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Tubular and cellular localization of the cardiac L-type calcium channel in rat kidney

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Background. The mRNAs of several types of calcium channels have been identified in intact rat kidney, and L-type calcium channels cause changes in intracellular calcium in primary cultures of distal tubule cells. The aim of this study was to evaluate the tubular and cellular distribution of the α_{1C} subunit of the L-type calcium channel in intact kidney.

Methods. RT-PCR and Northern blot analysis were used to assess the regional abundance of the mRNA of this channel. Immunocytochemistry combined with confocal microscopy and surface biotinylation were applied to determine the tubular and cellular localization of the protein.

Results. Northern blot and RT-PCR analysis indicated that the mRNA of the α_{1C} subunit of the cardiac L-type calcium channel was present in whole rat kidney, kidney tubules and kidney cell lines. Western blot of lysates from whole kidney, kidney tubules or cell lines revealed bands of ~190 kD for the α_{1C} subunit and ~60 kD for the β_3 subunit. Confocal immunohistochemistry indicated that the α_{1C} subunit of this channel was co-expressed in cells of the distal tubule that express calbindin-D_{28K}, but not in intercalated cells. The α_{1C} subunit was also highly expressed in both outer and inner medullary collecting ducts. Serial confocal microscopic images or surface biotinylation experiments determined that the channel was predominantly on the basolateral membrane but had some distribution on the apical membrane.

Conclusions. The distribution and cellular localization of the α_{1C} subunit of cardiac L-type calcium channel suggest it is probably involved in intracellular and membrane calcium signaling.

The family of depolarization-gated calcium channels is distributed in excitable tissues including heart, smooth and skeletal muscle, brain and endocrine organs where they function to increase calcium influx or to transduce voltage changes [1]. Six types of α_1 subunit have been cloned including three L-type channels (α_{1S}, α_{1C}, and α_{1D}), the ω-conotoxin-GIVA-sensitive N-type (α_{1B}), the ω-agatoxin-IVA-sensitive P/Q-type (α_{1A}), and the resistant R-type (α_{1E}). The L-type calcium channel complex is composed of multiple subunits [2] but the α_1 subunit comprises the main conduction core, the voltage sensor and also contains the drug binding sites for dihydropyridine antagonists such as nitrendipine or nifedipine or the agonist Bay K 8644, for phenylalkylamines such as verapamil, and for benzothiazipines such as d-cis-diltiazem [1, 3]. When the α_1 subunit is expressed alone, the membrane current density is low and voltage activation is slower than in endogenous tissue. The other subunits (α_2, β, γ, and δ) increase current activation and inactivation of the conduction core. Calcium channels also are expressed in non-excitable cells [4–8] and the mRNA of several types of calcium channels have been identified in both the cortex and medulla of intact rat kidney [9]. In distal tubule cells, Barry et al [10] found that antisense to the α_{1D} subunit inhibits a parathyroid hormone (PTH)-mediated calcium influx, whereas antisense to the α_{1C} subunit inhibits the diuretic-mediated calcium influx. In the cortical collecting duct calcium signaling is mediated by β-adrenergic receptors [11]. In medullary thick ascending limb cells, L-type calcium channels also have been implicated in increases in intracellular calcium after volume expansion [12]. Finally, in inner medullary collecting ducts, vasopressin receptors cause elevation of intracellular calcium [13].

Recently, two other calcium channels from the TRP family have been cloned and localized to the distal tubule of the kidney [14, 15]. Both ECaC [14] and CAT-2 [15] are localized in the principal cells of the distal tubule where 1α,25-dihydroxyvitamin D_3 responsive transepithelial calcium transport occurs, and both of these proteins colocalize with the major 1α,25-dihydroxyvitamin

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D_3-responsive component of the transepithelial calcium transport system, the intracellular calcium buffering protein calbindin-D_{28K}. Evidence suggests that these channels are the entry pathway for transepithelial calcium transport including calcium permeability, and activation by hyperpolarization, inhibition by cadmium and low pH, but little effect of blockers of L-type calcium channels [15]. However, because the α_{1C} subunit has been implicated by Barry et al to cause changes in intracellular calcium in primary cultures of distal tubule cells [10], we wondered whether the channel also would be colocalized with other components of the transepithelial calcium-transporting pathway in the intact kidney. Such colocalization would suggest that transepithelial calcium transport mediated by ECaC or CAT-2 may be modulated by calcium influx via L-type calcium channels. However, since the mRNA of the α_{1C} subunit was localized in the medulla where the highest abundance was in the inner medulla [9], calcium influx via this channel also may function to alter transport in tubule segments in this region. In this regard, the mRNA of the pore forming subunit of the α_{1G} channel (a T type channel) is distributed in high abundance in the inner medulla and in IMCD tubules [16], and using immunohistochemistry this channel was localized to the membranes of connecting tubules cells, distal convoluted tubule cells and the inner medullary collecting duct cells, suggesting that more than one pathway for calcium influx exists in these cells.

Our results show that the mRNA for the α_1 subunit of the cardiac L-type calcium channel is present in the cortex and medulla of the intact rat kidney and in a mouse inner medullary collecting duct cell line. Using reverse transcription-polymerase chain reaction (RT-PCR), mRNA was found in the proximal straight tubule, the cortical collecting duct and in medullary collecting duct tubules. The protein was localized in cells of the distal tubule that also express calbindin D_{28K} and in cells of the medullary collecting duct where its apical and basolateral membrane distribution would allow it to participate in calcium signaling. The β_3 subunit also is present in inner medullary collecting duct tubules, in the mouse inner medullary collecting duct cell line, and in a mouse medullary thick ascending limb cell line.

METHODS

Cells

The mouse medullary thick ascending limb cell line (mTAL), derived from an SV40-transgenic mouse, was grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 50 U/mL penicillin and 50 μg/mL streptomycin. The mouse inner medullary collecting duct cell line (IMCD; ATCC CRL-2123; ATCC, Rockville, MD, USA) derived from an SV-40 transgenic mouse, was grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 10% FBS and 50 U/mL penicillin and 50 μg/mL streptomycin. For extraction of RNA for Northern blot or proteins for Western blot, cells were split in a 1:4 ratio and plated onto T75 flasks without coating and grown to two days post-confluence. For immunocytochemistry or surface biotinylation cells were split and plated at 1:4 density on high pore density PET track-etched transwells (Beckton Dickinson Falcon, Franklin Lakes, NJ, USA) and grown three to five days after confluence.

Northern blot

The probe for the calcium channel was generated by RT-PCR from rat brain. Primers for α_{1C}, bp 5851-5871 and bp 6360-6341 were designed using sequence from rbC-II α_{1C} Accession # M67515, to give an expected PCR product of 510 bp. The area chosen for the probe between amino acid 1864 to 2033 is relative to Accession # AAA18905. This region was chosen because it shared only 51 amino acids with α_{1D} and 22 amino acids with α_{1S}. Our procedures for probe preparation, RNA sample preparation and blot hybridization have been previously described [17, 18].

RT-PCR

Reverse transcription-polymerase chain reaction was performed on cDNA made from RNA isolated from rat kidney cortex, outer medulla, and inner medulla. Rat brain was used as a positive control. In addition RT-PCR was performed on isolated rat kidney tubules. In order to isolate the tubular RNA, male Sprague-Dawley rats, about 150 g, were anesthetized and then the left kidney was perfused via the abdominal aorta using a perfusion/dissection solution containing 133 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L Na_2HPO_4, 3 mmol/L Na acetate, 1 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 0.5 mmol/L HEPES and 5.5 mmol/L glucose in 0.1% diethylpyrocarbonate treated water adjusted to pH 7.4, followed by 30 mL perfusion/dissection solution supplemented with 100 mg collagenase B and a 0.1% bovine serum albumin (BSA; Boehringer Mannheim, Mannheim, Germany). Once the tubules were dissected they were added to a pre-chilled sterile 1.5 mL Eppendorf tube containing 1 mL TRIzol. The tubules were homogenized immediately upon dissection using a tissue homogenizer (Kontes Glass Co, Vineland, NJ, USA) designed for Eppendorf tubes. The RNA was isolated according to the TRIzol protocol (Gibco BRL, Gaithersburg, MD, USA).

The primers for amplifying PCR products were taken from the nucleotide sequence of the mRNA as outlined for the calcium channel probe used in the Northern blot. The area chosen for these primers, amino acids YQDD ENR and APGRQF relative to Accession number AAA 18905, is not shared by α_{1D}, which has the sequences TWSRQNY and NKNSDK, respectively, in the same

area or α_{1S} that has HVDKLER, and the reverse primer has a deleted sequence in this region. RT-PCR for α_{1C} subunit of L-type calcium channel was performed following the protocols described previously [17, 18].

Western blot

The calcium channel α_{1C} subunit antibody (ACC-003) was obtained from Alomone Labs Ltd. (Jerusalem, Israel). This affinity-purified rabbit polyclonal antibody was raised to the peptide sequence TTKINMDDLQP SENEDKS corresponding to amino acids 818-835 of rat α_{1C} subunit (Accession # JH0426). A BLAST search at the National Center for Biotechnology Information revealed that this amino acid sequence was specific for α_{1C}. When the sequences of the other L-type calcium channel isoforms were lined up no more than seven dispersed amino acids matched this sequence for α_{1S} and no more than five dispersed amino acids matched for α_{1D}. A like sequence was not found in any other voltage-gated calcium channel including N, R or P/Q types. The antibody to the β_3 subunit of the calcium channel (ACC-008) was also obtained from Alomone Labs Ltd. This affinity purified rabbit polyclonal antibody was raised to the peptide sequence DRNWQRNRPWPKDSY corresponding to amino acids 463-477 of the rat brain β_3 subunit (Accession # AAB32350.1). Rat heart [2] or rat brain [19] was used as a positive control tissue for the α_{1C} and β_3 subunit antibodies, respectively.

Our procedures for protein sample preparations and Western blot have been described previously [18, 20]. To evaluate the distribution of protein in individual kidney tubules, male Sprague-Dawley rats weighing about 150 g were anesthetized, then the left kidney was perfused via the aorta using Hank's balanced salt solution (GIBCO BRL) containing a cocktail tablet of protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). After this, the tubules were dissected on ice in the same solution, and then the isolated kidney tubules were homogenized in Eppendorf tubes in the RIPA buffer [18, 20]. The proteins were separated by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; α_{1C} subunit) or 7.5% SDS-PAGE (β_3 subunit). Then proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) in a Tris/glycine transfer buffer (Bio-Rad) containing 5% methanol and 0.007% SDS (α_{1C} subunit) or 10% methanol (β_3 subunit). Membranes were blocked with 5% nonfat milk in Tris buffered saline containing 0.05% Tween 20 (TTBS) for one hour, and then were incubated overnight with primary antibody at 1.5-3 μg/mL (α_{1C} subunit) or 0.3 μg/mL (β_3 subunit) diluted with blocking buffer. After washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Life Science, Arlington Heights, IL, USA) diluted 1:10,000 with blocking buffer for one hour at room temperature. The blots were visualized by the SuperSignal®West Dura extended duration substrate (Pierce, Rockford, IL, USA). The free peptide utilized to generate each antibody was pre-incubated with the primary antibody at 5 μg/mL to determine the specificity of the labeling.

Immunocytochemistry

The identification of various tubular segments in the kidney was primarily based on their general anatomical and histological features combined with co-immunolocalization of several specific markers. The anatomical and histological features of various tubular segments were examined on hematoxylin and eosin (H&E) stained sections (data not shown) that were adjacent to the immunostained sections. The criteria used to identify the tubular segments were done according to definitions outlined by Tisher and Madsen [21]. In addition, we used calbindin-D_{28K} as a marker for calcium transporting distal tubules. Calbindin-D_{28K} was chosen since it is well documented that calbindin-D_{28K} is expressed in the distal tubule of many species including chicken, rat, rabbit, pig and human [22–26]. In human and rat kidney, calbindin-D_{28K} has been immunolocalized in the distal convoluted tubule, the connecting tubule and the initial segment of the cortical collecting duct. Connecting tubules and cortical collecting tubules were recognized by the appearance of the intermingled intercalated cells [27, 28], which were identified using fluorescence staining with peanut lectin [29, 30]. Discrimination between connecting tubules and cortical collecting tubules, though sometimes difficult, was based on their location in the cortex and in particular by the superficial T branches, indicating transitions between these segments [31]. Furthermore, we co-localized α_{1C} L-type calcium channel with Tamm-Horsfall protein using a sheep anti-Uromucoid antibody (Biodesign International, Kennebunk, ME, USA) to identify the medullary thick ascending limbs (mTALs; data not shown).

The calcium channel α_{1C} subunit antibody (ACC 003) used for immunocytochemistry was the same as described above for the Western blot. Tissue fixation, section preparation and immunostaining were performed following routine protocols as described previously [18, 20]. Rat kidney and brain were fixed by perfusion through the aorta with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, followed by a post-fixation in the same solution overnight. Both frozen sections and paraffin sections were used for immunostaining. To determine specificity of the staining, 1 μg of the antigenic peptide was added per microgram of primary antibody (3 or 6 μg/mL) in a three hour preincubation. The subcellular distribution of this subunit was analyzed with a confocal scanning unit (LSM 410; Carl Zeiss, Oberkochen, Germany) by evaluating at least 10 tubule segments in a series of x-y cuts (z sections) at 1 μm through the tubules.

The mouse monoclonal antibody to calbindin-D_{28K} obtained from Sigma (St. Louis, MO, USA) was generated using purified chicken gut calbindin-D_{28K} as the immunogen. This antibody reacts specifically with calbindin-D_{28K} in brain and kidney tissues from human, monkey, rabbit, rat, mouse, chicken. Rat cerebral cortex was used as positive control because the easily identified Purkinje cells (data not shown) are well established to have calbindin-D_{28K}. This antibody was used at a dilution of 1:500 with a secondary FITC-conjugated donkey anti-mouse IgG at a dilution of 1:200 (Jackson Immunoresearch, West Grove, PA, USA). The tubular colocalization of the antibodies to calbindin-D_{28K} and to the α_{1C} subunit were done by scoring about 2000 tubules in coronal sections of rat kidney cortex. The cellular colocalization of the antibodies to calbindin-D_{28K} and to the α_{1C} subunit was determined by scoring over 500 random cells in the cortex. The fluorescein-labeled, affinity-purified, peanut agglutinin was obtained from Biomeda (Foster City, CA, USA) and used at a dilution of 1:300. The tubular and cellular colocalization of peanut lectin and the α_{1C} subunit were preformed as described above for calbindin-D_{28K} and the α_{1C} subunit. Peanut lectin and calbindin-D_{28K} were expressed in tubules together 87% of the time. Double fluorescence sections were analyzed with an inverted microscope coupled to a confocal scanning unit (LSM 410; Carl Zeiss).

Cell surface biotinylation

Inner medullary collecting duct (IMCD) cells were plated on six-well cell culture inserts (Beckton Dickinson Falcon). The cells were analyzed three to five days after confluence. The cell surface biotinylation assay was carried out essentially as previously described [32]. The filter-grown monolayers were placed on the ice and washed three times with ice-cold Dulbecco's phosphate buffer saline (Gibco BRL) containing 1.36 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ (DPBS-Ca-Mg). Then the cells were incubated with 1 mg/mL NHS-SS-biotin (Pierce) in DPBS-Ca-Mg for 30 minutes at 4°C with gentle shaking. For apical biotinylation, 1 mL biotin solution was added to the apical chamber, and the basolateral compartment received DPBS-Ca-Mg lacking NHS-SS-biotin. For basolateral biotinylation, 2 mL biotin solution was added to the basal chamber, and the apical compartment received DPBS-Ca-Mg lacking NHS-SS-biotin. After Biotinylation, the cells were rinsed once and washed twice with 100 mmol/L glycine in DPBS-Ca-Mg to quench the reaction. After an additional two washes with cold DPBS-Ca-Mg, the monolayers were excised from the filter cup with a razor blade. The monolayers were then scraped and solubilized with RIPA buffer containing the protease inhibitors as used for protein isolated for the Western blots. The lysates were centrifuged at 10,000 $\times$ *g* for 10 minutes at 4°C. The supernatants were collected, from which 1/20 was taken as an estimate of the input, and the rest was incubated with 200 μL streptavidin-agarose beads (Pierce). To ensure the complete recovery of biotinylated protein, the extraction with streptavidin-agarose beads was performed two times. The first extraction was performed overnight and the second for two hours. After washing the beads with RIPA buffer five times, 50 μL of 2 $\times$ Laemmli sample buffer (Bio-Rad) containing 200 mmol/L dithiothreitol (DTT) was added and incubated at 37°C for one hour to elute the biotinylated protein. The elution process was also performed two or three times to ensure complete recovery. After centrifugation at 14,000 $\times$ *g* for two minutes, the eluates were collected for Western blot analysis using the α_{1C} subunit antibody (ACC 003) as described above and a monoclonal antibody to the α1 subunit of Na,K-ATPase (Upstate Biotechnology, Lake Placid, NY, USA). The densitometry analysis was performed using the Scion Image (Scion Corporation, Frederick, MD, USA).

RESULTS

mRNA of α_{1C} in kidney and kidney cell lines

Hybridization by Northern blot probe specific to the α_{1C} subunit of the L-type calcium channel showed two bands one at 9.5 kb and another at about 12 kb compared to the top molecular weight marker at 9.5 kb and the lower molecular marker at 7.5 kb (Fig. 1A, lane M). Hybridization of these bands was weak in the cortex (Fig. 1A, lanes C and C), stronger in the outer medulla (Fig. 1A, lane OM) and the strongest kidney expression was in the inner medulla (Fig. 1A, IM). Inner medullary collecting duct cells expressed comparable mRNA to the kidney cortex (Fig. 1A, lanes IMCD cells). The heart expressed similar mRNA when the total RNA was greatly diluted compared to that used in the kidney lanes. The mRNA for the α_{1C} subunit was previously reported in both cortex and medulla of the kidney with a lesser amount in the cortex and a larger amount in the inner medulla [9]. Figure 1B, top panel, shows RT-PCR products of the α_{1C} subunit of the L-type calcium channel from rat brain, kidney cortex, outer medulla and inner medulla. The size of the product was 510 bp as expected from the primer sequences and sequencing the products in both directions determined that the sequence was that of rat α_{1C}. RT-PCR products from tubules segments isolated from rat kidney are shown in Figure 1B, bottom panel. The lane labeled M shows the 100 bp ladder. The brightest molecular weight marker in the middle of the blot is 600 bp. The RT-PCR products ran near the 500 bp marker as expected. The product was present in all tubule segments tested, including the glomeruli, proximal convoluted tubules (PCT), proximal straight tubules (PST), medullary thick ascending limb (mTAL), cortical collecting ducts (CCD), outer medullary collecting ducts

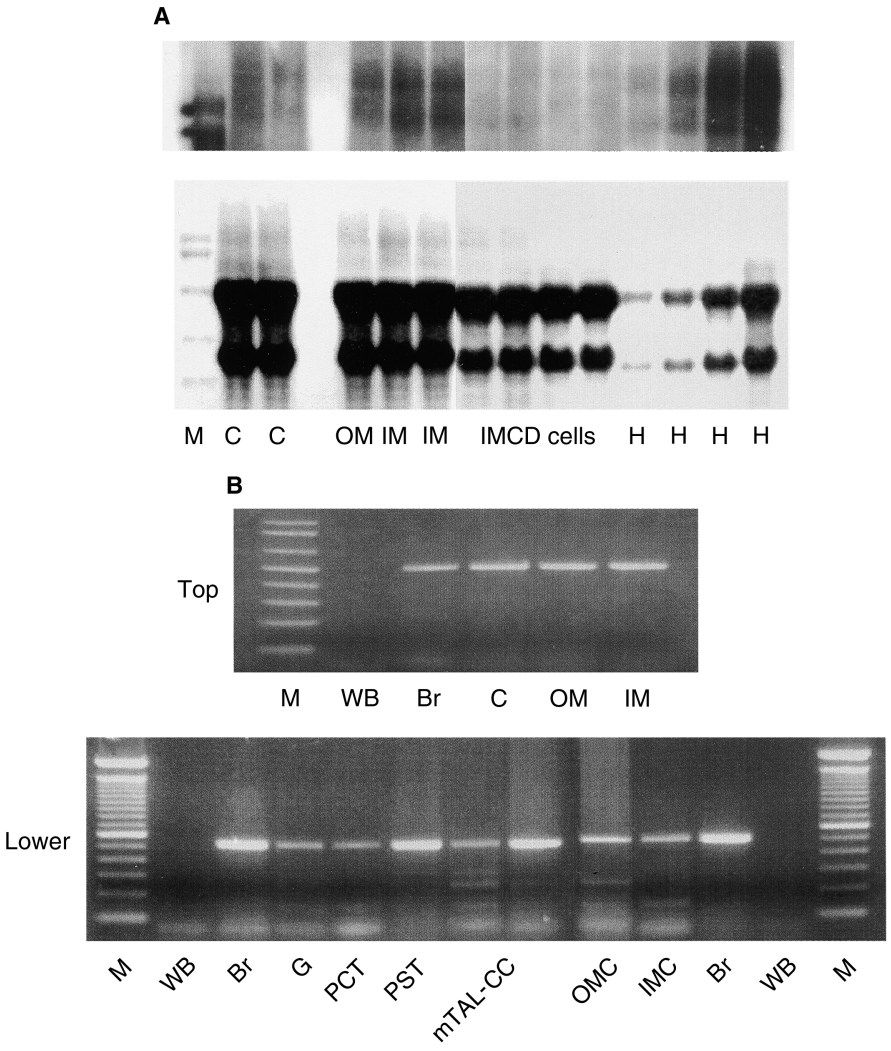


Fig. 1. (*A*) Northern blot of α_{1C} in rat heart, rat kidney, and kidney cell lines using a cDNA probe. The probe was made from bp 5851-6360 relative to Accession #M67515. The top panel shows the Northern blot hybridization showing two hybridization bands at 9.5 kb and another above 12 kb. The molecular makers on the left (M) are 7.5 and 9.6 kb. In the bottom panel the RNA is stained with methylene blue to show the 28S and 18S bands. In this lower blot the molecular marker on the left (M) shows the 28 S band at about 4.4 kb and the 18S band between 2.4 kb and 1.8 kb. Abbreviations are: C, rat kidney cortex; OM, rat outer medulla; IM, rat inner medulla; IMCD cells, the mouse inner medullary collecting duct cell line; H, rat heart total RNA. There is much less mRNA for the α_{1C} subunit in kidney when compared to heart because the hybridization was weaker in kidney compared to heart when less total mRNA was loaded. Forty micrograms of total RNA was loaded in each lane of kidney RNA whereas the four lanes of rat heart (H) had 2, 5, 10 and 20 μg of RNA loaded. (*B*) Distribution of mRNA of α_{1C} in whole kidney and tubule segments of the kidney as demonstrated by RT-PCR. (Top) PCR products were amplified from all areas of the kidney. Abbreviations are: M, molecular weight marker showing that the PCR product runs at 510 bp as expected; WB, the PCR reaction run with water instead of cDNA as a negative control; Br, brain; C, cortex; OM, outer medulla; IM, inner medulla. (Lower) PCR products were amplified from each tubule segment of isolated tubules of the rat kidney and also from rat brain (Br). Abbreviations are: M, molecular weight marker (the bright band in the middle of the gel is the 600 bp marker); WB, water blank (this PCR was run with water rather than cDNA as a negative control); Br, rat brain as a positive control; G, glomerulus; PCT, proximal convoluted tubule; PST, proximal straight tubule; mTAL, medullary thick ascending limb; CC, cortical collecting duct; OMC, outer medullary collecting duct; IMC, inner medullary collecting duct.

(OMCD), and inner medullary collecting ducts (IMCD). The assay was not optimized to be quantitative.

α_{1C} subunit in heart, kidney and kidney cell lines

Immunoblots of rat kidney tubules and rat heart lysates revealed a band about 190 kD (Fig. 2A). The intensity of the band in rat mTAL tubules (lane 1) and rat CCD tubules (lanes 2, 3) was much less than that of IMCD tubules (lanes 4, 5). Mouse mTAL cells grown in culture (lane 6) and mouse IMCD cells grown in culture (lane 7) also have less intense bands, whereas rat heart (lane 8) had the most intense band even though half the amount of protein compared to the kidney was loaded. The most prominent band was about 190 kD in all preparations and the second upper band above the 220 kD marker was consistent with a molecular weight of about 240 kD, which corresponded to the full length molecule [2]. These two molecular weights also corresponded to the two molecular weight mRNAs in the Northern blot, suggesting that there is some full length protein in the IMCD and mTAL cells resulting in the higher molecular weight protein. Lanes 9 to 14 show that the peptide to which the antibody was raised, completely displaced the bands near 190 and 240 kD, suggesting that the antibody was specific.

β_3 subunit in brain, kidney and kidney cell lines

Figure 2B is a Western immunoblot of the β_3 subunit of the L-type calcium channel. This antibody stained a band at about 60 kD in IMCD tubules (lane 1), mTAL cultured cells (lane 2), IMCD cultured cells (lane 3), and rat brain as a positive control (lane 4) [19]. The bands stained by the anti-β_3 antibody were displaced by the peptide to which the antibody was raised in mTAL cells (lane 5), IMCD cells (lane 6) and brain tissue (lane 7).

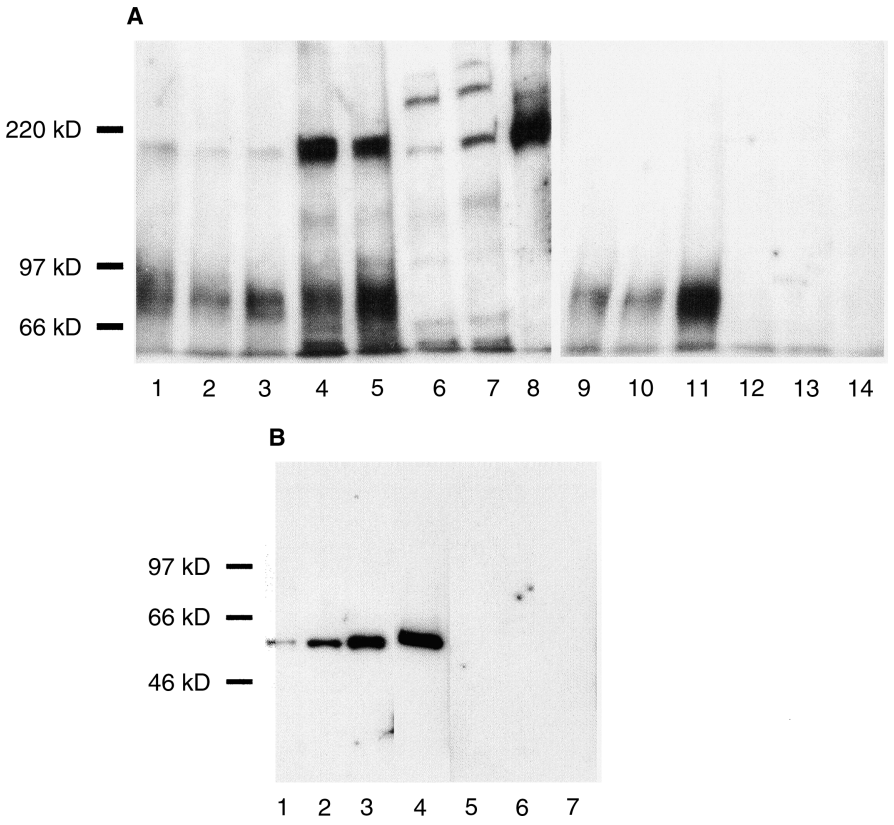


Fig. 2. (*A*) Identification of the α_{1C} subunit of the L-type calcium channel in an immunoblot of rat kidney tubules and kidney cell lines and rat heart using an antibody to the intracellular loop between domains II and III (ACC-003). Lanes 1 and 9, medullary thick ascending limb (mTAL) tubules. Lanes 2, 3 and 10, cortical collecting duct (CCD) tubules. Lanes 4, 5 and 11, inner medullary collecting duct (IMCD) tubules. Lanes 6 and 12, mTAL cells. Lanes 7 and 13 are IMCD cells. Lanes 8 and 14, rat heart (15 μg per lane) as a control. All other lanes had 30 μg of protein added per lane. As shown in lanes 1 through 8, the α_{1C} protein is about 200 kD, and there was more expression of the α_{1C} subunit in IMCD tubules and IMCD cultured cells. Lanes 9 through 14 show that the peptide, to which the antibody was raised, completely displaced the bands near 200 kD. (*B*) Western blot of the calcium channel β_3 subunit protein in rat IMCD tubules and cultured mTAL and IMCD cells. Lane 1, the affinity purified anti-β_3 subunit antibody stained a 60 kD protein in rat IMCD tubules. The antibody stained a similar molecular weight protein in mTAL cells in Lane 2 and in IMCD cells in Lane 3. Lane 4, rat brain tissue used as a positive control for the β_3 subunit. This band is about 60 kD as previously shown. There was stronger expression in IMCD cells than in mTAL cells. The bands stained by the anti-β_3 subunit antibody were displaced by the peptide to which the antibody was raised in mTAL cells (lane 5), IMCD cells (lane 6) and brain tissue (lane 7). Each lane had 10 μg of total protein added.

Immunocytochemical localization of the α_{1C} subunit in kidney

Immunocytochemical localization of the α_{1C} subunit of the L-type calcium channel in the kidney indicates a distribution to tubule segments in both the cortex and medulla (Fig. 3). To confirm the identity of the various tubule segments, H&E staining (not shown) was performed in the adjacent sections to the immunofluorescent section. Two types of immunostaining for the α_{1C} subunit were easily identified. The first type of staining was bright, intense, and sharp and closely associated with membranes (Fig. 3A, II, III, IV; Fig. 3B, V, VI), whereas the second type of staining was weak and distributed evenly in the cytoplasm (Fig. 3A, II). Both the bright and diffuse staining was completely displaced by peptide pre-absorption (Fig. 3B, VII), which suggests that these two types of staining were specific for the α_{1C} subunit.

In the kidney cortex, the bright staining was localized to the distal convoluted tubules (DCT), the connecting tubule (CT) or the cortical collecting duct (CCD; Fig. 3A, II, III, IV). In these cells, the staining was mainly localized to the basolateral membrane. but some was also present in the apical membrane. The weaker, more diffuse staining in the cortex was primarily distributed in the cytoplasm of the proximal tubules (PT; Fig. 3A, II). The staining in the cortical thick ascending limbs was comparable to that in PTs and appeared intracellular. In the medulla (Fig. 3B), the bright staining was in various segments of the OMCD (panel V) and the IMCD (panel VI), which were identified as tubules with a thin cytoplasm and a large inner tubular diameter. The staining in the medullary thick ascending limb was weak and distributed in the cytoplasm. Little staining was found in the thin limbs of Henle's loop, but it could be that the fluorescence was below the limits of detectability in these thin segments.

Double labeling of α_{1C} and calbindin-D_{28K} on the same kidney section showed that most of the cortical bright tubules that stained for α_{1C} colocalized with tubules that express calbindin-D_{28K}. It is well known that calbindin-D_{28K} is expressed in the distal tubule of many species including chicken, rat, rabbit, pig and human [22–25]. Murine calbindin-D_{28K} mRNA has been localized to the distal convoluted tubule, the cortical connecting tubule, and the cortical collecting ducts [26]. In human and rat kidney, calbindin-D_{28K} has been immuno-localized in the distal convoluted tubule, the connecting tubule and the initial segment of the cortical collecting duct. Rat calbindin-D_{28K} was previously localized, in addition to the distal tubule of the kidney, to cerebellum Purkinje cells and

to dentate gyrus granule cells [33]. As a positive control for the calbindin-D_{28K} antibody used in this study, we localized staining to Purkinje cells (not shown). Of all the α_{1C} bright-stained tubules in the cortex 71% were colocalized with calbindin-D_{28K} staining (Fig. 4A) and the remaining 29% of the red staining for the α_{1C} subunit was alone, mainly distributed in the medullary rays (Fig. 4A, DCT). Of the total population of calbindin-D_{28K}-labeled tubules, 48% were not colocalized with the bright α_{1C} stained tubules. These tubules that stained only for calbindin-D_{28K} were mainly distributed in the cortical labyrinth. These results suggest that in the cortex, the bright α_{1C} staining was localized to the late portion of the DCT, the CT and most of the length of the CCD.

In those tubules where calbindin-D_{28K} and α_{1C} colocalized, 92% of the cells had staining for both calbindin-D_{28K} and the α_{1C} subunit (Fig. 4B, C and D), suggesting that α_{1C} was mainly distributed in the calbindin-D_{28K} positive cells. The calbindin in these cells buffers the hormone-regulated calcium entry that is part of active transepithelial calcium transport [14]. As shown in Figure C and D, the subcellular localization of calbindin-D_{28K} and the α_{1C} subunit was different. Whereas calbindin-D_{28K} is known to be primarily in the cytoplasm [22–25], the α_{1C} subunit was localized more in the plasma membrane. The distribution of the α_{1C} subunit was mainly in the basolateral membrane but also in the apical membrane; however, as revealed in multiple x-z sections that were analyzed by confocal microscopy, the α_{1C} subunit appeared to be most often in basolateral membranes. Further confirmation of the cell type where the α_{1C} subunit resides was illustrated by double-labeling the α_{1C} subunit with peanut lectin. On the same section, only 20% of the cells showed colocalization of both the α_{1C} subunit and peanut lectin. Peanut lectin has been used previously as a marker of intercalated cells in the cortical collecting duct [29, 30]. In the rat kidney, these intercalated cells are present in the later portion of the distal convoluted tubule, the connecting tubules and the cortical collecting duct [27, 28]. Of the total tubules that stained for peanut lectin, 89% were colocalized in tubules that also contained α_{1C} staining (Fig. 4 E, F). The fact that there was a higher percentage of tubular, although not cellular, colocalization of the α_{1C} subunit with peanut lectin as compared to calbindin-D_{28K} also supports the idea that the α_{1C} staining is localized in the later portion of distal convoluted tubule, the connecting tubule and most of the cortical collecting duct in rat kidney cortex.

The staining of α_{1C} subunit of the calcium channel in the outer and inner medullary collecting duct cells was very bright and appeared to be both intracellular and in the plasma membrane. Because the staining in the IMCD cells appeared to be both in the membranes and inside the cell and the intracellular staining could be a result of fluorescence bleeding in these small cells, we investigated this distribution in a more quantitative manner using surface biotinylation. In order to determine the percentage of the channel that is targeted to the plasma membranes compared to that inside IMCD cells, surface biotinylation was performed on both the apical and basolateral membranes of polarized, cultured IMCD cells grown to five days post-confluence on permeable transwell supports. Surface biotinylation of IMCD cells showed that although some of the α_{1C} subunit was distributed in the membrane fractions a major portion of the channel protein was present inside the cell (Fig. 5A). Figure 5A lanes 2 and 3 show 1/20 of the total cellular α_{1C} subunit in the apical and basolateral biotinylation experiments, respectively. Figure 5A lanes 4 and 6 show the total apical biotinylated α_{1C} subunit and lanes 5 and 7 show the total basolateral biotinylated α_{1C} subunit. In this experiment about 1% of the total α_{1C} subunit is on the apical membrane whereas 5.4% is on the basolateral membrane. (The quantitative details of the densitometry analysis are shown in the legend to Fig. 5). Figure 5B lanes 2 and 3 show 1/20 of the total cellular Na,K-ATPase in the apical and basolateral biotinylation experiments, respectively. Figure 5B lanes 4 and 6 show the total apical biotinylated Na,K-ATPase and lanes 5 and 7 show the total basolateral biotinylated Na,K-ATPase. In this experiment about 4.1% of the total Na,K-ATPase is on the apical membrane whereas 36% is on the basolateral membrane. The densitometry analysis of immunoblots from three separate experiments revealed that $5.3 \pm 0.8\%$ (mean $\pm$ SE) of the total cellular content of the α_{1C} subunit resided in either the apical or basolateral plasma membranes, whereas $40 \pm 1.8\%$ of the total Na,K-ATPase resided in the membrane fractions. Of the α_{1C} subunit that was inserted into the plasma membrane, $17 \pm 1.1\%$ resided at the apical membrane whereas $83 \pm 1.1\%$ was inserted into the basolateral membrane. In the same three experiments, $6.7 \pm 3.6\%$ of the Na,K-ATPase was in the apical membrane and $93 \pm 2.9\%$ was on the basolateral membrane, suggesting that the surface biotinylation assay itself has a small finite leak or error because it is well documented that the Na, K-ATPase is predominantly in the basolateral membrane [34]. The estimate of the apical membrane distribution of the Na,K-ATPase using the surface biotinylation assay was probably an overestimate for two reasons. First, because the density of the Na,K-ATPase is so high on the basolateral membrane not all of the basolateral Na,K-ATPase was removed in the three elutions (not shown), although this efficiency is greater than other reports of 5% efficiency [35], probably resulting in an underestimate of the membrane fraction of Na,K-ATPase compared to total Na,K-ATPase. This also causes an overestimate of the apical distribution compared to basolateral distribution. In comparison, all of the α_{1C} subunit protein was removed by the second elution (Fig. 5, lanes 6, 7) and all of the apical Na,K-ATPase was removed

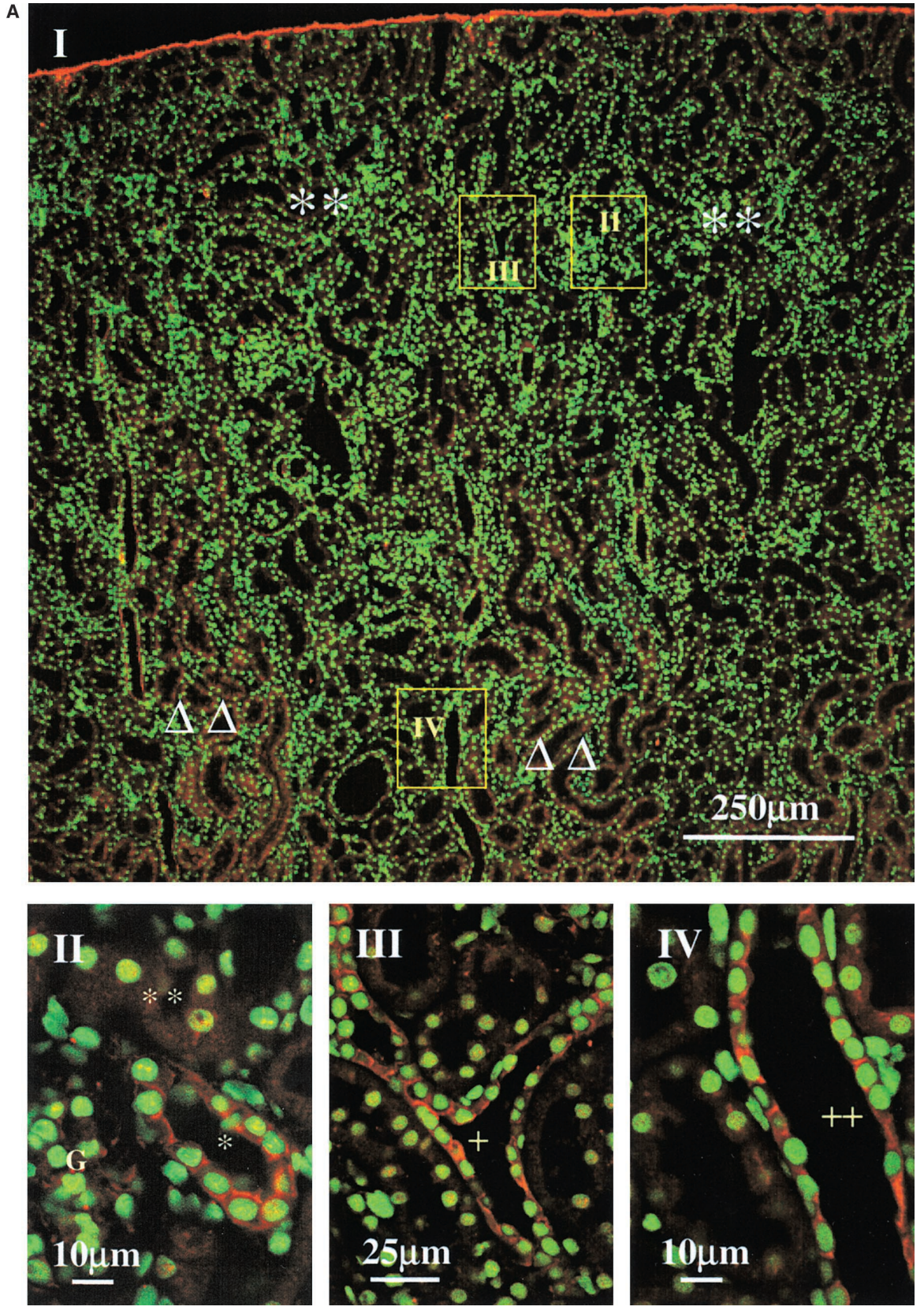


Fig. 3. See legend next page.

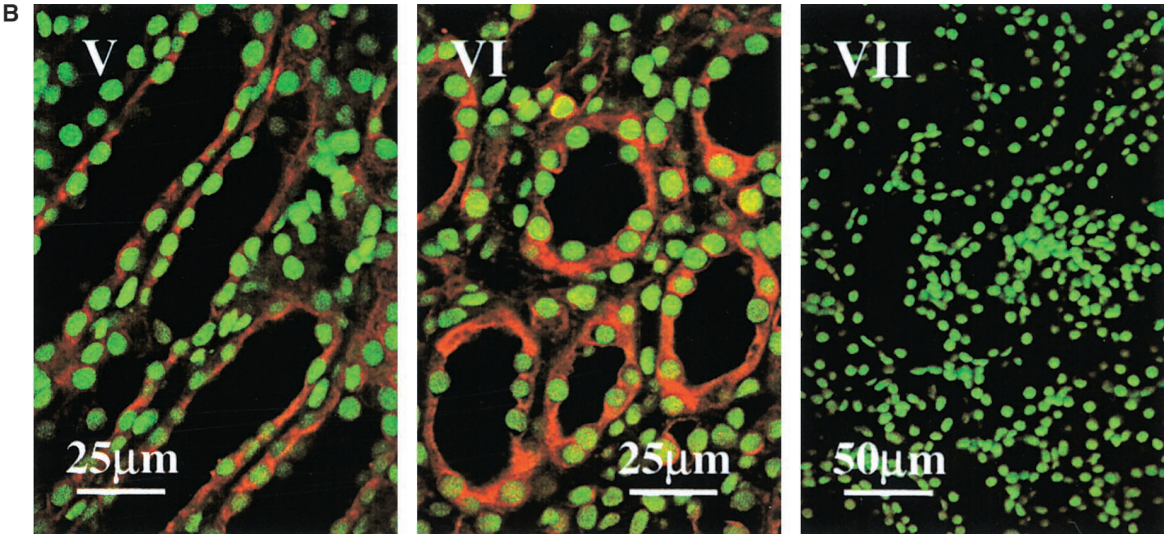
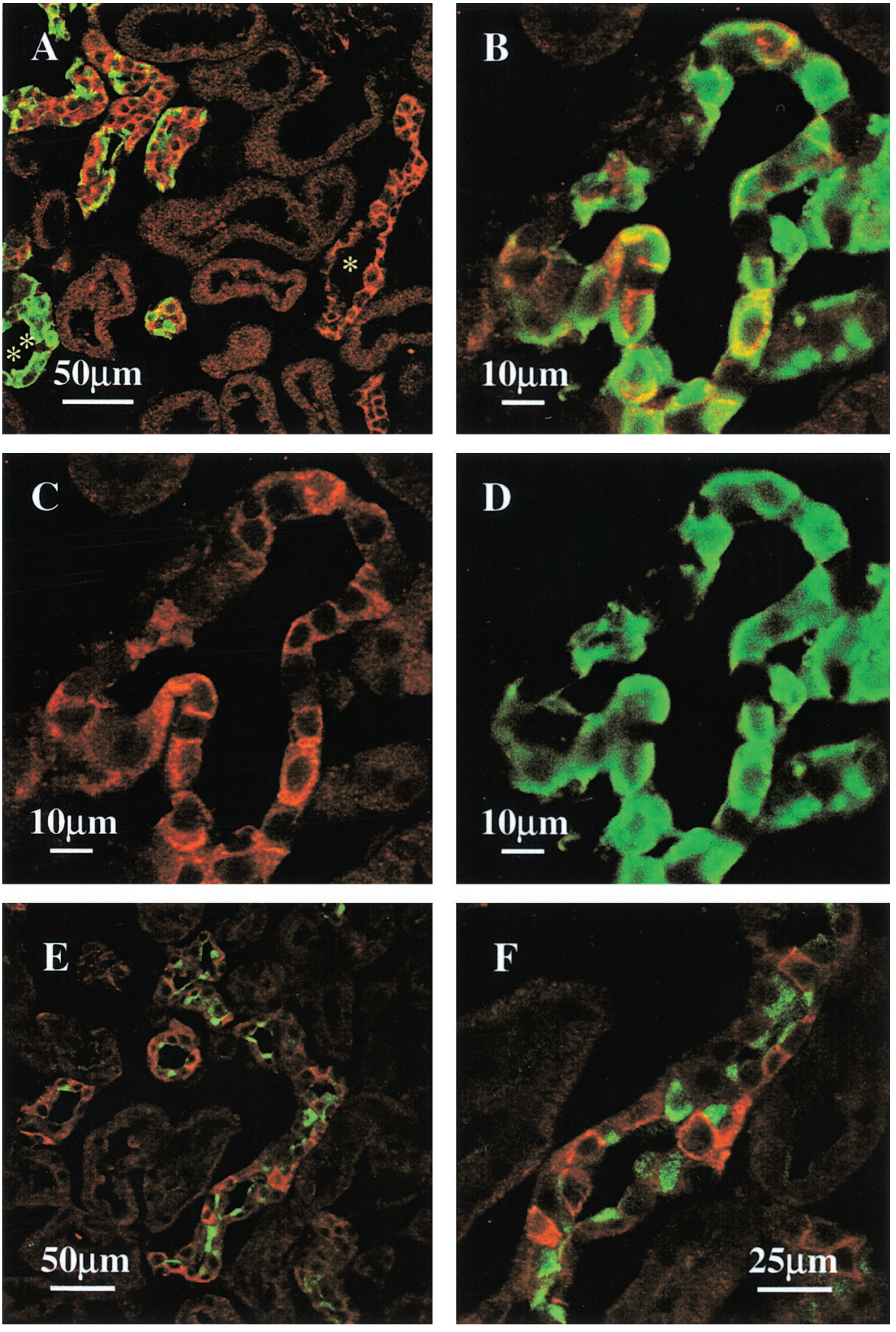


Fig. 3. (*A*) Immunohistochemical localization of the α_{1C} subunit of the L-type calcium channel in whole rat kidney. The primary antibody, a peptide purified rabbit polyclonal antibody (ACC-003) to α_{1C} (Alomone, Israel) was diluted 1:100 (3 μg/mL) and the secondary antibody goat anti-rabbit IgG cy3 (red) was diluted 1:200. The nuclei are counterstained with Hoechst 33342 (green). (panel I) Localization of the α_{1C} subunit of the L-type calcium channel in the kidney cortex at a low-magnification illustrates that positive staining of the α_{1C} subunit antibody distributes in the cortical labyrinth (**) around the corpuscles and in the medullary ray (ΔΔ). The boxes in Fig. 3A panel I that are labeled II, III, and IV are shown in higher magnification in Fig. 3A panels II, III, IV, below. (Panel II) The bright staining of α_{1C} was in the cortical labyrinth in isolated short tubule segments of small diameter near the glomerulus indicating the distal convoluted tubule (*). This staining was mainly distributed in the basolateral membranes as analyzed by X-Z sections (not shown). The staining in tubules of larger diameter that indicated proximal tubules (**) was less intense, diffuse and located in the cytoplasm. G denotes a glomerulus that also shows diffuse staining. (Panel III) The bright staining of α_{1C} was localized mainly in the plasma membrane of the tubules of the cortical connecting tubule (+) that were recognized by the position in the cortex and by the superficial T branch indicating transition from connecting tubules to cortical collecting ducts. The staining also was localized primarily in basolateral membranes as analyzed by X-Z sections (not shown). (Panel IV) In the medullary rays, bright staining was distributed in tubules with a large lumen diameter suggesting tubules of the cortical collecting duct. The lumen is marked (++). (*B*) High power magnification of tubules from the medulla of the rat kidney. (Panel V) Tubules of the OMCD and (panel VI) tubules of IMCD. In the medulla, the brightest staining was in various segments of the medullary collecting duct. The staining in IMCD was most intense and distributed not only in the membrane fraction but also in the cytoplasm. (Panel VII**)** The peptide to which the antibody was raised displaced all of the bright staining for α_{1C} in the cortex and the medulla including all of the weaker staining in the proximal tubules.

in two elutions (not shown). Secondly, using confocal microscopy we confirmed that the distribution of the Na,K-ATPase is solely in the basolateral membrane with no apical membrane distribution (cultured kidney cells grown one week post-confluence on permeable transwells). In this case, the immunofluorescent staining using the same antibody to the Na,K-ATPase used in these biotinylation experiments was analyzed using confocal microscopy (data not shown). Under the same growth conditions (almost one week post-confluence grown on transwells) IMCD cells also showed apical tight junctions as indicated by ZO antibody staining visualized by confocal microscopy (data not shown). These additional confocal experiments suggest that the cells grown in culture have established a normal apical and basolateral polarity and complete basolateral distribution of the Na,K-ATPase. Therefore, the apical distribution of the Na,K-ATPase measured in the biotinylation experiment is probably a result of the limits of the biotinylation assay itself and similar apical biotinylation results for the Na,K-ATPase has been observed before [32]. However, because the amount of distribution of the α_{1C} subunit of the calcium channel in the apical membrane is greater than that of the Na,K-ATPase, it suggests that there is a finite distribution of the calcium channel in the apical membrane of IMCD cells. Gottardi, Dunbar and Caplan previously showed that biotinylation efficiency sometimes depends on pH and that alkaline pH often improved biotinylation efficiency, but that some proteins were better biotinylated at neutral pH [32]. Given that the biotinylation of basolateral Na,K-ATPase was not completely efficient, the main focus of biotinylation in this study was to determine the percentage of the α_{1C} subunit of the L-type calcium channel protein present on the apical versus basolateral surface, rather than the total amount of α_{1C} subunit in the plasma membrane. While the total amount of α_{1C} subunit or the Na,K-ATPase in the plasma membranes might be underestimated, the ratio of α_{1C} subunit on the apical and basolateral plasma membrane would not change with pH. By comparing the ratio of apical versus basolateral biotinylation of α_{1C} with that of Na,K-ATPase on the same cells (Fig. 5B), we could determine whether there was a significant fraction of α_{1C} on the apical membrane.


A
50μm
B
10μm
C
10μm
D
10μm
E
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F
25μm

In summary, the α_{1C} subunit of the cardiac isoform of the L-type calcium channel in rat kidney was distributed through all kidney tubules except the thin limbs of Henle's loop. The staining in glomerulus, proximal tubule and thick ascending limb of Henle was weak and diffuse, whereas the staining in the late distal convoluted tubule, the connecting tubule, the cortical collecting duct, outer and inner medullary collecting ducts was bright, intense and mainly membrane associated. The most intense staining was seen in the IMCD, where surface biotinylation of IMCD cells indicates a greater basolateral than apical membrane distribution.

DISCUSSION

Yu et al showed that rat kidney expresses mRNA transcripts of the three α_1 isoforms of the L-type calcium channel as well as the P-type calcium channel, but that the distribution of each of these is different [9]. The channel mRNA was located primarily in the cortex and by PCR it showed a distribution in the distal convoluted tubule. The mRNA of the α_{1D} isoform was equally distributed in the kidney cortex, and in the inner and outer medulla. Yu et al also found very small amounts of the α_{1S} subunit mRNA in the inner medulla [9]. They found that the α_{1C} isoform mRNA was located primarily in the medulla with the greatest hybridization in the inner medulla. The Northern blot of α_{1C} mRNA in rat kidney (Fig. 1) also suggests that the major distribution of the mRNA for the α_{1C} subunit is in the inner medulla, but that this amount of mRNA is significantly less than that in the heart. Using RT-PCR, we also found that the α_{1C} subunit calcium channel mRNA is present in cortex, outer medulla and inner medulla as well as along each tubule segment and in the glomerulus. However, based on the Northern blot the amount of mRNA in the glomerulus, proximal tubule, and mTAL is probably much less than in the inner medulla. Interestingly, two L-type calcium channel mRNA transcripts (Dmca1D and Dmca1A) have been found by RT-PCR in *Drosophila melanogaster* Malpighian tubules, suggesting that L-type calcium channels must have functional significance in many species [36]. The unique distribution of the mRNA for each calcium channel isoform in the kidney suggests that each calcium channel α subunit isoform is probably coupled to unique calcium signaling pathways that in turn affect epithelial function or blood flow.

Sequencing of a subcloned transcript from rabbit proximal tubules indicates that the mRNA of the α_{1C} subunit is identical to the rabbit cardiac calcium channel α_{1C} except for a 33 base pair deletion in the motif 4 S3-S4 linker region [37]. Investigations by O'Neil et al indicate high affinity [^{3}H]dihydropyridine binding sites in apical, basolateral and microsomal membrane preparations of cultured rabbit renal proximal tubules [38], which would be consistent with the distribution found in proximal tubules by the immunostaining. Cell swelling of rabbit proximal tubules induced by hypo-osmotic medium causes an increase in intracellular calcium that is inhibited by the dihydropyridine or verapamil classes of calcium channel blockers [39, 40], and this increase in calcium in response to hypo-osmotic medium is also inhibited by antisense oligo-nucleotides to the α_{1C} subunit [37]. Pertinent to this observation, we found that the staining for the protein of the α_{1C} subunit in the proximal tubule is mainly intracellular. This raises the interesting possibility that the distribution of the channel could change from intracellular to membrane associated, for example, with hypo-osmotic medium or that the channel could have a heretofore unexpected intracellular function. Cell swelling of mTAL cells caused by hypo-osmotic medium results in an increase in intracellular calcium and a compensatory volume regulatory decrease that are inhibited by the presence of the calcium channel blockers verapamil or nitrendipine, or by removal of extracellular calcium or by buffering intra-

Fig. 4. Double immunofluorescent staining of the α_{1C} subunit of the L-type calcium channel and calbindin-D_{28K} or peanut lectin in rat kidney. The rabbit polyclonal antibody to α_{1C} was recognized by an anti-rabbit cy3-conjugated secondary antibody that produced the red staining. The mouse monoclonal antibody to calbindin-D_{28K} was recognized by a FITC-conjugated secondary antibody to produce a green stain. Overlap of the two stains produces yellow. (*A*) This view shows a low magnification of α_{1C}-labeled tubules in the rat kidney cortex. Of the tubules that were brightly stained for the α_{1C} subunit, 71% were colocalized with calbindin-D_{28K} staining and 29% of the tubules stained for the α_{1C} subunit alone (*). Of all the calbindin-D_{28K} positive tubules in cortex, about 48% of the tubules expressed calbindin-D_{28K} alone (**) whereas 52% of the tubules had calbindin-D_{28K} colocalized with the α_{1C} subunit. This colocalization suggests that many tubules that contain high amounts of α_{1C} in the kidney cortex are located in the distal tubule. (*B*) High power view of the cellular distribution of α_{1C} and calbindin-D_{28K} together. This shows that 92% of the cells stained for both α_{1C} and calbindin-D_{28K}. α_{1C} is localized primarily to the basolateral membrane (*C*) whereas calbindin-D_{28K} is mainly localized in the intracellular compartment (*D*). Overlap of the two antibodies produces a yellow stain. (C) The same view as B showing the cellular distribution of α_{1C} is mainly in the membranes of the cells (the green channel is subtracted). (D) The same view as B, showing the cellular distribution of calbindin-D_{28K} is mainly in the cytoplasm of the cells as previously shown (the red channel is subtracted). (*E* and *F*) Double immunofluorescence staining for the α_{1C} subunit of the L-type calcium channel and peanut lectin in rat kidney. The rabbit polyclonal antibody to α_{1C} was recognized by the cy3-conjugated secondary antibody producing a red stain. A FITC-conjugated peanut lectin produces a green stain. In all of the tubules stained by α_{1C} subunit, 88% are colocalized in the tubules that show peanut lectin staining. Panel F is a high magnification of panel E. The peanut lectin staining was located mainly in the apical region of the intercalated cells. Of the double-positive stained tubules, little of the staining was in the same cells as noted by the lack of yellow stain, which would indicate overlap. The staining of each color was quite distinct. This adds an additional indication that the calcium channel was distributed in the principal cells.

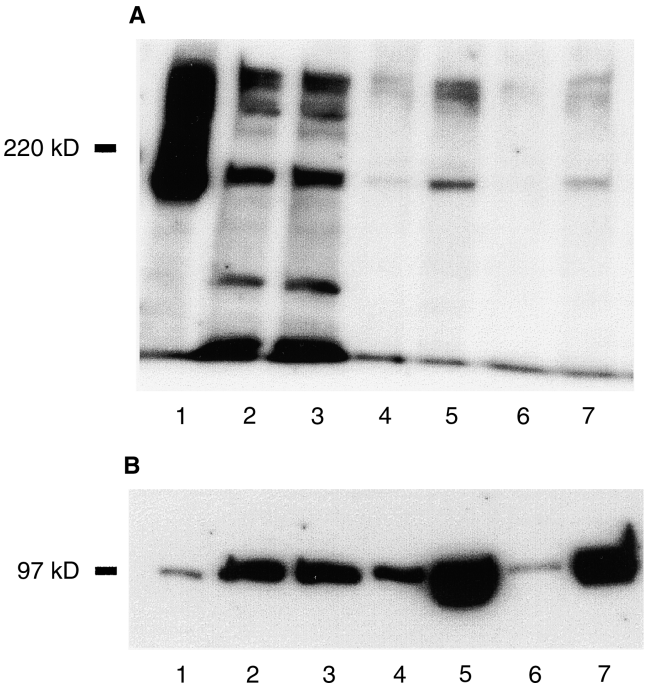

Fig. 5. (*A*) Western blot analyses of a representative biotinylation experiment examining the amount of apical and basolateral membrane-biotinylated α_{1C} subunit of the L-type calcium channel in IMCD cells. These results show one representative experiment of three separate experiments performed. Lane 1, 20 μg rat heart homogenate shown as control; lane 2, 1/20 of input, 114 μg of total IMCD protein for the apical biotinylation experiment (density 2306); lane 3, 1/20 of input, 121 μg of total IMCD protein for the basolateral biotinylation experiment (density 2335); lane 4, apical biotinylated α_{1C} from the first elution and first extraction (density 297); lane 5, basolateral biotinylated α_{1C} from the first elution and first extraction (density 1579); lane 6, apical biotinylated α_{1C} from the second elution and first extraction (density 131). The density of the third elution (not shown) is 15. Lane 7, basolateral biotinylated α_{1C} from the second elution and first extraction (density 832); the density of the third elution (not shown) is 82. The calculated total input density of α_{1C} for this apical biotinylation experiment is 46,120 arbitrary units and the amount of apical biotinylated α_{1C} subunit density from the three elutions is 443 arbitrary units. About 1% of the total α_{1C} subunit found in the apical membrane of these IMCD cells. The calculated total input density of α_{1C} subunit for this basal biotinylation experiment is 46,700 arbitrary units. The total amount of basolateral density of the α_{1C} subunit from the three elutions is 2493 arbitrary units. About 5.4% of the total α_{1C} subunit of the calcium channel is found at the basolateral membrane of IMCD cells. (*B*) Western blot analyses of a representative biotinylation experiment examining the amount of apical and basolateral membrane-biotinylated Na,K-ATPase in IMCD cells. This is the same blot as used for the experiment above but it has been stripped and reprobed with the antibody to the Na,K-ATPase. Lane 1, 20 μg rat heart homogenate shown as control; Lane 2, 1/20 of input, 114 μg of total IMCD protein for the apical biotinylation experiment (density 3651). Lane 3, 1/20 of input, 121 μg of total IMCD protein for the basolateral biotinylation experiment (density 3758), Lane 4, apical biotinylated Na,K-ATPase from the first elution and first extraction (density 2501). Lane 5, basolateral biotinylated Na,K-ATPase from the first elution and first extraction (density 12338). Lane 6, apical biotinylated Na,K-ATPase from the second elution and first extraction (density 573). The density of the third elution (not shown) is 15. Lane 7, basolateral biotinylated Na,K-ATPase from the second elution and first extraction (density 8309). The total amount of apical Na,K-ATPase density from the three elutions is 3089 arbitrary units and comparing this with the input density of 73,202 arbitrary units means that about 4.1% of the total Na,K-ATPase is found in the apical membrane of these IMCD cells in this experiment. The calculated total input density is for the Na,K-ATPase in this basolateral biotinylation experiment is 75,160 arbitrary units. The total amount of basolateral Na,K-ATPase from the three elutions is 26,626 arbitrary units. About 35.4% of the total cellular Na,K-ATPase is found at the basolateral membrane of IMCD cells in this experiment.

cellular calcium with BAPTA, suggesting that calcium influx via L-type calcium channels is a necessary component of the volume regulatory machinery [12]. L-type calcium channels also have been identified in the mesangial cells of the glomerulus using patch clamp recordings and fura 2 fluorescence [41], but because the staining of the α_{1C} subunit protein was quite weak in the glomerulus perhaps another isoform may account for L-type calcium channel activity in these cells.

Investigations by Barry et al suggested that the activity of the α_{1D} subunit is correlated to increases in intracellular calcium caused by parathyroid hormone (PTH) [10]. On the other hand, antisense oligonucleotides directed to the α_{1C} subunit inhibited the elevation of intracellular calcium caused by chlorothiazide. These authors hypothesized that the two isoforms of the L-type calcium channel cause calcium entry in the distal tubule. However, it is well established that both PTH and thiazides cause hyperpolarization of the apical membrane of the distal tubule. Since the L-type calcium channel is not stimulated by membrane hyperpolarization but rather by membrane depolarization, if this channel were in the apical membrane, calcium influx there would be inhibited by PTH and chlorothiazide. By contrast, the recently identified ECaC1/CAT channels are activated by hyperpolarization and are located in the apical membrane of distal tubules where calbindin-D_{28K} and the sodium calcium exchanger exist [42]. Using Z cuts through many tubules, we found that the protein of the α_{1C} subunit of the L-type calcium channel is primarily located on the lateral and basal membranes, but some of the staining also resides in the apical membrane of the distal tubule. Based on this distribution, this channel would cause the greatest calcium influx when the basolateral membrane of the distal tubule is depolarized. Because intracellular calcium causes inactivation of the ECaC channel [42], calcium influx via the α_{1C} subunit of the L-type calcium channel could actually block calcium entry and transepithelial calcium transport in this tubule segment. Because cyclic AMP (cAMP) is known to phosphorylate the cardiac α_{1C} subunit of the L-type calcium channel enhancing channel opening, in the presence of PTH a depolarizing membrane potential at the basolateral membrane would enhance opening of the cardiac α_{1C} subunit of the L-type calcium channel causing even greater increases in intracellular calcium. Similarly in the cortical collecting duct, calcium influx via the α_{1C} subunit of the L-type calcium channel would inhibit the amiloride-sensitive sodium

channel, ENaC, by blocking at the intracellular face of this channel [43]. Likewise, the flow-dependent calcium-activated potassium channel in the apical membrane of the cortical collecting duct [44] would have increased gating upon elevation of intracellular calcium via the α_{1C} subunit of the L-type calcium channel situated in the principal cells of this segment.

The major distribution of the protein of the α_{1C} subunit of the L-type calcium channel is in the cells of the outer and inner medullary collecting ducts. In these cells the V2 vasopressin receptor is known to mobilize calcium in addition to cAMP [13]. Star et al found that vasopressin increases calcium in the terminal part of the IMCD, but that the change in cAMP—not calcium—is responsible for the changes in urea permeability [45]. In addition, Mooren et al found that calcium influx was increased by hypoosomolality in IMCD cells [46]. On the other hand, the calcium influx caused by membrane associated L-type calcium channels may not cause global increases in intracellular calcium, but these increases in calcium may be localized to near the membrane where the activity of the calcium-activated potassium channel [47] or other calcium-mediated membrane events could be stimulated. Given the technical limits of immunolocalization in small cells and the inefficiency of biotinylation, we conclude that IMCD cells have both a membrane and intracellular localization of α_{1C}, suggesting that the L-type calcium channel may play an additional intracellular function as well as apical and basolateral membrane calcium signaling.

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