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Inferences on the Nature of the Apical Sodium Entry Site in Frog Skin Epithelium[1]

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ABSTRACT

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The inhibition and stimulation of short-circuit current in *in vitro* frog skin by a series of structural analogs of the diuretic drug amiloride were studied. Also, the inhibitory profile of a new experimental diuretic, 3-(3-amino-1,2,4-oxadiazol-5-yl)-5-chloro-2,6-pyrazinediamine, or CGS 4270, was determined. The major conclusions of our studies are: 1) amiloride remains the most effective inhibitor of Na^+ entry, with both the pyrazine ring and the acylguanidine moiety being required for maximum activity; 2) CGS 4270 also inhibits Na^+ transport in frog skin; it acts only from the external solution, is uncharged, is noncompetitive with Na^+ and interacts noncompetitively with amiloride; the inhibition is independent of external calcium; and 3) benzimidazole increases amiloride-sensitive short-circuit current and this compound is competitive with amiloride. These results indicate that the putative Na^+ entry protein contains multiple inhibitory sites and that compounds which stimulate Na^+ entry may also bind at the same molecular locus as an inhibitor.

Sodium entry through the outer apical membrane of all electrically high resistance epithelia is thought to occur passively, *i.e.*, it is driven by the existing electrochemical potential gradient. Although this entry step is passive, it is nonetheless facilitated. To date, the most potent and specific inhibitor of this entry mechanism is the diuretic agent, amiloride (fig. 1). The variety of species and tissues in which this drug is able to inhibit sodium influx demonstrates the ubiquity as well as the similarity in the chemical aspects of this permeation mechanism throughout the animal kingdom (Bentley, 1979).

In recent years, two different types of studies designed to identify the chemical nature of the functional ligands involved in both the transport of Na across the apical membrane and in the specific binding and subsequent inhibition of transport by amiloride have been reported. First, chemical modification of the frog skin with group-specific reagents revealed that sulfhydryl groups are components of the apical sodium translocation pathway, but not of the amiloride receptor site (Benos *et al.*, 1980), which supports the idea that the locus of action of amiloride and of Na^+ are spatially distinct. Second, extensive structure-function studies of amiloride analogs have been performed using adrenalectomized rats (Cragoe *et al.*, 1967) and isolated frog skin (Benos *et al.*, 1976; Cuthbert and Fanelli, 1978; Li and deSousa, 1979). According to the results of Cragoe and collaborators (1967), the acylguanidine moiety is that portion of the amiloride molecule which is the least sensitive to molecular manipulation. Thus, substituted benzyl groups could be attached to the terminal amine of the guanidine side chain with complete retention of activity. Incorporation of an additional NH group into the side chain to give a carboxamidoguanidine derivative also resulted in complete retention of activity (Shepard *et al.*, 1969). It was found also that a halogen atom in position 6 was required for diuretic activity. The bromo and iodo analogs were somewhat less active than amiloride. Either one or both of the hydrogen atoms of the 5-amino group could be replaced with various hydrocarbon residues (up to four carbon atoms) with little diminution of activity.

The specificity of the interaction between amiloride and the apical Na^+ transport site in frog skin appears to be more restrictive (Benos *et al.*, 1976). As with the diuretic activity, addition of an NH group to the guanidine residue produced no change in potency. Analogs with substituents on the 5-amino group were inactive, although the 5-dimethylamino compound stimulated transport at concentrations greater than 10^{-6} M (Li and deSousa, 1979; D. J. Benos, unpublished observation). With regard to substitution on position 6, activity was retained with the bromo analog, but the iodo analog had only approximately 50% of the activity of amiloride. The deschloro analog was inactive. The results of Cuthbert and Fanelli (1978) are essentially similar to those of Benos *et al.* (1976).

Other compounds also have been shown to stimulate sodium

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ABBREVIATIONS: BIG, 2-benzimidazolylguanidine; I_{sc}, short-circuit current; J^i_{Na}, net sodium influx; TAP, 2,4,6-triaminopyrimidine.

transport in frog skin, *e.g.*, BIG (Zeiske and Lindemann, 1974). These authors found that the 5-nitro analog and the substance in which the guanidine function was replaced by hydroxy group (*i.e.*, 2-hydroxybenzimidazole) displayed little activity. However, elimination of the guanidine group and simultaneous addition of either one or two methyl groups of the benzene ring resulted in only a very small decrease in the stimulatory effect.

Garcia-Romeu (1970) also observed stimulation of I_{sc} subsequent to external addition of substances containing guanidine and/or imidazole groups. The most effective compounds in stimulating I_{sc} were phentolamine, BIG and benzimidazole. Like Zeiske and Lindemann (1974), Garcia-Romeu (1970) concluded that the guanidine moiety was not essential for stimulatory activity, but that this property resided in the imidazole group.

These observations may be of critical importance in understanding the structural, physiological and biochemical basis of sodium entry into epithelial tissues. Apparently, similar compounds can either increase or decrease Na migration through these apical entry pathways. Perhaps this means that there are two regions of the entry site with which guanidine-containing molecules can interact.

The objective of the present study was to gain further insight into the properties of the Na^+ entry site using *in vitro* frog skin epithelium as a model system. Several amiloride-like compounds were screened for their ability either to increase or decrease Na^+ transport. Particular emphasis was given to two such compounds, CGS 4270 and benzimidazole. The results show that although CGS 4270 displays an inhibitory profile similar to amiloride, it is not mutually exclusive with amiloride, *i.e.*, it binds to a different molecular locus than does amiloride. Benzimidazole, on the other hand, while stimulating I_{sc}, is mutually exclusive with amiloride.

Methods

Abdominal skin of the bullfrog, *Rana catesbeiana*, was mounted as a flat sheet (3.14 cm^2 in area) between Lucite chambers equipped with glass solution reservoirs. The solutions in each chamber (12 ml each) were stirred and oxygenated by bubbling with room air. All experiments were performed at 19°C.

The open circuit potential across the skin was measured with calomel electrodes and current was passed through the skin *via* platinum electrodes. The outside bathing solution always served as ground. The potential sensing electrodes were connected to the solution reservoirs through 4% agar bridges having a composition identical to that of the bathing solution in the chambers. An automatic voltage clamp was used to pass and monitor the appropriate amount of current through the skin to clamp the transepithelial membrane potential to 0 mV. The voltage clamp circuit also compensated for the resistance of the solution between the agar bridges. In all experiments (except those in which the external calcium concentration was reduced to zero), both sides of the skin were bathed with identical solutions. Under these conditions, the magnitude of the I_{sc} and the net active transport of sodium are equivalent (Cereijido *et al.*, 1974; Candia and Reinach, 1977; Benos *et al.*, 1979).

Considerable variations in the absolute values of I_{sc} and transepithelial potential (V_m) were observed. The frogs were obtained from West Jersey Biological Supply Co. (Wenonah, N.J.). In order to minimize experimental variation, each series of experiments under a given set of conditions was performed on one batch of frogs. Experimental manipulations were initiated only after the transepithelial electrical properties of the *in vitro* frog skin had stabilized (30–60 min). Each skin preparation served as its own control. All results have been normalized to the values obtained under control conditions and expressed as the mean value ± 1 S.E.M. The probability that the difference between two population means was significant was assessed by computing the *t*-statistic for paired data, if the population variances were comparable; otherwise, the Fischer-Behrens test for significance was applied (Armitage, 1971). Differences between the means of three different populations were compared by a one-way analysis of variance.

The composition of the standard Ringer's solution used was as follows (in millimolars): NaCl, 110; KCl, 2.5; $CaCl_2$, 1.0; and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.4), 5. In experiments in which the sodium concentration was varied, the NaCl was replaced with an equimolar amount of recrystallized choline chloride. The sodium concentrations were 110, 55, 20, 6 and 3 mM. In experiments in which the external calcium concentration was reduced to zero, $CaCl_2$ was omitted from the Ringer's solution and 0.5 mM ethylene glycol bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid was added. The internal solution contained the standard (110 mM Na) Ringer's solution under all conditions.

All chemicals were of reagent grade and all solutions were made with doubly glass distilled water which was first passed through an ultra high purity demineralizer cartridge (Corning 3508A). Amiloride was obtained as a gift from Merck Sharp, & Dohme Research Laboratories (West Point, PA). All other organic compounds were obtained from Aldrich Chemical Company (Milwaukee, WI).

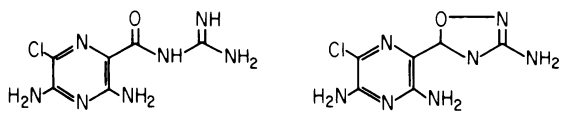
CGS 4270 or 3-(3-amino-1,2,4-oxidiazol-5-yl)-5-chloro-2,6-pyrazinedimine (Watthey *et al.*, 1980) is a diuretic agent in which the potentially conformationally mobile acylguanidine moiety of amiloride has been replaced by the rigid 1,2,4-oxidiazol-3-amine system (fig. 1).

In rats and dogs, CGS 4270 exhibited a profile of diuretic activity similar to that of amiloride. The substance was synthesized from 3,5-diamino-6-chloro-2-pyrazinecarbonitrile *via* the iminoether hydrochloride and the N-cyanoamidine.

We also performed ^{22}Na influx experiments under standard short-circuit conditions on frog skins either stimulated by benzimidazole or inhibited by CGS 4270 to confirm the equivalence of J^i_{Na} and I_{sc} under these conditions. The flux experiments were performed as previously described (Benos *et al.*, 1976). In the presence of 5 mM external benzimidazole, the ^{22}Na influx averaged 4.78 (±0.45) × 10^{-10} mol/cm^2·sec, whereas the simultaneously measured I_{sc} was 4.28 (±0.53) × 10^{-10} mol/cm^2·sec ($N = 7$). These numbers are not significantly different from one another ($P > 0.4$). This concentration of benzimidazole stimulated I_{sc} by an average of 104.8 ± 16.5% with respect to control, while increasing the mean transepithelial conductance from 0.34 ± 0.2 to 7.0 ± 1.1 mS/cm^2. Likewise, in eight additional frog skins, the equivalence of J^i_{Na} and I_{sc} was also demonstrated at two different concentrations of CGS 4270 (table 1). We assume in this paper that the identity between I_{sc} and J^i_{Na} is valid under all experimental conditions and drug treatments.

Results

Amiloride analog studies. The specificity of the interaction of amiloride and the sodium entry site was assessed by examining the effect upon I_{sc} produced by a series of heretofore untested analogs at pH 7.4 at an external concentration range of 10^{-6} to 10^{-4} M (table 2). Pyrazine compounds lacking an



Amiloride CGS 4270

Fig. 1. The structures of amiloride and CGS 4270.

TABLE 1

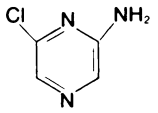
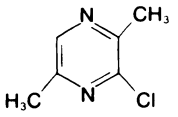
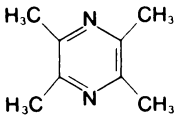
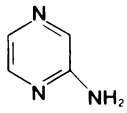
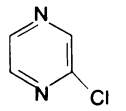
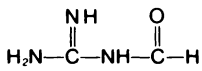
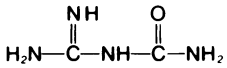
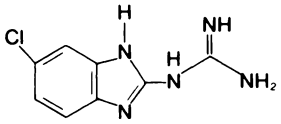
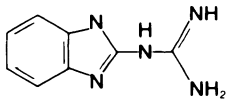
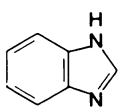
The effect of CGS 4270 on unidirectional $^{22}Na^+$ influx in frog skin epithelium

Condition	I_{sc}	Tracer Na^+ Influx
Control	3.23 ± 0.40	3.10 ± 0.44
CGS 4270 (1 × 10^{-5} M)	2.28 ± 0.37	2.17 ± 0.36
CGS 4270 (2 × 10^{-4} M)	0.93 ± 0.19	0.99 ± 0.24

TABLE 2

The effect of various amiloride analogs on I_{sc} of bullfrog skin

All compounds were added to the outside bathing solution at the concentrations indicated. The results are expressed as the steady-state I_{sc} observed after exposure to the compound normalized to the control value of I_{sc} measured just before compound addition. The probability that drug addition caused a significant effect on I_{sc} (either stimulation or inhibition) is indicated in the footnote.

Analog No.	Compound and Structure		10^{-7} M	10^{-6} M	10^{-5} M
1	2-Amino-6-chloro-pyrazine		0.99 ± 0.04 (*N* = 6)	1.14 ± 0.04 (*N* = 3)	1.05 ± 0.08 (*N* = 6)
2	3-Chloro-2,5-di-methylpyrazine		0.94 ± 0.03 (*N* = 3)	0.94 ± 0.04 (*N* = 3)	0.95 ± 0.05 (*N* = 3)
3	Tetramethyl pyra-zine		0.99 ± 0.02 (*N* = 3)	0.99 ± 0.02 (*n* = 3)	0.98 ± 0.01 (*N* = 3)
4	Aminopyrazine			0.93 ± 0.09 (*N* = 3)	0.91 ± 0.10 (*N* = 3)
5	Chloropyrazine			0.95 ± 0.02 (*N* = 3)	1.02 ± 0.05 (*N* = 3)
6	1-Formylguanidine		1.06 ± 0.04 (*N* = 3)	0.98 ± 0.04 (*N* = 6)	0.88 ± 0.03* (*N* = 3)
7	Guanylurea			1.03 ± 0.04 (*N* = 3)	0.98 ± 0.01 (*N* = 3)
8	5-Chloro-2-benz-imidazolylguanidine			1.04 ± 0.02 (*N* = 3)	1.07 ± 0.02 (*N* = 3)
9	BIG			0.87 ± 0.01*** (*N* = 3)	0.88 ± 0.04* (*N* = 3)
10	Benzimidazole			1.08 ± 0.01** (*N* = 3)	1.12 ± 0.03 (*N* = 3)

* $P < .05$; ** $P < .025$; *** $P < .001$.

acylguanidine group (analogs 1–5) had no significant effect on I_{sc} in this concentration range. Likewise, acylguanidines lacking a pyrazine ring, *i.e.*, 1-formylguanidine and guanylurea (analogs 6 and 7), had little influence on I_{sc}.

Three imidazole derivatives were also tested for their ability to alter I_{sc} (analogues 8–10). These experiments were prompted by the observations of Garcia-Romeu (1970) who first suggested that the imidazole group, when interacting with the putative sodium entry protein, results in a stimulation of I_{sc}. However, under the conditions used in the present study, BIG (analog 9) inhibited I_{sc}. This compound differs from analog 8 only in lacking the chloro substituent on the benzene ring.

The effect of CGS 4270 on I_{sc} in frog skin. Recently, the synthesis of an analog of amiloride in which the acylguanidine moiety was replaced by a 1,2,4-oxidiazol-3-amine unit was reported (Watthey *et al.*, 1980). This compound (CGS 4270) was found to act as a potassium-sparing diuretic with an overall excretion profile similar to that induced by amiloride in whole-animal studies using rats and dogs (Watthey *et al.*, 1980). We decided to examine the effect of CGS 4270 on I_{sc} not only because of its potential clinical application but also with the aim of gaining further insight into the properties of the apical sodium entry site in frog skin epithelium.

Figure 2, A and B displays log-dose response curves for the CGS 4270-induced inhibition of I_{sc} at external pH 6.0, 7.4 and 9.5. Acidifying the external medium had no effect on the inhibition of I_{sc} by CGS 4270. Raising the external pH to 9.5 slightly shifted the dose-response curves to the right, but the lowered mean inhibition of I_{sc} at any concentration of CGS 4270 is not significant ($P > 0.5$), Determination of the pK_a of CGS 4270 in Ringer's solution by fluorescent spectrometry was not possible due to the limited solubility of the compound in aqueous solution, but the pK_a for CGS 4270 in 80% aqueous dimethyl sulfoxide was found to be less than 2. Hence, it is unlikely that CGS 4270 is protonated in the pH range 6.5 to 9.5.

The inhibition of I_{sc} by CGS 4270 could be reversed by washing the apical surface of the frog skin with drug-free Ringer's solution. For example, in 19 separate skins, the ratio of I_{sc} after washing to I_{sc} measured just before addition of 2×10^{-4} M CGS 4270 was 0.956 ± 0.062, a value not significantly different from 1 ($P > 0.4$). Addition of CGS 4270 to the inside bathing solution had no effect on I_{sc}, even at 3×10^{-4} M.

The effect of external Ca^{++} on CGS 4270 inhibition was studied because it has been shown that Ca^{++} noncompetitively inhibits Na^+ transport from the external solution in frog skin (Mandel, 1978; Benos *et al.*, 1979) and also because Ca^{++} may be necessary for amiloride to inhibit Na^+ entry effectively in the skins of some species of frogs (Cuthbert and Wong, 1972; Benos *et al.*, 1976). Removal of external Ca^{++} had no effect on the inhibitory action of CGS 4270 (fig. 3).

The kinetic properties of CGS 4270 inhibition of I_{sc} were investigated, assuming that the rate of Na^+ transport as a function of $[Na^+]$ would be adequately described by Michaelis-Menten kinetics. The theoretical basis for analyzing steady-state Na transport data in frog skin in this way has been thoroughly discussed (Mandel, 1978; Benos *et al.*, 1979; Balaban *et al.*, 1979). Experiments were first performed to examine the effect of lowered external $[Na^+]$ on CGS 4270 inhibition characteristics (fig. 4). If CGS 4270 were competitive with Na^+, a leftward shift (*i.e.*, an increase in compound affinity) in the dose-response curve would be predicted. However, the inhibition curves obtained at either $[Na^+]$ = 110 or 3 mM were

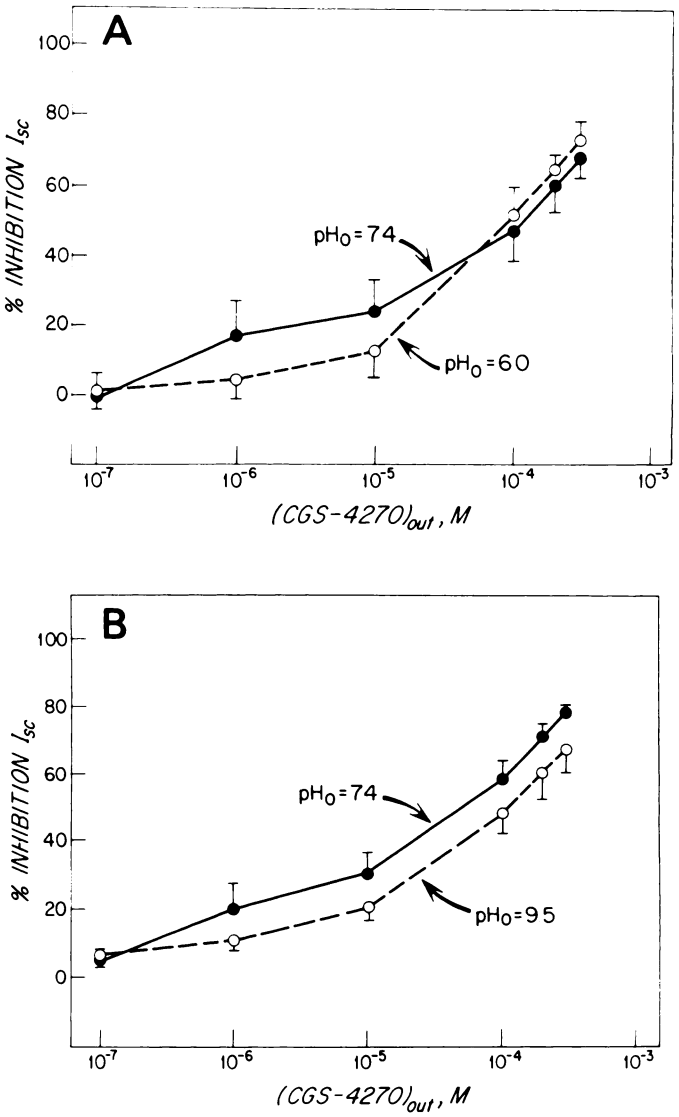


Fig. 2. Log-dose response curves of CGS 4270 inhibition of I_{sc} of bullfrog skin epithelium at 110 mM Na at external pH 7.4 and 6.0 (A) or pH 7.4 and 9.5 (B). The serosal medium pH was always 7.4. Each point represents the mean of 12 experiments and the vertical lines indicate 1 S.E.M. Changing the external pH to either 6.0 or 9.5 did not affect the transepithelial electrical properties of the epithelium measured at pH 7.4. The mean I_{sc} and conductance values measured before CGS 4270 addition for the skins used in (A) were 30.5 ± 3.0 $\mu A/cm^2$ and 0.51 ± 0.04 mS/cm^2, respectively, and for (B), 31.2 ± 4.2 $\mu A/cm^2$ and 0.76 ± 0.12 mS/cm^2, respectively.

indistinguishable (fig. 4), suggesting that CGS 4270 does not compete with Na^+. Figure 5 presents a summary of experiments in which the I_{sc} was measured as a function of $[Na^+]$, both in the absence and presence of 10^{-5} M CGS 4270. All results have been normalized to the value of I_{sc} at $[Na^+]$ = 110 mM (107 ± 18 μA) in the absence of drug. The data were recast as Eadie plots to allow calculations of the kinetic parameters K_t (defined as concentration of Na^+ required to maintain I_{sc} at one-half its maximum value) and I_{max} (the maximum value of I_{sc}). In the absence of drug, the K_t for Na^+ was 11.8 ± 2.2 mM. The addition of 10^{-5} M CGS 4270 resulted in no significant change in K_t (14.0 ± 2.7 mM), $P > 0.2$, but I_{max} was reduced from 105.6 ± 2.8 to 88.9 ± 4.5 μA ($P < 0.001$). These data show that CGS

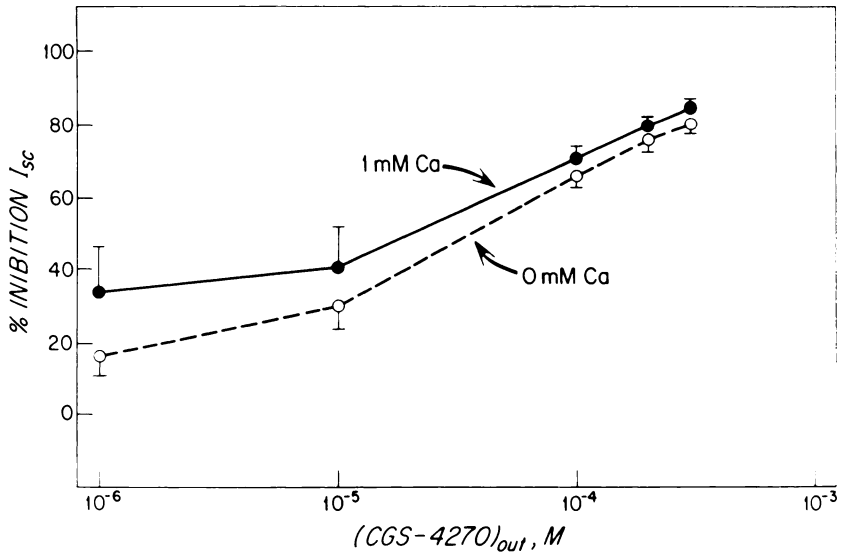


Fig. 3. The effect of Ca^{++} on the CGS 4270 inhibition of I_{sc} of bullfrog skin at 110 mM Na at pH 7.4 Each point is the mean of four skins and the bars represent 1 S.E.M. Removal of external Ca^{++} resulted in an average increase of I_{sc} of 38.9 ± 6.8%. The control values for L_{sc} and conductance in these experiments were 25.1 ± 3.5 $\mu A/cm^2$ and 0.45 ± 0.18 mS/cm^2, respectively.

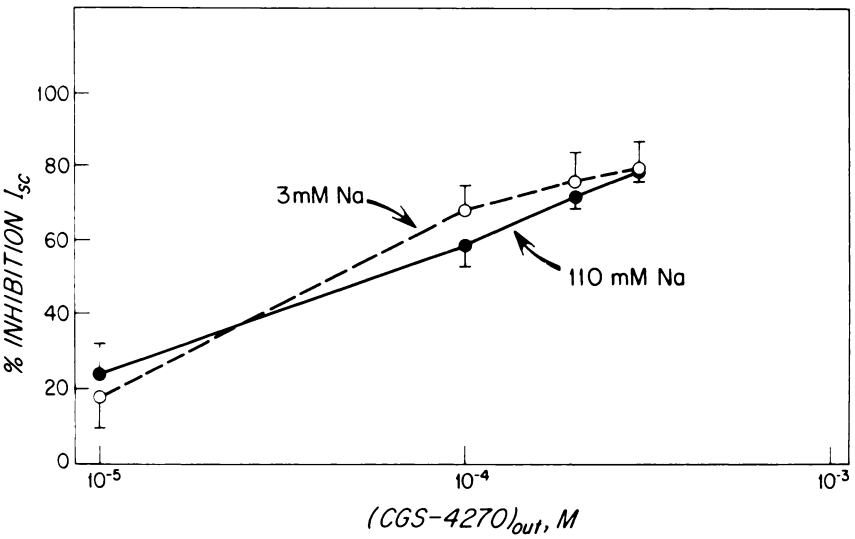


Fig. 4. Log dose-response curves of CGS 4270 inhibition of I_{sc} of bullfrog skin epithelium at external $[Na^+]$ of 110 and 3 mM (choline substitution). Each point was averaged from 12 skins (±1 S.E.M.). The initial I_{sc} (before changes in $[Na^+]$ or drug addition) averaged 34.1 ± 5.7 $\mu A \cdot cm^2$ and conductance values 0.58 ± 0.09 mS/cm^2. CGS 4270 always decreased the transepithelial skin conductance (G_T). For example, at $[Na^+]$ = 110 mM, 10^{-5} M CGS 4270 decreased G_T to 0.36 ± 0.05 mS/cm^2 ($P < 0.01$) and 2 × 10^{-4} M CGS 4270 decreased G_T to 0.25 ± 0.06 mS/cm^2 ($P < 0.001$).

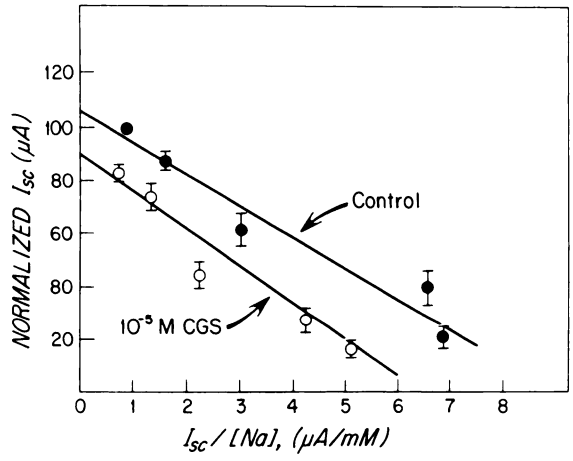


Fig. 5. Single reciprocal (Eadie) plot (I_{sc} *vs.* $I_{sc}/[Na^+]$) of I_{sc} *vs.* $[Na^+]$ data obtained from isolated bullfrog skin in the absence and presence of 10^{-5} M CGS 4270. The ionic strength was maintained constant with choline chloride. Each point represents the mean of 12 experiments. Mean values (±S.E.M.) of K_t and I_{max} were computed by averaging each K_t and I_{max} measured for every individual skin. Curves were fit by linear regression and r^2 was always greater than 0.96.

4270 is a noncompetitive inhibitor of Na^+ transport in bullfrog skin epithelium.

The results described thus far for CGS 4270 indicate that its inhibitory action has similar properties to those of amiloride and TAP in this preparation (Benos *et al.*, 1979; Balaban *et al.*, 1979). We next decided to examine the inhibition of I_{sc} by mixtures of CGS 4270 and amiloride in order to study the interaction between these two agents. The two simplest possibilities are that CGS 4270 and amiloride bind to the same molecular locus and are thus mutually exclusive or that CGS 4270 and amiloride bind to spacially distinct sites which do not interact. To investigate these possibilities, a kinetic model based upon noncompetitive inhibition was derived (see Segel, 1975; Balaban *et al.*, 1979). In the case of mutual exclusively, the equation for the Dixon plot is:

$$\frac{1}{I_{sc}} = \frac{K_t}{[Na^+]I_{max}K_{CGS}}[CGS] + \frac{1}{I_{max}}\left(1 + \frac{K_t[amiloride]}{[Na^+]K_{AM}}\right) \quad (1)$$

where K_{CGS} and K_{AM} are the equilibrium dissociation constants for CGS 4270 and amiloride, respectively. Thus, the plot $1/I_{sc}$ *vs.* [CGS 4270] at a fixed [Na] and [amiloride] is linear with a

slope of $K_t/[Na^+]I_{max}K_{CGS}$. The slope is independent of [amiloride] if the inhibitors are mutually exclusive.

If the inhibitors are noncompetitive, both with Na^+ and with each other, the equation for the Dixon plot is:

$$\frac{1}{I_{sc}} = \frac{K_t}{[Na^+]I_{max}K_{CGS}}\left(1 + \frac{[\text{amiloride}]}{\alpha K_{AM}}\right)[CGS] + \frac{1}{I_{max}}\left(1 + \frac{K_t}{[Na^+]} + \frac{K_t[\text{Amiloride}]}{[Na^+]K_{AM}}\right) \quad (2)$$

where α is measure of the interaction between the two inhibitors, *i.e.*, the binding of one inhibitor may ($\alpha \neq 1$) or may not ($\alpha = 1$) affect the binding of the other (Segel, 1975) and the family of curves obtained at different fixed concentrations of amiloride would not be parallel.

Figure 6 illustrates that two fixed concentrations of amiloride, 5×10^{-8} and 1×10^{-7} M, resulted in significant increases in the slopes of the Dixon plots (the probability that the mean values of the three slopes are the same is less than .005). This behavior is indicative of two inhibitors which interact at different molecular loci. The fact that the family of lines intersect to the left of the $1/I_{sc}$-axis and above the horizontal axis may indicate that CGS 4270 and amiloride do not have an allosteric effect on the binding of each other, *i.e.*, $\alpha = 1$ (Segel, 1975).

Stimulation of I_{sc} by benzimidazole. The effect of 1 and 5 mM benzimidazole on the external Na^+ activation curve of I_{sc} in bullfrog skin is shown in figure 7. Benzimidazole stimulated I_{sc} at all external [Na^+], above 6 mM. These increases in I_{sc} produced by 1 or 5 mM benzimidazole were significant at the .05 level. Reciprocal plots for these data revealed that benzimidazole increased I_{max} as well as decreased K_t slightly.

In order to decide whether benzimidazole interacts with the putative Na^+ entry protein at the same or at a different molecular locus than amiloride, experiments in which I_{sc} is measured as a function of amiloride concentration at different fixed concentrations of benzimidazole were performed and the data recast into a Dixon plot (*i.e.*, $1/I_{sc}$ *vs.* [amiloride]). If amiloride acts noncompetitively with respect to Na^+, but if amiloride and benzimidazole are mutually exclusive, the equation for the Dixon plot is (Segel, 1975):

$$1/I_{sc} = \frac{\left(1 + \frac{\alpha K_t}{[Na^+]}\right)}{\alpha K_{AM}I_{max}\left(1 + \frac{[\text{benz}]}{K_B}\right)}[\text{amiloride}] + \frac{1}{I_{max}}\left(1 + \frac{K_t}{[Na^+]}\right) \quad (3)$$

where K_B is the equilibrium dissociation constant for the reaction between benzimidazole and its interaction site on the apical membrane. Figure 8 illustrates that increasing benzimidazole concentrations resulted in parallel displacements of the amiloride Dixon plots with the slope remaining constant at approximately 3.0×10^{-2} M ($P > 0.25$ that the slopes are the same).

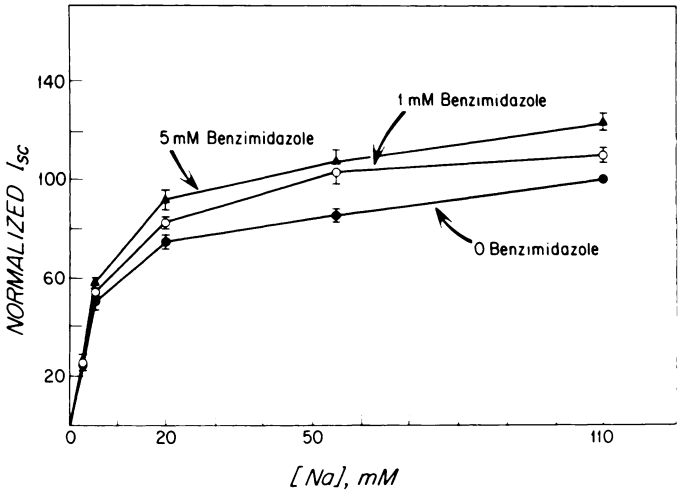


Fig. 7. I_{sc} *vs.* [Na^+] in the absence and presence of 1 and 5 mM benzimidazole in the external solution. Ionic strength was maintained with choline chloride. Each point represents the mean of 12 experiments and the bars indicate 1 S.E.M. The absolute values of I_{sc} and conductance measured under control conditions averaged 41.8 ± 2.2 $\mu A/cm^2$ and 0.56 ± 0.07 mS/cm^2, respectively. At [Na^+] = 110 mM, 5 mM benzimidazole caused an increase in the mean tissue conductance (0.77 ± 0.07 mS/cm^2, $P < 0.001$). There was no significant change in conductance values with respect to control produced by 1 mM benzimidazole under these conditions (0.58 ± 0.06 mS/cm^2, $P > 0.5$).

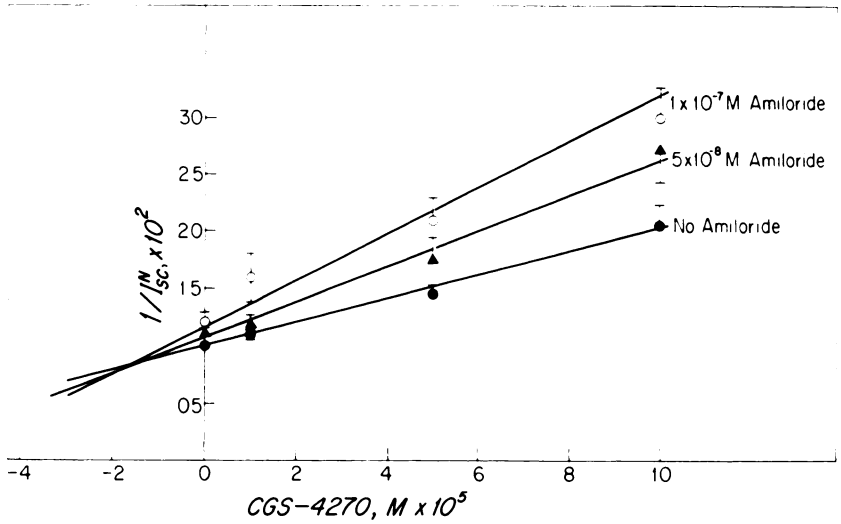


Fig. 6. Dixon plot of $1/I^n_{sc}$ *vs.* CGS 4270 at 0, 5 × 10^{-8} and 1 × 10^{-7} M external amiloride concentrations ([Na^+] = 110 mM). I^n_{sc} represents the I_{sc} normalized to 100 at I_{max}. Data points represent the mean of seven frog skins; bars indicate 1 S.E.M. In these experiments, the initial values of I_{sc} and conductance averaged 21.9 ± 2.2 $\mu A/cm^2$ and 0.57 ± 0.13 mS/cm^2, respectively.

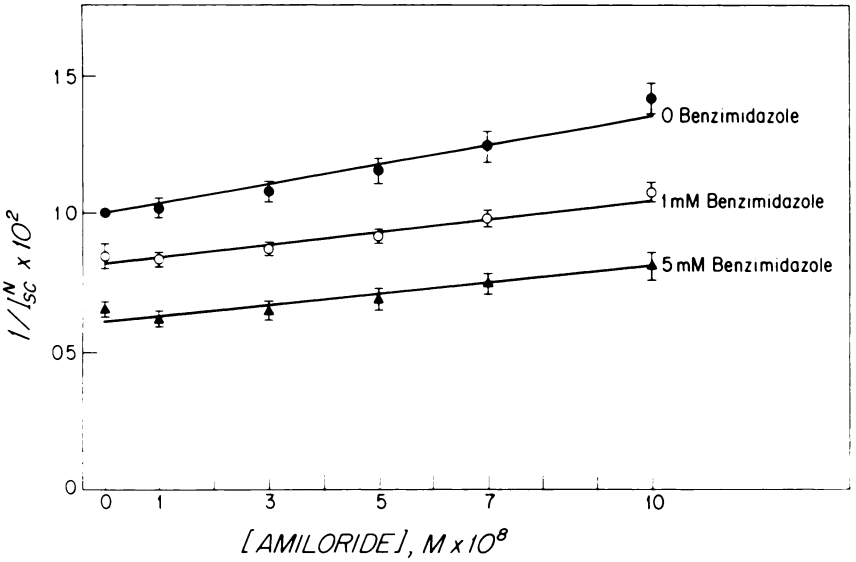


Fig. 8. Dixon plot of $1/I_{sc}^{n}$ *vs.* amiloride concentration at 0, and 5 mM benzimidazole. Data points represent the average of eight experiments and the bars indicate 1 S.E.M. Initial values of I_{sc} and conductance were 33.8 ± 5.5 $\mu A/cm^2$ and 0.41 ± 0.05 mS/cm^2, respectively, in these experiments. The slopes of each of these lines were not significantly different from one another ($P > 0.25$, analysis of variance).

The behavior conforms to equation 3 and thus indicates that benzimidazole and amiloride affect the binding of each other and that they may interact at the same site.

Discussion

The studies reported in this paper were designed with two purposes in mind. First, we wanted to obtain further information on the nature of the Na entry pathway in the apical membrane of the bullfrog skin epithelium by study of structure-activity patterns observed with inhibitors and activators of I_{sc}. Secondly, we desired to examine in a model system the pharmacological characteristics of a newly synthesized potassium-sparing diuretic, CSG 4270.

The inhibition of Na entry on bullfrog skin by CGS 4270 displays remarkable similarities to the inhibitory action of both amiloride (Benos *et al.*, 1979) and TAP (Balaban *et al.*, 1979). All three compounds inhibit I_{sc} rapidly and reversibly from the external solution. All drugs are noncompetitive inhibitors of Na entry, implying that amiloride, TAP and CGS 4270 interact with a site or sites spatially distinct from the site at which the transported Na^+ interacts. Amiloride, TAP and CGS 4270 do not require the presence of external calcium to potentiate their inhibitory activity in bullfrog skin.

There are several important differences between these three drugs, however. First, the K_Is are different; amiloride has an equilibrium dissociation constant of approximately 5×10^{-7} M in bullfrog skin (Benos *et al.*, 1979), TAP has a K_I of 10^{-3} M (Balaban *et al.*, 1979) and GCS 4270 possesses a K_I of about 5×10^{-5} M (figs. 2-4). Secondly, the inhibitory effect of CGS 4270 is independent of external pH in the range 6 to 9.5 and inasmuch as the pK_a of CGS 4270 in 80% aqueous dimethyl sulfoxide is <2, this drug is essentially unprotonated under the conditions of this study. Thirdly, both CGS 4270 and amiloride have Hill coefficients of around 0.5 (for CGS 4270, the calculated Hill coefficient was 0.55 ± 0.08, $N = 11$), whereas TAP has a Hill coefficient of approximately 1. These data indicate that for CGS 4270 and amiloride negative cooperatively is present in their interaction with bullfrog skin, but TAP interacts with its site in a one-to-one fashion with little or no cooperativity. Fourthly and most important, whereas amiloride and TAP are mutually exclusive (Balaban *et al.*, 1979), amiloride and CGS 4270 are not (fig. 6). The simplest explanation for these observations is that amiloride and TAP may be interacting at identical sites, whereas CGS 4270 binds to a different molecular locus. Because the diuretic activity of amiloride and CGS 4270 are similar (Watthey *et al.*, 1980), protonation is apparently not necessary for diuretic activity but may be required for maximum inhibition of I_{sc} in the frog skin preparation. Benos *et al.* (1979) have shown that amiloride may have more than one locus of action. It is possible that one of these sites has a relatively low affinity for amiloride and may correspond to the CGS 4270 binding site, a site in which protonation would not be required. This hypothesis may be tested by investigating the inhibitory activity of these drugs before and after treatment with chemical group specific reagents, as has been reported previously for amiloride (Benos *et al.*, 1980).

The specificity of the interaction between amiloride and the apical Na transport site was probed by examining the inhibition of I_{sc} produced by a number of structural analogs of the parent compound (table 2). These experiments extend the original studies of Benos *et al.* (1976) and others (Bentley, 1968; Cuthbert and Fanelli, 1978; Li and deSousa, 1979). From this earlier work, it appeared that both the substituted pyrazine ring and the acylguanidine portion of the molecule were required for maximal inhibitory activity. The results listed in table 1 confirm that neither pyrazine compounds lacking an acylguanidine group nor acylguanidines lacking a pyrazine ring have any influence on I_{sc}. Further, various combinations of these compounds (*e.g.*, analog 1 and analog 6) are also ineffective in inhibiting I_{sc}.

Two imidazole-containing molecules were found to stimulate I_{sc} (table 2) and hence to act as activators of Na entry (Segel, 1975). None of these compounds were capable of influencing I_{sc} when added to the serosal bathing medium. These observations confirm the previous reports of I_{sc} stimulation by imidazole derivatives (Garcia-Romeu, 1970; Zeiske and Lindemann, 1974; Cuthbert, 1976). The increases in I_{sc} of bullfrog skin were not as large as those reported by Zeiske and Lindemann (1974) in other species. It is noteworthy that in keeping with the results of the present study, Cuthbert (1976) observed inhibition of I_{sc}

by BIG at pH 7.6. Stimulation of I_{sc} by BIG was only evident at external pH values less than 4 and greater than 10. Cuthbert stated that BIG acted to stimulate I_{sc} by causing a nonspecific increase in membrane permeability. He argued that this was true because the increased I_{sc} (at pH > 10) was not amiloride sensitive, hence Na^+ entry was not occurring through "normal" sodium channels. However, this lack of sensitivity to amiloride would be anticipated because of the dimunition in the concentration of the biologically active protonated form at pH > 10 (Benos *et al.*, 1976).

Apparently, similar compounds can either increase or decrease Na^+ flow through these apical entry pathways. From the results presented here, one stimulator of I_{sc}, benzimidazole, is competitive with amiloride, possibly indicating a similar binding site. Also, the 5-dimethylamino analog of amiloride increases I_{sc} in frog skin (Li and deSousa, 1979). It would be interesting to determine if this compound and amiloride were mutually exclusive.

It appears that the electron distribution of amiloride and the effects that various substituent groups have upon this distribution are critical in determining whether I_{sc} is inhibited or stimulated. Theoretical calculations of electronic wave functions for a number of molecules have shown that a charge is not localized on a specific atom as is usually depicted, but is spread over the entire molecule (Richards and Wallis, 1977). An important implication of this is that it is untenable to think of localized negative charges on receptor sites positioned directly opposite a localized positive charge on an incoming molecule such as amiloride. The chemistry of drug receptor interactions is extremely complex; changes in the electronic distribution of an interacting molecule may perhaps profoundly alter a response of a receptor to that molecule.

The presence of a 5-dimethylamino substituent in the amiloride molecule thus might be expected to exert a profound influence on the interaction of the compound with the receptor. Amiloride itself may be regarded as an essentially planar molecule (Smith *et al.*, 1979). However, because of the adjacent chloride atom, the dimethylamino substituent in the analog will rotate so that the methyl groups lie above and below the plane of the aromatic ring to avoid volume strain. Furthermore, because of this rotation, there will be less electron donation to the aromatic ring and this will tend to render the nitrogen atom more basic.

From the steady-state kinetic experiments reported here and earlier (Balaban *et al.*, 1979), it is clear that CGS 4270 and amiloride (and possibly TAP) interact at different subsites on the Na^+ entry sites. Although benzimidazole and amiloride are mutually exclusive, it is not possible to state whether these molecules act at the same locus on the membrane.

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