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Amiloride and amiloride analogs inhibit Na^+/K^+-transporting ATPase and Na^+-coupled alanine transport in rat hepatocytes

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Amiloride, a commonly used inhibitor of Na^+–H^+ exchange, has been shown to exhibit a variety of nonspecific effects. Recently, the more potent amiloride analogs, 5-(*N*,*N*-dimethyl)amiloride hydrochloride (DMA) and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIA), have been used to control for the nonspecific effects of the parent compound. In the present study, we have explored the effects of these analogs on Na^+/K^+-transporting ATPase (Na^+/K^+-ATPase) and Na^+-coupled alanine transport in primary rat hepatocyte cultures and rat liver plasma membranes, and we have compared the effects of these analogs with the effects of amiloride and ouabain. Amiloride, DMA, and EIA increased staedy-state Na^+ content and inhibited ouabain-sensitive $^{86}Rb^+$ uptake in a reversible, concentration-dependent, ouabain-like manner, with estimated 50% inhibitory concentrations (IC_{50}) of $3.0 \cdot 10^{-3}$ M, $5.2 \cdot 10^{-4}$ M, and $1.2 \cdot 10^{-4}$ M, respectively. Amiloride, DMA and EIA also inhibited ouabain-sensitive ATP hydrolysis in rat liver plasma membranes with similar potency (IC_{50} values of $2.2 \cdot 10^{-3}$ M, $2.2 \cdot 10^{-3}$ M, and $1.7 \cdot 10^{-4}$ M, respectively). In separate experiments, amiloride ($5 \cdot 10^{-3}$ M), DMA (10^{-3} M), and EIA ($2.5 \cdot 10^{-4}$ M) decreased the uptake into hepatocytes of alanine by 20%, 61%, and 59%, respectively, and further studies with DMA (10^{-3} M) demonstrated that this inhibition was largely due to a decrease in the Na^+-dependent fraction of alanine uptake. These findings indicate that amiloride, DMA, and EIA inhibit hepatic Na^+/K^+-ATPase directly, reversibly, and with a relative rank order potency of EIA > DMA > amiloride. All three compounds also inhibit the hepatic uptake of alanine, and presumably could indirectly inhibit other Na^+-coupled transport processes as well.

Introduction

Amiloride in millimolar concentrations is a commonly used inhibitor of Na^+–H^+ exchange [1,2]. At these high concentrations, however, this compound also exerts nonspecific effects, including inhibition of Na^+/K^+-ATPase [3–7]. Certain amiloride analogs, including 5-(*N*,*N*-dimethyl)amiloride hydrochloride (DMA) and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIA) are up to 100–200-times more potent [8–16] and they are also generally considered to be more selective with respect to inhibition of Na^+–H^+ exchange than amiloride [16,17]. DMA and EIA have therefore recently been used to control for the nonspecific

Abbreviations: Na^+/K^+-ATPase, Na^+/K^+-transporting ATPase (EC 3.6.1.37); DMA, 5-(*N*,*N*-dimethyl)amiloride hydrochloride; EIA, 5-(*N*-ethyl-*N*-isopropyl)amiloride.

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effects of amiloride in studies exploring the role of Na^+/H^+ exchange in a variety of processes, including epithelial transport [18,19], biotransformation [20], and regulation of intracellular Na^+ concentration [10], cell volume [21], intracellular pH [11,13], and cell growth and differentiation [16,17,22].

Interpretation of such studies critically depends on the assumption that these inhibitors are selective in their effects. Recently, however, some of the more potent amiloride analogs, including DMA and EIA, have been shown to inhibit purified dog kidney Na^+/K^+-ATPase [7] and to impair ouabain-sensitive oxygen consumption and mitochondrial ATP production in rabbit proximal kidney tubules [4], thus raising questions regarding their selectivity for Na^+/H^+ exchange. In the present studies on rat liver, in which Na^+/K^+-ATPase exhibits less ouabain-sensitivity than in other species, we have confirmed an effect of amiloride and its analogs on cation transport and extended previous observations by demonstrating: (a) a dose-dependent decrease in ouabain-sensitive $^{86}Rb^+$ uptake in intact cells, and a very similar dose-dependent inhibition of Na^+/K^+-ATPase in isolated membranes, making primary inhibition of Na^+/K^+-ATPase, (rather than secondary inhibition due to decreased mitochondrial ATP production) the most likely mechanism by which these agents affect active cation transport; (b) that the effects of these agents on the Na^+/K^+-ATPase-mediated cation transport are evident within minutes and are completely reversible; and (c) that the consequences of Na^+/K^+-ATPase inhibition include an increase in intracellular Na^+ content and decrease in Na^+-coupled amino-acid transport.

Materials and Methods

Chemicals and solutions

Amiloride (3,5-diamino-6-chloro-*N*-(diaminoethylene)pyrazine carboxamide · HCl · $2H_2O$), 5-(*N*,*N*-dimethyl)amiloride hydrochloride (DMA), and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIA) were synthesized as previously described [23]. Appropriate stock solutions of these compounds in incubation medium (cultured hepatocyte studies, see below) or Tris buffer (liver plasma membrane studies, see below) were freshly prepared on each study day. Amiloride and DMA were readily dissolved by stirring and heating; for dissolution of EIA, a slight molar excess of isethionic acid was added. The final concentration of isethionic acid never exceeded $3.5 \cdot 10^{-4}$ M in cultured cell studies and $1.1 \cdot 10^{-3}$ M in plasma membrane studies, respectively. In preliminary experiments (data not shown) these concentrations of isethionic acid did not affect the measurements of Na^+/K^+-ATPase activity in either cultured hepatocytes or isolated liver plasma membranes, and much higher concentrations of isethionic acid have been shown previously to be non-toxic in rat liver [24]. ^{22}Na, ^{86}Rb, and L-[^{3}H]alanine were obtained from New England Nuclear (Boston, MA); L-alanine, (aminooxy)acetic acid, and ouabain from Sigma, St. Louis, MO; $Na_2ATP \cdot 3H_2O$ from Boehringer-Mannheim, F.R.G. All other chemicals used were of the highest purification grade commercially available.

Cultured hepatocytes

Hepatocytes were isolated from livers of male Sprague-Dawley rats (250–300 g from Bantin Kingman, Fremont, CA) by a modification of the method of Berry and Friend [25], plated on collagen-coated plastic dishes, and maintained in modified 199 OR medium supplemented with amino acids, insulin, and corticosteroids for 48 h before use, as described previously [26–28]. Cell viability was assessed in triplicate in one batch of cells after a 30-min exposure to the highest concentrations of inhibitors used, i.e., $5 \cdot 10^{-5}$ M amiloride, 10^{-3} M DMA, $2.5 \cdot 10^{-3}$ M EIA or $5 \cdot 10^{-3}$ M ouabain using Trypan blue exclusion. Trypan blue-excluding cells averaged 99% in controls, and 97%, 96%, 95% and 96% in the presence of amiloride, DMA, EIA or ouabain, respectively.

Liver plasma membranes

For these studies, we used a liver plasma membrane preparative technique which yields a mixture of both sinusoidal and canalicular membranes. Each batch of liver plasma membranes was prepared from livers of three male Sprague-Dawley rats (250–300 g, from Bantin Kingman,

Fremont, CA) by discontinuous sucrose density gradient centrifugation according to a modification of the methods of Song [29] and Boyer [30], as described previously [31].

Isotope uptake by cultured hepatocytes

^{86}Rb, ^{22}Na, and [^{3}H]alanine uptake studies were performed as described previously [26,27]. In brief, following a 30 min preincubation in a balanced electrolyte solution (130 mM NaCl/5 mM KCl/0.8 mM $MgSO_4$/1.2 mM $CaSO_4$/0.8 mM Na_2HPO_4/5 mM $NaHCO_3$/10 mM Hepes/5 mM glucose adjusted to pH 7.4 with NaOH or HCl) in the presence or absence of amiloride (10^{-6}–$5 \cdot 10^{-3}$ M), DMA (10^{-6}–10^{-3} M), EIA (10^{-6}–$2.5 \cdot 10^{-4}$ M), or ouabain (10^{-6}–$5 \cdot 10^{-3}$ M), cells were incubated in medium identical to that used for preincubation, except for the presence of radiolabel, and in selected studies, with replacement of Na^+ by choline. Na^+/K^+-ATPase-mediated ^{86}Rb uptake, i.e., Na^+/K^+ pump activity, was taken to be that fraction of total ^{86}Rb uptake inhibitable by ouabain ($5 \cdot 10^{-3}$ M). For determination of alanine uptake (20 μM), alanine metabolism was blocked by $2.5 \cdot 10^{-3}$ M (aminooxy)acetic acid present in preincubation and incubation medium [27].

At the end of the incubation period, uptake was stopped and extracellular isotope removed by dipping the dishes for 10 s in each of eight consecutive beakers containing 200 ml of ice-cold (4° C) balanced electrolyte solution without radioisotope. Previous studies have demonstrated that this wash procedure efficiently removes extracellular isotope, while causing minimal loss of intracellular isotope [26,32]. The cells were then scraped from the dishes directly into 2% (w/v) Na_2CO_3 in 0.1 M NaOH. Radioactivity was measured in an aliquot of the scrapings by liquid scintillation counting using external standardization for quench correction, and total cell protein was determined in another aliquot according to Lowry et al. [33].

Ouabain-sensitive ATP hydrolysis

Ouabain-sensitive ATP hydrolysis was measured as described previously [31]. The assay was run in the presence and absence of amiloride (10^{-5}–10^{-2} M), DMA (10^{-9}–10^{-3} M), or EIA (10^{-9}–10^{-3} M) in a total of 3.93 ml of reaction mixture containing 3.78 ml Tris buffer (165.32 mM Tris base/1.32 mM EGTA/158.80 mM NaCl/16.50 mM KCl adjusted to pH 7.4 with NaOH or HCl), 100 μl of substrate containing 200 mM of each $MgCl_2$ and ATP and 50 μl of liver plasma membranes (corresponding to about 100–200 μg of membrane protein). The reaction was begun by the addition of liver plasma membranes to the reaction mixture preincubated for 10 min at 37° C in a shaking water bath with or without $5 \cdot 10^{-3}$ M ouabain. The reaction was terminated after 10 min by addition of 1 ml of 35% (w/v) ice-cold (4° C) trichloroacetic acid and immediate chilling on ice. All samples were then centrifuged at $12\,350 \times g$ for 10 min, and 2 ml of supernatant were analyzed for inorganic phosphate according to Bartelett's modification [34] of the method of Fiske and SubbaRow [35]. In some studies employing DMA, the reaction was alternatively terminated, and inorganic phosphate was determined as described [36], with essentially similar results as with the earlier mentioned method for phosphate determination. Results obtained by both methods were therefore pooled and analyzed together. Blank incubations were performed identically except that liver plasma membranes were added only after termination of the reaction with the 'stop' solution. Na^+/K^+-ATPase activity was determined as the ouabain- ($5 \cdot 10^{-3}$ M) suppressible fraction of total ATPase activity. As previously shown [31], both the Mg^{2+} and the Na^+/K^+-ATPase reactions are linear for at least 10 min within the range of protein concentrations used, and preliminary studies (not shown) established that amiloride, DMA, EIA and ouabain did not interfere with the methods used for determination of inorganic phosphate.

Calculations and statistics

Initial ^{86}Rb uptake rates and steady-state ^{22}Na content, determined as described above, were expressed as nmol/min per mg cell protein and nmol/mg cell protein, respectively. Rates of ATP hydrolysis, determined as described above, were expressed as μmol inorganic phosphate/h per mg membrane protein. Na^+/K^+-ATPase activity under the different experimental conditions was calculated as the difference in the rate of ^{86}Rb uptake or ATP hydrolysis in the presence and

absence of $5 \cdot 10^{-3}$ M ouabain, and expressed as percentage of the values in concurrent controls. Initial rates of alanine uptake were determined from three time points (10, 40 and 60 s) using linear regression analysis and were expressed as pmol/min per mg protein. To determine the effects of DMA on Na^+-dependent and Na^+-independent alanine uptake, initial alanine uptake rates were determined from a single 60 s time point and were expressed as percentage of the values in concurrent controls. Na^+-dependent alanine uptake was taken as the difference of alanine uptake in Na^+-containing and Na^+-free solution. Dose-response curves were performed, and 50% inhibitory concentrations (IC_{50}) of the various compounds were calculated with a Hewlett-Packard 9825B computer (Hewlett-Packard, Palo Alto, CA) using a non-linear least-squares procedure.

All experiments were performed in duplicate or triplicate in between one and six different batches of cells or liver plasma membrane preparations and the data are presented as means ± S.E. A *t*-test was used to test the significance of the effects of DMA on total, Na^+-dependent and Na^+-independent alanine uptake, and *P* values of less than 0.05 were considered statistically significant.

Results

^{86}Rb uptake by cultured hepatocytes

Preliminary studies (not shown) established that, in the presence and absence of amiloride, DMA, EIA or ouabain (a) ouabain-sensitive ^{86}Rb uptake was linear for at least 10 min, and (b) the effects of amiloride, DMA and EIA on ouabain-sensitive ^{86}Rb uptake were independent of the length of the preincubation period (0–60 min). Thus, for all further experiments, a 30 min preincubation period was used and ouabain-sensitive initial ^{86}Rb uptake rates were determined from a single 10 min time point.

Under control conditions, initial ouabain-sensitive ^{86}Rb uptake rates determined in 15 different batches of hepatocytes averaged 2.83 ± 0.14 nmol/min per mg protein and accounted for 75 ± 1.3% of total initial ^{86}Rb uptake. These values are in good agreement with previous results in the same culture system [27]. Because of considerable interbatch variation [27], the effects of various inhibitor concentrations were expressed as a percentage of the values in concurrent controls. Amiloride (10^{-6}–$6 \cdot 10^{-3}$ M), DMA (10^{-6}–10^{-3} M), and EIA (10^{-6}–$2.5 \cdot 10^{-4}$ M) produced a concentration-dependent inhibition of initial ouabain-sensitive ^{86}Rb uptake rates with estimated 50% inhibitory concentrations (IC_{50}) of $3.0 \cdot 10^{-3}$ M, $5.20 \cdot 10^{-4}$ M, and $1.2 \cdot 10^{-4}$ M, respectively (Fig. 1). The inhibitory effects of amiloride and its analogs were qualitatively similar to those of ouabain (10^{-6}–$5 \cdot 10^{-3}$ M), which exhibited an estimated IC_{50} of $3.4 \cdot 10^{-4}$ M (Fig. 1). The inhibitory effects on ouabain-sensitive ^{86}Rb uptake rates produced by even higher concentrations of all compounds (present for 30 min of preincubation) were reversible following washing of the cells in ice-cold balanced electrolyte solution and a 30-min recovery period at 37 °C in inhibitor-free solution (Table I).

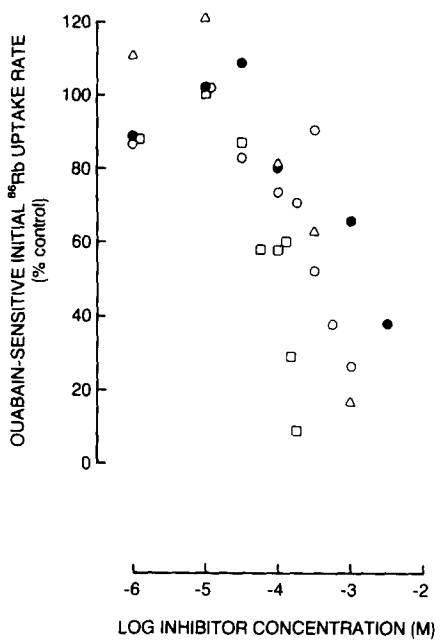

Fig. 1. Dose-dependent effects of amiloride (●), DMA (○), EIA (□) and ouabain (△) on ouabain- ($5 \cdot 10^{-3}$ M) sensitive ^{86}Rb uptake rates in 48-h-old rat hepatocyte cultures determined as described in Materials and Methods. Individual data points represent average values of triplicate determinations in 1–4 batches of cells (S.E. (not shown) ranged from 1 to 26%).

TABLE I

REVERSIBILITY OF THE EFFECTS OF AMILORIDE, DMA, EIA AND OUABAIN ON OUABAIN-SENSITIVE ^{86}Rb UPTAKE AND STEADY-STATE ^{22}Na CONTENT

Duplicate or triplicate incubations were performed in 2–4 different batches of cells and the results are expressed as percent of the values in concurrent controls. Numbers represent average values (S.E. is given in parentheses).

	Initial ouabain-sensitive ^{86}Rb uptake (% of control)		Steady-state ^{22}Na content (% of control)	
	inhibitor present [a]	after washout of inhibitor [b]	inhibitor present [a]	after washout of inhibitor [b]
Amiloride $5 \cdot 10^{-3}$ M	38 (1.4)	115 (27)	143 (0.7)	94 (4.3)
DMA 10^{-3} M	27 (0.4)	131 (9.6)	238 (34)	119 (4.5)
EIA 10^{-4} M	59 (3.2)	92 (5.5)	131 (1.4)	100 (7.1)
EIA $2.5 \cdot 10^{-4}$ M	9 (5.2)	155 (31)	383 (187)	166 (14)
Ouabain $5 \cdot 10^{-3}$ M	–	135 (14)	449 (34)	105 (1.5)

[a] Initial ouabain-sensitive ^{86}Rb uptake rates and steady-state ^{22}Na content were measured as described in Materials and Methods in the presence of inhibitor.

[b] To test reversibility, cells preincubated at 37 °C for 30 min in the presence of the indicated concentration of inhibitor were washed in the usual fashion (10 s in each of eight beakers containing ice-cold balanced electrolyte solution; see Materials and Methods). The cells were then allowed to recover for 30 min at 37 °C in balanced electrolyte solution without inhibitor before measuring uptake (see Materials and Methods). Controls were handled identically except that no inhibitor was present during the preincubation period. Control values for ouabain-sensitive ^{86}Rb uptake (2.99 nmol/min per mg protein $\pm$ 0.23, $n = 5$) and steady-state $^{22}Na^+$ content (32.3 nmol/mg protein $\pm$ 1.98, $n = 3$) determined using this modified protocol agreed well with those determined using the standard protocol (see Materials and Methods and Results, including Figs. 1 and 2).

Steady-state ^{22}Na content in cultured hepatocytes

Intracellular Na^+ concentration has been shown to be a major regulator of hepatic Na^+/K^+-ATPase activity [27]. In order to determine whether amiloride and its analogs might have decreased the intracellular Na^+ concentration by inhibiting Na^+–H^+ exchange, thus leading to a regulatory decrease in Na^+/K^+ pump function, intracellular Na^+ concentration was determined as steady-state $^{22}Na^+$ content in the cell batches used for measurement of ^{86}Rb uptake rates. In preliminary experiments (not shown) it was established that, in the presence or absence of amiloride, DMA, EIA or ouabain, ^{22}Na uptake had reached steady state by 30 min of incubation. Thus, in all experiments, steady-state ^{22}Na content was determined from a single 30 min time point.

Under control conditions, steady-state ^{22}Na content determined in 14 different batches of cells averaged 29.9 $\pm$ 1.1 nmol · mg protein^{-1}, which is in good agreement with previously reported results in a similar culture system [27]. Amiloride, DMA and EIA produced a concentration-dependent increase in steady-state ^{22}Na uptake of up to 142%, 238% and 386%, respectively, of the values in concurrent controls (Fig. 2). These effects of amiloride and its analogs were qualitatively similar to that of ouabain, which increased hepatocyte steady-state ^{22}Na content up to 449% of the values in concurrent controls (Fig. 2).

These increases in steady-state ^{22}Na content, like the inhibition of Na^+/K^+ pump activity, were reversible after washing the cells free of inhibitor, in ice-cold balanced electrolyte solution followed by a 30 min recovery period at 37 °C in inhibitor-free solution (Table I).

Ouabain-sensitive ATP hydrolysis in liver plasma membranes

To provide additional evidence for a direct action of amiloride and its analogs on hepatic Na^+/K^+-ATPase, the effects of various inhibitor concentrations in isolated liver plasma membranes were explored. In control experiments using four different membrane preparations, ouabain-sensitive ATP hydrolysis averaged 16.9 $\pm$ 1.2 μmol inorganic phosphate/h per mg protein and accounted for 32.4 $\pm$ 2.3% of total ATP hydroly-

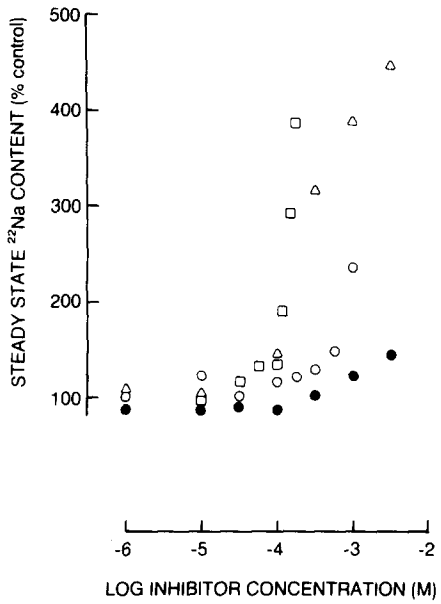

Fig. 2. Dose-dependent effects of amiloride (●), DMA (○), EIA (□), and ouabain (△) on steady-state ^{22}Na content in 48-h-old rat hepatocyte cultures determined as described in Materials and Methods. Individual data points represent average values of triplicate determinations in 1–4 batches of cells (S.E. (not shown) ranged from 1 to 33%).

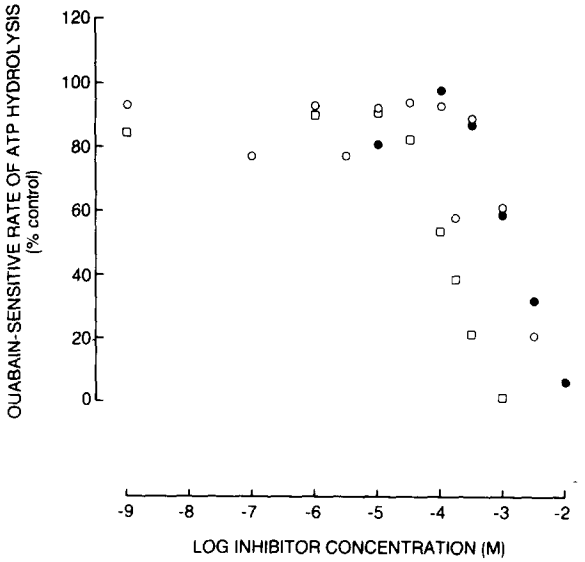

Fig. 3. Dose-dependent effects of amiloride (●), DMA (○), and EIA (□) on ouabain-sensitive ATP hydrolysis in rat liver plasma membranes determined as described in Materials and Methods. Individual data points represent average values of triplicate determinations in 1–6 different membrane preparations (S.E. (not shown) ranged from 4 to 20%).

sis. Because the ATPase measurements exhibited an intra-assay coefficient of variation of 0.128 and an inter-assay coefficient of variation of 0.220, the effects of various inhibitors were expressed as a percentage of the values in concurrent controls. Amiloride (10^{-5}–10^{-1} M), DMA (10^{-9}–$5 \cdot 10^{-3}$ M), and EIA (10^{-9}–10^{-3} M) produced a dose-dependent inhibition of ouabain-sensitive ATP hydrolysis with estimated IC_{50} values of $2.2 \cdot 10^{-3}$ M, $2.2 \cdot 10^{-3}$ M, and $1.7 \cdot 10^{-4}$ M, respectively (Fig. 3).

[^{3}H]Alanine uptake by cultured hepatocytes

Hepatic uptake of L-alanine is a largely Na^+-coupled process and therefore depends on the electrochemical Na^+ gradient established by the Na^+/K^+-ATPase [27]. We therefore measured uptake of L-alanine by cultured rat hepatocytes in order to determine whether amiloride and its analogs could indirectly affect Na^+-coupled transport processes.

In the presence and absence of inhibitors,

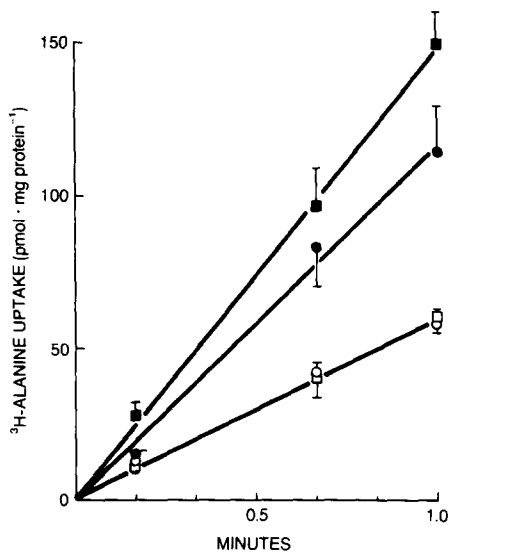

Fig. 4. Effects of $5 \cdot 10^{-3}$ M amiloride (●), 10^{-3} M DMA (○), and $2.5 \cdot 10^{-4}$ M EIA (□) on [^{3}H]alanine uptake in 48-h-old rat hepatocyte cultures as compared with control incubations (■) in the absence of inhibitor. Uptake was measured as described in Materials and Methods, and the results shown represent the average ± S.E. of triplicate determinations in two or three batches of cells.

[^{3}H]alanine uptake was linear for at least 60 s (Fig. 4) and initial [^{3}H]alanine uptake rates were calculated by linear regression analysis of uptake measurements at 10, 40 and 60 s. In three different batches of control cells, initial [^{3}H]alanine uptake rates averaged 145 ± 12 pmol/min per mg protein, which is consistent with previously reported results in a similar culture system [27]. Amiloride ($5 \cdot 10^{-3}$ M), DMA (10^{-3} M), and EIA ($2.5 \cdot 10^{-4}$ M) decreased initial [^{3}H]alanine uptake rates by about 20%, 61% and 59% to 116 ± 13, 57 ± 3 and 59 ± 4 pmol/min per mg protein, respectively.

In order to define more precisely the mechanism of this inhibition of alanine uptake, we measured the Na^+-dependent and Na^+-independent fractions of alanine uptake, following both acute and prolonged (30 min) exposure to 10^{-3} M DMA (Table II). Under the conditions used, total alanine uptake averaged 129 ± 17 pmol/min per mg protein and was decreased by about two-thirds in the absence of Na^+ (to 47 ± 1 pmol/min per mg protein). While DMA (10^{-3} M) decreased Na^+-independent alanine uptake after acute as well as prolonged exposure by 28% ($P = 0.06$) and 53% ($P < 0.05$), respectively, suggesting a direct effect on the Na^+-independent alanine transport system, most (61%) of the decrease in total uptake was attributable to a decrease in the Na^+-dependent component (46% inhibition, $P < 0.02$). Moreover, unlike the effect on Na^+-independent uptake, inhibition of Na^+-dependent alanine uptake was observed only after prolonged (30 min) exposure of hepatocytes to the inhibitor. The latter finding is consistent with a DMA-induced decrease of the Na^+ electrochemical gradient, rather than a direct effect of DMA on Na^+-coupled alanine transport. The inhibition of total alanine uptake observed with amiloride and EIA (Fig. 4), is, by analogy, also likely to be due to a time-dependent decrease of the Na^+ electrochemical gradient.

TABLE II

EFFECTS OF DMA ON ALANINE UPTAKE

Following a 30 min preincubation in balanced electrolyte solution, [^{3}H]alanine (20 μM) uptake was measured in Na^+-containing or Na^+-free (choline-containing) electrolyte solution, as described in Materials and Methods. The presence or absence of DMA (10^{-3} M) in the preincubation and uptake media is shown above. Since alanine uptake was linear for at least 60 s in the presence and absence of DMA (10^{-3} M) (Fig. 4), initial alanine uptake rates were calculated from single 60 s time points and are expressed as a percent of uptake in concurrent controls (no exposure to DMA at any time) which averaged 129 ± 17 and $47 \pm$ pmol/min per mg in the presence and absence of Na^+, respectively. Numbers present the average of three triplicate determinations in two different cell batches (S.E. is given in parentheses).

	Alanine uptake rate (% of control)	
	DMA present in uptake media only	DMA present in preincubation and uptake media
Na^+-containing media	84 (6)	50 (2) [b]
Na^+-free media	72 (12)	47 (8) [c]
Na^+-dependent uptake [a]	95 (15)	54 (2) [b]

[a] Difference between alanine uptake rates determined in Na^+-containing and Na^+-free solution.
[b] $P \leq 0.002$, compared to concurrent control.
[c] $P \leq 0.05$, compared to concurrent control.

Discussion

Certain amiloride analogs bearing substituents at the N-5, including 5-(*N*,*N*-dimethyl)amiloride (DMA) and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIA), are up to 100–200-times more potent than amiloride [8–16], and they are also considered to be more selective with respect to inhibition of Na^+–H^+ exchange than is amiloride [16,17]. The aim of the present study was to explore the specificity of two such potent Na^+–H^+ exchange inhibitors of the amiloride series, i.e., DMA and EIA, by assessing their effects on hepatic Na^+/K^+-ATPase activity and Na^+-coupled transport, and to compare them with those of amiloride and ouabain. Our findings complement recent observations of others which indicate that these agents inhibit purified dog kidney Na^+/K^+-ATPase [7] and ouabain-sensitive oxygen consumption in rabbit proximal tubules [3,4]. Apart from demonstrating inhibition of active cation transport in another tissue, they also extend these earlier observations in several respects. First, the concentrations (IC_{50}) at which amiloride, DMA and EIA inhibited ouabain-sensitive $^{36}Rb^+$ uptake in intact hepatocytes were very similar to the concentrations at which they inhibited Na^+/K^+-ATPase in isolated membranes. This suggests that

direct and primary inhibition of Na^+/K^+-ATPase is the most likely mechanism for the effect of these agents on Na^+/K^+-ATPase-mediated cation pumping. Although our studies do not exclude the possibility that altered cation transport reflects, in part, diminished mitochondrial ATP production [4,7] or nonspecific toxicity, such effects are not necessary to explain our results and might be expected to be associated with altered Trypan-blue exclusion or not to be completely reversible.

Second, our results indicate that the consequences of Na^+/K^+-ATPase inhibition include an increase in intracellular Na^+ content and presumably concentration (ouabain does not alter cell volume in this system [27]), as well as a decrease in the Na^+-coupled transport of amino acids and probably other solutes as well. Third, because the relative rank order potency (EIA > DMA > amiloride) with which these agents inhibit the Na^+/K^+-ATPase is similar to that reported in other systems for inhibition of Na^+–H^+ exchange [8–16], and because the inhibitory effects of amiloride and its analogs on Na^+/K^+-ATPase, like their effect of Na^+–H^+ exchange, are evident within minutes and are completely reversible, inhibition of Na^+/K^+-ATPase could easily remain undetected in the usual experimental setting.

While the present findings indicate that amiloride, as well as certain of its more potent analogs, inhibit the Na^+/K^+ pump in a variety of cell types, it is of interest that this effect may not be evident in some cell types or biologic preparations. For example, EIA in concentrations as high as 10^{-4} M has no effect on intracellular Na^+ concentration in chicken myocytes [10]. Moreover, in the isolated perfused rat liver, we were recently unable to detect inhibition of Na^+/K^+-pump activity, assessed by measurement of perfusate K^+ concentration and Na^+-dependent hepatic ^{86}Rb uptake, by concentrations of amiloride (10^{-3} M), DMA (10^{-3} M) or EIA ($2.5 \cdot 10^{-4}$ M), which inhibited pump activity in cultured hepatocytes by up to 90% [38]. The reason(s) for this difference in susceptibility to the inhibitory effects of amiloride and its analogs of the intact organ as compared with cellular and subcellular systems is at present unclear. Potential explanations may include metabolism and/or biliary excretion of the inhibitors by the isolated perfused rat liver cells [39], or a lower mass ratio of inhibitor to tissue in these cells as compared with cultured cells and isolated membranes, and thus a greater lipid phase available in the isolated perfused rat liver cells for partition of these rather hydrophobic compounds.

Finally, it is noteworthy that the differences in the potency with which DMA and EIA, as compared with amiloride, inhibit Na^+–H^+ exchange (up to 100–200-fold) is greater than their difference in potency (10- to 30-fold) with respect to inhibition of Na^+/K^+-ATPase. Thus, in the appropriate model and at the appropriate concentration, the use of DMA, EIA, and possibly other analogs may still offer advantages, in terms of selectivity, over the use of amiloride.

In summary, our findings dictate caution in interpretation of studies using not only amiloride, but also the more potent analogs DMA and EIA, in exploring the role of Na^+–H^+ exchange in various cellular functions. Moreover, since the concentrations at which 'nonspecific' effects of these agents are manifested appear to vary among different cell types and experimental models, their specificity for selective inhibition of Na^+–H^+ exchange vs. nonselective inhibition of cellular processes (e.g., Na^+/K^+-ATPase-mediated cation pumping and Na^+-coupled transport) needs to be established for each model system under study.

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