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THE EFFECT OF AMILORIDE ON SODIUM AND POTASSIUM FLUXES IN RED CELLS

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SUMMARY

1. The effect of amiloride on the influx and efflux of ^{24}Na and ^{42}K in red cells was studied. The drug was added to the bathing Ringer or else incorporated in resealed ghosts.

2. Amiloride does not inhibit the active or the passive (ouabain insensitive) extrusion of ^{24}Na.

3. Amiloride inhibits the influx of ^{24}Na into red cells by 70 %.

4. Whether added to the inside or to the outside of the cells amiloride has no effect on the efflux of ^{42}K.

5. Amiloride does not modify the uptake of ^{42}K from control Ringer. This uptake is strongly inhibited by the removal of Na. Amiloride has no effect on the extent of this inhibition.

6. It is concluded that amiloride specifically inhibits the passive penetration of Na, and has no effect on the Na-K-pumping mechanism. However, at the concentration which inhibits 70 % of the influx, amiloride fails to produce an observable effect on the ouabain-insensitive Na-efflux.

7. On the basis that the information obtained could be extrapolated to other membranes, the effect of amiloride on epithelial membranes is discussed.

INTRODUCTION

Amiloride is a diuretic substance that produces elimination of Na without significantly affecting the excretion of K (Baer, Jones, Spitzer & Russo, 1967). Studies carried out on isolated epithelia *in vitro* have shown that this substance inhibits the translocation of Na across the outer barrier of the epithelium if added to the outer bathing solution, i.e. by acting on the same side that Na uses to gain access to the translocating

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mechanism (Eigler, Kelter & Renner, 1967; Crabbé & Ehrlich, 1968; Bentley, 1968; Herrera, 1972). The nature of the mechanism inhibited by amiloride at this level is not known. This uncertainty stems from our ignorance of the mechanism used by Na to cross the outer barrier. The study of epithelia mounted between two Ringers with 115 mM-Na led to the opinion that, in order to penetrate into the epithelium, Na could use a passive mechanism (Fig. 1*a*). Amiloride would thus act on a passive mechanism. This is in keeping with the observation that enzymes such as ATPase, adenylcyclase and carbonic anhydrase are not inhibited by amiloride (Baer *et al.* 1967). However, recent observations have shown that Na penetration across the outer barrier is not only inhibited by amiloride added to the outer bathing solution, but also by ouabain added to the inside (Cereijido, Moreno, Rodriguez & Rotunno, 1971; Biber, 1971; Moreno, Reisin, Rodriguez, Rotunno & Cereijido, 1972). Since ouabain is known to be a highly specific inhibitor of the active transport of Na, its effect on the penetration of Na might be taken as an indication that the outer barrier possesses an inward orientated pump (Fig. 1*b*). Therefore, if one had more information on the sort of mechanism acted upon by amiloride, it would not only clarify the effect of this increasingly useful drug, but would also cast light on the mechanism of Na translocation across epithelial membranes. In the present paper the effect of amiloride was assayed on the movement of sodium and potassium in the red cell. This preparation was chosen because the addition of amiloride to the bathing Ringer, or its incorporation into resealed ghosts, offer the possibility of testing the effect of this drug on the same side used by Na to penetrate the membrane, i.e. a passive movement when entering, and mainly active step when leaving the cell. It is shown that amiloride stops the passive penetration of Na into the red cell without directly affecting the active extrusion of Na or the movement of K. On the assumption that the nature of the effect of amiloride is essentially the same in other cell membranes, a discussion of its activity on epithelia is made.

METHODS

Preparation of resealed ghosts. This method is essentially the one used by Glynn & Hoffman (1971). Freshly drawn human blood was used. Coagulation was prevented with heparin (15 mg/100 ml.). The cells were lysed by squirting 6 ml. of packed cells, obtained by spinning at 0° C, 5 min at 27·000 *g*, into 100 ml. ice-cold haemolysis solution contained in Erlenmeyer flasks and vigorously stirring with magnetic stirrers. The haemolysis solutions contained 2 mM-ATP, 3 mM-$MgCl_2$ and enough NaCl to give a concentration of Na 6 mM. The pH was 7·4. After the haemolysates had stood at 0° C for 5 min isotonicity was restored by the addition of 2·75 ml. 4 M-KCl to each flask. When cation efflux was to be studied ^{24}Na or ^{42}K was added before increasing the tonicity. Also, in cases where the effect of amiloride (*n*-amidino, 3,5-diamino-6-chloropyrazine carboxamide) within the cells was tested, this drug was

added to the haemolysate (final concentration 10^{-4} M) before increasing the tonicity. Experiments were run to insure that with this procedure amiloride achieves a concentration around 10^{-5} M. These experiments are described below. The flasks were transferred to a water-bath at 37° C and gently shaken for 40 min. At the end of this period the ghosts were centrifugated at 5° C and washed, four to five times in 50 ml. polypropylene centrifuge tubes with an ice-cold solution containing 151 mM-NaCl, 2 mM-$MgCl_2$ and 17 mM-Tris (pH 7·5 at room temperature). Between each wash the ghosts were spun for 5 min at 27,000 *g*.

Na or K efflux. Suspensions of ghosts preloaded with tracer were prepared by adding about 0·2 ml. of ghosts to 30 ml. of different incubation media in 50 ml. Erlenmeyer flask. The final haematocrit during incubation was around 0·7 %. The incubation medium contained (mM): 141 NaCl; 2 $MgCl_2$; 17 Tris (pH 7·5 at room temperature); 10 KCl. The flasks were then transferred to a water-bath and shaken at 37° C. Samples were withdrawn periodically and immediately centrifugated for 5 min at 18,000 *g*. The radioactivity in the supernatant in each sample was compared with that in an equal volume of suspension. For each set of experimental conditions a graph of tracer loss *vs.* time was extrapolated to zero time and the amount of tracer that was extracellular at the beginning of the incubation was thus measured. This initial loss was subtracted from the measured losses, and the fraction of the initial intracellular radioactivity that remained inside the cells at each time was calculated, and plotted on a logarithmic scale against time. The rate constant for the efflux was thus calculated.

K uptake. Red cell ghosts with or without amiloride and with or without NaCl present in the solution, resealed as described above, were added to 30 ml. of different incubation media. ^{42}K was then added to the bathing solution, the flasks were transferred to a water-bath at 37° C and gently shaken. 5·0 ml. samples were withdrawn periodically and spun at 0° C, 5 min at 27,000 *g*. The ghosts were washed four times with about 50 volumes of ice cold, unlabelled Ringer solution. The radioactivity and nitrogen content of the samples were measured.

Na uptake. Cells were washed three times in ice-cold Ringer solution, the supernatant and buffer coat were removed by aspiration. The washed red cells were suspended in Ringer solution (final haematocrit 1 %), ^{24}Na was added, the flasks were transferred to a water-bath at 37° C and gently shaken. Samples of 5·0 ml. were withdrawn periodically and spun at 0° C for 5 min. The cells were washed four times with about 50 volumes of ice-cold unlabelled Ringer solution. The activity of ^{24}Na was then measured.

Measurement of radioactivity. ^{24}Na and ^{42}K were measured using a Nuclear Chicago Auto Gamma counter set as spectrometer. All samples were counted for 10 min.

Fluorescence measurements. The fluorescence of the amiloride molecule was measured in a Farrand Spectrofluorometer (Farrand Optical Co., Inc. New York). The excitation wave-length, 368 nm, was selected by a monochromator and a Wratten glass filter (379 nm), from the emission of a xenon light source. The emitted light was filtered (374 nm) and received in a second monochromator.

Sources of material. ^{24}Na and ^{42}K was obtained from the Atomic Energy Commissions of Mexico and Argentina. Adenosintriphosphate and Tris were from Sigma Chemical Co. (St Louis, Mo), amiloride from Merck, Sharp & Dome (West Point, Pa.); choline chloride was purchased from Eastman Organic Chemicals (Rochester, N.Y.) and inorganic salts were of 'analytical reagent' grade.

The concentration of amiloride inside the resealed ghosts. This series of experiments was run to check whether amiloride is trapped within the ghosts and achieves there an appreciable concentration. (*a*) *Ghosts with amiloride added before resealing.* They were rehaemolysed with distilled water, treated with $ZnSO_4$ and $Ba(OH)_2$ and the

intensity of the fluorescence of the supernatant was measured. The amount of water originally contained in the cells was calculated through the difference between wet and dry weight in an aliquot of the cell suspension. The final concentration of amiloride in the supernatant was obtained by comparing the intensity of the sample with a standard curve of amiloride solutions at several concentrations. With this data the concentration of amiloride in the intracellular water was $0{\cdot}9$–$1{\cdot}3 \times 10^{-5}$ M (three determinations). (*b*) *Ghosts with amiloride added after resealing.* These cells were incubated with Ringer containing 10^{-4} M amiloride. They were then washed five times with ice-cold solutions as described above and treated as the ghosts in the preceeding group. The spectra indicated that the amount of amiloride contained in these cells was at least two orders of magnitude smaller than in the ghosts resealed in the presence of amiloride. The comparison of the two groups indicates that the amiloride not trapped inside the resealed cells is washed away and, conversely, when it is added before resealing, it is effectively retained inside the cells at a suitable concentration level.

RESULTS

Na efflux

The efflux of Na was studied in five groups of ^{24}Na-loaded ghosts: (1) *control ghosts*, which consists of resealed ghosts loaded, resealed and incubated as described in the Methods section; (2) *ghosts containing amiloride:* in this case amiloride was incorporated before resealing but was not present in the incubating Ringer solution, (3) *control ghosts resealed in the absence of amiloride* but in contact with this drug during the incubation. The results of these three groups are shown in Fig. 2. The experimental points can be fitted by essentially the same exponential curve with a rate constant of $0{\cdot}72$ h^{-1}; (4) *control ghosts with ouabain* added to the incubating Ringer ($7{\cdot}5 \times 10^{-5}$ M). The purpose of this group was twofold. First it was to check that the absence of effect of the amiloride may not be ascribed to an exaggerated leakiness and/or damage of the transporting mechanism of the ghosts which might have been produced during the experimental procedure. Secondly, to inhibit with ouabain the active component of the Na extrusion, so any effect that the amiloride might have on the passive movement would become more noticeable; (5) *ghosts containing amiloride and inhibited with ouabain.* The results of groups 4 and 5, which are shown in Fig. 2, can be fitted by the same exponential curve. The rate constant is $0{\cdot}102$ h^{-1}, i.e. much lower than the control, indicating that the outflux of Na from the resealed ghosts is due, in large part, to a pumping mechanism. The general conclusion from the results with the five groups of ghosts shown in Fig. 2 is that amiloride does not seem to affect the active nor the passive extrusion of Na. This observation is in keeping with the results obtained by Baer *et al.* (1967) showing that amiloride does not inhibit the activity of enzymes such as ATPase, adenylcyclase, or carbonic anhydrase. Dunn (1970) has reported that amiloride, at a concentration 100-fold

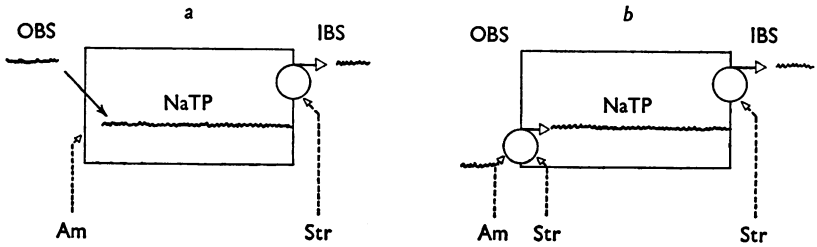

Fig. 1. Transcellular models of Na transport across epithelial membranes. OBS: outer bathing solution. IBS: inner bathing solution; NaTP: Na transporting pool. Am: amiloride; Str: ouabain. The wiggly line represents the level of the concentration of Na. In model *a* Na diffuses passively across the outer barrier and is pumped across the inner border. In model *b* Na is pumped across the outer border from a solution with low concentration of Na. In the first case amiloride stops a passive mechanism. In the second case amiloride inhibits a pump.

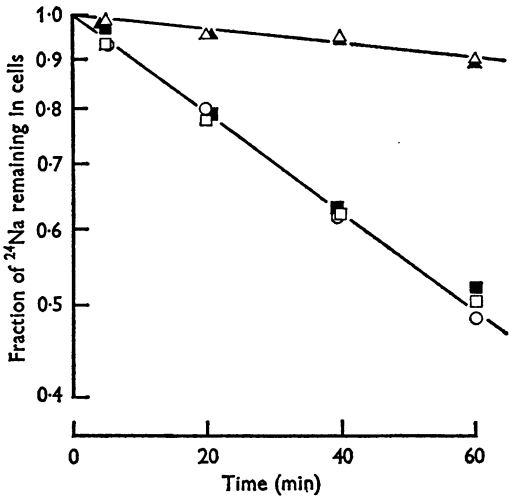

Fig. 2. Efflux of Na from red cell ghosts incubated at 37° C. The upper line describes the efflux in the presence of ouabain ($7{\cdot}5 \times 10^{-5}$ M) both in the absence (△) and in the presence (▲) of amiloride (10^{-5} M) *inside* the cells. The lower line corresponds to the efflux under control conditions (○), with amiloride *inside* (□), and with amiloride *outside* (■) the cells.

higher than the one used in the present work (10^{-3} *vs.* 10^{-5} M used here), exerts no effect on Na efflux when it was used in the presence of ouabain, although it reduced sodium efflux by some 20 % (three studies) when it was used in the absence of ouabain. The effect was not noticed at the lower concentration used in the present work (10^{-5} M). As shown below this concentration produces a profound (70 %) inhibition of the influx of Na. Also

the possibility exists that the decrease in the outflux observed by Dunn (1970) was a consequence of the Na depletion inside the ghost produced by the reduction of the influx.

Sodium influx

Fig. 3 shows the penetration of Na into two groups of red cells as a function of time. As judged by the difference in the initial slope of the curves, the addition of amiloride (10^{-5} M) to the bathing medium inhibits the penetration of Na by 70 %.

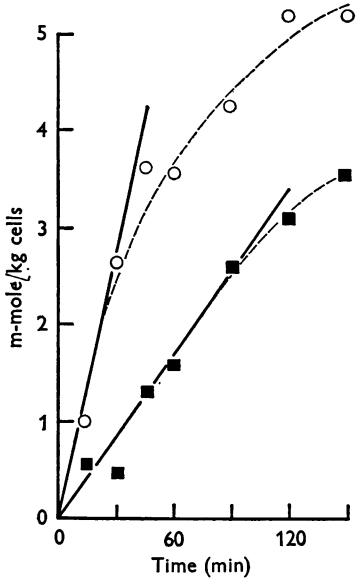

Fig. 3. Influx of Na in red cells incubated at 37° C under control conditions (○) and with amiloride (10^{-5} M) added to the bathing medium (■).

Potassium efflux

Fig. 4 shows the loss of ^{42}K from resealed ghosts incubated in control Ringer. One of the groups is control, a second group contains amiloride inside the ghosts, and the third group is in contact with amiloride added to the bathing solution. In the three cases potassium efflux follows first order kinetics for at least 50 min. The same curve can fit the three groups of experimental results. It is clear that amiloride whether added to the inside or to the outside of the cell has no effect on the efflux of ^{42}K.

K influx

The effect of amiloride on the uptake of ^{42}K by red cell ghosts incubated under control conditions and in the absence of Na is shown in Fig. 5. In the

last case isotonicity was maintained with choline. The fact that the experimental points in the control (circles) and amiloride treated ghosts (squares) can be fitted by the same experimental curve, indicates that amiloride, at the same concentration that inhibits 70 % of the Na influx (Fig. 3), does not modify the uptake of ^{42}K. The substitution of Na by choline severely

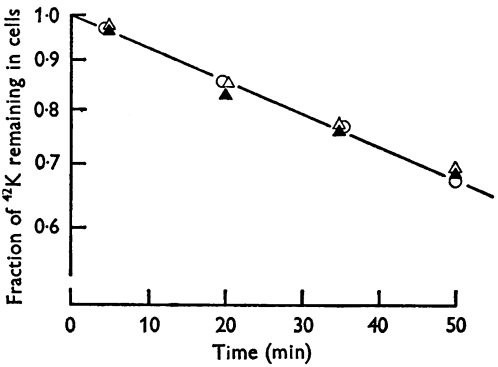

Fig. 4. Efflux of K from red cell ghosts. Control (○); with amiloride (10^{-5} M) inside the ghosts (▲) and with amiloride added to the bathing solution after resealing and before the start of the washout of ^{42}K (△).

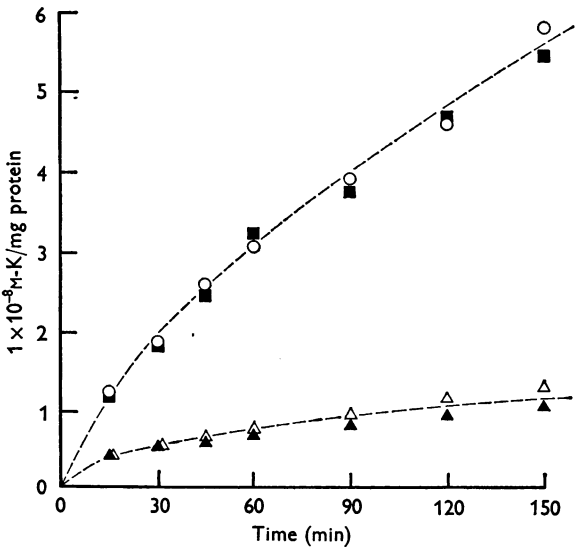

Fig. 5. Uptake of K in red cell ghosts. *Upper curve:* control (○) and amiloride (10^{-5} M) (■). *Lower curve:* ghosts resealed and incubated without Na in the medium. The group marked (▲), contains amiloride inside the ghost. In the group marked (△), which was resealed in the absence of amiloride, the drug was added to the incubating medium after resealing and just before adding ^{42}K.

decreases the influx of ^{42}K (Fig. 5, lower curve). This inhibition is not different when amiloride is present inside (full triangles) or outside (open triangles) the ghost.

DISCUSSION

The fact that once resealed, the ghosts can retain amiloride at a suitable concentration, together with the observation that it does not inhibit the active extrusion of Na, indicates that it does not prevent the access of Na to a pump, nor inhibits the activity of the pump itself. The pump was insensitive to amiloride regardless of whether this drug was added to the outside or to the inside of the cells. Amiloride did not impair the effect of ouabain. This is in keeping with the lack of effect of amiloride on ATPase activity (Baer *et al.* 1967). The (passive) flux remaining after the addition of ouabain is not sensitive to amiloride at the concentration 10^{-5} M. At this concentration amiloride produces a 70 % inhibition of the influx of Na. The absence of effect on the outflux would indicate that the ouabain-insensitive component of the outflux might not be a truly passive process (Hoffman & Kregenow, 1966).

Most of the efflux of K as well as the influx of Na are considered to be passive processes (see Garrahan, 1970). Amiloride affects the permeability to Na without affecting the permeability to K. These observations agree with the natriuretic, but K-sparing, action of amiloride. This could be due, in principle, to the existence of two kind of channels, only one (the Na one) being blocked by amiloride. Another possibility is the following. As the effective field of a site is reduced, the affinity of the site shifts to a lower order of selectivity in which the ratio Na-over-K permeability decreases (Eisenman, 1962). Amiloride, a molecule with net positive charges, could shield the field of the sites in the membrane producing a decrease of Na permeability. However, no increase of the permeability of K was observed (Figs. 4 and 5).

As mentioned in the Introduction, the net movement of Na across epithelial membranes is generally interpreted on the basis of the model depicted in Fig. 1*a*. This model envisages Na penetration across the outer border as a passive step. Amiloride acts at the level of the outer barrier (Biber, 1971; Moreno *et al.* 1972). The existence of a passive step at this level would be in keeping with the present results on the effect of amiloride. Yet several observations, which are not accounted for by this model, lead to the assumption that a Na pump is located at the outer border (Fig. 1*b*). These observations may be summarized as follows. (1) The frog skin, for instance, can transport a net amount of Na from an outer solution with 1 mM-Na, in spite of the fact that the whole epithelium and the epithelial cells have a much higher concentration of Na (Cereijido, Reisin & Rotunno,

1968; Aceves & Erlij, 1971; Cereijido, Moreno, Reisin, Rodriguez, Rotunno & Zylber, 1972). (2) Ouabain inhibits the penetration of Na at this level (Cereijido *et al.* 1971; Biber, 1971; Moreno *et al.* 1972). (3) The model of Fig. 1*a* assumes that the Na-transporting pool, contained between the step inhibited by amiloride and the step inhibited by ouabain, is constituted by the Na contained in, at least, one layer of cells. However, recent studies by Moreno *et al.* (1972) demonstrated the absence of a sodium transport pool with these characteristics. These observations would be consistent with the presence of a pump at the outer border as depicted in Fig. 1*b*. However, the observations of the present study indicate that amiloride does not inhibit a Na-K-pump, and (as long as this effect can be extrapolated to other membranes) this suggests that the model in Fig. 1*b* might not be correct either. A required modification may be that a passive diffusion process controls access to the pump, with the former being rate limiting.

Another consideration that can be made with regard to epithelial membranes is based on the observation of Biber, Aceves & Mandel (1972) that Na transport across the epithelium is related to K transport from the inner solution to the cell, but when Na transport is inhibited by amiloride this does not modify the pumping of K. This may indicate that the inhibition of Na transport produced by amiloride, although exerted on a passive step, has the peculiarity of breaking the linkage between the Na and K pumps, so that the pump keeps translocating K. This has raised the possibility that one of the properties of amiloride would be to replace Na at the Na-K pump. Yet, if the present results obtained with red cells are valid in epithelial cells, such replacement is unlikely as it is demonstrated (Fig. 5) that amiloride does not exempt K transport from the inhibition produced by the removal of Na.

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