PMCA4b. Basolateral localization of the Na\(^+\)/Ca\(^{2+}\) exchange; confocal microscopy

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Nephron segments, the DCT expresses PMCA transcripts encoding PMCA1 and 8.5 and 7.5 kb for PMCA4. We conclude that the PMCA isoforms and subtypes expressed in mouse distal convoluted tubule cells and Na\(^+/\)Ca\(^{2+}\) exchanger protein expression in mDCT cells. In lysates of mDCT cells, immunoprecipitation and Western blot analysis, performed with a monoclonal antibody to PMCA, revealed a 140-kDa protein consistent with PMCA. Laser-scanning confocal fluorescence microscopy indicated that PMCA and NCX1 expression is restricted to basolateral membranes only in confluent mDCT cells, because subconfluent cultures predominately express intracellular localizations. PMCA isoform-specific PCR primers generated appropriately sized products only for PMCA1 and PMCA4 from DCT cells but PMCA1–4 from whole mouse kidney. Assessment of splice site C in the calmodulin-binding domain demonstrated the presence of PMCA1b and PMCA4b mRNAs in mDCT cells. Northern blot analysis of mDCT cDNA revealed transcripts of 7.5 and 5.5 kb for PMCA1 and 8.5 and 7.5 kb for PMCA4. We conclude that DCT cells express PMCA transcripts encoding PMCA1b and PMCA4b. Basolocalization of the Na\(^+/\)Ca\(^{2+}\) exchanger and PMCA1b and PMCA4 change in mDCT cells, in concert with the Na\(^+/\)Ca\(^{2+}\) exchanger, mediate basal or hormone-stimulated Ca\(^{2+}\) efflux by distal tubules.

calcium transport; kidney; plasma membrane calcium-adenosine 5'-triphosphatase; sodium-calcium exchange; confocal fluorescence microscopy

RENAL CA\(^{2+}\) ABSORPTION OCCURS primarily in proximal tubules, thick ascending limbs, and distal convoluted tubules (DCTs); and in rabbit connecting tubules) through distinct pathways in each nephron segment. In proximal tubules, Ca\(^{2+}\) movement is primarily mediated by passive, paracellular mechanisms (49). Absorption in DCTs, in contrast, is an active, transcellular process that is stimulated by parathyroid hormone (PTH), 1,25-dihydroxyvitamin D\(_3\), and calcitomin (24). In cortical thick ascending limbs (CALs), Ca\(^{2+}\) transport is a hybrid of resting Ca\(^{2+}\) absorption that is passive and paracellular, whereas active absorption is transtemular (17, 18, 23). Cellular Ca\(^{2+}\) absorption is a two-step process wherein Ca\(^{2+}\) first enters the cell down its electrochemical gradient through apical membrane Ca\(^{2+}\) channels (3, 45) and then exits across the basolateral membrane into the peritubular fluid. This latter step depends on direct or indirect metabolic energy to overcome the thermodynamic barrier opposing Ca\(^{2+}\) efflux and is thought to be mediated by a plasma membrane Ca\(^{2+}\)-ATPase (PMCA) (5, 19) or an Na\(^+/\)Ca\(^{2+}\) exchanger (15, 16).

PMCA1s regulate intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) by extruding Ca\(^{2+}\) ions across plasma membranes. In the kidney, PMCA1s may have a specialized role in Ca\(^{2+}\) transport by participating in net absorptive movement. Although PMCA1 protein and transcripts are found in proximal tubule cells, they are expressed at higher levels in distal portions of the nephron (57, 61). Compared with other nephron segments, the DCT possesses the highest Ca\(^{2+}\)-ATPase activity (19) and exhibits the strongest immunocytochemical reactivity for PMCA1 protein expression (5, 6, 39). The PMCA isoforms expressed by Ca\(^{2+}\)-transporting DCT cells are subject to conflicting reports and contradictory information, and the PMCA1 subtypes are uncertain.

The PMCA enzymes are P-type ATPases that are encoded by four homologous genes designated PMCA1–4 (54). The isoforms are expressed in a tissue-dependent manner with PMCA1 and PMCA4 present in virtually all organs (51, 52), whereas PMCA2 and PMCA3 are expressed predominantly in brain and striated muscle (54).

The term PMCA isoform employed herein is used to distinguish PMCA gene products, such as PMCA1 and PMCA2. The term PMCA subtype refers to PMCA1s that arise from alternative splicing of an individual isoform, such as PMCA4a and PMCA4b (54).

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alternative splicing within regulatory sites to generate multiple subtypes of each isoform. Site A is located in the NH$_2$-terminal half of the enzymes in a phospholipid-sensitive region (64). Site C, near the COOH terminus of the enzymes, is present within the calmodulin-binding domain (53), with alternatively spliced subtypes exhibiting functional differences (21).

The NCX1 Na$^+$/Ca$^{2+}$ exchanger is similarly highly expressed in kidney and may also play a role in mediating Ca$^{2+}$ absorption. The exchanger has been mapped to basolateral membranes of distal nephron cells in humans (43), rats (43), mice (11, 37), dogs (7), and rabbits (46, 47). Within distal segments, additional molecular and functional evidence supports NCX1 localization in the CAL (18), DCT (60), connecting tubule (CNT) (20), and cortical collecting duct (57).

The goals of the present studies were to characterize the PMCA isoforms present in mouse DCT (mDCT) cells and to assess PMCA and NCX1 Na$^+$/Ca$^{2+}$ exchanger protein expression. As the establishment of cell polarity is necessary for vectorial transport in renal epithelial cells, we also examined the localization of PMCA and NCX1 in unpolarized and polarized mDCT cells.

METHODS

Cell culture. DCT plus CAL cells were isolated by immuneoselection using Tamm-Horsfall protein antiserum (44). Primary cultures of this mixed-cell population, referred to herein as distal tubule cells, were immortalized by exposure to chimeric adenovirus 12-simian virus 40 (AD12/SV40), and DCT cells were subcloned by limiting dilution. These immortalized cells, described here as mDCT cells, express a phenotype that includes increased cAMP in response to treatment with PTH or calcitonin (26), and thiazide-sensitive, but not thiazide-resistant, Ca$^{2+}$ transport (28). mDCT cells were subcloned by limiting dilution. These immortalized cells, described here as mDCT cells, express a phenotype that includes increased cAMP in response to treatment with PTH or calcitonin (26), and thiazide-sensitive, but not thiazide-resistant, Ca$^{2+}$ transport (28). mDCT cells were grown on 100-mm dishes (Corning Glass Works; Corning, NY) grown on 100-mm dishes (Corning Glass Works; Corning, NY) in DMEM/F-12 media (Sigma, St. Louis, MO) supplemented with 5% heat-inactivated FCS (Sigma) and an antibiotic mixture of 50 

Immunoprecipitation and Western blot analysis. mDCT cells (10$^7$) were washed three times with 3 ml of 1× PBS. The cells were lysed on ice in 1.0 ml of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS, 1.0 μg/ml aprotonin, and 75 μg/ml of 4-(2-aminoethyl)benzenesulfonyl fluoride) for 5 min. The suspensions were transferred to 1.5-ml Eppendorf tubes and centrifuged at 13,000 g for 5 min at 4°C. The supernatant was stored at −70°C until use. For precipitation, a monoclonal antibody (class IgG) recognizing all PMCA isoforms (5) (Affinity BioReagents, Golden, CO) or nonspecific mouse IgG (Sigma) was added to 1.0 ml of mDCT lysate to a final concentration of 0.5 or 0.25 μg/ml and incubated at 4°C for 2 h on a rocking platform. Twenty microliters of 1:1 ratio of 1× PBS/protein A Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ) mixture were added to the tube and incubated at 4°C for an additional hour. The reactants were then centrifuged for 30 s to pellet the Sepharose. The supernatant was removed and set aside on ice, and a Lowry protein assay was performed by using BSA as a standard. The pellet was washed three times with RIPA buffer. Fifty microliters of 2× SDS sample buffer were added to the Sepharose, and the mixture was heated for 5 min at 100°C. The Sepharose and sample buffer were centrifuged for 2 min, and the supernatant was removed. Twenty-five microliters of the supernatant and 10 μl of the precipitant were electrophoresed (Hoefer Scientific, San Francisco, CA) on a 7.5% polyacrylamide gel (SDS-PAGE) at 25 mA/gel. Prestained markers (Bio-Rad Laboratories, Hercules, CA) were electrophoresed in parallel and used for protein mass determination. The protein was transferred to nitrocellulose (Bio-Rad) in an electroblotting apparatus (Hoefer Scientific) for 2 h at 400 mA.

After being blocked for 18 h in 5% Blotto [instant nonfat dry milk (Carnation) in 1× Tris-buffered saline, pH 7.4] at 4°C, the membrane was probed with a 1:1,000 dilution of the monoclonal antibody to PMCA (Affinity BioReagents) for 90 min at room temperature. The blots were then incubated with a 1:3,000 dilution of a horseradish peroxidase-labeled goat anti-mouse IgG (Bio-Rad) in 1% Blotto for 60 min at room temperature and then developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions with Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

Immunofluorescence labeling and laser scanning confocal microscopy. mDCT cells were added to the apical chamber of rat-tail collagen-coated 12-mm-diameter Transwells (Corning-Costar, Cambridge, MA) at 50,000 cells/well. After 48 and 72 h, cells were fixed and processed with the use of a pH-shift protocol (2). Cells were incubated for 1 h at 37°C [with a 1:100 dilution of a mouse monoclonal antibody against PMCA (Affinity BioReagents) and a rabbit polyclonal antibody against NCX1 (Swant, Bellinzona, Switzerland)], washed, and then incubated for 1 h at 37°C [with a 1:200 dilution of Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes, Eugene, OR)]. Imaging was performed on a TCS confocal microscope equipped with krypton-, argon-, and helium-neon lasers (Leica, Deerfield, IL). Images were acquired with the use of a ×100 plan-achromat objective (1.4 numerical aperture) and the appropriate filter combination. Settings were as follows: photomultipliers set to 600–800 mV, 1.0-μm pinhole, zoom = 1.7–2.5, and Kalman filter (n = 4). The images (1,024 × 1,024 pixels) were saved in TIFF, with the contrast level of the images adjusted in the Photoshop program (Adobe, Mountain View, CA). The contrast-corrected images were imported into FreeHand (Macmedia, San Francisco, CA) and printed with a Kodak 8670PS dye sublimation printer (Rochester, NY). Cross-fluorescence was negligible when cells were labeled with either antibody alone.

RNA isolation. mDCT cells (1 × 10$^7$) were washed three times with 3 ml of 1× PBS. Cells were then solubilized and scraped in the presence of 1.0 ml of 1 M 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyamate, layered onto a 1.5-ml CaCl$_2$ gradient in 3-ml TL-100 centrifuge tubes (Beckman; Fullerton, CA), and overlaid with 0.15 ml of 20% sarcosyl. Gradients were centrifuged for 2 h at room temperature, and pellets were washed with 70% ethanol and resuspended in 100 μl of sterile water. Quantitation of yield was determined by absorbance at 260 and 280 nm.

RT-PCR. Total RNA (1.0 μg) from mDCT cells or 250 ng of mouse kidney mRNA (Clontech, Palo Alto, CA) was reverse transcribed by using MuMLV RT and random hexamers (GeneAmp RNA-PCR kit; PerkinElmer, Foster City, CA) for 10 min at room temperature and then for 15 min at 42°C in the presence of 5 mM MgCl$_2$. As a control for genomic DNA contamination of the RNA preparations, duplicate samples were not reverse transcribed. The cDNA was amplified with Tag polymerase in the presence of 2 mM MgCl$_2$ in the same tube as the RT reaction. PCR primers were specific for each
PMCA gene product and subtype (Table 1). β-Actin primers (Table 1) were based on the human genomic β-actin sequence (41). PCR was performed at 94°C for 1 min, annealed at the specific temperature for each primer pair (Table 1) for 1 min, and extended for 2 min at 72°C for 35 cycles, with a final extension of 7 min. The individual reactions were performed on at least two independent RNA samples, and the results were identical for each primer pair. The products were electrophoresed on a 1% agarose gel (FMC, Rockland, ME) and stained with ethidium bromide.

DNA sequencing. DNA sequencing was performed with the PRISM DyeDeoxy Sequencing Kit (ABI, Foster City, CA) as described by the manufacturer. The cDNA products were sequenced with the Applied Biosystems model 373A DNA sequencing system. Briefly, products were cut from a low-melting-temperature agarose gel (FMC), the cDNA was isolated (Wizard Prep; Promega, Madison, WI), and then 100 ng were sequenced with 3.2 pM of the top or bottom oligodeoxynucleotide PCR primer. At least two independent PCR experiments were sequenced in both directions to control for Taq polymerase incorporation errors. Sequence comparisons between cDNA products and previously identified PMCA sequences were carried out with GCG (Genetics Computer Group, Madison, WI) and GeneWorks (IntelliGenetics, Mountain View, CA) software.

Northern blot analysis. mDCT cell total RNA (5.0 μg) was electrophoresed on a 1.2% agarose-formaldehyde gel and electrotransferred overnight to GeneScreen Plus Membrane (DuPont-NEN, Wilmington, DE). The bands were prehybridized in a solution of 1 M NaCl, 1% SDS, and 10% dextran sulfate for 60 min at 60°C and then probed with 2 × 10⁶ cpm/ml of the randomly primed (Prime-it II kit; Stratagene), [32P]dCTP (ICN Pharmaceuticals, Costa Mesa, CA) mDCT PMCA1 or PMCA4 PCR products with the addition of 100 μl of a 10 mg/ml stock solution of salmon sperm DNA, where cpm is counts per minute. The bands were washed at high stringency with 50 ml of 2× sodium chloride-sodium citrate, 0.1% SDS three times at room temperature and then with 0.1× sodium chloride-sodium citrate, 0.1% SDS three times at 60°C and exposed to Kodak X-AR film for 24–48 h at –70°C.

RESULTS

Analysis of PMCA protein expression in mDCT cells. To assess PMCA protein expression in mDCT cells, immunoprecipitation and Western blot analysis were performed on total cellular lysates by using a monoclonal antibody that recognizes the conserved hinge domain present within all PMCA isoforms (5) (Fig. 1). Lysates not exposed to specific or nonspecific antibody produced no precipitants (Fig. 1A, lane 1). Nonspecific mouse IgG (0.5 μg/ml) exhibited only the IgG heavy chain band at 50 kDa (Fig. 1A, lane 2). The PMCA antibody produced concentration-dependent precipitation, where the 0.5 μg/ml concentration of antibody precipitated more PMCA than the 0.25 μg/ml concentration (Fig. 1A, lanes 3 and 4). Both PMCA antibody dilutions, 0.5 and 0.25 μg/ml, precipitated a polypeptide of 140 kDa, as well as the 50-kDa band from the IgG heavy chain. The 140-kDa mass of the reacting polypeptide in mDCT cell lysates agrees with reported PMCA molecular masses (5, 51). The parallel supernatants displayed a predictable pattern of PMCA content. Both control and nonspecific mouse IgG exhibited 140-
kDa PMCA bands of similar intensity (Fig. 1B, lanes 1 and 2), whereas the corresponding supernatants from lysates treated with the PMCA antibody showed light bands at 140 kDa (Fig. 1B, lanes 3 and 4), confirming the immunodepletion of PMCA. Also, lane 4 showed a slightly darker 140-kDa band in the supernatant compared with lane 3, indicating that the greater dilution of the antibody precipitated less PMCA protein; nonspecific IgG did not precipitate the enzyme. Thus the antibody to PMCA precipitated and recognized a single reacting species by Western blot analysis, which demonstrates that mDCT cells express PMCA protein.

Analysis of PMCA isoforms in mDCT cells. RT-PCR was used to characterize the PMCA mRNA isoforms expressed in mDCT cells. RNA from mDCT cells and mouse kidney was reverse transcribed and amplified by PCR in paired, separate experiments (each paired experiment produced the same result) with primers specific for the four PMCA isoforms (Table 1). The primers used for these studies are known to amplify the four PMCA isoforms from mouse kidney RNA (61) and were confirmed by sequencing (61). The primers to PMCA1 mRNA revealed a product of 550 bp in mouse kidney and mDCT cells (Fig. 2A). The primers specific for PMCA2 and PMCA3 generated appropriately sized products from whole kidney of 427 and 392 bp (Fig. 2, B and C, Mouse Kidney +), respectively. No products were detected in mDCT cells (Fig. 2, B and C, DCT +). The primers targeting PMCA4 transcripts generated an appropriately sized product of 546 bp in mouse kidney and in mDCT cells (Fig. 2D, Mouse Kidney + and DCT +). Samples that were run in the absence (−) of RT showed no products (Fig. 2, A–D), indicating that the cDNAs arose from amplification of RNA and not genomic DNA.

To confirm that the mRNA used in these reactions was intact, RT-PCR was performed with primers specific for β-actin (Fig. 2E). Products of 370 bp were detected in mouse kidney and mDCT cells. The actin primers used herein are known to span an intron. Hence, products arising from genomic DNA would be 790 bp long, whereas those formed from mature mRNA are 370 bp (41). Because only the 370-bp product was formed, it arose from mRNA and not genomic contamination. Moreover, the absence of PCR products for PMCA2 and PMCA3 is not due to breakdown of the mRNA, because the PMCA1 and PMCA4 products were amplified from the same RNA samples (Fig. 2, A and D). These results show that all four PMCA isoforms are present in mouse kidney RNA; however, in mDCT cells, only transcripts encoding PMCA1 and PMCA4 were detected.

Analysis of regulatory domain primary structure. PMCA transcripts are alternatively spliced to generate tissue-specific subtypes. Site C, within the calmodulin-binding domain in the 3′ portion of the PMCA transcript, undergoes splicing that leads to functional and regulatory differences among subtypes (21, 32). With the inclusion of an entire downstream exon, the “a” subtype is produced; whereas, when it is excluded, the “b” subtype is formed (53). RT-PCR was performed with primers that encompass site C (Table 1) within the PMCA1 and PMCA4 transcripts to determine PMCA subtype expression by mDCT cells. Mouse kidney mRNA was used as a positive control. The primers targeting PMCA1 revealed the presence of a single product of 430 bp in both mDCT cells and whole kidney (Fig. 3A, Mouse Kidney + and DCT +). The mDCT product was sequenced, and its identity as PMCA1b was confirmed by comparison with the corresponding rat 1b and human 1b sequences (Fig. 4A). The PMCA4 primers disclosed a single product of 443 bp from mDCT cells and from whole kidney RNA, which is consistent with the predicted size for a PMCA4b product (Fig. 3B, Mouse Kidney + and DCT +). PMCA4b expression was confirmed by sequence analysis (Fig. 4B). All reactions performed in the absence (−) of RT (Fig. 4) yielded no products, indicating specific amplification of mRNA. Thus mDCT cells express transcripts encoding the PMCA1b and PMCA4b subtypes.

Northern blot analysis of mDCT RNA. To determine the sizes of the PMCA1 and PMCA4 transcripts expressed in mDCT cells, Northern blot analysis was performed on total RNA with [32P]dCTP PCR products for mDCT PMCA1 or PMCA4. The PMCA1 probe hybridized with two distinct transcripts of 7.5 and 5.5 kb in mDCT cell RNA (Fig. 5). The probe specific for PMCA4 hybridized with 8.5- and 7.5-kb transcripts in RNA from mDCT cells (Fig. 5). The origin of multiple transcripts hybridizing with the PMCA1 and PMCA4 probes is unknown. However, the mRNA sizes in
mDCT cells are similar to doublets described in other reports (35, 36, 57) and may arise from splicing of untranslated portions of the mRNAs (10).

Analysis of NCX1 Na\(^+/Ca^{++}\)/exchanger and PMCA protein expression in distal tubule cells. The NCX1 Na\(^+/Ca^{++}\)/exchanger is principally localized to cortical distal nephrons (7, 20, 47). Primary cultures of distal tubule cells, a mixture of CAL and DCT cells (44), express the NCX1 exchanger (60) and, as shown here (Fig. 1), high levels of PMCA in mDCT cells as assessed by Western blot analysis.

The expression of PMCA and NCX1 was examined by simultaneous, dual fluorescence laser scanning confocal microscopy in subconfluent and confluent mDCT cells. In a subconfluent culture grown for 48 h on collagen-coated Transwell filters (Fig. 6), PMCA and NCX1 were colocalized and found predominately to be perinuclear, near the apex of the cells (Fig. 6, A, B, E).

**Fig. 3.** Analysis of PMCA1b and PMCA4b site C alternative splicing in mDCT cells. Primer sets (Table 1) encompassing the 3' splicing within site C were used to amplify transcripts from mouse kidney and from mDCT cells. Gel was stained with ethidium bromide. Left: 123-bp molecular size ladder (M). Right: PCR product sizes.

**Fig. 4.** Sequence analysis of PMCA1b and PMCA4b subtypes in mDCT cells. mDCT PCR products from Fig. 3 were isolated and sequenced. One-letter amino acid codes are shown. Boxed, shaded residues denote exact amino acid conservation, and boxed, very lightly shaded residues denote similar amino acid conservation, and unboxed residues denote amino acid mismatches among rat, human, and mouse PMCA subtypes. -, sequence gaps. A: mouse, rat, and human PMCA1b isoforms share exact identity within the amplified region. Rat PMCA1b sequence is Swissprot accession no. P11505, and human PMCA1b sequence is Swissprot accession no. P20020. B: PMCA4b subtypes share 75–80% similarity among mice, rats, and humans. Rat PMCA4b sequence is Swissprot accession no. Q64542, and human PMCA4b sequence is Swissprot accession no. P23634.
The DCT absorbs Ca\(^{2+}\) by active cellular transport in response to calcitropic hormones and to thiazide diuretics. Efflux of Ca\(^{2+}\) across basolateral membranes requires energy-dependent movement, because extrusion is opposed by an appreciable thermodynamic barrier. Although abundant evidence supports the functional activity and expression of PMCA in DCTs, the particular isoforms expressed by the cells forming the distal nephron are controversial. PMCA isoforms are known to be expressed in a tissue-dependent manner, with functional activity and regulation potentially varying with primary structure. In DCTs, one or more of these PMCA isoforms may mediate Ca\(^{2+}\) efflux. Therefore, the purpose of the present studies was to determine the PMCA isoforms and subtypes expressed by DCT cells. We also tested the hypothesis that DCT cells express both PMCA and NCX1 Na\(^+/Ca^{2+}\) exchanger. The results demonstrate that mDCT cells express mRNA transcripts for PMCA1b, PMCA4b, and PMCA protein. In addition, laser scanning confocal microscopy revealed that only mDCT cells grown to confluence express the NCX1 Na\(^+/Ca^{2+}\) exchanger and PMCA protein in basolateral membranes. By using immunoprecipitation and tandem Western blot analysis with a monoclonal antibody to the hinge region of the erythrocyte PMCA (5), we verified the presence of PMCA protein in mDCT cell lysates (Fig. 1). The results demonstrate that clonal mDCT cells express one or more PMCAs exhibiting a molecular mass of 140 kDa, similar to that of other PMCA enzymes (5, 13, 51). The antibody used in Western blot analysis in this report cannot distinguish among the four PMCA isoforms. Therefore, we turned to RT-PCR using isoform-specific PMCA primers. Although transcripts encoding PMCA isoforms 1 and 4 were evident by RT-PCR and Northern blot analysis (Figs. 2 and 3), only a single reacting protein was detected by Western blot analysis (Fig. 1). The similar migration of PMCA isoforms during SDS-PAGE (51) most likely accounts for the appearance of a single band. The confocal data (Figs. 6 and 7) demonstrate that PMCA and NCX1 expression is polarized to the basolateral surface in cultured mDCT cells only after confluence is achieved. This is consistent with studies of the development of basolateral membrane polarity in MDCK cells, a distal-like cell culture model. In one study, Na,K-ATPase distribution (another P-type ATPase) exhibited exclusive basolateral staining only after extensive cell-cell contacts were formed (42), and in another, basolateral polarity was not observed by 48 h of growth but was only observed after 72 h (59), which is similar to the results presented here. These observations demonstrate that not only cell density but also polarized cell development must be considered in studies involving colocalization of membrane proteins.

The presence of basolateral PMCA in mDCT cells is consistent with the hypothesized role of PMCA in DCTs to extrude Ca\(^{2+}\) from the cell at the basal surface against a concentration gradient. Polarization of PMCA in mDCT cells also agrees with previous observations that the same antibody identifies PMCA localized to the basolateral surface of DCTs in paraffin-embedded rat kidney sections (5). Thus PMCA shows similar polarization in cultured confluent mDCT cells as occurs in vivo. The results also incidentally verify that cultured mDCT cells exhibit a polarized phenotype when grown to confluence.

The observations reported herein of PMCA protein expression in mDCT cells are consistent with immuno-

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**Fig. 5.** Northern blot analysis of mDCT cell RNA. Each lane contained 5 \(\mu\)g of total RNA. A: PMCA1 cDNA probe hybridizes with appropriately sized transcripts of 7.5 and 5.5 kb. B: PMCA4 probe detects transcripts of 8.5 and 7.5 kb.
Fig. 6. Distribution of PMCA and NCX1 in subconfluent mDCT cells. Cells were grown for 48 h and fixed with the use of a pH-shift protocol, incubated with PMCA- and NCX1-specific antibodies, and then incubated with appropriate secondary antibodies conjugated to Alexa 488 (green) or 594 (red). Images were captured with the use of a laser scanning confocal microscope, and the Alexa signals were merged. A–D: PMCA appears green. E–H: NCX1 appears red. I–L: colocalization appears yellow in the merge. Shown are stacks of 4 optical sections (0.5 μm each) beginning from the apex of the cells (A, E, and I), continuing through the midregions (B, F, and J, and C, G, and K), and completing at the bases (D, H, and L).
Fig. 7. Distribution of PMCA and NCX1 in confluent mDCT cells. Cells were grown for 72 h and fixed with the use of a pH-shift protocol, incubated with PMCA- and NCX1-specific antibodies, and then incubated with appropriate secondary antibodies conjugated to Alexa 488 or 594. Images were captured with the use of a laser scanning confocal microscope, and the Alexa signals were merged. A–D: PMCA appears green. E–H: NCX1 appears red. I–L: colocalization appears yellow in the merge. Shown are stacks of 4 optical sections (0.5 μm each) beginning from the apical pole of the cells (A, E, and I), continuing through the lateral regions (B, F, and J, and C, G, and K), and completing at the bases (D, H, and L).
localization studies of rat kidney, in which high amounts of PMCA protein were expressed in distal nephrons (5, 38), as well as recent studies demonstrating PMCA and NCX1 immunostaining along basolateral regions of the mouse distal nephron (37). Furthermore, the clonal mDCT cell line used here expresses low levels of the Ca^{2+}-binding protein calbindin-D_{28k} (Christakos S and Friedman PA, unpublished observations), which is shown to colocalize with PMCA in rat distal tubule cells (5, 38) and with both PMCA and NCX1 in mouse distal tubules (37).

By using RT-PCR, we showed that mDCT cells express transcripts encoding two PMCA isoforms, PMCA1 and PMCA4 (Fig. 2). Northern blot analysis performed on RNA isolated from the mDCT cells corroborates the conclusion that PMCA1 and PMCA4 isoforms are present in these cells (Fig. 5). We previously found that renal proximal tubule cells derived from mouse S1, S2, and S3 segments similarly express PMCA1 and PMCA4 (61). Some other cells, such as UMR-106 osteosarcoma cells, are also known to express multiple PMCA isoforms, i.e., PMCA1, PMCA2, and PMCA4 (1). The consequences of expressing multiple PMCA isoforms within a single cell are not known, and further investigation will be required to resolve the roles of the individual DCT PMCA isoforms.

PMCA transcripts undergo tissue-specific alternative splicing within the calmodulin-binding domain site C (35, 51, 52). By using RT-PCR with primers encompassing this site, we determined that mDCT cells contain transcripts encoding PMCA1b and PMCA4b subtypes (Figs. 3 and 4), which most likely arise from the complete deletion of a single exon. Full-length mouse PMCA1 and PMCA4 genes have not been cloned; therefore, the exact gene structures are presently unknown. However, mouse PMCA1 and PMCA4 cDNA sequences and their respective intron-exon boundaries are highly homologous to those in rats and humans (Fig. 4, A and B) (34, 35) and suggest that the mouse possesses similar gene structures. Analysis of the site C splicing of PMCA1 and PMCA4 transcripts in human (52) and rat (34) kidney RNAs supports the view that the dominant renal isoforms are the b subtypes. It was reported that the b form of the enzyme may have a higher affinity for calmodulin and exhibit greater autoinhibition of Ca^{2+}-ATPase activity than the a form (21). The activities of the PMCA are regulated by protein kinase A (PKA) and protein kinase C (PKC) (14, 62), and a recent study provided evidence that phosphorylation by PKC increased the activity of PMCA4b (22). Ca^{2+} entry into primary cultures of DCT cells and the clonal mDCT cell line is stimulated by PTH and requires activation of PKA and PKC (25). Thus, hypothetically, downstream signaling mediated by PKC could stimulate the activity of PMCA4b through a phosphorylation event, leading to increased PMCA activity and Ca^{2+} efflux from DCT cells.

Magocsi et al. (39) reported a different PMCA isoform localization in the kidney than is described here. Gross identification of PMCA isoforms by RT-PCR demonstrated that PMCA1 was found in cortex, outer medulla, and inner medulla; PMCA2 in cortex and outer medulla; and PMCA3 in outer medulla (39). PMCA4 was not analyzed because its cDNA sequence was not then known. More precise localization was achieved by microdissecting individual rat tubules followed by RT-PCR. They found PMCA2 exclusively in proximal tubules, CALs, and distal tubules (39). In more recent studies, mRNAs encoding the four PMCA isoforms in the rat kidney were reanalyzed (12). mRNAs for PMCA1 and PMCA2 were particularly abundant in glomeruli, proximal convoluted tubules, descending thin limbs of Henle’s loop, DCTs, and cortical collecting ducts. Transcripts for PMCA3 were located in the descending thin limbs and CALs. PMCA4 was found throughout the nephron.

In contrast, we failed to detect PMCA2 by RT-PCR in mDCT cells (Fig. 2). The absence of PMCA2 most likely does not involve the inability of the PMCA2 primers to amplify the transcript, because cDNA of the appropriate size was found in mouse kidney mRNA (Fig. 2B).

The reasons for the apparent discrepancy in isoform expression between our studies and those of Magocsi et al. (39) and Caride et al. (12) are unknown at this time. Several possibilities may explain this inconsistency. The present work was conducted with an immortalized cell line, whereas the earlier observations were made with dissected nephron segments (39) or sections of kidney (5, 6). Nephron segment heterogeneity may also contribute to the disparity. Studies using markers for specific distal nephron membrane proteins (Na-CI co-transporter, Na^{+}/Ca^{2+} exchanger, Tamm-Horsfall protein, and band 3 anion exchanger) showed that the DCT and CNT partially overlap in the late DCT in rat kidney (43). In this case, CNT and DCT cells may have been analyzed together and revealed the PMCA2 isoform. Moreover, by using a microdissected nephron segment composed of many cells, it is impossible to determine whether one cell type expresses multiple isoforms or whether distinct isoforms are expressed in different cells within the same nephron segment. We have shown that clonal mDCT cells express multiple PMCA isoforms (Figs. 2 and 3). It is also known that PMCA2 is largely expressed in nervous tissues (52), and the DCT is highly innervated (56). Thus PMCA2 PCR products may have arisen from renal nerves isolated in conjunction with microdissected DCTs. Finally, the PCR products described in the Magocsi et al. (39) report were not sequenced; therefore, the exact isoform amplified could not be identified.

The findings now reported are consistent with a study that examined PMCA mRNA levels in human and rat kidney (52). These investigators showed that PMCA1 and PMCA4 are the major transcripts, whereas PMCA2 mRNA constituted <2% of the total PMCA mRNA in the kidney and PMCA3 could not be detected (52). These latter results were confirmed by Western blot analysis with PMCA isoform-specific antibodies (51). PMCA1 and PMCA4 were found to be the dominant isoforms expressed in human kidney (51), although nephron localizations were not performed. The present results provide direct, molecular evidence
by RT-PCR (Fig. 2) and Northern blot analysis (Fig. 5) for the expression of PMCA1 and PMCA4 isoforms in mDCT cells.

The expression of PMCA in the DCT may indicate its participation in net Ca\textsuperscript{2+} absorption. This idea is based on several observations. First, confocal and epifluorescence microscopy show that PMCA protein is localized to the basolateral membrane of distal tubules (Fig. 7, A–D) (37), which is the primary site of transcellular Ca\textsuperscript{2+} absorption. Second, the PMCA K\textsubscript{m} for Ca\textsuperscript{2+} is 0.1–0.7 μM (8, 9, 31, 58), which is within the range of free [Ca\textsuperscript{2+}]	extsubscript{i}; stimulated by PTH, calcitonin, and thiazide diuretics in DCT cells (28–30). However, lower affinities have also been reported (9, 55). Third, PMCA activity has been assessed in membrane vesicles prepared from distal and proximal cortical nephrons (46) and in microdissected nephron segments (19). Activity in distal segments is two to five times greater than that in proximal nephron segments or membrane vesicles derived from them. Finally, kinetic evidence points to the existence of multiple renal PMCA with different affinities and velocities for Ca\textsuperscript{2+}. Brunette et al. (9) identified low- and high-affinity PMCA in rat renal membranes. Similarly, Sugimura et al. (55) demonstrated that rat basolateral membrane vesicles possess both a high-affinity (K\textsubscript{m} for Ca\textsuperscript{2+} = 0.16 μM), low-capacity and a low-affinity (K\textsubscript{m} for Ca\textsuperscript{2+} = 4.7 μM), high-capacity PMCA. Intriguingly, the mDCT cells studied here express transcripts encoding two isoforms, PMCA1b and PMCA4b (Fig. 3). It will be interesting to learn whether one isoform is the high-affinity PMCA and the other is the low-affinity pump. Hypothetically, the high-affinity isoform could be responsible for mediating changes in [Ca\textsuperscript{2+}]\textsubscript{i} near resting cellular levels as a housekeeping isoform, whereas the low-affinity PMCA might be involved in hormone- or diuretic-stimulated Ca\textsuperscript{2+} absorption.

Kinetic studies of renal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange yield a K\textsubscript{m} of 0.1–1.0 μM, which is comparable to that of the PMCA (31, 33, 48, 58), whereas the V\textsubscript{max} of the PMCA is 20–40 times greater than that for Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (2–4 nmol·min\textsuperscript{-1}·mg protein\textsuperscript{-1}). Assuming that intracellular Na\textsuperscript{+} concentration is 17.5 mM (63) and [Ca\textsuperscript{2+}]\textsubscript{i} is 120–300 nM (27), the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger operates in the forward direction at no more than 4–10% of its V\textsubscript{max}, i.e., 3–8 nmol·min\textsuperscript{-1}·mg protein\textsuperscript{-1}. Under the same conditions, the PMCA works at 30–70% of its V\textsubscript{max}, or 24–56 nmol·min\textsuperscript{-1}·mg protein\textsuperscript{-1}. Thus, under resting conditions, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange would account for only some 15% of the Ca\textsuperscript{2+} efflux, the remainder being due to the activity of the PMCA. Other, greater contributions of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger to Ca\textsuperscript{2+} efflux have also been estimated (4). Thus it is reasonable to imagine that basal Ca\textsuperscript{2+} efflux is mediated by the PMCA, and, at stimulated levels of Ca\textsuperscript{2+} transport after hyperpolarization, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange energizes efflux. This is especially the case when Ca\textsuperscript{2+} transport is associated with membrane hyperpolarization, which elevates the rate of forward electrogenic Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange.

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