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APPARENT INHIBITION OF Na^+/H^+ EXCHANGE BY AMILORIDE AND HARMALINE IN ACRIDINE ORANGE STUDIES

IVAN SABOLIĆ * and GERHARD BURCKHARDT **

Max-Planck-Institut für Biophysik, Kennedyallee 70, 6000 Frankfurt am Main 70 (F.R.G.)

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Amiloride and harmaline were tested as inhibitors of proton movements in brush-border membrane vesicles from rat kidney cortex. Transmembrane pH differences were visualized using acridine orange. Fluorescence quenching due to Na^+ gradient-driven intravesicular acidification was inhibited by amiloride and harmaline. However, a similar inhibition was observed for the Na^+ gradient-driven electrogenic proton movements in the presence of gramicidin. Moreover, amiloride and harmaline decreased the fluorescence signal of electrogenic proton movements driven by a K^+ gradient in the presence of valinomycin. The degree of inhibition of intravesicular acidification by both drugs was concentration dependent. Half-maximal inhibition (I_{50}) of Na^+/H^+ exchange and K^+ gradient-driven proton movements occurred at 0.21 and 0.6 amiloride, respectively. The I_{50} for harmaline was 0.21 mM in both cases. Amiloride also decreased the initial quenching of acridine orange fluorescence due to a preset pH gradient without affecting the rate of dissipation of the pH gradient. This effect was independent of the buffer capacity. In contrast, harmaline seemed to dissipate pH gradient in the same way as a permeant buffer. Amiloride and harmaline led to a concentration-dependent fluorescence decrease even in aqueous solution. The results suggest an interaction of amiloride and harmaline with acridine orange which overlaps a possible specific inhibition of Na^+/H^+ exchange by these drugs.

Introduction

An electroneutral Na^+/H^+ antiporter is presumed to be responsible for H^+ secretion and Na^+ absorption in the kidney proximal tubule. Various techniques, including direct measurement by pH electrode [1,2], distribution of dimethyloxazolidine-2,4-dione [3], filter technique [4,5], flow dialysis [4,5] and quenching of acridine orange fluorescence [6–8], have been used to prove an Na^+/H^+-exchange mechanism in proximal tubular cells [2,3] and brush-border membrane vesicles derived from proximal tubules [1,4–8].

The diuretic, amiloride, has been described as a specific inhibitor not only of Na^+ channels but also of Na^+/H^+ exchange in various experimental models [9]. The inhibition of Na^+/H^+ exchange by amiloride has been observed both in renal cells [2] and in brush-border membrane vesicles [4,5,7,8]. The same was found for the halucinogen harmaline [10], a potent inhibitor of various Na^+-dependent transport processes in small intestine and renal proximal tubules [10–12].

However, a specific inhibition of Na^+/H^+ ex-

* Permanent address: Department of Physiology, Faculty of Medicine, University of Zagreb, Zagreb, Yugoslavia.
** To whom correspondence should be addressed.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid; Mes, *N*-morpholinoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

change by amiloride has been questioned recently by Dubinsky and Frizzell [13]. Working on intestinal brush-border membranes vesicles and purified phospholipid vesicles, they reported that amiloride can act as a permeant weak buffer, thus dissipating transmembrane pH gradients. Using the same technique, a ΔpH-dependent quenching of acridine orange fluorescence, we reinvestigated the effects of both amiloride and harmaline on electroneutral Na^+/H^+-exchange mechanism and on electrogenic proton movements in the brush-border membrane vesicles isolated from the rat kidney cortex.

Material and Methods

Brush-border membrane vesicles from rat kidney cortex were prepared by a calcium-precipitation technique [14]. With respect to the homogenate, the final membrane preparations were enriched in specific activity of leucine amidopeptidase (EC 3.4.11.-), a marker for luminal membranes, 10–15 times, whereas the enrichment for $(Na^+ + K^+)$ATPase (EC 3.6.1.3), a marker for basolateral membranes, was less than 1.0.

The vesicles were preloaded with a buffer of desired ionic composition by two washings followed by an incubation in the same buffer at room temperature for 2 h. The compositions of the preloading buffers are described in the legends to the figures. The protein concentration of the final vesicle preparations was adjusted to 16 mg/ml and was measured with Bio-Rad protein assay kit using bovine serum albumin as a standard.

The changes in transmembrane ΔpH were visualized by using the fluorescence quenching of acridine orange [6–8]. In all the experiments, 10 μl of brush-border membrane vesicles were diluted into 2.0 ml buffer containing 6 μM acridine orange. The fluorescence was continuously recorded at 25°C in the Shimadzu RF-510 spectrofluorophotometer (exitation, 493 nm; emission, 525 nm). During the measurement, the samples were constantly stirred. Ionophores (final concentrations: gramicidin, 1 μg/ml; valinomycin, 2.5 μM; FCCP, 5 μM) were added from ethanol stocks before additions of vesicles. The final concentration of ethanol was less than 0.5%. Amiloride and harmaline were added from water stocks prior to vesicles addition in the final concentrations indicated in the figure legends. Under all experimental conditions the fluorescence of acridine orange was adjusted to the same level by changing the instrument gain. The fluorescence quenching was expressed relatively (in %) to the initial fluorescence observed in the absence of transmembrane pH and ion gradients. The initial rate of vesicle acidification was estimated by drawing a tangent to the initial linear portion of the fluorescence signal and was expressed as fluorescence change per min. In the experiments with a preset ΔpH (pH-jump), the recordings of fluorescence changes were started 3 s after addition of the vesicles. If not stated otherwise, the data shown in the text are from single experiments, but are representative of two to three repetitions with separate membrane preparations.

Acridine orange was obtained from Eastman Kodak (Rochester, NY, U.S.A.), gramicidin from Fluka (Buchs, Switzerland), valinomycin and FCCP from Boehringer (Mannheim, F.R.G.), harmaline from Roth (Karlsruhe, F.R.G.), amiloride was a gift from Merck Sharp and Dohme Research Laboratories (West Point, PA, U.S.A.). All other chemicals were reagent grade. Tetramethylammonium gluconate was made by titrating tetramethylammonium hydroxide pentahydrate with gluconic acid (50% solution in water).

Results

When Na^+-preloaded vesicles were diluted into buffer containing acridine orange and the same concentration of Na^+, no change of fluorescence was observed (Fig. 1, $Na_i^+ = Na_o^+$). In contrast, by diluting Na^+-preloaded vesicles into Na^+-free buffer, a time-dependent drop of acridine orange fluorescence occurred (Fig. 1, E) indicating an intravesicular uptake of acridine orange due to a Na^+ gradient-driven intravesicular acidification [6–8]. This drop in fluorescence was much smaller in the presence of 1 mM amiloride (Am) and harmaline (Ha), which could suggest a specific inhibition of the Na^+/H^+ exchange. The addition of a channel-forming ionophore, gramicidin, resulted in a signal of a strong intravesicular acidification (Gr) due to electrogenic proton movements (artificial Na^+/H^+ exchange). However this signal was also decreased by amiloride (Gr + Am) and harmaline (Gr + Ha), indicating a

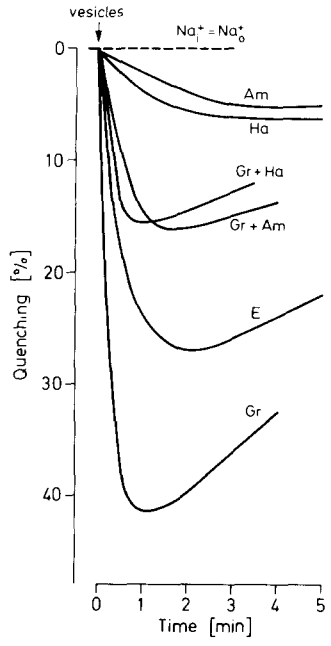

Fig. 1. Na^+ gradient-dependent quenching of acridine orange fluorescence: effect of amiloride and harmaline. Vesicles were preloaded with 100 mM mannitol, 100 mM tetramethylammonium gluconate, 150 mM sodium gluconate, 10 mM Hepes/Tris, pH 6.55, and diluted into buffer containing 6 μM acridine orange, 100 mM mannitol, 250 mM tetramethylammonium gluconate (100 mM tetramethylammonium gluconate and 150 mM sodium gluconate in the experiment indicated by $Na_i^+ = Na_o^+$), 10 mM Hepes/Tris, pH 6.55. Additions: E, ethanol; Gr, gramicidin; Am, amiloride (final concentration, 1 mM); Ha, harmaline (final concentration, 1 mM).

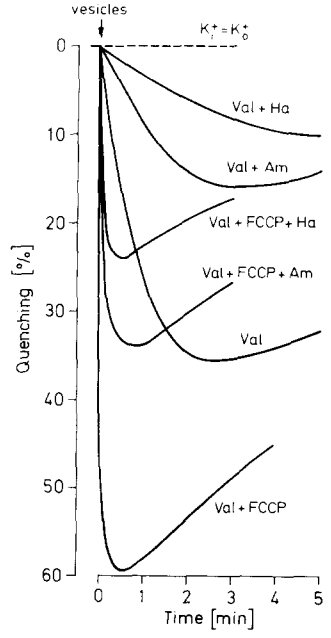

Fig. 2. K^+ gradient-dependent quenching of acridine orange fluorescence: effect of amiloride and harmaline. Vesicles were preloaded with 100 mM mannitol, 100 mM tetramethylammonium gluconate, 150 mM potassium gluconate, 10 mM Hepes/Tris, pH 6.55, and diluted into buffer containing 6 μM acridine orange, 100 mM mannitol, 250 mM tetramethylammonium gluconate (100 mM tetramethylammonium gluconate and 150 mM potassium gluconate in the experiment indicated by $K_i^+ = K_o^+$), 10 mM Hepes/Tris, pH 6.55. Additions: Val, valinomycin; Am, amiloride (final concentration, 1 mM); Ha, Harmaline (final concentration, 1 mM).

nonspecific effect of these compounds on the Na^+ gradient-dependent acridine orange quenching in brush-border membrane vesicles.

Indeed, amiloride and harmaline also interfered with acridine orange fluorescence signals obtained in the complete absence of sodium. When K^+-preloaded vesicles were diluted into K^+-free buffers in the presence of the K^+ ionophore valinomycin, the fluoroscence of acridine orange was transiently quenched (Fig. 2, Val). This quenching is due to an uptake of protons into the vesicles by a conductance pathway in consequence of an inside negative potassium diffusion potential. The signal was also decreased by amiloride (Val + Am) and harmaline (Val + Ha). Moreover, the quenching signal due to electrically coupled K^+/H^+ exchange in the presence of valinomycin and the protonophore, FCCP, was also much smaller in the presence of amiloride (Val + FCCP + Am) and harmaline (Val + FCCP + Ha). Here, harmaline was a more potent inhibitor of transmembrane proton movements than was amiloride.

The inhibition of the initial rates of acridine orange quenching due to electroneutral Na^+/H^+ exchange and K^+-driven proton uptake exhibited a sigmoid dependence on the concentrations of amiloride and harmaline (Fig. 3A and B, respectively). The electroneutral Na^+/H^+ exchange was measured in the presence of valinomycin and 100 mM K^+ inside and outside the vesicles to minimize electrically coupled Na^+ and H^+ movements. The Na^+ gradient-dependent acridine orange quenching obtained under this condition was about 35% smaller than the signal observed in the ab-

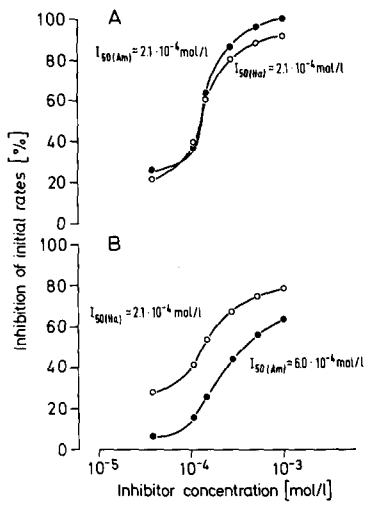

Fig. 3. The initial rates of Na^+/H^+ exchange-dependent (A) and of K^+ gradient-dependent (B) acridine orange uptake: inhibition by amiloride (●) and harmaline (○). A, Vesicles were preloaded with 100 mM mannitol, 100 mM potassium gluconate, 150 mM sodium gluconate, 10 mM Hepes/Tris, pH 6.55, and diluted into buffer containing 6 μM acridine orange, 2.5 μM valinomycin, 100 mM mannitol, 100 mM potassium gluconate, 150 mM tetramethylammonium gluconate, 10 mM Hepes/Tris, pH 6.55, and the indicated concentrations of inhibitors. B, Vesicles were preloaded with 100 mM mannitol, 100 mM tetramethylammonium gluconate, 150 mM potassium gluconate, 10 mM Hepes/Tris, pH 6.55, and diluted into buffer containing 6 μM acridine orange, 2.5 μM valinomycin, 250 mM tetramethylammonium gluconate, 10 mM Hepes/Tris, pH 6.55, and the indicated concentrations of inhibitors. Each point represents the mean of duplicate determinations.

sence of valinomycin and K^+, indicating that Na^+ gradient-driven intravesicular acidification, shown in Fig. 1, E, is partially due to electrogenic Na^+/H^+ exchange (unpublished data). The rest of the signal represented an electroneutral Na^+/H^+ exchange. As shown in Fig. 3, half-maximal inhibition (I_{50}) of electroneutral Na^+/H^+ exchange and of K^+ gradient-driven proton movements occurred at 0.21 and 0.6 mM amiloride, respectively. The I_{50} for harmaline was 0.21 mM in both cases.

The previous experiments indicated a non-specific effect of amiloride and harmaline on the quenching of acridine orange. This effect could be caused by an intravesicular accumulation of these weak bases, leading to a buffering of transmembrane ΔpH, as already suggested by Dubinsky and Frizzell [13] for amiloride. If amiloride and harmaline act as permeant buffers, their effects should be smaller at high intravesicular buffer capacities. We tested this possibility in experiments with a preset transmembrane ΔpH (pH-jump experiments) in the absence of Na^+ and K^+.

Dilution of vesicles of internal pH 5.5 into a buffer of pH 8.0 resulted in an immediate drop in fluorescence followed by a slower increase in fluorescence, indicating the dissipation of the pH gradient (Fig. 4A, 0). No fluorescence change was observed in the absence of a transmembrane ΔpH. Fig. 4 shows that, at low buffer capacity, increasing concentrations of amiloride decreased the initial quenching of acridine orange fluorescence. However, the rates of dissipation of quenching remained unaffected by different concentrations of amiloride, as demonstrated by parallel dissipation curves. At high buffer capacity (Fig. 4B), various amiloride concentrations had the same effect on initial quenching as at low buffer capacity. The

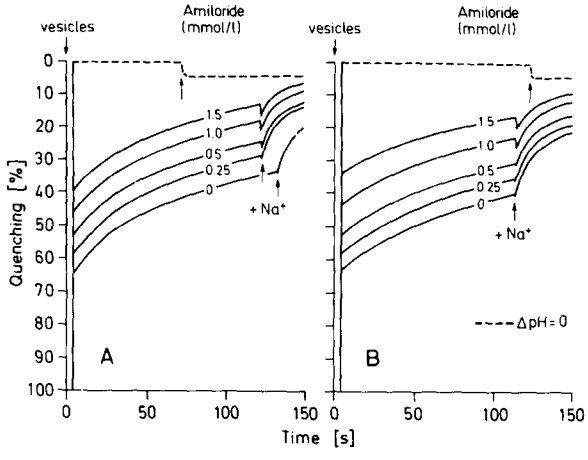

Fig. 4. Effect of amiloride on the dissipation of a transmembrane pH difference at low (A) and high (B) buffer capacities. A, Vesicles were preloaded with 299 mM mannitol, 150 mM tetramethylammonium gluconate, 10 mM Mes, 1 mM Hepes, pH 5.5, and diluted into buffer containing 6 μM acridine orange, 299 mM mannitol, 150 mM tetramethylammonium gluconate, 1 mM Mes, 10 mM Hepes, pH 8.0. B, Vesicles were preloaded with 200 mM mannitol, 150 mM tetramethylammonium gluconate, 100 mM Mes, 10 mM Hepes, pH 5.5, and diluted into buffer containing 6 μM acridine orange, 200 mM mannitol, 150 mM tetramethylammonium gluconate, 10 mM Mes, 100 mM Hepes, pH 8.0. The pH of the buffers was adjusted by titration with gluconic acid. The arrows indicate an addition of 50 μl 3 M NaCl into external buffer to collapse the pH gradient.

rates of dissipation of quenching were also unchanged by amiloride. These results indicate that amiloride did not affect transmembrane ΔpH, but instead interacted with acridine orange quenching.

In order to quantify this interaction, we evaluated the effect of 1 mmol/l amiloride on the initial quenching of acridine orange fluorescence at various preset transmembrane pH differences (Fig. 5). In the absence of amiloride, acridine orange fluorescence quenching showed an S-shaped dependence on transmembrane ΔpH. With amiloride, the curve is shifted to the right. Thereby, the effect of amiloride on fluorescence quenching depends on ΔpH being relatively bigger for small ΔpH.

pH-jump experiments with preset ΔpH of 2.5 units in the presence of increasing harmaline-concentrations (Fig. 6A and B) showed a decrease both in initial quenching and in the rate of dissipation of quenching. At high buffer capacity, the same concentration of harmaline was less effective than at low buffer capacity, suggesting that harmaline, in addition to an interaction with acridine orange quenching, can dissipate transmembrane pH gradients as a permeant weak buffer. Therefore, a quantitation of the harmaline effect on ΔpH-dependent fluorescence quenching is probably not possible.

An interaction of amiloride with acridine orange, as suggested from the pH-jump experiments, can be demonstrated by testing the fluorescence of acridine orange in aqueous solutions in the presence of various amiloride concentrations. Fig. 7 shows a concentration-dependent quenching of acridine orange fluorescence not only for amiloride but also for harmaline.

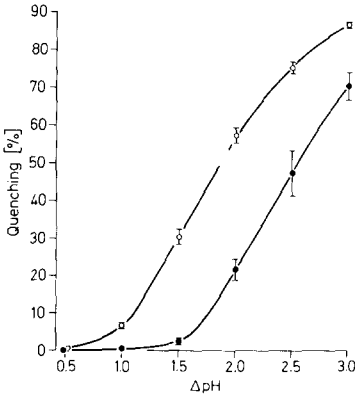

Fig. 5. The effect of amiloride on ΔpH-dependent quenching of acridine orange fluorescence. Vesicles were preloaded with 200 mM mannitol, 150 mM tetramethylammonium gluconate, 100 mM Mes, 10 mM Hepes, pH 5.5, and diluted into buffers containing 6 μM acridine orange, 200 mM mannitol, 150 mM tetramethylammonium gluconate and either 100 mM Mes, 10 mM Hepes, pH 6.0–7.0, or 10 mM Mes, 100 mM Hepes, pH 7.5–8.5, without (○) or with (●) 1 mmol/l amiloride. The pH of the buffers was adjusted by titration with gluconic acid. Each datum represents mean ±S.E. of three independent experiments.

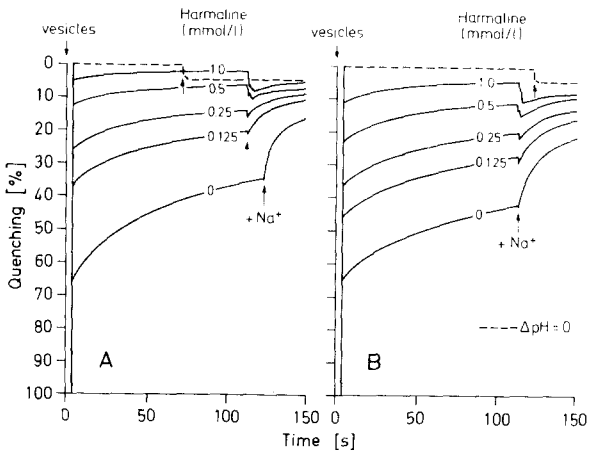

Fig. 6. Effect of harmaline on the dissipation of a transmembrane pH difference at low (A) and high (B) buffer capacities. Experimental details are given in the legend to Fig. 4.

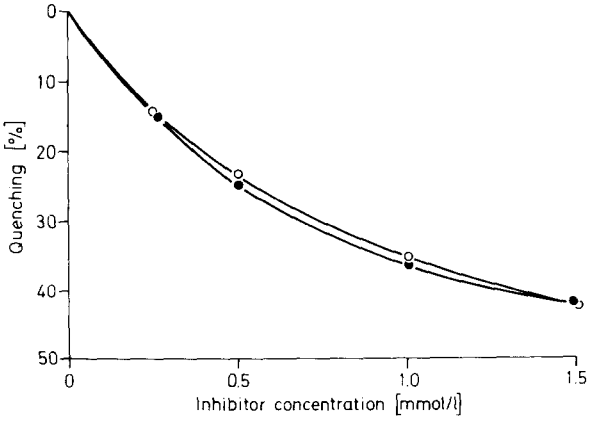

Fig. 7. Quenching of acridine orange fluorescence (6 μM acridine orange in water) in the presence of various concentrations of amiloride (●) and harmaline (○). Each point represents the mean of triplicate determinations; the S.E. values are within the symbols

Discussion

Recently it was claimed that amiloride dissipates a transmembrane ΔpH as a permeant buffer, thus simulating a specific inhibition of the Na^+/H^+ exchange [13]. Using ΔpH-dependent quenching of acridine orange fluorescence, we reevaluated the effect of amiloride and harmaline on Na^+/H^+ exchange in renal brush-border membrane vesicles.

In agreement with earlier observations [6–8], a quenching of acridine orange fluorescence was found in the presence of a Na^+ gradient (in > out). This quenching is due to an intravesicular acidification caused by the action of an electroneutral Na^+/H^+-exchange mechanism and electrically coupled Na^+ and H^+ movements through parallel conductance pathways ([7,8] and our own unpublished data). The Na^+ gradient-dependent quenching of acridine orange fluorescence was inhibited by amiloride, in accordance with the results of others [7,8], and also by harmaline. Both drugs were also effective when electrically coupled net Na^+ and H^+ movements were minimized in the presence of valinomycin and equal K^+ concentrations inside and outside the vesicles. Here, 1 mM amiloride was sufficient to block Na^+ gradient-dependent acridine orange fluorescence quenching completely; 1 mM harmaline inhibited it 92%. These results could indicate a specific interaction of both drugs with the electroneutral Na^+/H^+ exchanger, as also suggested by others [2,4,5,10], using different techniques.

However, a number of our observations show that the inhibition of Na^+ gradient-dependent acridine orange fluorescence quenching by amiloride and harmaline cannot be due only to an inhibition of Na^+/H^+ exchange. In agreement with the inhibition of nigericin-mediated Na^+/H^+ exchange in phospholipid vesicles by amiloride [13], we found an inhibition of gramicidin-induced Na^+/H^+ exchange in renal brush-border membranes by amiloride as well as by harmaline. The artificial cation-exchangers, nigericin and gramicidin, are not expected to be sensitive to amiloride and harmaline. Moreover, we found that these drugs were also effective in the complete absence of Na^+, i.e., conditions under which the intrinsic Na^+/H^+ exchanger does not operate [4]. Thus, amiloride and harmaline also inhibited K^+ gradient-dependent acridine orange fluorescence quenching in the presence of valinomycin ($[K^+_{in}] > [K^+_{out}]$). In these experiments an inside negative K^+ diffusion potential is created by valinomycin, which leads to an uptake of protons into the vesicles via proton-conductance pathways [7,8]. A faster proton uptake in the presence of an inside negative K^+ diffusion potential is observed after increasing the intrinsic proton conductance of the brush-border membrane by the uncoupler FCCP. Even under this condition, amiloride and, more effectively, harmaline inhibited the quenching of acridine orange fluorescence. These inhibitions are concentration-dependent, with the same I_{50} for the fluorescence signals of both electroneutral Na^+/H^+ exchange and proton uptake by conductance pathways, when harmaline is used. This suggests the same manner of action of harmaline, in both cases being nonspecific for the Na^+/H^+ exchanger. However, a smaller I_{50} of amiloride for Na^+/H^+ exchange than for conductive proton movements indicates, besides a nonspecific action, also a specific component in the inhibition by this drug.

Several reasons for the nonspecific effects of both drugs in acridine orange experiments can be considered. Firstly, both drugs could cross the membrane as cations dissipating Na^+ and K^+ diffusion potentials, as already suggested for harmaline by Alvarado et al. [12]. However, amiloride and harmaline inhibited Na^+ gradient-dependent acridine orange fluorescence quenching also in the absence of a membrane potential. Secondly, both drugs could act as permeant buffers, thus dissipating pH gradients [13]. As in our pH-jump experiments the inhibition of ΔpH-dependent acridine orange quenching by amiloride is independent of the buffer capacity, amiloride most likely does not dissipate a ΔpH as a permeant buffer. This conclusion is in disagreement with that of Dubinsky and Frizzell [13]. Instead, harmaline action depends on the buffer capacity and is, therefore, consistent with a dissipation of transmembrane pH gradient due to buffering. Thirdly, amiloride and harmaline could interfere with the ΔpH-dependent quenching of acridine orange fluorescence. This explanation seems to be the most plausible, because both drugs decrease

ΔpH-dependent acridine orange quenching in experiments with preset ΔpH even in the absence of Na^+. Acridine orange is less sensitive to ΔpH in the presence of amiloride and harmaline. Below a ΔpH of 1.3 no fluorescence quenching can be observed in the presence of 1 mmol/l amiloride. Unfortunately, the ΔpH developed by the electroneutral Na^+/H^+ exchange ranged between 1.1 and 1.3 units, and therefore its signal was completely abolished by the non-specific interaction of amiloride with dye quenching. The interpretation of harmaline effects on the electroneutral Na^+/H^+ exchange is even more complicated because of an additional dissipation of transmembrane pH differences. At present, the detailed mechanism by which both drugs decrease the sensitivity of acridine orange is unknown. A possible explanation is a direct interaction of both drugs with acridine orange itself, as indicated by a concentration-dependent quenching of acridine orange fluorescence in aqueous solutions.

In conclusion, acridine orange experiments with amiloride and harmaline should be interpreted with great caution as these compounds strongly interact with the dye, leading to an overestimation of the specific inhibition of the Na^+/H^+ exchange. In addition, harmaline dissipates the transmembrane pH gradients as a permeant buffer, an effect which compromises its use in all experiments with proton gradients.

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