MOLECULAR PHARMACOLOGY, 20:543–550

Kinetic Analysis of the Amiloride-Sodium Entry Site Interaction in Rabbit Colon

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Received October 6, 1981; Accepted June 16, 1981

SUMMARY

The diuretic amiloride inhibits apical sodium entry in isolated epithelia of rabbit decending colon primarily by decreasing the maximal sodium transport capacity of the tissue. Since in addition the apparent affinity of sodium to the transport system is also reduced, the type of inhibition exerted by amiloride has to be termed mixed. Increasing sodium concentrations cause a rightward shift of the amiloride concentration-response relationship. Elevation of cellular sodium by ouabain inhibits the action of amiloride noncompetitively. The concentration-response relationships of amiloride in the presence of various sodium concentrations yield Hill coefficients between 0.93 and 1.03, indicating a stoichiometry of the amiloride-sodium entry site interaction of 1:1. It is concluded that the sodium translocation site and the amiloride binding site are separate and that extracellular sodium decreases the apparent affinity of amiloride to its binding site, whereas increased intracellular sodium depresses the maximal amiloride response.

INTRODUCTION

Amiloride, a pyrazine-guanidine diuretic (1), has proved to be a very valuable tool for the investigation of the sodium transport mechanism in tight epithelia such as frog skin, toad bladder, and colon of various species. The fact that this agent is effective only when present on the outer, or luminal, side of the tissue and the rapid onset and reversibility of action suggest that amiloride inhibits apical sodium entry into the sodium-transporting cells by binding to superficial bindings sites (2–5). The mechanism of the inhibitory effect of amiloride on apical sodium entry, which is a saturable electrodiffusion process (6–8), does not involve a reduction in the driving forces for influx such as chemical and electrical gradients; rather, these gradients are increased in the presence of amiloride: the apical cell membrane is hyperpolarized [the cell interior becomes more negative (7, 9)] and the cellular sodium pool derived from the outer solution is reduced (8, 10, 11). In addition, amiloride was shown not to affect the ouabain-sensitive membrane (Na^+-K^+)-ATPase (1, 10, 11) but to inhibit sodium influx by decreasing the sodium conductance of the apical barrier (6–9, 12).

Although amiloride has been widely used for many years, there is still great uncertainty concerning the type of inhibition exerted by amiloride in kinetic terms. The amiloride action on sodium transport has been reported in various tissues to be competitive (13, 14), noncompetitive (2, 15), and mixed-type (15). The present study was conducted to characterize the macroscopic kinetics of the blocking action of amiloride on apical sodium entry in rabbit descending colon, which resembles the distal nephron inasmuch as the transepithelial electrical potential difference is dependent on the luminal sodium concentration and is increased by basolateral addition of aldosterone, whereas it is blocked by amiloride in the luminal solution or ouabain in the basolateral solution. Therefore, the rabbit descending colon may be considered to be a useful and readily accessible model system for the distal nephron, which was shown to be site of the diuretic action of amiloride (1, 16).

The experiments were designed in an attempt to answer the following questions: (a) How can the type of inhibition exerted by amiloride on sodium entry be classified? (b) Does sodium influence the amiloride effect? (c) What is the stoichiometry of the amiloride-sodium entry site interaction? (d) How many classes of binding sites mediate the amiloride effect? The results obtained from cumulative concentration-response studies of amiloride in the presence of various sodium concentrations provide evidence that in rabbit descending colon amiloride both depresses the maximal transport capacity for sodium and decreases the affinity of sodium for its transport system and that sodium has an inhibitory effect on the action of amiloride. The stoichiometry of the amiloride-sodium entry site interaction appears to be 1:1 with only one class of amiloride binding sites.

MATERIALS AND METHODS

Experimental procedure. White rabbits of either sex, weighing between 2 and 3 kg, were killed by an i.v. injection of pentobarbital. "Partial mucosal strip" prep-

This study was supported by Research Grant 3688 from the Fond zur Förderung der wissenschaftlichen Forschung in Österreich (Austrian Research Council).

0026-895X/81/030543-08 $02.00/0$02.00/0

arations of descending colon were obtained by removing the outer muscle layers by blunt dissection and mounted in a short-circuit apparatus as described by Frizzell and co-workers (5). Briefly stated, 1.27 cm^2 of tissue (serosal surface) was held between two halves of a Plexiglas chamber and exposed on each surface to 10 ml of a buffered electrolyte solution at 37°. The composition (millimolar) of the standard electrolyte solution was as follows: sodium, 140; chloride, 124; HCO_3, 21; potassium, 5.4; HPO_4, 2.4; H_2PO_4, 0.6; magnesium, 1.2; calcium, 1.2; glucose 10. Incubation media with lower sodium concentrations were prepared by isosmotic replacement of sodium with choline. The pH of all electrolyte solutions was 7.4 when equilibrated with a gas mixture of 95% O_2 and 5% CO_2.

The ψ_{ms}[1] with respect to the luminal solution was recorded with a Keithley 610C electrometer. In order to clamp ψ_{ms} at 0 mV, an external electrical current (I_{sc}) was passed across the tissue from a variable direct-current source.

After the I_{sc} had stabilized, cumulative concentration-response relationships of amiloride were obtained by adding increasing concentrations of amiloride to the luminal solution every 7 min, so that a complete concentration-response study was carried out on each tissue. Control experiments had shown that the amiloride effect is complete after 2–3 min.

Mathematical treatment of data. Both the concentration dependence of the inhibitory effect of amiloride on I_{sc} and the dependence of I_{sc} on the sodium concentration in the presence of various amiloride concentrations were evaluated according to the receptor theory by use of equations which are analogous to those used in enzyme kinetics. Indeed, both net transepithelial sodium transport and unidirectional luminal sodium entry are accurately described by saturation kinetics (17, 18). Furthermore, as is shown below, the inhibitory effect of amiloride on I_{sc} also conforms to the Michaelis-Menten equation. The concentrations at which the effects are half-maximal reflect dissociation constants if it is assumed that the extent of ligand saturation is measured by $\Delta/\Delta_{(max)}$, the effect expressed as the fraction of the maximal effect attainable.

The parameters (constants) of nonlinear enzyme kinetics are usually estimated by use of graphic methods (19). However, the linearization techniques make specific assumptions about the distribution of variance or are likely to introduce bias in the estimates unless carefully considered corrections are made. Clearly superior to the graphic methods are mathematical curve-fitting procedures to obtain accurate estimates of the parameters of enzyme kinetics (20). Therefore, the kinetic data of the present study were evaluated by using nonlinear regression analysis, underlying model equations which are specified in each case. In principle, the nonlinear regressions were calculated by adjusting initial parameter estimates, obtained by standard graphic procedures, according to the Gauss-Newton iterative procedure until the sum of squares of the residuals has converged to a minimum (21). The adjustments of the parameter estimates were the partial derivatives of the model equations with respect to each parameter. The iterative procedure was discontinued when no further improvement could be achieved, i.e., when the partial derivatives approached zero, so that the deviation between the calculated and the experimental data became constant with further iterations. No weighing factors were used, all points being considered to be equally precisely measured. The calculations were performed on a small-core (16 K) computer (pdp 11/10; Digital Equipment Corporation, Maynard, Mass.); the programs were written in FOCAL-11.

RESULTS

Classification of the inhibitory mechanism of amiloride on I_{sc}. Cumulative concentration-response relationships of amiloride were obtained with different sodium concentrations. By this procedure it is possible to study both the I_{sc} as a function of the sodium concentration at constant amiloride concentrations and, vice versa, as a function of the amiloride concentration at constant sodium concentrations. For better comparability of the amiloride effects at the different sodium concentrations, sets of experiments were performed in which four preparations from the same animal were incubated with 5, 15, 45, or 140 mM sodium.

For the kinetic analysis of the amiloride-sodium interaction, only the fraction of I_{sc} which is inhibitable by amiloride is dealt with, since only this fraction is a saturable function of the sodium concentration (18), whereas total I_{sc} may also include asymmetries in transepithelial ion fluxes other than active sodium absorption, for instance active chloride secretion which is not inhibitable by amiloride (5). Furthermore, only the amiloride-sensitive fraction of I_{sc} can be considered to reflect amiloride binding and may therefore be used for the Hill and Scatchard analyses. However, as has repeatedly been shown previously (5, 17) and as is also apparent from the present results (Table 1), the fraction of I_{sc} inhibitable by maximal concentrations of amiloride (1×10^{-4} M) does

TABLE 1

Comparison of total I_{sc} and the maximal amiloride-sensitive I_{sc} in the presence of different sodium concentrations

Values are means ± standard error of the mean of seven experiments.

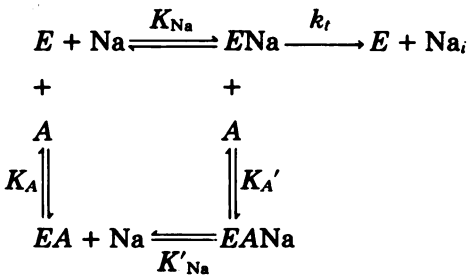
[Na]	Total I_{sc}	Maximal[a] amiloride-sensitive I_{sc}
mM	$\mu Eq/cm^2 \cdot hr$	$\mu Eq/cm^2 \cdot hr$
5	0.86 ± 0.13	0.77 ± 0.15
15	1.79 ± 0.32	1.89 ± 0.37
45	2.42 ± 0.25	2.45 ± 0.32
140	2.69 ± 0.75	2.42 ± 0.70

[a] The maximal amiloride-sensitive I_{sc} is the total I_{sc} minus the I_{sc} in the presence of 1×10^{-4} M amiloride.

[1] The abbreviations and symbols used are: ψ_{ms}, open-circuit transepithelial electrical potential difference; I_{sc}, short-circuit current; $I_{sc(max)}$ maximal I_{sc} in the absence of amiloride; E, apical sodium entry mechanism; [Na], [A], sodium and amiloride concentration, respectively; $[Na]_{0.5}$, sodium concentration at which I_{sc} is half-maximal; $[A]_{0.5}$, amiloride concentration at which the amiloride effect is half-maximal; K_{Na}, K_{Na}, dissociation constant of the E-Na and the EA-Na complex, respectively (see reaction Scheme (I); K_A, K'_A, dissociation constant of the E-A and the ENa-A complex, respectively; Δ, amiloride effect, expressed as percentage of I_{sc}; n, Hill coefficient of the interaction between amiloride and the apical sodium entry mechanism.

not differ markedly from total I_{sc} in this tissue, the mean of the maximal amiloride-sensitive I_{sc} at all four sodium concentrations used being 97 ± 4% of total I_{sc}.

Since the I_{sc} is a saturable function of the sodium concentration, the type of inhibition exerted by amiloride may be diagnosed from the effects of amiloride on the maximal I_{sc} and $[Na]_{0.5}$, the sodium concentration at which I_{sc} is half-maximal. When I_{sc} is plotted as a function of the sodium concentration in the incubation medium in the absence and presence of various amiloride concentrations (Fig. 1a), it is clear that in the presence of amiloride the maximal current decreases. The parameters of the inhibition kinetics of amiloride were calculated by nonlinear regression analysis (see Materials and Methods), employing an equation derived from the equilibria of the following reaction scheme with reversible and random binding:



SCHEME I

where E stands for the apical sodium entry mechanism, without implying a particular mechanism of translocation or saturation, and A for amiloride. K_{Na}, K'_{Na}, K_A, and K_A' are the dissociation constants of the indicated reactions; k_t represents the rate constant of the actual sodium translocation mechanism which moves sodium to the inside of the epithelium. The translocated Na, Na_i, which gives rise to I_{sc}, is proportional to the concentration of ENa, as only this complex is assumed to be sodium-conducting, whereas $EANa$ is not. Hence, the velocity equation may be derived from rapid equilibrium assumptions and the mass-action equilibria given in the reaction scheme

$$I_{sc} = \frac{I_{sc(max)}[Na]}{K_{Na}(1 + [A]/K_A) + [Na](1 + [A]/K_A')} \quad (1)$$

This equation, which is analogous to the general equation for linear mixed-type enzyme inhibition (19), includes all of the common types of inhibition as asymptotic or special cases. The classification of the type of inhibition exerted by amiloride follows from the values of K_A and K_A': when K_A' is infinite, $[A]/K_A'$ approaches zero and Eq. 1 reduces to the formalism of competitive inhibition, whereas when $K_A = K_A'$ the equation for noncompetitive inhibition ensues; when $K_A \neq K_A'$ but both have finite values, the type of inhibition is mixed.

The approximation of the parameters of Eq. 1 to the experimental data gave values of 8.5 ± 2.1 mM for K_{Na}, 0.11 ± 0.02 μM for K_A, 0.30 ± 0.08 μM for K_A', and 2.6 ± 0.7 μEq/cm^2·hr for $I_{sc(max)}$. Thus, the inhibition of I_{sc} by amiloride is not purely noncompetitive, but in addition there is a competitive component so that the amiloride-induced inhibition has to be termed mixed-type. In other words, amiloride not only depresses the maximal transport capacity of the epithelium, it also decreases the affinity of sodium to its transport system. The mixed-type inhibitory action of amiloride on I_{sc} is also apparent from the curvilinear relationship between $[Na]_{0.5}$ and the amiloride concentration, $[A]$, as illustrated in Fig. 1b. By definition, in the case of competitive inhibition, $[Na]_{0.5}$ has to increase linearly with $[A]$, whereas in the case of pure noncompetitive inhibition, $[Na]_{0.5}$ is constant at all $[A]$. The asymptote which is approached by the relationship between $[Na]_{0.5}$ and $[A]$ represents K'_{Na}.

Although the inhibitory effect of amiloride on I_{sc} has to be classified as mixed-type, the action is primarily noncompetitive since 1×10^{-5} M amiloride reduced the I_{sc} practically to zero, whereas $[Na]_{0.5}$ was only doubled.

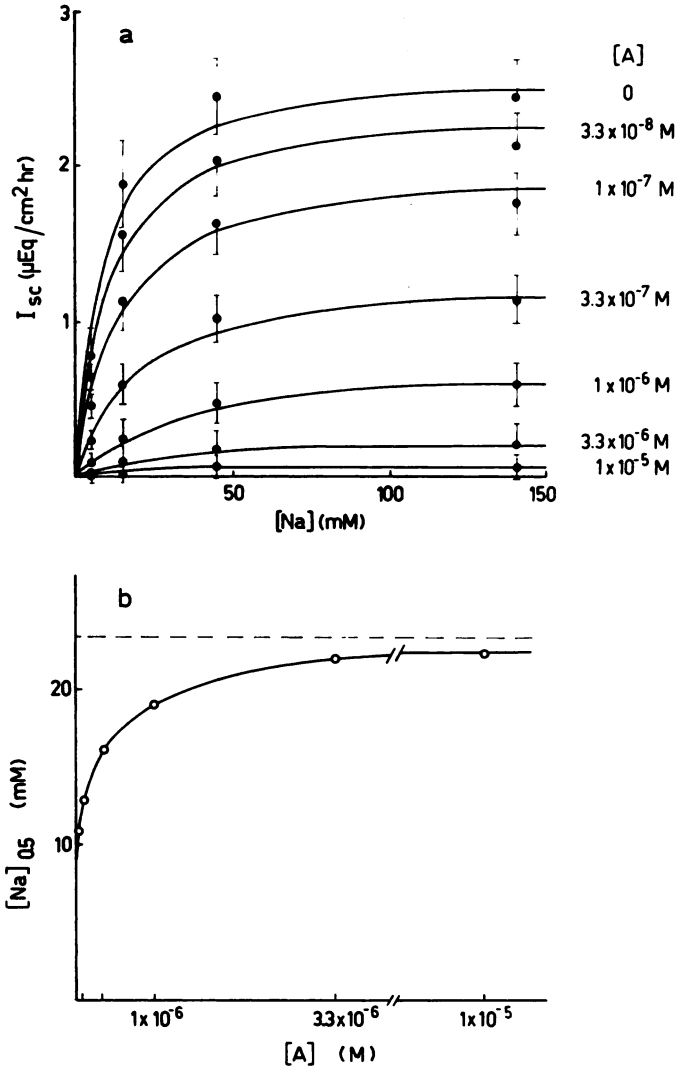


FIG. 1. *Effect of amiloride on the relationship between I_{sc} and the sodium concentration*

a. The data represent means ± standard error of the mean, based on seven experiments, of the steady-state I_{sc} in the absence and presence of the indicated amiloride concentrations, $[A]$. The curves were calculated by nonlinear regression analysis of the data underlying Eq. 1.

b. Dependence on $[Na]_{0.5}$ on $[A]$. - - - represents K'_{Na}, calculatd from $K_{Na}K'_A/K_A$.

The predominant noncompetitive nature of the inhibitory effect of amiloride is also indicated by the fact that the values of K_A and K_A' are quite similar.

Effect of sodium on the action of amiloride. In order to elucidate the influence of sodium on the effect of amiloride, the concentration-response relationship of amiloride was evaluated in the presence of four different sodium concentrations (Fig. 2a). Clearly, increasing sodium concentrations shift the concentration-response relationship of amiloride to the right, indicating inhibition of the amiloride action by sodium. Figure 2b depicts the dependence of $[A]_{0.5}$, the amiloride concentration at which the amiloride action is half-maximal, on the sodium concentration. If sodium is a competitive antagonist of the amiloride action,

$$[A]_{0.5} = K_A + \frac{K_A}{K_i}[\mathrm{Na}] \qquad (2)$$

Hence, in the case of a competitive sodium-amiloride interaction, $[A]_{0.5}$ would be a linear function of the sodium concentration (Fig. 2b, - - -), K_i representing the dissociation constant of the reaction of sodium with the amiloride binding site or, in phenomenologic terms, the sodium concentration which doubles K_A. Using eq. 2, K_i can be calculated to be approximately 110 mM. However, the relationship between $[A]_{0.5}$ and the sodium concentration may not be linear, as is apparent from Fig. 2b; therefore, interpretations of the inhibition of the amiloride action by sodium other than competition between sodium and amiloride for binding at the amiloride site are possible. This point is discussed in more detail below.

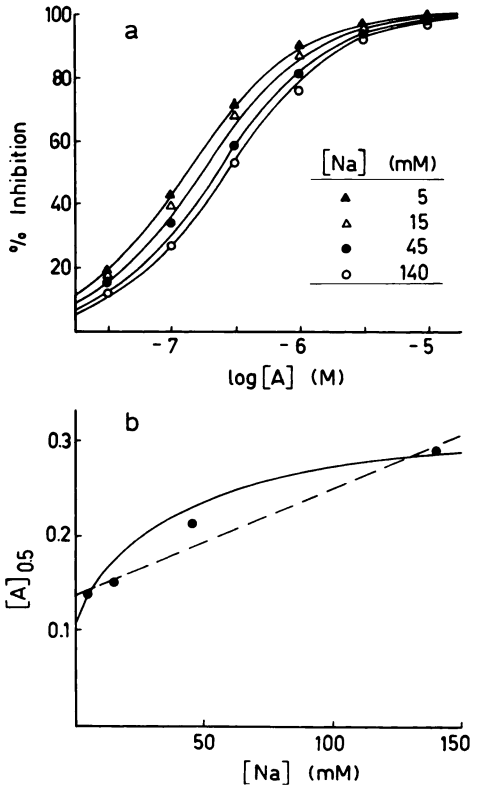


FIG. 2. *Cumulative concentration-response relationship of the inhibitory amiloride effect on I_{sc} in the presence of four different sodium concentrations*

a. The inhibitory effect of amiloride is expressed as the percentage decrease of the original uninhibited I_{sc}. The data represent the means ± standard error of the mean of seven experiments. The individual curves, representing the fractional occupancy of the amiloride receptor by amiloride, were calculated according to reaction Scheme I using 0.11 μM for K_A, 0.30 μM for K'_A, 8.5 mM for K_{Na}, and 23.2 mM for K'_{Na}.

b. Dependence of $[A]_{0.5}$ on the sodium concentration, [Na]. - - - was obtained by linear regression analysis of the data. The full curve represents the change in $[A]_{0.5}$ as a function of [Na], calculated according to reaction Scheme I by use of the dissociation constants given above.

Effect of ouabain on the action of amiloride. The results given in Fig. 2a suggest that sodium is an inhibitor of the action of amiloride; however, it is unclear whether this effect is exerted by extra- or intracellular sodium. In order to test the effect of elevated cellular sodium on the action of amiloride, 0.6 or 2.5 μM ouabain, which brings about an increase in cellular sodium by inhibiting the basolateral sodium extrusion mechanism, was added to the solution bathing the serosal side of the epithelium. These concentrations of ouabain caused declines in I_{sc} by 35 ± 6% and 53 ± 6%, respectively. After the I_{sc} had reached a new steady state, usually 60 min after the addition of ouabain, cumulative concentration-response studies were performed with amiloride (Fig. 3). Ouabain decreased primarily the maximal inhibitory effect of amiloride, whereas $[A]_{0.5}$ was unchanged. In other words, maximal concentrations of amiloride completely inhibited I_{sc} under control conditions (zero ouabain), whereas I_{sc} was not completely inhibitable in the presence of ouabain.

The kinetic parameters of the inhibitory effect of elevated cellular sodium on the action of amiloride were calculated by nonlinear regression analysis of the amiloride concentration-response relationships in the absence and presence of the two ouabain concentrations, using

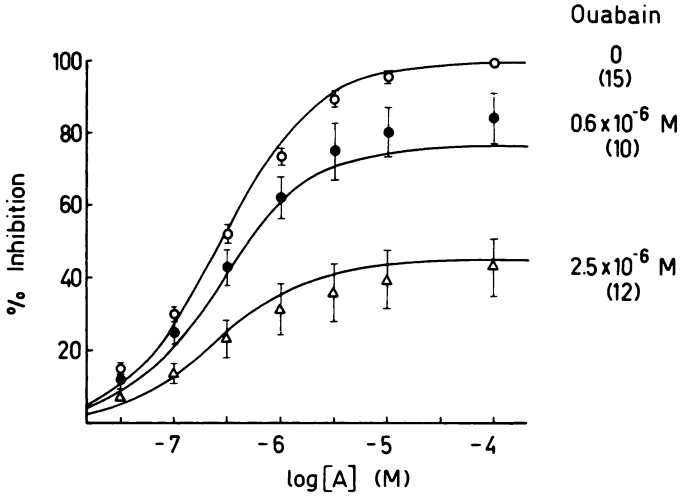


FIG. 3. *Effect of ouabain on the amiloride concentration-response relationship*

The inhibitory effect of amiloride is expressed as the percentage decrease of the I_{sc} before addition of amiloride. The data represent means ± standard error of the mean in the absence and presence of the indicated ouabain concentrations, added to the serosal solution. The numbers of experiments are given in parentheses. The curves were calculated by nonlinear regression analysis of the data, underlying the equation for linear mixed-type inhibition.

the general equation for linear mixed-type inhibition (19). Since the actual ouabain-induced increases in cellular sodium were not measured, the inhibitory constants of cellular sodium have to be expressed in terms of ouabain equivalents. These phenomenologic inhibition constants of ouabain, K_O and K_O', were calculated to be 2.35 ± 0.70 μM and 2.01 ± 0.93 μM, respectively. Since $K_O \approx K_O'$, the inhibition of the amiloride action by ouabain has to be classified as noncompetitive.

Stoichiometry of the amiloride-sodium entry site interaction. The stoichiometry and possible cooperativity of the amiloride-sodium entry site interaction were determined by use of the Hill equation, which is the simplified velocity equation for multi-site enzymes assuming strong cooperativity in substrate binding (19). The Hill equation can be converted to a useful linear form by logarithmic transformation (Hill plot), which in the case of the amiloride effect on I_{sc}, Δ, is given by

$$\log \frac{\Delta}{\Delta_{(max)} - \Delta} = n \log [A] + \log K \qquad (3)$$

where n represents the number of binding sites per receptor unit and K is the nth power of $[A]_{0.5}$.

The Hill plot of the inhibitory effect of amiloride on I_{sc}

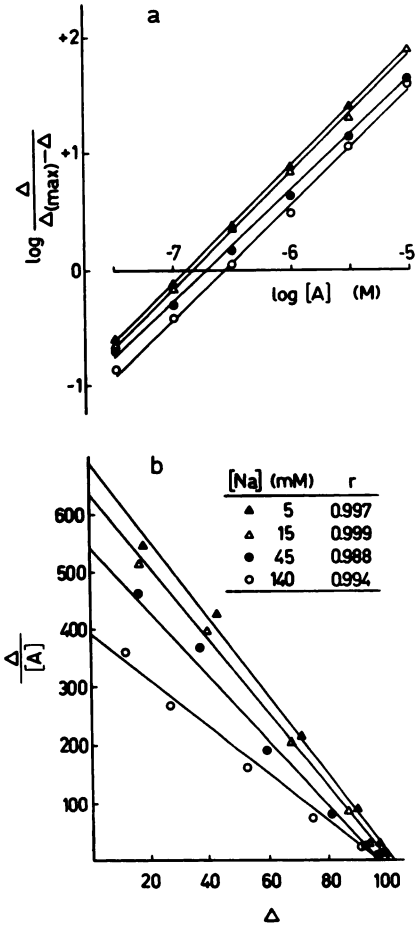


FIG. 4. *Hill plots (a) and Scatchard plots (b) of the amiloride effects in the presence of different sodium concentrations*

Δ, amiloride effect as used in Fig. 2a. The Hill coefficients calculated from the Hill plots are given in Table 2. The linear correlation coefficients (r) of the Scatchard plots are compiled in the *inset* of b; degrees of freedom = 6.

TABLE 2

Hill coefficients (n) of the concentration-response relationship of amiloride at different sodium concentrations

[Na]	n	r^a
mM		
5	1.03	0.999
15	1.02	0.999
45	0.93	0.999
140	0.99	0.999

[a] Linear correlation coefficient.

in the presence of four different sodium concentrations is given in Fig. 4a. As is apparent, the lines are shifted to the *right* with higher sodium concentrations, again illustrating the inhibitory effect of sodium on the action of amiloride. The values for n as obtained from the Hill plots at the individual sodium concentrations are compiled in Table 2. Clearly, the Hill coefficients do not markedly deviate from 1 at any sodium concentration, the regressions exhibiting a high degree of linearity as judged from the correlation coefficients. These data suggest that (a) there is no cooperativity among the amiloride binding sites and (b) one amiloride molecule binds per sodium entry site.

Are there different classes of amiloride binding sites? From a Hill coefficient of exactly 1 it must be concluded that only a single class of binding sites[2] exists in the system under investigation. However, the Hill plot is not well suited to exclude the existence of more than one class of binding sites. Theoretically, a Hill coefficient of 0.93, which is at the lower end of the values given in Table 2, is consistent with the existence of two classes of binding sites with dissociation constants differing by a factor of 10, when appropriate maximal binding capacities are assumed. More useful than the Hill plot or other graphic methods in detecting the presence of more than a single class of binding sites is the Scatchard (or Eadie-Scatchard) plot (19). Accordingly, the Michaelis-Menten formalism of the amiloride effect on I_{sc}, as illustrated by Fig. 2a, may be rearranged to

$$\frac{\Delta}{[A]} = -\frac{1}{K_A}\Delta + \frac{\Delta_{(max)}}{K_A} \qquad (4)$$

Figure 4b is a plot of Δ/[A] versus Δ in the presence of the four different sodium concentrations. Two or more classes of amiloride binding sites are reflected in the Scatchard plot by a curvilinear relationship which is convex toward the abscissa, whereas if there is only a single class of amiloride binding sites a linear relationship would result. Hence the critical point in detecting the presence of one or more classes of binding sites is the linearity of the relationship Δ/[A] versus Δ. Therefore, the linear correlation coefficients of each regression are given in the *inset* of Fig. 4b. As is clear from these values and from inspection of the individual relationships, there is no marked deviation from linearity with any of the sodium concentrations used. Thus there is no reason to assume the existence of more than one class of amiloride binding sites mediating the inhibitory effect on I_{sc}.

[2] A class of binding sites is defined to comprise all sites with the same affinity for the ligand.

DISCUSSION

Previous experiments have shown that in rabbit descending colon ψ_{ms} and I_{sc} are entirely attributable to active sodium transport from lumen to blood (J^{Na}_{net}) (5). Furthermore, in this epithelium, luminal sodium entry is rectified; i.e., luminal sodium efflux is undetectably small, so that under steady-state conditions luminal sodium influx into the sodium-transporting cells (J^{Na}_{mc}) equals J^{Na}_{net} (18). Therefore, in the rabbit descending colon, I_{sc} is identical with J^{Na}_{mc}. In other words, the reported effects of amiloride on I_{sc} reflect changes in luminal sodium entry.

Kinetics of the inhibitory effect of amiloride on sodium entry. According to the present results, the concentration dependence of the inhibitory effect of amiloride on luminal sodium entry in rabbit descending colon can be described by conventional enzyme kinetics. The type of inhibition exerted by amiloride in this tissue has to be termed mixed, since both the maximal transport capacity and the affinity of sodium for the transport system were decreased. This observation is in agreement with findings in the abdominal skin of the north European frog (*Rana temporaria*) and toad (*Bufo marinus*), whereas the effect of amiloride is purely noncompetitive in bullfrog (*R. catesbeiana*) and grassfrog (*R. pipiens*) (15). In the toad bladder amiloride also acts as a noncompetitive inhibitor of sodium transport (2). However, in the skin of *R. esculenta* and *R. ridibunda*, amiloride was reported to increase $[Na]_{0.5}$ without changing the maximal sodium current, which is characteristic of competitive inhibition (13).

According to the present results in rabbit colon, amiloride binds both to E and the ENa complex, indicating that the sodium translocation site and the amiloride binding site (the "amiloride receptor") are separate. This notion is also supported by the noncompetitive type of inhibition exerted by amiloride in several other tight sodium-transporting epithelia. The only mechanism whereby binding of agonist and antagonist to the same site would be consistent with noncompetitive inhibition is irreversible binding of the antagonist to the common site without altering the affinity of the vacant sites to the agonist. This possibility can be excluded, since the effect of amiloride was repeatedly shown to be rapidly and totally reversible by rinsing the tissue (2, 3, 17). The competitive component of the action of amiloride is in accord with separate sites for amiloride binding and sodium entry, because a decrease in the apparent affinity of sodium to the translocation site may not be brought about only by amiloride binding at the same site but also at a separate site, if the EA complex has a lower affinity for sodium than for E. This seems to be the case, since $K_{Na} < K'_{Na}$.

The stoichiometry of the interaction between amiloride and the sodium entry mechanism in rabbit descending colon appears to be 1:1 as judged from the slope of the amiloride inhibition curves (Hill plots)[3]. Although the amiloride binding site and the sodium translocation site seem to be separate, as argued above, the stoichiometry of 1:1 of the amiloride-sodium entry mechanism interaction suggests that the amiloride binding site is in close proximity to, if not on, the sodium entry mechanism. Equivalence in the number of amiloride binding sites and sodium entry sites was also proposed by Cuthbert and Shum (4) because (a) there was reasonable agreement between the affinity constants calculated from the concentration dependence of amiloride binding and inhibition of I_{sc} in frog skin, and (b) amiloride inhibition kinetics yielded Hill coefficients of 0.96 in toad bladder and 0.92–1.22 in skin of *R. temporaria* (22). On the other hand, there are also reports that the stoichiometry of the amiloride-sodium entry site interaction is lower than unity in skins of some frog species. From the data of Salako and Smith (3) a Hill coefficient of 0.81 may be derived, and Benos and co-workers (15) calculated Hill coefficients for the inhibitory effect of amiloride on sodium transport in various frog species and in toad ranging from 0.34 to 0.77. However, the very low Hill coefficients given in the paper by Benos and co-workers (15) result in part from the fact that, in the presence of low sodium concentrations, amiloride did not completely inhibit I_{sc}. This unexplained phenomenon causes a progressive decrease of the slope of the Hill plot with increasing amiloride concentrations, so that the Hill relationship is not linear. But even if only the amiloride-sensitive fraction of the data of Benos and co-workers (15) is subjected to the Hill analysis, the apparent Hill coefficients of the amiloride interaction with frog skin remain well below 1 (n increases from 0.54 to 0.64 with 100 mM and from 0.46 to 0.77 with 6 mM sodium), suggesting negative cooperativity among the amiloride receptor sites. One possible explanation for the low Hill coefficients emerges from the finding of Macchia and Helman (23) that there may be two classes of amiloride receptors in frog skin as concluded from a Scatchard analysis of the concentration dependence of the amiloride effect on I_{sc}; the existence of two populations of receptors in frog skin with different affinities for amiloride was also reported by Cuthbert and Shum (4). It is easy to show that the presence of two or more classes of binding sites leads to Hill coefficients of less than 1 with a progressive decrease in the value of the Hill coefficient as the difference in affinities of the binding sites increases or as the difference in the maximal binding capacities decreases. In short, the existence of two classes of amiloride binding sites in frog skin appears at present to be the simplest explanation for the low Hill coefficients obtained in this tissue. In contrast to frog skin, there is no evidence for the existence of more than one amiloride binding site in rabbit descending colon.

Inhibition of the action of amiloride by sodium. As is clear from the present results, the amiloride-induced inhibition of sodium transport is only one facet of the amiloride-sodium interaction; the other is an inhibitory effect of sodium on the action of amiloride. This effect could come about by competition between sodium and amiloride for binding at the amiloride receptor. However, it is also possible that sodium does not react with the amiloride receptor and that the rightward shift in the amiloride concentration-response relationship is merely a consequence of the fact that ENa has a lower affinity

[3] However, a Hill coefficient of 1 is also consistent with multiple binding sites per receptor unit as long as there is no cooperativity among the sites. In this case the binding of n molecules to n single-site receptors will have the same effect as the binding of n molecules to one n-site receptor unit (19), so that again a 1:1 stoichiometry of the amiloride-sodium entry site interaction would result.

for amiloride than does E. According to reaction Scheme I the percentage inhibition of I_{sc} is proportional to the fractional occupancy of the amiloride receptor by amiloride; i.e., the sum of $[EA] + [EA\text{Na}]$. When the dependence of the fractional occupancy on the amiloride concentration is calculated in the presence of 5, 15, 45, and 140 mM sodium, using the dissociation constants of the individual reactions obtained by approximating the parameters of Eq. 1 to the results given in Fig. 1a, the curves illustrated in Fig. 2a are obtained. As is apparent, increasing sodium concentrations shift the relationship between ($[EA] + [EA\text{Na}]$) and the amiloride concentration to the right with an excellent fit to the experimental data. Since we are dealing with mixed-type inhibition, the dependence of $[A]_{0.5}$ on the sodium concentration is not expected to be linear, as would be the case with competitive inhibition, but rather curvilinear, $[A]_{0.5}$ approaching K_A' at high sodium concentrations.

The dependence of $[A]_{0.5}$ on the sodium concentration, calculated according to reaction Scheme I, is given by the full curve in Fig. 2b. Although the four data points are insufficient to make a definite conclusion, the fit of the calculated curve, assuming mixed-type inhibition, to the experimental values is just as good as when the relationship between $[A]_{0.5}$ and the sodium concentration is assumed to be linear. In short, although it cannot be excluded that sodium binds at the amiloride receptor in rabbit colon, at present there is no evidence supporting such an assumption. The simplest system consistent with the present results is one in which sodium does not bind at the amiloride binding site and amiloride does not bind at the sodium translocation site.

Inhibition of the effect of amiloride by sodium has also been observed in frog and toad skin (4, 10, 14, 15). In these tissues sodium may interact with the amiloride receptor, since in skin of *R. temporaria* the relationship between $[A]_{0.5}$ and the sodium concentration appeared to be linear (4), and in bullfrog skin inhibition of I_{sc} by amiloride was reported to be noncompetitive (10, 15). In the case of pure noncompetitive inhibition, $[A]_{0.5}$ would not increase with increasing sodium concentrations. The decrease in the amiloride response with increasing sodium concentrations observed in frog skin therefore indicates that sodium not only binds in this tissue to its translocation site but in addition binds to a site which modifies the action of amiloride. This latter site may well be the amiloride receptor with competition between sodium and amiloride for binding at this site (4, 10). Benos and co-workers (15) have proposed that the differences in the amiloride inhibition kinetics observed in skin of different frog species may be attributed to variations in the affinity of sodium to the amiloride binding (or modifier) site: competitive inhibition would result if sodium and amiloride interact competitively with the modifier site, whereas noncompetitive inhibition would be obtained if sodium does not affect amiloride binding at this site. Sodium may not only prevent amiloride binding at the modifier site, but could also exert an "amiloride-like" action at this site to decrease the conductance of the sodium entry process. Indeed, Fuchs and co-workers (8) have suggested that the saturability of sodium transport in frog skin is due to binding of sodium to an inhibitor site at the external surface of the apical membrane, because the steady-state permeability of this membrane decreased with increasing sodium concentrations in the outer bathing solutions, whereas cellular sodium was calculated from the sodium-reversal potential to be nearly constant.

However, the inhibition of the effect of amiloride by sodium does not necessarily have to be a consequence of sodium binding at the external surface of the apical membrane, but may be brought about by an increase in cellular sodium, since it was shown in frog skin that inhibition of the serosal sodium extrusion mechanism by ouabain reduces amiloride binding at the outer face of this tissue (24). This effect was caused by a reduction in the number of amiloride binding sites, whereas there was no change in the affinity of amiloride. In agreement with these results, ouabain inhibits noncompetitively the action of amiloride in rabbit colon (see Fig. 3), indicating that an increase in cellular sodium (or possibly a decrease in cellular potassium or an increase in cellular calcium) reduces the sensitivity of the tissue to amiloride. The role of celluar sodium as the mediator of the effect of ouabain is supported by the finding that ouabain reduces only apical amiloride binding in the presence of high sodium concentrations (24). In frog skin, ouabain was also reported to decrease the inhibitory effect of amiloride on I_{sc} (14), as do other manipulations known to raise cellular sodium, such as serosal addition of aldosterone (14) and antidiuretic hormone (3, 4, 14). Although the rightward shift of the amiloride inhibition curves with aldostrone and antidiuretic hormone may be due to an increase in the proportion of sodium entry mechanisms in the conductive configuration (4), at least the data with ouabain support the notion that cellular sodium not only modifies apical sodium conductance (17) but also the amiloride action. Shum and Fanelli (25) have reported experiments which may provide direct evidence for the interference of high cellular sodium with the action of amiloride: in frog skin with a functionally removed serosal barrier, transepithelial sodium gradients produced amiloride-sensitive diffusion potentials, $[A]_{0.5}$ being 0.4 μM with a sodium gradient in the direction mucosa-to-serosa, but 2.3 mM when a sodium gradient was applied from serosa to mucosa. Thus, high cellular sodium appears to alter the effect of externally added amiloride, but in contrast to the results in rabbit colon, primarily by decreasing its affinity.

CONCLUSION

The molecular mechanism whereby amiloride blocks apical sodium entry is not resolved. At present there are two concepts: (a) amiloride acts on a regulatory mechanism or modifying center which is close to but spatially distinct from the sodium translocation site and which modulates the apical sodium conductance (6, 15, 22); and (b) the charged guanidinium group of amiloride plugs the sodium entry mechanism (26, 27) in a manner similar to the blockade of the selectivity filter of the sodium channels in excitable cell membranes by the guanidinium moieties of tetrodotoxin and saxitoxin (26). The present results in rabbit colon favor separate sites for sodium translocation and amiloride binding. Furthermore, if the "plug theory" is accepted, it is difficult to explain why dimethylamiloride (28) and another guanidinium com-

pound, benzimidazole-guanidine, at least when added in low concentrations, do not impede sodium entry but stimulate it (13, 26). The model of a regulatory mechanism, on the other hand, is fully consistent with the effects of stimulators and inhibitors of apical sodium entry (positive and negative effectors). This regulatory mechanism, which appears to be blocked by sulfhydryl reagents (29), may be acted upon both by intra- and extracellular factors which increase [low ambient sodium concentrations, aldosterone, certain anions (30)] or decrease (high sodium concentrations, amiloride) apical sodium conductance.

ACKNOWLEDGMENTS

Amiloride was a generous gift of Merck Sharp & Dohme, Rahway, N. J. The technical assistance of Mr. P. Krivanek is gratefully acknowledged.

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