Biochemical Status of Renal Epithelial Na^+ Channels Determines Apparent Channel Conductance, Ion Selectivity, and Amiloride Sensitivity

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ABSTRACT Purified bovine renal papillary Na^+ channels, when reconstituted into planar lipid bilayers, reside in three conductance states: a 40-pS main state, and two subconductive states (12–13 pS and 24–26 pS). The activity of these channels is regulated by phosphorylation and by G-proteins. Protein kinase A (PKA)-induced phosphorylation increased channel activity by increasing the open state time constants from 160 ± 30 (main conductance), and 15 ± 5 ms (both lower conductances), respectively, to 365 ± 30 ms for all of them. PKA phosphorylation also altered the closed time of the channel from 250 ± 30 ms to 200 ± 35 ms, thus shifting the channel into a lower-conductance, long open time mode. PKA phosphorylation increased the P_{Na}:P_K of the channel from 7:1 to 20:1, and shifted the amiloride inhibition curve to the right (apparent K_i^{amil} from 0.7 to 20 μM). Pertussis toxin-induced ADP-ribosylation of either phosphorylated or nonphosphorylated channels decreased the P_{Na}:P_K to 2:1 and 4:1, respectively, and altered K_i^{amil} to 8 and 2 μM for phosphorylated and nonphosphorylated channels, respectively. GTP-γ-S treatment of either phosphorylated or nonphosphorylated channels resulted in an increase of P_{Na}:P_K to 30:1 and 10:1, respectively, and produced a leftward shift in the amiloride dose-response curve, altering K_i^{amil} to 0.5 and 0.1 μM, respectively. These results suggest that amiloride-sensitive renal Na^+ channel biophysical characteristics are not static, but depend upon the biochemical state of the channel protein and/or its associated G-protein.

INTRODUCTION

In the kidney, apically located amiloride-sensitive cation channels act as highly regulated effectors of whole-body Na^+ balance (Smith and Benos, 1991; Benos et al., 1992, 1995; Garty, 1994). The rate of Na^+ entry into the cellular compartment through these ion channels is rate-limiting for net transepithelial Na^+ transport. The activity of these channels can be modulated by agents such as mineralocorticoids and antidiuretic hormones, G-proteins, and by Na^+, Ca^{2+}, and protons themselves (Garty and Benos, 1988; Ismailov et al., 1995; Benos et al., 1995). We recently reported that protein kinase A (PKA) plus ATP activated renal Na^+ channels after reconstitution into planar lipid bilayers, and that pertussis toxin-induced ADP-ribosylation either activated or inactivated these channels, depending on their previous phosphorylation state (Oh et al., 1993; Ismailov et al., 1994a). We found that these biochemical modifications primarily affected channel open probability (P_o). However, during the course of these experiments, we noticed the frequent appearance of long-lived, lower-conductance states after phosphorylation of the channel. This phenomenon was not explored in those earlier studies because of the preponderance of multiple-channel incorporations that made definitive kinetic analysis of this behavior unfeasible. Thus, in addition to the more typical alterations in single-channel P_o and/or number of active channels that underlie the regulation of most ion channels (Andersen and Koeppe, 1992; Levitan, 1994), physiological regulation of amiloride-sensitive Na^+ channel function can occur at yet another, more complex, level.

In this paper we report the results of single-channel experiments that explore the biochemical mechanisms of regulation of renal amiloride-sensitive Na^+ channels by protein kinase A and by the channel-associated $G_{\alpha i3}$ protein. We hypothesized that biophysical characteristics, i.e., P_o, open and closed times, cation selectivity, apparent single channel conductance, and amiloride sensitivity of this purified renal Na^+ channel could be changed by PKA-mediated phosphorylation and by the state of the channel's associated G-protein. We found that protein kinase A-induced phosphorylation of immunopurified Na^+ channels reconstituted into planar lipid bilayers by increased P_o and shifted the channels into long-lived lower-conductance states (12–13 and 24–26 pS). PKA phosphorylation and modulation of the channel-associated $G_{\alpha i3}$ protein by pertussis toxin (PTX) or GTP altered the channel's cation selectivity and inhibition by amiloride. These findings demonstrate that the biophysical characteristics of renal amiloride-sensitive Na^+ channels are not fixed but can be modulated by various biochemical reactions.

MATERIALS AND METHODS

Materials

QUAT (quaternary amine) and SP (sulfopropyl) ion-exchange cartridges were purchased from FMC BioProducts (Rockland, ME). Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Sigma (St. Louis, MO) and was oxidized by bubbling oxygen through a 4% cholesterol solution in octane for 8 h at 126°C, the

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octane boiling point (Tien et al., 1966). Pertussis toxin was obtained from RBI (Natick, MA). All other chemicals were reagent grade and were purchased from Fisher (Norcross, GA) or Sigma. All solutions were made with Milli-Q water and filter sterilized before use (Sterivex-GS, 0.22 μm filter, Millipore Corp., Bedford, MA).

Methods

Immunopurification of epithelial Na^+ channel protein from bovine kidney papillary collecting tubules was done as described previously (Oh and Benos, 1993a). Reconstitution of channel protein into proteoliposomes was made by passing detergent-solubilized, purified channel proteins through an Extracti-Gel D column (Pierce) as described previously (Oh and Benos, 1993a). Proteoliposomes were diluted with buffer and sonicated at 4°C for 30 s to yield a preparation producing primarily only single-channel incorporations.

Planar lipid bilayers

Bilayer membranes were constructed from a mixture of diphytanoyl-phosphatidylethanolamine, diphytanoyl-phosphatidylserine, and oxidized cholesterol in *n*-octane (2:1:2, w/w/w; final lipid concentration, 25 mg/ml). The lipid solution was spread over a 200-μm aperture drilled in a polystyrene cup with a fire-polished glass capillary. Bilayer formation was indicated by the subsequent increase in membrane capacitance to its final value of 300–400 pF. Membranes were formed in salt solutions of varying composition, according to the specific experimental design (see figure legends). In all cases, however, bathing solutions were buffered to pH 7.4 with 10 mM MOPS. In experiments in which the Na^+ concentration was varied, NaCl was replaced with equimolar *N*-methyl-D-glucamine chloride (NMDG-Cl), thus keeping identical the osmolarity in the *cis* and *trans* chambers. All experiments were performed in the presence of a minimum 10 μM Ca^{2+} in both solutions. Reconstituted proteoliposomes, appropriately diluted to produce single-channel incorporations, were spread over a preformed bilayer with a fire-polished glass capillary from the *trans* side, and the membrane was voltage-clamped to −40 mV. This incorporation procedure ensured that the channel was aligned with its cytoplasmic surface facing the *cis* bathing solution. Current was measured with a high-gain amplifier circuit (Alvarez et al., 1985), with electrical contact with the aqueous bathing solutions made by Ag-AgCl electrodes through 3% agar bridges made up with 3 M NaCl. The *trans* chamber was connected to the input of the current-to-voltage converter, and thus this chamber served as a virtual ground. Current digitization and storage were done as previously described (Ismailov et al., 1994a).

Phosphorylation and ADP-ribosylation experiments

The catalytic subunit of cAMP-dependent PKA (final concentration, 1.85 ng/ml), purified by Dr. Gail Johnson (University of Alabama at Birmingham), and ATP at different concentrations were added to the *cis* side of a channel-containing bilayer. Two microliters of activated pertussis toxin (final concentration, 100 ng/ml) containing 10 mM dithiothreitol, 1 mM ATP, and 1 mM NAD was added to one side of a channel-containing bilayer (chamber volume, 4 ml) to a final concentration of 100 ng/ml (Ausiello et al., 1992). Control experiments revealed that neither dithiothreitol, nor ATP, nor NAD alone used in the above final concentrations had any significant effect on single Na^+ channel activity. GTP-γ-S was added to a final concentration of 100 μM.

Data analysis and presentation

Only single-channel records were chosen for analysis. We have previously reported that PKA-induced phosphorylation and/or PTX-induced ribosylation could reveal the presence of "silent" channels in bilayers (Ismailov et al., 1994a). In these earlier studies PKA-mediated phosphorylation with maximal concentrations of ATP (i.e., 200 μM) resulted in quantal conductance transitions in increments of 40 pS. We interpreted this observation as an indication of multiple channels in the membrane. Therefore at the end of each experiment PKA plus 200 μM ATP was added to the *cis* (or cytoplasmic) bathing surface to determine the total number of Na^+ channels present. Bilayer membranes containing more than one Na^+ channel were observed in less than 2% of the total number of successful incorporations reported herein (7 of 354). These membranes were not used for analysis. Single-channel analysis was performed on records 3–15 min in length. The mean current ($\bar{I}$) over the period of observation was calculated using the events list generated by pCLAMP software and the equation

$$\bar{I} = \frac{\sum_{m=0}^{M} i_m \cdot t_m}{\sum t_m} \quad (1)$$

where i_m is the event current (all levels, including zero); t_m is an event dwell time, and M is the total number of events.

Single-channel open probability was calculated as follows:

$$P_o = \bar{I}/(N \cdot i) \quad (2)$$

where N is the total number of channels (always equal to 1 in these experiments), and i is the unitary current determined from all points current amplitude histograms produced by pCLAMP. The dashed line in each figure indicates the zero current level. Each experimental condition was repeated a minimum of three times.

RESULTS

Protein kinase A activates an epithelial sodium channel by converting its subconductive states into a long-lived condition

Typically, immunopurified renal Na^+ channels reconstituted into planar lipid bilayers displayed a very low open probability (~0.02 ± 0.01) (Oh and Benos, 1993b; Ismailov et al., 1994a,b). Under these circumstances it was difficult to investigate in detail the kinetics of this mostly closed channel. Our recent studies of the ionic regulation of renal Na^+ channels revealed two important observations that are germane to the present study (Ismailov et al., 1995). First, in most incorporations (~90%), the channels displayed a specific orientation upon reconstitution in the bilayer. The *cis* side was sensitive to protein kinase A, alkaline phosphatase, carboxymethyltransferase, and pertussis toxin, but not to amiloride (Ismailov et al., 1994a,b). The *trans* side, in contrast, was amiloride-sensitive, but insensitive to the other agents that are known to act from the cell interior. This orientation undoubtedly resulted from the electric field imposed during incorporation because if incorporation was done at 0 mV, the channels would orient randomly. Second, reduction of $[Na^+]_{cis}$ to 30 mM increased single-channel P_o by a factor of 5 (to $P_o = 0.11 \pm 0.02$) (Ismailov et al., 1995). The portion of time spent by a channel in one of the lower conductance states, however, remained unchanged under these conditions. Thus, both of these observations allowed us 1) to know a priori that this amiloride-sensitive sodium channel exposes its cytoplasmic surface to the *cis* compartment and its extracellular surface to the *trans* compartment; and 2) to resolve the kinetics of

the channel because of an increase in its activity due to a reduction of $[Na^+]_{cis}$.

Fig. 1 *A* depicts representative current traces at ±80 mV before (0 μM ATP) and subsequent PKA-mediated phosphorylation of the channel at increasing concentrations of ATP. As shown previously, PKA mediated increases in channel P_o saturated with increasing Mg-ATP concentration (Ismailov et al., 1994a). Control traces (on the top) displayed discrete channel openings with a P_o of 0.11 ± 0.02 at both ±80 mV when the membrane was bathed with a $[Na^+]_{trans}$ of 100 mM and a $[Na^+]_{cis}$ of 10 mM (n = 13). Both mean and unitary current-voltage (*I/V*) relationships of the channel were linear and intersected the voltage axis at +53 mV (data not shown). This observation indicated that the channel had a cation-to-anion permeability ratio of 9:1. The most frequently observed open-state unitary conductance of the channel was 40 pS; however, less frequent single-channel openings of ~⅓ and ~⅓ of this main conductance state were also consistently detected. Moreover, as noted earlier (Ismailov et al., 1994a), PKA-mediated phosphorylation converted the channel from one whose gating was independent of applied potential into one displaying voltage-dependent gating, i.e., channel P_o was significantly greater at negative applied potentials.

Phosphorylation by PKA resulted in the channel spending more time in its lower conductance states, even though the main 40 pS conductance state that was present before phosphorylation remained. The mean open time of the 40 pS conductance state also increased at 200 μM ATP (Fig. 1 *A*, bottom traces). Channel P_o was maximal at 200 μM ATP (see Fig. 2 *A*). All points and event amplitude histograms (Fig. 1 *B*) were binomially distributed after phosphorylation with 50 and 100 μM ATP, suggesting independent behavior of each of the channel conductance states. Event dwell time histograms were fitted to single exponentials with a time constant of ~210 ms for the closed state, and 335–405 ms for all of the three conductive states at 100 μM ATP (Fig. 1 *C*, Table 1). At 200 μM ATP, the mean open times for the 12–13, 24–26, and 40 conductive states were 350, 840, and 860 ms, respectively. These characteristics were very dif-

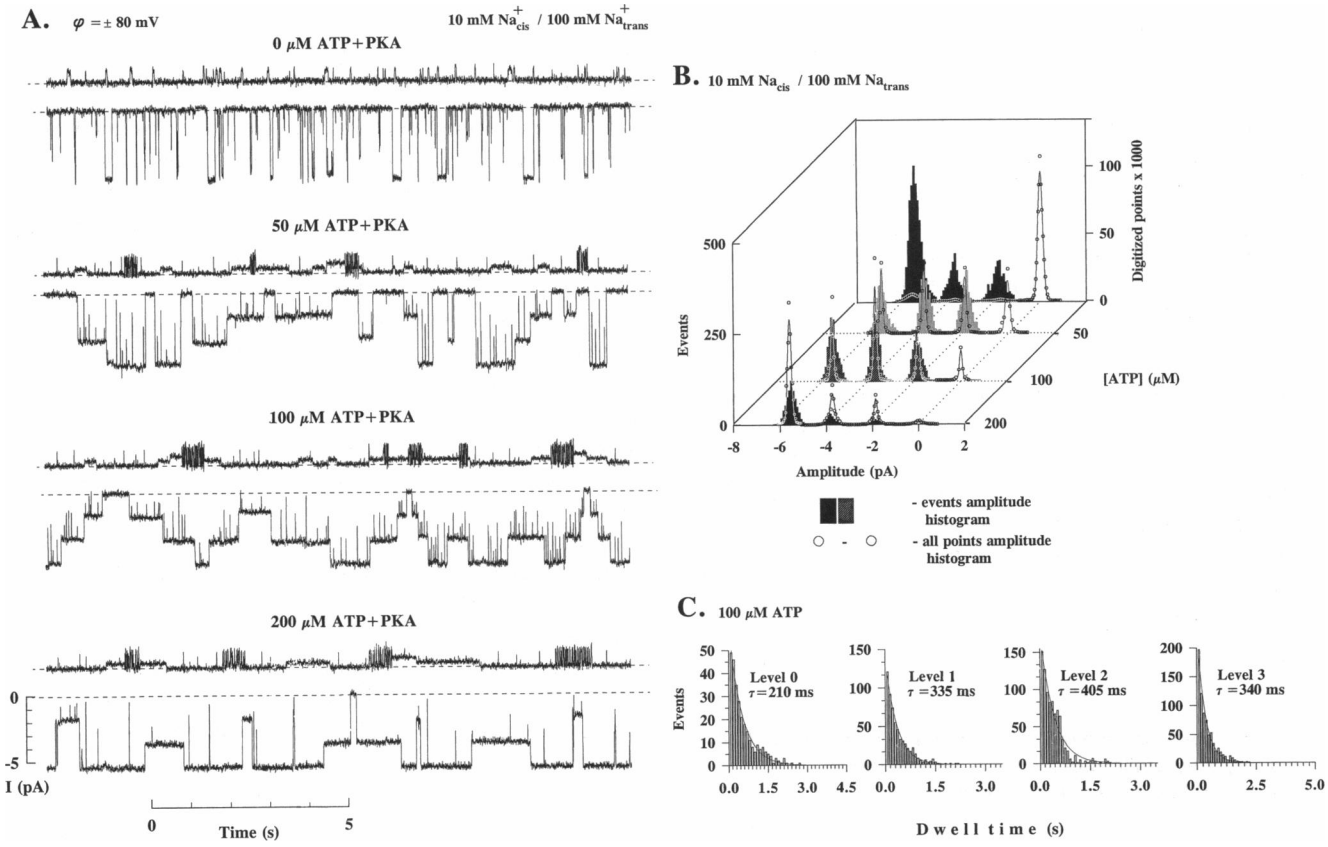

FIGURE 1 (*A*) Protein kinase A induced phosphorylation of immunopurified amiloride sensitive sodium channel reconstituted into planar lipid bilayer. Holding potential ±80 mV. Traces shown are representative of 13 independent experiments performed following the same sequence of ATP addition. Bathing solution in the *trans* compartment contained 100 mM NaCl, 50 mM NMDG-Cl, 10 mM MOPS, pH 7.5; the *cis* compartment contained 10 mM NaCl, 140 mM NMDG-Cl, 10 mM MOPS, ATP as indicated for each trace, 10 mM MOPS, pH 7.5. Records were filtered at 300 Hz using 8-pole Bessel filter before the acquisition and were sampled at 1000 Hz using Digidata 1200 interface. Plotted record was filtered at 100 Hz using the built-in filter of the pCLAMP software. (*B*) All points and events amplitude histogram of a single channel current recording. Events list and all points amplitude histogram were generated by pCLAMP software from a record of 15 min recorded as described for (*A*). Event detection thresholds were 50% of amplitude and 5 ms for every event duration. Numbers next to graphs correspond to current levels as indicated in (*A*). Histograms were fitted with Gaussian functions. (*C*) Single-channel events dwell time histograms. Values were calculated from single exponential fits.

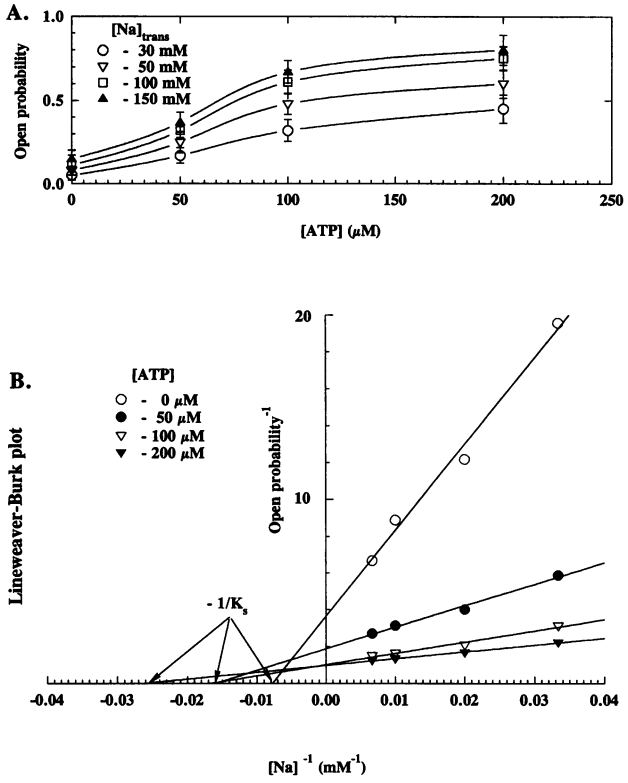

FIGURE 2 ATP dose-response curves of renal amiloride-sensitive Na^+ in the presence of protein kinase A. Bilayers containing reconstituted channel were bathed with asymmetrical salt solutions (see symbols on the plot), and increasing concentrations of Mg-ATP were added to the *cis* bathing compartment. PKA (1.85 ng/ml) was added to the *cis* compartment before ATP addition (*A*). (*B*) Lineweaver-Burk double reciprocal plot of data presented in (*A*). Points in the plots are mean ± SD for 3–7 experiments performed at each $[Na^+]$.

ferent from those observed under nonphosphorylated conditions (Table 1). All points and event amplitude histograms of nonphosphorylated channels and channels phosphorylated in the presence of 200 μM ATP were not binomially distributed, suggesting that two lower conductance levels represented subconductive channel states (Fox, 1987). Event dwell time histograms for nonphosphorylated channels (not shown) displayed a single exponential decay with a time constant of 250 ± 35 ms for the closed state, and single exponential decays with time constants of 135 ± 18 ms and ~9 ± 5 ms for the main open and both subconductive states, respectively. Neither PKA or ATP alone, added to either the *cis* or *trans* compartment or to both together, altered channel kinetics, suggesting that the modulatory effect was due to the phosphorylation reaction itself but not to ATP.

TABLE 1 Open and closed times of nonphosphorylated and phosphorylated amiloride-sensitive Na^+ channels reconstituted into planar lipid bilayers

	t_c (ms)	t_o^1 (ms)	t_o^2 (ms)	t_o^3 (ms)
Control				
	250 ± 35	8 ± 5	9 ± 3	135 ± 18
PKA + 50 μM ATP	215 ± 20	225 ± 23	200 ± 19	210 ± 22
PKA + 100 μM ATP	200 ± 21	350 ± 29	400 ± 30	350 ± 27
PKA + 200 mM ATP	150 ± 18	350 ± 24	840 ± 34	860 ± 32

Mean closed (t_c) and open (t_o) times of each conductance level (1, 12 pS; 2, 24 pS; 3, 40 pS) were determined from single channel event dwell time histograms, as shown in Fig. 1 *C*. Analyses were performed on 15 min of channel record for six separate experiments.

ATP dose-response curves in the presence of PKA for different "*trans*" sodium concentrations are presented in Fig. 2 *A*. These experiments were performed when $[Na^+]_{cis}$ was maintained at a low concentration (10 mM). P_o versus [ATP] curves were sigmoid and showed that at any [ATP], channel P_o increased as $[Na^+]_{trans}$ increased. Linearization of these data in Lineweaver-Burk plots (Fig. 2 *B*) showed that the apparent K_s of the channel for Na^+ (i.e., the $[Na^+]$ at which P_o was one-half of its maximal value; K_s was determined from the extrapolated x-intercept $-1/K_s$ of this plot at zero inhibitor concentration) decreased from 125 ± 7 mM for nonphosphorylated channels to 38 ± 2 mM for the channel phosphorylated at a saturating ATP concentration (200 μM).

Effects of protein kinase A-induced phosphorylation on channel cation selectivity

We next performed experiments that were designed to test the hypothesis that phosphorylation changes the relative permeability of this renal channel to monovalent cations, specifically Li^+, Na^+, and K^+. Reversal potentials determined from current-voltage curves measured under biionic conditions were used to assess the relative cation permeability of the channel phosphorylated in the presence of increasing ATP concentrations (Coronado et al., 1980). Fig. 3 depicts current-voltage curves measured after biochemical modifications of the channel bathed with asymmetrical solutions (Na^+_{trans} and K^+_{cis}). We used mean current-voltage relationships because of the observed change in apparent unitary current. Under nonphosphorylating conditions, the channel displayed a $P_{Li}:P_{Na}:P_K$ of 8:7:1 (n = 11; Table 2). When the channel was phosphorylated by PKA in the presence of either 100 μM or 200 μM Mg-ATP, the $P_{Li}:P_{Na}:P_K$ increased to 20:20:1 (n = 5 and 3, respectively). Thus, PKA-mediated phosphorylation converted this renal channel into one highly selective for Li^+ and Na^+ over K^+. This figure also shows permeability data after G-protein modification (see below).

Effects of protein kinase A-induced phosphorylation on inhibition of the channel by amiloride

As noted above, the initial level of channel activity was very low after reconstitution of this immunopurified renal Na^+ channel into planar lipid bilayers with symmetrical 100 mM NaCl solutions (Oh and Benos, 1993b; Ismailov et al., 1994a). Amiloride dose-response curves could be generated

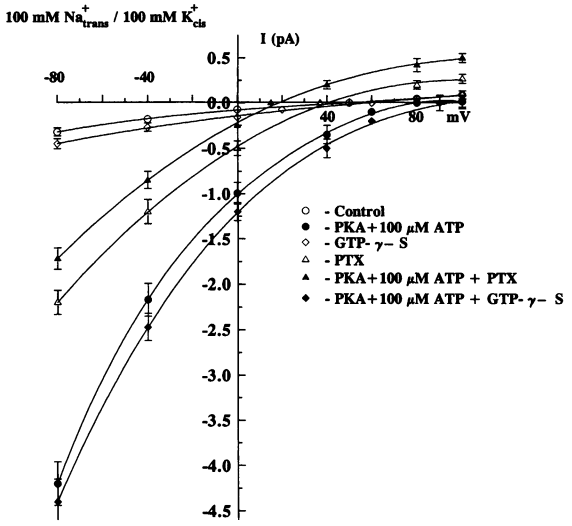

FIGURE 3 Effects of biochemical modifications of single renal Na^+ channel on mean current-voltage curves under biionic conditions. Points in plots are mean ± SD for $n > 4$ (see n in Table 2). *Trans* and *cis* bathing solutions contained 100 mM sodium and potassium chloride salts, respectively. Points were fitted to third-order regression equation of the type $y = b_0 + b_1x + b_2x^2 + b_3x^3$ using Sigmaplot 5.0 scientific graphing software.

under these conditions, but only if very long (>10 min) stretches of record were analyzed at each drug concentration. Under these conditions, the apparent inhibition constant ($K_i$amil) of amiloride was 0.7 ± 0.1 μM (n = 24). A high initial channel P_o of nonexogenously phosphorylated channels (P_o = 0.05 − 0.15) was observed only rarely (i.e., in 12 of 354 successful incorporations). In these 12 experiments, the apparent inhibition constant ($K_i$amil) of amiloride could be divided into two groups and varied from 0.3 ± 0.1 μM (n = 3) to 1.7 ± 0.3 μM (n = 9). This subset of higher initial P_o Na^+ channels, demonstrating a highly variable apparent $K_i$amil, indicated that the amiloride sensitivity of the channel was not constant and that the variability was dependent upon the biochemical state (i.e., phosphorylated or not) of the purified channel.

We next determined the inhibitory potency of amiloride on single-channel P_o at different *trans* sodium concentrations in experiments in which $[Na^+]_{cis}$ was lowered to 10 mM to increase the basal activity of the channel (Ismailov et al., 1995). Fig. 4 shows a typical current recording of such an experiment. The top trace represents channel activity recorded using asymmetrical sodium solutions in the absence of amiloride. Addition of 0.5 μM amiloride to the *trans* bathing solution (bottom trace) reduced channel P_o from 0.10 ± 0.05 in control to 0.05 ± 0.03 (n = 8). We did not note any difference in amiloride sensitivity of nonphosphorylated channels regardless of the $[Na^+]_{cis}$. Fig. 5 summarizes amiloride dose-response curves obtained from these types of experiments. Amiloride inhibition curves were also performed at different $[Na^+]_{trans}$ to determine the type of inhibition (i.e., competitive, noncompetitive, mixed, etc.) produced by the drug (Segel, 1975). Linear regression analysis of the transformed data presented as Dixon plots (Fig. 5 *B*) revealed a single intersection point on the abcissa, suggesting noncompetitive inhibition (true = 0.5 ± 0.2 μM). Lineweaver-Burk analyses of these same data (Fig. 5 *C*) also indicated noncompetitive inhibition of the channel by amiloride and further yielded a value of K_s of 125 ± 15 mM for $[Na]_{trans}$, in excellent agreement with the results presented in Fig. 2 *B*.

PKA phosphorylation of renal Na^+ channels previously activated by reducing $[Na^+]_{cis}$ to 10 mM further increased channel P_o. Fig. 6 displays current recordings of such a phosphorylated channel (100 μM ATP) in the absence and presence of amiloride at 100 μM $[Na]_{trans}$. The phosphorylated channel fluctuated between what appear to be three long-lived, 12–13-pS conductive states, as described above in Fig. 1 *A*. The channel was in a closed state 40 ± 6% and 70 ± 5% of the time when $[Na]_{trans}$ was 100 mM and 30 mM, respectively. Amiloride inhibited channel activity but only when the drug was added to the *trans* compartment at concentrations exceeding 0.5 μM. The bottom trace of each set records shows current recordings of the same channel obtained after *trans* addition of 5 μM amiloride.

Similar to the kinetic analysis performed on nonexogenously phosphorylated Na^+ channels, we determined in more detail to what extent the inhibitory potency of amiloride was dependent upon $[Na]_{trans}$ subsequent to PKA-mediated phosphorylation. Fig. 7 *A* depicts amiloride dose-response curves performed at 100 μM ATP concentration. When these data were linearized on Dixon plots, the lines intersected at a single point to the left of the ordinate and above the abscissa. This behavior was suggestive of either competitive or mixed inhibition, with a true inhibition constant of amiloride of 4.2 ± 0.7 μM. Apparent amiloride inhibition constants, determined at the intercept of each line with the abscissa, shifted from 6.0 ± 0.5 μM at 30 mM $[Na]_{trans}$ to 10 ± 0.7 μM at 150 mM NaCl. Double-reciprocal (Lineweaver-Burk) plots of these data (Fig. 7 *C*) also suggested a mixed type of inhibition, with the true equilibrium constant of Na^+ being 65 ± 10 mM.

Fig. 8 shows inhibition kinetic analyses of comparable experiments performed at 200 μM MgATP concentration. It is apparent from both the amiloride dose-response curves (Fig. 8 *A*) and the Dixon plots (Fig. 8 *B*) that amiloride had a further diminished inhibitory effect on P_o when the channel was maximally phosphorylated, as compared to the nonphosphorylated or intermediate phosphorylated channel. The true $K_i$amil under these conditions was 7.5 ± 1.0 μM, a value significantly different from that obtained at 100 μM ATP (4.0 ± 0.7 μM; $p < 0.005$) or when the channel was not phosphorylated (0.5 ± 0.2 μM; $p < 0.001$). However, double reciprocal replots of these data indicated that the type of inhibition was purely competitive (Fig. 8 *C*). From these experiments the true equilibrium constant of Na^+ was determined to be 30 ± 4 mM.

TABLE 2 Effects of biochemical modification of purified renal Na^+ channels incorporated in planar bilayers on amiloride sensitivity and ion selectivity

Conditions	$P_{Li}:P_{Na}:P_K$*	Apparent K_i^{amil} (μM)*	True K_i^{amil} (μM)‡	Type of inhibition
Control§	8:7:1 (n = 11)	0.7 ± 0.1 (n = 24)	0.5 ± 0.2 (n = 31)	Noncompetitive
+ (PKA + 100 μM ATP)	20:20:1 (n = 5)	20.0 ± 3.1 (n = 10)	4.0 ± 0.7 (n = 7)	Mixed
+ (PKA + 200 μM ATP)	20:20:1 (n = 3)	50.0 ± 8.7 (n = 7)	7.5 ± 1.0 (n = 6)	Competitive
+ GTP-γ-S	15:10:1 (n = 6)	0.10 ± 0.04 (n = 7)	N/D	N/D
+ PTX	4:4:1 (n = 7)	2.0 ± 0.2 (n = 7)	N/D	N/D
(PKA + 100 μM ATP) + PTX	2:2:1 (n = 6)	8.0 ± 0.9 (n = 8)	N/D	N/D
(PKA + 100 μM ATP) + GTP-γ-S	50:30:1 (n = 5)	0.5 ± 0.1 (n = 11)	N/D	N/D

*Bilayers were bathed with 100 mM salt solutions. §Control refers to bilayers containing a single, unmodified Na^+ channel.
‡True K_i^{amil} were determined from Dixon plot analysis of channel open probability at different [Na_{trans}], 10 mM [Na_{cis}], while maintaining osmolality constant; number of independent experiments performed at a given [Na_{trans}], [Na_{cis}] condition.
§Effects of PKA + ATP, GTP-γ-S, and pertussis toxin (PTX) on channel properties were assessed in independent experiments.

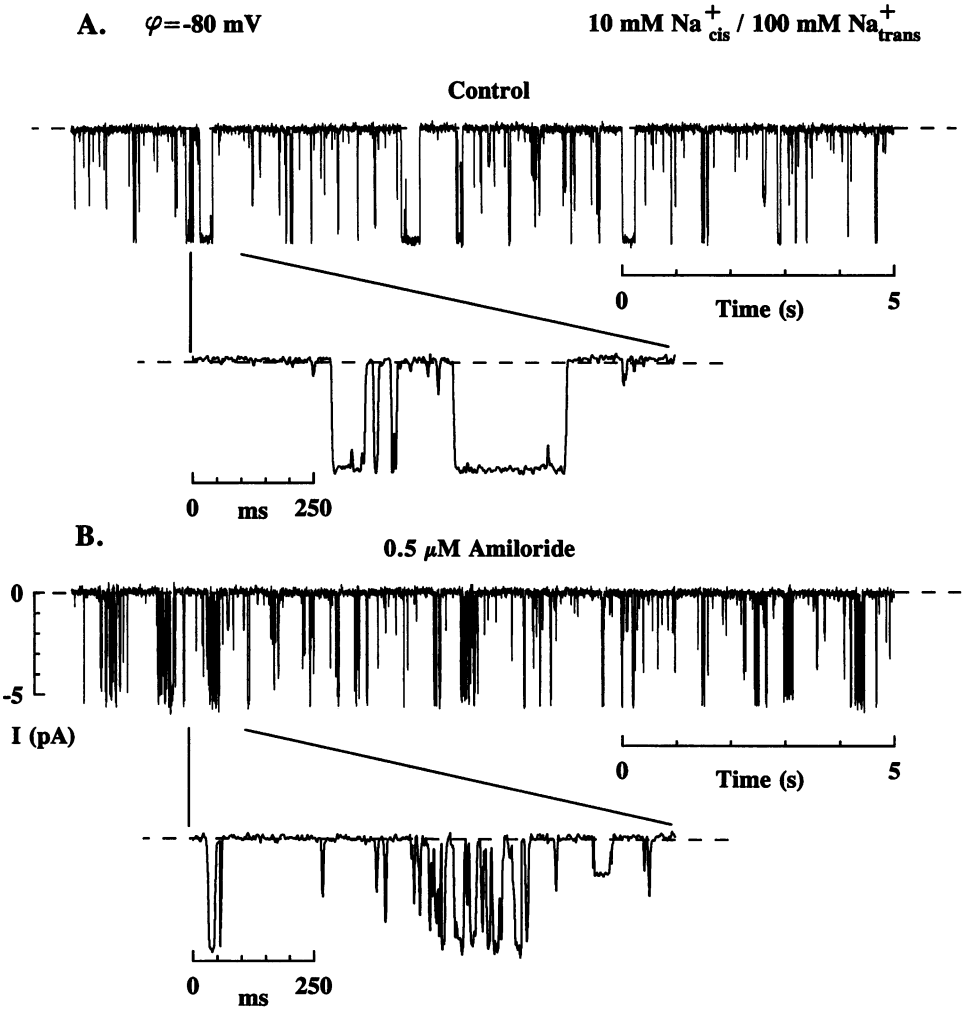

FIGURE 4 Effect of amiloride on single renal Na^+ channel reconstituted into planar lipid bilayers. Records shown are for −80 mV holding potential and are representative of 7 separate experiments performed in the absence (*A*) and presence (*B*) of the same concentration of amiloride. Records were filtered at 300 Hz with an 8-pole Bessel filter before the acquisition and were sampled at 1000 Hz with a Digidata 1200 interface. Bilayers containing reconstituted channels were bathed with asymmetrical salt solutions ([10 mM Na + 140 mM NMDG-Cl]$_{cis}$/[100 mM Na + 50 mM NMDG-Cl]$_{trans}$, 10 mM MOPS, pH 7, 5).

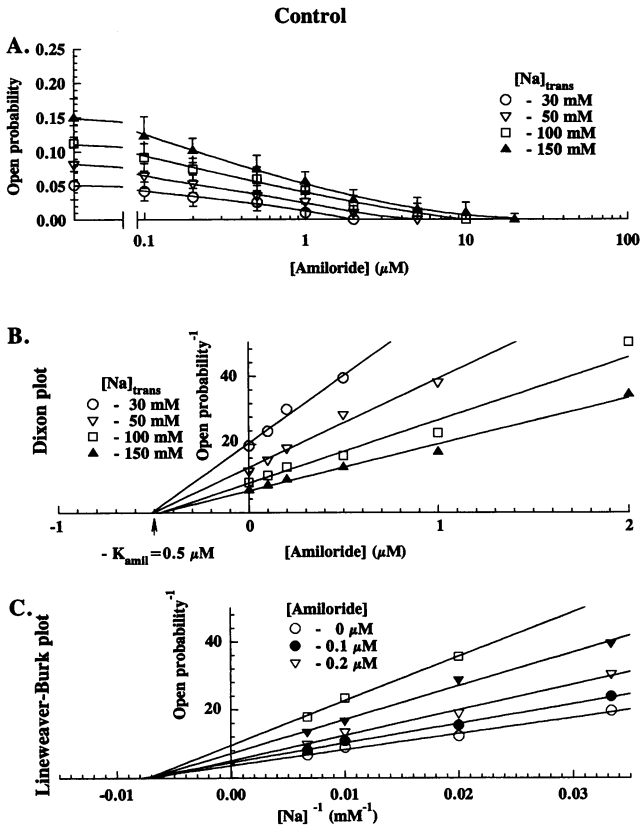

FIGURE 5 Amiloride dose-response curves of a single renal Na^+ channel and kinetic analyses of amiloride inhibition. The bathing solution contained [10 mM Na + 140 mM NMDG-Cl]$_{cis}$; [X mM NaCl (see plot legend) + (150-X) mM NMDG-Cl]$_{trans}$ and 10 mM MOPS both sides, pH 7.5. (*A*) Channel open probability versus amiloride concentration determined at various $[Na^+]_{trans}$. Each point represents mean ± SD for 7–8 separate experiments performed at each $[Na^+]_{trans}$. The entire range of amiloride concentration was used for each channel studied. (*B*) Dixon plot analysis of the data shown in (*A*). (*C*) Lineweaver-Burk analysis of the data presented in (*A*).

G-protein modulation affects Na^+ channel sensitivity to amiloride

We reported earlier the effects on channel activity after ribosylation of the channel-associated $G_{\alpha i3}$ protein by pertussis toxin (Ismailov et al., 1994a). The decrease in amiloride sensitivity of the channel subsequent to PKA phosphorylation prompted us to test the hypothesis that modulation of the channel-associated G-protein can also affect the extent to which amiloride can inhibit the channel. Two experiments were performed to test this hypothesis. First, the G-proteins of nonphosphorylated channels were ADP-ribosylated with pertussis toxin. ADP-ribosylation increased single-channel open probability (P_o = 0.41 ± 0.05, n = 4) relative to paired controls (P_o = 0.11 ± 0.03, n = 4). Channel P_o was decreased to 0.18 ± 0.02 by 2 μM amiloride as compared to channel activity in the presence of PTX. The apparent $K_i$amil was increased from 0.6 ± 0.1 μM to 2.0. ± 0.2 μM (n = 7) after PTX treatment (Table 1). Hence, amiloride was less effective as an inhibitor when the channel's associated G-protein was inactivated by ribosylation.

In separate experiments GTP-γ-S and GDP-β-S, nonhydrolyzable analogs of GTP and GDP, were added to the *cis* compartment. This treatment had no effect on single-channel kinetics. When the channel was exposed to 100 μM GTP-γ-S, amiloride still behaved as a flickering-type blocker. However, the potency of the drug was increased in that 0.3 μM reduced channel activity by 64%, to P_o = 0.05 ± 0.02 (n = 5), when $[Na^+]_{cis}$ was 10 mM. The apparent $K_i$amil decreased to 0.10 ± 0.04 μM from 0.5 ± 0.02 μM (n = 7, $p < 0.001$) after GTP-γ-S treatment. This effect was independent of $[Na^+]_{cis}$ (see Table 2). GDP-β-S (100 μM) addition to the *cis* bathing solution did not alter the amiloride sensitivity of the channel (not shown).

ADP-ribosylation by PTX is known to inactivate this renal Na^+ channel if the channel was previously phosphorylated (Ismailov et al., 1994a; Bubien et al., 1994). This observation formed the basis for the next series of experiments, where ADP-ribosylation and GTP-γ-S activation of the channel were performed after phosphorylation of the channel by PKA. PTX-induced ADP-ribosylation resulted in a decrease of channel activity as compared to the phosphorylated channel, as reported earlier (Ismailov et al., 1994a). However, after treatment of the previously PKA-phosphorylated channel with PTX, the channel reverted from residing primarily in the long-lived 12-pS conductance states back to residing in the 40-pS state. Amiloride (8 μM) produced a flickering-type block of this PKA-phosphorylated and PTX-treated channel, significantly decreasing its P_o from 0.32 ± 0.04 to 0.15 ± 0.03 ($p < 0.001$, n = 8). Similar experiments were performed where GTP-γ-S was added after PKA phosphorylation. Again, addition of GTP-γ-S did not affect channel kinetics as compared to those displayed by a phosphorylated channel (cf. Figs. 1 and 5). However, GTP-γ-S treatment of PKA-phosphorylated channels resulted in an enhancement of the efficacy of amiloride. Amiloride (0.5 μM) decreased channel P_o from 0.60 ± 0.09 to 0.31 ± 0.04 (n = 11). Thus, the biochemical configuration of the channel complex was specifically altered, and its biophysical fingerprint changed.

Table 2 summarizes the cation selectivity properties and nature of amiloride inhibition of these Na^+ channels when the channels were biochemically modified by these different perturbations. Treatment with PKA + ATP or GTP-γ-S both increased the Na^+/K^+ permeability of this renal Na^+ channel, whereas PTX treatment of either nonphosphorylated or PKA-phosphorylated channels decreased P_{Na}/P_K (see Fig. 3). PKA-mediated phosphorylation or PTX treatment both significantly reduced amiloride efficacy, and GTP-γ-S treatment of either nonphosphorylated or phosphorylated Na^+ channels increased the sensitivity of the channel to amiloride. Interestingly, GTP-γ-S treatment of PKA-phosphorylated channels produced channels of very high Na^+ selectivity and amiloride sensitivity, much like those recorded by patch clamp in renal collecting tubules (Palmer and Frindt, 1986; Ling et al., 1991; Silver et al.,

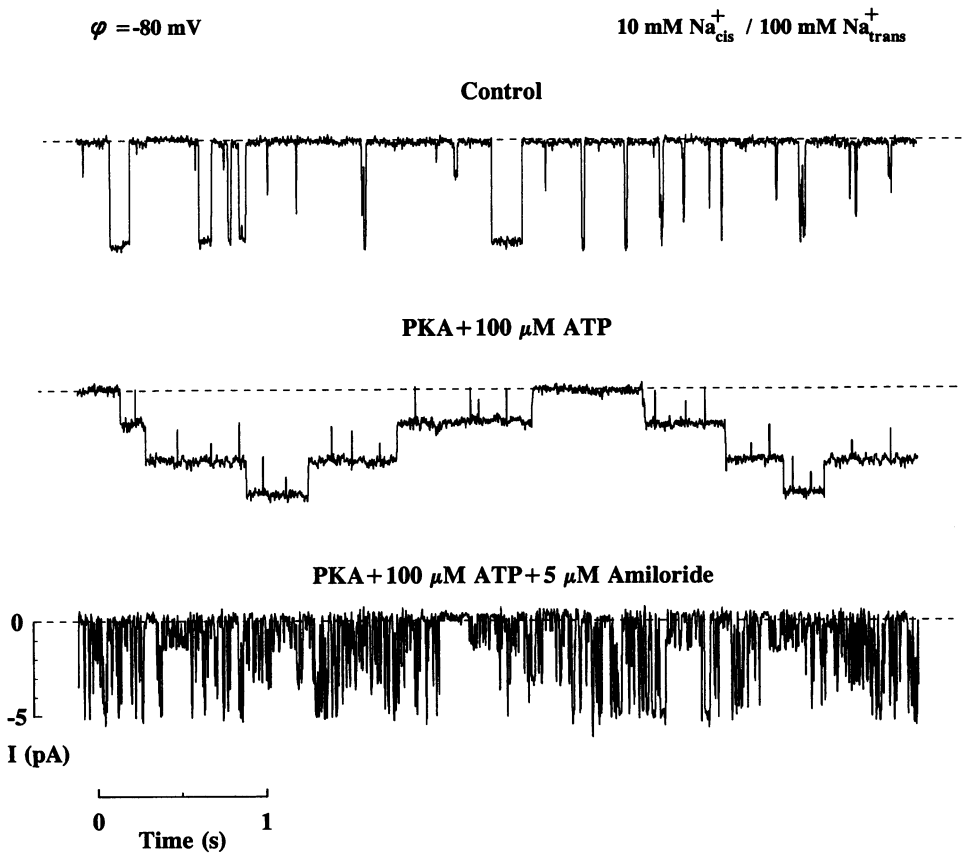

FIGURE 6 Effect of amiloride on single renal Na^+ channel reconstituted into planar lipid bilayers in the presence of protein kinase A + 100 μM ATP. Records shown are for a −80 mV holding potential. Bilayers containing reconstituted channels were bathed with asymmetrical salt solutions ([10 mM Na + 140 mM NMDG-Cl]$_{cis}$/[100 mM Na + 50 mM NMDG-Cl]$_{trans}$, 10 mM MOPS, pH 7.5). The *cis* solution contained 1.85 ng/ml PKA. In 19 experiments of this kind at each $[Na^+]_{trans}$ amiloride inhibited channel activity when added to the *trans* compartment at a concentration of >0.5 μM. A similar flickering-type block produced by amiloride was observed in 6 additional experiments after the channel was phosphorylated by 200 μM ATP.

1993). Moreover, these channels had relatively long open and closed times, and low apparent conductance.

DISCUSSION

Amiloride-sensitive Na^+ channels are present in many different cells and tissues, where they subserve the prime function of regulating Na^+ homeostasis. Patch-clamp and pharmacological observations indicate that these channels display a wide variety of biophysical characteristics including conductance, cation selectivity, and amiloride sensitivity (cf. Palmer, 1992; Garty, 1994; Benos et al., 1995). Several different classification schemes for these channels have been devised, based primarily on conductance and/or amiloride sensitivity (Smith and Benos, 1991; Palmer, 1992). However, Na^+ channels with different single-channel conductances, cation selectivities, and amiloride sensitivities, nonetheless can all be activated by PKA-mediated phosphorylation. The fact that many different biophysical types of amiloride-sensitive Na^+ channel can be identified contradicts the conclusion that there is only one class or family of channel.

In bilayers, purified and reconstituted Na^+ channels have conductance ranging from 4 to 80 pS, yet these different conductance channels display comparable Na^+/K^+ selectivity (5–7:1), amiloride sensitivity (100–700 nM), and kinetics (Olans et al., 1984; Sariban-Sohraby et al., 1984; Oh and Benos, 1993b; Oh et al., 1993). Because the prototypical type I Na^+ channel from renal epithelia has a very low conductance and high Na^+/K^+ permeability, can these reconstituted (or any other candidate) channel protein complexes exhibiting higher conductances and/or selectivity properties accurately represent so-called amiloride-sensitive Na^+ channels? Our experiments answer in the affirmative, because these single amiloride-sensitive, Na^+-selective ion channels still respond to the appropriate physiological regulatory inputs such as phosphorylation, G-protein activation or inhibition, and carboxyl methylation (Cantiello et al., 1990; Oh et al., 1993; O'Hara et al., 1993; Sariban-Sohraby et al., 1993; Ismailov et al., 1994a,b). This paper reports that PKA-mediated phosphorylation or G-protein modification of purified renal Na^+ channels reconstituted into planar lipid bilayers significantly alter the channel's biophysical fingerprint. These effects of phosphorylation occur directly on the channel complex itself rather than by recruiting latent channels with different properties. This statement is supported by the fact that only membranes containing single channels were used in these experiments, and that there is a direct correlation between $^{32}PO_4$ incorporation into the channel complex and stimulated macroscopic Na^+ transport (Benos, 1991). Also, channels were studied using protein preparations obtained by immunopurification employing antibodies highly specific to these renal Na^+ channels, rather than native membrane vesicles that undoubtedly contain different channel types. The main pharmacological and biophysical characteristics (i.e., amiloride sensitivity and

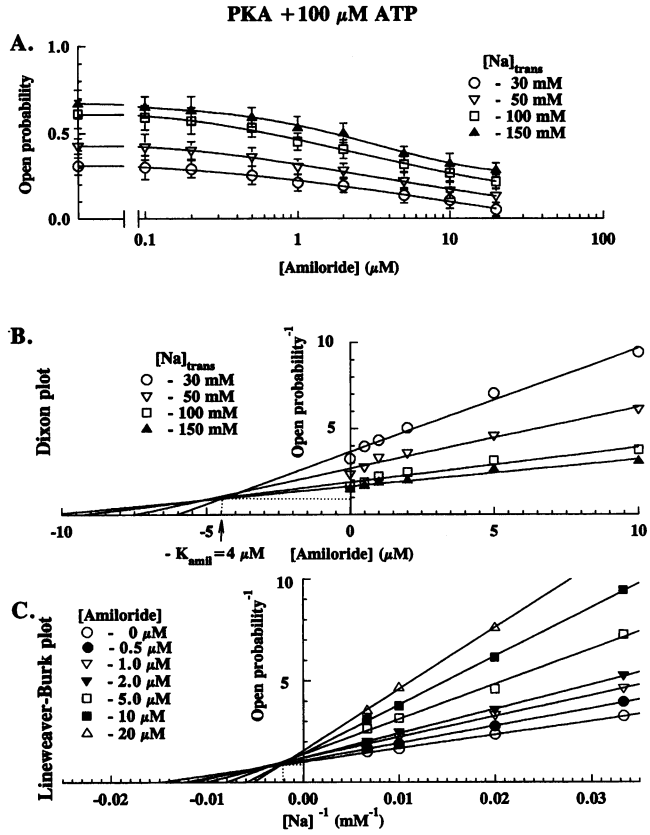

FIGURE 7 Amiloride dose-response curves of a single renal Na^+ channel in the presence of protein kinase A + 100 μM ATP and kinetic analyses of amiloride inhibition. The bathing solution contained [10 mM Na + 140 mM NMDG-Cl]$_{cis}$; [X mM NaCl (see plot legend) + (150-X) mM NMDG-Cl]$_{trans}$ and 10 mM MOPS both sides, pH 7.5. The *cis* solution contained 1.85 ng/ml PKA. (*A*) Channel open probability versus amiloride concentration determined at various $[Na^+]_{trans}$. Each point represents mean ± SD for 7–13 separate experiments performed at each $[Na^+]_{trans}$. The entire range of amiloride concentration was used for each channel studied. (*B*) Dixon plot analysis of the data shown in (*A*). (*C*) Lineweaver-Burk analysis of the data presented in (*A*).

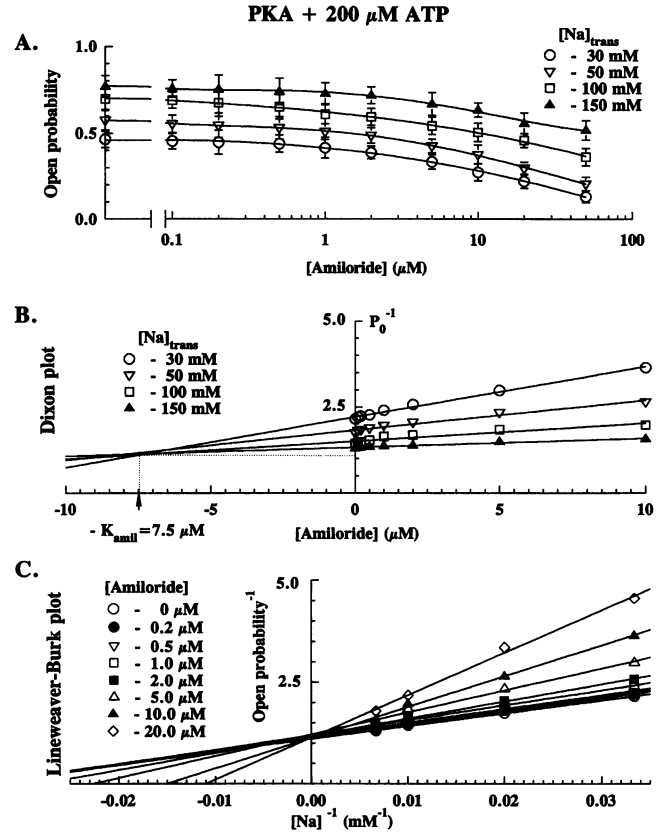

FIGURE 8 Amiloride dose-response curves of a single renal Na^+ channel in the presence of protein kinase A + 200 μM ATP and kinetic analyses of amiloride inhibition. The bathing solution contained [10 mM Na + 140 mM NMDG-Cl]$_{cis}$; [X mM NaCl (see plot legend) + (150-X) mM NMDG-Cl]$_{trans}$ and 10 mM MOPS both sides, pH 7.5. (*A*) Channel open probability versus amiloride concentration determined at various $[Na^+]_{trans}$. Each point represents mean ± SD for 7–13 separate experiments performed at each $[Na^+]_{trans}$. The entire range of amiloride concentration was used for each channel studied. (*B*) Dixon plot analysis of the data shown in (*A*). (*C*) Lineweaver-Burk analysis of the data presented in (*A*).

relative cation permeability) of the 40-pS and 12–13-pS and 24–26-pS conductance levels were identical under a given set of experimental conditions.

Phosphorylation of renal Na^+ channels by PKA has profound effects on the dependence of single-channel P_o on $[Na^+]_{trans}$ as well as on the nature and extent of amiloride inhibition. Under nonphosphorylated conditions, increasing $[Na^+]_{trans}$ (at constant 10 mM $[Na^+]_{cis}$) increases P_o. The $[Na^+]_{trans}$ at which one-half maximal P_o occurs (K_s) is 125 mM. The value of K_s decreases to 65 mM and 30 mM when the channel is PKA-phosphorylated by 100 μM and 200 μM ATP, respectively. Furthermore, PKA phosphorylation alters the nature of amiloride inhibition of this channel. Amiloride acts as a purely noncompetitive inhibitor of the nonphosphorylated channel, a mixed-type of inhibitor when the channel is phosphorylated at intermediate ATP concentrations, and a purely competitive inhibitor when the channel is phosphorylated in the presence of saturating ATP concentrations. The literature is replete with examples of amiloride inhibiting Na^+ channels in intact epithelia either competitively, noncompetitively, or in a mixed fashion (Bentley, 1968; Salako and Smith, 1970; Biber, 1971; Benos et al., 1976; Cuthbert and Shum, 1976; Sudou and Hoshi, 1977; Cuthbert, 1981). Although these observations may be explained in part based on species differences, alterations in the number of functional channels present in the apical membrane at different $[Na^+]$ or amiloride concentrations, or voltage-dependent effects of blockade (Palmer, 1984; Abramcheck et al., 1985; Helman and Baxendale, 1990), our observations strongly suggest that the phosphorylation and G-protein state of the channel are also important determinants of the nature of amiloride interaction with its binding site(s) on the channel. Presumably, these biochemical modifications produce an allosteric change in the channel complex, thereby altering its biophysical and pharmacological profile. Although the molecular

events mediating these actions of PKA-phosphorylation and G-protein modification are yet to be elucidated, it is apparent that these ion channels possess a large degree of flexibility in their single-channel properties.

Another significant effect of PKA phosphorylation is on the gating kinetics of this channel. Nonphosphorylated renal Na^+ channels in bilayers have a short-lived main state conductance of 40 pS at 100 mM [Na^+]. Nonetheless, this channel can fluctuate, albeit infrequently, between two smaller conductance levels. All of the current transitions between the closed state and any of the smaller conductance levels are rapid, with dwell times lasting no more than 10–100 ms. After phosphorylation, the current transitions become much more discernible, with the virtual disappearance of the 40-pS main conductance state at submaximal ATP concentrations. Moreover, dwell times in any conductance state increase significantly to the 0.3–1 s range, similar to what has been recorded in patch clamp experiments of native epithelia (Gogelein and Greger, 1986; Palmer and Frindt, 1986; Ling et al., 1991; Stoner et al., in press). Although amplitude histogram analysis of the 12–13-pS and 24–26-pS conductance states (at 100 μM ATP) seems to indicate that each state behaves as three independent channels with an equal frequency of appearance (Fig. 1 *B*), activation of the channel by PKA at 200 μM ATP shifts this kinetic behavior back to that of the nonphosphorylated channel, albeit with a dramatically increased P_o. Moreover, phosphorylation at saturating ATP concentration did not result in the appearance of conductance states of multiples of 40 pS, unlike the case when more than one channel was present in the membrane (cf. Ismailov et al., 1994a). This observation supports the conclusion that the 12–13-pS and 24–26-pS conductance levels are substates of the channel.

An alternative possibility that may account for the observation of multiple conductance levels is that other unique channels may have been simultaneously incorporated into the bilayer. We consider this possibility unlikely for the following four reasons. First, based on radioligand binding studies, the purity of the immunopurified matherial used in these experiments was greater than 95%. Second, if the 13-pS, 26-pS, and 40-pS channel represented separate channels, it would be expected that, at least occasionally, bilayer membranes containing only one of these putative channels should be seen after successful incorporation. However, in over 2000 experiments performed to date, these three channels have always occurred together and have not been observed separately from one another. Third, assuming that these three states represent individual channels, event amplitude histograms constructed from control recordings should be fit by binomial theory as predicted for independent channels (Fox, 1987). This was not the case (cf. Fig. 1 *B* and Ismailov et al., 1995). Fourth, because all of the conductance states were amiloride-sensitive and displayed identical cation selectivity under a specified set of experimental conditions, one would have to ascribe differential effects of PKA-mediated phosphorylation to each level (i.e., simultaneous stimulation and inhibition), rather than simply postulating a shift in gating mode. Thus, although we cannot definitely exclude the possibility that our proteoliposomes contained contaminating Na^+ channels, these channels would have to possess extraordinary interactive capabilities to account for the experimental observations.

PKA-mediated phosphorylation increased $P_{Na}+/P_K+$ while decreasing amiloride sensitivity, and virtually eliminated [Na^+] saturation behavior of the channel, at least in the physiological ranges of [Na^+]. G-protein modulation with GTP-γ-S subsequent to phosphorylation converted the channel back to one having high amiloride affinity and a very high permeability of Na^+ over K^+ (>30:1). Although many studies examining the effects of phosphorylation on ion channel function have been done (e.g., Ismailov and Benos, 1995; Levitan, 1994), very few have reported major changes in characteristic channel attributes, such as conductance, selectivity, or gating mode. Protein kinase C-mediated phosphorylation of voltage-dependent Na^+ channels abolished fast inactivation (West et al., 1991), and PKA phosphorylation of L-type Ca^{2+} channels induced a shift in gating modes (Yue et al., 1990). Phosphorylation by PKA of the cystic fibrosis transmembrane conductance regulator promotes a shift of this anion channel from an inactive to active mode (Welsh et al., 1992). Wilson and Kaczmarek (1993) have also reported that the gating kinetics of a voltage-dependent cation channel in *Aplysia* neurons can be modified by protein kinase A-dependent tyrosine phosphatase, but because this channel does not display any subconductance behavior, no effect on apparent single-channel conductance or selectivity was noted.

There are two major physiological implications to our findings. First, the observation that the same channel can be induced biochemically to manifest a panoply of biophysical properties suggests that many of the different types of amiloride-sensitive channels identified by patch clamp may simply represent different conformational states of the same protein. How all amiloride-sensitive cation channels are related to one another on a biochemical and molecular level is at present unclear, yet some evidence is available indicating that biochemically distinct channel protein complexes mediating amiloride-sensitive Na^+ transport exist (Benos et al., 1987; Canessa et al., 1993, 1994; Senyk et al., 1995; Bradford et al., 1995). Second, our results demonstrate for the first time that the biophysical characteristics of a specific ion channel are not invariant, but can be transformed, depending upon the biochemical state of the proteins comprising the channel complex.

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