Regulation by Na^+ and Ca^{2+} of Renal Epithelial Na^+ Channels Reconstituted into Planar Lipid Bilayers

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ABSTRACT Purified bovine renal epithelial Na^+ channels when reconstituted into planar lipid bilayers displayed a specific orientation when the membrane was clamped to -40 mV (*cis*-side) during incorporation. The *trans*-facing portion of the channel was extracellular (i.e., amiloride-sensitive), whereas the *cis*-facing side was intracellular (i.e., protein kinase A-sensitive). Single channels had a main state unitary conductance of 40 pS and displayed two subconductive states each of 12–13 pS, or one of 12–13 pS and the second of 24–26 pS. Elevation of the $[\text{Na}^+]$ gradient from the *trans*-side increased single-channel open probability (P_o) only when the *cis*-side was bathed with a solution containing low $[\text{Na}^+]$ (<30 mM) and 10–100 μM $[\text{Ca}^{2+}]$. Under these conditions, P_o saturated with increasing $[\text{Na}^+]_{\text{trans}}$. Buffering of the *cis* compartment $[\text{Ca}^{2+}]$ to nearly zero (<1 nM) with 10 mM EGTA increased the initial level of channel activity ($P_o = 0.12 \pm 0.02$ vs 0.02 ± 0.01 in control), but markedly reduced the influence of both *cis*- and *trans*- $[\text{Na}^+]$ on P_o . Elevating $[\text{Ca}^{2+}]_{\text{cis}}$ at constant $[\text{Na}^+]$ resulted in inhibition of channel activity with an apparent $[K]_{\text{Ca}^{2+}}^{\text{Ca}^{2+}}$ of 10–100 μM . Protein kinase C-induced phosphorylation shifted the dependence of channel P_o on $[\text{Ca}^{2+}]_{\text{cis}}$ to 1–3 μM at stationary $[\text{Na}^+]$. The direct modulation of single-channel P_o by Na^+ and Ca^{2+} demonstrates that the gating of amiloride-sensitive Na^+ channels is indeed dependent upon the specific ionic environment surrounding the channels.

INTRODUCTION

Channel-mediated apical Na^+ entry in Na^+ -reabsorbing epithelia such as renal collecting tubules, frog skin, and toad urinary bladder is rate limiting for net transepithelial Na^+ transport. In mammalian kidney these channels are under hormonal control (aldosterone, vasopressin) and play a prominent role in regulating whole body salt and water homeostasis. Patch clamp studies of native Na^+ reabsorbing epithelia have revealed a wide array of amiloride-sensitive Na^+ channels displaying different conductances, open and closed times, cation selectivities, and amiloride sensitivities (Benos, Awayda, Ismailov, and Johnson, 1995; Palmer, 1992; Smith and

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Benos, 1991). Expression cloning of a rat distal colon Na^+ channel yielded a channel possessing characteristics similar to those of native Na^+ channels found in renal cortical collecting tubule (Canessa, Horisberger, and Rossier, 1993; Canessa, Schild, Buell, Thoreus, Gantschl, Horisberger, and Rossier, 1994; Palmer and Frindt, 1986). The kinetic properties of purified and reconstituted bovine renal papillary Na^+ channels are distinctly different from those of either the cloned or native cortical collecting duct channel. However, most known regulatory pathways of epithelial Na^+ channels like protein kinase A and C phosphorylation and carboxymethylation remain intact after reconstitution of this biochemically purified protein complex (Oh, Smith, Bradford, Keeton, and Benos, 1993; Ismailov, McDuffie, and Benos, 1994a; Ismailov, McDuffie, Sariban-Sohraby, Johnson, and Benos, 1994b). Therefore, even though the exact molecular relationship between the various kinetic types of epithelial Na^+ channels is far from clear, the fact that biochemical regulation of purified renal Na^+ channel can be preserved in a physiologically relevant manner affords the opportunity to dissect these regulatory pathways in a direct and meaningful way.

Epithelial Na^+ channels are exposed to a highly variable $[\text{Na}^+]$ (0–150 mM) on their extracellular (luminal) surface, and to 5–30 mM Na^+ on their intracellular surface. Several studies have demonstrated that both extracellular and intracellular $[\text{Na}^+]$ modulate the activity of these channels. Increasing luminal Na^+ above 100–150 mM resulted in a saturation of Na^+ entry in frog skin (Fuchs, Larsen, Lindemann, 1977), toad urinary bladder (Li, Palmer, Edelman, and Lindemann, 1982; Palmer, Edelman, and Lindemann, 1980), *Necturus* urinary bladder (Thomas, Suzuki, Thompson, and Schultz, 1983), and rabbit descending colon (Thompson and Sellin, 1986; Turnheim, Thompson, and Schultz, 1983). This saturation of macroscopic Na^+ current by external $[\text{Na}^+]$ was called “ Na^+ self inhibition”. However, increasing luminal $[\text{Na}^+]$ in these experiments was inevitably associated with a rise in intracellular $[\text{Na}^+]$; hence, it was unclear from these findings whether external or internal $[\text{Na}^+]$ was responsible for the reduction in Na^+ transport. The experimental evidence for down regulation of apical Na^+ entry by cytoplasmic $[\text{Na}^+]$ (called feedback regulation) was provided in many other studies using frog skin (MacRobbie and Ussing, 1961; Biber, 1971; Erlij and Smith, 1973; Rick, Dorge, and Nagel, 1975; Cuthbert and Shum, 1977; Helman, Nagel, and Fisher, 1979), toad bladder (Hong and Essig, 1976), toad skin (Hviid Larsen, 1973), and rabbit urinary bladder (Lewis, Eaton, and Diamond, 1976).

This feedback regulation of apical Na^+ entry by cytosolic $[\text{Na}^+]$ is complicated by the fact that this effect requires Ca^{2+} . Inhibition of basolateral Na,K-ATPase with ouabain produced a decrease of apical Na^+ entry only if the serosal medium contained >0.1 mM Ca^{2+} (Chase, 1984; Garty and Lindemann, 1984; Palmer, 1985). Moreover, addition of ouabain to the solutions bathing rat renal cortical collecting tubules decreased single amiloride-sensitive Na^+ channel open probability (P_o) concomitant with an increase in intracellular $[\text{Ca}^{2+}]$ (Silver, Frindt, Windhager, and Palmer, 1993). This decrease in channel activity by ouabain was prevented if intracellular $[\text{Ca}^{2+}]$ was not permitted to increase. However, in all of the aforementioned studies using intact epithelia, several parameters changed simultaneously, such as intracellular pH, Na^+ , and Ca^{2+} , apical membrane voltage, et cetera. Thus, the de-

tailed mechanisms by which intra- or extracellular [Na⁺] and/or [Ca²⁺] ions regulate these channels remain unclear.

This report describes the findings of experiments that tested the hypothesis that [Na⁺] and [Ca²⁺] directly affect the function of renal amiloride-sensitive Na⁺ channels in a cell-free model system. Na⁺ channels were immunopurified from bovine renal papillary collecting ducts (Oh and Benos, 1993a) and reconstituted into planar lipid bilayers. These experiments were thus not confounded by uncontrolled changes in other variables such as pH, voltage, or number of functional channels. We found that (a) single-channel P_o increased with increasing [Na⁺]_{trans} but only if the *cis* compartment contained 30 mM Na⁺ and >5 μ M Ca²⁺; (b) P_o saturated with increasing [Na⁺]_{trans}; reducing [Na⁺]_{cis} increased P_o at any [Na⁺]_{trans}; (c) buffering of the *cis* [Ca²⁺] to <1 nM with 10 mM EGTA increased the initial level of channel activity ($P_o = 0.12 \pm 0.02$ vs 0.02 ± 0.01), but markedly reduced the influence of both *cis*- and *trans*-[Na⁺] on P_o ; and (d) protein kinase C (PKC)-induced phosphorylation shifted the dependence of channel P_o on [Ca²⁺]_{cis} from 10–100 μ M to 1–3 μ M.

MATERIALS AND METHODS

Materials

Chemicals were obtained from the following sources. QUAT (quaternary amine) and SP (sulfo-propyl) ion-exchange cartridges were purchased from FMC BioProducts (Rockland, ME). Phospholipids and dioctanoyl glycerol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO), and was oxidized by bubbling oxygen through a 4% cholesterol solution in octane at the octane boiling point of 126°C (Tien et al., 1966). Protein kinase C (mixture of α , β , and γ isoforms) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). All other chemicals were reagent grade, and all solutions were made with distilled, deionized water and filter sterilized before use (Sterivex-GS, 0.22- μ m filter, Millipore Corp., Bedford, MA).

Methods

Immunopurification of epithelial Na⁺ channel protein from bovine kidney papillary collecting tubules was performed as described previously (Oh and Benos, 1993a).

Reconstitution of Channel Protein into Lipid Vesicles

Proteoliposomes were made by passing detergent solubilized, purified channel proteins through a detergent removing Extracti-Gel D column (Pierce Chemical Co., Rockford, IL) as described previously (Oh and Benos, 1993b). Briefly, 0.5 ml of Extracti-Gel D suspension was poured into a Poly-Prep chromatography column (0.8 $\times$ 4 cm, Bio-Rad Laboratories, Hercules, CA), and the gel was equilibrated with 10 column volumes of 10 mM Na₂HPO₄ buffer (pH 7.4) containing 0.5% egg phosphatidylcholine. Solubilized proteins (0.15 ml) were then applied to the equilibrated Extracti-Gel D column and eluted with 10 mM Na₂HPO₄ buffer (pH 7.4) containing 0.5% egg phosphatidylcholine. Reconstituted proteoliposomes were stored at -70°C .

Planar Lipid Bilayers

Bilayer membranes, composed of a mixture of 2:1:2 (wt/wt/wt) diphytanoyl-phosphatidylethanolamine, diphytanoyl-phosphatidylserine, and oxidized cholesterol in *n*-octane (final lipid concen-

tration = 25 mg/ml), were formed with a fire polished glass capillary over a 200- μ m aperture drilled in a polystyrene cup. Bilayer formation was monitored by the increase in membrane capacitance (final value: 300–400 pF). Membranes were bathed with salt solutions according to the specific experimental design (see figure legends). In all cases, however, solutions were buffered to pH 7.4 with 10 mM MOPS. Na^+ gradient experiments were established by replacing NaCl with equimolar *N*-methyl-D-glucamine chloride (NMDG-Cl), thus maintaining identical the osmolarity in both the *cis* and *trans* chambers. Reconstituted proteoliposomes were spread over a preformed bilayer with a fire polished glass capillary from the *trans* side with the membrane being voltage-clamped to -40 mV (*cis* side with respect to *trans*). Channel incorporation was indicated by the appearance of quantal step increases in current with applied voltage.

Current measurements were performed with a high gain amplifier circuit (Alvarez, Benos, and Latorre, 1985). The *cis* chamber was connected to a pulse generator that controlled membrane potential vs ground. The *trans* chamber was held at virtual ground via connection to a current-to-voltage converter (OP 101, Burr-Brown) with a 1 G Ω feedback resistor. Electrical continuity was provided by a 3 M NaCl 3% agar bridge and Ag/AgCl electrode. Currents were monitored on a strip chart recorder (EasyGraf Recorder TA 240, Gould Electronics) and/or a digital storage oscilloscope, and were stored unfiltered on beta VCR tape using a Vetter model 20 digital data recorder (A. R. Vetter, Co., Inc., Rebersburg, PA). Current records were played back, low-pass filtered at the desired frequency through an 8-pole Bessel filter (902 LPF, Frequency Devices, Inc., Haverhill, MA), and sampled at 1 KHz using a Digidata 1200 interface and pCLAMP 5.6 software (Axon Instruments, Burlingame, CA).

Data Analysis and Presentation

Only records from preparations in which a single channel was incorporated into the bilayer were chosen for experimentation to simplify data analysis. This was confirmed for each recording by phosphorylating the channel at the end of each experiment to reveal the possible presence of silent channels as described previously (Ismailov et al., 1994a). If phosphorylation increased the total number of active channels in the membrane, the experiment was not included. This was an infrequent occurrence (24/253 experiments), because the channel-containing material (liposomes) was diluted and sonicated at 4°C for 30 s to produce empirically a vesicular preparation yielding primarily only single-channel incorporations. Single-channel analysis was performed on records 3–15 min in duration. 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 event current (all levels, including zero level); t_m is an event dwell time, and M is total number of events.

Single-channel open probability was calculated as follows:

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

where N is 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. All voltages in text and in figures are given for *cis* compartment with respect to virtually grounded *trans* compartment. Each experimental condition was repeated a minimum of three times.

Kinetic treatment of steady state membrane transport data is often done utilizing the Michaelis-Menten formulation (Lakshminarayanaiah, 1984). The observations that the curves describing the relationship between channel open probability and sodium concentration are rectangular hyperbolas, and that linearization of these plots (see Figs. 5 and 6 below) conform with a high degree of correlation to reciprocal rearrangements of the Michaelis-Menten equation substantiate the use of this formulation in the present study. There are other mathematical relationships (e.g., $P_o = a \log [\text{Na}]$) that can adequately describe the experimental P_o vs $[\text{Na}]$ curves. However, these formulations cannot account for the saturation of these curves and, most importantly, fail to describe competition effects with other cations. The basic Michaelis-Menten equation can be rewritten as:

$$P_o = \frac{P_o^{\max} \cdot [\text{Na}^+]}{K_s + [\text{Na}^+]} \quad (3)$$

where $P_o^{\max}$ represents the maximal (saturating) level of P_o that can be achieved under a given set of conditions, and K_s is operationally defined as the concentration value of Na⁺ required to maintain P_o at one half its saturating level.

RESULTS

Renal Amiloride-sensitive Na⁺ Channels Display Subconductive States

A representative single-channel recording after reconstitution of an immunopurified amiloride-sensitive renal Na⁺ channel into a planar lipid bilayer is presented in Fig. 1 A. Typically, when these channels are incorporated under symmetrical 100 mM NaCl conditions, they display a very low basal activity ($P_o \sim 0.02$). Close examination of the record reveals the existence of additional single-channel events that are smaller in amplitude and less frequent than the majority of channel openings (arrows). An all points amplitude histogram generated from such a record indicates only two peaks, one for an open and one for a closed state (Fig. 1 B, closed circles). These same two peaks were present in records of up to 20 min. However, a detailed examination of the histogram spreadsheets revealed a relatively increased number of points in bins that correspond to intermediate states. To examine this phenomenon more closely, an events analysis of a single-channel record of 15-min length was generated. Records were filtered at 200 Hz before the acquisition, and were sampled at 1,000 Hz. Event detection thresholds were set at 50% of the amplitude of the smaller events and 5 ms for each event duration. An events amplitude histogram of the single Na⁺ channel current is presented in Fig. 1 B. In addition to the peak main state current level of -1.8 pA, there were two additional current levels of (-0.65 and -1.2 pA). The event dwell time histograms for each of these conductance states are presented in Fig. 1 C. Level 0, or closed state dwell time histogram, was fitted by a single exponential with time constant of 650 ± 31 ms ($N = 4$). Dwell time histograms for each open state were best fitted by a single exponential. Time constants for levels 1 and 2 were not different from each other (11 ± 4 and 14 ± 3 ms, respectively; $N = 4$), but were substantially different from level 3 (52 ± 7 ms; $N = 4$). Constructing events amplitude and dwell time histograms from the same records with higher filter cut-off frequencies yielded essentially the same results, with the exception of a lowered signal-to-noise ratio.

FIGURE 1. (A) A typical single-channel recording of immunopurified amiloride-sensitive renal sodium channel reconstituted into planar lipid bilayer. Holding potential -40 mV (referenced to the *trans* compartment). Bathing solution contained symmetrical 100 mM NaCl, 10 mM MOPS, pH 7.5 . (Dashed line) Zero-current level; (dotted lines) channel open-state levels. This record was filtered at 200 Hz using an 8-pole Bessel filter before the acquisition and was sampled at $1,000$ Hz using a Digi-data 1200 interface. Arrows indicate lower conductance states. (B) All points and events amplitude histogram of a single-channel current recording. Events (bars) and all points (closed circles) amplitude histograms were generated by pCLAMP software from a record of 15 -min length recorded as described in A. Event detection thresholds were 50% of maximal 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 for closed and open states, respectively. Histograms were constructed from $1,339$, 286 , 292 , and 579 events for levels 0 through 3 , respectively.

Open Probability of Renal Na^+ Channel May Be Altered with $[\text{Na}^+]$

The existence and relative proportion of these three conductance states remained constant when $[\text{Na}^+]$ gradients of different magnitude and direction were imposed across a channel-containing bilayer (Fig. 2). Elevating the *trans*-to-*cis* $[\text{Na}^+]$ gradient by lowering $[\text{Na}^+]_{\text{cis}}$ resulted in an increase in the number of channel openings, as can be seen in single-channel records. However, at each $[\text{Na}^+]_{\text{cis}}$, the relative proportion of the two smaller current levels did not change (namely 17 , 17 , and 66% of the total, for levels 1 , 2 , and 3 , respectively). Single-channel open-dwell time his-

FIGURE 2. A single bovine renal amiloride-sensitive sodium channel in a planar lipid bilayer bathed with asymmetrical sodium solutions. Records for +80 mV (*top trace*, voltage is referenced to the *trans* compartment) and -80 mV (*bottom trace*) are shown. (*Dashed line*) Zero-current level. Bathing solution in *trans* compartment contained 100 mM NaCl, 10 mM MOPS, pH 7.5; *cis* compartment contained NaCl as indicated for each pair of traces, plus 10 mM MOPS, pH 7.5. Records were filtered at 100 Hz using an 8-pole Bessel filter before the acquisition and were sampled at 1,000 Hz using a Digidata 1200 interface.

tograms derived from the record of channel activity when the bathing solutions contained 100 mM [NaCl]_{trans} and 10 mM [NaCl]_{cis} revealed that the time constants for the two putative subconductive states remained the same as those observed under symmetrical [Na⁺] conditions ($\tau^1_0 = 8 \pm 5$ ms and $\tau^2_0 = 9 \pm 3$ ms ($N = 6$) vs $\tau^1_0 = 11 \pm 4$ ms and $\tau^2_0 = 14 \pm 3$ ms ($N = 4$), respectively). Interestingly, the open dwell-time constant for level 3 (τ^3_0 , main open state) increased threefold over that observed under symmetrical salt solutions [135 ± 18 ms ($N = 6$) vs 52 ± 7

ms ($N = 4$), respectively], while the closed time constant τ_c decreased from 650 ± 31 ms ($N = 4$) to 250 ± 35 ms ($N = 6$).

Mean current-voltage relationships of this renal Na^+ channel at constant $[\text{Na}^+]_{\text{trans}}$ (i.e., 100 mM) are plotted on the left side of Fig. 3. These curves shifted with the

FIGURE 3. Current-voltage relationships of single renal amiloride-sensitive sodium channels in planar lipid bilayers bathed with asymmetrical Na^+ solutions. $[\text{Na}^+]_{\text{cis}}$ was varied at constant $[\text{Na}^+]_{\text{trans}}$ (A) and $[\text{Na}^+]_{\text{trans}}$ was varied with $[\text{Na}^+]_{\text{cis}}$ held constant (B). Left side depicts mean current-voltage relationships, right side depicts single-channel unitary current-voltage relationships. Points in the plots are Mean $\pm$ SD for N of three to eight experiments at each concentration. Error bars for the right panels are smaller than symbol size.

Na^+ gradient in a manner such that the measured reversal potentials for each gradient condition were within one standard deviation of that calculated from the Nernst equation. Unitary current-voltage relationships (right side of Fig. 3) independently confirm that this shift of reversal potential was Nernstian. The slopes of

the individual mean current-voltage curves decreased with increasing $[\text{Na}^+]_{\text{cis}}$ when $[\text{Na}^+]_{\text{trans}}$ was held constant. This slope change in the mean current-voltage plots was not as pronounced when $[\text{Na}^+]_{\text{cis}}$ was maintained at 100 mM and $[\text{Na}^+]_{\text{trans}}$ was varied (Fig. 3 B). When unitary slope conductance was plotted vs either $[\text{Na}^+]_{\text{cis}}$ or $[\text{Na}^+]_{\text{trans}}$, the resultant curves show saturation (Fig. 4). The experimental points were fit with a Michaelis-Menten equation:

$$G_i = \frac{G_{\text{max}} \cdot [\text{Na}^+]}{K_s + [\text{Na}^+]} \quad (4)$$

where G_i is unitary slope conductance, and G_{max} is maximal conductance. These curves yielded K_s values for $[\text{Na}^+]_{\text{cis}}$ and $[\text{Na}^+]_{\text{trans}}$ of 9.72 ± 0.98 mM ($N = 5$) and

FIGURE 4. Semilogarithmic plots of renal amiloride-sensitive sodium channel slope unitary conductance vs sodium concentration. Points in plot were obtained from seven independent experiments. Within each figure, each symbol represents data acquired for the same channel. The points were fit with a Michaelis-Menten equation (see Eq. 4 in the text) with a G_{max} of 53.0 and 54.4 pS, K_s of 9.7 and 9.6 mM for plots vs $[\text{Na}^+]_{\text{cis}}$ and $[\text{Na}^+]_{\text{trans}}$, respectively.

9.63 ± 0.92 mM ($N = 5$), respectively. These K_s values are lower than the K_s values of 45–55 mM reported earlier for this same renal Na⁺ channel (Oh and Benos, 1993). In those earlier experiments, the $[\text{Na}^+]$ of both the *cis* and *trans* compartments were varied simultaneously, not independently as done here. In two additional experiments in which $[\text{Na}^+]_{\text{cis}}$ and $[\text{Na}^+]_{\text{trans}}$ were changed at the same time, K_s values of 41 and 46 mM were measured (not shown).

Plots of single-channel P_o vs $[\text{Na}^+]$ reveal a decrease of channel P_o with increasing $[\text{Na}^+]_{\text{cis}}$ (while maintaining constant $[\text{Na}^+]_{\text{trans}}$) (Fig. 5, *left upper panel*). P_o values were calculated for -80 mV. When the sodium gradient was directed from *trans* to *cis* (while maintaining constant $[\text{Na}^+]_{\text{cis}}$), saturation of channel P_o was ob-

served. P_o was maximal when $[\text{Na}^+]_{\text{cis}}$ was 30 mM (Fig. 5, *upper right panel*). Double reciprocal (Lineweaver-Burk) plots of these data (Fig. 5, *lower panel*) suggest an uncompetitive interaction between *trans* and *cis* sodium ions. These plots also demonstrate that elevating $[\text{Na}^+]_{\text{cis}}$ shifts the equilibrium constant (K_s) for $[\text{Na}^+]_{\text{trans}}$ to lower values, from $K_s = 100 \pm 12$ mM $[\text{Na}^+]_{\text{trans}}$ at 10 mM $[\text{Na}^+]_{\text{cis}}$, to $K_s = 25 \pm 4$ mM $[\text{Na}^+]_{\text{trans}}$ at $[\text{Na}^+]_{\text{cis}}$ 50 mM, thus prohibiting an increase in P_o with increasing $[\text{Na}^+]_{\text{trans}}$.

FIGURE 5. Sodium concentration dependence of renal amiloride-sensitive sodium channel open probability in presence of calcium. Bilayers containing a reconstituted channel were bathed with asymmetrical salt solution (see legends on the plot) and were held at -80 mV. Bottom panel depicts Lineweaver-Burk double reciprocal plot of data presented in the top left. Individual plots were fitted to linear regression. Points in the plots are Mean $\pm$ SD for N of three to five experiments performed at each $[\text{Na}^+]$.

Calcium Affects the Sodium Dependence of Channel Activity

All the above experiments were performed in solutions containing $10 \mu\text{M}$ Ca^{2+} as measured by Fura-2. To test the hypothesis that intracellular $[\text{Ca}^{2+}]$ may influence the activity of these amiloride-sensitive Na^+ channels, we reexamined the $[\text{Na}^+]$ dependencies of channel activity when $[\text{Ca}^{2+}]$ was reduced to <1 nM by buffering with 10 mM EGTA. Basal channel activity was significantly increased by this maneuver (to a P_o of ~ 0.1). Notably, the dependence of P_o on either *cis* or *trans* $[\text{Na}^+]$ was markedly reduced under these experimental conditions (Fig. 6).

FIGURE 6. Sodium concentration dependence of renal amiloride-sensitive sodium channel open probability in absence of calcium. Bilayers containing a reconstituted Na⁺ channel were bathed with 10 mM EGTA-buffered asymmetrical salt solution (see legends on the plot) and were held at -80 mV. Points in the plots are mean $\pm$ SD for an N of four experiments performed at each $[\text{Na}^+]$.

To further define the role of Ca^{2+} in modulating the effect of *trans* $[\text{Na}^+]$ on channel P_o , experiments in which $[\text{Ca}^{2+}]$ was varied were performed. Up to this point Ca^{2+} was either present ($[\text{Ca}^{2+}] = 10 \mu\text{M}$) or absent ($[\text{Ca}^{2+}] < 1 \text{ nM}$) on both sides of the bilayer. The effect of $[\text{Ca}^{2+}]$ on P_o was evident only from the *cis* side; reduction of $[\text{Ca}^{2+}]_{\text{trans}}$ to $< 1 \text{ nM}$ had no effect whatsoever on channel properties (data not shown). Thus, experiments were performed in which $[\text{Ca}^{2+}]_{\text{cis}}$ was varied, while maintaining the *trans* bathing solution at $< 1 \text{ nM}$ $[\text{Ca}^{2+}]$. Representative single-channel records of these experiments are shown in Fig. 7. Under control conditions ($< 1 \text{ nM}$ $[\text{Ca}^{2+}]$), single-channel P_o averaged 0.11 ± 0.02 for all tested $[\text{Na}^+]_{\text{cis}}$ and $[\text{Na}^+]_{\text{trans}}$. Closed and open dwell-time histograms for the main and two subconductive states in $< 1 \text{ nM}$ $[\text{Ca}^{2+}]$ conditions were virtually the same as was described for *trans*-to-*cis* sodium gradient experiments (see below). Inhibition of channel activity was observed when $[\text{Ca}^{2+}]_{\text{cis}}$ was increased above $5 \mu\text{M}$. Single-channel P_o decreased to 0.09 ± 0.01 and 0.02 ± 0.01 when $[\text{Ca}^{2+}]_{\text{cis}}$ was raised to 5 and 25 μM , respectively. Events amplitude histograms (Fig. 7B) derived from these records revealed that the relative proportion of each conduction state remained the same at any $[\text{Ca}^{2+}]_{\text{cis}}$, but that new shoulders appear on the peaks, suggestive of fast flickering behavior of the channel. Fig. 7C depicts closed and open time histograms of these channels in the presence of 25 μM $[\text{Ca}^{2+}]_{\text{cis}}$. These histograms indicate that channel inhibition occurred because of a decrease in both open and closed times of the channel. Interestingly, these effects of calcium were minimal when $[\text{Na}^+]_{\text{cis}}$ was maintained at 10 mM. Elevating $[\text{Na}^+]_{\text{cis}}$ resulted in a leftward shift of the P_o vs $[\text{Ca}^{2+}]_{\text{cis}}$ dose-response curve (Fig. 8).

Protein Kinase C Induced Phosphorylation of Renal Amiloride-sensitive Na⁺ Channels Shifts Their Sensitivity to Sodium and Calcium.

It has been reported that protein kinase C inhibits the activity of immunopurified renal Na⁺ channels subsequent to activation with protein kinase A (Oh and Benos,

FIGURE 7. (A) Effects of calcium on activity of immunopurified renal amiloride-sensitive sodium channel reconstituted into planar lipid bilayers. Records shown are for -80 mV holding potential. Bilayers containing reconstituted channels were bathed with asymmetrical salt solutions (10 mM Na_{cis}/100 mM Na_{trans}, 10 mM MOPS, pH 7.5). Medium was buffered with 10 mM Ca-EGTA to achieve the desired free Ca²⁺ concentration. (B) All points and events amplitude histogram of single amiloride-sensitive renal sodium channel current at different [Ca²⁺]_{cis}. Events list was generated by pCLAMP software from the record of 15-min length with 50% amplitude detection threshold and 5-ms duration threshold. All points amplitude histograms were fitted with Gaussian functions. (C) Events dwell time histograms of single amiloride sensitive renal sodium channel current at 25 μM [Ca²⁺]_{cis}. Histograms were constructed from 4,825, 877, 827, and 1,505 events for levels 0 through 3, respectively. Values were calculated from single and double-exponential fits of the data for levels 1 through 3 (*open states*) and double-exponential fits for level 0 (*closed state*).

B 10 mM Na_{cis} / 100 mM Na_{trans} **C** 25 μM Ca^{2+}

1993). To study the mechanism of PKC action in the absence of possible interactions between these two protein kinases, we used an $[\text{Na}^+]$ gradient to increase the initial level of activity. Fig. 9 depicts representative current traces from these experiments. Traces for ± 80 mV are presented. Under control conditions, the channel had an open probability of 0.11 ± 0.01 ($N = 3$). Addition of PKC while elevating $[\text{ATP}]$ in the *cis* bathing solution resulted in a progressive inhibition of channel activity. Moreover, there were bursts of flickery-type channel openings upon phosphorylation, in contrast to the relatively long events seen before ATP addition. This phosphorylation reaction required the presence of diacyl glycerol (DAG) and ATP in the *cis* compartment. Diacyl glycerol and ATP dose-response curves are presented in Fig. 10. These curves have a sigmoid shape and were used to choose the conditions for the subsequent $[\text{Ca}^{2+}]$ dose-response experiments. In separate ex-

periments ($N = 10$), addition of ATP or PKC alone, or in combinations with DAG to the *trans* bathing solution was without effect.

Current recordings of these latter experiments are shown as Fig 11. Again, Ca^{2+} was effective only when was added to the *cis* side of the bilayer. Characteristically, much lower concentrations of Ca^{2+} inhibited channel activity following PKC-mediated phosphorylation. Fig. 12 depicts the resulting $[\text{Ca}^{2+}]$ dose-response curves. A leftward shift of the curves towards more physiological $[\text{Ca}^{2+}]$ was observed for all of the tested concentrations of $[\text{Na}^+]_{\text{trans}}$, both in the absence and presence of PKC-mediated phosphorylation. However, it is also apparent from these data that $[\text{Na}^+]_{\text{trans}}$ has much less of an effect on channel open probability subsequent to treatment with PKC.

FIGURE 8. $[\text{Ca}^{2+}]$ dose-response curves of the activity of a single amiloride-sensitive sodium channel in a planar lipid bilayer bathed with asymmetrical sodium solutions. Bilayers containing a reconstituted channel were bathed with asymmetrical salt solutions (see legends on the plot). Points in the plots are Mean $\pm$ SD for $n \geq 3$ for each concentration.

DISCUSSION

Immunopurified Renal Na^+ Channels Have Multiple Subconductive States

The presence of subconductive states in current recordings of amiloride sensitive Na^+ channels incorporated into planar lipid bilayers has been previously reported (Sariban-Sohraby et al., 1984; Garty and Benos, 1988). This phenomenon remained largely unexplored in these earlier studies, although it was suggested that the variability in conductance could be related to the existence of possible different stable conductive states, as it is the case for the gramicidin A channel (Busath and Szabo, 1981). On the other hand, based on the conductance vs Na^+ activity curves constructed for two extremes of channel openings (namely, 4 and 44 pS), Olans, Sariban-Sohraby, and Benos (1984) proposed that two populations of amiloride sensitive Na^+ channels could also account for the data. Two channels with different conductances and Na^+ sensitivity were also reported by Hamilton and Eaton

FIGURE 9. Protein kinase C induced phosphorylation of immunopurified amiloride-sensitive sodium channel reconstituted into planar lipid bilayer. Holding potential ± 80 mV. Bathing solution in *trans* compartment contained 100 mM NaCl, 10 mM MOPS, pH 7.5; *cis* compartment contained NaCl as indicated for each trace, $2.3 \mu\text{M}$ $[\text{Ca}^{2+}]$, 5.4 ng/ml PKC, $5 \mu\text{M}$ DAG, $100 \mu\text{M}$ ATP, 10 mM MOPS, pH 7.5. EGTA (mM) was present on both sides. PKC, DAG, or ATP alone DAG in combination with ATP had no effect on single-channel properties ($N = 10$ for each condition, data not shown). Records were filtered at 100 Hz using an 8-pole Bessel filter before the acquisition and were sampled at 1,000 Hz using Digidata 1,200 interface.

(1986*a,b*) in patches of A6 cells, and by Ling, Hinton, and Eaton (1991) in cell-attached patches of rabbit cortical collecting tubule primary cultures grown on collagen supports in the presence of aldosterone. However, the alternate possibility that these channels are not different biochemical entities but result from a mani-

festation of different subconductive states of the same channel, should also be considered.

The results presented in this paper support this latter hypothesis. Events amplitude histograms (Fig. 1 *B*) constructed from single-channel recordings without and with a *trans*-to-*cis* $[\text{Na}^+]$ gradient suggest that at each $[\text{Na}^+]_{\text{cis}}$ the relative proportion of the two smaller current levels did not change (namely, 17, 17, and 66% of the total, for levels 1, 2, and 3, respectively). The overall distribution of channel openings is not binomial as would be expected for three independent channels with the same P_o (Hille, 1992). Moreover, these three conductance states were observed during every successful incorporation. If these were independent channels, we would expect situations in which only 12, 12, and 24, 12 and 40, 24, 24, and 40,

FIGURE 10. Dependence of single amiloride-sensitive sodium channel open probability on diacylglycerol and ATP concentrations in presence of protein kinase C. Bathing solution contained 100 mM $[\text{NaCl}]_{\text{trans}}$, 10 mM $[\text{NaCl}]_{\text{cis}}$, $[\text{Ca}^{2+}]_{\text{trans}} < 1 \text{ nM}$, 2.3 μM $[\text{Ca}^{2+}]_{\text{cis}}$, 10 mM EGTA and 10 mM MOPS, both sides, pH 7.5. 5.4 ng/ml PKC was added to *cis* compartment.

or 40 pS conductance channels were observed. Thus, it is likely that this epithelial amiloride-sensitive Na^+ channel has stable subconductive states.

Scrutiny of our experimental conditions reveals another important phenomenon that is typical for these renal amiloride-sensitive Na^+ channels. In 90% of all successful incorporations, the channel 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., 1994*a,b*). The *trans* side, in contrast, was amiloride-sensitive but insensitive to other agents that presumably act from the cell interior. This orientation undoubtedly occurs because of the procedure that we have developed for reconstitution of the channel into the bilayer. Bilayer membranes were composed of a mixture of negatively charged diphytanoyl-phosphatidylserine with neutral di-

FIGURE 11. Effects of $[Ca^{2+}]$ on the activity of a single amiloride-sensitive sodium channel in a planar lipid bilayer phosphorylated by protein kinase C. Records for -80 mV are shown. Dashed line indicates zero-current level. Bathing solution in *trans* compartment contained 100 mM NaCl, 10 mM MOPS, pH 7.5; *cis* compartment contained 10 mM NaCl, 5.4 ng/ml PKC, 100 μ M ATP, 5 μ M DAG, Ca^{2+} as indicated for each trace, 10 mM MOPS, pH 7.5. EGTA (10 mM) was present on both sides. Records were filtered at 100 Hz using an 8-pole Bessel filter before the acquisition and were sampled at 1,000 Hz using a Digidata 1200 interface.

phytanoyl-phosphatidylethanolamine and oxidized cholesterol in *n*-octane, and were voltage clamped to -40 mV (in *cis* compartment) while reconstituted proteoliposomes were spread over a preformed bilayer with a fire polished glass capillary (from the *trans* side). In experiments where the membrane voltage was held at 0 mV ($n > 50$), the channels oriented randomly in the bilayer. Thus, this simple manipulation allowed us to know a priori that this amiloride-sensitive sodium channel orients its cytoplasmic face toward the *cis* compartment and its extracellular surface toward the *trans* compartment.

Feedback Regulation of Na^+ Channels

There are three major conclusions regarding feedback regulation of Na^+ channels that can be drawn from our experiments. First, channel open probability was increased simply by lowering the *cis* (i.e., cytoplasmic) $[\text{Na}^+]$. These results are consistent with the theory of feedback regulation of epithelial sodium channels. The essential feature of this mechanism is that Na^+ entry channels are inhibited whenever intracellular Na^+ rises. Thus, in combination with a lower driving force for Na^+ entry, a decreased single-channel P_o would work to prohibit large increases in $[\text{Na}^+]_i$ and cell volume.

The observation of increased channel open probability upon elevation of $[\text{Na}^+]_{\text{trans}}$ is contradictory to the inverse relationship between macroscopic Na^+ permeability and mucosal $[\text{Na}^+]$ that was reported for frog skin (Fuchs et al., 1977, Els and Hel-

FIGURE 12. $[\text{Ca}^{2+}]$ dose-response curves of the activity of a single amiloride sensitive sodium channel in a planar lipid bilayer bathed with asymmetrical sodium solutions in presence and absence of protein kinase C. Bilayers containing a reconstituted channel were bathed with asymmetrical salt solutions (see legends on the plot). Points in the plots are mean $\pm$ SD for $n \geq 3$ for each concentration.

man, 1991), toad urinary bladder (Li, Palmer, Edelman, and Lindemann, 1982; Palmer et al., 1980), *Necturus* urinary bladder (Thomas et al., 1983), and rabbit descending colon (Thompson and Sellin, 1986; Turnheim et al., 1983). This discrepancy between the observations made in intact epithelia and those presented here may be reconciled by considering that increasing luminal $[\text{Na}^+]$ in these experiments inevitably elevated intracellular $[\text{Na}^+]$. Hence, in native tissues, it may be unclear whether external or internal $[\text{Na}^+]$ was downregulating Na^+ transport. Subsequent studies by Palmer and coauthors (Silver et al., 1993; Frindt, Silver, Windhager, and Palmer, 1993) confirmed the existence of negative feedback regulation of Na^+ channels by cytoplasmic Na^+ in rat cortical collecting tubules. In contrast, single cell-attached Na^+ channel recordings of K^+ -depolarized rat cortical collecting tubules failed to demonstrate a dependence of P_o on external (pipette)

[Na⁺] (Palmer and Frindt, 1988). However, in these experiments changes in external [Na⁺] were not made on the same cell and there was a wide variation in channel P_o , even under the same experimental conditions.

The second conclusion that can be drawn from our results is that [Na⁺]_{trans} affected channel P_o only when [Na⁺]_{cis} was below 30 mM. Under these conditions (while maintaining constant [Na⁺]_{cis} and elevating [Na⁺]_{trans}), a saturating increase of channel P_o was seen. Interaction between *trans* and *cis* sodium ions was uncompetitive, suggestive of multiple interaction sites for sodium on the channel complex itself. These sites may not necessarily lie within the conduction pathway, but may be located on the *trans*- and *cis*-facing surfaces of the protein, and thus be allosteric. These results are in agreement with the observed saturation of macroscopic Na⁺ currents with increasing external [Na⁺] in frog skin (Van Driessche and Lindemann, 1979). However, in contrast to the conclusion of these authors that only channel density decreases with increasing mucosal [Na⁺], single-channel current through these renal amiloride-sensitive channels in our experiments also demonstrated saturation behavior. This saturation behavior of single Na⁺ channel current agrees with earlier observations in bilayers (Olans et al., 1984; Oh and Benos, 1993b) and in patches of apical membranes from rat cortical collecting tubules (Palmer and Frindt, 1986). Moreover, elevating [Na⁺]_{cis} in our experiments shifts the apparent equilibrium constant (K_s) for [Na⁺]_{trans} to lower values, thus saturating Na⁺ flow at much lower [Na⁺]_{trans}.

The third conclusion of our study is that all the above effects of internal and/or external [Na⁺] are dependent on [Ca²⁺]_{cis}. Buffering of [Ca²⁺]_{cis} to <1 nM with 10 mM EGTA significantly increased the basal level of channel activity (from a P_o of ~0.02 to a P_o of ~0.1). Moreover, this maneuver eliminated the dependence P_o on either *cis* or *trans* [Na⁺]. These data are consistent with the observation in intact epithelia that inhibition of basolateral Na⁺, K⁺-ATPase with ouabain decreased the apical Na⁺ entry only if the serosal medium contained more than 0.1 mM Ca²⁺ (Chase, 1984; Garty and Lindemann, 1984; Palmer, 1985; Silver et al., 1993).

Our experiments, however, were unable to determine whether [Ca²⁺] or [Na⁺] plays the primary role in modulating channel P_o . [Ca²⁺] dose-response experiments revealed that both [Ca²⁺] and [Na⁺] affect channel kinetics, although their effects have different manifestations. When [Ca²⁺]_{cis} was increased <5 μ M a flickering behavior of current flow became apparent. Closed and open-time histograms of channel openings in the presence of 25 μ M [Ca²⁺]_{cis} indicate that inhibition occurs because of a decrease in both open and closed times of the channel. The closed time histogram is best fit by two exponentials. The fast exponent may be due to a fast flickering-type block produced by Ca²⁺, and the long one due to normal channel closures. Characteristically, inhibition of channel activity by [Ca²⁺]_{cis} was observed at concentrations far above the physiological range. Elevating [Na⁺]_{cis} resulted in a leftward shift of the P_o vs [Ca²⁺]_{cis} dose response curve, again suggesting that both [Ca²⁺] and [Na⁺] are involved in regulation of this channel. Further, protein kinase C induced phosphorylation of this renal amiloride-sensitive Na⁺ channel shifted these concentration dependence curves to values closer to the physiological range of Ca²⁺ concentrations. This observation, again, is consistent with the report that the increase in P_o upon reducing mucosal [Na⁺] could be pre-

vented by the Ca^{2+} ionophore A23187 or by activators of PKC (Ling and Eaton, 1989).

In conclusion, we provide direct evidence in support of the hypothesis that the gating behavior of single renal amiloride-sensitive Na^+ channels is regulated by Na^+ itself. We also show that intracellular Ca^{2+} can directly affect Na^+ channel activity, and that its availability is required for Na^+ to affect channel gating. Moreover, the inhibitory effects of Ca^{2+} are greatly enhanced by PKC-mediated phosphorylation. Thus, the inherent feedback control of epithelial Na^+ channels by small ions provides a hormonally independent means of modulating in a precise and instantaneous way the rate of Na^+ entry into epithelial cells.

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