MOLECULAR PHARMACOLOGY, 10, 880-891

Binding of Amiloride to Sodium Channels in Frog Skin

A. W. CUTHBERT AND W. K. SHUM[1]

Department of Pharmacology, University of Cambridge, England

(Received June 12, 1974)

SUMMARY

CUTHBERT, A. W., AND SHUM, W. K.: Binding of amiloride to sodium channels in frog skin. *Mol. Pharmacol.* **10**, 880-891 (1974).

Interactions between sodium ions and amiloride with the mucosal membrane have been studied using voltage-clamped frog skin. At sodium concentrations greater than 10 mEq/liter the interactions of amiloride with the mucosal channels appear to be competitive and have a stoichiometry of 1:1. Binding of amiloride to sodium channels has been measured using [^{14}C]amiloride. Reasonable agreement was found for the affinities of amiloride and triamterene derived from binding studies compared to values from inhibition studies. Antidiuretic hormone had no effect on the number of sodium channels in the mucosal surface of the skin, while the nominal currents passing the channels were doubled. The implications of the findings are discussed in relation to the mechanism by which antidiuretic hormone increases the mucosal permeability for sodium.

INTRODUCTION

Recently a method was described for estimating the upper limit for the number of sodium channels in the mucosal surface of frog skin epithelium (1). This paper describes further binding and inhibition, studies using the diuretic drugs amiloride and triamterene. The findings support the view that channel density in frog skin epithelium can be measured with reasonable accuracy. The labeling technique has also been used to investigate the effects of vasopressin on channel density in this epithelium. Brief accounts of some of the findings have been presented elsewhere (2, 3).

METHODS

Short-Circuit Current Measurements

Sodium transport across abdominal skin taken from frogs (*Rana temporaria*) was measured as short-circuit current. The area of skin used in all experiments was 9.6 cm^2, and the cells used for mounting the tissue were of the type described previously (1).

Labeling Experiments with [^{14}C]Amiloride

The method was essentially that described previously (1), with one minor modification. Basically the experiments consist of making pairs of determinations by measuring labeling of skins with low concentrations (less than 20 nM) of [^{14}C]amiloride in the absence and presence of a 100-fold excess of unlabeled amiloride, and with the simultaneous recording of SCC.[2] Five pairs of observations from a single skin are used to compute the channel density for that skin. In the present experiments six pairs of measurements have been made, but the calculations have been based only on the last five pairs, since it was

This work was supported by a grant from the Medical Research Council.

[1] Recipient of a Medical Research Council award for training in research methods.

[2] The abbreviations used are: SCC, short-circuit current; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; ADH, antidiuretic hormone.

found that the first pair of measurements gave a consistently low estimate of channel density.

In some experiments the channel density was determined in the presence and absence of ADH. The hormone (50 milliunits/ml; Pitressin; Parke Davis) was added to the serosal bathing fluid. [^{14}C]Amiloride had a specific activity of 54 mCi/mmole. All errors are given as standard errors.

Readers may omit the next portion without any loss of understanding of subsequent sections. This material is designed to give precise information about the method used for binding studies with [^{14}C]amiloride.

One important change which has been made from the original description (1) is that the sodium concentration of the mucosal bathing solution has been reduced from 2.5 mEq/liter to either 0 or 1.1 mEq/liter. This increases the apparent affinity of amiloride for the channel (see Figs. 1 and 2) and therefore the channel occupancy at a given amiloride concentration. Thus a somewhat more favorable ratio between bound and unbound amiloride is achieved.

It must be emphasized to others who might contemplate binding studies as described here that particular attention must be paid to the blotting procedure. Consideration of the data given below shows that we obtain blotting errors of less than 5 μl. This is not difficult to achieve if the skin is blotted firmly and the edges are probed with tissue folded to a point. Experiments in which plastic sheeting was substituted for skin showed that blotting errors of less than 5 μl were achieved; furthermore, they also showed that in the Perspex cells used in this study amiloride was not bound to sites from which it could be displaced by excess ligand.

To illustrate the way the raw data are analyzed, the results of three typical experiments are given below. A and B are data from binding experiments using 10 nM [^{14}C]-amiloride in the presence of 1.1 mEq/liter of sodium, whereas C is from a similar experiment in the absence of sodium. Column 1 shows the counts accumulated in 20 min in samples from skins exposed to 10 nM [^{14}C]-amiloride while column 2 shows counts for skins exposed to 1 μM amiloride with 0.01 times the specific activity of the low concentration. Skins were exposed alternately to label and label plus excess unlabeled drug, and five pairs of measurements were obtained from each skin.

	1	2	3	4	5
	1421	1248	89	69	20
	1406	1298	87	77	10
A	1424	1304	89	81	8
	1422	1347	94	81	13
	1398	1289	90	76	14
			90	77	13
	1437	1241	96	74	22
	1597	1501	113	98	15
B	1791	1634	130	113	17
	1924	1781	148	129	19
	1913	1723	143	130	13
			126	109	17
	2465	2150	151	126	25
	2482	2138	152	126	26
C	2438	2376	156	144	12
	2329	2117	140	124	16
	2439	2100	153	129	24
			150	130	20

The counts were converted to disintegrations per minute, making allowance for background (usually around 25 dpm) and using the machine (internal) standard. The counting efficiency was around 60%. Values for disintegrations per minute are shown in columns 3 and 4, and column 5 shows the paired differences, representing the amount of label bound to channels. In A the means of columns 3 and 4 are significantly different when compared by the t-test ($p < 0.001$), and in this experiment amiloride, 10 nM, caused 31% (mean of five values) inhibition of transport, giving a channel density of 217/μm^2. The calculation leading to this conclusion is as follows:

$$\text{Channel density} = \frac{13 \text{ dpm} \times 6.02 \times 10^{23} \text{ channels} \cdot \text{mole}^{-1}}{9.6 \times 10^{8} \mu\text{m}^2 \times 2.2 \times 10^{12} \text{ dpm} \cdot \text{curie}^{-1} \times 54 \text{ curie mole}^{-1} \times 0.31}$$

$$= 217 \text{ channels}/\mu\text{m}^2$$

The results of B show another and not uncommon feature, in that the counts retained by the skin rise gradually throughout the experiment, even though the paired differences remain tolerably constant. This variation makes the means of columns 3 and 4 not significantly different; however, the paired differences are significantly different ($p < 0.001$) from zero using the *t*-test for paired observations (4). Amiloride, 10 mM, caused 42% inhibition of transport in this experiment (mean of five values), giving a channel density of 210/μm^2. Experiment C illustrates a further feature seen with skins bathed in zero sodium and in newly molted skins. This is the increase in unbound material retained by the skin (see also DISCUSSION). The means of columns 3 and 4 are significantly different ($p < 0.005$) for experiment C.

This paper contains the results of 117 binding experiments using amiloride. In all but three of these the mean values of the counts retained by the skins in the presence of [^{14}C]amiloride were greater than the counts retained when the radiolabel was diluted with unlabeled drug. Furthermore, in all but 27 experiments the amount bound was significantly different from zero when tested by the *t*-test for means or for paired observations at the 95% level. Almost without exception the amount bound was insignificantly different from zero when the amiloride concentration was low (less than 5 nM) or when the triamterene concentration was high (2 μM or more), although individual values beyond these limits were sometimes significant.

Solutions

The Ringer's solution used throughout these experiments contained the following (millimolar concentrations): NaCl, 111; KCl, 2; $CaCl_2$, 1; glucose, 11.1; Tris buffer, 5. This solution was bubbled with air before and during the experiments, the pH remaining at 7.6. In some experiments the sodium content of the mucosal bathing solution was varied either by adding more or less NaCl, no correction being made for the change in tonicity. The serosal bathing solution was always normal Ringer's solution as described above. When sodium-free Ringer's solution was used for bathing the mucosal surface of the skins, glucose as well as NaCl was omitted.

In some experiments Ringer's solution containing EGTA (10 mM) was used. The EGTA was neutralized to pH 7.6 with KOH before addition to the Ringer's solution, from which $CaCl_2$ and KCl was omitted.

RESULTS

Inhibition Studies with Amiloride and Triamterene

Cumulative inhibition curves were obtained with respect to either amiloride or triamterene by adding these drugs in increasing amounts to the mucosal bathing solution. Each time a drug was added the SCC fell to a new steady-state value within seconds. The drugs were added in increasing amounts every 2 min, so that a complete inhibition curve could be obtained in about 15 min. The effects of both drugs were readily reversible by washing, and the degree of inhibition produced by a given concentration very reproducible. The extent of the inhibition for a given drug concentration was obtained simply by expressing the reduction of SCC as a percentage of the original uninhibited value.

The effect of changing the sodium concentration of the mucosal bathing solution on the inhibition of SCC by amiloride was studied in 16 experiments. In some of these experiments the sodium concentration of the mucosal solution was varied between 1.1 and 155 mEq/liter. Figure 1 shows a typical result, from which it is clear that sodium antagonizes the inhibitory effect of amiloride on sodium transport. In the example shown amiloride has an apparent affinity of 0.43×10^7 M^{-1} at 111 mEq/liter of Na^+, while at 1.1 mEq/liter of Na^+ the apparent affinity is 7.7×10^7 M^{-1}, an 18-fold increase in affinity. The form of the curves is suggestive of competitive antagonism, since they are tolerably parallel, but inspection shows that the slopes of the curves become less steep at low sodium concentrations, particularly when the concentration falls below 10 mEq/liter. By plotting the apparent IC_{50} (apparent equilibrium constant for amiloride) against sodium concentration (Fig. 2a) a straight-line plot was obtained, indicative of competitive antagonism (5). From this plot the IC_{50} at zero sodium concentration can be obtained by extrapolation. In turn, dose ratios

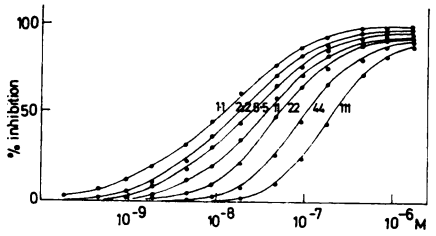

FIG. 1. *Curves relating percentage inhibition of SCC to amiloride concentration at different sodium concentrations for a single skin*

The sodium concentration (milliequivalents per liter) of the mucosal bathing solution is shown against each curve.

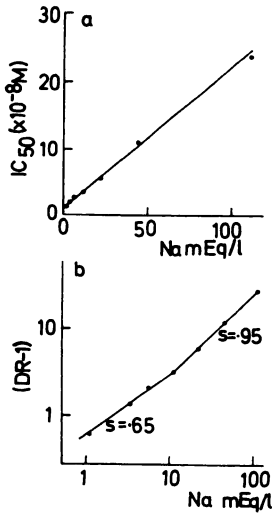

FIG. 2.

a. Hunter and Downs plot (IC_{50} for amiloride) plotted against Na^+ concentration. b. Plot of log (DR − 1) against log [Na^+]. Data for both parts of this figure were taken from Fig. 1.

(DR) for any given sodium concentration can be calculated. A plot of log (DR-1) vs. log Na^+ is shown in Fig. 2b. At concentrations of sodium above 10 mEq/liter a slope of nearly 1 was obtained, which is consistent with the stoichiometry of the reaction between sodium and amiloride being 1:1 (6). Below 10 mEq/liter Na^+ the slope becomes less steep, having a value of 0.65. Full analysis of the type just described was made in three experiments, the data from which are given in Table 1.

In three experiments the affinity of amiloride was measured in a different way. The variation of SCC with sodium concentration was first measured. Afterward the determinations were repeated in the presence of a fixed concentration of amiloride (either 10, 50, or 100 nM) and the affinity was derived from Lineweaver-Burk plots. When only the values for SCC at sodium concentrations greater than 10 mEq/liter were considered, straight-line plots were obtained. The values for the affinity of amiloride were comparable to those given in Tables 1 and 2 (1.01, 1.21, and 0.90 × 10^8 M^{-1} in three experiments). Combining these values with those in the tables gives a mean value for 10 observations of 1.16 × 10^8 M^{-1}. Incidentally, the log-log and reciprocal plots gave values of between 2 and 3 × 10^2 M^{-1} for the affinity of sodium for the transport mechanism in the mucosal surface of frog skin, values in line with those reported by others (7, 8).

To be sure that the increased effectiveness of amiloride in low sodium solutions was due to sodium removal and not to changes in tonicity, a number of experiments were performed in which inhibition by amiloride was measured in dilute and isotonic solution. Figure 3a shows means and standard errors from four experiments, using 5 mEq/liter of

TABLE 1

Interactions between sodium and amiloride

Expt.	Affinity constant for amiloride	Slope of log-log plots	
		Na > 10 mEq/liter	Na < 10 mEq/liter
	M^{-1}		
1	1.33 × 10^8	1.15	0.65
2	1.16 × 10^8	0.95	0.65
3	0.88 × 10^8	0.97	0.68
Mean ± SE	1.12 ± 0.13 × 10^8	1.02 ± 0.06	0.66 ± 0.01

TABLE 2

Binding constant of low-affinity channels

Expt.	Affinity constant
	M^{-1}
1	1.39 × 10^8
2	1.20 × 10^8
3	1.06 × 10^8
4	1.42 × 10^8
Mean ± SE	1.27 ± 0.08 × 10^8

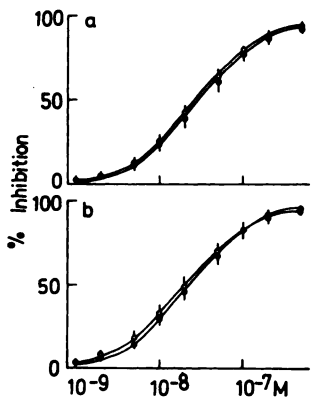

FIG. 3. *Inhibition of SCC by amiloride in the presence of 5 mM Na^+, skins bathed in hypotonic (○) and isotonic (●) mucosal solutions*

a. Solutions were made isotonic with sucrose (216 mM). Results are means of four experiments. Mean basal SCC values were 41.3 μamp in hypotonic and 39.6 μamp in isotonic solutions. b. Same as Fig. 3a, except that tonicity was restored with choline chloride (106 mM). Results are means of three experiments. Mean basal SCC values were 46.1 μamp in hypotonic and 47.3 μamp in isotonic solutions.

Na^+ in the mucosal solution. There was no significant difference between the amount of inhibition obtained with amiloride when the solution was dilute or made isotonic with sucrose (216 mM). A single experiment using 1.1 mEq/liter of Na^+ gave the same result. Furthermore, when tonicity was restored using choline chloride rather than sucrose, there was again no significant difference in the amount of inhibition caused by amiloride (Fig. 3b). It was also found that addition of choline chloride to the hypotonic mucosal solution made no significant difference to the basal short-circuit current. This is important, since with hypotonic mucosal solutions there are Na^+ and Cl^- gradients from inside to out, while when isotonic choline chloride is used there is only a sodium gradient. This suggests that the SCC is not significantly influenced by the serosal to mucosal ion gradients (see DISCUSSION).

In one particular batch of frogs, obtained in January 1973, the interaction between sodium and amiloride showed unusual features. Seven experiments were carried out, of which the result shown in Fig. 3 is typical. At low sodium concentrations the inhibition curves developed distinct inflections. A full analysis of four of these experiments was approached as follows. It was assumed that the inhibition curves with inflections represented the interactions of two populations of receptors with different affinities for amiloride. The proportions of the two receptor types were judged by eye and dotted onto the graph (Fig. 4). It was then possible to judge the concentrations of amiloride producing 50% inhibition of the low-affinity forms of the channel at different sodium concentrations. The calculations then proceeded as before. While this method of analysis is rather subjective, straight-line Hunter and Downs plots (5) were obtained, and the value for the affinity of the less sensitive channels for amiloride was close to those given in Table 1 (see Table 2). The behavior seen in this batch of animals has not reappeared in any subsequent batches.

A less extensive study was made of the interactions between triamterene and Na^+ with the mucosal sodium channels in frog skin. Triamterene showed the same type of interactions as did amiloride. By extrapolation, the concentration of triamterene which blocks 50% of channels at zero sodium concentration is 2.05 μM (mean of five measurements), making triamterene about 240 times less potent than amiloride (i.e., affinity 4.9×10^5 M^{-1}). Sodium ion–triamterene interactions were competitive, with a stoichiometry of 1:1 for the channel at sodium concentrations above 10 mEq/liter.

Binding Studies with [^{14}C]Amiloride

An upper limit for the number of sodium channels in frog skin epithelium was given in an earlier paper (1), but at that time we had no idea as to what fraction of the measured binding was to nonspecific sites not associated with sodium ion translocation. Our aim in these studies was to compare the values of binding constants determined directly with those measured indirectly from inhibition studies as described in the last section. Furthermore, we wanted to examine what effect antidiuretic hormone had on the number of functional sodium channels in the mucosal membrane.

It is worth remembering that since sodium

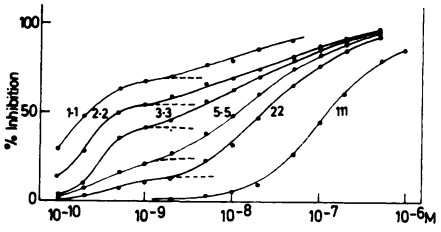

FIG. 4. *Curves relating percentage inhibition of SCC to amiloride concentration at different sodium concentrations for a skin showing anomalous responses*

The sodium concentration (milliequivalents per liter) of the mucosal bathing solution is shown against each curve.

interacts competitively with amiloride, higher receptor occupancies will be obtained at low sodium concentrations. Also, to calculate the sodium channel density it is necessary to know both the amount of amiloride bound and the amount of the inhibition of sodium transport caused by the bound label. If both are known, the amount of amiloride bound at 100% receptor occupancy can be calculated. For these reasons many of the binding experiments described below were made together with the simultaneous recording of sodium transport (SCC).

Amiloride binding curve. Using [^{14}C]amiloride at concentrations between 0.1 and 20 nM and in the absence of sodium, a binding curve for amiloride was constructed using 25 separate skins. Figure 5 shows the individual values for all the experiments, together with their means and standard errors at several concentrations. The curve appears to show saturation, although it would be better if values for concentrations higher than 20 nM had been available. Unfortunately, it is not possible to use higher concentrations, since the ratio of the amount of radiolabel bound to the amount trapped in the extracellular space and bound non-specifically becomes too small. In binding experiments the amount of radiolabel bound was typically some 20% of the total radioactivity retained by the skin. While the binding curve is not ideal, the concentration of amiloride producing 50% saturation of binding is about 7 nM. Thus the affinity for amiloride from the binding curve is 1.4×10^8 M^{-1}, a value not very different from the value of 1.16×10^8 M^{-1} obtained from inhibition studies.

Binding constant for triamterene. The binding constant of triamterene was determined indirectly by measuring the effect of increasing concentrations of triamterene on the binding of a fixed concentration of [^{14}C]-amiloride. The results of a series of binding measurements on 29 isolated skins are shown in Fig. 6. All the measurements were made

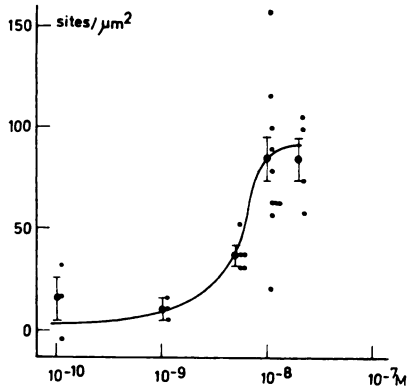

FIG. 5. *Binding curve for [^{14}C]amiloride constructed from results from 25 skins*

Means and standard errors for each amiloride concentration are shown together with individual values. Each individual value represents the mean of five determinations made on an individual skin.

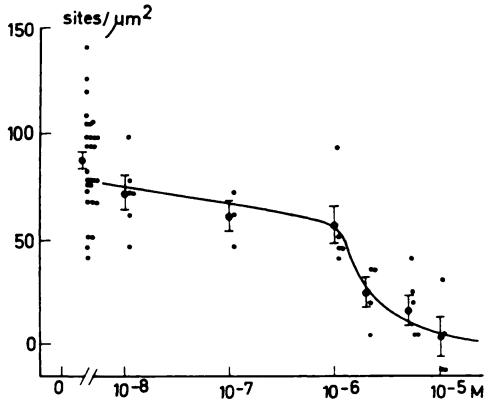

FIG. 6. *Binding of [^{14}C]amiloride (10 nM) in the presence of triamterene*

The binding capacity of 29 skins was determined first without and then in the presence of a fixed concentration of triamterene. Means and standard errors, together with individual values, are shown. Each individual value represents the mean of five determinations made on an individual skin.

in the absence of sodium in the mucosal bathing solution. Binding of amiloride was measured first in the absence of triamterene and then in the presence of a fixed concentration of this drug. It can be seen that increasing concentrations of triamterene reduced and finally abolished the binding of amiloride. The concentration of triamterene which caused 50% inhibition of amiloride binding was 1.4 μM. The affinity of triamterene was calculated from Eq. 1 (9):

$$AK_1 + 1 = TK_2 \qquad (1)$$

where A is the amiloride concentration (10 nM); T is the triamterene concentration causing 50% inhibition of amiloride binding; K_1 is the affinity of amiloride (1.16×10^8 M^{-1} from the inhibition curves); and K_2, the affinity of triamterene. Calculation gives K_2 as 15×10^5 M^{-1}, reasonably close to the value of 4.9×10^5 M^{-1} from inhibiton studies, considering the errors of the binding curve data.

Effects of vasopressin. A series of experiments was undertaken to measure the effect of vasopressin on the number of sodium channels in the mucosal surface of frog skin. Eleven experiments were attempted, and in some of these channel density was measured under control conditions and then after treating the serosal surface with hormone (50 milliunits/ml). In others the channel density in the presence of hormone was measured first, and after the hormone had been washed away the channel density in the unstimulated condition was measured. It was important that these experiments were carried out under voltage clamp conditions, since the apparent affinity of amiloride for the channels was altered by hormone. Figure 7 shows the concentration-inhibition curves for amiloride both before and after hormone in the presence of 1.1 and 111 mEq/liter of Na^+ in the mucosal bathing solution. At both sodium concentrations the inhibition curves were moved to the right by a factor of about 3. Thus only if the amount of inhibition produced by the label was known could the number of channels at 100% occupancy for the control and hormone-treated condition be calculated. Furthermore, since the inhibition curves were moved to the right by the same factor with either 1.1 or 111 mEq/liter of Na^+, the stoichiometry of the interaction between amiloride and sodium remained the same after hormone treatment.

Table 3 shows the effects of hormone on channel density. It can be seen that the number of channels remained constant after hormone. For six of the skins both parts of

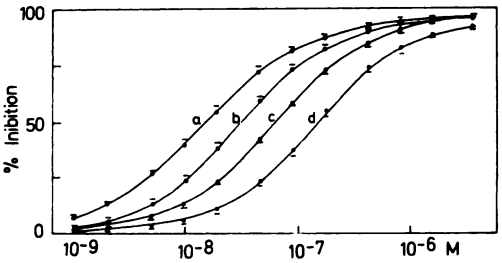

FIG. 7. *Curves relating percentage inhibition of SCC to amiloride concentration with either 1.1 mEq/liter (a, b) or 111 mEq/liter (c, d) of sodium in the mucosal bathing solution, before (a, c) and after (b, d) treatment of skins with ADH (50 milliunits/ml applied to serosal surface*

Each point shows the mean and standard error of three observations.

TABLE 3

Effect of ADH (50 milliunits/ml) on channel density and channel current in mucosal surface of frog skin

All experiments were carried out with the mucosal surface of the skins bathed with Ringer's solution containing 1.1 mEq/liter of Na^+. Binding was measured using 10 nM amiloride and with the skins short-circuited. Numbers in parentheses indicate the number of observations. Values are means ± standard errors. Confidence limits were calculated using Fieller's theorem (see ref. 4). All skins used in these experiments showed a significant ($p < 0.05$) binding of amiloride.

Skins	Channels/μm^2	Current
		$\times 10^{-16}$ *amp*
Controls (11)	187 ± 12	1.29 ± 0.17
ADH-treated (7)	170 ± 22[a]	2.55 ± 0.43[b]
Ratio (6)	1.09 ± 0.12	2.03 ± 0.27
95% confidence limits of ratio	0.89–1.29	1.87–2.31

[a] Not significantly different from controls.
[b] $p < 0.01$.

the experiment were completed without problems, and for these six the confidence limits of the ratio between the number of channels found after hormone compared to the control value are given. The ratio is close to unity. Since the SCC values of these skins were known, the nominal current passing through each channel can be calculated by dividing the SCC by the total number of channels in 9.6 cm^2. Table 3 shows that the "channel current" was doubled by ADH. The ratio for the six paired values is close to 2, and the 95% confidence limits are narrow.

Effects of calcium removal on amiloride binding. From previous reports it is known that when calcium is removed from the mucosal bathing solution (and probably from the mucosal surface, too) the inhibitory effect of amiloride on SCC and on oxygen consumption in transporting epithelia is reduced or abolished (10, 11). One possibility is that amiloride forms a ternary complex with calcium and the channel (11) to block transport.

An attempt to test this was made by measuring the binding of [^{14}C]amiloride in control skins and skins treated with EGTA (10 mM). Three experiments were made with sodium absent from the mucosal bathing solution. Amiloride binding was determined first under the control condition and then after exposing the mucosal surface of the skin to EGTA for 1 hr. The amount of amiloride bound per 9.6 cm^2 was measured; no change in binding capacity was recorded (Table 4). However, in these experiments there was no assurance that calcium removal had affected the inhibitory action of amiloride.

Further experiments were performed with 1.1 mEq/liter of Na^+ in the mucosal bathing solution and in which the SCC was recorded throughout. In some of the experiments amiloride binding was measured under both the control condition and after calcium removal, while in others only calcium-free binding was measured. The inhibitory effect of amiloride was significantly reduced (Table 4). (Amiloride binding is expressed as picomoles per 9.6 cm^2, as amiloride might still bind to channels in the absence of calcium without blocking the channel, and therefore it would be incorrect to calculate channel density.)

In this set of experiments treatment with EGTA significantly increased the binding capacity of the skins for amiloride, even though the inhibition produced was only 20% of that observed under control conditions. During these experiments four of the skins underwent spontaneous molting. When this happened, as much of the stratum corneum as possible was removed by gentle stroking with cotton wool soaked in Ringer's solution; then the skins were treated with EGTA and [^{14}C]amiloride binding was de-

TABLE 4

Effects of calcium removal on [^{14}C]amiloride binding by frog skin

All figures refer to measurements made using 10 nM amiloride. "Inhibition" indicates inhibition of resting SCC by 10 nM amiloride, where SCC is the resting short-circuit current. Figures in parentheses refer to the number of determinations. Means ± standard errors are given.

Sodium concentration	Control			Ca^{2+}-free			Ca^{2+}-free + molt		
	Binding	Inhibition	SCC	Binding	Inhibition	SCC	Binding	Inhibition	SCC
mEq/l	*pmole/9.6 cm²*	%	*μamp/cm²*	*pmole/9.6 cm²*	%	*μamp/cm²*	*pmole/9.6 cm²*	%	*μamp/cm²*
0.0	0.188 (2)			0.178 (3)					
1.1	0.10 ± 0.01 (4)	22.9 ± 1.4 (4)	1.7 ± 0.3 (4)	0.15 ± 0.01[a] (7)	4.0 ± 0.8[b] (7)	4.1 ± 0.3[b] (7)	0.44 ± 0.03[b] (4)	4.3 ± 0.7[b] (4)	18.9 ± 2.3[b] (4)

[a] $p < 0.05$ compared with corresponding control.

[b] $p < 0.001$ compared with corresponding control.

termined. From Table 4 it can be seen that the binding in these skins was significantly greater than that for EGTA-treated skins which had not molted. Furthermore, the binding capacity increased as the SCC increased, although the correlation is not close.

Channel density and sodium concentration. In this work amiloride binding was measured with 0 and 1.1 mEq/liter of Na^+ in the mucosal bathing solution. The experiments were performed during 1 year and perforce involved measurements on a number of different batches of animals. The data from different batches are given in Table 5, together with earlier data (1) obtained with 2.5 mEq/liter of Na^+ in the mucosal bathing solution. Although there is some variation between batches, the number of channels decreases as the sodium concentration falls. It must be remembered that at zero sodium the channel density is calculated assuming 100% occupancy with amiloride (10 nM), while at positive sodium concentrations the number of channels is calculated from the amount of amiloride bound together with the amount of inhibition caused.

DISCUSSION

The interactions between sodium ions and amiloride at the mucosal surface of frog skin has been shown to be competitive, at least for concentrations of sodium greater than 10 mEq/liter. Salako and Smith (8) also found a parallel shift in the concentration-inhibition curves for amiloride at different sodium concentrations, and although they did not explore such wide variations in concentration as in this work, their data agree quantitatively with those given here. Salako and Smith did not conclude that the sodium-amiloride interaction was competitive, basing this conclusion on results obtained by varying the sodium concentration in the presence of fixed concentrations of amiloride. Examination of their data shows that they obtained less than the expected amount of inhibition by amiloride using this method. In our hands both methods—variation of sodium concentration at a fixed amiloride concentration and vice versa—showed competitive interaction at sodium concentrations greater than 10 mEq/liter.

TABLE 5

Ionic strength and channel density

Individual values refer to means from different batches of animals.

Conditions	n	Channels/μm^2	
		Batch values	Mean ±SE
Zero sodium; 10 or 20 nM amiloride	14	82 ± 8	86 ± 4
	29	87 ± 4	(45)
	2	118	
1.1 mEq/liter of Na^+; 10 nM amiloride; skins short-circuited	4	270 ± 12	201 ± 14[a]
	11	187 ± 12	(15)
2.5 mEq/liter of Na^+; 10 or 20 nM amiloride; skins short-circuited	4	378 ± 98	378 ± 98[a] (4)

[a] Values differ from those in zero sodium ($p < 0.005$).

At sodium concentrations less than 10 mEq/liter the inhibition curves for amiloride became less steep, and in skins from one batch of animals definite inflections appeared. In these instances we considered the possibility that there are two distinct populations of receptors for amiloride. There is already evidence for multiple types of amiloride receptors in the mucosa of the toad colon (12).

We have sought a kinetic explanation for the results with frog skin. The scheme given below most closely fits the data. The scheme envisages two populations of receptors for amiloride, which differ in their affinity for the drug. The proportions of these two populations are considered to be dependent on the ambient sodium concentration, in such a way that the high-affinity form, R_1, is converted to the low affinity form, R_2, by increasing sodium concentration. Both forms of receptor are considered to interact with sodium or with amiloride with a stoichiometry of 1:1, both substances binding with the same sites (i.e., competitive interaction). The scheme may be represented thus:

$$\begin{array}{ccccc} \mathrm{Na}\,R_1 & \overset{K_2}{\rightleftharpoons} & R_1 & \overset{K_1}{\rightleftharpoons} & AR_1 \\ & & \mathrm{Na}\,\big\|\uparrow K_5 & & \\ \mathrm{Na}\,R_2 & \overset{K_4}{\rightleftharpoons} & R_2 & \overset{K_3}{\rightleftharpoons} & AR_2 \end{array}$$

where the constants are affinity constants, and A is amiloride. Assuming that the inhibition of transport is proportional to the total amount of drug-receptor complex formed (i.e., $AR_1 + AR_2$), then the fractional receptor occupancy is given by p, where

$$p = \frac{A(K_1 + K_1K_2A + K_1K_4\mathrm{Na} + K_2K_4\mathrm{Na} + K_1K_2K_5A\mathrm{Na} + K_2K_3K_5\mathrm{Na}^2)}{(1 + K_1A + K_2\mathrm{Na})(1 + K_3A + K_4\mathrm{Na})(1 + K_5\mathrm{Na})} \quad (2)$$

Solutions for Eq. 2 for a variety of sodium and amiloride concentrations, assuming values for the constants, are shown graphically in Fig. 8.

The general form of the theoretical "inhibition curves" is not unlike those found experimentally (see Fig. 4). At the extreme ranges of sodium concentration the receptors behave as a homogeneous population, with R_1 type receptors dominant at very low and R_2 type receptors dominant at high sodium concentrations, while in the intermediate ranges the population is heterogeneous. Log (DR−1) vs. log Na^+ plots derived from the theoretical curves have slopes of 1 at the extreme ranges of sodium concentration. Curiously, for the intermediate ranges, and calculating IC_{50} values as for Fig. 3, a slope of 0.7 was obtained for the heterogeneous region. The reduction in slope of both the inhibition curves and the log-log plots for normal skins at low sodium concentrations may have a basis similar to that described above. With regard to anomalous skins it would appear that a reduction of sodium concentration produced a large conversion to the R_1 type, an explanation which might have a seasonal basis. It must be remembered, however, that the model only fits the data but does not explain the phenomena. Paton (6) pointed out that if the stoichiometry between drug and antagonist is not 1:1, deviations of log-log plots from linearity will appear at low dose ratios. Thus if the model described above should prove incorrect it may mean that the stoichiometry between Na^+ and amiloride is not 1:1.

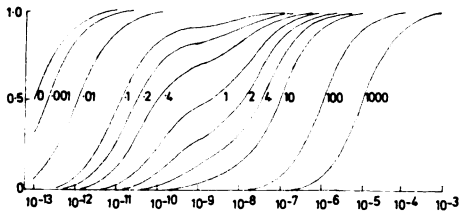

FIG. 8. *Theoretical curves showing fraction of channels bound by amiloride vs. amiloride concentration, derived from Eq. 2*

The numbers against each curve represent the relative sodium concentrations. Values chosen arbitrarily for K_1–K_5 were 10^{-13}, 10^{-6}, 10^{-8}, 10^{-3}, and 10^{-3}, respectively.

We feel that the binding data presented here support the view that the number of sodium channels in the mucosal membrane of frog skin can be measured with reasonable accuracy. However, there is no unequivocal evidence that the channels have a single binding site for sodium and amiloride, and perhaps the channel density will have to be divided by 2, 3, or 4 if the channel is found to be an oligomeric protein assembly. Reasonable agreement has been found for the values of affinity for amiloride determined by either binding or inhibition studies. Nevertheless, there may still be a real difference between the affinity of amiloride determined from inhibition and binding studies. The accuracy of the value from inhibition studies depends in the main on measurements made at sodium concentrations greater than 10 mEq/liter. Below 10 mEq/liter it has been suggested that there is partial conversion to a higher-affinity form of the channel. If this is true, then at zero sodium there may be a mixed population of channels with an apparent increase in average affinity. Until more accurate binding data can be obtained it will not be possible to resolve this problem. Furthermore, the accuracy of the binding data at finite sodium concentrations depends on the accuracy with which SCC can be measured. As pointed out under METHODS, there is a Na^+ and Cl^- gradient from the serosal to the mucosal solution, and if the permeability coefficients for Na^+ and Cl^- in the leak pathway are sufficiently different, an error of SCC would be recorded. However, when the Na^+ gradient was retained in the absence of a Cl^- gradient (i.e., with choline chloride addition) no

substantial change in SCC was recorded (see Fig. 3b), suggesting that no appreciable error occurred in the biophysical measurement of sodium transport. Another difficulty is that although amiloride was added to the short-circuited skin the tissue was open circuit when the solution was removed and the skin blotted. It is not possible to know whether some dissociation of the receptor complex occurred at this time. This difficulty does not apply to the experiments in zero sodium, where the short-circuited and open-circuited state are equivalent.

The data obtained with triamterene are internally consistent. This substance has actions similar to amiloride (13), and provided that the nonspecific sites to which triamterene binds are different from the nonspecific sites for amiloride, we can conclude that most of the amiloride binding is to sodium channels.

Variation of the sodium concentration in the mucosal bathing solution apparently alters the number of channels. At zero sodium the channel density is 86 $\pm$ 4/μm^2, a figure obtained using 10 nM amiloride and assuming 100% receptor occupancy. From inhibition studies this concentration of amiloride is expected to give 50% occupancy, and therefore if the value at zero sodium is doubled a result comparable to that obtained at 1.1 mEq/liter of Na^+ is obtained. However, the binding curve appears to approach saturation at a drug concentration of 10 M. These discrepancies might result from genuine variations between skins (see Figs. 5 and 6), a real effect of sodium concentration on channel density, and undoubtedly from limitations on the sensitivity of the labeling technique. It is of interest that Cuatrecasas (14) noted a decrease in the number of insulin binding sites as the ionic strength was decreased. One further point of interest which can only be seen with the unworked data is that the amount of unbound amiloride (i.e., amount of radiolabel retained by the skin in the presence of excess unlabeled drug) increases as the sodium concentration decreases. At its simplest this means that the extracellular space to the outside of the skin increases as the ionic strength is reduced, an observation reported previously by others (8).

If amiloride forms a ternary complex with calcium and the channel, as has been suggested (11), the removal of calcium might affect the binding of amiloride. Kinetic studies have shown that the affinity of amiloride was not changed by calcium removal, and these studies have shown that calcium removal does not decrease binding. Although amiloride binds equally well under calcium-free conditions, it is not able to prevent sodium ion translocation through the membrane.

It was fortuitous that in a few instances skins molted spontaneously after setting up. Frogs do this normally every 2 weeks or so, and we were able to show that there was a significant increase in the number of binding sites after it happened. It has been shown (15) that transport through freshly molted skins is rather insensitive to inhibition by amiloride. It is possible that calcium removal causes small patches of molting, exposing fresh epithelium with a high channel density. However, this is not to say that molting is responsible for the insensitivity to amiloride seen under calcium-free conditions. In the isolated toad bladder, which does not molt, removal of calcium from the mucosal bathing solution still inhibits the effects of amiloride (2).

Our primary aim in this work was to examine the effects of ADH on channel density, using amiloride as a probe. It is known for both frog skin and toad bladder that although the hormone acts on receptors located in the serosal surface the rate-limiting change in sodium permeability occurs at the mucosal face (e.g., ref. 16). Ignoring the complex series of events initiated by interaction of hormone with serosal receptors, what is the nature of the mucosal permeability change as revealed by these results?

The results show clearly that ADH causes no significant increase in the number of channels, and since the hormone increases SCC there is an increase in the nominal current per channel. The possibility that the hormone creates new channels in proportion to the increase in SCC can be rejected. The hormone apparently alters the channel in such a way that the current passing in the channels is increased, and at the same time the amiloride inhibition curve is moved to

the right along the concentration axis. As discussed previously (1), the current passing through each channel is calculated by assuming that the channels are permanently open, but if they were to open only intermittently the actual currents would be larger than those calculated. Thus a simple way in which ADH might increase the total current would be to increase the fraction of time the channels remained in the open configuration. For a fixed number of channels this is phenomenologically equivalent to increasing the ratio of open to closed channels at any instant. A two-state model for the channel, of the type proposed by Monod, Wyman, and Changeux (17), in which ADH increases the proportion of channels in the open configuration, would also explain the shift in the inhibition curve as well as the increase in current. In this situation ADH, or the agency generated by its interaction with the serosal membrane, would act as an allosteric activator of sodium transport. We do not favor a simple widening of a fixed number of channels, as it is difficult to see how specificity for sodium could be maintained. Furthermore, it is clear that the membrane "handles" the sodium ions in their passage through the membrane, even after treatment with ADH (18), making enlargement of pre-existing pores an unlikely explanation for the action of the hormone.

ACKNOWLEDGMENTS

We are grateful to Merck Sharp & Dohme for a supply of [^{14}C]amiloride and to Smith Kline & French for triamterene.

REFERENCES

1. Cuthbert, A. W. (1973) *J. Physiol. (Lond.)*, **228**, 681–692.
2. Cuthbert, A. W. (1974) in *Drugs and Transport Processes* (Callingham, B. A., ed.), Macmillan, London, in press.
3. Cuthbert, A. W. & Shum, W. K. (1974) *Naunyn-Schmiedebergs Arch. Pharmacol.*, **281**, 261–269.
4. Goldstein, A. (1964) *Biostatistics*, Macmillan, New York.
5. Hunter, A. & Downs, C. E. quoted by Dixon, M. & Webb, E. C. (1945) in *Enzymes*, Ed. 2, p. 330, Longmans, London.
6. Paton, W. D. M. (1970) *Ciba Found. Symp. Molecular Properties of Drug Receptors*, 3–32.
7. Cereijido, M., Herrera, F. C., Flanigan, W. J. & Curran, P. F. (1964) *J. Gen. Physiol.*, **47**, 879–893.
8. Salako, L. A. & Smith, A. J. (1970) *Br. J. Pharmacol.*, **39**, 99–109.
9. Gaddum, J. H. (1937) *J. Physiol. (Lond.)*, **89**, 7–9P.
10. Cuthbert, A. W. &. Wong, P. Y. D. (1971) *Biochim. Biophys. Acta*, **241**, 713–715.
11. Cuthbert, A. W. & Wong, P. Y. D. (1972) *Mol. Pharmacol.*, **8**, 222–229.
12. Cuthbert, A. W. (1973) *Eur. J. Pharmacol.*, **23**, 187–190.
13. Salako, L. A. & Smith, A. J. (1971) *Br. J. Pharmacol.*, **41**, 552–557.
14. Cuatrecasas, P. (1971) *J. Biol. Chem.*, **246**, 7265.
15. Nielsen, R. & Tomlinson, R. W. S. (1970) *Acta. Physiol. Scand.*, **79**, 238–243.
16. Curran, P. F., Herrera, F. C. & Flanigan, W. J. (1963) *J. Gen. Physiol.*, **46**, 1011–1027.
17. Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol.*, **12**, 88–118.
18. Frazier, H. S., Dempsey, E. F. & Leaf, A. (1962) *J. Gen. Physiol.*, **45**, 529–543.