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AMILORIDE: A POTENT INHIBITOR OF SODIUM TRANSPORT ACROSS THE TOAD BLADDER

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SUMMARY

1 Amiloride inhibits Na transport and short-circuit current (SCC) across the toad bladder. It is 1000 times more active at the mucosal than serosal surface. The lowest effective concentration was 10^{-7} M.

2. The inhibition was non-competitive with the sodium on the mucosal side of the bladder.

3. Vasopressin, cyclic adenosine monophosphate (AMP) and aldosterone increased Na transport and SCC across the bladder and these effects were inhibited by amiloride.

4. The antagonism of amiloride for vasopressin was non-competitive.

5. Amphotericin B also increases Na transport across the bladder but its action was not changed by amiloride.

6. Amiloride was without effects on SCC and diffusion potentials in bladders metabolically inhibited with CN^- and iodoacetic acid (IAA).

7. Neither plasma albumin, Ca^{2+} nor adenosine triphosphate (ATP) altered the effects of amiloride.

8. The only structural analogue of amiloride found to reduce SCC similarly was guanidine which was 1000 times less active. Pyrazine and a substituted pyrazine analogue were without effect. Neither guanidine nor the substituted pyrazine compound were competitive with amiloride.

9. Amiloride had no effect on the osmotic permeability of the toad bladder either in the presence or absence of vasopressin.

10. Na transport across the toad colon was also reduced by 10^{-5} M amiloride at the mucosal surface.

11. The possible mechanism of action of amiloride is discussed.

INTRODUCTION

The urinary bladder of the toad actively transports sodium from its mucosal to serosal side and is osmotically permeable to water, both processes being increased by vasopressin (Leaf, Anderson & Page, 1958;

Bentley, 1958) while sodium transport is also stimulated by aldosterone (Crabbé, 1961). These effects are reminiscent of functions occurring in the distal parts of the mammalian nephron for which in many respects the toad bladder *in vitro* provides a living model. However, the intimate nature of the processes of sodium transport and hormone action on such epithelial membranes is poorly understood and information about the mechanism of action of chemically characterized materials on such membranes may be illuminating. The potassium 'sparing' pyrazine diuretic 3,5-diamino-6-chloropyrazinoylguanidine (generic name, *amiloride*) reduces sodium transport across the renal tubule (Baer, Jones, Spitzer & Russo, 1967) and, as will be shown, in concentrations as low as 10^{-7} M also reduces sodium transport across the toad bladder. It decreases actions of vasopressin and aldosterone and appears to block entry of sodium through the mucosal side of the cell in a manner reminiscent to that of another compound containing guanidine, tetrodotoxin, which acts on nerve axons (Narahashi, Moore & Scott, 1964).

METHODS

Toads (*Bufo marinus*) were obtained from an animal supplier and kept on damp sand in a room maintained at 25° C.

Preparation of the bladder. This has been described in detail by Bentley (1958, 1960). Each lobe of the urinary bladder was dissected out and placed in frog Ringer solution of the following composition mM: NaCl 111, KCl 3·35, $CaCl_2$ 2·7, $NaHCO_3$ 2·38 and glucose 5·5. It was aerated and the pH was 7·8–8·0. Each bladder lobe, or its portion, was tied to the end of a piece of glass tubing, mucosal side facing inwards. It was filled through this tubing with 1 ml. of the solutions to be described. The outside serosal surface normally was bathed with the aerated Ringer solution. The temperature was 25° C.

The effects of aldosterone on the bladder are accentuated if the toads are kept for a couple of days in dilute saline (Crabbé & De Weer, 1965) and the excised bladders are preincubated for about 18 hr in substrate and steroid-free Ringer solution (Porter & Edelman, 1964). In the present experiments the toads were kept for 48 hr in 0·65 % NaCl solution before they were pithed and the bladders removed. The bladders were placed in substrate-free Ringer solution and kept at 5° C for 18 hr. They were then mounted on to the glass tubes and bathed in Ringer solution containing glucose (5·5 mM) and Na pyruvate (2 mM). The experiments were begun 2 hr later. Such bladders were in excellent condition, the initial p.d. being between 50 and 98 mV and they subsequently showed a good response to aldosterone.

Water transfer. This was measured gravimetrically by weighing the bladder preparations rapidly to 0·1 mg on a highly damped balance.

Short-circuit current and sodium transport. The short-circuit current (SCC) across the bladder of *Bufo marinus* normally and in the presence of adrenocortical and neurohypophysial hormones and various metabolic inhibitors reflects the net sodium transport from the mucosal to serosal surface (see Leaf, 1965). The method for measuring this was originally described by Ussing & Zerahn (1951) and essentially the same procedure has been used here. The p.d. across the bladder was measured with a voltmeter (Keithley Model 200b) connected to each side of the preparation through calomel cells and agar-saturated KCl bridges. The short-circuit current was applied through similar bridges and Ag–AgCl cells.

Drugs and reagents. The amiloride (3,5-diamino-6-chloropyrazinoylguanidine hydrochloride) and one of its structural analogues, 3,5-diamino-6-chloropyrazinecarboxylic acid were given to me by the Merck Institute for Therapeutic Research of West Point, Pennsylvania. Other reagents used were (+)-aldosterone (CIBA Pharmaceutical Co.), pyrazine (Mann Research Laboratories), ouabain, iodoacetic acid, sulphaguanidine, bovine plasma albumin (Sigma Chemical Co.), adenosine-5′-triphosphate, cyclic AMP (as Na salts) and guanidine hydrochloride (Calbiochem), amphotericin B (Squibb and Sons) and 8-arginine vasopressin (Parke, Davis and Company).

RESULTS

Short-circuit current (sodium transport). When amiloride (10^{-3} or 10^{-4} M) was present at the serosal surface of the toad urinary bladder the short-circuit current (SCC) was decreased (Table 1). When it was placed at the mucosal surface, however, it was far more effective, reducing the SCC at concentrations of 10^{-7} M.

The effects of amiloride at the mucosal surface were rapid, the p.d. being seen to plunge downwards immediately when it was added to this fluid. The p.d. and SCC recorded at short intervals after the addition of amiloride to the mucosal side of the bladder are shown in Fig. 1. After

TABLE 1. Effects of amiloride on SCC across the toad urinary bladder

		μA/100 mg bladder			*P vs.*
		Initial	10 min	Diff.	control
Controls	(24)	396	422	+28±10	—
Amiloride					
Serosal side					
10^{-3} M	(6)	683	427	−256±70	< 0·001
10^{-4} M	(6)	491	407	−86±27	< 0·001
10^{-5} M	(6)	338	356	+18±12	n.s.
Mucosal side					
10^{-5} M	(6)	393	66	−327±73	< 0·001
10^{-6} M	(6)	408	145	−263±43	< 0·001
10^{-7} M	(6)	534	336	−198±64	< 0·001
10^{-8} M	(6)	265	228	−37±16	n.s.

Results are given as means and differences ± S.E. of the number of experiments in parentheses. n.s., not statistically significant ($P > 0·05$).

removal of the amiloride the return of the SCC towards normal levels was recorded and found to be 88±7% of the initial level after 1 min and 82±5% after 5 min (6 experiments). The actions of amiloride at the serosal surface were in contrast not reversible when the serosal surface was washed in fresh Ringer solution containing no amiloride or if the mucosal fluid was replaced also. It is possible that under these conditions the amiloride becomes firmly trapped in the tissue.

The ratio of the SCC to the p.d. gives an indication of possible changes in the d.c. resistance of the membrane. Initially this ratio, p.d./SCC μA. 100 mg bladder (6 experiments) was 1·10 ± 0·1, 1 min after adding amiloride

(10^{-5} M) to the mucosal side it was $1{\cdot}4 \pm 0{\cdot}14$ and 9 min later it was $1{\cdot}68 \pm 0{\cdot}16$. The ratio of p.d./SCC thus increased by about 50% after 10 min exposure to amiloride suggesting that the d.c. resistance of the membrane was also increased.

Net sodium transport. The SCC across the toad urinary bladder has been repeatedly found to reflect the net sodium transport from the mucosal to serosal side under a variety of conditions and when various hormones and

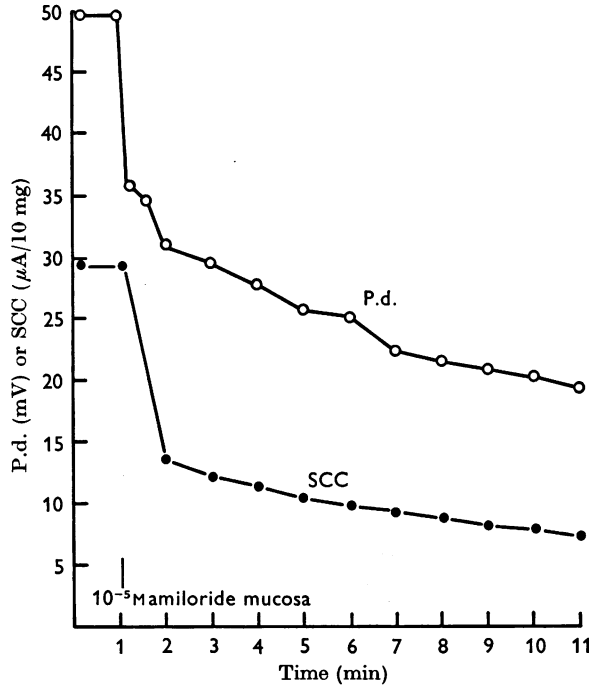


Fig. 1. Effects of 10^{-5} M amiloride at the mucosal surface of the toad bladder. Each point is the mean of six different experiments. ○, p.d., ●, SCC.

metabolic inhibitors are present (see Leaf, 1965). Measurement of the precise equivalence of SCC and net sodium transport requires experiments with radioactive isotopes. These were not performed in the present work, but measurements of the effects of amiloride on net sodium transport from the bladder were made using a flame photometer to measure sodium. Large bladders holding 3 ml. of Ringer solution were incubated for a 5 hr period. One lobe from each toad served as a control for the other lobe which was exposed to 10^{-5} M amiloride on its mucosal surface. The initial and final concentrations of sodium were determined and the fluid loss calculated gravimetrically. The six control bladder halves lost 10·8 μ-equiv/100 mg while those exposed to amiloride gained 2·6 μ-equiv/100 mg, the

mean difference being $13{\cdot}4 \pm 4{\cdot}0$ ($P < 0{\cdot}05$). Thus the amiloride abolished net sodium transport across the bladder.

In order to investigate the nature of the interaction of amiloride and sodium transport the SCC was measured in the presence of three different concentrations of amiloride at four different mucosal levels of sodium (Fig. 2). The normal SCC seen with different concentrations of sodium at the mucosal side obeys saturation kinetics reaching a maximum when the

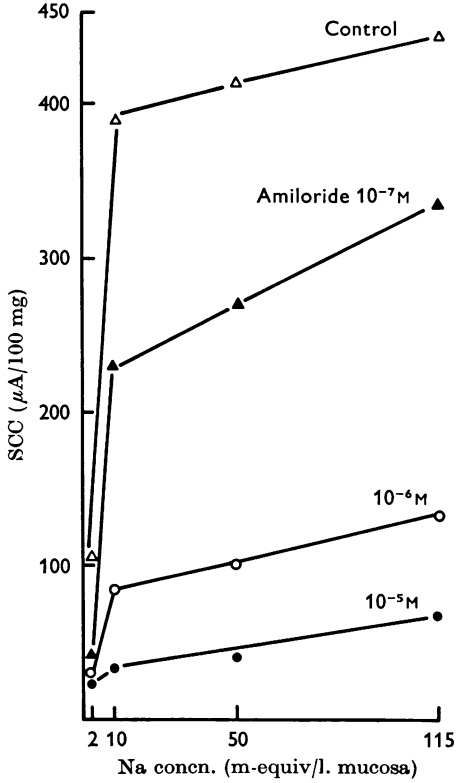


Fig. 2. Effects of different concentrations of amiloride (10 min exposure) on the SCC across the toad bladder with the mucosal surface bathed with different concentratlons of NaCl. Isotonicity with the serosal Ringer solution was maintained by substituting choline for Na. Each point represents the mean of six different experiments. △, control; amiloride, ●, 10^{-5} M; ○, 10^{-6} M, ▲, 10^{-7} M.

sodium concentration is about 10 m-equiv/l. Amiloride at concentrations of 10^{-7}, 10^{-6} and 10^{-5} M progressively lowers the SCC at each level of sodium in a manner indicating that the antagonism of amiloride for sodium transport is non-competitive.

Action of vasopressin, cyclic AMP, amphotericin B and (+)-aldosterone. Vasopressin and cyclic AMP increase sodium transport and SCC across the

toad bladder (Leaf *et al.* 1958; Orloff & Handler, 1962), their actions probably being mediated through the same effector mechanism, which is thought to be present at the mucosal side of the bladder epithelial cells (Leaf, 1965). When amiloride (10^{-5} M) was present at the mucosal side of the bladder both of these substances failed to increase the SCC (Table 2). The nature of the inhibitory effects of amiloride on the actions of vasopressin were examined in detail by comparing the SCC (sodium transport) after exposure of the bladder to various combinations of three different concentrations of amiloride and four of vasopressin. As shown in Fig. 3 their actions were non-competitive suggesting differences in precise receptor sites and demonstrating the complete inability of vasopressin to overcome the inhibitory action of amiloride.

Amphotericin B when present at the mucosal side of the bladder also increases SCC and sodium transport, an action thought to result from increased permeability of this surface of the cell (Lichenstein & Leaf, 1965). Amiloride in contrast to its effects on the actions of vasopressin and cyclic AMP did not alter the response to amphotericin B (Table 2).

TABLE 2. Effects of amiloride on the actions of vasopressin, cyclic AMP and amphotericin B on SCC across the toad bladder

	μA/100 mg bladder			
	Initial	10 min	Diff.	*P*
I.				
Vasopressin ($2{\cdot}1 \times 10^{-9}$ M)	224	288	$+64 \pm 13$	< 0·01
Vasopressin + amiloride (10^{-5} M)	27	33	6 ± 2	
II.				
Cyclic AMP (2×10^{-3} M)	204	276	$+72 \pm 9$	< 0·001
Cyclic AMP + amiloride (10^{-5} M)	57	45	-12 ± 6	
III.				
Amphotericin B ($1{\cdot}3 \times 10^{-5}$ M)	154	398	$+244 \pm 60$	n.s.
Amphotericin B + amiloride (10^{-5} M)	33	411	$+378 \pm 47$	

Results are given as measured differences ± S.E. of 6 experiments. The bladder preparations were exposed to the amiloride for 10 min before adding the other compounds.

Aldosterone increases sodium transport and SCC across the toad bladder but its effects in contrast to the previous agents tested take place slowly (Crabbé, 1961) so that a different experimental design had to be used. The toads and bladders were prepared as described in the Methods section and exposed to (+)-aldosterone for 4 hr before exposing them to amiloride. At the end of the 4 hr period a marked stimulation of the SCC was seen. In both the control bladders and those preincubated with aldosterone, amiloride produced a considerable reduction in SCC (Table 3).

Comparison of the actions of amiloride and ouabain in the presence of amphotericin B. Sodium transport across the toad bladder is inhibited by ouabain (Bonting & Canady, 1964), an action mediated by inhibition of

ATPase (Skou, 1965) which has been located histochemically at the serosal border of the bladder epithelial cells (Keller, 1963) where ouabain is also considered to act (Herrera, 1966). Thus the effects of ouabain and amiloride in relation to the mucosal action of amphotericin B may provide information relevant to their sites of action.

In the presence of 10^{-5} M amiloride, amphotericin B had an undiminished effect in stimulating the SCC while if ouabain (10^{-4} M) was present the

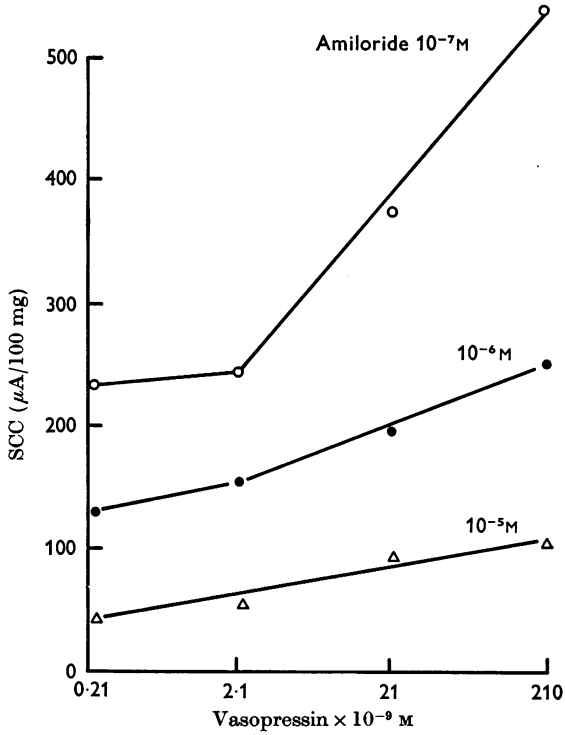


Fig. 3. Effects of different concentrations of amiloride on SCC across the toad bladder in the presence of different concentrations of arginine-vasopressin Each point is the mean of six different experiments. Amiloride: ○, 10^{-7} M; ●, 10^{-6} M; Δ, 10^{-5} M.

TABLE 3. Effects of amiloride (10^{-5} M) on SCC in toad bladders exposed to (+)-aldosterone (10^{-5} M) for 4 hr

	μA/100 mg bladder				
	Initial I	4 hr II	Diff. I−II	10 min amiloride III	Diff. II−III
Aldosterone	147	409	+262±74	195	−214+47
Control	170	177	+ 7±23	64	−113±33

The results are given as means and differences ±S.E. for 6 experiments.

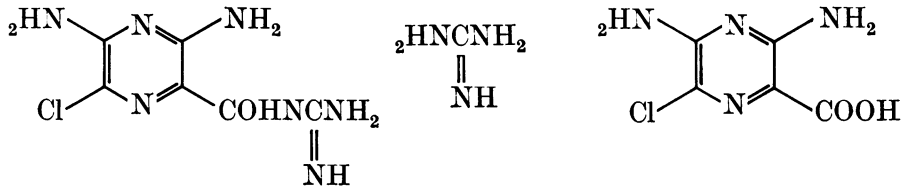
increase was nearly abolished (Table 4). This suggests that amiloride and ouabain are acting at different sites, that of the former being more proximate to that of the amphotericin B. These results are thus consistent with the possibility that amiloride like amphotericin B alters the permeability of the mucosal side of the cell rather than inhibiting the serosal sodium 'pump'.

Actions of some structural analogues of amiloride. Amiloride consists of a substituted pyrazine ring with an attached guanidine group (see Fig. 4). The actions of related compounds on the SCC across the toad bladder may provide information relevant to the mechanism of its effect.

TABLE 4. Comparison of the inhibitory effects of amiloride and ouabain on the actions of amphotericin B on SCC across the toad bladder

	μA/100 mg bladder			
	Initial	10 min	Diff.	*P*
I.				
Amphotericin B (6) ($1{\cdot}3 \times 10^{-5}$ M)	154	398	$+244 \pm 60$	
Amphotericin B (6) + amiloride (10^{-5} M)	124	468	$+344 \pm 93$	n.s.
II.				
Amphotericin B (10) ($1{\cdot}3 \times 10^{-5}$ M)	264	416	$+152 \pm 28$	
Amphotericin B (10) + ouabain (10^{-4} M)	274	303	$+29 \pm 30$	< 0·001

The results are given as means and differences ± S.E. of the number of experiments in parentheses.



Amiloride Guanidine 3,5-Diamino-6-chloro pyrazinecarboxylic acid

Fig. 4. Structures of amiloride, guanidine and 3,5-diamino-6-chloropyrazine-carboxylic acid.

Guanidine was also found to decrease the SCC across the toad bladder but was ineffective at concentrations less than 10^{-4} M at the mucosal surface and had no effect at the serosal surface (Table 5). The effects of guanidine like those of amiloride were reversed when guanidine-free Ringer solution was placed at the mucosal side of the bladder. Sulphaguanidine had no effect on the SCC across the bladder even at a concentration of 10^{-3} M (Table 5).

Some structural analogues of the substituted pyrazine ring, lacking the guanidine side chain, were next tested on the bladder (Table 5). Pyrazine (10^{-3} M mucosal side) did not change the SCC across the bladder. The substituted pyrazine ring of amiloride, 3,5-diamino-6-chloropyrazinecarboxylic acid, similarly did not alter the SCC.

TABLE 5. Effects of some structural analogues of amiloride on SCC across the toad bladder

		μA/100 mg bladder			*P vs.*
		Initial	10 min	Diff.	control
Controls	(18)	215	210	-5 ± 5	—
Guanidine					
Serosal 10^{-4} M	(6)	366	365	-1 ± 44	n.s.
Mucosal 10^{-4} M	(6)	377	172	-205 ± 44	< 0·001
10^{-5} M	(6)	485	486	$+1 \pm 35$	n.s.
Sulphaguanidine					
Mucosal 10^{-3} M	(4)	298	360	$+62 \pm 25$	n.s.
10^{-4} M	(6)	255	276	$+21 \pm 10$	n.s.
Pyrazine					
Mucosal 10^{-3} M	(6)	196	186	-10 ± 33	n.s.
3,5-Diamino-6-chloropyrazine					
Mucosal 10^{-3} M	(6)	156	144	-12 ± 14	n.s.

Results are given as means and differences ± S.E., number of experiments in parentheses. n.s. not statistically significant ($P > 0{\cdot}05$).

Thus the guanidine moiety of amiloride is essential for its effects, but the substituted pyrazine ring greatly increases the molecule's activity. It is possible that this part of the molecule as well as the guanidine moiety interact with the receptor. If this were so then it is conceivable that the substituted pyrazine ring may inhibit the actions of amiloride. The actions of amiloride (10^{-5} M mucosal surface) were thus compared in paired bladder halves with the actions of amiloride (10^{-5} M) plus the 3,5-diamino-6-chloropyrazinecarboxylic acid (10^{-3} M). The reduction in SCC with amiloride alone was 159 ± 18 μA/100 mg bladder (6 experiments) while in combination with the substituted pyrazine ring it was 138 ± 13 μA/100 mg, the difference not being significant statistically ($P > 0{\cdot}05$). The effects of amiloride (10^{-5} M) compared with the combined actions of amiloride (10^{-5} M) and guanidine (10^{-4} M) were also examined; with amiloride alone the reduction in SCC was 195 ± 30 μA/100 mg (6 experiments) while the two compounds together resulted in a reduction of 166 ± 23 μA/100 mg (6 experiments) which is also not a significant difference. This suggests that the intact amiloride molecule has a far greater affinity for the receptors involved than guanidine.

Actions of amiloride in the presence of ATP, plasma albumin and calcium. Several attempts were made to find agents which may modify the effects of amiloride.

Addition of 10 mM ATP to both sides of the bladder did not alter the actions of amiloride. Thus in six experiments the control SCC dropped

from 148 to 42 μA/100 mg in 10 min (difference -106 ± 9) when amiloride (10^{-5} M) was placed at the mucosal surface, while if ATP was also present it changed from 112 to 29 μA/100 mg (difference -83 ± 19).

Amiloride carries a positive charge and it seemed possible that this may interact with plasma proteins and influence the drug's action. No evidence of this was seen. Thus bovine plasma albumin (500 mg/l.) was added to the fluid bathing the mucosal surface in one group of five bladders and when amiloride (10^{-6} M) was added to this fluid the SCC dropped from 460 to 174 μA/100 mg (difference -286 ± 68). In control bladder halves with no albumin present it dropped similarly; from 380 to 153 μA/100 mg (difference -227 ± 35).

The presence of 10 mM-Ca^{2+} at the mucosal surface also failed to alter the actions of amiloride on the SCC.

Effects of amiloride on Na diffusion potentials and SCC across the bladder. It is possible that amiloride reacts with some structural components of the bladder epithelial cells to restrict access of sodium to the sodium 'pump'. In an attempt to investigate this and separate the passive and metabolically dependent components, the effects of amiloride on Na diffusion potentials and SCC were measured.

Bladders were exposed to 5 mM sodium cyanide and 2 mM iodoacetate (serosal side) till no p.d. could be recorded across the wall; active sodium transport then being probably abolished. This usually required about 1 hr exposure to the inhibitors. When this had been accomplished the bladders were transferred to a Ringer solution in which most of the sodium had been replaced by choline (but still containing the cyanide and iodoacetate). The difference in sodium concentration between the mucosal and serosal side was 122 m-equiv/l. The bladders were allowed 15 min to equilibrate to these conditions and the p.d. and SCC were then recorded. Amiloride (10^{-5} M) did not change the p.d. or SCC (Table 6) in marked contrast to the immediate effects of amiloride in bladders that had not been exposed to these metabolic inhibitors.

These experiments only provide equivocal evidence for lack of a simple structural effect of amiloride. Thus abolition of metabolism could lead to autolysis and influence the bladders ability to maintain its structure. Sodium may then diffuse through different channels. That substantial cell break-down had not taken place was indicated by measurements of osmotic water transfer which did not change for a period of 1 hr after complete abolition of the p.d. had been achieved. It is also interesting that such treatment does not abolish the passive effects of amphotericin B (P. J. Bentley, unpublished observations).

Absence of effects of amiloride on osmotic permeability. The toad bladder is osmotically permeable to water and this, like sodium transport, is

greatly increased in the presence of vasopressin (Bentley, 1958). It was thus of interest to know if amiloride could affect the permeability of the bladder to water as well as sodium and whether the action of vasopressin on water was inhibited in a similar way to its action on sodium transport.

Amiloride did not alter the osmotic permeability to water in the presence or absence of vasopressin (Table 7). The bladders in these experiments were filled with 10 mM choline chloride solution so that no sodium transport, which could have complicated interpretation of the results, was taking place.

TABLE 6. Electrical p.d. and SCC across the toad bladder in presence of amiloride after prolonged exposure to 5 mM-NaCN and 2 mM-Na iodoacetate. Sodium concentration gradient mucosa→ serosa 122 mM

	Initial	1 min exposure	10 min exposure
Amiloride 10^{-5} M			
P.d. mV (8)	9·9 ± 1·1	10·3 ± 0·9	10·4 ± 0·9
SCC μA/100 mg (8)	51 ± 13	48 ± 10	51 ± 11
Control			
P.d. mV (8)	11·7 ± 1·3	11·7 ± 1·3	11·8 ± 1·4
SCC μA/100 mg (6)	47 ± 18	47 ± 18	47 ± 19

Results are as means ± S.E., number of experiments in parentheses. For further explanation see text p. 326.

TABLE 7. Effects of amiloride on osmotic water transfer across the toad bladder in the absence and presence of vasopressin

	Period I	Period II + amiloride	Diff.
Water loss mg/hr vasopressin absent			
Serosal side 10^{-3} M (6)	30	25	−5 ± 4
Controls (6)	28	27	−1 ± 2
Mucosal side 10^{-5} M (6)	39	38	−1 ± 1
Controls (6)	32	31	−1 ± 4
Water loss mg/30 min vasopressin present $2·1 \times 10^{-9}$ M			
Serosal side 10^{-3} M (6)	155	220	+65 ± 31
Mucosal side 10^{-4} M (6)	207	171	−36 ± 18
10^{-5} M (6)	224	184	−40 ± 18
Controls (18)	180	179	−1 ± 11

Results are means and differences ± S.E. for number of experiments in parentheses. None of the changes differs significantly ($P < 0·05$) from the controls.

Thus the effects of amiloride on the permeability of the bladder are not sufficiently gross to influence its osmotic permeability to water but are directed towards sodium transport.

Effects of amiloride of SCC across the toad colon. The SCC across the toad colon also reflects the active net sodium transport from the mucosal to serosal surface (Cofre & Crabbé, 1967). The actions of amiloride on SCC across the colon were also examined in preparations of the toad colon set up in the same manner as the urinary bladders. Five preparations were used. When amiloride (10^{-5} M) was placed in the fluid at the mucosal

surface the SCC dropped from 47 to 15 μA/g colon after 10 min (difference $-32 \pm 6{\cdot}6$). In control experiments the SCC increased from 46 to 47 μA/g (difference $+1 \pm 6{\cdot}0$). The difference between these changes is statistically significant ($P < 0{\cdot}01$). When the amiloride was removed from the mucosal surface by replacing the Ringer solution the SCC returned to 61 μA/g.

DISCUSSION

Amiloride depresses sodium transport and the SCC across the toad bladder (and colon) but has no effect on water transfer whether vasopressin is present or not. The stimulating effects of vasopressin, cyclic AMP and aldosterone on sodium transport are inhibited while the action of amphotericin B is unaffected.

Amiloride consists of a substituted pyrazine ring attached to a guanidine group (Fig. 4). Guanidine, but not pyrazine analogues, was found to have a similar action to amiloride but was about 1000 times less effective. The results suggest that the interaction of the amiloride and its receptor site is primarily dependent on the guanidinium moiety of the molecule and may involve the positive charge that it carries. The neurotoxin tetrodotoxin reduces the passage of sodium ions across nerve axons (Narahashi *et al.* 1964) and it has been suggested that the guanidinium moiety plays a vital role in this action also (Kao & Nishiyama, 1965). Tetrodotoxin does not affect the bladder in a manner comparable to amiloride (Marumo, Asano, Sasoka & Koshikawa, 1967) but the similarity of their actions suggests that they may each ultimately act on biochemically identical processes in each tissue.

The available information suggests that sodium transfer across the toad bladder takes place principally in two successive steps; passive movement across the mucosal boundary into the cell followed by its expulsion against an electrochemical gradient at the serosal side, the site of the so called sodium 'pump' (Leaf, 1965). Vasopressin, aldosterone and amphotericin B are thought to increase the permeability of the mucosal barrier to sodium (Leaf, 1965; Crabbé & De Weer, 1965, Lichenstein & Leaf, 1965) and ouabain inhibits the serosal sodium 'pump' (Herrera, 1966). Amiloride inhibited the actions of the hormones but not that of amphotericin B. The latter's action could however be reduced by ouabain. Thus the present evidence suggests that amiloride restricts the entry of sodium across the mucosal side of the toad bladder epithelial cells.

Frazier, Dempsey & Leaf (1962) demonstrated that sodium transfer across the mucosal side of the bladder obeyed saturation kinetics suggesting its interaction with a substrate there. The non-competitive antagonism of sodium and amiloride does not indicate that the latter directly interacts

with such a substrate but conceivably it could interfere with the supply of such a material. For instance, competition of amiloride for some metabolic compound similar to guanidine could be occurring. Amiloride does not combine with sulphydryl groups or inhibit the actions of the enzymes, carbonic anhydrase, adenyl cyclase or ATPase (see Baer *et al.* 1967; Merck Laboratories, unpublished observations) thus excluding a number of potentially important metabolic sites of action. Alternatively, simple steric interference with sodium movement shielding the site of the mucosal sodium-substrate interaction from sodium could be occurring. Blocking a 'pore' leading to such a substrate would be conceptually fashionable but not necessarily correct. Whatever the mechanism of action of amiloride may be, such information will undoubtedly be relevant to elucidation of the process of active sodium transport across epithelial membranes.

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