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Associated Proteins and Renal Epithelial Na^+ Channel Function

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Abstract. The hypothesis that amiloride-sensitive Na^+ channel complexes immunopurified from bovine renal papillary collecting tubules contain, as their core conduction component, an ENaC subunit, was tested by functional and immunological criteria. Disulfide bond reduction with dithiothreitol (DTT) of renal Na^+ channels incorporated into planar lipid bilayers caused a reduction of single channel conductance from 40 pS to 13 pS, and uncoupled PKA regulation of this channel. The cation permeability sequence, as assessed from bi-ionic reversal potential measurements, and apparent amiloride equilibrium dissociation constant (K_i^{amil}) of the Na^+ channels were unaltered by DTT treatment. Like ENaC, the DTT treated renal channel became mechanosensitive, and displayed a substantial decrease in K_i^{amil} following stretch (0.44 ± 0.12 μM versus 6.9 ± 1.0 μM). Moreover, stretch activation induced a loss in the channel's ability to discriminate between monovalent cations, and even allowed Ca^{2+} to permeate. Polyclonal antibodies generated against a fusion protein of αbENaC recognized a 70 kDa polypeptide component of the renal Na^+ channel complex. These data suggest that ENaC is present in the immunopurified renal Na^+ channel protein complex, and that PKA sensitivity is conferred by other associated proteins.

Key words: Membranes — Reduction — Ion selectivity — Mechanosensitivity — Planar lipid bilayers — Protein kinase A

Introduction

Amiloride-sensitive Na^+ channels play an important role in Na^+ reabsorption by the kidney. Dysfunction of cAMP-regulatory pathways of these Na^+ channels has been implicated in at least one form of genetic hypertension, namely, Liddle's disease [37]. In spite of the preeminence of these channels for Na^+ homeostasis, there is much debate on their biochemical composition and their coupling to various regulatory pathways [3]. Work performed in our own and other laboratories has identified Na^+ channel protein complexes from mammalian renal tubules and amphibian A6 cells [5, 27, 28, 35], rabbit alveolar type II cells [36], and rat lymphocytes [7]. Each of these channel complexes has been functionally reconstituted into planar lipid bilayer membranes where they display appropriate sensitivity to the diuretic amiloride, a larger Na^+ to K^+ permselectivity, and an increase in channel activity following phosphorylation by protein kinase A (PKA) plus ATP. However, the overall biochemical composition of each of these channel complexes is unique and consists of multiple components [3–10].

Recently, an amiloride-sensitive epithelial Na^+ channel has been cloned from rat distal colon [8, 9], human lung [26], bovine renal papilla [13], *Xenopus* kidney [32, 33], and mouse lung [10]. The channel encoded by this clone (termed ENaC, for epithelial Na^+ channel) consists of three homologous subunits, α, β, and γ, with the conductive properties most probably residing within the α-subunit [8]. The functions of the β and γ subunits are unknown, but their presence is required for maximal channel activity, at least in the oocyte heterologous expression system [9]. In contrast to the aforementioned biochemically purified Na^+ channels, the ENaC channel is not cAMP-activated, but is mechanosensitive [2].

The purpose of the present work was to elucidate the relationship between the biochemically purified renal Na^+ channel and the ENaC clone. We began with the hypothesis that the channel complex immunopurified from bovine renal papillary collecting tubules contains as its central element an ENaC subunit(s). Specific regulatory inputs (e.g., sensitivity to PKA-induced phosphorylation) are provided by its associated protein components. We reasoned that if the channel protein complex

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was disrupted, the resultant single channel properties should be dissociated from PKA regulation and adopt ENaC characteristics. Indeed, we found that disulfide bond reduction with low concentrations of dithiothreitol (DTT) completely uncoupled channel activity and PKA-sensitivity. Moreover, DTT-treatment converted these purified renal Na^+ channels into ones with a relatively low conductance. These DTT-treated channels also displayed stretch-activation, with characteristics similar to those observed for αbENaC channels [2].

Materials and Methods

Immunopurification of Renal Na^+ Channel Protein Complex

Purification of amiloride-sensitive Na^+ channel protein complexes from bovine kidney papillary collecting ducts was performed as previously described [27]. Excised papillae were homogenized in the presence of protease inhibitors, and membranes were isolated by differential centrifugation, and solubilized using 10 mM CHAPS. Solubilized proteins were purified by sequential anion and cation exchange chromatography. The final protein complex was obtained by immunopurification using specific polyclonal anti-Na^+ channel antibodies. The channel purity of this preparation was determined by [^{3}H]-methylbromoamiloride binding as described previously [27], and was greater than 1000 pmoles/mg.

Reconstitution of Immunopurified Channel Proteins into Lipid Vesicles and Planar Lipid Bilayers

Liposomes into which channel protein was reconstituted were constructed as described earlier [28]. Briefly, they were made by passing purified channel proteins through an Extracti-Gel D column (Pierce) to remove detergent. The column was equilibrated with 10 mM Na_2HPO_4 plus 0.5% egg phosphatidylcholine. Reconstituted proteoliposomes were stored at −80°C until use.

Planar lipid bilayers were composed of a mixture of 2:1:2 (*w/w/w*) diphytanoyl-phosphatidylethanolamine, diphytanoyl-phosphatidylserine, and oxidized cholesterol (final phospholipid concentration = 25 mg/ml) in *n*-octane. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was oxidized as described by Tien et al. [39]. Membranes were routinely bathed in a solution containing symmetrical 100 mM NaCl, 10 mM MOPS buffer, pH 7.4. Reconstituted proteoliposomes were spread over a preformed bilayer with a fire-polished glass rod from the *trans* side with the holding potential set at −40 mV. As described earlier [e.g., 19, 20], under these conditions channels oriented in such a way that the amiloride-sensitive side faced the *trans* solution while the PKA-sensitive side faced the *cis* side. In the experiments reported in this manuscript, the proteoliposome preparation was diluted appropriately such that only membranes containing a single ion channel were routinely obtained. The total number of channels incorporated into any given bilayer membrane was determined by PKA-induced phosphorylation performed at the conclusion of each experiment. This maneuver reveals the presence of any ''silent'' channels resident in the bilayer membrane [19, 21]. Briefly, PKA + ATP acts by increasing the open probability of immunopurified amiloride-sensitive renal Na^+ channels in planar lipid bilayers rather than recruiting new channels because no channel-containing vesicles are present in the bathing solutions. This conclusion is also supported by the fact that entire substrate ultrastructure of these channels, i.e., a main conductance level with two additional conductance levels, always occurs in multiples of three [18, 19, 22]. The few membranes in which more than one channel was present (5/157) were excluded from analysis.

Current measurements and data acquisition and analysis were accomplished and performed as previously described [20]. Because the single channel open probability (P_o) was very low under the conditions used in these experiments, at least 10 min of recording for each condition or perturbation was used for analysis. The catalytic subunit of cAMP-dependent PKA (final concentration 1.85 ng/ml) was a gift Dr. Gail Johnson, University of Alabama at Birmingham. The dashed line in each figure represents the zero current level.

Hydrostatic Pressure Experiments

A hydrostatic pressure gradient (ΔP) across a channel-containing bilayer membrane was imposed by elevating the level of the solution in one of the two compartments of the bilayer chamber. Each compartment initially contained 4 ml of bathing medium, and bilayer formation and channel incorporation were done under these conditions. Following successful channel incorporation and subsequent to acquisition of basal activity, the fluid level on one side was either increased or decreased. To establish the sidedness of the effect of ΔP, 20 experiments were performed when the pressure gradient was applied from *trans* to *cis* and 20 experiments were done when the gradient was directed *cis* to *trans*. In all 20 experiments in which ΔP was *trans* to *cis*, an increase in channel activity was observed. However, when the gradient was directed to *cis* to *trans*, the results were less consistent. In four experiments, channel P_o increased; in nine experiments, channel activity was unaffected; in five experiments P_o decreased; and in two cases, channel activity was irreversibly lost. In all other cases, the effect of ΔP was reversible.

Ion Selectivity Measurements

Relative cation permeability measurements were based on 3-min recordings of single channel activity at different holding voltages under biionic conditions, and mean currents ($\bar{I}$) over this period of observation at each voltage were used to construct current-voltage curves. The Goldman-Hodgkin-Katz potential equation was used to determine P_X/P_{Na}, where X was either Li^+, K^+, or Ca^{2+}, from the measured zero current (reversal) potential.

Mean current is the product of the single channel unitary current (i), the number of active channels (N), and their P_o, given that the channels operate independently. Immunopurified bovine renal Na^+ channels have a main state conductance of 40 pS and two additional subconductance levels of ⅓ of the main state level [20]. These lower conductance states do not gate independently, and thus complicate the decision to use mean rather than unitary current in the computation of P_o and construction of current/voltage curves. However, simple calculation shows that using either $\bar{I}$ or i results in the same P_o, as long as the subconductance levels are all of equal magnitude.

$$\bar{I} = i\,N\,P_o$$

If i = main state current level at any given applied potential and $N = 1$

$$P_O = \frac{\bar{I}}{i}$$

If $i = i/3$ and using $N = 3$

$$P_o = \frac{\bar{I}}{i/3 \cdot 3} = \frac{\bar{I}}{i}$$

This formalism is thus valid if these two lower conductance states act as independent channels, as long as they are of equivalent magnitudes. For our purposes, we used the largest and most frequently observed main transition state as the unitary current for the calculation of channel P_o.

Antibody Production

Fusion protein was made utilizing the pET system (Novagen). Briefly, the full α-bENaC open reading frame was subcloned into a pET vector, which was subsequently transformed into *E. coli.* Fusion protein expression was induced with T7 RNA polymerase. The vector was engineered to produce a stretch of eight histidines (His-Tag) as part of the *N*-terminus of the target protein. A one step purification by metal chelation chromatography using a nickel-bound resin, which binds histidine, was subsequently used. After washing away the unbound protein, the tagged protein was eluted with imidazole. The eluted protein was concentrated and dialyzed against distilled H_2O, and stored at −80°C until used.

Fusion protein was diluted in sterile water to a final concentration of 200 μg/ml. One ml was injected into a White New Zealand rabbit at 10 intradermal dorsal sites, as described by Sorscher *et al.* (38). Rabbits were boosted every three weeks. Following the second boost, fusion protein was dissolved in an equal amount of sterile water and incomplete Freund's adjuvant to enhance its intradermal retention. Antibody titer was determined 10 days after each boost using an enzyme-linked assay (ELISA) using the original fusion protein as an antigen, as described by Sorscher et al. [38]. Briefly, a 96-well microtiter plate was coated with fusion protein, excess protein washed away, and serum then added at the appropriate dilution. An alkaline phosphatase-conjugated secondary antibody was added after washout of the unbound primary antibody (serum). Alkaline phosphatase development was carried out in the usual manner at room temperature. The absorbance data reported were collected after a 30-min development period but were essentially identical to the values recorded after 10 min of development. The rabbit exhibited the highest antibody titer after the fifth boost. Blood was then collected from the anesthetized rabbit followed by euthanasia. Blood was allowed to clot overnight at 4°C, and then the serum removed. IgG class I antibody was purified from rabbit serum using Protein A beads (BioRad), and was aliquoted and stored at −20°C. All animal treatments complied with the UAB animal resources protocols.

The specificity of both rabbit polyclonal antibodies generated against purified renal amiloride-sensitive Na^+ channel (α-NaCh) and against α-bENaC (α-αbENaC) was routinely tested using five independent techniques: ELISA, immunoblot analysis, Western blot analysis (in addition to determining which subunit or subunits the particular batch of antibody can detect), immunoprecipitation, and immunocytochemical localization. As additional controls, Western blot analysis of these antibodies was performed against several commercially available proteins that potentially may have been copurified with our preparation. We found that our α-NaCh antibody did not recognize fibrinogen, fetuin, BSA, or protein kinase A inhibitor. We found that the antibodies specifically localize to the apical membrane of A6 epithelia, to the principal cells of the rat cortical collecting tubule, as well as to the apical membrane of toad urinary bladder [6, 23, 40]. We have never observed any staining to rat or bovine renal proximal tubule, epithelia rich in Na^+/H^+ exchanger. Also, we have not seen any specific α-NaCh antibody staining to CHO cells or lung macrophages, cells devoid of amiloride-sensitive Na^+ conductance pathways [36, 40]. To determine the antigenic purity of the isolated bovine Na^+ channel protein complex, we performed the following series of Western blot analysis: immunopurified bovine Na^+ channel protein complex was separated on SDS-PAGE, transferred to nitrocellulose, and blotted with polyclonal antibodies made against four different potentially contaminating proteins, namely, fetuin, fibrinogen, bovine serum albumin, and α-acid proteoglycan. These antibodies did not recognize any protein in the purified channel complex.

Western Blot Analysis

Partially purified Na^+ channel protein complex (i.e., that obtained after the ion exchange chromatography steps) was used for Western blot analysis. Sodium dodecylsufate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Biorad Miniprotean II slab gel apparatus using the discontinuous buffer system of Laemmli [24]. Procedures for SDS-PAGE and Western blotting were described in detail previously [4, 27]. Approximately 20 μg of polyclonal IgG antibody raised against either αbENaC, immunopurified bovine renal Na^+ channel complex, or nonimmune IgG was used for Western blot analysis of the kidney Na^+ channel protein complex.

Results

Effect of Dithiothreitol on Na^+ Channel

We have previously reported that when immunopurified renal Na^+ channel protein complex was incorporated into planar lipid bilayer membranes, amiloride-inhibited channels with very low P_o of 0.02–0.05 were observed [20, 21, 28]. These channels displayed a specific orientation in that the *trans*-facing portion was sensitive to amiloride, and the *cis*-facing surface was the side from which the channel could be activated by protein kinase A-mediated phosphorylation. The same situation was observed here (Fig. 1, top trace). These Na^+ channel complexes consist of at least six major polypeptide components held together by disulfide bonds, the functions of each of these polypeptides are not entirely known [3, 15]. We postulated that if the complex was subjected to disulfide bond reduction, a dissociation between the conduction properties of the channel and its regulatory pathways may occur. Therefore, we tested the effects of increasing concentrations of the reducing agent dithiothreitol (DTT) on immunopurified renal Na^+ channel function in bilayers. Figure 1 presents the results of a typical experiment. DTT, at concentrations below 25 μM, had little effect on single channel properties. From 25-100 μM DTT, the major 40 pS conductance state of the channel was abolished, and was replaced by one to three transition states of 12–13 pS in size (*see* associated all-points amplitude histograms). These lower conductance states were present in the non-DTT treated channel (top trace in Fig. 1, and Refs. 15, 20–22, 35). Single channel P_o increased from an average of 0.03 ± 0.01

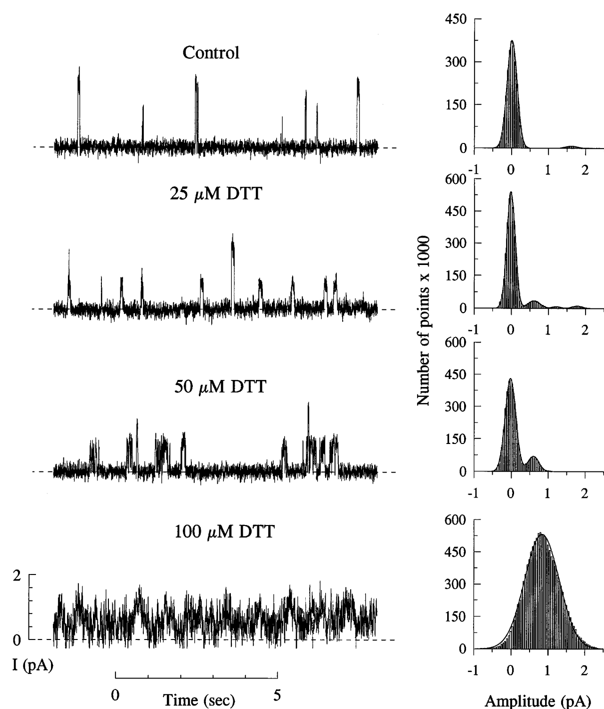

Fig. 1. Effect of dithiothreitol (DTT) on single-channel properties of immunopurified amiloride-sensitive Na^+ channels reconstituted into planar lipid bilayers. Holding potential + 40 mV. Traces shown are representative of 34 independent experiments performed following the same sequence of DTT addition to either compartment. Bathing solution in both *cis*- and *trans*-compartments contained 100 mM NaCl, 10 mM MOPS, pH 7.5. DTT was added to both compartments at the final concentrations indicated from a 25 mM stock aqueous solution. Records were filtered at 300 Hz using 8-pole Bessel filter prior to the acquisition and were sampled at 1000 Hz with Digidata 1200 interface. Plotted records were filtered at 100 Hz with the built-in filter of the pCLAMP software. Associated all points amplitude histograms were generated by pCLAMP software from records of 10-min length and were fitted with Gaussian functions.

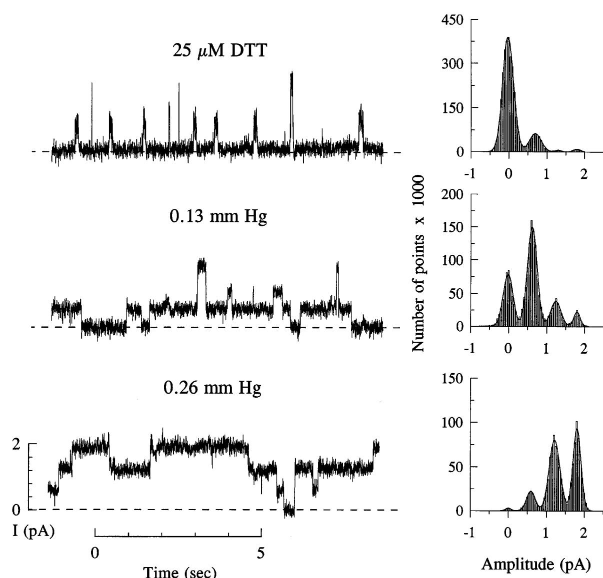

Fig. 2. Effect of hydrostatic pressure on renal amiloride-sensitive Na^+ channels in bilayers in the presence of 25 μM DTT. Holding potential + 40 mV. Traces shown are representative of 7 experiments. Bathing solution in both *cis*- and *trans*-compartments contained 100 mM NaCl, 10 mM MOPS, pH 7.5. Hydrostatic pressure upon the bilayer was established by raising the solution level in the *trans*-compartment. Records were filtered at 300 Hz using 8-pole Bessel filter prior to the acquisition and were sampled at 1000 Hz with Digidata 1200 interface. Plotted record was filtered at 100 Hz using the built-in filter of the pCLAMP software. Associated all points amplitude histograms were generated by pCLAMP software from a record of 10-min length and were fitted with Gaussian functions.

under control (i.e., zero DTT) conditions to 0.08 ± 0.02 following treatment with 25 μM or 50 μM DTT (N = 34). As these channels contain subconductive states of 12–13 pS magnitude (20), it appeared as if DTT promoted a shift into these lower conductance states. At DTT concentrations of 100 μM or above, channel activity became uncoordinated, with eventual loss of activity altogether (Fig. 1, bottom trace). To determine the sidedness of action of DTT, we performed a series of experiments in which 50 μM DTT was added only to the *cis* compartment (N = 10), only to the *trans* compartment (N = 9), or to both compartments (N = 7). Identical results as shown in Fig. 1 were obtained under all conditions. We conclude, therefore, that DTT is effective from either side of the bilayer.

Effect of Stretch on DTT-treated Na+ Channels

Because the apparent single channel conductance of these DTT-modified Na^+ channels was lowered to 12–13 pS and thus approached that of the cloned colonic ENaC channel [9, 18], we next examined whether any other biophysical or biochemical properties of the DTT-treated channels coincided with those of αbENaC [13]. One characteristic of αbENaC, incorporated into planar lipid bilayers, was that it displayed mechanosensitivity [2]. However, non-DTT-treated renal Na^+ channels in bilayers were not stretch-activated [2]. Figure 2 shows similar stretch-activated behavior of these DTT-modified Na^+ channels. Bilayer stretch was achieved by raising the fluid height in the *trans* compartment of the bilayer chamber by 0.5 or 1 ml, yielding a hydrostatic pressure differential (ΔP) of 0.13 and 0.26 mm Hg, respectively, given the geometric configuration of our bilayer system. As shown in Fig. 2, single channel P_o increased with increasing hydrostatic pressure, from a value of 0.08 ± 0.01 at ΔP = 0, to 0.24 ± 0.04 and 0.80 ± 0.05 at ΔP of 0.13 and 0.26 mm Hg, respectively (N = 7). Application of the pressure gradient in the opposite direction yielded variable changes in P_o (*see* Materials and Methods).

PKA Sensitivity of DTT-treated Na+ Channels

We next tested whether DTT-treated renal Na^+ channels could be activated by phosphorylation mediated by pro-

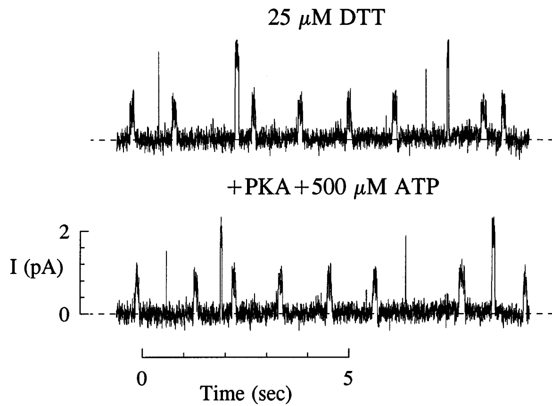

Fig. 3. Protein kinase A phosphorylation of a renal Na^+ channel reconstituted into planar lipid bilayers and treated with 25 μM DTT. Records shown are for + 40 mV holding potential and are representative of 5 separate experiments. Record was filtered at 300 Hz using 8-pole Bessel filter prior to the acquisition and was sampled at 1000 Hz with Digidata 1200 interface. Bilayers containing reconstituted channels were bathed with salt solutions as described in Fig. 1.

tein kinase A (PKA) plus ATP. The results of a typical experiment ($N = 5$) are shown in Fig. 3. There was no significant change in P_o following addition of the purified catalytic subunit of PKA plus saturating concentrations of ATP (0.08 ± 0.01 versus 0.09 ± 0.02). Thus, it is apparent that addition of PKA + ATP did not alter basal channel activity whatsoever, in contrast to the native immunopurified non-DTT-treated Na^+ channel complex reconstituted into planar bilayers [21].

Cation Permeability of DTT-treated Na^+ Channels

To assess whether DTT treatment affected the cation permeability properties of these renal channels, we performed bi-ionic reversal potential measurements. First, the cation vs. anion selectivity properties of these channels, determined from reversal potential measurements made when the bilayer was bathed with asymmetric NaCl solutions, remained unchanged with DTT treatment, i.e., $P_{Na^+}:P_{Cl^-}$ was greater than 10:1 (*data not shown*). Figure 4 summarizes the results of a total of 36 experiments in which the $P_{Li^+}:P_{Na^+}$, $P_{K^+}:P_{Na^+}$ and $P_{Ca^{2+}}:P_{Na^{2+}}$ were measured for channels in bilayers under the following conditions: nontreated, DTT-treated, and DTT-treated in the presence of a hydrostatic pressure gradient. In the absence of a hydrostatic pressure gradient, the relative cation permeability sequence of Li^+ (2.00 ± 0.08) > Na^+ (1.0) > K^+ (0.17 ± 0.05) > > Ca^{2+} (<0.001) was unaffected by DTT treatment. However, subsequent to the application of a 0.26 mm Hg hydrostatic pressure difference across a bilayer containing a DTT-modified Na^+channel, the channel's ability to discriminate among the above-tested cations was virtually

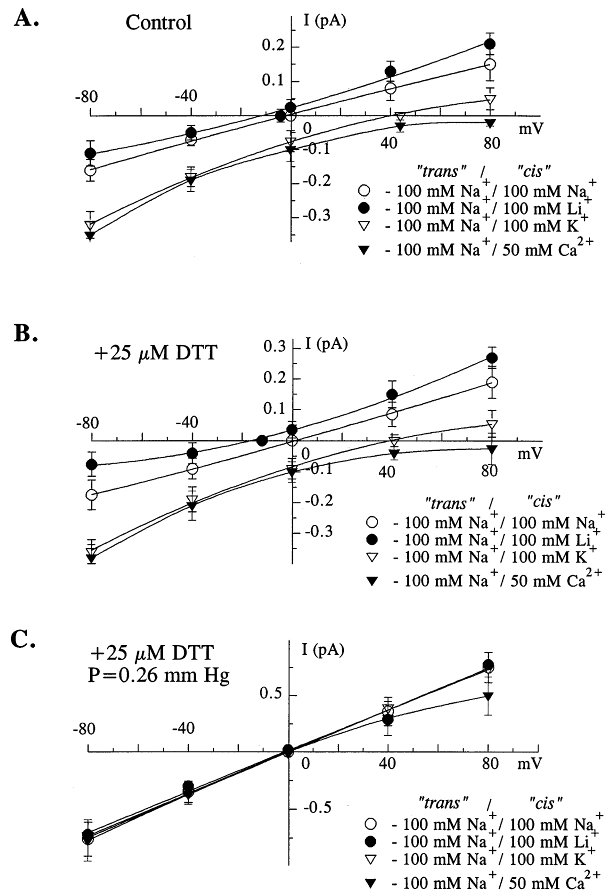

Fig. 4. Mean current-voltage curves of single renal Na^+ channel under symmetrical and bi-ionic conditions in the absence and presence of 25 μM DTT. Points in plots are Mean ± SD for $n = 4$. *Cis* and *trans* bathing solutions contained chloride salts as indicated in the figure. Additions of DTT were made to the *cis* compartment. Hydrostatic pressure upon the bilayer was applied by raising solution level in *trans* compartment. Plots were fitted to a third order regression equation of the type $y = \beta_o + \beta_1 X + \beta_2 X^2 + \beta_3 X^3$ with Sigmaplot 5.0 Scientific graphing software.

abolished (Fig. 4*C*). While the stretched channel remained cation selective, the previously impermeable Ca^{2+} could now be conducted through the channel under these stretched conditions.

Amiloride Sensitivity of DTT-treated Na^+ Channels

The effect of amiloride on single, DTT-treated renal Na^+ channels is shown in Fig. 5*A*. Amiloride produced a flickering-type block, with an apparent inhibitory equilibrium dissociation constant (K_i^{amil}) of 0.44 ± 0.12 μM ($N = 6$), a value indistinguishable from control (i.e., non-DTT-treated: $K_i^{amil} = 0.42 \pm 0.09$ μM, $N = 6$), or control + ΔP of 0.26 mm Hg ($K_i^{amil} = 0.37 \pm 0.13$ μM, $N = 6$) channels (*see* dose-response curves in Fig. 6). However, when a 0.26 mm Hg hydrostatic pressure difference was

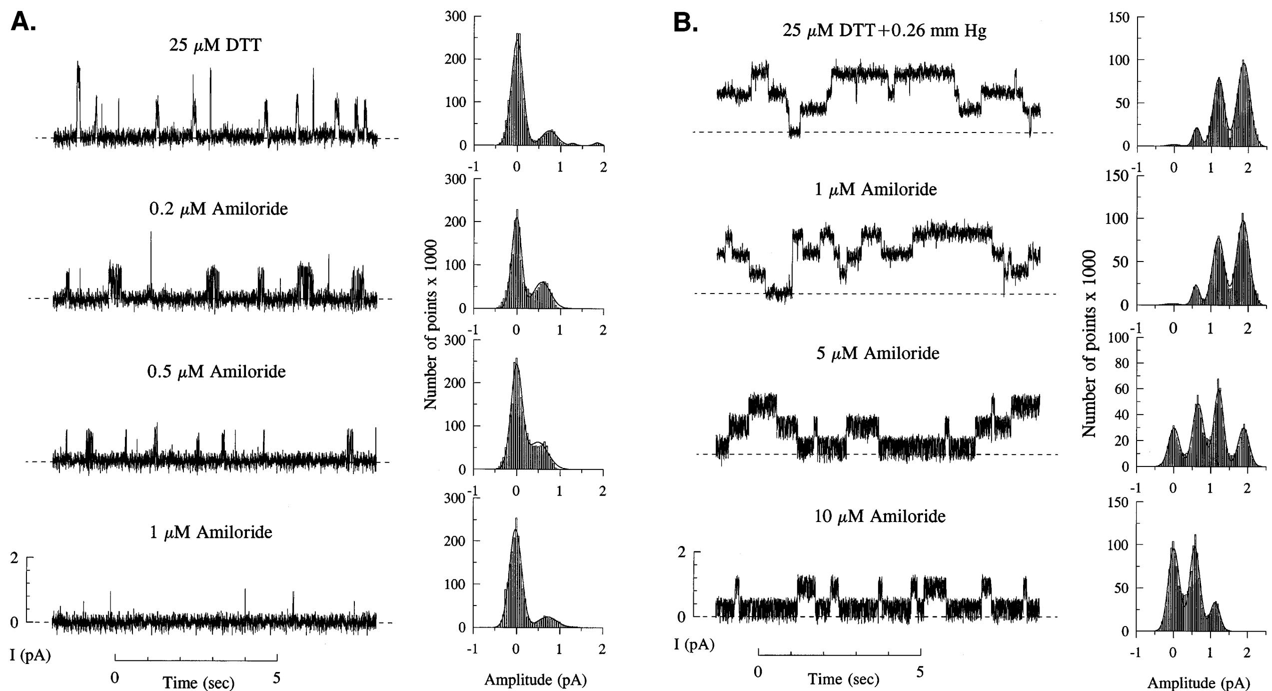

Fig. 5. (*A*) Effect of amiloride on a single DTT-treated renal Na^+ channel reconstituted into planar lipid bilayers. Holding potential + 40 mV. Traces shown are representative of 6 independent experiments. Bathing solution in both *cis-* and *trans-*compartments contained 100 mM NaCl, 10 mM MOPS, pH 7.5. Additions of amiloride were made to *trans* compartment to the final concentrations as indicated for each trace. Records were filtered at 300 Hz using 8-pole Bessel filter prior to the acquisition and were sampled at 1000 Hz using Digidata 1200 interface. Plotted record was filtered at 100 Hz with the built-in filter of the pCLAMP software. Associated all points amplitude histograms were generated by pCLAMP software from a record of 10-min length and were fitted with Gaussian functions. (*B*) Effect of amiloride on a single DTT-treated renal Na^+ channel reconstituted into planar lipid bilayers in the presence of a hydrostatic pressure gradient. Holding potential + 40 mV. Traces shown are representative of 6 independent experiments. Bathing solution in both *cis-* and *trans-*compartments contained 100 mM NaCl, 10 mM MOPS, pH 7.5. Hydrostatic pressure gradient across the bilayer was established by raising the solution level in *trans-*compartment. Additions of amiloride were made to *trans-*compartment to the final concentrations as indicated for each trace. Records were filtered at 300 Hz using 8-pole Bessel filter prior to the acquisition and were sampled at 1000 Hz with Digidata 1200 interface. Plotted record was filtered at 100 Hz with the built-in filter of the pCLAMP software. Associated all points amplitude histograms were generated by pCLAMP software from records of 10-min length and were fitted with Gaussian functions.

imposed across bilayers containing a DTT-modified channel, the ability of amiloride to inhibit was greatly diminished (Fig. 5*B*). The K_i^{amil} of amiloride under these conditions was shifted to the right, to a value of 6.9 ± 1.0 μM ($N = 6$; Fig. 6), very similar to the shift in amiloride's inhibitory capabilities that was observed for αbENaC after stretch [2].

Western Blot Analysis of Renal Na^+ Channels

To verify that an ENaC subunit was present in the purified renal Na^+ channel complex, we performed Western blot analyses of this channel complex using antibodies generated against a fusion protein of αbENaC. The main fusion protein product generated by *E. coli* expression of the full-length αbENaC cDNA was a 70 kDa protein. As seen in Fig. 7, when the fusion protein was subjected to SDS-PAGE and Western blotted with αbENaC antibodies, a 73 kDa protein was identified (*A*). The preim-

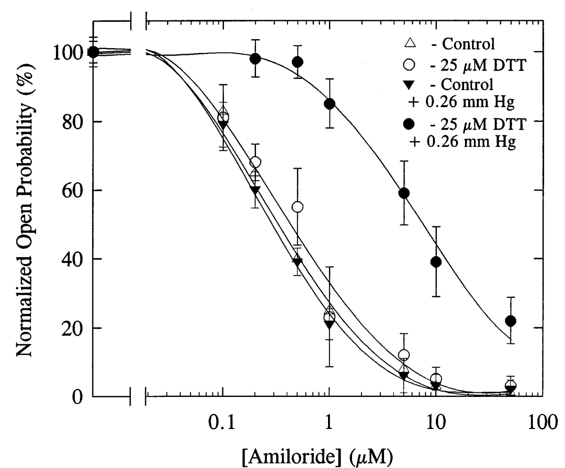

Fig. 6. Amiloride dose-response curves of a single renal Na^+ channel in absence and presence of 25 μM DTT and hydrostatic pressure gradient across the bilayer. Points in plots are Mean ± SD for $n = 6$ for experiments presented in Figs. 5 and 6.

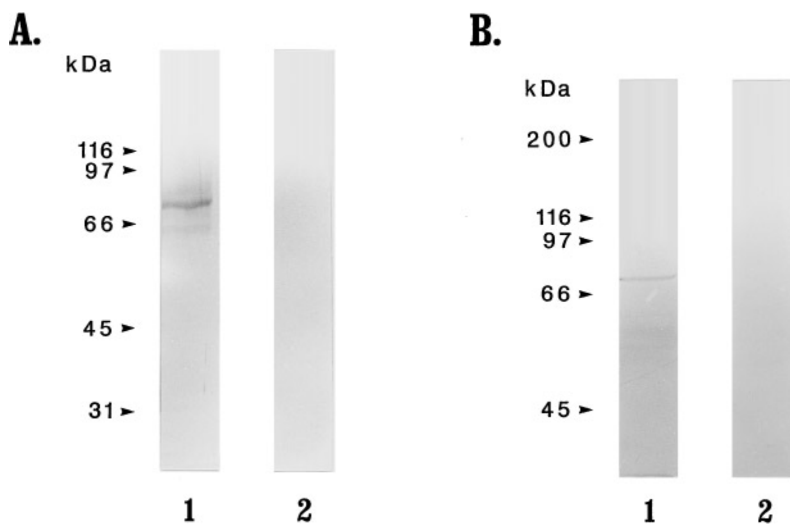

Fig. 7. Western blot analysis using polyclonal antibodies raised against a full-length fusion protein of αbENaC. The fusion protein was generated from the full-length bovine kidney clone of αENaC (2094 bp) encoding a protein with a molecular weight of ~80 kDa served as the antigen for raising a rabbit polyclonal antibody against this cloned Na^+ channel. In *A,* 10 μg/lane of the fusion protein were run on a 10% SDS polyacrylamide gel under reducing conditions (50 mM DTT). Western blots were performed as described in Materials and Methods. The primary antibodies used were 5 μg/ml of α-αbENaC IgG (lane 1) and 5 μg/ml of rabbit pre-immune IgG (lane 2). In *B,* αbENaC cDNA was in vitro transcribed and translated, and the resulting protein was separated by SDS-PAGE on an 8% gel with 50 mM DTT in the sample buffer. Western blots were performed as described. Lane 1 was blotted with αbENaC IgG (5 μg/ml) and lane 2 with preimmune IgG (5 μg/ml).

mune IgG did not react with the fusion protein. We also tested the antibody for reactivity with the product of an in vitro translation of αbENaC cRNA (*B*). The translated product has an apparent Mr of 80,000. The translation reaction mixture was separated by SDS-PAGE, transferred to PVDF-membranes, and blotted with the αbENaC antibodies. A prominent band running at ~80 kDa was identified.

We also performed a Western blot analysis of purified bovine renal Na^+ channel protein with antibodies generated against the immunopurified channel complex (α-Na^+ Channel), as well as against αbENaC. These results are shown in Fig. 8. As seen in the first lane, the αbENaC antibody recognized a single band at 70 kDa, indicating at least an immunological similarity between one component of the purified bovine renal Na^+ channel protein complex, and the bovine α-subunit isoform of ENaC. For comparison, lane 2 shows a Western blot of the same partially purified renal Na^+ channel material using a polyclonal antibody generated against highly purified renal Na^+ channel complexes [38]. The same 70 kDa band as well as a higher one at 150 kDa were recognized by these α-Na^+ Channel antibodies. The third lane demonstrates the lack of reactivity of nonimmune rabbit IgG with the renal Na^+ channel protein complex.

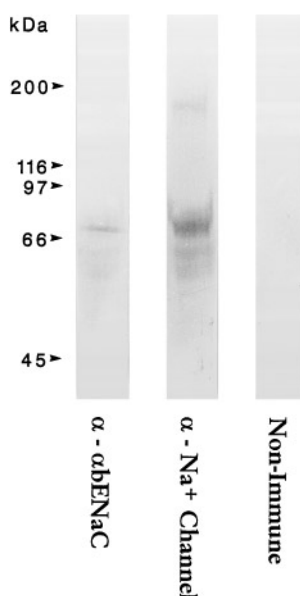

Fig. 8. Western blot analysis of partially purified bovine renal Na^+ channel protein complex. Ion-exchange purified bovine kidney protein was run under reducing conditions (50 mM DTT) on 8% SDS polyacrylamide gels. Protein was transferred to PVDF membranes, and blotted with specific antibodies generated against αbENaC or immunopurified bovine renal Na^+ channel protein complexes. The left lane shows that the antibody raised against a fusion protein of αbENaC recognizes a protein of 70 kDa. In the same material, the antibody raised against the large (700 kDa), highly purified bovine kidney protein complex identifies polypeptides of 55, 70, and 150 kDa (middle lane). The right lane shows that nonimmune rabbit IgG does not display any immunoreactivity with this channel protein preparation.

Discussion

Regulation of transepithelial Na^+ transport occurs at the level of the apically located Na^+ channel [8]. Most investigators have argued that regulation of the rate of Na^+ transport results from alterations in channel number rather than in single-channel properties [14, 15], although controversy on this point does exist. Moreover, different tissues display Na^+ channels of widely differing single-channel characteristics as well as differential hormonal regulation of Na^+ transport. With the initial cloning of an epithelial Na^+ channel from rat distal colon [8, 25], molecular studies of Na^+ channel function and com-

parisons with data obtained in native epithelia and channel protein reconstitution systems can now be performed. Na^+ channel complexes immunopurified from native bovine renal papillary collecting tubule epithelia have a molecular mass of 730 kDa and are composed of at least six nonidentical subunits of molecular masses 300, 150, 95, 70, 55, and 41 kDa [1, 4]. These channels, reconstituted into planar lipid bilayers display several unique characteristics: (i) a major conductance level of 40 pS in 100 mM NaCl and two 12–13 pS subconductive states [20]; (ii) are inhibited with high affinity by the diuretic amiloride [19, 28, 35]; (iii) can be activated by PKA-mediated phosphorylation [19, 21, 29]; and (iv) are not responsive to stretch [2]. The cloned ENaC channels reconstituted into planar lipid bilayers also have a major conductance level of 40 pS in 100 mM NaCl and two 12–13 pS subconductive states and are inhibited with high affinity by the diuretic amiloride [2, 18]; however, they cannot be activated by PKA-mediated phosphorylation; but do activate with stretch [2, 18]. Conductance values measured in bilayer experiments for both of these channels are different from patch-clamp measurements [9, 12, 16, 17, 18, 32] and from the prototypical type I epithelial Na^+ channel [31]. The reasons underlying these differences are not apparent, but may relate to the microenvironment of the channel complex. Because a variety of epithelial Na^+ channel protein complexes have been isolated [4, 5, 7, 8, 36], the key issue is to determine what the relationships are among the different polypeptide components and the cloned ENaC subunits, and how these polypeptides relate to the amiloride-sensitive channels characterized in native epithelia by patch clamp. One possibility is that different amiloride-sensitive channels exist. The channels would thus be encoded by entirely different genes and possess unique protein components. A second, more parsimonious hypothesis, is that the α, β, and γ ENaC subunits are part of the native channel protein complexes isolated, and that the functional diversity in single channel characteristics result from an association with different regulatory proteins.

The immunopurified 730 kDa renal protein complex forms an amiloride-sensitive Na^+ channel in planar lipid bilayers with appropriate responses to physiologically relevant regulatory inputs such as regulation by Na^+ and Ca^{2+} [20, 30], PKA-mediated phosphorylation [19, 20, 29], PKC-mediated phosphorylation [21, 29], activation/inactivation of associated G-protein [1, 19, 21], and carboxyl methylation [22, 34]. Because the immunopurified renal Na^+ channel protein complex consists of multiple components held together by disulfide bonds, we hypothesized that many of the aforementioned regulatory characteristics of the channel would be altered by disulfide bond reduction. Indeed, results reported in this paper show that subsequent to reduction with low concentrations of DTT, this purified renal Na^+ channel acquired a biophysical signature very comparable to the ENaC channel, namely, relatively low single channel conductance, loss of PKA sensitivity, stretch-activation, and decrease in amiloride sensitivity and loss of cation discrimination upon stretch.

Another important parallel between DTT-reduced immunopurified renal Na^+ channels and ENaCs is a shift of immunopurified renal Na^+ channels from concerted gating into a state in which the lower conductance states are preferred. The subconductive behavior of purified amiloride-sensitive Na^+ channels in planar lipid bilayers has been previously reported [15, 35]. Recent studies have shown that these conductance levels do not result from a manifestation of different biochemical entities, but are in fact subconductive states of the same channel [19, 20]. One alternative explanation of multiple conductance levels is the possibility that other unique channels may have been simultaneously incorporated into the bilayer. We do not think this possibility is tenable because of the following reasons: (i) based on radioligand binding studies, the purity of the immunopurified material used in these experiments was greater than 95%, assuming that one amiloride molecule binds per channel [4]; (ii) if each of the individual conductance levels represented separate channels, it would be expected that, at least occasionally, bilayer membranes only containing one of these putative channels should be seen independent of the others which was not the case [19, 21, 22]; (iii) event-amplitude histograms constructed from control recordings cannot be fit by binominal theory as predicted for independent channels [12; *cf.* Fig. 1, control trace, and refs. 19, 20]; (iv) all of the conductance states were amiloride-sensitive and displayed identical cation selectivities under a specified set of experimental conditions. Thus while we cannot totally exclude the possibility that our proteoliposomes contained other contaminating Na^+ channels, it appears unlikely that this is the case.

At least one or two associated proteins of the purified channel complex are recognized by specific antibodies generated against the αENaC subunit (Fig. 8 and refs. 2 and 13). Moreover, these immunoreactive polypeptides migrate on SDS-PAGE at apparent molecular masses identical to the unglycosylated and glycosylated forms of αbENaC [13]. Thus, our results are consistent with the hypothesis that the core conduction element of at least one purified type of epithelial Na^+ channel is ENaC, and that second messenger regulatory coupling occurs through association with specific polypeptide components. It is likely that these polypeptide components may be tissue specific. For example, it is a 300 kDa associated protein that is specifically phosphorylated by protein kinase A in the bovine papillary epithelial and A6 cells [29]. However, there is no 300 kDa protein that has been purified from rabbit alveolar type II cells, in spite of the fact that this protein complex can also be phosphorylated by protein kinase A + ATP [36].

In summary, we show that disulfide bond reduction of immunopurified bovine renal Na^+ channel protein complexes with DTT in planar lipid bilayers promotes a permanent transition of the channel into a lower conductance state. The cation permeability sequence or the amiloride-sensitivity of this DTT-modified channel is not significantly different from that of the untreated channel. However, DTT treatment results in a loss of PKA + ATP sensitivity, and the acquisition of mechanosensitivity. There is also a significant decrease in the ability of amiloride to inhibit the channel following stretch activation. All of these properties are comparable to those displayed by ENaC. Therefore, we suggest that this renal Na^+ channel has as its main conduction component ENaC, and that post-translational modifications such as PKA sensitivity are provided by association of this core ENaC conduction element with other cellular proteins.

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References

1. Ausiello, D.A., Stow, J.L., Cantiello, H.F., de Almeida, J.B., Benos, D.J. 1992. Purified epithelial Na^+ channel complex contains the pertussis toxin-sensitive $G_{i\text{-}3}$ protein. *J. Biol. Chem.* **267:**4759–4765
2. Awayda, M.S., Ismailov, I.I., Berdiev, B.K., Benos, D.J. 1995. A cloned renal epithelial Na^+ channel displays stretch activation in planar lipid bilayers. *Am. J. Physiol.* **268:**C1450–C1459
3. Benos, D.J., Awayda, M.S., Ismailov, I.I., Johnson, J.P. 1995. Structure and function of amiloride-sensitive Na^+ channels. *J. Membrane Biol.* **143:**1–18
4. Benos, D.J., Saccomani, G., Sariban-Sohraby, S. 1987. The epithelial sodium channel: subunit number and location of amiloride binding site. *J. Biol. Chem.* **262:**10613–10618
5. Blazer-Yost, B., Recio-Pinto, E. 1994. Structural and functional analysis of immunopurified toad urinary bladder Na^+ channels. *FASEB J.* **8:**A292 (*Abstr*)
6. Bradford, A.L., Ismailov, I.I., Achard, J.-M., Bubien, J.K., Benos, D.J. 1995. Immunopurification and functional reconstitution of an amiloride-sensitive sodium channel protein from rat lymphocytes. *Am. J. Physiol.* **269:**C601-C611
7. Brown, D., Sorscher, E.J., Ausiello, D.A., Benos, D.J. 1989. Immunocytochemical localization of sodium channels in rat kidney medulla. *Am. J. Physiol.* **256:**F366–F369
8. Canessa, C.M., Horisberger, J.-D., Rossier, B.C. 1993. Epithelial sodium channel related to proteins involved in neurodegeneration. *Nature* **361:**467–470
9. Canessa, C.M., Schild, L., Buell, G., Thoreus, B., Gatschl, I., Horiberger, J.-D., Rossier, B.C. 1994. Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. *Nature* **367:**463–467
10. Dagenais, A., Kothary, R., Berthiaume, Y. 1994. Ontogenesis and expression of an α-MENAC, an amiloride-sensitive sodium channel cloned from mouse lungs. Symposium on epithelial sodium channels and mechanosensitive channels: a new gene superfamily. Villars-sur-Ollon, Switzerland, (*Abstr.*)
11. Eaton, D.C., Hamilton, K.L., 1988. The amiloride-blockable sodium channel of epithelial tissue. *In*: Ion Channels (T. Narahashi, editor), Vol. 1, pp. 251–282, New York—London, Plenum Press
12. Fox, J.A. 1987. Ion channel subconductance states. *J. Membrane Biol.* **97:**1–8
13. Fuller, C.M., Awayda, M.S., Arrate, P., Bradford, A., Morris, R.G., Canessa, C.M., Rossier, B.C., and Benos, D.J. 1995. Cloning of a bovine epithelial Na^+ channel subunit. *Am. J. Physiol.* **269:**C641–C654
14. Garty, H. 1994. Molecular properties of epithelial amiloride-blockable Na^+ channels *FASEB J.* **8:**522–528
15. Garty, H., Benos, D.J. 1988. Characteristics and regulatory mechanisms of the amiloride-blockable Na^+ channel. *Physiol. Rev.* **65:**309–373
16. Hamilton, K.L., Eaton, D.C. 1985. Single channel recordings from amiloride sensitive sodium channel. *Am. J. Physiol.* **249:**C200–207
17. Hamilton, K.L., Eaton, D.C. 1985. Single channel recordings from two types of amiloride sensitive sodium channel. *Membr. Biochem.* **6:**149–171
18. Ismailov I.I., Awayda, M.S., Berdiev, B.K., Bubien, J.K., Lucas, J.E., Fuller, C.M., Benos, D.J. 1996. Triple-barrel organization of ENaC, a cloned epithelial Na^+ channel. *J. Biol. Chem.* (*in press*)
19. Ismailov, I.I., Berdiev, B.K., Benos, D.J. 1995. Biochemical status of renal epithelial Na^+ channels determines apparent channel conductance, ion selectivity, and amiloride sensitivity. *Biophys. J.,* **69:**1789–1800
20. Ismailov, I.I., Berdiev, B.K., Benos, D.J. 1995. Regulation by Na^+ and Ca^{2+} of renal Na^+ channels reconstituted into planar lipid bilayers. *J. Gen. Physiol.* **106:**445–466
21. Ismailov, I.I., McDuffie, J.H., Benos, D.J. 1994. Protein kinase A phosphorylation and G protein regulation of purified renal Na^+ channels in planar lipid bilayer membranes. *J. Biol. Chem.* **269:**10235–10241
22. Ismailov, I.I., McDuffie, J.H., Sariban-Sohraby, S., Johnson, J.P., Benos, D.J. 1994. Carboxyl methylation of purified renal amiloride-sensitive Na^+ channels in planar lipid bilayers. *J. Biol. Chem.* **269:**22193–22197
23. Kleyman, T.R., Smith, P.R., Benos, D.J. 1994. Characterization and localization of epithelial Na^+ channels in toad urinary bladder. *Am. J. Physiol.* **266:**C1105–C1111
24. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680–5
25. Lingueglia, E., Voilley, N., Waldemann, R, Lazdunski, M., Barbry, P. 1993. Expression cloning of an epithelial amiloride-sensitive Na^+ channel. *FEBS Lett.* **318:**95–99
26. McDonald, F.J., Snyder, P.M., McCray, P.B., Welsh, M.J. 1994. Cloning expression, and tissue distribution of a human amiloride-sensitive Na^+ channel. *Am. J. Physiol.* **266:**L728–L734
27. Oh, Y., Benos, D.J. 1993*a*. Rapid purification of an amiloride-sensitive Na^+ channel from bovine kidney and its functional reconstitution. *Protein Exp. Purif.* **4:**312–319
28. Oh, Y., Benos, D.J. 1993*b*. Single-channel characteristics of a purified bovine renal amiloride-sensitive Na^+ channel in planar lipid bilayers. *Am. J. Physiol.* **264:**C1489–C1499
29. Oh, Y., Smith, P.R., Bradford, A.L., Keeton, D., Benos, D.J. 1993. Regulation by phosphorylation of purified epithelial Na^+ channels in planar lipid bilayers. *Am. J. Physiol.* **265:**C85–C91
30. Olans, L., Sariban-Sohraby, S., Benos, D. 1984. Saturation behavior of single, amiloride-sensitive Na^+ channels in planar lipid bilayers. *Biophys. J.* **46:**831–835
31. Palmer, L.G. 1992. Epithelial Na^+ channels: function and diversity. *Annu. Rev. Physiol.* **54:**51–66
32. Palmer, L.G., Frindt, G. 1986. Amiloride sensitive channels from

the apical membrane of the rat cortical collecting tubule. *Proc. Natl. Acad. Sci. USA* **83:**2767–2770

33. Puoti, A., May, A., Canessa, C.M., Horisberger, J.D., Schild, L., Rossier, B.C. 1994. Cloning and functional analysis of the amphibian renal epithelial sodium channel. Symposium on epithelial sodium channels and mechanosensitive channels: a new gene superfamily. Villars-sur-Ollon, Switzerland (*Abstr.*)
34. Sariban-Sohraby, S., Fisher, R.S., Abramov, M. 1993. Aldosterone induced and GTP-stimulated methylation of a 90-kDa polypeptide in the apical membrane of A6 epithelia. *J. Biol. Chem.* **268:**26613–26617
35. 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
36. Senyk, O., Ismailov, I.I., Bradford, A.L., Baker, R.R., Matalon, S., Benos, D.J. 1995. Reconstitution of immunopurified alveolar type II cell Na^+ channel protein into planar lipid bilayers. *Am. J. Physiol.* **268:**C1148–1156
37. Shimkets, R.A., Warnock, D.G., Bositis, C.M., Nelson-Williams, C., Hansson, J.H., Schambelan, M., Gill, J.R., Ulick, S., Milora, R.V., Findling, J.W., Canessa, C.M., Rossier, B.C., Lifton, R.P. 1994. Liddle's syndrome: heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. *Cell* **79:**407–414
38. Sorscher, E.J., Accavitti, M.A., Keeton, D., Steadman, E., Frizzell, R.A., Benos, D.J. 1988. Antibodies against purified epithelial sodium channel protein from bovine renal papilla. *Am. J. Physiol.* **255:**C834–43
39. Tien, H.T., Carbone, S., Dawidowicz, E.A. 1966. Formation of ''black'' lipid membranes by oxidation product of cholesterol. *Nature* **215:**5106:1199–1200
40. Tousson, A., Alley, C., Sorscher, E.J., Brinkley, B., Benos, D.J. 1989. Immunocytochemical localization of amiloride-sensitive sodium channels in sodium-transporting epithelia. *J. Cell Sci.* **93:**349–362