Impaired ability of the Na\(^+\)/Ca\(^{2+}\) exchanger from the Dahl/Rapp salt-sensitive rat to regulate cytosolic calcium


Nephrology Research and Training Center, Departments of Medicine and Physiology, Division of Nephrology, University of Alabama at Birmingham, Alabama 35294

Submitted 25 March 2002; accepted in final form 10 January 2003

Impaired ability of the Na\(^+\)/Ca\(^{2+}\) exchanger from the Dahl/Rapp salt-sensitive rat to regulate cytosolic calcium. Am J Physiol Renal Physiol 284: F1023–F1031, 2003. First published January 14, 2003; 10.1152/ajprenal.00121.2002.—We previously cloned Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1) from mesangial cells of salt-sensitive (SNCX = NCX1.7) and salt-resistant (RNCX = NCX1.3) Dahl/Rapp rats. The abilities of these isoforms to regulate cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{I}) were assessed in fura 2-loaded OK cells expressing the vector (VOK), RNCX, and SNCX (SOK). Baseline [Ca\(^{2+}\)]\text{I} was 98 ± 20 nM (n = 12) in VOK and was significantly lower in ROK (44 ± 5 nM; n = 12) and SOK (47 ± 13 nM; n = 12) cells. ATP at 100 μM increased [Ca\(^{2+}\)]\text{I} by 189 ± 55 nM (n = 12), 21 ± 9 nM (n = 12), and 69 ± 18 nM (n = 12) in VOK, ROK, and SOK cells, respectively. ATP (1 mM) or bradykinin (0.1 mM) caused large increases in [Ca\(^{2+}\)]\text{I}, and ROK but not SOK cells were much more efficient in reducing [Ca\(^{2+}\)]\text{I} back to baseline levels. Parental Sprague-Dawley rat mesangial cells express both RNCX (SDRNCX) and SNCX (SDSNCX). SDRNCX and RNCX are identical at every amino acid residue, but SDSNCX and SNCX differ at amino acid 218 where it is isoleucine in SDSNCX and not phenylalanine. OK cells expressing SDSNCX (SDSOK) reduced ATP (1 mM)-induced [Ca\(^{2+}\)]\text{I} increase back to baseline at a rate equivalent to that for ROK cells. PKC downregulation significantly attenuated the rate at which ROK and SDSOK cells reduced ATP-induced [Ca\(^{2+}\)]\text{I}; increase but had no effect in SOK cells. The reduced efficiency of SNCX to regulate [Ca\(^{2+}\)]\text{I} is attributed, in part, to the isoleucine-to-phenylalanine mutation at amino acid 218.

sodium/calcium exchanger; mesangial cells; hypertension

IN RECENT STUDIES, Na\(^+\)/Ca\(^{2+}\) exchange (NCX) activity has been shown to exist in renal afferent and efferent arterioles and cultured mesangial cells (2, 20, 23, 33). It most likely plays an important role in the regulation of cytosolic calcium concentration ([Ca\(^{2+}\)]\text{I}) by serving as a Ca\(^{2+}\) efflux pathway. Studies in vascular smooth muscle (36) have further suggested that the exchanger has an important role in lowering agonist-induced elevations in [Ca\(^{2+}\)]\text{I}; and it is possible that it serves a similar function in the renal microcirculation. We 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 afferent arterioles and mesangial cells and this may be due to PKC-induced translocation of the exchanger to the plasma membrane (20). It is also well known that vasoconstrictive agonists, which elevate [Ca\(^{2+}\)]\text{I}, also activate PKC through the Ca\(^{2+}\)-diacylglycerol pathway. Although PKC can have multiple cellular effects, one effect may be to enhance NCX, which then serves to return agonist-induced elevations in [Ca\(^{2+}\)]\text{I} back to baseline levels.

In other studies, we found that this Na\(^+\)/Ca\(^{2+}\) exchanger pathway may be defective in the Dahl/Rapp salt-sensitive (S) rat (33), a genetic model of salt-dependent hypertension (3, 30). Both freshly dissected afferent arterioles from the Dahl/Rapp salt-resistant (R) rats and from rabbit kidneys as well as cultured mesangial cells from R rats all responded to the phorbol ester PMA with enhanced NCX activity (2, 8, 20, 23, 33). In afferent arterioles and cultured mesangial cells from S rats, PMA failed to increase Na\(^+\)/Ca\(^{2+}\) exchange activity. In S mesangial cells, PMA also failed to stimulate translocation of the exchanger to the plasma membrane, whereas in R mesangial cells, PMA promoted translocation (20).

To determine whether this difference between R and S rats was due to intrinsic differences in the exchanger protein, NCX was cloned from cultured mesangial cells from R and S rats (33). The clone from S rats denoted SNX and from R rats denoted RNCX differs at the amino acid level. One difference occurs at amino acid residue 218, where it is isoleucine in RNCX but is phenylalanine in SNX. The significance of this single amino acid difference is presently unknown. However, it may affect the regulation of SNX since 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\(^{2+}\) (25). The other difference between RNCX and SNX occurs within the cytosolic loop at the alternative splice site. This site is encoded by six different exons denoted A–F (16, 29). In the RNCX clone, the alternative splice site is encoded by exons B and D, and in SNX it is encoded by exons...
B, D, and F (33). Although there is some indication that the alternative splice site may be important in NCX regulation (27), the exact consequence of the differences in exons expressed by these two clones at this site remains unknown.

RNCX and SNCX were expressed in an opossum kidney cell line (OK-PTH) that does not express endogenous exchange activity. It was found that the exchanger activity of RNCX but not SNCX was enhanced by PMA (33). Thus, the PKC-Na+/Ca2+ exchanger 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 (3). It has been suggested that this progressive renal vasoconstriction is due to a dysregulation of [Ca2+]i in contractile elements of the renal microcirculation of the S rat. One possible explanation for this derangement in [Ca2+]i could be defective PKC regulation of the Na+/Ca2+ exchanger. However, whether expression of RNCX vs. SNCX actually results in differences in the regulation of [Ca2+]i has not yet been addressed. Therefore, the purpose of these studies was to express RNCX and SNCX in identical cellular environments (OK-PTH cells) and to determine whether these two isoforms differ in their ability to help regulate [Ca2+]i, in response to agonist-induced elevations in [Ca2+]i. Because RNCX and SNCX are both expressed in mesangial cells of Sprague-Dawley rats, these results were also compared with those obtained for OK-PTH cells expressing the SNCX that was cloned from mesangial cells of Sprague-Dawley rats (SDSNCX). For these studies, we used ATP to elevate [Ca2+]i in response to agonist-induced elevations in [Ca2+]i. Because RNCX and SNCX are both expressed in mesangial cells of Sprague-Dawley rats, these results were also compared with those obtained for OK-PTH cells expressing the SNCX that was cloned from mesangial cells of Sprague-Dawley rats (SDSNCX). For these studies, we used ATP to elevate [Ca2+]i via the activation of purinergic receptors; several isotypes of this receptor family were confirmed to be present in OK-PTH cells. In addition, bradykinin was also used to elevate [Ca2+]i to ensure that the findings obtained with ATP were not peculiar to purinergic receptor pathways.

METHODS

Cell cultures. Opossum proximal tubule kidney cells (OK-PTH, ATCC) were grown in MEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% Fetalclone III (Cellgro), 240 μg/ml l-glutamine (Gibco), 82 U/ml penicillin, and 82 μg/ml streptomycin (Sigma) in a humidified atmosphere under 95% air-5% CO2 at 37°C. Media were 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 SDSNCX. OK-PTH cells were transfected with pCDNA3.1/V5-His-TOPO (Invitrogen) containing RNCX (pCDNA3.1-RNCX), SNCX (pCDNA3.1-SNCX), or SDSNCX (pCDNA3.1-SDSNCX) cDNA using lipofectin (BRL) according to the manufacturer's instructions. Transfectants were selected for using geneticin at 500 μg/ml for 3 wk. After 3 wk, transfected cells were incubated in the presence of 500 μM Ca2+ and 20 μM ionomycin for 30 min, washed, and resuspended in complete media. This maneuver stimulates a significant rise in [Ca2+]i, and only cells with functional exchangers will be able to lower [Ca2+]i sufficiently to survive (Ca2+ killing) (15). This process was repeated every 3 days to enrich the population of OK-PTH cells that express functional Na+/Ca2+ exchanger.

Immunoblotting with Na+/Ca2+ exchanger-specific antibody. Cells were lysed in a buffer containing 10 mM Tris, 0.5 mM NaCl, 0.05% Triton X-100, 50 μg/ml aprotinin (Sigma, St. Louis, MO), 100 μg/ml leupeptin (Sigma), and 100 μg/ml pepstatin A (Sigma) adjusted to pH 7.2–7.4. Fifty micrograms of protein were run per lane and separated on 8% SDS polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Osmonics, Westbrook, MA). Immunoblotting was performed with a mouse monoclonal Na+/Ca2+ exchanger antibody with a dilution of 1:5,000 (SWant, Bellizona, Switzerland). Reactivity was detected by horseradish peroxidase-labeled goat anti-mouse secondary antibody. ECL chemiluminescence was used to visualize the secondary antibody.

Na+/Ca2+ exchanger activity measurement. Vector-transfected OK-PTH cells (VOK), or transfected OK-PTH cells expressing RNCX (ROK), SNCX (SOK), or SDSNCX (SDOK) were grown to 80% confluency in 100-mm cell culture dishes with MEM 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, Austin, TX) for 1 h at 37°C to allow loading of the dye into cells. Fura 2-loaded cells were pelleted at 700 g for 3 min and resuspended in Ringer solution (150 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1.6 mM Na2HPO4, 0.4 mM NaH2PO4, 5 mM glucose, 1.5 mM CaCl2, 10 mM HEPES).

A small quantity of cells was then transfected to a chamber that was mounted on an inverted microscope. After several minutes, OK cells would settle to the bottom of the chamber and adhere to the coverslip that formed the chamber’s bottom. After a period of 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 [Ca2+]i measurements were performed using dual-excitation wavelength fluorescence microscopy (Photon Technologies, Princeton, NJ) with a Leitz compact photometer that had been converted to perform photon counting. An adjustable photomultiplier window was placed over a single cell with magnification of ×400 using an Olympus X40 UPLFLens. Excitation wavelengths were set at 340 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 before the experimental measurements.

Baseline fura 2 ratios were measured for at least 100 s in cells that were bathed in Ringer solution at a rate of 1.7 ml/min. Cells were discarded if the baseline drifted either up or down. After a stable baseline reading was obtained, either 100 μM or 1 mM ATP or 100 μM bradykinin was added to the chamber at a rate of 1.7 ml/min to elevate [Ca2+]. The fura 2 ratio was monitored continuously before, during, and after addition of ATP or bradykinin until the ratio returned to a stable baseline. All solutions had a pH of 7.4 with temperature maintained at 37°C. In addition, there was no evidence of dye leakage throughout the experiment.

Effect of PKC downregulation on Na+/Ca2+ exchanger activity. Previous studies showed that PKC plays an important role in the activation of NCX. To examine the role that PKC might play in the regulation of RNCX, SNCX, or SDSNCX, cells expressing RNCX (ROK), SNCX (SOK), or SDSNCX (SDSOK) were treated with 300 nM PMA for 24 h to downregulate PKC before single-cell [Ca2+]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 using the equation described by Grynkiewicz et al. (10)

\[
[Ca^{2+}]_i = K_d \times \left(\frac{S_f}{S_b}\right) \times \left(\frac{R - R_{min}}{R_{max} - R}\right)
\]

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 340/380 nm, \(R_{min}\) and \(R_{max}\) are the ratios in absence and presence of \(Ca^{2+}\), respectively, and \(S_f\) and \(S_b\) are the emissions at 380 nm in the absence and presence of \(Ca^{2+}\), respectively. VOK, ROK, SDSOK, or SOK cells were loaded with 24 \(\mu\)M fura 2 for 1 h, followed by resuspension in Ringer solution. Calibration was accomplished after permeabilization of the cells with 5 \(\mu\)M imomycin and measurement of 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 \(R_{min}\), \(R_{max}\), \(S_f\), and \(S_b\).

**Genomic DNA sequencing.** The cDNAs for RNCX and SNCX show differences at amino acid 218, where it is isoleucine in RNCX but is phenylalanine in SNCX, and at the alternative splice site, where it is encoded by exons B and D and B, D, and F in RNCX and SNCX, respectively. To address the question of whether the mutation observed at the cDNA level occurs at the genomic level, genomic DNA was isolated from kidneys of S and R Dahl/Rapp rats using the Easy-DNA kit (Invitrogen) according to manufacturer’s instructions. A 100-ng aliquot of the genomic DNA was used to amplify the NH\(_2\) terminus of the \(Na^+/Ca^{2+}\) exchanger gene using a forward and a reverse primer (5'-gagggactcgcaaggggatctcctttt-3'//5'-gaggagaattctgaaccaaggacac-3'). PCR was carried out using the High Fidelity Supermix kit (Invitrogen). With the use of a minicycler (MJ Research), PCR was carried out at 94°C for 2 min, 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 10 min at 68°C. Ten microliters of each PCR product were fractionated on a 1% Tris acetate-EDTA gel, and the band was excised and gel purified using Gene Clean (Amersham). A 2-\(\mu\)l aliquot of the purified PCR fragment was ligated into pcDNA3.1GFPTOPO using the TOPO TA Cloning Kit (Invitrogen). The entire ligation mix was used to transform Top 10-competent *Escherichia coli* (Invitrogen) that were subsequently screened (34), and plasmid was isolated from six positive colonies containing the NH\(_2\) terminus for RNCX or SNCX. The plasmids were sent to Seqwright (Houston, TX) for sequencing, and the DNASIS Max program (Hitachi Software Engineering) was used for sequence alignments and manipulations.

**RESULTS**

**SNCX and RNCX exchanger levels.** With the use of immunoblot analysis, the level of protein expression in VOK, ROK, and SOK cells was evaluated and the results are shown in Fig. 1. A low level of NCX protein was detected in VOK cells, and this level was at least 10-fold less than that seen in ROK and SOK cells. We reported in an earlier study that OK-PTH cells did not functionally express the exchanger as evaluated by reverse mode (33). 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 VOK, ROK, and SOK cells. The results are presented in Fig. 2 and demonstrate that the expression of \(Na^+/Ca^{2+}\) exchanger in OK cells resulted in a significant reduction in \([Ca^{2+}]_i\). Baseline \([Ca^{2+}]_i\) was 98 ± 20 nM in VOK cells, whereas it was 44 ± 5 and 47 ± 13 nM \((n = 12)\) in cells expressing RNCX (ROK) and SNCX (SOK), respectively. Thus, the presence of the \(Na^+/Ca^{2+}\) exchanger 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 \(Na^+/Ca^{2+}\) exchanger 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; at least it is a low concentration for OK-PTH cells) 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 VOK and ROK cells. As shown in the example in Fig. 3A and in the summary in Fig. 3B, the largest Ca\(^{2+}\) transients in response to ATP administration were observed in VOK cells; i.e., [Ca\(^{2+}\)]\(_i\) increased by 189 ± 55 nM (n = 12). 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 69 ± 18 nM (n = 12) in the SOK group with ATP administration was significantly greater than the 21 ± 9 nM (n = 12) elevation in [Ca\(^{2+}\)]\(_i\) obtained in the ROK group. Thus, OK cells expressing the RNCX clone had a greater ability to buffer changes in [Ca\(^{2+}\)]\(_i\) compared with cells expressing the SNCX clone. The expressions of RNCX and SNCX were comparable in the two cell lines (Fig. 1), so it is highly unlikely that the difference in their abilities to buffer ATP-induced changes in [Ca\(^{2+}\)]\(_i\) was due to 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 Na\(^+\)/Ca\(^{2+}\) exchanger activity. Another means of assessing exchanger activity is to measure the initial rate of return (by taking the slope of the line) 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 but, by comparing the responses of OK-PTH cells expressing RNCX vs. SNCX, it is possible to determine whether there are differences in the rate of Ca\(^{2+}\) extrusion by these two isoforms. For these experiments, ATP and bradykinin were used at concentra-

Fig. 3. OK-PTH cells expressing RNCX (ROK) showed greater ability to buffer ATP-induced [Ca\(^{2+}\)]\(_i\) increase compared with vector-transfected (VOK) and transfected OK-PTH cells expressing SNCX (SOK). The abilities of ROK and SOK to buffer ATP-induced [Ca\(^{2+}\)]\(_i\) increase were assessed in VOK, ROK, and SOK cells following treatment with 100 μM ATP to elevate [Ca\(^{2+}\)]\(_i\). A: representative [Ca\(^{2+}\)]\(_i\) tracings of VOK, ROK, and SOK in response to 100 μM ATP. B: bar graph showing ∆[Ca\(^{2+}\)]\(_i\), in VOK, ROK, and SOK cells. ROK and SOK had lower ∆[Ca\(^{2+}\)]\(_i\), or greater capacity to buffer ATP-induced [Ca\(^{2+}\)]\(_i\) increase compared with VOK, with ROK having the greatest [Ca\(^{2+}\)]\(_i\), buffering ability. Data were analyzed for statistical significance using ANOVA. Values are means ± SE (n = 12); ∗P < 0.05 compared with VOK.
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 Fig. 6, A-C, there is a distinct difference in the effects of PKC down-regulation in ROK and SDSOK vs. SOK cells. As shown in Fig. 6, A and B, the rate of return of \([\text{Ca}^{2+}]_i\) in ROK and SDSOK cells, after administration of ATP, was dramatically reduced with PKC inhibition. However, the rate of return of \([\text{Ca}^{2+}]_i\) in SOK cells (Fig. 6 C) was not greatly affected by prior treatment with PMA. As shown in the summary in Fig. 6 D, there was a highly significant decrease in the rate of \([\text{Ca}^{2+}]_i\) recovery with PKC downregulation in ROK and SDSOK cells but not in SOK cells.

Genomic DNA sequencing. The amino acid difference between RNCX and SN CX at residue 218 occurred as the result of an A-to-T transversion that changed the codon for isoleucine (ATT) to that for phenylalanine (TTT). To determine whether this difference occurs at the genomic level, the coding region of the \(\text{Na}^+/\text{Ca}^{2+}\) exchanger gene from nucleotide 1 to 747 was sequenced in both directions using genomic DNAs isolated from kidneys of S and R Dahl/Rapp rats. The sequence data (Fig. 7) show that the difference between RNCX and SN CX that we observed in the cDNAs does not occur at the DNA level.

DISCUSSION

The \(\text{Na}^+/\text{Ca}^{2+}\) exchanger (NCX) is a 120-kDa transmembrane protein that consists of five \(\text{NH}_2\)-terminal and four COOH-terminal membrane-spanning domains and a cytosolic loop that comprises over 50% of the protein (11). There are at least four different NCX exchangers encoded by separate genes (18, 24, 26). To date, only NCX1 (which is also the cardiac isoform) has been demonstrated to occur in the tubules (4, 5), blood vessels (2, 23), and mesangial cells of the kidney (2, 20, 22, 33). RNCX (NCX1.3) and SN CX (NCX1.7) were previously cloned from mesangial cells of the Dahl/Rapp R and S rat, respectively (33). They are 100% homologous at the COOH-terminal membrane-spanning domains and nearly identical in the \(\text{NH}_2\)-terminal membrane-spanning domains except for a single amino
acid difference at residue 218, where it is isoleucine in RNCX and phenylalanine in SN CX. It should be noted that all other NCX1 isoforms that have been cloned have isoleucine at this site. Both RNCX and SN CX are also highly homologous in the large cytoplasmic loop except for the alternative splice site where RNCX is encoded by exons B and D, whereas SN CX is encoded by exons B, D, and F. Thus, these differences in amino acid sequence may cause the lack of PKC sensitivity of the Na+/Ca2+/H+ exchanger in afferent arterioles and cultured mesangial cells from the Dahl/Rapp S rat. This PKC sensitivity of RNCX but not SN CX 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 RNCX (ROK) and SN CX (SOK) was examined using a 45Ca2+/H+ influx assay that involves sodium-loading cells followed by extracellular sodium removal. This stimulates the reverse-mode NCX (Ca2+/Na+ entry and Na exiting the cell) that is opposite to how the exchanger normally operates under physiological conditions. Nevertheless, these studies indicated that 1) nontransfected OK-PTH cells had virtually no exchanger activity; 2) basal exchanger activity was similar in ROK and SOK cells; 3) RNCX activity increased with acute PMA treatment; and 4) SOK exchanger activity was insensitive to PKC activation or PKC downregulation by 24-h pretreatment 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 [Ca2+]i.

It has been suggested that NCX is involved in the extrusion of Ca2+ after agonist-induced elevations in [Ca2+]i (6). This issue was addressed in a recent study by Slodzinski and Blaustein (31), in which knockdown of exchanger activity was achieved by pretreatment with antisense oligodeoxynucleotides of NCX. They found that recovery from elevations in [Ca2+]i was substantially prolonged in cells that were treated with antisense compared with control cells. This exchanger-mediated reduction in [Ca2+]i represents the forward mode of the exchanger and more closely resembles the physiological role of NCX in [Ca2+]i homeostasis. In the present study, we used a similar approach except that we compared the regulation of [Ca2+]i in cells that were either vector transfected or transfected with the RNCX, SN CX, or SDSNCX clone. In this manner, we could directly determine whether there were differences in [Ca2+]i regulation between RNCX and SN CX and whether those differences could be attributed to the differences at the amino acid level.

Initial studies were performed to measure baseline [Ca2+]i in these three cell types. We found that cells expressing Na+/Ca2+ exchange isoforms had lower baseline [Ca2+]i compared with nontransfected cells. [Ca2+]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, Ca2+-ATPase, a high-affinity and low-ca-
pacity Ca\(^{2+}\)-extrusion mechanism, was important for setting the resting \([\text{Ca}^{2+}]_{i}\), whereas NCX, a low-affinity and high-capacity Ca\(^{2+}\)-extrusion mechanism, primarily extruded Ca\(^{2+}\) when \([\text{Ca}^{2+}]_{i}\) was elevated above baseline levels (1, 6, 9). Our studies indicate that NCX does contribute to the nonstimulated level of \([\text{Ca}^{2+}]_{i}\) at least in this cell type and under these conditions.

In most studies of agonist-induced alterations in \([\text{Ca}^{2+}]_{i}\), the initial paradigm is to invoke Ca\(^{2+}\) transients by rapidly applying sufficient hormone or agent to achieve a maximum \([\text{Ca}^{2+}]_{i}\) 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. We found that OK-PTH cells express P2X4, P2X5, and P2Y2 (data not shown) and that a concentration of 100 \(\mu\)M ATP produced optimal discrimination between the results obtained in the vector-transfected group vs. those obtained in ROK cells. In other cell types, 100 \(\mu\)M ATP may produce maximal effects; however, this may depend on, among other things, the number of P2 receptors that are on the cell membrane. The important point is that during this exposure to micromolar levels of ATP, cells expressing NCX were much better able to prevent or minimize the increase in \([\text{Ca}^{2+}]_{i}\).

Other studies were performed to assess the ability of RNCX and SNCX to extrude \(\text{Ca}^{2+}\) after maximal agonist-induced increases in \([\text{Ca}^{2+}]_{i}\). Similar to what was observed in the preceding experiments, the rate at which \(\text{Ca}^{2+}\) declined after administration of 1 or 0.1 mM ATP or bradykinin, respectively, was slower and took longer to occur in SOK cells vs. ROK cells. These results further support our conclusion that SNCX is much less efficient in regulating \([\text{Ca}^{2+}]_{i}\).
ations in \([\text{Ca}^{2+}]\). RNCX and SNCX are both expressed in the parental strain, Sprague-Dawley rat and are designated SDRNCCX and SDSNCX, respectively. RNCX and SDRNCX are identical at every amino acid residue, whereas SNCX and SDSNCX differ at amino acid 218, where it is isoleucine in SDRNCX but is phenylalanine in SNCX. To determine whether the single amino acid difference at 218 contributes, in part, to the inability of SNCX to regulate ATP-induced \([\text{Ca}^{2+}]\); increase efficiently, we next examined the ability of cells expressing SDRNCX (SDSOK) to regulate the ATP (1 mM)-induced \([\text{Ca}^{2+}]\); increase. SDSOK cells reduced ATP-induced \([\text{Ca}^{2+}]\), at a rate that was comparable to that for cells expressing RNCX. Thus, the isoleucine-to-phenylalanine change in SNCX appeared to contribute, in part, to the inability of this isoform to regulate agonist-induced elevations in \([\text{Ca}^{2+}]\), levels.

Because of the profound effect of the isoleucine-to-phenylalanine difference at amino acid 218 on the ability of SNCX to regulate agonist-induced \([\text{Ca}^{2+}]\); increase, we next determined whether the mutation occurred at the genomic level. This was accomplished by sequencing the coding region of the Na+/Ca2+ exchanger gene from genomic DNA of S and R Dahl/Rapp rats spanning nucleotides 1 to 747. Our data showed that the A-to-T transversion in the SNCX cDNA did not occur at the genomic level. However, because we took great care in eliminating PCR artifacts in our original study where we sequenced these isoforms, we feel confident that this mutation is not likely to be a PCR artifact. We suggest the possibility that this mutation might occur in the S mesangial cell as a result of mRNA editing. Although rare, studies have shown that this does occur in the eukaryotic cell and affects a number of proteins including Apo B (32), glutamate receptor (17), and oxytocin receptor (7). Apo B has two isoforms, Apo B-100 and Apo B-48. Apo B-100 is a 512-kDa protein that is synthesized in the liver, whereas Apo B-48 is a 240-kDa protein that consists of the NH2 terminus of Apo B-100. Apo B-48 is synthesized in the small intestine, is developmentally regulated, and is the result of a CAA-to-UAA mutation that generates this truncated form of Apo B. The glutamate receptor has also been shown to undergo mRNA editing, which results in a Q-to-R mutation that arises as the result of a CAG-to-CCG codon change. Studies showed that differences in oxytocin receptor mRNA sequences for different oxytocin receptor populations in the endometrium are due to mRNA editing. mRNA editing of the oxytocin receptor transcripts leads to differences in the observed responses to an oxytocin challenge. Therefore, although rare, the possibility exists that NCX mRNA editing may occur in addition to alternative splicing in mesangial cells of the S Dahl/Rapp rat.

Presently, the role that PKC plays in the activation of the Na+/Ca2+ exchanger is not clearly understood. Our laboratory demonstrated that PKC activates NCX in the renal microcirculation of rabbits and normotensive rats (8) and in the cloned exchanger RNCX (33). Also, work by Vigne et al. (35) found that phorbol esters stimulated the exchanger in cultured aortic smooth muscles. However, other studies in human mesangial cells by Mene et al. (21) failed to demonstrate enhanced reverse-mode NCX. In studying the regulation of NCX1, 2, and 3, Linck et al. (19) found that NCX1 and 3 showed modest stimulation by both PKA and PKC agonists. Exchanger activity appeared to be much more sensitive to PMA downregulation. Finally, Iwamoto et al. (12) demonstrated that activation of PKC by agonists enhanced exchanger activity and phosphorylation of NCX1 (12-14), 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 (12). Thus, the bulk of the present evidence favors PKC activation of NCX1, but the mechanism by which this occurs remains unclear. Our laboratory proposed that PKC may induce translocation of the exchanger, but whether this 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, which hydrolyses phosphatidylinositol 4,5-bisphosphate and elevates diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 binds to its receptor on the endoplasmic reticulum and stimulates the release of Ca2+, thereby initiating the rise in \([\text{Ca}^{2+}]\). With an elevation of \([\text{Ca}^{2+}]\); and DAG, there is activation of PKC, which, in turn, enhances NCX. Thus, PKC increases the rate at which the Na+/Ca2+ exchanger extrudes Ca2+ in exchange for Na+ thereby reducing \([\text{Ca}^{2+}]\); back to baseline levels.

In the present studies, this scenario was, in part, tested by 24-h pretreatment with PMA, a maneuver that is known to downregulate PKC activity. Studies in other cell systems (28) showed that extracellular ATP via P2 receptors does increase PKC activity. We demonstrated that inhibition of PKC activity greatly affected the abilities of ROK and SDSOK cells to rapidly return \([\text{Ca}^{2+}]\); to control levels. In contrast, PKC inhibition did not affect the rate of return in \([\text{Ca}^{2+}]\); in SOK cells. It should be mentioned that the \([\text{Ca}^{2+}]\); transients obtained in the presence of PKC inhibition were much more labile and variable compared with cells that were not treated with PMA. This may reflect the fact that PKC has multiple cellular effector sites including effects on other cellular Ca2+ regulatory processes. Nevertheless, these results are in agreement with our previous work examining reverse-mode NCX in ROK and SOK cells (33). They extend this previous work by suggesting that this difference in PKC sensitivity may have important consequences in terms of the cellular regulation of \([\text{Ca}^{2+}]\). Furthermore, these results indicate that insensitivity of SNCX to PKC may be attributed, in part, to the single amino acid difference at residue 218. However, future mutational analysis is needed to determine the exact mechanism that underlies the basis of SNCX insensitivity to PKC.
In conclusion, we found that an isoform of the Na+/Ca2+ exchanger that was cloned from mesangial cells of the Dahl/Rapp S rat has an impaired ability to regulate agonist-induced changes in [Ca2+]i. The cause of this reduced efficiency in Ca2+ extrusion appears to be due to a defect in PKC activation of SNX, which may be attributed, in part, to a single amino acid mutation at amino acid 218. We suggest that this reduced ability of the Na+/Ca2+ exchanger to regulate [Ca2+]i in the renal microcirculation may be responsible, in part, for the increased vascular resistance and reduced glomerular filtration rate that are hallmarks of this form of hypertension.

This work was supported by Grants 2R01-HL-07457-20 (P. D. Bell), 1K01-HL-67718-01 (M. T. Unlap), and T32-HL-07457-20 (E. Hwang) from the National Heart, Lung, and Blood Institute. J. P. Peterdi is a National Kidney Foundation postdoctoral fellow.

REFERENCES


2. Bell PD, Mashburn N, and Unlap MT. Renal sodium/calciumpmter charge: a vasodilator that is defective in salt-sensitive hyperten-


8. Fowler BC, Carmine PK, Nelson LD, and Bell PD. Charac-
terization of sodium-calcium exchange in rabbit renal arte-

9. Friedeman PA, Figueiredo JP, Maack T, and Windhager EE. Sodium-calcium interactions in the renal proximal con-


15. Kamendulis LM and Corcoran GB. Independence and addi-


20. Mashburn NA, Unlap MT, Runquist J, Alderman A, John-


22. Mene P, Pugliese F, Faraggiana T, and Cinotti GA. Identifi-

23. Nelson LD, Unlap MT, Lewis JL, and Bell PD. Renal arte-


29. Quednau BD, Nicoll DA, and Philipson KD. Tissue specific-


32. Smith HC and Sowden MP. Base-modification mRNA editing through deamination—the good, the bad and the unregulated. Trends Genet 12: 418–424, 1996.

33. Unlap MT, Peti-Peterdi J, and Bell PD. Cloning of mesangial cell Na+/Ca2+ exchangers from Dahl/Rapp salt-sensitive/resis-


35. Vigne P, Breittmayers JP, Duval D, Frelin C, and Lazdun-