Characterization of Na\textsuperscript{+}-permeable Cation Channels in LLC-PK1 Renal Epithelial Cells*

Received for publication, October 31, 2003, and in revised form, February 16, 2004
Published, JBC Papers in Press, February 24, 2004, DOI 10.1074/jbc.M311946200

Malay K. Raychowdhury§§*, Cristina Ibarra***, Alicia Damiano****, George R. Jackson, Jr.‡, Peter R. Smith‡‡, Margaret McLaughlin‡, Adriana G. Prat‡§, Dennis A. Ausiello§§, Alan S. Lader§§, and Horacio F. Cantiello§§***§§

From the ‡Renal Unit, Massachusetts General Hospital East, Charlestown, Massachusetts 02129, the §Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, the ¶Department of Fisiología, Facultad de Medicina, Universidad de Buenos Aires 1121, Argentina, the **Laboratorio de Canales Iónicos, Química General e Inorgánica, Departamento de Fisicoquímica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires 1113, Argentina, and the §§Departments of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama 35294

In this study, the presence of Na\textsuperscript{+}-permeable cation channels was determined and characterized in LLC-PK1 cells, a renal tubular epithelial cell line with proximal tubule characteristics derived from pig kidney. Patch-clamp analysis under cell-attached conditions indicated the presence of spontaneously active Na\textsuperscript{+}-permeable cation channels. The channels displayed nonrectifying single channel conductance of 11 pS, substates, and an \textasciitilde\textasciitilde 3:1 Na\textsuperscript{+}/K\textsuperscript{+} permeability-selectivity ratio. The Na\textsuperscript{+}-permeable cation channels were inhibited by pertussis toxin and reactivated by G protein agonists. Cation channel activity was observed in quiescent cell-attached patches after vasopressin stimulation. The addition of protein kinase A and ATP to excised patches also induced Na\textsuperscript{+} channel activity. Spontaneous and vasopressin-induced Na\textsuperscript{+} channel activity were inhibited by extracellular amiloride. To begin assessing potential molecular candidates for this cation channel, both reverse transcription-PCR and immunocytochemical analyses were conducted in LLC-PK1 cells. Expression of porcine orthologs of the αENaC and ApxL genes were found in LLC-PK1 cells. The expression of both gene products was confirmed by immunocytochemical analysis. Although αENaC labeling was mostly intracellular, ApxL labeled to both the apical membrane and cytoplasmic compartments of subconfluent LLC-PK1 cells. Vasopressin stimulation had no effect on αENaC immunolabeling but modified the cellular distribution of ApxL, consistent with an increased membrane-associated ApxL. The data indicate that proximal tubular LLC-PK1 renal epithelial cells express amiloride-sensitive, Na\textsuperscript{+}-permeable cation channels, which are regulated by the cAMP pathway, and G proteins. This channel activity may implicate previously orphaned epithelial channel proteins, although this will require further experimentation. The evidence provides new clues as to potentially relevant Na\textsuperscript{+} transport mechanisms in the mammalian proximal nephron.

* This work was supported in part by National Institutes of Health Grant DK48040 (to H. F. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by National Institutes of Health Training Grant T32DK07540C15.

§§ To whom correspondence should be addressed: Renal Unit, Massachusetts General Hospital East, 149 13th St., Charlestown, MA 02129. Tel.: 617-726-5640; Fax: 617-726-5669; E-mail: cantiello@helix.mgh.harvard.edu.

LLC-PK1 cells constitute an established cell line derived from normal pig kidney displaying several characteristics of the proximal tubule (1–3). Na\textsuperscript{+} transport by LLC-PK1 cells has been associated with coupled mechanisms, including amino acids (2), glucose (4), and inorganic phosphate co-transport (5). Na\textsuperscript{+} exchange with hydrogen ions mediated by the Na\textsuperscript{+}/H\textsuperscript{+} exchanger has also been described (6). Radioisotopic Na\textsuperscript{+} fluxes in LLC-PK1 monolayers provided the first evidence for the presence of an amiloride-sensitive electrodiffusional Na\textsuperscript{+} transport pathway (7) with several similarities to that observed at the apical membrane of tight epithelia (8). At least one other study determined that electrically sensitive, but pH-insensitive Na\textsuperscript{+} transport in LLC-PK1 cells displays pharmacological characteristics different from those expected from ENaC channels (9). A preliminary patch-clamp study suggested, however, the presence of amiloride-sensitive, Na\textsuperscript{+} channel activity in LLC-PK1 cells (10). In the present report, patch-clamping techniques and membrane reconstitution assays were applied to subconfluent LLC-PK1 cells, and membranes, respectively, to determine whether this renal tubular epithelial cell model indeed expresses Na\textsuperscript{+}-permeable cation channels. The data indicate the presence of amiloride-sensitive Na\textsuperscript{+} -permeable, 11-pS cation channels, whose activity is modulated by the cAMP pathway and G proteins. The presence of αENaC and ApxL was determined by RT-PCR. The expression of the gene products was also confirmed in the LLC-PK1 cells by immunocytochemical and Western blot analyses. The present data suggest that the mammalian equivalent of Apx, ApxL, and/or αENaC may be components of a Na\textsuperscript{+} channel complex involved in Na\textsuperscript{+} reabsorption in the mammalian proximal nephron.

EXPERIMENTAL PROCEDURES

Cell Cultures—LLC-PK1 cells (ATCC CRL1392) were grown and kept in a Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% t-glutamine as previously described (6, 7). The cells were grown on glass coverslips until partially confluent and kept in humidified atmosphere at 37 °C in 5% CO\textsubscript{2} gassed air. The 293 human embryonic kidney cells (ATCC CRL1573) were also grown and kept in humidified atmosphere at 37 °C in 5% CO\textsubscript{2} gassed air. A6 renal epithelial cells derived from Xenopus laevis (ATCC CCL106) were grown as previously described. Briefly, the cells were kept in a Coon’s modification of Ham’s F-12 and Liebovitz’s F15 media modified to

This paper is available on line at http://www.jbc.org
content 105 mM NaCl and 25 mM NaHCO₃. This mixture was supplemented with 10% fetal bovine serum (Invitrogen). A6 cells were grown and kept in humidified atmosphere at 27 °C in 5% CO₂, gassed air.

Single Channel Studies—Cell-attached and excised, inside-out patch-clamp experiments were carried out as previously described (11, 12). Currents and command voltages were obtained and driven with a PC-501 patch-clamp amplifier using a 10 gigahm head stage (Warner Instruments, Hamden, CT). The signals were filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). The data were stored in a hard disk of a personal computer and analyzed with pClamp 6.0.3 (Axon Instruments, Burlingame, CA). The data were further filtered at 200 Hz for display purposes. For excised patches, upward and downward deflections indicated the channel open state at the respective side of the chamber. This was done to ensure that only channels incorporated in the cis-side of the chamber were further studied. This was done to ensure that only channels incorporated in the "correct" orientation for activation/inhibition were studied. PKA-activated channels were active for longer than 30 min, and run-down was never observed. On the contrary, some experiments were unfinished because high channel activity was observed (up to six channel levels), the open probability (pₒ) was calculated as previously reported (36). Briefly, the pₒ of the channel was obtained from the following equation,

\[ pₒ = \frac{w}{N} = \frac{\sum w_(n,t)}{N} \]  

(1)

where \( t \) equals the total time at a given level (n) from a total channel number (N). The pₒ was confirmed by approximation (10%) to the value \( pₒ = 1 - (t_j/t_i)N \), where \( t_j \) is the time as calculated from the inverse of the total closed time (tᵢ), and N reflects the total channel number, as before.

For multichannel records, the mean membrane current from various reconstituted membranes were averaged and compared as indicated below.

Immunocytochemistry—Immunocytochemical analysis of αENaC and ApXL was performed as previously described (12). Briefly, immunocytochemistry analysis was conducted as follows. LLC-PK1 cells were fixed in 5% paraformaldehyde in 0.05% phosphate buffer for 20 min at room temperature. A goat anti-rabbit secondary Alexa fluor 594 (Molecular Probes) antibody was used at 1:800. Conversely, LLC-PK1 cells were grown on glass coverslips for 2–4 days (80% confluent). The cells were fixed in 5% paraformaldehyde in 0.05% phosphate buffer for 20 min at room temperature. Conversely, the cells were fixed with 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.5% sucrose in phosphate-buffered saline (PBS) for 40 min at room temperature, followed by cell permeabilization with 0.1% Triton X-100 for 5 min. After incubation with PBS containing 1% bovine serum albumin to block non-specific binding (10 min), coverslips were incubated for 1 h with the primary antibody diluted 1:100 in PBS. After extensive washing, either goat (or rabbit) anti-rat IgG coupled to the channel antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) was applied (1:400) or a goat anti-rabbit secondary Alexa fluor 594 (Molecular Probes, 1:800). LLC-PK1 cells were counterstained with Evans Blue (1:800) to assess the cellular morphology along with channel protein staining. After further washing in PBS, the coverslips were mounted in Vectashield anti-fading medium (Vector Labs, Burlingame, CA) diluted 1:1 in 0.3% Tris base, pH 8.9, sealed, and examined with a Nikon FXA fluorescence microscope. The images were captured using an Optronics 3-bit CCD color camera (Optronics Engineering, Goleta, CA), and IP Lab Spectra (Scanalytics, Vienna, VA) acquisition and analysis software running on a Power PC 8500 (Apple Computer, Cupertino, CA). The images were analyzed with anti-αENaC and anti-ApXL antibodies separately (both antibodies were a kind gift of Dr. Tom Kleyman).

Antibodies—The primary anti-ApXL antibody was a rabbit polyclonal serum raised against an Apx fusion protein containing the Apx COOH terminus (amino acids 1194–1385) (12). Anti-αENaC antibodies were from CalBiochem (San Diego, CA). Primary antibodies were used at a 1:1000 dilution as previously described (12). Rabbit anti-rat αENaC antibodies were raised against an immunogenic synthetic peptide (LMNGKNNKREEQGLGPEPAAQKPTC) corresponding to amino acid residues 20–42 of the human αENaC. Otherwise, rabbit anti-rat antibodies were raised against an immunogenic peptide corresponding to residues 44–57 (GLGKDKREEQGLO) within the NH₂-terminal intracellular domain of rat αENaC (14).

Total RNA Isolation—Total RNA was isolated from LLC-PK1, 293, and A6 cultured cells using a TRIZol RNA extraction kit (Invitrogen) following the manufacturers recommended procedures. The isolated RNA was quantified by absorbance at 260 nm and stored at −80 °C until further use.

RT-PCR Analysis—The RT-PCR assay was performed in two steps using ThermoScript RT-PCR System (Invitrogen). In the first step, total RNA (nearly 2 µg) was incubated for 60 min at 55 °C with reverse primers (0.2 µg each) for first strand cDNA synthesis. For ApXLp, a second step was conducted, including 35 PCR cycles at 96 °C (1 min). This was followed by 55 °C (1 min) and 72 °C (3 min), and a final extension at 72°C for 10 min at 72°C. For αENaC, an additional step was performed 96 °C (6 min). Forward and reverse primers (0.5 µg each) for ApXLp were 5’-TGCCGACCCATTCTCCTA-3’ (residues 4265–4283) and 5’-GCCGACTTATCTTCCAGGA-3’ (residues 4840–4821), respectively, from the COOH terminus of human Apx, sequence (15) and were used to amplify a 576-bp band. For αENaC, forward and reverse primers (0.5 µg each) were 5’-CCCTGCGCTTGGAGGATGCT-3’ (residues 2341–2361) and 5’-CCCTGCGCTTGGAGGATGCT-3’ (residues 2445–2425), respectively, in the conserved region of bovine αENaC (16) were used to amplify a 105-bp band. RT-PCR products were separated on either 1.5 or 3% agarose gels. The amplified bands were...
subjected to automated DNA sequencing analysis at the Massachusetts General Hospital DNA Sequencing Center.

Other Reagents—Both lysine vasopressin and arginine vasopressin (AVP; Sigma), were kept in 0.1 mM stock solutions in distilled water and used at 10 mM and 10 μM final concentration, respectively. The catalytic subunit of the PKA was obtained from Sigma and used at a final concentration of 10 μg/ml. MgATP (Sigma) was used at a final concentration of 1 mM. Pertussis toxin (PTX) was obtained from Peninsula Labs. (Belmont, CA) and stored at -20 °C. The toxin was activated in a stock solution containing 1000 mM NaCl, 20 mM Na2HPO4, pH 7.0. The solution also contained 10 mM dithiothreitol and 1 mM NAD.

The subscripts denote the fractional decrease in mean current as a function of the amiloride concentration. The spontaneous Na+ channel activity of LLC-PK1 channels in cell-attached and excised inside-out patches, spontaneous Na+ channel activity in asymmetrical Na+/K+ channels in LLC-PK1 cells, the plasma membranes from these cells were reconstituted into lipid bilayers to assess for cation-selective ion channel activity (Fig. 2). Channel activity was observed in 11 of 14 experiments in either asymmetrical NaCl (150/15 mM; Fig. 2a) or NaCl-aspartate. Spontaneous Na+-permeable channel activity increased by 191% (n = 25, p < 0.005; Fig. 2) (or otherwise activated) by the addition of PKA (10 μg/ml) and MgATP (1 mM) to the cis-side but not the trans-side of the reconstituted membranes (Fig. 2a, inset). Reconstituted channels had a single channel conductance of 15.6 pS (n = 3) and a lower subconductance state (Fig. 2b). The PKA-activated Na+-permeable channel activity decreased by 80% after the addition of amiloride (1 μM; Fig. 2c). To assess the affinity for the amiloride inhibition of the Na+-permeable channel activity in LLC-PK1 cell membranes, various concentrations of the drug were added to the trans-side of the chamber (Fig. 2d). The addition of 50 mM amiloride was sufficient to decrease the open probability of the channel by 30% (n = 3) as calculated under “Experimental Procedures.” The data were obtained from open and closed states at any given current level, from best fittings of open and closed dwell histograms (Fig. 2e). The mean currents were also obtained before and after the addition of increasing concentrations of the drug in multichannel records. The fractional data (as percentage) were fitted to an equation rendering a best fit of K_i = 50.4 nM (n = 7; Fig. 2f) for a single amiloride binding site. The data were also fitted with two putative binding sites (data not shown), which was not statistically different from the single site. The data would suggest the possibility that either one or two K_i, namely K_i = 74.2 nM and K_i = 3.99 μM, respectively, may be involved in binding and inhibiting 94 and 6% of the total current, respectively.

Regulation of Na+-permeable Channels in LLC-PK1 Cells—To assess whether vasopressin, known to activate the cAMP pathway in LLC-PK1 cells, regulates the Na+-permeable channel activity in these cells (20, 21), AVP was added to cell-attached patches. The addition of either lysine vasopressin (10 nM) or AVP (10 μM) readily induced Na+ channel activity on otherwise quiescent cell-attached patches from LLC-PK1 cells in 5 of 7 experiments (71%; Fig. 3a). The current-voltage relationship of the AVP-induced Na+ channel activity was highly similar to that obtained for AVP-stimulated Na+ channels of A6 cells (Fig. 3b) (19). To further assess the effect of the cAMP pathway on Na+ channel activity of LLC-PK1 cells, PKA (10 μg/ml) was also tested on quiescent excised inside-out patches (Fig. 3c). The addition of PKA plus ATP (1 mM) induced single channel currents (Fig. 3c, inset). Single channel open states often showed a smaller subconductance indicated by the open state noise distribution (Fig. 3c, histogram). Single channel currents of both AVP stimulation and/or PKA addition showed identical single channel conductance (Fig. 3d): 8.29 ± 3.05 pS (n = 13) versus 8.11 ± 0.98 pS (n = 17). These values were slightly lower but not statistically different from that obtained under spontaneous conditions (p < 0.2). The single channel currents observed after AVP activation, however, showed higher dispersion than that after PKA stimulation, suggesting a difference in the substrate residence times between the two activating methods. To test whether Na+ channel activity of LLC-PK1 cells is regulated by G proteins, the effect of PTX (100 ng/ml) was assessed in excised in inside-out patches (n = 4). Activated PTX completely blocked the spontaneous Na+ channel activity (Fig. 3e, middle tracing), which reversed after the addition of the nonhydrolyzable GTP analog, GTPγS (1 mM; Fig. 3e, bottom tracing). This is consistent with the inhibitory effect of PTX on the Na+ channel activity (22) but not the lower conductance (22) Na+ channels present in A6 renal epithelial cells.

RT-PCR Detection of αENaC and ApxL in LLC-PK1 Cells—To begin an assessment of potentially relevant proteins associated with the Na+ channel activity in LLC-PK1 cells, the presence of porcine orthologs of both ENaCα and Apx were explored. The expression of αENaC in LLC-PK1 cells was conducted with primers specific for the bovine αENaC subunit as previously reported (16). An expected band of 105 bp was ob-
served after one RT-PCR cycle, consistent with the presence of this ENaC subunit (Fig. 4a). The aENaC mRNA of LLC-PK1 cells was highly homologous (>90%) to the human aENaC subunit. The mammalian isoform of the Apx gene (ApxL) was also examined in LLC-PK1 cells by RT-PCR with specific primers from the COOH-terminal end of the human ApxL sequence (15). PCR products of appropriate mobility were determined on a 1.5% agarose gel using A6 cell mRNA as a positive control (Fig. 4b). The expected band of 575 bp for the ApxL primers was amplified from the LLC-PK1 material. The primers were also able to detect amphibian Apx (A6 cell). The LLC-PK1 product was more than 90% homologous to human ApxL (Fig. 4b), suggesting the presence of a porcine ortholog of this protein in the pig kidney cells.

Immunolocalization of aENaC in LLC-PK1 Cells—The presence of aENaC in LLC-PK1 cells was also determined by immunolocalization with anti-aENaC antibodies. Despite strong nonspecific immunodetection in the presence of antigenic peptide (Fig. 5b), aENaC labeling was observed in control LLC-PK1 cells (Fig. 5a), in particular, at the intracellular level.
Highly transporting cells (domes; Fig. 5c) showed somewhat stronger ENaC expression. However, no redistribution of ENaC labeling was detected after activation with either lysine vasopressin (10 nM) or AVP (10 \( \mu \text{M} \)) for 5 to 15 min (Fig. 5d).

**Immunolocalization of ApxL in LLC-PK1 Cells**—The presence of ApxL in LLC-PK1 cells was determined by immunolocalization with an anti-Apx-specific antibody. This antibody was previously used to detect Apx expression in A6 cells (23) and Apx-transfected human melanoma cells (12). Apx labeling was observed in control LLC-PK1 cells, in particular, at the subapical level (Fig. 6A). Labeling was performed with anti-Apx antibody (FITC, green labeling) and Evans Blue (red) to stain for cellular morphology and thus assess cell integrity. In most cases, Apx labeling was observed at the periphery of subconfluent LLC-PK1 cell islands (Fig. 6A), suggesting that cell growth and spreading of LLC-PK1 cells may affect ApxL expression. AVP treatment (10 \( \mu \text{M} \); Fig. 6) of LLC-PK1 cells for 15 min prior to Apx labeling displayed an increased membrane staining for Apx. AVP treatment showed more Apx-labeled cells (Fig. 6B). Thus, AVP not only increased Apx labeling but also induced a redistribution of the protein in LLC-PK1 cells.

**Apx(L) and \( \alpha \)ENaC Western Blot Analysis**—The possible interaction between Apx(L) and \( \alpha \)ENaC is an issue that will further clarify the molecular structure and functional dynamics of the Na\(^{-}\)-permeable cation channels of LLC-PK1 cells. To assess whether Apx(L) peptides and ENaC subunits may interact with each other, Western blot analysis of plasma membranes was conducted with antibodies used for the immunolocalization studies. The membranes were prepared as for the channel reconstitution assays. The membranes were separated into aliquots and subjected to gel electrophoresis, blotted, and labeled with Apx and \( \alpha \)ENaC antibodies in the absence or presence of competing peptides. Several peptides were ob-
Fig. 3. Regulation of Na⁺ channel activity in LLC-PK1 cells. a, addition of AVP (100 nM) to quiescent cell-attached patches (top tracing) induced Na⁺-selective single channel activity (bottom tracing). b, single channel current-voltage relationship of LLC-PK1 Na⁺ channels under cell-attached conditions. The data are the means ± S.E. obtained from nine experiments. c, addition of PKA (10 μg/ml) plus ATP (1 mM) to quiescent excised inside-out patches (top tracing) induced Na⁺-selective single channel activity (bottom tracing). The data are representative of 10 experiments obtained in symmetrical Na⁺. Expanded tracing indicates the single channel current (left bottom panel), and all-point histogram
served (Fig. 7), which were specific for either protein, including 90, 155, and 178 kDa for \( \alpha \)-ENaC labeling, and 118, 151, 178, and 220 kDa for Apx(L). These data suggest that both proteins (and/or peptides related to them) are present in the same membranes. At present, the nature of the antibodies precluded us from obtaining a co-labeling of both proteins. However, affinity-purified actin complexes co-precipitated both proteins after purification. This possible interaction with cytoskeleton-associated complexes is consistent with previous reports on Apx-ENaC complexes in A6 cells (24) and will be further explored elsewhere (data not shown).

**DISCUSSION**

The first step in electrodiffusional \( \text{Na}^+ \) transport across distal renal epithelia entails its selective movement into the cytosol through apical cation-selective channels. Little information is available, however, about the presence and potential physiological roles of ion channels responsible for apical \( \text{Na}^+ \) channel activity in proximal tubular cells. As much as 10% of the apical \( \text{Na}^+ \) conductance in the proximal nephron may be accounted for by electrodiffusional pathways (25). However, the possibility that the mammalian proximal tubule expresses a subconductance state. \( d \), current-voltage relationship of AVP-induced (circles) and PKA-induced (triangles) \( \text{Na}^+ \) channel activity in LLC-PK1 cells. AVP data were obtained after excision of AVP-treated cells under cell-attached conditions and direct addition of PKA and ATP under excised conditions. Both single channel conductances were identical, although higher dispersion is observed for the AVP-treated data. This is suggestive of frequent presence of subconductance states. The data are the means ± S.E. from 13 and 17 experiments for AVP and PKA treated cells, respectively. \( e \), \( \text{Na}^+ \) channel activity obtained under spontaneous conditions (top tracing) and/or AVP treatment was completely inhibited by the addition of activated pertussis toxin (100 ng/ml, middle tracing). This effect was rapidly reversed after the addition of GTPyS (1 mM, bottom tracing). The data are representative of four experiments.

**FIG. 4.** RT-PCR of LLC-PK1 mRNA. **a**, the presence of \( \alpha \)-ENaC in total RNA from LLC-PK1 cells was determined by RT-PCR (Left) with primers selective for the \( \alpha \) subunit of bovine ENaC (16). Positive control was conducted with mRNA from human 293 embryonic kidney cells. PCR products were separated on 3% agarose gel, indicating the 105-bp expected band. **Right panel**, sequence comparison of the RT-PCR product from LLC-PK1 and human \( \alpha \)-ENaC, indicating very high homology. **b**, left panel, the presence of ApxL in total RNA from LLC-PK1 cells was determined with primers selective for the human homolog of Apx (ApxL, as originally reported (15)). Lane 1, nucleotide ladder; lane 2, negative control; lane 3, LLC-PK1 RT-PCR material; lane 4, RT-PCR material from A6 cells. The amplified products of RNA isolated from LLC-PK1 and A6 cells is further shown in the **bottom panel**, indicating that the ApxL primers effectively recognized amphibian Apx. The arrow indicates the predicted 575-bp band. **Right panel**, nucleotide sequence comparison of LLC-PK1 RT-PCR product with human ApxL. High homology is observed.
Na+-permeable cation channels is still an open question. Early evidence suggested the presence of Na+ channels in brush border membranes of the renal proximal tubule. This includes NMR measurements of rapid Na+ exchange (26) and the hyperpolarizing effect of amiloride on the apical membrane of mouse straight proximal tubules (27). More direct evidence for the presence of amiloride-sensitive Na+ channels (12 pS) has been provided by patch-clamp studies of apical membranes from rabbit late (pars recta) (28) and rat (29) proximal tubules. The molecular nature of Na+-permeable cation channels in proximal tubule preparations is still unknown. A cGMP-gated, amiloride-sensitive, nonselective, 28-pS channel has been reported in proximal tubules (30). Further, Willmann et al. (29) demonstrated the presence of an amiloride-sensitive Na+-permeable conductance in rat proximal tubules. In that study, the presence of α, β, and γ-ENaC message was determined. The single channel conductance and the actual involvement of ENaC subunits in this Na+ conductance, however, were not assessed. The consensus and previous evidence would indicate that the mammalian proximal convoluted tubule does not express a functional ENaC channel (31–33).

LLC-PK1 cells are a useful in vitro renal tubular epithelial cell model with several properties of the pars recta of the proximal tubule (1). Several Na+ transport mechanisms have been previously described in LLC-PK1 cells (2, 3). Earlier studies from our laboratory determined the presence of electrodiffusional Na+ transport in LLC-PK1 cells (7). Based on its contribution to the resting membrane potential, its high affinity for amiloride, and blockage by extracellular La3+, this Na+ pathway was deemed consistent with the possible presence of Na+-permeable channels in these cells. At least one earlier preliminary patch-clamping study supported this contention (10).

To investigate whether Na+-permeable channels are present in LLC-PK1 cells, in the present study we applied patch-clamping techniques to these cells. Single channel currents were observed in cell-attached and excised, inside-out patches of LLC-PK1 cells. Spontaneous Na+-selective channel currents displayed an 11-pS single channel conductance and a 3:1

---

**Fig. 5. Immunocytochemical labeling of αENaC in LLC-PK1 cells.** a, subconfluent monolayers of LLC-PK1 cells were immunolabeled with the anti-αENaC antibody. The αENaC labeling was largely intracellular. Images were obtained at 20×. b, nonspecific labeling was observed, however, in the presence of αENaC immunogenic peptide (15 μg/ml). c, αENaC labeling was also observed in domes of fluid transporting cells. d, αENaC immunolabeling was not modified by the presence of AVP (10 μM). The data are representative of two or three experiments under each condition.

---

**Fig. 6. Immunocytochemical labeling of ApxL in LLC-PK1 cells.** a, subconfluent monolayers of LLC-PK1 cells were immunolabeled with anti-Apx antibody (FITC, green) and Evans Blue to counterstain for cellular morphology. b, labeling in the presence of immunogenic peptide (15 μg/ml) indicated low nonspecific immunoreactivity. The images were obtained at 40×. c, strongest ApxL labeling was observed in peripheral cells. d, Apical ApxL was clearly observed (X60). B, a, ApxL labeling of control, subconfluent cell islands was stronger in peripheral and weaker in internal cells. b, dramatic Apx redistribution was observed after treatment with AVP (10 μM). AVP-treated cells displayed an increase in both apical and intracellular ApxL staining. The images were observed at 40×. The data are representative of at least three experiments.
channel properties and regulation are most consistent with Apx(L) in LLC-PK1 cells is of particular interest, because the associated with the apical epithelial Na⁺ channel activity, similar to that reported in the present study. This channel is also functionally similar to the 9-pS apical Na⁺ channel of A6 cells (11, 17–19, 36). Apx-mediated Na⁺-permeable ion channel currents are regulated both by PKA and actin (12). Thus, the AVP and PKA regulation of the 11-pS Na⁺ channel in LLC-PK1 cells share similarities with the 9-pS Na⁺ channels observed in A6 amphibian epithelial cells (11, 19, 37). Although Apx is an amphibian protein, a mammalian homolog of the Apx gene, ApxL, has been cloned from human retina, which was also detected in brain, placenta, lung, pancreas, and kidney (15). The ApxL gene encodes a 1616-amino acid protein sharing significant sequence homology with Apx. The sequence homology of the LLC-PK1 gene product indicates the presence of a porcine ortholog of ApxL. It is important to indicate, however, that ApxL channel function is not demonstrated in this study. Further investigation will be required to assess whether the sequence homology between the amphibian Apx and the porcine ortholog of ApxL extend to their functional properties.

Because of the presence of both αENaC and ApxL gene products in LLC-PK1 cells, the possibility exists for the Na⁺ channel observed to be a reflection of either one of these channel proteins and/or a complex including both proteins. Several peptides of putative similarity with both ENaC and ApxL were co-expressed in LLC-PK1 membranes. This is in agreement with studies by Smith and co-workers (24), who determined the presence of a cytoskeletal complex associated with both ENaC and Apx in A6 renal epithelial cells. In conclusion, the present data determined the presence of an amiloride-sensitive 11-pS Na⁺-permeable cation channel in LLC-PK1 cells. Potential limitations arise from studies on cultured cell lines as it pertains to extrapolations to in vivo tissues and the kidney. Nevertheless, the presence of Na⁺-permeable channels in LLC-PK1 cells may be relevant for a better understanding of Na⁺ reabsorption in the mammalian proximal nephron previously thought to be devoid of Na⁺-permeable channels.

Acknowledgments—We thank Valeria C. Primo and Gayle Hawthorn for technical support, Dr. Tom R. Kleyman for useful discussions concerning various aspects of the manuscript, Drs. Tom R. Kleyman and Jonathan Zuckerman for providing antibodies for staining cells. We also acknowledge Dr. Marcello D. Carattini for help conducting some of the patch-clamping experiments and Nicolás Montalbetti, Gustavo Timpanaro, and Jimena Semprone for help with the reconstitution studies.

REFERENCES

1. Hull, R. N., Cherry, W. R., and Weaver, G. W. (1976) In Vitro Cell Dev. Biol. 12, 670–677.
2. Rabito, C. A., and Karish, M. V. (1982) J. Biol. Chem. 257, 6802–6808.
3. Rabito, C. A. (1986) Am. J. Physiol. 250, F734–F743.
4. Rabito, C. A., and Ausiello, D. A. (1986) J. Membr. Biol. 54, 31–38.
5. Rabito, C. A. (1983) Am. J. Physiol. 245, F22–F31.
6. Cantillo, H. F., Scott, J. A., and Rabito, C. A. (1986) J. Biol. Chem. 261, 3252–3258.
7. Cantillo, H. F., Scott, J. A., and Rabito, C. A. (1987) Am. J. Physiol. 252, F959–F977.
8. Rabito, C. A., and Ausiello, D. A. (1987) Am. J. Physiol. 253, F590–F597.
9. Moran, A., Asher, C., Crapoe, E. J. Jr., and Garty, H. (1988) J. Biol. Chem. 263, 19586–19591.
10. Moran, A., and Moran, N. (1984) Fed. Proc. 43, 447.
11. Cantillo, H. F., Swor, J. F., Prat, A. G., and Ausiello, D. A. (1991) Am. J. Physiol. 261, C882–C888.
12. Prat, A. G., Holtzman, E., Brown, D., Cunningham, C., Reisin, I., Klemey, T. M., Laughton, M., Jackson, G. J., Lydon, J., and Cantillo, H. F. (1996) J. Biol. Chem. 271, 18045–18053.
13. Gonzales-Perrett, S., Kim, K., Ibarra, C., Damiano, A. E. Zotta, E., Batelli, M., Harris, P. C., Reisin, I. L., Arnaout, M. A., and Cantillo, H. F. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1182–1187.
14. Smith, F. R., Mackler, S. A., Weiser, P. C., Brooker, D. R., Ahn, Y. J., Harte, B. J., McNulty, K. A., and Kleyman, T. R. (1998) Am. J. Physiol. 274, F91–F96.
15. Sciuffo, M. V., Bassi, M. T., Rugari, E. I., Renieri, A., Galli, L., and...
16. Fuller, C. M., Awayda, M. S., Arrate, M. P., Bradford, A. L., Morris, R. G., Canessa, C. M., Rossier, B. C., and Benos, D. J. (1995) *Am. J. Physiol.* **272**, C641–C654
17. Cantiello, H. F., Patenaude, C. R., and Ausiello, D. A. (1989) *J. Biol. Chem.* **264**, 20867–20870
18. Hamilton, K. L., and Eaton, D. C. (1985) *Am. J. Physiol.* **249**, C200–C207
19. Prat, A. G., Ausiello, D. A., and Cantiello, H. F. (1995) *Am. J. Physiol.* **265**, C218–C223
20. Goldring, S. R., Dayer, J. M., Ausiello, D. A., and Krane, S. M. (1978) *Biochem. Biophys. Res. Commun.* **83**, 434–440
21. Ohara, A., Matsunaga, H., and Eaton, D. C. (1980) *Am. J. Physiol.* **264**, 3552–C360
22. Hager, H., Kwon, T. H., Vinnikova, A. K., Masilamani, S., Brooks, H. L., Frokiaer, J., Knepper, M. A., and Nielsen, S. (2001) *Am. J. Physiol.* **280**, F1093–F1106
23. Duc, C., Farman, N., Canessa, C., Bonvalet, J.-P., and Rossier, B. (1994) *J. Cell Biol.* **127**, 1907–1921
24. Cantiello, H. F., Codina, J., Birnbaumer, L., and Ausiello, D. A. (1990) *J. Biol. Chem.* **265**, 21624–21628
25. Prat, A. G., Bertorello, A. M., Ausiello, D. A., and Cantiello, H. F. (1993) *Am. J. Physiol.* **265**, C224–C233
26. Elgavish, G. A., and Elgavish, A. (1985) *Biochem. Biophys. Res. Commun.* **128**, 746–753
27. Volkl, H., and Lang, F. (1988) *Biochim. Biophys. Acta* **946**, 5–10
28. Gogelein, H., and Gregor, R. (1986) *Pflugers Arch.* **406**, 198–203
29. Willmann, J. K., Bleich, M., Rizzo, M., Schmidt-Hieber, M., Ullrich, K. J., and Gregor, R. (1997) *Pflugers Arch.* **434**, 173–178
30. Cansepillo, F., McCoy, D. E., Green, R. B., Karlson, K. H., Dagenais, A., Molday, R. S., and Stanton, B. A. (1996) *Am. J. Physiol.* **271**, C1303–C1315
31. Hager, H., Kwon, T. H., Vinnikova, A. K., Masilamani, S., Brooks, H. L., Frokiaer, J., Knepper, M. A., and Nielsen, S. (2001) *Am. J. Physiol.* **280**, F1093–F1106
32. Sariban-Sohraby, S., and Fisher, R. S. (1992) *Am. J. Physiol.* **263**, C1111–C1117
33. Cantiello, H. F., Patenaude, C. R., Codina, J., Birnbaumer, L., and Ausiello, D. A. (1999) *J. Biol. Chem.* **274**, 21624–21628
34. Benos, D. J., Saccomani, G., Brenner, B. M., and Sariban-Sohraby, S. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 8526–8529
35. Sariban-Sohraby, S., and Fisher, R. S. (1992) *Am. J. Physiol.* **263**, C1111–C1117
36. Elgavish, G. A., and Elgavish, A. (1985) *Biochem. Biophys. Res. Commun.* **128**, 746–753
37. Volkl, H., and Lang, F. (1988) *Biochim. Biophys. Acta* **946**, 5–10
38. Gogelein, H., and Gregor, R. (1986) *Pflugers Arch.* **406**, 198–203
39. Willmann, J. K., Bleich, M., Rizzo, M., Schmidt-Hieber, M., Ullrich, K. J., and Gregor, R. (1997) *Pflugers Arch.* **434**, 173–178
40. Cansepillo, F., McCoy, D. E., Green, R. B., Karlson, K. H., Dagenais, A., Molday, R. S., and Stanton, B. A. (1996) *Am. J. Physiol.* **271**, C1303–C1315
41. Hager, H., Kwon, T. H., Vinnikova, A. K., Masilamani, S., Brooks, H. L., Frokiaer, J., Knepper, M. A., and Nielsen, S. (2001) *Am. J. Physiol.* **280**, F1093–F1106
42. Sariban-Sohraby, S., and Fisher, R. S. (1992) *Am. J. Physiol.* **263**, C1111–C1117
43. Cantiello, H. F., Patenaude, C. R., Codina, J., Birnbaumer, L., and Ausiello, D. A. (1999) *J. Biol. Chem.* **274**, 21624–21628
44. Prat, A. G., Bertorello, A. M., Ausiello, D. A., and Cantiello, H. F. (1993) *Am. J. Physiol.* **265**, C224–C233
45. Benos, D. J., Saccomani, G., Brenner, B. M., and Sariban-Sohraby, S. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 8526–8529
46. Sariban-Sohraby, S., and Fisher, R. S. (1992) *Am. J. Physiol.* **263**, C1111–C1117
47. Cantiello, H. F., Patenaude, C. R., Codina, J., Birnbaumer, L., and Ausiello, D. A. (1999) *J. Biol. Chem.* **274**, 21624–21628
48. Prat, A. G., Bertorello, A. M., Ausiello, D. A., and Cantiello, H. F. (1993) *Am. J. Physiol.* **265**, C224–C233
Characterization of Na⁺-permeable Cation Channels in LLC-PK1 Renal Epithelial Cells
Malay K. Raychowdhury, Cristina Ibarra, Alicia Damiano, George R. Jackson, Jr., Peter R. Smith, Margaret McLaughlin, Adriana G. Prat, Dennis A. Ausiello, Alan S. Lader and Horacio F. Cantiello

J. Biol. Chem. 2004, 279:20137-20146.
doi: 10.1074/jbc.M311946200 originally published online February 24, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311946200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 26 references, 13 of which can be accessed free at http://www.jbc.org/content/279/19/20137.full.html#ref-list-1