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Structure-Activity Relationship of Amiloride Analogs as Blockers of Epithelial Na Channels: I. Pyrazine-Ring Modifications

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Summary. The overall on- and off-rate constants for blocking epithelial Na channels by amiloride analogs were estimated by noise analysis of frog skin epithelium. The substituents at position-5 and -6 of the pyrazine ring of amiloride were varied in order to obtain the structure/rate constant relationship. (1) The off-rate constant increases with halo-substitutions at position-6 in the order Cl < Br < I < F < H. Substitution of Cl by H lowers the standard free energy of activation of the off-step by 2.3 kcal mol^{-1}. The on-rate constant is not affected. Apparently the substituent at ring position-6 controls the duration of attachment in the blocking position. pK_a considerations show that the duration is longer when the 6-substituent is more negatively polarized. We suggest that this substituent binds to the receptor by virtue of its electronegativity. (2) In contrast, replacement of the adjacent 5-amino group (electron donor) by H or Cl affects both the on-rate and the off-rate. The dual effect may be explained by a decrease of the electronic charge at more remote parts of the molecule (on-rate decrease), as well as at the 6-position (off-rate increase). Apparently the 5-amino group stabilizes the blocking position by increasing the electron density on the 6-ligand.

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

Introduction

The apical membranes of "distal" epithelia (like amphibian skin and urinary bladder, the mammalian distal nephron, urinary bladder and colon and the coprodaeum of birds) contain Na channels which provide a pathway for cellular Na uptake from the luminal compartment. Their channel nature is indicated by the high rate of Na translocation per conducting unit (e.g. Lindemann & Van Driessche, 1977; Christensen & Bindslev, 1982) and by determinations of the Na flux ratio exponent (Palmer, 1982; Benos, Hyde & Latorre, 1983). Amiloride, 3,5 - diamino - 6 - chloro - N - (diaminomethylene) pyrazinecarboxamide (*see* Fig. 1) is an effective reversible blocker of these channels.

Twenty years ago the potency of this drug was revealed by a biological screening process in which more than 300 compounds were administered to adrenalectomized rats and their diuretic effects compared (Bicking et al., 1965). Subsequently, a more sensitive indicator, the inhibition of Na uptake by frog skin *in vitro*, was used in several new investigations of the effectiveness of some of the analogs. These macroscopic studies showed that efficient blocking of Na channels requires a positively charged carbonylguanidinium "side chain" and an amino group at position-5 as well as a medium-sized halogen atom at position-6 of the pyrazine ring (Benos et al., 1976). It also was shown that replacement of one of the protons on the terminal nitrogen atom of the amidino group by lipophilic groups, like alkyl or benzyl, increases the blocking potency (Cuthbert & Fanelli, 1978).

Further elucidation of the molecular requirements for channel blockage calls for a distinction of effects of drug structure on the process which leads to the blocked channel and on the stability of the blocked state. This distinction is provided by the rate constants of the blocking process. To measure them, experiments based on macroscopic perturbations of drug concentration are not suitable because of the presence of unstirred layers which delay the response of the membrane. Therefore, we used steady-state analysis of Na current shot-noise, induced by the amiloride analogs to estimate the blocking rate constants from the dependence of spectral corner frequencies on blocker concentration. The present paper deals with analogs which differ in their ring substituents. A subsequent paper will deal with analogs which differ in their side chains.

Our results were, in part, reported at the 1979 spring meeting of the Deutsche Physiologische Gesellschaft (Li & Lindemann, 1979) and the VII In-

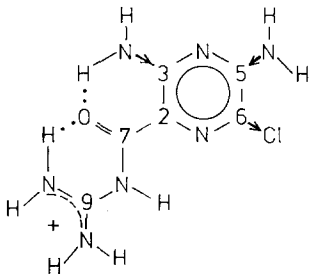

Fig. 1. Structure of the protonated form of amiloride. The planar conformer is characterized by two intramolecular hydrogen bonds and represents the most populated state in aqueous solution of pH < 7 (F1-tautomere of Smith et al., 1979). The numbers indicate carbon atoms. The arrows at pyrazine ring position-3, -5 and -6 point in the direction in which electrons are displaced by the ligand. Those pointing towards the ring indicate electropositive, those pointing away electronegative ligands compared to hydrogen

ternational Biophysics Congress (Li, Cragoe & Lindemann, 1981).

List of Symbols

Na_o	Na activity in apical (mucosal) solution (mM)
A_o	Amiloride concentration in apical solution (μM)
AA	Amiloride analog (Tables 1 and 2)
K^{ma}	Macroscopic inhibition constant, obtained from the inflection point of a macroscopic concentration-response curve
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}/k_{on})
k_{off}, k_{on}	Apparent off-rate constant (sec^{-1}) and second-order on-rate constant ($sec^{-1}\ \mu M^{-1}$) at room temperature as obtained by noise analysis from a rate-concentration plot. They are "overall" rate constants comprising one or more reaction steps
f_c^{AA}	Corner frequency (Hz) of a Lorentzian current power density spectrum induced by blocker AA
τ	= $(2\pi f_c^{AA})^{-1}$, relaxation time constant (sec) = inverse chemical rate close to equilibrium
$\Delta G_{on}^{\ddagger}$, $\Delta G_{off}^{\ddagger}$	Standard Gibbs free energies of activation (kcal mol^{-1}) for creation (association) and decay (dissociation) of the blocking state [overall reaction, Eq. (2)]
ΔG_o	Equilibrium free energy referenced to ground state (= $RT \ln K^{mi}$)
R, k, T, h, κ	Gas constant, Boltzmann constant, absolute temperature, Planck constant, transmission coefficient
σ_5, σ_6	Hammett substituent constant for substitutions at ring position-5 and -6
ρ	Hammett reaction constant
r	Correlation coefficient
n	Number of observations
SEM	Standard error of the mean

Materials and Methods

Rana ridibunda of East European origin (R. Stein, Lauingen, FRG) were kept in tanks at 10°C, unfed but having free access to running tap water. A few days before being sacrificed, the animals were put into deionized water of 18 to 22°C. After double-pithing the excised abdominal skin was mounted with the serosa backed by a filter paper on a Lucite® ring of 3 cm^2 of inner cross-sectional area. This ring was then inserted between two Lucite half-chambers. A hydrostatic pressure of 10 cm water column was applied to hold the preparation constantly against the filter paper and thus reduce microphonic artifacts in the noise records. Between the Lucite and the apical side of the epithelium an undercured silicon washer was inserted to achieve good sealing with minimal mechanical force. All experiments were carried out at room temperature (20 to 24°C). During noise recordings aeration of the serosal solution was stopped and superfusion of the outer skin surface was lowered to about 1 ml/min. The clamp voltage was set to zero mV. Data collection for noise analysis was done as described before (Li & Lindemann, 1983*a*,*b*).

When information on the noise components at higher frequencies was required, a mounting ring of only 0.12 cm^2 free cross-sectional area was used. With the smaller area and, thereby, a high absolute value of tissue impedance, the background noise introduced by the clamp amplifier at high frequencies was lowered significantly, as shown in Fig. 2*A*. Even in the absence of *edge damage*, small area preparations tend to show *edge effects*. These result from current loops which extend under the sealing material and cause the tissue conductance to be reduced to a smaller extent than the free area. Furthermore, the Na and amiloride concentrations under the sealing edge are not necessarily identical with those in the outer bulk solution. In order to learn about the influence of these effects on spectral corner frequencies, we compared corner frequencies of amiloride-induced Lorentzians in preparations which were first mounted as large areas and then remounted as small areas. As shown in Fig. 2*B*, the corner frequencies were practically identical. Spectral "peaking" near 80 Hz, as described by Hoshiko (1978), was not observed.

During the preliminary stage of this study, both serosally K-depolarized and nondepolarized skins were used. Later on, only skins that had been depolarized by a high K serosal solution for more than 1 hr were employed. The serosal K-Ringer's was of the same composition as that used by Fuchs, Hviid Larsen and Lindemann (1977). Unless stated otherwise, the outer solution used during noise measurements was a Na_2SO_4 Ringer's of 60 mM Na activity, buffered with 3.5 mM K-phosphate at pH 5.5.

The evaluation of the microscopic rate constants of the channel blocking process was based on the 2-state model (overall reaction):

$$A_o \Big|_{k_{on}} \qquad E \underset{k_{off}}{\longleftarrow} AE$$

where A denotes amiloride or one of its analogs, E the channel in the open state and AE the channel in the blocked state. For pseudo first-order conditions, i.e. $E \ll A_o$, the chemical rate (τ^{-1}) and the blocker concentration A_o are related by

$$\tau^{-1} = 2\pi f_c = k_{on}A_o + k_{off}. \qquad (1)$$

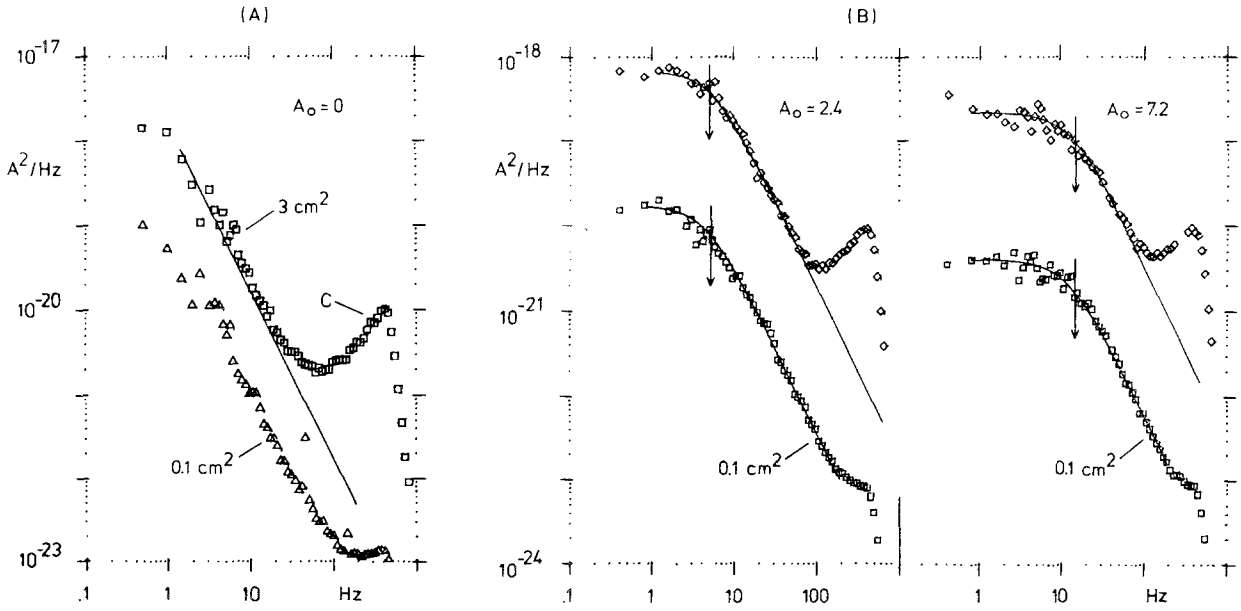

Fig. 2. Effects of exposed membrane area on current power density spectra. Nondepolarized short-circuited frog skin, Na_o = 60 mM, pH 5.5. (*A*) Current power density spectra obtained from one preparation first from 3 cm^2 (upper data) and subsequently from 0.1 cm^2 of nominal membrane area in the absence of amiloride. The same piece of tissue was used in both cases. The solid line has a slope of −2, i.e. spectral power decreased with $1/f^2$. For comparison the same anti-aliasing filter setting of 500 Hz was used in the two measurements. In the upper spectrum, the high frequency limit of evaluation is 25 Hz. At higher frequencies (i.e. lower tissue impedances) the clamp input stage noise (labeled *C*) dominates. For the lower spectrum the high frequency limit is increased due to the smaller membrane capacitance. (*B*) Amiloride-induced current power density spectra obtained at two amiloride concentrations and two membrane areas as described for panel *A*. The solid curves are fitted Lorentzians. In the case of 3 cm^2 of nominal area (upper spectra), the high frequency limit used for fitting was 50 Hz; Lorentzians were drawn beyond this limit to facilitate comparison. Note the large difference in plateau power and the small difference in corner frequency for amiloride-induced Lorentzians recorded from large and small membrane areas

As discussed previously (e.g. Lindemann & Van Driessche, 1978; Li & Lindemann, 1983*b*), a 3-state model which takes the competition between Na and amiloride or its analogs into account is more appropriate in describing the blocking kinetics. However, due to the small on-rate constant of the Na self-inhibition (Fuchs et al., 1977; Van Driessche & Lindemann, 1979), the microscopic rate constants of the blocking process of amiloride and the analogs are only marginally affected by this competition and the rate-concentration relationship derived from a 2-state model can be used as a good approximation. However, should Na_o in addition act as a high-rate competitor of amiloride (Frehland, Hoshiko & Machlup, 1983), then it would depress the apparent k_{on} of amiloride significantly [*see below*, Eq. (1a)].

Power density spectra were usually obtained for 4 to 8 different drug concentrations and—in the appropriate frequency range—fitted with single Lorentzian functions. The on-rate and off-rate constants of the pseudo-first-order blocking kinetics were obtained from rate-concentration plots (e.g. Fig. 3*B*) using Eq. (1). Analogs of weak macroscopic inhibitory activity often have off-rate-dominated characteristic rates which are too high to be directly observed in the frequency range 0.1 to 500 Hz. In such cases only the inhibition constant (K_{AA}), i.e. the ratio of on- and off-rate constants of AA could be obtained. K_{AA} was estimated by comparing the apparent on-rate constant of amiloride

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

in the absence ($AA_o = 0$) and presence of the fast analog (*see* Li & Lindemann, 1983*b*; Frehland et al., 1983).

The standard (Gibbs) free energies of activation for the creation and decay of the blocking state (overall reaction), shown schematically in the energy barrier diagram of Fig. 9*A*, were calculated from

$$\left.\begin{aligned} &\Delta G_o = RT \ln(K_{AA}^{mi}) \\ &\Delta G_{off}^{\ddagger} = RT \ln(\kappa kTh^{-1} \cdot k_{off}^{-1}) \\ &\text{and} \\ &\Delta G_{on}^{\ddagger} = \Delta G_o + \Delta G_{off}^{\ddagger} \end{aligned}\right\} \tag{2}$$

where h, k, R and T are, respectively, the Planck constant, the Boltzmann constant, the gas constant and the absolute temperature, $(RT) = 0.6$ kcal mol^{-1} and $(kT/h) = 6.25 \times 10^{12}$ sec^{-1} at room temperature, with the transmission coefficient κ set to unity. K_{AA}^{mi} and k_{off} are the measured microscopic inhibition constant and the off-rate constant of the channel-blocking process of amiloride or its analogs. A smaller off-rate constant reflects a larger standard free energy of "dissociation," $\Delta G_{off}^{\ddagger}$, i.e. a longer blocking time and—if the binding is paralleled by blocking—a longer dwell time of the blocker in the blocking position.

The methods of preparation of amiloride and most of its

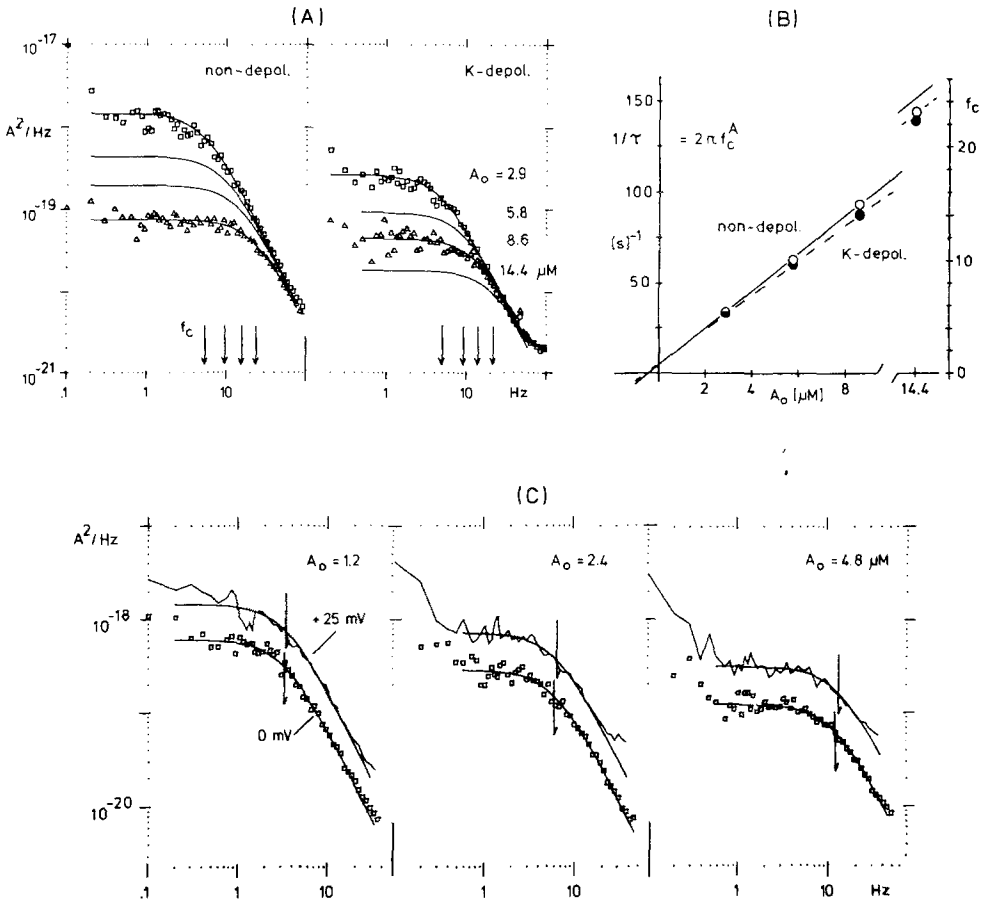

Fig. 3. Effects of apical membrane potential on the channel-blocking kinetics of amiloride in frog skin. 3 cm^2, Na_o = 60 mM, pH = 5.5. (*A*) Current power density spectra of amiloride-induced Lorentzians taken at the concentrations indicated. *Left:* the transepithelial potential was clamped to zero, the serosal side of the skin being in contact with Na Ringer's. In this state a voltage of more than 60 mV (apical side positive) across the apical membrane can be expected. *Right:* the same skin was then exposed to high potassium Ringer's from the serosal side for 60 min while being continuously short-circuited. By this "K-depolarization," the apical membrane potential is expected to approach the transepithelial potential, i.e. zero mV. Note the significant decrease in plateau powers. (*B*) Rate-concentration plot $1/\tau = 2\pi f_c^A = k_{on}A_o + k_{off}$ of the data from panel *A*. At each A_o the corner frequency of the Lorentzian does not change significantly by K-depolarization. (*C*) Current power density spectra taken from a K-depolarized skin at two transepithelial electrical potentials and three amiloride concentrations as indicated. The smooth lines are the fitted Lorentzians. An apical positive potential increases the plateaus of the Lorentzians without causing a significant change in the corner frequencies

analogs have been described previously (Bicking et al., 1965; Bicking et al., 1967; Cragoe et al., 1967; Jones & Cragoe, 1968; Shepard, Halczenko & Cragoe, 1969; Shepard et al., 1969; Bicking & Cragoe, 1970; Cragoe & Bicking, 1971; Cragoe & Shepard, 1971; Shepard, Halczenko & Cragoe, 1977; Cragoe & Woltersdorf, 1978; Cragoe, Woltersdorf & Bock, 1979).

Analog-10 (m.p. 194 to 195°C) was prepared as described for analog-9 (Bicking et al., 1967), except that methyl pyrazinoate was used in place of methyl 3-aminopyrazinoate.

Results

Amiloride

For reference, the kinetics of Na channel blockage by the parent molecule amiloride were first studied as a function of membrane voltage and mucosal pH.

Effect of Serosal Depolarization with K_2SO_4

To study the apical Na channels, we have, for want of a better apical membrane preparation, used serosal K depolarization (e.g. Fuchs et al., 1977) to make the basolateral membranes electrically transparent. (The literature on K-depolarization was recently reviewed (Lindemann, 1984; *see also* Garty & Lindemann, 1984).) However, the epidermis is usually studied in the natural nondepolarized state, i.e. with a high serosal Na activity. Therefore, we compared the kinetics of channel blockage under both conditions. Figure 3*A* shows the amiloride-induced Lorentzian spectra obtained from the same skin at the same amiloride concentrations before and after K depolarization. In spite of the large

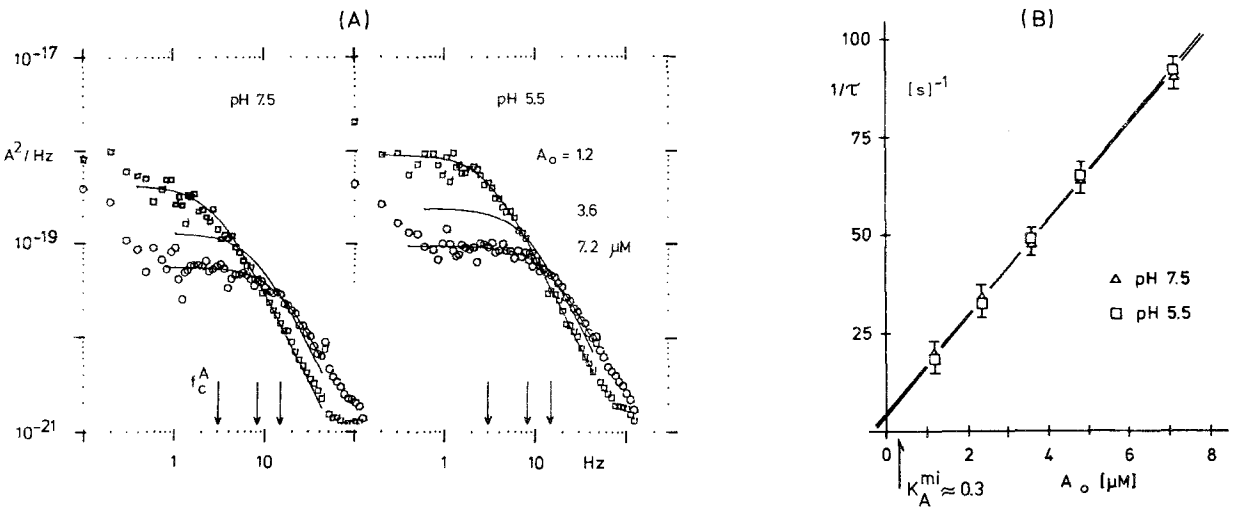

Fig. 4. Effects of apical pH on the channel-blocking kinetics of amiloride. K-depolarized, short-circuited frog skin, 3 cm², Na_o = 60 mM. (*A*) Amiloride-induced Lorentzians obtained from one skin at pH 7.5 (left) and at pH 5.5 (right) at the amiloride concentrations indicated. Although there is a clear difference in the plateaus of the corresponding Lorentzians, the corner frequencies do not differ significantly. (*B*) The rate-amiloride concentration plot for the mean corner frequencies (± SEM) from five skins. The extrapolated rate constants of amiloride at these two pH values do not show significant differences

change in the Lorentzian plateaus the change in corner frequencies was small to negligible. Thus the microscopic rate constants obtained from the rate *vs.* concentration plot, Fig. 3*B*, had similar values under nondepolarized and K-depolarized conditions.

This observation suggests that the effect of serosal depolarization with K_2SO_4 on the apical membrane is mainly a decrease of the driving force for the Na channel inward current. The computed single-channel current was in nondepolarized skins nearly twice as large as after depolarization. A change in the number of electrically detectable channels due to the depolarization may also occur (*see* Cuthbert & Shum, 1976; Henrich & Lindemann, 1984). The rate of blockage by amiloride is seen to be only weakly dependent upon membrane voltage. In six other skins measured with Na_o = 60 mM at pH 7.2, the on-rate and off-rate constants of the amiloride block were estimated to, respectively, $k_{on} = 12.88 \pm 0.57$ sec^{-1} μM^{-1} and $k_{off} = 5.34 \pm 1.19$ sec^{-1} (mean + SEM) for nondepolarized and $k_{on} = 12.69 \pm 0.69$ sec^{-1} μM^{-1} and $k_{off} = 5.28 \pm 1.63$ sec^{-1} for the same skins after K depolarization.

Insensitivity of the amiloride blocking kinetics to voltage changes was also observed when the same K-depolarized skin was subsequently clamped to +25 mV (apical solution positive with respect to serosal solution). As shown in Fig. 3*C* the electrical potential increases Na inward flow, the resulting Lorentzians having higher plateaus, but the corner frequencies show insignificant differences from those at 0 mV. This observation agrees with the finding of Henrich and Lindemann (1984), that the on-rate constant of amiloride and triamterene increases little with voltage below 60 mV. However, at more positive apical membrane voltages k_{on} increases significantly.

Effect of Apical pH

Macroscopic studies of the effectiveness of amiloride to block Na current have led to the suggestion that the protonated form of amiloride is the effective blocker (e.g. Benos et al., 1976; Cuthbert, 1976). It is desirable, therefore, to use apical solutions of low pH to assure a high degree of protonation of the blocker molecules. On the other hand, pH values below 5 are known to inhibit Na transport in frog skin (e.g., Ussing, 1949; Zeiske & Lindemann, 1975; Cuthbert, 1976). As a compromise we chose pH 5.5 at which amiloride and many of its analogs will be completely or almost completely protonated.

At an apical pH of 5.5 the net Na transport in the epidermis of *R. ridibunda* is larger than at pH 7.5 (e.g. Lindemann & Voute; 1976) indicating that the channel system as such is affected by pH. We have previously shown that at pH 5.5 the membrane contains more conducting channels (Li & Lindemann, 1981). Figure 4 shows the current power density spectra of a skin in the presence of submaximal concentrations of amiloride at pH 7.5 (based on a pK_a of 8.7: 94% protonated) and pH 5.5 (almost completely protonated), respectively. The plateaus

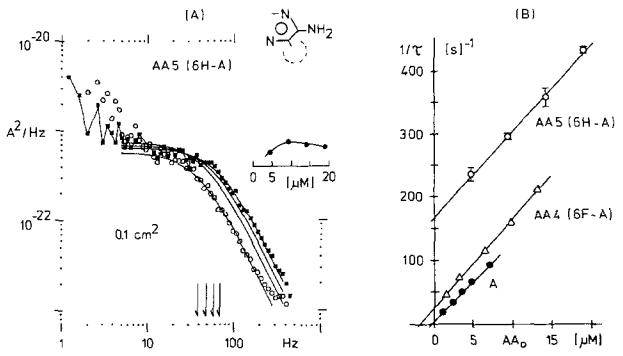

Fig. 5. Channel-blocking kinetics of two amiloride analogs with modifications at position-6 of the pyrazine ring. K-depolarized, short-circuited frog skin, 60 mM Na_o, pH 5.5. (*A*) Current power density spectra of 6H-amiloride (AA5). Membrane area 0.1 cm². Only the data points for the lowest and the highest concentration of this compound are displayed. The smooth curves are the fitted Lorentzians. The plateau-concentration plot (inset) shows a convex form since the concentration range encompasses the value of the microscopic inhibition constant. (*B*) Rate-concentration plot. The mean chemical rates (± SEM, $n = 3$) of AA5-induced Lorentzians (like those shown in panel *A*) are plotted. The upper line is a least-squares fit to these data. For comparison, the mean chemical rates and the regression lines for 6F-amiloride (AA4, $n = 6$ except for the first and the last data point for which $n = 2$) and for amiloride ($n = 5$) are also shown. The SEM of these data are smaller than the size of the symbols used (compressed ordinate scale). The regression lines show similar slopes but different ordinate intercepts, indicating that the substitution of 6-Cl by F or by H changes mainly the off-rate constant of blocking

of Lorentzians of corresponding amiloride concentrations are higher at pH 5.5, but the corner frequencies show only marginal changes. Thus the microscopic rate constants derived from the rate-concentration plot shown in Fig. 4*B* for the same preparation, are nearly identical at the two values of pH.

It appears, therefore, that the effect of pH on the Na channel system does not involve a change of the blocking process. This is an important conclusion which implies that the apparent increase in k_{on}, observed with analogs of low pK_a when the pH is lowered, is merely due to the increase in the concentration of the positively charged blocker species. Therefore this charge is one of the molecular features which contribute to the potency of amiloride. The uncharged species may either block not at all, or, more likely, act as a high-rate, low-potency competitor of the charged species.

The lipid solubility of amiloride, measured as the distribution ratio between CH_3Cl and a buffered aqueous solution, decreases fourfold when the pH is lowered from 7.4 to 5.5 (*see* Aceves, Cuthbert & Edwardson, 1979; Cragoe, 1979). The pH-insensitive rate constants of amiloride indicate, therefore, that lipophilicity is not essential for channel blockage by this molecule.

The on-rate and off-rate constant of amiloride at pH 5.5 for five different skins measured in a period of 10 days are 12.42 ± 0.26 sec^{-1} μM^{-1} and 4.10 ± 0.73 sec^{-1}, respectively. The values of these two rate constants measured in a 2-year period for 58 different skins are 13.17 ± 0.25 sec^{-1} μM^{-1} and 3.98 ± 0.19 sec^{-1}.

Halo-Substitution at Ring Position-6

Modifications at ring position-6 were done by replacing the Cl atom of amiloride by other halogen atoms or by hydrogen. The current power density spectra obtained in the presence of these analogs showed Lorentzian components similar to the amiloride spectra, and were readily analyzed in the low-frequency range. Only with a hydrogen at position-6 did the off-rate constant become so high that small area preparations had to be used to extend the upper frequency limit to 500 Hz.

The spectra in Fig. 5*A* were obtained with concentrations of 6H-amiloride of 4.8 to 19.2 μM. The corner frequency increased with concentration as in the case of amiloride, but the plateau of the Lorentzian went through a maximum (inset). The rate-concentration plot of 6H-amiloride is linear (Fig. 5*B*, upper data points). Corresponding data of amiloride and of 6F-amiloride are also shown. The dominant effect of modifications at position-6 is on the off-rate (ordinate intercept); the on-rate (slope) remains nearly unchanged.

Table 1 lists the rate constants obtained with the Cl-substituted compounds. While the on-rate constants are similar in value, the off-rate constants vary in the order Cl < Br < I < F < H. The mean time which the Na-channel spends in the blocked state ($1/k_{off}$) follows the inverse sequence, which is also the order of the macroscopic efficacy of these analogs (e.g. Benos et al., 1976), and of the *in vivo* efficacy when used in low concentrations (e.g. Cragoe, 1979). In aqueous solution the pK_a value of the side-chain terminal increases in the *same order* as k_{off} in response to modifications at position-6 (Table 1). This order warrants some comment.

While the basic electronegativity of the substituents increases in the order H < I < Br < Cl < F, the actual electron-withdrawing activity may follow a different series when ligands attached to a resonance system, as at ring position-6, exert both inductive and mesomeric effects. For instance, the Hammett substituent constants for these ligands in meta position of benzoic acid follow the sequence H < F < I < Cl < Br (e.g. McDaniel & Brown, 1958).

Table 1. Hammett substituent constant (σ) and blocking rate constants of amiloride and analogs with substitutions at pyrazine ring position-6

Analog number	Synthesis[a]	*X* pos.6	*Y* pos.5	pK_a[b]	σ_6[c]	On-rate constant ($sec^{-1}\ \mu M^{-1}$)	Off-rate constant (sec^{-1})	Block time (msec)	*n*[e]
1 Amiloride	b	Cl	NH_2	8.67	0.63	13.17 ± 0.25[d]	3.93 ± 0.19[d]	255.	58
2	b	Br	NH_2	8.72	0.58	14.19 ± 1.09	5.58 ± 0.92	179.	9
3	b	I	NH_2	8.85	0.45	11.43 ± 0.90	17.41 ± 0.40	57.	7
4	k	F	NH_2	9.00	0.3	13.54 ± 0.65	32.20 ± 1.57	31.	9
5	b	H	NH_2	9.30	0.0	14.47 ± 0.68	176.25 ± 17.73	6.	5

[a] b = Cragoe et al. (1967); k = Cragoe & Woltersdorf (1978).
[b] Proton gained in all cases.
[c] σ_6 refers to 6-substitutions at pyrazine rings where all other ligands are those of amiloride.
[d] Mean ± SEM.
[e] *n* = number of observations.

To give another example, according to Rowbotham and Schaefer (1974) the reactivity of intramolecular hydrogen bonding of *o*,*o'*-halophenols is I < F ≪ Br ≤ Cl. For the amiloride analogs the measured changes in pK_a allow to estimate the relative electron-donating or electron-withdrawing activity of the 6-ligands, by calculation of the Hammett substituent constants from

$$\sigma_6 = pK_a^{6-X} - pK_a^{6-H} \quad (3)$$

using H as the reference ligand. The values found are listed in Table 1. On plotting the logarithms of the rate constants against σ_6 (Fig. 6*A*), it was found that k_{off} obeys approximately a linear free-energy relationship. The slope (Hammett reaction constant ρ) is in the order of −2.6.

Substitutions at Ring Position-5

For the four analogs of this category (AA1, AA6-8) Hammett constants were calculated with

$$\sigma_5 = pK_a^{5-Y} - pK_a^{5-H} \quad (4)$$

using the 5-H analog for reference. Table 2 shows that the pK_a value is lowered (and σ_5 increases) when the 5-amino group is replaced by the weaker electron-donor H (AA6) or the electron-withdrawer Cl (AA7). This demonstrates the strong positive inductive effect which the 5-amino group exerts onto the side-chain of amiloride.

Analogs bearing a 5-H or 5-Cl in place of 5-NH_2 in the parent molecule yielded analyzable Lorentzians and linear rate-concentration plots (e.g. Fig. 3 in Li & Lindemann, 1983*b*). In Fig. 6*B* the ligands at position-5 were ordered with respect to σ_5. With increasing electronegativity of this ligand (more positive σ_5) k_{off} becomes larger. The behavior of k_{on} is more complex, but in clear contrast to replacements of 6-Cl, which increase the electronic charge of ring and side chain and leave k_{on} unaltered, the electronic charge on ring and side chain is now lowered and k_{on} is *decreased* compared to amiloride.

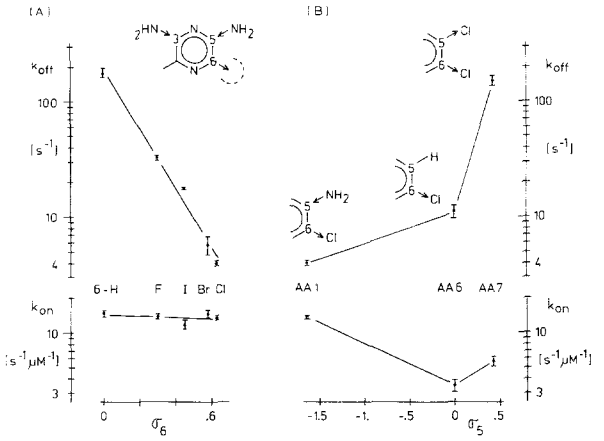

Fig. 6. The overall on-rate and off-rate constants are plotted against the Hammett substituent constant σ. In the insets, the arrows point in the direction in which electrons are displaced by the ligand (compare Fig. 1). The 5-H bond has no arrow since H is the reference ligand. The bars indicate one SEM. (*A*) Substitutions at position-6 (halo series): k_{on} is nearly independent of the 6-ligand while k_{off} decreases when the 6-ligand bears more electronic charge. The least-squares regression lines of the mean values indicate $\Delta\log k_{off}/\Delta\sigma_6 = -2.59 \pm 0.17$, $r = 0.994$, and $\Delta\log k_{on}/\Delta\sigma_6 = -0.056 \pm 0.086$, $r = 0.353$. Thus $\log k_{off}$ but not $\log k_{on}$ correlates linearly with σ_6. (*B*) Substitutions at position-5: k_{off} increases when the 5-ligand decreases the electron density of the pyrazine ring, (σ_5 changes towards positive values). The corresponding change of k_{on} is a decrease relative to k_{on} of amiloride (AA1)

Table 2. Hammett substituent constant (σ) and blocking rate constants of amiloride and analogs with substitutions at pyrazine ring position-5, or -6 and -5, or -6, -5 and -3

Analog number	Synthesis[a]	X pos.6	Y pos.5	Z pos.3	DR[b]	pK_a[c]	σ_5[d]	On-rate constant[e] ($sec^{-1}\ \mu M^{-1}$)	Off-rate constant (sec^{-1})	Block time (msec)	n
1 Amiloride	b	Cl	NH_2	NH_2	0.01	8.67	−1.64	13.17 ± 0.25[f]	3.93 ± 0.19[f]	255	58
6	a	Cl	H	NH_2	0.02	7.03	0.0	3.32 ± 0.44 (3.42)	10.89 ± 1.35	92	9
7	b	Cl	Cl	NH_2	—	6.60	0.43	5.16 ± 0.46 (5.57)	151.10 ± 16.48	7	6
8	b	Cl	$N(CH_3)_2$	NH_2	0.06	8.76	−1.73	$K_{AA} \approx 0.50 \pm 0.05$ mM		—	4
9	c	H	H	NH_2	—	7.4	—	$K_{AA} = 0.54 \pm 0.03$ mM		—	5
10	d	H	H	H	—	6.2	—	$K_{AA} > 4$ mM		—	3

[a] a = Bicking et al. (1965); b = Cragoe et al. (1967); c = Bicking et al. (1967); d = *see* Materials and Methods.
[b] Distribution ratio CH Cl_3 / 7.4 buffer.
[c] Proton gained in all cases.
[d] σ_5 refers to 5-substituents of pyrazine rings where all other ligands are those of amiloride. *See* Eq. (4).
[e] On-rate constants without brackets as measured at pH 5.5, using the total blocker concentration in the calculation. Those in brackets are the same mean values corrected for the concentration of positively charged blocker molecules.
[f] Mean ± SEM.

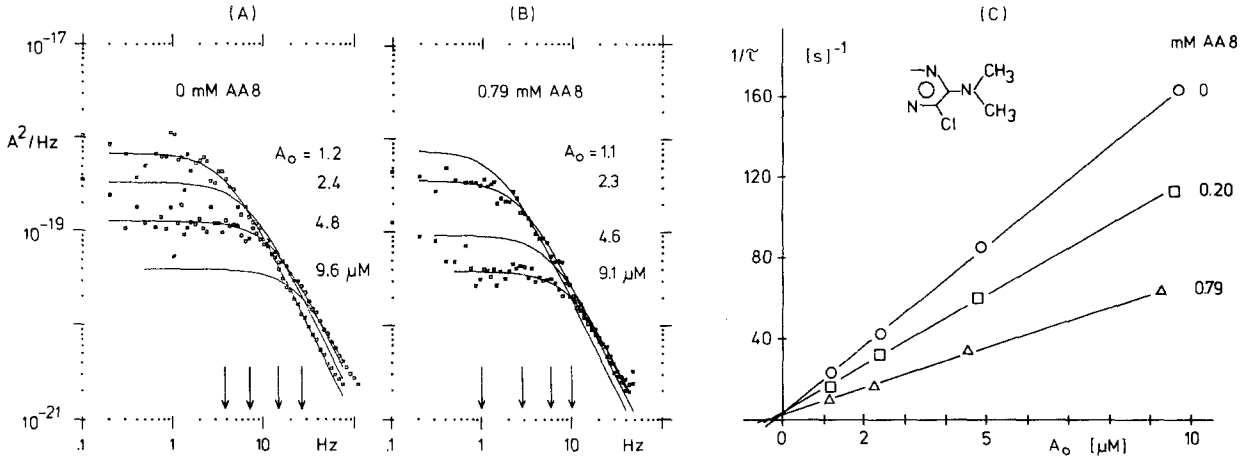

Fig. 7. Effect of the macroscopically stimulatory 5-dimethyl-amino-amiloride (analog 8) on the current power density spectra induced by Na and amiloride. K-depolarized, short-circuited frog skin, 3 cm^2, Na_o = 60 mM, pH 5.5. (*A*) and (*B*) Current power density spectra in the absence and presence of 0.79 mM AA8 plus amiloride at the concentrations indicated. In each spectrum only one Lorentzian is discernible. The corner frequencies are lowered by the presence of AA8. (*C*) Rate-amiloride concentration plot for different concentrations of AA8. All data are from one preparation. Note the decrease in the slope with increasing concentration of AA8 and the small changes in the ordinate intercept. This plot suggests that AA8 is a channel-blocking high-rate competitor of amiloride. Evaluation of the change in slope yielded $K_{AA8} \approx 0.5$ mM

The substitution of both hydrogen atoms on the 5-amino nitrogen by methyl groups yields a compound of net *stimulatory* activity in the concentration range from 1 μM to 1 mM (Li & DeSousa, 1979; Li & Lindemann, 1983*a*). The spectra taken in the presence of this analog (AA8) alone did not show a clear Lorentzian component in the frequency range of 0.1 to 100 Hz. However, when amiloride was present in addition, the corner frequencies of amiloride-induced Lorentzians were depressed (Fig. 7*A*,*B*). The slope of the rate-concentration plot of amiloride then became smaller (Fig. 7*C*), indicating a decrease in the apparent on-rate constant. This finding implies that the 5-dimethyl-amino analog, apart from its stimulatory action, is also a higher-rate blocker competitive to amiloride.

From the decrease in slope, the microscopic inhibition constant of AA8 was estimated to 0.5 mM

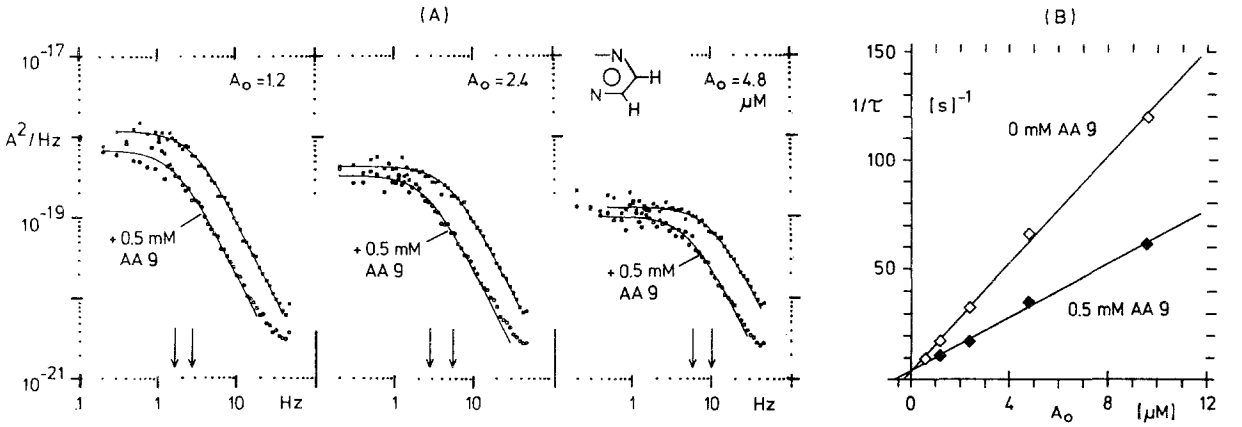

Fig. 8. Effects of the low potency analog 5,6-dihydroamiloride (AA9) on the blocking kinetics of amiloride. K-depolarized short-circuited frog skin, 3 cm², Na_o = 60 mM, pH 5.5. (*A*) Lorentzian current power density spectra induced by amiloride in the presence and absence of 0.5 mM AA9. Although two blockers are present, only one Lorentzian is discernible in each spectrum. Note the systematic decrease in corner frequency caused by the presence of the analog. (*B*) Rate-concentration plot for amiloride in the absence and the presence of 0.5 mM AA9. As in Fig. 7*C*, the presence of this analog decreases the slope without affecting the ordinate intercept. This effect is typical for the presence of a higher-rate competitive blocker. Evaluation of the decrease in slope yielded K_{AA9} = 0.54 mM

(Table 2 and Li & Lindemann, 1983*a*). The high chemical rate of blockage [Eq. (1)] inferred for AA8 suggests that the presence of the two methyl groups causes k_{off} to increase. This effect is not explicable by inductive effects onto other groups since the electropositive character of the 5-ligand is not decreased by this substitution. In fact, it is slightly increased, the Hammett σ of 5-$N(CH_3)_2$ being somewhat more negative than that of 5-NH_2 (Table 2).

Multiple Substitutions

When the ligands at ring position-5 and -6 or at ring position-3, -5 and -6 were jointly replaced by hydrogen (AA9, AA10), the pK_a decreased as expected from the net loss in positive inductive effects. With these analogs Lorentzians were not found in the frequency band 0.1 to 500 Hz. However, as described above for the 5-dimethylamino analog, the apparent on-rate of amiloride was depressed by these compounds (Fig. 8). They are, therefore, higher-rate blocking competitors of amiloride. Our estimates of their K_{AA} values are given in Table 2.

Discussion

Previously, the efficacy of an amiloride analog for *in vitro* systems was described in terms of one macroscopic inhibition constant (e.g. Benos et al., 1976; Cuthbert & Fanelli, 1978; for reviews *see* Cuthbert, 1981; Benos, 1982). Noise analysis now provides two "rate constants" by which structural influences on the on- and off-process of blocking may be distinguished. The blocking process is likely to be more complex than suggested by the reaction scheme of Eq. (1). Therefore, the rate constants found are "overall" constants which later on may be resolved into true rate constants, as has been done for many other systems (e.g. King & Burgen, 1976). For such an analysis the finding that the rate-concentration plots (of each analog tested) are linear, i.e., that the overall k_{on} is not concentration dependent, will be significant. Nevertheless, it may be supposed that the structure dependence of the overall k_{on} provides information about the process which leads to the blocked state, while the structure dependence of the overall k_{off} provides information about the stability of the blocked state.

With single substitutions like 6-Cl to 6-Br the change made in the blocker molecule is small. One is tempted, therefore, to attribute the resulting changes in rate constants to effects at the locus of the substitution, while in reality a substituent often affects remote parts of the molecule. This becomes particularly obvious when the pK_a changes observed in aqueous solution in response to substitutions at ring position-5 and -6 are noted (Tables 1 and 2). With this problem in mind we shall now try to find a hypothesis which attributes changes in k_{on} and k_{off} to analog structure. We shall discuss three alternatives:

1) Remote effects: Suppose binding were to occur through atoms of the side-chain and substitutions of ring ligands affect this process merely by altering the electron density on the chain. Position-5 and -6 ligands are almost equally remote from the side-chain. Therefore, replacement at position-5 or

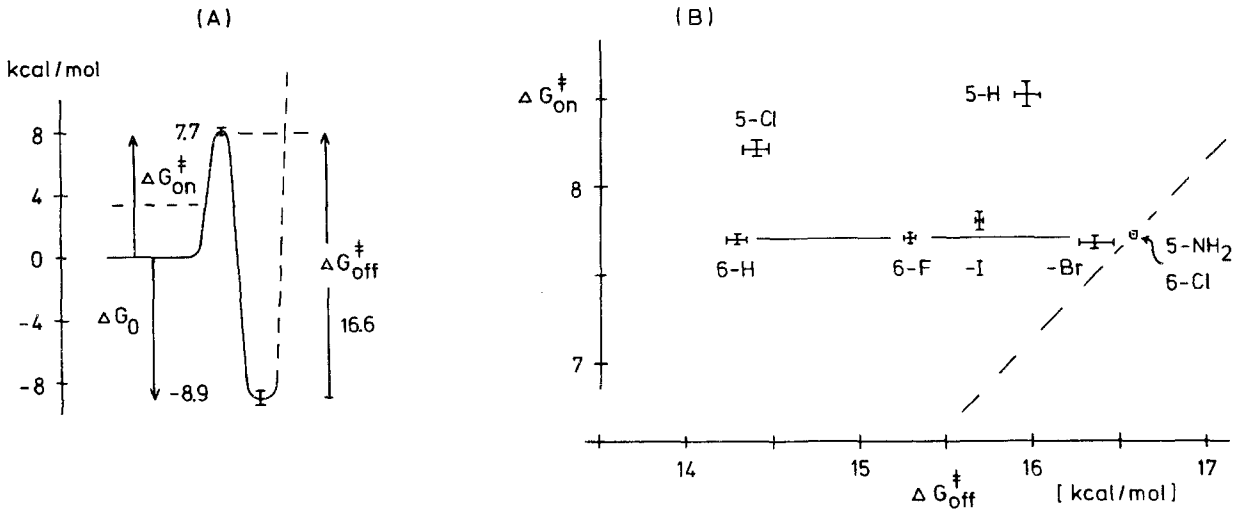

Fig. 9. (*A*) Profile of standard free energies relative to ground state (zero kcal mol^{-1}) for the channel block by amiloride at 22°C, calculated with Eq. (2) from the apparent rate constants of the overall blocking reaction [Eq. (1)]. Abscissa arbitrary. The left dashed line indicates an activation energy for diffusion limited encounters (k_{on} = 10^{10} liter mol^{-1} sec^{-1}). The minimum designates the equilibrium free energy ΔG_0. The bars denote one SEM. (*B*) Plane of standard free energies of activation [overall reaction, Eqs. (1) and (2)] for amiloride and analogs 2-7, which have single substitutions at ring position-5 or -6. The oblique dashed line is the locus of equal affinities for an equilibrium free energy ΔG_0 = −8.9 kcal mol^{-1}. The bars denote one SEM

-6 by ligands which are, for instance, weaker electron-withdrawers should affect the rate constants in the same way. This, however, is not the case. We find from Fig. 6 that 6-substitutions which increase the pK_a (i.e. lower σ_6) increase k_{off}, while 5-substitutions which increase the pK_a decrease k_{off}. Thus ring ligand substitutions which are synergistic with respect to electron density changes at the side-chain affect k_{off} differently. This observation excludes binding models based only on remote effects of ring ligands. Therefore, pyrazine ring ligands must themselves attach to the receptor.

2) Binding through 6-ligands: Figure 6*A* shows that the position-6 substituents affect, apart from the pK_a, only one rate constant: k_{off}. Thus, the 6-substituent obviously controls the stability of the blocked state. Furthermore, the order of position-6 substituents, with respect to pK_a- and k_{off}-changes, is the same, indicating that a long period of blocking requires a 6-substituent of high electronegativity. Furthermore, 5-substituents which decrease the electronic charge of pyrazine ring and side chain and, of course, also that of the 6-substituent, increase k_{off} (Fig. 6*B*), i.e. they decrease the period of blocking. These observations lead us to the hypothesis that a high electron density of the 6-substituent is essential for the stability of the blocked state. In fact, the 6-substituent may attach the analog to an electropositive residue of the receptor.

Binding through the 6-substituent is plausible also since the 6-Br and 6-I analogs were found to block irreversibly after UV-irradiation (Benos & Mandel, 1978; Cobb & Scott, 1981). The covalent bond then established may be close to the normal binding site of the 6-substituent. (However, Cuthbert et al., 1982, were unable to confirm these results with frog skin.

3) Binding through 5-ligands: The alternative that binding occurs predominantly through a 5-substituent of low electron density is less convincing because the sensitivity of k_{off} to 5-substituents is significantly smaller than to the *same* substituents at position-6. This can best be seen from the energies of activation which were calculated from the rate constants [Eq. (2)]. In Fig. 9*B* the activation energies of the on-process (ordinate) and the off-process are plotted against each other. The diagram shows that changing 6-Cl to 6-H decreases $\Delta G^{\ddagger}_{off}$ by 2.3 kcal mol^{-1} while the substitution 5-Cl to 5-H increases $\Delta G^{\ddagger}_{off}$ by only 1.6 kcal mol^{-1}. In conclusion, then, binding of the pyrazine ring to the receptor occurs through the 6-ligand and electron-donating 5-ligands stabilize the blocking position by increasing the electron density of the 6-ligand.

In Fig. 9*A* the activation energies are plotted against an arbitrary space coordinate, yielding the energy profile of channel blockage by amiloride (overall reaction). The distance of the two dashed horizontal lines shows that $\Delta G^{\ddagger}_{on}$ is much larger than expected from a diffusion limited on-process.[1] As

[1] Should mucosal Na ions act as high-rate competitors of the organic blockers, as recently proposed by Frehland et al., 1983, then the true $\Delta G^{\ddagger}_{on}$ will be smaller than the apparent value derived from our data, which were obtained at 60 mM Na_o [*see* Eq. (1a)].

shown in Fig. 9*B*, $\Delta G_{on}^{\ddagger}$ is almost unaffected by those substituents to which the off-process is most sensitive. These, however, are the substituents which *increase* the pK_a. When the pK_a is *lowered*, as by the 5-substituents used, $\Delta G_{on}^{\ddagger}$ increases. It appears, therefore, that the molecular process which leads to the blocked state is facilitated when the electronic charge on pyrazine ring and/or side-chain is comparatively large. The structure dependence of $\Delta G_{on}^{\ddagger}$ will be considered in more detail in the next paper of this series (Li, Cragoe & Lindemann, *in preparation*).

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