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Interactions of Sodium Channels in Transporting Epithelia: a Two-State Model

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SUMMARY

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A two-state (allosteric) model based on that for allosteric proteins is proposed for the sodium ion-translocating mechanism of transporting epithelia. The model supposes that the mechanism can exist in two alternative configurations: one form which can translocate sodium ions through the cell membrane, and another form which is unable to do this. Interactions between a number of substances which either increase or decrease sodium fluxes through the mucosal membranes are considered in terms of the model. The behavior of the model was found to mirror that of actual epithelia for a number of experimental situations. At the present time the following interactions with the translocation mechanism can be best explained in terms of the two-state model: amiloride–Na^+, amiloride–Na^+–antidiuretic hormone, Ca^{++}, Ca^{++}–antidiuretic hormone, and Ca^{++}–amiloride.

INTRODUCTION

This paper attempts to explain how the interactions of a number of substances with the mucosal membranes of sodium-transporting epithelia affect the translocation of sodium ions through those membranes. In particular attention will be focused on the properties of the mucosal membranes of two amphibian epithelia, the ventral skin of the frog and the urinary bladder of the toad.

The need to consider a two-state model for the sodium channel arose when data had accumulated which were not consistent with the Michaelis-Menten approach to interactions at drug receptors; that is, in this instance, at the sodium ion-translocating mechanism. After the model had evolved it was found to accommodate other data which otherwise fitted within a more conventional framework. Thus the model appears to have more general applicability than was anticipated.

The body of the paper consists of two main parts. The first deals with difficulties associated with the Michaelis-Menten approach to drug interactions at sodium channels, while the second part presents the model and discusses its applicability. The term channel is used in this paper as a phenomenological concept to mean the mechanism translocating sodium ions across the membrane. Further consideration of the nature of the translocation mechanism is given under GENERAL DISCUSSION.

METHODS

Details of the methods used for measuring sodium transport and channel density in

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amphibian epithelia are presented in detail elsewhere (1), including the preceding paper (2).

RESULTS AND DISCUSSION

Difficulties with the Michaelis-Menten Approach

Certain findings described in the preceding paper (2) are difficult to reconcile with the Michaelis-Menten hypothesis when considering interactions of sodium channels in transporting epithelia. These findings are that antidiuretic hormone does not increase the channel density (more strictly, the number of amiloride binding sites) in the mucosal face of frog skin, while the concentration-inhibition curve for amiloride is moved to the right along the concentration axis (see Fig. 7 of ref. 2).

Given these findings, the inadequacies of the mass-action type of approach become clearer as possible explanations for the findings are explored. There are three explanations to be considered for the lack of effect of ADH[1] on the number of channels, while allowing for the increase in transport following hormone, as follows.

1. Hormone increases the fraction of channels which are operative (open), or increases the proportion of time during which they are operative. Phenomenologically these two options are indistinguishable, given a fixed number of channels.

2. Hormone increases the rate of handling of sodium by each channel. This option could be equivalent to the second alternative of option 1 under some circumstances.

3. Hormone causes formation of a small (undetectable) number of new channels which handle sodium at an extremely high rate.

Option 3 can be eliminated, since the formation of a few new channels would need to be responsible for the shift of the inhibition curve to amiloride, yet the inhibition curve after hormone is a monotonic function of concentration, suggesting a homogeneous population of channels. Option 2 is equally untenable, although a simple "widening" of the channels, together with reduced affinity to amiloride, is appealingly simple. However this mechanistic view is too naive, since the channels remain specific for sodium after ADH. Furthermore, amiloride inhibition curves were always monotonic after ADH, requiring complete conversion of the channels to the widened form, a very unlikely situation. Finally, the stoichiometry between Na^+ and amiloride is 1:1 both before and after hormone. If the channels are widened to handle more sodium ions, this should be reflected by an increase in the number of amiloride binding sites.

An alternative form of option 2 is that the mechanism translocating sodium ions operates at a faster rate, without altering the proportion of time during which the mechanism is operative. If the translocation mechanism involves a mobile carrier, the hormone might influence the environment of the carrier in such a way that mobility increases. Under these circumstances the reduced affinity for amiloride might be due to a reduction in the statistical chance of a successful interaction between amiloride and the carrier at the outer surface of the membrane. On the other hand, increasing fluidization of the membrane would also reduce the chance of the carrier picking up a sodium ion, so that the degree of inhibition by amiloride, and hence its affinity, would not necessarily be altered by fluidization. This argument implies that hormone must effect alterations which result in a differential change in affinity of the translocation mechanism for sodium and amiloride.

One further, extreme alternative of option 2 is that the channels operative after ADH are not the same as in the control state; that is, ADH temporarily inactivates one set of sites and activates a second set which handle ions faster and have a reduced affinity for amiloride. This can probably be rejected,

[1] The abbreviations used are: ADH, antidiuretic hormone; SCC, short-circuit current; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; MWC, Monad, Wyman and Changeux.

since there would need to be complete conversion between two states if the inhibition curves for amiloride were to remain a monotonic function of concentration. Furthermore, this makes it difficult to understand how graded responses to hormone are obtained, and it would be curious if the number of sites in the two sets were equal.

Option 1 would appear to be the most plausible explanation for the effects of ADH. This supposes that a fixed number of channels can exist in open or closed forms and that ADH alters the proportion which are open (or alternatively increases the time spent in the open configuration). This does not, however, explain the shift in the amiloride inhibition curve caused by ADH. Assuming that amiloride binding occurs by Langmuir type adsorption, four types of interaction are possible. Amiloride might bind (a) to open channels only, (b) to closed channels only, (c) to open and closed channels equally, or (d) to open and closed channels unequally. Alternative (b) can be discarded within the Michaelis-Menten framework, since no inhibition of transport would occur, unless combination with closed channels leads to alteration of the equilibrium between the two forms (see below). Alternative (a) is also unacceptable, since ADH would cause an increase in the estimate of channel density, and the inhibition curve would not be shifted. Alternative (c) would give no increase in the number of channels after ADH, nor would the inhibition curve be shifted. Alternative (d) gives either an increase or decrease in channel density (depending on whether the affinity for amiloride is greater for the open or closed channels), but again the inhibition curve would be unaltered. To explain the experimental data it is necessary to assume both that amiloride binds unequally to open and closed channels and that there is a dynamic equilibrium between the two forms.

The two-state model outlined below is compatible with the experimental data, and forms a framework in which to consider other interactions with the sodium channel.

The Allosteric Model

The proposal is that the channel is an allosteric protein possessing multiple stereospecific sites for several ligands and is based on the simple model discussed by Monod, Wyman, and Changeux (3). The features of the model are that protein can exist in one of two conformational states and that binding sites remain equivalent upon transformation between the two states, but that the microscopic dissociation constants for a given ligand may be different in the two states. Mathematical approaches to two-state models to explain the actions of drugs and hormones have been made by several authors (4–8). Formulation of a model for the sodium channel may be considered as follows. Let there be two conformational states of the channel, the open (R) form and the closed (T) form. It is assumed that channels in the R form are able to translocate sodium ions, irrespective of what ligands are bound, while the channels in the T form are unable under any circumstances to translocate sodium ions. Thus the translocation of sodium ions will depend on the relative proportions of R and T forms. The relative proportions of the R and T forms will be altered by ligands which combine preferentially with one form; for example, ligands with a higher affinity for the R form will increase the proportion of that form.

Let each channel have n equivalent sites for binding amiloride (A), and let the microscopic dissociation constants be K_{AR} and K_{AT} for the R and T forms, respectively. The fraction of channels in the R form is given by the state function (3)

$$\bar{R} = \left[1 + L\left(\frac{1 + a\alpha}{1 + \alpha}\right)^n\right]^{-1} \qquad (1)$$

where $L = T_0/R_0$ and is the equilibrium constant of the two states in the absence of ligand, and $a = K_{AR}/K_{AT}$ and $\alpha = [A]/K_{AR}$.

It was shown (2) that sodium competitively inhibited the actions of amiloride and that the apparent stoichiometry with the channel for these two substances is 1:1. Modifying Eq. 1 to include the competitive

interaction between amiloride and Na^+, we obtain

$$\bar{R} = \left[1 + L\left(\frac{1 + a\alpha + b\beta}{1 + \alpha + \beta}\right)^n\right]^{-1} \quad (2)$$

where $b = K_{NaR}/K_{NaT}$ and $\beta = [Na]/K_{NaR}$.

When ADH reacts with the serosal surface of epithelia like frog skin and toad bladder the permeability of the mucosal face to sodium increases. The general view is that ADH generates a second (intracellular) messenger (9), which then either directly or indirectly is responsible for the increase in permeability. Without specifying the nature of the messenger, X, which eventually interacts with the mucosal membrane, let it bind to m sites (other than the n sites which interact with Na^+ and amiloride), with microscopic dissociation constants K_{XR} and K_{XT}, with the R and T forms of channel. The prediction is that in the presence of hormone the fraction of channels in the R form will be given by

$$\bar{R} = \left[1 + L\left(\frac{1 + a\alpha + b\beta}{1 + \alpha + \beta}\right)^n \cdot \left(\frac{1 + c\gamma}{1 + \gamma}\right)^m\right]^{-1} \quad (3)$$

where $c = K_{XR}/K_{XT}$ and $\gamma = [X]/K_{XR}$.

Since amiloride interacts with the mucosal membrane to inhibit sodium entry, the value of a will be greater than 1, but c will have a value less than 1, since ADH increases sodium permeability.

Three sets of solutions to Eq. 3 were computed for a range of amiloride concentrations (Fig. 1a–c). If it is assumed that Na^+ transport is a function of $\bar{R}$, such that $Z = f(\bar{R})$, where Z is the cellular response, then the results shown in Fig. 1 should mirror the inhibition of sodium transport by amiloride, in the presence and absence of hormone and at varying sodium concentrations. The theoretical curves clearly resemble the experimental findings (see Fig. 7 of ref. 2).

Comment must be made on the various parameters of Eq. 3 and how they were chosen to produce Fig. 1. It has been assumed throughout that $L = 1$; i.e., equal numbers of open and closed channels exist in the resting state. It is usual for ADH to cause a doubling or trebling of sodium transport in frog skin and toad bladder. If values of L were chosen much larger than 1, bigger increases in transport with ADH would be possible. With values of L smaller than 1 the response to ADH would be correspondingly small.

The ratio of the dissociation constants for the R and T forms of the channel with

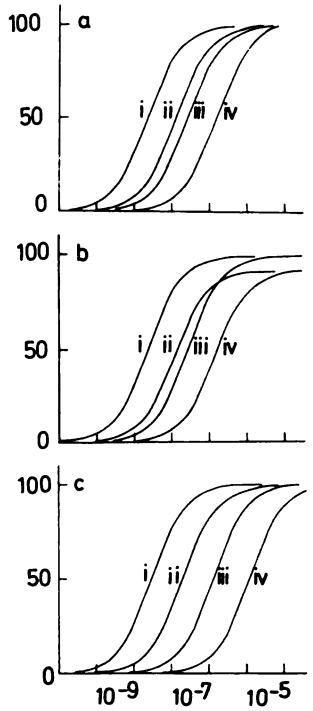

FIG. 1. *Theoretical inhibition curves for amiloride in the presence of low (i, ii) and high (iii, iv) sodium concentrations and in the absence (i, iii) and presence (ii, iv) of antidiuretic hormone, computed from Eq. 3*

The ordinate expresses the percentage reduction of $\bar{R}$, and the abscissa, the amiloride concentration. K_{AR}/K_{AT} was 1000 in a and c and 100 in b. Values of K_{AT} were taken as 10^{-8}. $K_{NaR} = K_{NaT} = 10^{-2}$ in a and b; in c, $K_{NaT} = 10^{-2}$ and $K_{NaR} = 10^{-3}$. Throughout $(1 + c\gamma)/(1 + \gamma)$ was taken to equal 0.1; m and $n = 1$; and low and high sodium concentrations were taken as 10^{-2} and 120×10^{-3}, respectively. The percentage increases in $\bar{R}$ in the absence of amiloride caused by hormone were 82% in a and b and 47% (10^{-2} Na) and 10% (120×10^{-3} Na) in c.

amiloride (K_{AT}/K_{AR}) were taken as 0.010 in Fig. 1a and c, and as 0.01 in Fig. 1b. This assumes that the affinity of amiloride for the closed (T) form of the channel is greater than for the open (R) form by a factor of either 1000 or 100. Nearly parallel shifts of the inhibition curves were obtained in all instances, but when the ratio was 0.01 (Fig. 1b) amiloride caused less inhibition in the hormone-stimulated condition than in the absence of hormone. (In fact, this was also true when the ratio was 0.001, but the difference is not obvious.) It should be remembered that if a ligand binds equally to both R and T forms, it would have no effect on the proportions of the two forms. Reference to the results of Salako and Smith (10) and those of the preceding paper (2) will show that it is common for high concentrations of amiloride to fail to cause complete inhibition of sodium transport in the presence or absence of ADH. However, the differences between complete inhibition and the amounts of inhibition obtained at high drug concentrations are so small that it is not possible to say whether these differences are real or due to experimental error.

The experimental results show that ADH caused approximately a 3-fold shift in the inhibition curve whether or not the sodium concentration was 1 or 110 mEq/liter (2). Whether the shift at different sodium concentrations is exactly the same is difficult to establish experimentally. By assuming that the affinity of Na^+ is the same for the R and T forms of the channel (i.e., $K_{NaR}/K_{NaT} = 1$), exactly the same shift is obtained (Fig. 1a and b). If, however, the affinity of Na^+ varies by as little as 10-fold, unequal shifts are obtained (Fig. 1c, $K_{NaR}/K_{NaT} = 0.1$). Thus, if the model is correct, the affinities of Na^+ for the R and T forms must be, at least, nearly equal. A further consequence of having equal affinities between Na^+ and the R and T forms is that the percentage increase in transport caused by ADH is the same at all sodium concentrations. This was found to be so experimentally (11).

In the computations used for Fig. 1, a value of 0.1 was given to the fraction $(1 + c\gamma)/(1 + \gamma)$. Thus X, the unknown factor generated by hormone, would be required to have only a modestly higher (10-fold) affinity for the R over the T form of the channel.

The factors m and n have been taken as 1 for the results shown in Fig. 1. If indeed m and n are 1, there can be no cooperativity. If there is cooperativity, it will be marked if L is large (>1) (3). In an attempt to ascertain whether or not interactions at sodium channels in transporting epithelia involve cooperative effects, Hill plots were made from the data contained in a number of amiloride inhibition curves selected at random.

For toad bladder the slopes of Hill plots were always approximately 1, and this was true too of frog skin when the sodium concentration was 1 mEq/liter. For frog skin at 111 mEq/liter of Na^+, the mean value of the slopes was greater than 1, although individual values were not invariably so. The data are summarized in Table 1. From these findings it is considered that no cooperativity was present under the conditions in which amiloride binding was measured as described in the preceding paper (2). The cooperativity between channels in frog skin at high sodium concentrations is small and, when present, might indicate that some of the channels occur in clusters. Figure 2 shows a Hill plot for a frog skin showing a maximal change in slope caused by changing the sodium con-

TABLE 1

Hill coefficients from amiloride inhibition curves

Tissue	[Na^+]	Hill coefficient		
		Mean ± SE	No. of observations	Range
	mEq/l			
Toad	111	0.96 ± 0.02	8	0.92–1.03
bladder	1.1	0.95	2	
Frog	111	1.22 ± 0.13	5	0.9–1.64
skin	1.1	0.92 ± 0.02	6	0.86–1.0

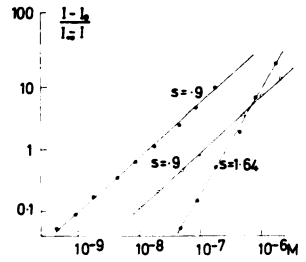

FIG. 2. *Hill plots constructed from amiloride inhibition curves*

●, frog skin in the presence of 1.1 (left) and 111 (right) mEq/liter of Na^+; ○, toad bladder in the presence of 111 mEq/liter of Na^+.

centration. It must be remembered, however, that a somewhat different explanation for the change in slope of the inhibition curves was described previously (2).

Interactions with calcium. It has been known for some time that when calcium is removed from the mucosal solution bathing sodium-transporting epithelia the inhibitory effect of amiloride is reduced or abolished (12–14). However, in order to alter the sensitivity of the mucosal membrane to amiloride in this way it is necessary to remove both calcium from the solution and that bound to mucosal sites by using a chelating agent. When calcium is restored to the bathing solution the inhibitory effect of amiloride reappears; indeed, other polyvalent cations (Mg^{2+}, Sr^{2+}, and La^{3+}) can restore the sensitivity to amiloride (15). When the mucosal surfaces of epithelia are treated with calcium-chelating agents the SCC increases (16), although this increase may not be maintained, perhaps as a result of loosening between the epithelial tight junctions. Thus, as in other biological situations, it is considered that the extent of monovalent cation permeability may be controlled by polyvalent cations, such as calcium (15). In general, sodium transport through toad bladder is not affected by changes in calcium concentration around the physiological range (17). However, in frog skin there is some dependence of sodium transport on calcium concentration at physiological levels (18–20), but it is unlikely that the sites involved are the same as those from which calcium has to be removed to affect the response to amiloride. There is evidence that sodium gains access to the transport apparatus by different mechanisms at high and low salt concentrations (21). The effect of calcium removal on the amiloride response is present at low (2) and high (13) sodium concentrations, and thus the amiloride effect in frog skin is dependent on high-affinity sites for calcium, which are relevant at environmental salt concentrations. In toad bladder we have shown that sodium transport, measured as sodium-dependent oxygen consumption, is sensitive to calcium, but at concentrations far below the physiological (15). The puzzle is how high-affinity sites for calcium can be relevant to the control of sodium permeability with calcium concentrations in the physiological range. The answer might be, if the two-state model of the sodium channel is correct, that the affinity of calcium for the open and closed forms is similar, but in favor of the closed form. In this way sodium transport would be sensitive to calcium at low concentrations, while increasing concentrations would be without any further effect.

Considering the effects of calcium alone on epithelia, the proportion of channels in the open form will be given by $\bar{R}$, where

$$\bar{R} = \left[1 + L\left(\frac{1 + d\delta}{1 + \delta}\right)^p\right]^{-1} \qquad (4)$$

where $d = K_{CaR}/K_{CaT}$ and $\delta = [Ca]/K_{CaR}$. Assuming that the amount of sodium transport is proportional to $\bar{R}$, the fractional reduction in transport from a maximal value will be given by $(1 - \bar{R})$. Values of $(1 - \bar{R})$ against calcium concentration calculated from Eq. 4 are given in Fig. 3a–c.

Figure 3a shows curves obtained assuming that the affinity of calcium for the T form of the channel is 100 times greater than for the R form. Results for values of L between 1 and 0.001 are shown. If calcium does indeed act as an allosteric inactivator of the sodium channel, removal of calcium would

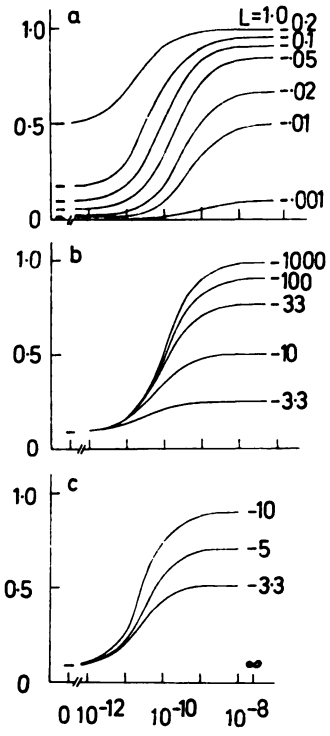

FIG. 3. *Fractional changes in $\bar{T}$ i.e. $(1 - \bar{R})$ with calcium concentrations computed from Eq. 4*

a. Variations of L with $K_{CaR} = 10^{-9}$, $K_{CaT} = 10^{-11}$, and $p = 1$. b. Variations in d with L fixed at 0.1. Throughout $K_{CaT} = 10^{-11}$ while K_{CaR} was greater by 3.3–1000-fold, and $p = 1$. c. As for b, with K_{CaR} 3.3–10 times greater than K_{CaT}, but with $p = 2$. The solid bars show the limits for zero and infinite calcium concentrations.

increase the proportion of the R form. The value of $L = 1$ taken in an earlier section would correspond to the normal physiological condition, with calcium present, and with ADH able to increase transport 2- or 3-fold. Under the abnormal condition, in which calcium is removed, a lower value of L is appropriate. It can be seen from Fig. 3a that with values of $L < 0.05$ and in the absence of calcium $\bar{R}$ approaches 1. Removal of calcium would be expected to increase sodium transport or sodium-dependent oxygen consumption, as has been discussed previously. Furthermore, it would be expected that the hormone would be unable to cause any further increase in transport in the complete absence of calcium, as has been found (15), although alternative biochemical explanations may be forwarded for this result. Figure 3b shows the effects of the variation of d on the inhibition curves after fixing L at 0.1. Quite small differences in affinity (10–33-fold) of calcium for the two forms of the channel produce inhibition curves of the form seen experimentally (15), when L is taken as 0.1. However, it should be noted that even smaller differences in affinity (3.3–10-fold) can produce the same pattern of inhibition with $L = 0.1$ if the value of p is increased from 1 to 2 (Fig. 3c).

ADH-calcium interactions. Combining Eq. 4 with the hormonal factor from Eq. 3 gives

$$\bar{R} = \left[1 + L\left(\frac{1 + d\delta}{1 + \delta}\right)^{p} \cdot \left(\frac{1 + c\gamma}{1 + \gamma}\right)^{m}\right]^{-1} \quad (5)$$

If the factor $[(1 + c\gamma)/(1 + \gamma)]^m$ is given the value of 0.1 as before, the effect of hormone on the calcium inhibition curves can be seen from Fig. 3a. Addition of hormone effectively reduces the value of L by 10 times. Thus hormone moves the calcium inhibition curve to the right. Using toad bladder it was found that hormone did indeed affect the calcium inhibition of oxygen consumption in this way. Over a limited concentration range the calcium inhibition curve was moved to the right in a roughly parallel manner and by about one order of magnitude (15).

Polyvalent metal ions other than calcium (Mg^{2+}, Sr^{2+}, La^{3+}, Eu^{3+}) were also shown to have effects like calcium—that is, they inhibited sodium-dependent oxygen consumption—and the inhibition curves were modified by hormone. Figure 4 is constructed from data of Cuthbert and Wong (15). The curves show the percentage inhibition of sodium-dependent oxygen consumption caused by different concentrations of metal ions (measured using EGTA buffers). The arrows indicate the direction and extent of the shift in the inhibition curves caused by a maximally

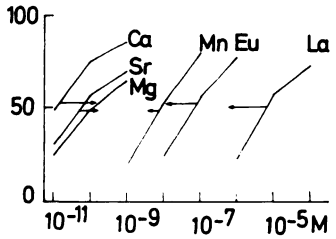

FIG. 4. *Effects of metal ions on inhibition of sodium-dependent oxygen consumption in toad bladders*

Bladders were treated first with EGTA and then bathed in EGTA-buffer solution containing metal ions at controlled concentrations. The arrows show the direction and extent of the shift caused by a maximally effective concentration of ADH. Constructed from data given by Cuthbert and Wong (15).

effective concentration of hormone. While Ca^{2+} and Sr^{2+} displace the curves to the right, La^{3+}, Eu^{3+}, and Mn^{2+} displace the curves to the left, the hormone being without effect on the inhibition curve to magnesium. These results too can be accomodated within the two-state model.

It may be supposed that the binding of one ligand to a protein molecule will affect the binding site for other ligands via conformational changes in the macromolecule. Since ADH does not affect the inhibition curve for magnesium, one explanation is that the hormonal factor, X, has an equal affinity for its sites on both the R and T forms of the macromolecules in the presence of Mg^{2+}. On the other hand, X would be required to have a higher affinity for the R form in the presence of Ca^{2+} and Sr^{2+}, while in the presence of La^{3+}, Eu^{3+}, or Mn^{2+} the T form would possess the higher affinity for X. Bentley (22, 23) found that toad bladders bathed in solutions in which magnesium replaced calcium were unable to respond to ADH, a finding in accord with the suggestions made above.

Calcium release from binding sites. In the model proposed in this paper calcium acts as an allosteric inhibitor of sodium ion translocation by increasing the proportion of the T forms of the channel. Conversely ADH, acting as an allosteric activator, will increase the proportion of R form, at the expense of the T form. Since the affinity of calcium is lowest for the R form, the net conversion of T to R channels must result in the release of part of the bound calcium.

It has been shown (24) that ADH causes an increased efflux of ^{45}Ca from the mucosal surface of toad bladders previously loaded with this isotope. Similar results were obtained for frog skin.[2] While it cannot be proved unequivocally that the increased calcium efflux is from membrane receptors, the detection of an increased efflux is in accord with the predictions of the two-state model.

Calcium-amiloride interactions. In the previous sections it was proposed that amiloride and calcium act in concert to increase the proportion of the T form of the channel. The effects of calcium are considered to be maximal at physiological concentration, a result of proposing a high affinity for calcium for both forms of the channel, but with a relatively small preference for the T form. If this is so, calcium is bound so effectively to the membrane that it might be considered part of the membrane structure. Recently Oschman and Wall (25) demonstrated calcium binding activity by electron microscopy in a number of epithelial cell types, which appeared to saturate at low calcium concentrations. Although the binding sites appeared to be on the inside of the membrane, they disappeared in the presence of the chelating agent EDTA.

An equation relating interactions of amiloride and calcium with the epithelial surface may be adduced by combining Eqs. 1 and 4. The relevant equation is

$$\bar{R} = \left[1 + L\left(\frac{1 + a\alpha}{1 + \alpha}\right)^{n} \cdot \left(\frac{1 + d\delta}{1 + \delta}\right)^{p}\right]^{-1} \quad (6)$$

Amiloride-sodium interactions have been

[2] Unpublished observations.

ignored for simplicity. Thus it can be seen that the effect of calcium removal is effectively to reduce the value of L, that is, to increase the proportion of the R form with respect to the T form. Figure 5a and b shows two solutions of Eq. 6 for the percentage inhibition of $\bar{R}$ in the presence and absence of calcium. The model predicts that calcium removal increases the SCC, decreases the maximal inhibition produced by amiloride, and in addition increases the macroscopic binding constant, the so-called K_m of the Michaelis-Menten terminology.

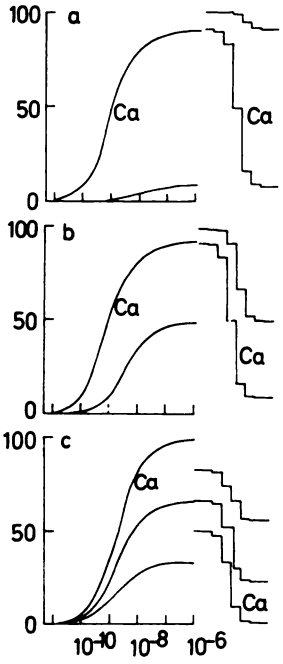

FIG. 5. *Effects of calcium on response to amiloride*
The ordinate shows the percentage reduction of $\bar{R}$ vs. amiloride concentration calculated from Eq. 6. In each set of curves the uppermost represents responses in the presence of calcium. Lower curves represent responses in the presence of a chelator which is assumed to remove calcium completely (a and b) and partially (c) from binding sites on or near the channel (see the text for full explanation). a and b. $K_{AT} = 10^{-10}$ and $K_{AR} = 10^{-8}$, $p = n = 1$. a. $L = 0.001$ and $(1 + d\delta)/(1 + \delta) = 100$ maximally. b. $L = 0.01$ and $(1 + d\delta)/(1 + \delta) = 10$ maximally. c. $K_{AT} = 10^{-9}$ and $K_{AR} = 10^{-7}$, $L = 1$. To the right of each pair of curves is a simulated concentration-response curve. Each step represents a 10-fold increase in amiloride concentration. These show how calcium removal affects the basal sodium transport. The scale is as for the curves. This figure should be compared with the experimental results in Fig. 6.

Normal frog skins show an altered responsiveness to amiloride when the mucosal surface is treated with a calcium-chelating agent. Under these conditions the SCC is increased and maximal inhibition is reduced, sometimes to zero (i.e., amiloride is without effect on transport), but with no appreciable effect on apparent affinity. In an earlier paper (13) it was suggested that amiloride formed a ternary complex with calcium and the channel and that only this complex was capable of blocking sodium entry. This restriction did not preclude the formation of a binary complex of amiloride and the channel, except that this complex was not able to block the translocation of sodium ions. This conventional explanation, based on Michaelis kinetics, fits the experimental data well, but if a similar restriction is applied the data can be explained using the two-state model. Thus, if the restriction is introduced that the R form can be easily converted to the T form only with calcium bound, no change in apparent affinity for amiloride will be obtained. Consider a partial reaction of the system thus:

$$n\mathrm{Ca}T \rightleftharpoons n - y\mathrm{Ca}R + yR + y\mathrm{Ca}$$

With calcium present, amiloride can inhibit transport completely by converting all the channels to the T form. In the presence of a chelating agent Eq. 6 would still apply; that is, L would not be changed, but only a fraction of the channels could be easily converted to the T form. Figure 5c illustrates solutions of Eq. 6 with this restriction, and shows that increased SCC and reduction of maximal inhibition with no change in apparent affinity are expected. Figure 6 shows the result of a typical experiment on frog skin. Exposure of the mucosal surface to calcium-free solution containing 1 mM EGTA reduced the maximal inhibition and increased SCC. The effect of EGTA was

progressive but could be reversed by adding calcium. The progressive effect is what might be reasonably expected if EGTA slowly removed calcium from high-affinity binding sites. Curiously, although calcium restores the blocking effect of amiloride, its action is rapidly reversed again by EGTA. As Fig. 6 shows, the apparent affinity of amiloride remains constant in situations where appreciable blockade of transport can be obtained. There is some suggestion that the affinity is reduced at low levels of blockade (curve *4*, Fig. 6b) although accurate measurement is difficult at this level.

Recently it was found that the response of newly molted skins to amiloride under calcium-free conditions resembles the results given in Fig. 5a and b; that is, both maximal inhibition and affinity are reduced whereas the SCCs are consistently high. When calcium is then added, the response to amiloride becomes normal within a few hours.[2] It appears that binding of calcium to newly molted skins may be part of an aging process required before the channels behave normally to amiloride. Thus subtle differences between newly molted and normal skins remain to be explored. It would appear that the two-state model might explain the behavior of both types of skin under nominally calcium-free conditions, even though the behavior of normal skin in this condition can also be understood equally well with the conventional approach.

In the preceding paper (2) it was shown that while calcium removal reduced the inhibitory effect of amiloride the amount of label bound to channels was not reduced. One of the properties of the two-state model is that the state function ($\bar{R}$) and the binding function ($\bar{Y}$) do not necessarily coincide. The binding function $\bar{Y}$ (i.e., fraction of ligand sites occupied in R and T forms) is given by

$$\bar{Y} = \frac{\alpha(1 + La)}{1 + \alpha + L(1 + a\alpha)} \qquad (7)$$

Figure 7 shows how both $\bar{R}$ (shown as fractional reduction in $\bar{R}$) and $\bar{Y}$ vary with the concentration of amiloride in the presence and absence of calcium. While there is a close correspondence between the fractional reduction in $\bar{R}$ and $\bar{Y}$ in the presence of calcium, this does not hold for calcium-free

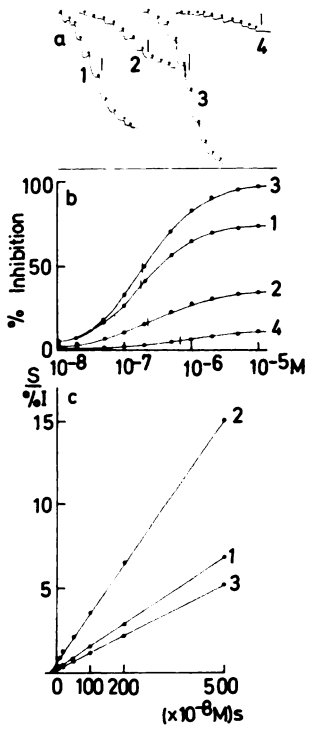

FIG. 6. *Calcium-amiloride interactions in frog skin*

a. Cumulative concentration-response curves to amiloride on SCC of frog skin. Successive curves were obtained at approximately hourly intervals. The sequence of amiloride concentrations used was 1, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 × 10^{-8} M. The vertical bars represent 20 μamp, and the horizontal line is zero current. Skin area was 7.1 cm^2. In curves *1*, *2*, and *4* the mucosal solution was calcium-free and contained 1 mM EGTA. Normal Ringer's solution was used for curve *3*. Initial currents were 256, 256, 176, and 216 μamp, respectively, for curves *1*–*4*.

b. Log concentration-inhibition curves for the results of Fig. 6a. Concentrations causing 50% of the maximal inhibition possible for each condition are marked with a vertical line.

c. Plots of amiloride concentration against ratio of amiloride concentration to percentage inhibition for curves *1*, *2*, and *3*. No change in apparent K_m is seen.

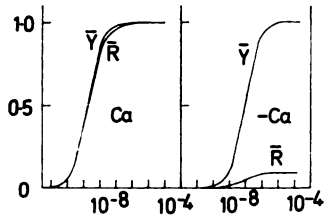

FIG. 7. *Fractional reduction in state function ($\bar{R}$) and the binding function ($\bar{Y}$) vs. amiloride concentration in the presence and absence of calcium, calculated from Eqs. 6 and 7*

The calcium factor $(1 + d\delta)/(1 + \delta)$ was taken as 1000, and L as 0.001. Thus the effect of removing calcium is to reduce L from 1 to 0.001. Throughout K_{AT} and K_{AR} were taken as 10^{-9} and 10^{-7}, respectively, and p and n were taken to equal 1.

conditions. Under this condition the conversion of R to T by amiloride is impaired, while the relation between $\bar{Y}$ and amiloride concentration is moved to the right. Thus, under calcium-free conditions, the R form binds amiloride but the complex is unable to prevent sodium ion translocation. This possibly provides a satisfactory explanation for the lack of effect of calcium removal on amiloride binding at the concentrations studied previously (2).

Incidentally, when cooperative effects are present, $\bar{R}$ and $\bar{Y}$ functions cannot coincide. Since all the previously reported binding studies were carried out under conditions in which no cooperative effects were discernible, a 1:1 relationship between number of channels and number of binding sites is more tenable.

Translocation of sodium. The competition between sodium and amiloride has been referred to earlier. One unanswered question is whether the sodium which competes with amiloride is the same sodium destined for translocation. From binding studies (2) it was shown that calcium removal did not affect amiloride binding, yet sodium transport was not greatly inhibited. The conclusion was that under these circumstances amiloride was bound to the R form of the channel, from which it must be concluded that Na^+ need not bind to this site for translocation. Alternatively there may be multiple attachment sites for amiloride, one of which is the site of Na^+ binding. If this is so, the lowered affinity of amiloride for the R form may result from the unavailability of this site, which is then free to interact with Na^+.

The relation between sodium ion penetration (26, 27), sodium transport (11, 28), and sodium concentration is a curvilinear one, which could represent mass-action kinetics between Na^+ and the R form of the channel.

GENERAL DISCUSSION

A plausible model of the sodium channel in transporting epithelia has been presented which is based on the MWC model for allosteric proteins. The MWC model is one of a large class of two-state models (see ref. 7) which predict results of the same form as given in this paper. An important task for the future is to devise experiments which might distinguish among various two-state models and between them and classical models.

Throughout this paper the term channel has been used to mean the mechanism by which sodium ions are translocated across the mucosal membrane of epithelia. Similarly, the term open indicates when this mechanism is operative, while the term closed indicates inactive mechanisms. It must be realized that detailed knowledge of transport mechanisms for ions across biological membranes is not available, but something may be learned from results with model membranes. In lipid bilayers a number of macrocyclic compounds can facilitate ion movements across bilayers, and these compounds can function either as carriers (e.g., valinomycin) or as channels (e.g., gramicidin and alamethicin).

In this paper the term carrier might have been used as a substitute for channel. For the model to apply to a carrier type of system there would need to be two conformations of the carrier with different affinities for a given ligand, and hormone would be

required to increase the proportion of those conformers able to translocate sodium ions across the membrane. It must be emphasized that the approach made in this paper does not distinguish between carrier and channel mechanisms.

In summary, the model proposed here suggests that sodium movement across the mucosal membrane of epithelia depends on the relative proportions of the two conformations of a transport mechanism, with the R form designated as active. Substances which may affect the state function $\bar{R}$, such as calcium, ADH, and amiloride, thus affect the level of sodium transport. In contrast, binding studies using [^{14}C]amiloride are considered an indication of the binding function, $\bar{Y}$, and are not necessarily related to the level of transport.

Three types of binding sites on the mechanism are necessary in the model. First is the binding site for sodium and amiloride, the latter acting as a competitive antagonist. A second binding site is required for the ligand generated by the hormone, this ligand behaving as an allosteric activator. Calcium is considered to be an allosteric inhibitor acting at a third independent site.

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