Vol. 108, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1982 Pages 738-745

AMILORIDE BLOCKS CELL-FREE PROTEIN SYNTHESIS AT LEVELS ATTAINED INSIDE CULTURED RAT HEPATOCYTES

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Received August 13, 1982

SUMMARY - Amiloride, a passive Na^+ influx inhibitor, lowers initial rates and plateau levels of [^{35}S]met uptake into proteins in cell-free rabbit reticulocyte lysates ($ID_{50} \sim 0.4$ mM). Isolated hepatocytes take up amiloride through a saturable ($K_m \sim 0.02$ mM; $V_{max} \sim 1.43$ nmol/ 10^6 cells/min) Na^+-dependent process. Similar temperature dependent uptake occurs in cultured hepatocyte monolayers. In chemically defined media, under growth reinitiation conditions, amiloride lowers overall rates of cellular protein and albumin synthesis ($ID_{50} \sim 0.4$ and ~ 0.028 mM, respectively). Amiloride concentrations (0.02 mM) that half-maximally inhibit reinitiation of hepatocyte DNA synthesis reach, within 30 min, cellular levels (~ 0.14 mM) that block reticulocyte lysate protein synthesis by 25%. These findings complicate interpretations, from studies in many eukaryotic systems, of cause and effect between mitogen-activated membrane Na^+ influxes and the reinitiation of DNA synthesis.

Studies with sea urchin eggs (1), hepatocytes (2,3), fibroblasts (4), neurons (5), and regenerating limbs (6) suggest that increased Na^+ influxes contribute to the initiation of DNA synthesis. This view (7,8) largely stems from experiments with amiloride (N-amidino-3,5-diamino-6-chloropyrazinecarboxamide), a specific reversible inhibitor of passive Na^+ uptake (9).

In stationary phase hepatocyte cultures, amiloride blocks mitogen stimulated Na^+-dependent amino acid transport and protein synthesis (2,10). However, only 20% of amiloride-sensitive amino acid transport can be attributed to Na^+ influx blockade (11). Additional experiments show that amiloride delays prereplicative activation of amino acid transport and DNA synthesis in a dose-dependent manner (2,8,10,12) that mimics the pattern of inhibition of both

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0006-291X/82/180738-08$01.00/0

processes by cycloheximide (10,13). These results imply that, if permeant, amiloride might act directly as a translational inhibitor of protein synthesis. Evidence presented here suggests that this occurs.

MATERIALS AND METHODS - Animals and chemicals. Adult livers were obtained from ♂ 250g Wistar or Fisher/344 rats (2). Standard tissue culture reagents were used (2). Radioisotopes (L-4,5-[^{3}H]leu [sp. act. 40-60 Ci/mM]; [^{35}S]met [sp. act. 1100 Ci/mM) were from NEN (Boston, MASS) and Amersham (Arlington, ILL). Amiloride® and [^{14}C]Amiloride® (sp. act. 5-7 mCi/mM) were gifts from Merck, Sharpe and Dohme (West Point, PA). Other chemicals were from Sigma (St. Louis, MO). Hepatocyte culture. Procedures to maintain adult rat hepatocytes as suspensions (10,11) or primary monolayers (2,10) have been published (14). Protein synthesis. Cellular rates of overall protein and albumin synthesis were measured in hepatocyte monolayers as described (2,15,16). Rabbit reticulocyte lysates were used to evaluate drug effects on cell-free protein synthesis (17). Rates were estimated by computer extrapolations of linear portions of the curves. [^{14}C]Amiloride uptake. Cultures were incubated with [^{14}C]drug for varying times in appropriate fluids. Free drug was separated from cells by centrifugation (suspensions) or aspiration (monolayers) with repeated washing using cold buffers (see Figs. 2,3). Under these conditions, sequestered extracellular radioactivity was undetectable. A value of 2.5µl $H_2O/10^6$ cells (2) was used to estimate intracellular drug "concentrations" (assuming negligible drug metabolism ([9]). This average volume was relatively invariant. Statistical tests. Two way analyses of variance were performed (2) using a Tektronix computer, Model No. 4051.

RESULTS - Amiloride inhibited cell-free protein synthesis in rabbit reticulocyte lysates (Fig. 1). Initial rates and plateau levels of [^{35}S]met uptake into proteins were reduced dose-dependently. Both parameters fell to 50% of controls with 0.4 mM amiloride and to 9-25% of controls with 0.03 mM cycloheximide (Fig. 1). Analysis of time courses in Fig. 1 showed that $\leq$0.05 mM amiloride was not inhibitory through 60 min, whereas concentrations $\geq$0.1 mM inhibited protein synthesis after 20 min ($p<0.001$).

Isolated hepatocytes took up amiloride (Fig. 2, top). When incubated with 0.04 mM [^{14}C]amiloride and Na^+, uptake proceeded linearly at 37°C and reached a steady state by 5-10 min ($t_{1/2} \sim 2$ min). If Na^+ was replaced by choline$^+$ or by Li^+ (data not shown), amiloride uptake fell >90%. Uptake consisted of a non-saturable Na^+-independent and a saturable Na^+-dependent component (Fig. 2, lower left). The latter was characterized by a $K_m \sim 0.021$ mM and $V_{max} \sim 1.43$ nmol amiloride/10^6 cells/min (Fig. 2, lower right).

Na^+-dependent amiloride uptake was seen in monolayer cultures (Fig. 3). Within 20 min, intracellular drug levels ($\sim$0.25 nmol [^{14}C]amiloride/10^6 cells; see control curve [156 mM NaCl] in left panel) exceeded ambient levels more

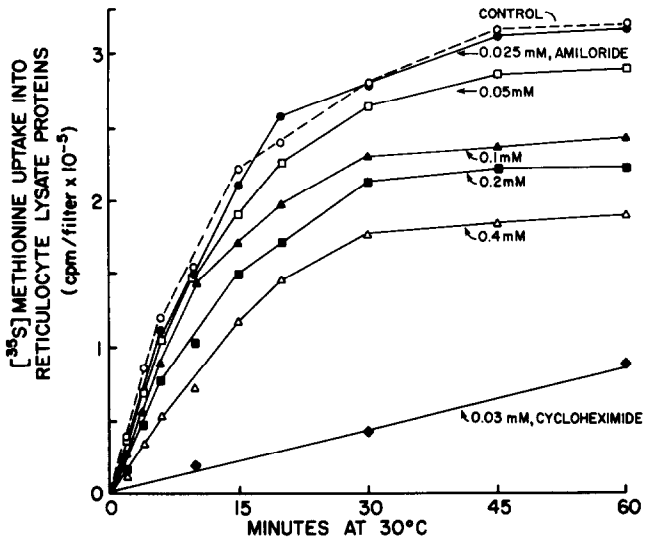

Fig. 1. Effects of amiloride on protein synthesis in rabbit reticulocyte lysates. Lysate reaction mixtures (final vol. 24 µl) and drug were incubated at 30°C. [^{35}S]met (sp. act. 26 Ci/mmol) was added (0 min). At varying times, samples were diluted into a 1 ml alkaline solution of bovine serum albumin, met and H_2O_2; incubated 15 min at 37°C; precipitated with 10% TCA, and collected on Millipore® filters, washed, dried and counted. Controls received H_2O instead of drug.

than 10-fold. If $[Na^+]_{out}$ was reduced to 25 or 0 mM, or if monolayers were first exposed to deionized H_2O for 1 hr in order to rupture cell membranes (data not shown), then initial rates and steady state levels of drug uptake fell 80-90% and >99%, respectively (Fig. 3). Lowering incubation temperatures from 37°C to 4°C (Fig. 3) or adding peptide mitogens (Fig. 3, right) also lowered uptake parameters. If control cultures in Fig. 3 were washed 2 hr after fluid change and then incubated at 37°C with fresh buffer, radioactivity exited from monolayers with 1st-order kinetics ($t_{1/2} \sim 19$ min, $k_{-1} \sim 0.03$ min^{-1}; data not shown). Monolayers concentrated less drug less rapidly than did cell suspensions. For example, at 0.011 mM amiloride, monolayers took up 3.3% as much drug (0.013 nmol/10^6 cells/min; Fig. 3, left) as did suspensions ($\sim$0.4 nmol/10^6 cells/min; Fig. 2, lower left).

When amiloride was added to monolayers under defined growth reinitiation conditions (2), the rates of [^{3}H]leu uptake into proteins were reduced dose-dependently (Fig. 4, top left). Since [^{3}H]leu uptake into acid-soluble pools was not affected by the drug (2,11), and because 0.8 mM unlabelled leu was

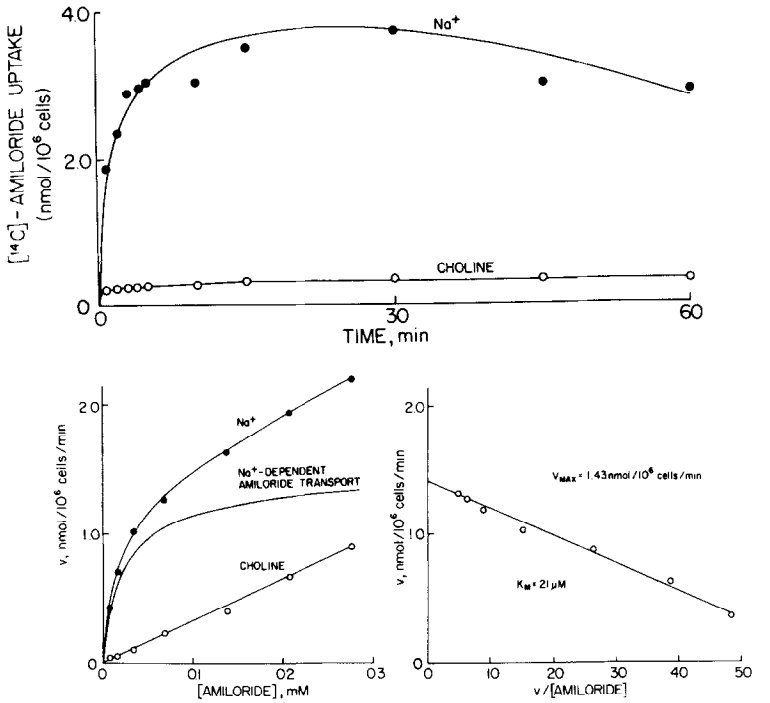

Fig. 2. Isolated adult rat hepatocytes: Kinetics of [^{14}C]amiloride uptake (top). 1.5 x 10^6 cells/ml were incubated at 37°C with 0.04 mM [^{14}C]amiloride (0.05 μCi/tube) in Krebs-Ringer bicarbonate buffers (10,11) ±Na^+. At varying times, cells were centrifuged, resuspended in 4°C NaCl, recentrifuged and counted. Concentration dependence of amiloride uptake (lower left). 1.5x10^6 cells/ml were incubated at 37°C for 2 min in buffer ±Na^+. The Na^+-dependent component of amiloride transport was obtained by subtracting, at each substrate concentration, the Na^+-insensitive part from the total. (lower right) Woolf-Augustinsson plot of the Na^+-dependent part of amiloride transport.

present during incubations, the measurements reflected relative rates of de novo protein synthesis (11,16). Extracellular amiloride concentrations of 0.004 mM amiloride did not affect overall rates, in contrast to drug levels of 0.04 mM and 0.4 mM which did ($p<0.02$ and $p<0.0001$, respectively). The latter dose reduced overall rates ca. 50% (Fig. 4, top left).

Amiloride did not block cellular protein synthesis immediately. More than 1 hr elapsed before overall rates changed (Fig. 4, right). Low concentrations of cycloheximide (0.0002-0.002 mM), by contrast, abolished synthesis within 30 min.

Amiloride reduced relative rates of cellular albumin synthesis; the ID_{50} was ~0.028 mM (Fig. 4, bottom left). Higher doses (0.04 and 0.4 mM) lowered synthesis rates between 1-2 hr ($p<0.003$ and $p<0.0001$, respectively), whereas

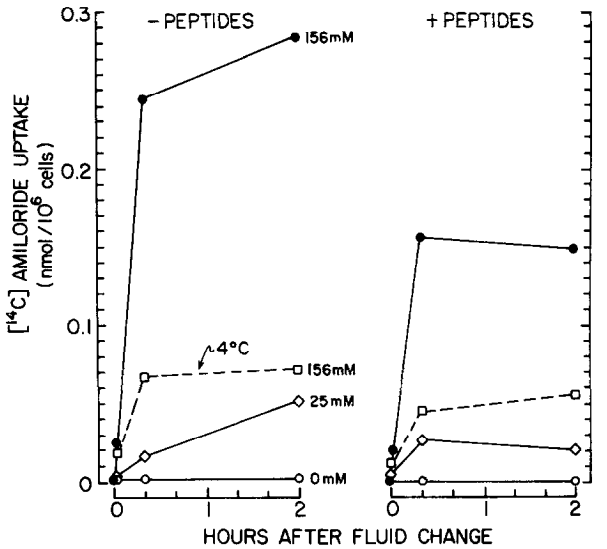

Fig. 3. Kinetics of [^{14}C]amiloride uptake by hepatocytes in monolayer culture. 12-day old stationary phase cultures (5-7x10^6 cells/dish) were washed x2 and fluid changed into 2 ml buffer (preincubated at 4° [dashed lines] or 37°C [solid lines]) minus (left) or plus 50 ng each ml^{-1} of EGF, insulin, and glucagon (right). Buffers (pH 7.4) contained varying levels of Na^+ (mM, as indicated) or choline$^+$ (to make final osmolarity equivalent to 156 mM Na^+), $CaCl_2$ (2 mM), KCl (5 mM), $MgSO_4$ (0.8 mM), glucose (5 mM), HEPES (50 mM), and 0.011 mM [^{14}C]amiloride (53000 dpm/µg). At varying times monolayers were processed as described for $^{22}Na^+$ uptake (2) and counted.

low doses (0.004 mM) were ineffective. At the lowest dose, however, [^{3}H]leu uptake eventually fell at 4 hr.

DISCUSSION - Amiloride inhibits protein synthesis directly in rabbit reticulocyte lysates (ID_{50} ~0.4 mM). Although external amiloride levels that fail to block cell-free protein synthesis (4 µM) inhibit reinitiation of hepatocyte DNA synthesis by 30% (2), the present findings indicate that even at such low ambient drug levels, cellular concentrations might rise enough (to ~0.04 mM) to block protein synthesis directly. Under such conditions, direct blockade would produce initial rate reductions of only 10-15%; however, 4 µM amiloride lowered rates of protein synthesis in monolayers at later times (4 hr). In addition, many investigators have used amiloride at 0.05-0.5 mM (1-3,5,6,18,19) which, if concentrated intracellularly, would attain levels that directly block protein synthesis ≥40%. Reductions in protein synthesis rates to this extent would certainly inhibit initiation of DNA synthesis (13). Therefore,

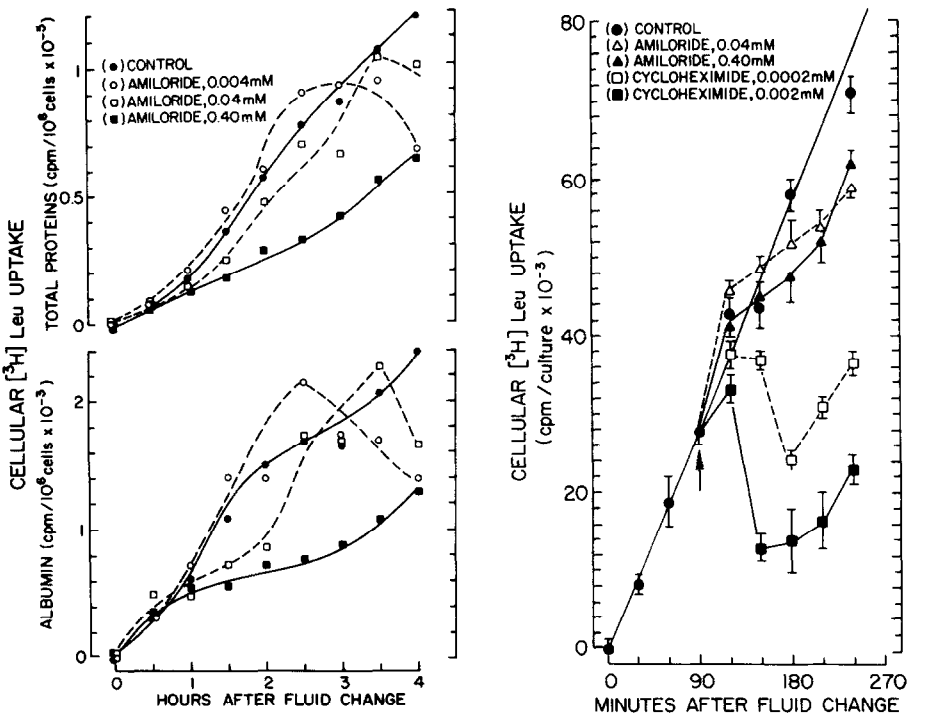

Fig. 4. Effects of amiloride on hepatocyte protein synthesis rates (left). Cultures (see Fig. 3) were washed x2 with 2 ml prewarmed (37°C) chemically defined growth medium (2); fresh medium plus peptides (see Fig. 3), [^{3}H]leu (20 μCi/ml), amiloride (0.004, 0.04, or 0.4 mM as indicated) or H_2O "control" was then added. At varying times, [^{3}H]leu uptake into overall cellular proteins (top) or albumin (bottom) was determined (16). Kinetics of drug induced reductions in overall hepatocyte protein synthesis rates (right). Culture conditions were as described above except that amiloride or cycloheximide (mM, as indicated) were not added until 90 min after the fluid change (arrow), and [^{3}H]leu was 5 μCi/ml.

amiloride might block proliferation, not by interacting with membrane receptors (18,20) through which mitogens accelerate inward Na^+ fluxes (1-8) but, rather, because it attains levels inside hepatocytes that effectively interfere with protein synthesis at some point(s) during the prereplicative phase.

Cellular albumin synthesis rates were ~14-fold more amiloride-sensitive than were overall protein synthesis rates. Since albumin's intracellular turnover time is < overall protein (our unpublished results), rapidly turning over proteins whose involvement is postulated for growth control (12,13) might be even more amiloride-sensitive than albumin. Thus it is difficult to accurately guage "safe" amiloride doses, i.e., levels which block Na^+ uptake and DNA synthesis initiation without blocking protein synthesis directly.

Unexpectedly (see refs. 9,20,21), amiloride uptake into hepatocytes was dependent upon and stimulated specifically by Na^+. Uptake exhibited membrane transport "carrier" properties since amiloride was concentrated intracellularly in a saturable and temperature-dependent manner. Na^+-dependent amino acid cotransporters do not seem to be involved since alanine, AiB and $N\text{-}CH_3$-AiB failed to inhibit amiloride uptake (our unpublished results). Although the nature of such carriers is unknown, differences in their structural or kinetic properties, as well as culture conditions, might explain why isolated hepatocytes concentrated more amiloride 5-fold faster than hepatocyte monolayers. Though amiloride extrusion rates were measured in monolayers, similar experiments were not performed with suspensions. Thus, discrepant uptake behaviour between the two systems might also be due to different drug efflux rates.

Drug "binding" to cellular components is an unlikely explanation of the findings presented here, for several reasons. First, amiloride uptake was virtually lost after prolonged hypotonic treatment of monolayers. Second, uptake was concentrative - cellular levels reached 10-30-fold higher values than ambient levels. In fact, at extracellular amiloride levels of 0.02 mM, the observed intracellular amiloride concentration was more than 170-fold higher than that predicted for passive uptake (pK_a[amiloride] ~8.7). This behaviour is unlike that expected for the saturable binding of small ligands to cell surface macromolecules. In this regard, unlabelled amiloride was a poor "competitor" for tracer level uptake (10^{-6}M) of [^{14}C]amiloride (50% reduction was not seen until ambient amiloride levels exceeded 0.1 mM; our unpublished results). Third, mitogenic peptides that do not compete with amiloride for peptide binding to the cell surface (2,10) reduced [^{14}C]amiloride uptake in monolayer cultures by 40-50%. This latter effect also was Na^+ and temperature-dependent. These observations all seem consistent with the conclusion that amiloride uptake observed in both systems reflects active accumulation within intracellular water.

An important difference remains between the way in which amiloride and cycloheximide block mitogen stimulated DNA synthesis. Amiloride is effective

mainly during the prereplicative phase, a 12-16 hr period after a mitogenic stimulus (2,8,12,19), whereas cycloheximide inhibits throughout the G_0, G_1 and S-phases of the cell "cycle" (13). Whether these differences reflect cell-cycle dependent differences in drug permeability or Na^+ influx processes required for DNA synthesis initiation is unknown. The latter hypothesis still remains attractive because mitogen-initiated DNA synthesis is blocked when $[Na^+]_{out}$ is <40 mM (2,19,22).

ACKNOWLEDGEMENTS - This work was supported by grants from the USPHS (AM28215, AM28392). We thank S. Dutky for assistance.

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