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Chemical Stimulation of Na Transport Through Amiloride-Blockable Channels of Frog Skin Epithelium

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Summary. The stimulation of apical Na permeability caused by a number of reagents effective from the outer side of the membrane was investigated by fluctuation analysis. In the epidermis of *R. ridibunda,* parachloromercuriphenyl sulfonate (PCMPS) and benzimidazolyl guanidine (BIG) increase the number (N_0) of conducting Na channels by releasing channels from Na self-inhibition. As a consequence, the apparent macroscopic affinity for amiloride is increased. 5-dimethyl amiloride and trinitrobenzene sulfonate (TNBS) also cause reversible stimulation by increasing N_0; here release from self-inhibition is less clear. With each of the four stimulators investigated, the Na channel current remained unaffected or was only marginally increased. In addition to its stimulatory effect, TNBS caused irreversible blockage of Na channels. Apart from their stimulatory effects, BIG and 5-dimethyl amiloride, both of which have a side-chain terminated with an amidino group, are high rate-blocking competitors of amiloride.

Key Words epithelial ion transport · apical Na channels · frog skin · fluctuation analysis · extrinsic blockers · extrinsic stimulators · amiloride analogs · PCMB · TNBS

Introduction

There are indications that the pyrazine-diuretic amiloride not only blocks apical Na channels, but in addition stimulates transport at low concentrations, a phenomenon to which incidental reference has been made in the literature (Cuthbert & Wong, 1972; Cuthbert & Shum, 1976; Bevevino & Lacaz-Vieira, 1982). Recently Thurman and Higgins (1982) showed that in the toad urinary bladder amiloride develops a strong stimulatory effect on Na uptake when applied in low concentrations in the presence of Ca ions and a divalent cation chelator. The amiloride analog benzamil was also seen to have an additional stimulating effect, at least with spironolactone-pretreated frogs (Cuthbert & Shum, 1976), while the 5-dimethyl analog is even predominantly stimulatory in frog skin (Li and DeSousa, 1979). Benzimidazolyl guanidine (BIG) was found to stimulate strongly up to concentrations of 1 mM (Zeiske & Lindemann, 1974). Furthermore, a variety of other chemicals, seemingly unrelated, cause a reversible increase of Na transport while present in the outer solution (*see* Table 1). Several multivalent cations are also stimulatory (*see*, e.g., Grinstein, Candia & Erlij, 1978).

The nature of the stimulation exerted by these agents can be further investigated by fluctuation analysis of the blocking action of amiloride. The method permits computation of the Na channel current and the density of conducting Na channels, and, thereby, reveals which of these parameters increases during stimulation. We investigated four stimulating agents: PCMPS, trinitrobenzene sulfonate (TNBS), 5-dimethyl amiloride and BIG. We found that neither PCMPS nor 5-dimethyl amiloride increases the Na channel current. With BIG a small increase was noted, which, however, cannot account for the net stimulation of transport. Instead, each of these chemically rather different agents was found to increase the density of conducting channels. In the case of PCMPS and BIG, both of which increase the macroscopic sensitivity to amiloride, the gain in conducting channels occurs predominantly at the expense of those channels previously blocked by the Na self-inhibition.

Provided that the stimulators of Table 1 all act by this kind of disinhibition, our result agrees with the previous conclusion of Kirschner (1955), that atropine stimulates by releasing from the "autoinhibition" which causes saturation of Na transport with increasing Na concentration. The same conclusion was reached by Johnston and Hoshiko (1971) with respect to novobiocin.

Our results have been reported at the 53rd Meeting of the Deutsche Physiologische Gesellschaft (Li & Lindemann, 1980).

Table 1. Stimulation of apical Na transport of frog skin by various organic agents present in the outer solution

Tissue	Agent	Reference
	fluorescein derivatives	Barnes, 1939
R. esculenta	atropine, curare, histamine	Kirschner, 1955
R. esculenta	local anaesthetics	Skou and Zerahn, 1959
R. pipiens	novobiocin	Johnston and Hoshiko, 1971
R. ridibunda	diphenylhydantoin	DeSousa and Grosso, 1973
R. esculenta	furosemide	Fülgraff et al., 1973
R. esculenta	BIG, phentolamine (regitin)	Garcia-Romeau, 1974
R. esculenta	BIG	Zeiske and Lindemann, 1974
	kallikrein	Haberich, 1976 (*personal communication*)
R. temporaria	bumetanide	Kramer, 1976
R. ridibunda	harmaline	DeSousa and Grosso, 1978
R. ridibunda	5-dimethyl amiloride	Li and DeSousa, 1979
R. temporaria	PCMP	Janaček, 1962
R. esculenta	PCMPS	Dick and Lindemann, 1975

List of Symbols

PCMB	*p*-chloromercuribenzoate
PCMPS	*p*-chloromercuriphenyl sulfonate
NEM	*n*-ethylmaleimide
TNBS	2,4,6-trinitrobenzene sulfonate (picrylsulfonic acid)
BIG	Benzimidazolyl guanidine
TAP	Triaminopyrimidine
5-DMA	5-dimethyl amino analog of amiloride
CTR	Control
Na_o	Na activity in the outer (apical) solution [mM]
A_o	Amiloride concentration in the apical solution [µM]
I_{sc}	Short-circuit current
I_{Na}	Amiloride-blockable component of macroscopic Na current through the apical membrane [$\mu A\ cm^{-2}$]
V	Transepithelial voltage (clamp voltage) [mV]
P_{Na}	Permeability of the apical ensemble of amiloride-blockable Na channels [$cm\ sec^{-1}$]
P'_{max}	P_{Na} value extrapolated to $A_o = 0$ µM at $Na_o =$ 60 mM
k_{off}, k_{on}	Apparent offrate constant [sec^{-1}] and onrate constant [$sec^{-1}\ \mu M^{-1}$] at room temperature as obtained by noise analysis from a rate-concentration plot; the values of these rate constants may include competitive effects
K_A^{mi}	k_{off}/k_{on} for amiloride, microscopic inhibition constant
K_A	Ratio of the true blocking rate constants (off/on) of amiloride
K_{BIG}	Ratio of the blocking rate constants of BIG
K_N	Inhibition constant [mM] of the Na self-inhibition obtained macroscopically but in the absence of added organic blockers
K_A^{ma}	Macroscopic inhibition constant [µM] of amiloride as obtained from the inflection point of a dose-response curve
f_c	Corner frequency [Hz] of a Lorentzian current power density spectrum
τ	$=[2\pi f_c]^{-1}$, relaxation time constant [sec] = inverse chemical rate of blockage close to equilibrium
i	Computed Na current [pA] passing a single channel when it is not blocked (state 0)
N_0, N_1, N_2	Area density [cm^{-2} or μm^{-2}] of Na channels in state 0 (conducting), state 1 (blocked by Na) and state 2 (blocked by amiloride); the total density is $N = N_0 + N_1 + N_2$
r	Regression coefficient
n	Number of observations
SEM	Standard error of the mean

Materials and Methods

Rana ridibunda of East European origin, bought from Stein, Lauingen-Donau (W. Germany), were kept in tanks at 10 to 15 °C; they were unfed but had free access to running tap water. A few days before the experiment the frogs were transferred into deionized water of room temperature (18 to 22 °C). After double-pithing, the abdominal skin was removed and mounted with the serosa backed by a filter paper on a ring of 3 cm^3 free cross-sectional area. The ring was 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 microphonics. Between the Lucite and the apical side of the epithelium an undercured silicone washer was inserted to achieve good sealing with minimal edge damage.

If not otherwise stated, skins were depolarized with a serosal K_2SO_4 solution for more than 1 h before measurements. 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 during noise measurements was a Na_2SO_4 solution of 60 mM Na activity buffered with 3.5 mM K-phosphate at pH 5.5. All solutions were used at room temperature. During noise recordings aeration of the serosal solution was stopped and perfusion of the mucosal chamber was lowered to about 1 ml/min.

For the recording of Na current fluctuations, the chamber was placed inside a grounded metal box shock-mounted on 4 springs. This box and the voltage clamp were enclosed by a large Faraday's cage. The low-noise transistor-input clamp described by Van Driessche and Lindemann (1978) was used with a high-pass *R/C* filter set at 0.07 Hz and an anti-aliasing filter set at 140 Hz. The clamp voltage was set to $V = 0$ mV. Both the short-circuit current and the filtered noise signal were monitored continuously on a strip chart recorder and an oscilloscope, respectively. I_{sc} was sampled at the beginning of each noise record. The noise signal was digitized by the 10-bit A/D

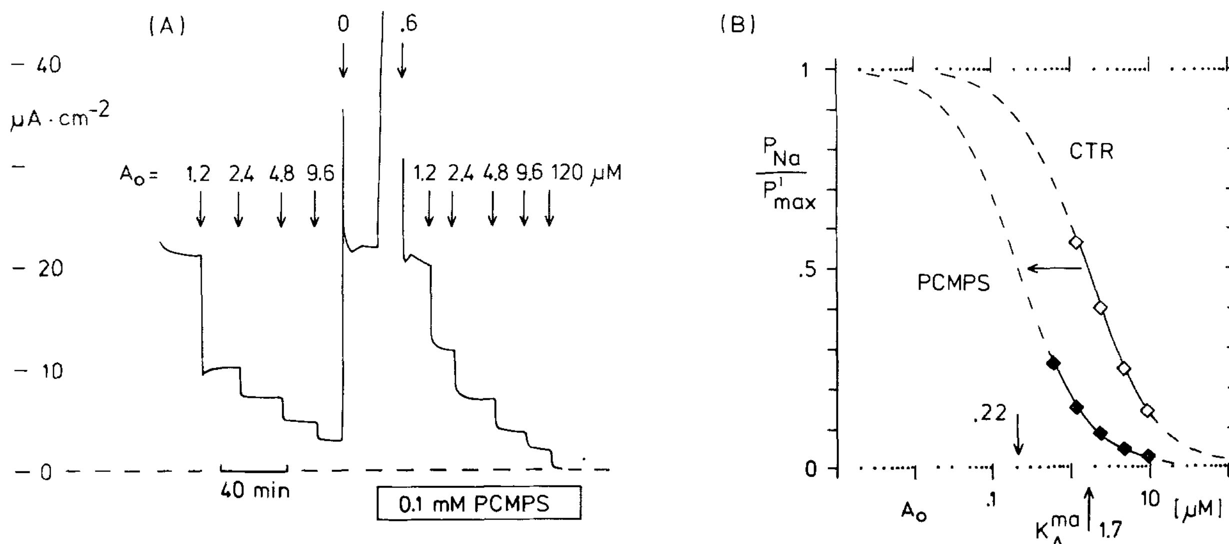


Fig. 1. *A*) Current response to increasing concentrations of amiloride observed in the absence and presence of PCMPS. Serosally K-depolarized short-circuited frog skin, outer solution 60 mM Na activity, pH 5.5. *B*) Amiloride dose-response curves computed from the data of panel (*A*). P_{Na} was estimated from $I_{Na}/(F \cdot Na_o)$; P'_{max} is the P_{Na} value at zero amiloride concentration. The macroscopic inhibition constants are indicated by vertical arrows. CTR = control data obtained before addition of 0.1 mM PCMPS

converter of a minicomputer (NOVA 1230, Data General) at intervals of 2.44 msec for a period of 10 sec. The resulting time series arrays of 4096 data points were Fourier-transformed and the current power density was computed on line and monitored on an XY-display. Twenty or more spectra were generally recorded, averaged and condensed as described before (Li, Palmer, Edelman & Lindeman, 1982). The processed spectral data were then stored on disk for further evaluation.

Current power density spectra taken at several blocker concentrations were fitted with a computer program which permitted the subtraction of a low frequency spectral component from the Lorentzian component. However, this subtraction was seldom necessary. In most cases it was sufficient to enter the frequency limits for fitting of the Lorentzian component. Plateau power and corner frequency were then obtained as fitting parameters of a least-squares procedure. The chemical rate of extrinsic blockage was obtained from the corner frequency of the Lorentzian multiplied by 2π. The onrate and offrate constants of the pseudo-first-order kinetics assumed for the chemical blocking process were obtained from the linear plot of rate *vs.* concentration, as shown under Results. I_{Na} was computed by subtracting the shunt current observed in the presence of 30 to 120 μM amiloride from the total current. Unless stated otherwise, values reported are the means ± standard deviation.

Amiloride-HCl-dihydrate was a gift from Sharp and Dohme GmbH, München, Germany, and its 5-dimethyl analog was a gift from Dr. E.J. Cragoe, Jr., of Merck, Sharp, Dohme Research Laboratories, West Point, Pennsylvania, to whom our thanks are due. The other stimulating reagents were obtained from commercial suppliers.

Results

Group-'Specific' Reagents

Sulfhydryl Group Reagents: Para-Chloromercuriphenyl sulfonate (PCMPS). SH-group reagents like PCMB and PCMPS, added to the outer bathing medium of the epidermis of *R. ridibunda,* have been shown to reversibly abolish the saturation of the steady-state Na current which occurs at large Na concentrations. Thereby these reagents stimulate Na transport. Reversibility is demonstrated by washing with a cystein solution (Dick & Lindemann, 1975; Dick, 1977; *see also* Lindemann & Voûte, 1976). If this stimulation results from an interference of the reagent with the Na self-inhibition mechanism and if, in addition, the latter is competitive with the Na channel blockage by amiloride, then the apparent macroscopic inhibition constant of amiloride, K_A^{ma} should decrease in the presence of PCMPS.

Figure 1 *A* shows the response of the short-circuit current (I_{sc}) to apical amiloride before and after addition of PCMPS. The solutions contained 60 mM Na_o (activity). In the absence of amiloride PCMPS strongly stimulated I_{sc}, in the example shown beyond the current limit of the voltage clamp (250 μA). However, in the presence of amiloride the absolute and relative increase in I_{sc} elicited by PCMPS was far less, and sometimes absent. This observation is explained when the normalized dose-response curves of amiloride in the absence and presence of PCMPS are compared. As shown in Fig. 1 *B*, 0.1 mM PCMPS shifts the dose-response curve of amiloride to the left. The K_A^{ma} is reduced from a mean value (± SEM) of 2.59 ± 0.28 to 0.59 ± 0.18 μM ($n = 5$). Thus the stimulation elicited by PCMPS in the presence of amiloride is partly or completely balanced by an increased sensitivity to amiloride. This is expected if the stimulation is based on release from the Na

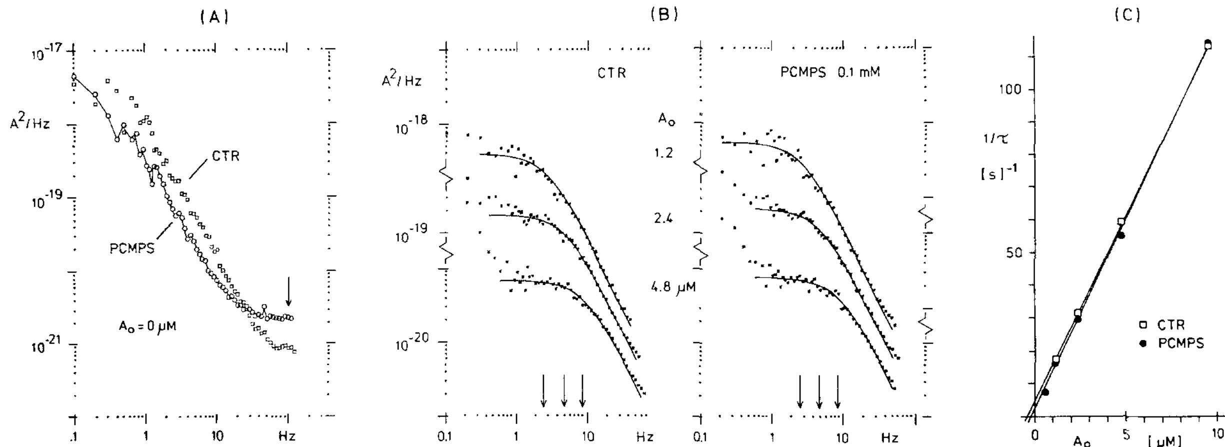


Fig. 2. *A*) Effect of 0.1 mM PCMPS (outer solution) on Na-current power density spectra taken in the absence of amiloride. 3 cm^2 of K-depolarized short-circuited frog skin; outer solution of 60 mM Na_o, pH 5.5. *B*) Amiloride-induced Lorentzians obtained in the absence (control (CTR), left) and presence of 0.1 mM PCMPS. Preparation of panel (*A*). The offsets in the ordinate scales are the same for left and right spectra. Note that plateaus and corner frequencies are hardly changed by PCMPS. *C*) Rate-concentration plot from Lorentzians obtained before and during exposure to 0.1 mM PCMPS

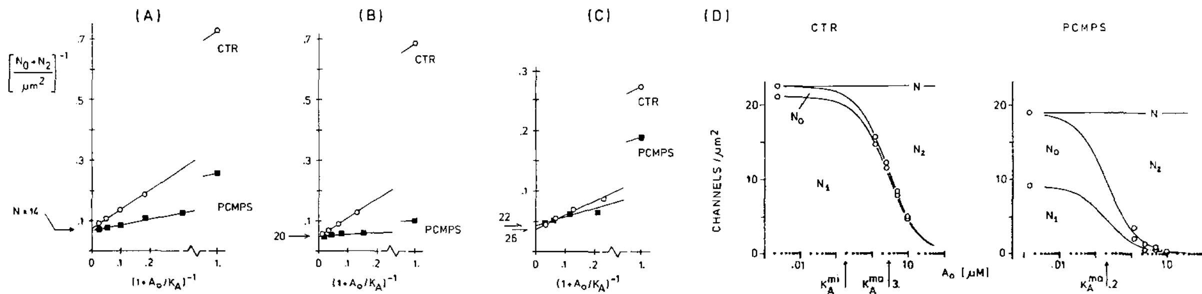


Fig. 3. *A*, *B*, *C*) Graphical estimation of the total number (N) of electrically detectable Na channels in the absence and presence of 0.1 mM PCMPS (3 preparations). The plots show that PCMPS increases N_0+N_2. In (*A*) and (*B*) N remained constant at 14 and 20 channels per μm^2, respectively. In (*C*) N decreased from 26 to 22 μm^{-2}. CTR = control (no PCMPS). *D*) Estimated change of N_0, N_1 and N_2 with the amiloride concentration. In the presence of 0.1 mM PCMPS (right panel) K_A^{ma} is lowered towards K_A^{mi}, and at a given A_o both N_0 and N_2 appear increased at the expense of N_1

self-inhibition with which amiloride competes in this species of frog.

At low frequencies the Na current power density spectrum in the presence or absence of PCMPS does not contain a clear Lorentzian plateau. Figure 2*A* shows that below 20 Hz the power is decreased by PCMPS, while at higher frequencies the power increases. The increase is probably caused by the input stage noise of the voltage clamp (*see* Van Driessche & Lindemann, 1978). The input stage noise will be larger when the resistance of the preparation decreases in the presence of PCMPS (*see* Lindemann & DeFelice, 1981). The same phenomenon was observed with two other agents which decrease the apical membrane resistance (Fig. 7*B* and Fig. 9*A*). It is not expected that this artifact affects the amiloride-induced Lorentzians because 1) the relatively high amiloride concentrations used keep the membrane resistance high, and 2) the induced noise-power is large.

The amiloride-induced Lorentzian components recorded in the presence and absence of PCMPS (Fig. 2*B*) show only small differences in corner frequency. Therefore, the blocking kinetics of amiloride do not seem to be modified by PCMPS, except for a small decrease in the offrate constant (ordinate intercepts, Fig. 2*C*; *see also* Table 2). The spectral plateaus (Fig. 2*B*) reflect the changes in I_{sc} caused by PCMPS in the presence of amiloride: the increase in plateau power is small and decreases with increasing amiloride concentration. This is expected if the stimulatory effect (increase in i or N_0) is counterbalanced by the increased macroscopic sensitivity to amiloride caused by an increase in K_N.

The single-channel current i and the total chan-

Table 2. Effects of 0.1 mM PCMPS on the Na transport parameters and amiloride blocking parameters of the apical membrane of skins from *R. ridibunda*

	I_{Na} (μA cm^{-2})	K_N (mM)	K_A^{ma} (μM)	K_A^{mi} (μM)	k_{on} (μM^{-1} sec^{-1})	k_{off} (sec^{-1})	i (pA)	N_0 (μm^{-2})	N (μm^{-2})
CTR	15.65 ±2.35	6.73 ±0.98	2.59 ±0.28	0.26 ±0.04	12.61 ±1.76	3.13 ±0.33	0.085 ±0.009	1.97 ±0.50	19.21 ±2.70
PCMPS (0.1 mM)	53.02 ±13.76	41.31 ±20.66	0.59 ±0.18	0.16 ±0.03	13.46 ±1.66	2.09 ±0.27	0.086 ±0.007	5.96 ±1.14	18.09 ±2.30

Means ±SEM, $n=5$. Laterobasal membranes depolarized with high K_2SO_4 Ringer's, apical Na_2SO_4 Ringer's: $Na_o = 60$ mM, pH 5.5. I_{Na}, K_N, K_A^{ma} and N_0 show increases of statistical significance ($P<0.05$) after PCMPS addition. When I_{Na}, in the presence of PCMPS and the absence of amiloride, exceeded the current limit of the voltage clamp, its value was estimated by extrapolating the amiloride dose-response curve to the point of zero amiloride. K_N was estimated as $Na_o/(K_A^{ma}/K_A^{mi}-1)$. N_0 for the absence of amiloride was estimated from I_{Na}/i.

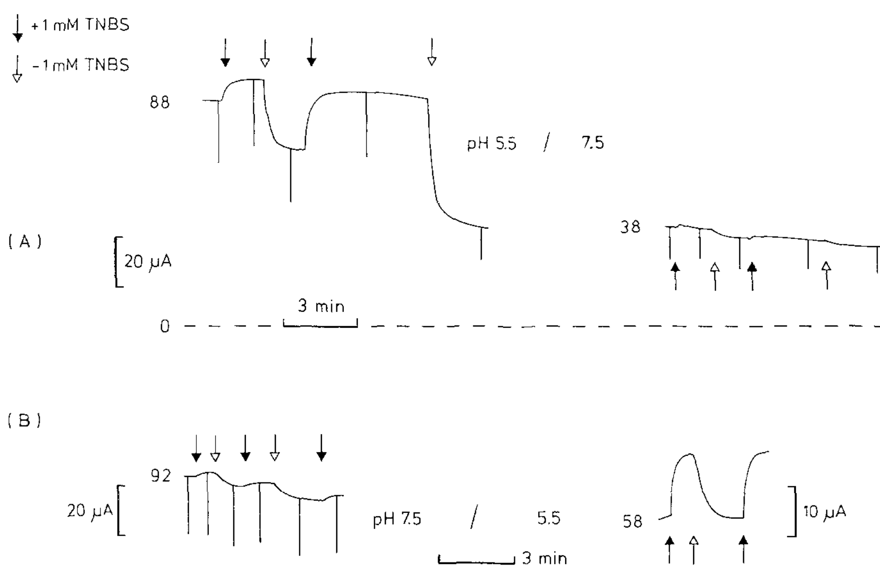


Fig. 4. Effect of addition and removal of TNBS (1 mM in the outer solution) on the short-circuit current of 3 cm^2 of K-depolarized frog skin. The numbers at the beginning of each current trace indicate the current value observed at this time. The spikes indicate conductance (current response to voltage displacements of −10 mV). Two preparations (*A*, *B*) at two pH values each. Outer Na activity 60 mM

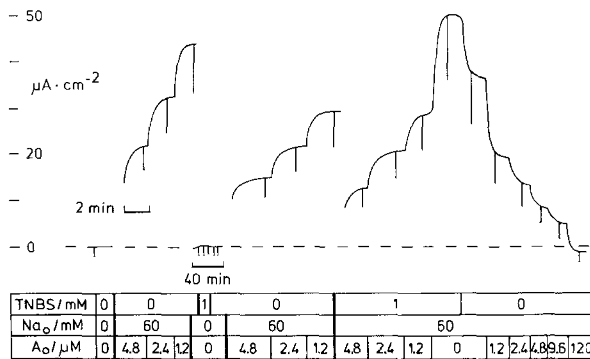
nel density N was calculated as described previously (Li et al., 1982). Figure 3*A*, *B* and *C* provide examples of how N is obtained graphically. Table 2 summarizes the effects of PCMPS on the Na transport parameters of the apical membrane of skins from *R. ridibunda*. Clearly, the stimulation of Na transport by PCMPS is not due to an increased channel current, but is based on an increase in the number of open channels. The increase occurs at the expense of those channels previously blocked by the Na self-inhibition mechanism (Fig. 3*D*). N_0 increased by a factor of 3 (mean of 5 experiments). The mean total density of electrically detectable channels (N) was not significantly changed.

Amino Group Reagent: 2,4,6-Trinitrobenzene Sulfonate (TNBS). For comparison with the SH reagent discussed above, effects of the NH_2 reagent TNBS were also investigated. It was previously noted that at pH 7.2 this compound causes a decrease of Na transport across the apical membrane of skins from *R. ridibunda*. The decrease of current has an exponential time course, the time constant being 6 min at 2 mM TNBS and 20 mM Na_o (Fallenstein, 1980).

In addition to the inhibitory effect, TNBS has a stimulatory effect on I_{Na}. As shown in Fig. 4, addition of TNBS at pH 5.5 causes an increase of current followed by a slow decrease observable when the exposure is prolonged. Removal of TNBS unmasks the inhibitory effect, which is obviously less reversible. The stimulatory effect is well developed at pH 5.5, but small and often insignificant at neutral pH. It appears that the stimulation by TNBS is faster, and reversible, while the inhibitory effect is slower and poorly reversible.

In the absence of apical Na ions, the addition of TNBS does not change the short-circuit current but the inhibition of Na transport develops never-

theless. In the experiment of Fig. 5, I_{Na} measured in the absence of amiloride exceeded 80 μA cm^{-2} (*not shown*). It decreased to 47 μA cm^{-2} after 15 min of exposure to 1 mM TNBS in Na-free Ringer's. Figure 5 illustrates the effect of TNBS on the response of I_{Na} to amiloride blockage. Analysis of these 4 dose-response curves showed K_A^{ma} to be only slightly reduced by TNBS. Irrespective of the absence or presence of sodium ions, the reagent decreased I_{Na} at each level of amiloride blockage, and this reduction improved with the time of exposure to TNBS.



TNBS/mM	0	0			1	0			1			0					
Na$_o$/mM	0	60			0	60			60								
A$_o$/μM	0	4.8	2.4	1.2	0	4.8	2.4	1.2	4.8	2.4	1.2	0	1.2	2.4	4.8	9.6	120

Fig. 5. Response of short-circuit current to different amiloride concentrations before, after and during exposure to TNBS (1 mM in the outer solution, pH 5.5). K-depolarized frog skin. The spikes indicate conductance (current response to voltage displacements of −10 mV). After 15 min of exposure to TNBS in a Na-free outer solution (Na replaced by K), currents were smaller at all amiloride concentrations. In the subsequent period the amiloride exposure was repeated in the presence of TNBS. At each A_o the current was similar in value to that obtained in the absence of TNBS. However, the final removal of TNBS reveals the presence of a reversible stimulatory effect

Further information on the effects of TNBS was obtained by analysis of amiloride-induced Na current fluctuations. It was difficult to apply steady-state noise analysis in this case, since the irreversible blocking effect of TNBS caused a continuous decrease of the macroscopic current. Figure 6*A* shows in each panel (from above to below) the amiloride-induced spectra before, during and after exposure to TNBS. The plateaus of Lorentzians at a given amiloride concentration decreased in the presence of TNBS, but the corner frequencies did not significantly change (vertical lines). Apparently the molecular changes which cause the reversible stimulation and those which cause the irreversible inhibition do not affect the blocking rate constants of amiloride. In Figure 6*B* the Lorentzian plateaus of panel *A* are plotted against the respective Na current in double logarithmic coordinates. At each amiloride concentration the changes in plateau power caused by TNBS are found to be almost linearly related to the corresponding changes in Na current (slope of approximately unity in the double-logarithmic plot). A change in channel current only would have caused a quadratic dependence. Therefore, in the absence of an effect on corner frequencies, a predominant change in the density of open channels rather than in the channel current is implicated for both the stimulatory and the inhibitory effect of TNBS.

In Table 3 the kinetic constants of amiloride blockage and the computed single-channel current (i) in the presence and absence of 1 mM TNBS are summarized. Only the Na channel current shows a small change of statistical significance ($P<0.05$ by Student's paired t-test) in response to TNBS.

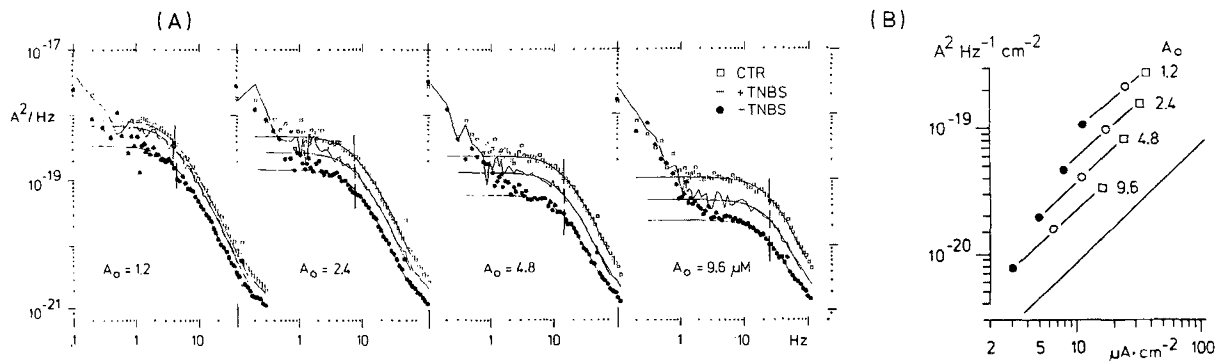


Fig. 6. *A*) Amiloride-induced Lorentzians in power density spectra obtained before exposure to TNBS (control (CTR), upper curves), in the presence of 1 mM TNBS (drawn-out curves) and after removal of TNBS (lower spectra). 3 cm^2 of K-depolarized short-circuited frog skin, outer Na activity 60 mM. The corner frequencies (vertical lines) are not affected by TNBS. The plateaus are decreased, and decreased further on removal of TNBS. This indicates reversible stimulation and additive irreversible inhibition during the presence of TNBS. After removal of TNBS the irreversible inhibitory effect remains. *B*) The plateau power density (ordinate) of amiloride-induced Lorentzians (panel *A*) is plotted against the macroscopic Na current. (□) indicates the control, (○) data in the presence of 1 mM TNBS and (•) data after removal of TNBS. A slope of approximately unity (lower line) is obtained at each amiloride concentration, indicating that the predominant effect of TNBS is not an increase (for the stimulation) or decrease in the channel current. The mean i of 3 experiments increased slightly (*see* Table 3)

Table 3. Effects of 1 mM TNBS on amiloride blocking kinetics and Na-channel currents

	K_A^{mi} (μM)	k_{on} ($\mu M^{-1}\ sec^{-1}$)	k_{off} (sec^{-1})	i (pA)
CTR (pH 5.5)	0.43 ±0.13	13.34 ±0.80	5.86 ±2.09	0.083 ±0.005
CTR (pH 7.5)	0.33	14.04	4.65	0.098
TNBS (1 mM, pH 5.5)	0.51 ±0.04	13.56 ±0.98	6.83 ±0.59	0.103 ±0.005
TNBS (1 mM, pH 7.5)	0.49	13.60	6.65	0.125

Means ±SEM. These data were obtained from serosally K-depolarized short-circuited preparations: 3 skins at a mucosal pH of 5.5 and 1 skin at a mucosal pH of 7.5 with amiloride (1.2 to 9.6 μM) either alone (CTR) or in the presence of 1 mM TNBS. Outer Na activity 60 mM. Measurements performed in the absence of TNBS with a skin pretreated with TNBS also showed only insignificant changes in the amiloride kinetics (as in Fig. 6), and a slight increase in the channel current.

The moderate increase in i can be expected from the action of a reagent that predominantly impairs the entry of Na through the apical membrane, but leaves the extrusion of Na through the basolateral membrane unchanged. The transapical Na gradient would thus be increased. Since the TNBS data in Table 3 were taken before the completion of the inhibitory action of TNBS, the quantitative aspect of the change in the total Na channel density was not examined in detail; nevertheless, it could be noted that both the density of open Na channels and the total channel density are invariably reduced after prolonged exposure to TNBS.

Stimulating Analogs of Amiloride

5-Dimethyl Amiloride. In this close structural analog of amiloride, both protons of the amino group at position 5 of the pyrazine ring are replaced by methyl groups. The compound stimulates Na transport across the skin of *R. ridibunda* in the concentration range from a few micromolar to nearly one millimolar (compound LT2 of Li and DeSousa, 1979). On the other hand, the compound has also been reported to inhibit Na transport. With a nominal concentration of 1 μM the short-circuit current in the isolated skin of *R. pipiens* is decreased by 4% (Benos, Simon, Mandel & Cala, 1976). Thus, the response of the macroscopic Na transport to 5-dimethyl amiloride appears to be qualitatively different in the two species of frogs investigated. However, the difference could be quantitative rather than qualitative, if the analog had intrinsically both a stimulatory and an inhibitory potency. These two putative effects of 5-dimethyl amiloride may be expected to change the macroscopic and microscopic blocking kinetics of amiloride, and were investigated accordingly.

At the concentrations used (0.2 and 0.79 mM), the analog caused a net reversible stimulation of the macroscopic Na current. The stimulation may well be due to an increase in K_N since in one experiment K_A^{ma} was found to be decreased (Fig. 7*A*). Lack of analog material prevented further experiments of this kind. The same effect on K_A^{ma} was previously found with PCMPS (Fig. 1*B*) and it was also found with BIG (*see* Fig. 9*C*).

5-dimethyl amiloride itself does not give rise to Lorentzian spectra of corner frequencies between 0.1 and 100 Hz (Fig. 7*B*) but it does systematically decrease the corner frequencies of the amiloride-induced Lorentzians, mainly by lowering the apparent onrate constant of the blocking kinetics of amiloride. The same effect was previously described for triaminopyrimidine (Li & Lindemann, 1981). It is not found with PCMPS and TNBS and, according to our previous analysis, explained by the analog acting as a higher rate blocking competitor of amiloride[1]. By using competition kinetics we calculated a microscopic inhibition constant of about 0.5 mM for 5-dimethyl amiloride. The corresponding macroscopic inhibition constant at 60 mM Na activity would be 4.3 mM, provided K_N retained the typical value of about 8 mM, i.e. were not increased by the stimulatory effect of the analog. Since K_N probably increases, K^{ma} will be somewhat smaller than 4 mM.

The single-channel current in the simultaneous presence of 5-dimethyl amiloride and amiloride was calculated with an equation similar to Eq. (13) of Lindemann and Van Driessche (1978) which is applicable since the analog showed clear competitive interaction with amiloride, the plateaus observed being those of the Lorentzians of the lower corner frequencies. The following assumptions en-

[1] *Compare* J.H.-Y. Li and B. Lindemann. Competitive blocking of epithelial Na channels by organic cations: The relationship between macroscopic and microscopic inhibition constants. *J. Membrane Biol.* (*in press*).

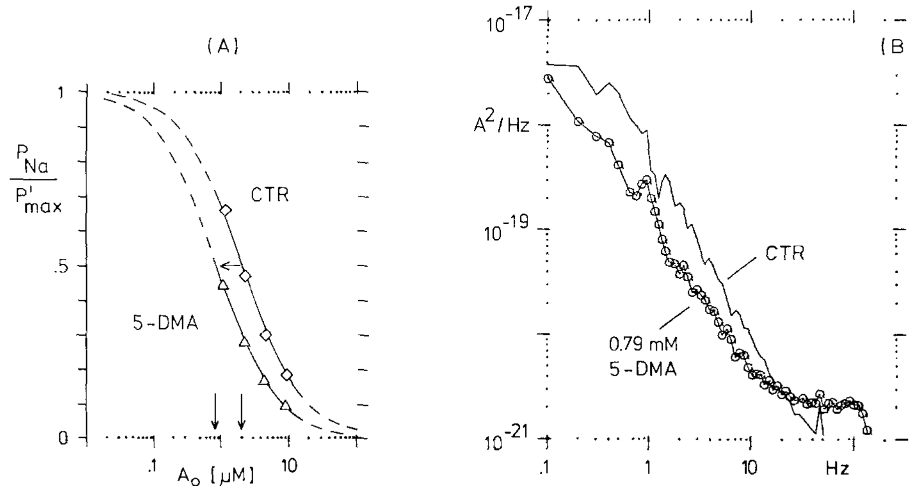


Fig. 7. *A*) Macroscopic dose-response curves of amiloride taken in absence (CTR) and presence of 0.79 mM 5-dimethyl amiloride (5-DMA) (pH 5.5, Na_o = 60 mM). *B*) Effect of 0.79 mM 5-dimethyl amiloride on Na current power density spectra taken in the absence of amiloride. 3 cm^2 of K-depolarized short-circuited frog skin; outer solution of 60 mM Na_o, pH 5.5. At this concentration the analog elicits a transient increase of I_{Na} of more than 2.5-fold, but does not induce a Lorentzian in the frequency range used. The apparent decrease in spectral power is similar to that observed with PCMPS (*see* Fig. 2*A*)

tered the calculation: (1) the Na self-blockage has a negligible influence on the interaction of amiloride and of the analog with the channel; (2) the true rate constants of the amiloride blocking process are not affected by the presence of the analog, and (3) the onrate constant of the blockage by the analog is similar to that of amiloride. Assumption (1) permits the use of a 3-state competitive reaction scheme to describe the interactions of Na, amiloride and its analog with the channel. Assumptions (2) and (3), together with the estimated microscopic inhibition constant of the analog, allow the calculation of the offrate constant of the analog. The value of the offrate constant thus obtained is orders of magnitude greater than that of amiloride and this difference justifies the approximations implicit in using the equation.

The single-channel current thus estimated for the presence of the analog and amiloride (0.123±0.004 pA, 3 experiments) was close to the control value obtained in the presence of amiloride alone (0.111±0.008 pA), although the stimulation of the macroscopic Na current was nearly threefold. In view of the unchanged channel current, the stimulatory action of 5-dimethyl amiloride, like that of PCMPS, must be based on an increase in the number of open channels. However, unlike PCMPS, the analog also exerts a high rate-blocking effect which competes with blockage by amiloride.

Benzimidazolyl Guanidine (BIG). This compound has been claimed to stimulate Na current through the apical membrane of frog skin epithelium (*R. ridibunda*) by preventing – like PCMPS – the decrease of Na permeability caused by extracellular Na ions. In contrast to PCMPS, an additional inhibitory effect on the Na current was noted at concentrations higher than 1 mM (Zeiske & Lindemann, 1974). In the epidermis of *R. temporaria,* the Na current response to BIG is more variable: depending upon the pH of the apical solution and the concentration of the compound, either net stimulation or net inhibition of the Na current was observed; the mixed stimulatory-inhibitory response prevented a quantitative study of BIG effects in this species (Cuthbert, 1976). In the toad urinary bladder the response of the short-circuit current indicates domination of the inhibitory effect of BIG in the concentration range 0.1 to 2 mM (*unpublished observations*).

In the epidermis of *R. ridibunda* the net response of I_{Na} to 1 mM BIG is stimulatory. However, the simultaneous presence of an inhibitory effect is noticeable from the transient increase of current observed right after removal of BIG from the outer solution. The typical time course shown in Fig. 8*A* suggests that the inhibitory effect has a higher offrate than the stimulatory effect, but requires larger concentrations of BIG. The stimulation is also evident in dose-response curves of amiloride obtained in the presence of BIG. As shown in Fig. 8*B* the plot of $1/P_{Na}$ *vs.* the amiloride concentration is shifted downward in the presence of BIG. Therefore, the Na permeability is increased and the apparent macroscopic inhibition

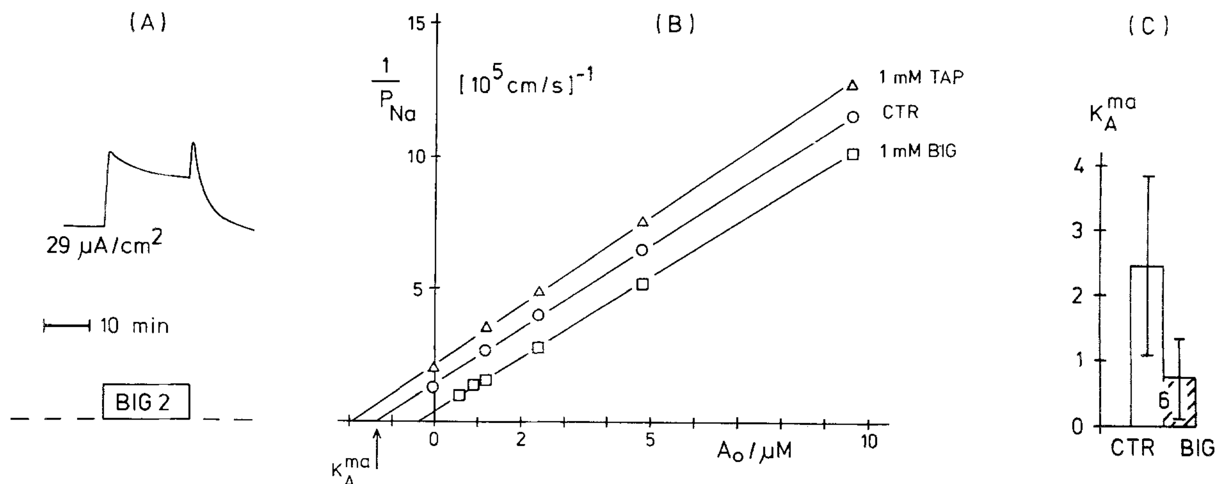


Fig. 8. *A*) Response of short-circuit current to addition and removal of BIG (2 mM in the outer solution). K-depolarized frog skin, outer Na activity 20 mM. *B*) Effect of BIG and TAP, present in the outer solution, on Na permeability at 60 mM Na_o, pH 5.5. P_{Na} was estimated as $I_{Na}/(F \cdot Na_o)$ for short-circuited K-depolarized skins. *C*) Apparent macroscopic inhibition constant of amiloride obtained from dose-response curves (like panel *B*) of 6 paired experiments without and with 1 mM BIG. Experimental conditions as for panel *B*

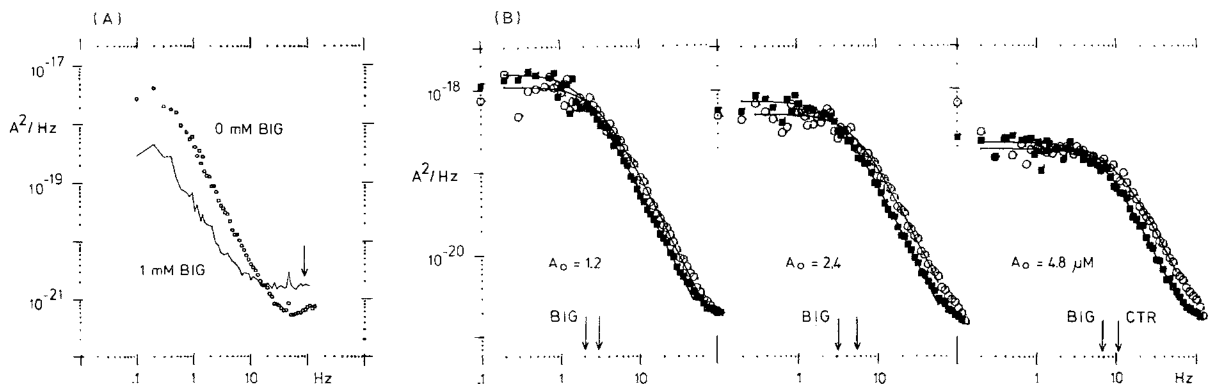


Fig. 9. *A*) Effect of 1 mM BIG (outer solution) on the Na power density spectrum taken in the absence of amiloride. 3 cm² of K-depolarized short-circuited frog skin, outer solution of 60 mM Na_o, pH 5.5. *B*) Amiloride-induced Lorentzians obtained in the absence (o) and presence (■) of 1 mM BIG. A decrease in corner frequency and a small increase in plateau power is noticeable

constant of amiloride is decreased (*compare* Lindemann, 1978). Had BIG merely a competitive inhibitory effect, the plot would have been shifted upward as indicated in Fig. 8*B* for the presence of triaminopyrimidine (TAP). This compound has been shown to possess a predominant inhibitory potency (Zeiske, 1975; Balaban, Mandel & Benos, 1979; Li & Lindemann, 1981, 1983[2]). The P_{Na} extrapolated to zero amiloride concentration was increased by the presence of BIG (1 mM), although the degree of this increase varied considerably (6 paired experiments). In this series of experiments the mean macroscopic inhibition constant of amiloride decreased from 2.45 to an apparent value of 0.74 µM by addition of BIG (Fig. 8*C*). The observed decrease of K_A^{ma} indicates that the stimulatory effect of BIG involves release from an inhibitory process competitive to channel blockage by amiloride. The value of 0.74 µM is an overestimate since the inhibitory effect of BIG is not taken into account.

In the presence of BIG the Na current power density shows a decrease at low frequencies but an increase at high frequencies (Fig. 9*A*, arrow). The increase will have the same explanation as that of Fig. 2*A*. We were unable to decide whether this spectral component contains, in addition, noise from a blocking process of higher chemical rate.

[2] *See* footnote 1, p. 185.

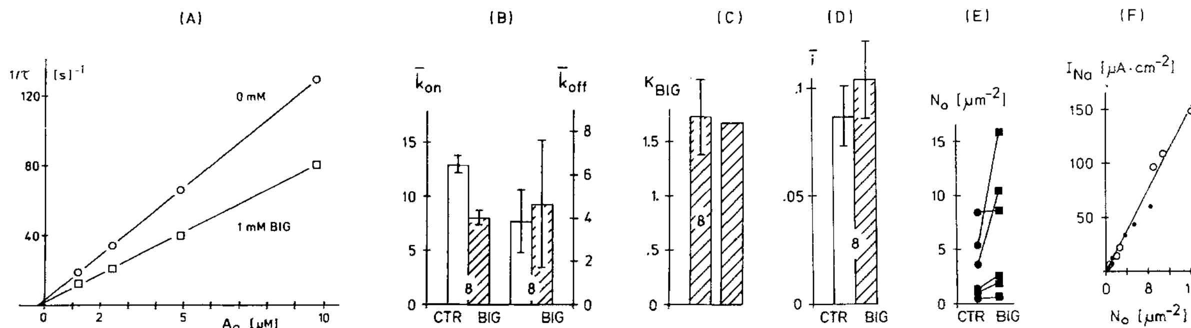


Fig. 10. *A*) Effect of 1 mM BIG (outer solution) on the rate concentration relationship of amiloride. Experimental conditions as in Fig. 9. The predominant decrease in slope indicates that BIG is a high rate blocker competing with amiloride. A predominant release from Na self-inhibition would have caused a parallel downward shift, which cannot be large, however, in view of the small onrate constant of the Na self-inhibition kinetics. *B*) Apparent rate constants of the amiloride block obtained in the presence and absence of 1 mM BIG. Mean ±SEM of 8 experiments, conditions as in Fig. 9. *C*) Microscopic inhibition constant (ratio of blocking rate constants) calculated from the change in slope of the amiloride rate concentration plot. The left value was calculated as the mean ±SEM of 8 separately evaluated experiments, the right value from the mean rate concentration plot of these experiments. *D*) Na channel currents calculated for the presence and absence of BIG (mean ±SEM of 8 experiments). *E*) Density of conducting Na channels (estimated as I_{Na}/i) for 6 paired experiments in the absence (control = CTR) and presence of 1 mM BIG. *F*) Correlation of macroscopic Na current and N_0 taken before (•) and during exposure (o) to 1 mM BIG. The slope of the regression line indicates a channel current of 0.095 pA. The separate slopes for control and BIG data are 0.072 and 0.098 pA

Amiloride-induced Lorentzians (Fig. 9*B*) were systematically displaced in the presence of BIG, such that the slope of the rate *vs.* amiloride concentration plot decreased significantly[3] (Fig. 10*A, B*), indicating that BIG is a higher rate blocker in competition with amiloride. From the change in slope the microscopic inhibition constant of BIG is estimated to be in the order of 2 mM (Fig. 10*C*).

The single-channel current in the presence of BIG was calculated from amiloride-induced Lorentzians as mentioned above for the presence of 5-dimethyl amiloride. With 1 mM BIG, the channel current shows a slight increase from a mean value of 0.087 to 0.104 pA (Fig. 10*D*). This increase cannot account for the large increase in the Na permeability observed at the same time. The stimulation, therefore, must mainly be due to an increase in the number of conducting Na channels. Except for one experiment, the calculated values of N_0 were increased by BIG, the absolute gain in conducting channels being larger when the control value was already high (Fig. 10*E*). The macroscopic Na current correlated well with N_0 ($r = 0.982$, Fig. 10*F*), indicating a mean channel current of 0.095 pA.

Discussion

Of the four agents investigated, only PCMPS (0.1 mM) appears to have a pure stimulatory effect on the apical Na transport system. The large increase in the macroscopic sensitivity to amiloride in combination with almost unchanged microscopic blocking kinetics of amiloride points to a release of Na channels from the Na self-inhibition which, in the epidermis of *R. ridibunda*, is kinetically competitive to the block by amiloride. This finding is in agreement with the increase in K_N noted by Dick (1977; *see also* Lindemann and Voûte, 1976) and by Fuchs et al. (1977). Further support for this conclusion is provided by our finding of an unchanged Na channel current and a large increase in N_0 at the expense of N_1, with N remaining essentially constant.

In the toad urinary bladder, PCMPS elicits a complex response which begins with a stimulation (e.g. Spooner & Edelman, 1976; Harms and Fanestil, 1977). In the skin of *R. catesbeiana* the response is similar. However, when PCMB is made nonpermeant by linkage to dextran it becomes purely stimulatory (Benos, Mandel & Simon, 1980). The authors report that in this tissue the efficacy of amiloride remains unchanged during the stimulation. This fits well with their previous finding that amiloride is not competitive to Na in *R. catasbeiana*.

The total channel density was extrapolated from a plot of $[N_0+N_2]^{-1}$ *vs.* $[1+A_o/K_A]^{-1}$, assuming that the real value of K_A is not changed by the presence of BIG. The results of an exceptionally good experiment are shown in Fig. 11*A*,

[3] *See* footnote 1, p. 185.

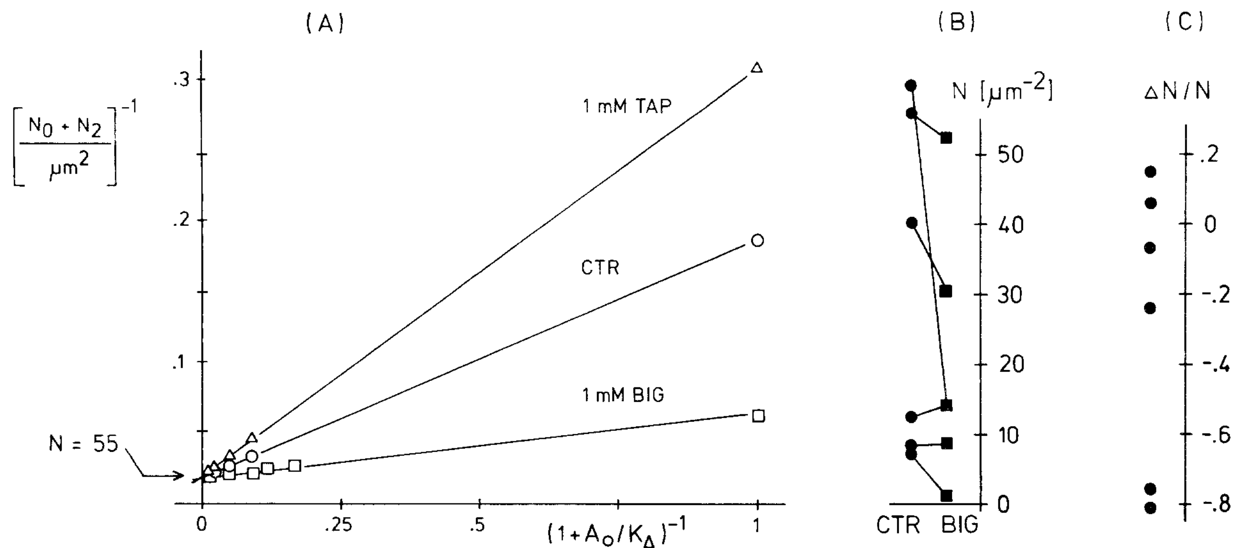


Fig. 11. *A*) Graphical estimation of the total number of electrically detectable Na channels. In this experiment a value close to 55 channels per μm^2 was found under control conditions and in the presence of 1 mM BIG or 1 mM TAP in the outer solution. Experimental conditions as in Fig. 9. The data for $A_o = 0$ μM (rightmost points, $N_2 = 0$) show clearly that TAP decreases N_o while BIG evokes an increase (net stimulation). *B*) Values of *N* obtained in the 6 paired experiments of Fig. 10*E* for the absence (CTR) and presence of 1 mM BIG in the outer solution. *C*) Relative change of the *N* values displayed in panel *B* in response to 1 mM BIG

where the data for the presence of BIG are plotted together with those for the presence of TAP. The close agreement of the ordinate intercepts shows that neither BIG nor TAP, which evoked opposite macroscopic Na current responses, altered the total channel density significantly. More typically the total channel density in the presence of BIG was found to be decreased (Fig. 11*C*), even though BIG stimulated Na transport. This decrease in the total channel density could be due to spontaneous deterioration of the preparation and/or the increase in cellular Na which may have caused – directly or indirectly – feedback inhibition from the intracellular compartment (e.g. Taylor & Windhager, 1979; Chase & Al-Awqati, 1981).

In conclusion, BIG releases channels from the Na self-inhibition and thus increases the macroscopic affinity for amiloride, as described above for PCMPS. Unlike PCMPS, BIG is a high-rate channel blocker competitive to amiloride, as described above for 5-dimethyl amiloride.

PCMPS is almost exclusively SH reactive (e.g. Means & Feeney, 1971; Friedman, 1973). Therefore, its stimulatory effect is very likely to involve a primary modification of an SH group. It is interesting to note that the SH reagent N-ethylmaleimide (NEM) does not evoke stimulation (Dick, 1977). Therefore, if PCMPS reacts with a SH group, this group is likely to be located in a niche which bars access of NEM (buried group, *compare* Angelone, 1965; Skou & Hilberg, 1965).

The net response to the amino-group reagent TNBS is more complex. We found that TNBS, apart from its reversible inhibitory effect at pH 7.5 (Fallenstein, 1980), evokes reversible stimulation at pH 5.5. The macroscopic sensitivity to amiloride is only slightly reduced by this reagent. Steady-state noise analysis of the stimulatory effect was difficult due to the progression of inhibition in the presence of TNBS. The data indicate, however, that the amiloride blocking kinetics remain unchanged. The Na channel current shows a small increase (20%) which may in part be explained by an increase of the transapical Na gradient caused by a drop of the cellular Na activity in response to the inhibition of apical entry of Na ions. The increase in Na channel current with increasing amiloride concentration has found a similar explanation (*see* Li et al., 1982).

In view of the only small decrease in K_A^{ma} evoked by TNBS it remains uncertain whether the stimulatory effect of this reagent involves release from Na self-inhibition. TNBS reacts covalently by nitrobenzylation of unprotonated terminal amino groups (e.g. ε-NH_2 groups of lysine). This reaction, which is expected to proceed more rapidly above pH 7, occurs already at low reagent concentrations and is irreversible after completion (Means

& Feeney, 1971; for further literature *see* Knauf & Rothstein, 1971). In view of its poor reversibility, the inhibitory effect of TNBS may be based on this reaction. In the skin of *R. ridibunda* other NH_2 reagents, like glutardialdehyde, also cause blockage of apical Na transport (Fallenstein & Lindemann, 1979). In the case of glutardialdehyde it could be shown that amiloride protects from the inhibitive effect (Fallenstein, 1980).

The reversible stimulatory effect of TNBS, which occurs predominantly at acid pH, will involve a different reaction. Reversible complexing of TNBS with amino-acid residues is possible. However, in view of the stimulatory effect of the SH-specific PCMPS, a reversible reaction of TNBS with an SH group (Freedman & Radda, 1968; Arrotti & Garvin, 1972) is the more likely possibility.

The 5-dimethyl amino analog of amiloride was found to cause both reversible stimulation and reversible inhibition of Na transport. The inhibition is of low efficacy, the K^{mi} being about 1000-fold larger than that of amiloride. In view of the structural change at position 5 of the pyrazine ring, this analog should have a very short block time (high offrate)[4]. Its effect on the kinetics of amiloride blockage (decrease in the apparent onrate) supports this proposal and characterizes the analog as a high rate-blocking competitor of amiloride.

At concentrations below 0.8 mM the net effect of 5-dimethyl amiloride was stimulatory. Unfortunately, the paucity of analog material prevented further experimentation aimed at answering whether release from Na self-inhibition is involved in this effect. In one experiment K_A^{ma} decreased, implying an increase in K_N as expected. The question was studied in more detail with BIG which is structurally related to 5-dimethyl amiloride in that it has an amidino group at the end of the side-chain as well as (unlike amiloride) a hydrophobic function at the other end of the molecule.

Like 5-dimethyl amiloride, BIG evokes both reversible stimulation and reversible inhibition. As observed also with 5-dimethyl amiloride, the inhibitory effect causes a decrease in the apparent onrate of amiloride. This is expected of a high rate blocker competitive to amiloride. BIG does not possess the chlorine and amino group substituents at its ring structure which are responsible for the long block time of amiloride.[4] Therefore, its high chemical rate of blockage is likely to be due to a high offrate. The microscopic inhibition constant of BIG was estimated to be in the order of 2 mM, i.e. fourfold larger than that of 5-dimethyl amiloride and 7000-fold larger than that of amiloride.

The net stimulation caused by BIG at concentrations of 1 mM resembles that observed with PCMPS. The macroscopic inhibition constant of amiloride is clearly decreased, implying release of channels from Na self-inhibition, as previously suggested by Zeiske and Lindemann (1974), and Fuchs et al. (1977). In contrast, the apparent microscopic inhibition constant of amiloride is increased. The effect is caused by a decrease in the apparent onrate of amiloride which is ascribed to competitive blocking (*see above*). Since the amiloride offrate is not significantly changed, we assume that the true rate constants of the amiloride block are not affected by BIG. The Na channel current shows a small increase which clearly cannot account for the net stimulation. N_0 increased in each case and N remained constant or decreased. The fact that N_0 increased but N did not, is compatible with our conclusion that the stimulatory effect involves release from Na self-inhibition.

The decrease in N observed in most cases during stimulation with BIG might be caused by feedback inhibition from the cellular side of the apical membrane, evoked directly or indirectly by the increase in cellular Na in response to the stimulated Na uptake (e.g. Cuthbert & Shum, 1978; Chase & Al-Awqati, 1981). The decrease in N was less apparent with PCMPS (Table 2). It appears possible, therefore, that PCMPS but not BIG protects from feedback inhibition. Indeed, Bevevino and Lacaz-Vieira (1982) have recently shown that in the skin of *Bufo marinus ictericus* PCMB overcomes the feedback inhibition of Na-preloaded epithelia.

Competitive Blocking Mechanisms

At present it is not certain where amiloride exerts its blocking effect. Both a direct obstruction of the outer channel opening and binding to a site near the Na channel followed by a conformational change which closes the channel appear possible. Fuchs et al. (1977) suggested that the Na self-inhibition effect, which is characterized by a long time constant, may involve binding of extracellular Na ions to a 'modifier site' followed by channel isomerization and delayed closure. Lindemann and Voûte (1976) postulated that amiloride might bind to the same modifier site, but with higher affinity, acting like a 'super Na ion.' This mechanism explained the competition of Na and amiloride observed by Cuthbert and Shum (1974) and others in a straightforward way (isosteric competition).

[4] J.H.-Y. Li, E.J. Cragoe, Jr., and B. Lindemann. Structure activity relationship of amiloride analogues. *J. Membrane Biol.* (*to be submitted*)

The isosteric competition model lost some of its attractiveness when fluctuation analysis showed that the rate constants of the block by amiloride are much larger than those of the block by Na_o (Lindemann & Van Driessche, 1978). In view of these results direct channel obstruction by amiloride, as already suggested by Cuthbert (1976), is a reasonable alternative. In this case, where Na and amiloride bind to different sites, competition with the Na self-inhibition would involve a change of channel conformation which assures that the blocking by Na_o and amiloride is (at least to a large degree) mutually exclusive (allosteric competition). In the more simple variant of pure rather than mixed allosteric competition a channel blocked by amiloride would be conformationally open and a channel closed by Na_o through the modifier site mechanism could not, in addition, be blocked by amiloride.

To account for the stimulating effects of BIG, some amiloride analogs and amiloride itself, the isosteric competition model has to be expanded by postulating additional binding sites which, when occupied by these agents, prevent or modify the isomerization of the channel which leads to closure. For the allosteric competition model, stimulation was suggested to occur by screening: the stimulating agent interferes sterically with the access of Na ions to the modifier site (e.g. Lindemann, 1977, 1978). In a subsequent paper we shall discuss another possibility.[2] Based on a study of the blocking rate constants of structural analogs of amiloride we propose that these blockers first occupy a pre-block position at the channel opening before attaining the blocking position. During occupation of the pre-block position the channel may still conduct. If occupation of the pre-block position involves allosteric competition with the Na self-inhibition, then each amiloride or analog molecule would stimulate before blocking. The dwell times in the two positions would determine whether the net effect is stimulatory or inhibitory.

This model was conceived for amiloride analogs. Stimulators which are certain (PCMPS) or likely (TNBS) to react primarily with SH groups, and which do not show competitive effects with respect to amiloride, may well use an entirely different reaction mechanism.

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