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Structure-Activity Relationship of Amiloride Analogs as Blockers of Epithelial Na Channels: II. Side-Chain Modifications

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Summary. The overall on- and off-rate constants for blockage of epithelial Na channels by amiloride analogs were estimated by noise analysis of the stationary Na current traversing frog skin epithelium. The (2-position) side chain structure of amiloride was varied in order to obtain structure/rate constant relationships. (1) Hydrophobic chain elongations (benzamil and related compounds of high blocking potency) increase the stability of the blocking complex (lowered off-rate), explained by attachment of the added phenyl moiety to a hydrophobic area near the site of side chain interaction with the channel protein. (2) Some other chain modifications show that the on-rate, which is smaller than a diffusion-limited rate, varies with side chain structure. In several cases this effect is not attributable to steric hindrance on encounter, and implies that the side chain interacts briefly with the channel protein (encounter complex) before the main blocking position of the molecule is attained. The encounter complex must be labile since the overall rate constants of blockage are not concentration-dependent. (3) In two cases, changes at the 2-position side chain and at other ring ligands, with known effects on the blocking rate constants, could be combined in one analog. The rate constants of blocking by the resulting compounds indicate that the structural changes have additive effects in terms of activation energies. (4) Along with other observations (voltage dependence of the rate constants and competition with the transported Na ion), these results suggest a blocking process of at least two steps. It appears that initially the 2-position side chain invades the outward-facing channel entrance, establishing a labile complex. Then the molecule is either released completely (no block) or the 6-ligand of the pyrazine ring gains access to its receptor counterpart, thus establishing the blocking complex, the lifetime of which is strongly determined by the electronegativity of the 6-ligand.

Key Words amiloride · analogs · blocking kinetics · structure-activity relationship · Na channels · frog skin · fluctuation analysis

Introduction

The diuretic amiloride reversibly inhibits several Na transport mechanisms, but with highest efficacy the electrodiffusional ion flow through epithelial Na channels (e.g. Benos, 1982). The drug is a substituted pyrazine with a carbonylguanidinium side chain. By analyzing the Na current fluctuations induced by analogs of amiloride, a structure-effect relationship can be constructed for the two overall rate constants (k_{on}, k_{off}) of the blocking process. With this approach, we have previously shown that the blocking time ($1/k_{off}$) increases with the electronegativity of the 6-substituent of the pyrazine ring (Li, Cragoe & Lindemann, 1985). We now present results with analogs obtained by modification of the carbonylguanidine side chain which is attached to position 2 of the pyrazine ring of amiloride. A tentative blocking model is derived from these observations.

Our results have in part appeared in abstract form (Li & Lindemann, 1979; Li, Cragoe & Lindemann, 1981).

List of Symbols

Na_o	Na activity in apical (mucosal) solution (60 mM).
I_{Na}	Amiloride-blockable component of the short-circuit current passing the epithelium in the mucosal → serosal direction.
A_o	Amiloride concentration (A = AA1 = analog 1) in apical solution (μM).
AAn_o	Concentration of amiloride analog *AAn* (Table 1 to 3)
AA(*x*,*y*)	Analog bearing structural changes of AA*x* *and* AA*y*.
K_{AA}^{ma}	Macroscopic inhibition constant, obtained from the inflection point of a macroscopic I_{Na} dose-response curve at blocking equilibrium.
K_{AA}^{mi}	Microscopic inhibition constant (μM or mM) of the extrinsic blocker AA, obtained from noise analysis as the intercept of a linear rate-concentration plot with the abscissa ($= k_{off}^{AA}/k_{on}^{AA}$).
$k_{off}^{AA}, k_{on}^{AA}$	Apparent off-rate constant (sec^{-1}) and second order on-rate constant ($sec^{-1}\ \mu M^{-1}$) of analog AA at room temperature, as obtained by noise analysis from a rate-concentration plot. These

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are "overall" (global) rate constants, comprising at least two reaction steps (Fig. 4).

k_{on}^{A+AA} Apparent on-rate constant of amiloride in the presence of AA. Used when AA is a high-rate competitor of A [Eq. (2b)]. In addition, Na_o is considered to be another high-rate competitor of A.

k_a On-rate constant of A or AA obtained by correcting for the presence of high-rate competitors including Na ions [Eq. (2b)].

f_c^{AA} Corner frequency (Hz) of a Lorentzian current power density spectrum induced by blocker AA.

λ $= 2\pi f_c$, relaxation rate constant (1/sec or rad/sec)
= chemical blocking rate close to blocking equilibrium.

σ Hammett substituent constant, calculated from $pK_a^H - pK_a^X$ for replacement of H by X.

$\Delta G_{on}^{\neq}$, $\Delta G_{off}^{\neq}$ Standard Gibbs free energies of activation (kcal mol^{-1}) for creation (association) and decay (dissociation) of the blocking state [overall reaction, Eq. (1)].

$\Delta\Delta G_{on}^{\neq}$ $= (\Delta G_{on}^{\neq})_{AA} - (\Delta G_{on}^{\neq})_{amiloride}$.

ΔG_o Equilibrium free energy referenced to ground state ($= R\,T \ln K^{mi}$).

R, k, T, h, κ Gas contant, Boltzmann constant, absolute temperature, Planck constant, transmission coefficient.

k_2, k_{-2}, α, β Rate constants for a two-barrier two-site blocking model (Fig. 4*B*).

TAP Triaminopyrimidine.

BIG Benzimidazolylguanidine.

SEM, n Standard error of the mean, number of observations.

Materials and Methods

Abdominal skins of *Rana ridibunda*, which contain a relatively high density of amiloride-blockable apical Na channels, were used. Mounting of the tissue, serosal K depolarization (Fuchs, Hviid Larsen & Lindemann, 1977), fluctuation analysis and data evaluation closely followed the description given previously (Li et al., 1985). In short, the steady-state short-circuit current was recorded with a mucosal sulfate solution of pH 5.5, with a Na activity of 60 mM and amiloride or analogs of amiloride at submaximal blocking concentrations. The fluctuating component of the current (0.2 to 100 or 1 to 500 Hz) was amplified and sampled. After Fourier transformation of the sampled data, power-density spectra were computed and averaged.

The blocker-induced spectral components could be recognized as Lorentzians. Their corner frequencies (f_c) increased with increasing blocker concentration. When the relaxation rate constant (chemical rate) $\lambda = 2\pi f_c$ was plotted against the blocker concentration, a linear relationship was found in all cases under pseudo first-order conditions. From this plot, the overall rate constants of blockage were calculated with

$$\lambda = k_{on}\,AA_o + k_{off} \tag{1}$$

where AA_o denotes the mucosal concentration of amiloride or one of its analogs.

Some analogs inhibited the short-circuit current slightly but did not generate fluctuations in the frequency band observed. Several of these compounds were identified as *high-rate blocking competitors* of amiloride in that they depressed the apparent on-rate constant of amiloride. In such cases, the inhibition contant ($K_{AA} = k_{off}^{AA}/k_{on}^{AA}$) of the high-rate analog was previously estimated with

$$k_{on}^{A+AA} = k_{on}^{A}/(1 + AA_o/K_{AA}) \tag{2}$$

where k_{on}^{A} refers to amiloride in the absence of AA [e.g. Li & Lindemann, 1983*b*, Eq. (16)]. However, since then the proposition of Frehland, Hoshiko and Machlup (1983), that the transported Na ion is also a high-rate competitor of amiloride, has received increasing experimental support (Palmer, 1984, 1985; Lindemann & Warncke, 1985). In this case

$$k_{on}^{A} = k_a/(1 + Na_o/K_S) \tag{2a}$$

where k_a is the on-rate constant of amiloride corrected for high-rate competition with Na_o, and K_S a lumped dissociation constant for a single Na ion present within the channel. For the *simultaneous* presence of amiloride and its two high-rate competitors Na_o and AA_o in the mucosal solution, the apparent on-rate constant of amiloride becomes

$$k_{on}^{A+AA} = k_a/(1 + Na_o/K_S + AA_o/K_{AA}) \tag{2b}$$

given here without derivation. Rearrangement yields

$$K_{AA} = AA_o \left(\frac{k_{on}^{A}}{k_{on}^{A+AA}} - 1\right)^{-1}\left(1 + \frac{Na_o}{K_S}\right)^{-1} \tag{2c}$$

which may be used to estimate K_{AA} if K_S is known. With 60 mM Na_o and K_S between 10–20 mM (discussed below), our previous use of Eq. (2) instead of Eq. (2b) overestimated K_{AA} by a factor of four to seven for AA8, 9, 10 as well as TAP and BIG (*see* Li & Lindemann, 1983*b*; Li et al., 1985).

The methods of preparation of amiloride and most of its analogs have been described previously (references in Li et al., 1985). Of the newly used compounds, AA14 was prepared and converted to the dihydrochloride salt (m.p. 192–194°C) using a procedure similar to that described for amiloride (Cragoe et al., 1967), except that *t*-octylguanidine was used in place of guanidine. AA16 (m.p. 88°C) was also prepared as described for amiloride, except that 1,1,2,2-tetra-methylguanidine was used in place of guanidine. AA20 was prepared and converted to the hydrochloride salt (m.p. 310°C) by a procedure similar to that described for AA22 (Shepard, Halczenko & Cragoe, 1977), except that ethylenediamine was used in place of hydrazine. AA26 (m.p. 227–228°C) was prepared as described for AA25 (Bicking et al., 1965) except that *p*-chlorobenzylguanidine was used in place of (2-phenylethyl)guanidine. Triamterene was a gift of Röhm Pharma GmbH, Darmstadt.

Results

Chain Elongation by Terminal Substitutions

Amiloride analogs in which one or more protons at terminal guanidino nitrogens have been substituted

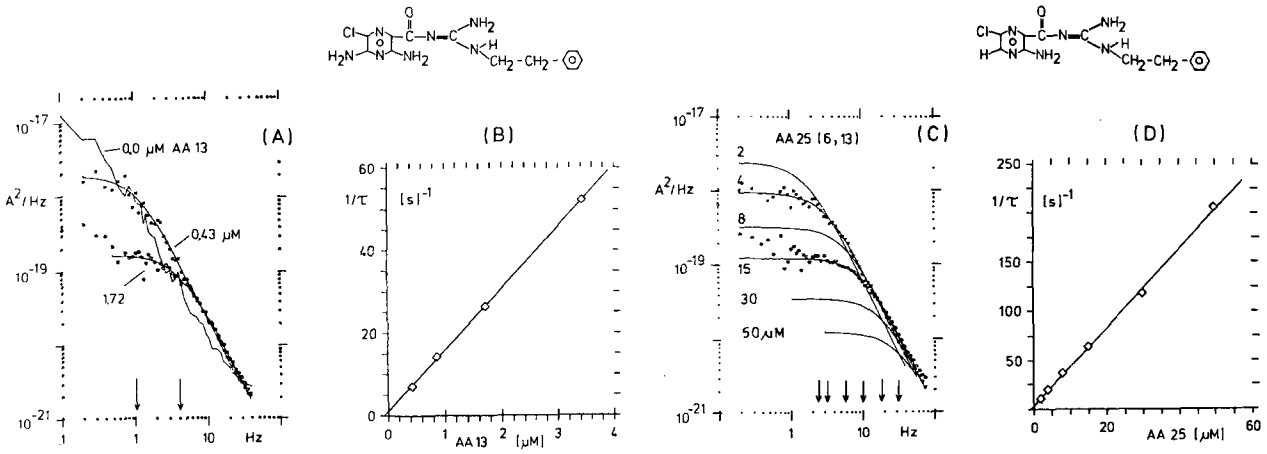


Fig. 1. Channel-blocking kinetics of the high potency analog phenethyl amiloride (AA13) and its 6-H derivative (AA25). K-depolarized, short-circuited frog skin, 3 cm^2, Na_o = 60 mM, pH 5.5. (*A*) Current power density spectra in the presence of AA13 at the concentration indicated. The smooth curves are the fitted Lorentzians. AA13 at 0.43 μM reduced the macroscopic current to 18% of the control. The induced Lorentzian has a corner frequency of only about 1 Hz. (*B*) Rate-concentration plot for the experiment shown in *A*. Note that both the ordinate and abscissa scales are expanded relative to those used with amiloride and ring-substituted analogs. The off-rate and on-rate constants extrapolated from this plot are 1.06 sec^{-1} and 14.9 sec^{-1} μM^{-1}, respectively. (*C*) Current power density spectra of analog 25, which combines the changes of AA6 and AA13, at the concentrations indicated. The smooth curves are the fitted Lorentzians. Data points are shown only for two of them. (*D*) Rate-concentration plot for *C*

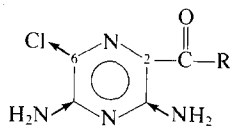
are listed in Table 1. By such changes, the distance between the positive charge and the center of the pyrazine ring remained nearly unaltered. Most of these compounds induced Lorentzian current noise in the 0.2 to 100 Hz or the 1 to 500 Hz band, from which linear rate-concentration plots were obtained. In analogs 11–14 only one proton on a terminal $-NH_2$ group is substituted. When the substituent is a small hydrophilic group, like –OH in analog 11, the pK_a drops below 6. The Hammett constant σ for this substitution is 3.19, i.e., the –OH acts as a strong additional electron sink. k_{on} appears to be reduced, but the pK_a-corrected value, which is based on the concentration of the protonated compound only, is close to that of amiloride (number in brackets in Table 1). k_{off} appears to be slightly increased but this change is at the limit of significance.

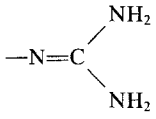
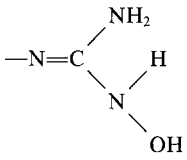
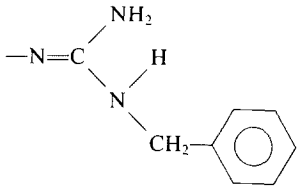
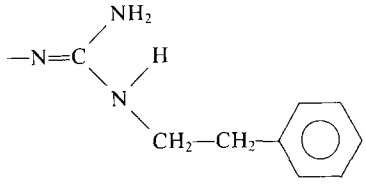
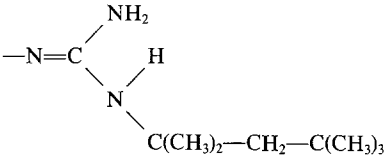
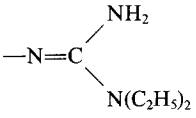
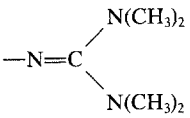
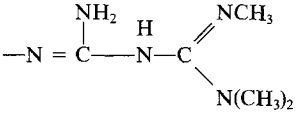
In contrast, when the substituent at the side chain terminal is highly lipophilic (benzamil type, AA12, 13 of Table 1, *see also* Table I of Cuthbert & Fanelli, 1978), the compounds are macroscopically *more* potent than amiloride (Aceves & Cuthbert, 1979; Aceves, Cuthbert & Edwardson, 1979). The Lorentzian spectra have much lower corner frequencies than amiloride-induced spectra and had to be pushed into the observed frequency band by increasing the concentration AA_o considerably above the known value of K_{AA}^{ma}. While the blocking rate of benzamil itself (AA12) was too low to be evaluated reliably, that of AA13 could be obtained (Fig. 1*A*). At 0.43 μM AA13, a concentration which blocked 82% of I_{Na}, the induced Lorentzian had a corner frequency of only 1.1 Hz. Lorentzian spectra with lower plateaus and higher corner frequencies were obtained by raising the blocker concentration further; the quality of the spectra, however, deteriorated because of the diminishing ratio of Lorentzian noise to background noise.

Based on the blocking rates observed in the concentration range of 0.43 to 3.44 μM (Fig. 1*B*), the overall rate constants of AA13 were estimated to be 14.9 sec^{-1} μM^{-1} and 1.06 sec^{-1} from this experiment. Mean rate constants from four experiments are given in Table 1. It can be seen that the large macroscopic efficacy of AA13 is due to a small increase in the on-rate constant and a significant, more than threefold increase in the blocking time ($1/k_{off}$). Thus K_{13}^{mi} is estimated to be 0.07 μM, while K_{13}^{mi} was estimated to be 0.11 μM from I_{Na} dose-response curves at blocking equilibrium. Spectra obtained with benzamil and AA14 resembled those shown in Fig. 1*A*, but were not good enough for a reliable estimate of rate constants. However, k_{on} of AA12 and 14, like that of AA13, may be expected to be close to k_{on} of amiloride. We shall come back to this point below.

Analogs 15 and 16 of Table 1 have an enlarged amidino moiety, due to two or four alkyl substituents on the terminal nitrogen atoms. Although the basicity of these two analogs is somewhat reduced, neither has a pK_a below 7.5; therefore, the degree of protonation during measurements at pH 5.5 is nearly the same as with amiloride. Hence, a de-

Table 1. Structure, method of preparation, and blocking rate constants of amiloride and some of its analogs with modifications at the side chain terminal



Analog number	*R*	pK_a^*	σ or $\Sigma\sigma$	k_{on} ($sec^{-1}\ \mu M^{-1}$)	k_{off} (sec^{-1})	*n*	K^{mi} (μM)	K^{ma} (μM)
1 Amiloride[a]		8.67	0.	13.17 ±0.25	3.93 ±0.19	58	0.3 ±0.13	1.79 ±0.97
11[b]		5.48	3.19	5.63 ±0.44 (11.53)	6.13 ±2.12	4	1.10 ±0.39 (0.532)	3.74 ±1.89
12 Benzamil[a]		8.10	0.57	—	—	9	—	—
13[a]		8.24	0.43	17.13 ±1.01	1.22 ±0.48	4	0.07 ±0.02	0.11 ±0.02
14†			−0.1	—	—	4	—	0.08 (n = 1)
15[a]		7.52	1.15	2.82 ±0.42	5.39 ±1.15	4	2.06 ±0.73	5.70 ±1.1
16†		7.92	0.75	0.79 ±0.07	3.25 ±0.59	4	4.25 ±0.87	40.5 ±8.4
17[c]		10.53	−1.86	3.91	6.71	1	1.7	12.5

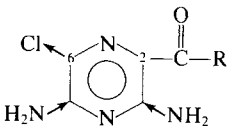
Mean ± SEM. * proton gained unless otherwise stated; † *see* Materials and Methods. On-rate constants without brackets as measured at pH 5.5. Those in brackets are the same mean values corrected for pK_a.

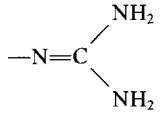
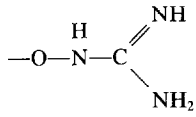
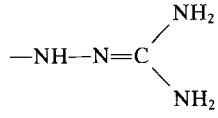
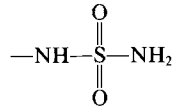
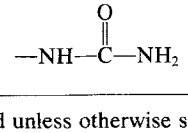
[a] Bicking et al., 1965; [a]Cragoe et al., 1967.

[b] Shepard, Halczenko & Cragoe, 1969*a*.

[c] U.S. Patent 3,531,484, Bicking & Cragoe, 1970.

Table 2. Structure, method of preparation, and blocking rate constants of amiloride and some of its analogs with single chain modifications



Analog number	*R*	pK_a^*	k_{on} ($sec^{-1}\ \mu M^{-1}$)	k_{off} (sec^{-1})	*n*	K^{mi} (μM)	$K^{m\alpha}$ (μM)
1 Amiloride[a]		8.67	13.17 ±0.25	3.93 ±0.19	58	0.3 ±0.13	1.79 ±0.97
18[b]		4.50	1.22 ±0.07 (13.4)	20.67 ±3.72	3	17.3 ±4.1 (1.54)	83. ±42.
19[c]		9.00	2.16 ±0.11	3.41 ±0.55	6	1.59 ±0.26	4.92 ±0.99
20[d]	—NH—CH_2—CH_2—NH_2	9.00	1.15 ±0.08	4.38 ±1.01	3	3.81 ±1.20	40.5 ±11.7
21[e]		5.83 (proton lost)	0.56 ±0.04 (0.82)	5.49 ±0.01	2	9.96 ±0.47 (6.7)	57.4 ±21.2
22[f]	—NH—NH_2	3.82	1.89 ±0.10 (92.3)	62.22 ±4.44	7	33.6 ±3.6 (0.67)	120. ±22. (*n* = 5)
23[g]		3.3	1.62 ±0.10 (258.)	27.80 ±2.15	5	17.6 ±2.4 (0.11)	57. ±21. (*n* = 4)

* Proton gained unless otherwise stated.
[a] Cragoe et al., 1967.
[b] U.S. Patent 4,145,551, Cragoe, Woltersdorf & Bock, 1979.
[c] Shepard et al., 1969.
[d] *See* Materials and Methods.
[e] U.S. Patent 3,573,305, Cragoe & Bicking, 1971.
[f] Shepard et al., 1977.
[g] U.S. Patent 3,575,975, Cragoe & Shepard, 1971.

crease in k_{on} would be due to changes in structure-related interactions with the receptor site on the Na channel. As shown in Table 1, AA15 and 16 have a markedly reduced on-rate constant but nearly the same k_{off} as amiloride.

Analog 17 is a pyrazinoyl*bi*guanidine with an exceptionally high pK_a. The compound may be viewed as resulting from substitution of one proton on a terminal guanidino nitrogen atom by a trimethylamidino moiety, $(CH_3)_2NC(=NCH_3)$—. Lorentzian spectra were obtained. Although the pK_a value is high, the linear rate concentration plot shows an on-rate constant smaller than that of amiloride, as was found with AA15 and 16, which have similar terminal substitutions.

Chain Elongation by Insertion

In analogs 18 and 19 (Table 2) the guanidino terminal is unchanged but the chain elongated by inser-

tion of either –O– (AA18) or –NH– (AA19) between the carbonyl group and the guanidino moiety. Thereby the distance from pyrazine ring to positive charge on the side chain is slightly increased (probably by less than 1 Å). As expected from the electron affinities of the inserted atoms, AA19 shows a slight increase and AA18 a drastic decrease in pK_a. Lorentzian spectra and linear rate-concentration plots were obtained. Both compounds have an apparent k_{on} much smaller than that of amiloride (*see* Table 2). However, if the on-rate constants of the protonated forms are compared (numbers in brackets), it can be seen that insertion of –NH– reduces k_{on} while insertion of –O– does not affect it. This structure-dependence of k_{on} points to chemical interaction with the receptor during the on-process.

The effect on k_{off} appears to be opposite to that k_{on}; i.e., insertion of –NH– hardly changes the off-rate but insertion of –O– results in a significant increase. The latter effect may have been expected because the electron acceptor –O– will decrease the electron density on the 6-C1 of the pyrazine ring, which is the major determinant of the off-rate (Li et al., 1985). The changes in rate constants are illustrated in terms of activation energies in Fig. 2*A*.

Replacements of the Amidino Terminal

With AA20 the side chain terminates in an amino rather than an amidino group. It is readily protonated, the pK_a being equal to that of AA19. The blocking kinetics of AA20 are also similar to those of AA19.

Analogs 21–23 of Table 2 are those where the amidino moiety was replaced by groups not readily protonated. These compounds are of low macroscopic inhibitory efficacy, which may be a mere consequence of their low pK_a values. In fact, AA21 is acidic. Lorentzian spectra could be obtained. The linear rate-concentration plots show small slopes and for AA22 and 23 high ordinate intercepts.

Because of the low pK_a values of AA22 and 23, a large concentration of unprotonated blocker will have been present at pH 5.5. It is quite conceivable that the unprotonated blocker acts as a high-rate competitor of the protonated form, decreasing its apparent k_{on} [Eq. (2)] just like k_{on} is decreased by Na_o [Eq. (2a)]. Then the listed k_{on}^{22} and k_{on}^{23} values will be underestimates even when pK_a corrected.

Double Substitutions: Ring and Side Chain

Analogs 24–26 (Table 3) have the 5-NH_2 of the ring replaced by 5-H and, in addition, a change at the side chain. Lorentzian spectra were obtained (e.g. Fig. 1*C*) and yielded linear rate-concentration plots in all cases. AA24 combines the modifications of AA6 (Li et al., 1985) and AA19 (Table 2). Compared to amiloride, a slightly increased k_{off} and, despite the still high pK_a value of 7.6, a k_{on} even smaller than that of AA6 or AA19 was found. In fact, k_{on}^{24} is smaller than k_{on}^{6} by about the same factor by which k_{on}^{19} is smaller than k_{on}^{1}, implying superimposed, synergistic effects of the 2- and 5-position substituents at the pyrazine ring.

The *superposition* of effects of such double substitutions can have practical advantages as shown in the following example. Replacement of a proton at the chain terminal $-NH_2$ by a methylene or ethylene spacer bearing a phenyl radical, as in AA13, increases the blocking potency by one order of magnitude. Table 1 shows that k_{on}^{13} is not much larger than k_{on}^{1}. Therefore, the increased efficacy of AA13 will be due to a lowered k_{off}. Unfortunately, such low k_{off} values are difficult to determine with sufficient precision. However, if a 5- or 6-position substituent were also changed, the resulting compound might show a higher off-rate than AA13. This would facilitate the analysis of side-chain alterations on k_{off}.

The ideal ring modification for this purpose would have been a replacement of 6-C1 by H, a change which increases the off-rate without affecting k_{on} (Li et al., 1985). Because this compound was not available, replacement of 5-NH_2 by H was used instead (AA25, 26 of Table 3); the analog with only this structural change (AA6) has a decreased mean k_{on} of 3.32 $sec^{-1}\ \mu M^{-1}$ and an increased k_{off} of 10.89 sec^{-1} compared to amiloride (Li et al., 1985).

Indeed, AA25, which combines the changes of AA6 and AA13, shows the low k_{on} characteristic of AA6 (Fig. 1*D*). Thus k_{on} was not much changed in the AA6 → AA25 alteration nor in the equivalent AA1 → AA13 alteration of the side chain. With respect to off-rate, it is interesting to note that k_{off}^{25} is smaller than k_{off}^{6} by roughly the same factor by which k_{off}^{13} is lower than k_{off}^{1}.

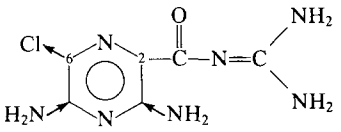
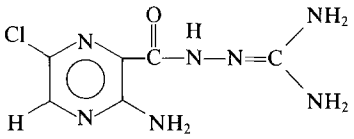
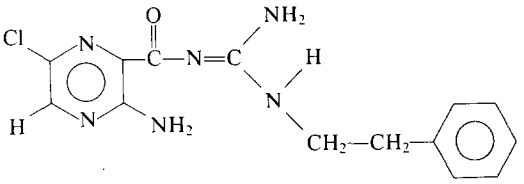
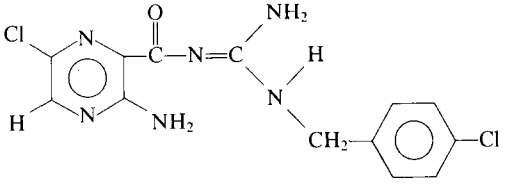
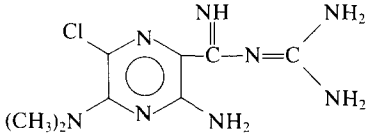
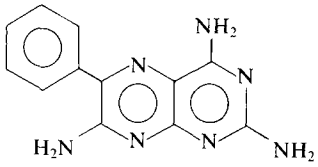
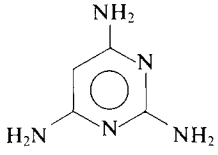
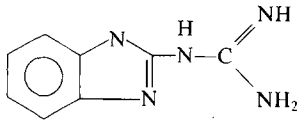
This observation may be given a physical basis as follows: Suppose AA1 is subjected to structural changes resulting in AAx and AAy and, when the two changes are combined, in AA(x,y). In simple cases the effects of these changes on the activation energies $\Delta G^{\neq}$ of chemical interactions with the receptor (on-process, off-process) may be additive, i.e.

$$\Delta G_{off}^{\neq,x,y} - \Delta G_{off}^{\neq,1} = (\Delta G_{off}^{\neq,x} - \Delta G_{off}^{\neq,1}) + (\Delta G_{off}^{\neq,y} - \Delta G_{off}^{\neq,1}). \quad (3)$$

Since $\Delta G_{off}^{\neq} = R\,T \ln (\text{const}/k_{off})$, we obtain by delogarithmation

$$k_{off}^{x,y} = k_{off}^{x}\, k_{off}^{y}/k_{off}^{1}. \quad (3a)$$

Table 3. Structure, method of preparation, and blocking rate constants of amiloride and some of its analogs with multiple modifications

Analog number	Structure	pK_a^*	k_{on} (sec^{-1} μM^{-1})	k_{off} (sec^{-1})	n	K^{mi} (μM)	K^{ma} (μM)
1 Amiloride[a]		8.67	13.17 ±0.25	3.93 ±0.19	58	0.3 ±0.13	1.79 ±0.97
24(6,19)[b]		7.61	0.57 ±0.04	6.45 ±0.89	6	11.3 ±1.2	59.2 ±6.8 (n = 5)
25(6,13)[c]		6.67	3.68 ±0.16 (3.93)	3.37 ±0.67	6	0.91 ±0.4 (0.86)	1.6 ±0.84
26[d]		6.40	0.24 ±0.05 (0.27)	2.78 ±0.63	3	11.6 ±0.3 (10.3)	30.4 ±7.8 (n = 2)
27[e]		6.00	9.45 ±0.70 (12.44)	91.03 ±7.61	6	9.91 ±1.2 (7.32)	60.8 ±9.7 (n = 4)
28[d] Triamterene		6.2	13.35 ±0.66 (16.02)	36.20 ±3.01	6	2.78 ±0.78 (2.26)	18.15 ±13.95 (n = 12)
29[d] TAP		6.72	—	—	4	83.0 ±20.	4150. ±133. (n = 11)
30[d] BIG		7.0	—	—	8	430. ±33.	—

On-rate constants in brackets are corrected for pK_a.
* Proton gained unless otherwise stated.
[a] Cragoe et al., 1967.
[b] Shepard et al., 1969*b*.
[c] Bicking et al., 1965.
[d] *See* Materials and Methods.
[e] Jones & Cragoe, 1968.

An equivalent relationship is derived for k_{on}. Applied to AA25, these equations allow the following predictions for rate constant changes resulting from the double substitution

$$k_{on}^{25} = k_{on}^{6}\, k_{on}^{13}/k_{on}^{1} = 4.45\ sec^{-1}\ \mu M^{-1}$$
$$k_{off}^{25} = k_{off}^{6}\, k_{off}^{13}/k_{off}^{1} = 3.38\ sec^{-1}. \quad (3b)$$

The measured values (Table 3) are in reasonable

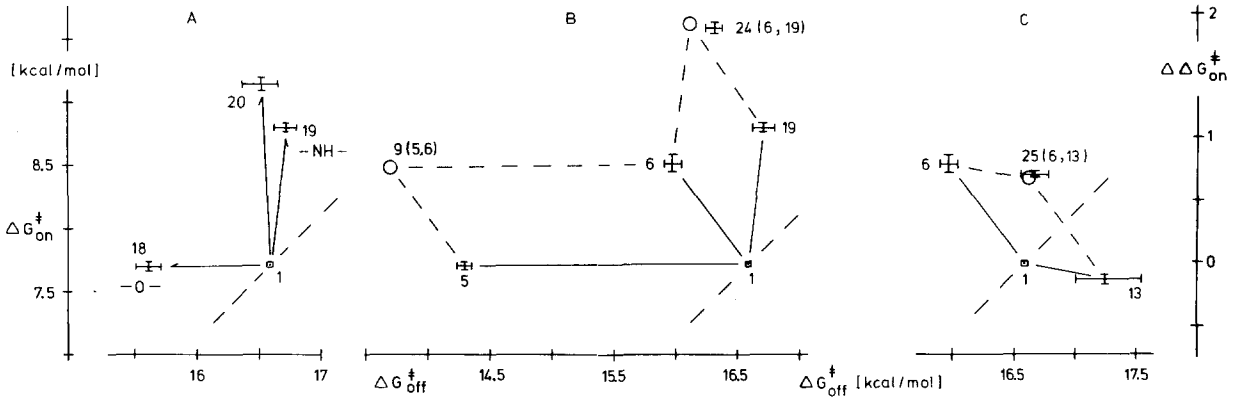


Fig. 2. Plane of standard free energies of activation (overall reaction, Eq. (1)) for amiloride (1) and some analogs. The oblique dashed lines indicate the locus of equal affinities (corresponding to an equilibrium free energy $\Delta G_0 = -9$ kcal mol^{-1}). pK_a-corrected on-rates were used in the calculation of $\Delta G^{\neq}_{on}$, but no correction was made for the presence of 60 mM Na_o, which depresses k_{on} (increases $\Delta G^{\neq}_{on}$) by high-rate competition with amiloride. For dual structural changes, the values predicted by superposition of single changes (Eq. (3)) are indicated by open circles. The bars indicate ± 1 SEM. (*A*) AA18, 19 and 20 have side-chain modifications (Table 2) which affect either the on process or the off process. (*B*) AA9, which combines the structural changes of AA5 and 6 (*see* Table 2 of Li et al. (1985)), had a blocking rate too high to be measured. AA24 combines the structural changes of AA6 and AA19. Its on-rate was correctly predicted by superposition. (*C*) For AA25, which combines the structural changes of AA6 and AA13, on- and off-rate were correctly predicted by Eq. (3)

agreement with these predictions. The superposition of substituent effects may be illustrated by plotting the $\Delta G^{\neq}$ values for on and off process against each other. Figure 2*B* and *C* shows that of the four predicted $\Delta G^{\neq}$ values which could be tested, only ΔG^{24}_{off} is not quite as expected.

When the superposition principle is applied to AA9 (*see* Table 2 in Li et al., 1985), the 5-deamino-6-dechloroamiloride which combines the nuclear substitutions of AA5 and 6, we obtain the predictions $k^9_{on} = 3.8$ sec^{-1} μM, $k^9_{off} = 488$ sec^{-1} (Fig. 2*B*). Clearly the resulting blocking rate will be too large to be observed in the frequency window employed. The ratio of these numbers is $K_9 = 130$ μM. Previously we noted that k^1_{on} is depressed by the presence of AA9, which we therefore classified as a high-rate blocking competitor of amiloride. Using Eq. (2) we estimated K_9 to be 540 μM (Li et al., 1985). When correcting this value for the additional high-rate competition by Na_o, we obtain from Eq. (2c) the predicted value of $K_9 = 130$ μM if $K_S = 19$ mM is used. This K_S value, in fact, is not unreasonable, being somewhat smaller than reported by Palmer (1984) for toad urinary bladder, but somewhat larger than found by Lindemann and Warncke (1985).

Because of the high-rate competitive effect of Na_o, all predicted and measured k_{on} values of this study are too small and should be corrected by multiplication with $(1 + 60/K_S)$ [*see* Eq. (2a)]. For values listed in Tables 1–3 we did not carry out this correction because the exact value of K_S is as yet uncertain and because, as will be shown, k_{on} is in any case a lumped constant comprising at least two reaction steps.

A general conclusion drawn from the blocking rates of AA13 and AA25, is that despite the considerable extension of the 2-position side chain by a spacer bearing a phenyl group, the on-rate is not decreased. In fact, it is slightly increased. An interesting exception is AA26 (Table 3), k^{26}_{on} being reduced by one order of magnitude with respect to k^{25}_{on}. This reduction is not explained by the low pK_a value of AA26, and will, therefore, be a more specific effect of the Cl atom attached to the phenyl moiety. The difference in k_{on} values found with AA25 and 26 would suggest steric hindrance on encounter, if the compound of lower k_{on} were bulkier. However, since the side chains of AA25 and 26 are comparable in length, the alternative must be considered, i.e., that chemical interaction of the side chain with the receptor takes place during the on-process (*see* Discussion).

Another general conclusion drawn from the blocking rates of AA13 and 25, is that the phenyl group, when attached to the terminal guanidino nitrogen atom through a spacer of proper length, decreases the off-rate considerably. Since the pK_a is little affected by this substitution, the decreased k_{off} will hardly be due to effects transmitted onto the pyrazine ring. Rather, interaction between phenyl group and a hydrophobic area of the receptor, providing a second attachment point, will be responsible (Li et al., 1981). However, the first attachment point, linking to the 6-Cl of the pyrazine ring (Li et al., 1985), remains in effect, as shown by the difference in k^{13}_{off} and k^{25}_{off}.

Finally, AA12 has a shorter spacer joining the phenyl group than AA13, and a higher blocking efficacy in that k_{off} of AA13 could be determined while k_{off} of AA12 was too small to be measured. This indicates that the hydrophobic area of the receptor is of restricted size. Furthermore, this area will be close to the channel entrance, because the entrance

is invaded by the positively charged side chain section (*see* Discussion). When the phenyl group is directly attached to the terminal guanidino nitrogen atom (phenamil), the binding becomes irreversible (Garvin et al., 1985).

In AA27 (Table 3) the 5-NH_2 group of the pyrazine ring is dimethylated as in AA8 and, in addition, the carbonyl oxygen at the side chain replaced by an imino group. The latter change decreases the pK_a to 6, showing that the imidocarbonyl group is an even stronger electron sink than the carbonyl group of amiloride. In contrast to AA8 (Li et al., 1985), which lacks the additional change at the chain, AA27 is macroscopically a weak inhibitor. At concentrations of 1–50 μM Lorentzian spectra were obtained and yielded linear rate-concentration plots. k_{on}^{27} becomes similar to that of amiloride when the low pK_a is taken into account. k_{off}^{27}, however, is increased above that of amiloride. Such an increase is found with all modifications investigated, which involve only one nuclear substituent (Li et al., 1985). In case of AA27, the change on the side chain, which lowers the pK_a and, therefore, may also lower the negative charge on the 6-Cl, would be expected to increase k_{off} even further. However, this does not seem to be the case since k_{off}^{27} could be measured while k_{off}^{8} was too large to be measured.

Less Closely Related Analogs

The last three members of Table 3, which have been widely used in the study of epithelial ion transport, are not true analogs of amiloride, although they share certain structural features with the amiloride molecule. Compound 28, triamterene, was shown to be a low efficacy substitute for amiloride in the ADX rat test (Cragoe, 1979) as well as in blocking the macroscopic Na current in amphibian epithelia (e.g., Cuthbert, 1976). Lorentzian spectra were obtained and, as reported previously (e.g., Hoshiko & Van Driessche, 1981; Christensen & Bindslev, 1982; Li & Lindemann, 1983*b*) yielded linear rate-concentration plots.

The blocking rate constants listed in Table 3 show that the comparatively weak inhibition exerted by triamterene is due to its large off-rate, the mean time of blocking being 1/10 of that of amiloride. This is understandable because triamterene lacks a regime of high electron density comparable to the 6-Cl atom of amiloride. Instead, it is probably the phenyl group which holds triamterene in its blocking position, using the hydrophobic receptor area which also serves as a (second) attachment point for AA12 and 13. Near neutral pH the effectiveness of triamterene is further reduced because of the low pK_a (*compare* Li & Lindemann, 1983*b*).

Compound 29, triaminopyrimidine (TAP), was first shown to be a blocker of the tight-junctional cation permeation pathway of leaky epithelia (Moreno, 1974). Later on, it was reported to block apical Na uptake in frog skin (e.g. Zeiske, 1975) and toad urinary bladder (Fanestil & Vaughn, 1979). Competition with amiloride was observed by Balaban, Mandel and Benos (1979). Lorentzian spectra induced by TAP were not obtained, because the blocking rate is too high. However, the high-rate competitive effect of TAP on the slower blocking kinetics of amiloride could be studied, since the apparent on-rate of amiloride was reduced by the presence of TAP. A microscopic inhibition constant of 330 μM, which is much smaller than the macroscopic value, was previously found by means of Eq. (2) (Li & Lindemann, 1983*b*). When this value is further corrected for the high-rate competition by Na_o, using Eq. (2c) and K_S = 20 mM (*see above*), we find 83 μM for K_{TAP}^{mi}. By this correction, the difference to K_{TAP}^{ma} becomes even larger.

Since TAP is a high-rate blocking competitor of amiloride, it must have a high k_{off}. This is understandable because the molecule has neither a zone of high negative charge, as the 6-Cl of amiloride, nor a zone suitable for hydrophobic attachment, as AA12, AA13 and triamterene.

The last compound in Table 3, benzoylimidazolylguanidine (BIG) bears structural similarity to amiloride only in having a guanidinium group on one end and a hetero-cyclic ring system on the other. The response of Na transport in the apical membrane of frog skin resembles that to AA8: stimulatory at low concentrations and inhibitory at high concentrations (*see* Zeiske & Lindemann, 1974). The two functions need not be exerted from the same receptor site.

The microscopic *inhibition* constant of BIG, estimated at a BIG concentration which still gives a net stimulation of I_{Na}, was previously reported to be near 1.7 mM (Li & Lindemann, 1983*a*). Using Eq. (2c) rather than Eq. (2) the corrected value is 430 μM. Thus BIG, as a blocker, is a very inefficient high-rate competitor of amiloride. It may attach in its blocking position through the imidazole ring, at the same hydrophobic receptor area that is also recognized by the phenyl groups of AA12, AA13 and triamterene.

Discussion

Complexity of the Problem

The relationship between blocker structure and the efficacy of Na channel blockage by molecules derived from amiloride was previously established with macroscopic dose-response curves (for re-

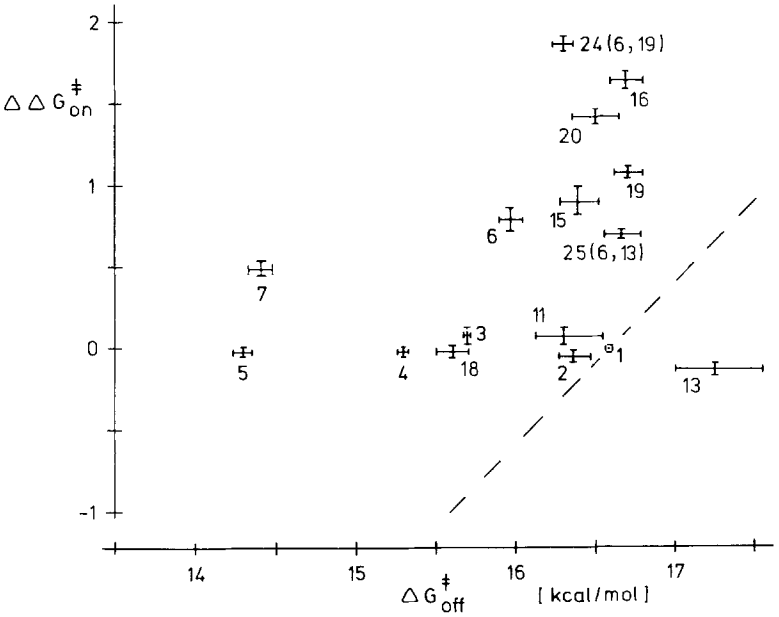


Fig. 3. Overview of activation energies calculated for 16 amiloride analogs from the overall rate constants k_{on} and k_{off}, using also data from Li et al. (1985). pK_a-corrected on-rates were used in the calculation of $\Delta\Delta G^{\neq}_{on}$. The bars denote SEM. The diagram shows that some structural changes (AA2-5, 11, 13, 18) affect mainly the off-rate while some other structural changes (AA15, 16, 19, 20) affect mainly the on-rate. To maintain clarity, results with AA17, 21–23 and 26–28 were left out (*see* Tables)

views *see* Cuthbert, 1981; Benos, 1982; Sariban-Sohraby & Benos, 1986). However, the inhibition constant retrieved from the inflection point of a dose-response curve is a function of two or more rate constants; in the simplest case it is the ratio of two blocking rate constants. These rate constants, and not the inhibition constant, may be expected to characterize the blocking process with respect to its mechanism.

In the present study, we attempted to establish structure-rate constant relationships by means of noise analysis. Blocker-induced fluctuations of Na current were recorded in the presence of submaximal concentrations of analogs of amiloride, and analyzed as described previously to obtain the global blocking rate constants (e.g. Lindemann & Van Driessche, 1977; Lindemann 1980, 1984). This approach was rewarding in that we found some structural changes of amiloride to affect merely one of the two rate constants. Thereby structural features of amiloride relevant to only the on-process or only the off-process of channel blockage, could be specified. (A graphical overview of our results is provided in Fig. 3.)

However, it turned out that the rate constants k_{on} and k_{off} [Eq. (1)] are themselves composed of a number of more basic rate constants. The structure dependence of these will have to be analyzed in the future before the blocking process can be understood in detail.

The Structure of Amiloride

Amiloride is a small molecule suitable for analysis with programs based on the self-consistent-field molecular orbital approach (e.g. Bingham, Dewar & Lo, 1975; Thiel, 1981). Such calculations iteratively yield the most likely bond angles, atomic distances and electronic charges. Using CNDO/2, Smith et al. (1979) found that the most likely conformer of amiloride is planar, stabilized by two intramolecular hydrogen bonds. Similar calculations with MINDO/3 yielded the charge distribution of this conformer (*see* Warncke & Lindemann, 1985*a*).

However, existing programs of this kind do not take solvation effects into account, but deal with molecules "in the gas phase." It is most disappointing to note that such calculations do not reproduce the pK_a increase of amiloride which is observed in aqueous solution when Cl atom at the 6 position of the pyrazine ring is replaced by F or H (C. Dorweiler and B. Lindemann, *unpublished*). This discrepancy points to the hydration shell as an essential part of the amiloride structure in aqueous solution. About the hydration shell nothing is known at present.

The On-Process

Insertion of –O– between the carbonyl and guanidino groups of the side chain leaves the (pK_a-corrected) on-rate unchanged (AA18), while insertion of –NH– at the same location (AA19) decreases the on-rate significantly (Fig. 2*A*). In view of the similar geometries of AA18 and AA19, the difference in on-rates will hardly be due to more pronounced steric hindrance encountered by AA19. Rather, the nature of the inserted atom determines the kinetic change, pointing to chemical interaction while the incoming side chain encounters the channel.

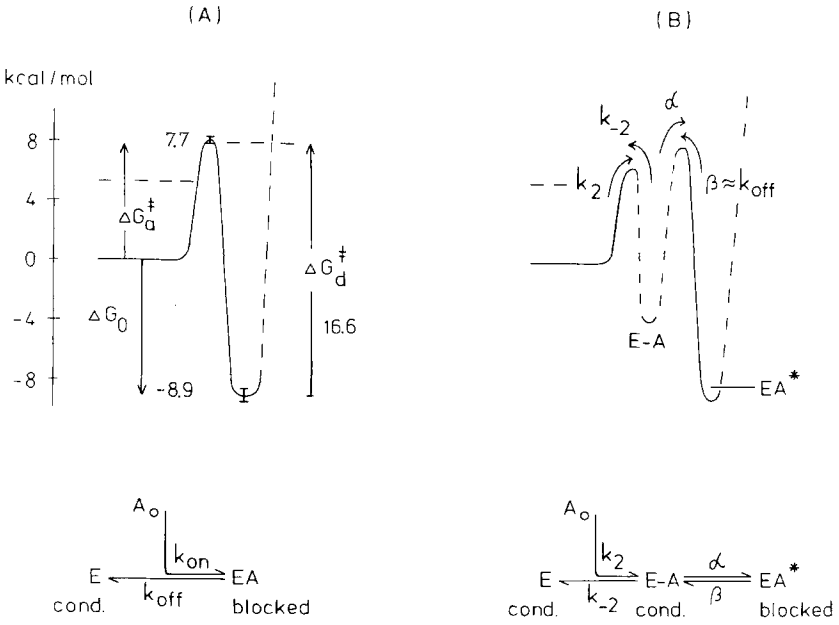


Fig. 4. Profile of standard free energies relative to ground state (0 kcal mol^{-1}) for the channel block by amiloride at 22°C, calculated from the apparent rate constants of the overall reaction (Eq. (1)) shown below. Abscissa, arbitrary. The left dashed line indicates an activation energy for diffusion-limited encounters ($k_{on} = 10^9$ liter mol^{-1} sec^{-1}). The minimum designates the equilibrium free energy ΔG_0. The bars denote SEM. (*B*) Energy profile for amiloride binding, including the preblock position (encounter complex *E-A*), based on the two-step reaction shown below. k_2 was chosen smaller than 10^9 liter mol^{-1} sec^{-1} to account for restrictions resulting from orientation, etc. (*see* text). States *E* and *E-A* of the channel could have equal Na conductivity, state *EA** is fully blocked

The structural transition from AA25 to AA26 also decreases the on-rate. Again this will not be due to steric hindrance, since the 2-position side chain of AA26 is no longer than that of AA25. Again chemical interaction during encounter appears to provide the explanation. Similar reasoning was used by King and Burgen (1976) to account for structure-dependent on-rates in another system.

In terms of rate theory (e.g. Eyring, Lumry & Woodbury, 1949; Johnson, Eyring & Polissar, 1954), the interaction on encounter may be modeled by splitting the peak of activation energy which determines k_{on} (7.7 kcal mol^{-1} in Fig. 4*A*) into two smaller peaks separated by a binding minimum which defines the stability of the encounter complex (Fig. 4*B*). In this scheme, a reaction sequence of at least two steps describes the on process: diffusional encounter → formation of the encounter complex → transition to the blocking complex.

One essential point is that the encounter complex is modeled distinct from the blocking complex. This seems quite justified since AA15, 16, 19 and 20 have decreased on-rates compared to AA1 but no appreciable change in the off-rate (*see* Fig. 3 for an overview). Similarly, AA2 to 5 (Li et al., 1985), and AA11 and 18 have decreased off-rates without appreciable changes in the on-rate. Therefore, a change in the encounter complex stability does not imply a change in the blocking complex stability and vice versa. This, of course, does in no way preclude the possibility of structural changes which affect both complexes, as in AA6 and 7.

Reactions of more than one step generally result in nonlinear rate-concentration plots (e.g. Eigen et al., 1964), while these plots were found to be linear (e.g. Fig. 1*B* and *D*) for all 23 analogs which generated Lorentzian noise spectra under pseudo first-order conditions. However, it can be shown that linear rate-concentration plots will result in cases where the encounter complex is of short life time. In the frame work of our model we may presume, therefore, that the encounter complex is labile.

The Value of k_{on}

For merely diffusion-limited encounters of small organic molecules with superficial receptor sites, high on-rates in the order of 10^3 sec^{-1} μM^{-1} are expected at room temperature, corresponding to activation energies of association of only 5.2 kcal mol^{-1} above ground state (e.g. Alberty & Hammes, 1958; Hammes, 1968). Furthermore, such diffusion-limited on-rates will be little affected by small structural changes of the molecule to be bound. Based on this reasoning, our data appear to show that the on-rate is limited by processes other than diffusion, as is common for the binding of small organic molecules to proteins (e.g. Haselkorn et al., 1974).

For a reasonably selective binding process, one expects that only a fraction of the incoming asymmetric molecules are of proper charge, conformation and orientation to be bound. The receptor can restrict the angle of access, can repel or attract the molecule electrostatically and, furthermore, may not at every time be receptive to bind the molecule. While it is not possible, at present, to account for all of these factors which may contribute to the observed low k_{on}, some of them can already be dealt with.

The pH-dependence of k_{on} observed with block-

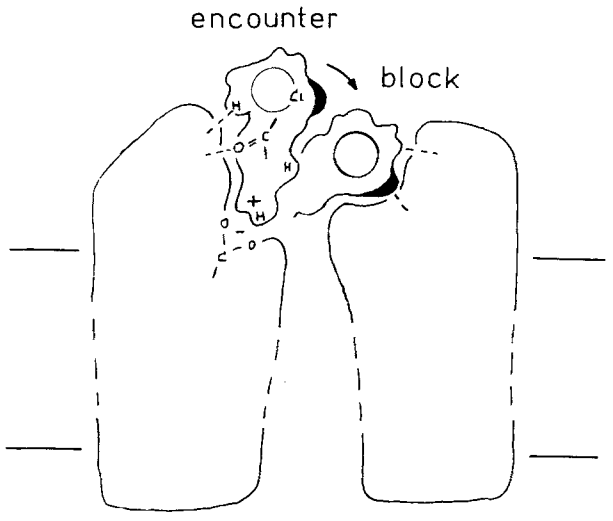


Fig. 5. Cartoon of a plug-type model for blockage of Na channels by amiloride (invasion hypothesis, Cuthbert (1976)), modified to account for the dependence of the on-rate on the side chain structure. The encounter complex (*E-A*) is shown on the left. Its transition to the more stable blocking position (rate constant α in Fig. 4*B*) may be aided by an adaptive change of the receptor conformation

ers of low pK_a suggests that the positively charged blocker species is much more effective than the uncharged species (e.g. Benos et al., 1976; Cuthbert, 1976; Li & Lindemann, 1981, 1983*b*). This conclusion received further circumstantial support by the observation that k_{on} of amiloride is increased and k_{off} decreased when the membrane voltage is made more positive at the outer side of the channel. This was found in relaxation experiments (e.g. Palmer, 1984, 1985; Warncke & Lindemann 1985*a,b*) and also by recording from single Na channels (e.g. Hamilton & Eaton, 1985; Reinhardt, Garty & Lindemann, 1985). The voltage dependence is compatible with invasion of the channel entrance by the protonated side chain of amiloride. The observation that the so-called uncharged analog CGS 4270 has a low on-rate (e.g. Abramcheck, Van Driessche & Helman, 1985) seems to support the *invasion hypothesis,* although the charge distribution on this molecule needs to be determined before a firm conclusion can be reached. In the present study we corrected all on-rates for the concentration of the charged species at the pH used. (The uncorrected values are also tabulated for reference.)

When the concentration of Na ions in the mucosal solution is increased, k_{on} appears to become smaller (Hoshiko & Van Driessche, 1981, 1986). Frehland et al. (1983) suggested that this is due to competition, in that amiloride cannot occupy its blocking position while a transported Na ion occupies the channel. Thereby the transported ion, which must invariably be present to observed the blocking effect, becomes a high-rate competitive antagonist of amiloride. Other groups have since confirmed this result (e.g. Palmer, 1984, 1985; Lindemann & Warncke, 1985). Thus the on-rate was previously underestimated and may become six to 10 times larger when corrected for competition with Na [Eq. (2a)], depending on the value of K_S used. When comparing analog data it is sufficient to use relative on-rates, for which the competition effect cancels out.

Apart from the Na-occupied transporting state, there may be other channel states which preclude binding of amiloride, e.g. the self-inhibition state (e.g. Fuchs et al., 1977; Lindemann & Van Driessche, 1978), the clogged state (Frehland et al., 1983) and the autoregulation state (Abramcheck et al., 1985). Kinetically such "lazy states" are not expected to affect the estimation of k_{on} and k_{off} as long as their rate constants are sufficiently small, as indeed they seem to be. However, the population of lazy states will increase K^{ma} above k_{off}/k_{on} (Li & Lindemann, 1983*b*).

The Off-Process

The atom bound to position 6 of the pyrazine ring (Cl in case of AA1) is of special significance in that its electronegativity determines the blocking time, i.e., $1/k_{off}$, but not the on-process leading to the blocked state. While the charge on the position-5 ligand also affects k_{off}, it does so to a lesser degree and in the opposite direction (*see* Fig. 6 in Li et al., 1985). We explained this by an inductive, electron-donating influence of the 5-ligand on the 6-ligand. The 5-ligand has an additional effect on k_{on} which is not shared by the 6-ligand. Comparing the two ligands, we find the effect of the 6-ligand on k_{off} to be both purer and stronger. Thus, it seems reasonable to propose that it is the 6-ligand which binds to a suitable (positively charged) area of the receptor. In view of the $\Delta G^{\neq}_{off}$ difference between AA5 and AA1 (Fig. 2*B*), a hydrogen bridge may be involved in this attachment.

Other bonds will contribute to the stability of the blocking complex. This is evident from the larger, but still measurable, off-rate of AA5. These bonds may in part also be provided by the ring moiety, since the off-rates of AA9 and 10 are substantially larger than those of AA5 (Li et al., 1985). Furthermore, the voltage dependence of k_{off} suggests that the positively charged side chain of amiloride is held in the channel entrance by the electrical field. Cuthbert (1976) suggested that a negatively

charged acidic group in the channel entrance attracts the side chain (invasion hypothesis). (Finally, in case of benzamil and AA13, 25 and 26 the phenyl group at the side chain terminal seems to attach to a hydrophobic receptor moiety, whereby k_{off} is lowered further.) Thus, k_{off}, like k_{on}, will be a composite rate constant which is to be modeled with more than one activation energy.

The Plug-Type Blocking Model

Cuthbert (1976) formulated his invasion hypothesis in analogy to the model of Na channel blockage by tetrodotoxin proposed by Hille (1975). Since then, several investigators have suggested that tetrodotoxin binds more superficially than originally thought (e.g. Kao & Walker, 1982; Green, Weiss & Andersen, 1986). For this problem the voltage dependence of the blocking rate constants of tetrodotoxin, and the effect of Na ions (e.g., Neumcke & Stämpfli, 1985) and high-rate blocking ions on these rate constants is of special significance (*see* Hille, 1984). For channel blockage by amiloride, the voltage dependence of k_{on} and k_{off} was convincingly shown and, like the competitive effect of the transported, channel-occupying Na ion (*see above* for references), is well compatible with an invasion mechanism.

As recently reviewed by Sariban-Sohraby and Benos (1986), some investigators favor interaction of amiloride with an allosteric binding site, because in some tissues amiloride was found to inhibit Na transport noncompetitively with respect to Na_o. However, these macroscopic studies did not distinguish high-rate and low-rate (lazy state-type) competitive effects of Na_o and generally did not provide for constancy of the membrane potential. Thus, only further experimentation can resolve the issue completely.

Our analog rate data are compatible with the invasion hypothesis and, together with other evidence, as quoted above, suggest a blocking mechanism (illustrated in Fig. 5) of the following features:

1) The positively charged 2-position side chain invades the outward-facing channel entrance, in part attracted by a fixed negative charge. This accounts for the voltage dependence of k_{on} and k_{off} and for the fact that the positively charged blockers have higher on-rates.

2) The 2-position side chain interacts with the channel, forming a labile encounter complex. Its stability codetermines the on-rate. This accounts for the structure dependence of k_{on} and the linearity of the rate-concentration plots.

3) The encounter complex decays by either releasing the blocker or by formation of the blocking complex. In the transition to the comparatively stable blocking complex an adaptive change of the receptor conformation (induced fit) is not excluded.

4) Invasion is impossible or release of the blocker is strongly favored when the channel contains a Na ion. This accounts for the high-rate competitive effect of Na_o.

5) Strong determinants of the blocking complex stability are the negative charge on the 6-ligand and the positive charge on the side chain.

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