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EFFECTS OF SOME PYRAZINECARBOXAMIDES ON SODIUM TRANSPORT IN FROG SKIN

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1 The inhibitory effect of amiloride (N-amidino-3,5-diamino-6-chloropyrazinecarboxamide) on sodium transport in isolated skin of frog has been compared with 17 of its analogues. The dissociation constant of amiloride for passive sodium channels was 181.9 ± 8.9 nM, and the maximal percentage inhibition of sodium transport was 101.3 ± 0.4% (means of 123 measurements) when measured at a sodium concentration of 111 mM.

2 The N-benzylamidino and N-*o*-chlorobenzylamidino compounds had affinities approximately 20 times larger than those for amiloride, and produced maximal inhibition of transport.

3 Substitution of chlorine in the 6-position by other halogens showed that the bromo-compound was equally active to amiloride, whereas the iodo derivative had an affnity equal to 15% of that for amiloride.

4 Substitution in the 5-amino group in 10 compounds reduced the affinities to less than 1% of that of amiloride, without affecting their ability to produce complete inhibition of transport.

5 N-Amidino-3,5-diaminopyrazinecarboxamide was unique in that it produced an unusual concentration-response relationship.

Introduction

The compound N-amidino-3,5-diamino-6-chloropyrazinecarboxamide (amiloride) is used as a potassium sparing diuretic acting on the distal tubule of the kidney. The mechanism of action of this substance has been worked out mainly from studies using amphibian sodium transporting epithelia (Bentley, 1968; Ehrlich & Crabbé, 1968; Salako & Smith, 1970; Dörge & Nagel, 1970; Biber, 1971; Cuthbert, 1976a). It is clear that amiloride blocks passive sodium entry into the transporting compartment of epithelia and not the processes of active sodium extrusion across the serosal surfaces.

Amiloride has been shown to affect passive sodium movement in a wide variety of tissues (see Cuthbert, 1974a) and the list is continually being extended. Two recent examples are the rat epididymis (Wong & Yeung, 1976) and the eggs of the sea urchin (Johnson, Epel & Paul, 1976). The latter indicates that passive sodium channels sensitive to amiloride are not confined to epithelia, and furthermore this type of channel is probably of considerable evolutionary antiquity, making studies on these channels of considerable biophysical interest.

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A number of studies with [^{14}C]-amiloride have made it possible to estimate the density of sodium channels in frog skin and toad bladder epithelial cells, and in turn, this has enabled investigations to be made of the ways in which the properties of the channels are changed when the tissues are perturbed with hormones, sodium deprivation, applied potentials etc. (Cuthbert, 1973a; 1974b; Cuthbert & Shum, 1974; 1975; 1976a, b, c).

In the papers describing the synthesis of a large number of pyrazine-carboxamides (Bicking, Mason, Waltersdorf, Jones, Kwong, Robb & Cragoe, 1965; Cragoe, Woltersdorf, Bicking, Kwong & Jones, 1967; Bicking, Robb, Kwong & Cragoe, 1967; Jones, Bicking & Cragoe, 1967) and diuretic activities were reported for dogs, rats and DOCA-treated rats. We have re-examined a selected number of these compounds using isolated skin of the frog to obtain precise quantitative data on the affinity of these compounds for sodium channels. The study was undertaken with four separate and clearly defined aims. These were (i) to compare the relative activities of compounds from measurements made with amphibian isolated epithelia with those obtained with DOCA-treated rats, (ii) to find more suitable ligands other than amiloride for use in binding studies, (iii)

to define more completely the chemical moiety required for sodium channel blockade, which may be of use in the design of polyvalent diuretic compounds, and (iv) to examine compounds for novel actions.

Methods

All experiments were carried out on the abdominal skin of the frog, *Rana temporaria*. The animals were kept partially immersed in tap water at room temperature. The experiments were carried out during May and June.

Skins were clamped between perspex chambers and bathed on both sides with Ringer solution (12 ml). Mucosal and serosal solutions were bubbled with air, which served both to aerate the solution and to mix small volumes of added drug solutions. Skins were voltage clamped at zero potential (short circuited) by an automatic voltage clamp (Schema Versatae S/V 360c) and the clamping current (short circuit current) was displayed continuously on a pen recorder (Bryans 27000 series). The electrodes for recording potential and passing current were conventional. The area of skin exposed was 7.5 cm^2. Under the conditions of these experiments the short circuit current (SCC) is equivalent to the net mucosal to serosal sodium flux (Ussing & Zerahn, 1951).

Inhibitors of transport (amiloride and analogues) were added to the mucosal bathing solution to give cumulative response curves. Time was allowed between each addition of drug for equilibrium to be achieved. Usually 2 min was sufficient, but with some analogues 5 to 10 min was required. In all experiments amiloride was used as a standard and a response curve to an analogue was bracketed by two amiloride response curves. As many as ten cumulative response curves were obtained with a single skin, such experiments taking about 6 h, allowing for the time required to wash away the drugs and for an equilibrium short circuit current to be re-established. Not less than six different concentrations of a drug were used to establish each curve, and inhibition of SCC by at least 90% was achieved.

Drug solutions were made either in distilled water, or for less soluble compounds the drug was dissolved in a few drops of 4N isethionic acid before dilution. Drug solutions were generally titrated to neutrality before use, but occasionally precipitation occurred in concentrated solutions above pH 6. These solutions were not sufficiently acid to alter the pH of the buffered bathing solution. For some of the less soluble and weakly active analogues there was some dilution of the mucosal sodium concentration (up to 20%). However, this change is not in the range of sodium concentration which affects the level of SCC. Equal volumes of bathing solution were added to the serosal solution when required to maintain zero hydrostatic gradient across the skin.

The response to each cumulative addition of drug was converted to percentage inhibition of initial SCC. A computer program was used to fit a hyperbola to the data from each cumulative response curve by the method of Wilkinson (1961). This is a weighted non-linear regression method, the standard errors being derived from the variance, and is particularly suited to fitting concentration-response or enzyme kinetic data. The data were fitted to an s/v versus s plot to obtain an estimate of Ki and V_{max}. These estimates were used to fit the data to a hyperbola and an iterative procedure used to minimise the weighted least squares derivation. The standard errors are indicative of the goodness of fit of the data. Values of Ki (concentration of inhibitor producing 50% inhibition of the maximal inhibition of SCC) and the percentage maximal inhibition of SCC, with their standard errors for each individual experiment, were obtained directly from the computer printout, together with a plot of the response curve in the form of concentration/% inhibition versus concentration.

The bathing solution used throughout these experiments had the following composition (mM): NaCl 111, KCl 2.0, $CaCl_2$ 1.0, glucose 11.0 and Tris buffer (pH 7.6) 5.0.

Results

Kinetic parameters for amiloride

Data from 123 concentration-response curves to amiloride in 37 different skins were analyzed as indicated in the methods section and the distributions of the values of maximal percentage inhibition of SCC and Ki are shown in Figure 1. The mean value for maximal percentage inhibition was $101.3 \pm 0.4\%$ (123 measurements). In 70% of the experiments the maximal percentage inhibition obtained from extrapolation of fitted curves was in the range 96–104%, and 97% of the values were in the range 90–110% (Figure 1a). The mean value for Ki in the same experiments was 181.9 ± 8.9 nM, but only 7% of the 123 values fell within 10% of the mean (Figure 1b), even though the standard errors of individual Ki values were small. In 84 experiments the standard error for individual measurements was less than 10 nM, and in only 5 experiments was this standard error more than 10% of the Ki value. Thus variation in Ki values is mainly dependent on variation between individual tissues, as the distribution of the mean Ki values for 37 skins shows a similar range and frequency distribution as does the individual values of Ki (Figure 1c).

To examine the possibility of a relation between the level of sodium transport, measured as SCC, and

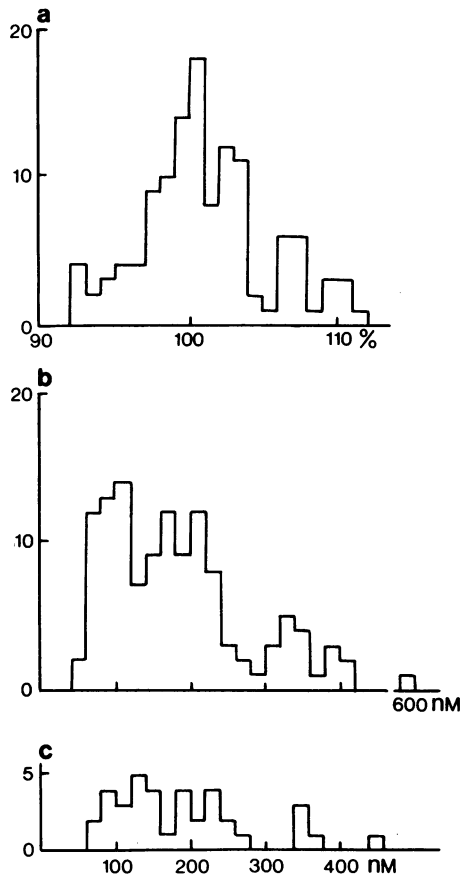


Figure 1. Frequency diagrams showing the distribution of kinetic parameters for 123 experiments with amiloride made using 37 different skins. The number of observations is given on the ordinate scale. On the abscissa scale is shown (a) % maximal inhibition (grouped into intervals of 1%); (b) individual values of *Ki* (grouped into intervals of 20 nM) and (c) mean *Ki* values for each skin (grouped into intervals of 20 nM). Values of maximal inhibition (*V*max) and *Ki* were obtained from data fitting procedures. (see Methods).

Ki values a plot was made of the mean values of *Ki* for each of 37 skins versus the initial SCCs (Figure 2). The correlation coefficient was only 0.3, and even this value was significant only at $P < 0.1$.

Previous observations have indicated that the relation between amiloride concentration and response approximates to a rectangular hyperbola, although occasional skins behave as if there are two populations of channels with different affinities for amiloride (Cuthbert & Shum, 1974). Recently two reports (Macchia & Helman, 1976; Benos, Simon, Mandel & Cala,

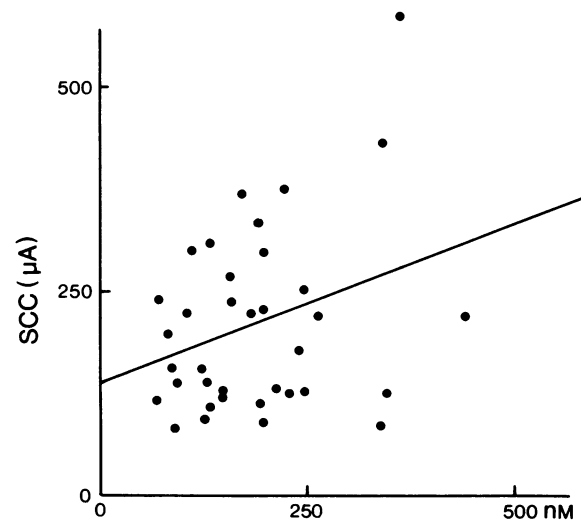


Figure 2 Mean values of Ki for amiloride (nM) in 36 skins plotted versus initial short circuit current (μA). Slope of regression line equals 0.37 ± 0.2 nM μA^{-1} (95% confidence limits −0.04 to 0.77), intercept is 140.5 ± 41.4 μA. Correlation coefficient of regression line is 0.3 ($0.05 < P < 0.1$).

1976) have indicated that some frog skins, particularly those of *R. pipiens*, behave as if they contain two populations of receptors, the more sensitive receptors controlling some 15% of the total SCC. For these reasons it was particularly important to demonstrate with *R. temporaria* skins, under the conditions we have used, that the presence of a small proportion of very sensitive receptors would make only a minimal difference to our estimates of *Ki*.

Figure 3 illustrates three computer-derived reciprocal polts which are typical. They were chosen to be representative of skins with low, medium and high *Ki*s, and within experimental error the reciprocal plots are linear. A second population of very sensitive receptors would cause the plots to curve toward the abscissa at low amiloride concentrations.

Experiments with amiloride analogues

From the results given for amiloride it is apparent that the *Ki* may vary with different tissues and therefore both absolute values of *Ki* for the analogues are given, together with the relative molar activity compared to amiloride. The latter was calculated from the mean value of *Ki* for amiloride for the two response curves which bracketed the response curve to the analogue. Similarly, values for maximal inhibition of SCC are given, both as a percentage and as a ratio, analogues causing less maximal inhibition having

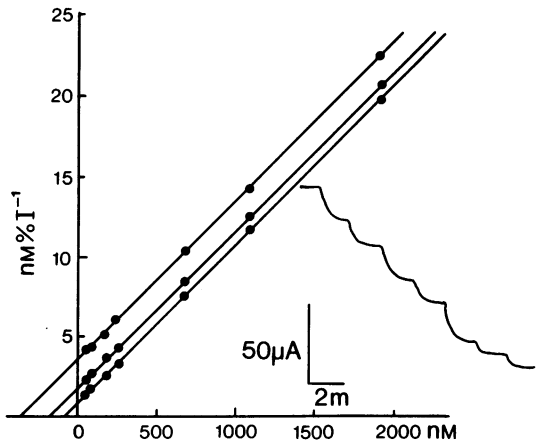


Figure 3 Reciprocal plots of concentration/% inhibition of SCC versus concentration for amiloride in three separate skins. The plots were derived by the method of Wilkinson (1961). The values for *Ki* and maximal inhibition of SCC for the three examples are 354.8 ± 12.8 nM and 100.3 ± 1.3%, 179.8 ± 1.9 nM and 100.5 ± 0.3%, 86.3 ± 2.6 nM and 100.9 ± 0.7%. Each reciprocal plot was derived from a single cumulative concentration-response curve, the one illustrated being used to derive the middle of the three plots. Amiloride concentrations were 41.6, 83.3, 167, 250, 667, 1082 and 1915 nM. Two minutes were allowed at each concentration for a steady state response to be achieved, the time marker corresponds to zero SCC. Skin area 7.5 cm².

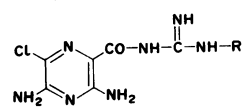
values of less than 100. Where the activity of amiloride analogues was examined on more than one occasion the mean values of the absolute values and the ratios are given.

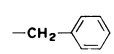
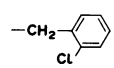
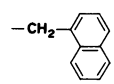
Table 1 gives the activities of four amiloride analogues with substituents on the terminal amino group in the guanidinium radical. All four analogues had greater affinity for the sodium channels in frog skin than amiloride, and like the latter all four produced complete inhibition of sodium transport. In particular, the benzyl and *o*-chlorobenzyl derivatives had about twenty times the affinity of amiloride. Introduction of an even larger hydrophobic grouping (α-naphthylmethyl) gave a compound which was only four times more active than amiloride, while a small hydrophilic grouping (hydroxyethyl) produced only a minimal increase in affinity.

Variation in halogen substitution in position 6 was also examined, and the results are summarized in Table 2. All three halogen substituted compounds were capable of causing complete inhibition of sodium transport. Activity was optimal with the chloro- and bromo-compounds which had approximately equivalent activity. The affinity fell to 13% of that of amiloride with the iodo-compound.

One of the most interesting compounds revealed by this study was the amiloride analogue with no substituent in position 6. This compound was examined on six occasions in 3 separate skins. The data were processed by computer in the normal way and revealed a compound with modest activity, but one

Table 1 Substitution on the terminal amino group in the guanidine radical



R	*Ki(nM)*	*Relative Molar activity*	*% Maximal inhibition*	*Relative maximal inhibition*	*DOCA-rat test*
H (amiloride) (123)	182.0	100	101.3	100	+4
 (benzamil) (a)	10.0	1820	100.0	99	+4
 (2)	12.4	2337	96.2	98	+4
 (3)	45.2	410	97.0	94	+3
$-CH_2-CH_2-OH$ (4)	64.1	139	98.1	98	+4

(a) Values for benzamil were taken from Cuthbert (1976a) and compared to the mean result for amiloride in this study. Numbers in parentheses indicate the number of measurements.

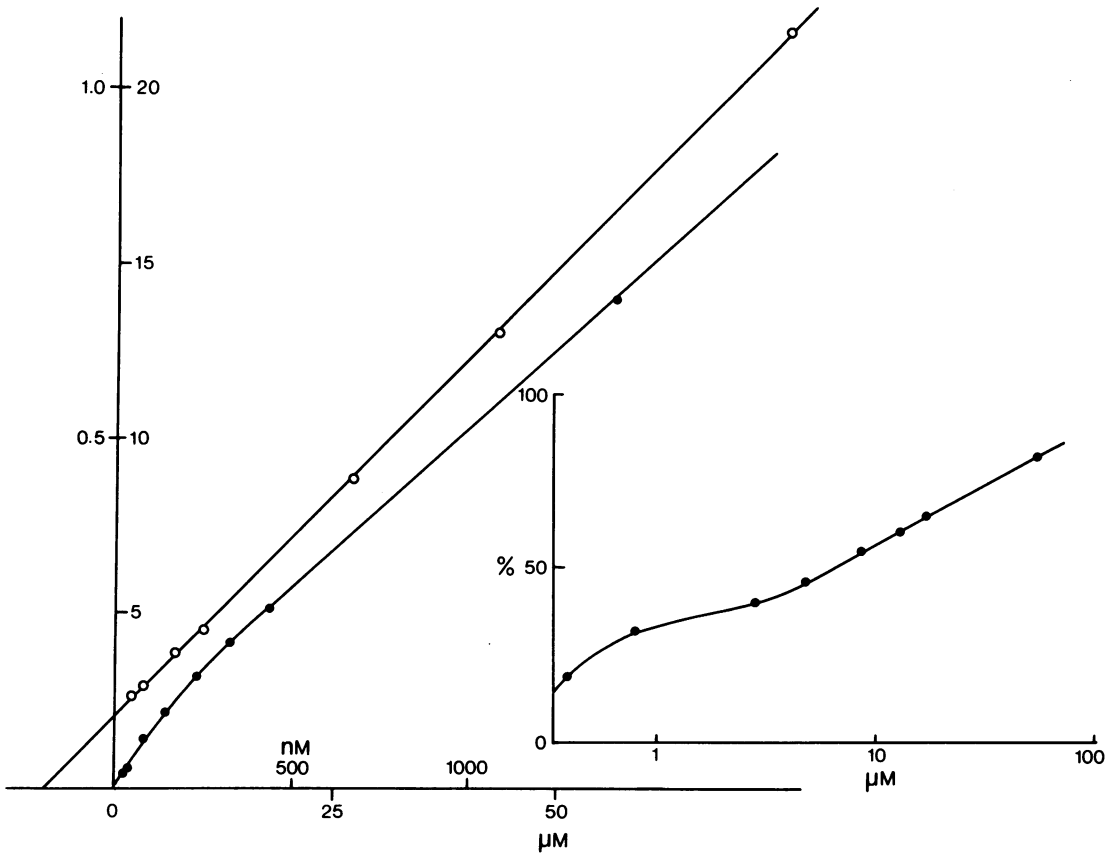
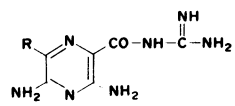


Figure 4 Reciprocal plots for amiloride (○) and N-amidino-3,5-diaminopyrazinecarboxamide (●) measured on the same skin. Calibrations on the inside of the axes refer to amiloride, while those on the outside refer to its analogue. The units for the ordinate scale are nM per % inhibition for amiloride, and μM per % inhibition for the analogue. The inset shows the log concentration-response curve for the analogue, the response being given as % inhibition of SCC. The value of *Ki* for amiloride was 200.6 ± 5.7 nM and for maximal inhibition was 98.6 ± 0.8%.

Table 2 Halogen substitution in position 6



R	*Ki(nM)*	*Relative Molar activity*	*% Maximal inhibition*	*Relative maximal inhibition*	*DOCA-rat test*
— (*6*)	[1049]	[14.8]	[80.7]	[79.8]	+1
Cl (123)	182	100.0	101.3	100.0	+4
Br (2)	179	118.0	97.9	96.8	+3
I (3)	1908	12.9	95.6	99.0	+3

Numbers in parentheses indicate the number of measurements. Amiloride is the chloro analogue. The values for N-amidino-3,5-diaminopyrazinecarboxamide (as all data in the Tables) were obtained by the fitting of the data to a single hyperbola. However (see text), it is considered that this fitting procedure is inappropriate in this case and the values are given in square brackets.

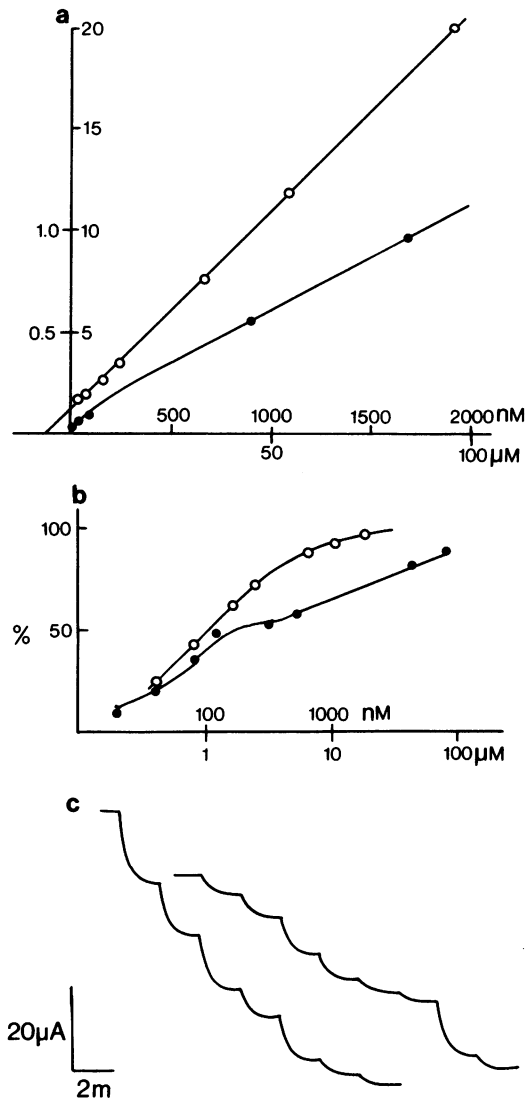


Figure 5. Responses to amiloride (○) and to N-amidino-3, 5-diamino-pyrazinecarboxamide (●) measured in the same skin. (a) Reciprocal plots. Units for ordinates are nM per % inhibition for amiloride and μM per % inhibition for the analogue. Calibrations inside axes refer to amiloride. Note not all the data for the analogue can be shown on the scale used. (b) Log concentration-response (% inhibition of SCC) for amiloride and its analogue. Same experiment as in (a). For the analogue the curve was computer-fitted to accommodate two pools of receptors (see text). (c) Actual responses used to construct (a) and (b). The left hand curve is for amiloride and the cumulative concentrations were 42, 83, 167, 250, 670, 1090 and 1920 nM. The cumulative concentrations of the analogue were 0.2, 0.4, 0.8, 1.2, 3.2, 5.2, 45 and 84.7 μM. The time marker also indicates zero SCC. Notice that steady state responses to both compounds were achieved within 2 minutes. The steady state current had fallen by about 15% between testing the two compounds, although the initial inhibition by amilioride was fully reversed.

which failed to give complete inhibition. However, the standard errors both for individual values of K_i and maximal inhibition were considerable, indicating a poor fit of the data to a hyperbolic function. For this reason the data were re-examined, and some most unusual features were found. As these results may be of considerable importance for the understanding of passive sodium channel mechanisms, two of the experiments, which are typical of the rest, are illustrated in some detail in Figures 4 and 5. In Figure 4 a semi-log plot of percentage inhibition of SCC versus concentration of the analogue (N-amidino-3,5-diamino-pyrazinecarboxamide) revealed a non-linear relationship. A reciprocal plot of the data gave a curve, concave towards the abscissa and perhaps indicating the presence of more than one receptor type, yet a similar plot for amiloride determined on the same epithelium gave a straight line indicating a single population of receptors. By extrapolation from the linear part of the reciprocal plot for N-amidino-3, 5-diaminopyrazinecarboxamide the value for maximal percentage inhibition was 86% compared to the value of 71% given by fitting a hyperbola in this particular experiment.

In a further experiment illustrated in Figure 5 the plateau part of the log concentration-response curve occurred at around 50% total inhibition of current compared to only 30% in the previous experiment. The initial part of the curve parallels the amiloride

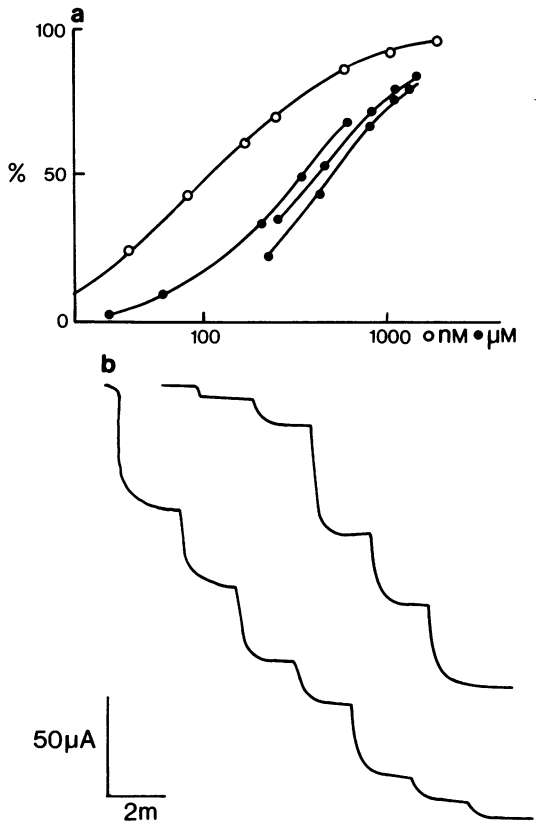


Figure 6 (a) Log concentration-response curves for amiloride (O) and for the 5-propylamino analogue (●) measured on the same skin. The response is expressed as % inhibition of SCC. (b) Actual responses to amiloride (left hand) and the 5-propylamino derivative. Note both materials produce responses with similar time courses. The time marker also indicates zero SCC. Solubility limited the maximal responses obtained with the 5-propylamino compound.

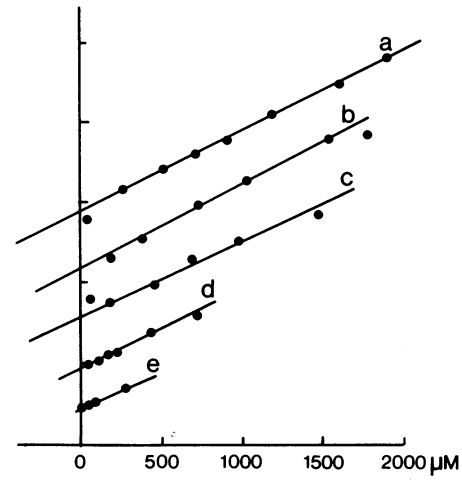


Figure 7 Reciprocal plots for five of the compounds given in Table 3. The abscissa scale gives the drug concentration in μM. The ordinate scale gives the ratio of the drug concentration (μM) to the percentage inhibition of SCC. Each calibration on the ordinate equals 10 units (μM/% inhibition). The analogues referred to in this figure are (from top to bottom), (a) ethylamino, K_i = 405.8 ± 38.0 μM, 99.8 ± 3.0% (b) hydroxyethylamino, K_i = 289.1 ± 66.4 μM, 96.0 ± 5.9% (c) methylethylamino, K_i = 308.6 ± 48.8 μM, 100.6 ± 5.5% (d) cyclopropylamino, K_i = 128.4 ± 16.9 μM, 99.3 ± 4.5% (e) 5-unsubstituted, K_i = 25.59 ± 1.2 μM, 104.9 ± 1.5%. The standard errors for the values of K_i and maximal % inhibition were derived by the method of Wilkinson (1961).

curve, and there is no indication of a non-linear response to amiloride, which is again emphasized by the reciprocal plots. The responses to amiloride were measured before those to the non-halogenated analogue, but an equally linear response to amiloride was obtained after the response curve to the analogue had been completed and the drug removed. Extrapolation from the linear part of the reciprocal plot for the analogue indicated that around 94% maximal inhibition of SCC could be achieved. In addition, the data from this experiment were fitted to a double hyperbolic function (Harwell Library Program MA14A and MA14C) which gave values of 0.75 ± 0.27 μM and 63.9 ± 9.8% for the values of K_i and maximal inhibition for the most sensitive population of receptors. Finally Figure 5 shows actual responses to amiloride and to N-amidino-3,5-diaminopyrazinecarboxamide obtained from the same epithelium. Both compounds produced rapid effects which reached steady state within 2 minutes. The effects of both compounds were readily reversible on washing.

Table 3 indicates the results obtained with ten analogues substituted in position 5. Clearly position 5 is of extreme importance for the activity of amiloride, as all the ten analogues had low activity compared to amiloride, some having affinities of less than 1000 times that of the parent compound.

When there was no substituent on position 5 the affinity fell to 1% of that of amiloride. Introduction of a benzyl group on the terminal guanidine moiety of 5-deamino amiloride increased affinity approximately eight-fold. The fractional increase is similar

to that obtained when the same grouping is introduced into the amiloride molecule. Substitution on the 5-amino position reduced the affinity of the analogues to less than 1% of that for amiloride. Thus an amino group in position 5 is optimal, any substitution producing a more drastic reduction in affinity than that caused by removing the amino group altogether.

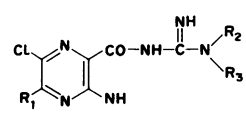
In spite of the low affinity of 5-amino substituted compounds they were able to produce complete inhibition of sodium transport. In two instances, with 5-propylamino and the 5-allylamino compounds, limited solubility made it impossible to obtain sufficient data for computer fitting. However, from the limited amount of data it appeared the compounds produced parallel log concentration-response curves to those for amiloride (Figure 6) suggesting that complete inhibition of transport was theoretically possible.

Figure 7 shows five reciprocal plots illustrating data for some of the compounds in Table 3. There is no evidence, except perhaps for the ethylamino compound, that more than one population of receptors is revealed, in contrast to the finding as before with N-amidino-3,5-diaminopyrazinecarboxamide (Table 2).

Discussion

In this study we have compared the activity of 17 amiloride analogues with the parent compound in isolated epithelium of an amphibian, the abdominal skin of the frog, *R. temporaria*. Comparisons were made of both the affinity of the compounds for the channels, as well as their ability to cause maximal inhibition. The affinity of a compound may be measured as the reciprocal of the concentration required to give 50% inhibition of sodium transport. However, this value will only have precise quantitative meaning provided the inhibitory process is the result of a combination of drug with a single population of receptors. In previous reports from this laboratory (Cuthbert, 1974b; Cuthbert & Shum, 1974) it has been shown that the relation between fractional inhibition and inhibitor concentration approximates to a single hyperbolic function, although whether the relationship is the result of simple mass action kinetics or due to a more subtle interaction mechanism based on a two-state model of the channel (Cuthbert, 1974b) cannot be distinguished experimentally. It was also demonstrated at the same time that occasional skins appeared to have two populations of channels, the relative ratio of these two populations being

Table 3 Substitution at position 5



R_1		R_2	R_3	Ki(μM)	Relative Molar activity	% Maximal inhibition	Relative maximal inhibition	DOCA-rat test
H	(3)	H	H	29.4	1.2	104.7	106	+3
H	(2)		H	4.0	8.9	97.5	101	+3
–NH–C_2H_5	(2)	H	H	585	0.034	108.8	107	+3
–NH–C_3H_7	(3)	H	H	437	0.048	—	—	+3
	(5)	H	H	349	0.029	104.7	105	+4
	(1)	H	H	344	0.040	107.8	108	+4
–NH– 	(2)	H	H	103.7	0.13	95.0	98	+3
–NH–CH_2– 	(2)	H	H	115.9	0.10	93.9	92	+4
–NH–CH_2–CH_2–OH	(1)	H	H	289	0.089	96.0	95	+1
–NH–CH_2–CH=CH_2	(1)	CH_3	CH_3	1500	0.00015	—	—	+4
–NH_2	(123)	H	H	0.182	100	101.3	100	+4

Numbers in parentheses indicate number of measurements.

dependent on the sodium concentration (Cuthbert & Shum, 1974). Two populations of amiloride-sensitive channels have also been demonstrated in the mucosal epithelium of the toad colon (Cuthbert, 1973b). Since then, other groups have shown that more than one population of channels may exist in amphibian epithelia. For example, Macchia & Helman (1976) found for skins of *R. pipiens berliendieri* and *R. temporaria* that the most sensitive receptor pool accounted for 16.5% and 12.5% of the SCC respectively, when measured in normal sodium Ringer solution. In the experiments of this series with *R. temporaria* skins and amiloride, no evidence for more than one receptor pool has been found, as indicated by the linearity of the reciprocal plots and the small standard errors for the kinetic parameters obtained when the data was fitted to a single hyperbolic function.

While these experiments were underway a report appeared from Benos *et al.* (1976) who have also examined the activity of a number of amiloride analogues on frog skin, some of which are common to this study. Unfortunately they studied the analogues at a single concentration only, making a comparison of relative potencies difficult. They also found that response curves to amiloride were non-linear, but used *R. pipiens* rather than *R. temporaria*, as in this study. This emphasizes that in pharmacological studies with frog skin it is important to specify species, season, temperature, pH and composition of the Ringer solution etc. and that comparisons made with different conditions may be invalid.

Under our conditions amiloride causes complete inhibition of SCC with rather little variation between different tissues (101.3 $\pm$ 0.4% inhibition). In contrast, the values of K_i varied quite considerably, the major part of this variation was between tissues, although variation within a single tissue was also observed. For this reason when the activity of analogues was examined, the data were calculated with respect to the responses to amiloride which bracketed the responses to the analogues.

It is pertinent to ask why the affinity for amiloride is variable. First, after frog skin has moulted it is virtually insensitive to amiloride (Nielsen & Tomlinson, 1970) but the sensitivity returns in a few hours, particularly when calcium is present in the bathing solution (Cuthbert, Okpako & Shum, 1974). Secondly, if the two-state model for the channel is correct, then the macroscopic affinity of amiloride for the channels will depend on the relative proportion of the two forms. For example, antidiuretic hormone which is believed to increase the ratio of open to closed forms, and hence increase transport, also reduces the affinity of amiloride (Cuthbert & Shum, 1974; 1975). We thought that the initial SCC might be an indicator of the relative proportion of open to closed channels, and therefore looked at the correlation between K_i and initial SCC. No correlation was found, perhaps indicating that resting SCC has multiple determinants.

Substitution in the guanidino moiety did not adversely affect activity, in fact with planar substituents such as benzyl and *o*-chlorobenzyl an increased hydrophobic binding capacity was probably responsible for the increased affinity, by raising the activation energy required for dissociation without affecting the rate of association, which is mainly diffusion limited. Clearly steric factors are also important as activity fell again when an even larger grouping, α-naphthylmethyl, was introduced. Small groups with little hydrophobic binding capacity would be expected to have rather minimal effects, as was found with the hydroxyethyl analogue. As was pointed out previously (Cuthbert, 1976a) substituents on the terminal amino of the guanidine moiety allow the stereochemistry of the charged guanidinium to be preserved, a component of the structure which appears to be essential for blocking activity of this type. For example 3,5-diamino-6-chloropyrazinecarboxylic acid is without activity (Bentley, 1968). High affinity compounds with substituents in the guanidinium grouping are potentially important for receptor binding studies.

Substitution at position 6 with different halogens indicates that probably both steric and electron withdrawing factors are important, and data for other analogues such as 6-cyano and 6-trifluoromethyl would be useful in deciding the relative importance of these two factors.

While the guanidinium group is essential for activity, position 5 on the pyrazine ring is crucial for conferring high affinity of these compounds, indicating that the 5-amino group of amiloride probably takes part in the receptor interaction. When there is no substituent in position 5 the activity falls to 1% of that for amiloride. However, addition of a guanidine substituent, such as benzyl, again increases activity as it does in the parent molecule. Substituents on the amino group at position 5 all reduce the affinity, yet remarkably these compounds are still able to cause complete inhibition of transport, although their affinities are often considerably less than 1% of that of amiloride.

The compound N-amidino-3,5-diaminopyrazinecarboxamide had apparently unique activity. With this analogue alone of the 17 examined, a non-linear concentration-effect relationship was found, while in the same tissues linear responses were obtained to amiloride. If the results are interpreted in terms of two receptor pools then the high affinity pool may account for as much as 60% of the SCC. It has already been pointed out that post-moult skins are very insensitive to amiloride and that sensitivity returns within a few hours of moulting. Extremely

subtle changes in receptor configuration, temporally dispersed among the population, must be occurring if different population kinetics are revealed by different drugs. It may be worthwhile investigating other substituents in the 6-position other than halogens to explore this possibility. An alternative explanation for the result is that the material contained a small proportion of highly active impurity, although it seems very unlikely this would be amiloride when the synthetic pathway is considered (Cragoe *et al.*, 1967). If, however, this is the explanation then it would mean that N-amidino-3,5-diaminopyrazinecarboxamide is a 'partial antagonist' capable of inhibiting SCC by 40–50%. This is an exciting prospect from the point of view of the two-state model of the channel (Cuthbert, 1974b). Compounds with similar microscopic affinities to the open and closed forms would not be expected to cause complete inhibition. This again indicates that studies on 6-substituted analogues may be revealing. Yet a further possibility is that the non-halogen compound produces a negatively co-operative effect, but that after the conducting channel density is reduced a normal, that is one to one, stoichiometry resumes.

Amiloride has a pKa of 8.7, benzamil has a pKa of 8.2, and the other analogues have values not very different from these. As all the experiments were performed at pH 7.6 a large proportion of the material was in the protonated form (Cuthbert, 1976a, b). Previous reports have shown it is the charged form of amiloride, which is the active moiety, that combines with an ionised receptor grouping with a pKa of around 5 (Cuthbert, 1976a). From this it is clear that changing pH will have only minimal effects on the ratios reported here.

All the analogues used in this study have been screened previously for their effects on the urinary Na/K ratio in DOCA-treated rats by the method of Marcus, Romanoff & Pincus (1952). They were scored depending on the dose (in μg per rat) required to cause a 50% reversal of the DOCA Na/K effect, the scores ranging from +4 (<10 μg/rat) to ± (>800 μg/rat). The scores are indicated in Tables 1 to 3. There is little parallelism between the two sets of data, although precise quantitative comparisons are not possible. Furthermore it is likely that many of the analogues had been partially metabolised, perhaps to amiloride, before acting on the kidney. Nevertheless amphibian isolated epithelium is a convenient and inexpensive way of screening for this type of activity *in vitro*.

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