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Importance of Guanidinium Groups for Blocking Sodium Channels in Epithelia

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SUMMARY

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Sodium transport in an isolated amphibian epithelium (skin of *Rana temporaria*) has been investigated as a function of pH. The evidence is consistent with the view that sodium entry into the epithelium is dependent on an acid grouping, which behaves as a singly ionizable grouping with a pK_a of around 5. The activities of three blocking drugs which prevent sodium entry have also been investigated as a function of pH. These were amiloride, triamterene, and *N*-(*N*-benzylamidino)-3,5-diamino-6-chloropyrazine carboxamide (benzamil). The variation in affinity of these compounds with pH was predictable from mass action kinetics if it was assumed that the positively charged inhibitors interacted with a negatively charged acid grouping in the mucosal membrane. With the three inhibitors the positive charge is located on a guanidinium (or isosteric) group, suggesting similarities with compounds which block sodium channels in excitable membranes. Tetrodotoxin had only weak blocking activity in this system ($K = 10^3$ M^{-1}) compared with amiloride ($K = 5 \times 10^6$ M^{-1}), triamterene ($K = 5 \times 10^5$ M^{-1}), and benzamil (5×10^7 M^{-1}). Guanidine had anomalous and unexplained blocking activity in this system, while 2-guanidinobenzimidazole had both stimulating and blocking activity. The former was probably due to the imidazoline ring structure, while the latter was dependent upon the guanidinium moiety.

INTRODUCTION

Variation of pH is a useful method for investigating the actions of drugs with pK_a values in the physiological range, particularly so when the system being investigated shows well-defined changes in biological activity with pH variation in the absence of drug. With these conditions the separate contributions caused by the changing ionization of both drug and receptor can be assessed, and a more complete account of the drug-receptor interaction may be possible. This paper reports on the effect of pH on the blocking actions of a number of drugs which inhibit sodium transport in epithelia. Sodium transport was studied in an amphibian skin which is tolerant to wide variations of pH without apparent damage.

The findings indicate the importance of the guanidinium grouping for drugs which block sodium entry into epithelia; furthermore; similarities between the behavior of sodium channels in epithelia and the voltage-dependent sodium channels of excita-

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ble membranes in response to blocking drugs have been found.

MATERIALS AND METHODS

Sodium transport was measured as short-circuit current by voltage-clamping pieces of frog abdominal skin (*Rana temporaria*) at zero transepithelial potential. Skin pieces were mounted vertically between Perspex hemichambers and bathed on both sides with Ringer's solution. (Each hemichamber contained 12 ml; skin area, 7.5 cm^2). The solutions were continously bubbled with air, which both aerated the solutions and ensured the rapid mixing of added drugs. Electrodes for recording potential and passing current were of conventional design, and the voltage was clamped automatically (Schema Versatae S/V-360 c). With both the serosal and mucosal solutions at neutral pH, the SCC[1] equals net mucosal to serosal sodium flux (1).

Throughout the experiments the serosal bathing solution contained Tris buffer (5 mM, pH 7.6) while the mucosal solution was unbuffered. The pH of the mucosal solution was controlled with a pH-stat (Radiometer), the electrode assembly of which was immersed directly in the mucosal bathing solution.

pK_a values for drugs used in this study were measured with an automatic titrator (Radiometer). The Ringer's solution used throughout contained NaCl, 111 mM; KCl, 2.0 mM; $CaCl_2$, 1.0 mM; and glucose, 11.0 mM.

RESULTS

Sodium transport, measured as SCC, was recorded from skins with the pH of the serosal solution controlled at 7.6, while the mucosal pH was changed stepwise from 3 to 10 in steps of 1 pH unit. The procedure used was to adjust the pH of the mucosal solution to a given pH using the pH-stat and, after the current had become steady, the next pH value was chosen, and so on. The "stirring in" of atmospheric CO_2, particularly at alkaline pH values, was automatically compensated by the addition of alkali from the pH-stat. In general, recovery from very acid (pH 3–4) conditions to more neutral conditions took about 15 min, whereas steady-state values were achieved quickly with pH variations on the alkaline side of neutrality. Although rapid equilibration occurred at alkaline pH values, there was a tendency for the SCC to fall slowly when very alkaline conditions were maintained for long periods. This condition was necessary only in the more lengthy experiments, described later.

Figure 1 shows a result for a single skin. Significant decreases in current were obtained only when the pH of the mucosal bathing solution was made acid, and maximal currents were achieved at alkaline pH. The minimal current was obtained at pH 4, further acidification causing an increase in SCC.

Amiloride, a potent inhibitor of sodium entry through the mucosal surface of epithelia (2–6), at least at near neutral pH values, was used to explore the variation

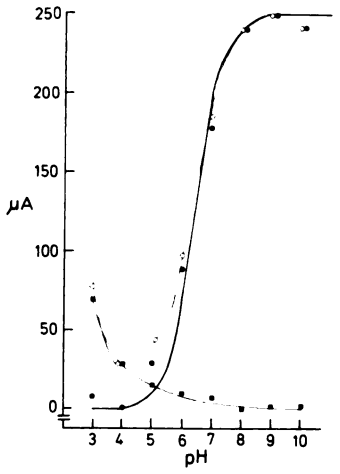

FIG. 1. *Short-circuit current recorded from frog skin, 7.5 cm^2*

The serosal bathing solution was buffered at pH 7.6, while the pH of the unbuffered mucosal solution was maintained at various pH values with a pH-stat. Currents were recorded in the absence (○) and presence (■) of amiloride, 200 μM. ●, differences in currents measured in the presence and absence of the inhibitor. The thick line is a theoretical curve for the ionization of a monobasic acid (pK_a 6.4), scaled to coincide with the maximal recorded current.

[1] The abbreviation used is: SCC, short-circuit current.

of current with pH after the initial determinations had been made in the absence of the drug. At pH 5–10 virtually all of the current was sensitive to amiloride; that is, amiloride (200 μM) caused abolition of SCC. Usually at pH 4 some fraction of the current was insensitive, while at pH 3 no part of the current was sensitive to the blocking drug. As the current at pH 3 is totally insensitive to amiloride, it may be due to a mucosal to serosal proton flux rather than sodium movement. Also shown in Fig. 1 is the amiloride-sensitive SCC as a function of pH. This function behaves as if a singly ionized acid grouping were responsible for controlling sodium entry, and hence sodium transport, in this tissue. Similar results were obtained for six separate skins, and the amiloride-sensitive SCCs in these experiments behaved as if they were controlled by acid groupings with pK_a values of 4.8, 6.4, 4.8, 4.8, 5.7, and 4.3. The mean hydrion concentration causing a 50% reduction of the amiloride-sensitive current in these experiments is 17 μM, corresponding to a pK_a of 4.8. When the pH of the mucosal solution bathing the skin was increased beyond 10.5, there was a dramatic fall in SCC, which rarely recovered when the pH was readjusted to neutral values. Thus pH 10.5 represents the practical upper limit of the experimental range in these experiments.

The next stage of the investigation was to examine in detail the effects of blocking drugs at different pH values. The protocol

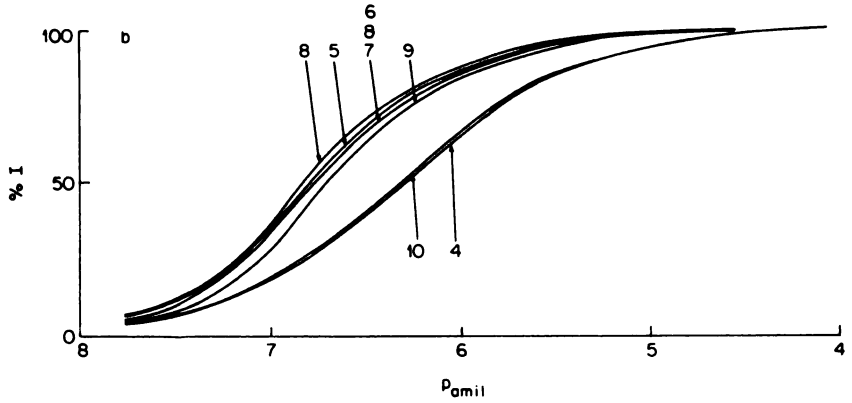

FIG. 3. *Percentage inhibition of SCC as a function of amiloride concentration at various pH values*
For clarity, experimental points are not shown. For only one experimental point was the distance from the appropriate curve greater than 2%. Data were taken from the results shown in Fig. 2.

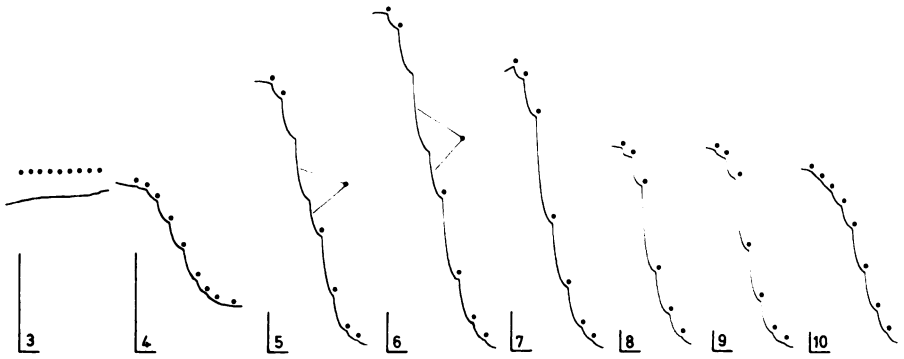

FIG. 2. *Effects of amiloride on SCC in frog skin (7.5 cm²) with the mucosal bathing solution controlled at various pH values*

Amiloride was applied cumulatively. The concentrations at the times indicated (by ●) were (from left to right) 0.015, 0.054, 0.21, 0.59, 2.13, 6.0, 21.4, 59.8, and 137 μM. Occasionally the concentration of amiloride was increased from 0.054 to 0.21 μM in two equal steps (as indicated). The calibrations are equal to 50 μamp and 2 min, and the time calibration also serves to indicate zero SCC. The pH of the mucosal solution is shown by the calibrations.

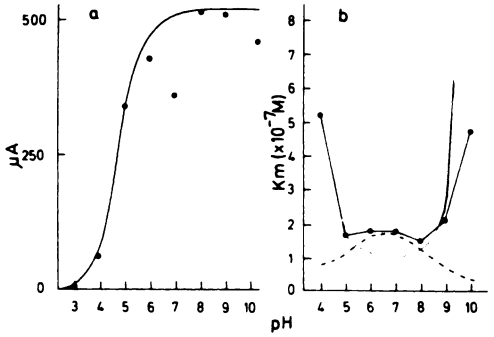

FIG. 4. *Variation of K_m with pH for amiloride*

a. Amiloride-sensitive SCC plotted against pH (●). The line represents the dissociation curve for a monobasic acid with pK_a 4.8.

b. K_m for amiloride plotted against pH (●). ——, predicted variation in K_m with pH; - - -, calculated value of K_{real} with pH (see the text for explanation). Data were taken from the experiments illustrated in Fig. 2.

of these experiments can be best illustrated by reference to the results shown in Figs. 2–4. Figure 2 shows a series of cumulative inhibition curves to amiloride obtained between pH 3 and 10. As expected, amiloride had no effect on SCC at pH 3 and caused maximally 70% inhibition at pH 4, while at all other hydrion concentrations complete inhibition by amiloride was achieved. Inhibition (as a percentage) of the amiloride-sensitive SCC is shown as a function of amiloride concentration in Fig. 3, from which the concentration causing 50% inhibition of the amiloride-sensitive current at each pH was measured. In Fig. 4a the amiloride-sensitive SCC is shown plotted against pH. The SCC-pH relationships obtained in these experiments were less accurate than those obtained in the earlier experiments (compare Fig. 1 with Figs. 4a and 7a). This was due to the considerable time required to complete an experiment when inhibition curves were determined. The determinations at different pH values were carried out in random order, the whole experiment taking about 4 hr compared to about 1 hr in the earlier experiments. Thus the gradual changes in SCC with time which occur at a single pH are superimposed upon changes due to pH; furthermore, the deleterious effects of prolonged exposure to alkaline solutions are apparent.

The changes in apparent K_m for amiloride at different pH values obtained from Fig. 3 are plotted in Fig. 4b. The apparent affinity of amiloride for sodium channels is reduced at both acid and alkaline pH values. The pK_a of amiloride was found to be 8.7, agreeing with the value given in the literature (7). As the receptor controlling sodium entry in frog skin appears to have a well-defined pK_a, and since the pK_a of amiloride is known, an attempt was made to develop a simple kinetic theory to explain the variation of K_m with pH.

The following treatment assumes that the protonated form of a drug reacts with the ionized acid grouping of the membrane component controlling sodium entry. Either protonation of the sodium entry mechanism or its combination with a charged drug molecule is assumed to block the sodium entry process.

K_D and K_R are the dissociation constants of the drug and membrane receptor, respectively. K_{real} is the true K_m for interaction of the charged drug with the ionized receptor; thus

$$K_{real} = \frac{[DH^+][R']}{DHR}$$

Presumably, K_{real} should remain constant at different pH values. The measured value of K_m ($K_{measured}$) will depend, however, on the total amount of drug rather than the fraction protonated.

Thus

$$K_{measured} = \frac{[D+DH^+][R'+RH]}{DHR}$$

Substitution gives

$$K_{measured} = \frac{[DH^+][R']}{[DHR]}\left[1 + \frac{K_D}{[H^+]} + \frac{[H^+]}{K_R} + \frac{K_D}{K_R}\right]$$

$$= K_{real}\left[1 + \frac{K_D}{[H^+]} + \frac{[H^+]}{K_R} + \frac{K_D}{K_R}\right]$$

Using this equation and the values of $K_{measured}$ for different pH values, the variation of K_{real} with pH was calculated, and is also shown in Fig. 4b. Little variation in this parameter was seen with pH. Similar results were obtained in two other experiments, the values of K_{real} being approximately 2.0×10^{-7} M and 1.4×10^{-7} M. One of these experiments is illustrated in Fig. 5.

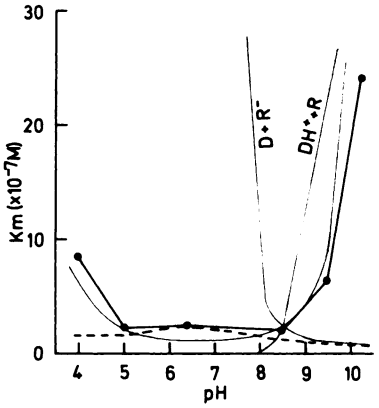

FIG. 5. *Effect of pH on K_m of amiloride*

●, measured values; two other curves refer to calculated values (——) and real values (- - -) of K_m. The pK_a of the membrane receptor was taken as 4.6, and was derived from measured values of the amiloride-sensitive current. The curves labeled $D + R^-$ and $DH^+ + R$ indicate the variation of K_m with pH which would be expected if the ionized drug reacted with a non-ionized receptor or vice versa (see the text for full explanation).

The same formalism described above can be used to test other possible reaction mechanisms. For example, suppose that the nonprotonated form of the drug is the reactive species and that the reaction is with the un-ionized receptor. In this situation an exactly similar variation in $K_{measured}$ with pH is predicted. However, the estimates of K_{real} for amiloride are then around 1×10^{-11} M. This low value is unlikely for two reasons. First, at neutral pH the offset of the blocking action of amiloride is rapid (4), even though the concentration of un-ionized drug is several orders of magnitude greater than 10^{-11} M. Second, at low pH, where the concentration of un-ionized drug approaches 10^{-11} M, the onset of the inhibitory effect is also extremely rapid (Fig. 2). Even if the rate of access of the drug to the receptor is simply diffusion-limited, the rapidity of the response is inexplicably great. Although the possibility of an interaction between non-ionized drug and receptor cannot be eliminated with certainty, economy of hypothesis forces the alternative view.

Other possibilities can be more readily dealt with, for example, the reaction of protonated drug with un-ionized receptor and vice versa. These possibilities have been explored, and some of the findings are shown in Fig. 5. The theoretical behavior of $K_{measured}$ with pH for the two situations given above is shown. The curves have been scaled to coincide with the experimental value at pH 8.5. Clearly there is little to support the view that either of these reactions dominates the biological response. While the interactions are best explained in terms of a protonated drug reacting with an ionized receptor, the contribution of other minor interactions of a different type cannot be assessed. The investigations were extended to other drugs known to block sodium entry; these were triamterene (8) and *N*-(*N*-benzylamidino)-3,5-diamino-6-chloropyrazine carboxamide (benzamil). The latter, a benzyl derivative of amiloride, differs from amiloride in two respects: its affinity for the sodium entry site is 10 times greater than that of amiloride, and its pK_a is 8.2. Like amiloride, however, benzamil caused complete inhibition of SCC at pH values between 5 and 10, partial inhibition at pH 4, and no effect at pH 3.

The results of an experiment with benzamil are shown in Fig. 6. K_{real} for this compound is around 10^{-8} M, that is, 10 times less than the value for amiloride. There is again agreement between the experimental and predicted behavior of this compound as an inhibitor of transport when pH is varied, suggesting that for this compound, too, the protonated

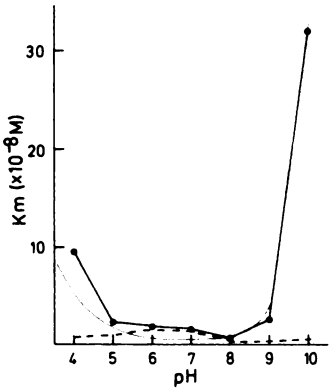

FIG. 6. *Effect of pH on K_m of benzamil*

●, measured values; the other two curves refer to calculated values (——) and real values (- - -) of K_m. The membrane receptor was assumed to have a pK_a of 5.

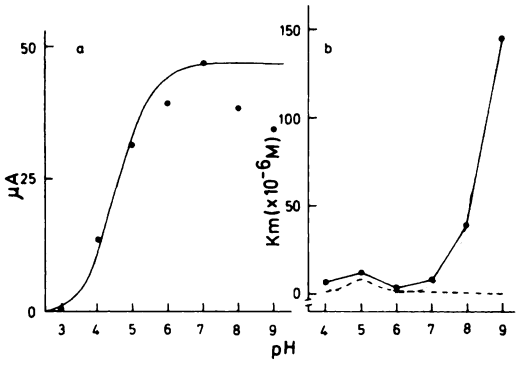

FIG. 7. *Effects of pH on actions of triamterene*

a. Amiloride-sensitive SCC vs. pH. The curve is a dissociation curve for a monobasic acid (pK_a 4.6) scaled to match the maximal SCC.

b. Values of K_m for triamterene vs. pH. ●, measured values; ——, predicted behavior of K_m with pH; - - -, calculated values of K_{real}.

form of the drug is the active species. In this experiment the pH vs. SCC curve was unsatisfactory for the reasons given earlier, and a pK_a of 5 was assigned to the membrane receptor for analysis.

Triamterene, in contrast to benzamil, has a much lower affinity for the receptor and is a much weaker base, with a pK_a of 6.2. This value was determined independently in this work and agrees with that given elsewhere (7).

Figure 7 illustrates one of two similar experiments carried out with triamterene. As this drug is less active than amiloride, complete inhibition curves were not obtained at the extreme values of pH owing to solubility limitations, but the amount of inhibition achieved was always greater than 50%. Amiloride was added in the presence of triamterene in these circumstances to make certain that the expected amount of inhibition could be achieved with these skins.

The pK_a of the membrane receptor was 4.6 for the experiment illustrated in Fig. 7a, and the behavior of theoretical and experimental values of K_m with pH again followed the now familiar pattern. The peak in the curve showing the calculated values of K_{real} for triamterene is a consequence of the apparently anomalous value of $K_{measured}$ for triamterene at pH 5.

Guanidine has a pK_a of 13.6 (9) and therefore will remain ionized over the complete pH range used in these experiments. Investigation of the blocking effects of guanidine presented some technical problems which stemmed from the very low activity of this material. Initially guanidine hydrochloride was used at a concentration of 9.9–213 mM. Curious behavior of the skins was observed when guanidine was applied at concentrations greater than 70 mM, which suggested that the effects were due to tonicity rather than to guanidine specifically. This was confirmed using sucrose to raise the tonicity. The effects of hypertonic mucosal solutions on the behavior of epithelia are well known (10, 11). The effects of guanidine therefore were examined at only three concentrations: 9.9, 19.6, and 65.4 mM. At these concentrations the effects of tonicity were minimal (i.e., caused less than 3% change in SCC). Reciprocal plots of inhibition vs. concentration are shown in Fig. 8. The results suggest that guanidine is capable of causing complete inhibition of SCC if a sufficiently high concentration can be employed. The variation in $K_{measured}$ with pH is totally unlike the expected variation based upon the theoreti-

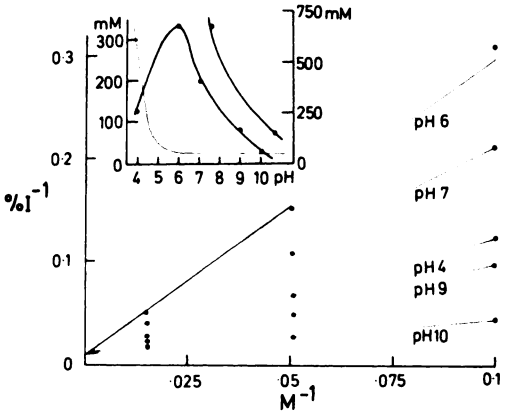

FIG. 8. *Effect of pH on actions of guanidine hydrochloride*

Reciprocal plots of percentage inhibition vs. concentration for different pH values. K_m values for guanidine were derived from the intercepts on the abscissa, and are plotted against pH (bell-shaped curve in inset). The curve to the right of the bell-shaped curve indicates K_m values for guanidine determined in earlier experiments. The thin line represents expected behavior of K_m with pH for guanidine.

cal considerations given earlier. The affinity of guanidine is greater at both extremes of the pH range investigated than at neutrality, as shown in the inset of Fig. 8. These experiments were performed in December 1975. The effects of guanidine were examined previously in *R. temporaria* skins in September 1972 at pH 7.6 and 10.6. Data from these earlier studies are also given in Fig. 8, and the same general pattern is seen, although the actual values are very discrepant to the recent estimates of K_m. In 1972 amiloride was compared with guanidine in the same skins, and the K_m for amiloride was 1.9×10^{-7} M at pH 7.6 and 5.0×10^{-6} M at pH 10.6. These values are very close to those obtained in the present experiments (see Fig. 4). Thus, in two sets of experiments, 3 years apart, the apparent K_m values for guanidine at different pH values were very discrepant, while those for amiloride remained constant.

A single experiment was carried out with tetrodotoxin to estimate its inhibitory action on this system. A concentration of 50 nM caused only 8% inhibition of SCC at pH 7.6. From the limited amount of information it was estimated that a concentration of around 1 mM is required to produce 50% inhibition of SCC at neutral pH. It was not considered worthwhile to expend further valuable material on this aspect.

The final compound to be investigated in this series of experiments was 2-guanidinobenzimidazole. Not only does this compound possess a guanidine group, but Zeiske and Lindeman (12) have shown that it stimulates sodium transport when applied to the mucosal surface of the skin of *Rana esculenta*.

The responses of *R. temporaria* skin to 2-guanidinobenzimidazole were somewhat variable, and both stimulation and inhibition of SCC by this agent were recorded, depending on pH. One example showing the variation in response with pH is given in Fig. 9. When the mucosal solution was adjusted to pH 10, 2-guanidinobenzimidazole produced stimulation of SCC. At a concentration of 5.5 mM there was a rapid increase in SCC superimposed on a more modest response (Fig. 9a). Addition of amiloride (200 μM) at this stage failed to cause complete inhibition of SCC, the amount of the current remaining insensitive to amiloride being equal to extra current added following the rapid increase in SCC caused by 2-guanidinobenzimidazole. Clearly the effect caused by high concentrations of 2-guanidinobenzimidazole is of little interest in this study, since sodium entry cannot occur by normal sodium channels, as the current is insensitive to amiloride. Results similar to those with 2-guanidinobenzimidazole have been obtained with polyene antibiotic amphotericin applied to the mucosal surface.[2] Here, too, the increase in SCC is not sensitive to amiloride, as would be expected with an agent known to cause a nonspecific increase in membrane permeability. At pH 7 only inhibition was observed, even at 5.5

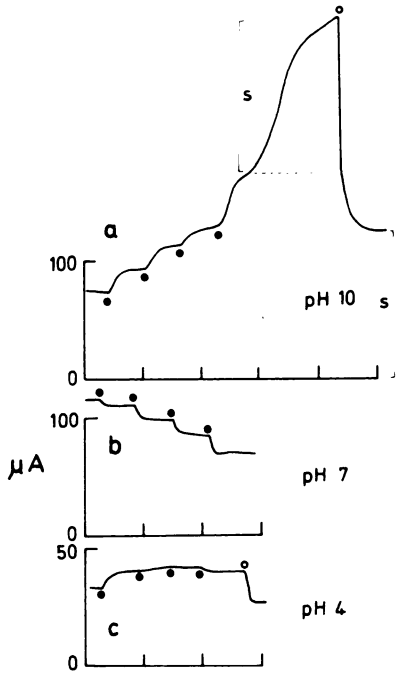

FIG. 9. *Effects of 2-guanidinobenzimidazole on SSC*

2-Guanidinobenzimidazole was added (●) to produce final concentrations of 0.5, 1.5, 2.5, and 5.5 mM. ○, addition of amiloride, 200 μM. The time divisions are 5-min intervals, and the time scale also indicates zero SCC. Note that the sudden increase in current which occurs in the presence of 5.5 mM 2-guanidinobenzimidazole at pH 10 (*s*) is not inhibited by amiloride. Results are shown for pH 10, pH 7, and pH 4 in a, b, and c, respectively.

[2] Unpublished observations.

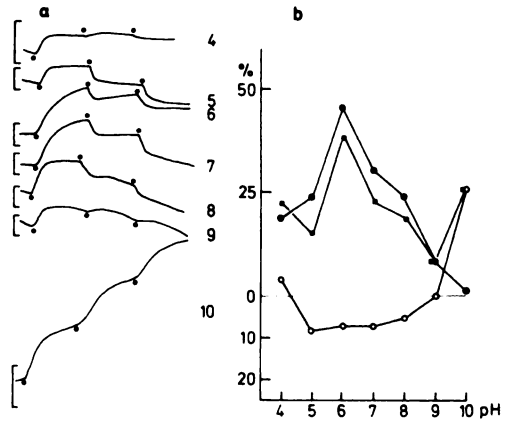

FIG. 10. *2-Guanidinobenzimidazole and pH*

a. Effects of 2-guanidinobenzimidazole (0.5, 1.5, and 2.5 mM) on SCC in a single skin as a function of pH (the pH value associated with each trace is indicated at the right). Calibration equals 10 μamp.

b. Percentage changes in SCC. ○, percentage decrease in SCC caused by increasing the 2-guanidinobenzimidazole concentration from 0.5 to 1.5 mM; ■, percentage increase in SCC caused by 0.5 mM 2-guanidinobenzimidazole; ●, percentage increase in SCC caused by 0.5 mM 2-guanidinobenzimidazole plus percentage decrease due to raising the concentration to 1.5 mM.

mM (Fig. 9b), while at pH 4 the effect was again a stimulation of SCC (Fig. 9c).

A further example of the effects of 2-guanidinobenzimidazole is shown in Fig. 10a. The highest concentration used in this experiment was 2.5 mM, in an attempt to avoid nonspecific effects. In this skin 2-guanidinobenzimidazole (0.5 mM) caused an increase in SCC at all pH values between 4 and 10. On increasing the concentration to 1.5 mM, inhibitory responses were recorded in the pH range 5–8, but further stimulation was seen at pH values outside these limits. Quantitative analysis is difficult with mixed excitatory-inhibitory effects, and only a simple treatment of the results was attempted. The percentage inhibition of SCC caused by increasing the 2-guanidinobenzimidazole concentration from 0.5 to 1.5 mM was taken as an indication of the inhibitory activity and is plotted against pH in Fig. 10b. The percentage increase in SCC caused by 0.5 mM 2-guanidinobenzimidazole showed no regular pattern when plotted against pH. It maybe that the lack of regularity arose because, although stimulation was seen at all pH values, the over-all stimulation was modified by varying degrees of inhibition. By adding the percentage inhibition caused by 1.5 mM 2-guanidinobenzimidazole to the percentage increase in SCC caused by this compound at 0.5 mM, the pattern shown in Fig. 10b was obtained.

The inhibitory response to 2-guanidinobenzimidazole (pK_a = 7) showed a pattern of activity with pH similar to that of amiloride; that is, activity was maximal at neutral pH but diminished at both extremes of the pH range investigated.

DISCUSSION

First consideration will be given to the effect of pH on ionization of the various inhibitors of transport, in particular to the disposition of charge on the guanidine grouping, which was a feature common to all the drugs examined. Table 1 gives the structures and first pK_a values for the various inhibitors. The values for amiloride, triamterene, benzamil, and 2-guanidinobenzimidazole were measured using an automatic titrator. The value for tetrodotoxin was taken from Camougis, Takman,

TABLE 1

Properties of inhibitors of sodium transport in epithelia

Compound	Approximate affinities for entry sites in presence of 111 mM Na^+ at pH 7	pK_a
	M^{-1}	
Amiloride (*N*-amidino-3,5-diamino-6-chloropyrazine carboxamide)	5×10^6	8.7
Benzamil [*N*-(*N*-benzylamidino)-3,5-diamino-6-chloropyrazine carboxamide]	5×10^7	8.2
Triamterene (6-phenyl-2,4,7-triaminopteridine)	5×10^5	6.2
Tetrodotoxin	$\sim 10^3$	8.8
Guanidine	5.0[a]	13.6
2-Guanidinobenzimidazole	—[b]	7.0

[a] Maximally.
[b] Very low.

and Tasse (13), and those for guanidine and all other values quoted in this discussion were taken from Perrin (9).

The pK_a for guanidine is 13.6, and therefore it will be protonated over the whole of the pH range investigated here. As the pK_a values of amiloride, benzamil, and 2-guanidinobenzimidazole are 6–7 units smaller than for guanidine, the groups attached to the guanidine moiety must be powerful electron sinks. The powerful electron-attracting ability of a carbonyl group adjacent to guanidine is illustrated by acetylguanidine (pK_a 8.26). Further addition of a benzyl group to the guanidine residue only reduces the pK_a by 0.5 unit, to 8.2 for benzamil. It is known that an aromatic ring adjacent to guanidine reduces its pK_a by about 3 units [for example, *N*-β-naphthylguanidine (pK_a 10.7) *N*-phenylguanidine (pK_a 10.8)], but the interpolation of a methylene bridge, as in benzamil, will weaken this effect.

Triethylamine has a pK_a of 10.9, yet 2-diethylaminoethylbenzimidazole has a pK_a of 2.1. It is entirely consistent that the benzimidazole moiety should reduce the pK_a of guanidine to 7, as in 2-guanidinobenzimidazole.

It seems likely that the 2-amino position in triamterene is protonated first. The pK_a values of 2-amino-, 4-amino-, and 7-aminopteridine are 4.26, 3.54, and 2.20, respectively, and 2,4-diamino- and 4,7-diaminopteridine have pK_a values of 5.30 and 4.96. 2,4,7-Triaminopteridine has a pK_a of 6.29, almost identical with that of triamterene (pK_a 6.2). With protonation in the 2-amino position in triamterene, the 1,2,3 portion of the pteridine ring is isosteric with the guanidine groups of benzamil and amiloride (Figs. 11 and 12).

It would appear that the pK_a values of all the compounds used in this study refer to the protonation of the guanidine moiety; furthermore, from Corey-Pauling-Koltun space-filling models of these compounds, it is clear that the guanidine group occupies a prominent and accessible position in each substance (Figs. 11 and 12). This is of interest, as it has been proposed that the guanidinium group of tetrodotoxin and saxitoxin is responsible for "plugging" sodium channels in excitable membranes (14). In benzamil, in which the guanidine is highly substituted, the phenyl ring can be almost coplanar with the pyrazine ring, providing considerable scope for short-range van der Waals interactions with hydrophobic surfaces (Fig. 12). This seems to be less possible with triamterene, as its guanidinium isostere is part of a rigid pteridine structure. This may explain the decreasing order of affinity found in passing from benzamil to amiloride to triamterene (Table 1).

Turning to the biological aspects of these results, it is necessary to comment on the rather wide variation in pK_a for the membrane receptors which appear to control sodium entry at the mucosal face of the epithelium (pK_a 4.6–6.4). The first reactive cell layer of frog skin lies immediately below a dead layer of cells, the stratum corneum (15). Frogs frequently shed the stratum corneum, and the underlying stratum granulosum becomes the new stratum corneum. It is possible in a skin which has molted recently that hydrion concentrations at the first reactive cell layer more truly represent those in the bulk phase than in a skin which is an intermolt phase. This, too, may explain the minor differences between the affinity of amiloride in different skins. However, there are also changes in the receptors for amiloride on molting. Nielsen and Tomlinson (16) found that freshly molted skins were insensitive to amiloride. We have confirmed this (17) and found that the normal range of sensitivity returns within 4 hr of molting, but this can be delayed by omitting calcium from the bathing solution. Thus variations in pK_a may be due to failure of the concentration at the first reactive cell layer to rise to that of the bulk phase, and also to unknown variations in the receptors themselves associated with the state of the skin.

With amiloride, benzamil, and triamterene, reasonable agreement has been found with the proposal that the charged (guanidinium) form of these drugs combines with a dissociated acid grouping in the sodium channel to block sodium entry. The agreement between experiment and theory is

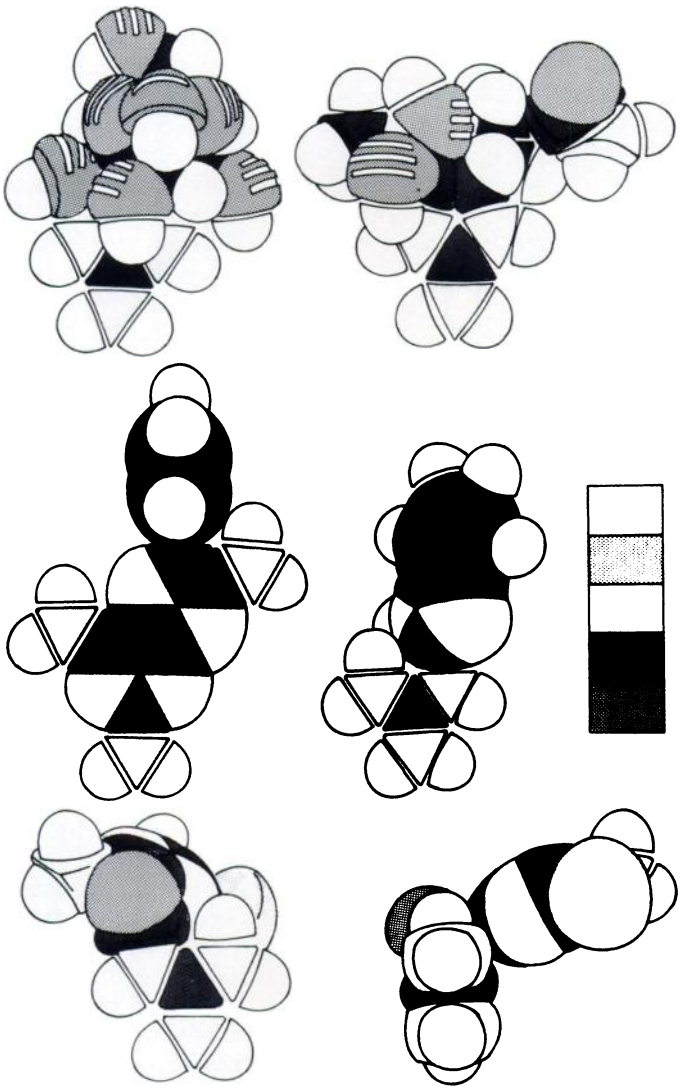

FIG. 11. *Diagrams traced from photographs of Corey-Pauling-Koltun space-filling models of (from top left) tetrodotoxin, saxitoxin, triamterene, 2-guanidinobenzimidazole, and amiloride (two views)*
The key shows the positions of (from the top) atoms of hydrogen, chlorine, nitrogen, carbon, and oxygen.

not perfect, which may mean that the theory is not totally adequate; for example, the nonprotonated drug molecules may have weak affinity. However, calculation of the predicted behavior of the drugs with pH requires accurate values of K_D, K_R, and K_{real}, and only the first of these can be measured with absolute precision.

Bentley (2) reported that 0.1 mM guanidine inhibited SCC in isolated toad bladder by 54%, while Garcia-Romeu (18) showed that the same concentration had no effect on the skin of *R. esculenta*. With skins of *R. temporaria* the inhibitory effects of guanidine are extremely weak and variable in extent. Furthermore, the variation in activity with pH shows an unexpected form. It appears that when the pH of the mucosal solution is moved far from neutrality, the inhibitory action of guanidine is increased. There is no obvious explanation for this behavior, and guanidine may inhibit transport by a totally different mechanism from that of amiloride. For ex-

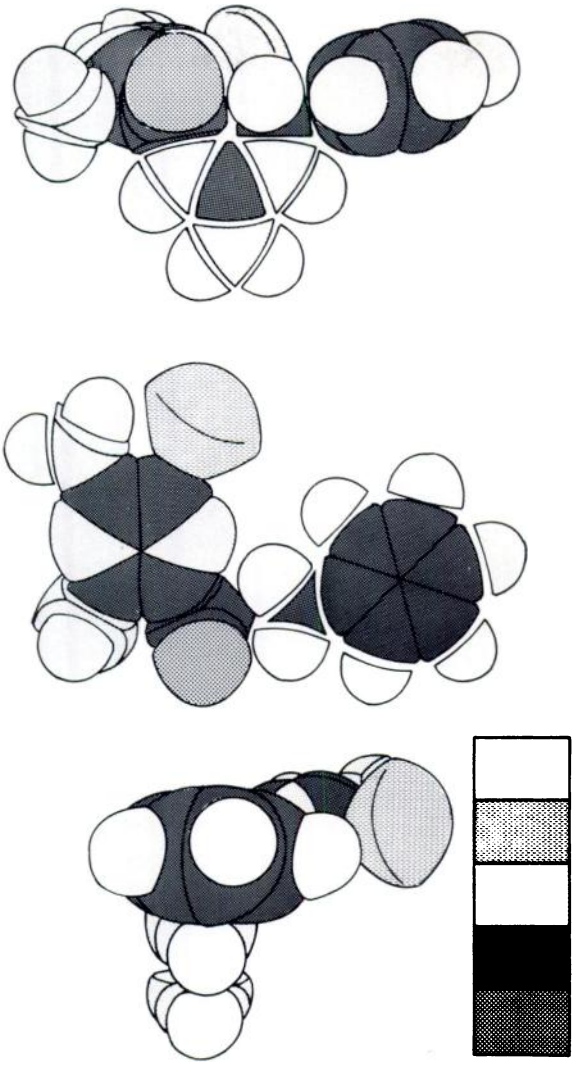

FIG. 12. *Benzamil, three views*

Diagrams were traced from photographs of a Corey-Pauling-Koltun space-filling model. The key shows the position of (from the top) atoms of hydrogen, chlorine, nitrogen, carbon, and oxygen.

ample, guanidine might be able to penetrate the mucosal sodium channel and accumulate in the transport compartment without being expelled by the serosal sodium pump, resulting in a reduction in current. On the other hand, this is unlikely, as ammonium ions (10 mM) do not affect the SCC of toad bladders when applied to the mucosal surface (19). It seems as if the results with guanidine are dependent both on species and perhaps on season, while bladders and skins of various amphibia respond uniformly well to amiloride, with variations of affinity within a 3-fold range at neutral pH. While reasonable agreement has been found for the suggestion that it is the charged (guanidinium) forms of the compounds which are active, it does not follow necessarily that the guanidinium group interacts directly with the active site. If this is so, the very low activity of guanidine may be explained. Alternatively, guanidine may be present at the active site so briefly that

effective inhibition of sodium ion translocation is prevented. Whatever the reason, it is clear that the high activity of some of the compounds does not depend on the guanidine moiety alone.

Although it is generally stated that tetrodotoxin has little or no effect on transport in epithelia, the evidence for this is hard to find. Failure to influence sodium transport when applied to the mucosal face (360 μM) has been reported for toad bladder (20). In a single experiment the toxin had a small, but nonetheless definite, effect, which indicated a probable affinity of 10^3 M^{-1}. If the guanidinium group is indeed the functional moiety, the specificity of the toxins for sodium channels in excitable membranes and of the diuretic drugs for epithelia must mean that the selectivity is very dependent on the non-guanidinium parts of the molecule. Goldberg and Kao (21) noted that chemical modification of tetrodotoxin led to profound loss of the biological activity, and found that 2-aminobenzimidazole and 2-amino-4,4,6-trimethyl-3,4-dihydropyrimidine, with guanidinium groupings in 5- and 6-membered rings, respectively, had little tetrodotoxin-like activity in nerve and muscle preparations.

The responses of epithelia to 2-guanidinobenzimidazole are more variable than one would wish for this compound to be studied quantitatively. However, the inhibitory component of its action appears to be due to a charged (guanidinium) grouping. The stimulating effects on SCC are more probably connected with the imidazole moiety, as phentolamine can also produce similar effects (18), at least in skins of *R. esculenta*. The over-all conclusion from this work is that guanidinium groups are important for the inhibitory actions of all the compounds studied.

During this study it became increasingly clear that the Kao-Nishiyama hypothesis (14) formulated to explain the effects of blocking drugs on sodium channels in excitable membranes, particularly as extended by Hille (22, 23), is remarkably similar to the situation in epithelia.

The conductance of maximally activated sodium channels in nerves is reduced by pH in a way which suggests that their permeability properties are controlled by a singly ionized acid grouping with a pK_a of about 5 (24). Tetrodotoxin is less active at alkaline pH, that is, in the dipolar ion form, than as the cationic species (3). Binding of radiolabeled tetrodotoxin to sodium channels in nerve membranes is antagonized at low pH in a way which suggests that protons compete with the toxins for the binding site (25). All these properties of the tetrodotoxin-receptor interaction are mirrored in the interactions of amiloride, benzamil, and triamterene with sodium channels in epithelia. In excitable membranes selectivity appears to be controlled by a filter which binds tetrodotoxin and which is in series with a voltage-dependent gating mechanism (26, 27). The selectivity process in sodium channels in epithelia seems to depend on the same general strategy as that in excitable membranes, but whether the process controls entry to a channel through which ion flow readily saturates, or to a carrier mechanism, or indeed forms part of a carrier mechanism, is not known. However, the similarities indicated by the present studies suggest that both types of entry mechanism, in epithelia and excitable membranes, may have a common ancestry.

A report of experiments to examine the effects of pH on the actions of amiloride appeared while this paper was in press (Benos, D. J., Simon, S. A., Mandel, L. J. & Cala, P. M. (1976) *J. Gen. Physiol.*, **68**, 43–63.) These authors considered only the effect of pH on the ionisation of the drug but not of the channel. This may explain some of the differences in detail from this study, although there is broad agreement in principle.

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