Renal Epithelial Protein (Apx) Is an Actin Cytoskeleton-regulated Na⁺ Channel*

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Apx, the amphibian protein associated with renal amiloride-sensitive Na⁺ channel activity and with properties consistent with the pore-forming 150-kDa subunit of an epithelial Na⁺ channel complex initially purified by Benos et al. (Benos, D. J., Saccomani, G., and Sariban-Sohraby, S. (1987) J. Biol. Chem. 262, 10613–10618), has previously failed to generate amiloride-sensitive Na⁺ currents (Staub, O., Verrey, F., Kleyman, T. R., Benos, D. J., Rossier, B. C., and Kraehenbuhl, J. P. (1992) J. Cell Biol. 139, 1497–1506). Renal epithelial Na⁺ channel activity is tonically inhibited by endogenous actin filaments (Cantiello, H. F., Stow, J., Prat, A. G., and Ausiello, D. A. (1991) Am. J. Physiol. 261, C882–C888). Thus, Apx was expressed and its function examined in human melanoma cells with a defective actin-based cytoskeleton. Apx-transfection was associated with a 60–900% increase in amiloride-sensitive (Kₐ = 3 μM) Na⁺ currents. Single channel Na⁺ currents had a similar functional fingerprint to the vasopressin-sensitive, and actin-regulated epithelial Na⁺ channel of A6 cells, including a 6–7 pS single channel conductance and a perm-selectivity of Na⁺/K⁺ of 4:1. Na⁺ channel activity was either spontaneous, or induced by addition of actin or protein kinase A plus ATP to the bathing solution of excised inside-out patches. Therefore, Apx may be responsible for the ionic conductance involved in the vasopressin-activated Na⁺ reabsorption in the amphibian kidney.

Sodium reabsorption by polarized renal epithelia is initiated by the activation of apical epithelial Na⁺ channels whose activity is controlled by hormones, including vasopressin. However, molecular information about the protein(s) responsible for the vasopressin-sensitive, and protein kinase A-regulated Na⁺ conductance is ill-defined (6, 7). A 130–160-kDa protein, most abundant in the apical membrane of A6 renal tubular epithelial cells, has been recently cloned (1). The apical protein from Xenopus laevis or Apx² may correspond to the 150-kDa subunit of the A6 cell Na⁺ channel complex (2), which is responsible for Na⁺ channel activity (8). Expression of Apx in Xenopus oocytes, however, failed to induce amiloride-sensitive Na⁺ channel activity (1). In this report, the possibility was explored that Apx, although representing the epithelial Na⁺ channel pore, may be under tonic inhibition by proteins associated with the Na⁺ channel complex, including the actin cytoskeleton (9). Actin, for example, activates epithelial Na⁺ channels in A6 cells, which are also inhibited by actin-cross-linking proteins such as actin-binding protein (ABP) and its homolog filamin (3). Therefore, Apx was expressed in human melanoma cells (ABP(−)), deficient in ABP-280 (homolog of filamin). ABP(−) cells are devoid of an organized actin cytoskeleton and have defective ion channel regulation (10, 11). Expression of Apx alone was sufficient to induce Na⁺-selective and amiloride-sensitive ion channel activity, which was regulated by the actin cytoskeleton.

MATERIALS AND METHODS

Cell Culture and Transfection of cDNA Encoding for Apx into Melanoma Cells

Cell Culture—The ABP-280-deficient human melanoma cell line (M2) was grown as described previously (10). Plasmid Construction—The Blu-Apx vector, containing the full-length cDNA encoding Apx and cloned to Bluescript M13-SK (Stratagene, CPI), was digested with the restriction enzymes NotI and XhoI. The 4.9-kilobase pair fragment containing the full-length Apx cDNA was purified and subcloned to the eukaryotic expression vector pcDNA3, containing a neomycin-resistant gene promoter (Invitrogen, CA), to form pcDNA3-Apx.

Melanoma Cell Transfection—Transfection of M2 cells with pcDNA3-Apx was conducted by the calcium phosphate precipitation technique as described previously (10). After recovery, cells were grown in a selection medium containing G418 (1 mg/ml) which efficiently kills these cells (10). Six G418-resistant clones were picked after 16–18 days and further cultured individually.

Immunocytochemistry

Immunocytochemistry was performed as described previously for cultured cells (12). Briefly, the various cell lines were grown on glass coverslips for 2–4 days (80% confluent), as for the patch-clamp experiments. Cells were fixed with either paraformaldehyde-lysine-periodate or 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at room temperature, followed by cell permeabilization with 0.1% Triton X-100 for 4 min. After incubation with PBS containing 1% bovine serum albumin to block nonspecific binding (10 min), coverslips were incubated with the primary antibody diluted in PBS. After extensive washing, goat anti-rabbit IgG coupled to Cy3 (indocarbocyanine, Jackson ImmunResearch Laboratories, West Grove, PA) was applied (1:400). After further washing in PBS, the coverslips were mounted in Airvol MOPS, 4-morpholinepropanesulfonic acid; rENaC, rat epithelial sodium channel.

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¶The abbreviations used are: Apx, apical protein from Xenopus laevis; ABP, actin-binding protein; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; rENaC, rat epithelial sodium channel.
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205 (polyvinyl alcohol; AIR Products, Allentown, PA), sealed, and examined with a Nikon FXA fluorescence microscope. Representative islands of cells were photographed using Kodak Tmax 400 film, push-processed to 1600 ASA. To quantify the amount of Axp labeled in Axp-7, M2, and A6 cells, pictures from immunocytochemistry studies were printed using identical exposure conditions, scanned, and gray-scale processed with NIH image software (version 4.1).

Antibodies

The primary antibody was an immunopurified rabbit polyclonal antibody raised against an Axp fusion protein containing Axp COOH terminus (amino acids 1194 to 1395), and diluted 1:10 (25 μg/ml, Fig. 1A). For Fig. 1B, the antibody-containing whole serum was used at a 1:100 dilution.

Whole-cell Current Studies

Actual currents and command voltages were obtained as described previously (11). Currents and command voltages were obtained and driven with a PC-501 patch-clamp amplifier using a 1 gigahertz head-stage (Warner Instruments, Hamden, CT) or with a Dagan 3900 (Dagan Corporation, Minneapolis, MN). Signals were filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). Data were stored in a hard disk of a personal computer and analyzed with PC-lamp 5.5.1 (Axon Instruments, Burlingame, CA). Holding potentials refer to the pipette-pipette and bathing solution was, in mM: 140 NaCl, 5.0 KCl, 0.8 MgSO₄, 1.2 CaCl₂, and 10 Heps, pH 7.4. When indicated, Na⁺ was replaced by an equimolar concentration of K⁺, and other solutes remaining the same. Likewise in some cases Cl⁻ was replaced by isethionate.

Single Channel Studies

The cell-attached and excised, inside-out patch-clamp configurations were carried out as described previously (3). Currents and command voltages were obtained and driven with a PC-501 patch-clamp amplifier using a 1 gigahertz head-stage (Warner Instruments, Hamden, CT) or with a Dagan 3900 (Dagan Corp., Minneapolis, MN) and further processed as indicated for the whole-cell experiments. Data were further filtered at 25-50 Hz for display purposes. Data from cell-attached or excised, inside-out patches were obtained between 120 mV, with upward and downward deflections indicating the channel open state at positive and negative holding potentials, respectively. Patch-pipette and bathing solutions were as described for the whole-cell experiments.

Membrane-enriched Preparations

Cultures of confluent M2 and Axp-7 cells were scraped, centrifuged at 3,000 rpm (4°C) for 10 min, and washed twice with PBS. The cell membrane pellets were resuspended in fresh buffer (1 ml) containing, in mM, sucrose 250, Tris-base (pH 7.6) 10, NaCl 50, phenylmethylsulfonyl fluoride 10 μM, and aprotonin 20 μM. The suspensions were sonicated at 45% duty cycle (Ultrasonic Processor, model W-375, Ultrasonic Inc.) for two 10-s runs. After addition of 9 ml of protease-inhibitor buffer, sonication was repeated, and the suspensions were centrifuged again at 3,000 rpm for 10 min. The supernatants were further ultra centrifuged at 25,000 rpm for 1 h at 4°C in an L8-80 M Ultracentrifuge (Beckman, Palo Alto, CA), using a swing rotor SW 41 Ti. Pellets were resuspended in 200 μl of protease-inhibitor buffer and stored at 80°C until further use. Protein content was determined using the Bradford method (Bio-Rad).

Planar Lipid Bilayer Studies

Membrane-enriched vesicles were either fused to planar lipid bilayers painted onto a 0.1-mm hole in a 13-mm reconstitution polyethylene cuvette (Warner Instrument Corp., Hamden, CT), as described by Alvarez (13), or painted directly onto the lipid bilayer. The phospholipid composition of the lipid bilayers was 1-palmitoyl-2-oleyl-sn-glycero-3-phosphatidyethanolamine:1-palmitoyl-2-oleyl-sn-glycero-3-phospho-tyldicholine (7:3, w/w; Avanti Polar Lipids, Alabaster, AL) in distilled water, and N-d-ethyl-N-isopropyl-N-amiloride (EIPA, Research Biochemicals Inc.) in dimethyl sulfoxide, were diluted in the bathing solution to final concentrations between 1 nm and 100 μM.

Actin and Associated Proteins

G-actin (Sigma) was used without further purification and was stored until the time of the experiment at ~80°C in 200-μl aliquots at approximately 5-10 mg/ml in a solution (depolymerizing buffer) containing, in mM: Tris-HCl, 2; ATP, 0.5; CaCl₂, 0.2; and β-mercaptoethanol, 0.5; pH 8.0.

Data Analysis

Data were expressed as the mean ± S.E., where n equals the number of patches analyzed. The mean conductance was obtained by regression analysis. Data were compared by Student's t test. The Goldman-Hodgkin-Katz equation was used to calculate the perm-selectivity ratio (PNa/PK) from the whole-cell and excised inside-out patch-clamp experiments where Na⁺ was equimolar replaced by K⁺: PNa/PK = K_p/(K_p + K_n); K_p(FeR/RT)/[Na_0(FeR/RT) - Na_0] where E is the measured reversal potential; PNa and K_p are the Na⁺ and K⁺ permeabilities, respectively; and K_p, Na_0, and K_p, Na_0 are the Na⁺ and K⁺ concentrations, respectively. For amiloride inhibition studies, the percent change of the whole-cell conductance as a function of the various concentrations of amiloride (A) was best-fitted to one binding site using the equation: 100 × [(1/Y_{t,ctrl} - 100)]/[Y_{t,ctrl} - 100(A + K)] where the inhibition constant Kₐ was obtained.

RESULTS

Six neomycin-resistant clones, named Axp-2, and Axp-4 through Axp-8, were produced under standard selection conditions (G418, 1 mg/ml) by stable transfection of M2 (ABP(−)) melanoma cells with the full-length cDNA for Axp. The presence of Axp in the G418-resistant clones was first determined by immunolocalization of Axp with immunopurified anti-Axp antibodies raised against a fusion protein containing the amino acid sequence 1194 to 1395 from Axp. Immunocytochemical analysis indicated that all six clones expressed various levels of the protein, and which displayed labeling comparable to that of Axp-7 (Fig. 1B). As a positive control, immunocytochemical analysis of Axp was also assessed in A6 epithelial cells, which expresses high levels of the protein, and which displayed labeling comparable to that of Axp-7 (Fig. 1B).

Functional expression of Axp was assessed in cells stably transfected with Axp, using the whole-cell patch-clamp technique. Wild-type M2 cells had a basal whole-cell conductance of 1.2 ± 0.4 nS/cell (n = 13) under symmetrical Na⁺ conditions (Fig. 2A), which decreased by only 3.2% (0.05 ± 0.01 nS/cell, n = 4; p < 0.01) in the presence of amiloride (10 μM). The basal whole-cell conductance of most transfected clones was higher than that of M2 cells (Table 1). Clones Axp-2 and Axp-6 did not show statistically different whole-cell conductances as compared to controls. However, Axp-6 whole-cell conductance
after replacement of Na\(^+\) by K\(^+\), thus consistent with a perm-selectivity ratio \(P_{Na^+}/P_{K^+}\) of 4.3. The dose-response decrease of the whole-cell currents by amiloride indicated an affinity of 3.3 \(\mu\)M (Fig. 3A), and a maximal inhibition of 62% (3.3 \(\pm\) 0.1 nS/cell, \(n = 6, p < 0.001\)). Apx-7 whole-cell currents were also inhibited by the amiloride analog benzamil (<1 \(\mu\)M) but not by N-(ethyl-N-isopropyl)-amiloride (100 \(\mu\)M; data not shown). A strong positive correlation was found between Apx expression by the various Apx clones and the whole-cell conductance (as indicated below), thus indicating a direct link between the two parameters (Fig. 2D). Although most clones (4/6) had a higher cationic conductance compared to M2 cells (Fig. 2D, Table I), no correlation was found between the whole-cell and the amiloride-sensitive conductance, thus most clones were insensitive to amiloride (Fig. 3B). Cyclic AMP stimulation of Apx-7 cells induced a 264 \(\pm\) 136% \((n = 8)\) increase of the whole-cell currents in 8 out of 14 experiments (57%, \(p < 0.05\)), although no effect was observed on M2 cells (47 \(\pm\) 11%, \(n = 6, N S\)). Addition of the actin cytoskeleton disrupter cytochalasin D (10 \(\mu\)g/ml), however, had no effect on the whole-cell currents of either M2 or Apx-7 cells (72 \(\pm\) 48%, \(n = 6, and 44 \(\pm\) 34%, respectively).

The molecular nature of the Apx-induced Na\(^+\) currents was further studied at the single channel level (Fig. 4). Spontaneous ion channel activity was observed in 50% \((n = 28)\) of cell-attached patches. The current-voltage relationship (Fig. 4C) was highly similar to that of Na\(^+\) channels previously reported in A6 epithelial cells (4, 15). After excision, Na\(^+\) channel currents had a single channel conductance of 6.2 \(\pm\) 0.6 pS \((n = 5)\) in symmetrical Na\(^+\) (140 mM NaCl or sodium isethionate, Fig. 4, A and B). In 3/8 quiescent patches (38%), addition of protein kinase A plus ATP (1 mM) induced Na\(^+\)-selective single channel currents of 7.2 \(\pm\) 0.2 pS \((n = 3, Fig. 4D)\). Addition of monomeric actin (1 mg/ml), in the presence or in the absence of ATP (1 mM), to excised, inside-out patches of Apx-7 cells, also induced and/or increased Na\(^+\) channel activity in 78% of the experiments tested (n = 9, Fig. 4, F and G). Actin-induced Na\(^+\) channels had a linear single channel conductance of 6.2 \(\pm\) 0.3 pS \((n = 6, Fig. 4H)\) and a perm-selectivity sequence of Na\(^+\) > Li\(^+\) > K\(^+\) (Fig. 4, H and I) with a 4:1 perm-selectivity ratio between Na\(^+\) and K\(^+\) (Fig. 4I).

Membrane-enriched fractions from Apx-7 cells were also reconstituted into lipid bilayers to assess for cation-selective ion channel activity (Fig. 5). Either spontaneous or actin-induced ion channel activity was observed in 4/4 experiments in symmetrical 150 mM NaCl (Fig. 5A) and 3/3 experiments in 300 mM NaCl (Fig. 5B). Reconstituted channels had a single channel conductance of 11–15 pS \((n = 4)\) and 18 pS \((n = 3)\) under either symmetrical 150 mM or 300 mM NaCl, respectively. Addition of 10 mM amiloride abolished ion channel activity under either condition. In asymmetrical NaCl (600:50 mM, cis/trans, respectively) spontaneous Na\(^+\) channel activity was observed only in 1/14 experiments but was readily activated in 9/14 experiments by addition of actin \(\pm\) ATP to the cis side of the chamber (Fig. 5C). Reconstituted channels had a single channel conductance of approximately 11 pS \((n = 3, Fig. 5C)\), rectified in a cation-selective manner, and were inhibited by addition of amiloride (0.1 \(\mu\)M) added to the trans but not to the cis side of the chamber (Fig. 5C). The reversal potential (E\(_r\)) of 60.9 \(\pm\) 7.7 mV \((n = 3)\), was consistent with the expected value of 63.6 mV for Na\(^+\)-selective currents. A perm-selectivity value \(P_{Na^+}/P_{K^+}\) of 4–6 was obtained in asymmetrical Na\(^+\)/K\(^+\) conditions (150 mM NaCl in cis, 150 mM KCl in trans). Membrane preparations from M2 cells showed no channel activity (n = 4).
DISCUSSION

The first step to the transepithelial Na⁺ movement of transporting epithelia entails the selective movement of this ion into the cellular compartment. The molecular nature of this first step, although known to involve apical ion channels, is largely unknown. Previous experimental evidence and current studies by various groups including ours indicate that three "types" of apical Na⁺ channels can be found in A6 and related epithelial cells (6), which have been recently characterized in the small conductance (~3–5 pS), highly selective Na⁺ channel with a Na⁺:K⁺ perm-selectivity ratio higher than 10 (16, 17), a higher mean conductance (7–15 pS) Na⁺ channel with a lower perm-selectivity ratio (3 to 5) (3, 15, 18, 19), and yet another nonselective cation channel with an even higher (23–28 pS) single channel conductance (20). No molecular information is yet available on the various molecular structures underlying the different functional apical channel fingerprints, except for recent studies providing strong evidence to suggest that the small channel type may represent the recently cloned, heterotrimeric ENaC (6). Functional expression of epithelial sodium channel homologs (14) is largely consistent with the channel(s) described by extensive studies of Palmer and Eaton's laboratories (16, 17, 21, 22). The molecular structure underlying the 9 pS Na⁺ channel, however, is apparently unavailable (for a review, see Ref. 6).

Whether one or all of the Na⁺-permeable apical epithelial...
channel types may represent different conductance states of the same structure, and/or can function in different "conductance" modes is, despite current interest, as yet unknown. The possibility exists, for example, that a particular ion channel may have more than one functional mode. Various functional studies of the apical channel complex originally described by Sariban-Sohraby et al. (23) and Benos et al. (24, 25) indicate that this channel structure may have more than one single channel conductance. Thus changes in the apical Na⁺ permeability of epithelial cells may be the reflection of one or more, not mutually exclusive, possibilities, including the fact that a particular Na⁺ channel may change its conductance properties depending on its environmental conditions (23, 24), and/or that different channels may be selectively expressed under different developmental conditions (6). Our studies on the A6 Na⁺ channel, for example, have been conducted on subconfluent cells grown on glass coverslips. Under these conditions single channel currents consistent with the "highly selective" Na⁺ channel are rarely observed which are a common observation of steroid-treated confluent A6 cells grown on permeable supports (16, 17, 26). Although further studies will be required to explore these various, yet not mutually exclusive, possibilities, previous independent studies have determined the presence of an apical epithelial multimeric protein complex (27). This channel complex contains at least one subunit (150 kDa) which has been directly implicated in the amiloride-sensitive Na⁺ channel activity (2).

Apx is a 120–170 kDa protein associated with the apical epithelial Na⁺ channel complex (1). The 150-kDa subunit of the A6 Na⁺ channel complex displays Na⁺ channel activity in lipid bilayers (8). Thus, Apx and the 150-kDa subunit may represent the same transmembrane protein. This is further suggested by the fact that antibodies raised against the 150-kDa subunit immunoprecipitate Apx (1) and conversely, antidiotypic antibodies to Apx immunoprecipitate the 150 kDa subunit of the Na⁺ channel complex (1). The possibility was raised that Apx may be the pore-forming component of the renal epithelial Na⁺ channel. However, previous studies failed to detect amiloride-sensitive Na⁺ channel activity after expression of Apx in Xenopus oocytes (1). This may be accounted for by two different possibilities. First, Apx might be a channel regulator. Another possibility is that although representing an ion channel, Apx could be functionally inhibited by other regulatory proteins. Actin, for example, not only activates Na⁺ channels in A6 epithelial cells (3) but is also responsible for the vasopressin-induced and protein kinase A-mediated Na⁺ channel activation (5). Further, actin-binding proteins, including the ABP-280 homolog filamin (3), inhibit spontaneous, protein kinase A- and actin-induced Na⁺ channel activity in A6 cells (3, 5). The cell volume-regulated activation of cation channels in human melanoma cells was also tonically inhibited by ABP-280 (11). Thus, human melanoma cells devoid of ABP-280 may provide a useful model to assess ion channel-actin cytoskeleton interactions.

The data in this report indicate that expression of Apx in cytoskeletonally deranged human melanoma cells is associated with ion channels having functional similarities with both the 9-pS apical Na⁺ channel of A6 cells (3, 4, 15, 18, 19), and with the pore-forming 150-kDa subunit of the epithelial Na⁺ channel complex originally purified by Benos et al. (2) and Sariban-Sohraby et al. (23). The functional similarities between Apx and the "slightly" Na⁺-selective apical channel of A6 cells previously reported by Hamilton and Eaton (15), and our own laboratory (3, 4, 18, 19), include an amiloride-sensitive single channel conductance of 6–9 pS, and a Na⁺:K⁺ perm-selectivity ratio of 3 to 5. Further distinction between Apx and the highly selective Na⁺ channel entails the perm-selectivity cation se-

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### Table 1

| Clone | Conductance | Percentage from M2 | p  |
|-------|-------------|--------------------|----|
| M2    | 1.2 ± 0.4 (13) | 58                 | NS |
| Apx-2 | 1.9 ± 0.3 (4)  | 483                | 0.01|
| Apx-4 | 7.0 ± 1.5 (7)  | 408                | 0.001|
| Apx-5 | 6.1 ± 0.1 (5)  | 183                | NS |
| Apx-6 | 3.4 ± 1.2 (12) | 550                | 0.01|
| Apx-6*| 7.8 ± 2.2 (5)* | 625                | 0.001|
| Apx-7 | 8.7 ± 0.7 (29) | 892                | 0.001|

*A Asterisk (*) indicates voltage activation by applying a depolarizing voltage step (120–150 mV) for 1 min. 
*b Numbers in parentheses indicate experiments analyzed.

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Fig. 3. A, effect of amiloride on the whole-cell currents of Apx-expressing cells. Percentage change in whole-cell conductance of Apx-7 (filled circles, n = 6) as a function of various concentrations of bathing amiloride. The solid line indicates the best nonlinear fit representing the percentage of inhibition of the conductance, \( 100 \times g_{\text{new}}/g_{\text{ctrl}} \), as a function of bathing amiloride concentration. The fitted \( K_i \) was 3.3 \( \mu \)M. B, comparison of amiloride effect on the various Apx-expressing clones. Percent inhibition by amiloride (10–100 \( \mu \)M) of whole-cell conductance was obtained for the various Apx-expressing clones. The numbers in parentheses indicate the number of whole-cell experiments analyzed.
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FIG. 4. A–C, Na⁺ channel activity of Apx-expressing melanoma cells. A, Apx-7 excised, inside-out patches displayed spontaneous Na⁺ channel activity. \( V_h \) indicates the holding potential applied to the pipette. B, current-voltage relationship was obtained in symmetrical Na⁺ (140 mM NaCl or sodium isethionate). Data are the mean ± S.E. obtained from \( n = 8 \) independent experiments. C, current-voltage relationship of spontaneous Na⁺ channel activity of Apx-7 under cell-attached conditions. Data are the mean ± S.E., \( n = 3 \). The dashed line represents the current-voltage relationship of the Na⁺ channel activity previously reported in epithelial A6 cells (4). D, effect of protein kinase A on Na⁺ channel activity of Apx-expressing cell membranes under excised inside-out patch-clamp conditions. Addition of protein kinase A (10 μg/ml) plus ATP (1 mM), to the cytoplasmic side of quiescent excised, inside-out patches from Apx-7 cells, induced or increased Na⁺ channel activity (top and middle tracings) within 2 min. The bottom tracing is the expanded segment marked with a bold line in middle tracing. The histogram further indicates the presence of substates along with the main conductance state of the channel. Data representative of \( n = 3 \) were obtained in symmetrical Na⁺. E, current-voltage relationship of protein kinase A-induced Na⁺ channels from Apx-7 cells. F–I, effect of actin on Na⁺ channel activity of Apx-expressing cell membranes under excised inside-out patch-clamp conditions. F, addition of actin (1 mg/ml) to the cytoplasmic side of quiescent excised, inside-out patches from Apx-7 cells, induced Na⁺ channel activity in positive (top tracing) and negative holding potentials (middle and bottom tracings) within 2 min. Data representative of \( n = 7 \) were obtained in symmetrical Na⁺ conditions. G, expanded segment, marked with a bold line in F, and total current histogram indicates the presence of substates of smaller conductance along with the main conductance state of the channel. H, current-voltage relationship of actin-induced Na⁺ channels from Apx-7 cells. Data are the mean ± S.E. of six, two, and two experiments for symmetrical Na⁺ (filled circles, solid line), Na⁺/K⁺ (open circles, dashed line), and Na⁺/NMG (open squares), respectively. I, current-voltage relationship of actin-induced Apx-7 Na⁺ channels observed in the presence of bathing Li⁺. The mean single-channel conductance was 7.8 ± 0.7 pS (\( n = 2 \) experiments).
sequence Na\(^+\) > Li\(^+\) > K\(^+\) observed, instead of Li\(^+\) > Na\(^+\) > K\(^+\)
as described for the highly selective Na\(^+\) channel (22). However, further support for functional similarities between the 9-pS Na\(^+\) channel and Apx are based on the fact that this channel protein is the target for protein kinase A activation, a regulatory process which is mediated by the actin cytoskeleton,
Reconstitution of Apx into lipid bilayers. Membrane-enriched preparations from Apx-7 cells were reconstituted into lipid bilayers using symmetrical 150 mM NaCl (A), symmetrical 300 mM NaCl (B), or asymmetrical NaCl (600:50 mM NaCl, cis:trans, respectively) (C). M2 membrane preparations showed no channel activity under the same conditions. A, actin-induced ion channel activity was inhibited by amiloride 10 μM (top tracing). The bottom tracing represents an expanded section from top tracing to indicate the presence of ∼10–15 pS ion channels. The amplitude histogram of the total current from bottom tracing indicates the presence of two channels. B, actin-induced ion channel activity (top tracing) was also inhibited by increasing amiloride concentrations (middle tracings) in symmetrical 300 mM NaCl. The bottom tracing represents the expanded section of the underlined tracing to indicate the presence of 18 pS ion channels. C, cation-selective ion channel activity was readily activated in 9/14 experiments by addition of actin to the cis side of the chamber (top tracing). Reconstituted channels rectified indicating cation selectivity, and a single channel conductance of 11 pS. Channel activity was inhibited by addition of amiloride (0.1 μM, bottom tracing) to the trans but not to the cis chamber.
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TABLE II

| Protein                | Amino acid | Sequencea | Similarity/Identity |
|------------------------|------------|-----------|---------------------|
| A.                     |            |           |                     |
| Human \(\beta\)-Spectrin| 130        | LENMGSHDIVD | 63.6/45.4           |
| Apx                    | 850        | :E:EEGTTDIVK | :           |
| Human Dystrophin        | 95         | LVNIGSTDIVD | 63.6/54.5           |
| B.                     |            |           |                     |
| Gelsolin               | 118        | VQHREVVQGF | 55.6/44.4           |
| Apx                    | 1067       | :V:DNQEVEGD | :           |
| Villin                 | 93         | VQHREVVQGN | 55.6/44.4           |

*a Asterisks (*) indicate positions in which amino acid substitution affects binding to actin.

which can also “bypass” the effect of protein kinase A phosphorylation on channel activation. This does not preclude that other channel proteins are also regulated by actin, nor that protein kinase A regulation is mediated by the cytoskeleton, as we have recently demonstrated for cystic fibrosis transmembrane conductance regulator (28).

Apx transfection into M2 human melanoma cells devoid of the filamin homolog ABP-280 was associated with amiloride-sensitive Na\(^+\) channel activity, which was further modulated by actin. This raised the possibility that Apx may directly interact with actin. A comparison between the actin-binding domain(s) of various actin-binding proteins and Apx indicated that at least two conserved actin binding consensus sites are present in Apx (Table II). Both domains are located close to the putative main cytoplasmic loop of Apx. The first putative actin binding domain showed strong homology with both spectrins, and dystrophins (29, 30). Interestingly, recent studies indicate that ENaC interacts with spectrin (31), thus suggesting that similar regulatory features may be shared by both epithelial ion channels. Another putative actin binding domain in Apx shared homology with the actin binding domains of the actin-severing proteins gelsolin and villin, in a region of Apx containing at least five amino acids essential for the actin-binding ability of these proteins. Thus, direct binding of actin to Apx may be critically involved in the regulation of its ion channel activity. The data in this report indicate that Apx may represent the vasoressin-sensitive, and actin-regulated renal Na\(^+\) channel of A6 cells (3, 5), whose regulation by actin may entail a novel feature of ion channels.

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