MOLECULAR PHARMACOLOGY, 8, 222-229

The Role of Calcium Ions in the Interaction of Amiloride with Membrane Receptors

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(Received November 9, 1971)

SUMMARY

When amiloride is added to the solution bathing the external surface of frog skin there is an immediate and reversible inhibition of the short-circuit current. However, if the external surface is bathed in calcium-free solutions containing ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid (EGTA), the inhibitory effects of amiloride are severely reduced or abolished. If calcium is readmitted to the external solution, the inhibitory effects of amiloride are restored. Other ions (Mg^{2+}, Sr^{2+}, La^{3+}, and Mn^{2+}) can substitute for calcium in these interactions. Quantitative considerations suggest that amiloride may form a ternary complex with Ca^{2+} and a membrane receptor controlling the access of sodium to the transport mechanism.

INTRODUCTION

The potassium-sparing diuretic amiloride [1-(3,5-diamino-6-chloropyrazinoyl)guanidine] inhibits sodium transport in epithelia such as frog skin and toad bladder. Amiloride is presumed to interact with receptors in the outer surface to prevent access of sodium to the transport mechanism (1–5). Amiloride can also inhibit the sodium-dependent oxygen consumption of toad bladders, although this inhibition requires the presence of Ca^{2+} in the bathing solution (6).

In this paper the requirement of Ca^{2+} for the effect of amiloride on sodium transport in frog skin is described. It is shown that calcium ions are necessary for amiloride to be effective in blocking the access of sodium to the transport mechanism. One difficulty with epithelia in calcium-free solutions is that the cells separate and transepithelial transport fails. These effects are hastened by calcium-chelating agents, such as EGTA,[2] and develop more quickly in toad bladder than in frog skin (7, 8). To overcome these difficulties the inner surfaces of epithelia were bathed with calcium-containing solutions, while the outer surfaces were bathed in calcium-free solutions containing EGTA. Under these conditions sodium transport remainded steady for several hours, while the concentration of calcium in the external solution was kept at a low value. Thus the procedure buffered any calcium penetrating to the outer surface from the inner solution and probably removed bound calcium from the outer surface of the cells.

METHODS

Experiments were performed on the abdominal skins of frogs (*Rana temporaria*). In addition a few experiments were performed using the isolated urinary bladders of toads

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[2] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid.

(*Bufo marinus*). Sodium transport was measured as short-circuit current (9). A Perspex chamber (skin area, 3 cm^2) was constructed so that the inner surface could be perfused with Ringer's solution previously equilibrated with air. The flow rate was adjusted so that the inner solution was changed every minute. This ensured that any EGTA penetrating from the outer solution would be quickly removed. The outer surface was bathed in 5 ml of Ringer's solution of various compositions and was bubbled with air. The mixing time of drugs added to this solution was virtually instantaneous. The outer solution could be withdrawn by suction and replaced within a few seconds.

Transepithelial potentials were measured using conventional KCl–agar bridges which led via calomel cells to a Radiometer pH meter. This potential could also be switched to a short-circuiting device, consisting of two operational amplifier feedback circuits which delivered current to the tissue through two AgCl electrodes and two KCl–agar bridges. The current delivered to the tissue was monitored on a potentiometric recorder (Bryan 27000 series) as the potential developed across an accurately known resistor.

Solutions. The Ringer's solution used had the following composition: NaCl, 112 mM; KCl, 3.5 mM; $CaCl_2$, 1 mM; NaH_2PO_4, 0.08 mM; $NaHCO_3$, 2.4 mM; and glucose, 11.1 mM. This solution had a pH of 7.6 when equilibrated with air. To make EGTA–Ringer's solution, $CaCl_2$ and glucose were omitted, and EGTA (5 mM, previously neutralized with NaOH to pH 7.6) was added. Glucose was omitted from this solution to keep the tonicity approximately constant. Tris–Ringer's solution was prepared by substituting Tris base for phosphate and bicarbonate, the pH being adjusted to 7.6 with HCl.

Solutions of amiloride (10 mM) (Merck Sharp & Dohme) in distilled water were prepared freshly for each experiment. The drug was added only to the solution bathing the outer surface of skins.

RESULTS

Role of calcium. When the outer surface of frog skin was bathed in EGTA–Ringer's while the inner surface was perfused with Ringer's solution, the inhibitory effect of amiloride on short-circuit current was greatly reduced. A typical experiment is illustrated in Fig. 1. In curve *a* the effect of cumulative doses of amiloride on short-circuit current for a skin bathed on both sides with Ringer's solution is shown. The responses to amiloride were readily reversed by rinsing with Ringer's solution. In curve *b* the outer solution had been changed to EGTA–Ringer's. Concentrations of amiloride identical with those given for curve *a* now produced only a minor decrease in short-circuit current. The addition of Ca^{2+} at this time produced a rapid increase in the amount of inhibition. The time course of the extra inhibition produced by Ca^{2+} in the absence of amiloride is shown in curve *c*. Finally, after bathing the outer surface in Ringer's solution for a further 45 min and after removal of the EGTA–Ringer's solution, the responses to amiloride were restored (curve *d*).

The results of 11 similar experiments have been collected and are shown as a scatter diagram in Fig. 2 The curves represent the lines of best fit calculated using the method of least squares. The slopes of the lines relating the percentage inhibition of short-circuit current to the concentration of amiloride are highly significantly different in the presence and absence of Ca^{2+} ($p < 0.001$). The slopes (percentage inhibition/[amiloride $\times 10^8$]M) are 28.41 ± 2.75 (SE) in the presence of Ca^{2+} (1 mM) and 12.15 ± 2.38 in the absence of Ca^{2+}. The concentration of amiloride producing 50% inhibition of short-circuit current was 0.24 μM in the presence of Ca^{2+} (1 mM), whereas the concentration required in the absence of Ca^{2+} was approximately 0.1 mM. From Fig. 2 it can be seen that some preparations failed to respond at all to amiloride following Ca^{2+} removal. In four experiments on toad bladders the results were similar to those with frog skin.

The most interesting results came from experiments in which the response to amiloride was not completely depressed by bathing the outer surface in EGTA–Ringer's solution. An example is shown in Fig. 3. Removal of Ca^{2+} moved the log dose-response

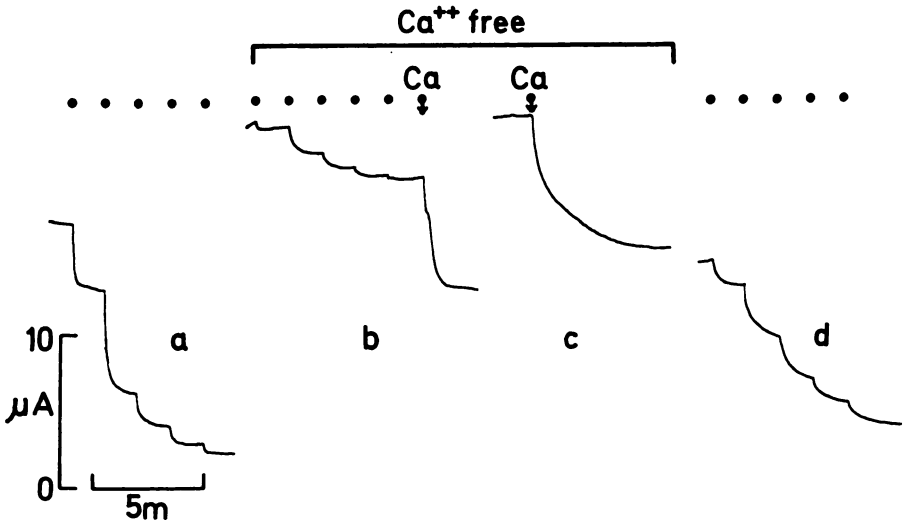

FIG. 1. *Effect of cumulative doses of amiloride on frog skin*

Amiloride was added at the times shown by the dots to produce final concentrations of 1, 6, 16, 36, and 136 × 10^{-6} M bathing the outer surface. In curve *a* the outer solution was Ringer's, whereas in curves *b* and *c* it was EGTA–Ringer's. $CaCl_2$ solution was added at the arrowed dots to produce a final concentration of 1 mM ionized calcium. The result shown in curve *d* was obtained after exposing the outer surface to Ringer's solution for 45 min after removal of EGTA–Ringer's solution. The time scale is in minutes (*m*).

curve to the right in a nonparallel manner. Also the maximal attainable response with amiloride was depressed. A double-reciprocal plot of the results produced straight lines which intersected on the abscissa. Further tests were made to see whether this result would be confirmed in other experiments. The data from the 11 experiments illustrated in Fig. 2 were used to construct plots of [amiloride]/percentage inhibition vs. [amiloride]. In five experiments the data gave straight lines when fitted by eye. In all these experiments the lines derived from data obtained in the presence and absence of calcium intersected on the abscissa. In four further experiments, in which amiloride produced very little or no inhibition in the absence of calcium, the values of [amiloride]/percentage inhibition could not be obtained. In the other two experiments the data could not be fitted to straight lines. Data from the first five experiments were further analyzed as follows. Lines of best fit using the least-squares method were obtained, together with the value of the mean ± standard error of the intercept on the abscissa. Student's *t*-test was used to compare the values of the intercepts in the presence and absence of calcium. In no experiment were the values of the intercepts significantly different. These data are summarized in Table 1.

Ions substituting for calcium. Experiments were carried out to see if other ions could substitute for Ca^{2+} in promoting the inhibitory action of amiloride. In these experiments Tris–Ringer's solution was used to prevent the precipitation of heavy metals. In the experiment illustrated in Fig. 4 the substitution of La^{3+} for Ca^{2+} was tried. In curve *a* the response of the skin to amiloride when bathed on both sides with Tris–Ringer's solution containing Ca^{2+} (1 mM) is shown. The skin was then exposed to EGTA–Ringer's solution on the outer surface only, while the inner surface continued to be bathed in Tris–Ringer's solution. After 30 min the responses to amiloride were tested again and found to be severely depressed (curve *b*). Tris–Ringer's solution containing La^{3+} (1 mM, as chloride) was then substituted for EGTA–Ringer's solution on the outer side of the skin. The responses to amiloride were fully restored after a 30-min exposure (curve *c*).

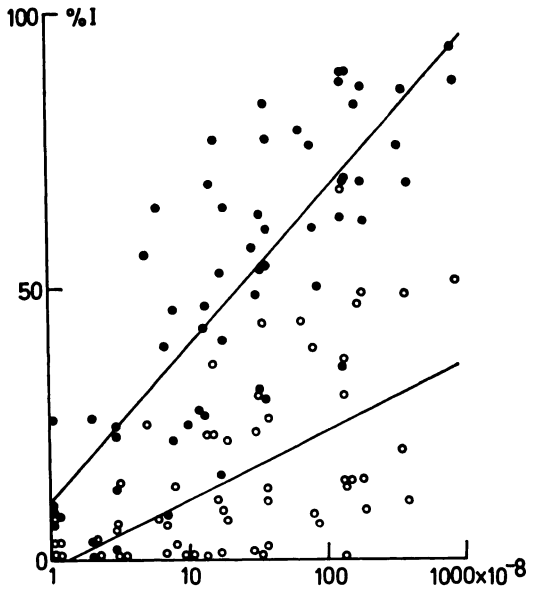

FIG. 2. *Scatter diagram showing percentage inhibition of short-circuit current (ordinate) as a function of concentration of amiloride (abscissa) for 11 experiments on frog skin*

●, Ringer's solution bathed the outer surface; ○, EGTA-Ringer's solution bathed the outer surface for 30 min. The mean short-circuit current before amiloride addition was 109.7 ± 40 μA/3 cm² in normal Ringer's solution, and 63.1 ± 13.3 μA/3 cm² after incubation for 30 min in EGTA-Ringer's solution.

The experiments in this series showed several interesting features. First, the resting short-circuit current values for the batch of frogs used in these experiments were much higher than those used for the first experiments, although the dependence on Ca^{2+} for the amiloride response remained the same. Second, with low concentrations of amiloride, the responses showed "fade" (see Fig. 4); in fact, the short-circuit current often rose to a higher value after the initial inhibitory effect of a low concentration of amiloride had disappeared. Fade was not apparent when high concentrations of the drug were used. Finally, La^{3+} increased the short-circuit current when applied to the outer surface. This was noted in other experiments when La^{3+} was applied to the external surface of isolated toad bladders (10).

Experiments such as the one just described may be criticized because trace amounts of Ca^{2+} occur in the Tris–Ringer's solution containing La^{3+}, and also Ca^{2+} may penetrate to the outer surface from the Ca^{2+}-containing inner solution. The restoration of the response may therefore have been due to Ca^{2+} arising from these sources, rather than to La^{3+}. This criticism can be met by consideration of the following experiment. Responses to amiloride in Ca^{2+} containing

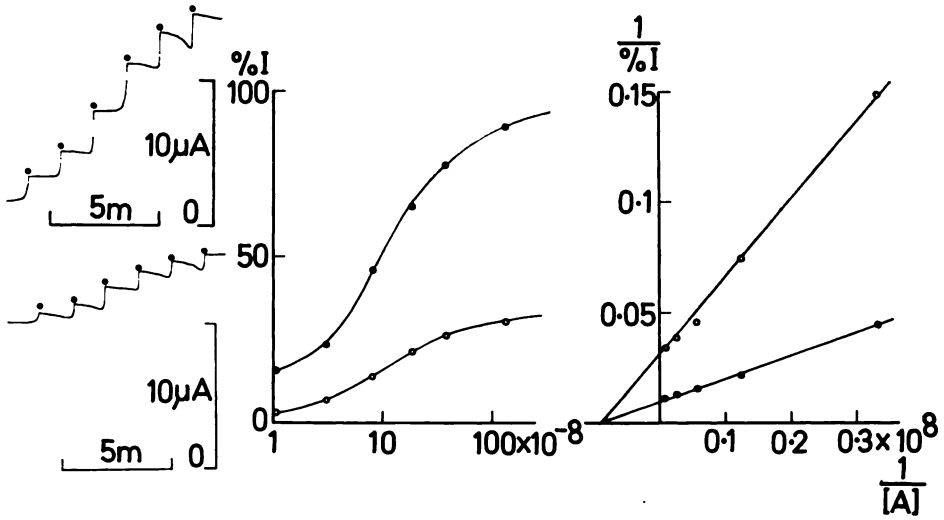

FIG. 3. *Results from a single experiment showing effects of amiloride on short-circuit current with outer surface bathed in Ringer's solution (upper left) and 30 min after exposure of outer surface to EGTA-Ringer's solution (lower left)*

Log concentration-response curves are shown in the middle panel, and a double-reciprocal plot of the results is depicted on the right. The latter gives a value of 0.9×10^7 M^{-1} for the affinity constant of amiloride (A). The time scale is in minutes (m).

Tris–Ringer's and EGTA–Ringer's solutions were obtained as before. The outer solution was then replaced with Ca^{2+}-free Tris–Ringer's solution but without EGTA. After 30 min the responses to amiloride were retested, and it was clear that some restoration of the responses had occurred. However, the restoration was smaller than those obtained following a further 30-min exposure to La^{3+}-containing Tris–Ringer's solution. This experiment is illustrated in Fig. 5, and indicates incidentally that EGTA per se is not responsible for inhibiting the response to amiloride.

TABLE 1

Values of intercepts from plots of [amiloride]/percentage inhibition vs. [amiloride]

Intercept × 10^{-6} M (±SE)		
Ringer's solution	EGTA–Ringer's solution	*p*
9.15 ± 0.52	10.15 ± 1.30	NS[a]
5.53 ± 1.17	6.35 ± 2.25	NS
2.33 ± 0.17	7.82 ± 3.82	NS
5.18 ± 1.41	9.20 ± 2.76	NS
6.29 ± 3.42	10.60 ± 7.26	NS
Mean 5.70 ± 1.09	8.8 ± 0.78	
Mean 6.76 ± 1.01		

[a] NS, not significant.

The inhibitory response to amiloride could also be restored by addition of ions other than Ca^{2+} or La^{3+}. Figure 6 shows a comparison of the results for La^{3+}, Mg^{2+}, and Mn^{2+} (1 mM). All three ions, at this concentration, were able to restore fully the inhibitory response to amiloride. Similar results were also obtained for Sr^{2+} (1 mM).

DISCUSSION

When frog skin is bathed on both sides with Ringer's solution, the net transport of sodium from outside to inside is given by the short-circuit current. Similarly, in the presence of amiloride and at steady state, the net

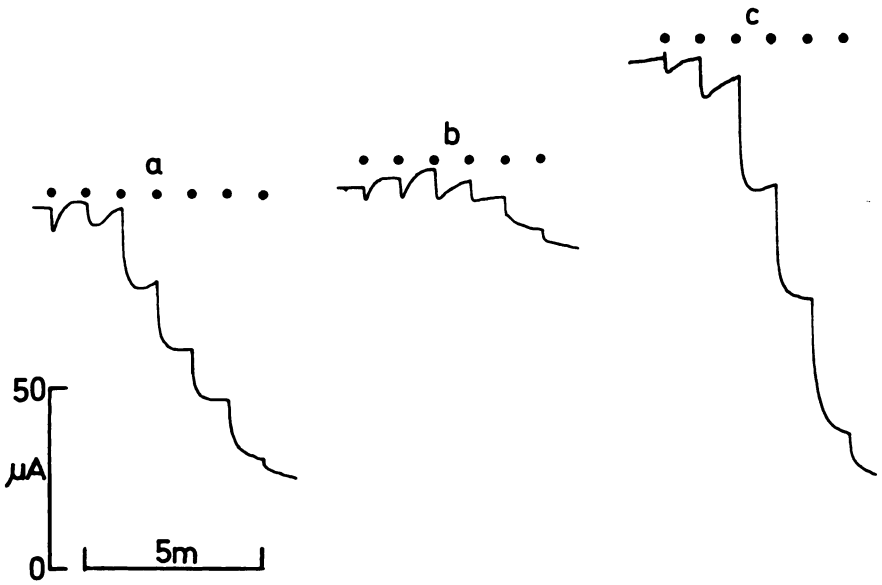

FIG. 4. *Results of an experiment illustrating effects of substitution of La^{3+} for Ca^{2+} on response to amiloride*

Amiloride was added (as shown by the dots) to the outer solution to produce final concentrations of 1, 3, 8, 18, 38, 138, and 338 × 10^{-6} M in curve *a*. In obtaining curves *b* and *c* the response to 1 × 10^{-6} M amiloride was not tested. For curves *a* and *c* the outer solutions were Tris–Ringer's containing 1 mM Ca^{2+} and 1 mM La^{3+}, respectively. For curve *b* the outer solution was EGTA–Ringer's. Thirty minutes were allowed for equilibration between curves *a* and *b* and between curves *b* and *c*. The time scale is in minutes (*m*).

sodium flux is given by the short-circuit current (2, 3, 11, 12). The system can be considered as a three-compartment one consisting of the outside solution, the skin, and the inside solution. When amiloride is added to the outer solution it produces its maximal effect in 30 seconds. However, sodium continues to move out of the middle compartment at a rate in excess of the short-circuit current for 10–15 min, until the middle compartment reaches a new steady state (3, 4). In these experiments cumulative doses of amiloride were added at 60- or 90-sec intervals, so that the whole sequence was completed in 10–15 min. At each dose level, therefore, the short-circuit current was at steady state while the efflux into the inside was not. If the efflux into the inside solution were allowed to reach a steady state between doses, the sequence would take 3–4 hr, during which time appreciable amounts of calcium would have been transferred to the outside (13, 14). Therefore the steady-state short-circuit currents recorded in these experiments represent the rate of sodium entry from the outside solution into the epithelium rather than the net transepithelial flux.

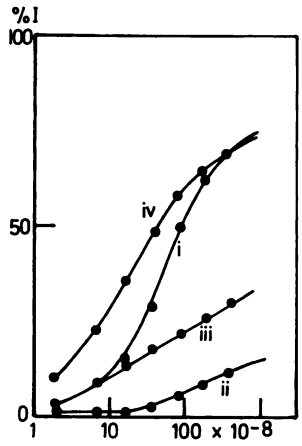

FIG. 5. *Log concentration–percentage inhibition curves for amiloride*

The outer solutions were Tris-Ringer's with 1 mM Ca^{2+} (curve *i*), EGTA-Ringer's (curve *ii*), calcium-free Tris-Ringer's (curve *iii*), and Tris-Ringer's with 1 mM La^{3+} (curve *iv*). Thirty minutes were allowed for equilibration after each solution change. The curves were determined in numerical order.

It has been shown that the inhibitory response to amiloride depends on the presence of calcium or some other polyvalent cation. Three equations describing the combination

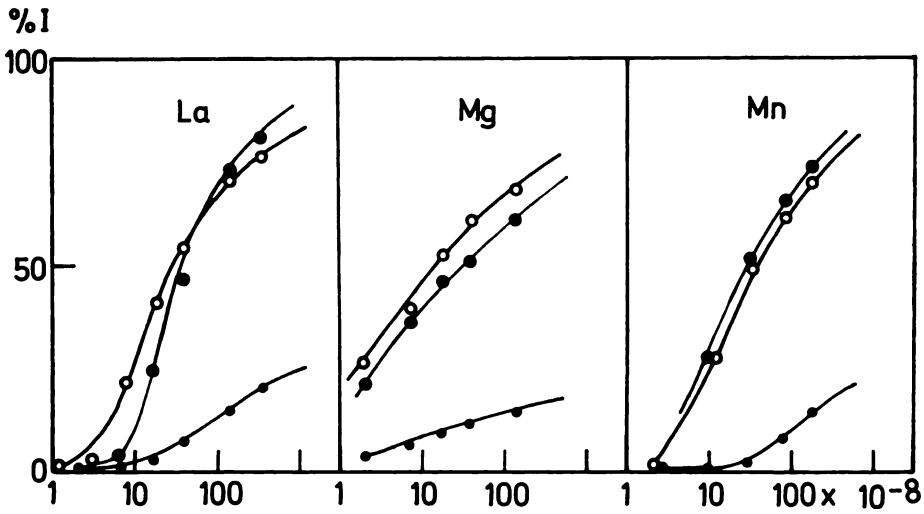

FIG. 6. *Log concentration–percentage inhibition curves for amiloride*

The curves were determined first with Tris-Ringer's solution containing 1 mM Ca^{2+} (○) bathing the outer surface, then with EGTA-Ringer's solution bathing the outer surface (●), and finally with Tris-Ringer's solution containing a 1 mM concentration of the appropriate ion (La^{3+}, Mg^{2+}, or Mn^{2+}) bathing the outer surface (●). Thirty minutes were allowed for equilibration after each solution change.

of amiloride and metal ions with receptors in the outer surface of frog skin may be envisaged:

$$A + R \rightleftharpoons AR$$
$$M + R \rightleftharpoons MR$$
$$A + MR \rightleftharpoons AMR$$

where A, M, and R refer to amiloride, metal ions, and receptors, respectively. These reactions can be described by kinetic equations as follows:

$$K_1 = \frac{y_1}{[A][1 - y_1 - y_2 - z]} \quad (1)$$

$$K_2 = \frac{y_2}{[M][1 - y_1 - y_2 - z]} \quad (2)$$

$$K_3 = \frac{z}{[A][y_2]} \quad (3)$$

where K_1, K_2, and K_3 are the affinity constants for the three reactions and y_1, y_2, and z are the fractions of receptors occupied respectively by amiloride, metal ions, and amiloride plus metal ions. If it is supposed that the inhibition of short-circuit current is caused only by the fraction of receptors in the ternary form, i.e., z, and Eqs. 1–3 are solved for z, then

$$z = \frac{AMK_2K_3}{1 + AK_1 + MK_2 + AMK_2K_3} \quad (4)$$

This equation predicts that at low metal ion concentrations the responses to amiloride at all concentrations will be less than the responses obtained at high metal ion concentrations. Also Eq. 4 predicts that the response curves for these two conditions will not be parallel.

A reciprocal plot of $1/z$ vs. $1/A$ will be a straight line of the form

$$\frac{1}{z} = \frac{1}{A}\left[\frac{1 + MK_2}{MK_2K_3}\right] + \left[\frac{K_1 + MK_2K_3}{MK_2K_3}\right] \quad (5)$$

The intercept on the abscissa will be given by

$$\frac{1}{A} = \frac{-(K_1 + MK_2K_3)}{1 + MK_2} \quad (6)$$

The analysis presented earlier suggests that the intercept is independent of the metal ion concentration, and requires that $K_1 = K_3$ = affinity constant for amiloride (Eq. 6). This would be so if calcium and amiloride interacted separately with the receptor controlling sodium entry, and provided that neither induced some form of allosteric change in the binding site for the other. If calcium does change the affinity of amiloride for the receptor, it must be by an amount undetectable by the present methods.

We have demonstrated (10) in EGTA-treated bladders that 10 pM Ca^{2+} is sufficient to reduce the sodium-dependent oxygen consumption by 50%. In the presence of antidiuretic hormone, which stimulates sodium transport in this tissue, a 10-fold increase in Ca^{2+} is required to inhibit the oxygen consumption to the same degree. We concluded that high-affinity calcium-binding sites may be involved in gating sodium entry to the transport mechanism. Yet, in the same tissue untreated with EGTA, amiloride was without effect on oxygen consumption when the Ca^{2+} concentration was reduced to 10 μM. In frog skin responses to amiloride often persisted even in the presence of EGTA, when the calcium concentration must have been considerably lower than 10 μM. It is probable that more than one type of calcium-binding site may be involved in regulating sodium entry to both these tissues, and there is evidence for more than one transport path for sodium in epithelia (15). Amiloride and Ca^{2+} could interact with the various pathways in different ways. Recently we summarized the differences between frog skin and toad bladder with respect to sodium transport, hydro-osmotic flow, and Ca^{2+} concentration (10). Finally, it is probable that the transport processes in frog skin are not carried out by the most superficial and cornified layers, but by deeper layers, perhaps the stratum granulosum (16). EGTA might hasten the removal of calcium from such deeper layers, while exposure to calcium-free solutions would have little effect since calcium-binding sites could be replenished from the massive stores in the submucosa [78.5 mmoles/kg in *R. temporaria* (14)] or from the inner solution. Clearly the relationship between Ca^{2+} and sodium transport in

epithelia is complex. In this study we have examined the effects of amiloride only at calcium concentrations of 1 mM or approaching zero. In addition, the experiments have been carried out rapidly to reduce changes in the calcium concentrations as a result of the steep calcium gradient from inside to outside. Even so, it must be remembered that in the presence of EGTA some Ca^{2+} will remain in solution, and since some sites have a very high affinity for calcium they may remain occupied even when the outer solution contains EGTA.

There have been many suggestions (for references, see ref. 10) that calcium is able to control the permeability of membranes to monovalent ions. A recent paper (17) suggests that Ca^{2+} must be displaced in order for Na^+ to gain access to the permeability channels. Such a mechanism may operate in the outer surface of sodium-transporting epithelia, and indeed in many experiments the addition of EGTA–Ringer's solution increased the short-circuit current, although this increase was generally followed by a decline to a steady value, lower than the initial short-circuit current. Amiloride may prevent sodium entry to the epithelium by binding in a way which prevents calcium from being displaced. This would suppose that amiloride binding could occur in the absence of calcium, but without affecting the sodium channels.

ACKNOWLEDGMENT

We are grateful to Merck Sharp & Dohme for a gift of amiloride.

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