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THE INTERACTION OF AMILORIDE ANALOGUES WITH THE Na^+/H^+ EXCHANGER IN KIDNEY MEDULLA MICROSOMES

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The effects of ten amiloride analogues on Na^+-H^+ exchange in rabbit kidney medulla microsomes have been examined. Most of the analogues appeared to inhibit Na^+ uptake into the microsomes more effectively than did amiloride either in the presence or absence of a pH gradient. However, the analogues were also capable of stimulating Na^+ efflux from the microsomes at concentrations somewhat higher than the concentrations at which they inhibited Na^+ influx. The concentrations at which the analogues stimulated Na^+ efflux were about 2–4-times higher than the concentrations at which they blocked influx. This suggested that the two processes were related. The analogues that stimulated efflux most effectively (the 5-*N*-benzylamino analogue of amiloride and the 5-*N*-butyl-*N*-methylamino analogue) were shown to induce completely reversible effects. These analogues did not stimulate L-[^{3}H]glucose efflux from medulla microsomes which ruled out nonspecific vesicle destruction or reversible detergent effects. These analogues also induced Na^+ efflux from microsomes in the presence of high concentrations of added buffer, which ruled out weak-base uncoupling effects. The possibility exists that these analogues are carried into the microsomes via the Na^+-H^+ exchange protein and that this permits them to both block Na^+ influx into the microsomes and stimulate Na^+ efflux as well.

Introduction

Microsomal membrane vesicles have been made from either the urinary bladder of the tropical toad, *Bufo marinus*, or the medulla of the rabbit kidney that were capable of amiloride inhibited Na^+-H^+ exchange [1–4]. The protein responsible for amiloride-inhibited Na^+-H^+ exchange in rabbit kidney medulla microsomes was extracted from the native membranes with octyl glucoside and incorporated into reconstituted proteoliposomes that were also capable of amiloride-inhibited Na^+ transport [3,5].

The drug amiloride was initially developed as one of a large series of pyrazinecarbonylguanidines that exhibited K^+-sparing diuretic properties [6]. The ability of these derivatives to reverse the enhancement of Na^+ retention and K^+ excretion produced in the rat kidney by the mineralcorticoid deoxycorticosterone acetate was determined by Cragoe et al. [6]. Amiloride was shown to be one of the most effective of these compounds [6]. After amiloride was shown to be capable of inhibiting Na^+ movement across the apical membranes of tight epithelia such as toad bladder and frog skin [7,8], the effects of a number of other pyrazinecarbonylguanidines on Na^+ movement

Abbreviations: Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid; Tris, tris(hydroxymethyl) aminomethane; Mes, 4-morpholineethanesulphonic acid.

across frog skin were determined. Most of the compounds were shown to be much weaker inhibitors of epithelial Na^+ transport than amiloride [9–11].

Amiloride has also been shown to inhibit Na^+-H^+ exchange across surface membranes of a variety of epithelial and non-epithelial tissues [12–17]. This Na^+-H^+ exchange process has been suggested to be important in the regulation of cellular volume [16] and pH [17], in the control of cell division [15], and to be required for kidney bicarbonate absorption [12]. Amiloride is not a very effective inhibitor of Na^+-H^+ exchange, since it normally needs to be used at rather high concentrations (10^{-5}–10^{-3} M) in order to block Na^+-H^+ exchange [12–17]. The Na^+ conductance in tight epithelia is sensitive to much lower amiloride concentrations (10^{-7}–10^{-6} M) [7–9]. It seemed likely, therefore, that certain pyrazinecarbonylguanidines other than amiloride might be more potent inhibitors of Na^+-H^+ exchange than was amiloride itself. We determined the effects of a series of such compounds on Na^+-H^+ exchange in rabbit kidney medulla microsomes in order to find such compounds and also to provide potential information concerning the molecular nature of the Na^+-H^+ exchange protein. Nearly all of the derivatives examined inhibited Na^+-H^+ exchange in rabbit kidney microsomes and some were more effective than amiloride. The relative sensitivity of these microsomes to amiloride derivatives was markedly different from the relative sensitivity of the Na^+ conductance in intact frog skin to the same derivatives [9–11], which was to be expected since the proteins responsible for Na^+ conductance and Na^+-H^+ exchange are most likely different.

We have also demonstrated that most of the amiloride analogues stimulate Na^+ efflux from medulla microsomes at concentrations higher than the concentrations at which they inhibit Na^+ influx. These effects are reversible, do not represent detergent effects, and most likely do not represent weak-base uncoupling effects. The analogues appear to stimulate Na^+ efflux from the microsomes by serving as substrates for the Na^+/H^+ exchanger, so that these organic cations are carried into the vesicles by the same protein that carries the Na^+ out.

Methods

Rabbit kidney medulla microsomes were formed as described in LaBelle and Lee [2]. Sodium transport into the microsomes was measured as described [2]. In order to detect leaks in the microsomes, we preloaded the microsomes with $^{22}Na^+$, added amiloride derivatives, and applied to Dowex columns as described previously [18]. The 11 amiloride derivatives were synthesized as described by Cragoe et al. [6].

The uptake of L-[^{3}H]glucose into medulla microsomes was measured by a modification of the procedure of LaBelle and Lee [2]. The microsomes (0.17 mg protein) were incubated for 30 min at 20°C with L-[^{3}H]glucose (6 μCi, 0.1 mM), NaHepps (6.4 mM, pH 8.2), and sucrose (0.25 M) in a total volume of 0.125 ml, then treated for 30 s with either 1 μl of dimethylsulfoxide or with an amiloride analogue dissolved in 1 μl dimethylsulfoxide, and then applied to a column of Sephadex G-50 in a syringe barrel (1 ml) and centrifuged as described in LaBelle and Lee [2]. The microsomes containing the L-[^{3}H]glucose passed through the Sephadex column while the extravesicular labeled compound was retained by the column.

Results

Sodium uptake into kidney medulla microsomes was very rapid, and almost entirely inhibited by amiloride (Fig. 1). In order to determine the effects of amiloride analogues on Na^+ transport into the microsomes, the microsomes were incubated with $^{22}Na^+$ for very short periods (30 s), so as to yield data that were as close as possible to initial rates. The average amount of ^{22}Na taken up by microsomes during a 30 s incubation in the absence of any analogue was 9.79 $\pm$ 1.41 nmol/mg protein ($n = 23$).

The effects of ten analogues of amiloride on Na^+ uptake into the microsomes were determined at a number of different concentrations. The structures of the analogues are shown in Table I. Nearly all of the compounds appeared to inhibit Na^+ transport more effectively than amiloride, but control experiments revealed that most of the compounds also stimulated Na^+ efflux from micro-

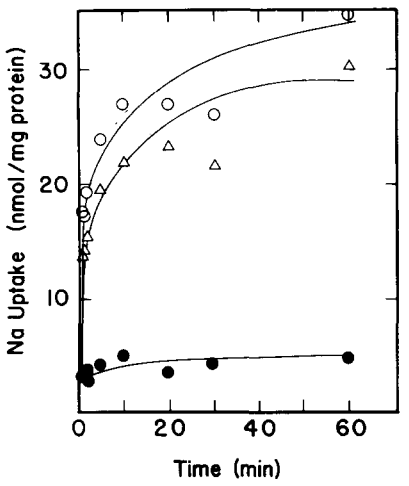
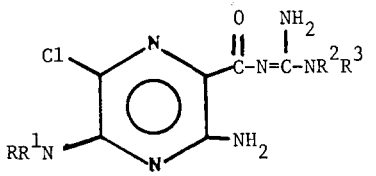
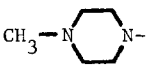


Fig. 1. Effect of time on uptake into medulla microsomes. Rabbit kidney medulla microsomes (0.18 mg protein) were incubated for the times indicated at 20°C with ^{22}NaHepps (0.5 μCi, 6.4 mM, pH 8.2) and sucrose (0.25 M), either with (●) or without (○) amiloride (2 mM) in a final volume of 0.25 ml and the incubation mixtures applied to Dowex columns and eluted as described [2]. The amiloride-sensitive Na^+ uptake is also shown (△).

TABLE I

STRUCTURE OF AMILORIDE ANALOGUES



Derivative code	RR^1N-	R^2R^3N-
A	H_2N-	-NH_2
B	H_2N-	-$NHCH_2C_6H_5$
C	$H_2NC(=NH)NH$-	-NH_2
D	$(CH_3)_2N$-	-NH_2
E	$(CH_3)_2CHNH$-	-$N(CH_3)_2$
F	$C_6H_5CH_2NH$-	-NH_2
G	$(C_2H_5)_2N$-	-NH_2
H	$H_2N(CH_3)N$-	-NH_2
I		-NH_2
J	$(CH_3)_2CHCH_2NH$-	-NH_2
K	$CH_3(CH_2)_3(CH_3)N$-	-NH_2

somes that had been pre-equilibrated with $^{22}Na^+$. These effects on efflux complicated the interpretation of the influx data. Since we used Dowex columns to separate extravesicular $^{22}Na^+$ from intravesicular isotope after incubating the microsomes with the $^{22}Na^+$, we could not incubate the microsomes with a compound that stimulated efflux, add $^{22}Na^+$, observe a decrease in intravesicular radioactivity, and conclude that the compound blocked influx. Efflux stimulation alone might yield results identical to results observed after influx inhibition. Therefore, we determined the ability of several concentrations of each analogue to both apparently inhibit Na^+ influx into the microsomes and to stimulate Na^+ efflux from the microsomes. We did not use analogue concentrations above 2 mM in any experiment, due to the limited water solubility of these compounds. Each compound was added to the microsomes by diluting concentrated dimethylsulfoxide stock solutions (50 mM). The final concentration of dimethylsulfoxide present with the microsomes was never greater than 2% and control determinations showed that such low dimethylsulfoxide concentrations failed to affect Na^+ transport. The data in Figs. 2 and 3 indicate that three amiloride analogues (analogues B, C, I) and amiloride itself (analogue A), were capable of substantially inhibiting Na^+ uptake into microsomes at several concentrations that failed to stimulate efflux from the microsomes by more than 20%. Analogues C and I inhibited Na^+ uptake by 50% at concentrations of 0.68 and 0.92 mM, while stimulating efflux by only 30% at a concentration of 2 mM. Amiloride inhibited Na^+ uptake by 50% at a concentration of 1.47 mM while only stimulating efflux by 20% at the 2 mM level. Benzamil (analogue B) inhibited influx by 50% at a concentration of 0.77 mM while the effects of this compound on Na^+ efflux were somewhat more severe than that of the other three compounds. We could determine the concentrations of analogues A, C, and I that inhibited Na^+ uptake into the microsomes with reasonable precision. Amiloride was the weakest of these compounds and the effects of the others on Na^+ influx were statistically indistinguishable. A non-linear, least-squares fitting algorithm [19] was used to fit the experimental data in Figs. 1–4 to expressions for simple single site binding. The influx data

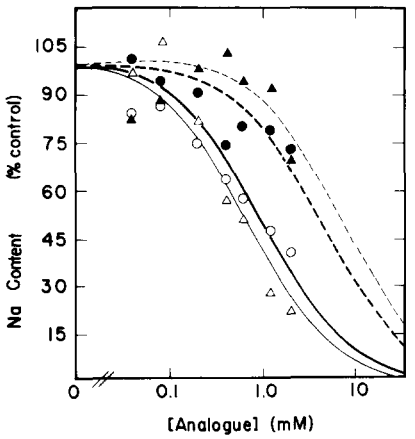


Fig. 2. Effect of analogues C and I on apparent sodium uptake into microsomes and on sodium efflux out of microsomes. Kidney microsomes were incubated with $^{22}Na^+$ for 30 s in the presence of the indicated amounts of analogue C (△) and analogue I (○), and the mixtures applied to Dowex columns as described in the legend of Fig. 1. Microsomes were also preincubated for 35 min with ^{22}Na as described in the legend of Fig. 1, and then the indicated amounts of analogue C (▲), and analogue I (●) were added for 30 s before the mixtures were applied to Dowex columns. The influx data points (open symbols) represent the means of triplicate determinations. The efflux data points (closed symbols) represent the means of duplicate determinations. Standard deviations were on the average 7.8% of the mean values.

points in Figs. 1–4 (open symbols) represent the means of triplicate determinations while the efflux data points (closed symbols) represent the means

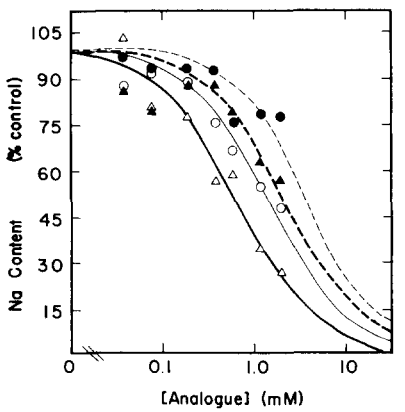


Fig. 3. Effect of analogues A and B on apparent sodium uptake into microsomes and on sodium efflux out of microsomes. Experimental conditions were the same as in Fig. 2 except that apparent Na^+ uptake was measured in the presence of analogues A (○) and B (△), and Na^+ efflux was measured in the presence of analogue A (●) and B (▲).

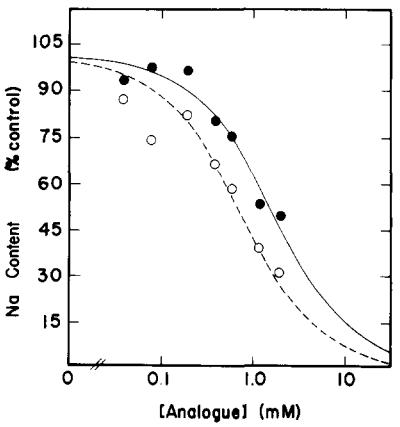


Fig. 4. Effect of analogue D on apparent sodium uptake into microsomes and sodium efflux out of microsomes. Experimental conditions were the same as in Fig. 2 except that apparent Na^+ uptake (○) was measured in the presence of analogue D and Na^+ efflux (●) measured in the presence of the same analogue.

of duplicate determinations.

Except for analogues E, F and K, all of the other compounds used in this study inhibited influx at lower concentrations than the concentrations at which they stimulated Na^+ efflux from the microsomes. Data obtained with analogues D and H are shown in Figs. 4 and 5. However, at each concentration examined, influx inhibition was

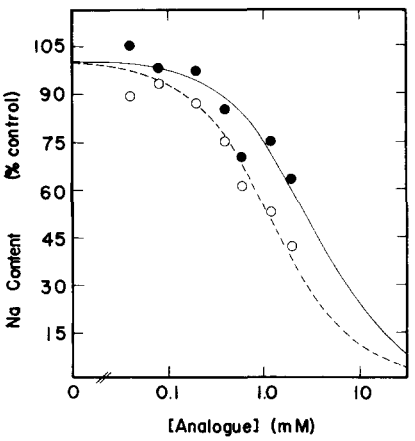


Fig. 5. Effect of analogue H on apparent sodium uptake into and sodium efflux out of microsomes. Experimental conditions were the same as in Fig. 2 except that apparent Na^+ uptake (○) was measured in the presence of analogue H and Na^+ efflux (●) was measured in the presence of the same analogue.

accompanied by efflux stimulation. This made it difficult to interpret the influx data. At the concentration of each analogue at which influx appeared to be 50% inhibited, there were also significant effects on efflux.

One of the compounds tested, analogue E, did not inhibit Na^+ uptake into the microsomes in the absence of a pH gradient at any concentration examined (up to 2 mM), nor did it stimulate Na^+ efflux from the microsomes in all experiments. The efflux of sodium from medulla microsomes was measured as a function of time and the results shown in Fig. 6. All of the amiloride analogues stimulated the rate of Na^+ efflux from the microsomes, provided the analogue concentration was 2 mM. Sodium efflux was stimulated the most by analogues B, D, F, G, J and K, while analogues A, C, E, H, and I exerted much smaller effects.

We have previously shown that amiloride-inhibited Na^+ uptake into medulla microsomes represented Na^+-H^+ exchange [3]. When microsomes were preincubated at pH 6.0 and incubated with $^{22}Na^+$ at pH 8.0 in order to induce a pH gradient, Na^+ transport into the microsomes was stimulated by 3–4-fold. All of the amiloride analogues inhibited the enhanced levels of Na^+ transport observed in the presence of a pH gradient. The effects of increasing amounts of analogues B, C, and I on Na^+-H^+ exchange in the microsomes are shown in Fig. 7.

The concentrations at which each analogue appeared to inhibit Na^+ uptake into microsomes by 50% both in the presence and the absence of a pH gradient are shown in Table II, together with the concentrations at which each derivative stimulated the efflux of 50% of the $^{22}Na^+$ from the microsomes. A nonlinear least squares fitting algorithm was again used to fit the data used to determine these values [19]. The regression coefficients for the curves obtained by this method were very high

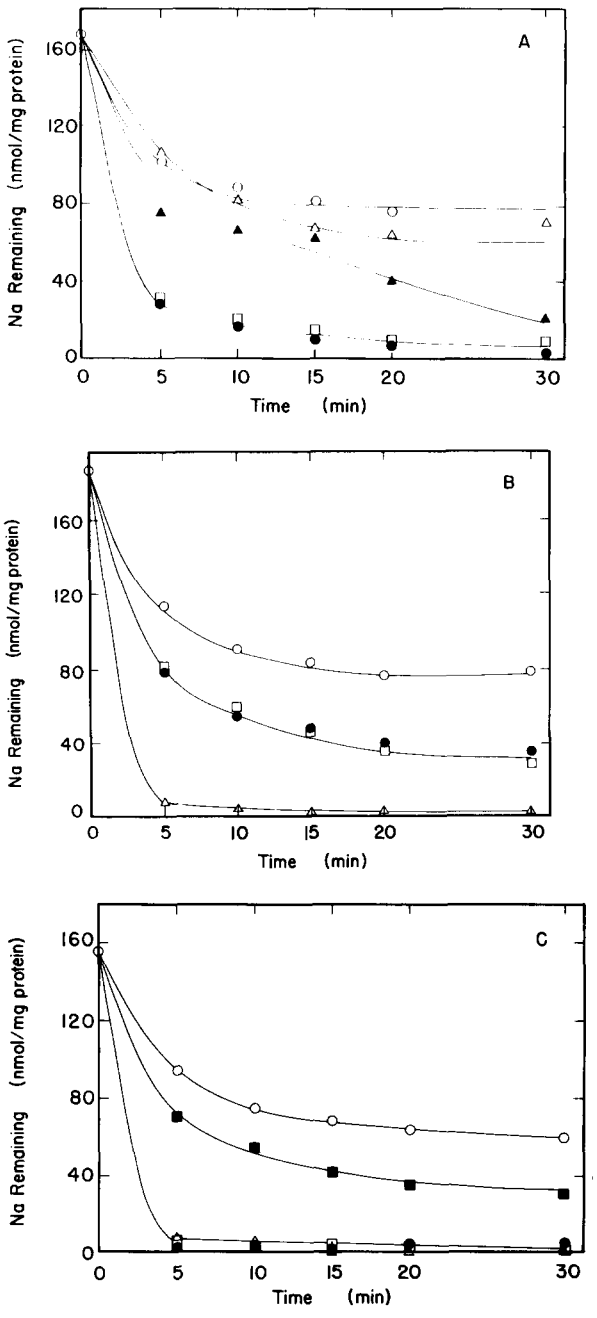


Fig. 6. Effect of amiloride analogues on Na^+ efflux from medulla microsomes. Aliquots of medulla microsomes (0.12 ml, 0.9 mg protein) were preincubated for 30 min at 20°C in a total volume of 0.3 ml with ^{22}NaHepps (2.4 μCi, 6 mM, pH 8.2) and sucrose (0.25 M) and then diluted with solutions (1.2 ml) containing unlabeled NaHepps (6 mM, pH 8.2), sucrose (0.25 M) and amiloride analogues (2 mM) and incubated at 20°C for the times indicated before aliquots (0.25 ml) of each solution were applied to Dowex columns, eluted and the radioactivity in the eluted microsomes determined. Each data point represents the mean of duplicate determinations and error bars indicating standard deviations would be smaller than the symbols. In part A, analogues included: control (no analogue (○), A (△), B (●), C (▲) and D (□). In part B, analogues included: control (no analogue (○), E (●), I (□), and K (△). In part C, analogues included: control (○), H (■), F (□), G (●), and J (△).

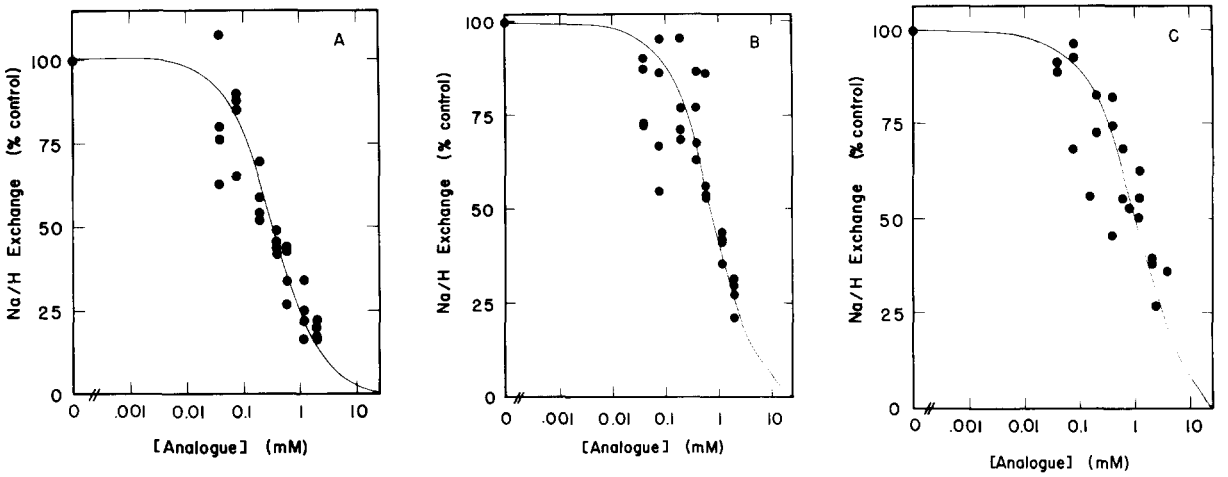


Fig. 7. Effect of analogues B, C, and I on Na^+-H^+ exchange in medulla microsomes. Aliquots (20 μl, 0.3 mg protein) of medulla microsomes were preincubated for 40 min at 20°C in a total volume of 40 μl with pH 6.0 buffer (45 mM Mes/14 mM Tris/7 mM Hepes/183 mM sucrose) and then diluted with solutions (0.21 ml) containing $^{22}NaCl$ (1 μCi, 4 mM) with increasing concentrations of amiloride analogues and pH 8.0 buffer (28 mM Tris/31 mM Hepps/190 mM sucrose), and then incubated at 20°C for 30 s, applied to Dowex columns, eluted, and the radioactivity in the eluted microsomes determined. (A) Analogue B, (B) Analogue C, (C) Analogue I.

(> 0.75 in most cases), which indicated that the analogues were most likely interacting at single sites. Na^+ uptake into the microsomes was slightly more sensitive to each analogue in the presence of a pH gradient than in its absence, but the concentrations at which each analogue inhibited Na^+ uptake both with and without the proton gradient were quite comparable. Only analogues B and D

TABLE II

AMILORIDE ANALOGUE CONCENTRATIONS THAT AFFECT MICROSOMAL SODIUM LEVELS BY 50%

Sodium uptake into the microsomes at 7 analogue concentrations was determined as described in the legends of Figs. 1 and 7. The concentrations of each analogue required to inhibit apparent Na^+ uptake by 50% in the presence or absence of a pH gradient are shown below. The ability of each analogue to stimulate Na^+ efflux from the microsomes was also determined and the concentrations required to permit the efflux of 50% of the microsomal Na^+ are shown below.

Analogue	Concentrations required to inhibit apparent uptake by 50% without pH gradient (mM ± S.D.) ($n = 3$)	Concentration required to inhibit apparent uptake by 50% with pH gradient (mM ± S.D.) ($n = 3$)	Concentration required to stimulate the efflux of 50% of the Na^+ (mM ± S.D.) ($n = 2$)
A	1.47 ± 0.38	0.74 ± 0.08	4.55 ± 0.05
B	0.77 ± 0.16	0.34 ± 0.03	2.3 ± 0.5
C	0.68 ± 0.1	0.89 ± 0.10	7.1 ± 1.6
D	0.76 ± 0.07	0.19 ± 0.05	1.7 ± 0
E	–	1.83 ± 1.3	–
F	0.19	0.13 ± 0.03	0.17
G	0.35 ± 0.05	0.20 ± 0.0	0.52 ± 0
H	1.23 ± 0.33	1.13 ± 0.13	2.94 ± 0.3
I	0.92 ± 0.34	1.07 ± 0.39	3.9 ± 0.25
J	0.64 ± 0.16	0.48 ± 0.13	1.4 ± 0.4
K	0.20	0.09 ± 0.01	0.24

inhibited Na^+ uptake in the presence of a proton gradient much better than in its absence. These results are in agreement with the evidence that sodium goes into the microsomes via the Na^+/H^+ exchanger either with or without a pH gradient [3,4]. The ability of each analogue to stimulate Na^+ efflux from the microsomes is qualitatively the same in Table II and Fig. 6. Since each analogue was tested in the experiment shown in Fig. 6 at a concentration of 2.0 mM, it was expected that each analogue that exerted a half-maximal effect in Table II at a concentration more than 2 mM would appear the least effective in Fig. 6, and this was indeed the case (except for analogue B, which was a borderline case).

Analogues F and K were shown to be much more effective at stimulating Na^+ efflux from the microsomes than the other derivatives (Table II). Microsomes were exposed for 30 s to either analogue F or K at a concentration shown in Table II to be sufficient to permit the efflux of more than 50% of the Na^+ from the microsomes (0.4 mM), and then the microsomes were either diluted by 1 to 12 with a solution containing $^{22}Na^+$ and no analogues for 30 min or diluted in the same way with a solution containing $^{22}Na^+$ and either analogue (0.4 mM). The effect of each analogue was shown to be completely reversible. Microsomes loaded with $^{22}Na^+$ in the complete absence of either analogue accumulated 11.6 ± 0.18 nmol Na^+/mg protein ($n = 3$), while microsomes preexposed to analogue F and treated with $^{22}Na^+$ after the concentration of analogue F had been reduced by 92% accumulated 10.5 ± 0.78 nmol Na^+/mg protein and microsomes pre-exposed to analogue K and treated with $^{22}Na^+$ after dilution with analogue-free solution accumulated 10.8 ± 0.78 nmol Na^+/mg protein. Microsomes pre-exposed to either analogue and then treated with $^{22}Na^+$ in the presence of analogue (0.4 mM) accumulated either 0.61 ± 0.07 nmol Na^+/mg protein or 0.67 ± 0.15 nmol Na^+/mg protein. These data indicated that both analogues could stimulate Na^+ efflux from the microsomes at a concentration of 0.4 mM. The effect of either analogue could be eliminated merely by lowering the concentration of the analogue by 92%.

In order to determine whether or not the amiloride analogues were exerting non-specific, detergent effects on the microsomes, we preloaded the microsomes with L-[^{3}H]glucose, and measured the ability of each derivative to stimulate L-glucose efflux from the microsomes. When used at concentrations that induced the loss of more than 50% of the Na^+ from the microsomes nearly all the analogues failed to significantly stimulate L-glucose efflux from the microsomes. This indicated that very few analogues were inducing non-specific, detergent-like leaks in the microsomes. Analogues B, D and J, however, did permit some L-glucose to leak out of the microsomes (30–50%) when used at a concentration of 2 mM. However, these analogues inhibited Na^+-H^+ exchange by 50% at much lower concentrations (0.19–0.48 mM) and most likely at the lower concentrations no detergent effects were exerted by these analogues.

In order to determine whether or not analogues F and K were stimulating Na^+ efflux from the microsomes via weak-base uncoupling effects, we preincubated the microsomes with $^{22}Na^+$ for 30 min in the presence of relatively high concentrations of pH 8.0 buffer (25.2 mM Tris, 34 mM Hepps), and then we added each analogue (0.4 mM) to the microsomes, waited 30 s, and applied to Dowex columns. The analogues were still capa-

TABLE III

EFFECTS OF AMILORIDE ANALOGUES ON THE NON-SPECIFIC EFFLUX OF L-[^{3}H]GLUCOSE FROM MEDULLA MICROSOMES

Medulla microsomes were preloaded with L-[^{3}H]glucose and treated for 30 s with the amiloride analogues as described in Methods.

Analogue	Analogue concn. (mM)	Amount of L-[^{3}H]glucose remaining after analogue treatment (pmol/mg ± S.D.)	n
None	0	11.4 ± 1.0	14
A	2	9.9 ± 0.4	4
B	2	7.5 ± 1.1	7
C	2	10.0 ± 0.7	3
D	2	7.9 ± 0.6	8
E	2	11.9 ± 0.7	4
F	0.2	11.5 ± 0.7	4
G	0.4	9.2 ± 1.0	8
H	2	10.5 ± 0.5	3
I	2	12.0 ± 0.8	4
J	2	5.6 ± 1.1	8
K	0.2	12.0 ± 2.5	8

ble of releasing 60% of the $^{22}Na^+$ from the microsomes in spite of the high buffering capacity of the solution, and this suggested that the analogues were not exerting their effects by acting as weak-base uncouplers.

Discussion

The amiloride analogues examined exert complex effects on the medulla microsomes, inhibiting Na^+ uptake into the microsomes either in the presence or absence of a pH gradient at generally lower concentrations than the concentrations required to stimulate Na^+ efflux. However, the concentrations required to stimulate Na^+ efflux are generally 2–4-times higher than the concentrations required to inhibit uptake (Table II), suggesting some relationship between the two processes. Since the concentration of each analogue required for 50% inhibition of $^{22}Na^+$ uptake into the microsomes was roughly the same in the presence of a pH gradient as in its absence (except for B and D), it seemed reasonable that the same protein was responsible for Na^+ uptake into the microsomes both in the presence of a pH gradient and its absence (as we have previously demonstrated [3]).

The two analogues that stimulated Na^+ efflux the most effectively (analogues F and K) did not induce irreversible leaks in the microsomes, which indicated that these compounds were not non-specifically destroying the microsomes. The fact that these analogues did not stimulate the efflux of L-[^{3}H]glucose from the microsomes also indicated that they were not destroying the microsomes and that they were not exerting reversible detergent effects (opening large holes reversibly). All of the other analogues (except B, D and J) also failed to stimulate L-[^{3}H]glucose efflux from the microsomes which indicated that these other analogues also failed to exert such detergent effects. Analogues B, D, and J only exerted slight detergent effects at concentrations 5–10-times higher than the concentrations at which they inhibit Na^+-H^+ exchange by 50%. These derivatives also stimulate $^{22}Na^+$ efflux from microsomes at lower concentrations than the concentrations required to permit [^{3}H]glucose efflux. These controls indicate that the analogues do not exert their effects on Na^+ transport via nonspecific detergent action on membranes. The possibility existed that these weakly basic analogues were diffusing across the microsomal membranes in the neutral form, picking up protons on the inside of the microsomes, and finding escape from the microsomes blocked due to the positive charge conferred by the proton. The analogues could then generate a pH gradient (inside alkaline) that would stimulate Na^+-H^+ exchange and drive Na^+ out of the microsomes. This theory has been proposed by Dubinsky and Frizzell to explain the inhibition of Na^+-H^+ exchange by amiloride itself [20]. However, we have shown that amiloride does not eliminate Na^+-H^+ exchange by this mechanism, since NH_3 is such a weak inhibitor of Na^+-H^+ exchange in medulla microsomes [3]. We have also shown that medulla microsomes have a substantial buffering capacity, since they are insensitive to added HCO_3^- and to low concentrations of such added buffers as Mes and Tris (5 mM) [2,3]. Therefore, we feel that this buffering capacity of the microsomes should eliminate any weak base uncoupling effects by the analogues. Since analogues K and F can still stimulate Na^+ efflux from the microsomes even after equilibration with a high concentration of added buffer, we feel that these analogues are not stimulating Na^+ efflux via weak base uncoupling effects.

To explain why the analogues stimulate Na^+ efflux from the microsomes, one could suggest that the analogues are carried across the microsomal membranes by the Na^+/H^+ exchanger itself. This would explain why the analogues stimulate Na^+ efflux, by exchanging for Na^+ either directly or indirectly (via exchange with protons). It would also explain why these analogues inhibit Na^+ influx, by competing for Na^+ as it interacts with the exchange protein. The effects of amiloride on Na^+-H^+ exchange have been shown to be competitive with respect to Na^+ [21], and amiloride has been shown to readily penetrate into intact cells [22,23]. Of course more work is needed to prove conclusively that the amiloride analogues can be carried into membrane vesicles via the Na^+/H^+ exchanger.

The analogues that stimulate Na^+ efflux most effectively were analogues F and K, the 5-*N*-benzylamino analogue of amiloride and the 5-*N*-butyl-*N*-methylamino analogue (50% efflux stimulated

by 0.2 mM concentrations). The next most effective analogue was analogue G, the 5-*N*,*N*-diethylamino analogue (50% efflux stimulated by 0.52 mM concentration). The 5-*N*-isobutylamino analogue (analogue J) the 5-*N*,*N*-dimethylamino analogue (analogue D), the amiloride analogue with a benzyl group on the terminal guanidine nitrogen atom (analogue B) and the 5-(1-methylhydrazino) analogue (analogue H) were less effective at inducing Na^+ efflux (50% effects exerted by concentrations between 1.4 and 2.9 mM). The 5-*N*-guanidino analogue (Analogue C), the 5-(4-methyl-1-piperazinyl) analogue (analogue I) and amiloride were very poor inducers of Na^+ efflux (50% effects exerted by concentrations between 4 and 7 mM).

The amiloride analogues could be ordered according to their effectiveness in apparently inhibiting Na^+ uptake into microsomes in roughly the same way. Using the values in Table II, these analogues fit into nearly the same effectiveness groups when examined for effects on either influx or efflux. However, due to the ability of these compounds to stimulate efflux, one cannot really determine the precise concentrations that are effective in inhibiting influx.

The relative effectiveness of the compounds in stimulating Na^+ efflux from microsomes appears to correlate with the presence of a large, hydrophobic group on the 5-amino nitrogen of the amiloride analogue. The largest such group is the benzyl group on analogue F, and the next largest number of carbons on the 5-amino nitrogen is provided by the butyl group and the methyl group on analogue K. These were the most effective compounds in stimulating efflux. Analogues with between two and four carbons attached to the 5-amino nitrogen were less effective in inducing efflux, (analogues G, J, and D) and amiloride (with no substituent on the 5-amino nitrogen) and analogues with a positively charged substituent on the 5-amino nitrogen (analogues C and I) were even less effective. These positively charged substituents would not be at all hydrophobic. The requirement for other portions of the amiloride molecule are more obscure. The relative inability of analogue E to stimulate efflux, suggests the requirement for an unhindered guanidine group but this is contradicted by the ability of analogue B (benzamil) to stimulate efflux more effectively than amiloride. More work with more analogues is required to further clarify these structure-activity relationships.

The work of Vigne et al. [24] has shown that an amiloride analogue with both an ethyl substituent and an isopropyl substituent on the 5-amino nitrogen is about 100 times as effective as amiloride in blocking Na^+-H^+ exchange in cultured cells. This correlates well with our evidence that large hydrophobic substituents on the 5-amino nitrogen increase the ability of the analogue to interact with the Na^+/H^+ exchanger.

There is no reason to expect the above compounds would inhibit Na^+-H^+ exchange into membrane vesicles with the same efficacy as they inhibit the Na^+ conductance across the apical membranes of tight epithelia, such as frog skin. Na^+-H^+ exchange has been shown to be much less sensitive to amiloride than the Na^+ conductance. Therefore, we were not surprised to find that some of the compounds examined above which bore substituents on the 5-amino nitrogen atom were shown to be totally incapable of inhibiting the Na^+ conductance in frog skin [9,11]. Benos et al. [9] found that neither derivative D nor derivative E inhibited Na^+ transport across frog skin when used at a concentration (10^{-6} M) at which amiloride inhibited by 72%. Li and De Sousa [11] found that derivative D actually stimulated Na^+ transport across frog skin when used at concentrations between 10^{-6} M and 10^{-4} M. Derivative D was much more effective than amiloride in interacting with the Na^+/H^+ exchanger in medulla microsomes (Table II), as were some other derivatives with substituents on the 5-amino group (derivatives F, K, G, J). The data of Benos et al. [9] showed that substitution of protons of the 5-amino group by alkyl groups prevented the derivative from inhibiting Na^+ transport across the frog skin and such substitutions were clearly tolerated by the microsomal Na^+-H^+ exchanger (Table II). Cuthbert and Fanelli [10] showed that benzamil (derivative B), was more effective than amiloride in inhibiting Na^+ transport across the frog skin, and this appears to be true for Na^+-H^+ exchange in the microsomes as well (Table II). Of course the k_i values determined for amiloride and benzamil in the frog skin preparation were about three

orders of magnitude lower than the effective concentrations in the microsomal system.

Cragoe et al. [6] determined the relative dose of each amiloride derivative required to reverse the effects of a mineralocorticoid on the Na^+/K^+ ratio as found in the urine of adrenalectomized (ADX) rats. The spectrum of effects exerted by the derivatives evaluated in this assay do not correlate at all with the inhibitory effects of the compounds on the Na^+/H^+ antiporter obtained in the present study. Thus, compounds B and E are equipotent to amiloride (+4) in the ADX rat assay, but B is more active while E is less active than amiloride in interacting with the Na^+/H^+ system. Compounds D, G, and K were somewhat less active (+3) than amiloride (+4) in the ADX rat assay; yet, D, J and K all interact better with the Na^+/H^+ exchanger than does amiloride. Compounds C, F, H, I, and J were much less active or inactive in the ADX rat assay (0 or +1) than amiloride; however, compounds F and J appear to inhibit Na^+-H^+ exchange better than amiloride, and compounds C, H and I are nearly equally effective. These results suggest that the diuretic and saluretic effects of amiloride and its derivatives are independent of their effects on the Na^+/H^+ exchanger and that the ratio of these effects is dependent on the particular structure of the compound. Amiloride most likely exerts diuretic effects by acting on the Na^+ conductance in the apical membrane of the cortical collecting tubule of the mammalian kidney [25].

The fact that alterations in the chemical structure of amiloride can be made without eliminating the ability of the compound to inhibit Na^+-H^+ exchange in vesicles indicates that some such compounds might prove useful as affinity reagents in the isolation of the Na^+-H^+ transport protein. Amiloride is a competitive inhibitor of Na^+-H^+ exchange (competitive with respect to Na^+) [21]. Therefore, its effects on Na^+-H^+ exchange are normally studied in vitro in the presence of low Na^+ concentrations and it is a fairly poor inhibitor at physiological extracellular Na^+ concentrations (150 mM). A more potent inhibitor of Na^+-H^+ exchange such as derivative G (the 5-*N*-diethylamino derivative) might inhibit Na^+-H^+ exchange better at physiological Na^+ concentrations and such a compound might eventually prove useful in the management of acid-base disorder clinically.

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