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Mode of Action of Amiloride in Toad Urinary Bladder

An Electrophysiological Study of the Drug Action on Sodium Permeability of the Mucosal Border

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Summary. The effect of amiloride on the sensitivity to Na of the mucosal border of toad urinary bladder was investigated by recording Na concentration-dependent transepithelial potential difference (V_t) and the intracellular potential. When mucosal Na concentration was normal, amiloride added to the mucosal solution at 10^{-4} M markedly reduced the mucosal membrane potential (V_m) and altered the potential profile from a two-step type to a well type. Similar changes were observed when Na was totally eliminated from the mucosal medium. The serosal membrane potential was insensitive to amiloride and elimination of mucosal Na. In the absence of amiloride, the V_t could be described by the Goldman-Hodgkin-Katz equation in the range of mucosal Na concentration from 0 to 16 mM, and amiloride extended this concentration range. By using the Goldman-Hodgkin-Katz equation, Na permeability was calculated from the data of V_t's obtained in the allowed ranges of Na concentration and compared before and after the addition of amiloride. The results show that Na permeability decreases to 1/600 of control when the maximum dose of amiloride (10^{-4} M) is applied. The relationship between Na permeability and amiloride concentration is well explained on the basis of assumptions that amiloride binds to the Na site of the mucosal border in one-to-one fashion and in a competitive manner with Na and that Na permeability reduces in proportion to increase in number of the sites bound with amiloride.

Amiloride (3,5-diamino-6-chloropyrazinoylguanidine) is known to have a moderate natriuretic effect [1, 16]. Studies in toad urinary bladder, frog skin and frog colon have shown that this drug profoundly inhibits transepithelial transport of Na (and Li) without affecting permeability to water, nonelectrolytes and hydrogen ion [2, 10, 15, 17]. The drug is effective only when added to the mucosal (or the outer) medium, and the time required for the onset of its action is very short. The drug has, therefore, been thought to inhibit Na entry from the mucosal medium into the

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epithelial cells. In fact, both influx of Na into the tissue and the transport pool of Na within the cells are considerably reduced by this drug [3, 8, 12, 23, 25, 26, 30, 32].

Despite such a distinct action of amiloride, the exact mechanism of its action is still obscure. Bentley [2], Cuthbert and Shum [9], and Zeiske and Lindemann [36] have attempted to obtain the stoichiometrical relationship between the dose of amiloride and transepithelial Na movement as measured by short-circuit current. However, no consistent results have been obtained in their studies.

The purpose of the present study is to gain further information about the mode of action of amiloride in the toad bladder. By using the Goldman-Hodgkin-Katz equation, Na permeability (P_{Na}) of the mucosal border was estimated by changing Na concentration of the mucosal medium and measuring the transepithelial potential difference (V_t). Microelectrode studies were also performed to determine the exact relationship of changes in V_t to those in the mucosal transmembrane potential (V_m). A simple stoichiometrical relationship was found between the dose of amiloride and P_{Na} of the mucosal border. Based on this finding, the mode of amiloride action was analyzed.

Materials and Methods

The urinary bladders were dissected from toads (*Bufo vulgaris*). After mounting the bladder between specially designed lucite chambers, the mucosal and serosal sides were perfused separately with the standard Ringer's solution, which had the following composition (in mM): NaCl 110, KCl 2, $CaCl_2$ 1, Tris-Cl 4, and glucose 5.5, pH 7.8. After the stabilization of V_t, Na concentration of the mucosal perfusion medium, $[Na]_m$, was changed by switching the perfusion fluid to a test solution of a desired Na concentration. The test solutions were prepared by mixing the standard solution with Tris-Cl-substituted Ringer's solution at desired volume ratios. The serosal side was usually perfused with the standard solution throughout the experiments. In some experiments, SO_4-substituted media were tested. Both Na_2SO_4-Ringer's and $(Tris)_2SO_4$-Ringer's solutions were prepared by replacing Cl totally with SO_4 and adding 80 mM mannitol. Amiloride was added only to the mucosal medium.

The chambers used in the present study are illustrated in Fig. 1. The preparation was clamped between the upper (the mucosal) and the lower (the serosal) half-chambers. The area of the window was 0.8 cm^2. The volumes of the mucosal and the serosal chambers were 1.0 and 2.2 ml, respectively. A special care was taken for protecting the preparations from the "edge damage" due to an excess force of clamping. After placing a ring-form cushion made of parafilm at the margin of the window, the preparation was clamped with a moderate force between the chambers, then recording of the V_t was started with the standard solution on both sides. The V_t gradually increased to reach a maximum steady level as four screws for the fixation of the chambers were gently turned. Further screwing with a strong force usually resulted in an abrupt decrease in V_t. Application of such an excess force was avoided.

The set of chambers was constructed with the following devices: 1) the exchange of the mucosal medium could be completed within a minute, and 2) a hydrostatic pressure difference

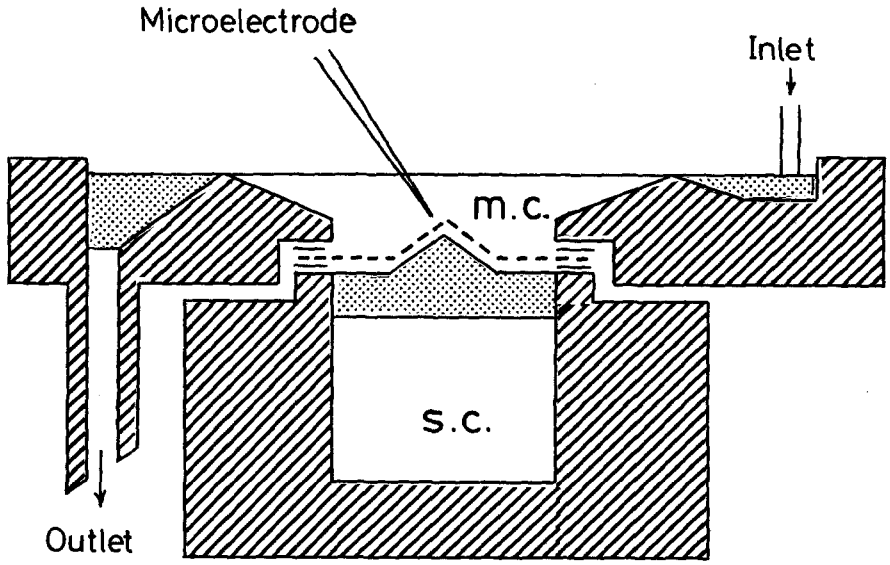

Fig. 1. A cross-sectional view of the chambers used in the present study. A bladder membrane (the broken line) was clamped between the mucosal half-chamber (*m.c.*) and the serosal half-chamber (*s.c.*). Dotted areas at the inlet and outlet of the *m.c.* are pieces of glass filter. The dotted area at the top of the *s.c.* is also glass filter which was used only in the microelectrode study. The inlet and outlet of the *s.c.* are located in the plane perpendicular to this figure (not shown in this figure)

across the bladder was nullified automatically. The rate of perfusion was set at 0.2 ml/sec on both sides. During the exchange of the mucosal medium, the rate of the mucosal perfusion was increased to 0.6 ml/sec for 5 min. Measurements of the time course of washout of lissamine green from the mucosal chamber revealed that the half-time of exchange was about 3 sec when the perfusion rate was 0.6 ml/sec.

For the microelectrode study, a glass filter of inverted V-shape was attached to the window of the serosal chamber, and the bladder was mounted on this filter (this filter was usually omitted in the experiments of V_t measurement only). The displacement of the epithelium due to contraction of smooth muscles was minimum at the top of the protrusion, where the cellular impalement with a glass microelectrode was carried out in the direction from the mucosal toward the serosal side. In some experiments, the V_m was recorded continuously from a single cell throughout several different experimental conditions. In such a case, the perfusion rate was reduced to about 0.01 ml/min. In other experiments, shortly recorded V_m's from many cells were compared before and after the change in conditions of the mucosal medium. In such a case, the perfusion was interrupted briefly during the recording of V_m for about 3 min. Ling-Gerard type glass microelectrodes filled with 3 M KCl were used. The electrical resistance of the electrodes ranged from 50 to 100 MΩ. The V_t was led out by use of a pair of 3 M KCl-agar bridges. Both V_t and V_m were recorded with a two-channel high-sensitivity DC-recorder (TOA, EPR-3T). The transepithelial resistance and the ratio of the mucosal to serosal membrane resistances (r_m/r_s) were measured by applying pulses of a DC current (1 to 16 μA, duration 0.6 sec) across the tissue through a pair of Ag-AgCl electrodes. Criteria for the correct intracellular location of the microelectrode tip were the same as those used by Civan and Frazier [7] and Reuss and Finn [27].

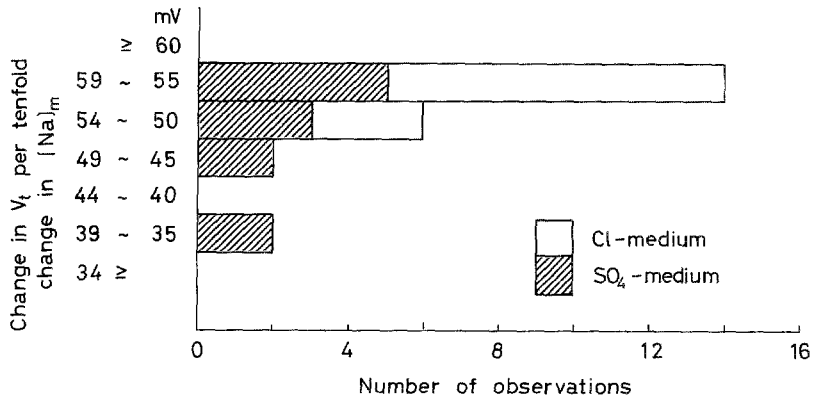

Fig. 2. Frequency distribution of the change in V_t per tenfold change in $[Na]_m$ in hibernating toads. The values presented here were determined at low Na concentrations around 4 mM

The quantitative analyses of amiloride effects necessitated the use of samples having uniform electrical properties. In summer, the Na sensitivity of the urinary bladder markedly varied from toad to toad. In contrast, the bladder of hibernating toads showed a relatively uniform and stable Na sensitivity. In all hibernating toads tested, the values of change in V_t per tenfold change in $[Na]_m$ (from 1 to 10 mM) ranged from 50 to 59 mV (Fig. 2). Replacement of Cl by SO_4 resulted in a reduction and a greater scattering of this value. Moreover, SO_4 substitution caused a rapid decrease in the Na sensitivity, while in Cl medium it remained constant for about 4 hr. Therefore, hibernating toads and Cl media were used in the subsequent experiments. The reason for the unfavorable effects of SO_4 is unknown, though Singer and Civan [33] showed that V_t and short-circuit current were more stable when an anion ranked at a higher position in the lyotropic series was employed as the principal anion.

Amiloride was kindly supplied by Merk Sharp and Dohme.

Results

Dependence of Amiloride Effect on the Presence of Na in the Mucosal Medium

As shown in Table 1, the transepithelial potential difference (V_t) and electrical conductance (g) of the urinary bladder were considerably greater in the presence of Na in the mucosal medium than in the absence of Na in the same medium. Amiloride added to the mucosal solution markedly decreased both V_t and g only when Na was present in the mucosal medium. Such a Na^+-dependent depressing effect of the drug was dose-dependent, and the effect reached a maximum at about 10^{-4} M (Fig. 3).

Table 1. Effects of amiloride on the transepithelial potential difference (V_t) and the transmural electrical conductance (g)

Mucosal medium	No. of observations	V_t (mV)		g (μmho/cm^2)	
		Control	Amiloride, 10^{-4} M	Control	Amiloride, 10^{-4} M
Standard sol.	23	89±8	−12±5	1045±227	224±20
Tris-Cl Ringer's sol.	25	−16±4	−16±4	154±9	153±9

The serosal surface was perfused with the standard solution throughout the experiments. Amiloride was added to the mucosal medium. The V_t is expressed as positive when the serosal side is positive with respect to the mucosal side. Each value is mean±SE.

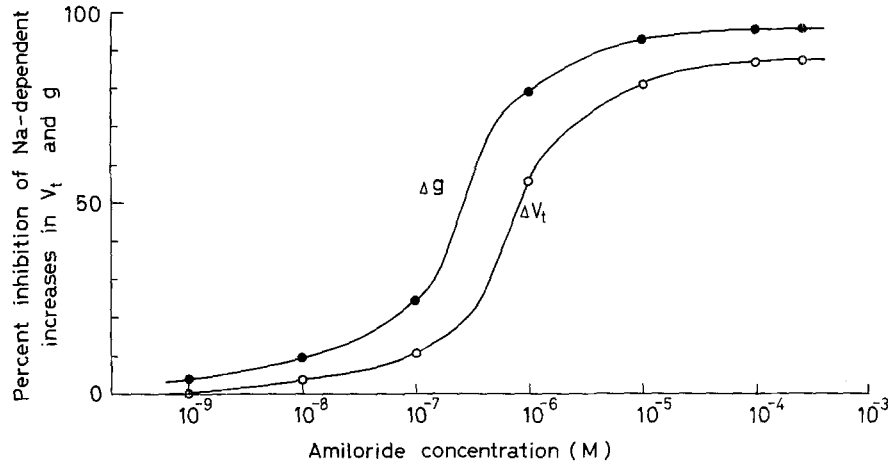

Fig. 3. Relations of amiloride-induced reductions in V_t (ΔV_t) and tissue electrical conductance (Δg) to the concentration of amiloride in the mucosal medium. ΔV_t and Δg are the differences in V_t and g before and after the replacement of Na-free mucosal medium (Tris-Ringer's solution) by the standard solution. The data are expressed as percent inhibition, and each value is the mean of data of five experiments

Effect of Amiloride on the Electrical Potential Profile

The V_t of the toad bladder has been shown to be a sum of the mucosal and the serosal transmembrane potentials (V_m and V_s) [13]. When a microelectrode was advanced from the mucosal toward the serosal side, two positive potential steps were recorded in most preparations bathed with normal Ringer's solution. The same type of potential profile has been observed by Frazier [13], Civan and Frazier [7] and Reuss and Finn

Table 2. Effects of 10^{-4} M amiloride on the transmembrane potentials and the membrane resistances of the epithelial cells

Mucosal medium	No. of observations	V_t (mV)	V_m (mV)	V_s (mV)	r_m/r_s
Standard sol.	41	79.1±3.4	45.4±2.8	34.3±2.6	2.0±0.1
Standard sol. containing amiloride (10^{-4} M)	32	−4.1±0.5	−31.7±1.9	27.6±2.0	4.5±0.2

Each value is mean±SE.

[27, 28]. The value of V_t obtained in the present study was 79.1±3.4 mV (mean ± SE, serosal side positive). The average values of the first step (V_m) and the second step (V_s) were 45.4±2.8 and 34.3±2.6 mV, respectively (Table 2). The addition of amiloride to the mucosal medium markedly altered the potential profile. Fig. 4 shows a typical record of the potential profile in the presence of 10^{-4} M amiloride. Upon penetration of the

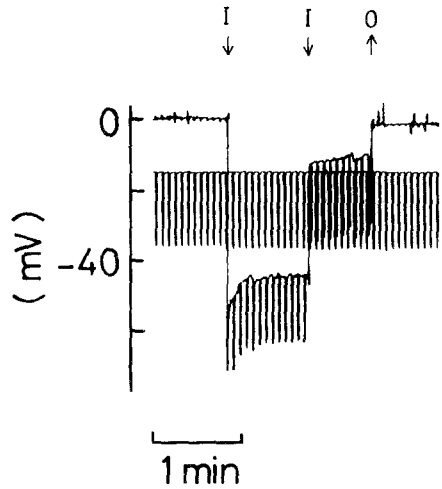

Fig. 4. A typical record of the potential profile of the bladder epithelium in the presence of 10^{-4} M amiloride on the mucosal side. Both the mucosal and serosal sides were bathed with the standard solution. The unchanged p.d. level at around −18 mV is the V_t recorded by means of 3 M KCl-agar bridges. The microelectrode was advanced from the mucosal solution toward the serosal side. Upon penetration of the electrode tip across the mucosal border (the first arrow *I*) the p.d. abruptly shifted to a negative value by about −45 mV, and then the p.d. spontaneously decreased slightly before attaining a steady level. Further advancement resulted in the second shift of the p.d. (the second arrow *I*) to a level which is nearly the same as that of V_t. At the arrow *O*, the electrode was withdrawn toward the mucosal solution. Vertical bars superposed on both p.d. tracings are *IR* drops caused by injected current pulses across the tissue

electrode tip through the mucosal border, the potential difference (p.d.) abruptly shifted from 0 to −44 mV (cell inside negative). Further advancement of the tip resulted in the second shift of the p.d. to reach the same level as that of V_t, showing a well-type potential profile. The average values of V_t, V_m, V_s and r_m/r_s in the presence of 10^{-4} M amiloride are shown in Table 2. Although amiloride caused a slight but statistically significant decrease in V_s, 92% of the change in V_t could be attributed to the change in V_m. The value of r_m/r_s increased from the control value of 2.0 to 4.5. These results indicate that the site of action of amiloride is the mucosal border.

In the absence of Na^+ in the mucosal medium, the average values of V_m, V_s and r_m/r_s were -27.5 ± 3.1 mV, 25.8 ± 2.6 mV and 4.6 ± 0.5, respectively, and the potential profile was well-type. The results show that about 90% of the Na-dependent increase in V_t can be attributed to the increase in V_m. In the absence of Na, both V_m and V_s remained unchanged before and after the application of 10^{-4} M amiloride.

Effect of Amiloride on Na Permeability

The change in V_t caused by the alteration of Na concentration of the mucosal medium was observed in the presence of amiloride at various concentrations. The Na concentration was elevated from 0 to 1, 4, 16, 64 and 110 mM, successively. The results obtained are summarized in Fig. 5, where the changes in V_t (ΔV_t's) are plotted against logarithm of $[Na]_m$. In the absence of amiloride (line *a*), the V_t increased linearly as Na concentration was increased from 0 to 16 mM. The slope of the linear segment of line *a* was 56.1 ± 1.2 mV per tenfold change in Na concentration. Amiloride reduced V_t at any Na concentration dose-dependently (lines *b* through *g*). However, about the same maximum slope was obtained when Na concentration was elevated. The higher the amiloride concentration, the higher the Na concentrations needed to obtain the maximum slope. Control curve (line *a*) showed a tendency toward saturation above Na concentration of 64 mM. Such a tendency disappeared when the drug was added at more than 10^{-7} M.

Permeability coefficient for Na (P_{Na}) of the mucosal border was estimated by using the Goldman-Hodgkin-Katz equation and the data presented in Fig. 5. Two exponential equations were derived in order to describe the V_m's under two different conditions. One is the V_m in the absence of Na (Tris substitution) in the mucosal medium and the other

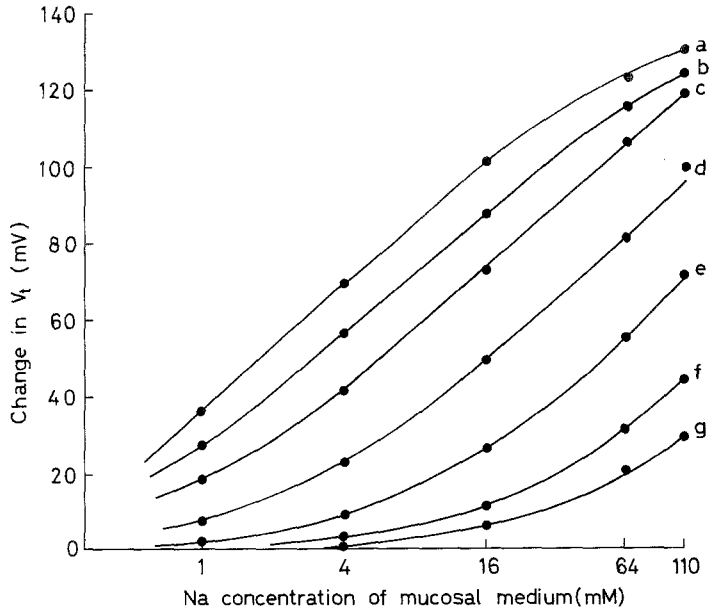

Fig. 5. The effect of amiloride on the relationship between V_t and $[Na]_m$. The increases in V_t (ΔV_t's) due to a successive increase in $[Na]_m$ from 0 to 1, 4, 16, 64 and 100 mM were plotted. Line *a* is control ($n=5$). Line *b* through line *g* are in the presence of amiloride at various concentrations: *b)* 5×10^{-8} M ($n=3$); *c)* 10^{-7} M ($n=5$); *d)* 5×10^{-7} M ($n=3$); *e)* 10^{-6} M ($n=4$); *f)* 5×10^{-6} M ($n=2$); *g)* 10^{-5} M ($n=4$)

for the V_m after a rapid replacement of the mucosal Na-free solution (Tris-Ringer's) with a test solution containing Na at a certain concentration. The former is denoted as V_m', and the latter as V_m''. V_m' and V_m'' can be described as

$$\exp\left(\frac{F\cdot V_m'}{RT}\right)=\frac{P_{\text{Tris}}\cdot 110+P_{\text{Cl}}[\text{Cl}]_c}{\alpha+P_{\text{Cl}}[\text{Cl}]_m} \tag{1}$$

and

$$\exp\left(\frac{F\cdot V_m''}{RT}\right)=\frac{P_{\text{Na}}[\text{Na}]_m+P_{\text{Tris}}(110-[\text{Na}]_m)+P_{\text{Cl}}[\text{Cl}]_c}{\alpha+P_{\text{Cl}}[\text{Cl}]_m} \tag{2}$$

where F is the Faraday constant, R the gas constant, T the absolute temperature, and P the permeability constant of the mucosal border to ion species denoted by the subscript. $[Na]_m$ is the Na concentration of the test solution and $[Cl]_c$ is the Cl concentration of the intracellular fluid. α is the sum of $P_{Na}[Na]_c$ and $P_{Tris}[Tris]_c$. Dividing Eq. (2) by Eq. (1), one can get the following equation describing the relationship between P_{Na}

and the changes in V_m (ΔV_m) or V_t (ΔV_t) caused by an increase in $[Na]_m$:

$$\exp\left(\frac{F}{RT}\Delta V_t\right)=\exp\left(\frac{F}{RT}\Delta V_m\right)=q_{Na}[Na]_m+1 \tag{3}$$

where

$$q_{Na}=\frac{P_{Na}-P_{Tris}}{P_{Tris}\cdot 110+P_{Cl}[Cl]_c}. \tag{4}$$

Fig. 6 shows an actually observed relationship between $\exp(F\cdot\Delta V_t/RT)$ and $[Na]_m$ in the absence and the presence of amiloride at various concentrations. Calculated values of q_{Na} are given in Table 3. Except for control (line *a*) and in the presence of 5×10^{-8} M amiloride (line *b*), all other lines were linear over the range of Na concentration examined. In control and in the presence of 5×10^{-8} M amiloride, the linearity of q_{Na} was observed below 16 mM $[Na]_m$. Above this, q_{Na} tended to decrease. A similar tendency toward saturation has been shown by previous authors [19, 21, 22] in the Na concentration-dependent increase in short-circuit current. In the following treatments, we used the values of q_{Na} estimated from the linear segments.

As clearly seen in Fig. 6, amiloride reduced the value of q_{Na} dose-dependently. The decrease in q_{Na} can be ascribed solely to the decrease in P_{Na}, since amiloride had no effect on V_t and V_m when $[Na]_m$ was zero. The mean value of q_{Na} in the presence of the maximum dose of amiloride (10^{-4} M) was 5.7 ± 0.4 M^{-1} ($n=9$), the value being about 1/600 of control value (3313 ± 230 M^{-1}, $n=5$).

Quantitative Relationship Between q_{Na} and Amiloride Concentration

A further analysis was made of the quantitative relationship between q_{Na} and amiloride concentration based on the following theoretical considerations. Amiloride applied in the mucosal medium was assumed to bind to some special site of the mucosal border, and P_{Na} of the mucosal border decreases in proportion to the increase in the number of sites bound with amiloride. The equilibrium of the binding reaction between amiloride (A) and the binding site (R) can be described as

$$AR \rightleftharpoons A+R, \qquad k_1=[AR]/[R]\,[A]_m \tag{5}$$

where $[R]$ is the number of the free sites, $[AR]$ the number of the sites forming the complex with amiloride (moles/cm^2), $[A]_m$ amiloride con-

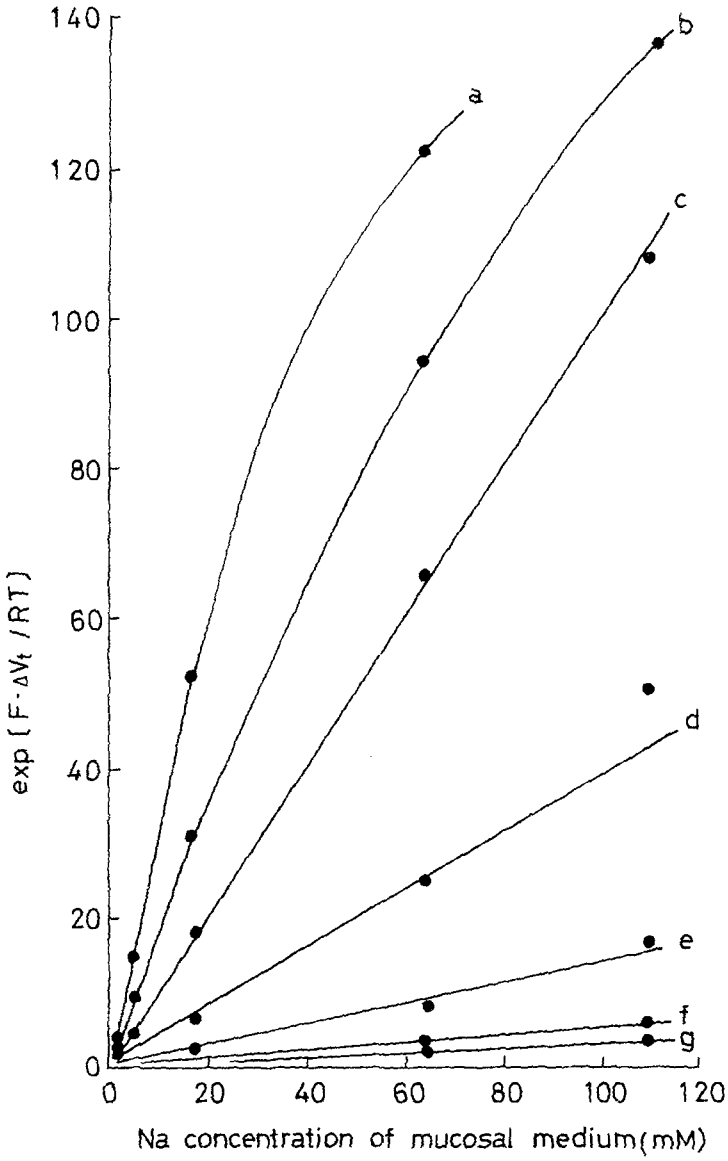

Fig. 6. Relationship between $\exp(F \cdot \Delta V_t/RT)$ and $[Na]_m$. For explanation *see text*. Line *a* is control and lines *b* through *g* are in the presence of amiloride (the drug concentrations are the same as in Fig. 5)

centration in the mucosal medium (moles/liter), and k_1 the association constant (moles/liter)$^{-1}$. Total number of sites r is given by

$$r = [R] + [AR]. \tag{6}$$

Table 3. Dependence of the value of q_{Na} on the mucosal Na concentration and the dose of amiloride

Concentration of amiloride (M)	No. of observations	q_{Na} (M^{-1})					
		Mucosal Na concentration					
		1 mM	4 mM	16 mM	64 mM	110 mM	Mean
0 (control)	5	3246±274	3383±209	3311±238	1919±190	1309±144	3313[a]
5×10^{-8}	3	1957	2087	1864	1461	1228	1969[a]
10^{-7}	5	1075±158	1131±127	1098±166	1011±137	979±112	1058
5×10^{-7}	3	320	349	378	354	450	310
10^{-6}	4		116± 28	126± 17	124± 13	131± 13	124
5×10^{-6}	2			34	38	41	37
10^{-5}	4			20± 3	19± 4	22± 4	20

The data are given by mean values of two to five experiments. Standard error of the mean was given when more than four observations were made.

[a] Mean values for control and 5×10^{-8} M amiloride were calculated from values for 1, 4 and 16 mM Na.

Based on the assumption described above, we can formulate the relation of P_{Na} to $[AR]$ under the effect of amiloride as follows

$$P_{Na}=(P_{Na})_o\left(1-\frac{[AR]}{r}\right)+P'_{Na} \tag{7}$$

where $(P_{Na})_o$ is the amiloride-sensitive component of P_{Na} under control conditions (without amiloride), and P'_{Na} is the residual permeability to Na under the maximum effect of amiloride (amiloride-insensitive component of P_{Na}). Eq. (5) can be rewritten as

$$k_1=\frac{[AR]}{(r-[AR])\cdot[A]_m}. \tag{8}$$

In the present study, we estimated the values of q_{Na} instead of P_{Na}. When we denote the q_{Na}'s under control conditions and under the maximum effect of amiloride as $(q_{Na})_o$ and $(q_{Na})_\infty$, respectively, Eq. (8) can be rewritten as

$$k_1=\frac{(q_{Na})_o-q_{Na}}{q_{Na}-(q_{Na})_\infty}\cdot\frac{1}{[A]_m}. \tag{9}$$

Eq. (9) can be rewritten as

$$\log\frac{(q_{Na})_o-q_{Na}}{q_{Na}-(q_{Na})_\infty}=\log[A]_m+\log k_1. \tag{10}$$

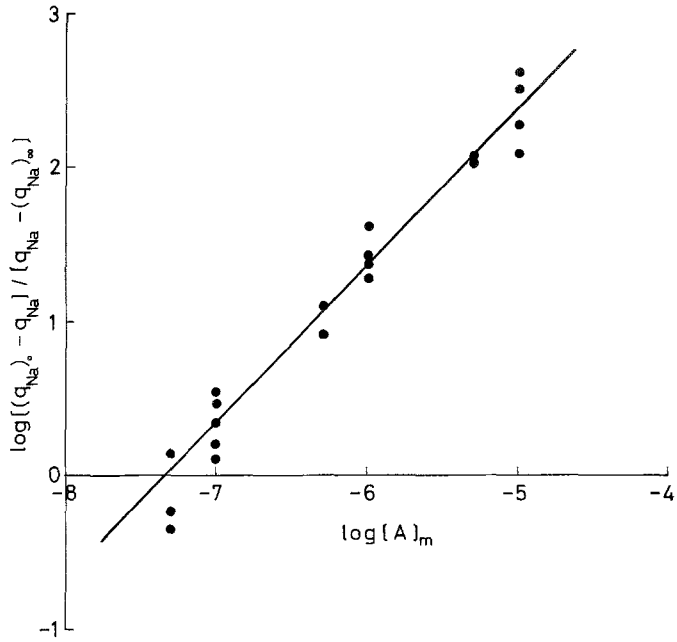

Fig. 7. Relationship between $\log[(q_{Na})_o - q_{Na}]/[q_{Na} - (q_{Na})_\infty]$ and $\log[A]_m$. For explanation *see text*

Eq. (10) means that the value of $\log[(q_{Na})_o - q_{Na}]/[q_{Na} - (q_{Na})_\infty]$ is directly proportional to $\log[A]_m$, and that the value of k_1 is equal to that of $[A]_m$ at which $(q_{Na})_o - q_{Na} = q_{Na} - (q_{Na})_\infty$. Fig. 7 shows the relationship between $\log[(q_{Na})_o - q_{Na}]/[q_{Na} - (q_{Na})_\infty]$ and $\log[A]_m$ obtained from the data shown in Table 3. As predicted from Eq. (10), a linear relationship was obtained, indicating that the assumptions made in the present analysis are valid. The regression line obtained from least-squares analysis was $Y = 1.04X + 7.62$ ($r = 0.92$). Estimated value of k_1 was 4.13×10^7 (moles/liter)$^{-1}$.

Discussion

The results of the present study show that amiloride markedly decreases the V_m and increases the ratio of the mucosal to serosal membrane resistances (r_m/r_s). These findings are in agreement with a view that amiloride inhibits Na permeation across the mucosal cell membrane [5, 12, 25]. Furthermore, the present theoretical treatment revealed that inhibitory action of amiloride on P_{Na} could be interpreted on the basis of

assumptions that amiloride binds to the Na-binding site of the mucosal membrane in one-to-one fashion and the amiloride-sensitive component of P_{Na} decreases in proportion to the increase in the number of sites bound with amiloride.

Before further discussing the nature of amiloride action, we must recall special features of Na permeation across the mucosal border. This process has been postulated, on the basis of the following facts, to involve an interaction of Na with some special sites of the membrane. (1) The process has a highly specialized selectivity to Na and Li [19, 21, 22]; (2) The activation energy for the Na permeation is 16.5 Kcal/mole, this being extremely greater than that predicted from simple diffusion [14]; and (3) Na penetration is competitively inhibited by Li and K [5, 31].

As to the nature of inhibitory action of the drug, either of the following mechanisms can be speculated, i.e. some conformational changes of the site and the competition between Na and amiloride for the common site. In regard to the mode of action it seems of interest that amiloride eliminates the saturable tendency of the Na-dependent increase in V_t and extends the range of Na concentration in which the V_t can be described by the Goldman-Hodgkin-Katz equation. Such effects can be explained on the basis of the competitive nature of action as described below.

Amiloride has a guanidine group in its molecule, therefore it behaves as a strong base. This substance, therefore, may compete with Na for a negatively charged common site. If this is the case, Eq. (6) should be rewritten as

$$r=[R]+[\mathrm{Na}R]+[AR]. \tag{11}$$

The association constant of the binding between the site and Na, k_2, is given by

$$k_2=[\mathrm{Na}R]/[\mathrm{Na}]_m\cdot[R]. \tag{12}$$

From Eqs. (5), (11) and (12), the following equation can be derived

$$[\mathrm{Na}R]=\frac{r\,k_2[\mathrm{Na}]_m}{1+k_2[\mathrm{Na}]_m+k_1[A]_m}. \tag{13}$$

On the other hand, P_{Na} can be defined as a coefficient proportional to medium-membrane distribution (partition) coefficient (β) for Na. Namely,

$$P_{Na}\propto\beta=[\mathrm{Na}R]/[\mathrm{Na}]_m. \tag{14}$$

As the amiloride-insensitive component of P_{Na} is negligibly small (about 1/600 of control P_{Na}) in the present preparations, we can derive the follow-

ing equation from Eqs. (13) and (14):

$$P_{Na} \propto \frac{[NaR]}{[Na]_m} \simeq \frac{r k_2}{1 + k_2 [Na]_m + k_1 [A]_m}. \quad (15)$$

Eq. (15) means that, in the presence of amiloride, P_{Na} is determined by both $[Na]_m$ and $[A]_m$ and that P_{Na} approaches a constant value determined by only $[A]_m$ when $[Na]_m$ is lowered. It is obvious that the higher the $[A]_m$, the higher the $[Na]_m$ needed to obtain a detectable decrease in P_{Na} from such constant value. This is in agreement with the obtained results (Fig. 6).

It is possible to calculate the $[Na]_m$ which causes an appreciable reduction, e.g. 10% reduction, of P_{Na} from the constant value if the values of k_1 and k_2 are known. From Eq. (15), such $[Na]_m$ is given by the following equation:

$$[Na]_m = (1 + k_1 [A]_m)/9 k_2. \quad (16)$$

The value of k_1 can be calculated by using Eq. (10) and the data presented in Fig. 7. The obtained value was 4.1×10^7 M^{-1}. Eq. (10) was derived to describe the binding reaction between the drug and the binding site without assuming the competition. However, the same k_1 can be used in the treatment where a competitive mechanism is assumed, since the values of q_{Na} used in the calculation of k_1 were obtained at very low concentrations of Na. On the other hand, the value of k_2 cannot be estimated directly. Nevertheless, the relationship between $\exp[F \cdot \Delta V_t/RT]$ and $[Na]_m$ presented in Fig. 6 indicates that an appreciable decrease in q_{Na} is seen when $[Na]_m$ is increased from 16 to 32 mM (lines *a* and *b*). Therefore, k_2 is considered to take a value within a range from 3.5 to 6.9 M^{-1}. It is also possible to calculate the lowest $[A]_m$ which causes an appreciable (10%) reduction of P_{Na} at normal $[Na]_m$ (110 mM). The estimated value ranged from 6.8×10^{-8} to 1.4×10^{-7} M, the range being in good agreement with the observed range from 5×10^{-8} to 1×10^{-7} M. Thus, the present results are well explained on the basis of the competitive nature of action. A further increase in the Na concentration above 110 mM was also tested in the presence of amiloride at a sufficiently high concentration. But the tests were unsuccessful because of alterations in electrical properties of the preparations due to the hypertonicity of the mucosal medium [34].

Guanidine has a relatively high affinity for the sites which have a high electric field strength, hence a higher affinity for Na (and Li) than for other alkali cations [11]. In a glass membrane of the Na electrode and the membranes of nerve axons, this compound mimics Na in regard to the

cation sensitivity [11, 18, 20, 35]. In toad bladder and frog skin, guanidine and some guanidine-related compounds, such as tetrodotoxin, benzoylthiazole-2-guanidine and benzoylimidazole-2-guanidine, inhibit transepithelial Na transport [2, 24, 36]. These facts seem to support the competitive nature of amiloride action.

In the present theoretical treatment, we assumed that the intracellular ionic composition did not change significantly during recording of changes in V_t caused by the perturbation in the mucosal compartment. We also assumed that all changes in V_t were solely due to the changes in V_m of the same nature. The device for rapid exchange introduced in the present study enabled us to change the mucosal solution with a half-time of 3 sec, and potential reading was made 1 to 1.5 min after switching of the perfusion fluid, when the V_t reached a maximum level. Thus, the possible effect of change in intracellular ionic composition was minimized as much as possible in this study. The saturable tendency of change in V_t at a higher range of Na concentration was unaffected by replacement of Cl with SO_4, suggesting that this tendency is due to a mechanism other than the change in the ionic composition within the cells.

In regard to the second assumption, the results of the microelectrode study seem to support such a simplification. However, small changes in V_s were always observed under the present experimental conditions as observed by previous investigators. Previously reported ratios of $\Delta V_s/\Delta V_m$ caused by changing $[Na]_m$ are considerably variable. For frog skin, Biber *et al.* [4] reported a negligibly small value, and Cereijido and Curran [6] a value of less than 0.1. On the other hand, the reported values for toad bladder are generally larger than those for frog skin, e.g. 0.17 [27] or about 0.5 [28]. As to the amiloride-induced changes in V_m and V_s, Reuss and Finn [29] reported a value of about 0.3. These values for toad bladder are considerably larger than that obtained in the present study (0.09). Furthermore, they showed a potential profile of two-step type even after the full development of the maximum effect of amiloride, while in our study only a well-type profile was observed under the effect of amiloride or Na-free conditions on the mucosal side.

There may be several possible mechanisms underlying the concomitant changes in V_s as discussed by Reuss and Finn [29], e.g. participation of an electrogenic Na pump at the serosal border or the microtubular system. However, we consider that some part of the change in V_s is the result of a technical fault; that is, cell damage caused by impalement with a microelectrode. Our preliminary experiments revealed that continuous recording with a microelectrode inserted into a single cell always yielded a higher

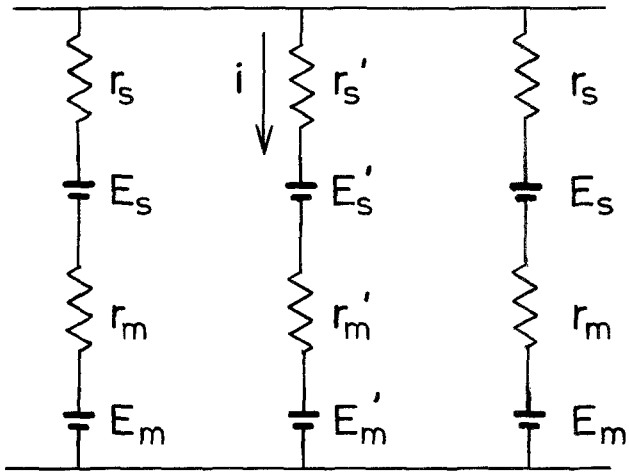

Fig. 8. An equivalent circuit of the toad bladder epithelium with a damaged cell due to microelectrode insertion. r_m and r_s are the membrane resistances of the mucosal and serosal borders, and E_m and E_s are the electromotive forces at the respective membranes. These electrical parameters of a damaged cell are denoted by r' and E'. The difference between E_m+E_s and $E'_m+E'_s$ generates a current flow (i). A possible explanation for the concomitant increase in V_s is as follows. Because of a decrease in Na sensitivity of the mucosal border of the damaged cell, E'_m may be less than E_m when Na is present in the mucosal medium; therefore, the current (i) flows through r'_s and r'_m from the serosal to mucosal side. Amiloride added to the mucosal medium may cause a more profound decrease in E_m of intact cells than E'_m and a greater increase in r_m than r'_m of the damaged cell. Consequently, preexisting current i may decrease. This results in a decrease in V_s, since V_s recorded from an impaled cell is dependent on both E'_s and $i \cdot r'_s$

value of amiloride-induced $\Delta V_s/\Delta V_m$ (0.27 on the average) as compared with those obtained by short insertions (for less than 3 min) of a microelectrode into different cells (0.09 on the average). Furthermore, the former type of experiments gave a smaller amiloride-induced increase in r_m/r_s than the latter. These suggest that leaving the microelectrode tip inside the cell for a long period of time causes some damage of the impaled cell. A possible cause of an increase in $\Delta V_s/\Delta V_m$ recorded from a damaged cell is explained in Fig. 8 (*see* legend for this figure). The greater values of $\Delta V_s/\Delta V_m$ for toad bladder as compared with that for frog skin may be due to higher vulnerability of the bladders to the microelectrode insertion because of movements of the tissue due to contractions of smooth muscles of the submucosal layer.

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