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Cloning and regulation of a renal mesangial cells sodium:calcium exchanger isoform.

Ianthalatres Williams
University of Alabama at Birmingham

CLONING AND REGULATION OF A RENAL MESANGIAL CELL SODIUM:CALCIUM EXCHANGER ISOFORM

by

IANTHALATRES WILLIAMS

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ABSTRACT OF DISSERTATION
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Name of Candidate Ianthalatres Williams

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The renal Na^+:Ca^{2+} exchanger (NCX) plays an important role in regulation of cytosolic calcium concentration $[Ca^{2+}]_i$ in contractile cells such as vascular smooth muscle and mesangial cells (MCs). NCX regulation of $[Ca^{2+}]_i$ is accomplished by exchanging one cytosolic Ca^{2+} ion for three extracellular Na^+ ions. Previously, we cloned, sequenced, and expressed MC exchangers from Dahl/Rapp salt resistant (R; RNCX1) and salt sensitive (S; SNCX1) rats. RNCX1 (alternative splice site encoded by B, D; isoleucine at amino acid 218), but not SNCX1 (B, D, and F; phenylalanine at 218), exhibited up-regulation following agonist-induced protein kinase C (PKC) activation. Using RT-PCR, we initiated studies to identify NCX1 isoform(s) from cultured MCs of the parental Sprague Dawley (SD) rat strain. We identified a novel isoform, denoted SDNCX1.10, that contains isoleucine at 218 and exons B, D, E, and F at the alternative splice site. SDNCX1.10 was then stably expressed in opossum proximal tubule kidney (OK-PTH) cells where we were able to evaluate functional activity by assessing, in forward and reverse mode, exchange activity. OK-PTH cells expressing SDNCX1.10 reduced elevations in $[Ca^{2+}]_i$, induced by 1 mM ATP via purinergic receptors, back to baseline levels 5-fold faster than cells expressing vector alone. Down-regulation of PKC, following prolonged exposure (24 h) to PMA resulted in a 66% reduction in ATP-

induced increases in $[Ca^{2+}]_i$ in SDNCX1.10 transfected cells compared to cells transfected with vector alone. This attenuation suggests that SDNCX1.10 is a PKC-sensitive isoform. KB-R7943, an exchanger-specific inhibitor, attenuated the reverse activity of SDNCX 1.10 (by 50% versus vector-only cells) and stimulated the forward mode. Further studies were performed to investigate some of the regulatory properties of SDNCX1.10. We found that this isoform was regulated by $[Ca^{2+}]_i$ and was inhibited by decreases in intracellular pH (pH_i) and that the activity of this exchanger was not altered by protein kinases A (PKA) and G (PKG). We also compared the regulation of $[Ca^{2+}]_i$ between the RNCX1 and SNCX1 isoforms. SNCX1 was much less effective in lowering agonist induced increases in $[Ca^{2+}]_i$ back to baseline, suggesting that cells expressing SNCX1 may have an impaired ability to regulate $[Ca^{2+}]_i$. Thus, this finding may help explain the hypertension and renal failure that are a hallmark of salt-sensitive hypertension.

DEDICATION

This dissertation is dedicated to all who helped make it happen. To my mother, Alice Williams, and my children, who stood by me from day one until the very last day, never doubting that I would make it. To my husband, Rodney McIntosh, for his undying support, love, and patience. And last, but certainly not least, to God, who carried me through.

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LIST OF ABBREVIATIONS

AA	afferent arteriole
AM	acetoxymethyl
ATP	adenosine triphosphate
BSA	bovine serum albumin
dNTP	2'-dideoxynucleoside-5'- triphosphate
IgG	immunoglobulin G
IgM	immunoglobulin M
IP_3	1,4,5-triphosphate
$[Ca^{2+}]_i$	cytosolic Ca^{2+} concentration
MC	mesangial cell(s)
$[Na^+]_i$	intracellular Na^+ concentration
Na_e	bath sodium
NCX	Na^+:Ca^{2+} exchanger
PBS	phosphate buffered saline
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PMA	phorbol 12-myristate 13-acetate
R	Dahl/Rapp salt-resistant rat

Rnase	ribonuclease
RNCX	Dahl/Rapp salt-resistant rat Na^+:Ca^{2+} exchanger
ROK	OK-PTH cells expressing the Dahl/Rapp salt-resistant Na^+:Ca^{2+} exchanger
S	Dahl/Rapp salt-sensitive rat
SD	Sprague Dawley rat
SDNCX	Sprague Dawley rat Na^+:Ca^{2+} exchanger
SNCX	Dahl/Rapp salt-sensitive rat Na^+:Ca^{2+} exchanger
SOK	OK-PTH cells expressing theDahl/Rapp salt-sensitive Na^+:Ca^{2+} exchanger
TMD	transmembrane spanning domain
XIP	inhibitory peptide

INTRODUCTION

The following dissertation encompasses three major objectives: first, clone a Na^+:Ca^{2+} exchanger (NCX) from cultured mesangial cells (MCs) of the Sprague Dawley (SD) rat; second, establish a stable cell line expressing this isoform of NCX; and third, provide evidence that this exchanger is functional and also elucidate some of the regulatory properties of this NCX.

NCX is a plasma membrane transport protein that plays an important role in the regulation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in a variety of cell types, including most contractile cells (1-9). NCX is electrogenic, transporting three Na^+ ions across the cell membrane for every one Ca^{2+} ion transported in the opposite direction. Gradients for Na^+ and Ca^{2+}; i.e. intracellular and extracellular concentrations, as well as membrane potential, determine direction of NCX transport of Na^+ and Ca^{2+} as well as the magnitude of transport (10). The normal gradients for Na^+ and Ca^{2+} across the cell are Na^+ = 140 $mM_{extracellular}$ and 10 $mM_{intracellular}$ and Ca^{2+} = 1.5 $mM_{extracellular}$ versus 100 $nM_{intracellular}$. Thus, NCX normally serves as a Na^+ influx and a Ca^{2+} efflux pathway. It is this function of NCX (as a Ca^{2+} efflux pathway) that normally defines its role in the regulation of $[Ca^{2+}]_i$. Various isoforms of NCX can be found in most cells throughout the body, including resistance vessels such as afferent arterioles (AA) and contractile cells of the kidney such as the MCs. Recently, considerable interest has been expressed in NCX's ability to regulate $[Ca^{2+}]_i$ and its potential involvement in the pathogenesis of certain disease processes, including hypertension (11-14).

Mesangial cells are located within each glomerulus of the kidney and subserve the important role of forming the structural support for the juxtaglomerular apparatus. However, these cells are multifunctional and exhibit contractile properties, as well as phagocytic-immunological functions. Like all cells, MCs possess multiple pathways for the entry and exit of Ca^{2+}. At the plasma membrane, there are ligand-gated and voltage-gated channels, as well as membrane transporters including the Ca^{2+}-ATPase and NCX. Within the cytosol there are Ca^{2+}-binding sites, as well as Ca^{2+}-storage sites, including the endoplasmic reticulum, which possesses both a Ca^{2+}-ATPase and an 1,4,5-triphosphate (IP_3) receptor. Because of the contractile properties of MCs, it has been proposed that these cells play a role in controlling glomerular filtration rate. Specifically, it has been suggested that contraction/relaxation of these cells may alter the glomerular ultrafiltration coefficient, which is one of the Starling forces that determines the rate of glomerular filtration. The inability of MCs to reduce increases in $[Ca^{2+}]_i$ could cause an increase in vascular tone, which would then cause a decrease in glomerular filtration rate. However, the exact mechanism by which these cells alter the ultrafiltration coefficient and, hence, glomerular filtration rate has not been determined with certainty. Nevertheless, regulation of $[Ca^{2+}]_i$ in MCs is important in the regulation of filtration and in the control of glomerular hemodynamics. Because renal function and glomerular hemodynamics play such an essential role in controlling blood pressure, it is possible that dysregulation of NCX may, therefore, contribute to certain forms of hypertension and renal disease (11-14).

Four genes are known to encode NCXand are denoted NCX 1-4 (14). NCX1, the most abundant and widely distributed gene, has been identified in almost all tissues,

including heart, brain, and kidney (15-17). NCXs 2 and 3 have been localized to the brain and skeletal muscle and share 70% homology to NCX1 at the amino acid level (15,16). Although very little is known about NCX4 (also denoted NCKX), it is a member of a closely related family, the Na^+:Ca^{2+}:K^+ exchanger. The Na^+:Ca^{2+}:K^+ exchanger was cloned from bovine rod outer segment in 1992 and is K^+ obligatory (18).

NCX1 was first cloned from canine cardiac sarcolemma (17) and has since been cloned from a variety of tissues (15,16,19-23). The DNA sequence, elucidated from the canine cardiac NCX, encodes a 108 kDa protein with 970 amino acids. NCX1 was predicted to consist of 12 exons encoding for an N terminus with five transmembrane domains (TMDs), a C terminus with six TMDs, and a large cytoplasmic loop domain between TMDs 5 and 6. However, Iwamoto *et al.* (24), using cysteine susceptibility and epitope tagging, recently reported that NCX1 consists of only 9 TMDs instead of 11. In Iwamoto *et al.*'s (24) model, there are five TMDs at the N terminus and four at the C terminus with a large cytoplasmic domain connecting the two termini (Fig. 1). Of special interest is a small region within the cytoplasmic domain near the C terminus that arises as the result of alternative splicing. This region is encoded by six exons denoted, A-F (Table 1). Alternative splicing of these six exons allows for different isoforms of NCX 1 (25-28). There are currently 12 isoforms of NCX1. Recent studies on various isoforms of NCX1 cloned from heart, brain, and *Drosphila melanogaster* suggest that the exchanger may have tissue specific characteristics and regulation (14,26). Before we successfully cloned exchangers from MCs, clones of three renal isoforms of NCX1 (NCX1.2, 1.3, and 1.7), each consisting of exon B and various combinations of exons C-F, had been reported (26-28). Although all three clones were isolated from renal tissues, their exact cellular origin remains unknown.

Extensive studies involving functional regulation of NCX1 have been done using the cardiac isoform(s). These studies have investigated the effects of agents such as Na^+, $[Ca^{2+}]_i$, ATP, intracellular pH, protein kinases, and vasoconstrictive hormones on the activity of NCX1 (2,10,29-32). However, little is known about regulators of the renal isoforms of NCX1. Renal NCX1, like the cardiac NCX isoform, may also be regulated by intracellular Na^+; Ca^{2+}; ATP; intracellular pH; PKA, PKC, and PKG, and hormones such as parathyroid hormone (PTH) and angiotensin II (Ang II) (33-37). Mene *et al.* (8) reported an Ang II-dependent increase in NCX1 activity in cultured human mesangial cells following stimulation of phospholipase C, an upstream mediator of PKC activation. However, an inhibitory effect on exchange activity was also noted for the same cells following acute treatment with either phorbol 12-myristate-13-acetate (PMA; also an activator of PKC) alone or in combination with an increase in $[Ca^{2+}]_i$ (8). Our laboratory demonstrated an increase in NCX1 activity in AAs of rabbit and rat, as well as in MCs from rats following acute PMA treatment (21,23,38). We also demonstrated an increase in exchanger activity following treatment with Ang II in cultured MCs from rat kidney (data not published).

Cardiac NCX1 was found to undergo Na^+_i-dependent inactivation, a phenomenon that occurs as the result of increases in $[Na^+]_i$, which acts as a feedback inhibitor to decrease NCX1 activity. Initial studies of Na^+_i-dependent inactivation found that limited proteolysis of the cytoplasmic side of the exchanger functionally removed inactivation of NCX1 in its stimulated state (39), implicating the cytosolic loop of NCX1 as the region involved in this regulation. Later, Hilgemann *et al.*(39) demonstrated Na^+_i-dependent inactivation by using patch clamping. When NCX1 was exposed to elevated Na^+, outward currents decreased exponentially. Na^+_i–dependent inactivation was also

influenced by intracellular pH, ATP, $[Ca^{2+}]_i$, and phosphotidylserine. Inactivation of NCX1 was augmented by cell acidification, whereas ATP, $[Ca^{2+}]_i$, and phosphotidyl - serine were all found to attenuate $[Na^+]_i$-dependent inactivation (39).

$[Ca^{2+}]_i$ has also been shown to regulate NCX 1 activity. Regulation by $[Ca^{2+}]_i$ is both a primary and a secondary process (40,41). Primary regulation by Ca^{2+} involves an initial increase in $[Ca^{2+}]_i$ that is then sensed by a Ca^{2+}-sensing region within the cytosolic loop (not yet identified) which initiates the exchange of Ca^{2+} for extracellular Na^+. In addition, with increases in $[Ca^{2+}]_i$, there is an increase in the affinity of the exchanger for the binding of Ca^{2+} at its regulatory binding site. This site is also located within the cytosolic loop and serves as a positive feedback signal to potentate NCX1 activity, thereby lowering $[Ca^{2+}]_i$ to baseline levels. This feedback process is called the secondary regulation of NCX1 by $[Ca^{2+}]_i$. Earlier studies suggested involvement of the cytosolic loop in Ca^{2+} regulation of the exchanger. This segment, which encompasses over half of the protein, is not essential in the Ca^{2+} transport function of this protein (42). Studies involving deletion of a 124 amino acid segment from the loop domain found that the deletion completely abolished all regulation by $[Ca^{2+}]_i$ (43). Recently, amino acids 371-508 have been identified and characterized as the Ca^{2+} regulatory binding site (42).

Another potential regulator of renal NCX1 is ATP. In a study using squid axons and cardiac myocytes, intracellular ATP caused an increase in NCX1 activity. ATP also increased the affinity of NCX1 for Ca^{2+} at its regulatory site and for Na^+ at its transport site (44-46). Dipolo and Beauge further reported that only in the presence of Mg·ATP was it possible for inorganic phosphate to stimulate NCX1 activity. ATP stimulation of NCX1 activity can also be inhibited by alkaline phosphatases, suggesting interplay between kinases and phosphatases in the regulation of exchanger activity (47). A number

of studies (10,44-46) have also provided evidence for a phosphorylation process involved in ATP stimulation of NCX1 activity in squid axons. These studies demonstrated strong stimulation of exchanger activity and increases in affinities for both Na^+ and Ca^{2+} at both transport and regulatory sites in response to ATP. In contrast, Berberian *et al.* (48) demonstrated that stimulation of NCX1 by Mg·ATP was not related to direct phosphorylation of the exchanger. Therefore, the exact mechanism of exchanger stimulation by ATP remains unclear.

The effect of intracellular pH on NCX1 activity has been recently examined (29,30). Cardiac NCX1 was found to be extremely sensitive to intracellular pH (pH_i). NCX1 activity is inhibited at pH 6 and stimulated at pH 9 (when compared to pH 7.4) (30). The sensitivity of NCX1 to pH_i (49) is evident when pH_i but not extracellular pH (pH_e) is lowered, resulting in reduced Ca^{2+} transport (30). Doering and Lederer and Philipson *et al.* (29,30) reported that protons inhibit NCX1 activity by competing with Ca^{2+} at intracellular Ca^{2+} transport sites. There is also speculation that the cytoplasmic loop domain may be a site of interaction for intracellular protons (29,30). There are also reports that Na^+_i binding to NCX1 increases the affinity of the exchanger for proton binding, which then inhibits the exchange cycle (31). Therefore, it is possible that pH_i effects are caused by direct interactions between protons and NCX1.

Besides the effects of ATP on NCX1 regulation, the effect of phosphorylation via various kinases on NCX1 regulation has been extensively studied. PKA, PKC and PKG may all serve as potential regulators of renal NCX1. There have been reports of increases in NCX1 activity caused by agents that activate kinases and increase phosphorylation of the exchanger (32,34,50-52). There also appears to be differences in the sensitivity of various NCX1 isoforms to various kinases. Because one or more of the NCX1 isoforms

can be expressed in a given tissue (15,16,26,53,54), kinases may give different responses because of the expression of different isoform(s) within tissue(s). In this regard, a limited amount of information suggests that differences in NCX regulation by kinases may be , in part, caused by the exons expressed at the alternative splice site. For instance, He *et al.* (34) reported that an astrocyte isoform containing exons B and D is insensitive to PKA, whereas a neuronal isoform with exons A and D was upregulated by this kinase. In contrast, Smith *et al.* (52) reported down-regulation of NCX1 activity in arterial myocytes because of PKA stimulation. Based on previous studies, which demonstrated marked differences in the response of NCX1 to PKA, more research in this area is needed.

Cyclic GMP (cGMP) an activator of PKG, was also found to stimulate NCX1 activity in vascular smooth muscle (vsm) cells (36). A 60% increase in Ca^{2+} uptake was demonstrated in smooth muscle cells receiving prior treatment with 8-bromo-cGMP, a cGMP analog (36). This finding may have important implications in the regulation of vascular resistance. Specifically, cGMP, in general, is a vasodilator and a mediator of nitric oxide-induced smooth muscle cell relaxation. Because NCX is a major Ca^{2+} efflux pathway, it is possible that at least part of the vasodilatory effect of nitric oxide may be caused by cGMP stimulation of the exchanger and a reduction in $[Ca^{2+}]_i$.

Other studies examining the involvement of phosphorylation of NCX1 suggest a potential role for PKC in regulation of exchanger function (51). Activators of PKC, as well as PKA, were reported by Linck *et al.* (33) to cause modest increases in exchanger activity following acute treatment with the phorbol ester, PMA, in baby hampster kidney (BHK) cells stably transfected with NCX1. In contrast, Smith *et al.* (52) reported a

decrease in NCX1 mRNA for kidney epithelial cells following 4 h of treatment with 10nM PMA. A decrease in mRNA was not seen, however, with 2 h of treatment.

A decrease in basal and agonist-stimulated NCX1 activity in cultured human renal MCs following PMA treatment has also been reported (8). Furthermore, it has been suggested that direct phosphorylation of the exchanger by PKC may not be required for stimulation of NCX1 activity in these cells (8). Iwamoto *et al.* (51) reported that, in primary cultures of rat aorta, NCX1 was phosphorylated and concomitantly activated in response to phorbol ester activation of PKC. Three serine residues (S249, S250, and S351) have been identified within the N-terminus of the cytoplasmic loop domain as sites for direct phosphorylation by PKC (51). The major PKC-dependent phosphorylation site appears to be serine 250, which may modulate the regulation of NCX1 by PKC. Furthermore, serine 249 can only be phosphorylated after serine 250 has been phosphorylated (51). Surprisingly, Iwamoto *et al.* demonstrated that PKC appears to be able to stimulate the activity of NCX1 even in mutants lacking S249, S250, and S351. These results suggest that direct phosphorylation of NCX1 by PKC may not be required for activation (51). Therefore, the exact mechanism(s) by which PKC stimulates certain isoforms of NCX 1 remains to be determined.

The use of functional assays, which measure changes in $[Ca^{2+}]_i$ in response to alterations in extracellular Na^+, established the presence of NCX1 in renal AAs of rabbit and rat kidney (21,23,55). NCX1 is also present in cultured MCs of Dalh/Rapp salt-resistant (R) and salt-sensitive (S) rats, as well as the parental strain, the SD rat (38,56). Its presence has also been confirmed in cultured human MCs using functional assays (8,57,58).

More recently, we have found PKC regulation of renal NCX1 to be different in isolated renal AAs from R and S rats. The S rat becomes hypertensive when fed a diet of 8% NaCl, whereas the R rat remains normotensive on a high-salt diet. Exchanger activity in the R rat arterioles appears to be stimulated by PMA, whereas exchanger activity in S rat arterioles shows no change in response to PMA treatment (38,56,59). We further found that PKC, either directly or indirectly, stimulates exchanger translocation from a cytosolic pool to the plasma membrane in MC from R rats. This translocation process appears to be defective in MCs from S rats. These findings are of great interest to us because the R isoform contains exons B and D, whereas the S isoform has exons B, D and F. The regulatory differences seen in the AAs and MCs of R and S rats following treatment with PMA could be caused by specific exons expressed at the alternative splice site in renal isoforms of NCX1.

Various combinations of six exons (A-F) encode for the alternative splice site of NCX1. Quednau *et al.* (54) used RT-PCR to examine eight previously reported NCX1 isoforms and four novel isoforms. Exons A and B were found to be mutually exclusive. Isoforms containing exon A are preferentially expressed in the heart, brain, and skeletal muscle. All other tissues, including kidney, contained isoforms with exon B (54). The presence of an isoform containing exon A has been reported to be present in distal convoluted tubule cells of mice (60); however, this isoform was not proven to be functional. Also, an isoform containing B was reported to be present in heart cells of mice (61). In a study using ribonuclease (RNase) protection assays, as well as RT-PCR, NCXs 1.1, 1.2, and 1.3 were compared (26). The three isoforms were found to be highly homologous at the amino acid level except in the C terminus of the intracellular loop (26). In addition, Lee *et al.* (15) and Lytton *et al.* (28) were able to independently

analyze a number of NCX1 isoforms using RT-PCR and rapid amplification of cDNA 5'-ends (5'RACE), finding that the transcripts from all isoforms contained a common NCX1 core and different 5' ends that corresponded to a region within the intracellular loop that undergoes alternative splicing (15,28). These findings suggest that the expression of various NCX1 isoforms may be controlled and regulated by different promoters within the gene, also allowing for tissue specificity and differential regulation. In more recent reports, alternative splicing of NCX 1 has been shown to affect regulation of the exchanger. Omelchenko *et al.* (35) reported that in *Drosophila*, two isoforms (CALX1.1 and CALX1.2), which are analogs to the mammalian NCX1 and differ by only five amino acids at the alternative splice site, were regulated quite differently by intracellular Na^+ and Ca^{2+}. Both CALX 1.1 and 1.2 undergo Na^+_i-dependent inactivation; however, CALX1.2 has an enhanced inactivation when compared to CALX1.1 (35). Surprisingly, both isoforms were downregulated by Ca^{2+}, which is in contrast to the mammalian NCX1. CALX1.2, which has a higher affinity for Ca^{2+} than CALX1.1, exhibited less inhibition to Ca^{2+} when the two isoforms were compared (35).

The maintenance of $[Ca^{2+}]_i$ homeostasis is critically important for contractile cells such as MCs. The NCX helps to maintain and regulate $[Ca^{2+}]_i$ in these contractile cells. Dysregulation of NCX1, therefore, can lead to various pathological conditions including salt-sensitive hypertension. To understand the role that NCX1 plays in salt-sensitive hypertension we need to better understand the regulation of the renal NCX1. The following studies allowed us to examine the presence, function, and effects of potential regulators on a renal NCX1 isoform isolated and cloned from MCs of the SD rat, denoted Sprague Dawley rat sodium:calcium exchanger (SDNCX)1.10. SDNCX1.10 contains exons B, D, E, and F and is 962 amino acids long. Previously, our laboratory

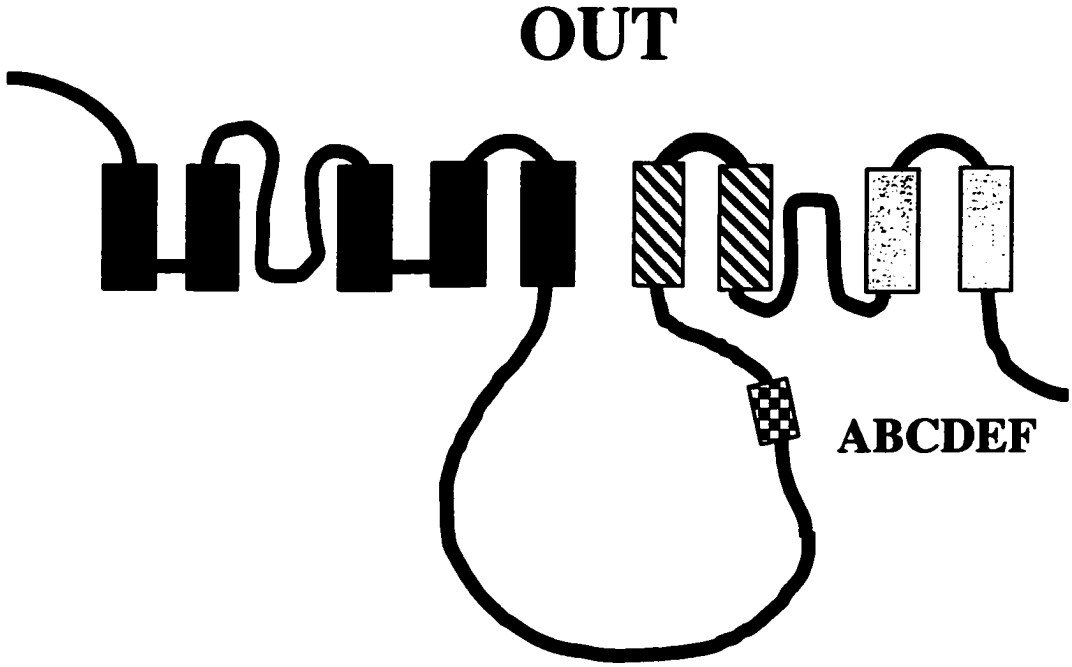
cloned and sequenced two other isoforms of renal NCX1 (RNCX1 and SNCX1), from MCs of R and S rats. RNCX1 contained exons B and D, whereas SNCX1 contained exons B, D, and F. RNCX1 and SNCX1 are 934 and 957 amino acids long, respectively. SDNCX1 and RNCX1 also have an isoleucine at amino acid residue 218, whereas SNCX1 has a phenylalanine. Functional studies were performed to compare the regulation of these three isoforms. RNCX1 and SNCX1 were cloned from the MCs of Dahl/Rapp R and S rats, respectively. R and S rats were derived from the SD rats. Therefore, we wanted to answer these questions: What isoforms of NCX are present in SD rat MCs and are they differentially regulated? Our results revealed that there were four isoforms of NCX present in MCs of SD rats and that there are differences at the amino acid level and in functional regulation between renal NCX isoforms. Therefore, we hypothesized that there are regulational differences in renal NCX isoforms RNCX1, SNCX1, and SDNCX1.10 and that these differences affect their abilities to regulate $[Ca^{2+}]_i$.

Table 1. The amino acid composition of exons expressed in the region of the cytosolic loop that undergo alternative splicing

Exon letter	Amino acid composition
A	KTISVKVIDDEEYEKNKTFFLEIGEPRLVEMSEKK
B	KIITIRIFDREEYEKECSFSLVLEEPKWIRRGMK
C	ALLLNEL
D	GGFT(I/L)T
E	GKKM(Y/V)
F	GQPVLRKVHAR(E/D)HPI(L/P)STVI(T/S)IS

Note: Parentheses represent degenerative amino acids.

Figure 1. A general topological model of NCX1 showing 12 exons that encode NCX 1. Exon 1 and the first 1/3 of exon 2 encode the 5' untranslated region. The remaining 2/3 of exon 2 encode the N terminus, including transmembrane domains (TMDs) 1-5 and most of the large cytoplasmic loop. The remainder of the cytoplasmic loop is encoded by exons 3-10. More specifically, exons 4-9, denoted A-F, code for the region of the cytoplasmic loop known to undergo alternative splicing. Exon 11 codes for TMDs 6 and 7 and their loop segments, whereas the first half of exon 12 codes for TMDs 8 and 9 and their loop segments. The first 1/2 of exon 12 also codes for a stretch of amino acids on the cytoplasmic face of the membrane between TMDs 7 and 8. The remaining 1/2 of exon 12 codes for the 3' untranslated region.



CLONING AND EXPRESSION OF A MESANGIAL CELL Na^+:Ca^{2+} EXCHANGER FROM SPRAGUE DAWLEY RATS

by

I. WILLIAMS MCINTOSH, E. HWANG, B. SIROKY, A. WESTBROOK, K. COGDELL, M. MOORE, G. KOVACS, T. UNLAP, AND P. D. BELL

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ABSTRACT

The Na^+:Ca^{2+} exchanger (NCX) is a major regulator of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in renal contractile cells such as vascular smooth muscle and mesangial cells (MCs). It also regulates$[Ca^{2+}]_i$ in afferent arterioles (AAs) of the kidney. Previously, we have cloned, sequenced, and expressed MC exchangers from Dahl/Rapp salt-sensitive (SNCX) and salt-resistant (RNCX) rats. RNCX (alternative splice site encoded by BD; isoleucine at amino acid position 218) but not SNCX (BDF; phenylalanine at 218) was upregulated by agonist-induced protein kinase C (PKC) activation. We initiated studies using RT-PCR to identify, from cultured mesangial cells of the parental Sprague Dawley (SD) rat strain, exchanger isoform expression and regulation. Four exchanger isoforms have so far been identified, including a novel isoform designated SDNCX1.10. SDNCX1.10 contained isoleucine at amino acid 218 and BDEF at the alternative splice site. SDNCX1.10 was then stably transfected into OK-PTH cells and exchange activity, in both the reverse and forward modes, were assessed in cells expressing either the vector or SDNCX1.10. Upon elevations in $[Ca^{2+}]_i$ induced by 1 mM ATP via purinergic receptors to assess the forward mode, OK-PTH cells expressing SDNCX reduced $[Ca^{2+}]_i$ (n = 19) back to baseline levels over 7 fold greater than cells expressing the vector alone. The role of PKC in this process was assessed by pretreatment of cells with phorbol 12-myristate 13- acetate (PMA) for 24 h to down-regulate PKC activity. In response to ATP-induced increases in $[Ca^{2+}]_i$, the rate of decline in $[Ca^{2+}]_i$ was attenuated 66% (n = 19), suggesting that SDNCX1.10 is a PKC-sensitive isoform. $^{45}Ca^{2+}$ uptake studies to assess the reverse mode showed that cells expressing SDNCX1.10 demonstrated exchange activity, which was over 7 fold greater (n = 19) than that for cells expressing the vector alone. This exchange activity is

attenuated nearly 50% by PKC down-regulation and KB-R7943, a specific exchange inhibitor. These results suggest that activation of SD Na^+:Ca^{2+} exchangers, at least in terms of SDNCX1.10, is mediated through PKC.

INTRODUCTION

Na^+:Ca^{2+} exchange is primarily a plasma membrane Ca^{2+}-extrusion pathway that plays an essential role in the regulation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (1-5). Three distinct exchanger proteins have been identified and are denoted Na^+:Ca^{2+} exchanger (NCX) 1 (6), NCX2 (7), and NCX3 (8); these proteins exhibit ~70% amino acid sequence homology and are encoded by three distinct genes (8). NCX1 is the most abundant and is widely distributed in a variety of tissues, including heart, brain, and kidney (9-12). NCX2 and 3 have been localized to the brain and skeletal muscle. NCX1 is a 108 kDa protein, which consists of 970 amino acids and is encoded by 12 exons (6). NCX1 consists of nine transmembrane domains (TMDs); five in the N terminus, four in the C terminus, and a large cytosolic loop that comprises over 50% of the protein (13).

A small region near the C terminus of the cytoplasmic domain undergoes alternative splicing of six exons, denoted A-F, which gives rise to at least 12 NCX1 splice variants (9-12). NCX1 splice variants that contain exon A and various combinations of exons C-F are preferentially expressed in heart, brain, and skeletal muscle, whereas those expressing exon B and various combination of exons C-F are preferentially expressed in kidney. The significance of this alternative splice site to the functional activity of the Na^+/Ca^{2+} exchanger is not well understood.

In previous work we have characterized Na^+:Ca^{2+} exchange activity in afferent arterioles (AAs) and cultured mesangial cells (MCs) from Dahl/Rapp salt-sensitive S

(hypertensive) and salt-resistant R (normotensive) rats. We found that protein kinase C (PKC) which has been shown to activate Na^+:Ca^{2+} exchange in certain tissues (14-17), up-regulated exchanger activity in cells from R but not S rats (4). Failure of PKC to regulate exchanger activity from the S rat may have implications concerning the ability of this renal isoform to regulate $[Ca^{2+}]_i$. We then cloned and sequenced the Na^+:Ca^{2+} exchanger from MCs of Dahl/Rapp S (SNCX) and R (RNCX) rats (18). The two isoforms are 100% homologous at the C terminus and differ in the N terminus at amino acid residue 218, where the amino acid is isoleucine in RNCX but is phenylalanine in SNCX. RNCX and SNCX also differ at the alternative splice region of the cytoplasmic loop, where RNCX is encoded by exons B and D and SNCX is encoded by B, D, and F. When these two isoforms were expressed in opossum proximal tubule kidney (OK-PTH) cells, RNCX, but not SNCX, was activated by PKC (18). Thus, the results obtained with cloned exchangers were the same as those characteristics of the exchangers obtained in native MCs and renal arterioles from R and S rats (4,5).

It should be noted that both the S and R rats were derived from Sprague Dawley (SD) rats (19), so there remains a question regarding what renal MC isoform(s) of NCX1 are expressed in the parental rat and whether this isoform is sensitive to PKC. Therefore, the purpose of the present studies was to clone Na^+:Ca^{2+} exchanger isoforms from MCs of the SD rat and to functionally characterize the regulation of one of these exchanger isoforms.

METHODS

Isolation and Culture of Glomerular Mesangial Cells

Kidneys from SD rats were aseptically removed, and glomerular MCs were isolated and cultured as previously described (20). In brief, renal cortical tissue was minced with a razor blade and passed through a no. 70 copper sieve (Fisher Scientific). Tissue was then passed through progressively smaller nylon sieves (Tetko) ranging in size from 315 μm to 75 μm to separate glomeruli from the remaining kidney tissue. Glomeruli were then treated with collagenase (Sigma) and plated onto 60 mm x 15 mm petri dishes (Costar). Cells were grown in RPMI 1640 media (Gibco) supplemented with 20% fetal bovine serum (Intergen), 240 μg/ml L-glutamine (Gibco), 82 units/ml penicillin, 82 μg/ml streptomycin (Sigma), and 2 μg/μl amphotericin B (Gibco). Cells were grown for 21 days in a humidified (95% air/5% CO_2) incubator at 37°C. Previous studies identified these cells as MCs; i.e. electron microscopy demonstrated prominent microfilaments, dense bodies, well-developed rough endoplasmic reticulum, gap junctions and attachment plaques. Vimentin, a cytoskeletal filament, was also found in these cells (20,21). Media was changed twice a week. Cells were subcultured and grown to confluency.

Reverse Transciptase and Polymerase Chain Reaction

mRNA was isolated from primary cultures of SD MCs, and 100 ng was used along with four sets of primers designed to amplify cDNAs corresponding to the N-terminal TMD (5'-gagaggatccgattgttcctttagaagcc-3'/5'-gaggagaattctgca acccaagcgaacaca-3'), the cytoplasmic domain (5'-gagaggatccgacaggcggcttctctttt-3'/5'-gaggagaattcgggcagct tctcctcccc-3'), the C-terminal TMD (5'-gagagggatcctcctgttttgattacgtga-3'/5'-gaggagaattcg

aggagaagaaatgtaca-3'), and the entire coding region (5'-gagagggatccaggttgaacaattggaagt-3'/5'-gaggagaattcgattgttcctttagaagcc-3') of the NCX. RT-PCR was carried out by using Titan One-Tube RT-PCR (Boehringer Mannheim) according to manufacturer's instructions. Using a minicycler (MJ Research), reverse transcription was carried out at 50°C for 35 min, 94°C for 2 min, and 10 cycles at 94°C for 30 s, 45°C for 30 s, and 68°C for 45 s. The PCR consisted of 40 cycles at 94°C for 30 s, 45°C for 30 s, and 68°C for 45 s, followed by a long extension time of 7 min at 68°C. To eliminate PCR generated mutations in the cDNAs, two different mRNA preparations were used for each PCR reaction. Ten microliters of each PCR product was fractionated on a 1% Tris acetate EDTA gel, and the proper DNA band was excised and gel purified using Gene Clean (Amersham). A 2 µl aliquot of the purified PCR fragment was ligated into pCRII-TOPO (for sequencing) or pCDNA3.1/V5-His-TOPO (for functional analysis) by using the TOPO TA Cloning Kit (Invitrogen). The entire ligation mix was used to transform Top 10 competent cells (Invitrogen) that were subsequently screened, and plasmids were isolated. Twelve plasmids representing cDNAs generated from two different mRNA preparations from MCs of SD rats were sequenced on an ABI Sequencer. The DANASIS program was used for sequence alignments and manipulations.

Transfection of OK-PTH Cells with SDNCX1.10

OK-PTH cells were transfected with either pCDNA3.1/V5-His-TOPO (Invitrogen) or pCDNA3.1-SDCX1.10 cDNA using Lipofectin (BRL) according to manufacturer's instructions and selected for transfectants using Geneticin at 500 µg/ml for 3 weeks. After 3 weeks, transfected cells were incubated in the presence of 500 µM of Ca^{2+} and 20 µM of ionomycin for 30 min, washed, and resuspended in complete

media. This maneuver stimulates a significant rise in $[Ca^{2+}]_i$, and only cells with functional exchangers will be able to lower $[Ca^{2+}]_i$ sufficiently to survive (Ca^{2+} killing) (22). This process was repeated every 3 days to enrich the population of OK-PTH cells that express functional NCXs.

Forward-mode exchanger activity measurements of $[Ca^{2+}]_i$

OK-PTH cells expressing either the vector or SDNCX1.10 were grown to 50% confluency in 100 mm dishes and treated with sodium butyrate (5 mM) to induce the expression of the NCX. Cells were harvested in 4 ml of 0.1% trypsin-EDTA for 1 min, resuspended in complete media, and loaded with 10 μM fura-2 in 10 ml of complete culture media for 1 hr. Fura-2 loaded cells were centrifuged at 700 x g for 10 min, resuspended in 5 ml of 150 Ringer's solution (148 mM NaCl, 5 mM KCl, 1 $MgSO_4$, 1.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES) and centrifuged as before. The final pellet was resuspended in 3 ml of 150 Ringer's solution and transferred to a quartz cuvette containing a magnetic stir bar. $[Ca^{2+}]_i$ measurements were performed by using dual excitation wavelength fluorescence microscopy (Photon Technologies International) while keeping the cells in suspension by constant agitation. Background correction was made in the absence of cells but in the presence of Ringer's solution. Baseline fura 2 ratios were measured with cells bathed in 150 Ringer's solution for at least 100 s, and experiments were then performed only if the ratio remained stable. Next, 1 mM ATP was added to the cuvette to elevate $[Ca^{2+}]_i$, and fura 2 ratio was monitored continuously before, during, and after the addition of ATP until the ratio returned to stable value. All solutions were adjusted to a pH of 7.4 and

temperature was maintained at 37°C. There was no evidence of leakage of fura 2 throughout the experiment.

The effect of PKC on exchanger activity was assessed by treating cells with 300 nM phorbol 12-myristate 13-acetate (PMA) for 24 h to down-regulate PKC, prior to the measurement of $[Ca^{2+}]_i$. In addition, the effect of the exchanger inhibitor, KB-R7943 (Kanebo, Japan), on the activity of SDNCX1.10 was also assessed by treating OK-PTH cells expressing either vector or SDNCX1.10 with 30 μM of KB-R7943 for 3 min prior to the addition of 1 mM ATP to elevate $[Ca^{2+}]_i$.

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

Calibrations were performed to convert Fura 2 ratios into $[Ca^{2+}]_i$ values. $[Ca^{2+}]_i$ was calculated by the equation described by Grynkiewicz *et al.* (23).

$$[Ca^{2+}]_i = Kd\ (\ (Sf2/Sb2)\ (\ (R-Rmin)/(Rmax-R)$$

where Kd is the effective dissociation constant of fura 2 and has a value of 224 nM; R is the fluorescence ratio obtained at 340 nm/380 nm, Rmin and Rmax are the ratios in absence and presence of Ca^{2+} respectively, and Sf2 and Sb2 are the emissions at 380 nm in the absence and presence of Ca^{2+}, respectively. Cells were loaded with 10 μM fura 2AM for 1 h, followed by resuspension in 150 Ringer's solution. The calibration was accomplished after permeabilizing the cells with 5 μM ionomycin and measuring fluorescence at both wavelengths, 340 and 380 nm, under Ca^{2+}-free (zero $[Ca^{2+}]_i$ and 2 mM EGTA) or Ca^{2+}-containing solutions (2 mM Ca^{2+}) to obtain Rmin, Rmax, Sf2 , and Sb2.

Reverse-mode exchanger activity: measurements of $^{45}Ca^{2+}$ influx

OK-PTH cells expressing SDNCX1.10 exchanger were assayed for exchanger activity by $^{45}Ca^{2+}$-uptake studies as follows. Cells were grown in 24 well plates to 80% confluency, washed twice with cold PBS and overlaid with 0.25 ml of 140 mM NaCl, 10 mM Tris-HCl (pH 7.4), 400 μM ouabain, and 10 μM monensin for 20 min to preload the cells with NaCl at room temperature. The loading solution was removed by aspiration and replaced with either 0.25 ml of 140 mM KCl or 140 mM NaCl and 10 mM Tris-HCl, pH 7.4 and 5 μM $^{45}CaCl_2$/ml for 1 min at room temperature. For selected samples, the exchanger inhibitor KB-R7943 (30 μM, 20 min) was added for the 20 min Na^+-loading period. For PKC down-regulation, the cells were treated with PMA (300 nm) for 24 h prior to the uptake studies. The $^{45}Ca^{2+}$-uptake solution was removed by aspiration and cells were washed three times, each time with 3 ml of cold 140 mM KCl. Cells were resuspended in 0.5 ml of 1 N NaOH and transferred to microcentrifuge tubes. Aliquots were used for liquid scintillation counting (0.25 ml) and Bradford protein assay (0.05 ml). The results are presented as percentage of control after exchange activity was calculated as nmoles $^{45}Ca^{2+}$/mg protein/min for cells in KCl solution minus that for cells in NaCl solution. The effect of each treatment was assessed for significance by using ANOVA.

RESULTS

Reverse transciptase and polymerase chain reaction

Two primary cultures of MCs from SD rats were used to isolate mRNAs. This was done to minimize PCR-generated errors. Using primers designed from the sequences

of renal isoforms of the NCX in RT-PCR reactions, fragments of approximately 0.7, 0.6, and 1.5 kb corresponding to the N-terminal and C-terminal membrane-spanning domains and the cytosolic loop, respectively, were generated. A 2.8 kb fragment that corresponds to the entire coding region of the exchanger was also obtained.

Nucleotide and amino acid sequence

Twelve clones generated from MCs of SD rats consisting of the entire coding region of the NCX were selected and sequenced in both directions. Nucleotide sequences indicated that the 12 clones consisted of four different NCX isoforms. These four isoforms were identical at the N- and C-terminal membrane-spanning domains. At amino acid residue 218, all four isoforms contained isoleucine. Thus, the phenylalanine at position 218 in SNCX is unique to the S MC NCX. The four isoforms were identical at most of the cytoplasmic domain, with the exception of the variable region that arises through alternative splicing. This site was encoded by exons B and D in four clones; by B, D, and F in four other clones; by B, C, D, E, and F in two clones; and B, D, E, and F in the final two clones. The clone that was encoded by exons B and D at the alternative splice site is identical to RNCX, a MC NCX that was cloned from MCs of R rats (18). The clone encoded by exons B, D, and F at the alternative splice site is identical to SNCX cloned from S rats (18), except at amino acid 218. As indicated, amino acid residue 218 is isoleucine in the SD isoform and phenylalanine in SNCX. Isoforms expressing B, C, D, E, and F (designated SDNCX 1.11) and B, D, E, and F (designated SDNCX1.10) were not seen in MCs of either S or R rats. The nucleotide and deduced amino acid sequences of SDNCX1.10, which consists of 962 amino acids, are presented in Figure 1A. SDNCX1.10 displays the consensus NCX topology, i.e. nine TMDs (underscores), five in

the N terminus and four in the C terminus, and a cytoplasmic domain that comprises over 50% of the protein. According to the previously used NCX nomenclature that classifies exchanger isoforms based on the exons expressed at the alternative splice site (10), SDNCX is NCX1.10, an isoform that has been detected in adult rat skeletal muscle and astrocytes.

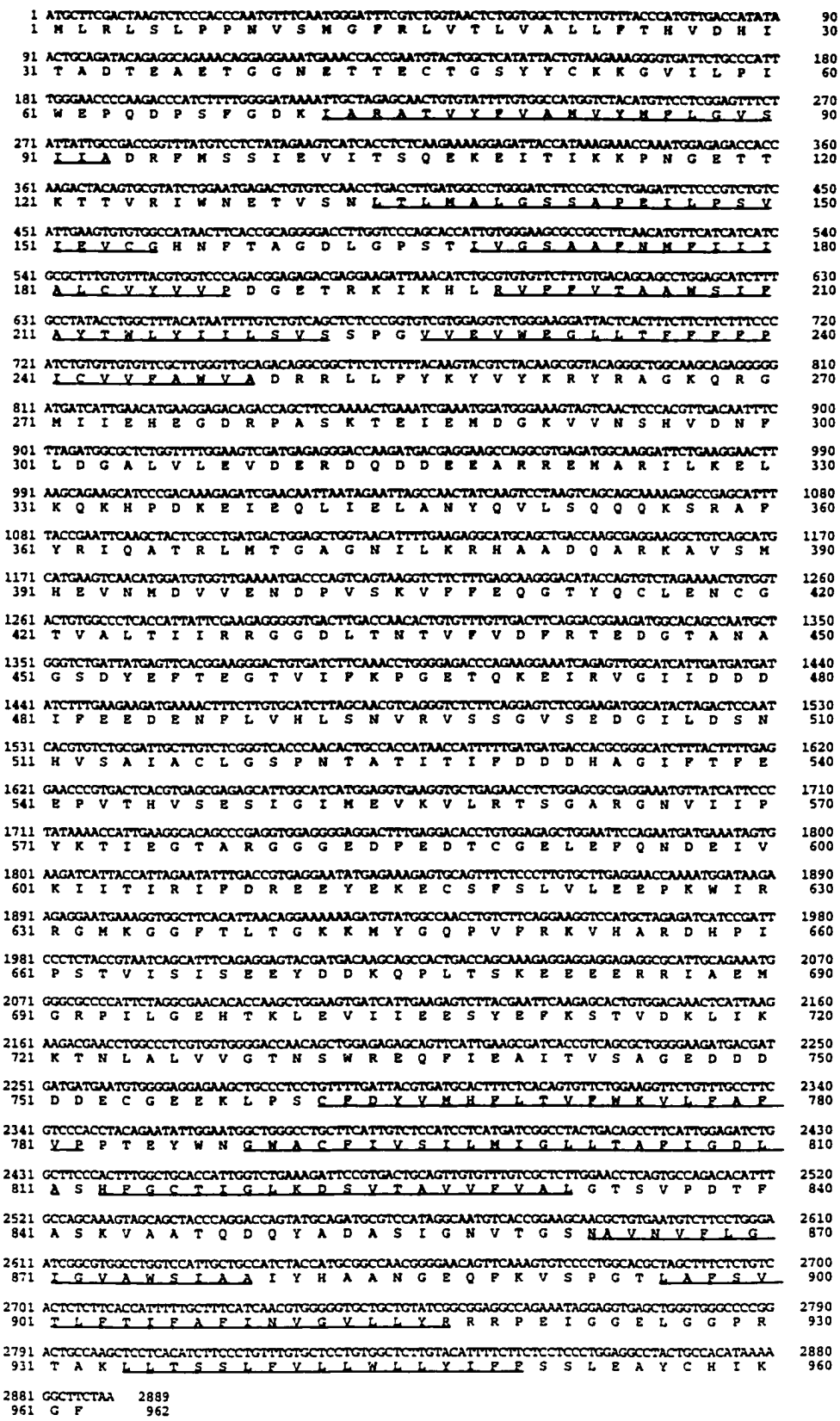
Functional assay of SDNCX

To determine whether SDNCX1.10 regulates $[Ca^{2+}]_i$, pCDNA3.1 and pCDNA3.1-SDNCX1.10 were transfected into a mammalian immortalized proximal tubule cell line (OK-PTH). Forward and reverse modes of exchanger activity were assessed by using fura 2-cytosolic Ca^{2+} measurements and a $^{45}Ca^{2+}$-uptake assay, respectively. These protocols are shown in Figure 2. The forward mode mimics the normal operation of the exchanger and is used to determine the ability of the exchanger to reduce $[Ca^{2+}]_i$. On the other hand, the reverse mode assay relies on prior cell Na^+ loading, and then removal of external Na^+, which then results in Na^+ exit and Ca^{2+} entry via the exchanger. By assessing the reverse mode, we were able to directly determine the effect of regulators on exchanger activity.

Using fura 2 we first measured nonstimulated baseline $[Ca^{2+}]_i$ in cells transfected with the vector alone and OK-PTH cells transfected with the exchanger. Baseline $[Ca^{2+}]_i$ averaged 133 ± 8 ($n = 19$) in control cells and 35 ± 8 ($n = 19$) in cells transfected with SDNCX1.10. Forward- and reverse-mode studies were then performed, and the results are shown in Figure 3A-E and Figure 4. In the forward-mode studies (Fig. 3A), ATP was added to increase $[Ca^{2+}]_i$ through purinergic receptors. The ability of OK-PTH cells to return $[Ca^{2+}]_i$ to control levels was then compared in cells expressing either the

Figure 1. Nucleotide sequence of a NCX cDNA isolated from MCs of SD rats. The cDNA was generated from poly A RNA isolated from primary mesangial cell cultures from Sprague Dawley rats and sequenced on an ABI Sequencer. The nucleotide sequence of the cDNA, designated SDNCX1, with the deduced amino acid sequence are shown (**A**). The numbering begins with the initiation site (ATG) and ends with the termination site (TAA). Underscores represent hydrophobic TMDs, five in the N terminus and six in the C terminus. Alignment of the amino acid sequences of SDNCX1.10, SNCX1, and RNCX1 (**B**) showing amino acid 218 where RNCX1 (bottom) and SDNCX1.10 (top) contain isoleucine but SNCX1 (middle) contains phenylalanine (box). Dotted lines represent the region of the exchanger which arises through alternative splicing of six exons and represents the absence of exon E in SNCX1 and exons E and F in RNCX1. This sequence has been deposited at GenBank under accession no.AY033398.

A



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B

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SDNXC1.10   MLRLSLPPNV SMGFRLVTLV ALLFTHVDHI TADTEAETGG NETTECTGSY YCKKGVILPI  60
SNCX        MLRLSLPPNV SMGFRLVTLV ALLFTHVDHI TADTEAETGG NETTECTGSY YCKKGVILPI  60
RNCX        MLRLSLPPNV SMGFRLVTLV ALLFTHVDHI TADTEAETGG NETTECTGSY YCKKGVILPI  60

SDNXC1.10   WEPQDPSFGD KIARATVYFV AMVYMFLGVS IIADRFMSSI EVITSQEKEI TIKKPNGETT 120
SNCX        WEPQDPSFGD KIARATVYFV AMVYMFLGVS IIADRFMSSI EVITSQEKEI TIKKPNGETT 120
RNCX        WEPQDPSFGD KIARATVYFV AMVYMFLGVS IIADRFMSSI EVITSQEKEI TIKKPNGETT 120

SDNXC1.10   KTTVRIWNET VSNLTLMALG SSAPEILPSV IEVCGHNFTA GDLGPSTIVG SAAFNMFIII 180
SNCX        KTTVRIWNET VSNLTLMALG SSAPEILPSV IEVCGHNFTA GDLGPSTIVG SAAFNMFIII 180
RNCX        KTTVRIWNET VSNLTLMALG SSAPEILPSV IEVCGHNFTA GDLGPSTIVG SAAFNMFIII 180

SDNXC1.10   ALCVYVVPDG ETRKIKHLRV FFVTAAWSIF AYTWLYIILS VSSPGVVEVW EGLLTFFFFP 240
SNCX        ALCVYVVPDG ETRKIKHLRV FFVTAAWSIF AYTWLYIILS VSSPGVVEVW EGLLTFFFFP 240
RNCX        ALCVYVVPDG ETRKIKHLRV FFVTAAWSIF AYTWLYIILS VSSPGVVEVW EGLLTFFFFP 240

SDNXC1.10   ICVVFAWVAD RRLLFYKYVY KRYRAGKQRG MIIEHEGDRP ASKTEIEMDG KVVNSHVDNF 300
SNCX        ICVVFAWVAD RRLLFYKYVY KRYRAGKQRG MIIEHEGDRP ASKTEIEMDG KVVNSHVDNF 300
RNCX        ICVVFAWVAD RRLLFYKYVY KRYRAGKQRG MIIEHEGDRP ASKTEIEMDG KVVNSHVDNF 300

SDNXC1.10   LDGALVLEVD ERDQDDEEAR REMARILKEL KQKHPDKEIE QLIELANYQV LSQQQKSRAF 360
SNCX        LDGALVLEVD ERDQDDEEAR REMARILKEL KQKHPDKEIE QLIELANYQV LSQQQKSRAF 360
RNCX        LDGALVLEVD ERDQDDEEAR REMARILKEL KQKHPDKEIE QLIELANYQV LSQQQKSRAF 360

SDNXC1.10   YRIQATRLMT GAGNILKRHA ADQARKAVSM HEVNMDVVEN DPVSKVFFEQ GTYQCLENCG 420
SNCX        YRIQATRLMT GAGNILKRHA ADQARKAVSM HEVNMDVVEN DPVSKVFFEQ GTYQCLENCG 420
RNCX        YRIQATRLMT GAGNILKRHA ADQARKAVSM HEVNMDVVEN DPVSKVFFEQ GTYQCLENCG 420

SDNXC1.10   TVALTIIRRG GDLTNTVFVD FRTEDGTANA GSDYEFTEGT VIFKPGETQK EIRVGIIDDD 480
SNCX        TVALTIIRRG GDLTNTVFVD FRTEDGTANA GSDYEFTEGT VIFKPGETQK EIRVGIIDDD 480
RNCX        TVALTIIRRG GDLTNTVFVD FRTEDGTANA GSDYEFTEGT VIFKPGETQK EIRVGIIDDD 480

SDNXC1.10   IFEEDENFLV HLSNVRVSSG VSEDGILDSN HVSAIACLGS PNTATITIFD DDHAGIFTFE 540
SNCX        IFEEDENFLV HLSNVRVSSG VSEDGILDSN HVSAIACLGS PNTATITIFD DDHAGIFTFE 540
RNCX        IFEEDENFLV HLSNVRVSSG VSEDGILDSN HVSAIACLGS PNTATITIFD DDHAGIFTFE 540

SDNXC1.10   EPVTHVSESI GIMEVKVLRT SGARGNVIIP YKTIEGTARG GGEDFEDTCG ELEFQNDEIV 600
SNCX        EPVTHVSESI GIMEVKVLRT SGARGNVIIP YKTIEGTARG GGEDFEDTCG ELEFQNDEIV 600
RNCX        EPVTHVSESI GIMEVKVLRT SGARGNVIIP YKTIEGTARG GGEDFEDTCG ELEFQNDEIV 600

SDNXC1.10   KIITIRIFDR EEYEKECSFS LVLEEPKWIR RGMKGGFTLT GKKMYGQPVF RKVHARDHPI 660
SNCX        KIITIRIFDR EEYEKECSFS LVLEEPKWIR RGMKGGFTLT -----GQPVF RKVHARDHPI 655
RNCX        KIITIRIFDR EEYEKECSFS LVLEEPKWIR RGMKGGFTLT ---------- ---------- 640

SDNXC1.10   PSTVISISEE YDDKQPLTSK EEEERRIAEM GRPILGEHTK LEVIIEESYE FKSTVDKLIK 720
SNCX        PSTVISISEE YDDKQPLTSK EEEERRIAEM GRPILGEHTK LEVIIEESYE FKSTVDKLIK 715
RNCX        --------EE YDDKQPLTSK EEEERRIAEM GRPILGEHTK LEVIIEESYE FKSTVDKLIK 692

SDNXC1.10   KTNLALVVGT NSWREQFIEA ITVSAGEDDD DDECGEEKLP SCFDYVMHFL TVFWKVLFAF 780
SNCX        KTNLALVVGT NSWREQFIEA ITVSAGEDDD DDECGEEKLP SCFDYVMHFL TVFWKVLFAF 775
RNCX        KTNLALVVGT NSWREQFIEA ITVSAGEDDD DDECGEEKLP SCFDYVMHFL TVFWKVLFAF 752

SDNXC1.10   VPPTEYWNGW ACFIVSILMI GLLTAFIGDL ASHFGCTIGL KDSVTAVVFV ALGTSVPDTF 840
SNCX        VPPTEYWNGW ACFIVSILMI GLLTAFIGDL ASHFGCTIGL KDSVTAVVFV ALGTSVPDTF 835
RNCX        VPPTEYWNGW ACFIVSILMI GLLTAFIGDL ASHFGCTIGL KDSVTAVVFV ALGTSVPDTF 812

SDNXC1.10   ASKVAATQDQ YADASIGNVT GSNAVNVFLG IGVAWSIAAI YHAANGEQFK VSPGTLAFSV 900
SNCX        ASKVAATQDQ YADASIGNVT GSNAVNVFLG IGVAWSIAAI YHAANGEQFK VSPGTLAFSV 895
RNCX        ASKVAATQDQ YADASIGNVT GSNAVNVFLG IGVAWSIAAI YHAANGEQFK VSPGTLAFSV 872

SDNXC1.10   TLFTIFAFIN VGVLLYRRRP EIGGELGGPR TAKLLTSSLF VLLWLLYIFF SSLEAYCHIK 960
SNCX        TLFTIFAFIN VGVLLYRRRP EIGGELGGPR TAKLLTSSLF VLLWLLYIFF SSLEAYCHIK 955
RNCX        TLFTIFAFIN VGVLLYRRRP EIGGELGGPR TAKLLTSSLF VLLWLLYIFF SSLEAYCHIK 932

SDNXC1.10   GF 962
SNCX        GF 957
RNCX        GF 934
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exchanger or the vector alone. As shown in the example in Figure 3A, the return $[Ca^{2+}]_i$ after ATP administration was far slower in vector-transfected cells when compared with cells transfected with the exchanger. In cells expressing SDNCX1.10, there was a 7-fold increase in the rate at which these cells were capable of reducing ATP-induced increases in $[Ca^{2+}]_i$ back to control levels when compared to cells expressing vector alone (Fig. 3E). The recovery rate was determined by using the initial slope of recovery curve.

To verify that SDNCX1.10 was in fact functional in these cells, we tested the effects of the exchange inhibitor KB-R7943 on forward- and reverse-mode NCX activity. The exact mechanisms of KB-R7943's actions have not been completely elucidated. It is believed that KB-R7943 acts directly on the transport machinery of the exchanger, with additional influences on ionic regulatory properties (24). The results are shown in Figure 3A, D, and E and Figure 4. Consistent with other reports (25), forward-mode NCX activity was not inhibited with KB-R7943 (Fig. 3C). Thus, when $[Ca^{2+}]_i$ was elevated by the addition of ATP, this drug did not attenuate the rate of return in $[Ca^{2+}]_i$ back to control levels (Fig. 3E). In fact, we found that this drug caused a significant stimulation of exchanger activity. However, as shown in Figure 4, reverse-mode NCX activity as assessed by $^{45}Ca^{2+}$-uptake was markedly attenuated (50%) by the addition of KB-R7943.

In previous studies, we have found that PKC stimulates exchanger function of RNCX but not SNCX (18). Thus, it was of interest to determine if the SDNCX1.10 isoform was sensitive to PKC. Because ATP stimulates NCX activity via PKC activation, this point could only be tested in forward-mode studies by inhibiting PKC activity through prior 24 h exposure to PMA. As shown in Figures 3B, D, and E, recovery of $[Ca^{2+}]_i$ in response to ATP was significantly attenuated (66%) with down-regulation of

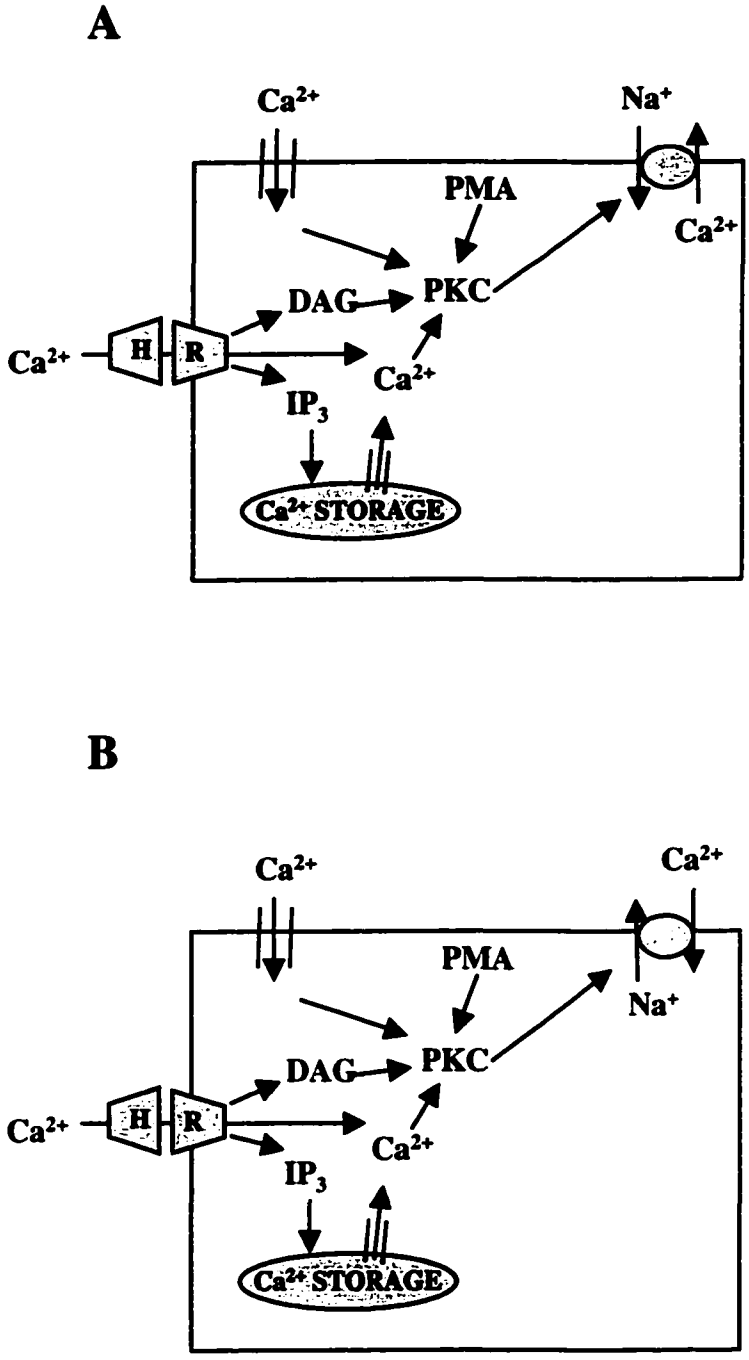
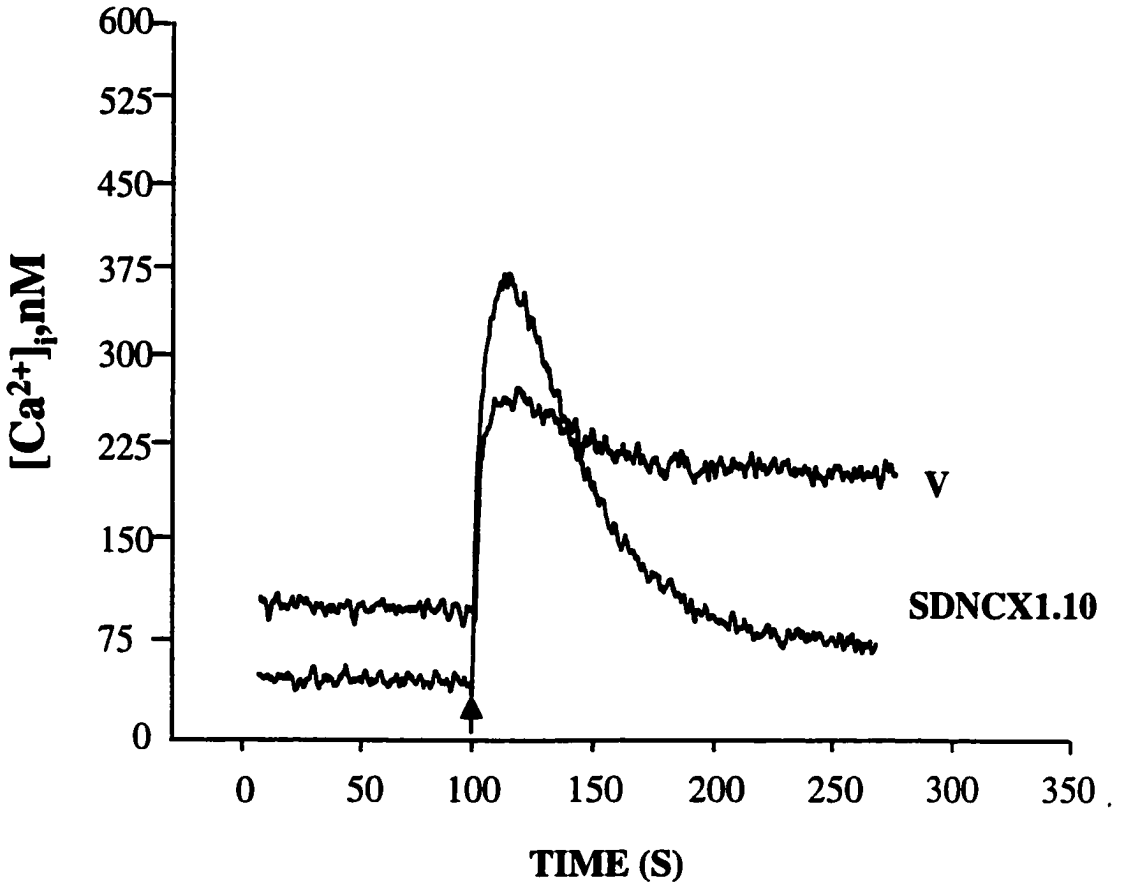


Figure 2. Schematic representing the two modes by which the NCX regulates $[Ca^{2+}]_i$. The binding of an agonist (H) to its receptor (R) triggers a rise in $[Ca^{2+}]_i$ (through Ca^{2+} channels), diacyl glycerol (DAG), and IP_3, which stimulates the release of Ca^{2+} from intracellular storage. Purinergic receptors can also act as Ca^{2+} channels in response to binding of ATP. DAG and Ca^{2+} stimulate PKC, which activates the exchanger to (**A**) extrude Ca^{2+} in exchange for Na^+ (forward mode) or (**B**) extrude Na^+ in exchange for Ca^{2+} (reverse mode) in Na^+-loaded cells in the absence of extracellular Ca^{2+}.

Figure 3. OK-PTH cells expressing SDNCX1.10 showed greater ability to buffer ATP-induced $[Ca^{2+}]_i$ increase when compared to cells expressing the vector alone. The abilities of cells expressing either the vector or SDNCX1 to buffer ATP-induced $[Ca^{2+}]_i$ increase were assessed following treatment with 1 mM ATP to elevate $[Ca^{2+}]_i$. (**A**) Representative $[Ca^{2+}]_i$ tracings of cells expressing the vector (V) or SDNCX1.10. (**B**) Representative $[Ca^{2+}]_i$ tracings showing the effect of PKC down-regulation after pre-treating cells expressing SDNCX1.10 with PMA (300 nM, 24 h), followed by elevation of $[Ca^{2+}]_i$ with 1 mM ATP. (**C**) Representative $[Ca^{2+}]_i$ tracings showing in the presence of the exchanger inhibitor, KB-R7943 (30 μM). (**D, E**) Bar graphs showing $\Delta[Ca^{2+}]_i$ in response to 1 mM ATP for VO or SDNCX1.10-expressing cells (**D**) and the rate of recovery following no treatment (CTL), treatment with PMA, or treatment with KB-R7943 (**E**) of ATP-induced $[Ca^{2+}]_i$ increase back to baseline levels following no treatment (CTL), treatment with PMA, or treatment with KB-R7943. Data were analyzed for statistical significance by using ANOVA. Means ± S.E.M. (n = 9). $^*p < 0.005$ compared with control.

A



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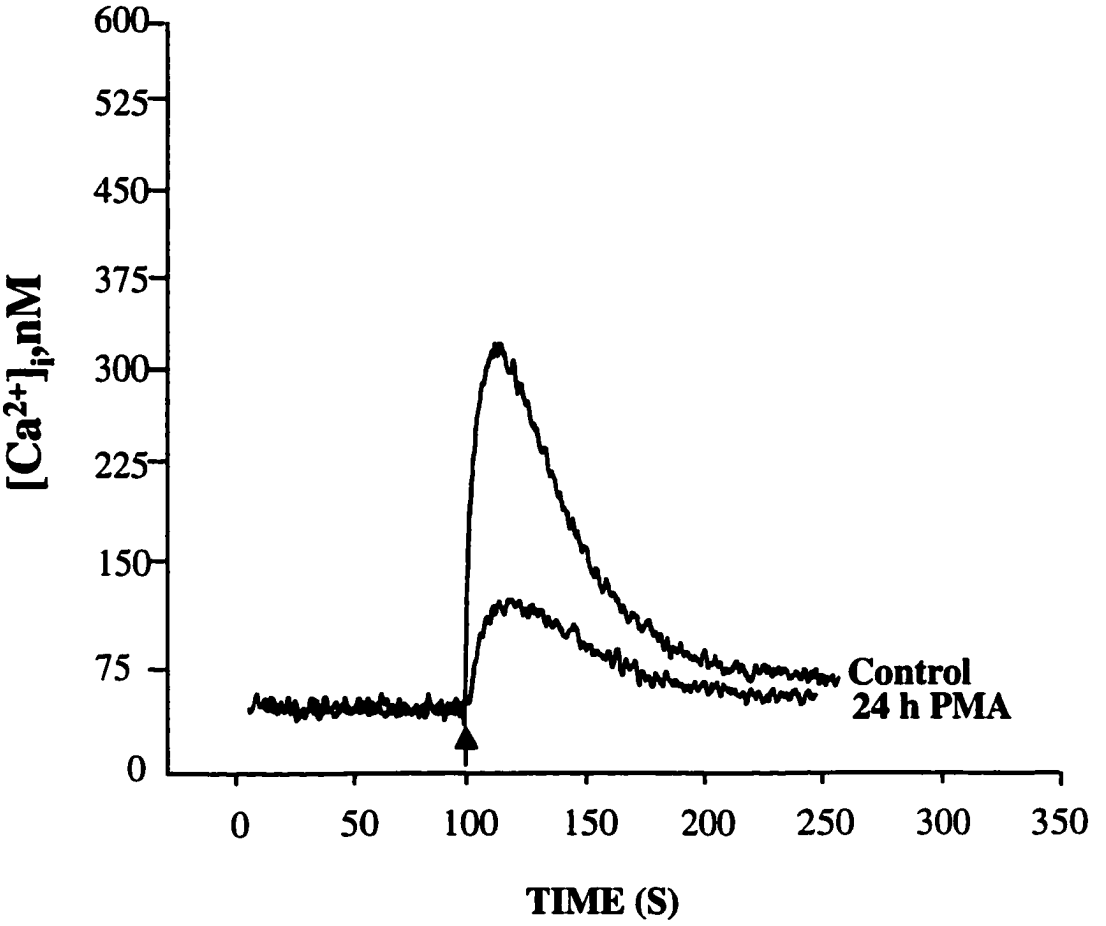

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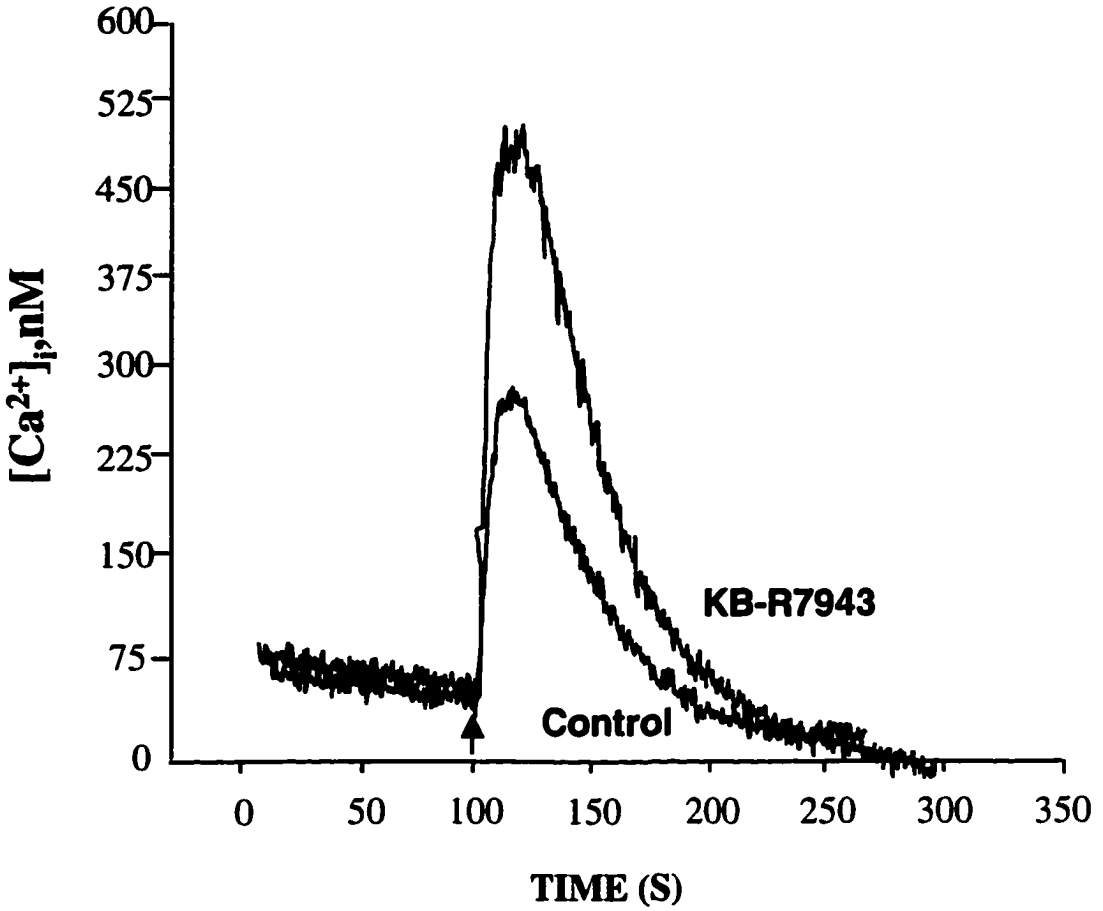

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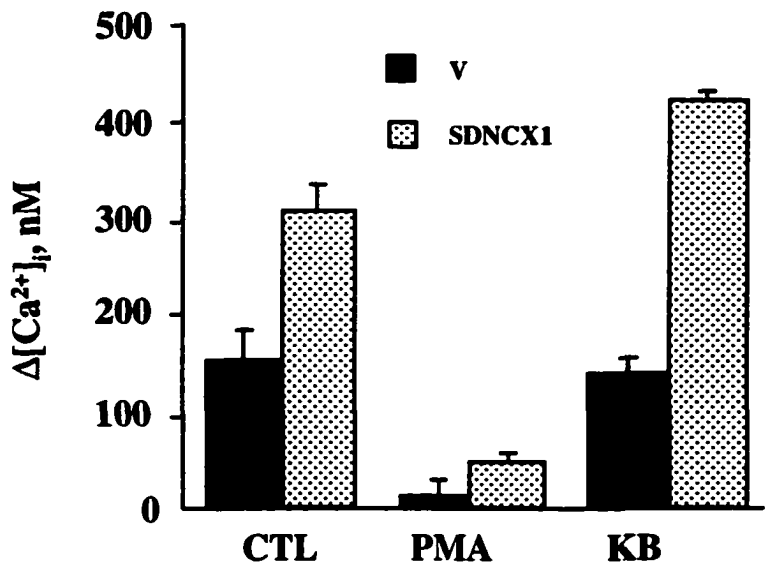

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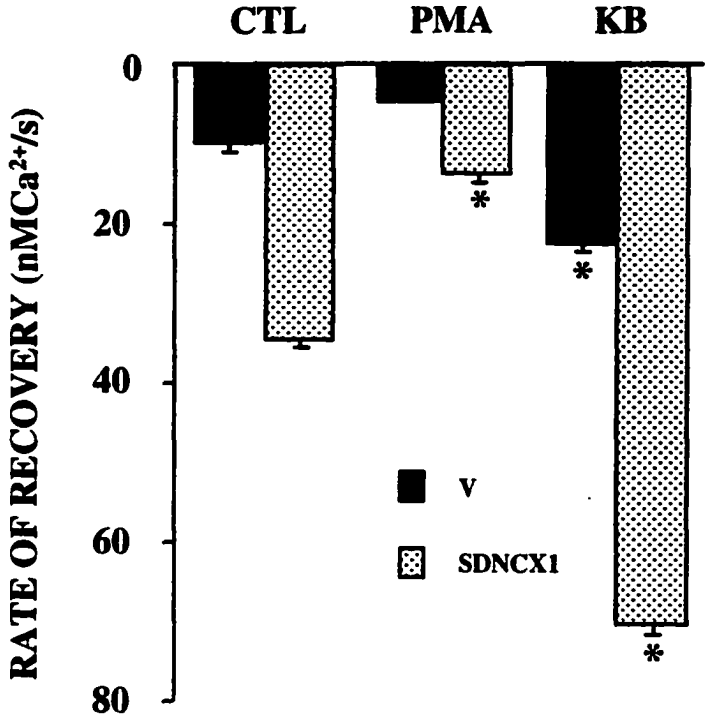

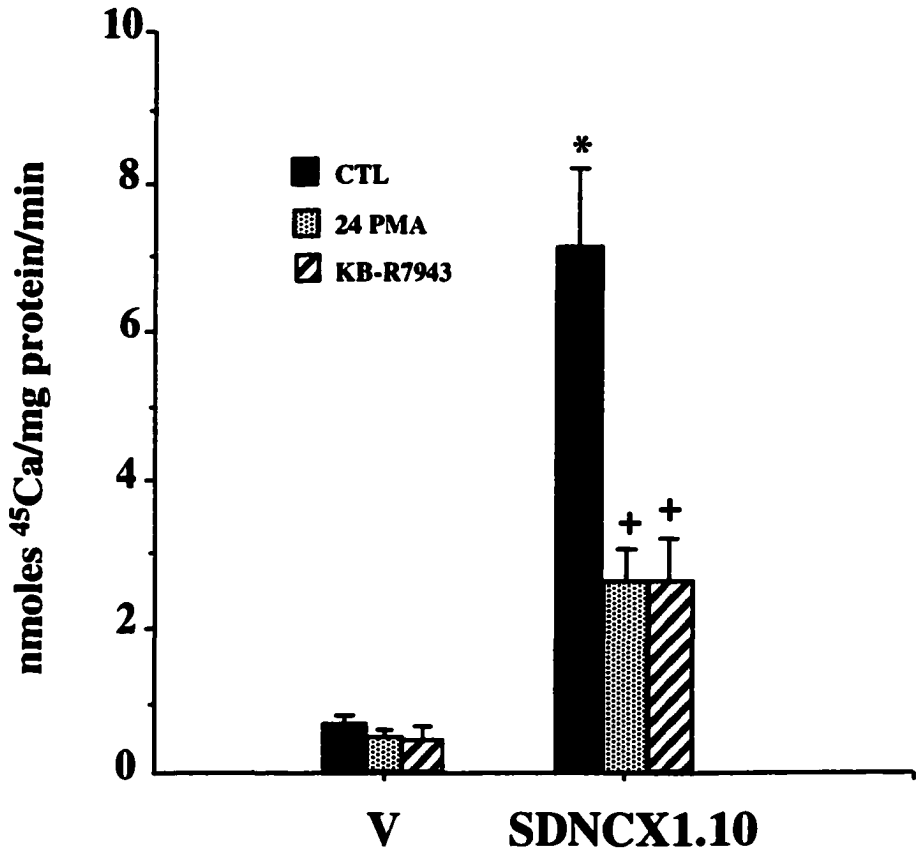

Figure 4. PMA and KB-R7943 attenuate exchanger activity (reverse mode) in OK-PTH cells expressing SDNCX1.10. Na^+-dependent $^{45}Ca^{2+}$ uptake was examined in Na^+-loaded cells to determine the ability of cells expressing vector alone (V) or SDNCX1.10 to regulate $[Ca^{2+}]_i$. Treatment of cells with KB-R7943 (30 μM, 20 min), an inhibitor of exchanger activity, and PKC down-regulation by PMA pre-treatment (300 nM, 24 h) attenuated exchanger activity. Data were analyzed for statistical significance by using ANOVA. Means ± S.E.M. (n = 12 for control and each treatment). $^*p < 0.05$ for cells expressing SDNCX1.10 when compared with cells expressing the vector; $^+p < 0.05$ KB-R7943- or PMA-treated cells expressing SDNCX1.10 when compared with control.

PKC activity. Also, in reverse-mode $^{45}Ca^{2+}$-uptake experiments, PKC down-regulation resulted in a substantial attenuation (50%) of the exchange activity.

DISCUSSION

We have previously shown that the NCX is present in AAs and MCs of S and R rats (4,5). Importantly, we demonstrated that PKC upregulated the MC NCX isoform from R but not S rats (4). Cloning and sequencing of SNCX and RNCX demonstrated that the two isoforms differ at amino acid residue 218, where the amino acic is isoleucine in RNCX but is phenylalanine in SNCX (18). The two isoforms also differ at the alternative splice site where RNCX is encoded by exons B and D, whereas SNCX is encoded by exons B, D, and F. Functional studies using OK-PTH cells expressing either RNCX or SNCX indicated that cells expressing RNCX efficiently regulated agonist-induced increases in $[Ca^{2+}]_i$ whereas cells expressing SNCX showed a diminished capacity to do so (18). Finally, the ability of cells expressing RNCX to regulate agonist-induced $[Ca^{2+}]_i$ increases was mediated through PKC (18). Thus, the difference in $[Ca^{2+}]_i$ regu- lation between these two isoforms resides in their differential sensitivity to PKC activation.

The S and R rats were originally derived from SD rats and were inbred based on their response to a high-salt diet (19). Thus, R rats were those in which blood pressure was the most unresponsive to a high-salt diet, and the S rats were those in which blood pressure increased the greatest on a high-salt diet. Both S and R rats are inbred strains; therefore both are genetically different from the parental SD rat. Therefore, one purpose of these studies was simply to characterize the exchanger isoform(s) expressed in MCs of SD rats.

We found that MCs cultured from SD rats expressed four different NCX isoforms. The salt-resistant isoform, RNCX, and the salt-sensitive isoform, SNCX, are expressed in MCs of SD rats and are designated SD-RNCX and SD-SNCX, respectively. RNCX and SD-RNCX are identical at every amino acid residue. SNCX and SD-NCX, however, are identical at every amino acid residue except at amino acid 218, where the amino acid is isoleucine in SD-SNCX but is phenylalanine in SNCX. The other two isoforms are identical to RNCX at the N- and C-terminal membrane-spanning domains and at most of the cytosolic domain, with the exception of the alternative splice site. RNCX is encoded by exons B and D; however, one SD isoform, designated SDNCX1.11, is encoded by exons B, C, D, E, and F and another, designated SDNCX1.10, is encoded by exons B, D, E, and F.

SDNCX1.10 encodes a protein that consists of 962 amino acids. A search of sequencing data available at Genbank revealed two partial NCX isoform sequences that exhibit a high degree of homology with SDNCX1.10 at the alternative splice site (26). These isoforms are designated NACA10 because both express exons B, D, E, and F at the alternative splice site. These isoforms were isolated and sequenced from odontoblasts of SD rats (accession no. AAD17214) and osteoblast-like osteosarcoma of humans (accession no. AAD17213) by Lundquist *et al.* (26). These two partial sequences span a region of the cytosolic domain corresponding to amino acids 456-670 of SDNCX1.10, which includes the alternative splice site (amino acids 601-668). At the alternative splice site, rat NACA10 and SDNCX1.10 isoforms are 100% homologous in the B, D, and F exons but differ by two amino acids in the E exon. Within this exon, there is a region that consists of amino acids G, K, K, M, Y in SDNCX1.10 but has G, K, Y, L, Y in NACA10. Human NACA10, on the other hand, is 100% homologous with SDNCX1.10

at exon B but shows differences at exons D, E, and F. Human NACA 10 and SDNCX1.10 differ by one amino acid at exon D (SDNCX1.10: GGFTLT vs NACA10: GGFTIT), three amino acids at exon E (SDNCX1.10: GKKMY vs NACA 10: GKYLF), and four amino acids at exon F (SDNCX1.10: GQPVFRKVHARDHPIP STVISIS versus NACA10:GQPVFRKVHAREHPILSTVITI A). In the non-alternative splice region of the cytosolic domain, rat NACA10 and SDNCX1.10 show 100% homology, whereas human NACA10 and SDNCX1.10 differ by 12 amino acids. The presence of NACA10 was also confirmed by homology-based RT-PCR in rat osteoblast-like UMR 106 cells, skeletal muscle of neonatal rats, and astrocytes (12).

The significance of co-expression of the four alternatively spliced NCX isoforms in MCs of SD rats is not known. However, multiple isoform expression has been observed in rat heart (27), where, through homology-based RT-PCR, it was discovered that cardiac myocytes expressed NCX1.1 (exons A, C, D, E, and F) as the major isoform and NCX1.3 (exons B and D) and NCX1.4 (exons A and D) as the minor isoforms. In cardiac fibroblasts, NCX1.3 and NCX1.4 are the major isoforms expressed, whereas NCX1.12 (exons A, D, E, and F) were expressed as minor isoforms (27). Also, the expression of NCX1 shows tissue specificity (12). Alternative splice variants containing exon A were found exclusively in the heart, brain, and skeletal muscle; isoforms containing exon B were found in non-neuronal and non-cardiac tissues with isoforms containing B and D and B, D, and F at the alternative splice being the predominant isoforms. Of the 12 clones that we sequenced from MCs of SD rats, four were SD-SNCX, four were SD-RNCX, two were SDNCX1.10, and two were SDNCX1.11. Therefore, SD-RNCX (exons B and D) and SD-SNCX (exons B, D, and F) may be the

predominant NCX isoforms in SD MCs, and SDNCX1.10 and SDNCX1.11 may be minor isoforms.

NCX is an integral part of the plasma membrane that is responsible for extrusion of one Ca^{2+} from the cytosol in exchange for three Na^{+}. This function is critical in all cells but especially in contractile cells such as MCs where the exchanger functions as a Ca^{2+} extrusion pathway. To assess the ability of SDNCX1.10 to regulate $[Ca^{2+}]_i$, OK-PTH cells expressing SDNCX1.10 or the vector alone were treated with ATP (1 mM) to elevate $[Ca^{2+}]_i$, and the ability (forward mode) of these cells to reduce ATP-induced $[Ca^{2+}]_i$ increase was examined. Our studies demonstrated that cells expressing SDNCX1.10 reduced ATP-induced $[Ca^{2+}]_i$ increase back to baseline at a significantly greater rate than cells expressing the vector alone. These results indicate that we have successfully transfected a functional NCX into this mammalian cell line. Also, using a $^{45}Ca^{2+}$-uptake assay, we demonstrated that cells transfected with the exchanger were capable of accumulating Ca^{2+} as Na^{+} moved down its concentration gradient. Additional evidence for a functional exchanger was provided by the inhibition of reverse-mode Na^{+}:Ca^{2+} exchange by the inhibitor KB-R7943. Interestingly, this agent failed to block and even significantly stimulated forward-mode Na^{+}:Ca^{2+} exchange. This finding, althought puzzling, is consistent with other studies in rabbit ventricular myocytes (24).

As indicated, in previous work, we found that exchanger activity was upregulated in the RNCX isoform but not in the SNCX isoform (18). Thus, two different isoforms, both derived from MCs exhibited differential PKC sensitivity. This leads to the question of whether other exchangers derived from MCs would be sensitive or insensitive to PKC. Accordingly, we tested for PKC sensitivity of our cloned SDNCX1.10 exchanger isoform. As discussed previously, ATP administration causes increases in $[Ca^{2+}]_i$

through the activation of puringeric receptors. Because both P2X and P2Y receptors are located in OK-PTH cells, this involves both Ca^{2+} mobilization, as well as Ca^{2+} influx. This increase in $[Ca^{2+}]_i$ will, among other things, result in activation of diacyl glycerol (DAG) and PKC activation. Thus, ATP, which turns on Ca^{2+} signaling, also initiates events that lead to the termination of this Ca^{2+} signal. Our results indicate that SDNCX1.10 is also upregulated by PKC. In forward-mode experiments, down-regulation of PKC by prior 24 h pre-treatment with the phorbol ester PMA markedly reduced the recovery of $[Ca^{2+}]_i$ after ATP adminstration. Also, in reverse-mode experiments, PKC down-regulation by PMA pre-treatment attenuated exchange activity.

Thus, we have cloned, sequenced, and expressed a NCX from cultured MCs of the SD rat. This isoform was active when expressed in a mammalian expression vector and was inhibited by the NCX inhibitor, KB-R7943. Its activity was also attenuated by PKC down-regulation, which suggests that the normal ensemble of exchanger isoforms that are expressed in MCs contribute to the regulation of $[Ca^{2+}]_i$ through activation by PKC.

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REGULATION OF MESANGIAL CELL Na^+:Ca^{2+} EXCHANGER ISOFORMS

by

I. WILLIAMS MCINTOSH, E.BATES, M.T. UNLAP, AND P.D. BELL

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ABSTRACT

Although regulation of the Na^+:Ca^{2+} exchanger (NCX) by a number of agonists has been well characterized in heart, little is known regarding the regulation of renal isoforms of the NCX. We studied the regulation, by intracellular Ca^{2+}, intracellular pH (pH_i) and extracellular pH (pH_e), and protein kinases A (PKA) and (PKG) G, of three renal isoforms that were isolated from mesangial cells of Dahl/Rapp salt-resistant (RNCX) and salt-sensitive (SNCX) rats and Sprague Dawley (SDNCX) rats. The three isoforms differ at the alternative splice site, where the site is encoded by exons B and D (RNCX), B, D, and F (SNCX) and B, D, E, and F (SDNCX). These isoforms also differ at amino acid 218, where the amino acid is isoleucine in RNCX and SDNCX but is phenylalanine in SNCX. Regulation of exchange activity was assessed in opossum proximal kidney tubule (OK-PTH) cells expressing RNCX, SNCX, or SDNCX by Na^+-dependent $^{45}Ca^{2+}$ uptake after Na^+ loading cells with ouabain and monensin. Elevating intracellular Ca^{2+} with 40 nM thapsigargin from 50 to 95 nM or reducing intracellular Ca^{2+} from 50 nM to 45 nM or from 50 nM to 40 nM with 10 or 20 μM BAPTA, respectively, showed that RNCX was fully functional at 40-95 nM of intracellular Ca^{2+} level; however, SNCX and SDNCX were only active within the 40-50 nM range. Regulation of exchange activities by pH_i and pH_e on exchange activities was assessed by using $^{45}Ca^{2+}$-uptake solutions of pH 6.2, 6.8, 7.4, and 8.0 in the presence or absence of 10 μM nigericin. In the presence of nigericin, RNCX and SDNCX were fully active at pH 7.4 and 8.0, whereas SNCX was fully active only at pH 7.4. In the absence of nigericin, RNCX demonstrated full activity at pH 6.8, 7.4, and 8.0; SDNCX was only fully active at pH 7.4 and 8.0; and SNCX was fully active only at pH 7.4. Finally, regulation of exchange activity by PKA and PKG using either CPT-cAMP or db-cGMP at 250 μM,

respectively, showed that both PKA and PKG activation enhanced SDNCX activity by 129 and 168%, respectively, while not affecting the activities of RNCX and SNCX. These studies demonstrate differential regulation of three renal NCX isoforms by intracellular messenger systems, including intracellular Ca^{2+} and pH and PKA and PKG.

INTRODUCTION

The Na^+:Ca^{2+} exchanger (NCX) is expressed in a variety of tissues, including kidney, smooth muscle, and brain, and serves the critical role of regulating intracellular calcium concentration ($[Ca^{2+}]_i$) (1-5). Regulation of $[Ca^{2+}]_i$ by the exchanger is achieved through counter-transporting three Na^+ for each Ca^{2+} that is extruded from the cell (6). The role of the exchanger in regulating $[Ca^{2+}]_i$ is especially critical in contractile cells such as cardiac, vascular smooth muscle, and mesangial cells (MCs) where contraction is facilitated by a sudden increase in $[Ca^{2+}]_i$, which occurs through Ca^{2+} entry and/or intracellular release mechanisms (7-9). Na^+:Ca^{2+} exchange plays the critical role of controlling $[Ca^{2+}]_i$ by decreasing $[Ca^{2+}]_i$ back toward basal levels.

Recently, a number of studies have focused on the physiological/patho-physiological regulation of NCX activity (10-12). Although the activity of NCX depends on the intracellular\extracellular gradients for Na^+ and Ca^{2+} and membrane potential, it is also appreciated that a wide range of factors can regulate exchanger activity. These include hormones (13-15), intracellular messenger systems such as cell pH (16,17), and other factors such as membrane fluidity (18,19) and potential interactions with other membrane proteins (20,21). Interestingly, there is also regulation of the exchanger by intracellular\extracellular $Ca^{2+}$and Na^+ (22-25). Current evidence indicates that this regulation occurs through the binding of $Ca^{2+}$and Na^+ to specific sites on the exchanger

protein, which, in turn, promotes up- or down-regulation of exchanger activity (22, 26,27).

NCX1, the most abundant of the NCXs, consists of nine membrane spanning domains, five in the N terminus and four in the C terminus, and a large cytosolic loop that comprises over 50% of the protein (28-30). NCX1 is encoded by 12 exons, six (denoted A-F) of which encode a small region within the cytosolic domain that arises through alternative splicing (31). Twelve splice isoforms of NCX1 (NCX1.1-NCX1.12) have been identified, and there appears to be a tissue-specific pattern of isoform expression (32-34). The physiological role of splice variants of NCX1 has not been elucidated with certainty, but one suggestion has been that differential regulation of the exchanger may result from the exons expressed at the alternative splice site (35). Thus, tissue-specific exchanger isoform expression may lead to tissue-specific regulation of exchanger activity. This possibility may be particularly important because the exchanger is expressed in a wide range of cells, tissues, and organs; therefore, there may be diverse mechanisms for the biological regulation of NCX.

We have previously cloned and sequenced three NCX1 isoforms from mesangial cells of Dahl/Rapp salt-sensitive (SNCX) hypertensive and salt-resistant (RNCX) normiotensive and Sprague Dawley (SDNCX) rats (36). According to the most recent nomenclature proposed, based on the presence of exons at the alternative splice site, RNCX is NCX1.3 (exons B and D), SNCX is NCX1.7 (exons B, D, and F), and SDNCX is NCX1.10 (exons B, D, E, and F). In addition to the difference at the alternative site, amino acid 218 is isoleucine in RNCX and SDNCX but is phenylalanine in SNCX. These exchanger isoforms have been transfected into a mammalian cell line, and functional studies have revealed important differences in exchanger regulation between

these three isoforms. Specifically, we have found that RNCX and SDNCX were upregulated by protein kinase C (PKC) but the activity of SNCX was unaffected by PKC (36). However, these studies did not investigate whether there are also differences in the response of these exchanger isoforms to other potential regulators of NCX. Accordingly, these studies were conducted to further characterize the response of RNCX, SNCX, and SDNCX to protein kinases, intracellular Ca^{2+}, and pH.

METHODS

Cell cultures

Opossum proximal tubule kidney (OK-PTH) cells were grown in MEM media (Life Tech) supplemented with 10% Fetalclone III (Cellgro), 240 µg/ml L-glutamine (Life Tech), 82 units/ml penicillin (Life Tech), and 82 µg/ml streptomycin (Sigma) in a humidified atmosphere consisting of 95% air/5% CO_2 at 37°C. Media was changed twice a week, and cells were routinely passaged 72 h after seeding. Each OK-PTH cell lot was used for no more than 30 passages.

Transfection of OK-PTH cells with RNCX, SNCX, and SDNCX

OK-PTH cells were transfected with pCDNA3.1/V5-His6 (Invitrogen) or pCDNA3.1/V5-His6 containing RNCX (pCDNA3.1-RNCX), SNCX (pCDNA3.1-SNCX) or SDNCX1.10 (pCDNA3.1-SDNCX) cDNA by using Lipofectin (Life Tech) according to manufacturer's instructions. Transfectants were selected using Geneticin (500 µg/ml, Sigma) for 3 weeks. After 3 weeks, OK-PTH cells transfected with the NCX isoforms were incubated in the presence of 500 µM $CaCl_2$ and 20 µM of ionomycin (Sigma) for 30 min, washed, and resuspended in complete media. This maneuver

stimulates a significant rise in $[Ca^{2+}]_i$, and only cells with functional NCXs will be able to lower $[Ca^{2+}]_i$ sufficiently to survive (Ca^{2+} killing). This process was repeated every 3 days to enrich the population of OK-PTH cells that express functional NCX.

$^{45}Ca^{2+}$-Uptake Assay

OK-PTH cells expressing the vector (pCDNA3.1) and RNCX, SNCX, or SDNCX were grown in 12 well plates to 80% confluency. Cells were then washed twice with cold phosphate-buffered saline (PBS) and overlaid, at room temperature with 0.50 ml of a Na^+-loading solution (140 mM NaCl, 10 mM Tris-HCl (pH 7.4), 400 μM ouabain (Sigma), and 10 μM monensin (Sigma) for 20 min to pre-load cells with Na^+. $^{45}Ca^{2+}$ uptake was assessed either in the absence (140 mM KCl, 10 mM Tris-HCl (pH 7.4), and 5 μM $^{45}CaCl_2$/ml) or presence (140 mM NaCl, 10 mM Tris-HCl, pH 7.4 and 5 μM $^{45}CaCl_2$/ml) of a Na^+ gradient. After incubation for 10 min in 0.50 ml of either the high-Na^+ or high-K^+ $^{45}CaCl_2$-uptake solutions, cells were washed extensively with a cold 140 mM KCl solution and lysed in 0.5 ml of 1 N NaOH, and aliquots were removed for liquid scintillation counting (0.25 ml) and Bradford protein assay (0.05 ml). Na^+:Ca^{2+} exchange activity was calculated as nmoles $^{45}Ca^{2+}$/mg protein/min in the absence of NaCl minus the activity obtained for cells in the presence of NaCl. The Na^+:Ca^{2+} exchange activity during each treatment was expressed as percentage of control.

The effect of reduced $[Ca^{2+}]_i$ on exchange activity was examined by incubating cells with the Ca^{2+} chelator BAPTA (10 or 20 μM)(Sigma) during the fura-2-loading and $^{45}Ca^{2+}$-uptake periods. The effect of increased $[Ca^{2+}]_i$ on exchange activity was assessed by incubating cells with 40 nM of thapsigargin (Sigma) for 20 min during the fura-2 loading period. The effects of PKA and PKG on exchange activity were assessed by

treating cells with 250 μM CPT-cAMP (Sigma) or DB-cGMP (Sigma), respectively, during the 10 min uptake period. The effects of pH on exchanger activity were assessed by setting extracellular at pH 6.2, 6.8, or 8.0 with or without nigericin (10 μM) during the $^{45}Ca^{2+}$-uptake procedure. The effect of each treatment was analyzed for statistical significance by using ANOVA.

Assay using BCECF

Cells expressing RNCX, SNCX, or SDNCX1.10 were grown on coverslips until 100% confluency in MEM media supplemented with 10% Fetalclone III and 82 μg/ml penicillin/streptomycin. Cells were then loaded with 12 μM BCECF in 10 ml complete media for 30 min at 37°C. The coverslip were then washed by immersion in 150 Ringer's solution (148 mM NaCl, 5 mM KCl, 1mM $MgSO_4$, 1.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES). The coverslips were next placed in a cuvette with 3 ml of Ringer's solution and attached to the Delta Scan. Solutions of 10 μM nigericin and Ringer's solution at pH 6.2, 6.8, 7.4, or 8.0 were perfused at a rate of 1.7 ml/min. Temperature was maintained at 37°C and there was no evidence of dye leakage.

RESULTS

Na^+:Ca^{2+} exchange activity in stably transfected OK-PTH cells

Na^+-dependent Na^+:Ca^{2+} exchange activity was assessed in stably transfected OK-PTH cells expressing RNCX, SNCX, or SDNCX (Fig. 1) by using a $^{45}Ca^{2+}$-uptake assay in the presence of 5 μM $^{45}CaCl_2$/ml. All three cell lines expressed significant Na^+-

dependent $^{45}Ca^{2+}$ uptake when compared to non-transfected or vector-transfected cells (data not shown).

Effect of increasing $[Ca^{2+}]_i$ on NCX activity

Na^+:Ca^{2+} exchange, through NCX, is regulated by the presence of Ca^{2+} on the intracellular side. Ca^{2+} binds to a site on the cytosolic loop, thereby changing the conformation of the exchanger protein and thereby controlling Ca^{2+} efflux and influx through the NCX (23,24). The effect of elevated $[Ca^{2+}]_i$ on NCX activity (Fig. 2B) was assessed by treating cells expressing RNCX, SNCX, or SDNCX with thapsigargin (40 nM) to mobilize Ca^{2+} from intracellular storage sites (Fig. 2A). Elevation of $[Ca^{2+}]_i$ from a baseline of 50-95 nM with thapsigargin increased RNCX activity (175%) while diminishing the activities of SNCX and SDNCX to 47% and 19% of control levels, respectively.

Effect of decreasing $[Ca^{2+}]_i$ on NCX activity

Next, we sought to examine the effects of reducing $[Ca^{2+}]_i$ on NCX activity (Fig. 3B) by treating stably transfected cells with BAPTA-AM (10 and 20 μM) to chelate intracellular Ca^{2+} (Fig. 3A). Treating cells with 10 μM BAPTA reduced $[Ca^{2+}]_i$ from 50 to 45 nM and increased the exchange activity of RNCX, SNCX, and SDNCX to 223, 279, and 220% over control, respectively. BAPTA at 20 μM reduced $[Ca^{2+}]_i$ from 50 to 40 nM and increased the exchange activities of RNCX (186%) and SNCX (354%) while not affecting the activity of SDNCX significantly.

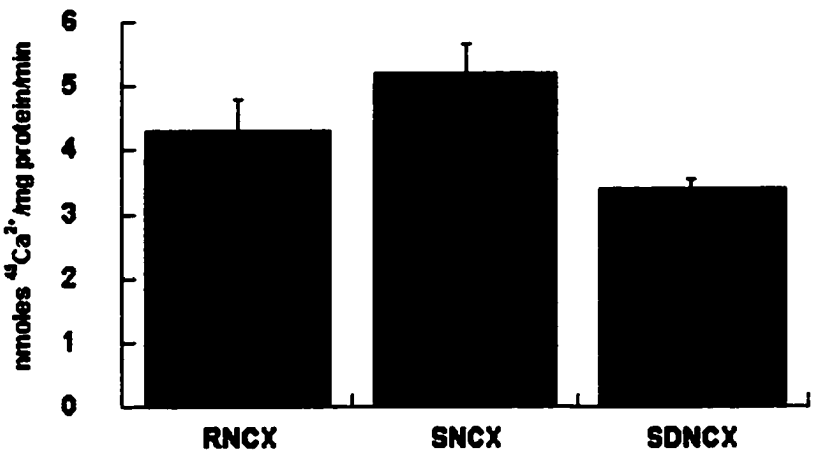

Figure 1. Stably transfected OK-PTH cells exhibit Na^+-dependent $^{45}Ca^{2+}$ uptake. Na^+-dependent $^{45}Ca^{2+}$ uptake was assessed in stably transfected cells expressing RNCX, SNCX, or SDNCX in the presence of 5 μCi of $^{45}CaCl_2$ and 50 μM $CaCl_2$. $^{45}Ca^{2+}$ uptake was carried out either in the presence or in the absence of NaCl. Na^+:Ca^{2+} exchange activity was calculated as nmoles of $^{45}Ca^{2+}$/mg protein/min in the absence of NaCl minus that in the presence of NaCl. The data were corrected for background $^{45}Ca^{2+}$ uptake (in the absence of a Na^+ gradient), which was minimal when compared to $^{45}Ca^{2+}$ uptake in the presence of a Na^+ gradient.

Figure 2. Increased intracellular calcium ($[Ca^{2+}]_i$) levels attenuate Na^+/Ca^{2+} exchange activity. The effect of increasing $[Ca^{2+}]_i$ on the activity of each NCX isoform was assessed by treating cells expressing RNCX, SNCX or SDNCX with 40 nM thapsigargin for 20 min to elevate intracellular calcium prior to $^{45}Ca^{2+}$ uptake. **(A)** Representative calcium tracing of fura 2-loaded OK-PTH cells expressing SDNCX following treatment with thapsigargin, which was added at 200 s. **(B)** The effect of thapsigargin-induced $[Ca^{2+}]_i$ increase on Na^+:Ca^{2+} exchange activity was assessed by $^{45}Ca^{2+}$ uptake which was carried out either in the presence or in the absence of NaCl. Na^+:Ca^{2+} exchange activity was calculated as nmoles of $^{45}Ca^{2+}$/mg protein/min in the absence of NaCl minus that in the presence of NaCl and expressed as % control. Data were analyzed for statistical significance by using ANOVA. Means ± S.E.M. (n = 9). $^*p < 0.05$ compared with control (untreated).

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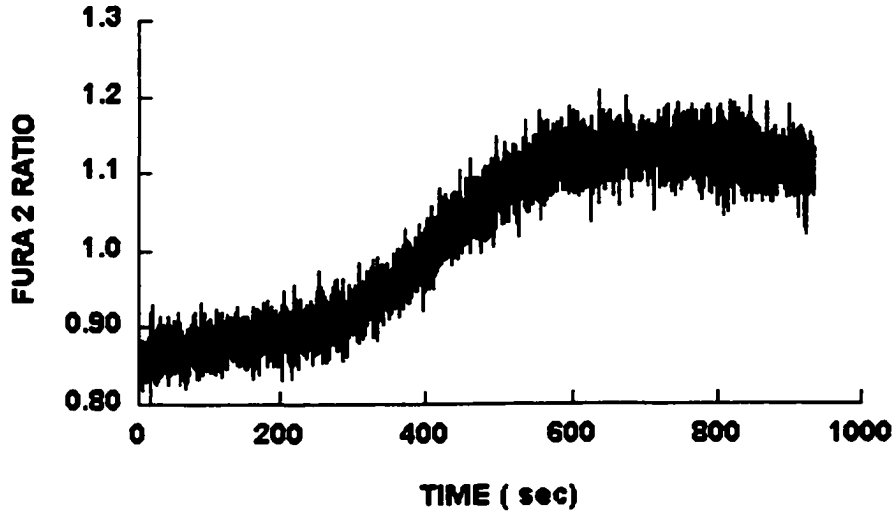

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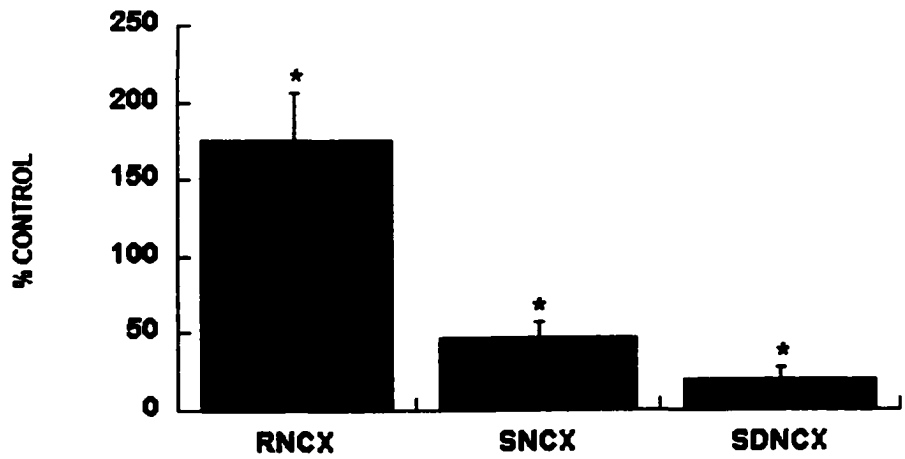

Figure 3. Decreased $[Ca^{2+}]_i$ levels stimulate $Na^+:Ca^{2+}$ exchange activity. The effect of decreasing $[Ca^{2+}]_i$ on the activity of each NCX isoform was assessed by treating cells expressing RNCX, SNCX, or SDNCX with BAPTA for 30 min at 10 (BA10) or 20 (BA20) μM to chelate intracellular calcium prior to and during $^{45}Ca^{2+}$ uptake. (**A**) Representative calcium tracing of fura 2-loaded OK-PTH cells expressing SDNCX following treatment with 10 μM (upper) or 20 μM (lower), BAPTA, which was added at 200 s until 800 s when it was replaced by normal Ringer's solution. (**B**) The effect of BAPTA-induced $[Ca^{2+}]_i$ decrease on $Na^+:Ca^{2+}$ exchange activity was assessed by $^{45}Ca^{2+}$ uptake, which was carried out either in the presence or in the absence of NaCl. $Na^+:Ca^{2+}$ exchange activity was calculated as nmoles of $^{45}Ca^{2+}$/mg protein/min in the absence of NaCl minus that in the presence of NaCl and expressed as % control. Data were analyzed for statistical significance by using ANOVA. Means ± S.E.M. (n = 9). $^*p < 0.05$ when compared with control (untreated).

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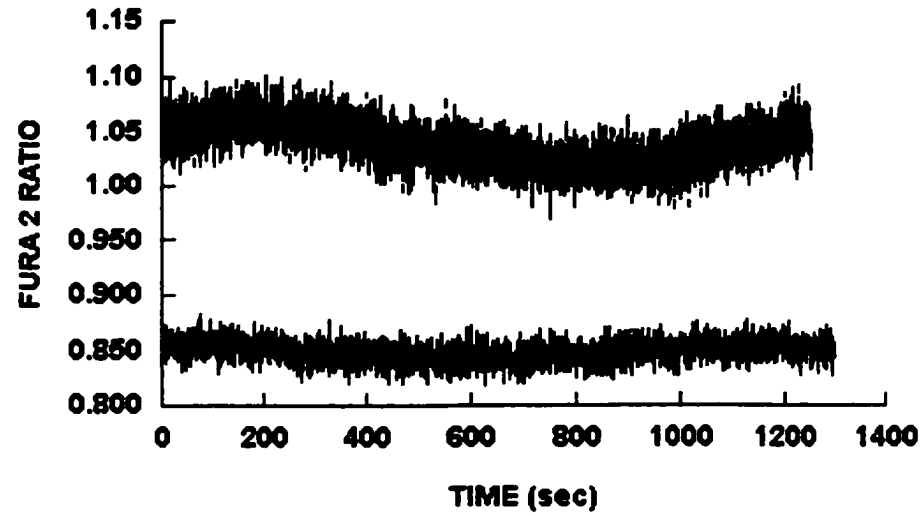

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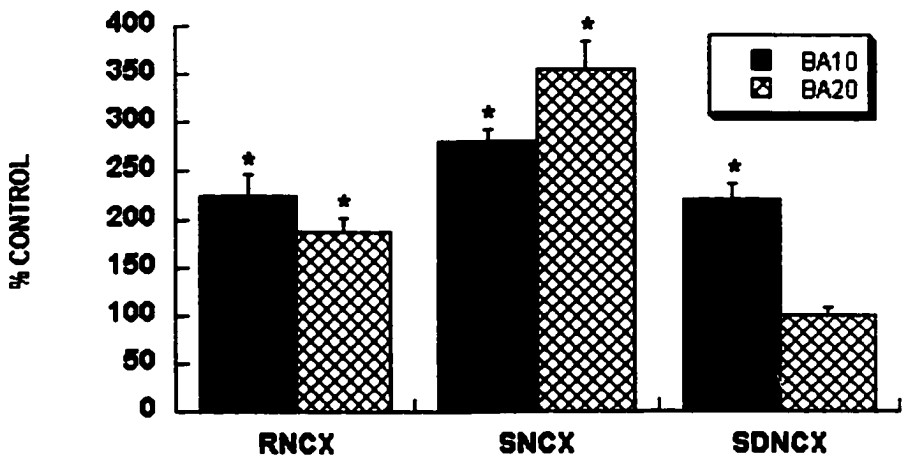

Effect of pH on NCX activity

Cell pH has been shown to have significant effects on the activities of NCX1, NCX2, and NCX3 (37). Acidic pH attenuates the activities of all three exchangers, whereas alkaline pH had no significant effect on the activities of NCX1 and NCX2 but stimulated NCX3 activity (37). The effects of pH on exchange activity (Fig. 4) was assessed by either treating or not treating stably transfected cells with nigericin (10 μM) at extracellular pH of 6.2, 6.8, 7.4, and 8.0. Data are expressed as percentage of control (the activity obtained at pH 7.4). In the absence of nigericin (Fig. 4C); pH 6.2 diminished the activities of RNCX (59%), SNCX (56%), and SDNCX (51%); pH 6.8 diminished the activities of SNCX (65%) and SDNCX (71%) but did not affect the activity of RNCX; and pH 8.0 attenuated the activity of SNCX (82%) but had no effect on RNCX and SDNCX activities. In the presence of nigericin (Fig. 4B), pH 6.2 diminished the activities of RNCX, SNCX, and SDNCX to 2.8, 6.5, and 31% of control, respectively. pH 6.8 also diminished the activities of all three isoforms to 21% (RNCX), 26% (SNCX), and 61% (SDNCX) of control, whereas pH 8.0 attenuated the activity of SNCX (77%) but did not significantly affect the activities of RNCX (89%) or SDNCX (90%).

Effect of PKA and PKG activation on NCX activity

Our previous studies have shown that PKC activation stimulates the activities of RNCX and SDNCX but not SNCX (36). To test the effects of PKA and PKG activation on NCX activity, cells expressing RNCX, SNCX, or SDNCX were treated with 250 μM of db-cGMP or CPT-cAMP to activate PKG and PKA, respectively. The results (Fig. 5) indicate that neither kinase activated RNCX or SNCX significantly but that both kinases increased the activity of SDNCX by 129% for PKG and 168% for PKA.

Figure 4. Variations in intracellular (pH_i) and extracellular (pH_e) pH attenuate Na^+:Ca^{2+} exchange activity. The effect of pH_i and pH_e on the activity of each NCX isoform was assessed by measuring $^{45}Ca^{2+}$ uptake in cells expressing RNCX, SNCX, or SDNCX at various pH's in the presence and absence of nigericin (10 μM), respectively. (**A**) pH_i tracing in cells loaded with the BCECF dye and incubated at various pH's in the presence of 10 μM nigericin in SDNCX1.10-expressing cells. $^{45}Ca^{2+}$ uptake was carried out at pH 6.2, 6.8, 7.4, or 8.0 either in the presence (**B**) Or in the absence (**C**) Of 10 μM nigericin and in the presence and absence of NaCl. Na^+:Ca^{2+} exchange activity was calculated as nmoles of $^{45}Ca^{2+}$/mg protein/min in the absence of NaCl minus that in the presence of NaCl. Exchange activities are expressed as % control (activity at pH 7.4). Data were analyzed for statistical significance by using ANOVA. Means ± S.E.M. (n = 9). $^*p < 0.05$ when compared with control (pH 7.4).

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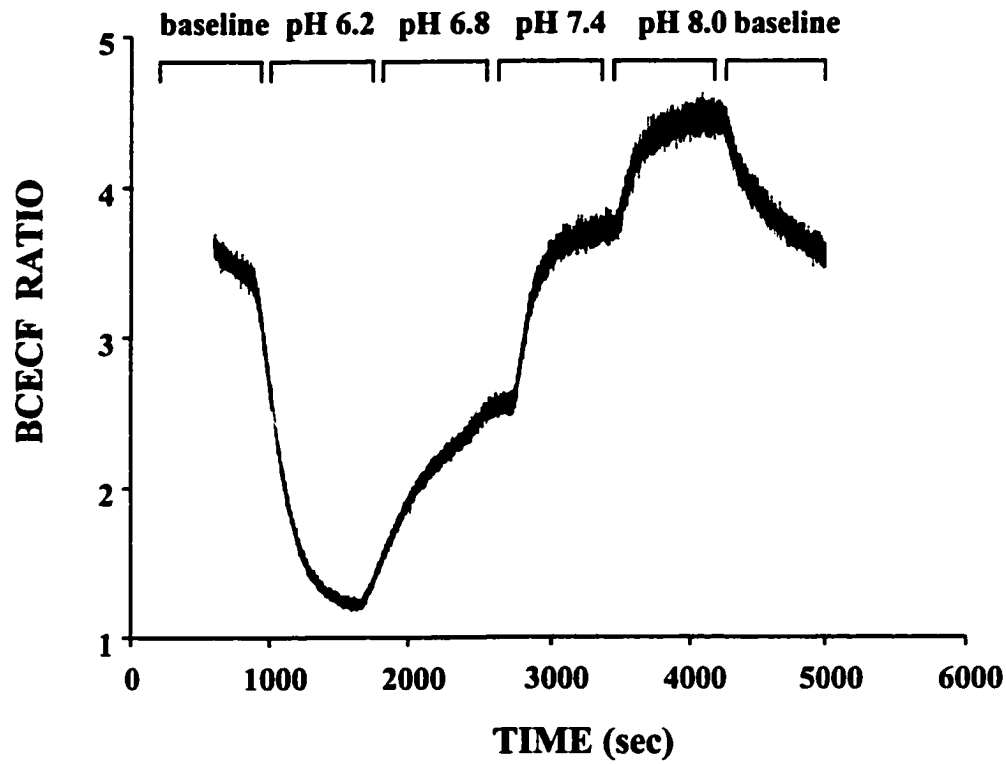

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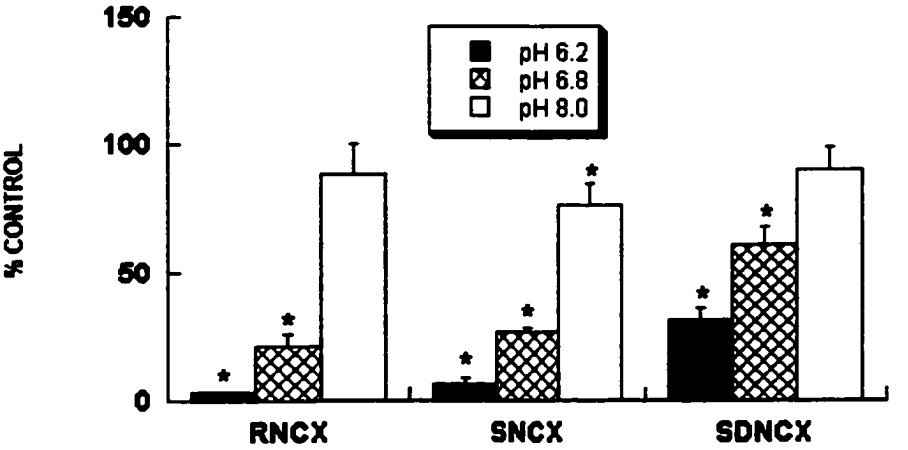

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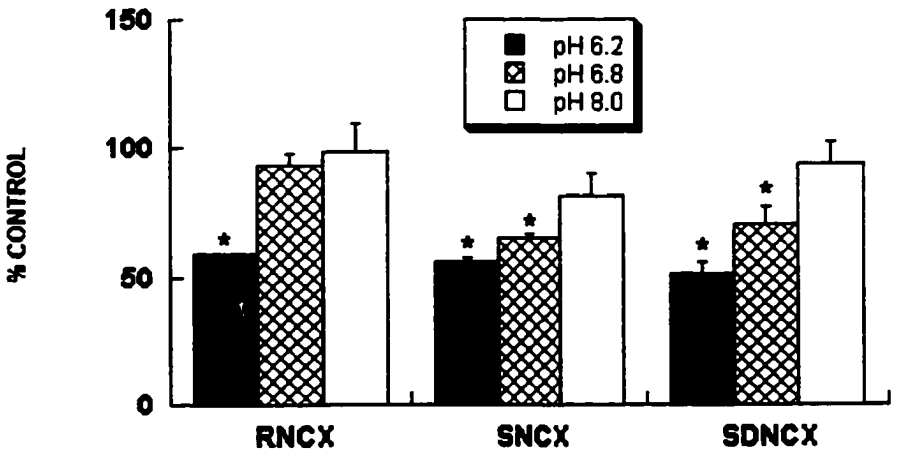

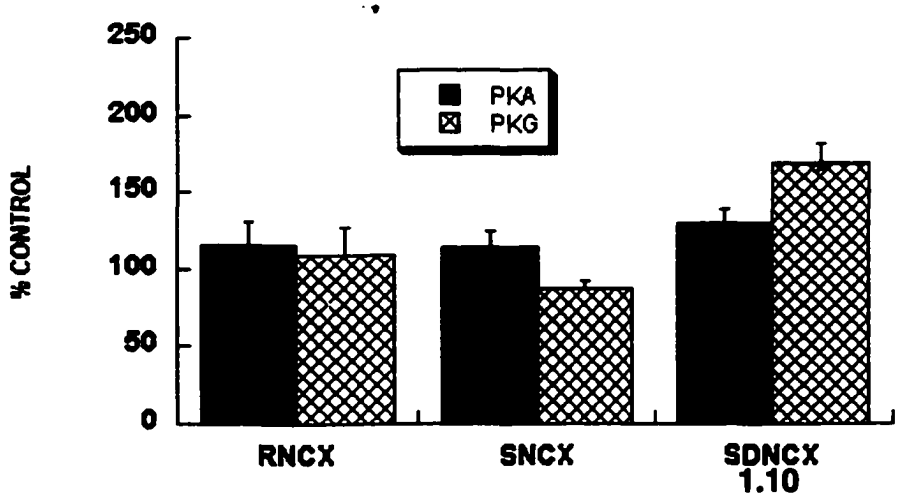

Figure. 5. PKA and PKG stimulate the $Na^+:Ca^{2+}$ exchange activities of SDNCX1.10, but not, RNCX and SNCX. The effect of PKA and PKG activation on the activity of each NCX isoform was assessed by treating cells expressing RNCX, SNCX, or SDNCX with 250 μM CPT-cAMP or dbcGMP, respectively, for 20 min prior to $^{45}Ca^{2+}$ uptake assays. $^{45}Ca^{2+}$-uptake was carried out either in the presence or in the absence of NaCl. $Na^+:Ca^{2+}$ exchange activity was calculated as nmoles of $^{45}Ca^{2+}$/mg protein/min in the absence of NaCl minus that in the presence of NaCl. Exchange activities are expressed as % control (untreated). Data were analyzed for statistical significance by using ANOVA. Means ± S.E.M. (n = 9). $^*p < 0.05$ when compared with control (untreated).

DISCUSSION

Regulation of the NCX by Ca^{2+}, pH, and phosphorylation has been extensively studied by using the cardiac isoform NCX 1.1, which consists of exons A, C, D, E, and F at the alternative splice site. However, regulation of the various isoforms of NCX1, including those that are expressed in renal tissues, is poorly understood. This study was performed to assess the roles that intracellular Ca^{2+}, pH, and phosphorylation by PKA and PKG may play in the regulation of the renal NCX isoforms RNCX, SNCX, and SDNCX.

Control of NCX activity by $[Ca^{2+}]_i$ is accomplished by the binding of low levels of Ca^{2+} (100-300 nM for giant patches or 20-50 nM for whole cells) to a regulatory region of the cytosolic domain, corresponding to amino acids 371-507 (23,25,38). The binding of regulatory Ca^{2+} alters the conformation of the exchanger, thus affecting the exchange rate of Ca^{2+} for Na^+, and ultimately resulting in an increase in intracellular Na^+ that leads to a period of Na^+-dependent inactivation. In the presence of high levels of regulatory $[Ca^{2+}]_i$, however, the Na^+-dependent inactivation phase is abrogated. In the present studies, the effect of $[Ca^{2+}]_i$ on RNCX, SNCX, or SDNCX activities was assessed by increasing or decreasing $[Ca^{2+}]_i$ with thapsigargin and BAPTA. Reverse-mode exchanger studies were then performed by assessing $^{45}Ca^{2+}$ uptake in exchange for Na^+ in Na^+-loaded cells.

Previously, we have shown that baseline $[Ca^{2+}]_i$ in stably transfected OK-PTH cells expressing RNCX, SNCX, or SDNCX was ~50 nM. Increasing $[Ca^{2+}]_i$ to 95 nM with thapsigargin reduced the activity of SNCX and SDNCX significantly but had no effect on RNCX activity. This effect is consistent with previous observations where regulatory Ca^{2+} levels of 25-50 nM (intact cells) or 100-300 nM (giant patches) were

required for the initiation of NCX activity (23,25). Previous studies in a number of cell lines yielded an EC_{50} of 1-80 nM for thapsigargin-induced Ca^{2+} release from intracellular storage (39); in this work, 40 nM thapsigargin elevated baseline $[Ca^{2+}]_i$ from ~50 nM to 95 nM. At this elevated level of $[Ca^{2+}]_i$, the activities of some NCX isoforms, including that for SNCX and SDNCX, are attenuated. However, the effects of increasing $[Ca^{2+}]_i$ on RNCX activity was different. Elevating $[Ca^{2+}]_i$ to 95 nM with thapsigargin did not inhibit but, rather, stimulated the activity of RNCX. Because the Ca^{2+}-binding region (amino acids 371-507) in these three isoforms is identical, the basis for this discrepancy is not immediately apparent. However, the lack of inhibition of high regulatory Ca^{2+} on NCX activity has been shown previously. Linck *et al.* (37) showed that inside-out vesicles from BHK cells expressing NCX1, NCX2, and NCX3 required regulatory Ca^{2+} concentration of up to 12 μM for optimal activity. Thus, there is a wide range of $[Ca^{2+}]_i$ that leads to regulatory Ca^{2+}-induced decreases in exchanger function. It is possible that $[Ca^{2+}]_i$ levels higher than 95 nM in cells expressing RNCX would have revealed a regulatory Ca^{2+}-induced decrease in exchanger function.

We also found that reducing regulatory Ca^{2+} below baseline levels (~50 nM) with 10 μM BAPTA to 45 nM had a mild stimulatory effect on the activities of all three NCX isoforms. Reducing the regulatory Ca^{2+} level further to 40 nM with 20 μM BAPTA still had a stimulatory effect on the activities of RNCX and SNCX but neither stimulated nor inhibited SDNCX activity. Therefore, reducing regulatory Ca^{2+} below resting levels in these cell lines had no inhibitory effects on RNCX, SNCX, and SDNCX. In the studies in which $[Ca^{2+}]_i$ is lowered, it is possible that some of the apparent stimulation of exchanger activity may have come through Ca^{2+} entry because of the increased driving force for Ca^{2+}. Nevertheless, our findings demonstrate for the first time, that renal

Na^+:Ca^{2+} exchange isoforms exhibit different patterns of regulation by $[Ca^{2+}]_i$. RNCX was functional at the entire range examined (40-95 nM), while SNCX and SDNCX were only functional at 40 nM to resting levels (~50 nM). Our findings may be important concerning the physiological function of these isoforms in controlling $[Ca^{2+}]_i$. Thus, RNCX may be an isoform that is particularly suited for removal of Ca^{2+}, even when $[Ca^{2+}]_i$ is markedly elevated. The other two isoforms may, in turn, be examples of isoforms that are more efficient at controlling $[Ca^{2+}]_i$ when it is near baseline levels. At the amino acid level, the Ca^{2+}-binding region that senses regulatory Ca^{2+} in RNCX is identical to that in SNCX and SDNCX and has 94%, 46%, and 66% identity with NCX1, NCX2, and NCX3, respectively. Therefore, other factors, including the alternative splice site, may play a role in affecting the level of regulatory Ca^{2+} for different NCX isoforms.

NCX activity has also been shown to be sensitive to pH_i and pH_e (37). NCX activity for NCX1, NCX2, and NCX3 was shown to be negligible at acidic pH's but increased as pH was raised to 9 (37). By using nigericin to equilibrate pH_i and pH_e, we found that the activity of all three renal isoforms were inhibited by acidic pH's, with RNCX and SNCX showing greater sensitivities than SDNCX. The greatest degree of inhibition was at pH 6.2, with lesser inhibition at pH of 6.8. At pH 8.0, all three isoforms' exchanger activities were not significantly different from those found at pH 7.4. Without nigericin, the activities of all three isoforms were inhibited at pH 6.2, whereas pH 6.8 inhibited the activities of SNCX and SDNCX but had no significant effect on RNCX activity. pH 8.0 had no effect on the activities of SNCX and SDNCX but attenuated the activity of SNCX. The effect of pH, especially pH_i, on NCX activity is believed to be the result of H^+ and Na^+ competing for similar sites on the exchanger (40,41). Hilgemann *et al.* (26) found that Na^+ binding to a site on the exchanger resulted

in Na^+ translocation or Na^+-dependent inactivation of the exchanger to a steady-state level. Matsuoka *et al.* (42) found that that the inhibitory peptide (XIP) of the exchanger was involved in mediating Na^+-dependent inactivation. The XIP region, however, is identical in RNCX, SNCX, and SDNCX and is 100%, 74%, and 72% identical to the XIP region in NCX1, NCX2, and NCX3. Therefore, if this region is involved in the interaction of Na^+ with the exchanger, other factors are likely involved in mediating the effects of pH on the activities of RNCX, SNCX, and SDNCX.

We have previously shown that PKC activation by acute treatment with phorbol esters upregulated Na^+:Ca^{2+} exchange activities of RNCX and SDNCX but not SNCX (36). Also, down-regulation of PKC by chronic treatment with phorbol esters attenuated the activities of these RNCX and SDNCX renal exchanger isoforms. The basis of this activation is not known, but others have shown that activation through PKC appeared to be independent of direct phosphorylation of the exchanger protein (43). However, recent evidence in squid indicates phosphorylation of a 13 kDa ancillary cytosolic protein that may interact with the exchanger. PKA has also been shown to activate NCX1 activity in BHK and Xenopus oocytes (37). Therefore, regulation of these three renal Na^+:Ca^{2+} isoforms by other kinases was assessed by examining the effects of PKA and PKG activation on Na^+:Ca^{2+} exchange activity. We found that activation of these two kinases slightly stimulated the activity of SDNCX but not RNCX or SNCX. The basis for this difference is not presently known but may be attributed, in part, to the presence of exon E in SDNCX but not in RNCX and SNCX. He *et al.* (35) have shown that differences by a few amino acid residues can have significant effect on activation of NCX by PKA in astrocytes and neurons. Also, Ruknudin *et al.* (44) showed that NCX1.1 was activated by

PKA, whereas NCX1.3 (RNCX) was not. The basis for this difference appeared to be that NCX1.1 was phosphorylated to a greater degree than NCX1.3.

These studies show that the three renal isoforms studied demonstrated different regulation by intracellular calcium, pH_i and pH_e , and PKA and PKG. RNCX was fully functional at intracellular calcium levels of 40-95 nM, but SNCX and SDNCX were only fully active within the 40-50 nM range. SNCX showed the greatest sensitivity to variations in pH_e and pH_i ,being fully active only at pH 7.4, followed by SDNCX (pH_e or pH_i 7.4 and 8.0), and RNCX (pH_e 6.8, 7.4, and 8.0 and pH_i 7.4 and 8.0). Finally, only SDNCX1.10 showed activation by both PKA and PKG.

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IMPAIRED ABILITY OF THE Na^+:Ca^{2+} EXCHANGER FROM THE DAHL/RAPP SALT-SENSITIVE RAT TO REGULATE CYTOSOLIC CALCIUM

by

E.F. HWANG, I. WILLIAMS MCINTOSH, G. KOVACS, J. PETI-PETERDI, B. SIROKY, W.C. RICE, E.M. SCHWIEBERT, M.T. UNLAP, AND P.D. BELL

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ABSTRACT

Previous studies in afferent arterioles and MCs found that the Na^+:Ca^{2+} exchanger (NCX) was upregulated by protein kinase C (PKC) in Dahl/Rapp salt-resistant (R) but not salt-sensitive (S) rats. Whether PKC sensitivity of the exchanger translates into differences in the regulation of cytosolic calcium concentration ($[Ca^{2+}]_i$) remains unknown. Na^+:Ca^{2+} exchanger 1 has been cloned from cultured MCs of Dahl/Rapp salt-sensitive S (SNCX1) and salt-resistant R (RNCX1) rats and differs at the amino acid level. To determine if these two isoforms regulate $[Ca^{2+}]_i$ differently, SNCX and RNCX were stably transfected into opossum proximal tubule kidney (OK-PTH) cells. Non-transfected (NT) OK-PTH cells and OK-PTH cells expressing RNCX1 (ROK) and SNCX1 (SOK) were loaded with fura 2 to measure $[Ca^{2+}]_i$. Baseline $[Ca^{2+}]_i$ was 98 ± 20 nM (n = 12) in NT cells and was significantly lower in cells expressing the exchanger isoforms (47 ± 13 nM (n = 9) in ROK and 44 ± 5 nM (n = 9) in SOK). ATP was used as the agonist to increase $[Ca^{2+}]_i$ because OK-PTH cells express purinergic receptors. ATP at 100 μM was used to assess the ability of NCX to buffer an ATP-induced Ca^{2+} load. ATP treatment increased $[Ca^{2+}]_i$ by 189 ± 55 nM (n = 12), 19 ± 7 nM (n = 9), and 72 ± 15 nM (n = 7, $p < 0.001$) in NT, ROK, and SOK cells, respectively. ROK was significantly more efficient at buffering changes in $[Ca^{2+}]_i$. ATP at 1 mM caused large increases in $[Ca^{2+}]_i$, and SOK cells were much less efficient in the rate of return of $[Ca^{2+}]_i$ to baseline levels when compared to ROK cells. The effect of protein kinase C (PKC) down-regulation on the $[Ca^{2+}]_i$ recovery rates to ATP administration was examined by treating cells with PMA (300 nM) for 24 h. PKC down-regulation significantly reduced the rate of return of $[Ca^{2+}]_i$ after ATP treatment in ROK cells but

had no effect in SOK cells. This reduced efficiency of SNCX to regulate $[Ca^{2+}]_i$ may contribute to increased pre-glomerular vascular resistance and MC contraction, and may contribute to the decreased GFR, renal blood flow, and eventual renal failure that are a hallmark of this model of salt-sensitive hypertension.

INTRODUCTION

In recent studies, Na^+:Ca^{2+} exchange (NCX) activity has been shown to exist in renal AAs and efferent arterioles and cultured MCs (1-4). This activity most likely plays an important role in the regulation of cytosolic calcium concentration ($[Ca^{2+}]_i$) by serving as a Ca^{2+}-efflux pathway. Other studies of vascular smooth muscle (5) have suggested that the exchanger has an important role in lowering agonist-induced elevations in $[Ca^{2+}]_i$, and NCX may likewise serve a similar function in the renal microcirculation. We have extended this proposal by suggesting that this process may also involve the activation of protein kinase C (PKC). Phorbol esters, which activate PKC, enhance exchanger activity in AAs and MCs and this may be caused by PKC-induced translocation of the exchanger to the plasma membrane (1-4). These results were seen in both the native tissue and in cultured MCs. It is also well known that vasoconstrictive agonists, which elevate $[Ca^{2+}]_i$, also activate PKC through the Ca^{2+}-diacyl glycerol pathway. Although PKC can have multiple cellular effects, one effect is to enhance Na^+:Ca^{2+} exchange, which then serves to return agonist-induced elevations in $[Ca^{2+}]_i$ back to baseline levels.

In other studies we have found that this NCX pathway may be defective in the Dahl/Rapp salt-sensitive (S) rat (2), a genetic model of salt-dependent hypertension (6,7).

Both freshly dissected AAs from the Dahl/Rapp salt-resistant (R) rats and from rabbit kidneys as well as cultured MCs from R rats, all responded to the phorbol ester, phorbol 12-myristate 13-acetate (PMA), with enhanced Na^+:Ca^{2+} exchange (1-4,8); however, in AAs and MCs from S rats, PMA failed to increase NCX activity. In S MCs, PMA also failed to stimulate translocation of the exchanger to the plasma membrane, whereas in R MCs, PMA promoted translocation (3).

To determine if this difference between R and S rats was caused by intrinsic differences in the exchanger protein, NCX was cloned from cultured MCs from R and S rats (2). The clones from S rats denoted, SNCX1, and from R rats, denoted RNCX1, differ at the amino acid level. One difference occurs at amino acid residue 218, where the amino acid is isoleucine in RNCX1 but is phenylalanine in SNCX1. The significance of this single amino acid difference is currently unknown. However, the variation may affect the regulation of SNCX1 because this difference occurs in a region that is 43% homologous to a region (amino acids 308-330) of the Na^+-K^+ ATPase that is responsible for binding Ca^{2+} (9). The other difference between RNCX1 and SNCX1 occurs within the cytosolic loop at the alternative splice site. This site is encoded by six different exons, denoted A-F (10,11). In the RNCX1 clone, the alternative splice site is encoded by exons B and D; in SNCX1, the alternative splice site is encoded by exons B, D, and F (2). Although there is some indication that the alternative splice site may be important in NCX regulation (12), the exact consequence of the differences in exons expressed by these two clones at this site remains unknown.

RNCX1 and SNCX1 were expressed in an opossum proximal kidney tubule (OK-PTH) cell line which does not express endogenous exchanger activity. It was found that

the exchanger activity of RNCX1 but not SNCX1 was enhanced by PMA (2). Thus, the PKC-NCX pathway in the S rat appears to be defective at the level of the exchanger protein.

The Dahl/Rapp rat model of hypertension is characterized by a marked increase in blood pressure, decreased renal blood flow, and a progressive fall in glomerular filtration rate when S rats are placed on an 8% NaCl diet (7). It has been suggested that this progressive renal vasoconstriction is caused by a dysregulation of $[Ca^{2+}]_i$ in contractile elements of the renal microcirculation of the S rat. One possible explanation for this derangement in $[Ca^{2+}]_i$ could be defective PKC regulation of the NCX. However, whether expression of RNCX1 versus SNCX1 actually results in differences in the regulation of $[Ca^{2+}]_i$ has not yet been addressed. Therefore, the purpose of these studies was to express RNCX1 and SNCX1 in identical cellular environments (OK-PTH cells) and to determine if these two isoforms differ in their ability to help regulate $[Ca^{2+}]_i$ in response to agonist-induced elevations in $[Ca^{2+}]_i$. For these studies we used ATP to elevate $[Ca^{2+}]_i$ via the activation of purinergic receptors; several isotypes of this receptor family were confirmed to be present in OK-PTH cells.

METHODS

Cell cultures

OK-PTH were grown in MEM media (Gibco) supplemented with 10% Fetalclone III (Cellgro), 240 µg/ml L-glutamine (Gibco), and 82 units/ml penicillin, 82 µg/ml streptomycin (Sigma) in a humidified atmosphere under 95% air/5% CO_2 at 37°C.

Media was changed twice a week, and cells were routinely passaged 72 h after seeding. Each OK-PTH cell lot was used for no more than 30 passages.

Transfection of OK-PTH cells with RNCX1 and SNCX1

OK-PTH cells were transfected with pCDNA3.1/V5-His-TOPO (Invitrogen) containing either RNCX1 (pCDNA3.1-RNCX1) or SNCX1 (pCDNA3.1-SNCX1) cDNA by using Lipofectin (BRL) according to manufacturer's instructions. Transfectants were selected for using Geneticin at 500 μg/ml for 3 weeks. After 3 weeks, transfected cells were incubated in the presence of 500 μM Ca^{2+} and 20 μM of ionomycin for 30 min, washed, and resuspended in complete media. This maneuver stimulates a significant rise in $[Ca^{2+}]_i$, and only cells with functional exchangers will be able to lower $[Ca^{2+}]_i$ sufficiently to survive (Ca^{2+} killing) (13). This process was repeated every 3 days to enrich the population of OK-PTH cells that express functional NCX.

Immunoblotting withh P2X receptor channel isoform and NCX -Specific antibodies

Cells were lysed in a buffer containing 10 mM Tris, 0.5 mM NaCl, 0.5% Triton X-100, 50 μg/ml aprotinin (Sigma), 100 μg/ml leupeptin (Sigma), and 100 μg/ml pepstatin A (Sigma) adjusted to pH 7.2-7.4. Twenty microgram (for P2X blots) or 50 μg (for exchanger blots) of protein was run per lane, separated on 8% SDS-polyacrylamide gels, and then transferred to PVDF membranes (Osmonics). Immunoblotting was performed with rabbit polyclonal antibodies to P2X1, P2X2, P2X4, and P2X7 at a dilution of 1:500 (Alomone Laboratories, Jerusalem, Israel), with anti-P2X5 at 1:1000 dilution (a generous gift of Drs. Mark Voigt and Terry Egan, St. Louis University, St. Louis, MO) or with mouse monoclonal NCX antibody with a dilution of 1:5000 (SWant,

Bellizona, Swizerland). For the P2X blots, reactivity was detected by horseradish peroxidase- labeled goat anti-rabbit secondary antibody (New England BioLabs) at a dilution of 1:3000; reactivity for the exchanger blots was detected by horse radish peroxidase labled goat anti-mouse secondary antibody. ECL chemiluminescence was used to visualize the secondary antibody.

Exchanger activity measurement.

Non-transfected (NT) OK-PTH cells or transfected OK-PTH cells expressing RNCX1 (ROK) or SNCX1 (SOK) were grown to 80% confluency in 100 mm cell culture dishes with MEM media supplemented with 10% Fetalclone III and 82 μg/ml penicillin/streptomycin. Cells were harvested with a cell scraper and incubated in media containing 24 μM fura 2-AM (TEF Labs) for 1 h at 37°C to allow loading of the dye into cells. Fura 2-loaded cells were pelleted at 700 x g for 3 min and resuspended in Ringer's solution (150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 5 mM D-glucose, 1.5 mM $CaCl_2$, 10 mM HEPES).

A small quantity of cells was then transferred to a chamber that was mounted on an inverted microscope. After several minutes, OK-PTH cells would settle to the bottom of the chamber and adhere to the coverslip that formed the chamber's bottom. After 5 to 10 min, it was possible to perfuse the chamber even at high flow rates with the cells remaining attached to the coverslip. Single cell $[Ca^{2+}]_i$ measurements were performed by using dual-excitation wavelength fluorescence microscopy (Photon Technologies) with a Leitz compact photometer that had been converted to perform photon counting. An adjustable photometery window was placed over a single cell with magnification of

400 X using an Olympus X40 UVFL lens. Excitation wavelengths were set at 340 nm and 380 nm and alternated at 25 Hz. Emission wavelength was set at 510 nm, with data collection at a rate of 5 points/s using PTI software. Background corrections were made prior to the experimental measurements.

Baseline fura 2 ratios were measured for at least 100 s in cells that were bathed in Ringer's solution at a rate of 1.7 ml/min. Cells were discarded if the baseline drifted either up or down. After obtaining a stable baseline reading, ATP at a concentration of either 100 μM or 1 mM ATP was added to the chamber at a rate of 1.7 ml/min to elevate $[Ca^{2+}]_i$. Fura 2 ratio was monitored continuously before, during, and after addition of ATP until the ratio returned to a stable baseline. All solutions had pH of 7.4 with temperature maintained at 37°C. In addition, there was no evidence of dye leakage throughout the experiment.

The effect of PKC down-regulation on exchanger activity

Previous studies have shown that PKC plays an important role in the activation of NCX. To examine the role that PKC might play in the regulation of RNCX1 or SNCX1, ROK and SOK were treated with 300 nM PMA for 24 h to down-regulate PKC prior to single cell $[Ca^{2+}]_i$ measurements. PMA was added directly to the culture media, and cells were maintained in the incubator throughout the 24-h period.

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

Calibrations were performed to convert fura 2 ratios into $[Ca^{2+}]_i$ values. $[Ca^{2+}]_i$ was calculated by using the equation described by Grynkiewicz *et al.* (14).

$$[Ca^{2+}]_i = K_d \times (S_{f2}/S_{b2}) \times (R-R_{min})/(R_{max}-R)$$

where K_d is the effective dissociation constant of fura 2 and has a value of 224 nM; R is the fluorescence ratio obtained at 340 nm/380 nm; R_{min} and R_{max} are the ratios in absence and presence of Ca^{2+}, respectively; and S_{f2} and S_{b2} are the emissions at 380 nm in the absence and presence of Ca^{2+}, respectively. NT, ROK, or SOK cells were loaded with 24 μM fura 2 for 1 h, followed by resuspension in Ringer's solution. Calibration was accomplished after permeabilizing the cells with 5 μM ionomycin and measuring fluorescence at both wavelengths, 340 and 380 nm, under Ca^{2+}-free (in 2 mM EGTA) or Ca^{2+}-saturated (in 0.25 M $CaCl_2$) conditions to obtain R_{min}, R_{max}, S_{f2}, and S_{b2}.

RESULTS

SNCX1 and RNCX1 exchanger levels

The levels of protein expression in ROK, SOK and NT cells were evaluated using immunoblot analysis, and the results shown in Figure 1. There was no appreciable detection of exchanger protein in NT cells, although it is not possible to rule out low levels of NCX expression completely. As reported in our earlier study, however, OK-PTH cells did not functionally express the exchanger as evaluated by the reverse mode of the exchanger using $^{45}Ca^{2+}$-uptake assays (2). The important point is that the expression of exchanger protein did not differ between ROK and SOK cells.

Baseline $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured under baseline conditions in NT and in OK-PTH cells transfected with RNCX1 or SNCX1. The results are presented in Figure 2 and demonstrate that the expression of NCX in OK-PTH cells resulted in a significant

reduction in $[Ca^{2+}]_i$. Baseline $[Ca^{2+}]_i$ was 91 ± 23 nM in NT cells and was 44 ± 5 nM and 47 ± 13 nM in ROK and SOK cells, respectively. Thus the presence of the NCX lowered resting $[Ca^{2+}]_i$, but there was no difference in baseline $[Ca^{2+}]_i$ between these two NCX isoforms.

$[Ca^{2+}]_i$ buffering capacity

The NCX is a Ca^{2+}-efflux pathway that contributes to the maintenance of low levels of $[Ca^{2+}]_i$. The rationale for this series of experiments was to perfuse a relatively low concentration of ATP (100 μM) and to assess the ability of the exchanger to extrude this ATP-mediated Ca^{2+} influx and mobilization. The concentration of ATP and the rate of perfusion were selected based on preliminary experiments that provided the optimal separation of Ca^{2+} transients between NT and ROK cells. As shown in the example in Figure 3A and in the summary in Figure 3B, the largest Ca^{2+} transients in response to ATP administration were observed in NT cells; i.e. $[Ca^{2+}]_i$ increased by 182 ± 52 nM. Thus, this group demonstrated the poorest Ca^{2+} buffering capacity in response to ATP. Both SOK and ROK cells were capable of maintaining lower levels of $[Ca^{2+}]_i$ with ATP administration. However, the increase in $[Ca^{2+}]_i$ of 72 ± 15 nM in the SOK group with ATP administration was significantly greater than the 19 ± 7 nM elevation in $[Ca^{2+}]_i$ obtained in the ROK group. Thus, OK-PTH cells expressing the RNCX1 clone had a greater ability to buffer changes in $[Ca^{2+}]_i$ when compared to cells expressing the SNCX1 clone. The expressions of RNCX1 and SNCX1 were comparable in the two cell lines

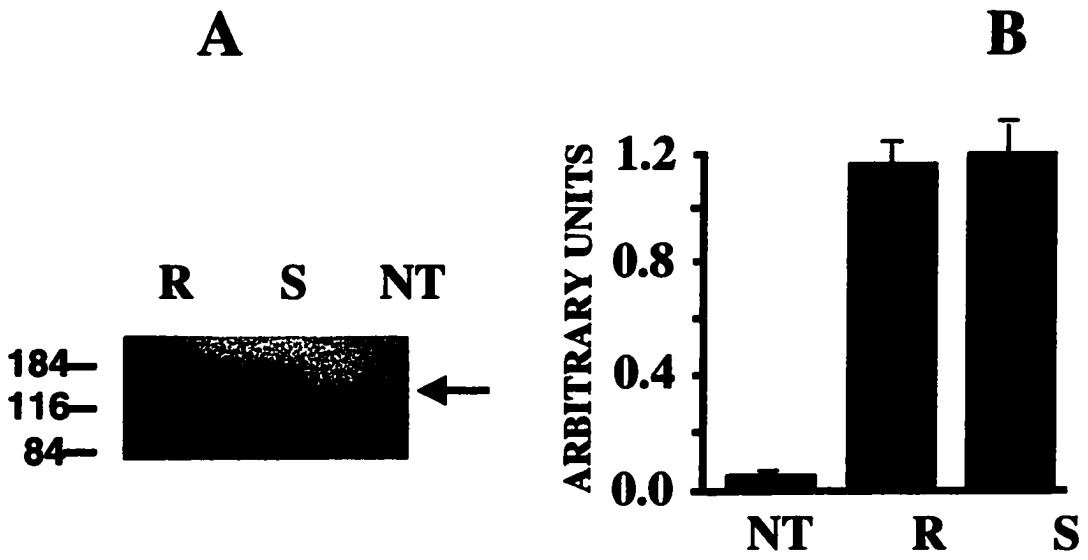


Figure 1. Stably transfected OK-PTH cells express comparable levels of RNCX1 and SNCX1 proteins. Cell lysate from OK-PTH cells expressing RNCX1 (ROK) or SNCX1 (SOK) was isolated as described in Methods, fractionated on an 8% SDS polyacrylamide gel, transferred to Immobilon and immunodetected with a monoclonal Na^+/Ca^{2+} exchanger antibody. (**A**) Western blot. (**B**) Protein expression as arbitary units.

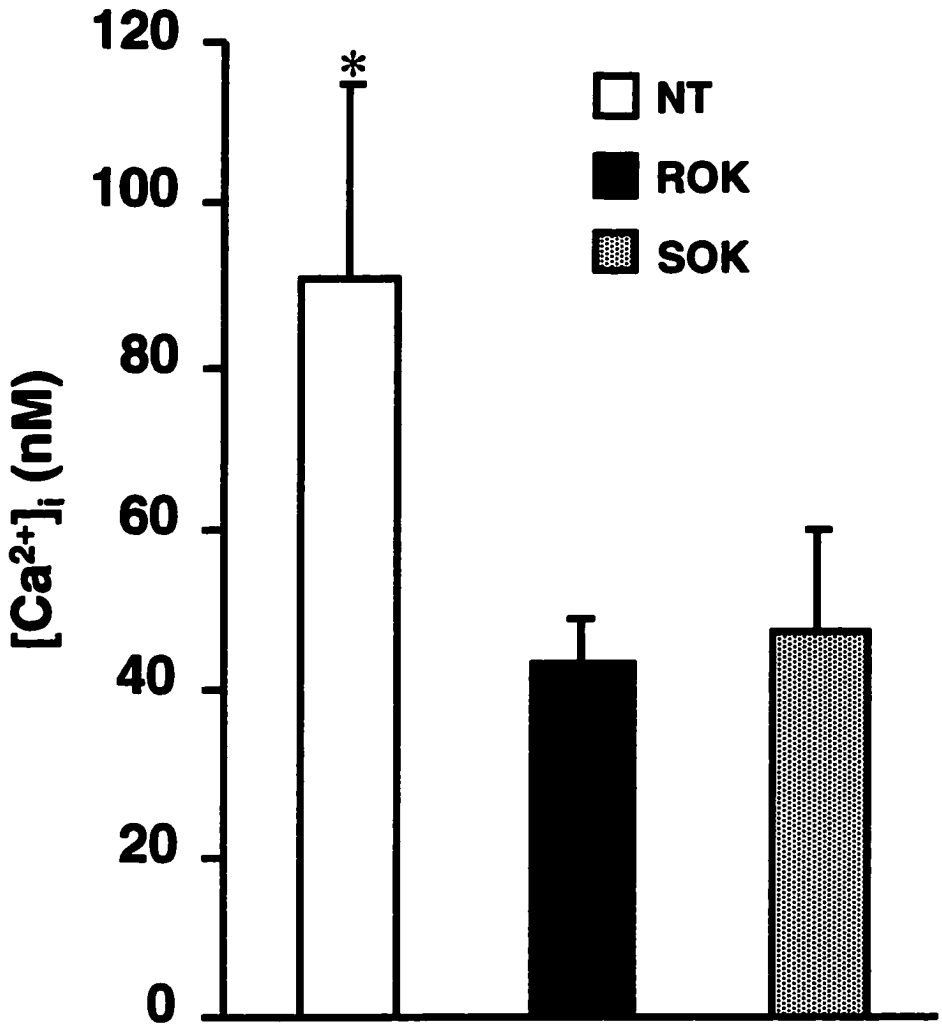


Figure 2. Baseline $[Ca^{2+}]_i$ is higher in non-transfected (NT) OK-PTH cells than in OK-PTH cells expressing RNCX1 or SNCX1. Baseline $[Ca^{2+}]_i$ in NT and transfected OK-PTH cells expressing RNCX1 (ROK) and SNCX1 (SOK) was measured using fura 2. Baseline $[Ca^{2+}]_i$ was significantly higher in NT cells than in ROK and SOK cells. Data were analyzed for statistical significance by using ANOVA. Means ± S.E.M. (n = 5 for NT and 9 for ROK and SOK). $^*p < 0.05$ compared with NT .

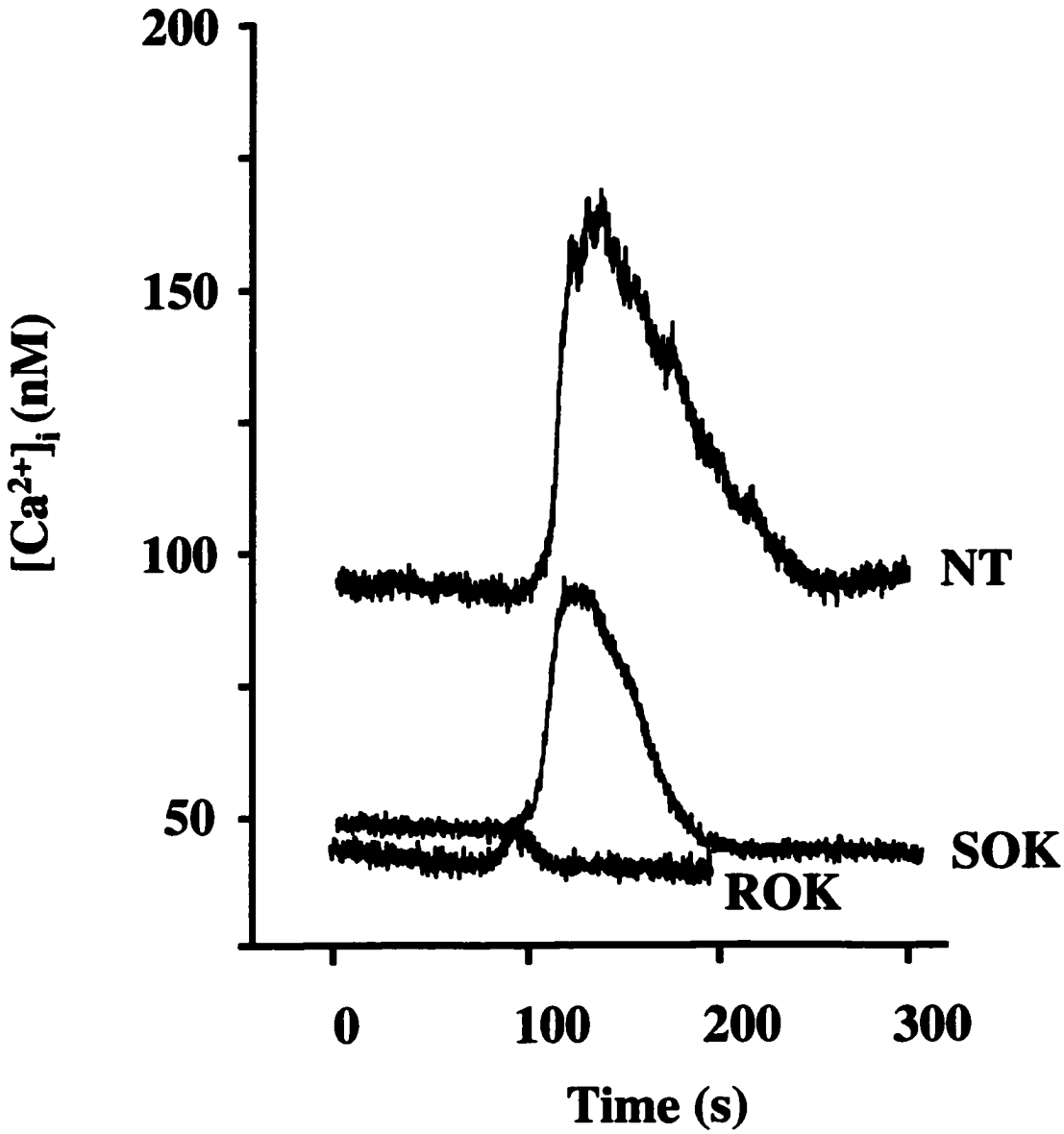
(Fig. 1), so that it is highly unlikely that the difference in their abilities to buffer ATP-induced changes in $[Ca^{2+}]_i$ was caused by differences in the expressions of these exchangers.

$[Ca^{2+}]_i$ recovery rate and time

The ability of the exchanger to buffer changes in $[Ca^{2+}]_i$ is one measure of NCX activity. Another means of assessing exchanger activity is to measure the initial rate of return and the total time that is required for $[Ca^{2+}]_i$ to approach baseline levels after agonist-induced increases in $[Ca^{2+}]_i$. Obviously, there are several Ca^{2+} extrusion/sequestering mechanisms; however, by comparing the responses of OK-PTH cells expressing RNCX1 versus SNCX1, it is possible to determine if there are differences in the rate of Ca^{2+} extrusion by these two isoforms. For these experiments, ATP was used at a concentration of 1 mM, which, in preliminary experiments, was found to produce rapid and maximal increases in $[Ca^{2+}]_i$. Presumably, at this concentration of ATP, the magnitude of Ca^{2+} influx and/or mobilization was sufficient to overwhelm the cellular extrusion/sequestering mechanisms. As shown in Figure 4A and summarized in Figures 4B and C, the presence of RNCX1 resulted in a significantly greater rate of return of $[Ca^{2+}]_i$ after the administration of 1 mM ATP when compared to cells expressing SNCX1. This increased rate of return can be seen in the example (Fig. 4A) but is also statistically significant when plotted as the initial rate of Ca^{2+} recovery (Fig. 4B) or as the time required for $[Ca^{2+}]_i$ to approach baseline levels.

Figure 3. OK-PTH cells expressing RNCX1 (ROK) showed greater ability to buffer ATP-induced $[Ca^{2+}]_i$ increase when compared to non-transfected (NT) and transfected OK-PTH cells expressing SNCX1 (SOK). The abilities of ROK and SOK to buffer ATP-induced $[Ca^{2+}]_i$ increase were assessed in NT, ROK, and SOK following treatment with 100 μM ATP to elevate $[Ca^{2+}]_i$. (**A**) Representative $[Ca^{2+}]_i$ tracings of NT, ROK, and SOK in response to 100 μM ATP. (**B**) Bar graph showing $\Delta[Ca^{2+}]_i$ in NT, ROK, and SOK. ROK and SOK had lower $\Delta[Ca^{2+}]_i$ or greater capacity to buffer $[Ca^{2+}]_i$ when compared to NT, with ROK having the greatest $[Ca^{2+}]_i$ buffering ability. Data were analyzed for statistical significance using ANOVA. Means ± S.E.M. (n = 9 for NT, n = 12 for SOK, and n = 12 for ROK). $^*p < 0.001$ when compared with NT .

A



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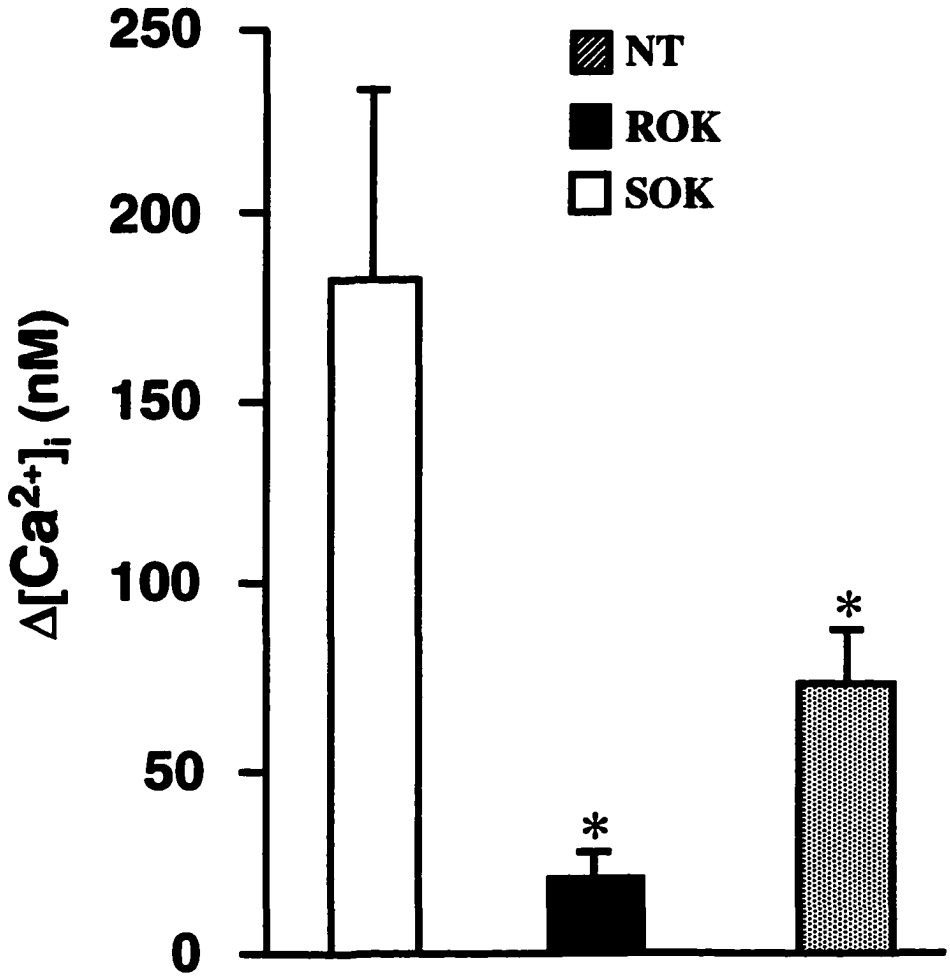
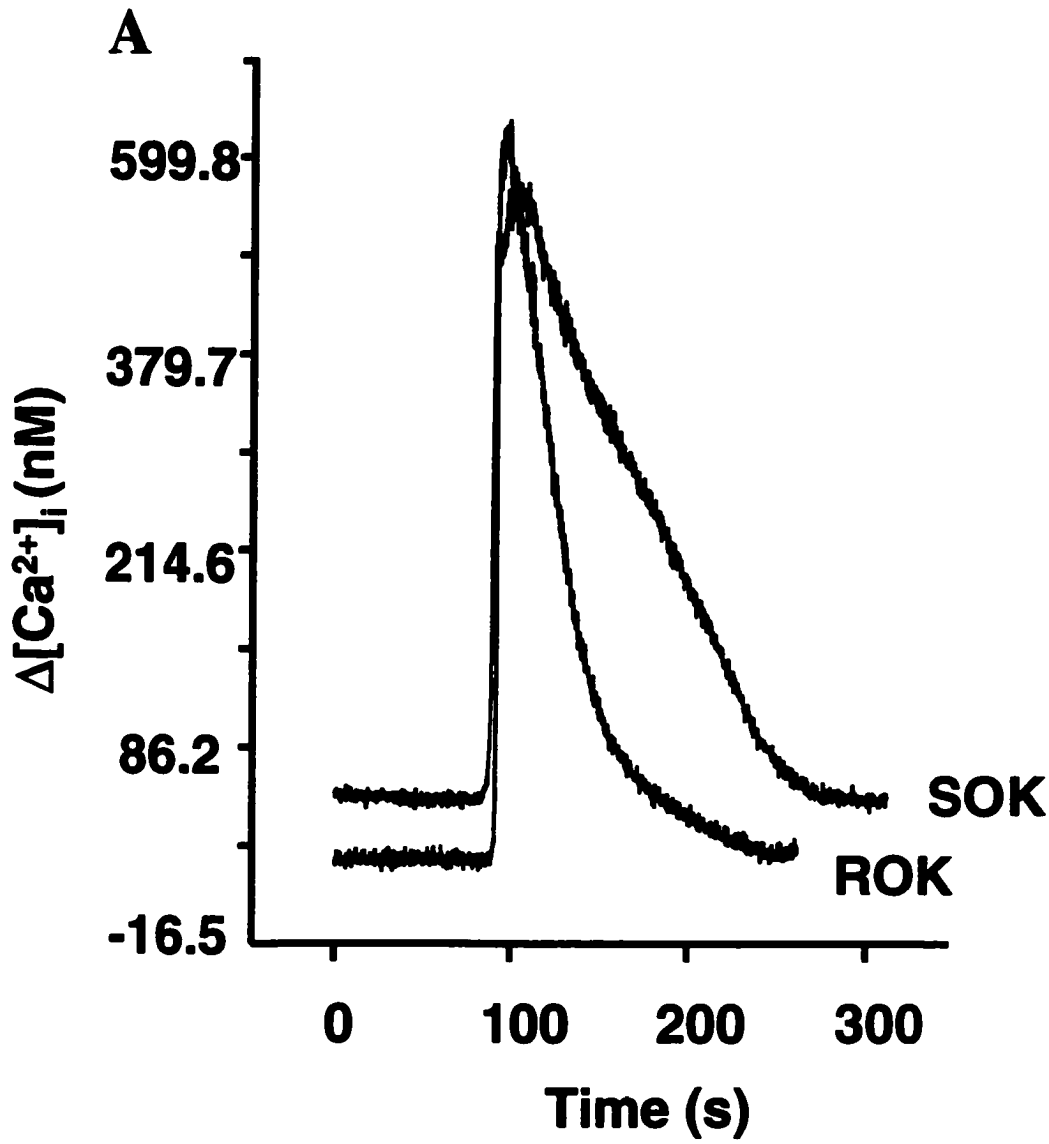
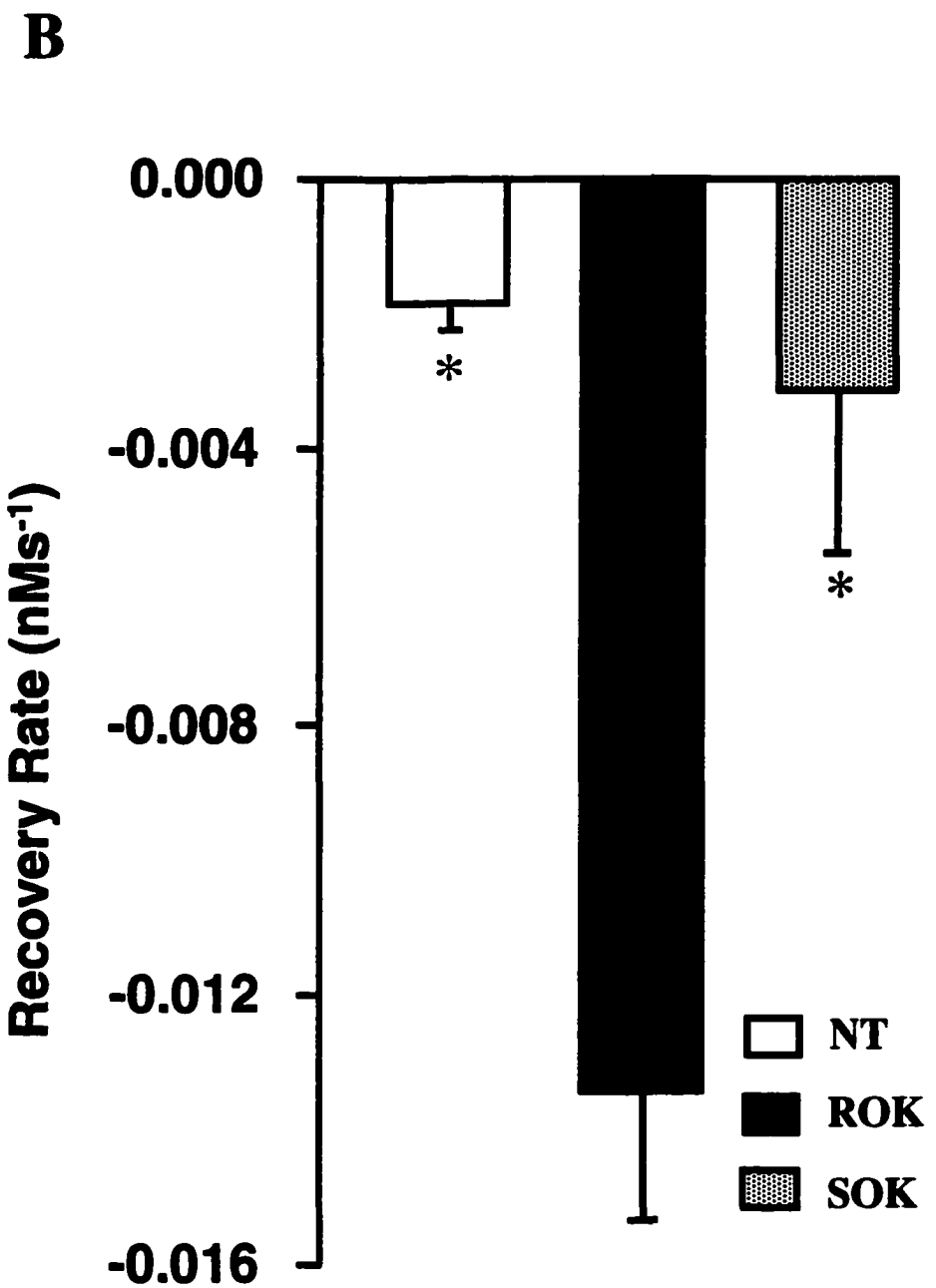


*** : P < 0.05 compared to NT**

Figure 4. OK-PTH cells expressing RNCX1 (ROK) reduced ATP-induced $[Ca^{2+}]_i$ increase at a significantly greater rate than cells expressing SNCX1 (SOK). The abilities of ROK and SOK to reduce ATP-induced $[Ca^{2+}]_i$ increase back toward baseline were assessed in fura 2-loaded ROK and SOK following treatment with 1 mM ATP to elevate $[Ca^{2+}]_i$. (**A**) Representative $[Ca^{2+}]_i$ tracings of ROK and SOK in response to 1 mM ATP. (B, C) Bar graphs showing $[Ca^{2+}]_i$ recovery rates. (**B**) And times. (**C**) In ROK and SOK. Data were analyzed for statistical significance by using ANOVA. Means ± S.E.M. (n = 12 for SOK and n = 12 for ROK). $^*p < 0.05$ when compared with NT .





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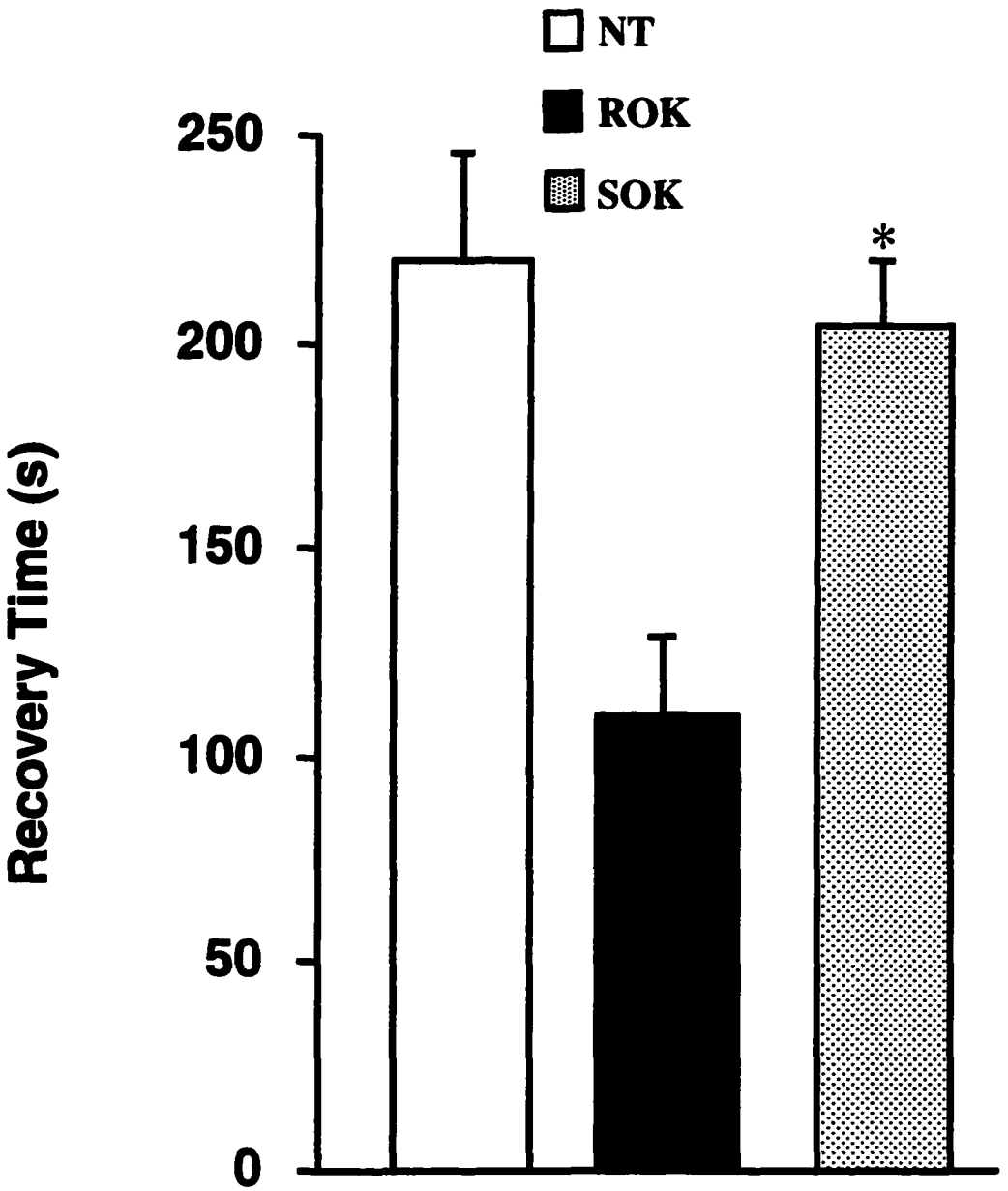

PKC regulation

Previous studies have shown that basal exchanger activity is similar between RNCX1 and SNCX1 isoforms and that PKC activation leads to enhanced exchanger activity in OK-PTH cells that expressed RNCX1 but not in SNCX1 (2). Because, in the presence of extracellular ATP, exchanger activity was higher in ROK cells when compared to SOK cells, this difference could be caused by ATP activation of PKC. To test this possibility, ROK and SOK cells were either left untreated or pretreated with 300 nM PMA for 24 h, a maneuver that is known to down-regulate PKC activity. As shown in Figures 5A-C, there is a distinct difference in the effects of PKC down-regulation in ROK versus SOK cells. As shown in Figure 5A, the rate of return of $[Ca^{2+}]_i$ in ROK cells after administration of ATP was dramatically slowed down with PKC inhibition; however, the rate of return of $[Ca^{2+}]_i$ in SOK cells (Fig. 5B) was not greatly affected by prior treatment with PMA. As shown in the summary in Figure 5C there was a highly significant decrease in the rate of Ca^{2+} recovery with PKC down-regulation in ROK but not in SOK cells.

DISCUSSION

The NCX is a 120 kDa transmembrane protein that consists of five N-terminal and four C-terminal membrane-spanning domains and a cytosolic loop that comprises over 50% of the protein (15). There are at least four different NCXs encoded by separate genes (16-18). To date, only NCX1 (which is also the cardiac isoform) has been demonstrated to occur in the tubules (19, 20), blood vessels (1, 21), and MCs of the kidney (1-3, 22). RNCX1 and SNCX1 were previously cloned from MCs of the S and R

rat, respectively (2). These clones are 100% homologus at the C-terminal membrane-spanning domains and nearly identical in the N-terminal membrane-spanning domains, except for a single amino acid transversion at position 218, where the amino acid is isoleucine in RNCX1 and phenylalanine in SNCX1. It should be noted that all other NCX1 isoforms that have been cloned have isoleucine at this site. Both RNCX1 and SNCX1 are also highly homologous in the large cytoplasmic loop, except at the alternative splice site where RNCX1 is encoded by exons B and D, whereas SNCX1 is encoded by exons B, D, and F. Thus, these differences in amino acid sequence may cause the lack of PKC sensitivity of the NCX in AAs and cultured MCs from the S rat. This PKC sensitivity of RNCX1 but not SNCX1 could also be clearly demonstrated when these two isoforms were cloned into OK-PTH cells.

In previous studies, exchanger activity in OK-PTH cells expressing RNCX1 and SNCX1 was examined by using a $^{45}Ca^{2+}$-influx assay that involves Na^+ loading the cells, followed by extracellular Na^+ removal. This maneuver stimulates the reverse-mode Na^+:Ca^{2+} exchange (Na^+enters and Ca^{2+} exits the cell), which is opposite of how the exchanger normally operates under physiological conditions. Nevertheless, these studies indicated that 1) NT OK-PTH cells had virtually no exchanger activity 2) basal exchanger activity was similar in ROK and SOK cells 3) RNCX1 activity increased with acute PMA treatment, and 4) SOK exchanger activity was insensitive to PKC activation or PKC down-regulation by 24 h pre-treatment with PMA. However, these studies did not provide insights into whether this difference in PKC sensitivity would have any consequences regarding the role of these exchangers in the regulation of $[Ca^{2+}]_i$.

It has been suggested that Na^+:Ca^{2+} exchange is involved in the extrusion of Ca^{2+} after agonist-induced elevations in $[Ca^{2+}]_i$ (23). This issue was addressed in a recent

Figure 5. PKC down-regulation attenuates the ability of cells expressing RNCX1 to reduce ATP-induced $[Ca^{2+}]_i$ back to baseline level. The effect of PKC down-regulation on $[Ca^{2+}]_i$ recovery rate in response to 1 mM ATP in ROK (**A**) And SOK. (**B**) Was examined in fura 2- loaded cells following ATP treatment with or without 24 h PMA (300 nM) pretreatment to down-regulate PKC by single cell $[Ca^{2+}]_i$ measurements in comparison with non-PMA-treated cells (CTL). (**C**) Bar graph showing the effect of PKC down-regulation on $[Ca^{2+}]_i$ recovery rate in ROK and SOK. Data were analyzed for statistical significance by using ANOVA. Means ± S.E.M. ($n = 12$). $^*p < 0.05$ when compared with non-PMA-treated cells.

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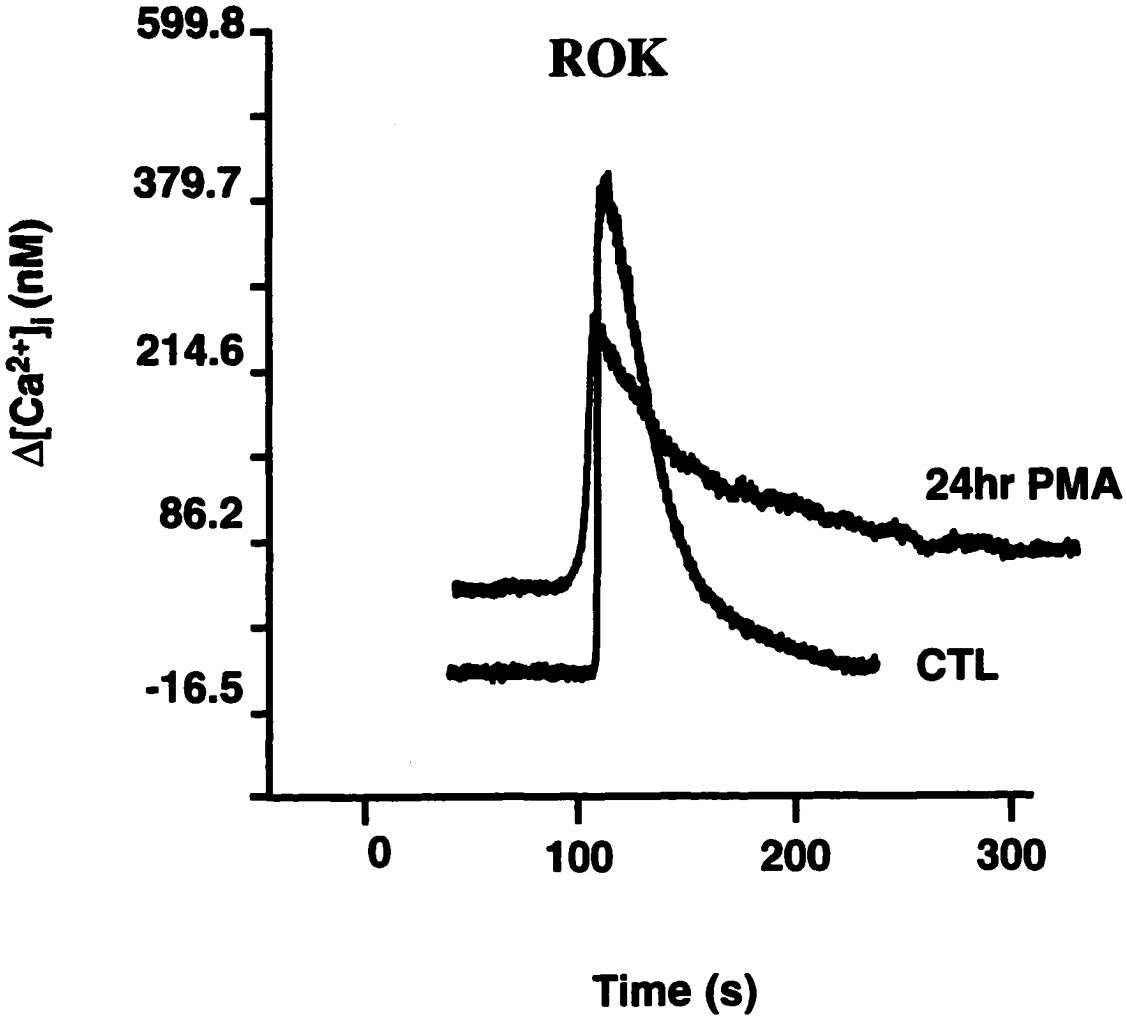

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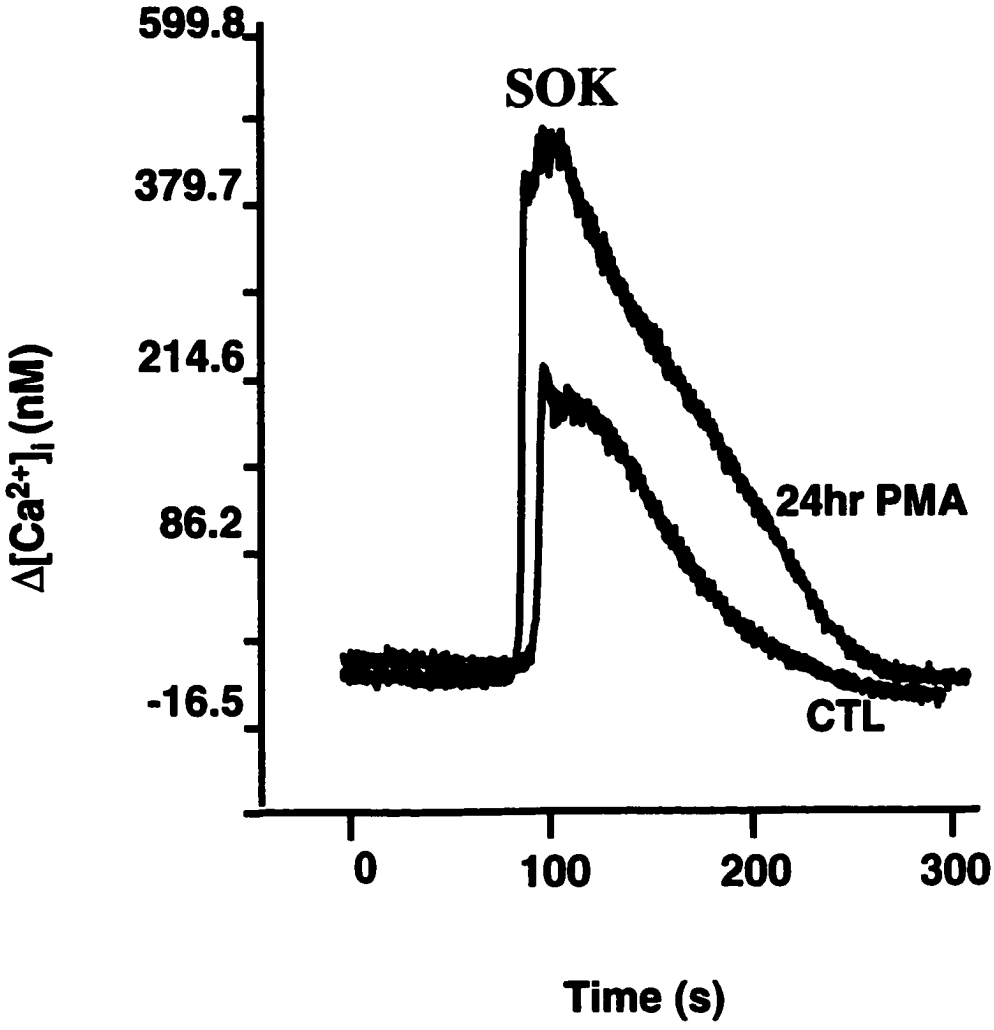

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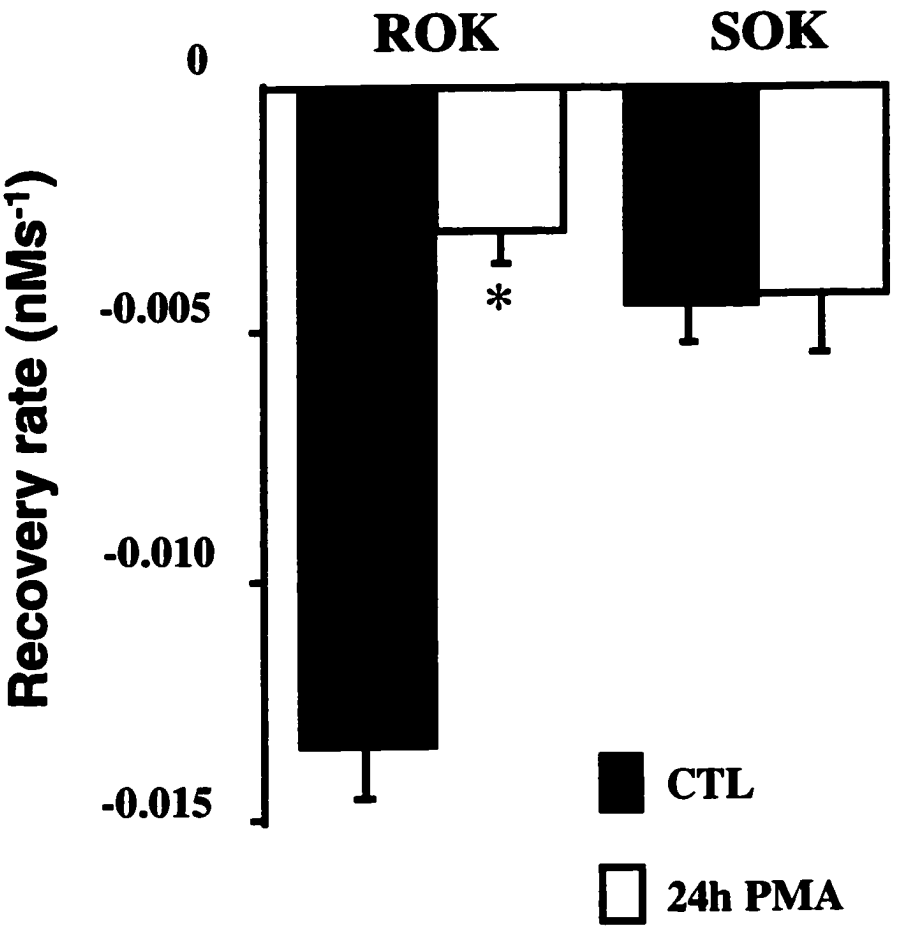

* : P < 0.05 compared to control

study by Slodzinski and Blaustein (24) in which knockdown of exchanger activity was achieved by pre-treatment with antisense oligodeoxynucleotides of NC; these authors found that recovery from elevations in $[Ca^{2+}]_i$ was substantially prolonged in cells that were treated with antisense when compared to control cells. This exchanger-mediated reduction in $[Ca^{2+}]_i$ represents the forward mode of the exchanger and more closely resembles the physiological role of Na^+:Ca^{2+} exchange in $[Ca^{2+}]_i$ homeostasis. In the present study, we used a similar approach, except that we compared the regulation of $[Ca^{2+}]_i$ in cells that were either non-transfected or transfected with the RNCX1 or SNCX1 clone. In this manner, we could directly determine if there were differences in $[Ca^{2+}]_i$ regulation between RNCX1 and SNCX1.

Initial studies were performed to measure baseline $[Ca^{2+}]_i$ in these three cell types. We found that cells expressing NCX isoforms had lower baseline $[Ca^{2+}]_i$ when compared to NT cells. $[Ca^{2+}]_i$ regulation is complex and is the result of an ensemble of receptors, channels, and transporters located at plasma and intracellular membrane sites. Nevertheless, the notion has been that at the plasma membrane, Ca^{2+}-ATPase, a high-affinity and low-capacity Ca^{2+}-extrusion mechanism, was important for setting the resting $[Ca^{2+}]_i$, whereas NCX, a low-affinity and high-capacity Ca^{2+}-extrusion mechanism, primarily extruded Ca^{2+} when $[Ca^{2+}]_i$ was elevated (23,25,26). Our studies indicate that NCX does contribute to the non-stimulated level of $[Ca^{2+}]_i$, at least in this cell type and under these conditions.

In most studies of agonist-induced alterations in $[Ca^{2+}]_i$, the initial paradigm is to invoke Ca^{2+} transients by rapidly applying sufficient hormone or agent to achieve a

maximal $[Ca^{2+}]_i$ spike. In the present studies, we wanted to examine the ability of the exchanger clones to handle a submaximal stimulus that would cause Ca^{2+} entry and mobilization. As shown in Figure 1, OK-PTH cells do express P2 receptors for ATP. We found that a concentration of 100 μM ATP produced optimal discrimination between the results obtained in the NT group versus those obtained in ROK cells. In other cell types, 100 μM ATP may produce maximal effects; however, this possibility may depend on, among other things, the number of P2 receptors that are on the cell membrane. The important point is that during this slow infusion of ATP, cells expressing NCX were much better able to prevent or minimize the increase in $[Ca^{2+}]_i$. Also, a major finding of this study is that ROK cells were much better at buffering changes in $[Ca^{2+}]_i$ when compared to SOK cells. Because these cell lines express comparable levels of NCX (Fig. 2), this evidence directly supports the notion that the SNCX1 isoform is not as efficient or as effective in regulating $[Ca^{2+}]_i$.

Other studies were performed to assess the ability of RNCX1 and SNCX1 to extrude Ca^{2+} after maximal agonist-induced increases in $[Ca^{2+}]_i$. Similarly to what was observed in the preceding experiments, the rate at which Ca^{2+} declined after administration of 1 mM ATP was slower and took longer to occur in SOK cells versus ROK cells. These results further support our conclusion that SNCX1 is much less effective in regulating agonist-induced alterations in $[Ca^{2+}]_i$.

Currently, the role that PKC plays in the activation of the NCX is not clearly understood. Our laboratory has demonstrated that PKC activates NCX in the renal microcirculation of rabbits and normotensive rats (8) and in the cloned exchanger RNCX1 (2). Also, work by Vigne *et al.* (27) found phorbol ester stimulation of the

exchanger in cultured aortic smooth muscles. However, other studies in human MCs by Mene *et al.* (28) failed to demonstrate enhanced reverse-mode Na^+:Ca^{2+} exchanger. In studying the regulation of NCX1, 2, and 3 Linck *et al.* (29) found that NCX1 and 3 showed modest stimulation by both PKA and PKC agonists. Exchanger activity appeared to be much more sensitive to PMA down-regulation. Finally, Iwamoto *et al.* (32) demonstrated that activation of PKC by agonists induced enhanced exchanger activity and phosphorylation of NCX1 (30-32), which occurred exclusively on serine residues. Mutational analysis, however, indicated that activation of the exchanger did not require direct phosphorylation of serine residues by PKC but did require the presence of the cytosolic loop (30). Thus, the bulk of the current evidence favors PKC activation of NCX1; however, the mechanism by which this activation occurs remains unclear. Our laboratory has proposed that PKC may induce translocation of the exchanger, but whether translocation is responsible for enhanced exchanger activity has also not been established.

The role of PKC in enhancing exchanger activity may be important based on the following scheme. The binding of a vasoactive hormone to its receptor triggers the activation of phospholipase C (PLC) which hydrolyses phosphatidyl inositol 4,5-bisphosphate (PIP_2) and elevates diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to its receptor on the endoplasmic reticulum (ER) and stimulates the release of Ca^{2+}, which thereby initiates the rise in $[Ca^{2+}]_i$. With an elevation of $[Ca^{2+}]_i$ and DAG, there is activation of PKC, which, in turn, enhances Na^+:Ca^{2+} exchange. Thus, PKC increases the rate at which the NCX extrudes Ca^{2+} in exchange for Na^+ thereby reducing $[Ca^{2+}]_i$ back to baseline levels.

In the present studies, this scenario was, in part, tested by 24 h pre-treatment with PMA, a maneuver that is known to down-regulate PKC activity. Studies in other cell systems (33) have shown that extracellular ATP via P2 receptors does increase PKC activity. We demonstrated that inhibition of PKC activity greatly affected the ability of ROK cells to rapidly return $[Ca^{2+}]_i$ to control levels. In contrast, PKC inhibition did not affect the rate of return in $[Ca^{2+}]_i$ in SOK cells. It should be mentioned that the Ca^{2+} transients obtained in the presence of PKC inhibition were much more labile and variable when compared to cells that were not treated with PMA, a finding that may reflect the fact that PKC has multiple cellular effector sites, including effects on other cellular Ca^{2+} regulatory processes. Nevertheless, these results are in agreement with our previous work examining reverse-mode Na^+:Ca^{2+} exchange in ROK and SOK cells (2) and extend this previous work by suggesting that this difference in PKC sensitivity may have important consequences in terms of the cellular regulation of $[Ca^{2+}]_i$.

As indicated, SNCX1 and RNCX1 differ at amino acid 218 and at the alternative splice site. Which of these differences is responsible for the lack of PKC sensitivity of SNCX1 is, at present, unknown. Future mutational analysis is needed to determine which of these two sites confers PKC insensitivity to the SNCX1 clone.

In conclusion, we have found that an isoform of the NCX, cloned from MCs of the S rat, has an impaired ability to regulate agonist-induced changes in $[Ca^{2+}]_i$. The cause of this reduced efficiency in Ca^{2+} extrusion appears to be a defect in PKC activation of SNCX1. We suggest that this reduced ability of the NCX to regulate $[Ca^{2+}]_i$ in the renal microcirculation may, in part, be responsible for the increased vascular resistance and reduced GFR that are hallmarks of this form of hypertension.

ACKNOWLEDGMENTS

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SUMMARY

In previous studies, we found functional evidence for the existence of NCX activity in AAs (21). Our work demonstrated that the exchanger may serve an important role in controlling $[Ca^{2+}]_i$ in these important renal resistance vessels. Other work from our laboratory confirmed the existence of the exchanger in cultured MCs (38). There has been considerable interest in altered regulation of $[Ca^{2+}]_i$ in genetic forms of hypertension and Blaustein *et al.* (4,13) suggested that the NCX might be involved in the pathogenesis of hypertension. Therefore, we investigated renal arteriolar exchanger activity in several forms of hypertension (23,38). We found that, in salt-sensitive hypertension, AA and MC basal exchanger activity was similar in S and R rats. However, PKC stimulation of exchanger activity was remarkably different between these two strains of rats. PKC clearly enhanced exchanger activity in AAs and MCs from R rats but not from S rats. Also, we found that PKC stimulated translocation of the exchanger from a cytosolic pool to the plasma membrane in MCs from R but not from S rats. The exchanger was then cloned from S and R MCs and we found that these two isoforms differed at one amino acid (218), in transmembrane-spanning domain 4, and at the alternative splice site (56). When expressed in OK-PTH cells, RNCX1 but not SNCX1 was PKC sensitive. Two questions that remained, at this point, were what isoform(s) of the exchanger were expressed in the parental strain that was used to derive the S and R rat, and was this isoform sensitive to PKC.

The S and R rats were both derived from the SD rat. The first article involved characterization of the exchanger isoform(s) expressed in MCs of SD rats. Our studies indicate that MCs from SD rats express four NCX isoforms. The R isoform, RNCX1, and the S isoform, SNCX1, are both expressed in MCs of SD rats and are designated SDRNCX1 and SDSNCX1, respectively. RNCX1 and SDRNCX1 are identical at every amino acid residue. SNCX1 and SDNCX1, however, differ at amino acid 218, where the amino acid is isoleucine in SDSNCX1 but is phenylalanine in SNCX1. The other two SD isoforms differ from RNCX at the alternative splice site, where one isoform is encoded by exons B, D, E, and F and the other is encoded by exons B, C, D, E, and F and are designated SDNCX1.10 and SDNCX1.11, respectively. SDNCX1.10 encodes a protein that consists of 962 amino acids and has a high degree of homology with two partially sequenced isoforms, designated NCX1.10 (54), which were isolated and sequenced from odontoblasts of SD rats and osteoblast-like osteosarcoma of humans. The presence of NCX 1.10 was also confirmed by homology-based RT-PCR of the alternative splice site in rat osteoblast-like UMR 106 cells, skeletal muscle of neonatal rats, and astrocytes (54).

The significance for the co-expression of the four alternatively spliced NCX isoforms in MCs of SD rats is not known. However, this co-expression has been observed previously in rat heart, where, through homology-based RT-PCR, it was found that cardiac myocytes expressed NCX1.1, as the major isoform, and NCX1.3 and NCX1.4 as minor isoforms (62,63). In cardiac fibroblasts, NCX1.3 and NCX1.4 are the major isoforms expressed, whereas NCX1.12 was expressed as the minor isoform. Also, the expression of NCX1 shows tissue specificity (54), where isoforms containing exon A were found exclusively in the heart, brain, and skeletal muscle. Isoforms containing exon B were found in non-neuronal and non-cardiac tissues, with isoforms containing B and D

and B, D, and F at the alternative splice being the predominant isoforms. Therefore, SD MCs express four different isoforms of NCX1, all of which contain exon B and various combinations of the other exons. At this time, it is not clear which of the isoform(s) is the most abundant or which play(s) the major physiological role in controlling $[Ca^{2+}]_i$. In the first study, we chose to determine if SDNCX1.10 could function as a NCX.

The NCX is an important regulator of $[Ca^{2+}]_i$. This function is critical in all cells but especially in contractile cells such as MCs. We assessed the ability of SDNCX1.10 to regulate $[Ca^{2+}]_i$ by using OK-PTH cells expressing SDNCX1.10 or the vector alone. Using forward- and reverse-mode exchanger techniques, we showed that SDNCX 1.10 was a functional NCX. Also, SDNCX1.10 regulated ATP-induced $[Ca^{2+}]_i$ through PKC. These results are consistent with the notion that the renal exchanger isoforms are PKC sensitive and that the lack of PKC sensitivity suggests an abnormality in functional activity.

Regulation of the NCX by Ca^{2+}, pH, and phosphorylation has been extensively studied by using the cardiac isoform NCX1.1 which consists of exons A, C, D, E, and F at the alternative splice site (7,33). However, regulation of the various isoforms of NCX1, including those that are expressed in renal tissues, is poorly understood. Therefore, the second article assessed and compared the effects of intracellular Ca^{2+} and pH and phosphorylation by PKA and PKG on the regulation of the renal NCX isoforms RNCX, SNCX, and SDNCX1.10.

Control of NCX activity by $[Ca^{2+}]_i$ is accomplished by the binding of low levels of Ca^{2+} (100-300 nM for giant patches or 20-50 nM for whole cells) to a regulatory region of the cytosolic domain, corresponding to amino acids 371-507 (64-66). The binding of Ca^{2+} alters the conformation of the exchanger, thus affecting the exchange rate

of Ca^{2+} for Na^+. In the present studies, the effect of $[Ca^{2+}]_i$ on RNCX, SNCX, or SDNCX activities was assessed by increasing or decreasing $[Ca^{2+}]_i$ with thapsigargin and BAPTA; reverse-mode exchanger studies were then performed by assessing $^{45}Ca^{2+}$ uptake in exchange for Na^+ in Na^+-loaded cells.

Increasing $[Ca^{2+}]_i$ in OK-PTH cells expressing RNCX, SNCX, or SDNCX 1.10 from ~50 nM to 95 nM with thapsigargin reduced the activity of SNCX and SDNCX significantly but had no effect on RNCX activity. This effect is consistent with previous observations where regulatory Ca^{2+} levels of 25-50 nM (intact cells) or 100-300 nM (giant patches) were required for the initiation of NCX activity. Previous studies in a number of cell lines yielded an EC_{50} of 1-80 nM for thapsigargin-induced Ca^{2+} release from intracellular storage (67); in our work, 40 nM thapsigargin elevated baseline $[Ca^{2+}]_i$ from ~50 nM to 95 nM. At this elevated level of $[Ca^{2+}]_i$, the activities of some NCX isoforms, including those for SNCX and SDNCX, are attenuated. However, the effects of increasing $[Ca^{2+}]_i$ on RNCX activity was different. Elevating $[Ca^{2+}]_i$ to 95 nM with thapsigargin did not inhibit but, rather, stimulated the activity of RNCX. Because the Ca^{2+}-binding region (amino acids 371-507) in these three isoforms is identical, the basis for this discrepancy is not immediately apparent. However, the lack of inhibition of high regulatory Ca^{2+} on NCX activity has been shown previously. Linck *et al.* (33) showed that inside-out vesicles from BHK cells expressing NCX1, NCX2, and NCX3 required regulatory Ca^{2+} concentration of up to 12 μM for optimum activity. Thus, there is a wide range of $[Ca^{2+}]_i$ that leads to regulatory Ca^{2+}-induced decreases in exchanger function. It is possible that $[Ca^{2+}]_i$ levels higher than 95 nM in cells expressing RNCX would have revealed a regulatory Ca^{2+}-induced decrease in exchanger function.

We also found that reducing regulatory Ca^{2+} with 10 μM BAPTA to 45 nM had a mild stimulatory effect on the activities of all three NCX isoforms. Reducing the regulatory calcium level further to 40 nM with 20 μM BAPTA still had a stimulatory effect on the activities of RNCX and SNCX but neither stimulated nor inhibited SDNCX activity. Therefore, reducing regulatory Ca^{2+} below resting levels in these cell lines had no inhibitory effects on RNCX, SNCX, and SDNCX.

NCX activity has also been shown to be sensitive to pH_i. NCX1, NCX2, and NCX3 are all inhibited at acidic pH's, but exchanger activity is increased as pH was raised to 9 (33). Using nigericin to equilibrate pH_i and pH_e, we found that the activity of all three renal isoforms were inhibited by acidic pH's with RNCX and SNCX showing greater sensitivity when compared to SDNCX. The greatest degree of in-hibition was at pH 6.2, with lesser inhibition at pH of 6.8. At pH of 8.0, all three isoforms' exchanger activities were not significantly different from those found at pH 7.4. Without nigericin, the activities of all three isoforms were inhibited at pH 6.2, whereas pH 6.8 inhibited the activities of SNCX and SDNCX but had no significant effect on RNCX activity. pH 8.0 had no effect on the activities of RNCX and SDNCX but attenuated the activity of SNCX. The effect of pH, especially pH_i, on NCX activity is believed to be the result of H^+ and Na^+ competing for similar sites on the exchanger. Hilgemann *et al.* (37) found that Na^+ binding to a site on the exchanger resulted in Na^+ translocation or Na^+-dependent inactivation of the exchanger to a steady-state level. Matsuoka *et al.* (43) found that the inhibitory peptide (XIP) of the exchanger was involved in mediating Na^+-dependent inactivation. The inhibitory peptide region, however, is identical in RNCX, SNCX, and SDNCX and is 100, 74, and 72% identical to the XIP region in NCX1, NCX2, and NCX3, respectively. Therefore, if this region is involved in the interaction of Na^+ with

the exchanger, other factors are likely involved in mediating the effects of pH on the activities of RNCX, SNCX, and SDNCX.

In other work, regulation of these three renal Na^+:Ca^{2+} isoforms by PKA and PKG was assessed using CPT-cAMP and db-cGMP. We found that activation of these two kinases slightly stimulated the activity of SDNCX but not RNCX and SNCX. The basis for this difference is not presently known but may be attributed, in part, to the presence of exon E in SDNCX but not in RNCX and SNCX. He *et al.* (34) have shown that differences by a few amino acid residues in the alternative splice site can have significant effects on activation of NCX by PKA in astrocytes and neurons. Also, Ruknudin *et al.* (68) showed that NCX1.1 was activated by PKA, but NCX1.3 (RNCX) was not. The basis for this difference appeared to be that NCX1.1 was phosphorylated to a greater degree than NCX1.3.

These studies indicate that the three renal isoforms demonstrated different regulation by intracellular Ca^{2+}, pH_i and pH_e, and PKA and PKG. RNCX was fully functional at intracellular calcium levels of 40-95 nM, but SNCX and SDNCX were only fully active within the 40-50 nM range. SNCX showed the greatest sensitivity to variations in pH_e and pH_i, being fully active only at pH 7.4, followed by SDNCX (pH_e or pH_i 7.4 and 8.0) and RNCX (pH_e 6.8, 7.4, and 8.0 and pH_i 7.4 and 8.0). Finally, only SDNCX showed activation by both PKA and PKG.

The S rat model of genetic hypertension mimics this condition in humans in that a high-salt diet leads to the development of hypertension and, over time, to renal failure. Therefore, the third article assessed the abilities of SNCX and RNCX exchanger isoforms to regulate agonist-induced $[Ca^{2+}]_i$ increase. Initial studies were performed to measure baseline $[Ca^{2+}]_i$ in OK-PTH cells expressing these exchanger isoforms. We found that

cells expressing NCX isoforms had lower baseline $[Ca^{2+}]_i$ when compared to non-transfected cells.

The $[Ca^{2+}]_i$ regulation is complex and is the result of an ensemble of receptors, channels, and transporters located at plasma and intracellular membrane sites. Nevertheless, the notion has been that, at the plasma membrane, a Ca^{2+}-ATPase, a high-affinity and low-capacity Ca^{2+}-extrusion mechanism, was important for setting the resting $[Ca^{2+}]_i$, whereas NCX, a low-affinity and high-capacity Ca^{2+} extrusion mechanism, primarily extruded Ca^{2+} when $[Ca^{2+}]_i$ was elevated (4). Our studies indicate that NCX does contribute to the non-stimulated level of $[Ca^{2+}]_i$, at least in this cell type and under these conditions. We next performed studies to assess the ability of RNCX1 and SNCX1 to extrude Ca^{2+} after maximal agonist-induced increases in $[Ca^{2+}]_i$. Similarly to what was observed in the preceding experiments, the rate at which Ca^{2+} declined after administration of 1 mM ATP was slower and took longer to occur in OK-PTH cells expressing SNCX when compared to cells expressing RNCX. These results further support our conclusion that SNCX is much less effective in regulating agonist-induced alterations in $[Ca^{2+}]_i$.

Currently, the role that PKC plays in the activation of the NCX is not clearly understood. As indicated, our laboratory has demonstrated that PKC activates NCX in the renal microcirculation of rabbits and normotensive rats (21,23) and in the cloned exchanger RNCX1. Also, work by Vigne *et al.* (50) found phorbol ester stimulation of the exchanger in cultured aortic smooth muscles. However, other studies in human MCs by Mene *et al.* (8) failed to demonstrate enhanced reverse-mode Na^+:Ca^{2+} exchange. In

studying the regulation of NCX1, 2, and 3, Linck *et al.* (33) found that NCX1 and 3 showed modest stimulation by both PKA and PKC agonists. Exchanger activity appeared to be much more sensitive to PMA down-regulation. Finally, Iwamoto *et al.* (59) demonstrated that activation of PKC by agonist-induced enhanced exchanger activity and phosphorylation of NCX1, which occurred exclusively on serine residues. Mutational analysis, however, indicated that activation of the exchanger did not require direct phosphorylation of serine residues by PKC but did require the presence of the cytosolic loop. Thus, the bulk of the current evidence favors PKC activation of NCX1, but the mechanism by which this occur remains unclear. Our laboratory has proposed that PKC may induce translocation of the exchanger, but whether this translocation is responsible for enhanced exchanger activity has also not been established.

The role of PKC in enhancing exchanger activity may be important based on the following scheme. The binding of a vasoactive hormone to its receptor triggers the activation of phospholipase C (PLC), which hydrolyses phosphatidyl inositol 4,5-bisphosphate (PIP_2) and elevates diacyl glycerol (DAG) and IP_3. IP_3 binds to its receptor on the endoplasmic reticulum (ER) and stimulates the release of Ca^{2+}, which initiates the rise in $[Ca^{2+}]_i$. With an elevation of $[Ca^{2+}]_i$ and DAG, there is activation of PKC which, in turn, enhances Na^+:Ca^{2+} exchange. Thus, PKC increases the rate at which the NCX extrudes Ca^{2+} in exchange for Na^+, thereby reducing $[Ca^{2+}]_i$ back to baseline levels.

In the present studies, this scenario was, in part, tested by 24 h pre-treatment with PMA, a maneuver that is known to down-regulate PKC activity. We demonstrated that inhibition of PKC activity greatly affected the ability of ROK cells to rapidly return $[Ca^{2+}]_i$ to control levels. In contrast, PKC inhibition did not affect the rate of return in

$[Ca^{2+}]_i$ in SOK cells. It should be mentioned that the Ca^{2+} transients obtained in the presence of PKC inhibition were much more labile and variable when compared to cells that were not treated with PMA, a finding that may reflect the fact that PKC has multiple cellular effector sites, including effects on other cellular Ca^{2+} regulatory processes. Nevertheless, these results are in agreement with our previous work examining reverse-mode Na^+:Ca^{2+} exchange activities of RNCX and SNCX. These results are also consistent with previous work by others (23,32,38) suggesting that this difference in PKC sensitivity may have important consequences in terms of the cellular regulation of $[Ca^{2+}]_i$. As previously indicated, SNCX and RNCX differ at amino acid 218 and at the alternative splice site. Which of these differences is responsible for the lack of PKC sensitivity of SNCX1 is, at present, unknown. Future mutational analysis is needed to determine which of these two sites confers PKC insensitivity to the SNCX1 clone.

In conclusion, we have found that an isoform of the NCX cloned from MCs of the S rat has an impaired ability to regulate agonist-induced changes in $[Ca^{2+}]_i$. The cause of this reduced efficiency in Ca^{2+} extrusion appears to be a defect in PKC activation of SNCX1. Therefore, the physiological relevance of this dissertation is as follows. SNCX has an imparied ability to regulate $[Ca^{2+}]_i$. This impaired ability causes the recovery of agonist-induced increases in $[Ca^{2+}]_i$ to baseline levels to be severely attenuated. The sustained increase in $[Ca^{2+}]_i$ leads to an increase in vascular tone. The increase in vascular tone causes a decrease in glomerular filtration rate (GFR) and renal blood flow (RBF). The decrease of GFR and RBF causes an increase in blood pressure that can lead to pathophysiological conditions such as end stage renal disease (ESRD) and salt-sensitive hypertension (Table 1).

Table 1. Generalized summary of significant results obtained from all three parts of the dissertation for isoforms RNCX, SNCX, and SDNCX1.10.

Isoforms	RNCX	SNCX	SDNCX1.10
Alt splice site	BD	BDF	BDEF
AA 218	I	F	I
$[Ca^{2+}]_i$ regulation	+++	+	+++
regulatory Ca^{2+}	40-95nM	40-50nM	40-50nM
pH_i	7.4-8.0	7.4-8.0	7.4-8.0
pH_e	6.8-8.0	7.4-8.0	7.4-8.0
PKC	+	-	+
PKA/PKG	-	-	+

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GRADUATE SCHOOL
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DISSERTATION APPROVAL FORM
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Name of Candidate Ianthalatres Williams

Graduate Program Physiology and Biophysics

Title of Dissertation Cloning and Regulation of a Renal Mesangial Cell Sodium:Calcium Exchanger Isoform

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Name		Signature
Phillip D. Bell	**, Chair**	
Catherine M. Fuller		
Pamela A. Lucchesi		
Lisa M. Schwiebert		
Peter R. Smith		
C. Roger White		

Director of Graduate Program

Dean, UAB Graduate School

Date 3/8/02