Blockade of the Na\(^{+}/H\(^{+}\) Antiport Abolishes Growth Factor-induced DNA Synthesis in Fibroblasts

STRUCTURE-ACTIVITY RELATIONSHIPS IN THE AMILORIDE SERIES*

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We have previously characterized in Chinese hamster lung fibroblasts a growth factor-activatable and amiloride-sensitive Na\(^{+}/H\(^{+}\) antiport (Pouyssegur, J., Chambard, J. C., Franchi, A., Paris, S., and Van Obberghen-Schilling, E. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3935–3939). In this report, we compared the affinity of 28 analogs of amiloride for inhibition of the Na\(^{+}/H\(^{+}\) antiport and inhibition of growth factor-induced DNA synthesis. We showed that the guanido moiety of amiloride must be protonated to elicit inhibition of the Na\(^{+}/H\(^{+}\) exchange. Substitutions within this moiety by methyl, phenyl, or benzyl groups reduced the activity 20- to 100-fold. On the contrary, substitution of the proton(s) of the 5-amino group of amiloride with alky1 or alkenyl groups increased the IC\(_{50}\) up to 100-fold (5-N,N-diethylamiloride has a K\(_{i}\) of 4 \times 10\(^{-8}\) M).

In HCO\(_{3}^{-}\)-free medium and at lower [Na\(^{+}\)]\(_{o}\) (25 or 50 mM) to reduce competition with amiloride, we found that growth factor-stimulated DNA synthesis of G0-arrested cells is inhibited by amiloride and its analogs with the same rank order as that for Na\(^{+}/H\(^{+}\) antiporter inhibition. Over a range of 3 logs of concentration, a tight correlation was established between IC\(_{50}\) for the blockade of both processes, Na\(^{+}/H\(^{+}\) exchange and percentage of cells entering the S phase upon growth factor action.

These findings indicate that, in HCO\(_{3}^{-}\)-free medium, the functioning of the Na\(^{+}/H\(^{+}\) exchange system is required for growth factor-induced DNA synthesis.

A Na\(^{+}/H\(^{+}\) exchange system previously characterized in brush-border membrane vesicles (1, 2) was recently identified in the plasma membrane of a wide variety of animal cells, including skeletal muscle cells (3-5), neuronal cells (6), erythrocytes (7), epithelial cells (8), and fibroblasts (9-13). This system uses the inward directed Na\(^{+}\) gradient to actively extrude intracellular H\(^{+}\) generated by cell metabolism. Therefore, it plays a major role in intracellular pH (pH\(_{i}\)) homeostasis (14). Indeed, recent experiments with cultured cells, showed that a specific blockade of the Na\(^{+}/H\(^{+}\) exchange activity, either with amiloride or by mutation,1 dramatically slowed down pH\(_{i}\) recovery after an acute acid load (15).

A second important feature of the Na\(^{+}/H\(^{+}\) antiporter is its rapid activation by growth factors in quiescent cells (9, 12, 13, 17-19). The resulting stimulated Na\(^{+}\) influx and intracellular alkalinization (13, 15) have been postulated as possible "messengers" of growth factor action (9, 15, 19, 20). To test this hypothesis and to obtain further insight into the biochemistry and regulation of Na\(^{+}/H\(^{+}\) antiporter activity of this system, we developed both a pharmacological and genetic approach. Amiloride, 3,5-diamino-6-chloro-N-(diaminomethylene)pyrazinecarboxamide, is an inhibitor of the Na\(^{+}/H\(^{+}\) antiporter, acting competitively on the Na\(^{+}\) site (10). In this paper, we report the structure-activity relationships of amiloride and some of its analogs for the blockade of the Na\(^{+}/H\(^{+}\) exchanger in Chinese hamster lung fibroblasts. A tight correlation between the inhibition of Na\(^{+}/H\(^{+}\) exchange activity and the inhibition of growth factor-induced DNA synthesis was found with amiloride analogs (K\(_{i}\) ranging from 4 \times 10\(^{-8}\) to 2 \times 10\(^{-5}\) M). These results suggest that the Na\(^{+}/H\(^{+}\) antiporter plays an important role in growth factor action.

EXPERIMENTAL PROCEDURES

Cell Culture—The Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection) was maintained in DMEM (Gibco H21) supplemented with 5% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 \mu g/ml) in a humidified atmosphere of 5% CO\(_{2}\), 95% air at 37°C. The other cell types were maintained under the same conditions except for serum which was raised to 10%. Secondary cultures of chick embryo fibroblasts were established from 10-day-old embryo and Fisher rat fibroblasts from newborn animals. Human fibroblasts were from a biopsy of normal adult forearm and corneal endothelial cells, lens epithelial cells, vascular smooth muscle cells, all of bovine origin were generously provided by Dr. Yves Courtois, Paris.

CCL39 cells were arrested in G0/G1 essentially as described (21); confluent cell monolayers grown in 24-well plates were washed with serum-free medium and incubated for 24 h in serum-free DME/Ham's F-12 medium (1:1).

\(^{3}H\)Thymidine Incorporation—For measurement of growth factor-induced DNA synthesis, G0/G1-arrested cells were incubated for 24 h at pH 7.4 in 30 mM Hepes-buffered DME (atmosphere 0% CO\(_{2}\)). The reinitiation medium contained 3 \mu M \(^{3}H\)thymidine (1 \muCi/ml), growth factors (a-thrombin and insulin or 10% dialyzed fetal calf serum), and varying concentrations of amiloride or amiloride analogs.

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3 The abbreviations used are: DME, Dulbecco's modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMA, 5-N,N-diethylamiloride.
Unless otherwise specified the Na+ concentration of DME medium was reduced either to 25 or to 0 mM, iso-osmolality being maintained with choline chloride. Following the 24-h incorporation, cells were then washed with phosphate-buffered saline at 0°C and fixed with 10% trichloroacetic acid (30 min at 0°C). Acid-precipitable material was solubilized in 0.1 M NaOH, and radioactivity was assayed by liquid scintillation spectrometry. For estimation of the number of cells entering into the S phase, cell monolayers were, after fixation, processed for autoradiography.

22Na Uptake Studies—Exponentially growing cells, in 24-well plates, were rinsed and incubated at 37°C for 2 h in 130 mM LiCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM glucose, buffered with 20 mM Hepes-Tris, pH 7.4. After this preincubation period intracellular [Li+] reached 80 to 90 mM, pH_t = 7.1. Cells were rapidly rinsed twice with 135 mM choline chloride and incubated in prewarmed (37°C) uptake medium containing 135 mM choline chloride, 1 mM MgCl2, 2 mM CaCl2, 1 mM ouabain, and 1 μCi/ml of 22NaCl (carrier free). Unless otherwise specified this uptake medium which contained varying concentrations of amiloride or amiloride analogs was buffered at pH 7.4 with 20 mM Hepes-Tris. 22Na influx was stopped by rinsing 4 times the cell monolayer with phosphate-buffered saline at 0°C (the 4 washes lasted 10 s). Under these conditions, 22Na influx rates were linear with time for 10 min; therefore, in routine experiments, 8-min uptake points on duplicate dishes were performed. The amiloride-sensitive 22Na influx rate was determined as the difference in the initial rate of 22Na uptake measured in the absence and in the presence of 100 μM DMA (analog 4). The amiloride-sensitive Na+ influx represents more than 95% of total Na+ uptake.

Cell-free Protein Synthesis—mRNA was purified from exponential cultures of CCL39 cells according to the protocol outlined in Ref. 22. Vanadyl-ribonucleoside complex (10 mM) was used in the lysis buffer to inhibit RNases. Polyadenylated RNA was purified on oligo(dT) matrix commercially available. Centre Amersham. All other chemicals were of the highest purity commercially available.

RESULTS

The Na+/H+ exchange system is reversible and highly activated by imposing either an inward or outward directed Na+ (or Li+ or H+) gradient across the plasma membrane. Fig. 1 shows a time course of total and amiloride-insensitive Na+ influx in Li-loaded CCL39 cells. This Na+/H+ exchange assay was chosen for routine experiments, because 1) Na+ uptake is linear with time, up to 10 min, 2) more than 95% of Na+ influx is mediated via the Na+/H+ exchanger, and 3) at low external [Na+] (22Na tracer) amiloride binding is not inhibited by Na+. Results to be published have revealed that this system does not operate directly as a Li+/Na+ exchange but as a coupled exchange system: Li+/H+, H+/Na+.

Na+/H+ Exchange Activity Is Blocked by the Protonated Form of Amiloride—Amiloride because of its guanidino moiety (Fig. 2) is a weak base, pK_a = 8.7 (27); therefore, in the physiological pH range, amiloride exists primarily as a monovalent cation. Substitution of the 5-amino group by a hydroxyl group leads to an analog with a pK_a of 7.03. It was, therefore, of interest to compare the capacity of both compounds to inhibit Na+/H+ exchange at two external pH values, pH_e = 7.4 and 8.4. Fig. 3 shows the concentration-response curve for the inhibition of the initial rate of 22Na+ efflux by amiloride and one of its analogs. The activity of amiloride in inhibiting Na+ uptake is almost insensitive to pH_e in the range of 7.4 to 8.4. IC_{50} concentrations inhibiting 50% of amiloride-sensitive Na+ influx vary from 3 to 4 μM. These values correspond to that previously reported for inhibition of Na+-dependent H+ efflux from CCL39 cells (9, 10). In contrast, the analog with a lower pK (7.03) shows an 8-fold increase in its IC_{50} (4 to 20 μM) when pH_e is shifted from 7.4 to 8.4. These results indicate that only the protonated molecule of amiloride is the inhibitory form toward the Na+/H+ exchange system.

Structure-Activity Relationships in the Amiloride Series—Twenty-eight analogs of amiloride with substitutions in 5- and 6-position of the pyrazine ring, as well as on the terminal guanidino nitrogen atom, have been tested. Concentration-response curves and IC_{50} (K_{50}) for the inhibition of the Na+/

![Fig. 1. Effect of an amiloride analog (DMA) on the time course of 22Na+ influx in CCL39 cells. Li+-loaded CCL39 cells cultured in 24-well plates were incubated for various periods of time with 22Na+ tracer (1 μCi/ml) pH_e = 7.4 with 1 mM ouabain in the absence (Δ) or in the presence (●) of 100 μM of the amiloride analog (DMA, compound 4). For details of the preincubation period in LiCl and for the uptake medium see under "Experimental Procedures."](http://www.jbc.org/content/158/13/4314/F1.large.jpg)

![Fig. 2. Structure of protonated amiloride.](http://www.jbc.org/content/158/13/4314/F2.large.jpg)

![Fig. 3. Concentration-response curves for the inhibition of Na+ influx by amiloride and an analog with a lower pK_a; effect of external pH. 22Na+ initial rates were determined as described under "Experimental Procedures" except that uptake experiments were conducted either at external pH (pH_e) = 7.4 (○) or at pH_e = 8.4 (△). Top, compound used is amiloride (no 6) with a pK_a of 8.7; bottom, compound used is an amiloride analog (no 11) where the 5-amino group is substituted by a proton. Its pK_a = 7.0.](http://www.jbc.org/content/158/13/4314/F3.large.jpg)
H⁺ exchange system by the most representative of these analogs are presented in Fig. 4 and Table I. Replacement of one or two protons on the terminal nitrogen atom of the guanidino group by either a phenyl (no. 13) or a benzyl (no. 3, benzamil) or two methyl groups (no. 5) strongly reduces the activity of these molecules.

Although the 5-amino group is not essential for activity (compare compounds no. 12 and no. 8, Table I), substitution of protons of the 5-amino group (by alkyl or alkenyl moieties) generates compounds with higher potency; 5-N,N-dimethyl amiloride (no. 4) has a $K_{o_s}$ of $2 \times 10^{-7}$ M; 5-N-methyl-N-allyl amiloride (no. 26) and 5-N,N-diethylamiloride (no. 17) have a $K_{o_s}$ of $4 \times 10^{-8}$ M (Table I).

Growth-Factor-Induced DNA Synthesis Is Inhibited by Amiloride and Its Analogs—Removal of calf serum for 24 h from CCL39 cell culture arrests more than 99.5% of the cell population in G0/G1 (21). Addition of either 10% fetal calf serum or purified growth factors (a-thrombin and insulin) reinitiates DNA synthesis of 40 to 60% of the cells in 24 h (28, 29). This DNA replication which takes place after a constant lag of 10 h is dependent upon external Na⁺ concentration [Na⁺]o. In Hepes-buffered medium, pH 7.4, it is optimal around 100 mM, reduced 2-fold at 50 mM, 4-fold at 25 mM, and abolished below 10 mM [Na⁺]o.

One of the first actions of serum or purified growth factors to G0/G1-arrested fibroblasts is the stimulation of the amiloride-sensitive Na⁺/H⁺ antiporter (9, 12, 13). If the functioning of this membrane-bound system is involved in growth factor-induced DNA replication, we predicted that: (a) blockade of the Na⁺/H⁺ antiporter activity should prevent growth factor-induced DNA synthesis, (b) increasing [Na⁺]o should antagonize this inhibition, and (c) a correlation should exist between the inhibitory doses of amiloride analogs altering Na⁺/H⁺ exchange activity and those inhibiting growth factor-induced DNA replication.

Fig. 5 shows that DMA (no. 4) like amiloride inhibits Na⁺/H⁺ exchange activity by competing with the Na⁺ site. With a $K_I$ of $1.5 \times 10^{-7}$ M, this analog is 17-fold more potent than amiloride. Both amiloride and DMA inhibited serum-induced DNA synthesis in G0/G1-arrested CCL39 cells (Fig. 6, A and B). Interestingly, at two external Na⁺ concentrations [Na⁺], = 25 mM, [Na⁺]o = 50 mM we observed that DMA is 17-fold more potent than amiloride in inhibiting serum growth factor action (Table II). A second point of interest shown in Fig. 6B is that increasing [Na⁺]o from 25 to 135 mM shifted the IC₅₀ (concentrations of DMA inhibiting 50% of serum-induced thymidine incorporation) toward higher values. This result was expected as a consequence of a direct block of the Na⁺/H⁺ exchange activity.

Fig. 7 depicts concentration-response curves for the inhibition of thymidine incorporation ([Na⁺]o = 25 mM) with the other amiloride analogs. With the exception of compounds 3 and 13 which turned out to be toxic for the cells, the other analogs inhibited serum-induced DNA synthesis with the same rank order as that found for inhibition of Na⁺/H⁺ exchange activity.

Five of the most representative analogs of amiloride were analyzed in more detail. G0-arrested CCL39 cells were reinitiated with the combination of purified growth factors a-thrombin and insulin in Hepes-buffered HCO₃⁻-free medium (pH 7.0 or 7.4) in which [Na⁺]o was lowered to 50 mM. Under these conditions 30% of the cells enter S phase in 24 h following growth factor addition. The presence of amiloride analogs during the 24 h of reinitiation reduces, in a dose-dependent manner, the percentage of cells entering the S phase.

Fig. 8 shows at two external pH values (7.0 and 7.4) a good correlation between IC₅₀ of these amiloride analogs for blocking both processes, Na⁺/H⁺ exchange and growth factor-induced DNA synthesis (per cent of labeled nuclei). A higher sensitivity (2- to 3-fold) of amiloride analogs is observed when pHₕ is reduced from 7.4 to 7.0.

Effects of Amiloride and of Its Analogs on Cell-free Protein Synthesis—It has previously been shown that amiloride inhibits cell-free protein synthesis in rabbit reticulocyte lysate (30). This secondary effect of amiloride could explain its inhibitory action on cell growth or DNA synthesis. We, therefore, analyzed whether the better analogs of amiloride for blocking Na⁺/H⁺ exchange and DNA synthesis are also better analogs for blocking protein synthesis. Fig. 9 confirms that amiloride inhibits cell-free protein synthesis with an IC₅₀ of 400 μM. More importantly the two amiloride analogs DMA and DEA (5-N,N-diethylamiloride), are, respectively, 17- and 75-fold more potent than amiloride in blocking both Na⁺/H⁺ exchange activity and DNA synthesis but are equivalent to amiloride for inhibition of protein synthesis (IC₅₀ = 400 μM, Fig. 9).

Ubiquity and Amiloride Specificity of the Na⁺/H⁺ Antiport in Animal Cells—The routine assay system depicted in Fig. 1 allowed a rapid screening to know whether the Na⁺/H⁺ membrane-bound exchange system is expressed in all animal cells. This system specifically enhanced in Li⁺-loaded cells and inhibited by amiloride was characterized in all the cultured cells we tested: chick embryo, hamster, rat and human fibroblasts; epithelial cells (bovine lens, hepatica, Buffalo rat.
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**TABLE I**
Structure-activity relationships of amiloride in relation to the blockade of the Na\(^{+}/H^{+}\) exchange system

Modifications in positions 6, 5, and in the guanidino group. (\(K_{50}\) were calculated from Fig. 4).

<table>
<thead>
<tr>
<th>Code number</th>
<th>X</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>(K_{50}) M</th>
</tr>
</thead>
<tbody>
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<td>1</td>
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<td>-NH-CH(_2)CH-</td>
<td></td>
<td></td>
<td>3 (\times) 10(^{-4})</td>
</tr>
<tr>
<td>13</td>
<td>Cl</td>
<td>-NH-CH(_2)CH-</td>
<td></td>
<td></td>
<td>3 (\times) 10(^{-4})</td>
</tr>
<tr>
<td>14(^a)</td>
<td>Cl</td>
<td>NH(_2)</td>
<td></td>
<td>H(^+)</td>
<td>9 (\times) 10(^{-4})</td>
</tr>
<tr>
<td>10</td>
<td>H</td>
<td>NH(_2)</td>
<td></td>
<td>H(^+)</td>
<td>2.5 (\times) 10(^{-4})</td>
</tr>
<tr>
<td>5</td>
<td>Cl</td>
<td>N(CH(_2))(_2)</td>
<td>CH(CH(_3))(_2)</td>
<td>H(^+)</td>
<td>1.5 (\times) 10(^{-4})</td>
</tr>
<tr>
<td>3</td>
<td>Cl</td>
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<td></td>
<td>H(^+)</td>
<td>8 (\times) 10(^{-5})</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
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<td></td>
<td>H(^+)</td>
<td>2 (\times) 10(^{-8})</td>
</tr>
<tr>
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<td>H(^+)</td>
<td>3 (\times) 10(^{-8})</td>
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<tr>
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</tr>
<tr>
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<td>H(^+)</td>
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</tr>
<tr>
<td>28</td>
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<td>H(^+)</td>
<td>1.5 (\times) 10(^{-7})</td>
</tr>
<tr>
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<td>-CH(_3)</td>
<td>H(^+)</td>
<td>1 (\times) 10(^{-7})</td>
</tr>
<tr>
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<td>-CH(_3)</td>
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</tr>
<tr>
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<td>-CH(_3)</td>
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<tr>
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<td>-CH(_3)CH(_2)</td>
<td>-CH(_3)CH(_3)</td>
<td>4 (\times) 10(^{-8})</td>
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</table>

\(^a\) The 5-amino group is replaced by a hydrogen.

\(^b\) Insertion of a nitrogen between the carbonyl carbon and the guanidino group.

**DISCUSSION**

The diuretic drug amiloride has proven to be a very potent and specific inhibitor of Na\(^{+}\) transport in a wide variety of cellular systems (reviewed in Ref. 27). It blocks 1) the conductive Na\(^{+}\) entry pathway found in electrically high resistance epithelia (27, 31), 2) Na\(^{+}/\)Ca\(^{2+}\) exchange (32), and 3) Na\(^{+}/H^{+}\) electroneutral exchange (10, 27). The voltage-dependent Na\(^{+}\) channels specifically blocked by tetrodotoxin (33) are insensitive to amiloride. However, both amiloride and tetrodotoxin share in common a guanidinium moiety which is the active group for a Na\(^{+}\) recognition site. We have shown here that only the protonated guanidinium form is inhibitory for Na\(^{+}/H^{+}\) exchange. Two active amiloride molecules with respective pK values of 8.7 and 7.0 behave differently when tested for Na\(^{+}/H^{+}\) exchange inhibition at pH, 7.4 and 8.4. Amiloride, with a pK of 8.7 is mainly in the protonated form at both pH values, and its potency is unaffected. For the other analog with a pK of 7.0, the protonated form decreases by an order of magnitude from pH 7.4 to 8.4; in parallel the corresponding IC\(_{50}\) is shifted from 3 to 40 \(\mu\)M. Therefore, as it has been shown by Cuthbert for inhibition of Na\(^{+}\) conductance in frog skin (27), amiloride has to be protonated to be biologically active in regard to Na\(^{+}/H^{+}\) antiport inhibition.

Results obtained using analogs substituted in the 5- and 6-position and guanidino nitrogen atom of the amiloride molecule, have led to the following conclusions. 1) The unsubsti-
tuated guanidino group is essential for activity of the molecule since introduction of a benzyl (benzamil) or a phenyl group reduces potency 27- and 1000-fold, respectively; 2) the halogen atom in the 6-position is also important for activity since its substitution by a proton reduces potency by 100-fold (compare analogs 10 and 12); 3) substitutions within the 5-amino group enhance the potency of amiloride by 70- to 100-fold. It seems that the steric environment around the 5-amino nitrogen atom is responsible for the increase in potency and, therefore, for stabilization of the molecule on the receptor. All these conclusions, as well as the IC50 values reported in Table I, are in good agreement with an extensive pharmacological study on skeletal muscle cells carried out by Vigne et al. in our institute; they used 38 analogs of amiloride and a different Na+/H+ assay system (34). The pharmacological features of the Na+/H+ antiport summarized in 1) and 3) and reported by Vigne et al. (34) contrast markedly with the effects observed with similar amiloride analogs on Na+ transport in frog skin (27, 31, 35) and Na+/Ca2+ exchange (32).

Another major point of interest concerning the Na+/H+ antiport is the growth control “Na+ trigger” hypothesis (19, 36). Briefly, this hypothesis is based on the following observations. 1) One of the earliest detectable actions of mitogens on quiescent cells is the stimulation of Na+ influx (9, 12, 17, 19); 2) this mitogen-stimulated Na+ influx results from the activation of the “quiescent” Na+/H+ antiport (9, 13, 15); and 3) amiloride, which blocks mitogen-stimulated Na+ influx (9, 12) and ΔpH (15), blocks mitogen-induced DNA synthesis (20, 37). However, the third point favoring this hypothesis has been seriously subjected to criticism (38, 39). In general, high concentrations of amiloride (0.1 to 0.5 mM) must be used in order to block either DNA synthesis or growth. At these concentrations amiloride is known to penetrate the cells and to directly inhibit protein synthesis (30, 40). Indeed, we have seen that amiloride, as well as the more potent analogs, inhibit cell-free protein synthesis with an equal potency (IC50 = 4 × 10–14 M). In this study we circumvented these difficulties, first by comparing more potent analogs than amiloride and secondly by reducing [Na+]o, a substrate which competes with amiloride. Under these conditions, lower concentrations of amiloride or analogs are required to elicit biological effects. Fig. 6, A and B, illustrates the difficulties in using amiloride at 135 mM [Na+]o and the incorrect conclusions that one can draw. Fig. 6A shows that at 135 mM [Na+]o, amiloride blocks serum-induced DNA synthesis with an IC50 of 250 μM. This IC50 is unchanged when [Na+]o is reduced to 75 mM. This observation, inconsistent with the competitive inhibition between amiloride and Na+ toward the Na+/H+ exchanger, indicates that the amiloride biological inhibition is due to secondary effects such as inhibition of protein synthesis. In contrast, at lower [Na+]o, IC50 of amiloride for DNA synthesis inhibition is shifted to the left as one would expect from a...

**Fig. 7.** Concentration–response curves for inhibition of serum-induced DNA replication by amiloride and its analogs at 25 mM [Na+]o. Conditions of reinitiation of G0/G1-arrested CCL39 cells were the same as those described under “Experimental Procedures.” Results are expressed as percentages of [3H]thymidine ([TdR]) reinitiated in the absence of drug. Circled numbers identify the amiloride analogs listed in Table I.

**Table II**

Comparison of the IC50 of amiloride and an analog (DMA) for inhibiting Na+/H+ exchange activity and serum-induced DNA synthesis in CCL39 cells

<table>
<thead>
<tr>
<th>Function inhibited</th>
<th>Amiloride</th>
<th>DMA</th>
<th>Ratio of IC50 amiloride to IC50 DMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na+/H+ exchange activity</td>
<td>IC50 (μM): [Na+]o = 25 mM</td>
<td>6</td>
<td>0.35</td>
</tr>
<tr>
<td>Serum-induced DNA synthesis</td>
<td>IC50 (μM): [Na+]o = 25 mM</td>
<td>36</td>
<td>2</td>
</tr>
<tr>
<td>IC50 (μM): [Na+]o = 50 mM</td>
<td>120</td>
<td>7</td>
<td>17</td>
</tr>
</tbody>
</table>
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**FIG. 8.** Correlation between the inhibition by amiloride and its analogs of Na⁺/H⁺ exchange and growth factor-induced DNA replication. G0/G1-arrested CCL39 cells were reinitiated with α-thrombin (2 units/ml) and insulin (10 μg/ml) in Hepes-buffered medium pHₐ = 7.4 (△) or 7.0 (□) containing 50 mM [Na⁺], (see under "Experimental Procedures"). Various concentrations of amiloride and analogs were added in presence of growth factors and [³H]thymidine. The percentage of cells entering the S phase during the 24 h of reinitiation were scored by autoradiography. In absence of drug and growth factors, we scored less than 0.1% labeled nuclei; in presence of growth factor we scored 31% labeled nuclei at pHₐ = 7.4 and 18% at pHₐ = 7.0. The concentration of amiloride analogs inhibiting 50% of Na⁺/H⁺ exchange at 50 mM [Na⁺], (IC₅₀ = 3 × Kₛₐ) are plotted against the corresponding IC₅₀ for growth factor-induced DNA replication (concentrations inhibiting 50% of labeled nuclei). Circled numbers identify the amiloride analogs listed in Table I.

**FIG. 9.** Effects of amiloride and its analogs on protein synthesis in rabbit reticulocyte lysate. 25-μl translation assay containing 1 μg of CCL39 mRNA, [³H]leucine, various concentrations of amiloride or analogs and rabbit reticulocyte lysate were incubated for 60 min at 37 °C. Protein synthesis was estimated as the radioactivity incorporated into the acid-precipitable fraction. Addition of CCL39 mRNA stimulated 12-fold the basal protein synthesis. Inhibition of protein synthesis by amiloride (△), 5-N,N-dimethylamiloride 4 (□) and 5-N,N-diethylamiloride 17 (□) is expressed as the percentage of the value obtained in the absence of drug.

Direct blockade of Na⁺/H⁺ exchange. Interestingly, this shift to the left is observed over the entire range of [Na⁺], when a 17-fold more potent analog, DMA, is used (Fig. 6B). The fact that at these lower [Na⁺], 25 or 50 mM, the relative potency between amiloride and DMA for blocking the Na⁺/H⁺ exchange (17-fold) and serum-induced DNA synthesis is conserved (Table II), is a strong argument indicating that DNA synthesis and Na⁺/H⁺ exchange activity are linked.

Two additional observations reinforce this conclusion. 1) The series of amiloride analogs inhibited DNA synthesis reinitiation at 25 mM [Na⁺], with the same rank order as inhibition of Na⁺/H⁺ exchange (Fig. 7); 2) at a higher [Na⁺], 50 mM, at which a large percentage of cells are reinitiated, a good correlation between blockade of Na⁺/H⁺ antiport and inhibition of the number of cells entering the S phase was established (Fig. 8). Because we had to lower [Na⁺], to reduce competition with amiloride, one might question whether the correlation could be extended to physiological [Na⁺]. We have checked that possibility at 140 mM [Na⁺], or 10 μM of the most potent analogs (n°17 and n°26) prevent growth factor-induced reinitiation. This result is confirmed by the fact that at physiological [Na⁺], CCL39-derived mutant cells lacking Na⁺/H⁺ exchange activity cannot reinitiate DNA synthesis at pHₐ ≥ 7.4 or below.

These findings converge to establish a link between Na⁺/H⁺ exchange activity and growth factor-induced DNA synthesis in a HCO₃⁻-free medium. However, when the medium is buffered with CO₂/HCO₃⁻, the role of the Na⁺/H⁺ antiport appears secondary since 1) concentrations of amiloride analogs which block more than 95% of Na⁺/H⁺ exchange activity do not prevent reinitiation of DNA synthesis (data not shown); 2) growth of CCL39-derived mutant cells lacking Na⁺/H⁺ antiport activity is not altered. These observations reconcile the apparent divergent conclusion reached by Stiernberg et al. (16) in their study, carried out in the presence of HCO₃⁻, and the present report. Preliminary results indicate that CCL39 cells possess a Cl⁻/HCO₃⁻ exchange system that could substitute in the presence of HCO₃⁻ for Na⁺/H⁺ antiport in pHₐ regulation.

In conclusion, we have found that in HCO₃⁻-free medium the functioning of the Na⁺/H⁺ antiport and, therefore, pHₐ regulation (15) is tightly linked to growth factor-induced DNA synthesis. However this conclusion does not answer the question as to whether the growth factor-stimulated Na⁺/H⁺ antiport and subsequent rise in pHₐ (9, 12, 15) is required for growth reinitiation. The results concerning this question will appear in a separate report.

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REFERENCES

Blockade of the Na+/H+ antiport abolishes growth factor-induced DNA synthesis in fibroblasts. Structure-activity relationships in the amiloride series.

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