Characterization of Potent Na^+/H^+ Exchange Inhibitors from the Amiloride Series in A431 Cells†

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ABSTRACT: Na^+/H^+ exchange is stimulated in a variety of cell types by addition of mitogenic polypeptides such as epidermal growth factor or platelet-derived growth factor. In order to assess the importance of Na^+/H^+ exchange in the mitogenic response, it is desirable to have available inhibitors of this process which exhibit high affinity and good specificity. We characterize in this report a number of 5-alkylamino-substituted derivatives of amiloride [3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazinecarboxamide] which show much higher affinity than the parent compound for the Na^+/H^+ antiporter in A431 cells. High affinity is conferred by substitution with two alkyl groups and is increased by introducing a branched alkyl chain. An analogue bearing a 5-anilino group is also very potent. These analogues effectively inhibit the elevation of intracellular pH upon stimulation of Na^+/H^+ exchange by growth factors. We have assessed other potential inhibitory effects of these compounds on cellular metabolism. In agreement with previous reports, we find that amiloride inhibits protein synthesis both in cells and in cell-free translation systems. While amiloride and its analogues show similar inhibition of protein synthesis in a cell-free system, most analogues inhibit cellular protein synthesis at much lower concentrations than does amiloride. These analogues are also potent inhibitors of purified Na,K-ATPase and cause a profound decrease in intracellular K^+ as well as ATP content. These latter effects, however, require analogue concentrations which are 5–7 times higher than those inhibiting cellular protein synthesis. Among the analogues tested, the one bearing a 5-*N*,*N*-dimethyl substitution is exceptional in being a potent inhibitor of Na^+/H^+ exchange ($K_I \simeq 0.17$ μM) while exhibiting side effects that are only slightly increased relative to amiloride. This compound may be useful for studies of the physiological function of the Na^+/H^+ antiporter.

A variety of cultured animal cells express on their cell surface a Na^+/H^+ antiporter which can function to extrude protons from the cell using the inwardly directed Na^+ gradient as a driving force (Moolenaar et al., 1981, 1983; Paris & Pouyssegur, 1983; Rindler & Saier, 1981; Rothenberg et al., 1983a,b; Schuldiner & Rozengurt, 1982; Vinge et al., 1982). The diuretic amiloride was initially shown to be a potent inhibitor of conductive Na^+ transport in electrically tight (high-resistance) sodium transporting epithelia (Cuthberg & Fanelli, 1978) but also inhibits Na^+/H^+ exchange though with lower potency [for review, see Benos (1982)]. Kinetic studies have suggested that amiloride inhibits Na^+/H^+ exchange by competing for the Na^+ site (Rindler et al., 1979; Kinsella & Aronson, 1980; Vinge et al., 1982, 1983; Paris & Pouyssegur, 1983). Thus, at physiological Na^+ concentrations, effective inhibition of Na^+/H^+ exchange requires rather high (~0.1–1 mM) amiloride concentrations.

Addition of a variety of polypeptide growth factors to cells stimulates an amiloride-sensitive and electroneutral Na^+ uptake, suggesting a stimulation of Na^+/H^+ exchange (Smith & Rozengurt, 1978; Koch & Leffert, 1979; Moolenaar et al., 1982; Rothenberg et al., 1982, 1983a; Pouyssegur et al., 1982; Owen & Villerial, 1983). The recent development of techniques for intracellular pH measurement in monolayer cultures has resulted in the demonstration that stimulation of Na^+/H^+ exchange by growth factors leads to cytoplasmic alkalinization (Schuldiner & Rozengurt, 1982; Rothenberg et al., 1983b; Moolenaar et al., 1983; Cassel et al., 1983; Burns & Rozengurt, 1983). While such cytoplasmic alkalinization can potentially stimulate a variety of cellular responses, its role in the mitogenic response to growth factors remains uncertain. Inhibitor studies using amiloride are complicated by the rather low affinity of this drug for the Na^+/H^+ antiporter, since the relatively high concentrations of amiloride that are required to block Na^+/H^+ exchange inhibit protein synthesis in cells and in cell-free systems (Fehlman et al., 1981; Leffert et al., 1982; Lubin et al., 1982) and may also inhibit the Na^+/K^+ pump (Soltoff & Mandel, 1983) and CO_2 evolution from pyruvate (Taub & Saier, 1981). Due to these side effects of amiloride, the interpretation of earlier findings (Koch & Leffert, 1979; Rozengurt & Mendoza, 1980: Rozengurt, 1981; Moolenaar et al., 1982) that amiloride blocks cellular proliferation is rather ambiguous.

In this paper we describe the interaction of a number of amiloride analogoues with the Na^+/H^+ antiporter and show that many of them exhibit a much higher affinity than amiloride for this molecule. We also assessed the specificity of the analogues for the antiporter based on measurements of the effects of the analogues on other activities in cells and in cell-free systems.

Materials and Methods

Amiloride derivatives were synthesized as previously described (Bicking et al., 1965; Jones et al., 1969; Cragoe & Waltersdorf, 1978; Cragoe, 1983). Methods of cell culture and sources of reagents can be found in Rothenberg et al. (1983a,b).

Salt Solutions. The basic physiological solution (solution A) contained 135 mM NaCl, 5.4 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1 mM P_i, 25 mM glucose, and 25 mM MOPS–NaOH[1] at pH values as indicated in individual ex-

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[1] Abbreviations: MOPS, 4-morpholinepropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DME, Dulbecco's minimum Eagle's medium; ATPase, adenosinetriphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Me_2SO, dimethyl sulfoxide.

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periments. Sodium-free solution A contained an isoosmotic replacement of NaCl and NaOH with tetramethylammonium chloride and tetramethylammonium hydroxide, respectively.

$^{22}Na^+$ Uptake Measurement. Confluent cultures of A431 cells in 35-mm dishes [(1–1.5) × 10^6 cells/dish] were preincubated for 3.5–4 h at 37 °C in solution A, pH 7.2, containing 0.5 mM ouabain. This incubation results in an equilibration of the intracellular Na^+ pool with extracellular Na^+ (Rothenberg et al., 1983a,b). $^{22}Na^+$ uptake was assayed in sodium-free solution A, pH 7.2, also containing 0.5 mM ouabain. Cells were briefly rinsed with the sodium-free solution and incubated at 37 °C in 0.9 mL of the same solution containing 1 × 10^6 cpm/mL $^{22}Na^+$. Uptake was terminated, and intracellular $^{22}Na^+$ content was determined as described (Rothenberg et al., 1983a).

pH_i Measurements. Fluorometric pH_i measurements were carried out as previously described (Rothenberg et al., 1983b; Cassel et al., 1983). Briefly, nearly confluent cells are loaded with 4,5-dimethylfluorescein–dextran by osmotic lysis of pinosomes (Okada & Rechsteiner, 1982). Cells are allowed to recover from the osmotic stress by incubation overnight in HCO_3^--free, Hepes-buffered DME (DME/Hepes) supplemented with 1% fetal calf serum. pH_i measurements were carried out at 37 °C in an SPF-500 spectrofluorometer. Calibration of pH_i vs. relative fluorescence was obtained after equilibrating pH_i with the extracellular pH in the presence of ouabain as described (Rothenberg et al., 1983b).

Protein Synthesis in Cell-Free Translation System. A rabbit reticulocyte lysate in vitro translation kit (Bethesda Research Laboratories, Gaithersburg, MD) was used to measure the incorporation of [^{35}S]methionine into polypeptides according to the manufacturer's instructions. Reaction mixtures in a final volume of 20 mL were incubated for 30 min at 30 °C in the presence of 0.1 μg of rabbit globin mRNA, 5 μCi of [^{35}S]methionine and varius concentrations of amiloride analogues. Background radioactivity (incubation at 0 °C) was 4% of the incorporation in the control system.

Cellular Protein Synthesis. Cells in Linbro 24-well trays (1 × 10^5 cells/well) were incubated for 2 h at 37 °C DME/Hepes containing 10 μCi/mL [^{3}H]leucine. At the end of the incubation period the cells were treated for 20 min with cold 5% trichloroacetic acid and then washed twice with trichloroacetic acid. Cell protein was solubilized by incubation for 15 min at 60 °C in 0.3 N NaOH, and radioactivity was assayed in a scintillation counter.

Na,K-ATPase Assay. Reaction mixtures in a final volume of 0.2 mL contained 40 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 0.15 mM EGTA, 4% (v/v) Me_2SO, 3 mM ATP (Sigma; vanadate free), 1 × 10^6 cpm of [γ-^{32}P]ATP (New England Nuclear), and 30 mM MOPS–KOH, pH 7.4. The reaction was initiated by addition of purified Na,K-ATPase from dog kidney (Sigma), 12 μg of protein per assay, and was proceeded for 20 min at 37 °C. ATP hydrolysis was linear with time for at least 30 min A total of 19% of the ATP was hydrolyzed during the assay period, and 89% of this hydrolysis was ouabain sensitive. The release of [^{32}P]P_i was determined after adsorption of the residual [γ-^{32}P]ATP to charcoal.

The ATP content of cells was determined by using the luciferin–luciferase reagent (Sigma) and monitoring the emitted light in a scintillation counter.

Results

Inhibition of Na^+/H^+ Exchange in Ouabain-Treated Cells. In order to assess Na^+/H^+ exchange activity in A431 cells, it is necessary to activate the antiporter which normally shows very low activity (Rothenberg et al., 1983a). We have previously observed maximal stimulation of Na^+/H^+ exchange after prolonged incubation in the presence of the Na^+,K^+-ATPase inhibitor ouabain (Rothenberg et al., 1983a,b). During this process cellular Na^+ increases and cellular K^+ decreases, but the mechanism of activation of the Na^+/H^+ exchanger by this treatment is unknown. Exposure of ouabain-treated cells to sodium-free medium results in rapid intracellular acidification (Rothenberg et al., 1983a; Figure 3) accompanied by Na^+ efflux from the cells which is partially inhibited by an amiloride analog (Figure 1). When carrier-free $^{22}Na^+$ is added to the sodium-free solution the tracer is taken up into the cells (Figure 1A). This uptake is linear for 6–8 min and is almost completely abolished by 0.2 mM amiloride. Thus, the measurement of Na^+ uptake under these conditions provides a sensitive assay for the Na^+/H^+ antiporter activity. It is possible that the observed $^{22}Na^+$ entry represents Na^+/Na^+ exchange rather than Na^+/H^+ exchange. Alternatively, external $^{22}Na^+$ could exchange for intracellular protons which rapidly accumulate in the cells due to the net efflux of internal Na^+ through the Na^+/H^+ antiporter. The results of the $^{22}Na^+$ uptake assay for Na^+/H^+ exchange are in good agreement with the results obtained independently by measurements of pH_i, as will be shown below.

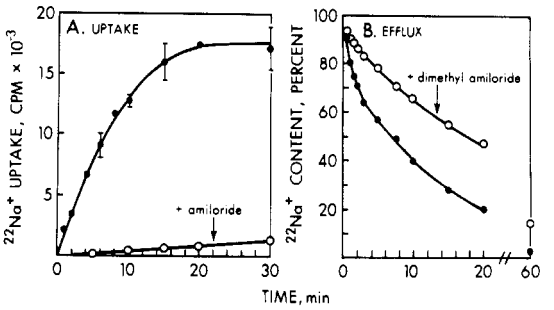


FIGURE 1: Kinetics of $^{22}Na^+$ uptake and efflux in ouabain-treated cells upon switching to Na^+-free solution. (A) $^{22}Na^+$ uptake was assayed in duplicate as described under Materials and Methods in the presence or absence of 0.2 mM amiloride. (B) Confluent cells in Linbro 24-well trays were loaded with $^{22}Na^+$ (150 mM, 1.5 × 10^8 cpm/mL) by incubation in the presence of ouabain as described under Materials and Methods. Subsequently the cells were rapidly washed 4 times at 22 °C in Na^+-free solution containing 0.3 mM amiloride and then washed once in the absence of amiloride. The entire washing procedure took approximately 10 s and resulted in less than 3% loss of intracellular $^{22}Na^+$. the experiment was initiated by the addition of 1 mL of Na^+-free solution at 37 °C with or without 50 μM N^5,N^5-dimethylamiloride (analogue A1). At each time point the medium was removed into a counting vial and replaced with another 1 mL of Na^+-free solution. After 60 min the cells were extracted with trichloroacetic acid to determine the residual $^{22}Na^+$. $^{22}Na^+$ content at each time point is calculated by subtracting the accumulative $^{22}Na^+$ released into the medium from the amount of radioactivity at zero time, calculated as the sum of the counts in all samples and is the trichloroacetic acid extract. Results are represented as percent of the total counts (7870 and 7548 cpm in the samples incubated with and without N^5,N^5-dimethylamiloride, respectively).

The effect of amiloride and its analogues on the rates of $^{22}Na^+$ uptake is shown in Figure 2, and the 50% inhibitory concentrations (IC_{50}) are summarized in Table I. These IC_{50} values are essentially equivalent to K_I values since the competing substrate (Na^+) is present at only trace amounts in the assay. In agreement with this assumption, the IC_{50} of 4 μM found for amiloride by using this assay is similar to K_I values reported for this drug in other cells by using different methods (Vinge et al., 1982, 1983; Paris & Pouyssegur, 1982). Substitutions of the hydrogen atoms on the 5-amino group of amiloride with different alkyl and aryl groups (see Table I) result in many cases in compounds demonstrating higher affinity (lower IC_{50} values) than the parent compound. In-

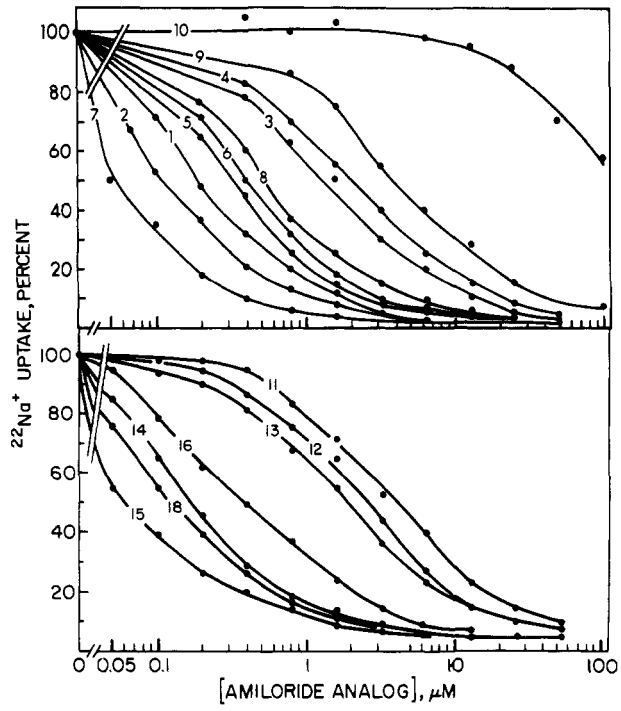


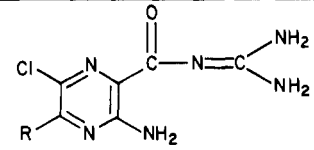
FIGURE 2: Concentration dependency of the inhibition of $^{22}Na^+$ uptake by amiloride analogues. Cells were preincubated in the presence of ouabain, and Na^+ uptake in carrier-free solution was subsequently assayed for 6 min as described in Figure 1A. The structure of the amiloride analogue (coded by analogue number) used in each experiment is indicated in Table I. Data are represented as percent $^{22}Na^+$ uptake relative to control systems incubated in the absence of amiloride analogue.

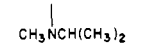
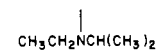
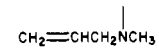
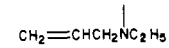
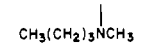
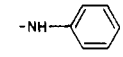
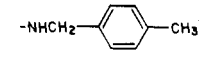
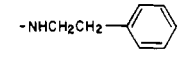
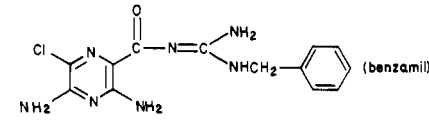
spection of the structure–activity relationship among these compounds suggests that analogues demonstrating high affinity for the antiporter have some specific structural features. While single substitution with straight chain alkyl groups (A11, A12, A13) has little effect on the affinity for the antiporter, disubstitution, e.g., introduction of two methyl groups (A1), results in a much more potent inhibitor. This is true even when the two substituents are joined to form a ring (A5). The presence of branched alkyl groups, either as a single substituent (*tert*-butyl, A2) or together with a second alkyl subsitutent (A7, A14) increases the inhibitory potency, as does the presence of an alkenyl group (A18). The compound bearing a 5-anilino group (A15) is among the most potent inhibitors, while the presence of a methylene or ethylene bridge between the amino and phenyl groups (A3, A4) strikingly reduces the inhibitory potency. Finally benzamil, which contains a benzyl substituent on the terminal nitrogen atom of the guanidino moiety of amiloride, is a very poor inhibitor in this system. Since benzamil is a very potent inhibitor of the Na^+ channel of tight epithelia (Cuthberg & Fanelli, 1978), it is clear that such a channel does not contribute to the observed $^{22}Na^+$ uptake in our assay.

Most potent among the analogues tested in A431 cells is the one bearing ethyl and isopropyl substituents (A7, K_I = 0.05 μM). Similar K_I values for this analogue have recently been reported for Na^+/H^+ exchange systems of 3T3 fibroblasts, chick skeletal muscle cells, and chick cardiac cells (Vinge et al., 1983).

Effects of Amiloride Analogues on Intracellular pH. Further evidence that the effect of inhibitors on $^{22}Na^+$ uptake in our assay is due to inhibition of the Na^+/H^+ antiporter comes from measurements of intracellular pH. As mentioned above, exposure of ouabain-treated, Na^+-loaded cells to Na^+-free medium results in rapid cytoplasmic acidification

Table I: Affinity of Amiloride Analogues for the Na^+/H^+ Antiporter in A431 Cells



analogue code	R	IC_{50}[a] (μM)
A9	$-NH_2$ (amiloride)	4.0
A11	$-NHCH_3$	4.0
A12	$-NHC_2H_5$	2.6
A13	$-NH(CH_2)_2CH_3$	2.0
A8	$-NHCH_2CH(CH_3)_2$	0.5
A2	$-NHC(CH_3)_3$	0.12
A1	$-N(CH_3)_2$	0.17
A14		0.16
A7		0.05
A16		0.40
A18		0.12
A6		0.30
A5		0.30
A15		0.06
A4		2.5
A3		1.6
A10	 (benzamil)	150

[a] IC_{50} values were determined by measurements of Na^+ uptake as shown in Figure 2.

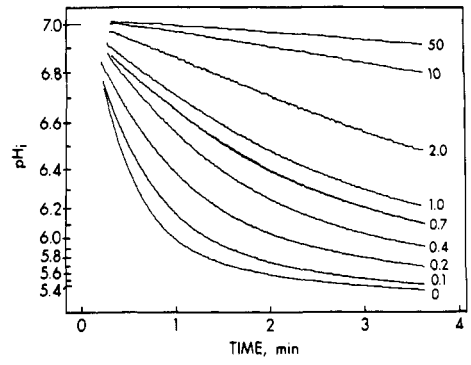


FIGURE 3: Concentration dependency of the inhibition by analogue A1 of intracellular acidification upon switching Na^+-loaded cells into Na^+-free solution. Cells that have been loaded with dimethylfluorescein–dextran were incubated for 4 h in the presence of ouabain at pH 7.0, as described in Figure 1A. This incubation results in an equilibration of intracellular and extracellular Na^+ and H^+ (Rothenberg et al., 1983a,b). At zero time the cells were switched to Na^+-free solution, pH 7.0, containing the indicated micromolar concentration of analogue A1, and the decline of cellular fluorescence was recorded by using signal damping of 1 s.

due to the exchange of internal sodium for external protons. As shown in Figure 3, addition of increasing concentrations of analogue A1 [R = $N(CH_3)_2$] leads to progressive inhibition of the rate of intracellular acidification.[2] Kinetic analysis of

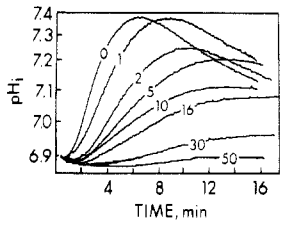


FIGURE 4: Inhibition by analogue A1 of growth factor promoted cytoplasmic alkalinization. Cells that have been loaded with dimethylfluorescein–dextran were preincubated for 3–4 h in solution A, pH 6.8. At zero time the cells were switched to the same solution containing 100 ng/mL EGF, 0.5% dialyzed fetal calf serum, and the indicated micromolar concentrations of analogue A1.

the data based on the rate constants for the exponential decay of fluorescence at each analogue concentration yields a K_i value of 0.2 μM. This value is very close to that found for analogue A1 by $^{22}Na^+$ uptake measurements (0.1m μM; Table I), suggesting that both assays are essentially equivalent.

Addition of EGF or serum to A431 cells in physiological solution results in cytoplasmic alkalinization due to the stimulation of Na^+/H^+ exchange (Rothenberg et al., 1983b). We found in this study that addition of EGF together with low serum concentration (0.5–1%) results in much faster and larger alkalinization than that obtained with each mitogen alone (Figure 4). Although alkalinization with EGF plus serum is partially transient, its initial phase can be used to obtain reproducible measurements of the effect of inhibitors. As shown in Figure 4, addition of increasing concentration of analogue A1 [R = $N(CH_3)_2$] results in a progressive inhibition of intracellular alkalinization. Addition of the inhibitor also extends the lag period that precedes pH_i elevation by the growth factors [see also Rothenberg et al. (1983b); Cassel et al., 1983]. Taking the maximal rates of pH_i increase following the lag period as a measure for the effect of the inhibitor gives an IC_{50} value of $\sim$2 μM for the inhibition of growth factor facilitated alkalinization by analogue A1. This value is 10–12 times larger than that found by using the assays described in Figures 2 and 3 in which Na^+-free media were employed. Measurement of the effect of analogues, A2–A9 on the rate of EGF plus serum promoted alkalinization (data not shown) also yielded IC_{50} values that are 7–13 times higher than those found in the assays employing sodium-free media. This is not due to possible sequestration of the analogue by serum, since we found that 2% serum has no effect on the IC_{50} values of the analogues as measured by $^{22}Na^+$ uptake into ouabain-treated cells in Na^+-free solution. The results would be consistent with the reported competition of sodium and amiloride for the antiporter (Rindler et al., 1979; Kinsella & Aronson, 1980; Vinge et al., 1982, 1983; Paris & Pouyssegur, 1983) and an apparent K_m value for sodium of about 15 mM (Vinge et al., 1982, 1983; Paris & Pouyssegur, 1983). However, due to the complex kinetics of pH_i elevation by the growth factors, it may not be appropriate to equate the IC_{50} of the analogues as estimated by the inhibition of the rate of alkalinization with the inhibition constant for the interaction of the analogue with the antiporter. It should be noted that analogue concentrations that are much higher than the IC_{50} value are required to

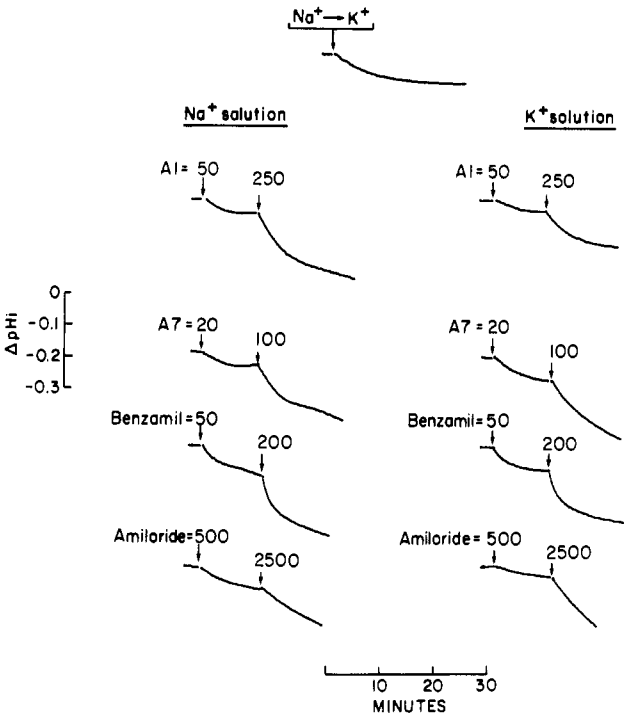


FIGURE 5: Effect of amiloride analogues on the apparent intracellular pH in high Na^+ and high K^+ solutions, pH 7.2. Top trace shows the effect on pH_i of switching cells from normal solution A to solution A in which Na^+ was replaced with an equimolar concentration of K^+. Left and right traces show the effect of amiloride analogues at the indicated concentrations (μM) on the apparent pH_i in normal solution A and in Na^+-free, high K^+ solution, respectively. Experiments in the high K^+ solution were initiated following preincubation in this solution for 45 min which allows pH_i to stabilize at a slightly lower value (see top trace).

completely inhibit growth factor dependent alkalinization (Figure 4). Even though at 50 μM analogue A1 there is no *net* alkalinization, the inhibition of growth factor dependent alkalinization is not complete since in the absence of growth factors the apparent pH_i decreases somewhat under these conditions (see Figure 5 below).

Role of Na^+/H^+ Exchange in the Maintenance of pH_i. Cells are apparently exposed to a continuous acid load from metabolism. If Na^+/H^+ exchange is required for the maintenance of intracellular pH, the inhibition of this activity should result in cytoplasmic acidification. The availability of amiloride analogues with high affinity for the Na^+/H^+ exchanger allows a test of this question. In Figure 5, we tested the effect of Na^+H^+ exchange inhibitors on pH_i in A431 cells incubated in the absence of mitogens and in the nominal absence of bicarbonate. Addition of amiloride analogues 1 and A7 at concentrations that almost completely inhibit Na^+/H^+ exchange results in an only slight apparent acidification of the cytoplasm which then reaches a new steady state. These results suggest that Na^+/H^+ exchange is not required for the maintenance of cytoplasmic pH under these conditions. If the cells are first preincubated in Na^+-free medium containing K^+, until pH_i reaches a new and somewhat lower steady state, a further slight acidification similar to that in Na^+ medium is noted upon subsequent addition of the amiloride analogues (Figure 5). Since, in the absence of Na^+, the Na^+/H^+ antiporter cannot function, these observations indicate that the apparent slight acidification of the cells upon addition of the analogues is probably unrelated to inhibition of Na^+/H^+ exchange. In fact, benzamil, a very weak inhibitor of Na^+/H^+ exchange, also causes cytoplasmic acidification at concentrations where it has no effect on Na^+/H^+ exchange and does so both in Na^+-

[2] Control experiments have shown that the amiloride analogues do not produce any significant fluorescence under the conditions of the fluorescence-based pH_i measurements. As previously reported for amiloride (Cassel et al., 1983), the analogues can similarly quench the dimethylfluorescein fluorescence in free solutions, but quenching of intracellular fluorescence is unlikely, in particular the low analogue concentrations used in most of the present studies.

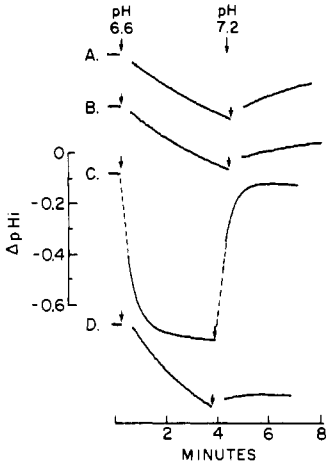


FIGURE 6: Effect of analogue A1 on the response of pH_i to changes in extracellular pH. Extracellular pH was first dropped from 7.2 to 6.6 and then brought back to 7.2 as indicated by arrows. (A, B) Cells in solution A; (C, D) cells that have been preincubated for 4 h and subsequently assayed in solution A containing 0.5 mM ouabain. (A, C) No amiloride analogue; (B, D) 50 μM analogue A1 was present in both pH 6.6 and pH 7.2 solutions.

containing and in Na^+-free medium (Figure 5). High concentration of analogues A1 and A7 also cause drastic acidification. The mechanism of this apparent acidification is not known.

We previously found that pH_i in cells partially tracks the extracellular pH (Rothenberg et al., 1983b; Cassel et al., 1983), and it was of interest to study the effect of Na^+/H^+ exchange on this response. As shown in Figure 6A, dropping the medium pH from 7.2 to 6.6 results in a considerable decrease in pH_i, which slowly increases when pH_0 is subsequently brought back to 7.2. This pattern remains unchanged in the presence of 50 μM analogue A1 (Figure 6B), suggesting that Na^+/H^+ exchange does not counteract the effect of low external pH under these conditions. When the same experiment is carried out in ouabain-treated cells in which Na^+/H^+ exchange is fully activated, pH_i rapidly and completely tracks pH_0 due to a rapid equilibration between pH_i and pH_0 through the Na^+/H^+ antiporter (Figure 6C; Rothenberg et al., 1983b). Unlike the untreated cells, addition of 50 μM analogue A1 to the ouabain-treated cells strongly inhibits the rate of the response to pH_0 changes (Figure 6D), demonstrating the effective inhibition of Na^+/H^+ exchange by the analogue.

Effect of Amiloride Analogues on Cellular Metabolism. The use of amiloride or amiloride analogues in long-term metabolic studies requires a critical assessment of their specificity. In this section we examine the effect of various amiloride analogues on protein synthesis, Na^+/K^+ ATPase activity, and the maintenance of cellular K^+ (measured by the use of $^{86}Rb^+$) as well as cellular ATP levels. Ideally, a suitable inhibitor of Na^+/H^+ exchange will not inhibit other cellular processes at inhibitor concentrations that effectively block Na^+/H^+ exchange. Previous studies (Fehlmann et al., 1981; Leffert et al., 1982; Lubin et al., 1982) have shown that amiloride inhibits protein synthesis in cells as well as in cell-free translation system. As shown in Figure 7, different amiloride analogues show either the same or slightly less inhibitory potency in an in vitro rabbit reticulocyte translation system. By contrast, incorporation of [^{3}H]leucine into cellular proteins during a 2-h pulse (Figure 8) is inhibited by analogue concentrations that are considerably lower than those of amiloride. Comparison of the IC_{50}'s of the analogues for the inhibition of [^{3}H]leucine incorporation into cellular proteins (Figure 8) with the relative affinities of these analogues for the Na^+/H^+ antiporter (Figure 2) shows a lack of correlation for at least some of the analogues (most notably with analogues A3, A5, and A6).

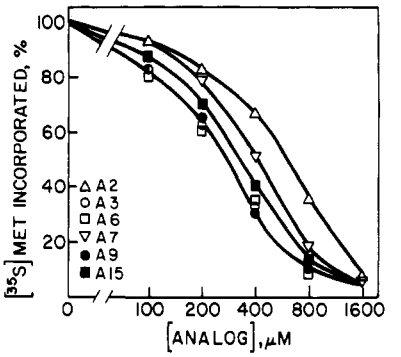


FIGURE 7: Effect of amiloride analogues on protein synthesis in a cell-free translation system. Translation of globin mRNA in the reticulocyte lysate system was assayed as described under Materials and Methods. In the absence of amiloride analogue, the incorporation into protein was 584 000 cpm (100%).

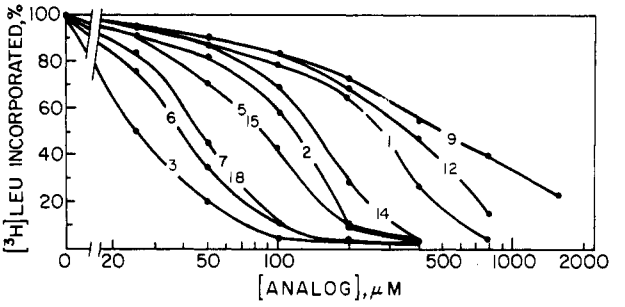


FIGURE 8: Effect of amiloride analogues on [^{3}H]leucine incorporation into cellular proteins. Incorporation was assayed by using a 2-h incubated in Hepes-buffered DME medium, as described under Materials and Methods. Control incorporated 30 600 cpm into protein (100%). Numbers in each experiment refer to the amiloride analogue used by using the designation shown in Table I.

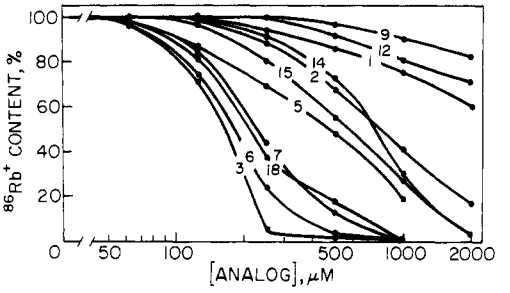


FIGURE 9: Effect of amiloride analogues on cellular $^{86}Rb^+$ content. Cells in Linbro 24-well trays (10^5 cells/well) were loaded to steady state with $^{86}Rb^+$ by preincubation for 3 h at 37 °C in bicarbonate-free, Hepes-buffered DME containing 1.4×10^6 cpm/mL $^{86}Rb^+$. Subsequently the medium was changed to an identical solution (also with $^{86}Rb^+$) containing analogues with the indicated code numbers, and incubation was continued for 2 h. $^{86}Rb^+$ content was determined after washing the cells as described for $^{22}Na^+$ uptake assays. The control system (no analogue) contained 30 800 cpm.

Soltoff & Mandel (1983) have reported that amiloride at relatively high concentrations inhibits the Na^+,K^+-ATPase and causes a decrease in cellular K^+ levels. Considering the well-known dependency of protein, synthesis on potassium ions, we tested the effect of the analogues on intracellular K^+ content using ^{86}Rb as a tracer. Cells were preequilibrated with the tracer in bicarbonate-free DME medium and subsequently incubated for 2 h in the same medium in the presence of different analogue concentrations. As shown Figure 9, many

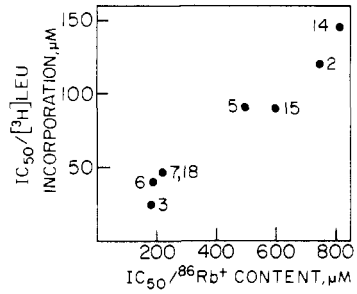


FIGURE 10: Comparison of IC_{50}'s of amiloride analogues, inhibition of cellular protein synthesis, and stimulation of $^{86}Rb^+$ loss from cells. Data are taken from Figures 8 and 9.

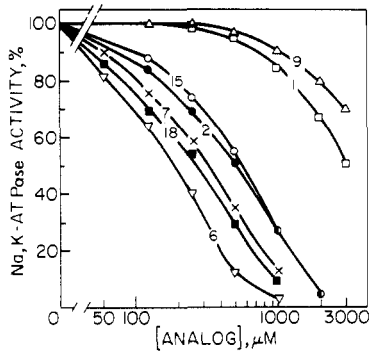


FIGURE 11: Inhibition of dog kidney Na,K-ATPase by amiloride analogues. Assays were carried out as described under Materials and Methods. Control rate was 440 nmol of P_i (mg of ATPase protein)$^{-1}$ min^{-1} (100%). Results are corrected for ouabain-insensitive ATP hydrolysis (11% of the total in the control system).

analogues cause a profound decrease in $^{86}Rb^+$ content whereas amiloride has only a small effect. Comparison of Figures 8 and 9 shows that the concentration of the analogues causing 50% decrease in $^{86}Rb^+$ content are 5–7 times higher than their IC_{50}'s for protein synthesis (Figure 10). These results suggest that depletion of intracellular K^+ cannot fully explain the inhibition of protein synthesis by the analogues.

The amiloride analogues also inhibit purified Na,K-ATPase from dog kidney with greater efficiency than does amiloride (Figure 11). The IC_{50}'s of the analogues for the Na,K-ATPase are quite similar to those affecting $^{86}Rb^+$ content (compare Figures 9 and 11). However, as shown in Figure 12, exposure of cells to analogues A2, A6, A7, and A15 also results in a decrease in ATP content while amiloride and analogue A1 at concentrations up to 2 mM have no effect. The concentrations of the analogues producing 50% decrease in cellular ATP are again similar to those producing a decrease in $^{86}Rb^+$ content. In fact, high concentrations of analogues A6, A7, and A15 (but not A2) caused significant release of ATP into the medium as well as profound alterations in cellular morphology. It is therefore unclear whether the decrease in $^{86}Rb^+$ content that is caused by the analogues is primarily due to inhibition of the Na^+,K^+-ATPase or whether it also reflects some general toxic effects of high concentrations of these analogues.

Discussion

Previous work with amiloride analogues has been primarily directed toward improving the diuretic properties of the compounds which reflect their effect on conductive Na^+ transport in the kidney. We have now characterized some of the structural features that confer on amiloride analogues high affinity for the Na^+/H^+ antiporter (up to 80 times higher than that of amiloride). These features are quite different from

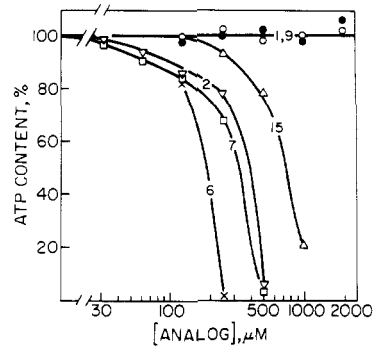


FIGURE 12: Effect of amiloride analogues on cellular ATP levels. A431 cell (2.5×10^5 per Linbro well) were incubated for 2 h in 0.5 mL of DME/Hepes containing amiloride analogues. The cells were than rapidly washed with phosphate-buffered saline and extracted for 5 min with 1 mL of cold 0.3 N $HClO_4$. The extract was neutralized with 0.21 mL of 1 M K_2CO_3 and diluted 1:600 for ATP determination. Results are expressed as percent of the control value (3.5 μmol of ATP/10^6 cells).

those present in potent inhibitors of the conductive Na^+ channel. Thus, alkyl substitutions at the 5-amino group increase the affinity of the analogues for the Na^+/H^+ antiporter (Table I) but decrease their affinity for the conductive Na^+ channel (Cuthberg & Fanelli, 1978), while benzamil is a poor inhibitor of Na^+/H^+ exchange but a potent one for the Na^+ channel (Cuthberg & Fenelli, 1978) as well as for Na^+/Ca^{2+} exchange (Kaczorowski et al., 1983).

The primary assay that we used to measure the effect of inhibitors on the Na^+/H^+ antiporter is based on our previous observation (Rothenberg et al., 1983a,b) that Na^+/H^+ exchange becomes very active following incubation of cells in the presence of ouabain. The activation of the antiporter in ouabain-treated cells is interesting in itself since it may reflect on the mechanism of the activation by physiological effectors such as growth factors. We found (unpublished observations) that activation by ouabain treatment can be mimmicked by incubation in K^+-free solution or in solution containing Li^+ which is not transported by the Na,K-ATPase but appears to be equivalent to Na^+ as a substrate for the Na^+/H^+ antiporter (Paris & Pouyssegur, 1983). All these treatments result in the replacement of intracellular K^+ with Na^+ (or Li^+); whether the depletion of intracellular K^+ or the increase in Na^+ levels causes the observed activation of Na^+/H^+ exchange or whether this activation is a secondary consequence of changes in the composition of intracellular monovalent ions will require further study.

Our finding that potent amiloride analogues effectively inhibt the cytoplasmic alkalinization induced by the addition of growth factor to cells lends further support to previous evidence (Schuldiner & Rozengurt, 1982; Rothenberg et al., 1983b; Cassel et al., 1983; Moolenaar et al., 1983; Burns & Rozengurt, 1983), suggesting that pH_i ele vation by these mitogens is due to a stimulation of the Na^+/H^+ antiporter. By contrast we found that the analogues have little effect on pH_i in the absence of mitogens, suggesting that Na^+/H^+ exchange is not a major mechanism for the basic maintenance of intracellular pH in A431 cells. Since cells are apparently exposed to constant acid load due to metabolic acid production as well as the inside-negative membrane potential (Roos & Boron, 1981), additinal acid-extruding mechanisms should exist. The nature of these hypothetical mechanisms is presently unknown.

Despite the increase in the affinity of some of the amiloride analogues for the Na^+/H^+ antiporter, our results indicate that the use of many of these analogues as specific inhibitors of

the antiporter may be complicated due to their increased side effects on cellular functions. These functions include protein synthesis and the ability of cells to maintain intracellular pH as well as K^+ and ATP levels. Inhibition of protein synthesis by amiloride was first reported by Fehlmann et al. (1981), by Leffert et al. (1982), and by Lubin et al. (1982). These investigators found that amiloride inhibits protein synthesis both in cells and in a cell-free translation system and suggested that the inhibition of cellular protein synthesis follows the accumulation of amiloride within the cells. Our findings show that both amiloride and its analogues produce similar inhibition of protein synthesis in the cell-free system while most analogues are much more inhibitory for cellular protein synthesis. This suggests that the incubation of cellular protein synthesis may be in part an indirect consequence of the inhibition of other cellular functions which are more sensitive to amiloride analogues than to the parent compound. Indeed we find that these analogues cause a depletion of the intracellular K^+ and ATP pools at concentrations that are much lower than those required to demonstrate similar effects by amiloride. However, the analogue concentrations required to produce these effects are severalfold higher than those producing inhibition of protein synthesis. Although inhibition of protein synthesis could be partially related to small reduction in K^+ concentration in view of the reported sharp dependency of the former on adequate K^+ levels (Lopez-Rivas et al., 1982), our data call for additional explanations. It is possible that the analogues affect [^{3}H]Leu transport into the cells, although this does not seem to be the case with amiloride (Fehlmann et al., 1981). Intracellular effects of the analogues could also depend on the rate and extent of their accumulation within the cell. Finally, the analogues may be indeed more inhibitory to cellular protein synthesis than they are under the conditions used for the assay of protein synthesis in vitro in the reticulocyte lysate system.

While the mechanism by which the analogues inhibit cellular functions requires further study, these side effects of many of the potent analogues may prelude their use in studies of the role of Na^+/H^+ exchange in cellular events such as the possible role of the stimulation of the antiporter by growth factors in mitogen action. These side effects of the analogues are not restricted to the A431 cell since we found a similar pattern of inhibition of protein synthesis by amiloride analogues in human foreskin fibroblasts (unpublished results). Exceptional among the analogues that we have studied is the one bearing a N^5,N^5-dimethyl substituent (A1). While this analogue has a rather high affinity for the Na^+/H^+ antiporter (~25 times better than amiloride), it has no effect on cellular ATP content and is not much more inhibitory than amiloride for the other cellular functions that we have tested. We are currently attempting to construct amiloride analogues that may be less toxic than the parent compound by introducing a second charged group which should render the molecule less permeable to cells. The potent amiloride analogues described in this paper will nevertheless be useful for the characterization of Na^+/H^+ exchange systems, and radioactivity labeling some of these analogues should provide a ligand that can be used in a binding assay to follow the purification of the Na^+/H^+ antiporter from the cell membrane.

Added in Proof

Since submission of this paper, similar observations have been reported by L'Allemain et al. (1984).

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Functional Characterization of Human Erythrocyte Spectrin α and β Chains: Association with Actin and Erythrocyte Protein 4.1†

Carl M. Cohen* and Robert C. Langley, Jr.

ABSTRACT: Human erythrocyte spectrin α and β chains were purified by preparative sodium dodecyl sulfate gel electrophoresis and also by DEAE-cellulose chromatography in the presence of urea. The purified chains behaved as individual monomers on sucrose gradients and did not form homodimers. Recombination of the chains led to the formation of α–β heterodimers with sedimentation characteristics identical with native α–β dimers. The binding of ^{125}I-labeled band 4.1 to α and β chains was measured by sucrose gradient rate zonal sedimentation and by quantitative immunoassay. It was found that both α and β chains associated with ^{125}I-labeled band 4.1 in a nearly identical manner over the range of band 4.1 concentration studied. The association was abolished by heat denaturation of the spectrin chains or by denaturation of band 4.1 with a 40-fold molar excess of *N*-ethylmaleimide. As expected, purified β chains but not α chains bound to ^{125}I-labeled ankyrin as measured by a quantitative radioimmunoassay. The binding of purified α chains, β chains, and recombinant α–β heterodimers to F-actin was measured in the presence of band 4.1. We found that α or β chains separately exhibited no band 4.1 dependent association with F-actin but that α–β heterodimers formed by recombination of the chains did. We conclude that spectrin binding to F-actin in the presence of band 4.1 requires the participation of both of spectrin's polypeptide chains.

The spectrin molecule is the major structural protein of the red cell membrane and constitutes approximately 75% by weight of red cell membrane skeletons obtained by Triton extraction of ghosts (Sheetz & Sawyer, 1978; Lux et al, 1976; Branton et al., 1981). Although the importance of spectrin to the maintenance of red cell shape and membrane integrity has been appreciated for some time, the biochemistry of this protein has only recently been explored in any detail [see Knowles et al. (1983) for a review]. Recent work has shown that spectrin is a heterodimeric protein containing a 240 000-dalton α chain and a 220 000-dalton β chain that associate with one another at multiple sites (Morrow et al., 1980) along their length. As visualized by low-angle rotary shadowing (Shotton et al., 1979; Tyler & Branton, 1980), the dimer has a contour length of 1000 Å, is 50 Å wide, and, because of its heterogeneous appearance as seen by low-angle rotary shadowing, is thought to be highly flexible. Under appropriate conditions, spectrin dimers readily self-associate in a head to head fashion to form tetramers (Shotton et al., 1979; Ungewickell & Gratzer, 1978) that are 2000 Å long, as well as various higher oligomeric structures (Morrow et al., 1981). Tetrmers and higher oligomers are thought to be the predominant form of spectrin in the membrane skeleton in situ.

Spectrin has two well-defined functions within the membrane skeleton. First, it fastens itself and the entire membrane skeleton to the membrane by its association with ankyrin, which in turn binds to the integral membrane protein band 3, thus securing the membrane skeleton to the lipid bilayer (Bennett, 1982). Second, spectrin tetramers bind to and cross-link short actin filaments and in this way produce the two-dimensional network that constitutes the membrane skeleton [see Cohen (1983) for a review]. The binding of spectrin to actin is promoted by the protein band 4.1, which is essential for stabilizing the otherwise weak association between these two proteins [reviewed in Cohen (1983)].

The partial localization of the functional sites on spectrin for these and other associations has been achieved through a combination of biochemical and electron microscopic techniques. It is known that spectrin's binding site for ankyrin is located on the β chain, about 200 Å from the head of the chain (that end which participates in the dimer–dimer association), and that spectrin's binding sites for actin and band 4.1 are located at the tail of the molecule (Branton et al., 1981; Cohen, 1983). Beyond this however, little is known. The focus of this work is to define which of the polypeptide chains of spectrin are responsible for binding to band 4.1 and actin. Our results show that while spectrin α and β chains may be different in many other respects, their association with band 4.1 and possibly actin seems to be remarkably alike.

Materials and Methods

Protein Preparations. Human erythrocyte spectrin and band 4.1 were prepared from freshly drawn human blood as described previously (Cohen & Foley, 1982; Tyler et al., 1972). Actin was extracted from rabbit muscle by the method of Spudich & Watt (1971) and handled as described by Cohen & Foley (1982). Ankyrin was prepared as a byproduct of band 4.1 purification as described by Tyler et al. (1979).

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