J. Membrane Biol. 127, 121–128 (1992)

The Journal of
Membrane Biology

Toad Bladder Amiloride-Sensitive Channels Reconstituted into Planar Lipid Bilayers

Luiz G.S. Branco and Wamberto A. Varanda
Department of Physiology, Faculty of Medicine of Ribeirão Preto, USP. 14049, Ribeirão Preto S.P., Brazil

Summary. In the present study we used established methods to obtain apical membrane vesicles from the toad urinary bladder and incorporated these membrane fragments to solvent-free planar lipid bilayer membranes. This resulted in the appearance of a macroscopic conductance highly sensitive to the diuretic amiloride added to the *cis* side. The blockage is voltage dependent and well described by a model which assumes that the drug binds to sites in the channel lumen. This binding site is localized at about 15% of the electric field across the membrane. The apparent inhibition constant ($K_{(0)}$) is equal to 0.98 μM. Ca^{2+}, in the micromolar range on the *cis* side, is a potent blocker of this conductance. The effect of the divalent has a complex voltage dependence and is modulated by pH. At the unitary level we have found two distinct amiloride-blockable channels with conductances of 160 pS (more frequent) and 120 pS. In the absence of the drug the mean open time is around 0.5 sec for both channels and is not dependent on voltage. The channels are cation selective (P_{Na}/P_{Cl} = 15) and poorly discriminate between Na^+ and K^+ (P_{Na}/P_K = 2). Amiloride decreases the lifetime in the open state of both channels and also the conductance of the 160-pS channel.

Key Words cation channels · amiloride · lipid bilayer · reconstitution · toad bladder

Introduction

Tight epithelial tissues are characterized by the presence of Na^+-specific pathways in their apical membranes whose permeability can be hormonally controlled, e.g., by aldosterone (Palmer et al., 1982; Asher & Garty, 1988) and oxytocin (Van Driessche, Aelvoet & Erlij, 1987). This entry pathway is highly sensitive to amiloride (Benos, 1982) and was strongly correlated with ion channels by Lindemann and coworkers using current fluctuation analysis (Lindemann, 1980, 1984). More recently, studies on the short-circuited toad bladder have shown that blockage by amiloride is voltage dependent. The drug may sense about 15% of the transmembrane electric field (Palmer, 1984). Ca^{2+} and other divalents also block the channel (Palmer, 1985*b*). In apical membrane vesicles the effect of Ca^{2+} has been shown to be dependent on the pH of the solutions (Garty, Civan & Civan, 1985). In reconstitution experiments into planar lipid bilayers Sariban-Sohraby et al. (1984) reported the appearance of channels with conductances ranging from 5 to 59 pS. These channels are sensitive to amiloride from both sides and poorly discriminate between Na^+ and K^+. This low selectivity is in agreement with results concerning apical sodium channels studied by Hamilton and Eaton (1985) with the patch-clamp technique. On the other hand, Frings, Purves and Macknight (1988), using the same technique, described highly selective Na^+ channels. However, their experiments were performed in the presence of bretylium which may have influenced the properties of the sodium channels. The conductances reported by the above authors also vary: Hamilton and Eaton (1985) describe channels with 7 to 10 pS while Frings et al. (1988) show values in the range of 5 to 59 pS and 86 to 490 pS when the patches were exposed to fluoride. Reinhardt, Garty and Lindemann (1985) reconstituted an amiloride-sensitive Na^+ channel from toad bladder into PE-decane lipid bilayers having 155 pS. Functional expression of mRNA into frog oocytes resulted in macroscopic currents or ^{22}Na fluxes inhibitable by amiloride with kinetic parameters similar to those observed in the intact tissue (George et al., 1989; Palmer et al., 1990). No data have been reported at the single-channel level. Amiloride-binding proteins were isolated both from A-6 cells (Benos et al., 1986) and porcine kidney (Frelin et al., 1988).

In this paper we present results concerning the incorporation of channels from the apical membrane of the toad bladder cells into planar lipid bilayers. Our results show the presence of cation channels highly sensitive to amiloride from both sides, which can be blocked by Ca^{2+} ions.

Materials and Methods

Toads (*Bufo paracnemis*) were double pitted and exsanguinated by transventricular perfusion with 200 to 300 ml of Ringer solution containing (in mM): 110.0 NaCl, 3.5 KCl, 2.5 $NaHCO_3$, 1 $CaCl_2$ and 5 glucose, pH 7.5. When the bladders became bloodless they were removed and placed in a beaker with ice-cold aerated Ringer, cut open and blotted on filter paper. Separation of apical membrane fragments was done according to procedures described by Chase and Al-Awqati (1983). Determination of ATPase activity by the method of Heinonen and Lahti (1981) in our preparation showed an enrichment of approximately six times in the apical fraction. After isolation the membranes were washed, repelleted and resuspended in a minimum volume of solution containing 500 mM sucrose and 10 mM Tris (pH 7.40). With this method each toad bladder yielded 81.2 ± 18.5 (n = 6) μg of protein. The samples were split into small aliquots and kept at −70°C. Prior to use each 10 μl of solution containing the apical membranes received 20 μl of 200 mM NaCl and 3 μl of 1% Triton X-100 (Sigma). This new solution was sonicated in a bath sonicator (Bransonic 220) for 90 sec and used immediately. Ten different preparations were tested in at least five membranes for each experimental condition.

Lipid bilayer membranes were formed by the apposition of two monolayers across a hole (150 to 200 μm in diameter) in a Teflon partition separating two aqueous solutions according to the method of Montal and Mueller (1972). Monolayers were spread from a solution of 1% (wt/vol) asolectin (Lecithin type II from Sigma) in hexane. The bare bilayer resistance was always higher than 2.5×10^{11} Ω, and the capacitance was between 0.7 and 0.9 $\mu F/cm^2$. The aqueous solutions (4 ml) contained 97.5 mM NaCl and 2.5 mM $NaHCO_3$ (pH 8.0), unless otherwise stated. Membrane electrical conductances were measured under voltage-clamp conditions as described elsewhere (Siqueira-Branco & Varanda, 1991). The ionic currents flowing through the membrane were sent to an oscilloscope (Gould OS-4000) and recorded on paper (Narco MK IV Physiograph). Voltage polarity refers to the side of the membrane containing vesicles (*cis* side). The vesicle-free side is called the *trans* side and was kept at virtual ground.

Fusion, evidenced by a sudden increase in the conductance of the membrane, was usually observed within 40 min after adding the vesicles (5 to 10 μl) to the *cis* solution, in the presence of a 3 : 1 NaCl gradient (0.3 M NaCl on the *cis* side). When desired the *cis* chamber was perfused with 40 ml of vesicle-free solution in order to interrupt fusion and/or to change the ionic composition across the membrane. Addition of calcium was not required, and channel incorporation was irreversible.

Data were analyzed by hand and digitized on an IBM-compatible PC/XT microcomputer for further processing. Single-channel conductances were determined by reading at least 100 events per voltage and averaging the amplitudes; then, an I-V plot was constructed and the conductance calculated. Mean open times were also calculated by averaging at least 100 readings, per voltage. All solutions were prepared with twice-distilled water and filtered through 0.2-μm filters (Millipore GSWP 02500). Amiloride (Merck Sharp and Dohme) was dissolved at a concentration of 1 mM and added to the chambers in small volumes as required.

Results

As a first approach to the reconstitution we incorporated as many channels as possible into a lipid bilayer and checked for the appearance of a macroscopic amiloride-blockable conductance. The results of these experiments are shown in Fig. 1. Figure 1*A* displays current *versus* time records for a membrane clamped at ±100 mV under control conditions and in the presence of 10^{-6} M amiloride on the *cis* side. It is clearly seen that for positive voltages the current rises and declines to a steady-state level within seconds. On the other hand, for negative voltages the block is partially reversed and the steady-state current level is higher than the instantaneous one. Figure 1*B* is a current *versus* voltage relationship for the experiment depicted in Fig. 1*A*. Amiloride blocks the conductance in a dose- and voltage-dependent manner. Under control conditions the membrane conductance at +100 mV is around 1200 pS, whereas it falls to about 600 pS in the presence of 10^{-6} M amiloride and to 250 pS in the presence of 10^{-4} M amiloride. Figure 1*B* also displays the characteristic rectifying behavior expected from a voltage-dependent blockade. Addition of amiloride at a final concentration of 10^{-4} M to both sides of the bilayer drastically reduces the conductance to values close to the bare bilayer (Fig. 2). If the *cis* side is subsequently perfused with drug-free solution (Fig. 2) the rectification is again apparent but in the opposite direction. These results suggest that either the channels are inserted without any orientation in the planar bilayer or that amiloride can block them from both sides. They also show that the blockade is a reversible process.

From the data in Figs. 1 and 2 we can now calculate the corrected amiloride-sensitive current by subtracting from the control I-V relationship in Fig. 1 the amiloride-insensitive portion in the presence of 10^{-4} M amiloride shown in Fig. 2. The same treatment is applied to the currents obtained in the presence of 10^{-6} M amiloride on the *cis* side. The result of these calculations is shown in Fig. 3*A*. Figure 3*B* is the fit of a sequential blocking model to the amiloride-sensitive fraction of the current as described in Woodhull (1973). As can be seen, the experimental points are adequately described by a relationship of the type:

$$\ln[(I_{Na}/I^B_{Na}) - 1] = \ln [B]/K_{(0)} + z\partial FE/RT \qquad (1)$$

where I_{Na} is the total amiloride-sensitive current, I^B_{Na} is the current obtained in the presence of the blocker, $[B]$ is the blocker concentration, $K_{(0)}$ is the apparent inhibition constant at zero voltage, ∂ is the fractional distance of the electric field sensed by the blocking site, E is the electric potential difference across the membrane, and z, F, R and T have their usual meanings. From the slope of the line we can

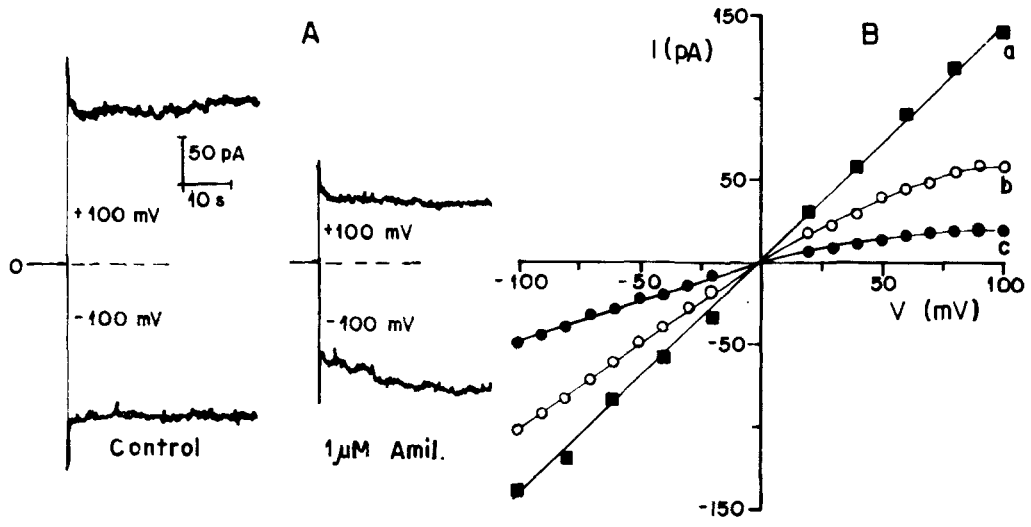

Fig. 1. Amiloride-blockable conductances. (*A*) A record of current *versus* time for a membrane clamped at ±100 mV (indicated on the side of each trace) under control conditions and with 10^{-6} M amiloride on the *cis* side. *0* indicates the current level with no potential applied. (*B*) An *I-V* plot constructed from records like those in *A*. Values of current refer to steady state. Curve *a* is control, and curves *b* and *c* are in the presence of 10^{-6} and 10^{-4} M amiloride, respectively. Traces in *A* were superimposed for presentation purposes. Bathing solutions were: 97.5 mM NaCl, 2.5 mM $NaHCO_3$, pH 8.0.

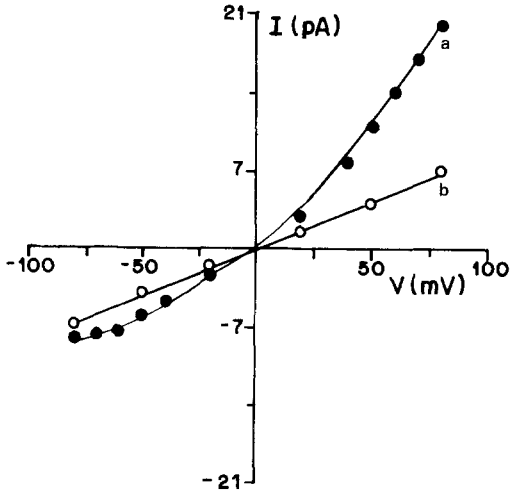

Fig. 2. Reversibility and sidedness of amiloride blockage. Amiloride was added at a final concentration of 10^{-4} M to both sides of a bilayer (curve *b*); the remaining conductance was taken to be amiloride-insensitive. The *cis* side was then perfused with amiloride-free solution, and a new *I-V* curve was obtained with the drug (10^{-4} M) on the *trans* side only (curve *a*). Electrolyte solutions were the same as in Fig. 1.

calculate the fractional distance of the electric field (∂) sensed by the blocker at its binding site. In this case $\partial = 0.15$. The intercept at zero voltage gives $K_{(0)} = 0.98\ \mu$M. Taken together these data are indicative that our preparation displays the essential features of the amiloride-blockable conductance present in the apical membrane of tight epithelia. In order to further strengthen this point we also tested the effect of Ca^{2+} ions on the currents. The reasoning for this experiment was based on (i) reports showing that Ca^{2+} blocks both the short-circuit current (Palmer, 1985*b*) and the fluxes of ^{22}Na in vesicles derived from the apical membrane of toad urinary bladder (Chase & Al-Awqati, 1983) and (ii) the fact that we never observed any measurable current through the bilayer if Ca^{2+} was present in the bathing solutions prior to fusing the vesicles to the planar bilayer. Figure 4 shows the same type of results as described in Fig. 1 but using Ca^{2+} on the *cis* side, instead of amiloride, as a blocker. The divalent ion induces a decrease in conductance which is both voltage and concentration dependent. Perfusion of the *cis* chamber with Ca^{2+}-free solution, plus EGTA, completely reverses the blockade. Also, addition of Ca^{2+} up to 1 mM to the *trans* side of the bilayer did not cause any decrease in the current. This is suggestive that the channels are preferentially oriented in the bilayer and that amiloride very probably interacts with the channel from both faces. Attempts to explain the Ca^{2+} blockade by assuming a simple sequential model were completely ineffective, as seen in Fig. 5. The relationship between $\ln[(I_{Na}/I^{B}_{Na}) - 1]$ and voltage is complex and could not be described even by assuming two binding sites for Ca^{2+}.

To further characterize this amiloride-blockable conductance, experiments were also done at the single-channel level. Thus, the amount of vesicles added to the *cis* side was decreased and the chamber

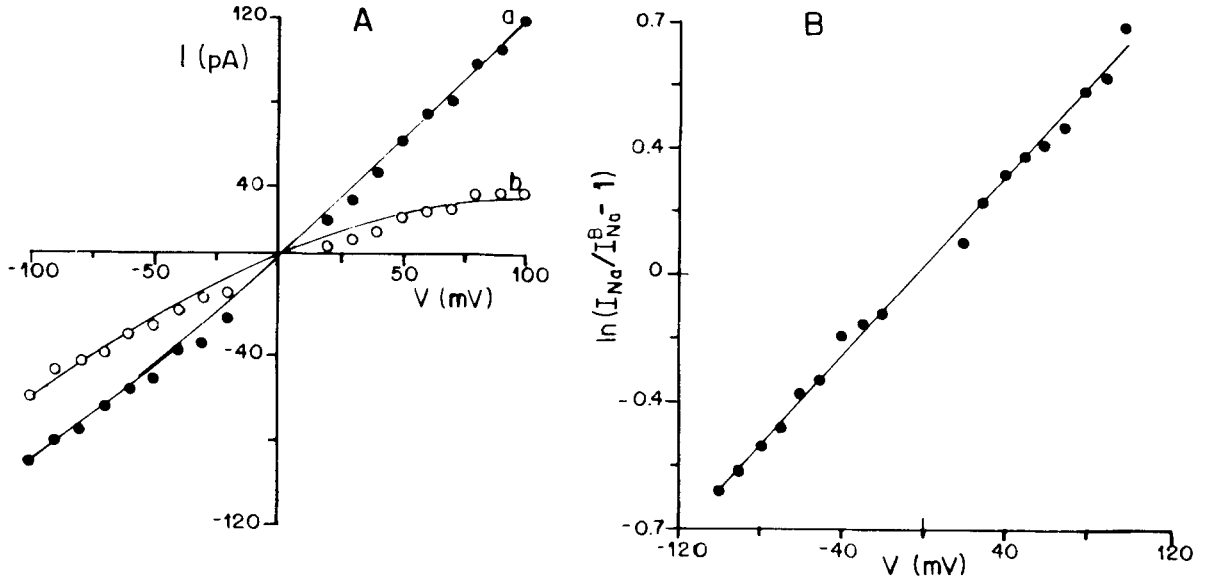

Fig. 3. Amiloride blockage is voltage dependent. (*A*) The *I-V* relationship for a bilayer under control conditions (curve *a*) but having subtracted the amiloride-insensitive portion of the current (curve *control* of Fig. 1 minus curve *b* of Fig. 2; this gives the corrected I_{Na} for each voltage). The same was done with curve *b* but in the presence of 10^{-6} M amiloride (i.e., curve *b* of Fig. 1 minus curve *b* of Fig. 2; this gives I^B_{Na}). (*B*) The fit of a sequential blocking model, as predicted by Eq. (1), to the data obtained with 10^{-6} M amiloride on the *cis* side. Solutions are the same as in Fig. 1.

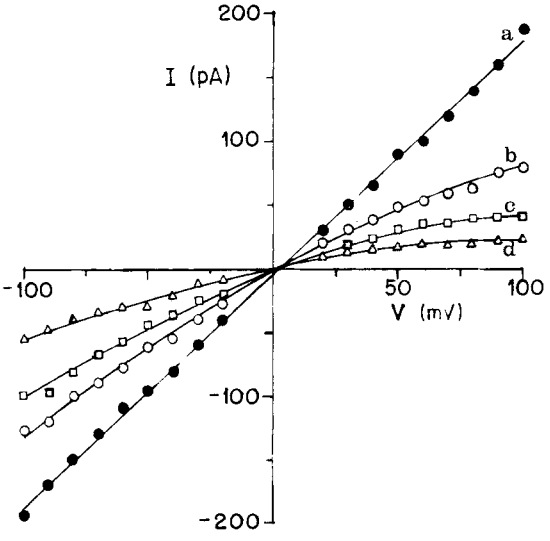

Fig. 4. *I-V* curves under control conditions (curve *a*) and with 10^{-6} M (curve *b*), 5×10^{-6} M (curve *c*) and 10^{-5} M (curve *d*) final concentration of $CaCl_2$ on the *cis* solution. Nominal Ca^{2+} concentration. Bath solution is 97.5 mM NaCl, 2.5 mM $NaHCO_3$, pH 8.0.

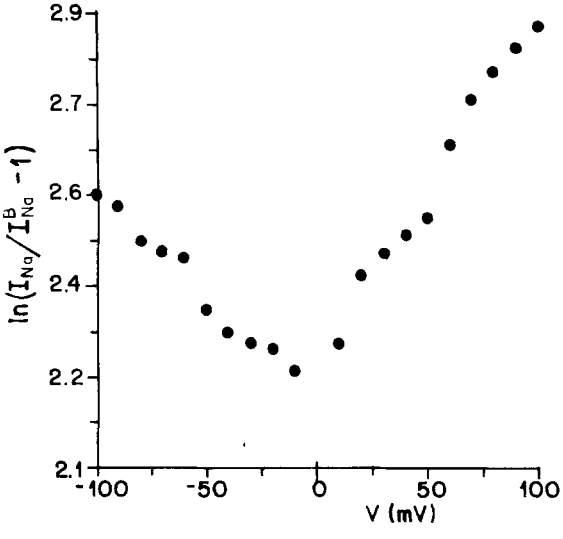

Fig. 5. Ca^{2+} blockade is a complex phenomenon. Data represent the ratios of currents under control conditions and in the presence of 50 μM $CaCl_2$ on the *cis* solution against voltage. Equation (1) does not fit the points.

perfused with vesicle-free 0.1 M NaCl solution as soon as one fusion event was detected. In general one fusion event leads to the incorporation of more than one channel into the membrane. Figure 6 shows typical records of current *versus* time for such a situation. Figure 6*A* shows single-channel currents in the absence of amiloride. The lifetime of the channel in the open state is voltage independent with a mean value around 0.5 sec. It displays an average conductance of 160 pS in a symmetrical 0.1 M NaCl solution (Fig. 7, curve *a*), and its selectivity is mainly

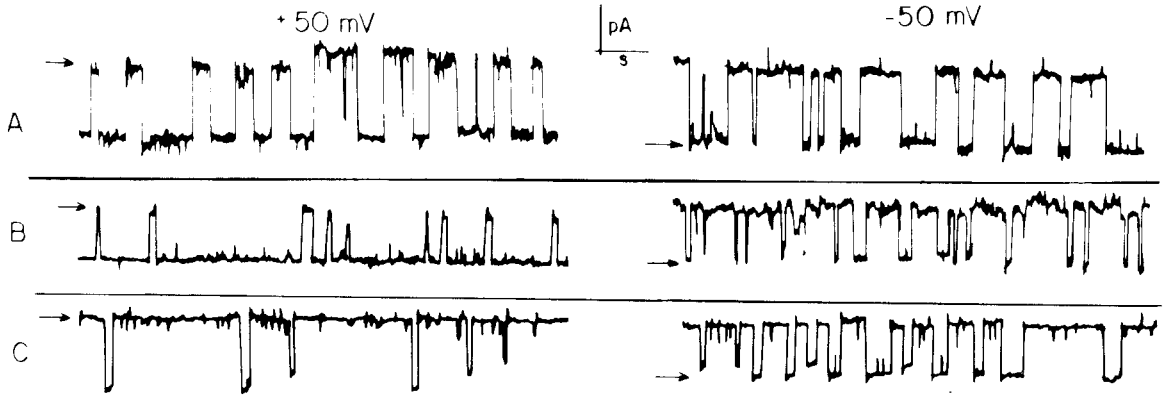

Fig. 6. Single-channel current *versus* time records at +50 mV (right) and −50 mV (left). (*A*) The control situation, (*B*) in the presence of 10^{-6} M amiloride on the *cis* side only and (*C*) with 10^{-6} M amiloride added to the *trans* side only. The vertical bar indicates 5 pA for all records. The time bar is 1 sec for the control traces (*A*) and 0.4 sec for the others (*B* and *C*). Arrows indicate the open state of the channels.

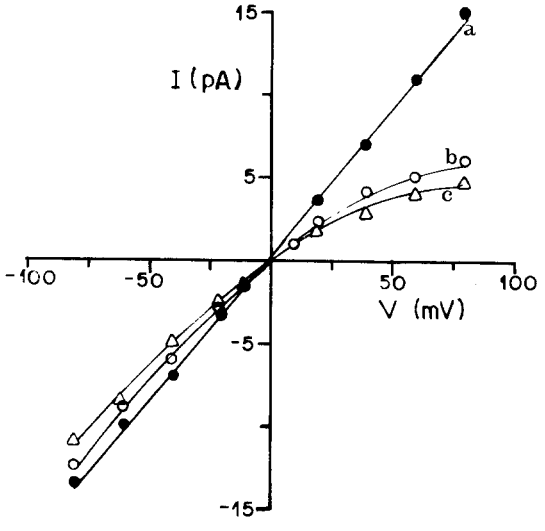

Fig. 7. Single-channel currents against voltage under control conditions (curve *a*) and in the presence of 10^{-7} M (curve *b*) and 2×10^{-7} M (curve *c*) amiloride on the *cis* solution. The decrease in conductance with voltage, in the presence of amiloride, indicates $\partial = 0.45$. Electrolyte solutions are the same as in Fig. 1.

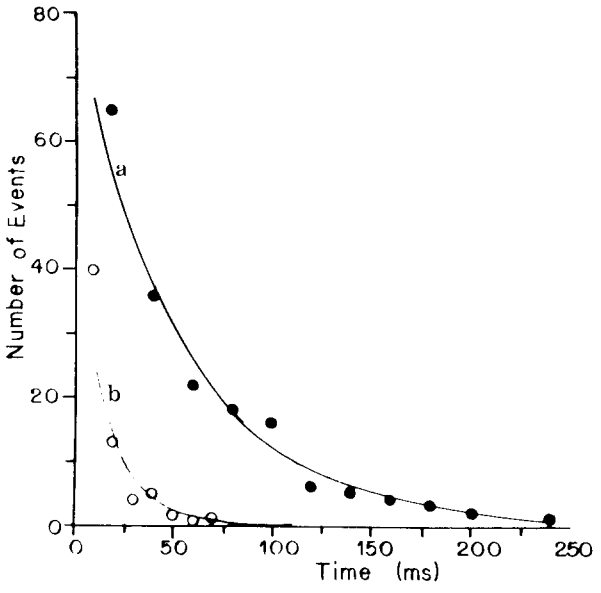

Fig. 8. Frequency distribution of open times observed at +20 mV under control conditions (curve *a*) and after the addition of 10^{-7} M amiloride on the *cis* side (curve *b*). Each point is the mean of at least 100 readings from the current × time record. The lines are single-exponential functions fitted to the data.

cationic ($P_{Na}/P_{Cl} = 15$), poorly discriminating between Na^+ and K^+ ($P_{Na}/P_K = 2.04$). These ratios were calculated by the Goldman-Hodgkin-Katz equation using values of reversal potentials measured in the presence of a 3 : 1 NaCl gradient across the membrane (0.1 M NaCl on the *cis* side and 300 mM NaCl on the *trans* side) or 0.1 M NaCl on the *cis* and 0.1 M KCl on the *trans* side. Amiloride on the *cis* side has a double effect on this channel (Fig. 6*B*). First, it decreases the single-channel conductance in a dose-dependent manner, transforming the linear *I-V* relationship in the control situation into a rectifying one (Fig. 7, curves *b* and *c*) and, second, it also decreases the open lifetime of the channel. For example, at +20 mV the mean open time is 0.57 sec in the control situation and 0.09 sec in the presence of 10^{-7} M amiloride (Fig. 8). This single-channel voltage-dependent conductance in the presence of amiloride can again be explained by assuming a sequential model of blockade, as was done for the macroscopic currents (*see* Figs. 2 and 3). Our calculations resulted now in a ∂ around 0.45. This site

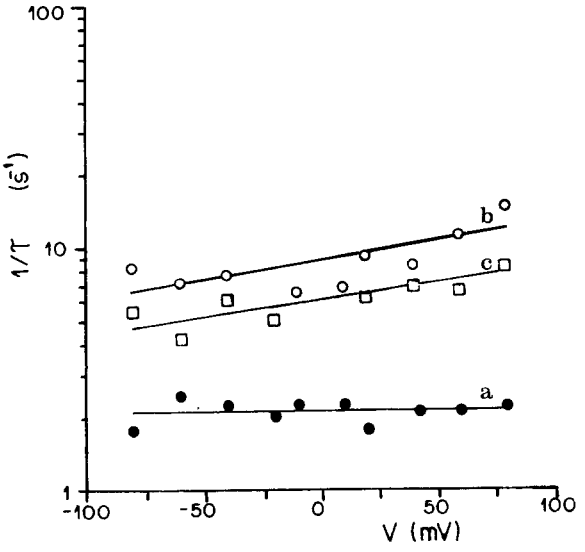

Fig. 9. Semi-logarithmic plot of the reciprocal of the mean open time ($1/\tau$) *versus* voltage under control conditions (curve *a*) and with 2×10^{-7} M (curve *b*) and 10^{-7} M (curve *c*) amiloride on the *cis* side only. $1/\tau$ varies *e*-fold/180 mV, indicating a value of ∂ around 0.14.

would be responsible for the decrease in conductance. Addition of amiloride to the *trans* side also affects the kinetics of the channel in a similar but less pronounced manner (Fig. 6*C*). Although this type of channel was the most commonly encountered in our experiments (70% of the time) we also observed the presence of another channel with conductance of 120 pS (*not shown*). Amiloride affects only the lifetime in the open state of this channel but not its conductance. Furthermore, the effect of the drug on this channel is not voltage dependent. From results like those in Fig. 8 we measured the open lifetime (τ) of the channel at several voltages and plotted the results in Fig. 9. As can be seen, in the control situation $1/\tau$ does not vary with voltage but in the presence of amiloride (10^{-7} M in this case) $1/\tau$ varies *e*-fold every 180 mV. This indicates that the binding site responsible for decreasing the lifetime of the channel is localized at about 14% of the electrical field. Increasing the amiloride concentration displaces the points upwards but does not alter the slope.

Discussion

The search for the amiloride-sensitive Na^+ channel ubiquitously present in the apical membrane of tight epithelia has led to a number of different results, depending on the particular tissue and technique used. Here we describe the incorporation of apical membrane fragments from toad urinary bladder into lipid bilayers and, as a consequence, the appearance of a conductance highly sensitive to amiloride and calcium. The results from macroscopic experiments, where many channels are incorporated into the bilayer, closely resemble results obtained in depolarized toad urinary bladder under short-circuit conditions. In this way, due to its cationic nature, the blocking action of amiloride is voltage dependent in both systems. This fact has permitted the calculation of the fractional distance along the electric field across the channel (∂) in which the binding site for the drug is localized (Woodhull, 1973; Latorre & Miller, 1983; Palmer, 1984, 1985*a*). Several papers report the fact that making the mucosal side more positive increases the blocking effect similar to results shown here in Figs. 1 and 2. They also claim that the guanidinium group of amiloride should sense 10 to 20% of the electric field (Palmer, 1984, 1985*a*,*b*). Our calculated value of ∂, around 15% (Fig. 3*B*), is well within this range. In our experiments the effect of amiloride is shown to be reversible, as well established in the field, and to act from both sides of the channel (Figs. 1, 2 and 6). Although this result may also indicate that the channel is inserted without any orientation into the planar bilayer, it is worth noting that the calcium effect is seen when the divalent ion is added only to the *cis* side. In fact, Sariban-Sohraby et al. (1984) have shown this kind of effect at the single-channel level in reconstitution experiments utilizing cultured A-6 cells. This double-sided effect has also been suggested from measurements of ^{22}Na fluxes in apical membrane vesicles (Garty, 1984). Another parameter derived from fitting the electrostatic blocking model to the data is the apparent inhibition constant at zero voltage, $K_{(0)}$. Our value of 0.98 μM indicates a relatively high affinity of amiloride for its binding site. Values of $K_{(0)}$ in the literature range from 0.104 μM for the isolated short-circuited toad bladder (Palmer, 1985*a*) to 0.11 to 0.35 μM from noise analysis experiments (Lindemann, 1984) and to 0.6 and 6.4 μM in experiments with apical membrane vesicles (Garty, 1984). The last two estimates were taken by Garty to reveal the presence of two distinct conductive pathways for sodium.

Functional expression of the channel from A-6 cells in oocytes resulted in amiloride-blockable ^{22}Na fluxes which were half-maximally inhibited at 6×10^{-8} M (George et al., 1989). An electrophysiological study of these expressed channels showed a ∂ of 0.41 and a $K_{(0)}$ of of 0.1 μM (Palmer et al., 1990). Our results pointing to a ∂ of 0.15 are more within the range found in the intact bladder, i.e., 0.15 to 0.3 (Palmer, 1984), whereas our $K_{(0)}$ value (0.98 μM) is somewhat larger than this but within the range published in the literature.

In our first attempts to reconstitute the apical Na^+ channel, Ca^{2+} ions were originally included both in the homogeneizing medium and in the solutions bathing the membrane. Needless to say, no conductance increment was ever observed in the planar bilayer due to addition of apical membrane fragments, even after more than 1 hr. The result in Fig. 4 indeed shows that Ca^{2+} blocks the conductive pathways present in the bilayer. The results also show that blockage is brought about by calcium present on only one side of the membrane, normally the *cis* side. Addition of Ca^{2+} to the *trans* side resulted in no effect. These results have two implications: (i) they assure us that the channels have a preferential orientation in the bilayer, confirming that amiloride acts from both sides of them, and (ii) the effect of Ca^{2+} cannot be explained by assuming screening of surface charges in the bilayer, since in that case we would expect a symmetry in the action of Ca^{2+}. The action of Ca^{2+} ions on the Na^+ movement has been well described in experiments where ^{22}Na fluxes have been measured in apical membrane vesicles (Chase & Al-Awqati, 1981; Garty, Asher & Yeger, 1987). There are even suggestions that only one Ca^{2+} ion interacts with one channel (Chase & Al-Awqati, 1983), a hypothesis not supported by our results in Fig. 5. The voltage dependence of Ca^{2+} blockade is complex and cannot be explained even by assuming two binding sites as suggested by Palmer (1984, 1985*b*). In fact this interaction should involve several charged groups in the channel-forming protein. This assumption is supported by the effectiveness of pH in modulating blockage by Ca^{2+} (Siqueira-Branco & Varanda, 1991), as seen also in experiments with vesicles (Garty et al., 1987). In cortical-collecting ducts Palmer and Frindt (1987) have shown evidence that intracellular Ca^{2+} acts to block Na channels, but the effect is not direct, contrary to our results.

At the single-channel level the present data do not support the current expectations for the amiloride-sensitive channel. The first problem arises with the measured single-channel conductance: 160 and 120 pS for the two types of channels most commonly seen. These values are far above the range of 4 to 80 pS reported for channels from A-6 cells reconstituted into lipid bilayers (Sariban-Sohraby et al., 1984) or the 7 to 10 pS observed with the path-clamp technique (Hamilton & Eaton, 1985). The latter approach applied to the toad urinary bladder disclosed several types of channels grouped by the authors into four classes with conductance varying from 5 to 59 pS (Frings et al., 1988). On the other hand, in accordance with our results Reinhardt et al. (1985) report a single-channel conductance of 155 pS for channels from toad bladder reconstituted into painted bilayers. A second point of interest is the selectivity of the channels. Our measured reversal potentials indicate that they are not exclusively cationic and that they poorly discriminate between Na^+ and K^+, a feature reported also by others (Sariban-Soraby et al., 1984; Hamilton & Eaton, 1985) but in clear contrast with results derived mainly from short-circuit current analysis in intact epithelia (Palmer, 1982).

Furthermore the channel described here is effectively blocked by amiloride from both sides, having both its conductance and lifetime decreased by the diuretic in a voltage-dependent manner. The decrement in lifetime can easily be explained by assuming a simple sequential model of open channel block, but the effect on conductance is an unexpected finding. Sariban-Sohraby et al. (1984) reported similar results for their 5-pS channel and explained the phenomenon by assuming that the amplifier could not adequately follow the amiloride-induced flickering in current. This possibility cannot be discarded in our case also. The kinetics of the channel is not voltage dependent (±100 mV), with a mean open time around 0.5 sec. Amiloride (10^{-7} M) decreases this value to approximately 0.09 sec, and in accordance with the macroscopic results its effect is voltage dependent ($\partial = 0.14$). Since the effect of amiloride on conductance and lifetime is characterized by distinct voltage dependencies, it is appealing to consider the presence of at least two binding sites for the drug. In fact the existence of more than one binding site has been already suggested by Benos, Mandel and Balaban (1979). On the other hand, the assumption of two distinct binding sites is somewhat contradictory to what was seen at the macroscopic level, since this fact would have to be reflected in the macroscopic kinetics also. This issue may have been obscured at the macroscopic level due to a nonuniform population of channels present in the bilayer. On the other hand, this may indicate that the voltage-dependent effect of amiloride in a multi-channel experiment may result essentially from a reduction in open lifetime brought about by the site localized at 14% of the electrical field.

The general characteristics of the channel described here could also be related to a Ca^{2+}-sensitive cation channel observed in depolarized epithelia through current fluctuation analysis (Van Driessche et al., 1988; Van Driessche, Erlij & Simaels, 1989). In both cases the channel is fully expressed when Ca^{2+} is absent from the bathing solutions and has poor selectivity for monovalent cations. On the other hand, a major difference exists between them: while our channel is effectively blocked by amiloride and Ca^{2+}, the previous one is not affected by the diuretic. In addition, Ca^{2+} seems not to permeate our channel, since bi-ionic experiments, where the

membrane had 0.1 M NaCl on the *trans* side and 0.05 M $CaCl_2$ on the *cis*, resulted in a Na^+-selective membrane. This is contrary to the suggestion of Van Driessche et al. (1989) that the channel is primarily involved in passing Ca^{2+} ions.

In summary, we have shown that a macroscopic conductance can be induced in planar lipid bilayers by the incorporation of apical membrane vesicles derived from toad urinary bladder. This conductance is highly sensitive to amiloride and can be blocked by Ca^{2+} ions. As evidenced by single-channel measurements this conductance seems to be mediated by poorly selective cationic channels also subjected to blockage by Ca^{2+} and amiloride. Due to their relatively high conductance and poor selectivity it is difficult to strictly correlate their function to what is classically expected from the amiloride-sensitive Na^+ channel from tight epithelia. Certainly more experiments are needed in order to fully understand the possible physiological role of these channels.

We thank Prof. F. Lacaz Vieira and Prof. J. Procopio for reading and criticizing the manuscript. This work was supported by FAPESP and FINEP. LGSB was the recipient of a fellowship from CNPq.

References

Asher, C., Garty, H. 1988. Aldosterone increases the apical Na^+ permeability of toad bladder by two different mechanisms. *Proc. Natl. Acad. Sci. USA* **85:**7413–7417

Benos, D.J. 1982. Amiloride: A molecular probe of sodium transport in tissues and cells. *Am. J. Physiol.* **242:**C131–C145

Benos, D.J., Mandel, L.J., Balaban, R. 1979. On the mechanism of the amiloride-sodium entry site interaction in anuran skin epithelia. *J. Gen. Physiol.* **73:**307–326

Benos, D.J., Saccomani, G., Brenner, B.M., Sariban-Sohraby, S. 1986. Purification and characterization of the amiloride-sensitive sodium channel from A6 cultured cells and bovine renal papilla. *Proc. Natl. Acad. Sci. USA* **83:**8525–8529

Chase, H.S., Al-Awqati, Q. 1981. Regulation of the sodium permeability of the luminal border of toad bladder by intracellular sodium and calcium. *J. Gen. Physiol.* **77:**693–712

Chase, H.S., Al-Awqati, Q. 1983. Calcium reduces the sodium permeability of luminal membrane vesicles from toad bladder. *J. Gen. Physiol.* **81:**643–665

Frelin, C., Barbry, P., Chassande, O., Vigne, P., Lazdunski, L. 1988. The structure of the amiloride sensitive apical Na^+ channel from pig kidney. *Kidney Int. (Suppl.)* **26:**S12–S13

Frings, S., Purves, R.D., Macknight, A.D.C. 1988. Single-channel recordings from the apical membrane of the toad urinary bladder epithelial cell. *J. Membrane Biol.* **106:**157–172

Garty, H. 1984. Amiloride blockable sodium fluxes in toad bladder membrane vesicles. *J. Membrane Biol.* **82:**269–279

Garty, H., Civan, E.D., Civan, M.M. 1985. Effects of internal and external pH on amiloride blockable Na^+ transport across toad urinary bladder vesicles. *J. Membrane Biol.* **87:**67–75

George, A.L., Staub, O., Geering, K., Rossier, B.C., Kleyman, T.R., Kraehenbuhl, J.-P. 1989. Functional expression of the amiloride-sensitive sodium channel in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **86:**7295–7298

Hamilton, K.L., Eaton, D.C. 1985. Single-channel recordings from amiloride-sensitive epithelial sodium channel. *Am. J. Physiol.* **249:**C200–C207

Heinonen, J.K., Lahti, R.J. 1981. A new and convenient colorimetric determination of inorganic ortophosphate and its application to the assay of inorganic pyrophosphatase. *Anal. Biochem.* **113:**313–317

Latorre, R., Miller, C. 1983. Conductance and selectivity in potassium channels. *J. Membrane Biol.* **71:**11–30

Lindemann, B. 1980. The beginning of fluctuation analysis of epithelial ion transport. *J. Membrane Biol.* **54:**1–11

Lindemann, B. 1984. Fluctuation analysis of sodium channel in epithelia. *Annu. Rev. Physiol.* **46:**497–515

Montal, M., Mueller, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA* **69:**3561–3566

Palmer, L.G. 1982. Ion selectivity of the apical membrane Na channel in the toad urinary bladder. *J. Membrane Biol.* **67:**91–98

Palmer, L.G. 1984. Voltage-dependent block by amiloride and other monovalent cations of apical Na^+ channels in the toad urinary bladder. *J. Membrane Biol.* **80:**153–165

Palmer, L.G. 1985*a*. Interactions of amiloride and other blocking cations with the apical Na^+ channel in the toad urinary bladder. *J. Membrane Biol.* **87:**191–199

Palmer, L.G. 1985*b*. Modulation of Na permeability of the toad urinary bladder by intracellular Na, Ca, and H. *J. Membrane Biol.* **83:**57–69

Palmer, L.G., Corthesy-Theulaz, I., Gaeggeler, H.P., Kraehenbuhl, J.P., Rossier, B. 1990. Expression of epithelial Na channels in *Xenopus* oocytes. *J. Gen. Physiol.* **96:**23–46

Palmer, L.G., Frindt, G. 1987. Effects of cell Ca and pH on Na channels from rat cortical collecting tubule. *Am. J. Physiol.* **253:**F333–F339

Palmer, L.G., Li, J.H.-Y., Lindemann, B., Edelman, I.S. 1982. Aldosterane control of the density of sodium channel in the toad urinary bladder. *J. Membrane Biol.* **64:**91–102

Reinhardt, R., Garty, H., Lindemann, B. 1985. Amiloride-blockable Na-channels observed after fusion of membrane vesicles from toad urinary bladder to planar phospholipid bilayers. *Pfluegers Arch.* **405(Suppl. 2):**R30

Sariban-Sohraby, S., Latorre, R., Burg, M., Olans, L., Benos, D. 1984. Amiloride-sensitive epithelial Na^+ channels reconstitution into planar lipid bilayer membranes. *Nature* **308:**80–82

Siqueira-Branco, L.G., Varanda, W.A. 1991. Ca^{2+}-induced down-regulation of epithelial amiloride-sensitive channels reconstituted into planar lipid bilayers. *Braz. J. Med. Biol. Res.* **24:**123–126

Van Driessche, W., Aelvoet, I., Erlij, D. 1987. Oxytocin and cAMP stimulate monovalent cation movements through a Ca^{2+}-sensitive channel in the apical membrane of the toad urinary bladder. *Proc. Natl. Acad. Sci. USA* **84:**313–317

Van Driessche, W., Erlij, D., Simaels, J. 1989. Role of an apical cation-selective channel in function of tight epithelia. *Biol. Cell* **66:**37–41

Van Driessche, W., Simaels, J., Aelvoet, I., Erlij, D. 1988. Cation-selective channels in amphibian epithelia: Electrophysiological properties and activation. *Comp. Biochem. Physiol.* **90A:**693–699

Woodhull, A.M. 1973. Ionic blockade of sodium channel. *J. Gen. Physiol.* **61:**687–708

Received 5 June 1991; revised 3 December 1991