the media at 160, 320 and 470 mosm, respectively. These media also contained 1-2 μCi/ml $^{86}RbCl$. The value of Na^+,K^+,Cl^- cotransport activity in control cells in isosmotic medium without heat stress was taken as 100%. Means ± S.E obtained in 2 experiments performed in quadruplicate are given.


Na+,K+,Cl- cotransport, %
400
350
300
250
200
150
100
50
0
160
320
470
Osmolality (mOsm)
Control
Mild HS
Severe HS
Mild+Severe HS

CHAPITRE 6

DISCUSSION GÉNÉRALE
ET
CONCLUSION

Régulation du co-transporteur Na^+, K^+, Cl^- dans les cellules MDCK

Peu de données existent sur la régulation du co-transporteur rénal Na^+, K^+, Cl^- chez les mammifères. Il a été démontré que la réabsorption du NaCl dans l'anse de Henlé est stimulée par une voie de signalisation dépendante de l'AMP cyclique (Greger 1985). Toutefois, la contribution relative du co-transporteur Na^+, K^+, Cl^- à un tel phénomène est controversée (Molony et al. 1989, Schlatter and Greger 1985, Vuillemin et al. 1992). Pour cette thèse de doctorat, nous avons d'abord examiné les voies de signalisation impliquées dans la régulation de l'influx de potassium dans des cellules d'épithélium rénal d'origine canine (MDCK) (Chapitre 3). Nous avons été les premiers à démontrer que, dans des cellules d'épithélium rénal de mammifère, le co-transporteur Na^+, K^+, Cl^- est complètement inhibé par l'activation de la protéine kinase C par le 4β-phorbol 12-myristate 13-acétate (PMA) et partiellement inhibé par l'activation des purinocepteurs-P_2. L'activité de ce transporteur demeure cependant insensible aux agonistes α-adrénergiques, cholinergiques et dopaminergiques, à la vasopressine et à l'angiotensine II. Nous avons également observé une légère stimulation de ce transporteur par l'activation de la voie de signalisation de l'AMP cyclique. Toutefois, l'activation de la signalisation dépendante du GMP cyclique n'a pas modifié l'activité de ce transporteur (Chapitre 3). La puissance de l'inhibition du co-transporteur par différents agonistes purinergiques suggère que cet effet est induit par les purinocepteurs-P_{2x} ou P_{2y}. Il a été démontré que les purinocepteurs-P_{2y} sont exprimés dans les cellules MDCK. Toutefois, leur rôle dans la régulation des fonctions cellulaires est peu connu.

Au cours des dix dernières années, il a été démontré, dans les cellules MDCK, que les purinocepteurs-P_2 jouent un rôle dans plusieurs fonctions cellulaires qui utilisent différentes voies de signalisation dont l'activation des protéines G_i/G_q, PLC, PKC, PLA_2, MAPK, l'adenylate cyclase et la PKA. Un résumé de ces voies de

signalisation est illustré à la figure 1 de ce chapitre. Afin de caractériser les mécanismes impliqués dans la régulation du co-transporteur Na^+, K^+, Cl^- par l'agoniste purinergique ATP, nous avons examiné l'implication potentielle de ces voies de signalisation.

Dans les cellules MDCK, la toxine de pertussis prévient la production d'$InsP_3$ induite par l'ATP (Paulmichl et al. 1991, Yang et al. 1997). Malgré une telle observation, nous n'avons pas observé d'effet de cette toxine sur la régulation du co-transporteur Na^+, K^+, Cl^- par l'ATP. De plus, le blocage de la production de l'$InsP_3$ induit par l'ATP par l'inhibiteur de la PLC, U73122, n'a pas modifié l'activité de ce transporteur. L'activation de la $PLC_{\beta1/\beta2}$ couplée aux protéines G_q/G_i n'est donc apparemment pas impliquée dans la régulation du co-transporteur Na^+, K^+, Cl^- par l'ATP.

Plusieurs laboratoires ont démontré que, dans les cellules MDCK, l'ATP provoque une élévation de la $[Ca^{2+}]_i$ induite par l'$InsP_3$. Celle-ci est conséquente à un relargage de Ca^{2+} provenant des réserves intracellulaires et à l'influx provenant des canaux calciques activés par la libération de Ca^{2+} (CRAC) (Paulmichl et al. 1991, Delles et al. 1995). Notre étude confirme cette observation. Toutefois, les résultats que nous avons obtenus en traitant les cellules MDCK avec plusieurs agents modulateurs de la $[Ca^{2+}]_i$ via différents mécanismes démontrent que cet ion n'est pas impliqué dans la régulation du co-transporteur Na^+, K^+, Cl^- par l'ATP.

Afin d'examiner l'implication de la PKC dans l'inhibition du co-transporteur Na^+, K^+, Cl^- induite par l'activation purinergique, nous avons étudié l'effet de l'inhibiteur sélectif de la PKC, la calphostine C, sur la régulation du co-transporteur Na^+, K^+, Cl^- par l'ATP et par le PMA. La calphostine C et un inhibiteur moins

sélectif, la staurosporine, ont aboli l'effet inhibiteur du PMA sans toutefois modifier la régulation du transporteur par l'ATP (Chapitre 4). De plus, la régulation du co-transporteur Na^+, K^+, Cl^- par l'ATP est insensible à la régulation à la baisse des isoformes de la PKC sensibles au PMA alors que la régulation par le PMA est abolie dans les mêmes conditions (Chapitre 3). L'ensemble de ces résultats suggèrent que les isoformes de la PKC sensibles au PMA ne sont pas impliqués dans la régulation du co-transporteur Na^+, K^+, Cl^- par les purinocepteurs. Toutefois, mis à part les isoformes classiques connus de la PKC (PKC-α, βI, βII et γ) reconnus pour leur sensibilité au PMA, au diacylglycerol et au Ca^{2+} et les plus récents isoformes de la PKC (PKC-δ, ε, η et ξ) sensibles au PMA et au diacylglycerol mais insensibles au Ca^{2+}, on connaît aussi les isoformes atypiques de la PKC insensibles au Ca^{2+} et au PMA/diacylglycerol (PKC-ζ, ι, λ et μ) plus résistants à la staurosporine et aux autres inhibiteurs de la PKC disponibles (Kikawa et al. 1989, Akimoto et al. 1994, Selbie et al. 1993). Le rôle des isoformes atypiques de la PKC dans la régulation du co-transporteur Na^+, K^+, Cl^- devrait donc être investigué.

Le rôle des récepteurs à activité tyrosine kinase dans l'activation des MAP kinases est très connu. Par ailleurs, des récepteurs couplés aux protéines G, dont ceux des acides lysophosphatidiques, de la prostaglandine F_{2A}, et les récepteurs α-adrénergique et cholinergique, activent aussi les MAP kinases (revue de la littérature, Force et Bonventre 1998). Les voies de signalisation de l'activation des MAP kinases, dépendantes mais aussi indépendantes de *ras*, sont déclenchées par les sous-unités α des protéines G et par le dimère βγ. La voie dépendante de $p21^{ras}$ implique l'hydrolyse des phosphoinositides stimulée par la PLC et la libération du Ca^{2+} induite par l'$InsP_3$. La voie indépendante de $p21^{ras}$ implique l'activation des isoformes de la PKC sensibles au diacylglycerol. Récemment, il a été démontré, dans les cellules MDCK, que l'activation purinergique induit la phosphorylation de la MAPK (Xing et

al. 1997). Nos résultats confirment, en partie, cette observation. En effet, nous avons observé une phosphorylation de la MAPK après l'ajout du PMA et celui de l'ATP, ce qui suggère l'implication de l'activation de la voie de signalisation des MAP kinases dans l'inhibition du co-transporteur Na^+, K^+, Cl^- induite par l'ATP et par le PMA. Toutefois, l'ensemble de nos résultats n'appuient pas cette hypothèse. Premièrement, tel que mentionné précédemment, l'inhibition de la MAPK par le PD98059 n'a pas empêché l'inhibition du co-transporteur Na^+, K^+, Cl^- par l'ATP. Deuxièmement, l'activation de la MAPK par le sérum de veau n'affecte pas l'activité du même co-transporteur. Troisièmement, l'activation de la voie de signalisation de la MAPK par l'ATP est diminuée après la régulation à la baisse de la PKC à la suite d'une pré-incubation de 24 h avec le PMA et après incubation avec le chélateur de calcium BAPTA-AM. Ces observations suggèrent que les voies de signalisation dépendantes de $p21^{ras}$ et de la PKC sont impliquées dans l'activation de la MAPK induite par les purinocepteurs. Cette conclusion est en accord avec nos données sur l'augmentation de la phosphorylation de la MAPK par la thapsigargine, un inhibiteur de la pompe à Ca^{2+} du réticulum endoplasmique. Par contre, ni la régulation à la baisse de la PKC par le PMA, ni la modulation de la $[Ca^{2+}]_i$ par l'addition de BAPTA ou de thapsigargin, n'ont affecté la régulation purinergique du co-transporteur Na^+, K^+, Cl^-.

Dans les cellules MDCK, l'activation des purinocepteurs P_2 augmente l'activité de la PLA_2 via les voies de signalisation dépendantes de la PKC et de la MAPK et sensibles à la toxine de pertussis (Firestein et al. 1996). Les résultats de nos travaux ont démontré que l'effet inhibiteur de l'ATP sur l'activité du co-transporteur Na^+, K^+, Cl^- est indépendant de l'activité de la PLA_2. En effet, la régulation du co-transporteur Na^+, K^+, Cl^- par l'ATP demeure inchangée malgré le traitement des cellules MDCK avec les toxines de pertussis et du choléra, l'ajout de l'acide arachidonique et des inhibiteurs de la PLA_2, la cyclooxygénase, la lypoxygénase et la

MAPK. Ces résultats ne mettent donc pas en évidence l'implication de la PLA_2 dans l'inhibition du co-transporteur Na^+, K^+, Cl^- induite par l'ATP.

L'implication de l'AMP cyclique dans la régulation du co-transporteur Na^+, K^+, Cl^- par les purinocepteurs-P_2 est aussi peu probable puisque, tel que mentionné précédemment, une légère augmentation plutôt qu'une inhibition de l'activité de ce transporteur est observée dans les cellules traitées avec la forskoline et dans les cellules traitées avec la toxine du choléra. De plus, nous avons obtenu des résultats négatifs avec un inhibiteur de la PKA, le H-89.

Il est bien connu que l'activité du co-transporteur Na^+, K^+, Cl^- peut être modulée par la variation des concentrations d'ions monovalents aussi bien que par le changement du volume cellulaire. La pré-incubation des cellules MDCK dans un milieu sans Na^+ et sans Cl^- est suivie d'une diminution marquée de la régulation du co-transporteur par le PMA alors que la régulation par l'ATP demeure inchangée. Ces observations suggèrent que les purinocepteurs-P_2 sont exclusivement impliqués dans la régulation du nombre de transporteurs fonctionnels, ou du nombre de transporteurs à être remplacés. De plus, la régulation par le PMA augmente aussi la sensibilité du co-transporteur à l'inhibition associée aux changements des concentrations intracellulaires de Cl^- et de Na^+. Quant au volume cellulaire, il ne semble pas avoir un rôle dans la régulation de l'activité du co-transporteur par le PMA ni par l'ATP (Chapitre 4).

En conclusion, nos résultats démontrent que l'inhibition du co-transporteur Na^+, K^+, Cl^- induite par les récepteurs purinergiques-P_2 est indépendante des protéines G sensibles aux toxines de choléra et de pertussis. L'activation de la voie de signalisation du GMP cyclique, par l'ajout du 8-Br-cGMP, du nitroprusside ou de

l'ANP (*atrial natriuretic* peptide), n'a eu aucun effet sur l'activité du co-transporteur Na^+, K^+, Cl^- (Chapitre 3). L'inhibition de ce transporteur par l'ATP n'implique apparemment pas les voies de signalisation via les récepteurs-P_2 connues dans les cellules MDCK qui sont l'augmentation de la $[Ca^{2+}]_i$, l'activation des PLC et PLA_2, l'activation des isoformes de la PKC sensibles au diacylglycerol, la phosphorylation des MAP kinases, les modifications des concentrations intracellulaires d'ions monovalents et du volume cellulaire. Dans leur ensemble, nos résultats suggèrent que l'inhibition du co-transporteur Na^+, K^+, Cl^- induite par l'ATP dans les cellules MDCK est initiée par une voie de signalisation que nous n'avons pas étudiée dans notre étude et qui n'est pas décrite dans ce type de cellules. Il est bien connu que l'activité des canaux ioniques dans des cellules électriquement excitables peut être l'objet d'une régulation par des voies de signalisation membranaire via une interaction directe des canaux avec des protéines G distinctes de G_s et de G_i (Brown et Birnbaumer 1990). Récemment, il a été démontré qu'une telle régulation par les protéines G est aussi impliquée dans la régulation de l'échangeur Na^+/H^+ par G_{12} et G_{13} (Lin et al.1996, Voyno-Yasentskaya et al 1994). Le rôle des protéines G autres que G_s et G_i dans les mécanismes d'inhibition purinergique du co-transporteur Na^+,K^+,Cl^- reste à élucider.

Les données sur la régulation purinergique du co-transporteur Na^+, K^+, Cl^- présentés dans cette thèse de doctorat peuvent avoir une importance clinique pour les raisons suivantes. Premièrement, aux terminaisons des systèmes nerveux central et périphérique, l'ATP et autres agonistes purinergiques sont co-localisés avec des neurotransmetteurs spécifiques tels les catécholamines et l'acétylcholine. De plus, l'hypoxie locale et autres stress cellulaires sont des sources de libération d'ATP initiant une activité purinergique (Motte et al. 1995). Nous proposons que l'inhibition du co-transporteur Na^+, K^+, Cl^- par l'activation des récepteurs-P_2, rapportée dans notre étude et révélée pour la première fois dans des cellules d'épithélium rénal de

mammifère, peut être impliquée dans la régulation de la fonction rénale dans des conditions aussi bien physiologiques que pathophysiologiques, incluant des maladies telles que l'hypertension artérielle et les complications qui y sont associées.

Modulation par le stress thermique de la régulation des co-transporteurs Na^+, K^+, Cl^- et Na^+, Pi dans les cellules MDCK

L'étape suivante dans notre étude était d'examiner l'effet potentiellement protecteur des protéines de choc thermique HSPs sur la régulation du co-transporteur Na^+, K^+, Cl^- et du co-transporteur Na^+, P_i dans les cellules MDCK (Chapitre 5). L'induction des HSPs par le stress thermique ou par d'autres inducteurs confère aux cellules une résistance accrue à un stress subséquent qui serait autrement létal (Beck and De Maio 1994, Marber et al. 1993, Brown et al. 1992). Le phénomène de thermoprotection est généralement démontré par une augmentation de la survie cellulaire. Toutefois, la protection de fonctions cellulaires spécifiques permet d'envisager un potentiel thérapeutique de l'utilisation du stress thermique pré-conditionnant. Les études sur le rôle potentiel du stress pré-conditionnant dans le rein sont contradictoires. À notre connaissance, nous sommes les premiers à avoir étudié l'implication potentielle des HSPs dans la régulation de transporteurs d'ions dans des cellules d'épithélium rénal de mammifère. Nos résultats ont démontré, dans le modèle d'épithélium rénal de mammifère utilisé, les cellules «Madin-Darby canine kidney» (MDCK), que le stress thermique sévère augmente l'activité des deux transporteurs d'ions étudiés, les co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i, et abolit leur régulation par la protéine kinase C, tout en préservant celle par l'ATP. Dans notre analyse des mécanismes pouvant expliquer l'activation de ces transporteurs, nous avons d'abord considéré l'effet direct potentiel du stress thermique sur l'organisation structurale des co-transporteurs. Toutefois, cette hypothèse est peu probable à cause du manque d'homologie entre ces deux protéines. L'explication la plus vraisemblable est que

l'activation des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i se fait par la modulation thermique d'un élément commun impliqué dans l'activité de ces deux transporteurs. Cet élément serait relié au réseau du cytosquelette. Plusieurs observations appuient cette hypothèse : 1) Dans plusieurs types de cellules à noyaux, dont les cellules du muscle lisse vasculaire de l'aorte et les cellules de l'épithélium rénal, la modification du cytosquelette affecte l'activité des co-transporteurs Na^+, K^+, Cl^- (Orlov et al. 1995) et Na^+, P_i (Friedlander et al. 1988, Hansch et al. 1993); 2) Le traitement de cellules du muscle lisse vasculaire de l'aorte et des cellules de l'épithélium rénal par un désintégrateur des faisceaux des microfilaments, par la cytocholasine B ou par la phalloïdine un stabilisateur de l'actine-F, empêche la régulation hormonale du co-transporteur Na^+, K^+, Cl^- ; 3) L'interaction de la PKC avec des protéines d'ancrage a un rôle important dans la régulation de l'activité d'enzymes et de transporteurs liés à la membrane. Nous avons démontré que le stress thermique sévère inhibe complètement la régulation des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i par un activateur de la PKC, le PMA; 4) Dans les érythrocytes de rats, la rupture du cytosquelette par 10 minutes de pré-incubation à 49°C abolit complètement l'activation du co-transporteur Na^+, K^+, Cl^- induite par le rétrécissement cellulaire (Orlov et al. 1990, 1993), ce qui est en accord avec nos résultats sur l'inhibition de la régulation par le volume du co-transporteur Na^+, K^+, Cl^- observée dans les cellules MDCK soumises à un stress thermique sévère.

Contrairement à l'inhibition complète de la régulation induite par le changement du volume cellulaire et par le PMA, le stress thermique sévère n'a pas modifié significativement la régulation des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i par l'ATP. Ces résultats démontrent que le mécanisme d'inhibition des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i par l'ATP est différent de celui du PMA. Cette conclusion est appuyée par les résultats discutés précédemment sur l'absence d'effet de la régulation

à la baisse de la PKC sur la régulation du co-transporteur par l'ATP, et par l'inhibition du co-transporteur par la staurosporine.

Toutefois, malgré l'induction des HSPs par un stress pré-conditionnant, nous n'avons pas observé de thermoprotection de l'activité basale des co-transporteurs Na^+, K^+, Cl^- and Na^+, P_i, ou de leur régulation par le volume et par la PKC. Ces résultats suggèrent que, dans des cellules d'épithélium rénal de mammifère, les voies biochimiques impliquées dans les fonctions cellulaires décrites précédemment sont résistantes à la modulation de la concentration des HSPs sensibles au stress thermique pré-conditionnant. Brown et al. (1992) ont démontré que le stress thermique pré-conditionnant protège l'activité des transporteurs rénaux de glucose et de sulfate de l'inactivation induite par le stress thermique sévère. L'induction parallèle de différentes HSPs suggère leurs implications dans cet effet protecteur observé dans un épithélium rénal de truite. L'absence de thermoprotection documentée dans notre étude peut s'expliquer par les raisons suivantes : la thermoprotection de l'activité des transporteurs d'ions peut être une caractéristique spécifique aux cellules d'épithélium rénal de poisson, limitée aux transporteurs de glucose et de sulfate ou spécifique aux cellules d'épithélium rénal provenant de tubules proximaux. De plus, nous avons utilisé, pour nos expériences, des cellules non-polarisées alors que Brown et al. (1992) ont utilisé des cellules cultivées sur filtres. Les résultats contradictoires obtenus dans notre étude par rapport à celle de Brown (1992) peuvent donc dépendre du fait qu'ils ont étudié des cellules polarisées. Les cellules non-polarisées et les cellules polarisées ont une organisation différente du cytosquelette.

Notre étude n'exclut pas la possibilité que les HSPs jouent un rôle dans la limitation des dommages causés à l'épithélium rénal en réduisant la mortalité cellulaire, ou en stimulant la réparation des dommages causés par le stress thermique.

Le fait que nous ayons observé, avec d'autres chercheurs (Moseley et al. 1994), un effet pré-conditionnant modéré sur la viabilité des cellules MDCK, évaluée par le contenu en LDH et en protéines totales, appuie cette affirmation. D'ailleurs, il a récemment été démontré, par les équipes des Docteurs Tremblay et Hamet, que le stress thermique modéré, accompagné d'une accumulation des HSP27 et HSP70, protège les cellules du muscle lisse vasculaire de l'inhibition de la prolifération subséquente au stress thermique sévère et à l'induction de la nécrose. Par contre, le stress thermique reste sans effet sur l'induction de l'apoptose (Champagne et al. 1998). Toutefois, un rôle majeur des HSPs dans les maladies associées aux co-transporteurs Na^+, K^+, Cl^- and Na^+, P_i, est peu probable. Dans leur ensemble, ces résultats suggèrent que les co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i sont importants dans la réponse au stress thermique sévère mais que, dans les cellules MDCK, la protection de fonctions cellulaires spécifiques ne peut être acquise par l'induction des HSPs.

CONCLUSION

La production de protéines de stress, les HSPs, et l'activité des transporteurs d'ions dont le NKCC1, le NKCC2, le NHE1 et le NHE3 sont modifiées dans l'hypertension génétique (pour une revue de la littérature récente, voir Orlov et al. 1998). Le but de cette thèse de doctorat était de déterminer l'implication de protéines de stress, les HSPs, sur la modulation de l'activité des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i dans des cellules d'épithélium rénal de mammifère. Nos travaux visaient d'abord à identifier les voies de signalisation impliquées dans la régulation du co-transporteur Na^+, K^+, Cl^- et du co-transporteur Na^+, P_i dans des cellules d'épithélium rénal de mammifère. L'induction des HSPs peut conférer aux cellules une protection contre un stress subséquent plus sévère (Beck et De Maio 1994, Marber et al. 1993, Brown et al. 1992). Nous avons donc tenté de déterminer si un tel phénomène de thermoprotection pouvait jouer un rôle dans le maintien de l'activité des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i. Nous avons aussi étudié l'implication potentielle des HSPs dans la régulation hormonale de ces transporteurs d'ions.

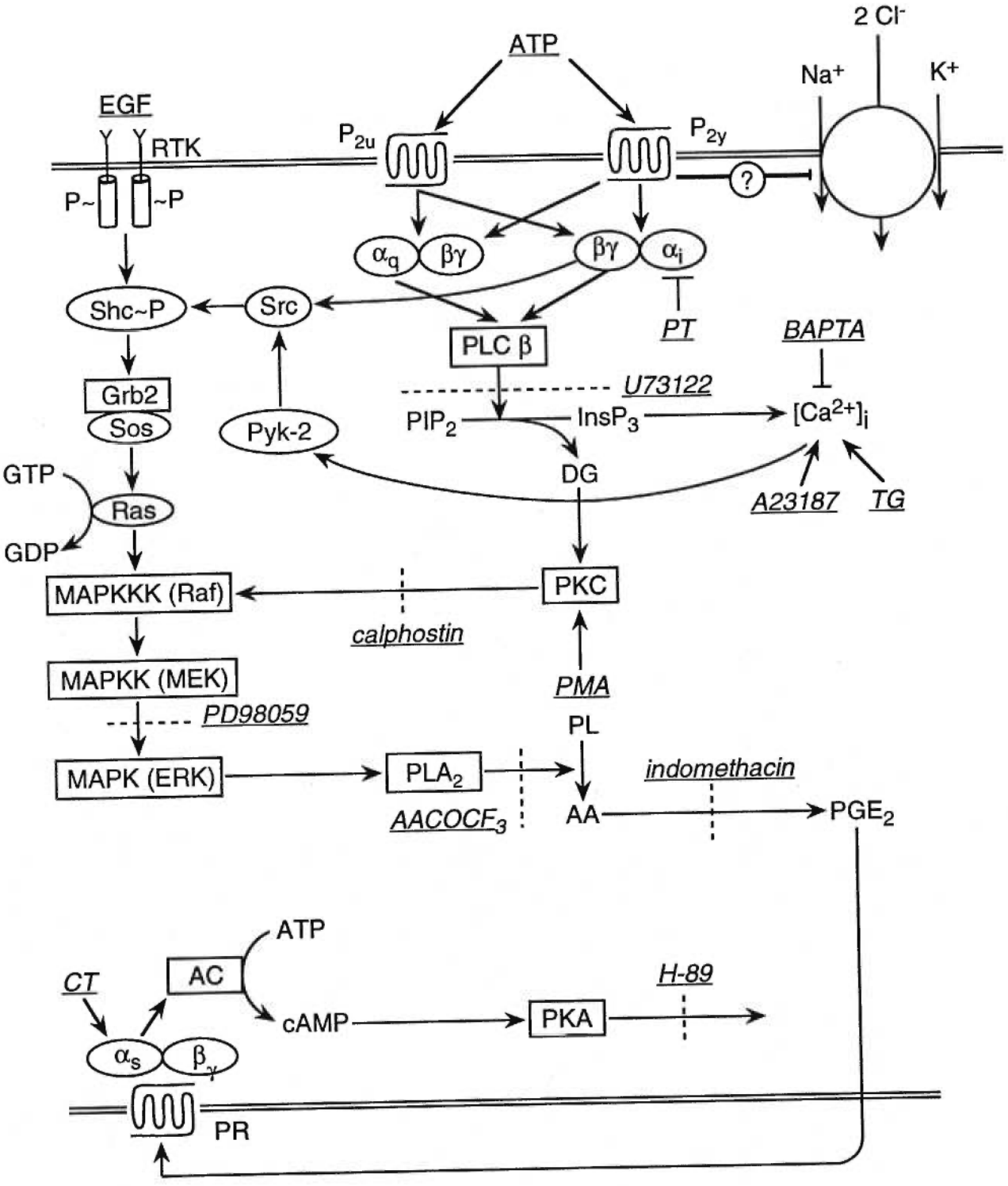
Les conclusions découlant de cette thèse sont les suivantes :

Dans les cellules MDCK,

1. l'activation des isoformes de la protéine kinase C sensibles au PMA inhibent complètement l'activité du co-transporteur Na^+, K^+, Cl^- et d'environ 30 % l'activité du co-transporteur Na^+, P_i.
2. l'activation des purinocepteurs-P_2 inhibe de 50 à 60 % l'activité des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i.
3. la régulation du co-transporteur Na^+, K^+, Cl^- par les purinocepteurs-P_2 ne se fait pas via les isoformes de la protéine kinase C sensibles au PMA ni par les mécanismes connus de la signalisation induite par l'ATP, c'est-à-dire par l'activation de la PLA_2, des kinases MAP, du relargage du $Ca^{2+}{}_i$ et du turnover du phosphoinositol (figure 1).

FIGURE 1

Signalisation purinergique dans les cellules MDCK. **AC** – adénylate cyclase; **CT** – toxine du choléra ; **DG** – diacylglycerol; **PL** – phospholipides; **PR** – récepteur du PGE_2 ; **PT** – toxine de pertussis; **RTK** – récepteur pour les tyrosines kinases ; **TG** – thapsigargine ; **?** – indéterminé. → – activation ; ———| ou - - - - - – inhibition. Les agents modulateurs utilisés dans notre étude sont en italique.


ATP
2 Cl-
Na+
K+
EGF
RTK
P2u
P2y
?
P~
~P
αq
βγ
βγ
αi
Shc~P
Src
PT
BAPTA
PLC β
U73122
Grb2
Sos
Pyk-2
PIP2
InsP3
[Ca2+]i
GTP
Ras
GDP
DG
A23187
TG
MAPKKK (Raf)
PKC
calphostin
MAPKK (MEK)
PMA
PD98059
PL
MAPK (ERK)
PLA2
indomethacin
AACOCF3
AA
PGE2
ATP
CT
AC
H-89
cAMP
PKA
αs
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4. l'activité basale des transporteurs d'ions étudiés est indépendante de l'induction de la HSP70 et de la HSP27 par le stress thermique pré-conditionnant.
5. le stress thermique sévère augmente l'activité basale des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i et abolit complètement la régulation de ces transporteurs par le PMA, sans modifier significativement la régulation par l'activation des purinocepteurs-P_2.
6. la production de la HSP70 induite par un stress thermique modéré ne protège pas les co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i de leur activation à la suite d'un stress thermique sévère ou de leur inhibition via les purinocepteurs-P_2.

Nos travaux ont donc contribué à identifier, pour la première fois, les voies de signalisation impliquées dans la régulation du co-transporteur basolatéral Na^+, K^+, Cl^- dans des cellules du tubule collecteur de mammifère. Nous avons aussi démontré que la régulation purinergique du co-transporteur Na^+, K^+, Cl^- se fait par une voie de signalisation jusqu'à présent non décrite dans les cellules MDCK.

Dans leur ensemble, les résultats présentés dans cette thèse de doctorat n'appuient pas l'hypothèse d'un rôle des HSP27 et HSP70 dans la modulation de l'activité basale et de la régulation hormonale des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i dans des cellules d'épithélium rénal de mammifère. Toutefois, nos données n'excluent pas la possibilité que la HSP27, la HSP70 ou d'autres HSPs jouent un rôle dans la modulation de transporteurs d'ions qui n'ont pas été étudiés dans cette thèse.

En conclusion, dans les cellules MDCK, les purinocepteurs-P_2 et certains des isoformes de la protéine kinase C sensibles au PMA sont impliqués dans la régulation de l'activité des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i. Deux mécanismes différents, indépendants de l'induction des HSP70 et HSP27, l'un insensible et l'autre sensible au stress thermique, sont responsables de la régulation de ces transporteurs. Nos résultats suggèrent qu'un rôle majeur des HSPs dans la modulation des co-transporteurs rénaux Na^+, K^+, Cl^- et Na^+, P_i dans l'hypertension génétique est peu probable.

PERSPECTIVES

Une particularité des cellules épithéliales est la polarité morphologique et fonctionnelle de celles-ci. Les travaux présentés dans cette thèse de doctorat ont tous été effectués sur des cellules cultivées sur un support imperméable. Les cellules polarisées ont une organisation du cytosquelette différente des cellules non-polarisées. La seule autre étude qui a étudié l'effet des HSPs sur l'activité de transporteurs membranaires a été effectuée avec des cellules cultivées sur un support perméable (Brown et al. 1992). Dans cette étude, un effet de thermoprotection de l'activité des co-transporteurs Na^+-sulphate et Na^+-glucose, associé à l'induction des HSPs, a été démontré. L'étude des co-transporteurs rénaux Na^+, K^+, Cl^- et Na^+, P_i dans des cellules polarisées mérite donc d'être considérée. De plus, avant d'exclure complètement l'implication des HSPs dans la modulation des co-transporteurs rénaux Na^+, K^+, Cl^- et Na^+, P_i , une étude portant sur les cellules des différents tubules pourrait être envisagée. En effet, l'abondance des différents transporteurs membranaires et la régulation hormonale de ceux-ci est très spécifique à l'espèce mais aussi au segment du néphron étudié.

Dans les cellules MDCK, l'activation des isoformes de la protéine kinase C sensibles au PMA et l'activation des purinocepteurs-P_2 inhibent l'activité des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i. Nous avons aussi observé l'inhibition du co-transporteur Na^+, K^+, Cl^- par le PMA dans des cultures primaires de tubules proximal et distal d'épithélium rénal de lapin que que l'activité de ce transporteur soit modulée par l'ATP dans ces cellules (Gagnon et al.1998). Ces résultats nous amènent à poser les questions suivantes :

1. Est-ce que l'inhibition purinergique des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i dans l'épithélium rénal est spécifique à l'espèce et limitée aux cellules d'origine canine?
2. Est-ce que l'inhibition purinergique des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i dans l'épithélium rénal est limitée aux cellules du tubule collecteur et plus particulièrement aux cellules intercalaires ou principales?
3. Quel est le type de récepteur-P_{2y} impliqué dans l'initiation du message?
4. Quels sont les mécanismes d'induction de l'inhibition purinergique des co-transporteurs Na^+, K^+, Cl^- et Na^+, P_i indépendants de la protéine kinase C (isoformes sensibles au PMA)?

L'activité de plusieurs transporteurs d'ions a été identifiée comme phénotype intermédiaire associé à l'hypertension artérielle (revue de la littérature, Orlov et al. 1998). Jusqu'à présent, aucune étude n'a permis d'identifier des mutations dans la région codante des gènes des transporteurs d'ions associés à l'hypertension primaire. Il est donc plus probable que l'augmentation de l'activité des transporteurs d'ions monovalents identifiée dans l'hypertension génétique soit secondaire à des anomalies des voies de signalisation impliquées dans leur expression et/ou dans leur fonctionnement. La poursuite de la caractérisation de la régulation hormonale et des voies intracellulaires impliquées dans la régulation des transporteurs membranaires est donc justifiée.

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ANNEXE I

CURRICULUM VITÆ