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Phenamil: An Irreversible Inhibitor of Sodium Channels in the Toad Urinary Bladder

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Summary. Several new amiloride analogues and two reported photoaffinity analogues were tested for irreversible inhibition of short-circuit current, I_{sc}, in toad bladder. Bromoamiloride, a photoaffinity analogue, induced 40% irreversible inhibition at 500 μM after irradiation with ultraviolet light $\geq$ 320 nm. Iodoamiloride caused no irreversible inhibition. Of the new analogues tested, only 3,5-diamino-6-chloro-N-[(phenylamino) aminomethylene] pyrazinecarboxamide, *phenamil,* irreversibly inhibited I_{sc} at concentrations of 0.05 to 5 μM when added to the mucosal solution. Irreversible inhibition of I_{sc} by phenamil may be attributed to specific blockage of the mucosal sodium channels, which depended on: 1) time of exposure; 2) mucosal pH; 3) mucosal sodium concentration. For example, 5 μM phenamil irreversibly inhibited I_{sc} by 38% in 103 mM Na at pH 8.6 and nearly 75% in 30 mM Na at pH 6.4 after a 40-min exposure. Irreversible inhibition occurred in two phases with time constants of $\leq$ 10 min and approximately 140 min. Due to its irreversible nature, phenamil may be used to measure channel density.

Key Words amiloride · toad bladder · phenamil · sodium channels · binding

Introduction

Amiloride has been extensively used to investigate sodium transport in tight epithelia. In these tissues, amiloride inhibits transepithelial sodium transport primarily by binding to a sodium channel located in the mucosal membrane. Although amiloride binds specifically and with high affinity (5×10^{-7} M) to this channel, the amiloride-channel complex rapidly dissociates, having a sodium-dependent dissociation constant of 100 msec (in 60 mM NaCl) (*see* Benos, 1982, for review). The reversibility of amiloride has been extremely useful for experiments involving noise analysis, which yield the total number of open channels. However, this property limits the use of amiloride to measure channel density and as a tool for the eventual isolation and reconstitution of the channel. Consequently, it would be advantageous to obtain a molecule that is specific for epithelial sodium channels yet binds irreversibly.

In an attempt to find such molecules, investigators have used both group specific reagents and photoaffinity amiloride analogues. These compounds have been tested for irreversibility by measuring the irreversible inhibition of short-circuit current, I_{sc}, which in toad bladder is entirely accounted for by sodium transport. Benos and Mandel (1978) first reported that 500 μM bromoamiloride was a photoaffinity analogue as it irreversibly inhibited I_{sc} of frog skin by 40% when irradiated with ultraviolet light at 250 nm. Subsequently, Cobb and Scott (1981) obtained similar results utilizing 500 μM bromoamiloride irradiated at $\geq$ 320 nm in toad bladder. They also reported that 100 μM iodoamiloride induced 100% irreversible inhibition using the same procedure. Although iodoamiloride would appear to be a most promising molecule to use for binding and reconstitution studies (despite the high concentration required), we have not been able to reproduce these results (as reported below). In addition to the photoaffinity analogues, several group specific reagents have been reported to irreversibly inhibit I_{sc} of frog skin at concentrations ranging from 25 to 500 μM (Benos et al., 1980; Park & Fanestil, 1980; Park et al., 1983). Although both classes of molecules irreversibly inhibit I_{sc} presumably by binding to the sodium channel, neither of these classes are likely to be useful in measuring channel density since the high concentrations required for irreversible inhibition of I_{sc} would result in a large amount of nonspecific binding.

In this paper, the behavior of an amiloride analogue, 3,5-diamino-6-chloro-N-[(benzeneamino) aminomethylene] pyrazinecarboxamide (phenamil), which irreversibly inhibits I_{sc} in toad bladder at con-

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Table 1. Composition of solutions

Solution	pH	Compound (mM)						
		NaCl	KCl	$CaCl_2$	$MgSO_4$	$NaHCO_3$	NaH_2PO_4	Gluc.
A	8.6	85.0	4.0	1.5	0.8	17.5	0.8	10.5
B	8.6	11.7	4.0	1.5	0.8	17.5	0.8	10.5
C	8.1	100.0	4.0	1.5	0.8	2.5	0.8	10.5
D	6.4–6.7	102.7	4.0	1.5	0.8	0.0	0.8	10.5
E	6.4–6.7	29.2	4.0	1.5	0.8	0.0	0.8	10.5
F	7.0	9.2	4.0	1.5	0.8	0.0	0.8	10.5

centrations ranging from 0.05 to 5 μM is described. It is shown that phenamil specifically binds to the mucosal sodium channel and that the affinity constant is enhanced by reducing mucosal sodium and/or pH.

Materials and Methods

Experimental Animals and Solutions

Toads, *Bufo marinus*, were obtained from Carolina Biological Supply (Burlington, N.C.) or National Reagents (Bridgeport, Conn.). Toads were kept in aquaria containing tap water until they were sacrificed by double pithing. For a given set of experiments animals from one source were utilized although no differences were noted between groups.

All chemicals were reagent grade. Amiloride and its analogues were synthesized and characterized as described earlier (Cragoe et al., 1967) and their pK_a's determined as described elsewhere (Benos et al., 1976). Nystatin (606 U/ml) (Calbiochem, LaJolla, Calif.) was added to the mucosal solution utilizing dimethylsulfoxide as a carrier.

The composition of solutions used in this study are given in Table 1. When solutions containing low sodium ion concentrations were used and I_{sc} measured, sodium was not replaced (unless otherwise specified) with other electrolytes or nonelectrolytes to balance the osmolality of the mucosal solution, since I_{sc} does not depend on the osmolality of the mucosal solution (Mandel & Curran, 1973). The basolateral solution always contained 103.5 mM sodium. All solutions were gassed with air and experiments were performed at 22 ± 2°C.

I_{sc} Measurements

Hemibladders were gently stretched across a 2-mm nylon mesh (Small Parts, Miami, Fla.) and mounted in modified Ussing chambers with quartz windows as previously described by Benos and Mandel (1978). Both sides of the chamber were perfused via a recirculating system containing 20 ml reservoirs filled with solution A. The area of the chamber was 1.77 cm^2, and the volume on each side was 2 ml. Circular pieces of Pt wire were used as current electrodes. To measure transepithelial voltage, calomel electrodes bathed in 3 M KCl were interfaced with the chamber through 4% agar bridges containing 100 mM NaCl. I_{sc} was obtained by clamping the membrane potential to zero with an automatic voltage clamp which compensated for series resistance and voltage asymmetries in the circuit. I_{sc} is defined positive when cations move inward (mucosal to serosal) and anions move outward.

Bromoamiloride (500 μM) or iodoamiloride (100 μM) was added to the mucosal bathing solution for 40 min, during which time the tissue was irradiated three times for 30 sec by a Hg arc lamp with a skylight filter absorbing all wavelengths below 320 nm. The drug was then washed from the chamber by perfusion with 50 ml of solution A. I_{sc} was continuously measured during these manipulations. The effects of ultraviolet light by itself on the toad bladder and 500 μM bromoamiloride without irradiation were also measured. After the bladders had been treated with bromoamiloride and irradiated, nystatin (606 U/ml) was added to the mucosal solution to determine whether the irreversible inhibition of I_{sc} was due to a blockage of mucosal sodium channels. This concentration of nystatin was utilized since it caused a maximal increase in I_{sc} as determined by a dose-response curve. Bladders treated with iodoamiloride were also irradiated with unfiltered and 366-nm light for up to 5 min. When I_{sc} reached steady state the experiment was terminated.

Other amiloride analogues were screened for irreversible inhibition of I_{sc} by adding 5 μM of drug to the mucosal side. Both the mucosal and the serosal surface were bathed with solution A. After 40 min of incubation, the drug was washed from the chamber using 50 ml of solution A. The experiment was terminated when I_{sc} reached steady state. A similar protocol was used to examine the effects of 30 mM mucosal Na (solution B) and reduced mucosal pH (solution D) on the irreversible inhibition of I_{sc} induced by 5 μM phenamil or bromophenamil. The experiments designed to study irreversible inhibition as a function of sodium concentration were performed by using either solutions C (Fig. 4) or D (Fig. 7) and varying the sodium concentration as needed. The remaining ingredients were left unaltered.

Statistics

All data are reported as the mean ± SEM. Data were analyzed using the student's t-test. Differences with $P < 0.1$ were considered significant. All hemibladders reaching a steady-state I_{sc} were used in these experiments.

Results

Photoaffinity and Other Amiloride Analogues

Figure 1 shows a typical experimental protocol in which I_{sc} was monitored as a function of time for a

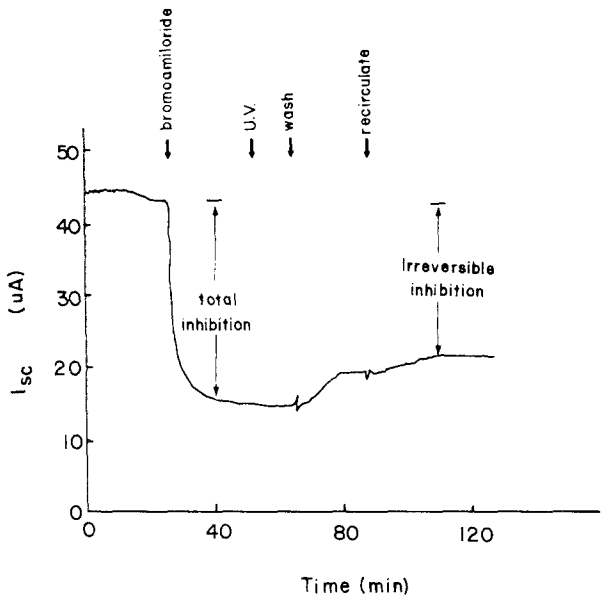
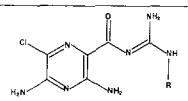


Fig. 1. Typical trace demonstrating irreversible inhibition of I_{sc} induced by bromoamiloride irradiated 3 times for 30 sec at wavelengths ≥ 320 nm. The arrows show the times at which the drug was added, irradiated, washed out and the mucosal solution recirculated. Wash and recirculation artifacts are also seen. Total inhibition is defined as the difference between the initial I_{sc} and the steady-state I_{sc} when the drug is present. Irreversible inhibition is defined as the difference between the initial I_{sc} and the steady-state I_{sc} after wash-out of bromoamiloride. In this experiment I_{sc} was irreversibly inhibited 50% in 103 mM sodium at pH 8.6

Table 2. Effect of various amiloride analogues on I_{sc} (*see* Fig. 2)

#	 R	Percent of initial I_{sc}[a]	
		Total inhibition	Recovery after wash
1	CH_2—CH_2—⬡	26 ± 3	92 ± 12
2	CH_3 ⬡ CH_3	52 ± 6	88 ± 10
3	—CH_3—⬡	20 ± 6	91 ± 11
4	—⬡F	27 ± 6	86 ± 7
5	—$C(CH_3)_2CH_2C(CH_3)_3$	26 ± 8	83 ± 12
6	—$CH(CH_3)$⬡	10 ± 8	107 ± 4
7	CH_2⬡CH_3	2 ± 1	84 ± 10
8	Brombenzamil[b]		
	CH_2—⬡	18 ± 6	90 ± 10
	+ UV	19 ± 7	102 ± 19

[a] Mean ± SEM. $n = 4$ except for bromobenzamil, where $n = 9$.
[b] In bromobenzamil, the 6-chloro group is replaced by 6-bromo.

bladder bathed in solution A. After I_{sc} reached steady state, 500 μM bromoamiloride was added to the mucosal bathing solution whereupon I_{sc} decreased from 44 to 16 μA. This effect was specific to the mucosal solution. After 30 min, the bladder was irradiated with ultraviolet light over a period of 10 min, as described in Materials and Methods. The difference between the initial steady-state I_{sc} and the steady-state I_{sc} in the presence of the drug was defined as "total inhibition." At the end of the irradiation period, bromoamiloride was washed from the mucosal half of the chamber by perfusing with 50 ml solution A. Fresh solution was then added to the reservoir and was recirculated through the chamber until I_{sc} reached a new steady state. "Irreversible inhibition" was defined as the difference between the initial steady-state I_{sc} and the steady-state I_{sc} after washout of the drug and recirculation of the mucosal solution. The I_{sc} of this tissue (Fig. 1) was inhibited by 50% (from 44 to 22 μA) and remained inhibited for the duration of the experiment, which was approximately 60 min after initiation of the wash.

In similar experiments, 500 μM bromoamiloride produced a total I_{sc} inhibition of 81 ± 6% ($n = 7$), and upon irradiation irreversibly inhibited I_{sc} by 36 ± 5%. This inhibition did not occur when the bladders were not irradiated, as evidenced by the 91 ± 9% ($n = 6$) ($P > 0.1$) recovery of I_{sc}. When nystatin was added to the mucosal solution (a maneuver that greatly increases the mucosal membrane permeability to monovalent ions) of the bromoamiloride-treated, irradiated bladders, the I_{sc} increased by 51 ± 25% ($n = 3$) over its initial value. This response was not significantly different from nystatin-stimulated controls in which I_{sc} increased by 44 ± 19% above the initial I_{sc} ($P > 0.1$). In contrast to these results with bromoamiloride, iodoamiloride (100 μM) inhibited I_{sc} by 79 ± 2% (total inhibition), but had no significant irreversible effect on I_{sc} upon irradiation since I_{sc} recovered 90 ± 6% ($n = 14$) after wash-out ($P > 0.1$).

Due to the high concentration required for irreversible inhibition, bromoamiloride was not deemed suitable for binding studies. Consequently, nine other amiloride analogues were screened for irreversible inhibition of I_{sc} at a concentration of 5 μM, a value more appropriate for binding studies. As seen in Table 2, eight of these compounds did not induce irreversible inhibition ($P > 0.1$). The structures of these molecules were identical to amiloride except that a terminal guanidino nitrogen atom bore the substituent indicated by "R" in the first column. For bromobenzamil (no. 8) the chloro group was replaced by bromo and the R group is as shown in the table. Bromobenzamil was tested with and without UV irradiation. At 5 μM, the total inhibition of I_{sc} by

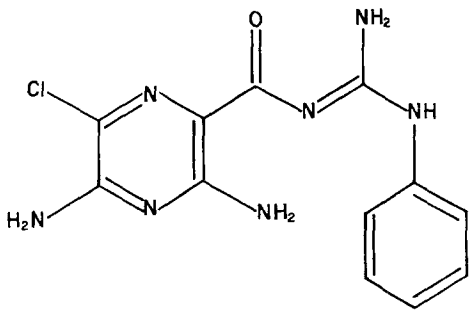


Fig. 2. Structure of phenamil in unprotonated form

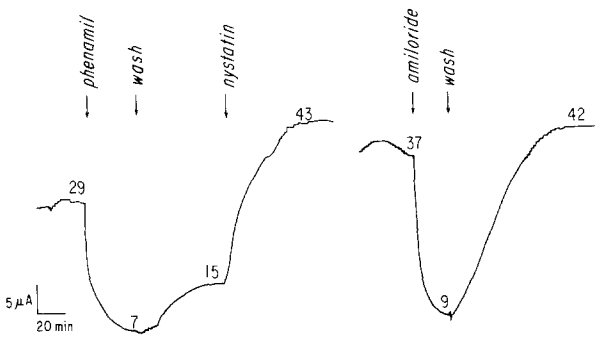


Fig. 3. Representative traces of phenamil and amiloride-induced inhibition of I_{sc} at a concentration of 5 μM. Also shown is the effect of nystatin (606 U/ml) on I_{sc} of the phenamil-treated bladder. Phenamil induced a total inhibition of 22 μA and an irreversible inhibition of 14 μA. In contrast, amiloride induced a total inhibition of 28 μA, none of which was irreversible. Mucosal sodium concentration was 103 mM at pH 8.6

these analogues ranged from 48% (no. 2) to 98% (no. 7).

Phenamil

One amiloride analogue, phenamil (Fig. 2), inhibited I_{sc} irreversibly. Figure 3 compares typical I_{sc} responses of bladders bathed in solution A to which 5 μM phenamil or 5 μM amiloride were added. In the phenamil experiment, I_{sc} was reduced from 29 to 7 μA, whereas in the amiloride experiment, I_{sc} was reduced from 37 to 9 μA. After exposure to phenamil or amiloride for 40 min, the drugs were washed from their respective mucosal chambers and the mucosal solutions were recirculated. The I_{sc} of the phenamil-treated bladder recovered to 52% of its initial value whereas the I_{sc} of the amiloride-treated bladder returned to 114% of its initial value. Thus phenamil irreversibly inhibited I_{sc} by 48% and amiloride induced no irreversible effect. The addition of nystatin to the mucosal solution of the phenamil-treated tissue stimulated I_{sc} to 43 μA.

In eight similar experiments, addition of 5 μM phenamil irreversibly inhibited I_{sc} by 38 ± 5% ($P < 0.001$). Addition of nystatin to phenamil-treated bladders increased I_{sc} to 191 ± 51% ($n = 8$) of initial values, whereas in control bladders, nystatin increased I_{sc} to 162 ± 36% ($n = 4$) of initial values. These increases are not significantly different from each other ($P > 0.1$). When added to the basolateral solution, 5 μM phenamil had no effect on I_{sc}.

Parameters Which Increase Irreversible Inhibition

Since mucosal sodium and amiloride both have been shown to inhibit apical sodium permeability (*see* MacKnight et al., 1980 for review), experiments were performed to measure the effect of reducing the mucosal sodium concentration on the phenamil-induced inhibition. The total inhibition of I_{sc} induced by phenamil was measured at three sodium concentrations: 103 mM; 35 mM; and 10 mM at pH 8.1. The first of these is the concentration of sodium in the serosal solution. The second approximates the lowest concentration which saturates the entry process, and the last concentration approximates the $K_{1/2}$ for sodium entry (*see* Bentley, 1968, for example). The percentage of the initial I_{sc} is plotted *vs.* the phenamil concentration at the above three Na^+ concentrations in Fig. 4. The inhibition caused by 10^{-8} M phenamil is not sodium dependent. In contrast, total inhibition caused by 10^{-7} M phenamil increased from 23 ± 4% to 54 ± 5% as the mucosal sodium concentration was reduced from 103 to 10 mM. At 10^{-5} M phenamil, altering the mucosal sodium concentration had no effect, since total inhibition was saturated at all three sodium concentrations. Under these conditions, there appears to be a phenamil-insensitive I_{sc}. However, as will be shown later, I_{sc} may be completely inhibited by phenamil by decreasing the mucosal sodium further as well as lowering the pH.

The interaction between mucosal sodium concentration and 10^{-7} M phenamil showed mixed inhibition kinetics as seen in the inset of Fig. 4, where 1/% I_{sc} is plotted *vs.* 1/Na. In terms of the Michaelis-Menten kinetic scheme, V_{max} was reduced by 33%. By extrapolating the lines to the intercept with the abscissa it can be shown that the $K_{1/2}$ increased from 8 to 20 mM sodium.

To study the effect of mucosal sodium concentration on phenamil-induced irreversible inhibition, a sodium concentration of 30 mM was selected since the I_{sc} is independent of external Na at this and higher concentrations. Therefore, at this sodium

Table 3. Inhibition of I_{sc} by 5 μM phenamil, bromophenamil, or amiloride in 30 mM sodium and the effects of increasing mucosal sodium to 103 mM (pH 8.6)

A. Total inhibition caused by phenamil or amiloride was not statistically different ($P > 0.1$). Only phenamil induced an irreversible effect. This irreversible inhibition was greater in 30 mM sodium than in 103 mM sodium ($P < 0.001$). Data are given as mean ± SEM.

	% of initial I_{sc}					(n)
	Initial	30 mM Na	Drug	Wash (30 mM Na)	Wash (103 mM Na)	
Phenamil (5 μM)	100	94 ± 4[a]	33 ± 4	40 ± 3[b,c]	68 ± 4[b,c]	(5)
Amiloride (5 μM)	100	100 ± 8[a]	30 ± 8	86 ± 8[a]	105 ± 9[a]	(4)

[a] Not significantly different from initial values ($P > 0.1$).
[b] Significantly different from amiloride-treated tissue ($P < 0.01$).
[c] Significantly different from initial values ($P < 0.001$).

B. Only bromophenamil irreversibly inhibited I_{sc} in 30 mM sodium. However, neither drug had an irreversible effect when the mucosal sodium concentration was restored to 103 mM. Data are given as mean ± SEM.

	% of initial I_{sc}					(n)
	Initial	30 mM Na	Drug	Wash (30 mM Na)	Wash (103 mM Na)	
Bromophenamil (5 μM)	100	85 ± 6[a]	18 ± 8[b]	55 ± 6[c]	93 ± 6[a,b]	(5)
Amiloride (5 μM)	100	92 ± 5[a]	17 ± 8	96 ± 12[a]	109 ± 11[a]	(4)

[a] Not significantly different from initial value.
[b] Not significantly different from amiloride-treated tissue.
[c] Significantly different from amiloride-treated tissue ($P < 0.02$).

concentration, changes in I_{sc} can only be attributed to differential blockage of the channel by phenamil. Reducing mucosal sodium from 103 to 30 mM sodium augmented irreversible inhibition of I_{sc} induced by 5 μM phenamil from the previously obtained value of 38 ± 5% to 60 ± 3% (Table 3A). As also seen in that table, returning the mucosal sodium concentration to 103 mM partially reversed the irreversible inhibition induced by phenamil but the irreversible inhibition in 103 mM sodium (32 ± 4%) was still significant ($P < 0.01$). In solution B total inhibition by amiloride and phenamil were similar (70 ± 8% and 67 ± 8%, respectively), although amiloride did not irreversibly inhibit I_{sc}.

In a further study, the irreversible inhibition induced by bromophenamil in 30 and 103 mM sodium was examined. In bromophenamil, 6-bromo is substituted for the 6-chloro group. In 30 mM sodium (solution B), 5 μM bromophenamil irreversibly inhibited I_{sc} by 45 ± 6% ($n = 5$) ($P < 0.02$). However, I_{sc} completely recovered when the mucosal sodium concentration was returned to 103 mM ($P > 0.2$) (Table 3B). The bladder was irradiated with ultravi-

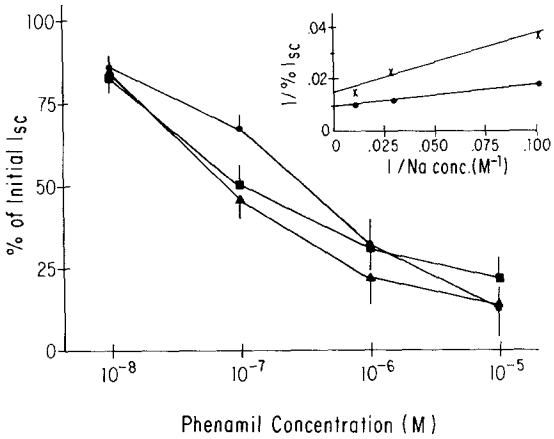


Fig. 4. Log dose-response of I_{sc} versus phenamil concentration. Total inhibition of I_{sc} is plotted at three mucosal sodium concentrations: 103 (●), 35 (■), and 10 (▲) mM. All data points of the dose-response were normalized to the initial I_{sc}. The inset shows a double reciprocal plot of $1/I_{sc}$ versus 1/[Na]. 10^{-7} M phenamil (x) increased the $K_{1/2}$ for sodium entry from 8 to 20 mM and decreased the maximum rate by 33% (*see* inset) as compared to controls (●). Each point represents the mean ± SEM of five experiments

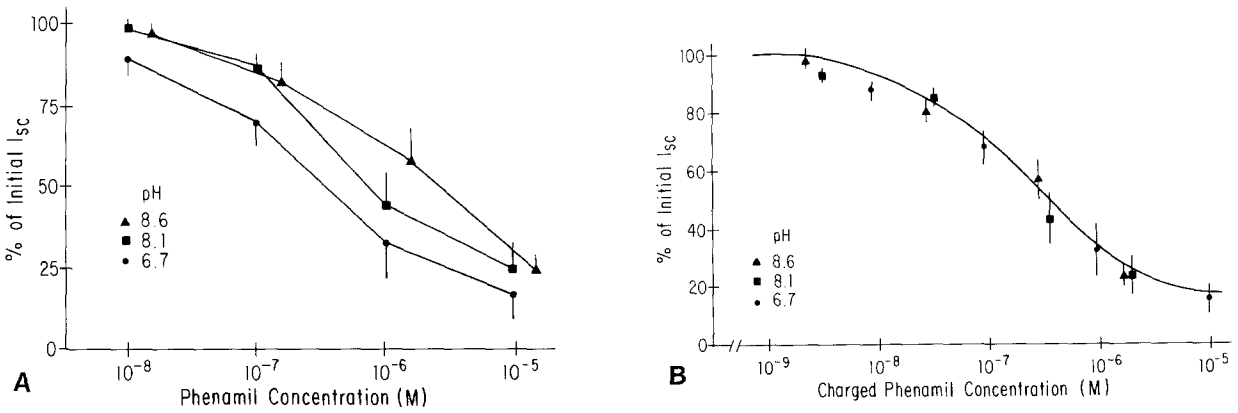


Fig. 5. *A*. Effect of reducing mucosal pH on total inhibition induced by phenamil. Log dose-response curves for three pH's are shown. Mucosal sodium concentration was 103 mM. Each point represents the mean ± SEM of 5 experiments. *B*. Log dose-response of I_{sc} versus concentration of phenamil in charged form. The data of Fig. 5*A* are replotted using the Henderson-Hasselbach equation and a pK_a of 7.8 to obtain the points in this figure. The solid line represents a theoretical dose-response curve with a K_I of 2×10^{-7} M and a maximum inhibition of 83%

olet light in 30 mM sodium and bromophenamil in an attempt to enhance the irreversible inhibition. However, no difference was noted between the irradiated and nonirradiated bromophenamil-treated tissues upon the return of mucosal sodium to 103 mM. Amiloride, as before, induced no irreversible inhibition at either sodium concentration.

The effects of reduced mucosal pH on the efficacy of phenamil were also tested. This avenue was pursued because inhibition of I_{sc} by amiloride was shown to be pH-sensitive in frog skin (Benos et al., 1976). Figure 5*A* shows the log dose-response curves of total inhibition by phenamil in 103 mM sodium at pH 8.6 (solution A), 8.1 (solution C), and 6.7 (solution D). As the pH of the mucosal solution decreases, the curve shifts to the left indicating that the drug is more potent at lower pH's. These results were replotted in Fig. 5B by calculating the concentration of phenamil in charged form using the measured pK_a of 7.8 and the Henderson-Hasselbalch equation. These data indicate that phenamil, as amiloride, acts in its charged form. The theoretical curve in Fig. 5B was drawn assuming a K_I of 2×10^{-7} M and a maximum inhibition of 83%. Consequently, reducing the mucosal pH increases the concentration of phenamil in charged form and thereby should increase both the total and irreversible inhibition. This prediction was borne out by experiments showing that the total inhibition (in 103 mM sodium) increased from 64 ± 2% (n = 16) at pH 8.6 to 94 ± 3% (n = 13) at pH 6.7 to 6.4. Irreversible inhibition by 5 μM phenamil also increased at lower pH (6.4 to 6.7), 52 ± 6% (n = 5) versus 38 ± 3% at pH 8.6 (n = 16). Both total and irreversible inhibition at pH 6.4 to 6.7 were significantly different from the inhibition at pH 8.6 ($P < 0.05$). Total inhibition induced by amiloride (5 μM) at pH 6.7 was 76 ± 11% and there was no irreversible inhibition at this pH.

Time Course of Inhibition

The time course of *irreversible inhibition* by 5 μM phenamil was studied under conditions that maximize its inhibitory effect, i.e., 30 mM sodium at pH 6.4 (solution E). Under these conditions phenamil produces 99 ± 1 (n = 16) percent total inhibition. Figure 6 shows the irreversible inhibition of I_{sc} as a function of time of incubation for a representative experiment performed in quarter bladders from the same toad. In one quarter bladder, after 10 min of exposure, I_{sc} was irreversibly inhibited by 62%. In the others, irreversible inhibition increased slowly during exposures of up to 80 min. The slow phase of irreversible inhibition can be fit by an exponential having a time constant of 140 min. Since the slow process does not extrapolate to 100% I_{sc} at time zero, a hypothetical line was drawn through the 10-min point and the zero-minute point. If it is assumed that an exponential process occurs between these two points, a time constant of 10 min or less would be calculated. This fast time constant could not be resolved to less than 10 min due to the physical constraints of the chambers.

The "fast" irreversible reaction appeared to be dependent on the mucosal sodium concentration. In Fig. 7, I_{sc} is plotted versus the mucosal sodium concentration after 10 min of exposure to phenamil and washout at the same sodium concentration. The experiments were performed in solutions D and E, as well as similar solutions containing other sodium

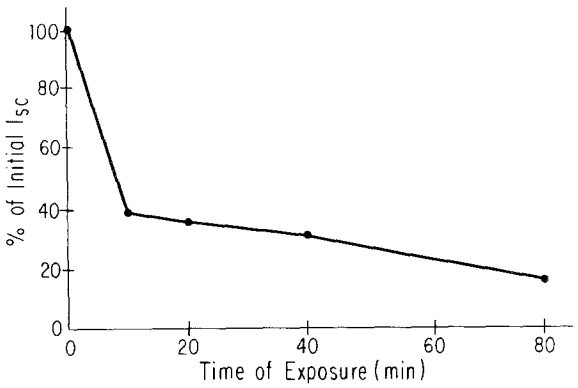


Fig. 6. Typical data showing the irreversible inhibition induced by 5 μM phenamil in 30 mM sodium at pH 6.4 versus time of exposure. The data were obtained in four parallel experiments performed in quarter bladders from the same toad

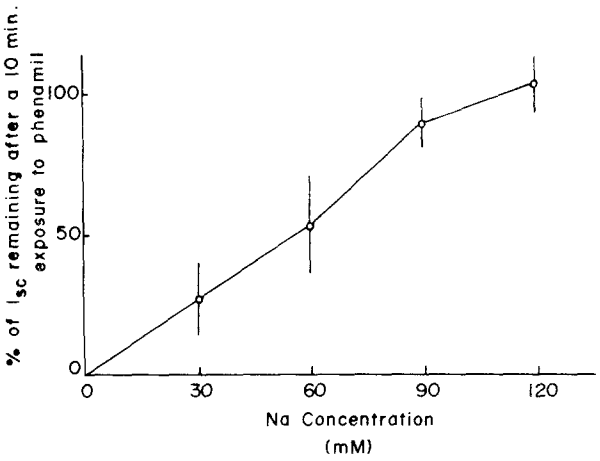


Fig. 7. Effect of mucosal sodium concentration on irreversible inhibition induced by 5 μM phenamil after a 10-min exposure. The mucosal pH is 6.4. At 60 mM sodium, I_{sc} is inhibited by approximately 50%. The effect saturates at about 90 mM sodium, since the means at 90 and 120 mM are not statistically different ($P > 0.1$). Each data point represents the mean ± SEM of four experiments

concentrations without cationic replacement. Identical curves were obtained when choline was added to the lower Na solutions to obtain a total choline plus sodium concentration of 120 mM. When extrapolated, the line passes through the origin, indicating that in zero sodium the "fast" reaction would induce 100% irreversible inhibition.

Amiloride-Phenamil Interaction

Table 4 demonstrates the effects of the addition of 5 μM amiloride on the irreversible inhibition of I_{sc} induced by 0.05 μM phenamil. These concentrations were selected as a prelude to ^{3}H phenamil binding studies. In these experiments, the mucosal side was bathed in solution F. Under these conditions 0.05 μM phenamil caused a total inhibition of 57 ± 12% and an irreversible inhibition of 25 ± 4% ($n = 4$). In the presence of 100-fold excess amiloride, irreversible inhibition induced by phenamil was prevented ($P < 0.05$).

Table 4. Interaction of amiloride and phenamil[a]

	Percent of initial I_{sc}			(*n*)
	Initial	Drug	Wash	
Phenamil (0.05 μM)	100	43 ± 12	75 ± 4	(4)
Phenamil (0.05 μM) plus amiloride (5 μM)	100	23 ± 5	98 ± 6	(3)

[a] Excess amiloride (5 μM) prevented phenamil (0.05 μM) from irreversibly inhibiting I_{sc} ($P < 0.05$). Mucosal solution was 10 mM sodium and pH 7.0.

Discussion

These studies were undertaken in an effort to find and characterize an irreversible amiloride analogue that could be used to quantitate and further characterize the sodium channels in the mucosal membrane of the toad urinary bladder.

Photoaffinity and Other Amiloride Analogues

Bromoamiloride at a concentration of 500 μM irreversibly inhibited the I_{sc} by 36 ± 5% after UV irradiation. These results are in agreement with those of Benos and Mandel (1978) and Cobb and Scott (1981). Both groups reported approximately 40% irreversible inhibition of I_{sc} by this compound. Nystatin, an ionophore that increases monovalent ion permeabilities of plasma membranes, increased I_{sc} when added to the mucosal side after the tissue had been irradiated in the presence of bromoamiloride. The I_{sc} reached the same values upon addition of nystatin, whether the tissue was treated with bromoamiloride or not. This shows that the irreversible inhibition induced by bromoamiloride was due to blockage of sodium entry channels in the mucosal membrane (Wills, 1981). If bromoamiloride were also inhibiting the Na,K-ATPase or metabolism, the rates of transport in the presence of nystatin would be significantly lower in bromoamiloride-treated bladders than controls.

The mechanism of bromamiloride's irreversible inhibition of sodium entry is unclear. Benos and

Mandel (1978) suggested that the irreversible inhibition may be due to formation of a covalent bond via nucleophilic substitution at the halogen moiety. Parenthetically, if nucleophilic substitution were occurring for the halogen, the order of reactivity would be I > Br > Cl (Morrison & Boyd, 1973). Therefore, iodoamiloride should be the most effective photoaffinity label. However, this compound in our hands caused no irreversible inhibition of I_{sc} upon irradiation. These data contrast with those of Cobb and Scott (1981) who obtained complete irreversible inhibition of I_{sc} in the toad bladder by 100 μM iodoamiloride. Three different lots of iodoamiloride were tested as well as several regimens of irradiation. No explanation for the differences in results can be offered.

Bromobenzamil was synthesized in an attempt to create a more potent analogue of bromoamiloride. Benzamil is approximately an order of magnitude more potent than amiloride in reversibly blocking the I_{sc} of frog skin (Cuthbert, 1976; Cragoe, 1979). It was thus hypothesized that bromobenzamil would be an order of magnitude more efficacious than bromoamiloride as a specific irreversible inhibitor. This was not the case since 5 μM bromobenzamil was ineffective as an irreversible inhibitor (Table 2).

Since bromobenzamil proved to be ineffective, several other amiloride analogues were tested for irreversible inhibition of I_{sc} at a concentration of 5 μM (Table 2). These compounds differed from amiloride in that a terminal guanidino nitrogen atom bore a hydrocarbyl substituent. Most of those screened did not induce an irreversible inhibition of I_{sc}. Increasing the potency or hydrophobicity of the analogue as compared to amiloride did not render the action of the molecule irreversible.

Phenamil

Phenamil was the only analogue that induced a significant irreversible inhibition of I_{sc} at 5 μM. The results with nystatin addition demonstrate that the irreversible inhibition of I_{sc} induced by phenamil is due to a process occurring at the mucosal membrane and not due to inhibition of the Na,K-ATPase or metabolism. The data shown in Table 4 demonstrate that amiloride prevents phenamil from irreversibly inhibiting I_{sc}. Although only reported at a single concentration (0.05 μM), other experiments (*data not shown*) show that this prevention of irreversible inhibition occurs at other concentrations of amiloride and phenamil. The ability of amiloride to prevent the action of another inhibitor of sodium entry has been used as a criterion to specify interaction with the mucosal sodium channel (Park & Fanestil, 1980; Garty & Edelman, 1983). If phenamil were acting by partitioning into a slowly exchangeable compartment (either the interior of the cell or the plasma membrane) it is unlikely that amiloride would prevent this. Therefore, the present data suggest that the effect of phenamil is due to its direct interaction with the sodium entry channel.

The properties which cause phenamil to irreversibly block the sodium channel are unknown. However, phenamil has two characteristics which, taken together, make it unique among those amiloride analogues tested. These are: 1) the conformation of phenamil; and 2) its electron cloud configuration. Using CPK models, it may be shown that phenamil can be arranged in a planar configuration, similar to amiloride (Smith et al., 1979). The phenyl ring may add destabilized π electrons to the compound, forming a nearly completely conjugated molecule. This hypothesis has been tested with analogues which only possess one of these characteristics but are otherwise very similar to phenamil (Table 2). Utilizing CPK molecular models it was found that many of the molecules in Table 2 cannot be arranged in a planar configuration. None of these molecules inhibited I_{sc} irreversibly. Molecules which are planar but lack the necessary electronic configuration, i.e., amiloride and 4-fluorophenamil, are also reversible. Fluorine, an electronegative element, disrupts the conjugation pattern by withdrawing electrons from the phenyl ring. Further requirements for irreversible inhibition would be difficult to predict, since the effects of the added phenyl ring on such parameters as internal hydrogen bonding and partition coefficients cannot be easily predicted (Wolfenden, 1983).

Effects of Mucosal pH and Sodium

It has been shown that amiloride inhibits I_{sc} only in its charged form in frog skin (Benos et al., 1976). This also appears to be the case for phenamil in toad bladder. A plot of log [phenamil] in charged form versus percent inhibition of I_{sc} can be fitted by the same theoretical curve for three different pH's (Fig. 5*B*). Since the charged form is the active moiety, decreasing the pH of the mucosal bathing solution also causes an increase in the percentage of irreversible inhibition. Another advantage of the decreased pH would be to reduce the flux of phenamil into the cell, an important consideration to minimize nonspecific binding of the radiolabeled compound. The flux is reduced at lower pH because the

charged form of drugs is usually less permeant than the unprotonated form (Benos et al., 1983).

The interaction between phenamil and sodium appears to be complex. No interaction was noticeable at low (0.01 μM) and high (10 μM) concentrations of phenamil. At the higher concentrations of phenamil the drug may overwhelm any competition with sodium, whereas at the lower concentrations the reverse may be true. At an intermediate concentration (0.1 μM), mixed inhibition kinetics between phenamil and sodium was observed, similar to that found by Benos et al. (1979) for amiloride in toad skin. This contrasts with the competitive inhibition of I_{sc} by amiloride in frog skin (Cuthbert & Shum, 1974; Cuthbert, 1976) or the noncompetitive interaction which has also been reported for toad bladder and frog skin (Bentley, 1968; Benos et al., 1979). On the basis of this apparent interaction between phenamil and sodium for total inhibition of I_{sc}, reduced mucosal sodium concentration was pursued as a means to enhance the irreversible inhibition of I_{sc} by phenamil. Irreversible inhibition of I_{sc} by 5 μM phenamil was almost doubled in 30 mM sodium as compared to 103 mM. Since at this phenamil concentration sodium has little effect on total I_{sc} inhibition, it seems that the process of "irreversible" binding of phenamil to the channel may be different from the mechanism which induces total inhibition.

Time Course of Inhibition

The irreversible inhibition caused by phenamil can be fit by two exponentials with a fast component of less than or equal to 10 min and a slow component with a time constant of 140 min. The irreversible inhibition obtained at 10-min incubation as a function of sodium concentration (Fig. 7) may be explained in one of two ways: 1) Mucosal sodium concentration may alter the rate of the process responsible for the "fast" time constant; or 2) The mucosal sodium concentration may affect the number of channels which participate in this process. Additional experiments are necessary to distinguish between these two possibilities.

The effect of mucosal sodium concentration on the slow time constant was not investigated. However, the sodium dependence of this slower process seems to be small, since the time constant for irreversible inhibition is longer than 70 min in 103 mM sodium and is 140 min in 30 mM sodium. Therefore, the differences in phenamil-induced irreversible inhibition obtained at 30 and 103 mM sodium may be completely explained by changes in the "fast" phase.

External Site of Action of Phenamil

According to the data presented in Fig. 7, phenamil would be expected to irreversibly inhibit 100% of the I_{sc} at zero sodium ion concentration. Assuming that this event is completed in 10 min (maximum estimate), the time constant for this process would be approximately 3 min (maximum estimate). It is likely that the fast phase of phenamil binding occurs on the outside of the membrane where changes in the sodium concentration would be most dramatic. As the sodium concentration never fell below saturating levels for the channel (30 mM), it is unlikely that the intracellular sodium concentration changed significantly for these conditions (Schultz, 1981). Therefore, changes in intracellular sodium could not explain the sodium-dependent changes in irreversible inhibition. The increased rate of irreversible inhibition caused by reduced mucosal sodium was not due to reductions in the ionic strength as determined by the choline additions. Thus the reaction from the blocked channel to the irreversibly blocked channel is not dependent on ionic strength.

In summary: 1) phenamil irreversibly inhibits I_{sc} specifically and with high affinity, presumably binding to the mucosal sodium channel; 2) the irreversible inhibition induced by phenamil is both pH and sodium sensitive; and 3) phenamil induces a new irreversibly blocked channel state.

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