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Changes in sodium pool and kinetics of sodium transport in frog skin produced by amiloride

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Summary

1. Amiloride produces a decrease in size of the active sodium transport pool of isolated frog skin.

2. Rate coefficients for sodium movement into and out of the transporting cells across the outside membrane are decreased by amiloride. The rate coefficient for sodium extrusion across the inside membrane is not significantly affected.

3. In the presence and in the absence of amiloride, the relation of sodium transport to outside sodium concentration exhibits similar saturation kinetics but amiloride reduces sodium transport rate at every sodium concentration of the outside solution.

4. Labelling of skin with ^{14}C-amiloride from the outside solution is significantly greater than labelling with ^{14}C-inulin.

5. The results of these studies suggest that amiloride reacts with sites on the outside membrane of the transporting cells as a result of which the rate of sodium movement across this membrane is diminished.

Introduction

Previous studies have shown that the new diuretic compound, amiloride (N-amidino-3,5-diamino-6-chloropyrazine carboxamide hydrochloride), added to the outside surface of the isolated amphibian skin or bladder, causes inhibition of active sodium transport across the tissue (Eigler, Kelter & Renner, 1967; Baba, Lant, Smith, Townshend & Wilson, 1968; Bentley, 1968; Ehrlich & Crabbé, 1968).

The outside passive membrane of the transporting cells has been suggested as the site of this inhibitory effect on active sodium transport (Bentley, 1968; Ehrlich & Crabbé, 1968).

However, some of the findings of these previous studies are consistent with direct inhibition of the sodium pump. Thus, the failure of amiloride to inhibit diffusion short-circuit current and potential difference in toad bladder (Bentley, 1968) would be expected if the action of the drug were on the sodium pump, and the reduction in the electromotive force of the active transport system (E_{Na}) in the frog skin would be compatible with such an action (Baba *et al.*, 1968).

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In this study, the rate coefficients for sodium movement across the outside and inside surfaces of the frog skin epithelium, the " active sodium transport pool " within the transporting cells and the unidirectional fluxes of sodium across the cell surfaces have been determined. The effect of amiloride on these parameters was studied in order to localize, more directly, the amiloride-sensitive step in the transepithelial transport of sodium. Experiments were also performed to determine the mechanism by which amiloride might produce its effect.

Methods

Abdominal skin of the frog, *Rana temporaria*, was mounted across a circular window, 2·85 cm^2 in area, between two Perspex half-cells, each containing 50 ml of bathing solution. The skin was then short-circuited and time allowed for the short-circuit current to reach a steady value before adding drugs or altering the conditions.

Standard Ringer solution was used in the studies but in some experiments solutions with lower sodium content were used. These were obtained by substituting choline chloride for sodium chloride in equimolar amounts. When a sodium-free solution was required, sodium bicarbonate was also replaced with the choline salt. The concentrations of calcium, chloride and potassium were constant in all solutions.

Estimation of the " active sodium transport pool "

The sodium content of the skin involved in the active transport process (the " active sodium transport pool "), was estimated by two separate methods. In the first, 50 μCi ^{24}Na were added to the Ringer solution bathing the outside surface of the skin. The inside solution was sodium-free. One hour after adding the isotope, the exposed skin was cut out, blotted carefully on a Whatman number 54 filter paper (taking care to avoid contamination of the inside skin surface with the outside solution), and counted for ^{24}Na activity. A sample of the outside solution was also counted and its chemical sodium content estimated. After counting, the skin was blotted again and weighed in a tared glass vessel to obtain its wet weight. It was then dried for 18 h in an oven at 210° C and re-weighed to obtain the dry weight.

In some experiments, the dried skin was dissolved by immersing in 0·5 ml concentrated nitric acid and allowing to stand for 72 h. The volume was then made up to 25 ml with distilled water and the sodium content estimated.

The " active sodium transport pool ", taken to represent the amount of sodium within the skin with which ^{24}Na added to the outside solution comes into equilibrium (Frazier, Dempsey & Leaf, 1962 ; Herrera, 1968), is given by

$$[Na]_t = \frac{^{24}Na_t \times [Na]_o}{^{24}Na_o}$$

in which, $[Na]_t$ = the " active sodium transport pool " (μequiv/g tissue water), $[Na]_o$ = concentration of sodium in the outside solution (μequiv/ml), $^{24}Na_t$ = skin counts per gram of tissue water per 100 s and $^{24}Na_o$ = (counts of outside solution per ml) per 100 s.

The experiments were performed in pairs using symmetrical halves of the same skin ; one half was treated with amiloride and the other served as the control.

The second method used for the estimation of the "active sodium transport pool" involved a kinetic analysis of the build up of ^{24}Na influx to a steady state. The method is based on a model for three-compartment systems first proposed by Schoffeniels (1957) and modifications of the method have been used by Hoshiko & Ussing (1960), Curran, Herrera & Flanigan (1963), and Herrera (1968). Using this method, the rate coefficients for the movement of sodium across the inside and outside membranes of the transporting cells and the unidirectional sodium fluxes across the individual membranes were also estimated. The theoretical considerations relevant to the method have been discussed in detail by Curran *et al.* (1963).

The experiments were performed in pairs using symmetrical halves of the same skin. One half served as the control and the other half was treated with amiloride 2×10^{-7}M. The skins were bathed on both surfaces with Ringer solution.

After an equilibration period, 100 μCi ^{24}Na were added to the outside solution and sampling was begun immediately from the opposite side. 5 ml samples were withdrawn at 2 min intervals for the first 10 min and subsequently at 5 or 10 min intervals for a total period of 1 h. Each sample was replaced with an equal volume of fresh solution and adequate mixing of the fluid in the two chambers was ensured by bubbling oxygen vigorously into them throughout the experiment. One hour after adding ^{24}Na to the outside solution, the exposed skin was cut out, blotted carefully and counted. A sample of the outside solution was also counted and its chemical sodium content estimated. After counting the skin, its wet weight, dry weight and tissue water weight were estimated as previously described.

The rate of appearance of ^{24}Na activity in the inside solution (dP_3/dt) was calculated for each sampling period and expressed as a fraction of the final steady state value $(dP_3/dt)\infty$. For each experiment the quantity, $1-(dP_3/dt)/(dP_3/dt)\infty$, was plotted on a semilogarithmic scale against time. A straight line was fitted by inspection and from its slope (λ), and the skin counts at the end of the experiment ($P_2\infty$), the active sodium transport pool and the rate coefficients for sodium movement across the epithelial cells of the skin were calculated (Curran *et al.*, 1963).

Effect of varying the sodium concentration of the outside solution

Two series of experiments were performed to study the possible interrelationships between sodium and amiloride. In the first, the short-circuit current (as the measure of active sodium transport) was determined in relation to the concentration of sodium in the outside solution. The skin was set up in the usual manner and, after stabilization, the short-circuit current was recorded. The outside solution was then changed to one having a different sodium concentration and, after allowing time for equilibration, the short-circuit current was again recorded. Measurements were made first in the absence of amiloride and then repeated in the same skin but with amiloride 2×10^{-7}M added to the outside solution.

In the second series of experiments the effects of six different concentrations of amiloride were tested at four different sodium concentrations of the outside solution.

Labelling of skin with ^{14}C-amiloride

Skins were mounted in the usual manner and after equilibration, 10^{-4}M ^{14}C-amiloride, having a total activity of 12·5 μCi, was added to the outside solution and

left for 1 h. The exposed skin was then cut out, blotted and weighed. It was homogenized in a glass tissue grinder with 0·25 ml 10% trichloroacetic acid. The homogenate was diluted with 0·5 ml of Ringer solution and centrifuged for 25 min at 4,000 rev/min. The activity of ^{14}C in the supernatant fluid as well as in the ouside and inside bathing solutions was then determined.

The water content of the frog skin, considered to be equal to the loss in weight after drying at 210° C for 18 h, averaged 82·1% of the wet weight in forty measurements. The weight of tissue water in this series of experiments was, therefore, estimated by multiplying the wet weight by 0·82. The activity of ^{14}C-amiloride per gram of tissue water divided by its activity per millilitre of outside solution, multiplied by 100, represented the percentage labelling of tissue water by ^{14}C-amiloride. Similar experiments were performed using ^{14}C-inulin in place of ^{14}C-amiloride.

The experiments with ^{14}C-amiloride were performed at two different concentrations of sodium in the outside solution (114·4 and 2·5 mM). The inside solution was standard Ringer solution in all cases.

Analytical methods

Chemical estimation of sodium was by flame photometry using an EEL flame photometer. Osmolality of solutions was measured with an Advanced Instruments Osmometer and pH with glass electrodes and a Pye pH meter. ^{24}Na-activity was counted with an Ekco well-type scintillation counter and ^{14}C-activity was counted with a Tri-carb liquid scintillation spectrometer (Packard Instruments Co., La Grange, Ill.).

Results

The active sodium transport pool estimated by adding ^{24}Na to the outside bathing solution showed a wide variation from one skin to another but there was good agreement between values obtained for paired skin halves (Table 1). When one skin half was exposed to amiloride and the other served as control, the sodium pool was found, in every instance, to be smaller in the presence of amiloride (Table 2). Tissue water weight was not affected by amiloride in twenty experiments in which the effects of 2×10^{-7}M and 2×10^{-6}M concentrations of the drug were tested. The mean tissue water in control skins was 82·1% ±3·2 (S.D.) of wet tissue weight; in amiloride-treated skins it was 82·1% ±2·2 (S.D.).

TABLE 1. *Short-circuit current and active sodium transport pool in paired skin halves (i and ii)*

Experiment Number	Short circuit current μA/cm²			Tissue pool μequiv/g tissue water		
	(i)		(ii)	(i)		(ii)
1	22·8		23·2	11·2		9·0
2	21·1		23·9	7·9		8·8
3	45·6		45·0	10·3		9·4
4	22·5		21·1	9·8		9·7
5	6·3		6·0	3·9		3·8
6	11·2		11·6	6·9		6·5
7	24·6		26·7	10·4		12·3
Mean	22·0		22·5	8·6		8·5
(±S.D.)	±12·4		±12·3	±2·4		±2·6
Difference (±S.E.)	0·5	±	6·6	0·1	±	1·3
P		n.s.			n.s.	

In nine control skin halves in which the active sodium transport pool (measured isotopically) was compared with the total tissue sodium (measured chemically), the isotopic method measured only $9{\cdot}5 \pm 1{\cdot}5$ (S.E.) μequiv of sodium per g of tissue water as opposed to $31{\cdot}0 \pm 5{\cdot}8$ (S.E.) μequiv/g of tissue water from direct chemical analysis. The total tissue sodium was not affected by amiloride, the value in the nine amiloride-treated skin halves being $32{\cdot}6 \pm 5{\cdot}9$ (S.E.) μequiv/g of tissue water.

Build-up of ^{24}Na influx to a steady state

The kinetic model used in this series of experiments is illustrated in Fig. 1, which also serves to define some of the parameters estimated.

A plot of the natural logarithm of $[1-(dP_3/dt)/(dP_3/dt)\infty]$ against time for control and amiloride-treated skin halves yielded two straight lines (Fig. 2) as predicted from the theoretical considerations (see Curran *et al.*, 1963), and the values of the various parameters of the kinetic model obtained in eight experiments are summarized in Table 3 and 4.

As in the first group of experiments the active sodium transport pool was significantly diminished by amiloride. Moreover, the pool sizes were of similar magni-

TABLE 2. *Effects of 2×10^{-7} M amiloride on short-circuit current and active sodium transport pool in frog skin*

Experiment number	Short-circuit current μA/cm^2		Sodium pool μequiv/g. tissue water	
	Control	Amiloride	Control	Amiloride
1	7·0	2·8	3·3	3·0
2	36·8	15·8	11·2	4·8
3	70·2	24·6	10·0	4·7
4	52·6	39·2	11·6	3·7
5	70·2	28·1	11·6	8·9
6	35·1	18·9	9·2	6·4
7	14·7	6·3	4·8	3·7
8	20·4	9·7	7·2	3·6
9	17·5	10·5	6·1	3·1
10	17·5	7·6	4·5	4·4
11	9·1	7·4	4·3	2·8
Mean	31·9	15·5	7·6	4·5
±S.D.	±23·4	±11·2	±3·2	±1·8
Difference ±S.E.M.	16·4	± 7·8	3·1	± 1·1
P		=0·05		<0·02

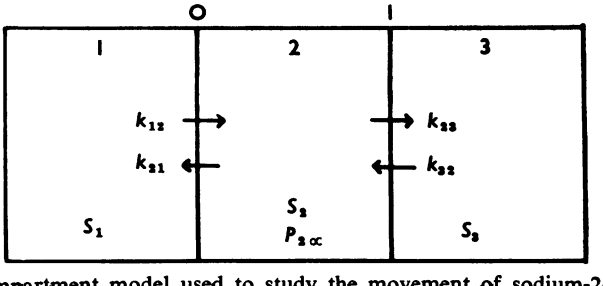

FIG. 1. Three-compartment model used to study the movement of sodium-24 across the frog skin. Compartment 1 represents the outside solution, 2, the skin and 3 the inside solution. O=outside membrane of skin epithelial cell. I=inside membrane of skin epithelial cell. K_{12}, etc.=rate coefficient for sodium movement from compartment 1 to compartment 2. S_1, S_2, S_3=total sodium content in compartments 1, 2 and 3 respectively. $P_{2\infty}$=sodium-24 content of the skin at steady state.

tude in the two types of studies, thus demonstrating the reproducibility of the methods.

The rate coefficients for sodium movement into and out of the cell across the outside membrane (k_{12} and k_{21} respectively; Fig. 1) were significantly reduced by amiloride, and, although the transfer coefficient for sodium extrusion across the

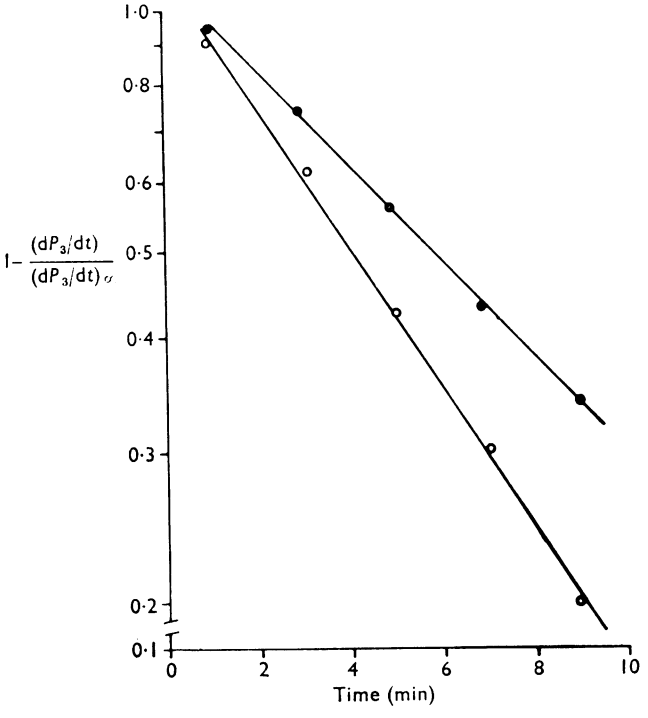

FIG. 2. Plot of the logarithm of $1-\frac{(dP_3/dt)}{(dP_3/dt)_\infty}$ against time for control (○——○) and amiloride-treated (2×10^{-7}M, ●——●) skin halves. The straight lines were fitted by inspection.

TABLE 3. *Effects of amiloride* 2×10^{-7}M *on transfer coefficients, net sodium transport and "active sodium transport pool" in frog skin*

Experiment number	Net Na transport μequiv/cm² per h		Active Na transport pool μequiv/g tissue water		k_{12} ($h^{-1}\times10^3$)		k_{21} (h^{-1})		k_{23} (h^{-1})	
	Control	Amiloride	Control	Amiloride	Control	Amiloride	Control	Amiloride	Control	Amiloride
1	1·4	0·5	6·2	5·2	1·5	0·4	5·3	2·1	5·9	3·8
2	1·3	0·5	6·1	4·4	1·2	0·6	4·7	1·0	5·2	7·2
3	1·8	0·9	8·6	7·1	1·9	4·1	6·5	4·5	5·7	3·2
4	1·2	0·4	6·4	4·0	1·6	0·7	8·3	5·6	5·0	3·5
5	1·8	0·5	8·2	3·1	1·6	0·5	4·7	3·6	6·8	3·6
6	1·4	0·7	5·3	3·3	1·3	0·6	5·8	3·8	6·7	4·9
7	0·7	0·1	6·4	2·5	1·0	0·4	6·8	6·4	3·7	4·8
8	1·0	0·2	5·7	4·2	0·9	0·3	5·9	2·9	6·0	3·4
Mean	1·3	0·5	6·6	4·2	1·4	0·6	6·0	3·9	5·6	4·3
(±S.D.)	±0·4	±0·3	±1·2	±1·3	±0·3	±0·3	±1·2	±1·8	±1·0	±1·3
Difference (±S.E.M.)	0·8±0·2		2·4±0·6		0·8±0·2		2·1±0·8		1·3±0·6	
P	<0·01		<0·01		<0·01		<0·05		>0·05	

inside membrane (k_{23}) was diminished in six out of eight experiments, the mean decrease was not significant at the 5% level (Table 3).

The unidirectional fluxes of sodium across the outside and inside membranes of the transporting cells, ϕ_{12}, ϕ_{21} and ϕ_{23}, were obtained by multiplying the corresponding rate coefficient by the total sodium content of the appropriate compartment (S). Thus, ϕ_{12} is given by $k_{12} \times S_1$, ϕ_{21} by $k_{21} \times S_2$ and ϕ_{23} by $k_{23} \times S_2$. ϕ_{32} was estimated by subtracting net flux (ϕ_{net}) from ϕ_{23}, net flux being equal to $\phi_{12} - \phi_{21}$.

ϕ_{12}, ϕ_{21} and ϕ_{23} were all significantly reduced by amiloride. Compared with the other fluxes, ϕ_{32}, the sodium flux from the inside solution into the cell across the inside membrane was very small and was unaffected by amiloride (Table 4).

The rate coefficient for sodium movement into the cell across the inside membrane (k_{32}), can be obtained by dividing the sodium influx across the inside membrane (ϕ_{32}) by the total sodium content of the inside solution (S_3). This procedure, in the eight experiments summarized in Tables 3 and 4 gave a mean value for k_{32} of $2{\cdot}5 \times 10^{-5}$ per hour, which is one fifty-sixth of the value of k_{12} in the same series of experiments. These findings therefore confirm the widely held view that the inside membrane is relatively impermeable to sodium.

Effect of varying the sodium content of the outside solution

When short-circuit current was determined as a function of the concentration of sodium in the outside solution, there was an initial rapid rise in current as the sodium concentration was increased. This was followed by a slower rise with attainment of a maximum at a sodium concentration of about 60 mM. The shape of the curve was similar in the presence of amiloride but with a smaller current at every sodium concentration (Fig. 3). A reciprocal plot (Lineweaver & Burk, 1934) of the results of these experiments yielded two straight lines showing different slopes and significantly different intercepts in the ordinate and abscissa. The values obtained after exposure to amiloride fell in the upper line with steeper slope (Fig. 4). The characteristics of these lines therefore suggest either non-competitive or uncompetitive interaction between amiloride and sodium.

TABLE 4. *Effect of* 2×10^{-7} M *amiloride on unidirectional sodium fluxes in frog skin. Sodium fluxes are expressed in μequiv/g tissue water per h*

Experiment Number	ϕ_{12}		ϕ_{21}		ϕ_{23}		ϕ_{32}	
	Control	Amiloride	Control	Amiloride	Control	Amiloride	Control	Amiloride
1	65·4	28·1	32·9	10·9	36·6	19·8	4·1	2·6
2	58·7	35·8	28·7	4·4	31·7	31·7	1·0	0·3
3	103·5	53·7	55·9	32·0	49·0	22·7	1·4	1·0
4	83·8	33·8	53·1	22·4	32·0	14·0	1·3	2·6
5	93·0	22·4	38·5	11·2	55·8	11·2	1·3	0
6	63·9	28·4	30·7	12·5	35·5	16·2	2·3	0·3
7	67·2	23·5	43·5	16·0	23·7	12·0	0	4·5
8	68·2	26·5	33·8	12·2	34·4	14·3	0	0
Total	603·7	252·2	317·1	121·6	298·7	141·9	11·4	11·3
Mean	75·5	31·5	39·6	15·2	37·3	17·7	1·4	1·4
(±S.D.)	±16·0	±10·1	±10·3	±8·5	±10·3	±6·8	±1·3	±1·6
Difference (±S.E.M.)	44±6·7		24·4±4·7		19·6±4·4		0±0·7	
P value	<0·001		<0·01		<0·01		n.s.	

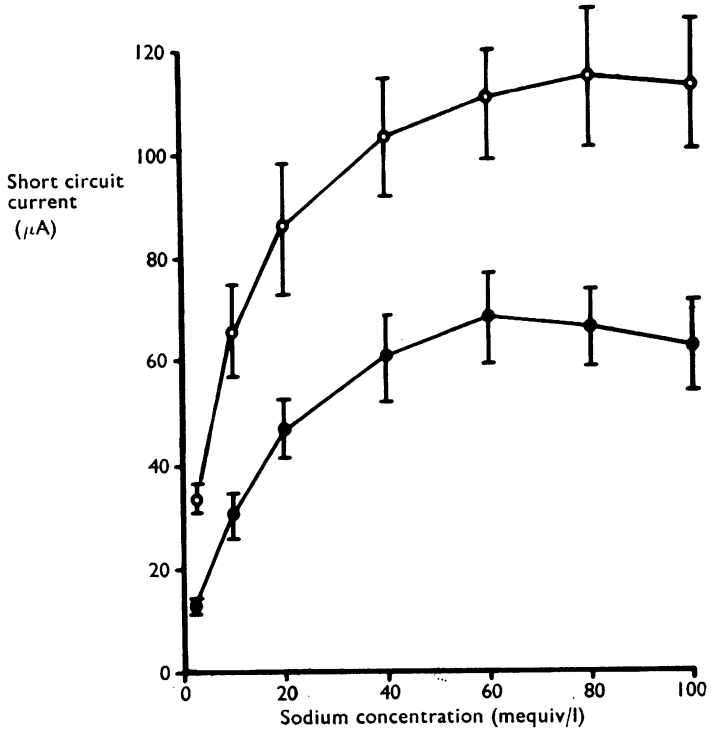

FIG. 3. Relation of short-circuit current to the concentration of sodium in the outside solution in the absence (○) and presence (●) of amiloride 2×10^{-7}M. Each point represents the mean ± standard deviation of five experiments.

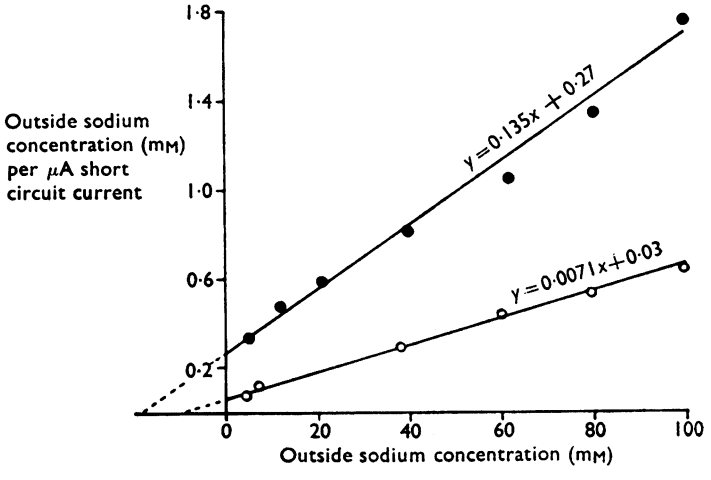

FIG. 4. Relation of short-circuit current to the sodium concentration of the outside solution using the reciprocal plot of Lineweaver and Burk (1934). Result for a typical experiment. ●, Amiloride ; ○, no amiloride.

The effects of six different concentrations of amiloride at four different concentrations of sodium in the outside solution are shown in Fig. 5. As sodium concentration was lowered below 60 mM the dose-response curve shifted to the left, indicating increased effectiveness of the compound at lowered sodium concentrations.

^{14}C-amiloride labelling of skin

The percentage labelling of frog skin by ^{14}C-amiloride added to the outside solution was significantly higher than labelling by ^{14}C-inulin. The labelling by ^{14}C-amiloride when the outside solution contained only 2·5 mM sodium was greater than when the outside solution was standard Ringer solution, but the difference was not significant (Table 5). No ^{14}C activity was present in the inside solution.

Discussion

Following the work of Koefoed-Johnsen & Ussing (1958), the frog skin epithelium is generally considered to present two main barriers to the transepithelial transport

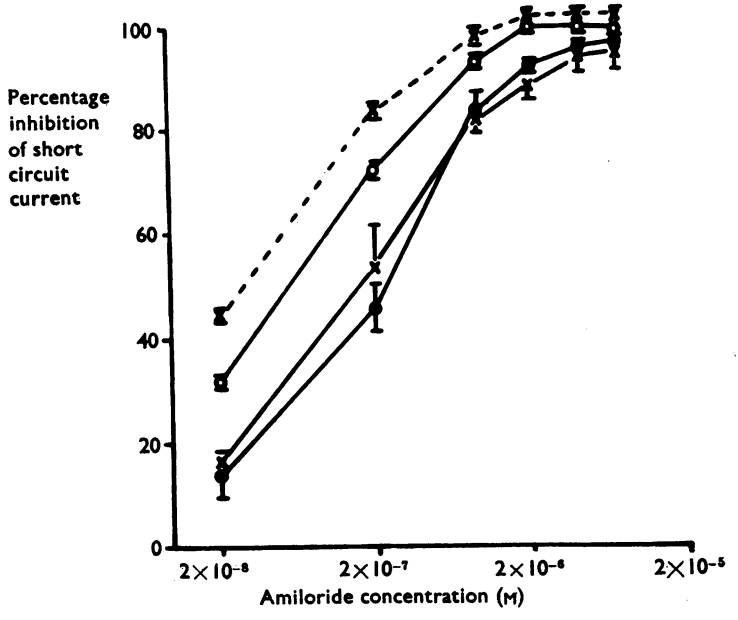

FIG. 5. Dose-response curves for amiloride at different concentrations of sodium in the outside solution: ×----×, 2·5 mM, $n=4\pm$S.E.; ○——○, 20 mM, $n=4\pm$S.E.; ●——●, 60 mM, $n=4\pm$S.E.; ×——×, 100 mM, $n=6\pm$S.E.

TABLE 5. *Labelling of frog skin with ^{14}C-amiloride*

Drug	Outside sodium concentration (mM)	Number of experiments	% labelling of tissue water (±S.E.)
^{14}C-amiloride 10^{-4}M	114·4	6	10·6±2·4
^{14}C-amiloride 10^{-4}M	2·5	6	19·9±7·0
^{14}C-inulin	114·4	6	1·4±0·3

of sodium—a passive outer barrier corresponding to the outside membranes of the cells and an inside membrane barrier presumed to be the site of the active extrusion mechanism. Bentley (1968) in the toad bladder, and Ehrlich & Crabbé (1968) in toad bladder and skin, have suggested that the effects of amiloride on sodium transport could be explained in terms of changes in the sodium permeability of the passive outer barrier. The results obtained in this study confirm these suggestions. Under the conditions of these experiments changes in the permeability of the outside membrane will be reflected by changes in the rate coefficients across the membrane. Amiloride decreases the rate coefficient for sodium movement into the cell from the outside solution (K_{12}). As a result of this change, the rate at which sodium enters the transporting cells from the outside solution is decreased and this leads to a decrease in the active sodium pool of the transporting system.

There is no evidence from this study that the effect of amiloride can be explained in terms of a direct alteration in the active transport mechanism presumed to be located at the inside membrane. The rate coefficient for sodium extrusion across this membrane (k_{23}) which is a reflection of the activity of the sodium pump was not consistently decreased, and the mean decrease was not significant. Hence the change in the unidirectional sodium flux from the cells toward the solution caused by amiloride is due to change in the intracellular active sodium pool rather than to changes in the active system which brings about the sodium extrusion across the membrane. Since the properties of the inner membrane are not altered by amiloride, the changes in the size of the sodium pool and in the rate of sodium transport result from the observed changes in the outside membrane. A similar mechanism has been suggested to explain the effects on sodium transport of antidiuretic hormone added to the inside solution and calcium added to the outside solution (Curran *et al.*, 1963). The stimulatory effect of local anaesthetics on sodium transport across the frog skin has also been attributed to an increase in the passive permeability of the outside membrane to sodium (Skou & Zerahn, 1959).

Cereijido, Herrera, Flanigan & Curran (1964) demonstrated that the relation of sodium transport to the concentration of sodium in the outside bathing solution obeys typical saturation kinetics and that this saturation phenomenon is probably due to interaction of sodium with a substrate at the outside membrane. The results presented in Figs. 3 and 4 confirm the saturation of sodium transport with increasing concentration of sodium in the outside solution and suggest that amiloride acts by inhibiting this initial step in the transepithelial transport of sodium.

The percentage labelling of frog skin by ^{14}C-amiloride was significantly greater than labelling by ^{14}C-inulin, suggesting that amiloride selectively accumulates in frog skin, either at the outside membrane or in a compartment beyond that membrane. Diffusion of amiloride across the tissue was ruled out by the absence of ^{14}C-activity in the inside solution 30 min after adding the ^{14}C-amiloride to the outside solution. Binding of amiloride to the tissue may have a direct bearing on its action, in which case the reduction in the effect of the compound as the outside sodium concentration is increased (Fig. 5) suggests that fewer sites of amiloride binding were available at the higher sodium concentrations. Indeed, the percentage labelling of ^{14}C-amiloride at an outside sodium concentration of 114·4 mM was approximately half of the percentage labelling at an outside sodium concentration of 2·5 mM ; the absolute difference in percentage labelling in the six experiments was, however, not significant (Table 5).

In conclusion, the results of this study suggest that amiloride inhibits sodium transport across the frog skin by an action on the passive outside membrane of the transporting cells and that it acts by attaching to sites on the cells as a result of which the rate of movement of sodium into the cells across their outer membrane is diminished.

Note added in proof. The interpretation of the results of sodium pool measurements and the kinetic analysis are based on the Koefoed-Johnsen and Ussing model in which the active sodium pool lies between a passive permeability barrier and an active transport barrier. This model has formed the basis for several investigations of the site and mode of action of substances on sodium transport in frog skin. In a recent paper by Zerahn (1969) this model has been challenged and it has been suggested that the labelled sodium pool " behaves as if it has passed the transport mechanism ". While these views are as yet unconfirmed, they may demand a reappraisal of present concepts of transport barriers in frog skin and in particular of the site of action of pharmacologically active substances.

Our view that amiloride reduces the permeability of a passive barrier to sodium is not incompatible with Zerahn's model.

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