Amiloride Analogs Inhibit L-type Calcium Channels and Display Calcium Entry Blocker Activity*

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Three structural classes of commonly used amiloride analogs, molecules derivatized at the terminal guanidino-nitrogen, the five-position pyrazinoyl-nitrogen, or di-substituted at both of these positions, inhibit binding of the L-type Ca\(^{2+}\) channel modulators diltiazem, gallopamil, and nitrendipine to porcine cardiac sarcolemmal membrane vesicles. The rank order of inhibitory potencies among the various derivatives tested is well defined with amiloride being the least potent. Saturation binding studies indicate that inhibition of ligand binding results primarily from effects on Ke. Ligand dissociation measurements suggest that amiloride derivatives do not associate directly at any of the known sites in the Ca\(^{2+}\) entry blocker receptor complex. In addition, these compounds do not compete at the Ca\(^{2+}\) coordination site" within the channel. However, studies with inorganic and substituted diphenylbutylpiperidine Ca\(^{2+}\) entry blockers reveal that amiloride analogs interact at a site on the channel where metal ions bind and occlude the pore. Photolysis experiments performed with amiloride photoaffinity reagents confirm that a specific interaction occurs between such probes and the channel protein. Upon photolysis, these agents produce concentration- and time-dependent irreversible inactivation of Ca\(^{2+}\) entry blocker binding activities, which can be protected against by either verapamil or diltiazem. \(^{45}\)Ca\(^{2+}\) flux and voltage-clamp experiments performed with GH\(_3\) anterior pituitary cells demonstrate that amiloride-like compounds inhibit L-type Ca\(^{2+}\) channels directly. Moreover, these compounds block contraction of isolated vascular tissue in pharmacological assays. Electrophysiological experiments indicate that they also inhibit T-type Ca\(^{2+}\) channels in GH\(_3\) cells. Taken together, these results demonstrate unequivocally that amiloride analogs display significant Ca\(^{2+}\) entry blocker activity in both ligand binding and functional assays. This property, therefore, can seriously complicate the interpretation of many in vitro and in vivo studies where amiloride analogs are used to elicit inhibition of other transport systems (e.g. Na-Ca and Na-H exchange).

The major pathway for Ca\(^{2+}\) entry into electrically excitable and certain nonexcitable cells is through voltage-dependent Ca\(^{2+}\) channels (1). Among the various types of Ca\(^{2+}\) channels that have been described (i.e., L-, T-, and N-types), the best characterized is the L-type, or high-threshold, slowly inactivating, Ca\(^{2+}\) channel (2). Extensive characterization of this system has been possible because of the fully developed pharmacology of the L-type channel; this protein is the target of the therapeutic class of drugs known as Ca\(^{2+}\) entry blockers. At least four different structural classes of Ca\(^{2+}\) entry blockers exist: dihydropyridines, aralkylamines, benzothiazepines, and substituted diphenylbutylpiperidines (3–5). Studies with purified cardiac sarcolemmal membrane vesicles have demonstrated that these agents bind with high affinity to distinct sites in a Ca\(^{2+}\) entry blocker receptor complex (6–8) which is functionally associated with the L-type Ca\(^{2+}\) channel in heart (9). Each site in this complex is coupled to other receptors by well defined positive or negative allosteric interactions (6–8). Therefore, by monitoring binding of known Ca\(^{2+}\) entry blockers, it is possible to determine whether previously uncharacterized compounds interact with L-type Ca\(^{2+}\) channels, and then to ascertain how these agents function. Mechanisms of action of several novel L-type Ca\(^{2+}\) channel modulators have been elucidated in this fashion (10–12).

The pyrazine diuretic amiloride, and various structurally related analogs, have been extensively employed as probes of a wide variety of transport systems (for a review see Ref. 13). Notably, terminal guanidino-nitrogen derivatives will block epithelial Na\(^{+}\) channels, while analogs substituted at the five-amino group of the pyrazine ring have been found to inhibit Na-H exchange. Both structural classes, as well as molecules di-substituted at these two positions, are also effective inhibitors of Na-Ca exchange. Amiloride analogs have been used primarily for two purposes: to investigate the activity of an isolated transport system, where in most cases they cause inhibition by mimicking Na\(^{+}\), and to elucidate the physiological role of a given transport reaction in either in vitro or in vivo studies. Examples of the latter usage include the study of amiloride analogs which block Na-H exchange to determine the function of that transport reaction in controlling ionic homeostasis (14, 15), mitogenesis (16, 17), chemotaxis (18), and stimulus-secretion coupling (19); the investigation of Na-Ca exchange inhibitors to ascertain that transport system's role in regulating Ca\(^{2+}\) homeostasis of cardiac (20, 21), and smooth muscle (22, 23) tissues.

Recently, electrophysiological experiments have demonstrated that amiloride and the analog 3,4-dichlorobenzamil will block the activity of L- and T-type Ca\(^{2+}\) channels (24–26). For this reason, the interaction between a number of commonly used amiloride derivatives and the L-type Ca\(^{2+}\)
channel was studied using combined ligand binding, Ca\textsuperscript{2+} flux, voltage clamp, and pharmacological techniques. All of these analogs have been found to be inhibitors of the L-type Ca\textsuperscript{2+} channel. Based on their modulation of Ca\textsuperscript{2+} entry blocker binding reactions, they most likely interact at the pore of the channel, the same site where inorganic channel inhibitors bind. The same amiloride derivatives block T-type Ca\textsuperscript{2+} channels. The Ca\textsuperscript{2+} entry blocker activity of the amiloride series precludes the use of such agents in physiological studies to determine the role of either Na-H or Na-Ca exchange, if block of Ca\textsuperscript{2+} channel activity will complicate the interpretation of experimental data. A preliminary report of these findings has been made in abstract form (27).

**EXPERIMENTAL PROCEDURES**

**Materials**—d-cis-[\textsuperscript{3}H]Diltiazem (60.0 Ci/mmol), \[\textsuperscript{3}H\]nitrendipine (87 Ci/mmol), \[\textsuperscript{3}H\]D-600 (87 Ci/mmol), \[\textsuperscript{3}H\]D-600 (47 Ci/mmol), and 4\textsuperscript{Ca}Cl\textsubscript{2} (0.7 Ci/mmol) were purchased from DuPont-New England Nuclear. All amiloride analogs and various other drugs were obtained from the chemical collection of Merck. Chemical structures of amiloride and the analogs used in these studies are shown in Table 1. All other reagents were purchased from commercial sources and were of the highest purity available.

**Preparation of Membrane Vesicles**—Purified porcine cardiac sarcosarcolmem membrane vesicles were prepared by discontinuous sucrose density gradient centrifugation of a crude membrane preparation using a modification of an established procedure as described (28). Vesicles were rapidly frozen in liquid nitrogen and stored at -80°C until use. Under these conditions, binding activities were stable for at least 6 months.

**Binding Assays**—d-cis-[\textsuperscript{3}H]Diltiazem, \[\textsuperscript{3}H\]nitrendipine, \[\textsuperscript{3}H\]D-600, and \[\textsuperscript{3}H\]D-600 associations with cardiac membrane vesicles were monitored as previously described (6-8). Triplicate assays were routinely performed under each experimental condition. The standard error of the mean of these results was typically less than 3.% Stock solutions of all drugs were prepared in MezSO. The final concentration of MezSO in the binding assays was never allowed to exceed 0.2%. Control experiments showed no effect of these MezSO concentrations on any of the binding reactions.

**Data Analysis**—Data from saturation experiments were subjected to a Scatchard analysis and linear regression was performed to yield the equilibrium dissociation constant (Kd) and maximum concentration of receptor sites (Bmax). Correlation coefficients were typically by the method of Cheng and Prusoff (29) to determine Kd values for concentration of 1 mg protein/ml in a medium of 40 mM

**RESULTS**

Effect of Amiloride Analogs on Ca\textsuperscript{2+} Entry Blocker Binding—Several structural classes of amiloride analogs have been used to study the mechanism and physiological role of various sodium transport systems in a wide variety of cells and tissues. To determine whether amiloride analogs interact with L-type Ca\textsuperscript{2+} channels, the actions of selected agents from each structural series were monitored on Ca\textsuperscript{2+} entry blocker binding in purified porcine cardiac sarcosarcolmem membrane vesicles. For these experiments, interactions with three types of well-characterized L-type Ca\textsuperscript{2+} channel modulators (i.e., d-cis-diltiazem, D-600, and nitrendipine, representing benzothiazepine, aralkylamine, and dihydropyridine classes of organic Ca\textsuperscript{2+} entry blockers, respectively) were investigated. The data illustrated in Fig. 1 demonstrate that members of three structural classes of amiloride analogs inhibit equilibrium binding of diltiazem, D-600, and nitrendipine with a defined rank order of potency. Amiloride, itself, is the least potent inhibitor in this series of compounds. Two guanidino-nitrogen substituted analogs, benzamil and naphthylmethylamiloride (L-595,187), display increased potency as inhibitors of ligand binding. Two five-position pyrazinoyl-nitrogen substituted analogs display several common properties in their action as is apparent from the data of Fig. 1. Ligand binding at each site in the Ca\textsuperscript{2+} entry blocker
receptor complex is affected by all amiloride derivatives tested with unitary Hill coefficients \((n_H = 1)\) for inhibition. When the three inhibitory patterns are compared, the rank order of potency of these compounds is the same at each site, and absolute effectiveness of any given derivative in inhibiting the three different binding reactions is nearly identical, with the exception of results obtained with amiloride. In addition, inhibition of diltiazem binding (Fig. 1A) and D-600 binding (Fig. 1B) is complete in every case, while inhibition of nitrendipine's interaction (Fig. 1C) is partial, except for the case of the di-substituted amiloride derivative (i.e., L-651,525) which produces complete block of dihydropyridine binding. These results suggest that several structurally different amiloride analogs interact with L-type \(Ca^{2+}\) channels in a similar fashion.

The structure-activity relationships within these different derivative classes have been further defined by investigating a number of commonly used amiloride analogs. Results were obtained by titrating the ability of various compounds to interfere with diltiazem, D-600, and nitrendipine binding, and then calculating respective \(K_i\) values according to the Cheng-Prusoff relationship (29). These data are listed in Table I. The first group of analogs investigated are guanidinium derivatives which have been shown to be effective inhibitors of Na-Ca exchange (32). Substitution at the terminal guanidino-nitrogen atom increases potency of these compounds over that of amiloride, with a maximal increase in activity of \(\sim 10-20\) fold when \(K_i\) values representing inhibition of D-600 binding are compared. Three photoreactive amiloride derivatives, which are potential photoaffinity probes for the \(Ca^{2+}\) channel (see below), have been tested: 2-methoxy-5-nitrobenzamil (L-648,865), 2-methoxy-5-nitro-benzamil (L-663,126), and bromobenzamil (L-647,367). All of these agents are good inhibitors of ligand binding. As found previously for inhibition of Na-Ca exchange, di-substitution of the terminal guanidino-nitrogen (i.e., L-663,128) abolishes the ability of this series to inhibit \(Ca^{2+}\) entry blocker binding.

The next group of analogs characterized are \(N^5\)-substituted inhibitors which are known to block both Na-Ca and Na-H exchange (32, 33). The most active of these derivatives are 20-40-fold more potent than amiloride in inhibiting D-600 binding. Increasing hydrophobicity by increasing chain length of \(N^5\)-alkyl substituents enhances the ability of these compounds to modulate \(Ca^{2+}\) entry blocker binding, and two widely used Na-H exchange inhibitors, \(N^5\)-(ethyl, isopropyl) amiloride (L-593,754) and \(N^5\)-(methyl, isobutyl)amiloride, interact with L-type \(Ca^{2+}\) channels over the same concentration range where they block other Na' transport systems. However, since \(N^5\)-aryl substituents are slightly less active than corresponding alkyl derivatives, the efficacy of molecules in this structural class does not strictly mirror general increases in hydrophobicity.

Molecules substituted at both the terminal guanidino-nitrogen and five pyrazinoyl nitrogen atoms are the most potent inhibitors of Na-Ca exchange of all amiloride analogs invest-

TABLE I

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>(R_1)</th>
<th>(R_2)</th>
<th>(R_3)</th>
<th>(K_i) VALUES ((\mu M))</th>
</tr>
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<tr>
<td>AMILORIDE</td>
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<td>(NH_2)</td>
<td>Cl</td>
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<tr>
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<td>Cl</td>
<td>NH_2</td>
<td>0.03 0.06 0.02</td>
</tr>
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<td>Cl</td>
<td>Nh_2</td>
<td>0.03 0.06 0.02</td>
</tr>
<tr>
<td>L-595,187</td>
<td>Nhch_2</td>
<td>Cl</td>
<td>Nh_2</td>
<td>0.03 0.06 0.02</td>
</tr>
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<td>Nh_2</td>
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</tr>
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<td>L-648,865</td>
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<td>Cl</td>
<td>Nh_2</td>
<td>0.03 0.06 0.02</td>
</tr>
<tr>
<td>L-652,165</td>
<td>Nhch_2</td>
<td>Cl</td>
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</tr>
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FIG. 1. Effect of amiloride analogs on \(Ca^{2+}\) entry blocker binding to porcine cardiac sarcolemmal membrane vesicles. Cardiac sarcolemmal vesicles were incubated at 25 °C with either 10 nM \([H]diltiazem\), 3 nM \([H]D-600\), or 0.17 nM \([H]nitrendipine\) (C) in the presence of increasing concentrations of either L-651,525, \(\Delta\), L-593,755 (■), L-654,250 (●), L-595,187 (□), benzamil (△), or amiloride (□) until equilibrium was achieved. Inhibition of binding was assessed relative to ligand association in untreated vesicles. The structures of the amiloride derivatives investigated are listed by L-number in Table I.
tigated to date (34). Representatives of this structural group are also the most potent modulators of Ca\(^{2+}\) entry blocker binding of the three amiloride derivative classes tested, with some compounds being 100-fold more effective than amiloride in blocking D-600 binding. The data of Table I suggest an interesting parallelism between the ability of different amiloride analogs to inhibit Na-Ca exchange (32, 34) and Ca\(^{2+}\) entry blocker binding activities.

**Mechanism by Which Amiloride Analogs Interact with L type Ca\(^{2+}\) Channels**—To investigate the mechanism by which amiloride analogs modulate binding of diltiazem, D-600, and nitrendipine to their respective receptors in porcine cardiac sarcolemmal membrane vesicles, saturation binding studies have been performed and the data were subjected to Scatchard-type analyses (Fig. 2). Scatchard representations of diltiazem (Fig. 2A), D-600 (Fig. 2B), and nitrendipine (Fig. 2C) binding reveal, in each instance, the existence of a single class of binding sites with affinities of 80, 10, and 0.24 nM, respectively, and site densities which are all equivalent (i.e., ~4 pmol/mg protein) at 25 °C. These values are in close agreement with those previously measured in other porcine cardiac sarcolemmal membrane preparations (7). When similar experiments are repeated in the presence of either N\(^5\)-(methyl, isobutyl)amiloride or 3,4-dichlorobenzamil (L-594,881), compounds representing two different structural classes of analogs, several effects on equilibrium ligand binding are noted. These agents inhibit diltiazem, D-600, and nitrendipine binding in nearly identical fashions by increasing ligand K\(_d\) values in each case. In addition, the guanidino-nitrogen substituted analog L-594,881 causes a small, but reproducible, decrease in the B\(_{max}\) of D-600 binding. These patterns suggest that the two types of amiloride analogs could affect benzothiazepine, aralkylamine, and dihydropyridine receptors in either a competitive or allosteric fashion.

To explore these possibilities, kinetics of ligand dissociation have been studied under the conditions described in Fig. 3. Since it is well established that distinct binding sites exist in the cardiac Ca\(^{2+}\) entry blocker receptor complex for benzothiazepine-, aralkylamine-, and dihydropyridine-like agents (6, 8), it follows that if amiloride derivatives were to interact in a simple competitive fashion at any one of these sites, they would not alter the dissociation of ligand from that site. For these experiments, cardiac sarcolemmal membrane vesicles were incubated with either diltiazem (Fig. 3A), D-600 (Fig. 3B), or nitrendipine (Fig. 3C), at 25 °C until equilibrium was achieved. Removal of \([\text{H}]\)ligand from each receptor was then initiated by addition of a large excess of the appropriate nonradioabeled congener. Dissociation rates are first order in every case, since semi-logarithmic representations of each of these data sets are linear. When the experiment is repeated in the presence of either N\(^5\)-(methyl, isobutyl)amiloride or 3,4-dichlorobenzamil, there is a marked increase in rates of dissociation of both diltiazem and nitrendipine. Interestingly, however, only 3,4-dichlorobenzamil increases the dissociation rate of D-600. To determine whether such lack of effects on aralkylamine dissociation kinetics is a general characteristic of N\(^5\)-substituted derivatives, D-600 dissociation has also been measured in the presence of N\(^5\)-(ethyl, isopropyl)amiloride, and N\(^5\)-(propyl, butyl)amiloride. In both cases, these agents increase the rate of D-600 loss from its receptor (Fig. 3B). Taken together, these data suggest that amiloride analogs do not bind competitively at benzothiazepine, aralkylamine, or dihydropyridine sites. Similar types of experiments have demonstrated that amiloride derivatives also do not interact competitively at a newly characterized site in the Ca\(^{2+}\) entry blocker receptor complex to which substituted diphenylbutyrylpiperidines bind (8). Therefore, amiloride derivatives modulate Ca\(^{2+}\) entry blocker binding reactions through allosteric mechanisms by apparently associating at a unique site on the L-type Ca\(^{2+}\) channel protein.

**Fig. 2. Effect of amiloride analogs on equilibrium binding of Ca\(^{2+}\) entry blockers to porcine cardiac sarcolemmal membrane vesicles.** A. diltiazem binding. Cardiac sarcolemmal vesicles were incubated with increasing concentrations of d-cis-[\(\text{H}\)]diltiazem in the absence (O), or presence of either 1.5 \(\mu\)M L-654,250 (b), or 4 \(\mu\)M L-594,881 (A) until equilibrium was achieved. B. D-600 binding. Membrane vesicles were incubated with increasing concentrations of [\(\text{H}\)]D-600 in the absence (O), or presence of either 2.5 \(\mu\)M L-654,250 (b), or 2.5 \(\mu\)M L-594,881 (A) until equilibrium was achieved. C. nitrendipine binding. Membrane vesicles were incubated with increasing concentrations of [\(\text{H}\)]nitrendipine in the absence (O), or presence of either 2.5 \(\mu\)M L-654,250 (b), or 2.25 \(\mu\)M L-594,881 (A) until equilibrium was achieved. All experiments were performed at 25 °C. In each case, specific binding data are presented in the form of a Scatchard representation.

**Fig. 3. Effect of amiloride analogs on dissociation kinetics of Ca\(^{2+}\) entry blockers from porcine cardiac sarcolemmal membrane vesicles.** A. diltiazem dissociation. Cardiac sarcolemmal vesicles were incubated with 50 nM d-cis-[\(\text{H}\)]diltiazem at 20 °C until equilibrium was achieved. Dissociation of ligand was initiated by addition of 10 \(\mu\)M d-cis-[\(\text{H}\)]diltiazem alone (O), or with either 25 \(\mu\)M L-654,250 (b), or 25 \(\mu\)M L-594,881 (A) also present. B. D-600 dissociation. Membrane vesicles were incubated with 3.4 nM [\(\text{H}\)]D-600 at 20 °C until equilibrium was achieved. Dissociation of bound ligand was initiated by addition of 5 \(\mu\)M (±) [\(\text{H}\)]D-600 alone (O), or with 25 \(\mu\)M of either L-654,250 (b), L-594,881 (A), L-593,754 (L), or L-593,755 (C). C. nitrendipine dissociation. Membrane vesicles were incubated with 0.29 nM [\(\text{H}\)]nitrendipine at 20 °C until equilibrium was achieved. Dissociation of ligand was initiated by addition of 1 \(\mu\)M [\(\text{H}\)]nitrendipine alone (O), or with either 25 \(\mu\)M L-654,250 (b), or 25 \(\mu\)M L-594,881 (A) also present. All time courses of dissociation were monitored at 20 °C. Data are presented as values of ligand binding at various times compared with the amount of ligand bound at equilibrium versus time.
of two types of ion-binding sites is possible; the "Ca"^{2+} coordination site" within the channel (35), or the site at the mouth of the channel where inorganic channel blockers function (36). To test the first of these possibilities, the potencies of N-(methylisobutyl)amiloride and 3,4-dichlorobenzamil have been determined as inhibitors of diltiazem, D-600, and nitrendipine binding, either in the absence of Ca"^{2+} (100 mM EGTA present), or with 10 or 100 μM Ca"^{2+} present. The presence of Ca"^{2+}, itself, has some effect on the different Ca"^{2+} entry blocker binding reactions (7). However, no significant differences are observed in the abilities of either of the two amiloride analogs to inhibit binding of Ca"^{2+} entry blockers at any of the levels of Ca"^{2+} tested (not shown). These findings imply that the Ca"^{2+} coordination site is not the site at which amiloride derivatives interact.

To test the second possibility, binding of fluspirilene, a substituted diphenylbutylpiperidine L-type Ca"^{2+} channel modulator, has been monitored with amiloride analogs present. It has previously been shown that Cd"^{2+}, La"^{3+}, Co"^{2+}, and Ni"^{2+}, inorganic Ca"^{2+} entry blockers which inhibit channel activity by occluding the pore, stimulate the association of fluspirilene with membranes (8). Enhanced binding is due to increased local affinity and receptor site density. Cd"^{2+} and La"^{3+} are unique in that they yield bell-shaped dependencies in their action: as the concentration of these metal ions is increased, fluspirilene binding first increases and then returns to the control level. If amiloride analogs were to interact at the site where inorganic cations function, they should alter the ability of these latter agents to modulate fluspirilene binding. Data from an experiment in which the fluspirilene binding is recorded as a function of Cd"^{2+} concentration, without or with 3,4-dichlorobenzamil present, are shown in Fig. 4A. Addition of 2 μM of this amiloride analog, a concentration that blocks fluspirilene binding by 50% (8), shifts the Cd"^{2+} concentration dependence such that greater amounts of metal ion are required to affect fluspirilene binding, but the shape of the complete binding profile remains unchanged. When the action of 3,4-dichlorobenzamil is monitored on fluspirilene binding over a wide range of ligand concentrations and at a stimulatory concentration of Cd"^{2+} (250 μM), data presented in the Scatchard representation of Fig. 4B are obtained. Cd"^{2+}, as found previously, increases fluspirilene binding through a combined K_d and B_{max} effect. However, when the experiment is repeated in the presence of 4 μM amiloride derivative, a Scatchard analysis indicates that this fixed concentration of 3,4-dichlorobenzamil inhibits the stimulation of fluspirilene binding characteristically elicited by 250 μM Cd"^{2+}. Lower concentrations of 3,4-dichlorobenzamil yield data displaying intermediate behavior in a Scatchard analysis (not shown). These results suggest that Cd"^{2+} and amiloride derivatives could compete for binding at a common site.

Another piece of evidence supporting this conclusion is derived from observations regarding the effects of metal ions on association of other classes of Ca"^{2+} entry blockers. Cd"^{2+}, as found previously, increases binding of diltiazem, D-600, and nitrendipine by decreasing ligand affinity (not shown), a similar pattern as is found with the amiloride analogs (Fig. 2). It is noteworthy that 3,4-dichlorobenzamil, by itself, causes complete inhibition of fluspirilene binding (8), rather than stimulation, as might be expected given the action of the inorganic pore blockers. Moreover, amiloride, a much less hydrophobic compound, also inhibits rather than stimulates this binding reaction (not shown). The reason for this apparent difference in organic versus inorganic cations as modulators of fluspirilene binding is unclear, but it may indicate that amiloride analogs and Cd"^{2+} share overlapping binding sites which are allosterically linked to the substituted diphenylbutylpiperidine receptor in different fashions.

To confirm that amiloride analogs interact with L-type Ca"^{2+} channels in a specific manner, rather than by simply affecting the protein through perturbation of the membrane environment, two different photoactive amiloride derivatives have been studied. Bromobenzamil and 2-methoxy-5-nitrobenzamil were utilized to determine whether photolysis of these compounds would produce irreversible inhibition of Ca"^{2+} entry blocker binding in cardiac sarcolemmal membrane vesicles, and then to characterize the properties of such an event. The photochemistry of these two agents is different: bromobenzamil contains a bromine in the 6-position of the pyrazinyl moiety which is a leaving group when the pyrazine ring is irradiated with monochromatic light at 365 nm (37); 2-methoxy-5-nitrobenzamil contains a methoxy group linked to a nitrophenyl moiety that can function as a leaving group when the nitrophenyl-ether chromophore is irradiated with monochromatic light at 313 nm (38, 39). Model photolysis reactions performed in solution with the latter amiloride analog are consistent with the photochemistry proceeding by a nucleophilic aromatic substitution reaction (not shown). It is possible for protein nucleophiles located at the site where amiloride-like compounds bind to form a covalent adduct with either of these derivatives and thereby yield stable incorporation of photo-probe into the channel protein.

When sarcolemmal membranes are incubated with either putative photoaffinity reagent in the dark, reversible inhibition of Ca"^{2+} entry blocker binding activity occurs (Table I). However, subjecting membranes to photolysis conditions in the presence of either bromobenzamil (Fig. 5A), or 2-methoxy-5-nitrobenzamil (Fig. 5B), as described under "Experimental Procedures," results in time- and concentration-dependent block of diltiazem, D-600, and nitrendipine binding reactions. Inhibition is irreversible in each case, since repeated washing of membranes does not restore the binding activities. Data portrays the time dependence of inactivation are illustrated in Fig. 5. Rates of photoinactivation in every case are first order. Interestingly, each binding reaction displays a characteristic rate of loss of activity, with D-600 being most sensitive and nitrendipine being least sensitive to photoinactivation.
activities--To determine whether amiloride analogs will block Ca\(^{2+}\) uptake into this cell line is inhibited by dihydropyridine Ca\(^{2+}\) channel agonists (8). When 45Ca\(^{2+}\) influx is measured in GH\(_4\) cells under depolarizing conditions in the presence of either amiloride or different amiloride analogs, these agents produce concentration-dependent inhibition of Ca\(^{2+}\) uptake (Fig. 6). This pattern compares qualitatively with the rank order of potency displayed by these analogs in inhibiting Ca\(^{2+}\) entry blocker binding reactions, and suggests that amiloride derivatives can block L-type Ca\(^{2+}\) channel activity in a direct fashion. The best Ca\(^{2+}\) channel inhibitor in this series is 20-fold less potent than nitrrendipine (8) at blocking Ca\(^{2+}\) uptake into GH\(_4\) cells.

To confirm the hypothesis that amiloride derivatives modify L-type Ca\(^{2+}\) channels directly, GH\(_4\) cells have been subjected to whole cell voltage-clamp using patch-clamp procedures under conditions where inward Ca\(^{2+}\) currents can be isolated. In order to accomplish this, cells were treated with TTX to abolish Na\(^{+}\) channel currents and perfused with Ca\(^{2+}\)-free and Mg\(^{2+}\)-free Tyrode solution. Cells were depolarized with a hyperpolarized membrane potential where both types of Ca\(^{2+}\) channels can be activated, and then stepped to different depolarized potentials, the inward current traces shown in Fig. 7A are obtained. In these traces, the rapidly activating and inactivating components of current represent primarily T-type Ca\(^{2+}\) channel activity, while the sustained component (measured at 100 ms) is due to this isolation procedure. The patterns illustrated in Fig. 7, D and E that result from a control experiment are obtained.

Amiloride Analogs Inhibit L-type and T-type Ca\(^{2+}\) Channel Activities--To determine whether amiloride analogs will block L-type Ca\(^{2+}\) channel function, three different experimental approaches have been employed. Channel activity has been monitored in the presence of amiloride derivatives using either standard voltage-clamp or whole cell voltage-clamp techniques. A major pathway for Ca\(^{2+}\) influx into GH\(_4\) rat anterior pituitary cells is through L-type Ca\(^{2+}\) channels (26). Supporting this conclusion are data which show that depolarization-mediated Ca\(^{2+}\) uptake into this cell line is inhibited by dihydropyridine Ca\(^{2+}\) channel blockers, while Ca\(^{2+}\) influx under normal membrane resting potentials is markedly enhanced by dihydropyridine Ca\(^{2+}\) channel agonists (8). When Ca\(^{2+}\) influx is measured in GH\(_4\) cells under depolarizing conditions in the presence of either amiloride or different amiloride analogs, these agents produce concentration-dependent inhibition of Ca\(^{2+}\) uptake (Fig. 6). The extent of drug block increases with time of exposure of cells

while diltiazem displays intermediate behavior. Such results confirm previous observations suggesting that benzoazepine and aralkylamine receptors are distinct entities in heart (7, 10). Control experiments demonstrate that illumination of vesicles in the absence of amiloride analogs has no effect on any of these binding reactions. Furthermore, the osmotic integrity of the membrane is not affected by photolysis procedures because passive fluxes of Ca\(^{2+}\) are not modified (not shown), suggesting that general membrane disruption is not occurring. Importantly, when similar experiments are performed in the presence of either excess verapamil (Fig. 5A), or excess diltiazem (Fig. 5B), the time course of photoactivation of the three ligand binding reactions is significantly slowed. Equivalent protection is afforded by either verapamil or diltiazem, no matter which amiloride analog is used to produce irreversible inhibition of ligand binding. Protection of binding activities from photoinactivation by inclusion of L-type Ca\(^{2+}\) channel modulators strongly suggests that amiloride analogs interact in a specific fashion with the channel protein and that their binding site is coupled allosterically to receptors for other Ca\(^{2+}\) entry blockers.

Amiloride Analogs Inhibit L-type and T-type Ca\(^{2+}\) Channel Activities--To determine whether amiloride analogs will block L-type Ca\(^{2+}\) channel function, three different experimental approaches have been employed. Channel activity has been monitored in the presence of amiloride derivatives using either standard voltage-clamp or whole cell voltage-clamp techniques. A major pathway for Ca\(^{2+}\) influx into GH\(_4\) rat anterior pituitary cells is through L-type Ca\(^{2+}\) channels (26). Supporting this conclusion are data which show that depolarization-mediated Ca\(^{2+}\) uptake into this cell line is inhibited by dihydropyridine Ca\(^{2+}\) channel blockers, while Ca\(^{2+}\) influx under normal membrane resting potentials is markedly enhanced by dihydropyridine Ca\(^{2+}\) channel agonists (8). When Ca\(^{2+}\) influx is measured in GH\(_4\) cells under depolarizing conditions in the presence of either amiloride or different amiloride analogs, these agents produce concentration-dependent inhibition of Ca\(^{2+}\) uptake (Fig. 6). The effectiveness of members of this series of compounds is: N-(2,4-dimethylphenyl)-N\(^{\prime}\)-(4-chlorobenzyl)-

amloride (IC\(_{50} = 180 \text{ nM}\) ) > naphthylmethylamiloride (IC\(_{50} = 2.4 \mu M\) ) ≈ 3,4-dichlorobenzamil (IC\(_{50} = 4.2 \mu M\) ) ≈ N\(^{\prime}\)-[methyl, isobutyl]amiloride (IC\(_{50} = 5.6 \mu M\) > N\(^{\prime}\)-[ethyl, isopropyl]amiloride (IC\(_{50} = 13 \mu M\) ) > benzamil (IC\(_{50} = 56 \mu M\) ) > amiloride (IC\(_{50} = 1.7 \text{ mM}\) ). This pattern compares qualitatively with the rank order of potency displayed by these analogs in inhibiting Ca\(^{2+}\) entry blocker binding reactions, and suggests that amiloride derivatives can block L-type Ca\(^{2+}\) channel activity in a direct fashion. The best Ca\(^{2+}\) channel inhibitor in this series is 20-fold less potent than nitrrendipine (8) at blocking Ca\(^{2+}\) uptake into GH\(_4\) cells.

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Calcium Channel Inhibition by Amiloride

FIG. 7. Effect of amiloride analogs on L- and T-type Ca\(^{2+}\) channel activity in GH3 cells. GH3 cells were bathed in a medium containing 135 mM NaCl, 5 mM KCl, 10 mM CaCl\(_2\), 1 μM TTX, 5 mM Hepes-NaOH, pH 7.3. The electrode solution contained 142 mM CsCl, 5 mM EGTA, 5 mM Hepes-KOH, pH 7.3. The volume of the experimental chamber was 1.5 ml, and the cells were superfused at a rate of 0.5 ml/min. The flow rate was increased 3-fold for the first few minutes after a change of solution. Data (A) were obtained for the plot of peak (D) or steady-state (E) Ca\(^{2+}\) currents versus voltage in a control cell by eliciting 200 ms depolarization steps (C) from a holding potential (V\(_{H}\)) of -80 mV (A). After 10 min of superfusion with the same solution containing 5 μM L-593,754 (B) data (D) depicting the effect of the amiloride analog on peak (D) and sustained (E) Ca\(^{2+}\) currents were collected.

TABLE II
Inhibition of Ca\(^{2+}\)-induced contraction in rat aorta by amiloride analogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiloride</td>
<td>10</td>
</tr>
<tr>
<td>Benzamil</td>
<td>44</td>
</tr>
<tr>
<td>3,4-dichlorobenzamil (L-594,881)</td>
<td>71</td>
</tr>
<tr>
<td>N(^5)-(propyl, butyl)amiloride (L-593,755)</td>
<td>80</td>
</tr>
<tr>
<td>N(^5)-(4-chlorobenzyl)amiloride (L-593,438)</td>
<td>83</td>
</tr>
</tbody>
</table>

The results presented in this study represent the first demonstration that all commonly used amiloride analogs possess significant Ca\(^{2+}\) entry blocker activity. The evidence supporting this claim is compelling. As predicted for any compound which modulates L-type Ca\(^{2+}\) channels, amiloride derivatives affect binding of nifedipine, D-600, diltiazem, and fluspirilene to their respective sites in the Ca\(^{2+}\) entry blocker receptor complex via a characteristic pattern of interactions. In addition, these compounds block depolarization-induced Ca\(^{2+}\) uptake into GH3 anterior pituitary cells mediated by L-type Ca\(^{2+}\) channels with a rank order of effectiveness similar to that displayed for inhibition of ligand binding. Several representative amiloride analogs also inhibit the GI3 L-type Ca\(^{2+}\) channel in voltage-clamp experiments with potencies resembling those observed in flux protocols, suggesting direct action of amiloride-like compounds at the level of this channel. Moreover, various amiloride derivatives block depolarization-induced contraction of aortic smooth muscle, a predicted pharmacological consequence of Ca\(^{2+}\) entry blocker activity, with the expected rank order of potency. Thus, these data demonstrate that Ca\(^{2+}\) entry blocker activity is an inherent pharmacological property of all of the amiloride analog structural classes investigated in this study.

DISCUSSION

The results presented in this study represent the first demonstration that all commonly used amiloride analogs possess significant Ca\(^{2+}\) entry blocker activity. The evidence supporting this claim is compelling. As predicted for any compound which modulates L-type Ca\(^{2+}\) channels, amiloride derivatives affect binding of nifedipine, D-600, diltiazem, and fluspirilene to their respective sites in the Ca\(^{2+}\) entry blocker receptor complex via a characteristic pattern of interactions. In addition, these compounds block depolarization-induced Ca\(^{2+}\) uptake into GH3 anterior pituitary cells mediated by L-type Ca\(^{2+}\) channels with a rank order of effectiveness similar to that displayed for inhibition of ligand binding. Several representative amiloride analogs also inhibit the GI3 L-type Ca\(^{2+}\) channel in voltage-clamp experiments with potencies resembling those observed in flux protocols, suggesting direct action of amiloride-like compounds at the level of this channel. Moreover, various amiloride derivatives block depolarization-induced contraction of aortic smooth muscle, a predicted pharmacological consequence of Ca\(^{2+}\) entry blocker activity, with the expected rank order of potency. Thus, these data demonstrate that Ca\(^{2+}\) entry blocker activity is an inherent pharmacological property of all of the amiloride analog structural classes investigated in this study.
Calcium Channel Inhibition by Amiloride

600, and nitrendipine association by lowering ligand affinity at each site in the Ca\(^{2+}\) entry blocker receptor complex. Similar effects are produced by the inorganic Ca\(^{2+}\) channel inhibitor Cd\(^{2+}\). Although all amiloride structural classes increase ligand \(K_d\) values, none bind competitively at any of the known Ca\(^{2+}\) entry blocker receptors because they increase rates of ligand dissociation from each of these sites. This pattern indicates that the amiloride-binding site on the channel must be allosterically coupled to other receptors in the Ca\(^{2+}\) entry blocker receptor complex. Additional evidence supporting this conclusion is supplied by studies with photo-reactive amiloride derivatives which produce irreversible inhibition of diltiazem, D-600, and nitrendipine binding upon photolysis. Since this process can be prevented by including either excess verapamil or diltiazem during the reaction, such findings would suggest that occupation of the aralkylamine or benzothiazepine site must prevent binding of amiloride derivatives to the L-type Ca\(^{2+}\) channel. This observation is also consistent with the idea of allosteric coupling between the different sites. The action of amiloride analogs on binding of substituted diphenylbutylpiperidine Ca\(^{2+}\) entry blockers is particularly interesting, given the effect which 3,4-dichlorobenzamil has on the ability of the inorganic pore blocker Cd\(^{2+}\) to modulate \(\nu\)-spiralike binding. The interaction between the amiloride analog and Cd\(^{2+}\) appears competitive. This implies that amiloride’s acyl-guanidino group, which is protonated at physiological pH, binds at the mouth of the channel where metal ions interact. Therefore, it is predicted that amiloride analogs should block channel activity by interfering with Ca\(^{2+}\) movement along the ion conduction pathway. Recent electrophysiological data with 3,4-dichlorobenzamil are consistent with this model (26). This study indicates that L-type Ca\(^{2+}\) channel inhibition is modulated by the functional state of the channel, being enhanced by channel opening. Together, these two separate lines of evidence suggest that amiloride derivatives possess Ca\(^{2+}\) entry blocker activity because they block the pore of the L-type Ca\(^{2+}\) channel. Analogous mechanisms can be envisioned for amiloride inhibition of other types of voltage-dependent Ca\(^{2+}\) channels.

The similarity in the ability of amiloride analogs to function as Ca\(^{2+}\) entry blockers and to block various Na\(^{+}\) transporting systems is striking. In most cases, effective inhibitors are hydrophobic cations which easily partition into the membrane to generate a high local concentration at the external face of the transporter. These agents then compete with metal ions for substrate-binding sites. This type of mechanism is envisioned for amiloride inhibition of the Na-Ca exchange transporter (40). Only in the case of epithelial Na\(^{+}\) channels does the interaction of amiloride with the target protein appear to be more selective (41). Thus, it is predicted that the inhibitory potency of amiloride derivatives should correlate, for the most part, with the lipophilicity of individual molecules. The structure-activity relationship presented in Table I depicting the Ca\(^{2+}\) entry blocker activity of different amiloride analogs supports this conclusion. Moreover, common features undoubtedly exist in the structure of various ion-binding sites on channels and transporters. Given that metal ions display little discrimination as inhibitors of different transport systems, it is not surprising that amiloride analogs would also show a similar lack of selectivity in their inhibitory action. Therefore, amiloride analogs would appear to be most useful for studying the properties of an individual transport system when its activity can be isolated. Nonetheless, the utility of these compounds as inhibitors can still be exploited. For example, in the case of the L-type Ca\(^{2+}\) channel, some question exists as to what subunits comprise the pore structure. Even though the \(\alpha_{1}\) subunit contains the receptors for Ca\(^{2+}\) entry blockers (42, 43), it is not clear that this entity is sufficient to establish channel activity, or if other components are necessary to construct a functional channel protein. The amiloride photoaffinity reagents described in the present study could be used to address this type of question. Since these agents bind at the external pore, radiolabeled derivatives could be useful in identifying those protein components which form this structure. Although both \(^{3}H\)bromobenzamil and \(^{14}C\)2-methoxy-5-nitrobenzamil are covalently incorporated into many different membrane proteins upon photolysis of cardiac sarcosomal vesicles,\(^{3}\) similar studies carried out with a purified L-type Ca\(^{2+}\) channel preparation are expected to result in labeling of the relevant peptides which comprise the pore region.

Amloride derivatives have been employed in numerous studies to investigate the role of Na-Ca and Na-H exchange in intact cells and tissues. Since Ca\(^{2+}\) channels are distributed in a wide variety of cell types, and Ca\(^{2+}\) plays such a fundamental role in many different cellular processes, the Ca\(^{2+}\) entry blocker activity of the amiloride series casts suspicion as to whether some of the effects reported with these compounds might not be related to block of Ca\(^{2+}\) influx, rather than to inhibition of the transport system that was under investigation. For example, the negative inotropic activity of 3,4-dichlorobenzamil in isolated cardiac muscle preparations could be a consequence of Ca\(^{2+}\) channel block, either alone, or in addition to inhibition of Na-Ca exchange as was originally postulated (20). Other investigators have recognized the limited selectivity of 3,4-dichlorobenzamil as a probe of Na-Ca exchange in heart and have suggested that 2-chlorobenzamil is a much more specific inhibitor (21). However, this compound blocks depolarization-induced Ca\(^{2+}\) uptake into G11 cells with a 6-fold higher potency than it inhibits Na-Ca exchange in cardiac sarcosomal membrane vesicles. Ca\(^{2+}\) entry blocker activity is an inherent pharmacological property of amiloride analogs that is derived from the joint hydrophobic and cationic nature of these molecules. Thus, their use in studying intracellular regulation, mitogenesis, chemotaxis, stimulus-secretion coupling, Ca\(^{2+}\) homeostasis, or any other biological phenomenon in intact systems must be viewed with extreme caution.

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