SATURATION BEHAVIOR OF SINGLE, AMILORIDE-SENSITIVE NA$^+$ CHANNELS IN PLANAR LIPID BILAYERS

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ABSTRACT Single epithelial Na$^+$ channels incorporated into planar lipid bilayers were studied to determine the effects of Na concentration on its own conductance. Amiloride-sensitive Na$^+$ channels were obtained from apical membrane vesicles made from A6 cells, a continuous epithelial cell-line derived from amphibian kidney. Single-channel conductance was found to be a saturable function of Na$^+$ concentration, with a Michaelis constant of ~17 or 47 mM, for a G_{max} of ~4 or 44 pS, respectively.

INTRODUCTION

Saturation of macroscopic Na$^+$ influx [or, alternatively, short circuit current (I_{sc})] with increasing external Na$^+$ concentration in in vitro frog skin epithelia was first described by Kirschner (7). There exist in the literature two interpretations of this saturating behavior of I_{sc}. In the first view, saturation occurs because of a direct interaction of Na$^+$ with the channel, the overall rate of transport being limited by the translocation step (7, 11). In the second view, saturation occurs as a result of Na$^+$ binding to a modifier site, in effect inhibiting its own flux (5, 9, 10). This second interpretation has profound implications concerning the mechanism of ion permeation through these entry channels, which are sensitive to inhibition by the diuretic compound amiloride (1, 2, 16). Saturation produced by self-inhibition means that the transport entities themselves do not saturate with increasing [Na_o^+].

This substrate inhibition hypothesis is supported by the results of experiments in which a time-dependent relaxation of I_{sc} following a step increase in [Na_o^+] was observed (5). First, the peaks of these current transients did not saturate with [Na_o^+], while the steady-state values of I_{sc} did. Second, it was found that certain molecules, benzimidazolyl-guanidine (BIG), *p*-chloromercuribenzoic acid (PCMB), and *p*-chloromercuribenzene sulfonic acid (PCMBS) that stimulated I_{sc} did so by eliminating the steady-state current component (4, 10). Third, Fuchs et al. (5) and Palmer et al. (12) found that the current-voltage relationship through amiloride-sensitive, Na$^+$ entry sites could be well described by continuum electrodiffusion theory. Fourth, experiments in which single Na$^+$ channel properties were deduced from amiloride-induced current fluctuations showed a decrease in conducting channel density with increasing [Na_o^+], with no evidence of saturation of individual channels up to 100 mM [Na_o^+]. These noise experiments were performed in frog skins depolarized with high serosal potassium.

For this study, we used the cultured established cell line A6, derived from toad kidney (13). Previous characterization of these cells (14) has revealed that (*a*) apical sodium uptake is a saturable function of sodium concentration in the mucosal bath; (*b*) this sodium transport is reversibly inhibited by the diuretic drug amiloride, with an apparent inhibitory dissociation constant of 0.05 μM; and (*c*) incubation of the epithelium with the steroid hormone aldosterone leads to a threefold increase in apical sodium uptake. These properties of the apical sodium transport system are similar to those found in naturally occurring sodium-transporting tissues such as frog skin and toad bladder, and thus make the A6 cell-line a good model system for characterizing the apical entry step for sodium in high resistance epithelia.

The goal of our study was to address the issue of whether individual amiloride-sensitive Na$^+$ channels saturate or not. After channel incorporation into planar lipid bilayer membranes, single channel conductance was measured as a function of Na$^+$ concentration. We found that individual amiloride-sensitive Na$^+$ channels saturated with increasing [Na$^+$] regardless of the absolute value of their maximal conductance.

MATERIALS AND METHODS

Cell Culture and Vesicle Preparation

Apical membrane vesicles were prepared from the continuous A6 epithelial cell line originally derived from *Xenopus laevis* kidney (13). Cells reach confluence by day 7 or 8, after plating at a density of $\sim 4 \times 10^4$ cells/ml. A6 cells were grown on 0.45-μm filters (Millipore/Continental Water Systems, Bedford, MA) as described by Sariban-Sohraby et al. (14). A6 cells grown in culture form confluent epithelia, with their basal surface oriented against the substrate. When grown on permeable supports, A6 cells possess amiloride-sensitive sodium transport activity, as assessed from $^{22}Na^+$ influx measurements. Such activity, however, was not detected if cells were grown on conventional plastic culture dishes (14). Cells were fed twice a week with growth medium consisting of seven parts Ham's F-12, three parts Liebovitz's L-15, 100 mU/ml penicillin, 100 μg/ml streptomycin, 1 mM glutamine, and 10% fetal bovine serum. This mixture was modified for amphibian cells to contain 90 mM NaCl and 8 mM $NaCO_3$, and adjusted to pH 7.4 with NaOH. A potential difference of at least 10 mV across the mucosal (outer) and serosal (inner) sides of the filter served to indicate a mature Na^+ transport system. This transepithelial potential was measured as the potential difference between two calomel electrodes connected to the mucosal and serosal sides of the filter via 3% agar bridges. A6 cells with a transepithelial potential difference were used for vesicle preparation as described earlier (16). Briefly, cells were rinsed with homogenization medium containing 10 mM Tris-Hepes, 10 mM $CaCl_2$, and 30 mM mannitol at pH 7.4, and then scraped in the same medium. Scraped cells were homogenized with a homogenizer (Vir Tis Co., Inc., Gardiner, NY) (10 min at a setting of 40, and 4 min at a setting of 100). $CaCl_2$ served to precipitate the cells' nuclei. Homogenization was followed by differential centrifugation to separate apical membrane fragments. The apical membrane-containing pellet was resuspended in a buffer containing 0.5 M sucrose, 1 mM NaCl, and 10 mM Tris-Hepes, pH 7.4. 50-μl aliquots of this apical membrane vesicle suspension were stored in cryotubes under nitrogen at $-80°C$.

Lipid Bilayer Formation

Experiments were performed using planar lipid bilayers formed from a mixture of 10 mg/ml bovine brain phosphatidylethanolamine (PE) and 10 mg/ml bovine brain phosphatidylserine (PS). The lipids were combined in a PE/PS ratio of 7:3. Since both PE and PS were originally stored in chloroform, the chloroform was evaporated off with nitrogen and replaced with decane, bringing the final concentration of the lipid mixture to 20 mg/ml. Using glass rods, lipid bilayers were painted onto polystyrene cups having circular holes 150–340 μm in diameter. The bilayer separated two aqueous chambers containing equal concentrations of salt solution. The two chambers were filled to the same height in order to eliminate hydrostatic pressure differences. The concentration of salt solution used depended upon the experiment to be carried out. Generally, the chambers initially contained symmetrical solutions of either 10 or 100 mM NaCl and 10 mM morpholinopropane sulfonic acid- (MOPS-) Tris adjusted to pH 7.0 with Tris Base. All solutions were sterilized by passage through a 0.22-μm Millipore filter. Sodium concentrations were measured using an atomic absorption spectrophotometer (model 5000, Perkin-Elmer Corp., Instrum. Div., Norwalk, CT).

Electrical Measurement

Electrical measurements of channel properties were made using a two-electrode voltage clamp (8). The *cis* component was connected to a function generator that controlled membrane potential. The *trans* chamber was connected to a current-to-voltage converter and to an operational amplifier in order to adjust the gain of the circuit. Consequently, the *trans* side was at virtual ground potential. Positive current was defined as the flow of positive ions from the *cis* side to the *trans* side. Both sides were connected to the electronics via 0.1 M NaCl agar bridges in series with Ag/AgCl electrodes.

Incorporation of Vesicles into Planar Bilayers

Once a stable membrane was obtained, NaCl was added to the *cis* side to create an osmotic gradient. The NaCl concentration was raised to 100–350 mM on the *cis* side. Concentrations were measured by removing solution samples from both the *cis* and *trans* chamber and determining sodium ion activity using a sodium-specific electrode (Orion Research, Inc., Cambridge, MA). Next, 15–30 μl of A6 vesicle suspension ($\sim$1 mg/ml protein) was added to the *cis* compartment. The chamber pools were stirred with small magnetic fleas until a conductance jump (indicating channel incorporation) was noted. Once incorporation occurred, stirring was stopped in order to prevent the incorporation of additional protein. In some experiments, the *cis* chamber was mechanically perfused with fresh salt solution to remove excess protein. The choice of replacement solution for the *cis* chamber upon perfusion depended upon the experiments to be performed. In general, the replacements were salt solutions containing 10 mM MOPS-Tris at pH 7.0.

Data Collection and Analysis

Currents were monitored simultaneously on an oscilloscope and a strip chart recorder. In some experiments channel data were recorded on FM tape for later analysis. Currents were low-pass filtered at 60–100 Hz unless otherwise noted. The resolution of the amplifier and recording circuit was 1 pA in current amplitude and 1 ms in time. Under conditions of symmetrical salt concentration, single-channel conductance values were determined using Ohm's law. At least 10 current fluctuations under a given set of experimental conditions were averaged.

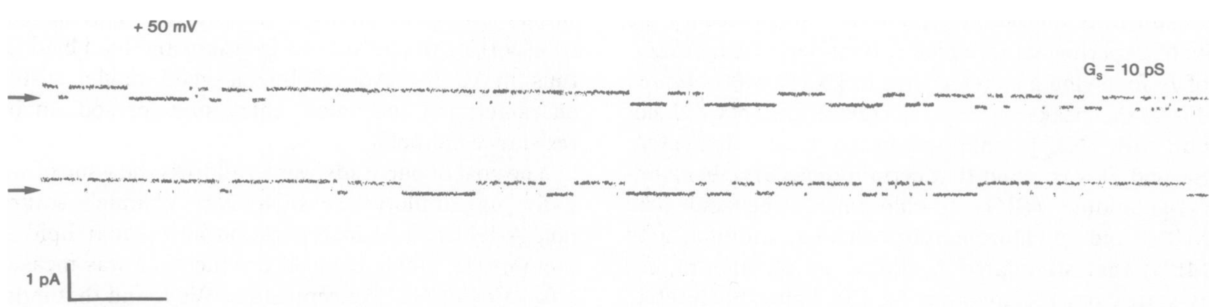

FIGURE 1 Single, amiloride-sensitive, Na^+ channel current records obtained after the fusion of A6 apical membrane vesicles into planar lipid bilayers. Salt concentrations were symmetrical at 10 mM NaCl buffered to pH 7.0 with 10 mM MOPS-Tris. The closed time of a fast state and of the long-lived closed state can also be distinguished.

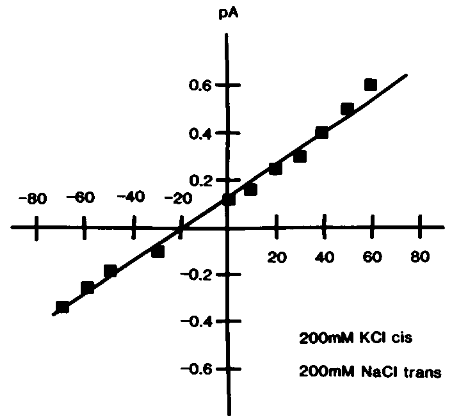

FIGURE 2 Bi-ionic potential measurement for amiloride-sensitive channel shown in Fig. 1. Salt concentrations were 200 mM KCl *cis* and 200 mM NaCl *trans*. Solutions were buffered to pH 7.0 with 10 mM MOPS-Tris. The zero current potential for this channel was −20 mV.

RESULTS AND DISCUSSION

Single-channel currents from A6 apical membrane vesicles incorporated into planar lipid bilayers are shown in Fig. 1. By convention, the arrow indicates the current level when the channel is in its closed state, whereas the upward deflection represents the open state. Hence, channel openings and closings trace out rectangular current pulses. In this experiment, the bilayer was bathed symmetrically with 10 mM NaCl. The slope of the channel open-state current-versus-voltage curve yielded a conductance of 7 pS (data not shown). This channel was similar in behavior to the Na^+ channel characterized by Sariban-Sohraby et al. (15) with regard to amiloride sensitivity, cationic vs. anionic selectivity, and closed and open dwell-times. Likewise, the channel conductance and probability of being in the open state were independent of voltage. Finally, the permeability of the channel for Na^+ relative to K^+ was 2:1,

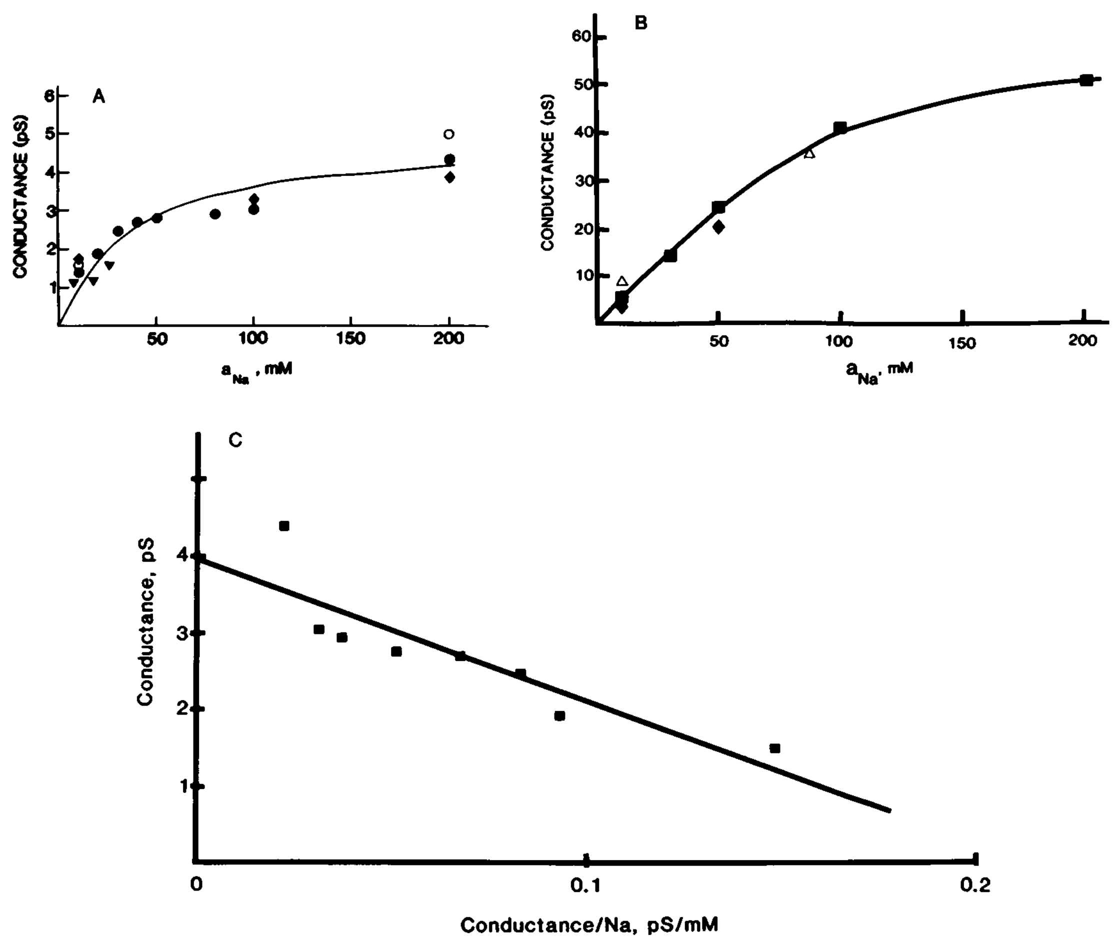

FIGURE 3 (*A*) Saturation of small conductance amiloride-sensitive channels with increasing symmetrical NaCl activities. Sample points were obtained from experiments with three different membranes. K'_m was 17 mM while G_{max} was 4 pS. (*B*) Saturation of large conductance amiloride-sensitive channels with increasing symmetrical concentrations of NaCl. Sample points were obtained from experiments with three different membranes. K'_m was 47 mM while G_{max} was 44 pS. K'_m represents that concentration at which conductance is 50% of its maximum value. G_{max} is analogous to V_{max} in enzyme substrate kinetics. (*C*) Single reciprocal plot of channel conductance vs. Na activity under asymmetrical NaCl conditions. *Trans* NaCl activity was maintained at 10 mM, while *cis* NaCl activity was increased from 10 to 200 mM. G_{max} was 4 pS and K'_m was 18.5 mM.

as previously described (15). This selectivity was determined by plotting a current vs. voltage relationship under the experimental conditions of 200 mM NaCl *trans* and 200 mM KCl *cis*, as Fig. 2 shows. A reversal potential of -20 mV was obtained from the current-versus-voltage plot. Using the Goldman-Hodgkin-Katz potential equation, this zero current measurement gives an Na^+/K^+ permeability ratio of 2:1. The same reversal potential, and hence permeability ratio, were obtained regardless of the absolute conductance of the amiloride-sensitive Na^+ channels (15).

Variations in channel open-state conductance with symmetrical changes in NaCl concentrations at a potential of $+50$ mV are depicted in Fig. 3. Channels with either low or high conductance levels but having otherwise similar properties (15) were studied. The upper curve (Fig. 3 *A*) shows values obtained from three different membranes in which low-conductance (G_{max} = 4 pS) amiloride-sensitive channels have incorporated. Fig. 3 *B*, on the other hand, shows conductance as a function of concentration for high conductance (G_{max} = 44 pS) amiloride-sensitive channels. Both curves reach a saturating conductance when the NaCl concentration is raised sufficiently high. Such saturation behavior can be analyzed in terms of Michaelis-Menten kinetics. Single reciprocal Eadie-Hofstee plots of the data yield a K'_m of 17 mM and a G_{max} of 4 pS for the channel shown in Fig. 3 *A*, and a K'_m of 47 mM and a G_{max} of 44 pS for the higher conductance channel (Fig. 3 *B*). K'_m represents that concentration at which the conductance is at 50% of its maximal value. Similar measurements of conductance were performed at a constant 10 mM NaCl on the *trans* side while varying the *cis* NaCl concentration from 10 to 200 mM. In this particular experiment, G_{max} was 4 pS and K'_m 18.5 mM (Fig. 3 *C*). The value of K'_m ($\sim$17 mM), obtained in both symmetrical and asymmetrical Na^+ concentrations in the low conductance channels, is in excellent agreement with the value of 18 mM, measured in intact A6 cells by varying the mucosal $[Na^+]$ (14). These data suggest that the affinity of the transporter for Na^+ is independent of the $[Na^+]$ on the opposite side.

It is clear that both high- and low-conductance amiloride-sensitive channels saturate in accordance with Michaelis-Menten kinetics. Conductance vs. concentration plots for sodium activities ranging from 10 to 200 mM (Fig. 3) reveal that individual amiloride-sensitive sodium channels saturate with increasing sodium activities. These data are not in accord with the proposed substrate inhibition model deduced from noise analysis measurements of single-channel properties (16). In the noise experiments of Van Driessche and Lindemann, amiloride-sensitive Na^+ currents were measured for Na^+ concentrations ≤ 110 mM. The results reported here for amiloride-sensitive channels reconstituted from A6 apical membrane vesicles are at variance with the noise results where the K'_m for Na^+ was independent of the absolute magnitude of the single-channel conductance.

The significance of transepithelial currents measured across amphibian skins in the presence of high serosal K^+ must also be reevaluated. Using a two-state channel model (i.e., block or unblocked), Van Driessche and Lindemann (17) found that the blocking (or association) rate constant for amiloride in *R. esculenta* skin epithelia in the presence of high serosal K^+ concentrations was independent of Na^+ concentration.[1] In contrast, in similar experiments carried out by Hoshiko (6) with *R. temporaria* skins and serosal Na^+ in place of K^+, the rate constant for channel blocking was found to depend upon Na^+ concentration. Clearly, different results were obtained in the presence of serosal K^+ Na^+. Furthermore, preliminary data relating single-channel Na^+ currents deduced from fluctuation analysis on *R. temporaria* skins in the presence of serosal Na^+ rather than K^+ suggest that individual amiloride-sensitive Na^+ channels do saturate (6). These data are in accord with our direct measurements of the saturation behavior of amiloride-sensitive Na^+ channels in bilayers. High serosal K^+, in addition to depolarizing the basolateral membrane, also results in a 7–10-fold increase in intracellular levels of cyclic AMP (3). Hence, single-channel properties measured in K^+-depolarized skins may be altered due to modifications in intracellular hormonal and/or ionic concentrations.

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