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Expression of Dihydropyridine Binding Sites in Renal Epithelial Cells

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It has recently been shown that rabbit kidney epithelial cells (proximal tubule) possess two dihydropyridine (DHP)-sensitive calcium entry channels (1, 2). To evaluate the properties of the DHP binding sites, the binding of the DHP, [^{3}H]PN200-110, was studied in epithelial cell membrane fractions (proximal tubule) of rabbit kidney cortex. High-affinity binding sites for the DHP were observed in both basolateral and apical membranes and in a membrane microsomal preparation from rabbit primary cultures of proximal tubule cells (cultured PT). In an extended analysis of the basolateral membrane preparation, two high-affinity binding sites were evident with binding dissociation constants, K_d, of 0.005 and 0.75 nM. The K_d values are similar to that observed for L-type calcium channel α_1-subunits. Using a homology-based cloning strategy, a 388-base fragment of an α_1-subunit was cloned from RNA isolated from rabbit cultured PT cells and freshly isolated proximal tubules and found to encode a protein identical to the cardiac form of the L-type α_1-subunit (α_{1C}-subunit). It is concluded that renal epithelial cells express high-affinity dihydropyridine receptors and that the receptors may be components of plasma membrane calcium channels, including L-type calcium channels, that control calcium entry in these cells.

A major class of calcium channel in excitable tissues is the voltage-activated, L-type calcium channel. This channel is activated by membrane depolarization and, as such, plays a central role in intracellular calcium signaling during cell activation by a variety of humoral and physical conditions that alter membrane voltage (see reviews, 3, 4, 5, 6, 7, 8). A characteristic of the L-type calcium channel is the sensitivity to the 1,4-dihydropyridines (DHP). DHPs are known to bind to these channels with high affinity and act as either agonists or antagonists of the channel (9, 10, 11, 12, 13). Pharmacologically the channels are classified, therefore, as DHP-sensitive. Furthermore, the DHP-binding site or receptor has been shown to be part of the α_1-subunit of the channel (14, 15, 16, 17, 18, 12, 19).

DHP-sensitive calcium channels have now been identified in a wide variety of cells. Recently, an osmo-mechanically activated calcium entry pathway which was sensitive to DHPs was identified in renal epithelial cells (20, 21, 2) and bone cells (22). In renal cells the calcium entry pathway was blocked by low concentrations of DHPs, such as nifedipine and nitrendipine, but was not readily activated by the typical DHP agonists, Bay K 8644 (23, 24, 2). Using the single-channel patch clamp technique in renal proximal tubule cells, two calcium-selective channels were identified which were sensitive to DHPs (1, 2). One of these channels was activated by membrane depolarization while the other was activated by membrane stress or treatment with protein kinase C (PKC) activators. Both channels were blocked by low concentrations (μM) of nifedipine while, the voltage-activated channel was activated by low concentrations of Bay K 8644 typical of L-type channels. Hence, DHP-sensitive calcium channels are expressed in epithelial cells although the relation to L-type channels is not fully known. Furthermore, the properties of the DHP-binding sites have not been elucidated.

The purpose of the present study was to provide an initial assessment of the properties of the DHP-binding sites in renal epithelial cells. Using an equilibrium binding assay for the high affinity DHP, PN200-110, two high affinity binding sites were identified in plasma membranes of the epithelial cells. Furthermore, expression of a cardiac form of an L-type calcium channel was confirmed in these cells using RT-PCR. Hence, at least two DHP binding sites are expressed in these cells, both of which may be associated with calcium channels. Part of this study has been published in abstract form (25).

METHODS

Membrane preparation. Cell membranes were isolated from New Zealand white rabbit kidneys. Kidneys were removed under anesthe-

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sia and placed in ice-cold isolation buffer (100 mM mannitol, 5 mM HEPES, 1 μg/ml each of protease inhibitors aprotinin, leupeptin, and pepstatin A, and the pH adjusted to 7.4 with KOH). Membranes were isolated and partially purified from cortical slices and, hence, primarily reflect proximal tubule membranes. Basolateral membranes were isolated using the procedure of Dubinsky and Monti (26). Briefly, cortical slices were minced and homogenized in a Potter-Elvejhem glass homogenizer with a teflon pestle at 1100 rpm. The homogenate was centrifuged at 3000 $\times$ g (Sorvall RC-5B, SS34 rotor) for 15 minutes, the supernatant recentrifuged at 10,000 $\times$ g for 10 minutes, and the final supernatant recentrifuged at 30,000 $\times$ g for 45 minutes. The resulting pellet was resuspended and homogenized (6-8 strokes, by hand) in 10 mls ice cold isolation buffer containing 1 M NaBr. The membrane preparation was then layered on a discontinuous sucrose gradient (25, 30, 35, 40, and 45% sucrose in isolation buffer with 1 M NaBr) and centrifuged in a Beckman L8-55 ultracentrifuge (SW 28 rotor) at 95,000 $\times$ g for 2 hours. The basolateral membranes were carefully aspirated from the 25-30% sucrose interface, diluted in 2$\times$ ice cold isolation buffer and centrifuged in the ultracentrifuge (Ti 70 rotor) at 75,000 $\times$ g for 1 hour. The membrane pellet was resuspended in isolation buffer, homogenized (6 strokes, by hand), aliquoted and stored at -80 °C until use. The purity of the basolateral membranes was verified using the Na-K-ATPase activity as a marker of the basolateral membrane (26). The basolateral membrane preparation was enriched by 10.1-fold over the original homogenate.

In a few studies, proximal tubule apical membranes were isolated from cortical slices using the Mg^{2+} precipitation methods which will preferentially precipitate proximal tubule brush border membranes (apical membrane) (27). Briefly, after an initial homogenization and centrifugation to obtain a crude membrane preparation as done above (30,000 $\times$ g spin), the pellet was resuspended in isolation buffer and $MgCl_2$ added to give a final concentration of 10 mM, and incubated on ice for 45-60 minutes. The suspension was centrifuged at 1500 g for 10 minutes, the supernatant saved and the pellet resuspended with added $MgCl_2$ for an additional 60 minutes. After a second centrifugation, the supernatants, containing the apical membranes, were combined and diluted in isolation buffer containing 1 mM EDTA. The mixture was centrifuged at 100,000 $\times$ g for 15 minutes, the pelleted resuspended in isolating buffer and centrifuged again at 100,000 $\times$ g for 15 minutes. The resulting pellet, containing apical membranes, was stored at -80 °C until use. The purity of the apical membrane preparation was verified using alkaline phosphatase activity as an apical membrane marker (27). Compared to the original homogenate, the apical membrane preparation was enriched 4.6-fold.

To determine if the DHP binding sites were also expressed in primary cultures of proximal tubules, primary cultures were grown to confluency in plastic tissue culture dishes (100 mm plates, 7-9 days) as described in detail previously (23, 1). A crude cell membrane preparation was prepared (cultured cell membranes) after washing the cells 2$\times$ with PBS containing protease inhibitors (1 μg/ml each of aprotinin, leupeptin, and pepstatin A). Cells were lysed by adding 5 ml of 5 mM TrisHCl (ice cold), pH 8.0, with protease inhibitors, to each dish and allowed to stand for 30 minutes at 4 °C. The sample was aspirated and centrifuged at low speed for 5 minutes. The pellet was homogenized (6 strokes by hand) and centrifuged at 3,000 $\times$ g for 10 minutes and the supernatant collected and re-centrifuged at 75,000 $\times$ g for 30 minutes as done above. The membrane pellet, defined as the cultured cell membranes, was resuspended in 50 mM MOPS and stored at -80 °C until use.

The protein content of all samples was determined using the methods of (28) using bovine serum albumin as a standard.

Dihydropyridine binding assay. Dihydropyridine binding sites were assayed by monitoring the specific binding of [^{3}H]PN200-110 to membranes of each of the above membrane preparations as done by others (9, 29, 30). Typically 75-150 μg of membrane vesicles (basolateral membranes, apical membranes, or cultured cell membranes) were added to 2 ml MOPS buffer (50 mM MOPS, 0.1 mM $CaCl_2$, pH 7.4) in a test tube under red light and 0.01 to 0.5 nM [^{3}H]PN200-110 (0 - 26 μCi from a 8 Ci/mmole stock) added to each tube and mixed. Specific binding was obtained from the difference in total binding of [^{3}H]PN200-110 and non-specific binding obtained in the presence of over a 1000-fold excess of cold PN200-110 (1 μM). Membrane preparations were incubated at room temperature in the dark for 2 hours and then washed 5$\times$ with 5 ml ice-cold MOPS buffer by filtration through Whatman GF/f filters. The filters were added to 10 ml Hydrofluor and radioactivity counted in a liquid-scintillation. Non-specific binding typically averaged 70-80% of total binding.

Reverse transcriptase-PCR. Total RNA was extracted using guanidine isothiocyanate (31) from rabbit isolated proximal tubules (isolated PT) or from confluent primary cultures of proximal tubules (cultured PT). The isolated PT and cultured PT cells were obtained from kidney cortical slices as described in detail previously (23). In a few cases RNA was extracted from samples of rabbit cardiac and aortic tissue.

A homology-based cloning strategy was used to clone cDNA encoding a fragment of the L-type calcium channel α_1-subunit (the pore forming subunit with the DHP-binding site) using reverse transcriptase-PCR (RT-PCR) to amplify the mRNA from kidney tissue as done before (32). Sense and antisense primers for PCR were selected from a region of the α_1-subunit mRNA that was relatively conserved between cardiac and skeletal α_1-subunits (see Figure 3). Based on the cardiac sequence, the sense primer was a 19 mer sequence starting at base location 3400 and the antisense primer was a 17 mer sequence ending at base location 3824. Using the Gene Amp RNA PCR kit (Perkin-Elmer-Cetus), complementary DNA was synthesized from each RNA sample via reverse transcription with random hexamer priming. The PCR reactions were performed on each cDNA using the following temperature protocol: denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, and elongation at 72 °C for 90 s for 40 cycles. The amplified PCR products were ligated into the PCR1000 vector (Invitrogen, Inc.). The cloned DNA was sequenced by chain termination (33) of ^{35}S-labeled molecules generated by modified T7 DNA polymerase (Sequenase, United States Biochemical Corp.).

Chemicals and drugs. Labelled (+)-[^{3}H]PN200-110 (isopropyl-4-(2,1,3,-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarbozylate) was obtained from Amersham and unlabelled PN200-110 from Sandoz Pharmaceuticals Corporation (Isradipine). Protease inhibitors (aprotinin, leupeptin, and pepstatin A) were from Boehringer Mannheim Biochemicals. Oligonucleotide primers were made on site. All other chemicals were obtained as specified above or from Sigma.

RESULTS

Dihydropyridine binding. The dihydropyridine, PN200-110, displayed high affinity binding to all renal cell membranes. As shown in Figure 1, binding of [^{3}H]-PN200-110 to basolateral membranes occurred in the subnanomolar range and appeared to approach saturation binding with nM concentrations of ligand. When the basolateral membrane data were replotted using a Scatchard plot analysis (34) to evaluate binding sites, a non-linear plot was obtained, indicative of more than one binding site (Figure 1 inset). The data could best be fit with a two-receptor binding model with dissociation constants, K_d, of 0.005 nM and 0.75 nM, respectively. While the maximal number of binding sites, B_{max}, could not be estimated reliably with the limited number of data points, the initial estimates from the Scatchard plots for B_{max} are 0.2 and 9.1 pmol/mg, respectively, for

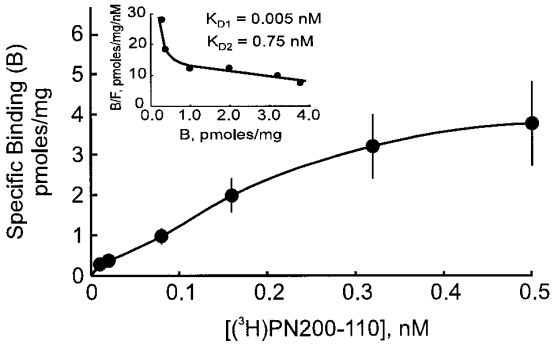

FIG. 1. Specific binding of (+)-[^{3}H]PN200-110 to partially purified basolateral membranes from the kidney cortex. The inset is a Scatchard plot showing specific binding (B) against specific binding/free (+)-[^{3}H]PN200-110 (B/f). The data were fit to a two site binding model with dissociation constants for site 1, K_{d1}, and site 2, K_{d2}, of 0.005 and 0.75 nM, respectively. The line is the best-fit to the model.

the two binding sites. Whether additional binding sites are also present in renal tissue cannot be determined from this analysis.

To determine if other proximal tubule cell membrane preparations also have high affinity dihydropyridine binding sites, specific binding of [^{3}H]PN200-110 to other membrane preparations using a constant concentration of [^{3}H]PN200-110 (0.32 nM) was also assessed. As shown in Table 1, all proximal tubule membrane fractions displayed high affinity dihydropyridine binding sites with a similar number of binding sites apparent at this level of PN200-110. Specific binding averaged 3.8, 2.0, and 8.2 pmol/mg for the basolateral membranes, apical membranes, and cultured PT membranes, respectively. Assessment of the number of binding sites will require a detailed receptor analysis for these other membrane fractions.

Expression of an L-type calcium channel. L-type calcium channels are known to possess high affinity Dihydropyridine binding sites (see above). It may be that the source of the DHP binding sites in the renal epithelial cells reflects expression of an epithelial L-type calcium channel. Using RT-PCR with primers for a 388-base fragment of cDNA encoding a conserved region of the cardiac L-type calcium channels (see METHODS and Figure 3), the PCR product using proximal tubule RNA yielded a DNA fragment that appeared to be identical in size to that obtained from RNA isolated from either cardiac or aortic tissue (Figure 2). Both cardiac and aortic tissue are known to express the cardiac form of the L-type calcium channel α_1-subunit (α_{1C}-subunit). An amplified DNA product of similar size was observed for RNA isolated from both cultured PT cells (cultured PT, lane 4) and freshly isolated PT cells (isolated PT, lane 5). The amplified product appeared less abundant in isolated PT, but was readily apparent upon density scanning (Figure 2, lower tracings) or by application of larger amounts of the PCR reaction volume to the gel.

The PCR product from the cultured PT cells was subcloned and sequenced. As shown by the base-sequence comparison in Figure 3, the sequence of the renal (cultured PT) fragment was 100% identical with the cardiac α_1-subunit (α_{1C}-subunit) demonstrating expres-

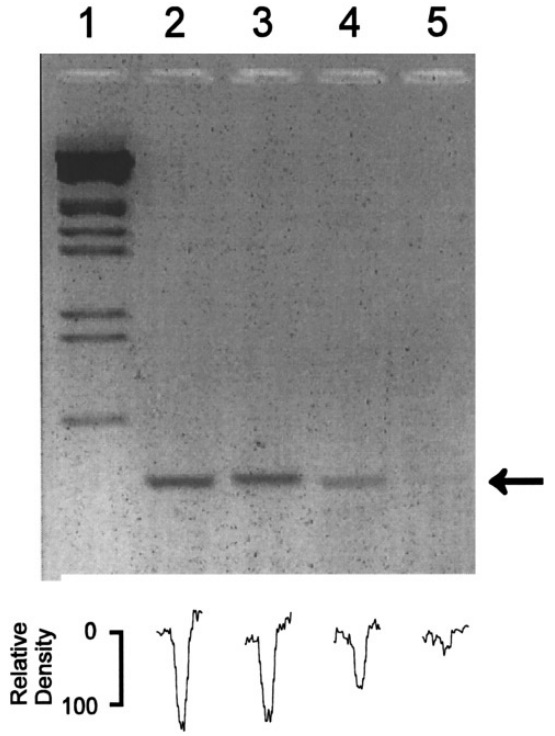

FIG. 2. Agarose gel electrophoresis of RT-PCR amplified DNA products stained with ethidium bromide (printed as the negative to facilitate density scanning of bands, below). Each well was loaded with 10 μl from a 100 μl reaction that contained cDNA generated from 5-10 μg total RNA. The traces at the bottom of each lane are the relative density scans of each reaction product. The lanes correspond to Lane 1, standards of Hind III digested λ DNA with the lowest band at 564 bp; Lane 2, rabbit cardiac muscle; Lane 3, rabbit aorta; Lane 4, rabbit cultured proximal tubule cells (cultured PT); and Lane 5, rabbit isolated proximal tubules (isolated PT).

TABLE 1

Specific Binding of [^{3}H]PN200-110 to Renal Epithelial Cell Membranes

	Cortical basolateral membranes (n = 7)	Cortical apical membranes (n = 2)	Cultured proximal tubule cell membranes (n = 3)
(+)-[^{3}H]PN200-110 Specific binding[a] (pmoles/mg)	3.8 ± 0.9	2.0 ± 0.9	8.2 ± 3.6

[a] Specific binding in the presence of 0.32 mM (+)-[^{3}H]PN200-110.

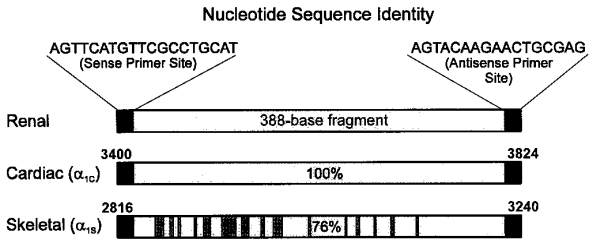

FIG. 3. Base-sequence comparison of the cloned RNA segments of rabbit proximal tubule cells with the corresponding regions of mRNA encoding the α_1-subunits from cardiac (α_{1C}-subunit) and skeletal (α_{1S}-subunit) muscle L-type calcium channel. The proximal tubule fragment was identical (100%) to the α_{1C}-subunit, but only 76% identical to the α_{1S}-subunit. The shaded areas reflect those segments with two or more base differences in sequence relative to the proximal tubule sequence. The location and sequence of the upstream primer and downstream primer used for the PCR are shown along with the corresponding base locations for the cardiac and skeletal sequences. The cardiac sequence is from Mikami *et al.* (16), Accession No. 15539. The skeletal muscle secquence is from Tanabe *et al.* (36), Accession No. A30063.

sion of the cardiac form of the L-type calcium channel in renal epithelial cells. Comparison to the skeletal form of the α_1-subunit (α_{1S}-subunit) demonstrates only a 76% base identity as has been previously shown for the cardiac channel. While this renal fragment is identical to the cardiac subunit, it is not known if the renal form is alternatively spliced, nor if it displays variations in hypervariable regions such as the voltage sensing region of the 4th membrane spanning region (S4).

DISCUSSION

The present study demonstrates that rabbit renal epithelial cells express both dihydropyridine binding sites and L-type calcium channels. High affinity binding sites appear to be expressed in native tissues, including both basolateral and apical cell membranes, and in membranes from cultured proximal tubule cells. The presence of these binding sites is consistent with the demonstration of dihydropyridine-sensitive calcium influx channels at both the basolateral border (24, 21) and apical border (1, 2) of proximal tubule cells.

Scatchard analysis of PN200-110 binding to renal cortical basolateral membranes revealed at least two high affinity, DHP binding sites. The basolateral membrane fraction will primarily reflect basolateral membranes of proximal tubule cells since the proximal tubule cells are the dominant cell type in the cortex, the source of the basolateral membranes (35). Similarly, the apical cell membrane preparation should primarily reflect brush-border membranes (i.e., proximal tubule apical cell membranes). However, membranes from other cell types may be a contaminant in the basolateral and apical membrane preparations and, hence, the binding may also reflect properties of other cell types. Nonetheless, using patch clamp analysis of rabbit cultured proximal tubule cells, two distinct dihydropyridine-sensitive calcium channels have been identified in these cells: a voltage-activated, L-type calcium channel (1), and a mechano- and protein kinase C- regulated calcium channel (2). Hence, the presence of two dihydropyridine binding sites in proximal tubule cells would be consistent with the presence of two separate channels. It is most probable that the identified Dihydropyridine binding sites do, indeed, reflect properties of the proximal tubule calcium channels although this remains to be demonstrated directly.

L-type calcium channel α_1-subunits are known to possess a high affinity binding site for dihydropyridines. The observed K_d range for PN200-110 binding is highly variable amongst tissues, ranging from 0.006 to 0.4 nM, depending on the specific α_1-subunits expressed, other subunits expressed as part of the channel complex, and the ionic composition (9, 29, 10, 11, 17, 30, 19). The K_d values for the two binding sites observed in the present study (0.005 and 0.75 nM) demonstrate that the renal cells express binding sites similar to that observed for L-type channels. Hence, it would appear that at least one of the high affinity binding sites is part of the L-type calcium channel expressed in renal epithelial cells (2). The second Dihydropyridine binding site is likely, therefore, to be part of the second, DHP-sensitive and PKC-regulated calcium channel observed in the renal epithelial cells (1, 23). Alternatively, this second binding site may be part of a yet undefined dihydropyridine receptor in renal cells.

In summary, the current study demonstrates for the first time the presence of high-affinity, dihydropyridine binding sites in renal epithelial cells. These binding sites would appear to be part of distinct calcium channels expressed in these cells.

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