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Effects of amiloride on Na^+ content and pinocytosis in *Entamoeba histolytica*

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Amiloride, a blocker of Na^+ leak and Na^+-H^+ exchange in animal cells, caused cells of *Entamoeba histolytica* to release Na^+ (up to 40% of their original Na^+ content within 90 min, at an amiloride concentration of 3 mM); K^+ content was not affected. By comparing the unidirectional uptake of $^{22}Na^+$ with that of the fluid-phase marker ^{125}I-labeled poly(vinylpyrrolidone) we established that the amiloride-induced Na^+ loss was due to inhibition of pinocytic Na^+ uptake rather than to blockage of an amiloride-sensitive transport system in the plasma membrane. Amiloride penetrated the cells, and both its intracellular concentration and its effect on pinocytosis increased with pH. The permeant weak base quinacrine similarly inhibited pinocytosis in a pH-dependent manner. We conclude that the effect of amiloride on pinocytosis and, consequently, on Na^+ content was due to its properties as a permeant weak base.

Introduction

We recently started to investigate cation transport pathways in the protozoan parasite *Entamoeba histolytica* [1]. From the effect of cytochalasin B, an inhibitor of pinocytosis, on the Na^+ content of the cells we concluded that (i) pinocytosis is an important component of the homeostatic system for Na^+, and (ii) the plasma membrane of *E. histolytica* must contain a Na^+'pump' [1]. However, we have no information yet on the nature of this pump (for instance, whether it is primary- or secondary-active), or on the existence of other Na^+ or K^+ pump- or leak-ways in the plasma membrane. In this framework we set out to investigate the effects of a range of transport inhibitors on cation homeostasis in the amoebae.

Amiloride, a diuretic drug, is a well-known inhibitor of conductive Na^+ entry in tight epithelia and of Na^+-H^+ antiport activity in leaky epithelia [2]. In the present paper, we report that in *E. histolytica* amiloride had a profound effect on Na^+ homeostasis. However, contrary to the situation in epithelia we found that this effect was not due to the inhibition of a trans-plasma membrane transport system for Na^+. Rather, amiloride blocked pinocytosis, apparently by acting as a permeant weak base. For animal cells, several other aspecific side-effects of amiloride have been related to its weak-base properties [3–5].

Methods and Materials

E. histolytica strain HM1:NIH was grown axenically at 36°C in TYI-S-33 medium [6] supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). After chilling, cells were harvested by centrifugation (10 min at 400 × *g*), and washed twice at room temperature in an incubation saline containing (mM): NaCl, 120; KCl, 40; $MgSO_4$, 1; $CaCl_2$, 1; ascorbic acid, 10; cystein, 6; sorbitol, 100; bis-Tris propane, 25, or Pipes, 10. Bovine serum albumin (Sigma Fraction

Abbreviations: ^{125}I-PVP, ^{125}I-labeled poly(vinylpyrrolidone); bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; Pipes, 1,4-piperazinediethanesulfonic acid.

V) was added at 1 mg/ml. pH was adjusted with either HCl or Tris to 6.8, unless indicated otherwise. This saline had approximately the same Na^+ and K^+ content and osmolarity as the growth medium [1], and allowed for a cell survival of at least 80% in incubations up to 90 min at 36°C (estimated with Trypan blue).

The washed cell pellet was suspended in incubation saline to a density of 20–50 μl packed cells/ml suspension. Na^+ and K^+ contents were determined by flame photometry as described in Ref. 1. Pinocytic activity was determined with either ^{125}I-PVP or horseradish peroxidase as fluid-phase markers as described in Ref. 1. For the simultaneous determination of unidirectional $^{22}Na^+$ influx and pinocytic activity, $^{22}Na^+$ and ^{125}I-PVP (both at 0.2–0.5 μCi/ml; Amersham) were added to the cell suspension, and uptake was started by transfer of the cells from room temperature to a 36°C shaker water bath. At the indicated time points, duplicate 0.2-ml aliquots were centrifuged and the cells were washed twice with 1 ml of a saline ('washing saline') containing (mM): $MgCl_2$, 100; sorbitol, 100 and Tris, 10, brought to pH 7.0 with HCl. Samples of the supernatant and the tubes containing the washed cell pellets were counted in a dual-channel Philips P4600 γ-counter.

Cell pellet water was determined with ^3H_2O, as in Ref. 1. Amiloride was the kind gift of MSD, München. It was added immediately before the start of the experimental incubation from a 0.3 M stock solution in dimethylsulfoxide; an equivalent amount of solvent was added to the control cells. The uptake of amiloride by the cells was determined fluorimetrically. To this end, cells were spun down at the indicated time points, washed twice with ice-cold washing saline, dissolved in Triton X-100 (1 mg/ml), the insoluble residue was removed by centrifugation, and the fluorescence was measured (λ_{ex}, 379 nm; λ_{em}, 422 nm) in an Amicon SPF-500 fluorimeter. Quinacrine dihydrochloride was purchased from Sigma, and dissolved in water.

Calculations. In *E. histolytica,* unidirectional $^{22}Na^+$ uptake takes place through two routes, a trans-plasma membrane route and a pinocytic route, the latter dominating under normal conditions (Ref. 1; see also Fig. 2). To assess directly the relative contributions of the two routes, we have, in Figs. 2 and 4, expressed both $^{22}Na^+$ uptake and ^{125}I-PVP uptake as ml/ml cells (referring to the pcked cell volume as determined with ^3H_2O at the start of the experiment). The pinocytic contribution to $^{22}Na^+$ uptake (in ml/ml cells) is given by the uptake of ^{125}I-PVP. Consequently, the trans-plasma membrane uptake of $^{22}Na^+$ (again in ml/ml cells) is equivalent to the difference between total $^{22}Na^+$ uptake and ^{125}I-PVP uptake. The absolute amount of Na^+ (in μmol/ml cells) unidirectionally taken up across the plasma membrane can be calculated as the product of this difference with the extracellular Na^+ concentration.

Differences between duplicates were less than 10% for the determination of ^3H_2O space and $^{22}Na^+$, ^{125}I-PVP or horseradish peroxidase uptake, and less than 5% for the determination of Na^+ and K^+ content.

Results and Discussion

As we have shown before [1], cells of *E. histolytica* in our synthetic saline were approximately in a steady state for Na^+ and K^+ (Fig. 1, open symbols; the 20% decrease in cation content over the 90-min incubation period can be largely accounted for by cell death, see Methods and Materials). Amiloride (3 mM) caused the cells to lose Na^+ specifically: after 90 min, the Na^+ content had dropped to 60% of the control value, whereas K^+ content of the amiloride-treated cells was equal to that of the control cells (Fig. 1, closed symbols).

Theoretically, this effect of amiloride on the steady state of Na^+ could be due to either an inhibition of Na^+ influx or a stimulation of Na^+ efflux. To decide which of these alternatives applied here, we measured the unidirectional $^{22}Na^+$ fluxes. As can be seen from Fig. 2 (triangles), amiloride strongly inhibited $^{22}Na^+$ uptake. This, and the fact that the drug decreased rather than stimulated $^{22}Na^+$ efflux (Fig. 3) argues for the inhibition, by amiloride, of Na^+ influx into the amoebae. This by itself would be in accord with the known effects of amiloride on Na^+ leak or exchange systems across the plasma membrane of animal cells [2].

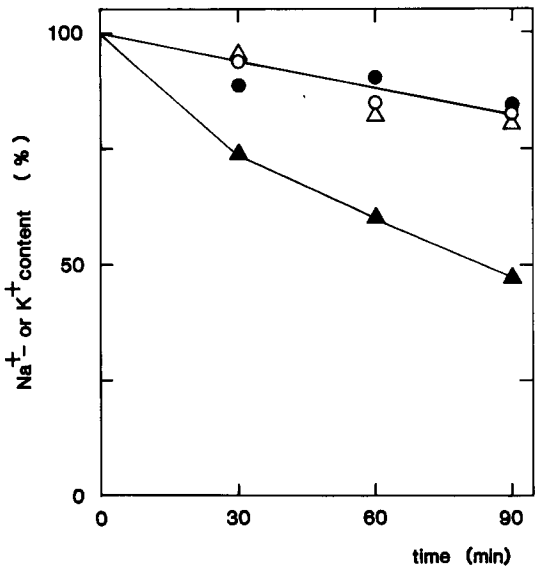

Fig. 1. Effect of amiloride on Na^+ and K^+ content of *E. histolytica*. Na^+ and K^+ content were determined by flame photometry as indicated in Methods and Materials. △, ▲, Na^+ content; ○, ●, K^+ content. Open symbols, controls; closed symbols, plus amiloride (3 mM). 100% values corresponded with 62 and 54 μmol/ml cells for Na^+ and K^+, respectively.

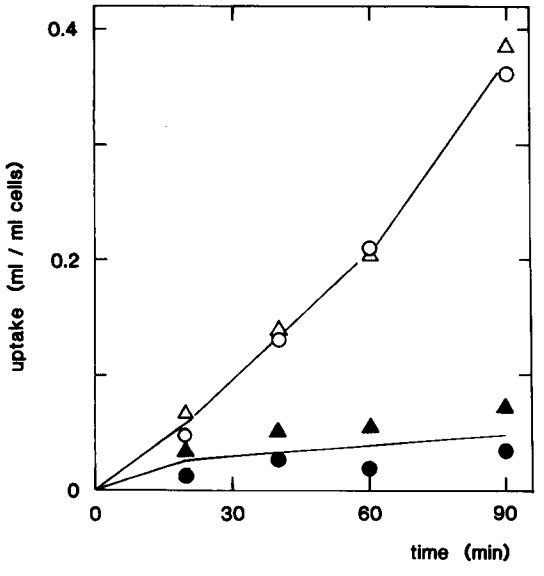

Fig. 2. Effect of amiloride on unidirectional Na^+ uptake and pinocytosis. The uptake of $^{22}Na^+$ and that of the fluid-phase marker, ^{125}I-PVP, were simultaneously followed as described in Methods and Materials. Both are expressed as ml/ml cells (see Methods and Materials, section Calculations). △, ▲, Na^+ uptake; ○, ●, ^{125}I-PVP uptake. Open symbols, controls; closed symbols, plus amiloride (3 mM).

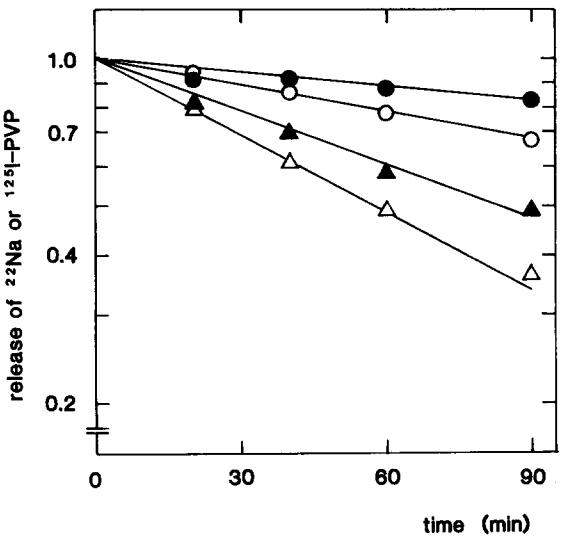

Fig. 3. Effect of amiloride on the release of $^{22}Na^+$ and ^{125}I-PVP from preloaded cells. Cells were preincubated in TYI-S medium for 2 h at 36°C with $^{22}Na^+$ (0.15 μCi/ml) and ^{125}I-PVP (0.3 μCi/ml), washed twice and then resuspended in incubation saline at 36°C. Duplicate samples were spun down at the indicated time points and the remaining radioactivity was determined as described for the uptake experiments (Methods and Materials). For symbols, see legend to Fig. 2; the amiloride concentration was again 3 mM. Radioactivity at $t = 0$ corresponded to 0.51 and 0.65 ml/ml cells for $^{22}Na^+$ and ^{125}I-PVP, respectively.

However, as can be appreciated from a direct comparison of the uptake of $^{22}Na^+$ with that of the fluid-phase marker, ^{125}I-PVP (Fig. 2), the amiloride-sensitive Na^+ uptake in the amoebae was pinocytic rather than transmembranous: virtually all of $^{22}Na^+$ uptake was accounted for by pinocytosis, and amiloride inhibited the latter process. Clearly, this experiment did not allow us to decide whether amiloride affected the transmembrane movement of Na^+ as well. To resolve better the transmembrane component, we substituted most of the Na^+ in the incubation saline by choline cation. The rationale for this is that the transmembrane uptake component, in contrast to the pinocytic component, is expected to exhibit saturation characteristics, which would enhance its relative contribution at low external Na^+ concentrations. Fig. 4A shows that, indeed, under those conditions the transmembrane uptake of $^{22}Na^+$ could be readily evaluated; it turned out to be completely insensitive towards amiloride (Fig. 4B).

Thus, the action of amiloride on Na^+ content was indirect: the drug affected pinocytosis and this, in turn, caused the cells to lose Na^+ specifically. This being established, two questions arise: first, how did amiloride inhibit pinocytosis, and second, why did inhibition of pinocytosis result in a specific Na^+ (as opposed to K^+) loss?

As to the first question, we reasoned that the answer might be that amiloride behaves as a permeant weak base [3–5], and that this category of compounds is known to inhibit pinocytosis in other cells [7]. The following observations confirm this notion. Fig. 5 shows that the effect of amiloride on pinocytosis increased with pH (pinocytosis itself between pH 5.5–7.5 was relatively pH-insensitive; results not shown). This suggests that the deprotonated, permeant form of the drug was the species that inhibited pinocytosis. In line with this, Fig. 6 shows that amiloride did permeate the cells, and that its rate of permeation and its intracellular accumulation increased with pH. Finally, an unrelated permeant weak base, quinacrine, similarly inhibited pinocytosis in a pH-dependent manner; for instance, at a concentration of 0.1 mM it

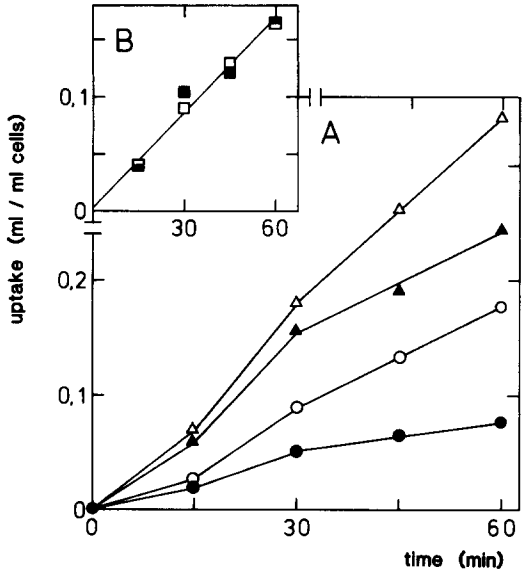

Fig. 4. Effect of amiloride on the transmembrane uptake of $^{22}Na^+$. Cells were washed and incubated in saline containing, instead of 120 mM Na^+ 3 mM Na^+ plus 117 mM choline cation (see text). (A) uptake of $^{22}Na^+$ and ^{125}I-PVP in the absence and presence of amiloride. For symbols, see Fig. 2; the amiloride concentration was 1 mM. (B) transmembrane uptake of $^{22}Na^+$ in the absence (□) and presence (■) of amiloride. The two sets of data in B were calculated from the data in A as the difference between the values for open triangles and circles and closed triangles and circles, respectively (see Methods and Materials, section Calculations).

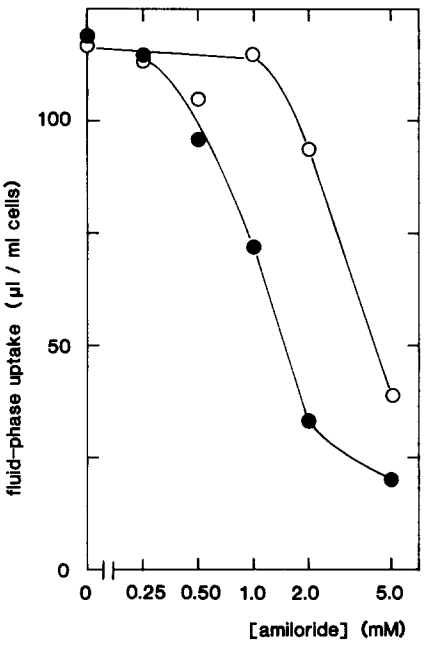

Fig. 5. Dose-response curves for the effect of amiloride on pinocytosis at pH 6.3 and pH 7.3. Cells were incubated for 90 min at 36°C with horseradish peroxidase (0.5 mg/ml) as a fluid-phase marker, and processed as indicated in Methods and Materials. ○, pH 6.3; ●, pH 7.3.

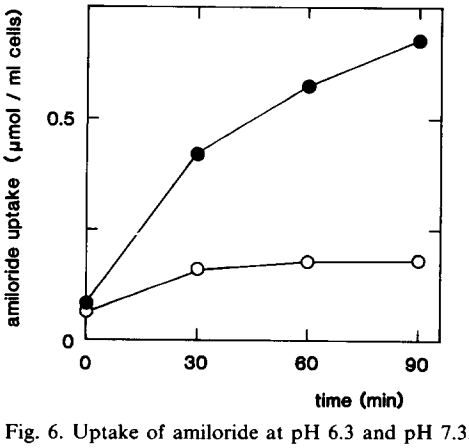

Fig. 6. Uptake of amiloride at pH 6.3 and pH 7.3. Cells were incubated at 36°C with 1 mM amiloride and spun down at the indicated time points. Amiloride content was determined fluorimetrically as described in Materials and Methods. ○, pH 6.3; ●, pH 7.3.

inhibited pinocytosis by 24% and 67% at pH 6.3 and pH 7.3, respectively.

Permeant weak bases presumably inhibit pinocytosis by accumulating in, and equilibrating the pH of, acidic subcellular compartments [7,8]. In *E. histolytica,* an acidic compartment has been visualized with the metachromatic vital stain acridine orange [9]. It appears as small (< 2 μm) vesicles that constitute at most a few percent of the total cell volume (see Ref. 9, Fig. 8). If, as seems reasonable to assume, amiloride specifically accumulates into this compartment, its concentration may well reach very high values there (up to tens of mM).

As to the second question: why the specific Na^+ loss? – we have previously reported [1] an analogous effect of cytochalasin B, a completely unrelated inhibitor of pinocytosis, on Na^+ content. Amiloride inhibited both pinocytosis (Fig. 2) and regurgitation (Fig. 3; the residual ^{125}I-PVP release in the presence of amiloride is probably due to cell death) as did cytochalasin B [1]. We therefore tend to explain the amiloride-induced Na^+ loss by the same model as that we proposed [1] previously to account for the effect of cytochalasin B. This model implies that pinocytically accumulated Na^+ leaves the cell by an in-series arrangement of an endosomal Na^+ leakway with a plasma membrane Na^+ pump. In other words, the amiloride-induced Na^+ loss would be transmembranous, and in the absence of the inhibitor would have been compensated for by ongoing pinocytic Na^+ uptake. The specificity for Na^+ (as opposed to K^+) would ultimately reside in the endosomal membrane compartment.

One final remark: in animal cells, amiloride in concentrations comparable to those employed here has been used to block Na^+-H^+ exchange [2]. Undoubtedly, amiloride will inhibit pinocytosis in those cells as well. Since fluid-phase uptake in animal cells (except macrophages) [10] is quantitatively insignificant compared to that in *E. histolytica,* this will probably not affect the interpretation of the data under most circumstances. Even so, the effect of amiloride on pinocytosis should be added to the list [3–5] of aspecific actions of the drug, and is one further reason to switch to the recently developed high-affinity inhibitors of Na^+-H^+ exchange [11].

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References

1 Bakker-Grunwald, T., Löhden, U. and Trissl, D. (1985) Biochim. Biophys. Acta 815, 170–174
2 Benos, D.J. (1982) Am. J. Physiol. 242, 131–145
3 Krulwich, T.A. (1983) Biochim. Biophys. Acta 726, 245–264
4 Dubinsky, W.P., Jr. and Frizzell, R.A. (1983) Am. J. Physiol. 245, 157–159
5 Davis, R.J. and Czech, M.P. (1985) J. Biol. Chem. 260, 2543–2551
6 Diamond, L.S., Harlow, D.R. and Cunnick, C.C. (1978) Trans. R. Soc. Trop. Med. Hyg. 72, 431–432
7 Dean, R.T., Jessup, W. and Roberts, C.R. (1984) J. Biochem. 217, 27–40
8 Ohkuma, S. and Poole, B. (1978) Proc. Natl. Acad. Sci. USA 75, 3327–3331
9 Aley, S.B., Cohn, Z.A. and Scott, W.A. (1984) J. Exp. Med. 160, 724–737
10 Steinman, R.M., Mellman, I.S., Muller, W.A. and Cohn, Z.A. (1983) J. Cell Biol. 96, 1–27
11 Vigne, P., Frelin, C., Cragoe, E.J. and Lazdunski, M. (1983) Biochem. Biophys. Res. Commun. 116, 86–90