Effect of Amiloride and Some of Its Analogues on Cation Transport in Isolated Frog Skin and Thin Lipid Membranes

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ABSTRACT The inhibition of short-circuit current (I_{sc}) in isolated frog skin and the induction of surface potentials in lipid bilayer membranes produced by the diuretic drug amiloride and a number of its chemical analogues was studied. The major conclusions of our study are: (a) The charged form of amiloride is the biologically active species. (b) Both the magnitude of I_{sc} and the amiloride inhibitory effect are sensitive to the ionic milieu bathing the isolated skin, and these two features are modulated at separate and distinct regions on the transport site. (c) Amiloride is very specific in its inhibitory interaction with the Na^+ transport site since slight structural modifications can result in significant changes in drug effectiveness. We found that substitutions at pyrazine ring position 5 greatly diminish drug activity, while changes at position 6 are less drastic. Alterations in the guanidinium moiety only diminish activity if the result is a change in the spatial orientation of the amino group carrying the positive charge. (d) Amiloride can bind to and alter the charge on membrane surfaces, but this action cannot explain its highly specific effects in biological systems.

INTRODUCTION

Amiloride (N-amidino-3,5-diamino-6-chloropyrazine carboxamide [Fig. 1]) is a very potent and specific inhibitor of sodium transport in a wide variety of cellular and epithelial transport systems (Aceves and Cereijido, 1973; Bentley, 1968; Crabbe and Ehrlich, 1968; Dunn, 1970; Nagel and Dorge, 1970; Salako and Smith, 1970 a,b; Kirschner et al., 1973). Amiloride is an effective inhibitor of sodium transport at very low concentrations (10^{-9}–10^{-7} M) when applied to the solution bathing the mucosal (outer or luminal) surface of epithelia. Amiloride's inhibition of sodium transport in the frog skin and toad bladder is rapid and reversible. Most of the delay in reaction time can be attributed to diffusion through the unstirred layer lying adjacent to the epithelial surface (Biber, 1971). Amiloride is thought to act by preventing sodium entry across the outside border rather than by direct inhibition of the energy-requiring active transport step which is believed to be located on the inside epithelial surface. Furthermore, this drug does not inhibit the enzymatic activity of carbonic anhydrase or the ouabain-sensitive membrane adenosine triphosphatase (Baer et al., 1967).

This paper reports studies that we have performed with the diuretic drug amiloride and several of its structural analogues on isolated frog skin and on planar phospholipid bilayer membranes. These systems were chosen for the following reasons: (a) the sodium entry step across the outside surface of the frog skin is believed to be a facilitated step (Biber and Curran, 1970; Ussing et al., 1974); (b) pharmacological studies may be readily made on the Na$^+$ entry step in frog skin with the intention of learning about the structure of this site; and (c) the effect of amiloride on nonactin (a known carrier molecule) conductance may be examined in an artificial bilayer and the results so obtained can then be correlated to the drug's physiological effects. The results which we present in this paper are consistent with the idea that in frog skin the active form of amiloride is the charged species. Amiloride's inhibitory interaction with the Na$^+$ transport mechanism appears to be very specific since slight modifications of the
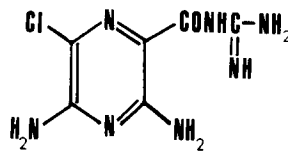
AMILORIDE

FIGURE 1. The primary structure of amiloride.

molecule result in significant changes in its effectiveness. Our results show that amiloride does induce a surface potential on black lipid membranes, but this cannot explain its highly specific effects in biological systems. We found that amiloride's inhibitory effect requires calcium; however, uranyl as well as hydrogen can substitute for calcium while magnesium is less effective. All of our information indicates that the sodium entry step into the sodium transport system in frog skin appears to have characteristics that do not allow categorization as a simple carrier or channel.

MATERIALS AND METHODS

Isolated Frog Skin Experiments

The abdominal skin of *Rana pipiens* was mounted as a flat sheet (3.14 cm^2 in area) between Lucite chambers equipped with solution reservoirs similar to those described by Schultz and Zalusky (1964). The solutions in each chamber (12 ml each) were stirred and oxygenated by bubbling with air.

The open circuit potential across the skin was measured with calomel electrodes, and

current was passed through the skin via Ag-AgCl electrodes. Both pairs of electrodes were connected to the solution reservoirs with 2% agar bridges having a composition identical to that of the bathing solution in the chambers. An automatic voltage clamp that compensated for the resistance of the solution between the agar bridges was used to pass the appropriate current through the skin to clamp the membrane potential at zero millivolts.

To measure unidirectional sodium influxes as a function of external pH, the skins were allowed to equilibrate until the open circuit potential reached a steady value and were then voltage clamped to 0 mV. Radioactive sodium (^{22}NaCl, 1 μC) was added to the outside solution and, after a 30-min equilibration period under short circuit conditions, 1-ml samples were withdrawn from the opposite solution at 20-min intervals and replaced with nonradioactive solution. At least three samples were taken at any given pH, after which the pH of the outside solution was titrated to a new value with an automatic pH-stat (Radiometer, Copenhagen, Denmark). The skin was allowed to equilibrate at the new pH for an additional 30 min before flux sampling was continued. At the end of each flux period at any given pH, a 1-ml sample was withdrawn from the hot outside solution. Samples were counted in Bray's solution using a liquid spectrometer (Intertechnique, model SL30, Dover, N. J.). Unidirectional influxes were calculated from the rate of tracer appearance in the inside solution, and from the specific activity of the outside solution.

The composition of the regular NaCl Ringer solution used in all of the present experiments was as follows: 112 mM NaCl, 2.5 mM KHCO$_3$, 2 mM urea, and 1.0 mM CaCl$_2$. The pH of this solution, when gassed with room air at room temperature, was pH 8.4. In some experiments in which the Ca^{++} concentration was desired to be zero, CaCl$_2$ was omitted from the Ringer solution, and 0.5 mM ethyleneglycol bis (β-aminoethylether)-N,N-tetraacetic acid (EGTA) added.

Bilayer Experiments

EXPERIMENTAL PROCEDURES Lipids were extracted from sheep red blood cells with chloroform-methanol as described by Andreoli et al. (1967). Membranes were formed at room temperature (20–22°C) from a solution of red cell lipids dissolved in n-decane (16 mg/ml). The lipid solution was brushed across a circular aperture (1.8 mm^2 in area) in a polyethylene partition separating two chambers, each chamber containing identical salt solutions (Mueller et al., 1964). Both chambers (1-ml vol each) were continuously stirred with magnetic fleas. The aqueous solutions (all 0.1 M KCl) were buffered at pH 7.4 with phosphate buffer, and were saturated with room air. The addition of amiloride did not appreciably alter the pH of the above solution.

In some experiments both membrane potential and current were measured with a four-electrode, voltage-clamp apparatus (Andreoli and Troutman, 1971). Calomel electrodes were used, and the front or outside chamber was always at ground potential. Current and voltage were recorded on a Varian G-2000 dual channel chart recorder (Varian Instruments, Palo Alto, Calif.). In other experiments, a voltage generator which swept the membrane voltage at a rate of 10 mV/s was used. Again, a four-electrode system was employed and current-voltage curves were traced directly on an X-Y recorder (Hewlett-Packard, model 7015A, Palo Alto, Calif.).

Nonactin was stored in ethanol at −20°C at a concentration of 10^{-5} M until it was ready to use, and small aliquots of this stock solution were injected into both chambers with a Hamilton syringe in a volume necessary to obtain the desired concentration. After the addition of nonactin we waited 30 min to obtain the current-voltage curve. The large area of the hole ensured that nonactin was not diffusing into the torus faster than it could be replaced by diffusion through the unstirred layer. Upon the addition of amiloride to the

above film we waited 20 min before obtaining the current-voltage curve. Amiloride, and all of the analogues used, were stored in powder form at room temperature; solutions of drug were made up fresh on the day of the experiment. Amiloride and its structural analogues were obtained from Merck, Sharp, and Dohme Research Laboratories, West Point, Pa.

THEORY

For a membrane separating identical salt solutions and containing the neutral (i.e., uncharged) carrier molecule nonactin, the rate-limiting step of transport is the translocation of the nonactin-K^+ complex across the membrane interior. The conductance, $G(o)$, in the limit of zero applied voltage, may be calculated from the following equation:

$$G(o) = (F^2/d)\omega C_m \qquad (1)$$

where ω is the mobility of the permeant monovalent species within the membrane, d the membrane thickness, F the Faraday constant, and C_m the concentration of the charged species within the membrane (McLaughlin et al., 1970). This equation assumes that the electric profile within the membrane is constant.

If the system is in equilibrium, the concentration of the permeant species in the membrane, C_m, is related to its bulk aqueous concentration by a partition coefficient, β, and the surface potential, ψ_s, defined here as the potential difference between the membrane interior and the bulk aqueous solution (McLaughlin et al., 1970):

$$C_m = \beta C_{bulk} \exp (\pm F\psi_s/RT) \qquad (2)$$

where R is the gas constant and T is the absolute temperature. Combining Eqs. 1 and 2, we obtain an expression of $G(o)$ in terms of the partition coefficient, the membrane mobility, the bulk aqueous concentration of the permeant species, and the surface potential:

$$G(o) = (F^2/d)\omega\beta C_{bulk} \exp (\pm F\psi_s/RT). \qquad (3)$$

Assuming that amiloride and its analogues do not affect the mobility of the nonactin-K^+ complex within the membrane or the partition coefficient, the change in the nonactin-K^+ conductance in the limit of zero applied voltage produced by amiloride or one of its analogues may be used to compute a change in the surface potential of the bilayer:

$$G(o)/G^+(o) = \exp (-F \Delta\psi_s/RT) \qquad (4)$$

where $G^+(o)$ is the zero-potential conductance of nonactin-K^+ in the presence of amiloride or one of its analogues. The assumptions used in this analysis are given further credence by the observations of Singer (1974) who demonstrated amiloride-induced changes in the zeta potential of phosphatidyl-choline liposomes of the same magnitude as reported in the present communication.

RESULTS

Effects of External pH, Divalent Cations, and Dibucaine

Since amiloride has an apparent pK_a value of 8.7 (E. J. Cragoe, personal communication),[1] most of the drug exists as a positively charged species under physiological conditions. To determine if the uncharged or the charged moiety is necessary for the inhibition of sodium transport in frog skin, we measured the short circuit current (I_{sc}), as a function of external bulk pH in the presence and absence of 10^{-6} M amiloride in the bulk solution (Fig. 2). In the absence of the drug, the I_{sc} was constant within the pH range 6.5 to 9, but increased monotonically above pH 9. This increase in I_{sc} with pH was variable; it occurred in most (about 75%) but not all experiments. Amiloride, at 10^{-6} M, produced a 65% inhibition of the I_{sc} from pH 6.5 to 8.7. However, above pH 8.7, the percent inhibition was progressively reduced, until it reached zero at pH 11. This result was independent of the behavior of the I_{sc} above pH 9 and is therefore consistent with the notion that the charged form of amiloride is the active one, as discussed more fully below.

Experiments were undertaken to determine whether the I_{sc} remained a measure of net Na^+ influx at high pH. The results are shown in Table I, in which the unidirectional ^{22}Na influx and I_{sc} were measured at bulk pH 8.4 (normal Ringer's), 9.5, and 10.5. The unidirectional sodium efflux was small (0.1–0.2 $\mu mol/cm^2 \cdot h$), and was constant at all of these pH values (Mandel, unpublished observations). From these data we conclude that the short circuit current is a good measure of active sodium transport over this pH range.

The results shown in Fig. 2 are more easily visualized if they are replotted as normalized percent inhibition versus pH (Fig. 3). The experimental points in Fig. 3 are shown as solid dots. The smooth curve was calculated as the percentage of amiloride in the charged form versus pH using the unmodified form of the Henderson-Hasselbalch equation, assuming a pK_a of 9.4. The discrepancy between this pK_a value and the measured value of 8.7 is a point that will be treated in the Discussion.

The inhibition of the I_{sc} as a function of amiloride concentration was measured at bulk external pH 8.4 and 10.7. The results, shown in Fig. 4, are plotted as percent inhibition versus the external amiloride concentration. At the high pH, the inhibition curve is shifted to the right, indicating a decrease in the potency of the drug. From this graph we can determine the total concentration of amiloride necessary to produce 50% inhibition at either pH (Table II). The concentration of the charged form of amiloride present at these pH's (at the 50% inhibition level) was computed from the Henderson-Hasselbalch equation, using pK_a values of either 8.7 or 9.4. At these two pH's the concentration of uncharged

moiety which is present at these total amiloride concentrations varies by more than two orders of magnitude, hence this reaffirms the notion that it is the charged form of amiloride which is active.

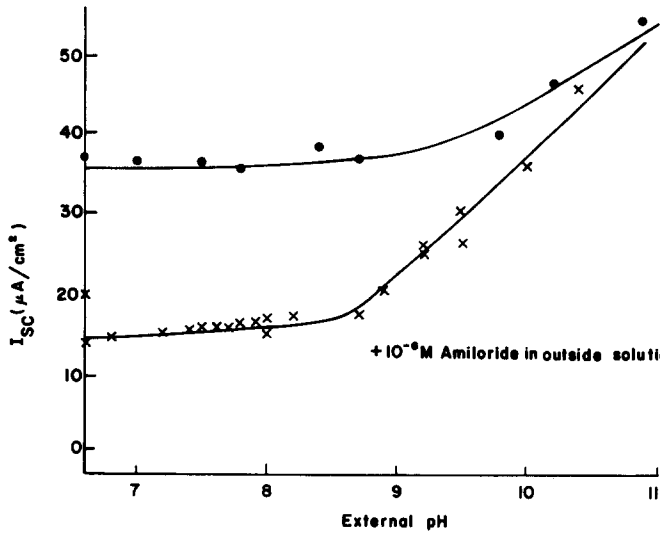Calcium has been shown to have profound effects on the I_{sc} in frog skin

FIGURE 2   FIGURE 3

FIGURE 2.   The effects of pH and amiloride on I_{sc} of isolated frog skin. Short-circuit current is plotted as a function of external pH in the presence (×) and absence (●) of 10^{-6} M amiloride in the outside solution. Normal Ringer solution bathed both sides of the frog skin. This experiment was performed on three separate preparations with essentially the same results. External pH was varied by the addition of dilute solutions of either HCl or NaOH; the internal solution was always held at pH 8.4. The surface area of the frog skin was 3.14 cm².
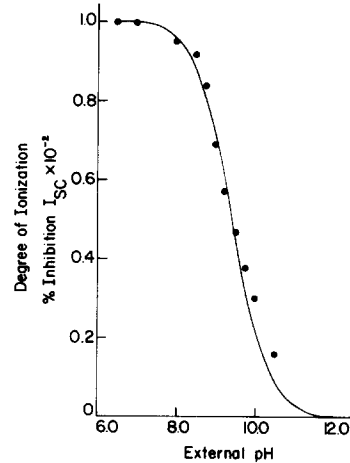
FIGURE 3.   Plot of percent inhibition of I_{sc} or fraction of the total concentration (10^{-6} M) of amiloride in the charged form versus external pH. This graph was computed from the data presented in Fig. 2. Percent inhibition has been normalized to that obtained at pH 6.5 by 10^{-6} M amiloride. The curve was drawn from the Henderson-Hasselbalch equation assuming a pK_a value of 9.4.

TABLE I

EFFECT OF pH ON I_{sc} AND UNIDIRECTIONAL ^{22}Na INFLUX
IN ISOLATED FROG SKIN

pH	Short-circuit current	Tracer Na⁺ influx	N
	$\mu mol/cm^2/h$	$\mu mol/cm^2/h$	
8.4	1.55±0.11	1.38±0.16	6
9.5	2.13±0.16	2.24±0.20	5
10.5	2.16±0.09	2.51±0.22	6

The values given are means ± 1 SEM.

(Curran and Gill, 1962). Furthermore, calcium has been shown to be important in promoting amiloride inhibition of I_{sc} (Cuthbert and Wong, 1972). We have extended these observations to include the effects of divalent cations, pH, and dibucaine on the I_{sc} in the presence and absence of amiloride. The results of
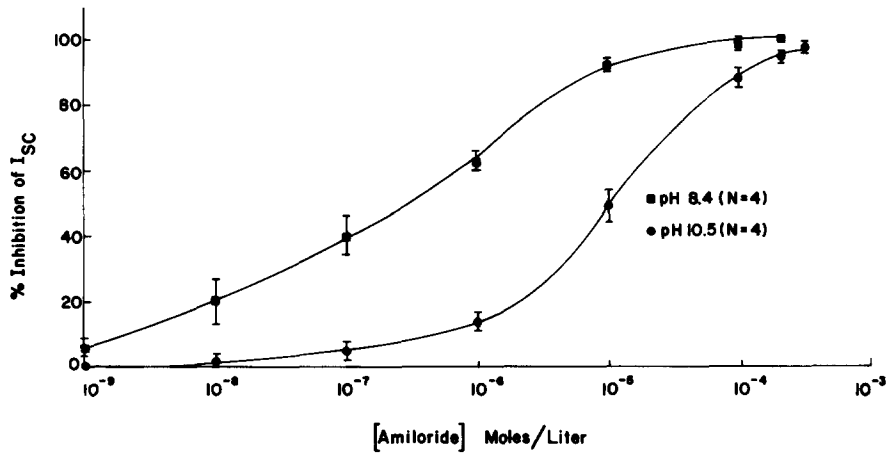

FIGURE 4. The effects of pH on amiloride inhibition of I_{sc} of isolated frog skin. The results are presented as percent inhibition of I_{sc} as a function of the amiloride concentration in the outside solution (logarithmic scale). The number of separate frog skins employed at each pH are given in parentheses; the points represent mean values, while the bars indicate 1 SEM.

TABLE II

CHARGED AMILORIDE CONCENTRATION NECESSARY TO PRODUCE 50% INHIBITION OF I_{sc} IN ISOLATED FROG SKIN AT TWO DIFFERENT EXTERNAL pH VALUES

External pH	Total amiloride concentration (at 50% inhibition of I_{sc})	Charged amiloride concentration (at 50% inhibition of I_{sc})
8.4	5.5×10^{-7} M	3.7×10^{-7} M (pK_a=8.7)
10.5	1.0×10^{-5} M	1.6×10^{-7} M (pK_a=8.7)
8.4	5.5×10^{-7} M	5.0×10^{-7} M (pK_a=9.4)
10.5	1.0×10^{-5} M	7.4×10^{-7} M (pK_a=9.4)

The data are taken from Fig. 4 and the concentration of charged amiloride was computed using the Henderson-Hasselbalch equation at two separate values of pK_a.

these experiments are shown in Figs. 5 and 6; in all of these experiments, the internal solution was maintained constant (regular Ringer's at pH 8.4 containing 1 mM CaCl$_2$). In the presence of Ca^{++} 0.1 mM amiloride completely inhibited the I_{sc} at either pH 6.0 or 8.4 as shown in Fig. 5. In addition it is evident that the I_{sc} is unaffected by lowering the external pH to 6.0; this is in agreement with the observations of Schoffeniels (1955) and Mandel (1975). Therefore any changes in the interaction between amiloride and Ca^{++} produced by the decreased pH

would be independent of the active transport system itself. In the absence of external Ca^{++}, the I_{sc} is observed to double at either pH 8.4 or 6.0. However, the inhibitory action of amiloride is significantly different at these two pH's in the absence of external Ca^{++}, since 10^{-4} M amiloride inhibits the I_{sc} by 65% at pH 8.4 and by 90% at pH 6.0. Therefore, it appears that protons in the external solution potentiate the inhibitory action of amiloride in the absence of external Ca^{++}.

Log-dose response curves of percent inhibition of I_{sc} produced by amiloride were measured in the presence and absence of external calcium at external pH 8.4 and 6.0 (Fig. 6 a). These results demonstrate that, at pH 8.4, removal of Ca^{++} from the external solution not only decreased the maximal percent inhibition

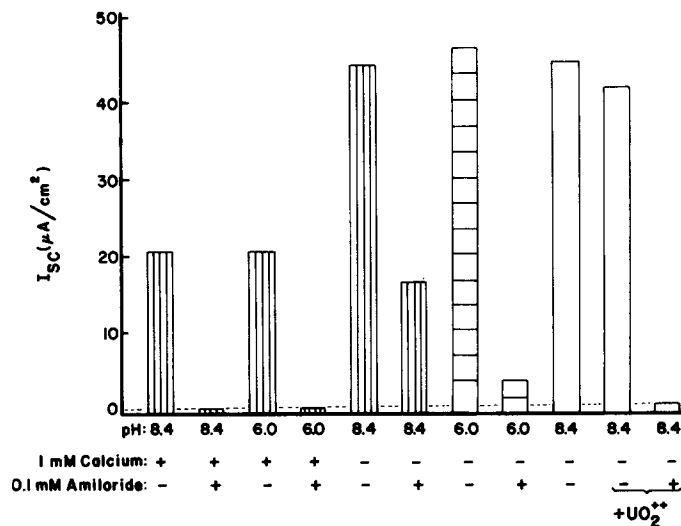| pH:	8.4	8.4	6.0	6.0	8.4	8.4	6.0	6.0	8.4	8.4	8.4
I mM Calcium:	+	+	+	+	−	−	−	−	−	−	−
0.I mM Amiloride:	−	+	−	+	−	+	−	+	−	−	+

$+UO_2^{++}$

FIGURE 5. Bar graphs of I_{sc} of isolated frog skin at pH 6.0 and 8.4 in the presence and absence of various divalent cations and amiloride. The results presented in this figure were obtained from three separate frog skin preparations, indicated by the different banding patterns. All of these experiments were performed a minimum of three times. The experiments shown in this figure were chosen because the initial level of I_{sc} was the same and hence are most comparable.

obtained by amiloride but also increased the amiloride concentration needed for 50% inhibition (I_{50}) from 10^{-7} to 2×10^{-6} M. A kinetic analysis of these results was made by means of a single reciprocal plot (Fig. 6 b); a linear extrapolation of data treated in this manner yields an affinity constant. The nonlinear character of the plots seen in Fig. 6 b indicates that affinity constants cannot be readily obtained in this system without a detailed knowledge of all the rate equations and kinetic parameters which describe the overall transport system.

At pH 6.0, similar log-dose response curves are obtained: the maximal percent inhibition is a function of external Ca^{++}; however, the I_{50} is not affected by Ca^{++}. Eadie plots of these data are also nonlinear.

Other divalent cations could substitute for external Ca^{++} in promoting the amiloride inhibition of I_{sc}. In the absence of external Ca^{++}, uranyl ion (UO_2^{++}) at

a concentration of 1 mM, increased amiloride inhibition from 65 to 100% while magnesium (1 mM) only increased inhibition to 90% (significantly different from 100%, since subsequent addition of 1 mM Ca^{++} produced 100% inhibition). The effects of external UO_2^{++} in the presence and absence of amiloride differentiate
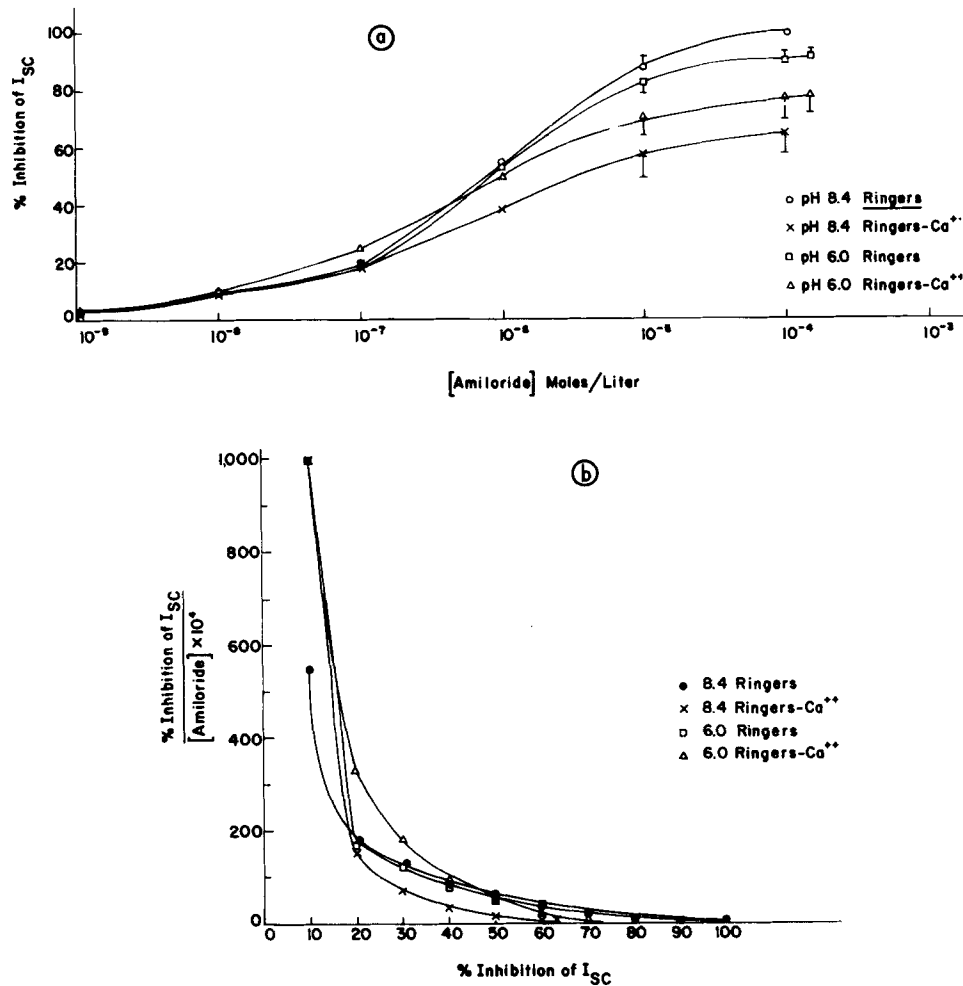

FIGURE 6. (a) The effects of external Ca^{++} on the amiloride inhibition of I_{sc} of isolated frog skin at pH 6.0 and 8.4. The points are mean values of (N) skins and the bars represent 1 SEM. (b) Single-reciprocal plots of the data presented in (a).

between the effects of divalent cations on the I_{sc} from those which promote the inhibitory action of amiloride. As seen in Fig. 5, 1 mM UO_2^{++} added to the external solution produced little inhibition of the I_{sc}. However, in its presence, 10^{-4} M amiloride produced 100% inhibition of I_{sc}.

In Ca^{++}-free Ringer solution, at pH 8.4, 1 mM dibucaine (a local anesthetic) decreased the I_{sc} by about 50%, whereas its presence did not promote the inhibitory actions of amiloride. All of these results indicate that the interaction

between amiloride and the entry step of sodium into the frog skin is sensitive to the external ionic environment in a manner which is different from the sensitivity of the active transport system itself to the same external solutions (see Discussion).
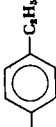
Amiloride Analogue Studies

The specificity of the interaction of amiloride and the outside sodium transport site was studied by examining the inhibition of the I_{sc} produced by a number of structural analogues at an external concentration of 10^{-6} M (Table III). Amiloride (Fig. 1) consists of a substituted pyrazine ring with a guanidinium group attached to ring position 2. Ring positions 3 and 5 are occupied by amino groups and a —Cl is attached at position 6. Lengthening the guanidine side chain with a —NH group at position 2 of the pyrazine ring produced no diminution in the compound's effectiveness as a sodium transport inhibitor (analogue 2). Shortening this chain either by making the carboxylic acid derivative (analogue 3) or by removing —NH (analogue 4), severely reduced the molecule's ability to inhibit the I_{sc}. Alteration of the 5-amino group eliminated the compound's effectiveness (analogues 5–8, and 12 through 15). At position 6, replacing the —Cl with —Br resulted in no effect (analogue 10) while replacement with —I reduced the effectiveness by about half. Substitution of —H for —Cl (analogue 9) severely reduced the inhibitory capability of the molecule.

Alterations of the amiloride molecule may change the pK_a to some value different from that of the parent compound. Only limited data are available on the apparent pK_a values of these analogues; these values were supplied by Merck, Sharp, and Dohme and were measured in 30% ethanolic solutions. If the pK_a of any of these analogues is less than the pK_a of amiloride, the concentration of the charged form of this analogue at pH 8.4 would be less than the charged amiloride concentration, even though the total concentration of drug would be the same. Under these conditions any decrease in the analogue's effectiveness may be due to a lower concentration of the charged form of the compound (the active component) rather than an altered chemical structure. Table IV presents the results of experiments done at the appropriate pH to yield the same concentration of charged species as amiloride at pH 8.4. Analogues whose pK_a values were supplied by Merck, Sharp, and Dohme were tested. As can be seen from Table IV, these compounds were still ineffective in reducing the I_{sc}, indicating that an altered chemical structure was responsible for the decreased effectiveness.

Fig. 7 displays a series of log-dose response curves of the amiloride-induced inhibition of I_{sc} in the presence of two amiloride analogues: N-amidino-3-amino-6-chloropyrazinecarboxamide and guanidine. The former is comparable to amiloride, except that the 5-amino portion is replaced by —H (analogue 6, Table III). Guanidine is considered an analogue of amiloride in that there is a guanidine moiety at position 2, and the positive charge is likely to be situated there. These two compounds do not inhibit the I_{sc} by themselves at the concentrations indicated in the figure. However, the presence of either analogue decreases the amiloride effectiveness at all concentrations, as seen by shifts of the

TABLE III
EFFECT OF AMILORIDE AND ITS CHEMICAL ANALOGUES ON THE INHIBITION OF I_{sc} OF ISOLATED FROG SKIN

Analogue number	Compound	R2	R3	R5	R6	% Inhibition of I_{sc}
1	Amiloride	-CONHCNHNH₂	-NH₂	-NH₂	-Cl	71.8±2.0 (N=21)
2	3,5-Diamino-6-chloropyrazine carboxamide guanidine hydrochloride	-CO(NH₂)CNHNH₂				74.9±1.5 (N=3)
3	3,5-Diamino-6-chloropyrazine carboxylic acid	-COOH				1.0±1.0 (N=3)
4	3,5-Diamino-6-chloropyrazinoyl hydrazine	-COCNHNH₂				5.4±0.9 (N=4)
5	3-Amino-6-chloropyrazine carboxyamido guanidine hydrochloride	-C(NH₂)CNHNH₂		-H		11.2±1.9 (N=6)
6	N-Amidino-3-amino-6-chloropyrazine carboxamide			-H		4.5±1.0 (N=3)
7	3-Amino-5-methylamino-6-chloropyrazinoyl guanidine			-NHCH₃		16.1±5.2 (N=4)
8	N-Amidino-3-amino-5-dimethylamino-6-chloropyrazine carboxamide			-N(CH₃)₂		9.7±0.2 (N=3)
9	N-Amidino-3,5-diaminopyrazine carboxamide				-H	12.0±0.5 (N=6)
10	N-Amidino-3,5-diamino-6-bromopyrazine carboxamide				-Br	68.0±3.8 (N=3)
11	N-Amidino-3,5-diamino-6-iodopyrazine carboxamide				-I	33.1±4.1 (N=3)
12	N-Amidinopyrazine carboxamide		-H	-H	-H	0(0;0) (N=2)
13	N-Amidino-3-aminopyrazine carboxamide			-H	-H	5.5±1.7 (N=4)
14	N-Amidino-3-amino-6-bromopyrazine carboxamide			-H	-Br	3.6±2.5 (N=3)
15	N(N,N-Dimethyl)-3-amino-5-isopropylamino-6-chloropyra-zine carboxamide	-CONHCNHN(CH₃)₂		-NHCH(CH₃)₂		1.6±0.4 (N=4)
16	N(N-Phenethylamidino)-3-amino-6-chloropyrazine carboxamide	-CONHCNHNH-⟨C₆H₄⟩-C₂H₅		-H		8.6±1.0 (N=4)

All of these compounds were added to the outside solution (pH = 8.4) at a concentration of 10^{-4} M.

log-dose response curves to the right. A potentiation of the inhibitory action of amiloride was observed in a single experiment in the presence (10^{-4} M) of the negatively charged carboxylic acid derivative (analogue 3).

Bilayer Experiments

The effect of 10^{-4} M amiloride on the conductance of lipid bilayers made from sheep red cell lipids bathed in a symmetrical solution of 10^{-1} M KCl and 10^{-8} M nonactin at pH 7.4 is shown in Fig. 8. The upper trace is the current-voltage curve obtained in the absence of amiloride, and the lower curve is that obtained in the presence of 10^{-4} M amiloride on both sides of the membrane. The presence of 10^{-4} M amiloride in the aqueous solutions decreased the nonactin-induced potassium conductance in the limit of zero applied voltage by a factor of 2. From the analysis given in the Methods section, this decrease in conductance

TABLE IV

EFFECT OF SEVERAL AMILORIDE ANALOGUES ON THE INHIBITION OF I_{sc}
OF ISOLATED FROG SKIN AT LOW EXTERNAL pH

Compound	External pH	pK$_a$	% Inhibition of I_{sc}
N-Amidino-3-aminopyrazine carboxamide (analogue 13)	6.7	—	1.7±0.7 (N=4)
N-Amidino-3-amino-6-chloropyrazine carboxamide (analogue 6)	6.7	7.0	3.6±0.9 (N=4)
N-Amidino-3-amino-6-bromopyrazine carboxamide (analogue 14)	6.8	7.1	0.4±0.4 (N=4)
N (N-Phenethylamidino)-3-amino-6-chloropyrazine carboxamide (analogue 16)	6.4	6.7	23.1±3.7 (N=4)
N (N,N-Dimethyl)-3-amino-5-isopropylamino-6-chloropyrazine carboxamide (analogue 15)	7.5	7.8	0.5±0.3 (N=4)
3-Amino-6-chloropyrazine (carboxamido) guanidine hydrochloride (analogue 5)	7.3	7.6	17.2±1.0 (N=4)

All compounds were added to the solution bathing the outside surface of the frog skin at a concentration of 10^{-6} M. The pH of the outside solution was adjusted to the desired value by titration with a dilute solution of HCl.

translates into an increase in the surface potential of 18 mV at room temperature. The above experiment was also performed on neutral egg phosphatidyl-choline bilayers with essentially the same results, indicating that this increase in surface potential was independent of the net charge on the lipid constituents of the bilayer (red cell lipids carry a net negative charge and contain cholesterol [White, 1973]).

Table V summarizes the results obtained from this analysis on the amiloride-nonactin-K$^+$ system. At pH 7.4, amiloride increased the surface potential of the net negatively charged sheep red cell lipid bilayers by 18 mV.

The specificity of amiloride in altering the surface potential was tested by studying two analogues of this compound (Table V). Both compounds differ from the parent molecule in the group attached to carbon ring position 5; the —NH$_2$ group is replaced by a dimethyl amino group (—N(CH$_3$)$_2$) in the first analogue, and by a —H in the second. The second analogue also has an

additional —NH group inserted into the guanidinium moiety, which is attached to the ring at position 2. As can be seen in Table V, a comparable increase in the surface potential of the bilayer is produced by both of these analogues. These two analogues display very little biological activity as assayed in the isolated frog skin (Table III).
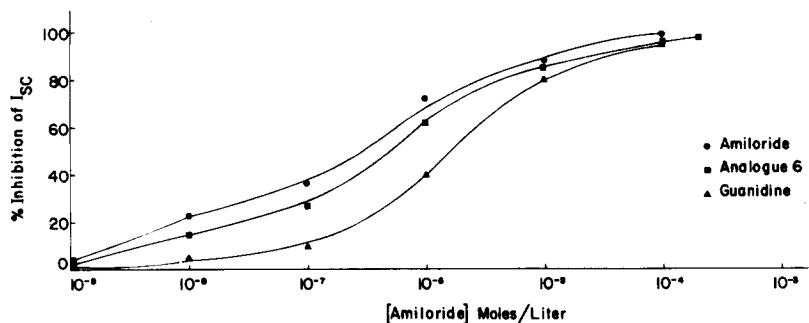

FIGURE 7. Log-dose response curves of the amiloride inhibition of I_{sc} of isolated frog skin in the presence of two structural analogues. This experiment was performed at pH 8.4; the analogues themselves produced no inhibition of I_{sc} at the concentrations indicated.
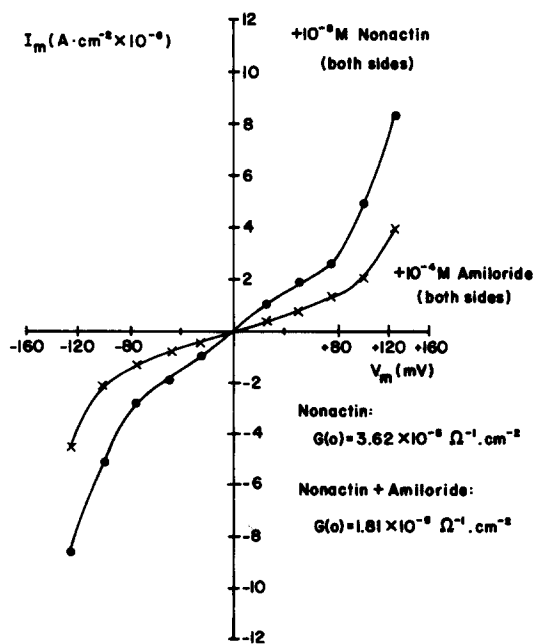

FIGURE 8. The effect of amiloride on the current-voltage curve of a nonactin-modified sheep red cell lipid bilayer membrane. The membrane separated identical solutions of 10^{-1} M KCl, 10^{-8} M nonactin, and was buffered at pH 7.4 with 10^{-3} M phosphate.

DISCUSSION

Previous experiments suggest that the sodium entry step into frog skin is not due to a simple diffusion process, but that the sodium ion must interact with some membrane component for translocation to occur (Biber and Curran, 1970; Biber, 1971; Mandel and Curran, 1973). The evidence for this contention is threefold: (a) Na⁺ influx is not a linear function of external sodium concentration, but displays saturation kinetics; (b) lithium competitively inhibits a portion of the sodium influx; and (c) external K⁺ competitively inhibits active Na⁺ transport (K⁺ is impermeant from the outside). Amiloride exerts its inhibitory effect on the saturable portion of the sodium influx. This portion is equal in magnitude to the short-circuit current (Biber, 1971). Therefore, it seems likely that amiloride blocks the external site for Na⁺ entry into the active transport system. The present study reports our investigation into the nature of this amiloride-sensitive transport site.

TABLE V

ALTERATION OF THE SURFACE POTENTIAL OF SHEEP RED CELL LIPID
BILAYERS BY AMILORIDE AND TWO OF ITS CHEMICAL ANALOGUES

Compound	Change in surface potential
	mV
Amiloride	+18
N-Amidino-3-amino-5-dimethyamino-6-chloropyrazine carboxamide (analogue 8)	+22
3-Amino-6-chloropyrazine-(carboxamido)guanidine hydrochloride (analogue 5)	+14

Both sides of the membrane were bathed with identical solutions of 10^{-1} M KCl and 10^{-8} M nonactin buffered at pH 7.4. Amiloride and its analogues were added to both sides of the membrane at 10^{-4} M. The results presented are the mean values of three separate experiments.

Amiloride (Fig. 1) consists of a substituted pyrazine ring with a guanidinium group attached to ring position 2. The 3 and 5 ring positions are occupied by amino groups and a —Cl is attached at position 6. Since amiloride contains a guanidinium moiety, it exists either as the positively charged amine or as the undissociated base. The relative proportions of these two forms at any given pH can be determined from the Henderson-Hasselbalch equation. If the charged form of amiloride is the physiologically active form increasing the external pH from 8.4 should result in a decreased amiloride effectiveness, since the drug will now be predominantly in the neutral form. This type of behavior is experimentally observed, as shown in Figs. 2 and 3. The results shown in Fig. 4 are also consistent with the notion that the charged form of amiloride is the active species, since the log-dose response curve of amiloride inhibition is shifted to the right at higher pH. As pH is increased by two units, the concentration of charged amiloride necessary to produce 50% inhibition of I_{sc} remains unchanged, while the total amiloride concentration varies by two orders of magnitude (Table II).

It is apparent from Fig. 3 that 50% inhibition of I_{sc} is obtained at pH 9.4, a value which differs from the pK_a value of 8.7 supplied by the drug company. This discrepancy can be rationalized in several ways. The pK_a of 8.7 was determined in a 30% ethanol-water solution of zero ionic strength (E. J. Cragoe, personal communication). The pK_a of a base is generally lower in an ethanolic solution than in a pure water system because of the difference in dielectric constants (Edsall and Wyman, 1958). Increases in ionic strength also increase the pK_a of a base (Edsall and Wyman, 1958). Both of these factors may contribute to the discrepancy between the pK_a values of 8.7 and 9.4. In addition, the pH in the vicinity of the sodium transport site may actually be lower than the bulk pH. This could be the result of the net secretion of hydrogen ions across the external surface of the frog skin (Friedman et al., 1967; Emilio et al., 1970; Emilio and Menano, 1975).

Another feature shown in Fig. 2 is that the short-circuit current of the untreated frog skin increases as the external pH is raised above 9. Depending upon the molecular nature of the Na^+ transport site, this observation could result from several possibilities. First, the number of functional transport sites could increase at high pH; removal of H^+ ion could expose more sites. Second, the number of sites could remain constant, but the turnover rate (i.e., the number of ions handled per unit time) could increase. If this site were pore-like, an increased turnover rate could result from the pore diameter becoming larger, from a shortened dwell time of the ion inside the pore, or from the channel remaining open longer. If there are multiple conductance states per channel (Cuthbert, 1974), increasing the pH could increase the possibility that the channel would be in a higher conductance state. In a carrier-like site, high pH could increase turnover by increasing transport through the rate-limiting step of the ion-carrier reaction sequence. A third possibility would be a reduction in the divalent calcium concentration at high pH by the association of calcium with hydroxyl ions (Sillen and Martell, 1964). It is noteworthy that increasing hydroxyl ions increases I_{sc} (Fig. 2), as does removal of calcium from the external solution (Fig. 5).

The results shown in Figs. 5 and 6 indicate that a complex interaction exists between amiloride, cations, and the sodium transport site. There are two effects of calcium: one in reducing the I_{sc} and the other in promoting amiloride inhibition. We have presented data showing that H^+ and UO_2^{++} are able to substitute for Ca^{++} in the second effect, while dibucaine but not UO_2^{++} can substitute for Ca^{++} in the first. These two separate actions are interpreted as occurring at two distinct sites, each site possessing different cation-binding specificities.

The lack of inhibitory action of uranyl ion on I_{sc} (in the absence of amiloride) is surprising in light of the known inhibitory effects of other divalent cations of similar size, such as barium and strontium (Curran and Gill, 1962; Mandel, unpublished observations). Since UO_2^{++} is known to profoundly alter surface charge in bilayers (McLaughlin et al., 1971) and nerve (D'Arrigo, 1975), it is unlikely that surface charge plays an important role in the active transport of sodium through the frog skin. If UO_2^{++}, at 10^{-3} M, acted on frog skin in the

same manner as it acts on phospholipid bilayer membranes, i.e., by inducing a positive surface potential of about 100 mV in Ringer solution (McLaughlin et al., 1971), the surface pH would be increased by about 1.7 pH units and the surface concentration of charged amiloride would be reduced from 6.7×10^{-5} to 4.0×10^{-8} M. At this amiloride concentration I_{sc} should be inhibited only by about 5–10% rather than the 100% observed. Hence, the action of UO_2^{++} as well as other cations in potentiating the inhibitory effect of amiloride probably occurs at a protein site rather than at a phospholipid region of the outer membrane of frog skin. This interaction presumably alters the proteins of the transport site in such a manner as to increase amiloride binding.

The results presented herein are consistent with those of Cuthbert and Wong (1972) who found that removal of Ca^{++} from the external bathing solution severely reduced the inhibitory effects of amiloride. However, some important discrepancies with their results were found: first, magnesium (at 1 mM) did not entirely substitute for calcium; second, the interaction between amiloride and the transport site is nonlinear when analyzed in terms of a single reciprocal (see Fig. 6 b) or a double reciprocal plot (Lineweaver-Burk plot) and, therefore, affinity constants cannot be readily obtained. Third, the present results, showing two distinct Ca^{++} sites, are inconsistent with the ternary complex model of Cuthbert and Wong (1972) wherein the calcium which inhibits Na^+ transport is the same calcium which potentiates the inhibitory action of amiloride.

We have demonstrated that amiloride can alter the surface charge on artificial bilayers (Table V). This action, however, appears to be a general property of various amiloride analogues, regardless of their effectiveness as short-circuit current inhibitors. Therefore, the inhibition of I_{sc} by amiloride cannot be attributed to surface charge effects, but it appears that amiloride specifically interacts with membrane components directly involved in the translocation of sodium across the external surface of the frog skin.

This interaction is extremely specific since slight modifications in the primary structure of amiloride can render the molecule ineffective. From an analysis of compound structure-activity relationships, we may be able to obtain some appreciation of what components are necessary to ensure proper drug action, and hence how the Na^+ transport site might be organized. As Table III indicates, halogenation of the 6-position on the pyrazine ring is essential for activity. If the —Cl group is replaced by —H, compound activity is virtually eliminated. Substitution by —Br or —I likewise decreases activity. Steric considerations may be important; for a proper "fit" or "approach" to the Na^+ transport site, this group may require a suitable size range. Chloride has a Van der Waals radius of 1.8 Å, while that for —Br is 1.95 Å, and —I, 2.1 Å. Hydrogen has a radius of 1.2 Å. The further away from a radius of 1.8 Å, the less effective the compound is in inhibiting the I_{sc}. These same analogues were tested in whole animal studies with essentially similar results (Cragoe et al., 1967) with the exception that the frog skin bioassay is more sensitive in discriminating between the actions of the various analogues.

If the amino group at position 5 of the amiloride ring is modified, the

resultant compound loses its potency (Table III, analogues 6–8). Substitutions at position 3 were not available.

Since the charged form of amiloride is essential for activity, this implies that for maximal inhibitory function, the charge must have a certain orientation with respect to the transport site, as well as a certain spatial separation relative to other portions of the amiloride molecule. Our conclusions concerning the localization of the positive charge are based upon the following considerations: *(a)* The charge is located on the guanidinium portion of the molecule, since any amino group on the ring would have a pK much lower than 8 (Handbook of Chemistry and Physics, 55th ed., Vol. 57). *(b)* The charge on the guanidinium group may be resonating (Edsall and Wyman, 1958), however, the double-bonded nitrogen could carry the charge, since methylation of the terminal nitrogen does not alter the diuretic potency of the drug (Cragoe et al., 1967). Lengthening of this guanidinium moiety by insertion of an additional —NH group (Table III, analogue 3) does not alter the effectiveness of the compound. Comparing the space-filling models of amiloride *(a)* and this analogue *(b)* presented in Fig. 9, we see that the orientation of the charged group does not change with respect to the rest of the molecule, and that the distance of this charge group from the center of the ring is not appreciably different. If instead of adding a —NH to the guanidine we remove one (Table III, analogue 4), the analogue inhibits the I_{sc} only 4% at 10^{-6} M. The space-filling model of this analogue (Fig. 9 c) shows that the charged portion of the guanidinium group is not in the same position as in the parent molecule. It is also possible that substitutions of this sort may change the pK_a of the compound, thereby decreasing the total concentration of charged molecules in solution. From these data we conclude that the guanidinium group has to be charged and that this charge must bear a given configuration relative to the rest of the molecule in order for the whole molecule to possess inhibitory activity.

We found that both guanidine (10^{-4} M) and analogue 6 (10^{-5} M) shift the amiloride log-dose response curve to the right (Fig. 7). Neither compound appreciably inhibits I_{sc} at the concentrations used, hence a competitive type of inhibition is probably occurring. From our structure-function analysis of the amiloride analogues, we concluded that for an analogue to possess inhibitory action it must simultaneously be charged in the guanidinium moiety, halogenated at position 6, and possess a —NH$_2$ group at position 5. This implies that all of these positions are involved in the molecule's interaction with the transport locus. Thus, any analogue with some but not all of the above groups should competitively inhibit amiloride.

Throughout this paper we have attempted to obtain ideas about the molecular architecture of the sodium transport site located on the outer surface of the frog skin by studying the interactions of this site with amiloride and a number of its structural analogues. Biological ion translocators are commonly categorized as being either carriers or pores. Comparisons between the sodium entry step in frog skin and known carrier molecules and pore-formers in both biological and model systems using the criteria of saturation kinetics, turnover rates, and
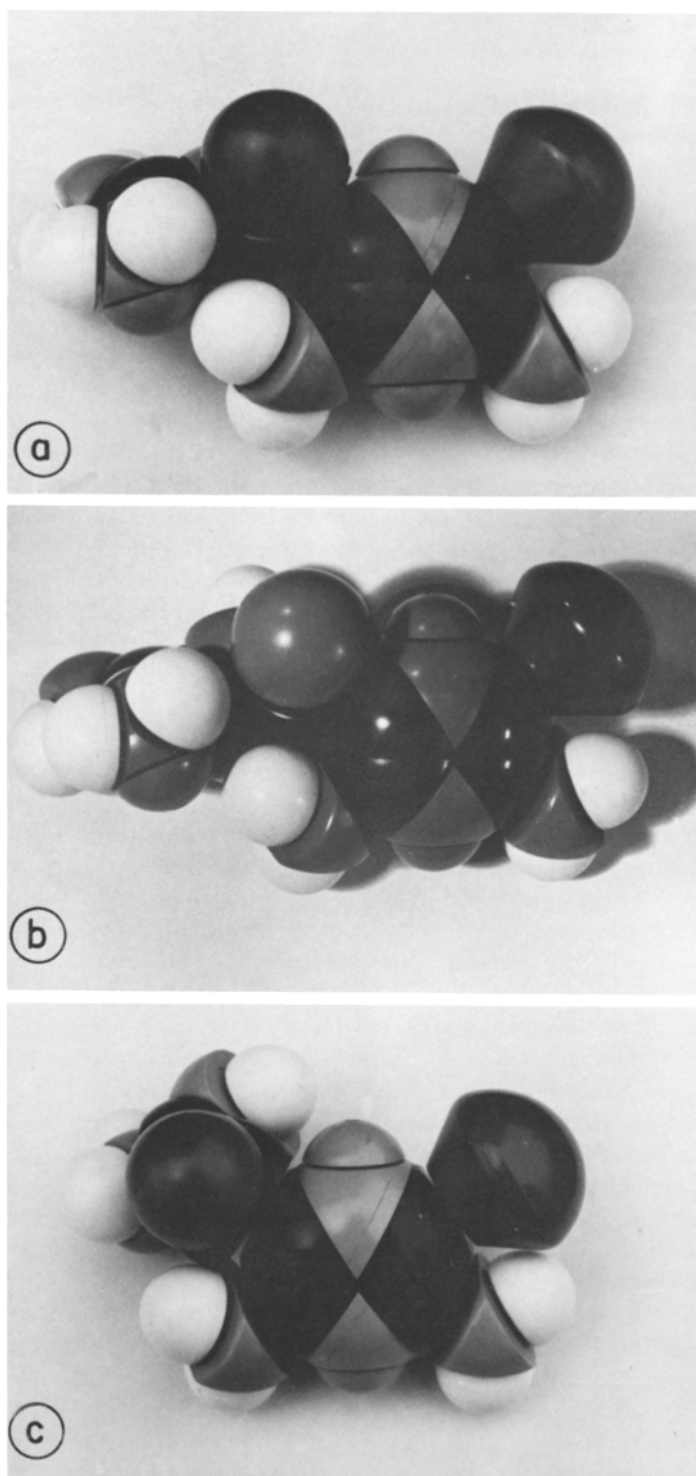
FIGURE 9. Corey-Pauling space-filling models of amiloride and two of its chemical analogues with modifications made in the guanidinium side chain (ring position 2). (a) N-amidino-3,5-diamino-6-chloropyrazine carboxamide (amiloride); (b) 3,5-diamino-6-chloropyrazine carboxamide guanidine hydrochloride (analogue 2); (c) 3,5-diamino-6-chloropyrazinoyl hydrazine (analogue 4).

activation energies do not allow a distinction to be made between these two modes of ion transport (Snell and Leeman, 1957; Hladky and Haydon, 1972; Haydon and Hladky, 1972; Lauger, 1972; Cuthbert, 1973; Cuthbert and Shum, 1974 *a,b;* Alvarez et al., 1975; Hille, 1975 *a,b*). Elucidation of the exact chemical nature of this sodium transport site must await further analysis.

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