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INDUCTION OF TRANSPORTING SITES IN A SODIUM TRANSPORTING EPITHELIUM

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SUMMARY

1. Frogs (*Rana temporaria*) were bathed for 1 week in solutions containing 1·1 mM sodium chloride and either one or both of amiloride (10^{-4} M) and spironolactone (10^{-5} M). This procedure was designed to deplete the sodium transporting compartment of the skin epithelium of sodium, while at the same time antagonizing the effects of endogenous aldosterone.

2. After 1 week the skins were used *in vitro* to measure the level of sodium transport (short-circuit current) and the density of sodium entry sites in the mucosal surface of the epithelium ([^{14}C]amiloride binding).

3. Sodium deprivation for 1 week caused approximately a doubling of both sodium transport and the density of sodium entry sites in the mucosal surface of the epithelium compared to control skins.

4. When the results for sodium deprived and control skins were pooled there was a highly significant correlation between the density of sodium entry sites and sodium transport.

5. Mechanisms by which sodium deprivation leads to an increase in the density of sodium entry sites are discussed.

INTRODUCTION

Many of the activities of cells are controlled or modified by interactions between endogenous substances, such as hormones and neurotransmitters, with receptors located in the cell membrane. It is a common finding that when tissues are removed from the influence of their normal activators, by for example denervation or the use of blocking drugs, the sensitivity of the tissue to the activator is increased (for reviews see Emmelin, 1961; Trendelenberg, 1963). One explanation of increased sensitivity, or supersensitivity as it is often termed, is that the number of receptor molecules is increased, so that at a given fractional occupancy the physiological response is enhanced (Raff, 1976). In some recent examples (see Discussion)

supersensitivity has been shown to be correlated with an increase in receptor density.

In the experiments reported here the ideas outlined above have been explored in an entirely new situation. A sodium transporting epithelium was deprived of sodium for a period of 1 week and at the end of this time the level of transport and the density of sodium entry sites in the epithelium were measured. It had already been established that adaptation of amphibian epithelia to high or low salinities influenced the subsequent level of sodium transport by a negative feed-back reaction, that is deprived tissues had elevated transport capability and vice versa (Bentley, 1973; Katz, 1975). In these experiments it was found that sodium deprivation increased both the level of sodium transport and the numbers of membrane macromolecules controlling sodium entry into the epithelium. When the results for control and sodium deprived epithelia were pooled there was a significant correlation between sodium transport and density of entry sites.

METHODS

All experiments were performed on the isolated abdominal skin of frogs (*Rana temporaria*). The animals were kept at room temperature (20° C) in the laboratory. Most of the animals were pre-treated for 1 week before use as set out below.

Group A. Pre-treated with amiloride and spironolactone. Animals were given a single injection of 0·2 μmole spironolactone (dissolved in 1 % alcohol) into the dorsal lymph space. The animals were placed in individual plastic boxes partly immersed in a solution containing sodium chloride, 1·1 mM, spironolactone, 10^{-5} M, and amiloride, 10^{-4} M. The solution was changed daily for 6 days.

Group B. Pre-treated with spironolactone. As for group *A* except the solutions in which the animals were kept contained no amiloride.

Group C. Pre-treated with amiloride. Animals were partly immersed in a solution containing sodium chloride, 1·1 mM, and amiloride, 10^{-4} M. The solution was changed daily for 6 days.

Control animals were kept in plastic tanks in tap water at room temperature.

Measurement of sodium transport

Sodium transport in abdominal skin was measured as short-circuit current. The area of skin used was always 9·6 cm^2. Electrodes for measuring skin potential and for passing current were conventional, and skin potentials were clamped automatically at zero by a voltage clamp, the short-circuit currents being recorded on a pen recorder. Details of the apparatus have been described previously (Cuthbert, 1973). When transport of sodium was stimulated with antidiuretic hormone it was added to the serosal bathing solution. Amiloride, a drug inhibiting the sodium entry process, was always added to the mucosal bathing solution.

Determination of the number of sodium entry sites

The number of sodium entry sites in the mucosal surface of frog skin epithelium was determined by measuring the binding of [^{14}C]amiloride. From measurement of the amount bound and the fractional inhibition of current caused by the radiolabelled ligand the total number of sodium entry sites in the mucosal surface of pieces of frog

skin was calculated. Details of methods used, together with a critical evaluation of possible sources of error have been given fully in earlier papers in which [^{14}C]amiloride binding has been described. It is considered that the amiloride binding site is identical to, or at least part of, the sodium entry site and that the density of sites can be estimated with reasonable accuracy. For a further account and discussion of these premises the reader should consult the sources cited below (Cuthbert, 1973; Cuthbert & Shum, 1974*a*, *b*, 1975, 1976*a*).

Experimental protocol

Animals were pre-treated in the ways described above for periods of 1 week. The treatments were designed to do one or both of two things: to prevent the entry of sodium through the mucosal surface of the skin and to prevent the actions of endogenous aldosterone on the epithelial cells. The former was achieved by addition of amiloride to the external fluid while the latter was achieved with the aldosterone antagonist, spironolactone. The plastic boxes in which frogs were treated were of a design which made it impossible for the animals to take up positions in which the abdominal parts of the skin were not immersed in fluid. The frogs survived the various pre-treatments without ill effects.

At the end of 1 week's treatment the animals were pithed and pieces of abdominal skin were mounted in the apparatus with Ringer solution bathing each surface. Skins were immediately short-circuited and then 30 min were allowed for the short-circuit current to stabilize. The value of this current at 30 min in Ringer solution was noted, after which a cumulative inhibition curve was determined by adding progressively increasing concentrations of amiloride to the mucosal bathing solution. The reciprocal of the concentration of amiloride causing 50 % inhibition of transport was taken as the apparent affinity of the binding site for amiloride. Following this the mucosal bathing solution was changed to low sodium Ringer solution. No correction was made for tonicity under these conditions as it has been shown that correction of tonicity with either choline chloride or sucrose does not affect the short-circuit current (Cuthbert & Shum, 1974*b*). With these conditions in which there is a gradient of sodium from inside to outside the short-circuit current still reflects the net mucosal to serosal sodium flux (Biber & Curran, 1970). After a further period of stabilization of 30 min the value of the current was noted under low sodium conditions, and the apparent affinity of amiloride under these conditions was determined exactly as before.

At this stage the density of amiloride binding sites was measured using [^{14}C]-amiloride and with a mucosal sodium concentration of 1·1 mM. The reason for making the binding measurements at low sodium concentrations is that the apparent affinity of amiloride for the binding sites is enhanced by at least one order of magnitude compared to that in Ringer solution. In fact, amiloride and sodium appear to compete for the binding site (Cuthbert & Shum, 1974*a*). The advantage obtained with low sodium conditions is that non-specific binding of the ligand is very small.

Four separate binding measurements were made for each skin with the low sodium solution bathing the mucosal surface. Mean values of the amount of [^{14}C]amiloride bound when the tissue was exposed to a 10 nM solution of this ligand, together with the mean value for the fractional inhibition of current caused by the ligand, allowed calculation of the density of the binding sites in each skin.

As the total number of amiloride binding sites is measured as well as the total current flowing through the skin the nominal current per channel was calculated, assuming that amiloride binding sites are equivalent to sodium entry sites and that each site is conducting continuously.

Materials used

The materials used and their sources were as follows. Antidiuretic hormone (Pitressin), Parke Davis and Co.; amiloride, Merck Sharp and Dohme Limited; spironolactone, Sigma Chemical Co.; [^{14}C]amiloride and *N*-(*N*-benzyl-amidino)-3,5-diamino-6-chloropyrazinecarboxamide were gifts from the Merck Institute, West Point, Pennsylvania. The Ringer solution used had the following composition (mM): NaCl, 111; KCl, 2; $CaCl_2$, 1; glucose, 11 and Tris buffer, pH 7·6, 5. The solution was oxygenated by bubbling with air before and during the experiments. When labelling with [^{14}C]amiloride was carried out the sodium content of the mucosal bathing solution was reduced to 1·1 mM. No correction was made for the change in tonicity of low sodium Ringer solution.

RESULTS

Table 1 gives the results for ten separate skins, taken from ten animals all from the same delivery batch. The table shows the values of short-circuit current for both high and low sodium conditions, the density of binding sites and the nominal current per binding site. As the method used to measure [^{14}C]amiloride binding is a difference method, that is the difference between the amount of [^{14}C]amiloride retained by the skin in the presence and absence of an excess of unlabelled amiloride, the four paired differences were tested for significance from zero. The significance of this test is also given in Table 1, where it is seen that in all but one skin, where both the resting short-circuit current and site density was low, statistically significant amounts of binding were detected.

The idea behind these experiments was to examine whether or not deprivation of sodium would affect the number of sodium entry sites in frog skin epithelium. Since sodium deprivation might stimulate endogenous aldosterone secretion which itself can stimulate the appearance of sodium entry sites in the skin (Cuthbert, Okpako & Shum, 1974) it was considered necessary to block the effects of endogenous aldosterone with spironolactone.

A cursory examination of the results given in Table 1 shows that sodium deprivation is associated with an increase in binding site density. In the two skins pre-treated with amiloride alone there was no obvious extra increase in site density although aldosterone was not inhibited. Thus the results for skins from animals in groups *A* and *C* have been combined in the mean values given in Table 2. From Table 2 it is concluded that deprivation of sodium *in vivo* for a period of one week increases the steady-state short-circuit current, and hence the steady-state level of sodium transport *in vitro* when the mucosal surface is bathed in either high or low sodium-containing solutions. Furthermore, the density of amiloride binding sites, and presumably the number of sodium entry sites, is correlated with level of sodium transport measured *in vitro*. This correlation is illustrated in

TABLE 1. Effect of pre-treatment with amiloride and spironolactone on the transport characteristics of frog skin

	SCC in high Na, 111 mM (μA/cm^2)	SCC in low Na, 1·1 mM (μA/cm^2)	Binding site density		Na current/ binding site (A $\times 10^{-16}$)	Apparent affinity of amiloride M^{-1}	
			Sites/μm^2	P		High Na (111 mM)	Low Na (1·1 mM)
Group A: pre-treatment with spironolactone and amiloride	32·8	6·2	793	<0.05	0·776	5.0×10^6	7.1×10^7
	27·8	4·3	774	<0.001	0·557	11.8×10^6	6.7×10^7
	29·2	4·6	641	<0.001	0·722	2.4×10^6	5.6×10^7
	27·9	3·0	510	<0.005	0·597	8.3×10^6	6.3×10^7
Group B: pre-treatment with spironolactone	8·3	1·7	383	<0.005	0·431	3.3×10^6	3.3×10^7
	13·1	2·8	347	<0.005	0·793	2.5×10^6	5.5×10^7
	9·7	1·4	324	<0.001	0·441	5.9×10^6	6.3×10^7
	6·5	1·2	165	n.s.	0·724	13.5×10^6	7.4×10^7
Group C: pre-treatment with amiloride	44·5	6·6	674	<0.005	0·978	9.1×10^6	4.3×10^7
	30·0	4·2	734	<0.001	0·573	9.5×10^6	4.5×10^7

SCC, short-circuit current. n.s., not significant.

TABLE 2. Mean values of the transport characteristics of skins treated with amiloride or amiloride and spironolactone (Groups *A* and *C*) compared with skins treated with spironolactone alone (Group *B*)

	SCC in high Na, 111 mM (μA/cm^2)	SCC in low Na, 1·1 mM (μA/cm^2)	Binding sites/μm^2	Current/site (A $\times 10^{-16}$)	Apparent affinity of amiloride M^{-1}	
					High Na (111 mM)	Low Na (1·1 mM)
Groups *A* and *C* ($n = 6$)	32.0 ± 2.6	4.8 ± 0.6	688 ± 43	0.701 ± 0.07	$7.7 \times 10^6 \pm 1.4 \times 10^6$	$5.8 \times 10^7 \pm 0.5 \times 10^7$
Group *B* ($n = 4$)	9.4 ± 1.4	1.8 ± 0.4	305 ± 48	0.597 ± 0.09	$6.3 \times 10^6 \pm 2.5 \times 10^6$	$5.6 \times 10^7 \pm 0.9 \times 10^7$
P	<0.001	<0.001	<0.001	n.s.	n.s.	n.s.

SCC, short-circuit current. n.s., not significant.

Fig. 1, which shows the relation between the density of binding sites and the short-circuit current in low sodium conditions. The regression line has a highly significant ($P < 0{\cdot}001$) correlation coefficient, 0·87. Although we have reported several times previously on the density of binding sites in frog skin under different conditions we have been unable to examine our data for correlation of this type as steady-state values for short-circuit current in a given batch of animals usually centre around a single mean. The values shown in Fig. 1 clearly group into two populations, those from skins deprived of sodium having a mean value for the short-circuit current of approximately 2·5 times those not subjected to sodium deprivation.

Although skins treated alone with spironolactone acted as controls for sodium depleted skins, it is of interest to compare spironolactone treated skins with skins untreated with any drugs. [^{14}C]amiloride binding was measured in twelve skins taken from frogs kept in tap water at room temperature. With low sodium Ringer bathing the mucosal surface the short-circuit current was $2{\cdot}08 \pm 0{\cdot}23$ μA/cm^2 and the binding site density was $333 \pm 53/\mu$m^2. These results are also incorporated in Fig. 1 where it is seen that these untreated skins have a basal transporting capacity comparable to those of spironolactone treated skins.

There was no statistical difference between the current/site in skins derived from sodium depleted and non-sodium depleted animals. We have commented before (Cuthbert & Shum, 1976*a*) that determination of current/site requires the determination of the short-circuit current, inhibition of the current by ligand and the amount of ligand bound. Each of these values will have inherent errors so that the final value of current/site may be rather insensitive to physiological perturbations. It is therefore not possible to exclude minor differences between current/site in skins from the two groups. The magnitude of the current/site measured in these experiments is comparable to previously recorded values (Cuthbert & Shum, 1974*b*, 1976*a*). There were no differences in the apparent affinities of amiloride, measured under either low or high sodium conditions, in the two groups of skins. In particular, the values obtained at a sodium concentration of 1·1 mM showed only a twofold variation in ten experiments. This finding indicates that in at least this respect the probe molecule, amiloride, does not distinguish between the receptors in sodium depleted and normal tissues.

There were some unusual features in the experiments described which require further comment. When skins were taken from animals in Group *A* or *B*, that is animals receiving spironolactone, the initial response to amiloride was an increase in short-circuit current. An example is shown in Fig. 2*A*. The increase in current appeared to be independent of the concentration of amiloride, although the rate of increase was increased

with increasing drug concentrations. After some time the current stabilized at a higher value than the steady value at 30 min. When amiloride was removed the current remained elevated and subsequent addition of amiloride gave either pure inhibition or very minor stimulation followed by inhibition. The values of current given in Tables 1 and 2 are those obtained after the current increase in response to an initial application of amiloride had taken place.

To investigate this phenomenon further eight frogs from a single batch were taken and treated for 1 week as before. At the end of 1 week skins from all eight animals were mounted so that their short-circuit currents could be recorded. This current was measured at the end of 30 min

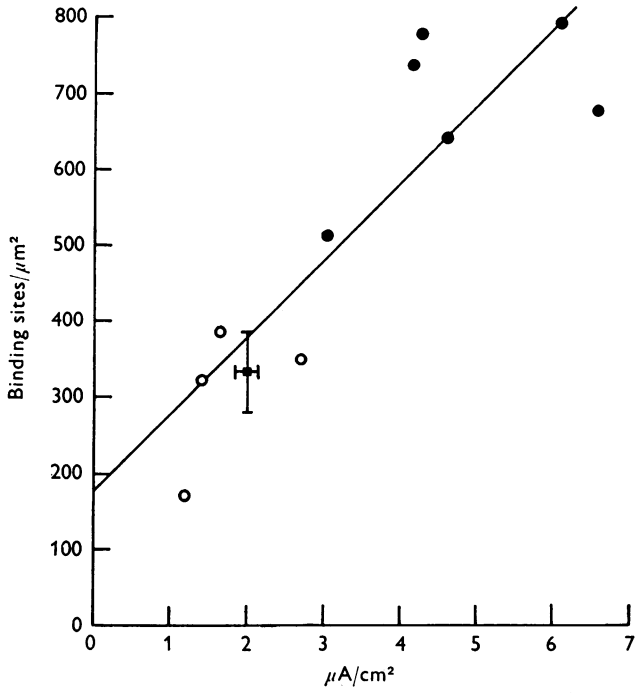

Fig. 1. Diagram showing relation between number of amiloride binding sites/μm^2 and short-circuit current ($\mu A/cm^2$) measured with the mucosal solution containing 1·1 mM sodium. The data were taken from Table 1. Filled circles are for skins from animals in Groups *A* and *C*, open circles for skins from animals in Group *B*. The correlation coefficient of the regression line shown is 0·87. The 95% confidence limits of the intercept are 93 to 253 binding sites/μm^2. The square symbol indicates mean values for twelve skins taken from untreated frogs kept in tap water at room temperature. Standard error bars for both current and binding site density are shown. The untreated control data are added for comparison and were not included in the regression analysis.

equilibration, then after exposure to a single concentration of amiloride. Antidiuretic hormone (300 mu./ml.) was then added to the serosal surface and sufficient time allowed for the short-circuit current to increase to a new steady-state value. One example, with skin taken from a frog treated with amiloride and spironolactone for 1 week, is shown in Fig. 2*B*.

These experiments, although rather limited in number, confirm what we had found in the earlier experiments (see Table 3). Initial stimulation by amiloride was only seen in those animals exposed to spironolactone. The initial stimulation increased the currents in amiloride and spironolactone treated skins to values comparable to those obtained from animals

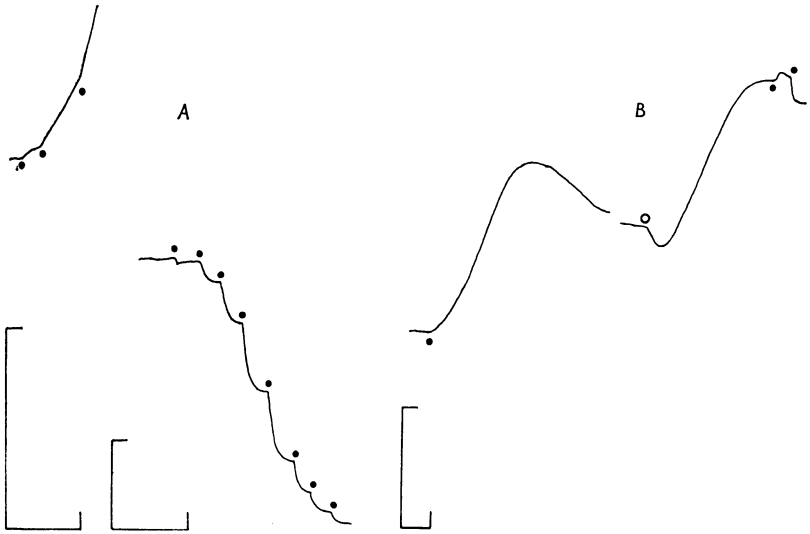

Fig. 2. Records of short-circuit current measured in isolated frog skin. The calibrations are 100 μA and 5 min, and the time calibrations also indicate zero current. The skins were taken from two frogs which had been pre-treated with amiloride and spironolactone for 1 week, as described in Methods. The Figure shows currents recorded when both the mucosal and serosal bathing solution contained 111 mM sodium.

In *A*, the responses to amiloride (10, 20 and 50 nM) at 30 min after mounting are shown. At this stage amiloride caused the current to increase from 182 to 315 μA. After the current became steady and the amiloride was removed subsequent additions of the drug caused inhibition. Responses to 10, 20, 50, 100, 200, 500, 1000, and 2000 nM are shown.

In *B* the response to a single application (50 nM) of amiloride is seen on a compressed time scale. The current, initially 165 μA, reached a steady state at 260 μA. After amiloride was removed the skin responded to ADH (300 mu./ml., open symbol) by an increase of current to 370 μA. Addition of amiloride at this point caused a further minor stimulation of current with 50 nM, while increasing the concentration to 100 nM caused an inhibition.

treated with amiloride alone. In some experiments we used another inhibitor of sodium transport, *N*-(*N*-benzylamidino)-3,5-diamino-6-chloro-pyrazine carboxamide, rather than amiloride. This compound behaves exactly like the parent compound but has an affinity about 10 times greater (unpublished). This compound also produced an initial stimulation of current in spironolactone treated animals.

TABLE 3. Effect of amiloride (5×10^{-8} M) and ADH (300 mu./ml.) on pre-treated skins

	Initial SCC after 30 min (μA)	Final SCC after amiloride (5×10^{-8} M, μA)	% increase in current after ADH (300 mu./ml.)
Group *A* (3) (amiloride and spironolactone)	185	245	29
Group *B* (2) (amiloride)	318	270	22
Group *C* (2) (spirono-lactone)	75	92	47
Control (1) (tap water)	147	139	34

Values given in the Table are mean values and the numbers in parentheses indicate number of animals. Values for short-circuit current (SCC) (μA) are for whole skins (9·6 cm^2). Amiloride was removed before ADH was added. In some experiments amiloride (5×10^{-8} M) was substituted by *N*-(*N*-benzylamidino)-3,5-diamino-6-chloro-pyrazinecarboxamide (5×10^{-9} M).

In the course of several years we have applied amiloride to the mucosal surface of hundreds of frog skins. In a very few skins we have seen a minor stimulation of current, but then only with very low concentrations of amiloride. One example can be seen in Fig. 4 of our 1972 paper (Cuthbert & Wong, 1972). Whether these rare observations have the same basis as the ones which are exaggerated by spironolactone treatment is unknown. Spironolactone treatment did not appear to modify the responses to ADH. Here the skins deprived of sodium showed somewhat smaller responses than non-deprived skins, whether or not they had been exposed to spirono-lactone. It might be expected that those skins with very large resting currents (Groups *A* and *B*) would show smaller responses to ADH, as there is necessarily an upper limit to the transporting capacity of the skin.

DISCUSSION

The object of the work reported in this paper was to examine whether or not the density of sodium entry sites in the mucosal surface of frog skin was influenced by the availability of sodium. There were two alternative approaches we might have used. Either frogs could have been exposed to

sodium-free solutions for prolonged periods or alternatively sodium entry to transporting cells could be prevented with amiloride. There is abundant evidence that this compound blocks sodium entry into the transporting compartment, although it does not prevent sodium efflux through the skin, which may occur via a route not involving the transporting compartment (Bentley, 1968; Salako & Smith, 1970; Ehrlich & Crabbé, 1968; Biber, 1971; Cuthbert, 1971; Dörge & Nagel, 1970). We chose the second of the two alternatives, as we felt that sodium escaping from the frog into sodium-free solution may reach sufficient concentration in the stationary layer covering the skin to allow recapture. The second alternative also allows the animal to replenish body sodium by drinking, although it is unusual for amphibia to do so (Krogh, 1939), while ensuring depletion in the transporting compartment of the epithelial cells. We were also concerned that if the animals became sodium deficient release of endogenous aldosterone might occur, particularly as we have shown already that aldosterone causes an increase in the density of sodium entry sites, both in frog skin and toad urinary bladder (Cuthbert *et al.* 1974; Cuthbert & Shum, 1975). As adrenalectomy is technically difficult in amphibia we used an aldosterone antagonist, spironolactone (Sharp & Leaf, 1966; Swaneck, Chu & Edelman, 1970; Funder, Feldman, Highland & Edelman, 1974). The steroid is easily able to penetrate the skin epithelium so that a high concentration was maintained in the epithelial cells by inclusion in the bathing fluid after an initial priming dose was given. Thus animals treated with spironolactone alone served as controls for the test animals. The results from two animals which received treatment with amiloride alone were not substantially different from those treated with amiloride and spironolactone, perhaps indicating that aldosterone concentrations were not elevated in sodium deprived animals, so that results from these two groups were pooled for analysis.

The important results from these experiments can be summarized very simply: sodium deprivation increases both the level of sodium transport, estimated as short-circuit current, and the density of sodium entry sites, estimated as amiloride binding sites. The correlation of these two parameters is significant, although it is notable that the regression line fails to pass through the origin. This may indicate that some fraction of the binding measured is to non-specific sites, that is unassociated with sodium entry. The density of binding sites recorded in controls in this study is somewhat higher than previously reported values. In fifteen determinations a mean value $201 \pm 14/\mu m^2$ was obtained in skins exposed to 1·1 mM sodium (Cuthbert & Shum, 1974*b*). In this previous study animals were stored in tanks at 4° C while in this study they were kept at room temperature (20° C) which may be responsible for the difference.

Bentley (1973) made a study in which he deprived anurans of three species (*Bufo marinus*, *Rana pipiens* and *Xenopus laevis*) of sodium by exposing to 10^{-5} M amiloride for 15 days in water containing 0·25 mM sodium. As the author points out this concentration of amiloride is adequate to prevent sodium entry *in vitro*. Only in *B. marinus* was a significant depletion in serum sodium recorded. Short-circuit current values measured *in vitro* from skins of *Rana* and *Xenopus* treated with amiloride were not different from controls, while bladders from *Bufo* showed a 60 % increase in current compared to their controls. Even in *Bufo* the serum sodium fell only to 92 mM, compared to control values of 101 mM; while in the other two species the serum sodium did not fall significantly, yet the levels of sodium in the transporting compartment of the skin epithelial cells must have been drastically reduced. At present it is not possible to resolve the differences in response between the four species for which information is now available.

In yet another species, *Bufo viridis*, it has been reported that adaptation of these animals to high salinities results in a reduction of amiloride sensitive sodium transport and a reduction of mucosal sodium influx (Katz, 1975). The author concludes that the effect of saline adaptation is to 'reduce the number of Na selective sites at the outer barrier', a conclusion which is in accord with our direct measurements of site density.

There are now many examples of situations in which the density of membrane macromolecules is governed by the availability of their normal activators. In some instances the activator exerts a negative feed-back control, as for example with nicotinic receptors in the neuromuscular junction (Axelsson & Thesleff, 1959; Miledi & Potter, 1971), muscarinic receptors in salivary glands (Emmelin, 1961), β-receptors in pineal tissue (Kebebian, Katz, Romero & Axelrod, 1974) and insulin receptors in fat cells (Soll, Kahn, Neville & Roth, 1975). In other situations control is by positive feed-back, as for example the effect of intracellular sodium on the density of pumping sites (Boardman, Huett, Lamb, Newton & Polson, 1974).

At this time little can be said about the way in which sodium lack increases receptor density. We feel it unlikely that it results from aldosterone secretion as steps were taken to block endogenous aldosterone.

It is difficult to know if the concentration of spironolactone used is sufficient to antagonize completely endogenous aldosterone, because of the paucity of quantitative information. Using an *in vitro* toad bladder preparation Porter (1968) studied the competitive interaction between aldosterone and spironolactone. From a plot of the data given by Porter in Table 6 of his paper it is found that spironolactone, 10^{-5} M, gives 60 % inhibition of the short-circuit current response to aldosterone, 70 nM. This concentration of aldosterone is twice that found in the blood of toads adapted to distilled water (32 nM) and four times that found in saline adapted animals (18 nM) (Crabbé, J. quoted by Denton, 1965).

We have been able to measure the rate constant for the disappearance of sodium channels in isolated cells from toad urinary bladder (Cuthbert & Shum, 1976*b*). The rate constant was 0·012 hr^{-1} and as far as we can tell the sodium sites in toad bladder are similar to those in frog skin. Making this assumption it seems unlikely that the extra sites in deprived tissues could result from a reduced rate of degradation alone.

In previous experiments we have shown that the number of sites which can be labelled by amiloride can be increased by raising the sodium concentration (Cuthbert & Shum, 1974*b*, 1975) or by increasing serosal negativity (Cuthbert & Shum, 1976*a*). Both of these effects are immediate, indicating that there are sites which are normally masked under the condition we have used (short-circuited and 1·1 mM sodium). It would seem that sodium deprivation stimulates amiloride binding either by *de novo* synthesis of binding sites or by unmasking pre-existing sites.

No discussion is possible of the initial stimulating effects of amiloride on the short-circuit current in spironolactone treated frog skin. This action of amiloride does not fit into the existing framework of our knowledge of this drug. The results are presented entirely for their curiosity.

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