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Effects of amiloride on active sodium transport by the isolated frog skin: evidence concerning site of action

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Summary

1. Amiloride reduces short-circuit current and potential difference across the isolated frog skin.

2. Isotopically measured sodium influx and efflux are diminished.

3. Total electrical conductance and partial sodium conductance are diminished, the reduction in total conductance being entirely accounted for by the reduction in partial sodium conductance.

4. The effect of antidiuretic hormone (ADH), cyclic 3′5′-adenosine monophosphate (cyclic AMP) and theophylline can be antagonized by pretreatment with amiloride but the antagonism can be abolished by increasing the concentration of these compounds.

5. Amiloride has no effect on oxygen consumption in concentrations which inhibit sodium transport. However, it prevents the stimulatory effect of ADH on oxygen consumption.

6. The results are consistent with an action of amiloride at the passive outside membrane of the transporting cells of isolated frog skin.

Introduction

The new diuretic drug amiloride (N-amidino-3-5-diamino-6-chloro-pyrazine carboxamide (Fig. 1)) has been shown not only to increase urinary sodium and water loss in animals and human subjects but also to inhibit active sodium transport in isolated frog skin (Baba, Lant, Smith, Townshend & Wilson, 1968). This effect on isolated tissue has also been demonstrated in toad skin, bladder and colon (Bentley, 1968; Ehrlich & Crabbé, 1968; Crabbé & Ehrlich, 1968). In this paper, the action of amiloride on the isolated frog skin is described in greater detail in order to clarify the site and mechanism of action of this compound.

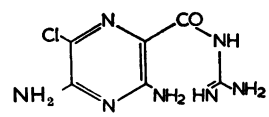

FIG. 1. Structural formula of amiloride.

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Methods

The experiments were performed on the isolated abdominal skin of the frog, *Rana temporaria*, during the period June 1967 to May 1969.

Electrical properties and sodium transport

Skins were mounted between two Perspex half-cells exposing an area of 2·85 cm^2 to the bathing solutions. The apparatus for mounting the skin and the electrical circuits for measuring the potential difference (p.d.) and short-circuit current (scc) of the skin were similar to those of Ussing & Zerahn (1951). The compartment on either side of the skin was filled with 50 ml of the bathing solution, through which oxygen was bubbled during each experiment. The skin was kept short-circuited in each experiment except for periods not exceeding 20 s, when the potential difference was measured. The scc and p.d. were measured at intervals of 10–15 min and test substances were not added to the solutions until steady readings had been obtained for a minimum period of 30 min.

Substances to be tested were dissolved in Ringer solution and added to the fluid bathing one side of the skin. An equal volume of the solvent was then added to the other side to maintain equal fluid volumes on the two sides of the skin.

Measurement of sodium fluxes

Sodium fluxes were measured using sodium-24 (^{24}Na). The isotope (approx. 100 μCi) was added to the solution on one side of the skin and after allowing 1 h for equilibration, 1 ml samples were removed for counting every 30 min from the solution on the opposite side. Flux in the absence of amiloride was first determined for two 30 min periods. Amiloride was then added to the outside solution and flux determined for two further periods.

At the midpoint of the experiment a 1 ml sample was taken from the side to which ^{24}Na had been added. This sample was diluted 1 : 200 for counting and the chemical estimation of sodium content. Isotope activity was counted with a well-type scintillation counter and an E.M.I. scaler. Calculation of sodium flux was made according to the method of Ussing (1949).

Oxygen consumption

The rate of oxygen consumption by pieces of abdominal skin was measured by standard manometric techniques (Umbreit, Burris & Stauffer, 1964). A constant volume respirometer of the rotary type was used (Circular Warburg Apparatus, II, Townson & Mercer, Ltd.). The shaking frequency was 112/min and the amplitude 5 cm. Oxygen uptake was not affected by increasing the shaking frequency or amplitude. The temperature of the apparatus was maintained at 24·5° C.

Three pieces of skin weighing between 100 and 200 mg were placed in 2·5 ml Ringer solution in the main compartment of the manometer flask. Carbon dioxide was absorbed with 0·3 ml 10% sodium hydroxide in the centre well of the flask, which also contained a pleated filter paper. The side arm of the flask contained 0·1 ml of the test solution (or Ringer's solution in control experiments). After coupling the flask to the manometer, the apparatus was gassed with pure oxygen for 3 min. Oxygen uptake was then measured for one control hour. The contents

of the side arm were subsequently tipped into the main compartment and measurement continued for a further hour.

At the end of each experiment, the skins were removed and blotted with soft filter paper (Whatman No. 54) and weighed in tared glass vessels. The skin was then dried overnight (approximately 18 h) in an oven at 210° C. The dried skins were re-weighed. This method produced complete drying and yielded values for tissue water of 82·1% ±2·7 (S.D.) of the wet weight (mean derived from forty skins).

The pH of the immersion fluid was measured both before and at the end of each experiment and the results were discarded of any experiment in which pH changed.

Solutions

Frog Ringer solution used in the experiments contained sodium chloride 112 mM, potassium chloride 2·0 mM, sodium bicarbonate 2·4 mM and calcium chloride 2·0 mM. The pH of the solution was 8·4 and the osmolality 225 mOsmol/kg water. In some experiments, sodium-free Ringer solution was used. This was obtained by substituting choline chloride and bicarbonate for the sodium salts in equimolar amounts. The pH of this solution was adjusted to 8·4 with a concentrated solution of choline bicarbonate. The osmolality of sodium-free Ringer solution was 240 mOsmol/kg water.

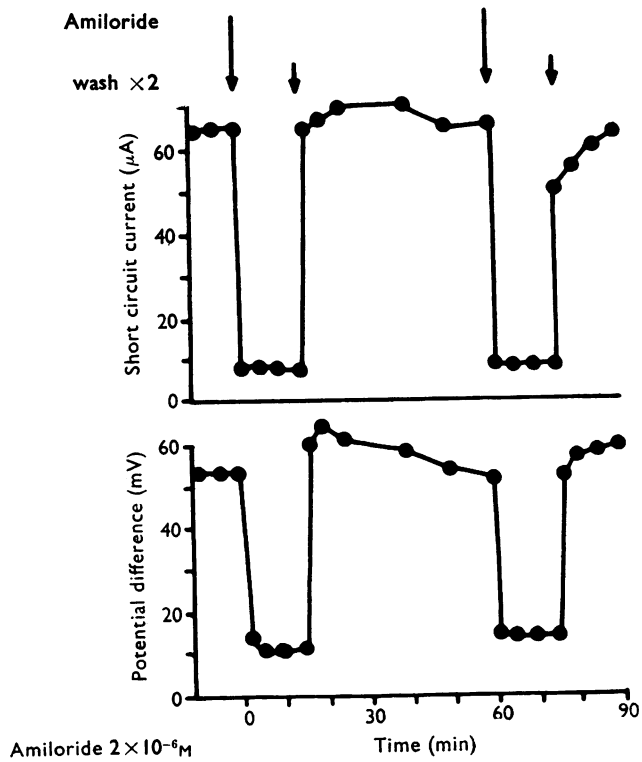

FIG. 2. Effect of amiloride added to the outside bathing solution on short-circuit current and potential difference in the isolated frog skin.

Analytical methods

The sodium concentration of all solutions was estimated by flame photometry using the E.E.L. apparatus. A Pye meter was used to measure pH and osmolality was recorded with an Advanced Instruments osmometer.

Reagents

Amiloride and the diuretic, ethacrynic acid, were obtained as gifts from Merck Sharp and Dohme Limited. Other reagents were mersalyl injection (B.P.), triamterene (Smith, Kline and French), the steroidal aldosterone antagonist SC 14266 (Searle and Co.), antidiuretic hormone (Vasopressin injection, B.P.) and cyclic 3'5'-adenosine monophosphate and the dibutyryl analogue (Boehringer Corporation), theophylline (B.D.H.) and ouabain (Sigma, Ltd.).

Results

Effects on electrical properties and sodium fluxes. Amiloride had no effect when added to the solution on the inside surface of the skin in concentrations of 2×10^{-6}M and 10^{-4}M. When added to the solution on the outside skin surface, however, concentrations of 2×10^{-8}M and above caused a fall in the scc. The effect started within 5 s of adding the drug and was usually complete within 10 min. The current remained steady at the new level for 2–3 h. Amiloride also caused a reduction in the potential difference across the skin. The change in p.d. was proportionately less than that in scc and this was reflected in an increase in the direct current (d.c.) resistance of the skin. If the amiloride was removed and the skin washed twice with fresh Ringer solution, there was an immediate return of the scc to the initial level (Fig. 2).

Dose-response relationships. The effect of amiloride was tested at different concentrations of the compound. The result of a representative experiment is shown in Fig. 3. Because of the ease with which the effect of amiloride could be reversed,

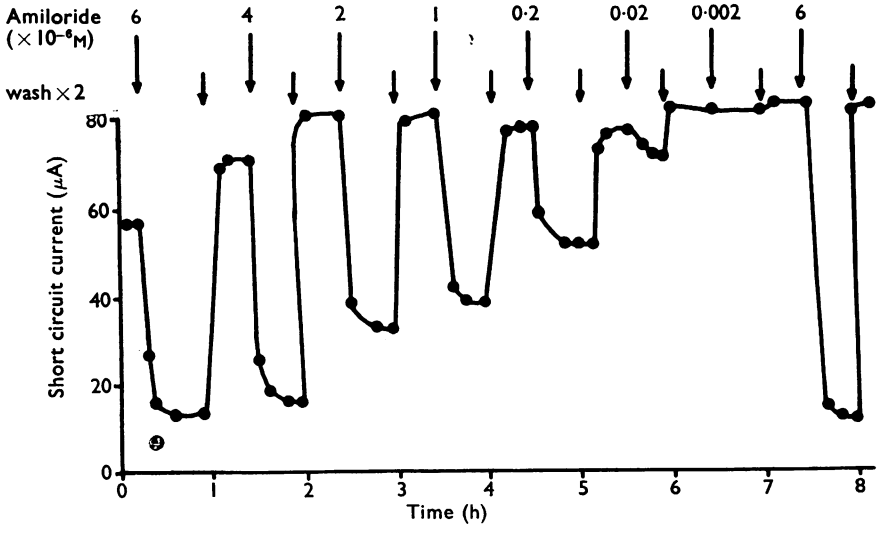

FIG. 3. Effects of different concentrations of amiloride (added to the outside bathing solution) on short-circuit current in the frog skin.

the six concentrations used were studied in the same skin, each test period being followed by a control period in which the outside solution contained no amiloride. This procedure did not affect the sensitivity of the skin to the drug and the scc fell to the same level following 6×10^{-6}M amiloride at the end of an 8 h experiment as at the beginning. The variation in the control level of scc shown in Fig. 3 was frequently observed, but there was no consistent trend. The results in this series of six experiments were expressed in terms of percentage inhibition of scc. In Fig. 4 this has been plotted against the logarithm of the concentration of amiloride, each point on the upper curve representing the mean ± standard error of six experiments. The minimum concentration of amiloride which consistently produced an effect on scc was 2×10^{-8}M. Maximal effects were produced by a concentration of 4×10^{-6}M or more.

Unidirectional sodium fluxes. In the absence of amiloride, sodium influx was usually 5–15% higher than the short-circuit current when both were expressed in the same units. Amiloride (10^{-6}M) caused parallel reductions in both measurements (Table 1). Sodium efflux values were usually a small percentage of the short-circuit

TABLE 1. *Effect of amiloride* (10^{-6}M) *on sodium influx in the frog skin*

Experiment number	Period		Mean scc (μA)	Na influx (μequiv./cm² per 30 min)	Na influx (mCoulombs/cm² per 30 min)	scc (mCoulombs/cm² per 30 min)
	Control	1	56·00	0·37	35·71	35·00
	,,	2	56·00	0·34	33·04	35·00
1	After drug	1	8·00	0·04	4·31	5·00
	,,	2	8·00	0·05	5·28	5·00
	Control	1	79·00	0·50	48·14	50·20
	,,	2	72·00	0·47	45·80	45·80
2	After drug	1	20·00	0·21	20·48	12·77
	,,	2	18·00	0·13	12·09	11·49
	Control	1	84·66	0·81	78·26	60·41
	,,	2	83·00	0·85	82·23	59·38
3	After drug	1	24·00	0·29	27·85	15·00
	,,	2	24·00	0·18	16·96	15·00
	Control	1	41·33	0·33	31·81	26·28
	,,	2	43·33	0·32	30·54	27·57
4	After drug	1	13·33	0·12	11·56	8·49
	,,	2	14·00	0·11	10·97	8·92

TABLE 2. *Effect of amiloride* (10^{-6}M) *on sodium efflux in the frog skin*

Experiment number	Period		Mean scc (μA)	Sodium efflux (μequiv. cm² per 30 min)	Sodium efflux (mCoulombs/cm² per 30 min)	Mean scc (mCoulombs/cm² per 30 min)	E_{Na} (mV)	R_{Na} (Ω/cm²)
	Control	1	40·67	0·027	2·64	25·87	61·1	1,502
	,,	2	44·00	0·025	2·40	27·98	65·2	1,481
1	After drug	1	9·00	0·022	2·16	5·73	33·2	3,691
	,,	2	11·33	0·019	1·80	7·21	41·3	3,658
	,,	3	12·00	0·019	1·80	7·63	42·5	3,642
	Control	1	44·33	0·053	5·07	27·71	47·9	1,081
	,,	2	43·00	0·056	5·42	26·88	45·8	1,065
2	After drug	1	9·00	0·029	2·81	5·63	28·2	3,137
	,,	2	11·00	0·034	3·25	6·88	29·2	2,653
	,,	3	11·67	0·034	3·25	7·29	30·2	2,597
	Control	1	86·00	0·051	4·92	53·75	63·6	740
	,,	2	86·00	0·057	5·53	53·75	60·9	708
3	After drug	1	10·00	0·022	2·13	6·25	35·2	3,520
	,,	2	10·00	0·022	2·13	6·25	35·2	3,520

current in the control periods. Amiloride (10^{-6}M) caused a small reduction in the sodium efflux (Table 2).

The electromotive force of the active sodium transport mechanism (E_{Na}) and the resistance to sodium (R_{Na}) in the active transport path were calculated from the efflux values by the method of Ussing & Zerahn (1951). Amiloride caused an increase in the R_{Na} and a decrease in the E_{Na} (Table 2). The change in E_{Na} was proportionately less than that in R_{Na}.

In four other efflux experiments, the change in partial sodium conductance caused by amiloride was compared with the change in total skin conductance. The drug caused a decrease in both total and partial sodium conductance and the decrease in total conductance was accounted for entirely by the decrease in partial sodium conductance (Table 3). Since the total electrical conductance of the skin is the sum of the partial conductances of the ions moving through the active transport path and the shunt path ($1/R=1/R_{Na}+1/R_{shunt}$ (Ussing, 1965)), these results suggest that movement of sodium alone was affected by amiloride.

Effect of varying the sodium concentration of the bathing solutions. Removal of sodium from the inside bathing solution had no significant effect on the scc and p.d. The effect of amiloride on scc was unchanged in these conditions. On the other hand, a concentration of mersalyl which normally produced 100% inhibition of scc produced only 52% ±5·5 (S.E., $n=10$) when sodium was removed from the inside solution. If the experiments were performed in reverse order, the inside solution being changed to sodium-free Ringer after the scc had been reduced to zero with 6×10^{-6}M amiloride or 4×10^{-4}M mersalyl, the scc remained at zero in the presence of amiloride, whereas with mersalyl there was an immediate rise in scc which became stable at a new level after 10–15 min. In six experiments the new steady level was 33% ±12·1 (S.D.) of the value before mersalyl was added. Subsequent addition of amiloride caused the scc to drop to zero again.

Removal of sodium from the outside solution caused a fall in the scc and p.d. to very low levels. Neither p.d. nor scc was further affected by the addition of amiloride to the outside solution. However, addition of mersalyl to the inside

TABLE 3. *Comparison of the effects of amiloride* (2×10^{-7}M) *on total and partial sodium conductance in the frog skin*

Experiment number	Period	scc (μA)	p.d. (mV)	Total conductance mmhos cm²	Sodium efflux mCoulombs/cm² per 30 min	scc mCoulombs/cm² per 30 min	E_{Na} (mV)	Partial sodium conductance (K_{Na}) mmhos cm²
1	Control 1	38·3	28·0	1·37	6·16	23·94	40·7	0·97
	,, 2	38·0	28·7	1·32	5·91	23·75	41·4	0·92
	After drug 1	20·5	21·0	0·98	4·58	12·81	34·2	0·60
2	Control 1	50·0	49·0	1·02	3·56	31·25	58·5	0·85
	,, 2	50·0	49·0	1·02	3·35	31·25	59·9	0·83
	After drug 1	24·0	44·0	0·55	1·20	15·00	65·2	0·37
3	Control 1	49·0	72·0	0·68	1·14	31·16	85·8	0·57
	,, 2	46·0	72·0	0·64	0·93	29·26	89·3	0·51
	After drug 1	24·0	57·0	0·42	0·71	15·27	79·9	0·30
4	Control 1	28·0	23·0	1·22	2·10	17·50	57·3	0·49
	,, 2	28·0	23·0	1·22	2·00	17·50	58·4	0·48
	After drug 1	17·0	16·0	1·06	1·46	10·63	54·3	0·31

solution caused a reversal in the direction of the electrical potential difference and current (Table 4).

Relationship between scc and sodium influx in the absence of sodium from the outside solution. The short-circuit current has been found to equal net sodium transport under a variety of conditions with identical solutions on both sides of the skin (Ussing & Zerahn, 1951). However, differing concentrations of sodium and bicarbonate in the media on the two sides of the skin might create diffusion currents leading to a disturbance of the normal relationship between current and sodium flux (Mullins, 1958). In three experiments in which sodium influx was measured in the presence of a sodium-free inside solution it was found to lie between 100 and 115% of the short-circuit current during control periods and after treatment with amiloride. These results thus suggest that the equality of net sodium transport and short-circuit current was largely undisturbed by the absence of sodium from the inside solution and, moreover, that valid conclusions may be drawn from measurements of scc under these conditions.

Interactions between amiloride and other substances

Interaction of amiloride and antidiuretic hormone. Antidiuretic hormone (ADH) stimulates short-circuit current in frog skin by an action on the outer membrane of the transporting cells (Ussing, 1960; Curran, Herrera & Flanigan, 1963). Interrelationships between ADH and amiloride were therefore examined in the hope that they might provide information on the site and mechanism of action of amiloride.

In the presence of ADH (0·04 unit/ml) amiloride (4×10^{-6}M) caused a reduction in scc which was greater than in the absence of the hormone. Addition of amiloride caused a decrease in scc which was reversed when the outside solution was replaced with fresh Ringer solution. ADH caused an increase in scc and subsequent addition of amiloride produced a fall in scc greater than that occurring before treatment with ADH (Table 5).

This procedure was reversed in a further series of experiments, the effect of ADH being tested in the presence and absence of amiloride. As the effect of ADH is not as easily reversed as that of amiloride, different skins were used in the paired studies. In the presence of amiloride (4×10^{-6}M) the stimulatory effect of ADH (0·04 u./ml) was reduced (Table 6).

TABLE 4. *Effects of amiloride* (4×10^{-6}M) *and mersalyl* (4×10^{-4}M) *on potential difference (inside positive) in seven skins bathed on the inside with standard Ringer and on the outside with sodium-free choline Ringer*

Experiment number	Initial p.d. (mV)	p.d. 15 min after adding amiloride (mV)	Mimimum p.d. after adding mersalyl (mV)
1	+1·5	+1·0	− 3·0
2	+0·5	−1·0	−17·0
3	0	0	−20·0
4	+2·0	+1·0	−20·0
5	+3·0	+1·5	−28·0
6	+3·0	+2·0	−55·0
7	+1·0	0	−58·0
Mean ± S.D.	+1·5 ± 1·2	+0·6 ± 1·0	−29·0 ± 20·4
Difference (mean ± S.E.M.)			
Due to amiloride		−0·9 ± 0·6 (N.S.)	
Due to mersalyl			−29·6 ± 7·7 ($P < 0·01$)

The results of these last two groups of experiments suggest a possible interaction between ADH and amiloride. This was examined in greater detail by studying the interrelationships between the two compounds at varying concentrations.

The effects of six concentrations of amiloride were studied in skins pretreated with 0·04 u./ml ADH and the results compared with the effects of the same concentrations of amiloride in untreated skins. The results are shown in Fig. 4, in which the percentage inhibition of scc has been plotted against the logarithm of the concentration of amiloride. Each point represents the mean ± S.E. of six experiments and the control curve is from the experiments discussed in the section on dose-response relationships. The curve in the presence of ADH lies to the right of the control curve and is parallel to it in its middle portion. Amiloride in a concentration of 4×10^{-6}M, which produced maximal inhibition of scc in control skins, was submaximal in the presence of ADH.

In the second series of experiments the effect of four concentrations of ADH was studied in the presence of three concentrations of amiloride. The results are shown in Fig. 5, in which increase in scc has been plotted against the logarithm of the concentration of ADH. At certain concentrations, the stimulatory effect of ADH was abolished by amiloride, but this antagonism could be overcome by increasing the concentration of ADH.

TABLE 5. *Effect of amiloride* (4×10^{-6}M) *on scc in frog skin before and after treatment with ADH*

Experiment number	Initial scc (μA)		Final scc (μA)		Fall in scc (μA)	
	No ADH	ADH 0·04 u./ml	No ADH	ADH 0·04 u./ml	No ADH	ADH 0·04 u./ml
1	85	162	10	26	75	136
2	30	63	2	4	28	59
3	62	150	5	10	57	140
4	70	150	5	14	65	136
5	70	147	3	11	67	136
6	92	160	24	32	68	128
Mean ± S.E.					60 ± 6·23	122·5 ± 11·68
Difference ± S.E.M.						62·5 ± 13·24
P value						< 0·01

TABLE 6. *Effect of ADH* (0·04 *u./ml*) *on scc in the presence and absence of amiloride* (4×10^{-6}M)

Experiment number	Increase in scc caused by ADH 0·04 u./ml (in μA)	
	No amiloride	Amiloride 4×10^{-6}M
1	57	14
2	41	24
3	78	14
4	60	0
5	64	14
6	70	4
Mean	62	12
S.E.	±5·1	±3·5
Difference (±S.E.M.)	50 ± 6·2	
P	< 0·001	

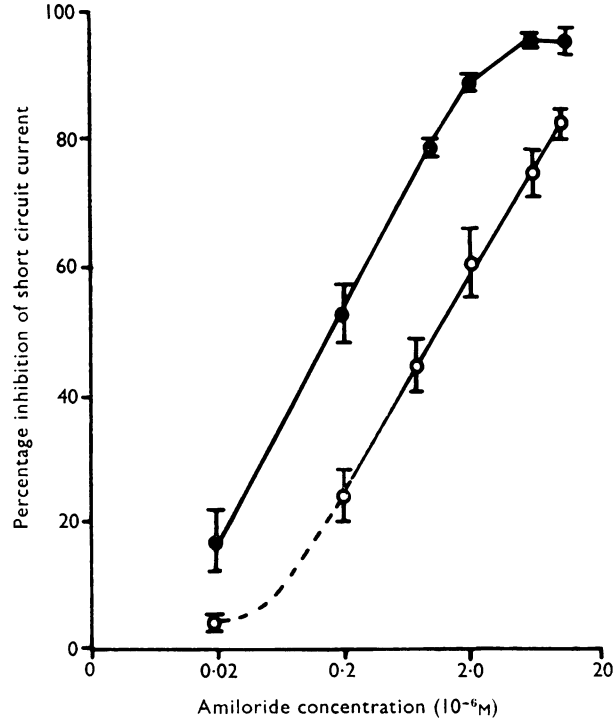

FIG. 4. Dose-response curves for amiloride in the absence (●——●) and presence (○——○) of ADH 0·04 u./ml. Each point represents the mean of six experiments ± standard error.

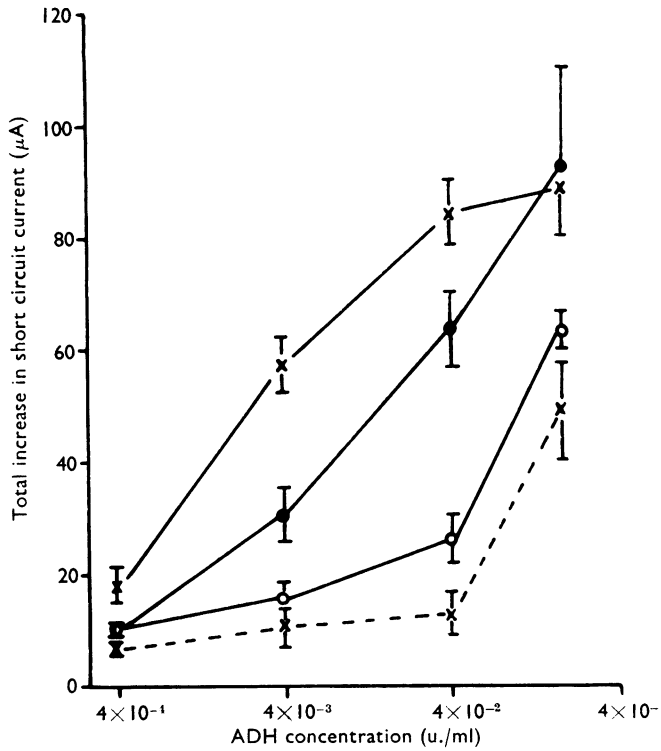

FIG. 5. Effect of three different concentrations of amiloride on the response of the frog skin to four different concentrations of antidiuretic hormone (ADH). ×——×, No amiloride; ●——●, amiloride 2×10^{-7}M; ○——○, amiloride 10^{-6}M; ×- - -×, amiloride 4×10^{-6}M. Each point represents the mean of six experiments ± standard deviation.

These studies were made over a period of several months during the spring, summer and autumn of 1968. Because of the known seasonal variation in the response of frog skin to ADH (Hong, Park, Park & Kim, 1968) and the possible contribution from this source to the observed differences, two smaller studies were performed, designed to eliminate seasonal differences. Each took 6 days and was made on the same batch of frogs using symmetrical halves of the same skin. In the first series of experiments one skin half was treated with 2×10^{-7}M amiloride followed, after 15 min, by ADH 0·04 or 0·2 u./ml. The other skin half was treated only with the corresponding concentration of ADH. Each concentration of ADH was tested in six paired experiments. The effect of 0·04 u./ml ADH was antagonized by amiloride, but the antagonism was abolished by increasing the concentration of ADH to 0·2 u./ml (Table 7).

In the second series, both skin halves were treated with 0·08 u./ml ADH and 30 min later one skin was treated with amiloride. After a further half hour both skins were finally exposed to 0·4 u./ml ADH (Fig. 6). The first addition of ADH produced a maximal increase in scc and this fell on adding amiloride. A considerably increased concentration of ADH failed to increase scc in the control skin but produced a significant reversal of the amiloride inhibition. Percentage increase in scc after 0·4 u./ml ADH in six skins was $38{\cdot}1 \pm 10{\cdot}6$ (mean $\pm$ S.D.). This result does not rule out the possibility that the action of amiloride on the permeability barrier occurs at a different site from that of ADH. At the same time, it would be compatible with competitive interaction between the two compounds.

Effects of amiloride on response to cyclic 3',5'-adenosine monophosphate (cyclic AMP) and theophylline. The foregoing results indicate that amiloride added to the outside solution and ADH added to the inside solution may share some common site of action. The possible location of such a site was examined by studying the interaction of amiloride with cyclic AMP and theophylline, which have been shown to mimic the action of ADH on sodium transport in toad bladder and frog skin (Orloff & Handler, 1962 ; Baba, Smith & Townshend, 1967).

Cyclic AMP alone had no consistent effect in frog skin, confirming a previous observation by Baba *et al.* (1967). However, the dibutyryl analogue consistently produced an increase in scc and p.d. and a decrease in d.c. resistance of the skin. These effects and their time course were similar to the effects of ADH. The stimulatory effect of dibutyryl-cyclic-AMP was antagonized in six skins, by amiloride

TABLE 7. *Increases in the scc produced by two different concentrations of ADH in the presence of and absence of amiloride*

Experiment number	ADH 0·04 u./ml Increase in scc (μA)		ADH 0·2 u./ml Increase in scc (μA)	
	No amiloride	Amiloride 10^{-6}M	No amiloride	Amiloride 10^{-6}M
1	32	11	45	41
2	25	5	54	51
3	31	17	59	37
4	32	26	65	65
5	30	15	45	50
6	30	11	40	44
Mean	30	17	51	48
$\pm$S.E.	1·1	3·8	4·0	4·0
Difference	$13 \pm 4{\cdot}0$ (S.E.M.)		$3 \pm 5{\cdot}6$	
P	$<0{\cdot}05$		N.S.	

(2×10^{-6}M) but this antagonism was overcome when the concentration of dibutyryl cyclic AMP was increased from 0·2 mM to 1·0 mM. The experiments were performed in pairs using symmetrical halves of the same skin (Table 8).

The influence of two concentrations of amiloride on the stimulatory effect of theophylline on scc is shown in Fig. 7, in which increase in scc has been plotted

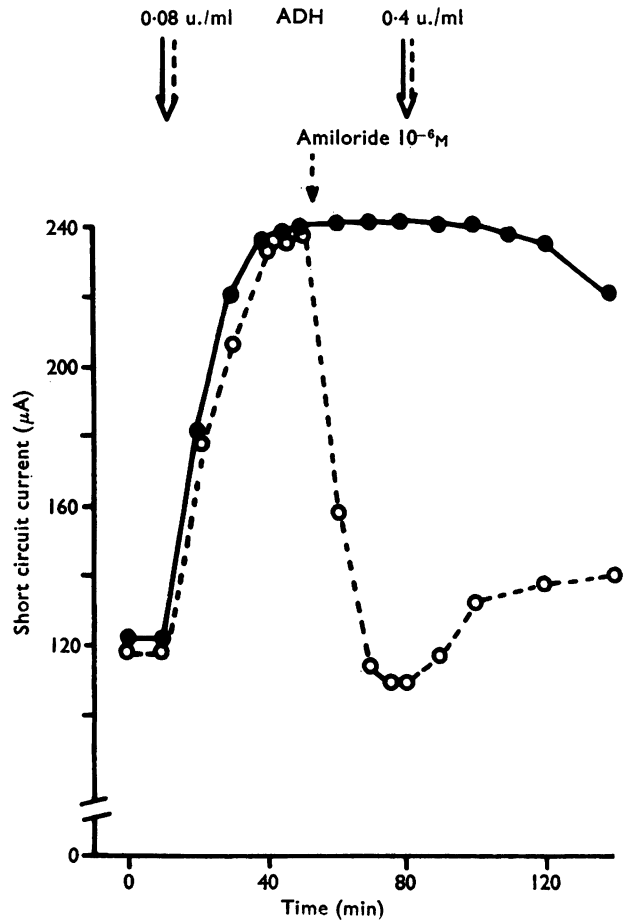

FIG. 6. Antagonism of the effect of amiloride by ADH. Result for two skin halves; ●——●, treated with ADH only, ○——○, treated with ADH and amiloride.

TABLE 8. *Effect of amiloride* (10^{-6}M) *on the stimulatory effect of dibutyryl cyclic AMP on short-circuit current in frog skin*

	Dibutyryl cyclic AMP (0·2 mM) Increase in scc in μA		Dibutyryl cyclic AMP (1·0 mM) Increase in scc in μA	
Experiment number	Without amiloride	Amiloride present (10^{-6}M)	Without amiloride	Amiloride present (10^{-6}M)
1	47	0	50	43
2	32	0	30	29
3	57	2	108	104
4	40	8	51	70
5	33	1	30	56
6	30	5		
Mean	40	3	54	60
S.E.(±)	4·1	1·3	14·3	11·4
Difference±S.E.M.	37±4·3		6±17	
P	<0·001		N.S.	

against the logarithm of the concentration of theophylline. The increase in scc produced by theophylline could be abolished by pretreatment with amiloride, but this effect could be overcome by increasing the concentrations of theophylline. In this respect the response resembles that to ADH and dibutyryl cyclic AMP in the presence of amiloride.

Specificity of the amiloride-ADH interaction. The specificity of the amiloride-ADH interaction was tested by studying the effect of ADH on the action of two other diuretics, ethacrynic acid and the steroidal spironolactone, SC 14266.

The effect of ethacrynic acid added to the inside solution depends on the concentration. $5{\cdot}6 \times 10^{-5}$M produced stimulation of scc, whereas 7×10^{-4}M and $1{\cdot}4 \times 10^{-3}$M produced a brief stimulation followed by a decrease. At all concentrations, however, subsequent addition of ADH had no effect on scc. If, on the other hand, ethacrynic acid was added after ADH, the stimulatory action of the diuretic was abolished but not the inhibitory effect.

The spironolactone, SC 14266, inhibited scc in skin from frogs kept before use in tap water and the magnitude of the response to the compound was the same whether ADH was present or not, suggesting that ADH and SC 14266 were acting independently of each other (Table 9).

Oxygen consumption

The oxygen consumption of frog skin was found to vary with the time of year and this variation paralleled a similar seasonal variation in scc (Fig. 8). Results from twenty-five control experiments showed that the oxygen uptake remained steady over the 2 h that each experiment lasted.

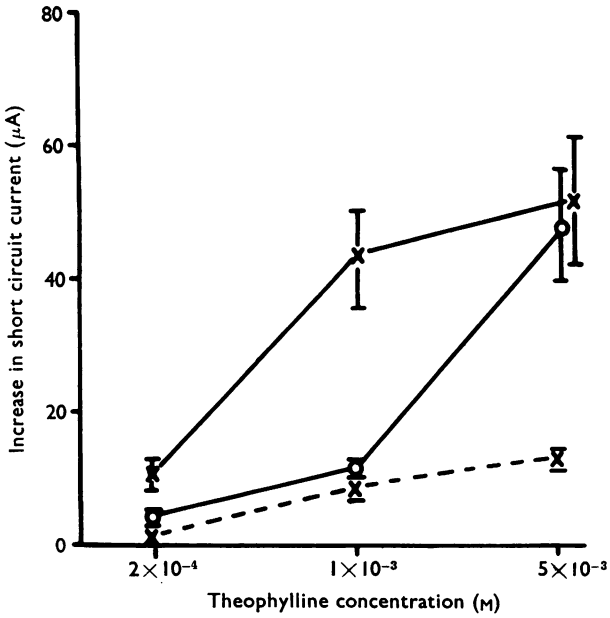

FIG. 7. Effects of three different concentrations of theophylline in control skins and skins pretreated with two different concentrations of amiloride. ×——×, No amiloride; ○——○, amiloride 10^{-6}M; ×- -×, amiloride 4×10^{-6}M. Each point represents the mean of six experiments ± standard deviation.

TABLE 9. *Effect of SC 14266 on short-circuit current in the presence and in the absence of ADH*

Experiment number	Decrease in scc caused by SC 14266 $1{\cdot}2\times10^{-3}$M	
	ADH not present	ADH present 0·04 u./ml
1	55	50
2	20	20
3	44	70
4	22	27
5	66	72
6	61	85
Mean	45	54
S.E.	8·2	10·6
Difference	$9\pm13{\cdot}4$ (S.E.M.)	
P	<0·6 (N.S.)	

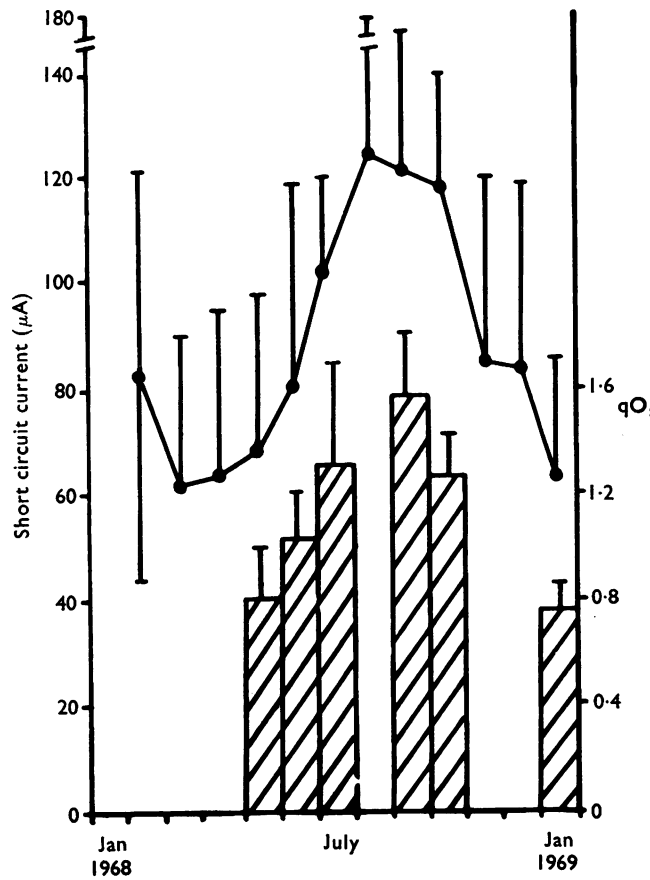

FIG. 8. Seasonal variations in sodium transport rate (represented by the short-circuit current, ●——●) and oxygen consumption rate (μl/mg dry weight per h; mean ± S.D.) in the frog skin (hatched columns).

Four concentrations of amiloride producing between 15 and 95% inhibition of scc had no effect on oxygen consumption (Table 10).

ADH (0·04 u./ml) caused an increase in oxygen consumption, but this increase was prevented by immersing the skins in Ringer solution containing 4×10^{-6} or 2×10^{-7}M amiloride. The effect was not prevented by 2×10^{-8}M amiloride (Table 11).

Discussion

The effect of amiloride added to the solution bathing the outer surface of the isolated frog skin is similar to that previously reported in this tissue (Eigler, Kelter & Renner, 1967; Baba *et al.*, 1968), toad bladder (Bentley, 1968) and toad skin, bladder and colon (Ehrlich & Crabbé, 1968; Crabbé & Ehrlich, 1968). The concentration range over which the compound was found to be effective was similar to that reported by Bentley (1968) in the toad bladder. Concentrations of up to 10^{-4}M were found to be ineffective from the inside solution. This conflicts with the observation of Bentley (1968) but accords with those of Ehrlich & Crabbé (1968) and Baba *et al.* (1968).

Amiloride reduces both sodium influx and efflux, the major effect being a reduction in influx and the equality of net sodium flux with scc appears to be undisturbed by treatment with amiloride.

According to the model of frog skin proposed by Koefoed-Johnsen & Ussing (1958), the primary action of any substance affecting sodium transport may be at the outside membrane, across which sodium is supposed to move into the cell by passive mechanisms, or at the inside membrane, the postulated site of the "sodium pump" which actively extrudes sodium from the cell.

TABLE 10. *Effect of amiloride on oxygen consumption rate (qO_2) in frog skin*

Concentration of amiloride (mol)	Number of experiments	qO_2 (μl/mg dry weight per h) ± S.E.		
		Before amiloride	After amiloride	Difference
4×10^{-6}	19	0·8±0·04	0·8±0·05	0±0·06
1×10^{-6}	10	1·5±0·10	1·4±0·06	−0·1±0·11
2×10^{-7}	12	1·3±0·06	1·2±0·06	−0·1±0·07
2×10^{-8}	10	1·5±0·10	1·6±0·09	+0·1±0·14

TABLE 11. *Effect of ADH (0·04 u./ml) on qO_2 in pieces of skin immersed in Ringer solution or in Ringer solution containing various concentrations of amiloride*

	Number of experiments	qO_2 (ml/mg dry weight per h) mean ±S.E.			
		Before ADH (1)	After ADH (2)	Difference (2)−(1)	*P* value
Ringer only	5	1·0±0·02	1·4±0·05	+0·4±0·06	<0·01
Ringer+ amiloride 4×10^{-6}M	6	0·9±0·05	0·9±0·04	0±0·07	>0·8 (N.S.)
Ringer+ amiloride 2×10^{-7}M	10	1·1±0·06	1·2±0·07	+0·1±0·09	>0·1 (N.S.)
Ringer+ amiloride 2×10^{-8}M	10	1·1±0·05	1·3±0·04	+0·2±0·06	<0·02

The fact the amiloride is effective only from the outside solution and the rapidity of the effect would suggest a dominant action at the outside membrane while not excluding a concomitant effect at the inner membrane. Action at the outer membrane would reduce the amount of sodium available to the pump, which would lead to the observed decrease in sodium influx. The reduction in sodium efflux and increase in the partial sodium resistance in the active transport path which were also observed accord with an action at the outer membrane (Schoffeniels, 1957).

However, the reduction in the electromotive force of the active sodium transport mechanism might indicate direct inhibition of the sodium pump. The estimation of this parameter from Ussing's work equation (Ussing, 1960) is beset with a number of uncontrollable variables (for example the presence of exchange diffusion and sodium movement along shunt paths (Ussing & Windhager, 1964 ; Civan, Kedem & Leaf, 1966)) which reduce the accuracy of the method. It is therefore not justifiable to base firm conclusions on this evidence alone.

Mersalyl has been shown to inhibit sodium transport by an action on the active mechanisms (Linderholm, 1952 ; Jamison, 1961). In the absence of sodium from the inside solution, a concentration of this diuretic drug which normally produced complete inhibition of scc caused only partial inhibition. This contrasted with the effect of amiloride, which was unaltered by reduction of the sodium concentration in the inner bathing solution. The effect of mersalyl is consistent with direct inhibition of the sodium pump, the residual scc possibly resulting from diffusion of sodium down a concentration gradient. The existence of such a gradient and diffusion current is theoretically possible as the inside membrane is permeable to sodium, if only to a small degree (Herrera, 1966).

On the other hand, the fact that a concentration of amiloride which normally inhibits scc completely still does so with sodium absent from the inside solution accords with inhibition of passive sodium movement across the outside membrane. Similarly, the reversal of the p.d. by mersalyl in the absence of sodium from the outside solution is consistent with direct inhibition of the metabolically dependent active extrusion mechanism which would again allow the production of a current of sodium diffusion in the direction of the concentration gradient. The lack of effect of amiloride in these conditions would be expected for any compound acting on the passive barrier alone.

Furthermore, the diffusion current produced by inhibiting the sodium pump completely with mersalyl and then substituting sodium-free Ringer in the inside solution can itself be inhibited by amiloride. This result differs from that of Bentley (1968), who found that amiloride had no effect on the diffusion current produced in a similar manner but using the metabolic inhibitors sodium cyanide and iodoacetate as pump inhibitors. These compounds are metabolic poisons and their effect on active sodium transport is not selective (Huf, Doss & Wills, 1957). Mersalyl, on the other hand, appears to have a selective action on the active transport mechanisms (Linderholm, 1952) and it may be that the discrepancies between Bentley's results and those presented here are a reflection of the choice of substance as pump inhibitor.

Antidiuretic hormone has been shown to stimulate active sodium transport in frog skin by an action at the passive outside membrane (Ussing, 1960 ; Curran *et al.*, 1963). This action may be mediated by cyclic AMP (Orloff & Handler, 1962 ; Baba *et al.*, 1967). The studies reported here show that amiloride may interact

competitively with ADH and also with both dibutyryl cyclic AMP and theophylline. Bentley (1968) found that ADH was unable completely to overcome the inhibitory action of amiloride and concluded that their interactions were non-competitive. Eigler *et al.* (1967), using frog skin, found that amiloride prevented the stimulatory effect of ADH. On the other hand, Ehrlich & Crabbé (1968), using toad bladder, found that the stimulatory effect of ADH was undisturbed by pre-treatment with amiloride. The differing results obtained by these authors is, in our opinion, a reflection of the concentrations of substances selected.

Amiloride had no effect on oxygen consumption in concentrations which produce inhibition of active sodium transport. This suggests that the effect of amiloride on sodium transport is a primary action and not secondary to inhibition of intracellular metabolism. In the presence of certain concentrations of amiloride, the stimulation of oxygen consumption by ADH was antagonized, but this antagonism could be overcome by increasing the concentration of ADH relative to that of amiloride. Since ADH stimulates qO_2 by allowing more sodium to pass into the cells across the outside membrane (Leaf & Dempsey, 1960) the antagonism of ADH by amiloride suggests that the diuretic prevents this increased entry of sodium into the cells.

The results of these studies therefore suggest that amiloride acts on the outside membrane of the epithelial cells of the skin as a result of which entry of sodium into the cells is reduced. This, in turn, leads to a reduction in the rate of active extrusion of sodium across the inside membrane. A similar site of action has been suggested for ADH (Curran *et al.*, 1963), local anaesthetics (Skou & Zerahn, 1959) and calcium added to the outside skin surface (Curran & Gill, 1962; Curran *et al.*, 1963). The action of amiloride appears to be selective for sodium rather than a non-specific "tightening" of the membrane to the passage of ions. In this respect the drug resembles ADH but differs from calcium (Herrera & Curran, 1963). However, the effects on the E_{Na} suggest the possibility of some direct action on the active extrusion mechanism.

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