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AN UPPER LIMIT TO THE NUMBER OF SODIUM CHANNELS IN FROG SKIN EPITHELIUM

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SUMMARY

1. Binding of [^{14}C]amiloride to the mucosal surface of frog skin epithelium has been measured in the presence and absence of an excess of unlabelled amiloride. Simultaneous records were obtained of the sodium transport through the skin as indicated by the short circuit current.

2. The number of binding sites with affinities for amiloride of 5×10^7 l./mole was around 400/μm^2.

3. The relation between the binding sites and sodium channels is discussed.

4. Making certain assumptions it is concluded that when frog skin epithelium is bathed in Ringer solution each sodium channel can handle 8000 sodium ions/sec.

INTRODUCTION

The surface density of sodium channels in sodium transporting epithelia is of interest since it might allow conclusions to be made about the handling of sodium by the ionophores. This paper describes experiments which have enabled an estimate to be made of the density of these channels in the mucosal surface of frog skin epithelium. Use has been made of [^{14}C]amiloride, an agent which selectively blocks the entry of sodium to the transport mechanism (Salako & Smith, 1970*a*, *b*; Nagel & Dörge, 1970*a*, *b*; Cuthbert & Wong, 1972). The method is non-destructive and allows repetitive determinations to be made in a single tissue. In addition, simultaneous determinations of the rate of sodium transport have been made using an automatic voltage clamp, so that the rate of sodium movement through each channel can be estimated.

The results indicate that the probable number of sodium channels in frog skin is about 400/μm^2, and that each channel is capable of handling at least 8000 ions each second.

METHODS

Experiments were performed on the abdominal skins of *Rana temporaria*. Skins were dissected and mounted horizontally in a chamber with the mucosal surface uppermost. The chamber was made of Perspex and is illustrated in Fig. 1. The area of skin was 8 cm^2.

The serosal surface was bathed in Ringer solution which was not changed throughout the experiment. The mucosal surface was bathed with 10 ml. Ringer solution and oxygenated by bubbling air through the Ringer. The mucosal solution could be rapidly removed by suction and replaced in a few seconds.

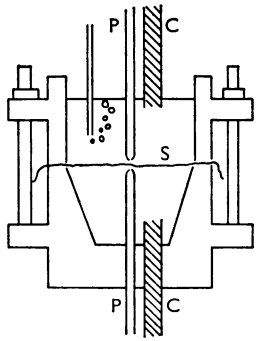

Fig. 1. Diagram of Perspex cell for holding skin (S) showing position of current passing (C) and potential measuring (P) electrodes.

Electrodes for measuring potential and passing current were inserted through the base of the chamber, and a similar pair were held in an assembly above the mucosal surface. Electrodes consisted of polyethylene tubes filled with agar containing 3 M-KCl. The mucosal electrode assembly, together with the aeration tube, were arranged so that they could be rapidly lifted above the mucosal chamber. The potential electrodes led via calomel cells to a millivoltmeter (Radiometer pH meter) and a short-circuiting device. This latter consisted of two operational amplifier (Analog Devices Type 144A) circuits so arranged that current was fed to the current electrodes to clamp the transepithelial potential at zero. The short circuit current (SCC), which is a measure of the net mucosal to serosal flux of sodium (Ussing & Zerahn, 1951) was displayed on a pen recorder (Bryan 2700 series).

The protocol used for labelling studies is illustrated in Fig. 2. After an initial period of stabilization (30 min) a cumulative dose–response curve to amiloride was recorded. This allowed the choice of a concentration of drug which caused between 30 and 50% inhibitition of SCC. The half-time for reversal of the chosen concentration of amiloride was next determined. This was usually between 10 and 30 sec.

The mucosal surface of the skin was then exposed to [^{14}C]amiloride (A^*) at the chosen concentration for exactly 2 min. This solution was removed, the electrode assembly raised, and the mucosal surface blotted three times with Kleenex tissue. The mucosal surface was then bathed with 10 ml. of a solution of amiloride at a hundredfold the chosen concentration (A) for exactly 3 min. This time was at least six times the half-time for reversal of the chosen concentration, and at least 98% of [^{14}C]amiloride retained by the skin will have moved into the mucosal solution after 3 min. At the end of this time the mucosal solution was removed and exactly

9 ml. taken for counting. An aliquot of the original [^{14}C]amiloride solution was also taken for counting. The foregoing procedure detects not only [^{14}C]amiloride bound to the skin but [^{14}C]amiloride trapped in the small extracellular space at the mucosal surface. After washing the procedure was repeated using [^{14}C]amiloride (A^*) at the chosen concentration together with a hundredfold excess of amiloride (A). Under these conditions only 1 % of the binding sites will be occupied by [^{14}C]amiloride, while the same concentration will be present in the extracellular space. The difference between the amount of [^{14}C]amiloride retained by the skin under the two conditions represents an upper limit of the amount bound to sodium channels.

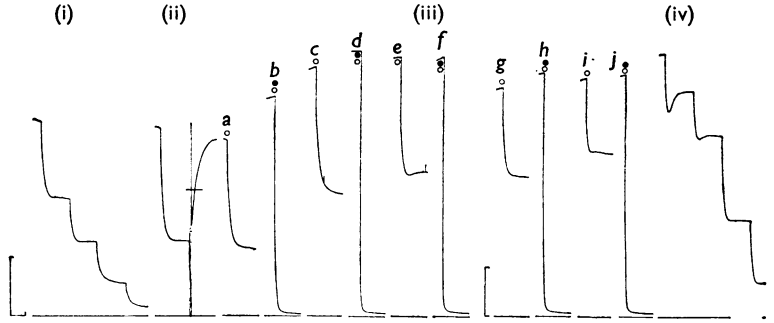

Fig. 2. Illustration of the protocol used for determining [^{14}C]amiloride binding to the mucosal surface of frog skin in normal Ringer. Records show the short circuit current of the skin. The panels illustrate, from left to right:

(i) a cumulative dose–response relationship to amiloride. The concentrations of amiloride were 5×10^{-8}, 10^{-7}, 3×10^{-7} and 10^{-6} M;

(ii) the determination of the half-time for offset of the response to 10^{-7} M amiloride. The $t_{\frac{1}{2}}$ is indicated by a horizontal bar and was 15 sec;

(iii) the next ten panels show the effects of [^{14}C]amiloride 10^{-7} M at ○ (panels *a*, *c*, *e*, *g* and *i*), and [^{14}C]amiloride 10^{-7} M plus amiloride 10^{-5} M at ○● (panels, *b*, *d*, *f*, *h* and *j*). Between each panel the skin was blotted and eluted with 10^{-5} M amiloride for 3 min, and then washed in Ringer solution;

(iv) the final panel shows a cumulative dose–response relation obtained using the same drug concentrations as in (i).

The vertical calibrations are 100 μA and the horizontal calibration is 1 min. Note the change in sensitivity after panel *f*. The horizontal line at the bottom of each panel indicates zero current.

The whole of the above procedure was repeated five times giving five paired differences from which calculations were made. There were three reasons for exposing the skin to high concentrations of amiloride during the washout of the radiolabel. They were (i) all determinations of binding were made following a complete inhibition of SCC, (ii) a concentration gradient for amiloride was maintained between the mucosal solution and the interior of the cells and (iii) the excess of amiloride acted as a carrier for the radiolabel.

The samples (9 ml.) of radioactive solutions were evaporated to dryness in an oven at 110° C overnight. The dried samples were then dissolved in 0·5 ml. distilled water and 10 ml. scintillation fluid (butyl PBD, 0·6 % w/v; methoxyethanol, 16 % v/v; ethoxyethanol, 32 % and toluene to 100 %). Samples were counted in a Packard

liquid scintillation spectrometer. The number of disintegrations/min in each sample were calculated by the usual procedures.

Each of the five determinations in each experiment yielded four counts as follows. DPM bound in the presence of A^*, DPM bound in the presence of $A^* + A$, plus counts of the activities of the radioactive solutions added to the skin. For comparison of the amounts bound in the absence and presence of excess unlabelled amiloride the counts were normalized, that is (DPM for A^*) $\times$ 10/9 was compared with

$$[\text{DPM for } (A^* + A) \times 10/9] \times \frac{\text{DPM for 100 } \mu\text{l. of solution containing [}^{14}\text{C]amiloride}}{\text{DPM for 100 } \mu\text{l. of solution containing [}^{14}\text{C]amiloride plus excess amiloride}}.$$

This procedure allowed for small differences between the amounts of [^{14}C]amiloride added, and for counting only 9 ml. of the 10 ml. of solution for washout.

The statistical significance of the five paired observations was determined using the method described by Goldstein, 1964, p. 60). The upper 95% confidence limit of the mean of the difference between the pairs was obtained from

$$\text{Upper } 95\% \; CL = (\bar{A}^* - \overline{[A^* - A]}) + 2{\cdot}306 \,.\, S \,.\, 0{\cdot}63$$

(Bailey, 1959, p. 172), where S is the standard deviation of ten observations.

The maximal number of molecules which can be bound by the skin was obtained from

$$T = N \times \frac{100}{\%I},$$

where N is either mean number of molecules bound or the upper 95% confidence limit of the number of molecules bound and $\%I$ is the mean percentage inhibition caused by the chosen concentration of [^{14}C]amiloride. Assuming this binding is to sodium channels (see discussion) the current flowing through each channel, I, was given by

$$I = \frac{\text{mean SCC before addition of [}^{14}\text{C]amiloride}}{\text{total number of sodium channels}}$$

and the number of ions, X, handled/sec by each channel is given by

$$X = \frac{I \times 6{\cdot}02 \times 10^{23}}{96{,}500}.$$

[^{14}C]Amiloride (0·35 mg, 0·054 c/m-mole) was supplied by Merck Sharp & Dohme. This was dissolved in a 3·85 ml. 100 mM-NaCl and 0·25 ml. distributed into fifteen bottles. These samples were lyophilized and stored at 0° C. Solutions of [^{14}C] amiloride either 10^{-5} or 10^{-6} M were reconstituted as required. Using unlabelled amiloride it was shown that the lyophilization procedure caused no loss of amiloride. Amiloride was assayed by measuring the emission spectrum at 418 nm. Checks were also made to show that the drying procedure for the samples caused no loss of radioactivity. The Ringer solution used had the following composition: NaCl, 112 mM; KCl, 3·5 mM; $CaCl_2$, 1 mM; NaH_2PO_4, 0·08 mM; $NaHCO_3$, 2·4 mM and glucose 11·1 mM. This solution had a pH of 7·6 when equilibrated with air. When low sodium Ringer was used (2·5 mM-Na) NaCl was omitted. All experiments were carried out at room temperature (20 $\pm$ 2° C).

RESULTS

Preliminary experiments were made to determine the characteristics of the onset and offset of the responses to amiloride. Fig. 3 shows records obtained by exposing a skin to different concentrations of amiloride for 2 min, after which the solution was rapidly removed by suction and replaced by fresh Ringer solution, but without blotting the skin. The records show that the maximal effects are reached well within 2 min, and that the

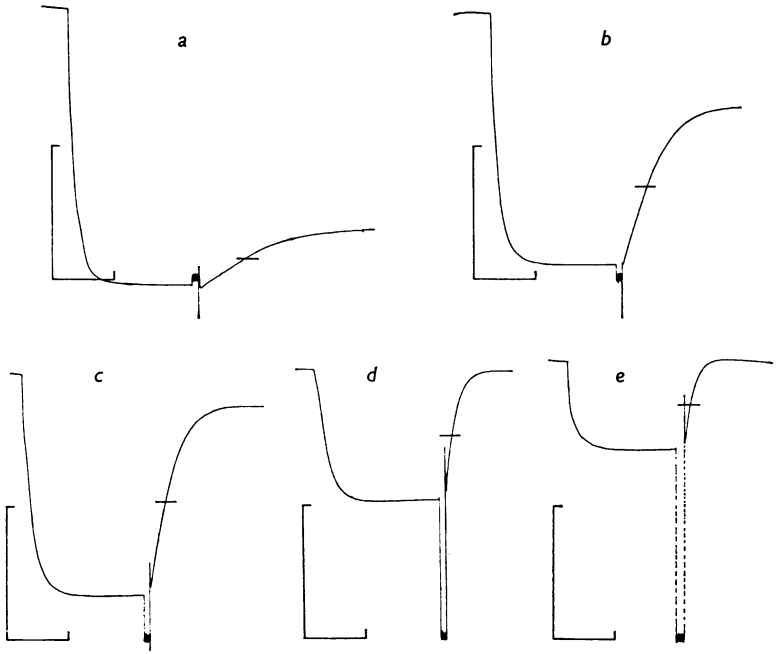

Fig. 3. Illustration of an experiment to determine the half-time for offset of the response to amiloride. The mucosal surface of the skin was exposed to the drug for exactly 2 min, after which the solution was changed to normal Ringer. The solution change took approximately 10 sec. The concentrations of amiloride used were 10^{-5}, 10^{-6}, 5×10^{-7}, 10^{-7} and 5×10^{-8} M in *a*, *b*, *c*, *d* and *e* respectively. Calibrations are 100 μA and 1 min. The half-times are marked with horizontal bars.

apparent half-time of offset increases with the drug concentration. It was also noted that there is not full recovery of the SCC after a single replacement of solution with the higher concentrations of drug. This is obviously due to drug remaining on the skin when the solutions were changed. Average results from three experiments such as the one illustrated are shown in Table 1.

In other experiments the apparent half-time of offset was measured at

a fixed concentration of amiloride but after exposure for different times. This is illustrated in Figs. 4 and 5. It is clear the apparent half-time for offset does not depend on the exposure time at a given concentration, but does depend on the concentration.

TABLE 1. Showing the effects of amiloride at different concentrations on the inhibition of SCC, the half-time for offset, and percentage recovery following a change of solution

Amiloride concentration (M)	% inhibition of SCC	Apparent half-time of offset (sec)	% recovery of SCC
10^{-7}	57·1	5·0	99·5
5×10^{-7}	83·8	13·0	90·4
10^{-6}	90·5	19·5	80·1
10^{-5}	99·0	37·0	30·7

Each value is the mean of three determinations.

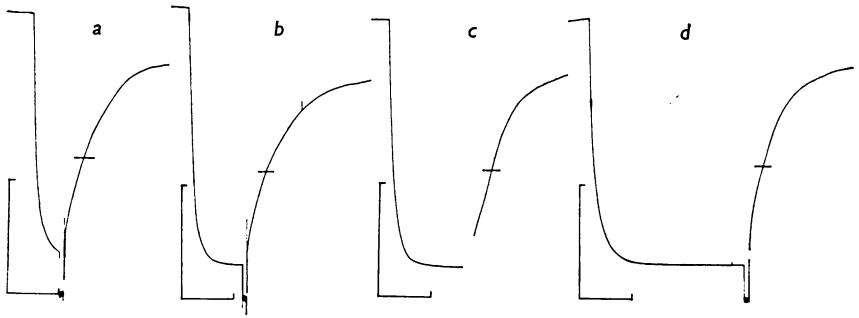

Fig. 4. Illustrations of an experiment in which the skin was exposed to 10^{-6} M amiloride for various times (30, 60, 90 and 180 s at *a*, *b*, *c* and *d* respectively). The half-times of offset are marked by horizontal bars. Calibrations are 100 μA and 1 min.

These preliminary experiments showed, for subsequent labelling experiments, that 2 min was sufficient time for equilibration with the receptors provided that the concentration of [^{14}C]amiloride was lower than about 2×10^{-7} M, and that 3 min was sufficient time to allow complete exchange of the bound label for non-radioactive amiloride.

Labelling experiments were carried out as described in the methods and as illustrated in Fig. 2. The results of six experiments are given in Table 2. In none of the six experiments was the difference between the amount of label retained by the skin in the absence and presence of excess unlabelled amiloride statistically significant. However, since in the presence of excess amiloride the binding of the [^{14}C]label to the receptors must have been depressed the maximal possible differences which could exist without

detection at the 5% level in the absence and presence of excess amiloride were calculated. These are shown as the upper 95% confidence limits in Table 2. An upper limit for the number of molecules bound to frog skin was calculated from this data as $2259 \pm 349/\mu m^2$.

Although the data obtained at this stage of the work were of interest ways were sought to obtain more accurate values. It were clear that an

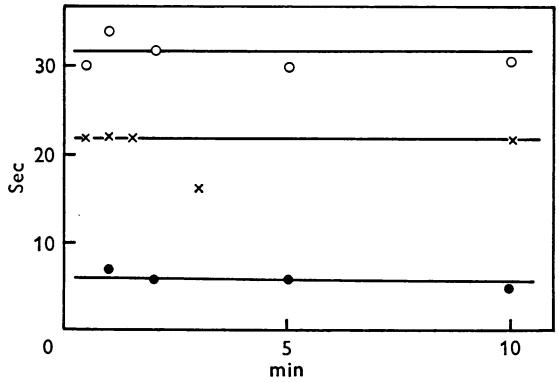

Fig. 5. Graph showing the half-time of offset (sec) of amiloride inhibition versus the duration of exposure (min) for different concentrations of drug. Amiloride concentrations were 10^{-7} M (●), 10^{-6} M (×) and 5×10^{-6} M (○).

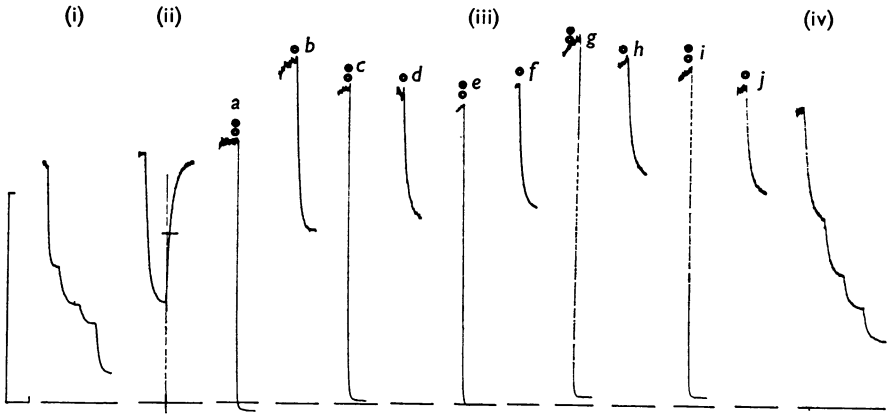

Fig. 6. Records from an experiment in which the uptake of [^{14}C]amiloride was measured with low sodium Ringer bathing the mucosal surface of the skin. The arrangement of the figure is as for Fig. 2. The details are as follows: (i) concentrations of amiloride used for the dose–response relationship determinations were 10^{-8}, 2×10^{-8}, 3×10^{-8} and 10^{-7} M; (ii) the half-time of offset for 2×10^{-8} M amiloride was 18 sec; (iii) [^{14}C]amiloride 2×10^{-8} M was added at ○ (panels *b*, *d*, *f*, *h* and *j*), 2×10^{-6} M amiloride was added at ●, together with the label (panels *a*, *c*, *e*, *g* and *i*). The calibrations are 100 μA and 2 min; (iv) as (i).

TABLE 2. Uptake of [^{14}C]amiloride in frog skin in normal Ringer (skin area 8 cm^2)

Expt.	A^* (M)	A (M)	DPM eluted: A^*	DPM eluted: A^*+A	DPM eluted: Δ	DPM eluted: P	Upper 95% C.L.	SCC (μA/cm^2)	% I by A^*
1	10^{-7}	10^{-5}	384 ± 32	391 ± 26	− 7	n.s.	88	31·0	39·5
2	2×10^{-7}	2×10^{-5}	527 ± 27	503 ± 25	+24	n.s.	110	51·6	40·4
3	10^{-7}	10^{-5}	376 ± 13	357 ± 22	+19	n.s.	78	52·8	45·4
4	2×10^{-7}	2×10^{-5}	730 ± 72	742 ± 49	−12	n.s.	188	29·9	60·4
5	2×10^{-7}	2×10^{-5}	1012 ± 110	1011 ± 69	+1	n.s.	303	34·1	51·1
6	2×10^{-7}	2×10^{-5}	1279 ± 67	1256 ± 53	+23	n.s.	231	27·2	47·2
						Mean value ± S.E.		37·8 ± 4·7	

TABLE 3. Uptake of [^{14}C]amiloride in frog skin in low sodium Ringer (skin area 8 cm^2)

Expt.	A^* (M)	A (M)	DPM eluted: A^*	DPM eluted: A^*+A	DPM eluted: Δ	DPM eluted: P	SCC (μA/cm^2)	% I by A^*	Total no. of channels /μm^2	I($\times10^{-6}$ A) per channel	Ions/sec per channel
1	10^{-8}	10^{-6}	59 ± 3	52 ± 4	+ 7	< 0·025	4·1	29·3	156	2·60	1620
2	2×10^{-8}	2×10^{-6}	164 ± 10	140 ± 6	+24	< 0·01	7·5	28·7	526	1·42	885
3	2×10^{-8}	2×10^{-6}	112 ± 11	85 ± 9	+27	< 0·005	11·5	31·2	557	2·06	1285
4	2×10^{-8}	2×10^{-6}	102 ± 9	86 ± 10	+16	0·01	19·4	38·9	273	7·12	4440
					Mean values ± S.E.		10·6 ± 3·3	—	378 ± 98	3·3 ± 1·3	2058 ± 808

increase in the specific activity of amiloride would not help and it was unlikely that the standard errors associated with the experimental determinations could be improved since they were surprisingly small. It was necessary to increase the potency of amiloride as an inhibitor of sodium transport. Fortunately, Salako & Smith (1970*b*) had shown that if the sodium concentration in the bathing solution was reduced then amiloride was a more effective inhibitor of SCC. Experiments were therefore made in which the mucosal sodium concentration was reduced to 2·5 mM from 115 mM. This increased the effectiveness of amiloride ten-fold as an inhibitor of sodium transport. In experiments with low sodium Ringer bathing the mucosal face the SCC was reduced to approximately 25% of the normal value. Four experiments carried out with these conditions are shown in Table 3. In each instance there was a highly significant difference between the amount of [^{14}C]amiloride bound in the absence and in the presence of excess unlabelled amiloride. The differences gave a value for the number of molecules bound as $378 \pm 98/\mu m^2$. The epithelia bathed in low sodium Ringer solution were as resilient to the frequent changes of bathing solution, blotting, etc., as were those bathed in normal Ringer. Fig. 6 illustrates the responses of a preparation in low sodium Ringer.

In general, it was noticed that during the course of experiments in which either high or low sodium solutions were used to bathe the mucosal surface the SCC increased while the percentage inhibition caused by a given concentration of amiloride became less. In all instances a cumulative dose–response curve was plotted at the end of the experiment and compared with the initial curve. The two curves were parallel, but that determined at the end of the experiment was moved to the right along the concentration axis by approximately one tenth of an order.

DISCUSSION

Preliminary experiments showed that the apparent half-time for offset of the inhibition caused by amiloride was independent of the length of exposure, but increased with concentration. It seems probable from this that the mucosal surface of frog skin presents a very tight structure against the penetration of amiloride, since the slowing of offset does not appear to be caused by leaching out of drug molecules from the cells when the solutions were changed. What is more likely is that when high concentrations of drug were applied binding occurred to low affinity sites, which provided a reservoir from which high affinity sites could be attacked after the solutions were changed. Salako & Smith (1970*b*) found that amiloride did not enter the serosal solution from the mucosal side even after exposure for 1 hr.

The important question raised by these results is whether the difference

between the amount of [^{14}C]amiloride bound in the absence and presence of excess amiloride is equivalent to the amount of drug bound to sodium channels. The usual requirements for molecules used in labelling studies is that they should show high specificity and affinity, and preferably react irreversibly with the target. The main problem with amiloride is that its inhibitory effects are rapidly reversible. The apparent half-times for offset were just a few seconds so that it was not possible to use any washing procedure. Fortunately the extracellular space of the mucosal surface of frog skin is only 1·3 % as measured with [^{14}C]inulin (Brash & Cuthbert, 1970) which means that the amount of radioactivity retained in the extracellular space by the tagged and blotted skin was small. However, Erlij & Smith (1971) showed that inulin was an unreliable indicator of the extracellular sodium space of the mucosal surface of frog skin, so it is probable that inulin would be an unreliable indicator of the mucosal amiloride space. For this reason amiloride itself was used to probe the size of the mucosal extracellular space. When unlabelled amiloride was added to the mucosal surface in hundredfold excess of the concentration of [^{14}C]amiloride the concentration of the radiolabel in the extracellular space would be unaltered compared to that found in the absence of excess amiloride.

The difference between the amount of radiolabel bound in the absence and presence of excess amiloride will represent drug bound to high affinity sites in the epithelium. The ideal way of showing that the radiolabel is bound exclusively to sodium channels would be by competition studies with other agents which are able to block the channels. Unfortunately no such agents are available, but clearly some of the bound material must have been attached to sodium channels since the SCC was inhibited. With low sodium Ringer any binding sites which were not sodium channels must have had affinity constants for amiloride of around 5×10^7 l./mole.

Suppose there is a great abundance of low affinity sites (10^6 l./mole) for amiloride which are not related to sodium channels. Then using A^*, 10^{-8} M and A^* plus A, 10^{-6} M the fractions of these non-specific receptors occupied by the radiolabel would be 1 and 0·5 % respectively and the difference detected by the present methods would be 0·5 %. Using A^*, 10^{-7} M and A^* plus A, 10^{-5} M the fractions occupied would be 9·1 and 0·9 % respectively and the difference detected would be 8·2 %, that is sixteen times greater than with the first combination. Since the absolute amount of bound radioactivity (columns 6 in Tables 2 and 3) is similar with different concentrations of amiloride it is concluded that the sites which bind amiloride when present in low concentration have affinities greater than 10^6 l./mole.

If it is assumed that all the bound material is bound to sodium channels then the number of channels can be calculated assuming a 1:1 stoichiometry. The figures given in Table 3 should be regarded therefore as an upper limit for the channel density. There is no absolute proof for the suggested stoichiometry but kinetic studies with amiloride (Cuthbert & Wong,

1972) are consistent with a 1:1 relationship. Although the estimates of the upper limit for the number of channels obtained in normal Ringer were on average six times greater than those found in low sodium Ringer it was surprising that values were of the same order, in spite of the failure to achieve significant difference.

With the foregoing assumptions it appears that each channel can handle about 2000 ions/sec when the mucosal solution contains 2·5 mM sodium. Since in normal Ringer values of the SCC were four times higher it appears that each channel can handle about 8000 ions/sec in the presence of 115 mM sodium. The non-linearity between the amount of sodium transferred and sodium concentration is consistent with the existence of a saturating mechanism operating at the mucosal face (Frazier, Dempsey & Leaf, 1962).

Application of Smoluchowski diffusion theory and assuming that the channels are similar in size to the sodium ion then the rate at which sodium ions bombard each channel can be calculated (Burgen, 1966). For low sodium Ringer (2·5 mM) the calculated value is $6{\cdot}25 \times 10^6$ ions/sec, that is three orders of magnitude higher than estimated from the experimental data. It would be unreasonable to believe that the density of channels had been over-estimated by 1000-fold, from which it is concluded that the sodium ions do not enter epithelium by a simple diffusion process, even at low sodium concentrations. It is, of course, possible that sodium channels open only intermittently so that transient current densities higher than the calculated average values may be achieved. Also, it might be more correct when calculating the value of the current in each channel to take a value of 105% SCC, since SCC represents only the net flux. The backflux is normally 5% of the forward flux of sodium in frog skin. However, until it is known whether or not backflux occurs through the same channels as the forward flux this refinement is unjustified.

It is of interest to compare the density of ionic channels in this situation with those found for other biological membranes. Using tetrodotoxin Moore, Narahashi & Shaw (1967) found the upper limit for sodium channels in lobster nerve was $13/\mu m^2$. Later Keynes, Ritchie & Rojas (1971) obtained values of $75/\mu m^2$ for rabbit vagus, and $49/\mu m^2$ and $36/\mu m^2$ for crab and lobster nerves respectively. In the motor end plate Miledi & Potter (1971) found using α-bungarotoxin that there were 10^5 ACh receptor sites/μm^2. This number is probably simply related to number of sodium and potassium ionophores in the end-plate.

Taking the value for the density of sodium channels in frog skin as $400/\mu m^2$ and assuming they are arranged as a mosaic, and not concentrated at the intercellular junctions (Cereijido & Rotunno, 1967) then the distance between channels would be approximately 500Å. In this work the surface area of the skin was calculated from the dimensions of the chamber.

Histologically the skin surface appears dimpled and Smith (1971) suggests the measured area should be increased by one third to give the true surface area. If this is done the upper limit for the number of channels becomes $300/\mu m^2$.

No explanation is available for the increase in SCC and the fall off in the potency of amiloride which was noted in the course of most experiments. The possibility cannot be ignored that the continual treatment with amiloride, together with washing and blotting removed or impaired the gating mechanism controlling sodium entry to the channels.

I am grateful to Merck Sharp and Dohme for a sample of [^{14}C]amiloride.

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