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CHARACTERISTICS OF THE ENTRY PROCESS FOR SODIUM IN TRANSPORTING EPITHELIA AS REVEALED WITH AMILORIDE

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SUMMARY

1. The permeation of sodium ions through the mucosal surface of frog skin epithelium at different transepithelial potentials has been investigated using the blocking drug amiloride.

2. An increase in serosal negativity in voltage-clamped skins was associated with an increase in the absolute amount of inhibition caused by a fixed concentration of amiloride. Hyperpolarizing or depolarizing skins with respect to the short-circuited condition did not affect the apparent affinity of amiloride for the entry sites.

3. When skins were voltage clamped at -50 mV (serosa negative) the specific binding of amiloride to sodium entry sites was increased by 77% compared to the short-circuited condition. Skins clamped at $+50$ mV had only 72% of the specific binding found in short-circuited skins. Experiments with a second blocking drug, triamterene, indicated that the extra binding sites appearing at -50 mV were similar to those found under short-circuit conditions. The appearance and disappearance of binding sites may reflect changes in cell volume.

4. The findings suggest that the increased sodium current which flows when skins are clamped at -50 mV results from an increase in the number of entry sites, and perhaps also to a voltage sensitive increase in flux through each entry site.

INTRODUCTION

The permeation of sodium ions across the mucosal membranes of transporting epithelia was originally thought to occur by free diffusion (Koefoed-Johnsen & Ussing, 1958; Gatzy & Clarkson, 1965; Leb, Hoshiko & Lindley, 1965). This concept appears to be no longer tenable, not only from biophysical measurements (Finn, 1974) but particularly from measurements

of sodium influx. If epithelia are exposed for brief times to mucosal solutions containing radiosodium then sodium influx into the tissue can be measured directly, assuming there is no backflux or transepithelial transport and provided saturation is not achieved. The results of sodium influx experiments in frog skins and toad bladders may be summarized as follows. There is a hyperbolic relationship between sodium influx (or at least that component of influx associated with sodium transport) and sodium concentration, indicative of a saturable phenomenon. At a given sodium concentration antidiuretic hormone increases influx, whereas amiloride inhibits influx (Rotunno, Villalonga, Fernandez & Cereijido, 1970; Biber & Curran, 1970; Cereijido & Rotunno, 1971; Biber, 1971; Ferguson & Smith, 1972; Moreno, Reisin, Rodriguez-Boulan, Rotunno & Cereijido, 1973; Biber & Cruz, 1973; Erlij & Smith, 1973; Biber & Sanders, 1973). One of the sources cited above (Biber & Sanders, 1973) is of particular interest since it was shown that the extent of sodium influx at a given sodium concentration can be influenced by the transepithelial potential. If the serosa was made negative with respect to the mucosa sodium influx was increased and vice versa, and the results were interpreted in terms of a mobile carrier model.

We have approached the problem of sodium ion penetration from a different angle. Amiloride is known to block sodium entry into epithelia and previously we have described methods for measuring the density of amiloride binding sites in the mucosal surface of frog skin (Cuthbert, 1973; Cuthbert & Shum, 1974*a*, *b*). We have studied the action and binding of amiloride at the mucosal surface of frog skin voltage clamped both at positive and negative potentials and under short-circuit conditions. Our findings are used to discuss various alternatives for the mechanism of sodium ion permeation across the mucosal border of frog skin.

METHODS

Pieces of frog (*Rana temporaria*) abdominal skin were clamped horizontally in cells with the mucosal surface uppermost. The skin areas were either 7·1 or 9·6 cm^2. The serosal bathing solution was always frog Ringer (see below), whereas the mucosal solution had the same composition as frog Ringer except for NaCl, the concentration of which was varied. The tissue holder, electrodes for measuring transepithelial potential and for passing current have been described in detail elsewhere (Cuthbert, 1973). The transepithelial potential could be controlled automatically at values between +100 and −100 mV (serosal potential with respect to mucosal potential) by a voltage clamp device. The current required to clamp the potential at a given value was recorded on a pen recorder (Bryans, 2700).

Methods for measuring binding of amiloride to membrane receptors have been given previously (Cuthbert, 1973; Cuthbert & Shum, 1974*b*) and readers should refer to these for details. In brief the method measures the amount of displaceable amiloride binding as follows. The skin is exposed to [^{14}C]amiloride 10 nM, 54 c M^{-1} for

2 min and reduction of clamping current noted. The radioactive solution is then rapidly removed, the skin blotted dry, the residual radioactivity removed by elution and the [^{14}C]amiloride measured. Following this the skin is exposed to a 100-fold greater concentration of amiloride (1·0 μM) but with a specific activity of only 0·54 c M^{-1}. The residual activity remaining on the skin is again measured. The difference between these two measures is taken as the amount of displaceable amiloride binding. Four of five pairs of similar measurements are made for a particular condition and the mean value, M, of the paired differences is used to calculate the total number of amiloride binding sites. If 10 nM amiloride reduces the sodium current by a fraction α (again the mean of four or five measurements) then at 100 % occupancy of the binding sites the amount of amiloride bound will be given by $M\alpha^{-1}$. Throughout the number of binding sites per square micron is given, assuming a stoichiometry of 1:1 (Cuthbert & Shum, 1974*b*).

The method we have used here and described previously (Cuthbert, 1973; Cuthbert & Shum, 1974*a*, *b*) is believed to measure the density of sodium entry sites with reasonable accuracy, although variations between individual skins is considerable. This claim is based on the result that there is agreement between the affinity of amiloride determined directly and when used to inhibit sodium transport. Furthermore there was little evidence to suggest that non-specific binding contributed substantially to the measured values. The variation between different skins is not of consequence in this work as the density of binding sites is always measured under short-circuited conditions and compared with the value obtained under other conditions in the same skin.

The composition of the frog Ringer used was as follows: (mM) NaCl, 111 mM; $CaCl_2$, 1 mM; KCl, 1 mM; Tris buffer, pH 7·6, 5 mM and glucose 11·1 mM. When low sodium solutions were used a portion of the sodium chloride was omitted. Tonicity was either maintained with chlorine chloride or the hypotonic solution was used. In labelling experiments the mucosal bathing solution was always hypotonic and contained 1·1 mM-NaCl; glucose was omitted from this solution.

We have not been able to measure specific amiloride binding when high sodium solutions are used to bathe the mucosal surface. The reason is that sodium and amiloride compete for the entry sites (Cuthbert, 1973) and in consequence higher concentrations of amiloride are required to inhibit transport (and to bind to receptors). As we employ a difference method the amount of displaceable ligand becomes insignificant compared to the amount of ligand retained in the presence of excess unlabelled drug. However, we would maintain that skins bathed in hypotonic, low sodium solutions on the outside with high sodium solutions on the inside more truly represents the physiological condition. In experiments not involving binding measurements we have used both low and high sodium solutions bathing the mucosal surface, the latter representing the more usual experimental condition.

RESULTS

Initial experiments were designed to examine the blocking action of amiloride on skins clamped at different potentials. As Biber & Sanders (1973) had shown, sodium influx was influenced by the transepithelial potential. It was considered that the absolute decrease in current caused by a fixed concentration of amiloride would vary in a similar way. The reasoning for this is as follows. Suppose the serosa is made negative with respect to the mucosa and that sodium ions are translocated through a

fixed number of sites then the flux through each site may be enhanced. Blockade of a given fraction of these sites will cause a greater absolute change in current than when the skin is short-circuited. This assumes that

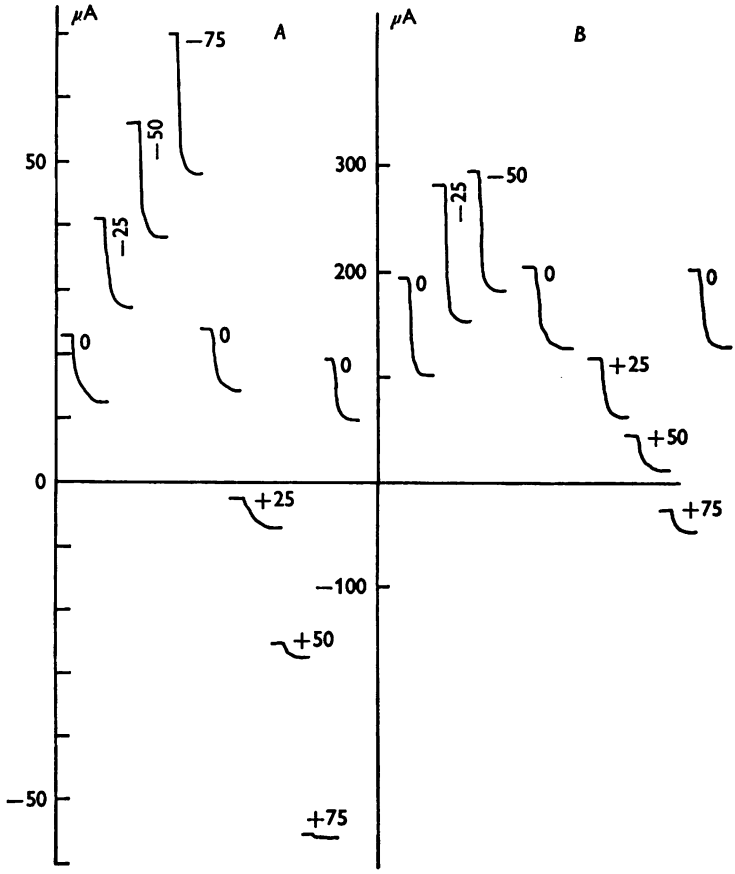

Fig. 1. The effect of amiloride on the current flowing through skins voltage clamped at different transepithelial potentials. The Figure illustrates results obtained when either low sodium (1·1 m-equiv/l.) (*A*) or high sodium (111 m-equiv/l.) solutions (*B*) bathed the mucosal surface of the skin. After the transepithelial potential had been set at a new value a single concentration of amiloride, 10^{-8} M in (*A*) or 10^{-7} M in (*B*), was added to the mucosal solution. The consequent effects on the transepithelial current are illustrated. The drug was allowed to act for approximately 1 min after each addition and was then washed away before the transepithelial potential was clamped at a new value. The transepithelial potential at which the skin was clamped is shown by each trace. The potentials given are of the serosal side relative to mucosal side of the skin. Positive current flows inward through the mucosal surface of the skin.

the applied potential changes neither the blocking actions of amiloride or number of sites available for sodium ion translocation.

Fig. 1*A*, *B* illustrates results from experiments in which the reduction in current (μA) caused by a fixed concentration amiloride was measured at different values of transepithelial potential. It is clear that as the serosal side was made more negative the absolute inhibitory effect increased, reaching a maximal value at a potential of about -75 mV. Alternatively when the potential across the skin was clamped at around $+75$ mV the inhibitory action of amiloride on the current was suppressed. Fig. 2 shows similar results from another experiment in which both the amiloride concentration and the transepithelial potential were varied.

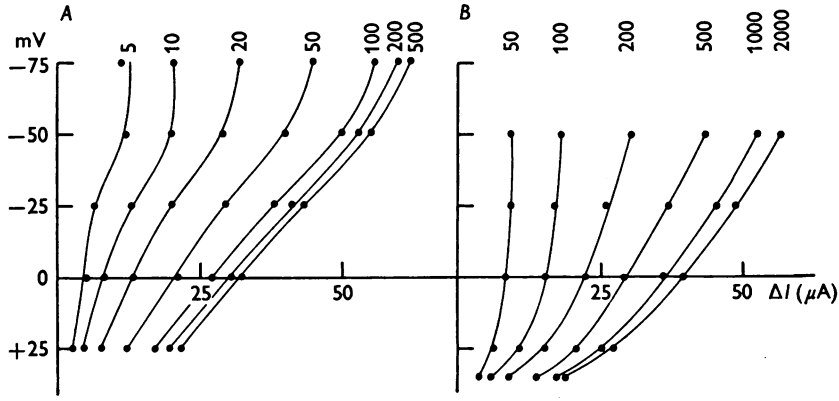

Fig. 2. Relation between change in current (ΔI, in μA) caused by amiloride at different transepithelial potentials (mV). The amiloride concentration (nM) is shown on individual curves. The experiments were made in the presence of 1·1 m-equiv/l. (*A*) or 111 m-equiv/l. (*B*) sodium in the mucosal solution. Potentials are those of the serosal surface compared to the mucosal surface.

While the experiments described above were being carried out it became obvious that the fractional increase in current measured at steady state caused by clamping the potential at negative values was greater when the mucosal sodium concentration was low (1·1 m-equiv/l.) compared to when the mucosal solution was Ringer (111 m-equiv/l.). This can be appreciated by reference to Fig. 3. At its simplest frog skin epithelium may be represented by the equivalent circuit shown in the inset to Fig. 3, where R_{Na} represents the resistance through which sodium ions must move to gain access to the transport mechanism, while R_L represents a parallel leak pathway. Under short-circuit conditions no current flows through R_L and the SCC is equivalent to the net mucosal to serosal flux of sodium. If the

serosa of the skin is now made negative an extra sodium current will flow through R_{Na}, but in addition current will flow through R_L. The amount of sodium flowing through the active pathway can be gauged by blocking

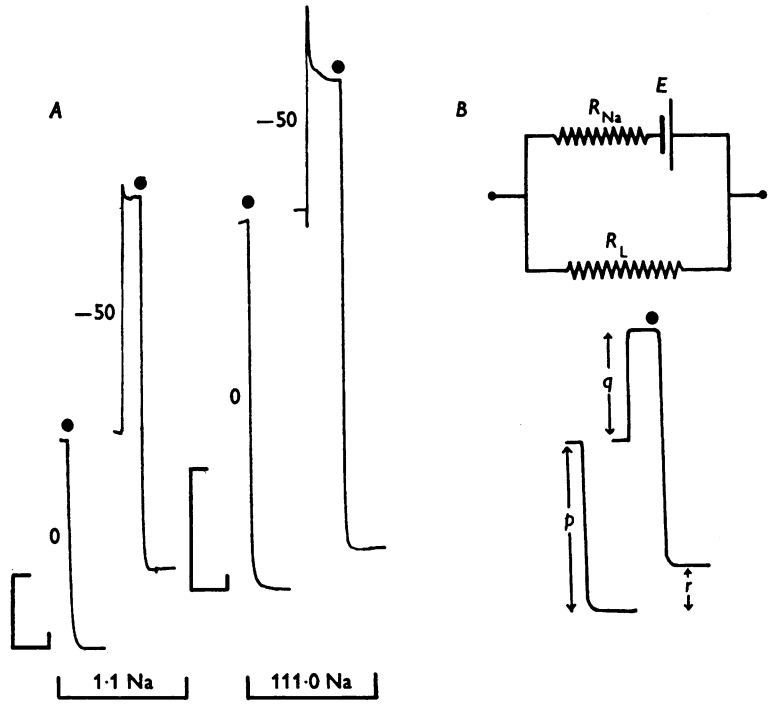

Fig. 3. *A*, the effect of changing the clamping potential from 0 to −50 mV in a frog skin with the mucosal surface bathed in solutions containing either 1·1 or 111·0 sodium m-equiv/l. At the dots amiloride, 10^{-4} M, was added to the mucosal bathing solution. Calibrations are 10 μA and 1 min for low sodium and 100 μA and 1 min for high sodium conditions. The time marker also indicates zero current. Area of skin 7·1 cm^2. In low sodium solution the open circuit potential was 26 mV and in high sodium solution it was 91 mV.

B, equivalent circuit of skin and indication of the way in which percentage increase in sodium current was calculated. p is the resting short-circuit current and q is the increase in current caused by clamping at −50 mV instead of 0 mV. Not all of the increase is due to sodium movement, and that not due to sodium is given by r, the residual current remaining after sodium entry has been blocked with amiloride, 10^{-4} M.

this pathway with amiloride at high concentration (10^{-4} M) leaving a residual non-sodium current. The percentage increase in sodium current caused by clamping the serosa at a negative potential is then given by $[(q-r)/p] \times 100$ (see Fig. 3 for explanation). Table 1 shows the percentage

increase in sodium current at low and high sodium concentrations, both with and without correction for tonicity. There is a significantly greater fractional increase in sodium current at low sodium concentrations.

TABLE 1. Percentage increase in sodium current caused by changing the clamp potential from zero to −50 mV

Low sodium (1·1 m-equiv/l.)	High sodium (111 m-equiv/l.)
78·1	16·3
58·5	9·5
54·3	27·3
75·8	23·5
Low sodium (1·1 m-equiv/l.) plus choline chloride (110 mM)	**High sodium (111 m-equiv/l.)**
63·2	25·0
61·1	13·1
56·3	15·3
Mean ± S.E. 63·9 ± 3·6	18·6 ± 2·5

$P < 0.001$

Seven separate skins were used. They were exposed either to high or low sodium solutions initially and then to the alternative solution. In three of the experiments tonicity in the low sodium solution was maintained with choline chloride.

So far we have assumed that the applied potential changes neither the affinity of the binding site for amiloride or the number of such sites. To test the former complete inhibition curves were constructed with the skin voltage clamped at a number of potentials, including zero. In this way we were able to measure the apparent affinity of amiloride for its binding site. Fig. 4 illustrates some of our results in which cumulative inhibition curves were obtained at a number of different potentials. The percentage inhibition of the sodium current was measured taking the maximal inhibition obtainable with amiloride as 100%. At high sodium concentration (111 m-equiv/l.) there was no difference between the inhibition curves at potentials greater and less than zero. With low mucosal sodium there were small but insignificant changes in the apparent affinity of amiloride with potential.

The small changes in apparent affinity were no greater than the differences found when repeated observations were made at 0 mV. However, similar small shifts were found in two other experiments, and these shifts were in the same direction – that is, to the left of the curve at 0 mV when the serosa was made negative, and vice versa for positive potentials. It would appear, therefore, that the apparent affinity of amiloride was not significantly influenced by transepithelial potential.

Next we attempted to test our second assumption, namely that the

number of sites available for sodium entry does not depend on the transepithelial potential. We have measured the number of amiloride binding sites in skins clamped at +50 and −50 mV and compared this with the amount bound by the same skins under short-circuit conditions. The results of nineteen separate experiments are given in Tables 2*A* and *B*. The results show that the amount of displaceable amiloride increases by nearly 80% when the serosa is made 50 mV negative, while displaceable

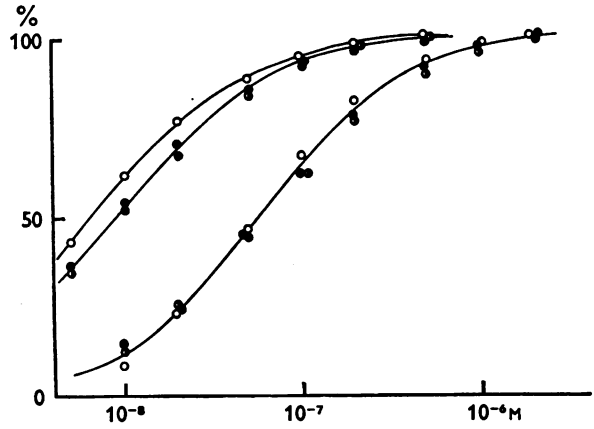

Fig. 4. The effects of amiloride on the inhibition of transepithelial current. Curves illustrate the percentage inhibition of the sodium current versus the concentration of amiloride. Total inhibition of the sodium current was taken as that inhibition produced by amiloride, 10^{-4} M. The left-hand set of curves refer to conditions in which the mucosal bathing solution contained sodium at only 1·1 m-equiv/l. while the right-hand curves refer to 111-m equiv/l. Filled circles are for short-circuit conditions, open circles indicate results for skins clamped at −50 mV and half-filled circles indicate results for skins clamped at +25 mV (1·1 m-equiv sodium/l.) or +50 mV (111 m-equiv sodium/l.). The values of short-circuit current were 44 μA (1·1 m-equiv sodium/l.) and 225 μA (111 m-equiv sodium/l.) (7·1 cm^2).

amiloride binding decreases by nearly 30% when the serosa is made 50 mV positive, compared to the amount bound under short-circuit conditions. These observations were made with a mucosal bathing solution containing only 1·1 m-equiv/l. sodium. The amount of displaceable amiloride only is shown in Table 2; however, there was no significant difference in the non-displaceable amiloride (that is, the radioactivity remaining on the skin in the presence of excess unlabelled drug) whatever potential was used to clamp the skin. The mean values for the inhibition of sodium current by amiloride (10 nM) were similar for short-circuited skins and skins clamped at either +50 and −50 mV. For skins clamped at +50 or

−50 mV the total sodium current was estimated by determining the inhibition caused by 1·0 μM amiloride. This concentration causes 100% inhibition of SCC in skins in the presence of sodium 1·1 m-equiv/l. Some of the skins used for these experiments were taken from aldosterone-treated animals. It has been shown previously (Cuthbert, Okpako & Shum, 1973) that the density of amiloride binding sites increases in freshly moulted skins from aldosterone-treated animals. This device was used in an attempt to increase the amount of binding sufficiently to increase the chances of showing a significant difference in the amount of amiloride bound under short-circuit and non-short-circuit conditions. In each of the experiments 6–9 shown in Table 2*A* the amount of amiloride bound under short-circuit conditions was significantly ($P < 0{\cdot}05$) less than the amount bound at −50 mV. The mean increase in amiloride binding found for the nine experiments of Table 2*A* (77%) was significantly greater than zero ($P < 0{\cdot}001$). When the serosa was voltage clamped 50 mV positive the decrease in the amount of amiloride bound compared to short-circuit conditions was also significantly greater than zero ($P < 0{\cdot}001$) for the ten experiments shown in Table 2*B*.

Since we know the total current attributable to sodium in the skins used in binding experiments it was possible to divide this current by the total number of sodium entry sites to obtain the nominal current flowing through each site. The values for this are given in Table 2*A* and *B*. Examination of these data shows there are no significant differences between the currents for sites in normal and aldosterone moulted skins, or between skins which are short-circuited and those clamped at either plus or minus 50 mV. It must not be assumed that no changes in current per channel occur even though statistical significance was not achieved in these experiments (see Discussion).

Whether changes in the density of amiloride binding sites with transepithelial potential really reflect changes in the availability of sites for sodium entry will be discussed fully later in this paper. However, it was possible that changes in transepithelial potential either created or removed amiloride binding sites which are unrelated to the sodium entry process. An attempt to check this has been made using triamterene, a drug which acts similarly to amiloride (Salako & Smith, 1971). The affinity constants for amiloride and triamterene for the sodium entry sites in frog skin were found to be $1{\cdot}4 \times 10^8$ and 15×10^5 M^{-1} respectively from previous binding studies (Cuthbert & Shum, 1974*b*). The occupancy of the receptors by amiloride in the presence of both drugs is given by p, where

$$p = \frac{[A]K_A}{1+[A]K_A+[T]K_T},$$

TABLE 2*A*. Amiloride binding and transepithelial potential. Effect of making serosa negative

	Short-circuited (0 mV)					Serosa negative (−50 mV)					
Experiment	Displaceable [^{14}C]amiloride (d.p.m./9·6 cm^2)	P	Inhibition of sodium current (%)	Binding sites/μm^2	Current/binding site (A × 10^{-16})	Displaceable [^{14}C]amiloride (d.p.m./9·6 cm^2)	P	Inhibition of sodium current (%)	Binding sites/μm^2	Current/binding site (A × 10^{-16})	Increase in binding (%)
1	17·3 ± 4·6	< 0·05	36·7	247	0·63	25·0 ± 7·6	< 0·05	34·0	383	0·55	55
2	17·2 ± 3·7	< 0·01	35·5	253	0·54	20·5 ± 3·5	< 0·01	37·2	288	1·16	13·5
3	11·8 ± 6·0	n.s.	31·8	194	0·62	25·0 ± 8·7	< 0·05	29·5	443	0·89	128·4
4	19·0 ± 9·5	n.s.	35·9	277	0·52	24·0 ± 5·8	< 0·01	34·1	368	0·72	32·9
5	12·0 ± 3·5	< 0·05	33·3	189	1·16	25·5 ± 9·5	n.s.	38·6	346	1·22	83·1
6*	23·0 ± 10·3	n.s.	36·3	331	0·80	47·0 ± 7·9	< 0·01	38·0	647	0·65	102·2
7*	37·4 ± 4·9	< 0·002	33·3	587	0·78	53·2 ± 5·6	< 0·001	34·8	800	1·25	36·1
8*	10·0 ± 6·1	n.s.	24·8	211	1·37	29·6 ± 6·2	< 0·01	35·9	431	0·83	104·3
9*	21·0 ± 8·7	= 0·05	31·1	353	0·43	67·4 ± 5·8	< 0·001	41·7	845	0·42	138
Mean ± S.E.	18·7 ± 2·8		33·2 ± 1·2	294 ± 41	0·76 ± 0·10	Mean ± S.E.	35·2 ± 5·5	36·0 ± 1·2	506 ± 69	0·85 ± 0·10	77·1 ± 14·8

Displaceable [^{14}C]amiloride refers to the amount of [^{14}C]amiloride which was displaced by 100-fold excess of unlabelled drug. The [^{14}C]amiloride (54 c/mole) was used throughout at a concentration of 10^{-8} M and was applied in the mucosal solution which contained sodium (1·1 m-equiv/l.). The mean value of difference between the counts retained by the skin in the absence and presence of excess unlabelled amiloride from the five pairs of observations was tested for significance from zero by the paired t test. The number of binding sites at 100 % occupancy was calculated after dividing the amount bound by the fractional inhibition of current caused by 10^{-8} M amiloride. Asterisks indicate freshly moulted skins taken from aldosterone-treated frogs. (0·2 μmole given 15 hr previously).

TABLE 2*B*. Amiloride binding and transepithelial potential. Effect of making serosa positive

	Short-circuited (0 mV)					Serosa positive (+50 mV)					
Experiment	Displaceable [^{14}C]amiloride (d.p.m./ 9·6 cm^2)	*P*	Inhibition of sodium current (%)	Binding sites/ μm^2	Current/ binding site (A × 10^{-16})	Displaceable [^{14}C]amiloride (d.p.m./ 9·6 cm^2)	*P*	Inhibition of sodium current (%)	Binding sites/ μm^2	Current/ binding site (A × 10^{-16})	Decrease in binding (%)
1	28·0 × 7·2	< 0·02	40·0	379	0·58	14·0 ± 7·6	N.S.	38·5	190	0·61	49·9
2	17·2 ± 4·6	= 0·02	35·1	256	0·64	17·6 ± 6·1	< 0·05	36·1	255	0·67	0
3	24·2 ± 3·4	= 0·005	42·7	296	0·69	16·4 ± 5·2	< 0·05	32·3	266	0·32	10·1
4	16·0 ± 5·9	= 0·05	31·6	265	1·12	15·0 ± 4·2	< 0·02	35·2	223	0·63	15·9
5	30·8 ± 7·7	< 0·02	36·8	438	0·68	17·0 ± 12·0	N.S.	30·5	292	0·47	33·3
6	24·2 ± 4·9	< 0·01	37·4	338	0·48	13·4 ± 5·0	< 0·05	31·3	224	0·31	33·7
7	59·4 ± 10·0	< 0·001	35·9	866	0·44	31·4 ± 10·0	< 0·05	34·0	483	0·61	44·2
8*	45·6 ± 6·2	< 0·002	27·9	855	0·74	24·2 ± 10·7	N.S.	24·7	513	0·47	40·0
9*	34·6 ± 4·2	< 0·002	30·6	592	0·47	17·6 ± 9·6	N.S.	28·2	327	0·22	44·8
10*	22·8 ± 6·1	= 0·02	28·1	424	0·43	19·0 ± 5·3	< 0·025	24·9	399	0·33	5·9
	Mean ± S.E. 30·3 ± 4·2		34·6 ± 1·6	471 ± 72	0·63 ± 0·07	Mean ± S.E. 18·7 ± 1·7		31·6 ± 1·5	317 ± 36	0·46 ± 0·05	27·8 ± 5·7

Conditions as for Table 2*A*.

and where K_A and K_T are affinity constants and $[A]$ and $[T]$ are concentrations of amiloride and triamterene respectively. Thus in the presence of triamterene (10^{-5} M), binding of amiloride (10^{-8} M) should be reduced to 8% of its normal value. The effect of triamterene (10^{-5} M) on the binding of amiloride in four skins clamped at -50 mV was studied, and the results are given in Table 3. It can be seen that the prediction is true within experimental error for skins clamped at -50 mV. The results suggest that the extra amiloride binding sites which appear at -50 mV behave similarly to 'normal' sites with respect to their interaction with the two ligands.

TABLE 3. Influence of triamterene (10^{-5} M) on displaceable [^{14}C]amiloride (10^{-8} M) binding with the transepithelial potential clamped at -50 mV

Expt.	Displaceable [^{14}C]amiloride (p-mole/9·6 cm^2)	P	Displaceable [^{14}C]amiloride in presence of triamterene (10^{-5} M) (p-mole/9·6 cm^2)	P
1	0·159	<0.05	0·054	n.s.
2	0·160	<0.02	-0.011	n.s.
3	0·264	<0.01	-0.052	n.s.
4	0·267	<0.001	-0.033	n.s.
Mean ± S.E.	0·213 ± 0·031	—	-0.011 ± 0.045	—
		$P < 0.01$		

Displaceable amiloride binding was measured as before making five pairs of measurements in each skin using 10^{-8} M [^{14}C]amiloride in the absence and presence of excess unlabelled amiloride. The P values indicate whether or not the amount bound was significantly greater than zero. Afterwards the measurements were repeated in each skin in the presence of 10^{-5} M triamterene. Note that there was no significant amount of amiloride bound in the presence of triamterene and that the amounts bound in the absence and presence of triamterene were significantly different.

DISCUSSION

Binding studies presented here show that the amount of displaceable amiloride binding is sensitive to the transepithelial potential. Evidence that amiloride binding sites are close to, or part of, the sodium entry mechanism have been presented elsewhere (Cuthbert, 1973; Cuthbert & Shum, 1974*a*, *b*, 1975). It is important to consider if changing the transepithelial potential actually alters the number of translocating sites in the membrane or whether other explanations are more likely.

The amount of non-displaceable binding was not affected by clamping the skins at potentials different from zero, suggesting that the procedure

did not alter the concentration of ligand in stationary layers adjacent to the mucosal membrane. Furthermore, if electrical gradients substantially altered the amiloride concentration at the interface then the apparent affinity of amiloride would be changed, which was not the case. Also it should be remembered that the method used to calculate the total number of binding sites at 100% occupancy was independent of the amiloride concentration, depending only on the amount bound and the inhibition recorded.

Some criticism of the method, however, may be made as follows. Although the radio-label was added to skins clamped at various potentials the clamp had to be removed to allow blotting and elution of the bound material. At this time the ligand may have dissociated from its binding site. We have had to assume that the ligand is retained in the stationary layer or in spaces between the stratum corneum and stratum granulosum during the interval between removing the clamp and elution.

Another severe difficulty encountered in interpreting the data arose from considering the effects of potential on charged groups in the membrane which are unrelated to sodium ion translocation. For example, when the serosa is made negative anionic groups normally embedded in the membrane may become available to bind amiloride. We approached this problem by using a second ligand triamterene with actions identical to those of amiloride (Salako & Smith, 1971). Using previously determined values for the affinities of amiloride and triamterene we calculated the concentration of triamterene which would be necessary to reduce the binding of amiloride to a low value in short-circuited skins. We found that the same concentration of triamterene also produced the predicted effect on amiloride binding in skins in which the number of amiloride binding sites had been increased by voltage clamping at -50 mV. This suggests that the extra binding sites appearing at -50 mV have an affinity for amiloride at least equal to or greater than the sites available in short-circuited skins. The likelihood that the extra binding sites appearing at -50 mV were non-specific low affinity sites can probably be eliminated.

Although we have not measured complete binding curves for amiloride there is no substantial evidence for a change in affinity with potential. To arrive at this conclusion from inhibition studies we have assumed that the amiloride-insensitive current was not due to sodium. It would require extreme coincidence if this procedure gave identical affinities with transepithelial potentials ranging from $+50$ to -50 mV.

The total number of binding sites at a given potential has been calculated from the amount of displaceable amiloride and the fractional change in sodium current caused by the ligand. The validity of this approach needs to be considered. It is proposed (Cuthbert, 1974) that the sodium

entry mechanism can exist in 'on' and 'off' forms, and that amiloride has a higher affinity for the 'off' form, and that the ratio of 'on' and 'off' forms without bound ligand depends on the allosteric constant, L. Inaccuracy in estimating the number of binding sites will occur if the ligand binds appreciably to the 'on' form of the mechanism as this fraction of bound ligand will not be associated with a reduction in current. Provided amiloride has an affinity of at least 20 times more for the 'off' form than the 'on' form the discrepancy will be slight. The reasoning to support this is given below.

A consequence of the argument given above is that the reduction in the fraction of 'on' channels caused by amiloride should be equal to the total fractional occupancy – that is, the fraction of both 'on' and 'off' sites occupied by ligand. The fractional reduction in 'on' sites can be calculated from the state function $\bar{R}$, where

$$\bar{R} = \left[1 + L\left[\frac{1+a\alpha}{1+\alpha}\right]\right]^{-1},$$

while the total fractional occupancy is given by the binding fraction, $\bar{Y}$, where

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

and where $a = K_{on}/K_{off}$ and $\alpha = [\text{amiloride}]/K_{on}$, K_{on} and K_{off} being the microscopic dissociation constants of amiloride for the 'on' and 'off' forms of the sites respectively (see Cuthbert, 1974). Fig. 5 shows that the binding function and the fractional reduction in state function correspond very closely when the two microscopic dissociation constants differ by 1000 and 100 times. Even when the difference in affinity is only 20 times the discrepancy is not great at low occupancies, such as used in this binding study. However, when affinities differed by only 10 or 15 times the ligand was unable to cause complete inhibition, that is some 'on' sites still existed at high ligand concentrations. Virtually complete inhibition is obtained with amiloride suggesting the affinity is much greater for the 'off' sites than the 'on' sites, and consequently that the method we have used to estimate the total number of channels is valid.

From what has been given above it is unlikely that changes in transepithelial potential affect the value of L as the affinity for amiloride does not change substantially with potential. The increase in the number of binding sites, when the serosa is made negative, may have a trivial explanation. For example, when frog skin is short-circuited or clamped at negative potentials cell swelling occurs (Voûte & Ussing, 1968). In this condition some unfolding of the membrane may occur so that sites previously unavailable to sodium ions and amiloride become accessible. Recently Finn & Reuss (1975) have shown that when the serosal surface is exposed to hypotonic solution, which causes the cells to swell, there is a reduction in the resistance of the mucosal membrane and an increase in shunt path resistance.

In earlier binding studies with amiloride (Cuthbert & Shum, 1974*a*, *b*,

1975) we found that the number of binding sites increased with the sodium concentration. In this situation the apparent affinity for amiloride changes since sodium ions and amiloride interact competitively. In other respects the effects of sodium and serosal negativity seem comparable. Pasternak & Snyder (1975) have proposed a similar mechanism for the effects of sodium

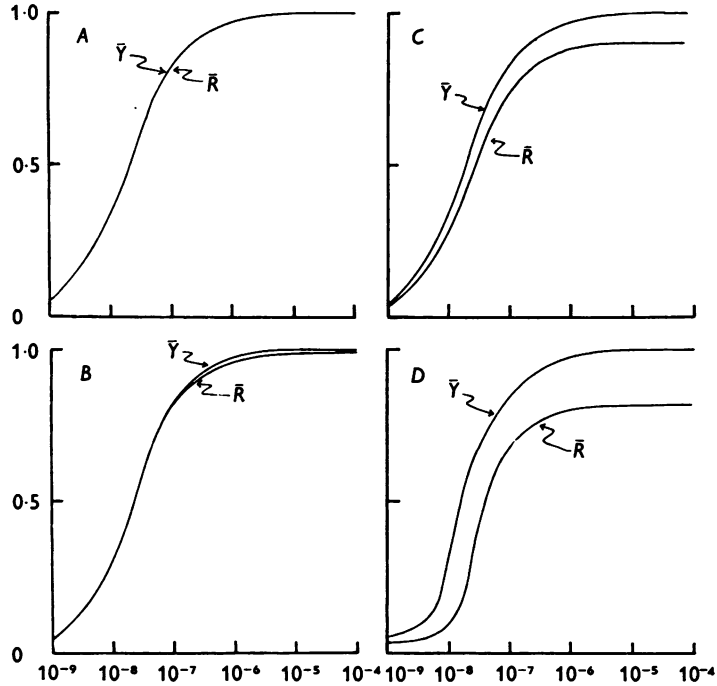

Fig. 5. Theoretical curves showing the fractional reduction in state function, $\bar{R}$ plotted versus the concentration of amiloride (in mole/l.), and the binding function, $\bar{Y}$, also plotted against amiloride concentration.

Throughout the affinity of amiloride for the 'off' form of the translocation mechanism was taken as 10^8 M^{-1}. The affinity of amiloride for the 'on' form was taken as 1000, 100, 20 and 10 times less than 10^8 M^{-1} in *A*–*D* respectively. Note that the discrepancy between the curves at around values of 0·3 (the approximate occupancy used in this study) is small or non-existent except in *D*. If the affinity for both forms was identical then the fractional reduction in $\bar{R}$ would remain constant at zero.

on the analgesic receptor. The effect of sodium appears specific in that it is not mimicked by potassium (Cuthbert & Shum, 1975). Thus, if sodium and serosal negativity are both increasing the number of entry sites it is to be expected that the effect of potential on sodium current will be less at high sodium concentrations (Table 1).

If an increase in serosal negativity increases the number of sodium entry

sites in the mucosal membrane it is reasonable to ask if this is associated with an increase in conductance. We abandoned studies of frog skin conductance with external electrodes as we were unable to reach a definitive interpretation of the results. From the equivalent circuit given in Fig. 3 the skin conductance is given by G, where

$$G = \frac{R_{Na} + R_L}{R_{Na} . R_L},$$

also the transepithelial potential V is given by

$$V = \frac{R_L}{R_L + R_{Na}} (I_e R_{Na} - E),$$

where I_e is the current flowing in the external circuit.

If potential changes the number of entry sites then we would not expect linear I/V relationships. Linear relationships, however, are commonly reported for both frog skin (Biber & Saunders, 1973) and toad bladder (Saito, Lief & Essig, 1974). However, the linearity might be a consequence of high leak pathways such that the conductance is dominated by R_L. From the linear I/V relationship of Biber & Saunders (1973) the skin potential was only +15 mV which may indicate a low value for R_L. In other instances non-linear I/V relationships have been reported for frog skin and toad bladder. Candia & Chiarandini (1973) described rectification in frog skin and Civan (1970) found a decrease in resistance in toad bladders clamped at negative potentials, in both instances the changes occurring in a direction consistent with our findings. The crucial part of the argument on the nature of the I/V relationship for the active pathway in these epithelia rests on the nature of shunt pathways. In frog skin Mandel & Curran (1972) found a decrease in shunt permeability in passing from −100 to 0 mV, whereas in toad bladder the permeability of the shunt pathway to sodium, at least, is independent of potential (Saito *et al.* 1974). Clearly the only way to establish the I/V relationship for the mucosal membranes of epithelial cells is to use micro-electrodes. Recently, Finn & Reuss (1975) have shown that the conductance of the mucosal membrane does increase when the cells are caused to swell. Furthermore, they argue that total conductance may, under some conditions, remain constant, while the conductances of leak and active pathways change in opposite directions.

From our data no significant differences between the current flowing through each site at different transepithelial potentials have been detected. However, the calculation of the current per site requires determination of amount of displaceable ligand, the transepithelial current, percentage inhibition of current and maximal inhibition of current by ligand, all with

their individual errors, which makes it unlikely that the final values for current per site will be very sensitive to the experimental conditions. For this reason it is not possible to make very definite conclusions about the mechanism of sodium entry. Biber & Sanders (1973) assumed that permeability of the skin remained constant at different transepithelial potentials, and that the sodium flux was potential sensitive. Alternatively the flux through each site might be insensitive to potential, while the number of sites is determined by potential. Yet again both flux and the number of sites may be potential dependent. While our changes in current per site do not have statistical significance, they are in the direction which suggests that the current through each site is potential dependent.

For example, when skins were clamped at +50 mV there was a reduction in current per site of nearly 30 % which just failed to achieve significance ($0{\cdot}05 < P < 0{\cdot}1$). On the other hand when skins were clamped at −50 mV the current per site was only 10 % greater than under short-circuit conditions. One reason for the failure to see a large increase in current per site in skins clamped at negative potentials may be as follows. Our calculations are based on transepithelial current and not mucosal sodium influx. Biber & Sanders (1973) showed that the mucosal influx may exceed the net transepithelial flux in skins clamped at negative potentials. This presumably indicates that at negative potentials there is an increased efflux of sodium out of the cell across the mucosal membrane, or that other anions or cations become involved. Therefore we cannot, from these results, conclude that the sodium influx through mucosal sites is potential dependent, although the indications are in support of the findings of Biber & Sanders (1973) that this is so. However, the assumptions they were forced to make for their computations that permeability remains constant with voltage is probably untrue, as the number of sites available for entry appears to be potential dependent.

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