J. Membrane Biol. 144, 147–156 (1995)

The Journal of
Membrane
Biology

Amiloride-sensitive Apical Membrane Sodium Channels of Everted *Ambystoma* Collecting Tubule

L.C. Stoner[1], B.G. Engbretson[1], S.C. Viggiano[1], D.J. Benos[2], P.R. Smith[3]

[1]Department of Physiology, State University of New York, Health Science Center, Syracuse, New York 13210
[2]Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama 35294
[3]Department of Biology, Syracuse University, Syracuse, New York, 13224

Received: 15 August 1994/Revised: 9 November 1994

Abstract. Patch clamp methods were used to characterize sodium channels on the apical membrane of *Ambystoma* distal nephron. The apical membranes were exposed by everting and perfusing initial collecting tubules in vitro. In cell-attached patches, we observed channels whose mean inward unitary current averaged 0.39 ± 0.05 pA (9 patches). The conductance of these channels was 4.3 ± 0.2 pS. The unitary current approached zero at a pipette voltage of −92 mV. When clamped at the membrane potential the channel expressed a relatively high open probability (0.46). These characteristics, together with observation that doses of 0.5 to 2 μM amiloride reversibly inhibited the channel activity, are consistent with the presence of the high amiloride affinity, high sodium selectivity channel reported for rat cortical collecting tubule and cultured epithelial cell lines.

We used antisodium channel antibodies to identify biochemically the epithelial sodium channels in the distal nephron of *Ambystoma.* Polyclonal antisodium channel antibodies generated against purified bovine renal, high amiloride affinity epithelial sodium channel specifically recognized 110, 57, and 55 kDa polypeptides in *Ambystoma* and localized the channels to the apical membrane of the distal nephron. A polyclonal antibody generated against a synthetic peptide corresponding to the C-terminus of Apx, a protein associated with the high amiloride affinity epithelial sodium channel expressed in A6 cells, specifically recognized a 170 kDa polypeptide. These data corroborate that the apically restricted sodium channels in *Ambystoma* are similar to the high amiloride affinity, sodium selective channels expressed in both A6 cells and the mammalian kidney.

Correspondence to: L.C. Stoner

Key words: High-selectivity sodium channel — Initial collecting tubule — Amphibian kidney — Amiloride-sensitive channels — Anti-sodium channel antibodies — Everted renal tubule

Introduction

The vertebrate distal nephron is known to be an important site for aldosterone-dependent sodium reabsorption [*for review see* ref. 42]. Distal nephron sodium reabsorption has traditionally been considered to be a two-step process [42]. An amiloride blockable, electroconductive pathway located in the apical membrane allows for the controlled entry of sodium into the cell. Once in the cytoplasm, the sodium can be extruded at the basolateral aspect of the cell by the ouabain-sensitive Na^+K^+ATPase. Thus, the Na^+K^+ATPase maintains the cellular sodium concentration well below that of the extracellular milieu, providing a chemical gradient for the entry of sodium at the apical membrane. The sum of the apical membrane entry and basolateral extrusion processes is the net reabsorption of sodium.

Considerable literature published in the last decade establishes that sodium channels represent the conductive pathway in the apical membrane [3, 31, 37]. Epithelial sodium channels have been characterized in experimental analogues of the distal nephron such as the toad bladder [11] and the frog skin [8] and in cultured cells derived from both the mammalian [23, 24] and amphibian distal nephron [7, 13, 14]. Sodium channels have also been extensively characterized in the native rat cortical collecting tubule [29, 30].

Several types of epithelial sodium channels have been described. Two types are blocked by low doses (<1

μM) of amiloride [*for review see* ref. 31]. One of these is a low conductance (5 pS) channel which is highly selective for sodium ($P_{Na}/P_K > 10$). Its characteristics include relatively long mean open times and a high open probability (0.3–0.5). This channel is found in frog skin [8], toad bladder [11], rat collecting tubule [29, 30], and tissue culture cells derived from the distal nephron of both rabbit [24] and the amphibian, *Xenopus* [13, 17, 26]. The sensitivity of this channel to antidiruetic hormone [26, 27] and aldosterone [10, 17], together with the fact that it is the predominant sodium channel found in the native rat collecting tubule [29, 30], make it a prime candidate for explaining net sodium reabsorption by the distal nephron. In addition, its high selectivity over potassium would make it ideal for establishing large sodium gradients between body fluids and urine.

Another sodium channel identified in cell cultures derived from the distal nephron of both the amphibian and the mammal has a conductance of 9 pS, a moderate selectivity for sodium ($P_{Na}/P_K \sim 3.0$), shorter mean open times (10–100 msec), and a low open probability (0.1) [7, 13, 16, 23]. At least in A6 amphibian epithelial cells, these channels appear to be predominantly expressed when cells are grown on plastic, whereas the 5 pS channel predominates when both mammalian [24] and A6 cells [26] are grown on filter supports in the presence of aldosterone. Whether these two channel types represent different entities or different forms of the same channel has not been established [31].

To date, the sodium channels of the amphibian initial collecting tubule itself have not been examined. It has been shown to actively reabsorb sodium [41] and to express a lumen-negative transepithelial voltage [41, 45, 46], both of which are inhibited by low doses of amiloride. One hallmark of sodium reabsorption by the distal nephron in amphibia is the ability to reduce urinary sodium and chloride to levels well below that of plasma [4, 33].

The initial collecting tubule of *Ambystoma tigerinum* can be dissected from the kidney [41, 45, 46] and studied in vitro. This particular renal tubule segment can also be turned inside out and perfused [9], exposing the apical membrane to electrophysiological techniques. In this study, we have used patch clamp methodology to identify a 5 pS amiloride-sensitive sodium channel expressed in the apical membrane of the everted tubule. This channel's electrical characteristics appear identical to those of the 5 pS channels reported for the rat collecting tubules [29, 30], cultured cells derived from rabbit collecting tubules [24], and A6 cells [13, 17, 26].

We have also used antisodium channel antibodies [38] to localize and biochemically characterize the epithelial sodium channel expressed in this nephron segment. Immunochemical analysis suggests that the biochemical characteristics of these apically restricted sodium channels in *Ambystoma* distal nephron are similar to those previously reported in A6 cells [1, 3] and the mammalian kidney [3].

Materials and Methods

Biological Preparations

A6 renal epithelial cells, derived from the distal nephron of *Xenopus laevis,* were obtained at passage 69 from the American Type Culture Collection (Rockville, MD). They were used through passage 84. Cells were cultured at 28°C in a humidified incubator gassed with 1.5% CO_2. Cells were fed twice a week with Dulbecco's Modified Eagle medium (amphibian formula: GIBCO, Grand Island, NY) supplemented with 40 mU/ml penicillin, 40 μg/ml streptomycin and 10% bovine calf serum (Hyclone, Logan, UT). A6 cells were subcultured onto either Millipore HAWP filter rings (Millipore, Burlington, MA) for membrane preparations or onto 24 mm Nunc tissue culture inserts (Nunc; Roskilde, Denmark) for patch clamping. These cultures were chronically exposed to aldosterone (10^{-7} M final concentration; Sigma, St. Louis, MO) from the time of seeding. Filters were used 5–7 days and 14–21 days after seeding for patch clamping and membrane preparations, respectively.

Land-phase tiger salamanders, *Ambystoma tigrinum,* were obtained from Charles Sullivan (Nashville, TN). Animals were kept in special aquaria (Aquaratron, Westminster Scientific, Westminster, MD) containing 1.5 in of circulating tap water at 70°C. Rocks were placed in the tanks to allow animals free access to air. Salamanders were fed crickets daily.

Patch Clamp Methods

Ambystoma were doubly pithed immediately prior to removal of the kidneys via an abdominal incision. Slices of kidney several mm thick were cut and immediately placed in room temperature saline for the dissection of initial collecting tubules. The saline contained in mM: NaCl, 105; KCl, 3.0; $CaCl_2$, 2.0; $MgSO_4$, 1.25; KH_2PO_4, 1.25; HEPES (N-[Hydroxyethylpiperazine-N-]2-ethanesulfonic acid), 5; and dextrose, 5.5. The osmolarity of this solution averaged 217.5 ± 1.6 mOsm/Kg H_2O [15]. The pH was titrated to 7.6. For dissection, one gm% of Fraction V bovine serum albumin (Sigma, St. Louis, MO) was added to this saline to reduce the tendency of dissected tubules to stick to glass and the dissection instruments. Unless stated otherwise, the saline solution minus the albumin was used to bathe both surfaces of the everted tubule and to fill the patch pipette.

The general features of everting amphibian renal tubule fragments have been described in a previous publication [9]. A brief description of the technique and modifications developed to successfully perfuse initial collecting tubules are presented here. Dissected tubules were transferred to a setup used to perfuse kidney tubule fragments in vitro.

Pipettes used to perfuse and evert *Ambystoma* collecting tubules were modified from those normally used to perfuse renal tubule fragments [9, 40, 41]. The inner perfusion pipette was pulled with a very long (3.0 to 4.0 mm) narrow parallel section. The outer diameter of this pipette averaged 10 to 15 μm. To prevent the basement membrane from sticking to the inner pipette it was pretreated with the albumin containing dissection solution for 10 min prior to mounting the tubule on the pipettes. The outer holding pipette was fabricated with an inner diameter of 85 to 100 μm to allow ample space to evert the tubule.

Eversion was initiated by first retracting the inner perfusion pipette to a point where a small patch of the basement membrane could be snagged and tucked into the lumen of the tubule. The perfusion

reservoir was turned off during eversion. After recentering the inner pipette, the fragment could be everted by slowly advancing the inner pipette while applying gentle suction to the outer pipette. Once the tubule was everted onto the inner perfusion pipette, the perfusion was restarted and a suction pipette mounted to the collection end v-track of the perfusion apparatus was used to gently pull the tubule off the inner perfusion pipette. About 200 μm of the inner pipette remained in the lumen of the everted tubule through which perfusate could be supplied to perfuse the basolateral surface of the everted tubule.

Methods for fabricating patch clamp pipettes and making seals were modified from those of Hamill et al. [12]. Pipettes were pulled from 150 μL Microcaps (Drummond Scientific, Broomall, PA) on a Brown-Flaming P-80/PC puller (Sutter Instrument, San Rafael, CA) immediately before use. Only pipettes whose resistance was between 2.5 and 3.3 MΩ routinely formed seals with the apical membrane. These pipettes form seals with the apical membrane 55% of the time. The tips of pipettes were firepolished on a Narishige Microforge (Narishige Co., Ltd., Tokyo, Japan) to minimize the capacitative properties of the electrode.

When patch clamping cells on a tubule suspended between perfusion and collection pipettes, the preparation was viewed via a high-resolution video monitor (Javelin Electronics, Torrance, CA). The perfused tubule was lowered to touch the surface of the coverslip which was pretreated with CellTac® (Collaborative Research, Boston, MA). This prevented the tendency of the tubule to roll during seal formation or fluid exchanges of the apical surface. To form a seal, the pipette was typically positioned directly above the center of the everted tubule with a mechanical micromanipulator. The final approach to the tissue was made with a Narishige Hydraulic Manipulator.

The data presented are from cell-attached patches. Patches with a seal resistance of less than 1.0 gΩ were discarded. Channel activity from a patch when the pipette and bathing solutions were identical and voltage-clamped to 0 mV was deemed to be from a cell-attached patch. To form a seal, pipettes were routinely positioned above areas of the everted collecting tubule which appeared to have a smooth, flat surface. Cells which appeared to bulge out from the surface were avoided.

In some experiments, we applied amiloride to the outside surface of the patch while recording from channels in a cell-attached patch. This necessitated the development of a low noise system which would allow us to change fluid inside the tip of the patch pipette. We used a modification of a fluid exchange system originally developed by Tang et al. [43] to perfuse whole cell preparations. This consisted of using .01 in I.D. × .03 in O.D. Microbore tubing (Thomas Scientific, Swedesboro, NJ) fitted with a fine quartz tip to deliver the drug to within 2.0 to 0.5 mm of the tip of the patch pipette.

This method involves fluid flow through long pieces of saline-filled tubing. The column of saline acts as an antenna increasing the noise level so that small channels cannot be monitored. To preclude the introduction of noise we filled the system initially with mineral oil, stained with sudan black, then aspirated a column of saline containing 2×10^{-6} or 5×10^{-7} M of amiloride 4 to 5 cm long into the tip of the exchange system. This approach produced recordings with low noise.

The patch clamp signal was monitored via an Axopatch 1-B amplifier (Axon Instrument, Burlingame, CA) equipped with a TMA-1 interface. A permanent record of experimental data was digitized (Model VR-10, Instrutech, Mineola, NY) and recorded on videotape for offline analysis. The signal was filtered to tape at 50 Hz. For analysis, data were fed into the computer at a sampling rate of 50 μsec/point and filtered at 50 Hz. Events shorter than 25 msec were discarded. p-CLAMP software (Axon Instruments) was used to analyze the data.

The chord conductance of channels was determined from the slope of the *I-V* relationship. Clamp voltages used typically ranged from −20 to +120 mV. The signal of an active patch was monitored for 60 to 180 sec at each voltage.

Ninety-seven percent of the seals ($n = 35$) obtained expressed channels. Many of the patches clearly possessed more than one type of channel. One quarter of the seals were patches that appeared to express a single channel type whose conductance and reversal potential were consistent with that reported for sodium channels. When more than one channel was evident in the patch, the open probability was computed as the fraction of time the individual channels were in the open state divided by the maximal number of levels observed. Mean open times were computed from the time a single channel spends in the level 1 state.

Results are presented as the mean value ± SEM (number of channels studied).

Membrane Preparations

Kidneys were immediately excised from ten doubly pithed *Ambystoma*, washed in phosphate-buffered saline (PBS) (pH 7.5) and cut into small pieces. Pieces were then homogenized with ten manual strokes of a Teflon glass homogenizer in 8 ml of ice-cold radioimmunoprecipitation (RIPA) buffer (in mM: 50 Tris-HCl, pH 7.4; 150 NaCl; 1 EGTA; 1% NP-40; 0.25% sodium deoxycholate) supplemented with 2 μg/ml deoxyribonuclease, 2 μg/ml ribonuclease, 175 μg/ml phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin (Sigma) and placed for 1.5 h on a rotator (4°C). This was followed by further homogenization with ten strokes of a Dounce homogenizer and centrifugation for 10 min at 5,000 × *g* (4°C). The supernatant was collected and concentrated to a final volume of 1 ml using Amicon Centricon 10 concentrators (Amicon, Beverly, MA). This concentrate was stored at −80°C until use.

Filter grown A6 cell monolayers were scraped into ice-cold, pH 7.4 buffer containing 60 mM sucrose and 10 mM tris(hydroxymethyl) aminomethane (Tris)-N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid HEPES supplemented with 2 μg/ml deoxyribonuclease, 175 μg/ml phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin and homogenized with ten manual strokes of a teflon glass homogenizer. $CaCl_2$ was added to a final concentration of 10 mM, the homogenate was spun for 10 min at 5,000 × *g* (4°C), and the resulting supernatant was subsequently spun for 1 hr at 43,000 × *g* (4°C). The pellet was then resuspended in ice-cold buffer containing 150 mM NaH_2PO_4, 10 mM 3-[-3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 75 μg/ml phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin for 1 hr at 4°C. The material was subsequently centrifuged for 1 hr at 43,000 × *g* (4°C) and the supernatant was collected and stored at −80°C.

Antibodies

Rabbit polyclonal antisodium channel antibodies generated against purified bovine papillary high amiloride-affinity sodium channel complex have been previously described by Sorscher et al. [38]. Rabbit polyclonal antibodies against Apx [39], a protein associated with the renal high amiloride affinity sodium channel complex characterized by Benos et al. [1, 2] and Kleyman et al. [18] from A6 renal epithelial cells and bovine kidney, were generated using a 16 mer synthetic peptide (K-V-Y-E-E-Q-F-E-S-I-H-N-S-L-P-P) corresponding to the C-terminus of Apx by Multiple Peptide Systems (San Diego, CA). The peptide was synthesized using standard solid-phase chemistry and its sequence was determined by mass spectroscopy and amino acid sequencing. Antibodies were affinity purified using the peptide coupled to a Proton-1 affinity column (Multiple Peptide Systems) following the manufacturer's protocol.

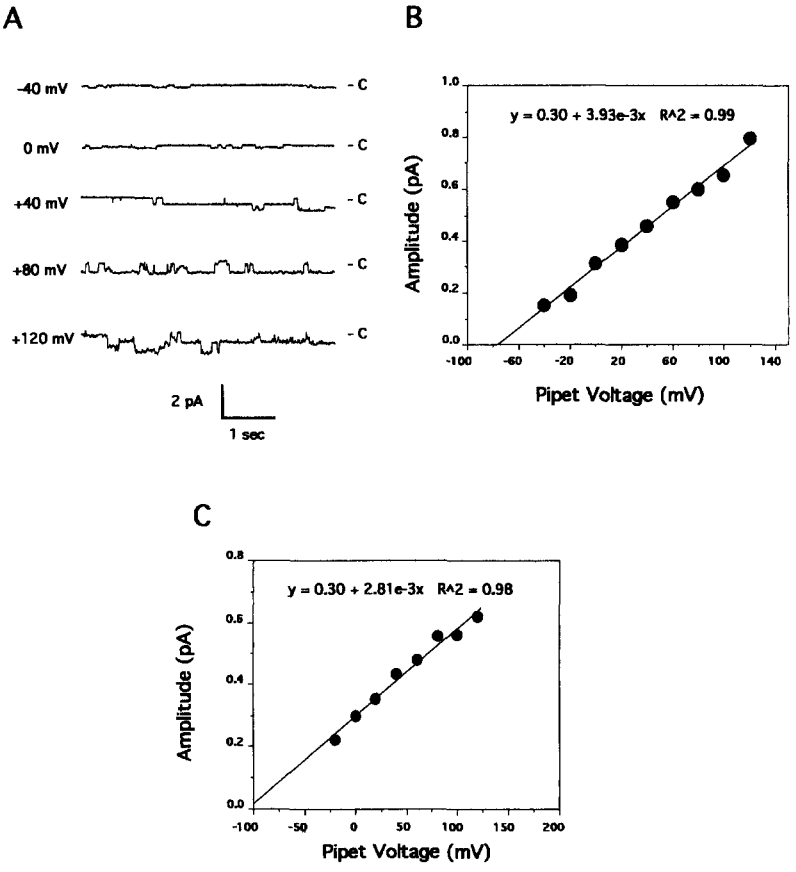

Fig. 1. Representative traces from cell-attached patch on apical membrane of *Ambystoma* initial collecting tubule (*A*). The closed state of each trace is indicated to the right of the panel and the pipette voltage to the left. The data were filtered at 25 Hz. (*B*) shows the current-voltage plot of the data from the same patch. (*C*) shows the current voltage plot of data collected from a cell-attached patch on the apical membrane of an A6 epithelial cell.

Immunoblots

RIPA solubilized *Ambystoma* kidney proteins were prepared for SDS-PAGE by diluting 1:5 with 2X SDS sample buffer [19] containing 5% β-mercaptoethanol (Sigma), heating at 95°C for 10 min and centrifuging at 14,000 rpm for 10 min in a microcentrifuge. A6 cell apical membrane proteins were prepared for SDS-PAGE by diluting 1:1 with 2X SDS sample buffer containing 10 mM dithiothreitol, heating at 95°C for 10 min and centrifugation as above. Following 7.5% SDS-PAGE, proteins were transferred to Immobilon PVDF paper (Millipore) using a Mini-Protean II electrophoretic system (BioRad, Hercules, CA) [44]. Blots were probed with a 1:200 dilution (10 μg/ml) of anti-Apx antibody, a 1:200 dilution (10 μg/ml) of anti-Apx antibody preincubated with a 1:100 dilution (20 μg/ml) of Apx peptide for 1 hr at 4°C prior to use, a 1:200 dilution (9.15 μg/ml) of rabbit polyclonal antisodium channel antibody, and a 1:200 dilution of nonimmune rabbit IgG in Rad-Free blocking solution (Schleicher & Schuell, Keene, NH) for 2 hr at room temperature. Blots were extensively washed and then incubated in 1:1000 dilution of alkaline phosphate conjugated goat anti-rabbit IgG (Schleicher & Schuell) for 30 min at room temperature. Following extensive washing, bound antibodies were detected using the Rad-Free chemiluminescent detection system (Schleicher & Schuell).

Immunolocalization of Sodium Channels

Excised *Ambystoma* kidneys were rinsed in PBS (pH 7.5), cut into small pieces and subsequently frozen in tissue freezing compound (Triangle Biomedical Services, Durham, NC) in gelatin capsules by submersion in liquid nitrogen. Frozen sections (5 μm thick) were cut using a Minotome cryotome (International Equipment, Needham, MA), collected on gelatin coated slides, and stored at −80°C. Prior to processing for immunofluorescence microscopy, sections were fixed for 1 min in ice-cold methanol and then allowed to air dry. Sections were rehydrated in PBS and then incubated for 1 hr in PBS containing 3% normal goat serum (Cappel/Organon Teknika, Durham, NC). Sections were incubated with polyclonal rabbit antisodium channel antibody diluted 1:50 in PBS-BSA (36 μg/ml final concentration) overnight at 4°C, washed six times in PBS, and then incubated in a 1:200 dilution of Texas red conjugated goat anti-rabbit IgG (Cappel/Organon Teknika) in PBS-BSA for 1 hr at room temperature. Following six washes in PBS, sections were mounted in Aqua Polymount (Polysciences, Warrington, PA). Controls consisted of substituting nonimmune rabbit IgG for the primary antiserum and secondary antibody alone. Photomicrographs were taken on a Nikon Optiphot UD microscope using Kodak T-MAX 400 ASA film push processed to 1600 ASA.

Results

Sodium Channels in the Apical Membrane of the Initial Collecting Tubule

The electrical characteristics of a low conductance channel found in cell-attached patches of the apical membrane of the amphibian initial collecting tubule are presented in Fig. 1. Panel *A* presents representative traces of channels clamped at voltages ranging from −40 to

Table. Characteristics of apical sodium channels in *Ambystoma* initial collecting tubule

Parameter	n	Value
Conductance (pS)	9	4.27 ± 0.22
Open probability	6	0.458 ± 0.074
Channel amplitude (pA)	9	0.390 ± 0.048
Reversal potential (mV)	9	−92.1 ± 11.3
Mean open time (msec)	6	446 ± 223
Number of channels per patch	9	3.11 ± 0.38

Values for open probability and channel amplitude were determined with the patch voltage-clamped to 0 mV. All values are presented as mean ± SEM.

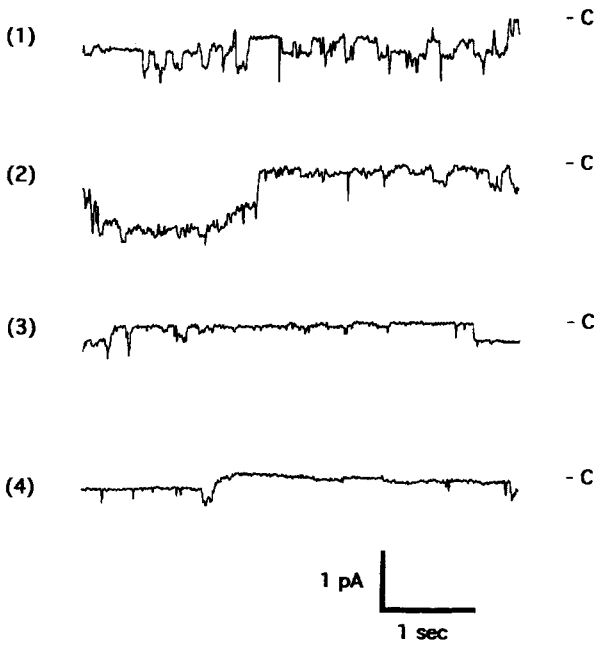

Fig. 2. Series of traces from cell-attached patch on apical membrane of *Ambystoma* initial collecting tubule illustrating the inhibition of sodium channels by 5×10^{-7} M amiloride. The patch was clamped to a pipette voltage of +40 mv. The closed state is indicated to the right of each trace. Top trace shows control conditions and the second, third and fourth are sequential traces showing inhibition of the channels by amiloride. The data were filtered at 25 Hz.

+120 mV. Panel *B* plots the current-voltage relationship for the channels in this patch. The slope was nearly 4 pS with an estimated pipette reversal potential of −72.2 mV. As shown in panel *C*, the current-voltage relationship is essentially identical to that of the predominant sodium channel expressed in A6 renal epithelial cells grown on permeable supports in the presence of aldosterone [17].

The averaged results of a series of such analyses are presented in the Table. These channels have a mean conductance of 4.3 pS and spend nearly 50% of their time in the open state. The unitary current at 0 mV is small at 0.39 pA. The pipette potential at which unitary current is zero, estimated by extrapolating the *I-V* relationship to zero current, averaged −92 mV. These values are nearly identical to those presented by others for A6 cells in culture [13, 17] and the rat cortical collecting tubule [29, 30].

In four experiments, we had a pipette in the lumen of the patch pipette capable of delivering an amiloride-containing saline to the extracellular surface of the patch. This pipette was placed within 0.5 to 2.0 mm of the tip of the patch pipette. In each case the channel activity initially observed in the patch was inhibited 1 to 4 min after the initiation of the recording. This lag time probably represents the time necessary for diffusion of the amiloride from the exchange pipette to the tip of the patch pipette to occur. An example of such inhibition is presented in Fig. 2. This patch possessed three channels whose characteristics were similar to those in the Table. The top trace shows the channel activity at pipette voltage of +40 mV. Just prior to amiloride inhibition, the activity of the channels was high and then stopped abruptly. Traces 2–4 in the figure show the 15-sec time period during which inhibition occurred. Laskowski and coworkers [20] have demonstrated that amiloride blockage of sodium channel activity is voltage dependent. At positive pipette potentials the sodium channel is blocked by amiloride, whereas at negative potentials the positively charged amiloride is pulled away from the blocking site, thereby allowing sodium ions to move through the channel [20]. In agreement with Laskowski et al [20], amiloride inhibition of the 5 pS channel was only observed at positive pipette potentials (*data not shown*).

In three of the four cases where inhibition occurred, we could reverse the inhibition by applying gentle suction to the fluid exchange pipette. This process aspirates the amiloride-containing saline out of the barrel of the patch pipette. An example of this experiment is presented in Fig. 3. Trace 1 illustrates the control trace at a pipette voltage of +60 mV. The second trace shows a relatively inactive patch after diffusion of amiloride has inhibited the channel. The last three sweeps illustrate reversal of the amiloride effect some 45 sec after exchange of the fluid with normal saline.

Immunoblot Analysis

To examine the biochemical characteristics of the epithelial sodium channel expressed in the distal nephron of *Ambystoma* by immunoblot analysis, we used two different antisodium channel antibodies: (i) an antibody generated against a high amiloride affinity renal epithelial sodium channel isolated from bovine renal papilla

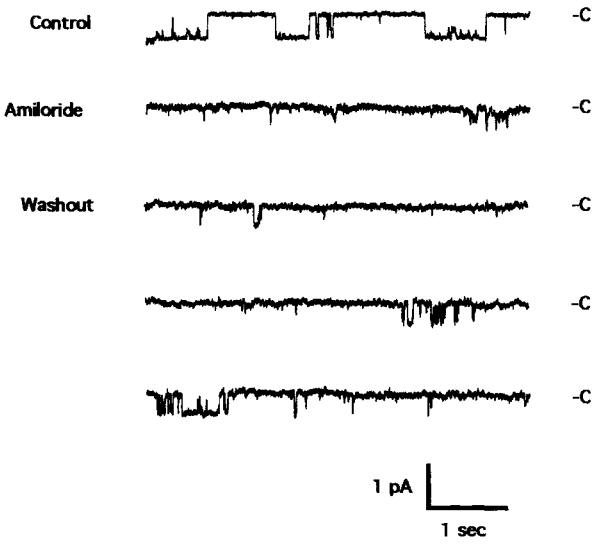

Fig. 3. Traces from cell-attached patch on apical membrane of *Ambystoma* initial collecting tubule illustrating the reversibility of sodium channel inhibition by 5×10^{-7} M of amiloride. The patch was clamped to a pipette voltage of +60 mv. The closed state is indicated to the right of each trace. The top trace shows control conditions. The second trace shows inhibition by 2×10^{-6} M amiloride and the third, fourth and fifth sequential 5-sec traces show reversal of amiloride inhibition. The data were filtered at 50 Hz.

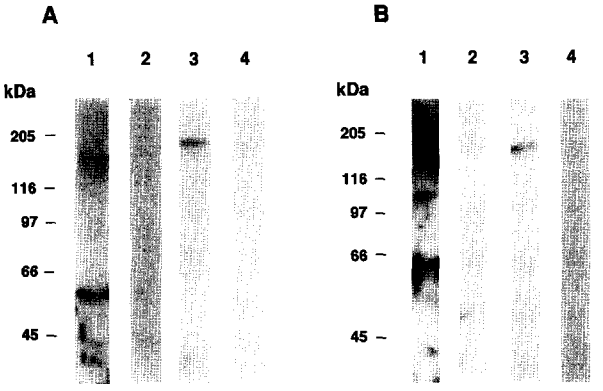

Fig. 4. Immunoblots of SDS-PAGE separated, detergent solubilized A6 renal epithelial cell apical membrane proteins (*A*) and RIPA solubilized *Ambystoma* kidney (*B*) proteins probed with anti-sodium channel and anti-Apx antibodies. *A:* A6 cell apical membrane proteins probed with rabbit anti-bovine renal epithelial sodium channel antibodies (1), nonimmune rabbit IgG (2), rabbit anti-Apx antibodies (3), or rabbit anti-Apx antibodies preincubated with excess free peptide (4). Anti-sodium channel antibodies label polypeptides of 150 and 55 kDa and anti-Apx antibodies label a 180 kDa polypeptide. *B:* RIPA solubilized *Ambystoma* kidney proteins probed with rabbit anti-bovine renal epithelial sodium channel antibodies (1), nonimmune rabbit IgG (2), rabbit anti-Apx antibodies (3), or rabbit anti-Apx antibodies preincubated with excess free peptide (4). Anti-sodium channel antibodies strongly recognize polypeptides with M_rs of 110, 57, and 55 kDa and anti-Apx antibodies recognize a single polypeptide with an M_r of 170 kDa.

(antisodium channel antibody) [38] and (ii) an antibody generated against a 16 mer synthetic peptide corresponding to the C-terminus of Apx, a protein associated with the renal high amiloride affinity sodium channel characterized from A6 epithelial cells and bovine papillary collecting ducts (anti-Apx antibody) [39]. The specificity of these antibodies was examined by immunoblotting detergent solubilized apical membrane proteins from the amphibian cell line A6, cultured in the presence of aldosterone. As shown in Fig. 4*A*, the antisodium channel antibodies recognize polypeptides with M_rs of 150 and 55 on immunoblots of A6 cell apical membranes. When nonimmune IgG is substituted for the antisodium channel antibodies, no polypeptides are recognized (Fig. 4*A*, lane 2). Affinity purified anti-Apx antibodies recognize a polypeptide with an M_r of ~180 kDa on immunoblots of A6 apical membranes, which corresponds to the M_r of (Apx 130–180 kDa) [39] (Fig. 4*A*, lane 3). When the anti-Apx antibodies are preincubated with excess peptide, no polypeptides are recognized (Fig. 4*A*, lane 4).

Immunoblot analysis of RIPA solubilized *Ambystoma* kidney proteins reveals that the antisodium channel antibodies specifically recognize a 110 kDa polypeptide and a doublet with M_rs of 55 and 57 kDa whereas the affinity purified anti-Apx antibody recognizes a single polypeptide exhibiting an M_r of ~170 kDa (Fig. 4*B*). In addition, the antisodium channel antibodies weakly recognized polypeptides with M_rs of ~116 and ~170 kDa, however this was not consistently observed.

Immunocytochemical Localization of Sodium Channels

We used the antisodium channel antibodies to determine the distribution of the epithelial sodium channels in *Ambystoma* kidney by indirect immunofluorescence microscopy. Examination of frozen sections incubated with antisodium channel antibodies and processed for immunofluorescence microscopy revealed that sodium channel immunostaining was restricted to regions which exhibited the characteristics of the amphibian distal nephron [15]. As illustrated in Fig. 5, immunoreactivity was localized to the apical membranes and subapical cytoplasm of the distal nephron cells. Within each distal nephron profile, there were also cells which did not exhibit apical immunoreactivity and thus were likely intercalated cells. There was no specific staining of the glomerulus, proximal tubule or the early distal nephron which is characterized by extensive infolding of the basolateral membrane [15]. In control sections, there was no staining of distal nephron cells when nonimmune serum was substituted for immune serum or with the secondary antibody alone (*data not shown*).

Discussion

Several types of ion channels that translocate sodium have been identified in the amphibian distal nephron cell

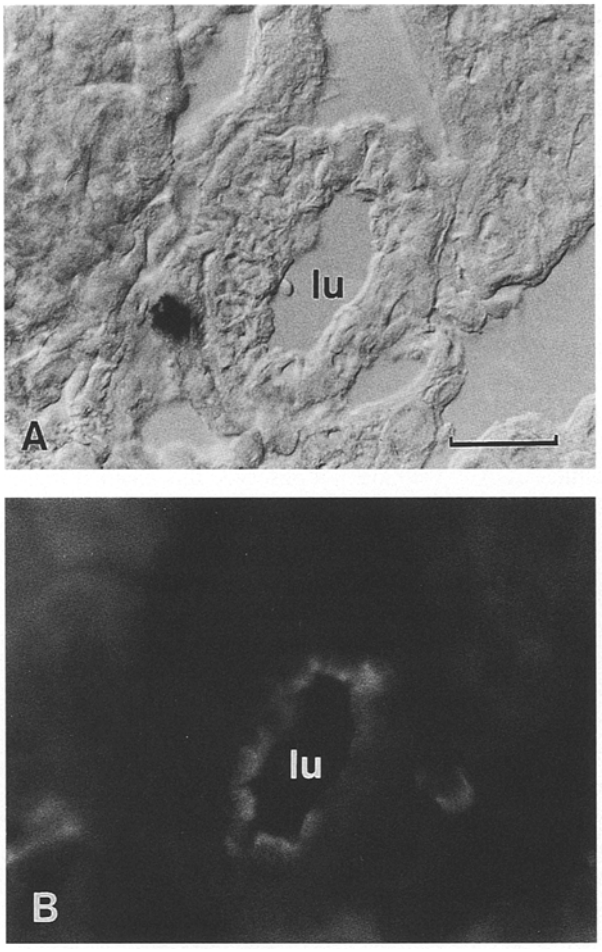

Fig. 5. Immunofluorescence localization of epithelial sodium channels within the distal nephron of *Ambystoma A:* Differential interference contrast photomicrograph of a frozen section through a distal nephron tubule. *B:* Immunofluorescence photomicrograph of the same tubule stained with rabbit anti-sodium channel antibodies followed by Texas-red conjugated goat anti-rabbit IgG secondary antibody. Note fluorescence is localized to the luminal membrane of the duct cells. Cells which do not exhibit immunoreactivity may represent intercalated cells. *lu* denotes the tubular lumen and the scale bar is 20 μm.

line A6: a 5 pS, a 9 pS, and a 30 pS channel [7, 13, 26, 31]. Only the 5 pS channel has been shown to exist in the native mammalian collecting tubule [29, 30]. The high conductance channel appears nonselective to sodium and potassium and its inhibition, or lack of it, by amiloride is less clear [29, 31]. The 9 pS channel is highly sensitive to amiloride and moderately selective to sodium [7, 13]. These characteristics have left open the possibility that it too is involved in sodium reabsorption.

We have previously demonstrated that both the diluting segments (early distal tubule) and the initial collecting tubule of the salamander, *Ambystoma,* can be everted and perfused in vitro [9]. This preparation not only exposes the apical surface of the initial collecting tubule to patch clamp methodology, it also allows the investigator to independently control the saline bathing the two surfaces of the perfused tubule. This tubule segment is known to actively reabsorb sodium [41]. Our goal was to determine which, if any, of the sodium channels found in A6 cells were present in the native amphibian epithelium.

This is the first study to characterize sodium channels on the apical surface of the native amphibian initial collecting tubule. The sodium channels we find have a conductance of 4.3 ± 0.2 pS (range 3.2–5.2 pS) and express an open probability of 0.46. These characteristics are nearly identical to that reported for the highly selective sodium channel observed in the A6 cell line by Eaton and his coworkers [13, 17, 25] and the sodium channel identified in the native rat cortical collecting tubule [29, 30].

The value of the pipette potential where the unitary current of this channel approaches zero was determined by linear extrapolation of the current-voltage relationship (Fig. 1). At –92 mV it approaches a reasonable value of E_{Na} across the apical membrane of the late distal nephron (17). This observation implies a high selectivity of the channel in question. This characteristic seems plausible for the initial collecting tubule of *Ambystoma* which reabsorbs substantial quantities of sodium chloride against a large chemical gradient [4, 33, 41]. Unlike the mammalian collecting tubule which secretes comparable quantities of potassium [40], the initial collecting tubule of *Ambystoma* secretes very little potassium and is relatively impermeable to water even in the presence of antidiuretic hormone [41]. The reabsorption of sodium independent of potassium secretion and water reabsorption would require a highly selective channel.

It is possible to estimate from our data whether or not the channel density is sufficient to explain the rate of sodium reabsorption observed in isolated *Ambystoma* collecting tubules. Based on measurements of the tip diameter of our pipettes we calculate the cross-sectional area to be 1.40 μm^2. Nearly 90% of our seals possess channels whose characteristics are consistent with the presence of the 5 pS sodium channel. From these observations and the values in the Table for unitary current, open probability and channel density per patch, we can estimate the current density per patch to be 0.397 pA/μm^2 of membrane. This corresponds to a sodium flux of 0.41×10^{-17} moles/μm^2 sec. The measured inner diameter of this tubular segment is 26 μm [41]. Assuming the lumen membrane to be a right cylinder there would be 0.8164×10^5 μ^2/mm tubular length. Multiplying the sodium flux rate by this conversion factor yields 18.2 pmol/mm · min, a value in reasonable agreement with 21.2 pmoles/mm · min, the published value for the rate of net sodium transport by this tubular segment [41].

Taken together, these properties are consistent with the notion that the 5 pS sodium channel we observe in the apical membrane of the initial collecting tubule could account for the flux of sodium into the cell during net

sodium reabsorption. It should be stressed that several assumptions and estimations have been made in this calculation. They include: lack of precise measurements of the cross-section area of the patch, a potentially significant underestimation of apical surface area and the fact that some of the cells in the initial collecting tubule, the intercalated cells, are not thought to contain sodium channels.

Inhibition by the potassium-sparing diuretic, amiloride, is considered to be an almost universal characteristic of the apical channels responsible for sodium reabsorption by the late distal nephron. Amiloride sensitivity has been shown for a variety of sodium selective channels found in epithelial cell cultures [13, 14, 16, 24, 25, 26], native kidney tubules [10], analogues of the distal nephron [8, 11], and sodium channels expressed in oocytes [32] and reconstituted into lipid bilayers [28, 34, 36].

In general, investigators have shown both the highly selective and the moderately selective sodium channel to be sensitive to low dose (<1 μM) of amiloride [8, 11, 13, 14, 23, 34, 36]. As expected for an inhibitor predicted to occlude the conductive pore itself, the drug decreases the mean open time and open probability of these channels. Kinetic analysis of the effect of amiloride on mean closed time histograms suggest that two closed states exist in the presence of submaximal doses of the drug, one the closed state of the channel not blocked by the drug and the other the amiloride-blocked channel [13, 24].

We used amiloride inhibition in this study to confirm the identity of the 4.3 pS channel characterized in the apical membrane of the *Ambystoma* initial collecting tubule. As illustrated in Fig. 3, inhibition was abrupt and reversible when fresh bath was washed into the tip of the patch pipette. We did not attempt to do long-term recordings to confirm the kinetic analysis of others.

As expressed by Palmer [31], several lines of evidence indicate that the 9 pS channel and 5 pS channel may be intraconvertable forms of the same channel. One of his arguments for this case is epithelial sodium channels purified from A6 cells grown on filter supports, a preparation which normally produces a predominance of the 5 pS channel, express the 9 pS channel when incorporated into lipid bilayers [36]. In addition, mRNA isolated from A6 cells grown on plastic, which usually possess the 9 pS channel, induces the production of 5 pS highly selective sodium channel in oocytes [32]. Taken together, these observations are consistent with the notion that the 5 pS and 9 pS channels are really different variants of the same channel. If so, a very interesting question for future research in native tissue is what physiological perturbations induce conversion of one type of channel to another.

Some authors have suggested that a third channel type may contribute to sodium reabsorption in the late distal nephron. A high conductance 28–30 pS, nonselective, cation channel has been observed in a number of epithelial tissue culture cell lines including: cell lines thought to represent the principal cells of the rabbit collecting tubule [24], cells of the rabbit medullary collecting duct [23], A6 cells [27], and vasopressin-treated toad bladder epithelial cells [11]. Its nonselectivity, lack of sensitivity to aldosterone, and questionable inhibition by low doses of amiloride make it difficult to assign a physiological significance to its presence [31]. In medullary collecting tubule cells, it has been shown to be inhibited by atrial natriuretic peptide [23] and in A6 cells it has been shown to be transiently activated by large doses of arginine vasotocin [27]. There have been no reports of the presence of this channel in the native rat collecting tubule. As yet, we have not observed this channel in the native tissue of the amphibian distal nephron.

Benos and coworkers [1, 2, 28, 35] have biochemically characterized a renal high amiloride affinity epithelial sodium channel complex purified from bovine collecting ducts and A6 cells. This sodium channel is a heteroligomeric complex consisting of six polypeptides with molecular masses of 300–315, 130–180, 90–110, 70–85, 55–65, and 41 kDa. The purified bovine channel complex forms highly sodium selective, amiloride-sensitive channels when incorporated into planar lipid bilayers [28] and the 130–180 kDa polypeptides isolated from A6 cell sodium channel complex yields a sodium selective channel in lipid bilayers with a conductance of 5 pS [36]. Kleyman and collaborators [18] have purified an essentially identical heteroligomeric sodium channel complex from A6 cells using a monoclonal antibody directed against the amiloride binding component of the sodium channel.

Recently an epithelial sodium channel protein (ENaC) has been cloned from the rat distal colon [5, 6, 20, 21]. This channel is a heteromultimeric complex consisting of three homologous subunits (α, β, γ) which have predicted molecular masses of 79, 72, and 75 kDa respectively [6]. In addition to the rat colon, message for this channel is expressed in kidney medulla and cortex and in lung [6]. When expressed in oocytes, this channel has a single channel conductance of 4 pS and exhibits ion selectivity, gating kinetics, and an amiloride pharmacological profile similar to that of the 4 pS, highly selective sodium channel expressed in native sodium reabsorbing epithelia [6]. At present the relationship between this channel and the channel biochemically purified from A6 cells and bovine collecting ducts [1, 2, 18] is unclear. However, it is conceivable that the biochemically purified sodium channel is either a different isoform of ENaC or the product of a different sodium channel gene.

In this study, we have used antibodies directed against the purified bovine renal sodium channel [38] to biochemically characterize the epithelial sodium channels in the distal nephron of *Ambystoma*. As shown in

Fig. 4, the anti-sodium channel antibody exhibited strong cross-reactivity with polypeptides exhibiting M_rs of 150 and 55 kDa on immunoblots of A6 cell apical membranes and 110, 57, and 55 kDa on immunoblots of RIPA solubilized *Ambystoma* kidney. Although the polyclonal anti-bovine renal epithelial sodium channel antibody was raised against the purified heteroligimeric complex, recognition of the individual polypeptides on immunoblots varies with titers. The molecular masses of the polypeptides recognized in *Ambystoma* are similar to two of the polypeptides of the renal high amiloride affinity sodium channel complex, i.e., the 90–110 kDa and the 55–65 kDa. We have also used an antibody directed against a synthetic peptide corresponding to the C-terminus of Apx, a polypeptide which may represent part of the sodium channel complex characterized by Benos et al [1, 2, 28] and Kleyman et al. [18]. This antibody recognized a single polypeptide on immunoblots of A6 cells and *Ambystoma* kidney exhibiting M_rs of 180 and 170 kDa respectively, which are in agreement with the reported M_r of Apx (130–180 kDa) [39]. Taken together, these data suggest that the renal sodium channel expressed in *Ambystoma* is a heteroligomeric complex similar to the channel characterized biochemically and electrophysiologically from A6 cells and bovine kidney [1, 2, 28].

In summary, we have demonstrated that the initial collecting tubule in *Ambystoma* expresses a 5 pS amiloride-sensitive channel in the apical membrane whose electrical characteristics appear identical to those of the 5 pS channels reported for the rat collecting tubules, cultured cells derived from rabbit collecting tubule and A6 cells. The density of these channels suggests that they may represent the channels primarily responsible for sodium reabsorption process in the *Ambystoma* initial collecting tubule. In addition, immunochemical analysis corroborates that these apically restricted sodium channels are similar to the high amiloride affinity, sodium-selective channels previously characterized from A6 cells and the mammalian kidney.

This work was supported by American Heart Association, New York Affiliate Grant 91007G (LCS) and National Institute of Diabetes and Digestive and Kidney Disease Grants DK-37206 (DJB) and DK46705 (PRS).

References

1. Benos, D.J., Saccomani, G., Brenner, B.M., Sariban-Sohraby, S. 1986. Purification and characterization of the amiloride-sensitive sodium channel from A6 cultured cells and bovine renal papilla. *Proc. Natl. Acad. Sci. USA* **83:**8525–8529
2. Benos, D.J., Saccomani, G., Sariban-Sohraby, S. 1987. The epithelial sodium channel: subunit number and location of amiloride binding sites. *J. Biol. Chem.* **262:**10613–10618
3. Benos, D.J., Cunningham, S., Baker, R.R., Beason, K.B., Oh, Y., Smith, P.R. 1992. Molecular characteristics of amiloride-sensitive sodium channels. *Rev. Physiol. Biochem. Pharmacol.* **120:**31–113
4. Bott, P.A. 1962. Micropuncture study of renal excretion of water, K, Na and Cl in *Necturus*. *Am. J. Physiol.* **203:**662–666
5. Canessa, C.M., Horisberger, J.-D., Rossier, B.C. 1993. Epithelial sodium channel related to proteins involved in neurodegeneration. *Nature* **361:**467–470
6. Canessa, C.M., Schild, L., Buell, G., Gautschi, I., Horisberger, J.-D., Rossier, B.C. 1994. Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. *Nature* **367:**463–467
7. Cantiello, H.F., Patenaude, C.R., Ausiello, D.A. 1991. G-protein subunit, ∂_i-3. activates a pertussis-sensitive Na^+ channel from the epithelial cell line, A6. *J. Biol. Chem.* **264:**20867–20870
8. Desmedt, L., Simaels, J., Van Driessche, W. 1991. Amiloride blockage of Na channels in epithelia does not require external Ca. *Pfluegers Arch.* **419:**632–638
9. Engbretson, B.G., Beyenbach, K.W., Stoner, L.C. 1988. The everted renal tubule: A method for direct assessment of the apical membrane. *Am. J. Physiol.* **255:**F1276–F1280
10. Frindt, G., Sackin, H., Palmer, L.G. 1990. Whole-cell currents in rat cortical collecting tubule: low-Na diet increases amiloride-sensitive conductance. *Am. J. Physiol.* **258:**F562–F567
11. Frings, S., Purves, R.D., Macknight, A.D.C. 1988. Single-channel recordings from the apical membrane of the toad urinary bladder epithelial cell. *J. Membrane Biol.* **106:**157–172
12. Hamill, O.P., Neher, M.E., Sakman, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100
13. Hamilton, K.L., Eaton, D.C. 1986. Single-channel recordings from two types of amiloride-sensitive epithelial Na channels. *Memb. Biochem.* **6:**149–171
14. Hamilton, K.L., Eaton, D.C. 1985. Single-channel recordings from amiloride-sensitive epithelial sodium channel. *Am. J. Physiol.* **249:**C200–C207
15. Hinton, D.E., Stoner, L.C., Burg, M.B., Trump, B.F. 1982. Structural heterogeneity in the distal nephron of the salamander (*Ambystoma tigrinum*): An electron microscopic study of isolated tubule segments. *Anatomical Rec.* **204:**21–32
16. Joris, L., Krouse, M.E., Hagiwara, G., Bell, C.L., Wine, J.J. 1989. Patch-clamp study of cultured human sweat duct cells: amiloride-blockable Na channel. *Pfluegers Arch.* **414:**369–372
17. Kemendy, A.E., Kleyman, T.R., Eaton, D.C. 1992. Aldosterone alters the open probability of amiloride-blockable sodium channels in A6 epithelia. *Am. J. Physiol.* **263:**C825–C837
18. Kleyman, T.R., Kraehenbuhl, J.-P., Ernst, S.A. 1991. Characterization and cellular localization of the epithelial Na^+ channel. Studies using an anti-Na^+ channel antibody raised by antiidiotypic route. *J. Biol. Chem.* **266:**3907–3915
19. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **222:**680–685
20. Laskowski, F.H., Christine, C.W., Gitter, A.H., Beyenbach, K.W., Gross, P., Fromter, E. 1990. Cation channels in the apical membrane of collecting duct principal cell epithelium. *Renal Physiol. Biochem.* **13:**70–81
21. Lingueglia, E., Voilley, N., Waldmann, R., Lazdunski, M., Barbry, P. 1993. Expression cloning of an epithelial amiloride sensitive Na^+ channel. *FEBS Lett.* **318:**95–99
22. Lingueglia, E., Renard, S., Waldmann, R., Voilley, N., Champigney, G., Plass, H., Lazdunski, M., Barbry, P. 1994. Different homologous subunits of the amiloride sensitive Na^+ channels are differently regulated by aldosterone. *J. Biol. Chem.* **269:**13736–13739
23. Light, D.B., Schwiebert, E.M., Karlson, K.H., Stanton, B.A. 1989.

Atrial natriuretic peptide inhibits a cation channel in renal inner medullary collecting duct cells. *Science* **243:**383–385

24. Ling, B.N., Hinton, C.F., Eaton, D.C. 1988. Amiloride-sensitive sodium channels in rabbit cortical collecting tubule primary cultures. *Am. J. Physiol.* **255:**F1276–F1280
25. Marunaka, Y., Hagikwara, N., Tohda, H. 1991. Insulin activates single amiloride-blockable Na channels in distal nephron cell line (A6). *Am. J. Physiol.* **261:**F933–F944
26. Marunaka, Y., Eaton, D.C. 1991. Effects of vasopressin and c-AMP on single amiloride-blockable Na channels. *Am. J. Physiol.* **260:**C1071–C1084
27. Marunaka, Y., Hagikwara, N., Tohda, H., Nakahari, T. 1994. Antidiuretic hormone-responding nonselective cation channel in distal nephron epithelium (A6). *Am. J. Physiol.* **266:**C1513–C1522
28. Oh, Y., Benos, D.J. 1993. Single-channel characteristics of a purified bovine renal amiloride-sensitive sodium channel in planar lipid bilayers. *Am. J. Physiol.* **264:**C1489–C1499
29. Palmer, L.G., Frindt, G. 1986. Amiloride-sensitive Na channels from the apical membrane of the rat cortical collecting tubule. *Proc. Nat. Acad. Sci. USA* **83:**2767–2770
30. Palmer, L.G., Frindt, G. 1987. Effects of cell Ca and pH on Na channels from the rat cortical collecting tubule. *Am. J. Physiol.* **253:**F333–F339
31. Palmer, L. 1992. Epithelial Na channels: function and diversity. *Am. Rev. Physiol.* **54:**51–66
32. Palmer, L.G., Corthesy-Theulaz, I., Gaeggeler, H., Kraehenbuhl, J., Rossier, B. 1990. Expression of epithelial Na channels in Xenopus oocytes. *J. Gen. Physiol.* **96:**23–46
33. Richards, A.N., Walker, A.M. 1937. Methods of collecting fluid from known regions of the renal tubules of amphibia and perfusing the lumen of a single tubule. *Am. J. Physiol.* **118:**111–120
34. Sariban-Sohraby, S., Latorre, R., Burg, M., Olans, L., Benos, D. 1984. Amiloride-sensitive epithelial Na channels reconstituted into planar lipid bilayer membranes. *Nature* **308:**80–82
35. Sariban-Sohraby, S., Benos, D.J. 1986. Detergent solubilization, functional reconstitution, and partial purification of epithelial amiloride-binding protein *Biochemistry* **25:**4639–4646
36. Sariban-Sohraby S., Abramow, M., Fisher, R.S. 1992. Single-channel behavior of a purified epithelial Na channel subunit that binds amiloride. *Am. J. Physiol.* **263:**C1111–C1117
37. Smith, P.R., Benos, D.J. 1991. Epithelial Na^+ channels. *Ann. Rev. Physiol.* **53:**509–530
38. Sorscher, E.J., Accavetti, M.A., Keeton, D., Steadman, E., Frizzell, R.A., Benos, D.J. 1988. Antibodies against purified epithelial sodium channel protein from bovine renal medulla. *Am. J. Physiol.* **255:**C835–C843
39. Staub, O., Verrey, F., Kleyman, T.R., Benos, D.J., Rossier, B.C., Kraehenbuhl, J.-P. 1992. Primary structure of an apical protein from *Xenopus laevis* that participates in amiloride-sensitive sodium channel activity. *J. Cell. Biol.* **119:**1497–1506
40. Stoner, L., Burg, M. 1975. Ion transport in cortical collecting tubule: Effect of amiloride. *Am. J. Physiol.* **227:**453–459
41. Stoner, L.C. 1977. Isolated, perfused amphibian renal tubules: the diluting segment. *Am. J. Physiol.* **233:**F438–F444
42. Stoner, L.C. 1985. The movement of solutes and water across the vertebrate distal nephron. *Renal Physiol.* **8:**237–248
43. Tang, J.M., Wang, J., Quandt, F.N., Eisenberg, R.S. 1990. Perfusing pipets. *Pfluegers Arch.* **416:**347–350
44. Towbin, H., Staehelin T., Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA* **76:**4350–4354
45. Yucha, C.B., Stoner, L.C. 1986. Bicarbonate transport by amphibian nephron. *Am. J. Physiol.* **251:**F865–F872
46. Yucha, C.B., Stoner, L.C. 1987. Bicarbonate transport by initial collecting tubule of aquatic- and land-phase amphibia. *Am. J. Physiol.* **253:**F310–F317