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AMILORIDE FLUXES ACROSS ERYTHROCYTE MEMBRANES

DALE J. BENOS [a], JUAN REYES [a] and DAVID G. SHOEMAKER [b]

[a] *Department of Physiology and Biophysics, Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, 45 Shattuck Street, Boston, MA 02115, and* [b] *Department of Physiology, Yale University School of Medicine, New Haven, CT 06510 (U.S.A.)*

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Amiloride is known to inhibit both the influx of Na^+ and the activation of mitogensis in many cultured cell lines. This paper describes experiments in which the permeability coefficient of amiloride was determined from measurements of tracer fluxes across human erythrocytes and resealed ghosts. From an analysis of these fluxes, a permeability coefficient of 10^{-7} cm/s for the uncharged form of amiloride was deduced. Based upon this measured permeability value, we present calculations of intracellular accumulation times of amiloride in cells of differing surface-to-volume ratio.

Introduction

Amiloride-sensitive sodium transport systems have been described in many different biological tissues obtained from nearly every phylum of the animal kingdom (see Benos [1] for a review). 5 years ago, it was generally accepted that amiloride at very low concentrations (under 10^{-4} M) could only block the Na^+ entry channel of the apical membrane of electrically high-resistance epithelia. However, numerous reports have appeared describing amiloride-inhibitable Na^+-transport systems in cells and even low-resistance epithelia, including fibroblasts [2–5], neuroblastoma [6,7], muscle [8], hepatocytes [9–12], gallbladder [13,14] and renal proximal tubules [15]. These latter Na^+ transport systems differ from the former in two major respects. First, their sensitivity to amiloride is much lower (50% inhibition occurring at amiloride concentrations greater than 1 μM) and second, the mechanism of transport being an electroneutral ionic exchange system (e.g., Na^+-H^+, Na^+-Na^+, or Na^+-Ca^{2+}) rather than a conductive ionic channel mechanism. These exchange systems have been implicated in cell volume regulation [7], growth [4,5,11], and differentiation [16].

The proposal that increased Na^+ influxes activate quiescent cells by initiating DNA synthesis with subsequent increases in protein synthesis and mitosis is supported by the observation that amiloride prevents mitogenesis [3,11,17]. One of the initial events in this triggered mitotic sequence is the functional appearance of an amiloride-sensitive Na^+-H^+ or Na^+-Ca^{2+} exchanger. Since the inhibitory effect on Na^+-cation exchange processes is routinely assessed using millimolar concentrations of amiloride and in some cases exposure times of hours or more, the possibility exists that amiloride can enter cells and exert additional effects, especially since this drug is a weak base. Indeed, amiloride (0.1–1 mM) has been shown to inhibit (Na^+-K^+)-ATPase activity of isolated renal proximal tubules [18], renal kallikrein enzymatic activity [19], and protein synthesis in hepatocytes and cell-free reticulocyte lysates [9,12,17].

Although several authors have demonstrated that amiloride can be taken up by cells [10,12,16,20], none has provided a quantitative measure of flux rate. We have determined the permeability coefficient of amiloride from mea-

surements of unidirectional amiloride influx and efflux in human intact red blood cells and resealed ghosts. We show that the uncharged form of amiloride can permeate biological membranes and, based upon its measured permeability coefficient, we present calculations of equilibration times within cells of differing surface-to-volume ratios. We further discuss situations in which the protonated moiety of amiloride may also cross membranes, leading to a net intracellular accumulation of drug.

Materials and Methods

Red cell experiments

Freshly drawn and heparinized human blood was washed five times with ice-cold, phosphate-buffered medium; the buffy coat was removed by aspiration after the first and second washes. This medium consisted of 140 mM NaCl buffered to pH 7.4 with KH_2PO/K_2HPO_4 (2.5 mM). Cells were spun for approximately 5 min at $1500 \times g$ in a Sorvall RC-5B refrigerated centrifuge. Intact erythrocytes were transferred to a 100 ml Erlenmeyer flask and suspended in control medium to a final hematocrit of 20%. The cells were then incubated for 3 h at 37°C with constant gentle agitation in a water bath prior to flux measurements. The control medium consisted of the following (in mM): NaCl (145); $CaCl_2$ (1.5); $MgCl_2 \cdot 6H_2O$ (1.0); KH_2PO_4 (2.0); K_2HPO_4 (0.5); Mops (10). This solution was buffered to pH 7.4 at 37°C with Tris base. Resealed red-cell ghosts were prepared essentially as described by Passow and coworkers [21]. The ghosts were prepared in antifreeze-cooled, jacketed glass flasks maintained at -0.5 ± 0.5°C. 12 ml of a 45–47% red-cell suspension were added to 912 ml of hemolysing solution (4 mM $MgCl_2$/0.1 mM EGTA (pH 3.6–4.0)) and allowed to incubate for 10 min at pH 6.0. Ghosts were then concentrated by centrifuging for 10 min at $30000 \times g$ and resuspending in 33 ml of hemolysis solution containing exactly $5.0 \cdot 10^{-5}$ M [^{14}C]amiloride (spec. act. 1.67 μCi/μmol). 3 ml of a 2 M NaCl/Tris-OH solution was added to restore tonicity and bring the pH to 7.2. The ghosts were then incubated for an additional 10 min at 0°C, and for 60 min at 37°C for resealing. Following resealing, the ghosts were centrifuged at $27000 \times g$ for 5 min, and the supernatant assayed for hemoglobin by absorbance measurements using a Gilford 300-N spectrophotometer. The packed ghosts were suspended in an equal volume of supernatant, and layered onto a 25 ml 43% sucrose cushion (50 mM NaCl/25 mM Tris-HCl (pH 7.2) at 0°C). The ghosts were spun on the cushion for 45 min. Those ghosts remaining on the top of the cushion were harvested and resuspended in 40 ml of phosphate-buffered saline, and washed three times by repetitive centrifugations (5 min at $27000 \times g$). After the final wash, ghosts were kept on ice until use.

Transport assays

After the initial preincubation period, the intact erythrocytes were washed four times with 60 vol. of ice-cold control solution. After the final wash, the cells were tightly packed and most of the supernatant was removed by aspiration. To initiate the experimental flux, cells (0.5 ml) were transferred to 6 ml of prewarmed control medium, containing [^{14}C]amiloride (1.67 μCi/μmol spec. act.; final concentration, $2 \cdot 10^{-4}$ M), and [^{3}H]poly(ethylene glycol) ($M_r = 4000$) as an extracellular space marker. Final hematocrit ranged from 5–8%. At appropriate time intervals (15–30 min), duplicate 0.5 ml samples were taken.

Samples were immediately placed into 40-ml centrifuge tubes containing 3 ml of dibutylphthalate and 35 ml of ice-cold, isotonic $MgCl_2$. The cells, upon centrifugation, passed through the phthalate leaving the incubation medium behind. In some experiments, the $MgCl_2$ wash solution contained $2 \cdot 10^{-4}$ M unlabeled amiloride. After aspiration of the aqueous medium above the phthalate, the inside of each tube was washed three times with the isotonic $MgCl_2$ solution, and the cells were lysed by addition of 3 or 4 ml 15 mM $LiNO_3$ containing 0.07% Acationox. The volume of this hemolyzing solution delivered was determined by weight. Two 1 ml aliquots were counted for radioactivity using a Beckman liquid scintillation counter, Model 6800. Three 20 μl samples of supernatant were also measured for ^{14}C and ^{3}H activity prior to cell addition and just after the conclusion of the experiment. All counts were corrected for quench and converted to disintegrations per minute (dpm). Extracellular [^{14}C]amiloride was calculated by multiplying the

total ^3H dpm in the cell sample by the $^{14}C/^3H$ ratio of the medium. Cellular uptake of amiloride was calculated from the total [^{14}C]amiloride dpm in the cells after subtraction of the extracellular ^{14}C dpm. All calculations and analytical procedures have been described before [22].

Amiloride efflux measurements

[^{14}C]Amiloride effluxes were measured from previously loaded red cell membrane ghosts, essentially as previously described [21,23]. After washing, 1 ml of packed ghosts was added to 40 ml of control medium at 37°C. Duplicate 1 ml samples were taken with time, placed into centrifuge tubes and centrifuged at $33\,533 \times g$ for 5 min at 0°C.

Cell number was routinely measured in all flux experiments by counting in a Coulter Counter. Efflux rate coefficients were determined from the slope of the plot of $-\ln(1-[(\text{cpm})_t/(\text{cpm})_\infty])$ versus time, where $(\text{cpm})_t$ represents all the counts of [^{14}C]amiloride appearing in the medium at time t, and $(\text{cpm})_\infty$ is the equilibrium number of ^{14}C cpm in the medium (measured 3 h after the start of the experiment).

Results

The uptake of [^{14}C]amiloride by human red blood cells as a function of time is presented in Fig. 1. At pH 7.4 and 37°C (Fig. 1A), the uptake of amiloride by human erythrocytes was linear only for the first 15–30 min; thereafter, the uptake curve tended towards saturation. The solid lines in Fig. 1 were drawn according to Eqn. 1 (see Discussion). Using the linear portion of the uptake curve, the unidirectional influx of ^{14}C of amiloride was 81.5 μmol amiloride/l red blood cells per h. Since the surface area of 1 liter of packed human red cells is $1.69 \cdot 10^7$ cm^2 [23], this flux can be expressed as $1.31 \cdot 10^{-15}$ mol/cm^2 per s. The total permeability coefficient for amiloride was thus $6.6 \cdot 10^{-9}$ cm/s.

Assuming that only the nonionic (i.e., uncharged) form of amiloride crosses the membrane and that the $pK = 8.7$ [1], we recalculated the permeability coefficient to nonionic amiloride in these influx experiments to be $1.37 \cdot 10^{-7}$ cm/s. We also measured the influx of amiloride into intact human red cells equilibrated and suspended

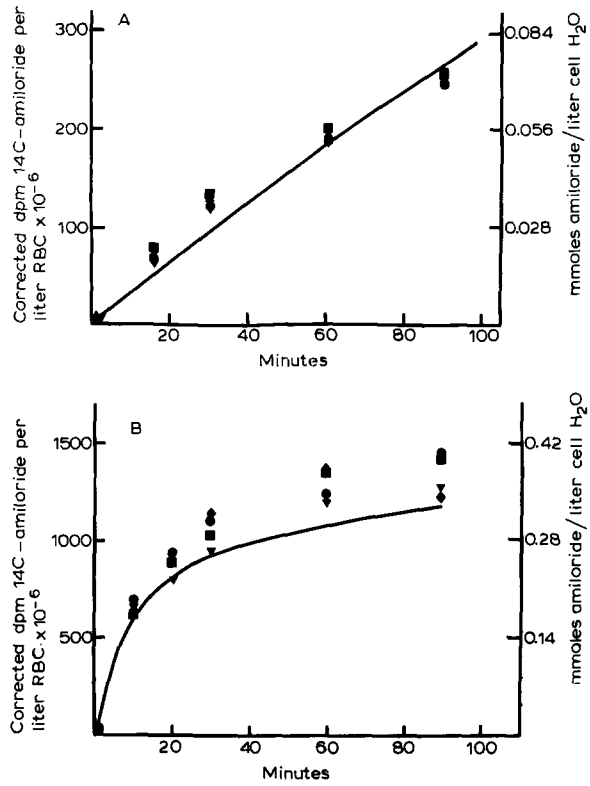

Fig. 1. Time-course of [^{14}C]amiloride uptake into human erythrocytes at pH 7.4 (A) and pH 9.0 (B) at 37°C. Each point represents the mean of duplicate samples obtained from the same flux flask. The counts of [^{14}C]-amiloride have been corrected for quench, and the results are expressed as dpm [^{14}C]amiloride per liter packed red blood cells (RBC). The solid lines were drawn according to Eqn. 1, with $P_B = 1.4 \cdot 10^{-7}$ cm/s, $V_c = 8.7 \cdot 10^{-11}$ cm^3, and $A = 1.42 \cdot 10^{-6}$ cm^2 [10]. An even better fit of the results can be obtained if a permeability of 10^{-9} cm/s is assumed for the charged form of amiloride, and the kinetic equation is extended to include a Nernst-Planck constant-field regime for the flux of charged amiloride.

in a medium of pH 9 (Fig. 1B). It is apparent that the rate of [^{14}C]amiloride uptake is much faster at pH 9 than at pH 7.4, suggesting that indeed the uncharged form of amiloride is the permeant species. From these experiments, we calculate a permeability coefficient of $1.40 \cdot 10^{-7}$ cm/s for uncharged amiloride.

In order to verify this value of permeability coefficient as well as to convince ourselves that amiloride was indeed crossing the red-cell membrane (as opposed to surface binding), we next measured the efflux of [^{14}C]amiloride from re-

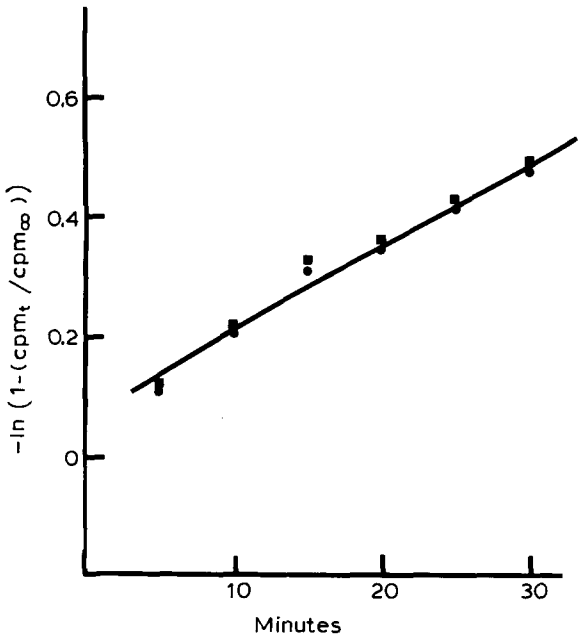

Fig. 2. Rate of [^{14}C]amiloride efflux from human red blood cell ghosts at pH 7.4 (37°C). The ordinate has a logarithmic scale, [cpm]$_t$ are the cpm of [^{14}C]amiloride in the medium at any time t and [cpm]$_\infty$ are the cpm in the medium after isotonic equilibrium has been attained. The rate of tracer efflux (k = 0.0357 min^{-1}) was determined from the slope of the line. Each point is the mean of duplicate determinations. Amiloride was initially incorporated into the ghosts at a concentration of $5.0 \cdot 10^{-5}$ M.

sealed human red cell ghosts to the medium. The results of two such experiments are shown in Fig. 2. From these data, a rate coefficient for efflux (k_0) of $1.16 \cdot 10^{-3}$ s^{-1} can be calculated. Since $P = k_0(V/A)$, where V/A is the ghost volume to surface area ratio ($V/A = 6.127 \cdot 10^{-5}$ cm for resealed ghosts, see Funder and Wieth [23]), an amiloride permeability coefficient of $0.71 \cdot 10^{-7}$ cm/s was computed. The similarity between the calculated permeability coefficients for uncharged amiloride in both influx experiments as well as that computed from the efflux experiment supports the contention that only the uncharged form of amiloride is crossing the erythrocyte membrane.

Discussion

The inhibition of Na^+ transport by the diuretic drug amiloride in various types of cells has led to remarkable conclusions concerning the role of Na^+-H^+ or Na^+-Ca^{2+} exchange processes as signals for mitogenesis or cell differentiation. However, one characteristic of amiloride inhibition of these ionic exchange processes is that relatively high concentrations of the drug are required (0.1–1 mM) to produce maximal inhibition. Although most experimental manipulations are done at external pH values of 7.4, a substantial fraction (0.05) of amiloride exists as the weak base and hence may permeate biological membranes, possibly exerting effects intracellularly. Implicit in this argument is the assumption that amiloride can permeate biological membranes. Here we demonstrate that the uncharged form of amiloride can indeed cross membranes; we measure a permeability coefficient of 10^{-7} cm/s for the uncharged form of this drug.

Further support for the idea that only the uncharged form of amiloride is permeable across lipid membranes comes from conductance measurements made either in solvent-containing or solvent-free planar lipid bilayer membranes (data not shown, see Refs. 24 and 25 for methods). In two separate experiments at pH 7.0 and 100 mM NaCl/0.2 mM amiloride produced no measurable change in base bilayer conductance. From the electrical measuring characteristics of our bilayer system, we estimate that the maximal permeability of charged amiloride is less than 10^{-8} cm/s. We also found that neither the charged nor neutral moieties of amiloride could alter, nonspecifically, other membrane properties such as capacitance, interfacial potentials, or the conductance induced by lipophilic anions or cations.

Estimations of the time-course of accumulation of amiloride within the intracellular water of different cells can be made using the following equation:

$$d/dt\left(\frac{C_i^{BH+} \cdot V_c}{A}\right) = \left[\frac{P_B C_o^{tot}}{1 + 10^{(pK - pH_o)}}\right] - \left[P_B \cdot 10^{(pH_i - pK)}\right] C_i^{BH+} \quad (1)$$

where C_o^{tot} is the total external concentration of amiloride, C_i^{BH+} the intracellular charged amiloride concentration, P_B the permeability coefficient of uncharged form of amiloride, V_c the

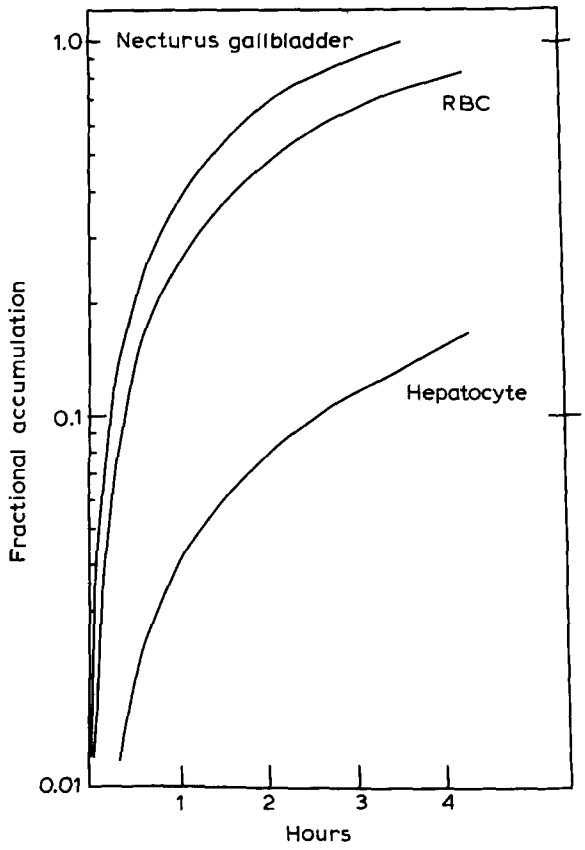

Fig. 3. Theoretical intracellular amiloride accumulation rates at external pH of 7.4. Calculations were made using Eqn. 1, which assumes that only the uncharged amiloride moiety is permeable ($P_B = 10^{-7}$ cm/s). Cellular volume and surface areas used in these computations were: red blood cell, $V_c = 8.7 \cdot 10^{-11}$ cm^3, $A = 1.42 \cdot 10^{-6}$ cm^2 [10]; hepatocyte, $V_c = 7.3 \cdot 10^{-9}$ cm^3, $A = 1.8 \cdot 10^{-5}$ cm^2 [12]; and for the *Necturus* gallbladder cell, $V_c = 2.2 \cdot 10^{-8}$ cm^3, $A = 2.64 \cdot 10^{-4}$ cm^2 [27]. Calculations for the *Necturus* gallbladder cell were done assuming mucosal drug exposure only, and that the mucosal surface area was 40-times greater than that measured microscopically due to microvilli and other surface infoldings.

cellular volume and A the area. pK and pH have their usual meanings. This equation was derived from Fick's Law and the Henderson-Hasselbalch equation, assuming that only the uncharged form of amiloride could penetrate biological membranes, and that the acid-base equilibrium is much faster than the transmembrane diffusion of amiloride. Fig. 3 plots the calculated fractional accumulation of amiloride (defined as the ratio of intracellular to extracellular drug concentration) versus time for cells with dimensions corresponding to the human erythrocyte, hepatocyte and a typical epithelial cell (*Necturus* gallbladder). If cells are suspended in a solution containing 1 mM amiloride, intracellular concentrations of drug would reach 0.4 mM after 65, 100 and 480 min for the gallbladder, red cell and hepatocyte, respectively. However, Leffert et al. [12] using hepatocytes and Smith et al. [16] using erythroleukemic cells measured intracellular accumulation ratios of 20–25 after 20 min and 4 h, respectively. Further, Leffert et al. [12] showed that this net uptake of amiloride is dependent upon external Na^+. We argue that net accumulation rates of about 10-times the external concentration can be achieved if, in these cells, the permeability of the charged form of amiloride becomes, at a minimum, equal to that of the uncharged form. In this case, cells would accumulate the drug in a net fashion because of the partitioning of the charged form according to the negative electrical potential difference across the cell membrane. In some cells, where Na^+-dependent amino acid transport systems exist, notably hepatocytes, proximal tubules and small intestine, amiloride may permeate in the charged form by substituting for the amino acids on these cotransport systems. Hence, our time estimations shown in Fig. 3 are minimum.

Some experiments have involved exposing biological preparations to amiloride for periods as long as 40 h [16]. Although these investigators realized that amiloride could enter the cells, they did not consider the possibility that amiloride could, in addition to inhibiting ionic fluxes, inhibit protein synthesis or other enzymatic activities. We suggest that caution be exercised in the interpretation that amiloride is a specific inhibitor of Na^+/cation exchange processes in these chronic experiments, or in drawing any mechanistic interpretations concerning the involvement of such ionic transport systems in the regulation of cellular growth and differentiation.

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