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INHIBITION OF Na^+/Ca^{2+} EXCHANGE BY AMILORIDE ACTING FROM OPPOSITE SIDES OF CARDIAC SARCOLEMMA

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Abstract—Amiloride inhibited the Na^+/Ca^{2+} exchange activity of cardiac sarcolemmal vesicles with similar affinities at the *cis* and *trans* sides of the membrane, estimated apparent K_i on both sides of the sarcolemma being similar.

The extent of amiloride inhibition on Na^+/Ca^{2+} exchange activity was decreased by alkaline pH only when the drug was acting from the external side of the vesicle sarcolemma, whereas when vesicles were preincubated with the drug at different pH values, amiloride appeared to act as a weak permeant base, being a more effective inhibitor at alkaline pH values. In fact, a rise in the pH of the preincubation medium may favour the entry and consequently the effect of the drug on the exchanger. The pH dependence of the inhibition of Na^+/Ca^{2+} exchange activity by either extravesicular or intravesicular amiloride was consistent with the hypothesis that in both cases the protonated drug was the active form.

Evidence is presented that the pattern of interaction of amiloride on the Na^+/Ca^{2+} exchange system strictly depended on the sidedness of drug action. In fact, while Na^+ protected against inhibition by amiloride when it was acting on the same side of the vesicle membrane as the drug, it synergically interacted with amiloride to inhibit exchange activity when it was acting on the opposite side of the sarcolemma as the drug. Furthermore, only extravesicular amiloride removed the stimulation of Na^+/Ca^{2+} exchange activity in Ca^{2+}-treated vesicles.

Amiloride, a pyrazine–guanidine compound widely used as a potassium-sparing diuretic, is a well-known inhibitor of passive Na^+-fluxes [1], Na^+/H^+ exchange carrier [2] and Na^+/Ca^{2+} exchange in several plasma membrane preparations, including cardiac sarcolemmal vesicles from different species [3–8]. Amiloride is a positive inotropic agent [6, 9–11] and protects against digitalis toxicity both *in vivo* [12] and *in vitro* [6, 10]. The cardiac effects of amiloride have been shown to occur in a range of concentrations close to those inhibiting Na^+/Ca^{2+} exchange in cardiac sarcolemmal vesicles [6, 8] and have been tentatively ascribed to inhibition of Ca^{2+} flux through the Na^+/Ca^{2+} exchange system [6, 10, 13]. This system catalyzes electrogenic counter-transport of Na^+ ions on either side of the plasma membrane for Ca^{2+} on the opposite side, with a probable stoichiometry of 3 to 1 [14]. The electrochemical gradients for Na^+ and Ca^{2+} and the transmembrane electrical potential determine the direction of net Ca^{2+} flux [15]. In cardiac muscle Na^+/Ca^{2+} exchange plays a key role in the regulation of intracellular calcium levels and thus in contractile control (for reviews, see Refs 16–18).

Since we have recently shown that, in cardiac sarcolemmal vesicles, amiloride binds to sarcolemma and also enters the vesicles, reaching intravesicular concentrations in the millimolar range [8], the interaction of amiloride with the exchanger system was investigated under experimental conditions in which the drug could act from either the external or internal side of the sarcolemma. In the present study, we show that amiloride displays similar apparent affinities for the exchanger at both sides of the sarcolemma. In addition, the pH dependence of the inhibition of Na^+/Ca^{2+} exchange activity by either extravesicular or intravesicular amiloride was consistent with the hypothesis that in both cases the protonated drug is the active form. However, the way in which stimulation of Na^+/Ca^{2+} exchange activity by intravesicular Ca^{2+} is affected by amiloride and the pattern of interaction between Na^+ and amiloride on the exchange system strictly depends on the sidedness of amiloride action.

MATERIALS AND METHODS

Materials. Amiloride was purchased from Merck-Sharp and Dohme S.p.A. (Rome, Italy). $^{45}CaCl_2$ was obtained from New England Nuclear (Florence, Italy). Alamethicin was kindly provided by Dr. J. E. Grady, Upjohn Company (Kalamazoo, MI). 9-Aminoacridine and MOPS† were purchased from Sigma Chemical Co. (St Louis, MO). All other reagents were pure-grade.

Cardiac sarcolemmal vesicle preparation. Cardiac sarcolemmal vesicles were prepared from bovine ventricular tissue, as previously described [6, 8]. The vesicles were suspended in ice-cold 160 mM NaCl in 20 mM MOPS/Tris (pH 7.4) at a final protein

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† Abbreviation used: MOPS, 4-morpholinepropanesulfonic acid.

concentration of about 10 mg/ml, apportioned into 50-μl aliquots, quick-frozen and stored at −40°. Each aliquot was used only once, immediately after thawing. It was calculated that about 60% of the vesicles were inside-out by stimulation of Na^+–K^+ ATPase activity by alamethicin [19].

The final protein concentration of the vesicle suspension was measured by the method of Lowry *et al.* [20] using bovine serum albumin as standard.

Vesicle treatment. Amiloride hydrochloride was dissolved in distilled water at 37° immediately before the experiment was started.

Experiments designed to examine the effects of amiloride at the external surface of the sarcolemma were performed by adding different amounts of the drug directly into the medium for $^{45}Ca^{2+}$ uptake assay. In order to examine the effects of amiloride at the internal surface of the vesicle membrane, 27 μg of Na^+-loaded vesicles were diluted 1:1.2 by adding aqueous solutions of amiloride in a final volume of 10 μl and incubated at 37° for 20 min before the $^{45}Ca^{2+}$ uptake assay. Control vesicles were preincubated with an equal volume of distilled water.

Assay of Na^+/Ca^{2+} *exchange activity.* To monitor Na_i^+-dependent Ca^{2+} uptake, 3-μl aliquots (8 μg of protein) of the vesicle suspension or the preincubated vesicles were diluted into 160 mM KCl in 20 mM MOPS/Tris (pH 7.4), containing 40 μM $^{45}CaCl_2$ (100–200 cpm/pmol) in a total volume of 321 μl, and incubated at 37°. The Ca^{2+}-uptake reactions were stopped at different periods of time (10 or 30 sec) by rapid Millipore filtration [8] of 100-μl aliquots. The dried filters were counted by liquid scintillation spectroscopy (Aquasol).

Na_i^+-dependent Ca^{2+} uptake was linear for up to 40 sec, so in all experiments Ca^{2+} uptake in the first 10–30 sec was measured. Values for $^{45}Ca^{2+}$ uptake were corrected by subtracting blanks for $^{45}Ca^{2+}$ associated with the vesicles in the absence of a Na^+ gradient (about 10%). Na_i^+-independent Ca^{2+} uptake was not significantly affected by amiloride.

For experiments in which the pH of uptake and preincubation media was varied, 20 mM MOPS/Tris at pH 6.8 and 20 mM Tricine/Tris at pH 8.0, 8.5 and 9.0 were used as buffers. Stock solutions of amiloride were adjusted to the desired pH immediately before starting the experiment.

The determination of △pH in vesicles preincubated in absence and presence of amiloride was performed by monitoring fluorescence of 9-aminoacridine [21].

Amiloride assay. The amount of amiloride associated with intact and osmotically shocked vesicles was estimated, as reported in Ref. 8, by measuring fluorescence intensity [22] (excitation wavelength, 363 nm; emission wavelength, 410 nm), under the same experimental conditions adopted for monitoring the concentration- and pH-dependence of amiloride effect on Na^+/Ca^{2+} exchange activity in preincubated vesicles. Upon preincubation with the drug, aliquots of vesicle suspension were immediately diluted 1:107 in KCl medium. After 30 sec at 37°, aliquots of 100 μl were rapidly filtered through a Millipore filter (0.45 μm) and washed twice with 2.5 ml of ice-cold 160 mM NaCl. The filters were then soaked in 3 ml of 1% sodium dodecylsulfate for 5 min. The fluorescence intensity was calibrated with standard solutions of amiloride dissolved in 1% sodium dodecylsulfate. The intravesicular contents of amiloride, expressed as μg of amiloride per mg of vesicular protein, were calculated by subtracting the amounts of amiloride associated to broken vesicles from those of intact vesicles.

Notations $amiloride_o$, $amiloride_i$, and Na_o^+, Na_i^+ refer to extravesicular and intravesicular amiloride and Na^+, respectively.

RESULTS

Na^+/Ca^{2+} *exchange inhibition by amiloride acting from outside or inside cardiac sarcolemmal vesicles*

In our previous work [8], amiloride appeared to be a more effective inhibitor of Na^+/Ca^{2+} exchange activity when acting from inside the vesicles, as judged by IC_{50} values. An estimate of the apparent K_i for amiloride on the two sides of the vesicle membrane was therefore made.

Dixon plots for Na^+/Ca^{2+} exchange activity in cardiac sarcolemmal vesicles in the presence of amiloride are shown in Fig. 1. In Fig. 1A, vesicles were directly assayed for $^{45}Ca^{2+}$ uptake in the presence of increasing amounts of amiloride (1–6 mM). The apparent K_i for amiloride, calculated from the least-squares regression line up to 4 mM, was 1.63 mM. However, at the highest concentrations of amiloride (4–6 mM), the Dixon plot appeared non linear. In Fig. 1B, vesicles were preincubated with the drug and then assayed for $^{45}Ca^{2+}$ uptake. The values for amiloride concentration on the abscissa refer to concentrations of amiloride inside the vesicles, calculated from the mean values for the amount of amiloride associated with the vesicles after 20-min preincubation at 37° reported in Table 1, assuming an internal vesicle volume of 8.5 μl per mg of protein [23]. The least-squares regression line shown in Fig. 1B yielded a K_i value for amiloride of 1.67 mM.

Influence of pH on Na^+/Ca^{2+} *exchange activity in absence and presence of amiloride*

It has been observed that amiloride fluxes across the plasma membrane of cells are strongly pH-dependent, the highest rate being at alkaline pH [24, 25]. Furthermore, amiloride has been shown to be a more effective inhibitor of Na^+/Ca^{2+} exchange at acid pH in synaptosomal membrane [26] and pituitary plasma membrane [7] vesicles, suggesting that the positively charged form of amiloride is required for inhibitory activity.

To determine whether the exchanger was inhibited by the charged or uncharged form of the drug at the two sides of the vesicle sarcolemma, the influence of pH on the effects of amiloride on the exchanger was investigated under conditions in which the pH of the KCl buffer for $^{45}Ca^{2+}$-uptake assay or of the vesicle preincubation medium, or both, were varied (Fig. 2). In the upper pannels of Fig. 2, values of Na_i^+-dependent Ca^{2+} uptake at different pH values are reported. Data are normalized, so that Ca^{2+} uptake at pH 7.4 is taken as 100% activity. In the lower panels, Na_i^+-dependent Ca^{2+} uptake values obtained in the presence of a fixed amiloride con-

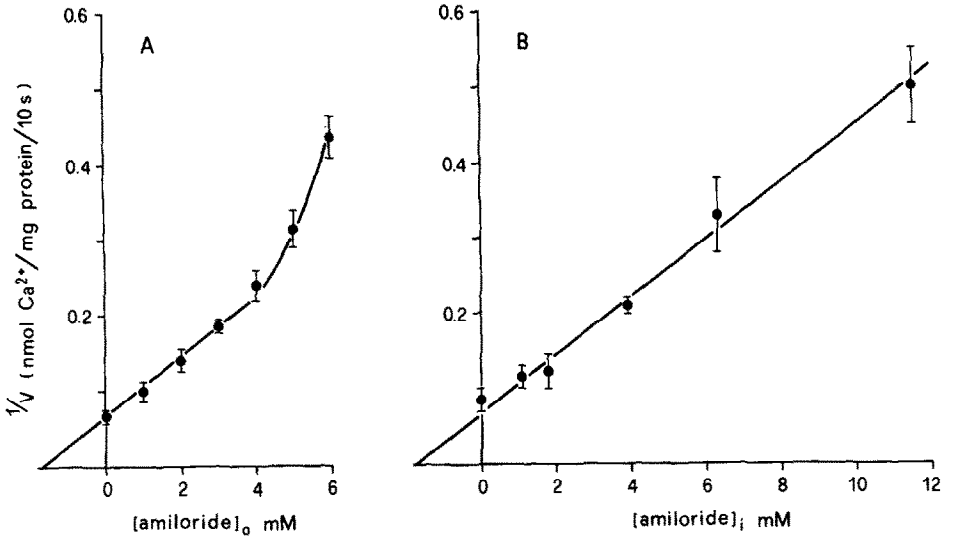

Fig. 1. Dixon plots for Na^+/Ca^{2+} exchange activity in cardiac sarcolemmal vesicles in presence of amiloride. (A) Na^+-loaded vesicles were directly assayed for $^{45}Ca^{2+}$ uptake (10 sec) in KCl buffer (pH 7.4) containing increasing amounts of amiloride (see Materials and Methods). (B) Na^+-loaded vesicles were preincubated for 20 min at 37° with increasing amounts of amiloride. 3-μl aliquots of preincubated vesicles were then assayed for $^{45}Ca^{2+}$ uptake (10 sec) by means of dilution into KCl buffer (pH 7.4) (see Materials and Methods). All data points are means ± SE of 4 experiments.

centration are compared with those from their respective control vesicles and expressed as percentage inhibitions. Data are plotted vs pH values either during $^{45}Ca^{2+}$ uptake (Fig. 2A) or during vesicle preincubation (Fig. 2B).

The modification of pH from 6.8 to 9.0 during the 30 sec of $^{45}Ca^{2+}$-uptake reaction markedly increased the activity of the Na^+/Ca^{2+} exchange system (Fig. 2A, upper panel), in agreement with previous data in the literature [23, 27, 28]. Similar pattern of pH-dependence was found also when vesicles were preincubated in media at various pH values and then assayed for Ca^{2+} uptake at the pH values used during vesicle preincubation (Fig. 2B, upper panel). However, it was evident that preincubation at pH 9.0 decreased stimulation of exchange activity to the values obtained at pH 7.4 (Fig. 2B, upper panel). When vesicles preincubated in media at various pH values were assayed for $^{45}Ca^{2+}$ uptake at pH 7.4 (Fig. 2B, upper panel), only slight differences in exchange activities both at acid and alkaline pH values compared to those obtained at neutral pH were found.

Table 1. Intravesicular levels of amiloride in cardiac sarcolemmal vesicles preincubated with increasing amounts of extravesicular amiloride

$[amiloride]_o$ (mM)	$[amiloride]_i$ (μg amiloride/mg prot)
2.5	2.49 ± 0.93
5.0	4.05 ± 0.65
7.0	8.72 ± 1.91
10.0	14.19 ± 2.30
15.0	25.90 ± 3.22

Na^+-loaded vesicles were preincubated with amiloride as in Fig. 1B. The amount of amiloride associated with the vesicles was estimated as described under Materials and Methods. Values are means ± SE of at least 3 separate experiments.

The inhibitory activity of 2.5 mM amiloride on Na^+/Ca^{2+} exchange appeared to be pH-dependent from pH 6.8–9.0, maximum percentage inhibition being obtained at pH 6.8 (Fig. 2A, lower panel), in agreement with the data of Schellenberg *et al.* [26] and Kaczorowski *et al.* [7]. By contrast, the pattern of amiloride inhibition of Na_i^+-dependent Ca^{2+} uptake in vesicles preincubated with the drug at different pH values appeared to depend on whether the Ca^{2+}-uptake assay was performed at pH 7.4 or at the pH values used during vesicle preincubation (Fig. 2B, lower panel). In the first case, there was a gradual increase in the inhibitory activity of amiloride as the pH of the preincubation medium became more alkaline. In the second case, only slight differences in the extent of amiloride inhibition at various pH values were found. In these experiments, Na_i^+-independent Ca^{2+} uptake was not affected by changes in the pH value of either preincubation or uptake medium.

To test whether the observed differences in amiloride inhibition of Na^+/Ca^{2+} exchange in preincubated vesicles could be ascribed to a different extent of amiloride uptake into the vesicles, the intravesicular levels of amiloride were determined under the same experimental conditions, as detailed under Materials and Methods. Table 2 shows that, when vesicles were preincubated with the drug at various pH values and, upon dilution in KCl medium buffered at pH 7.4, incubated for 30 sec at 37°, the intravesicular amiloride concentration increased from pH 6.8 to 8.0. By contrast, when preincubated vesicles were diluted in KCl medium buffered at the

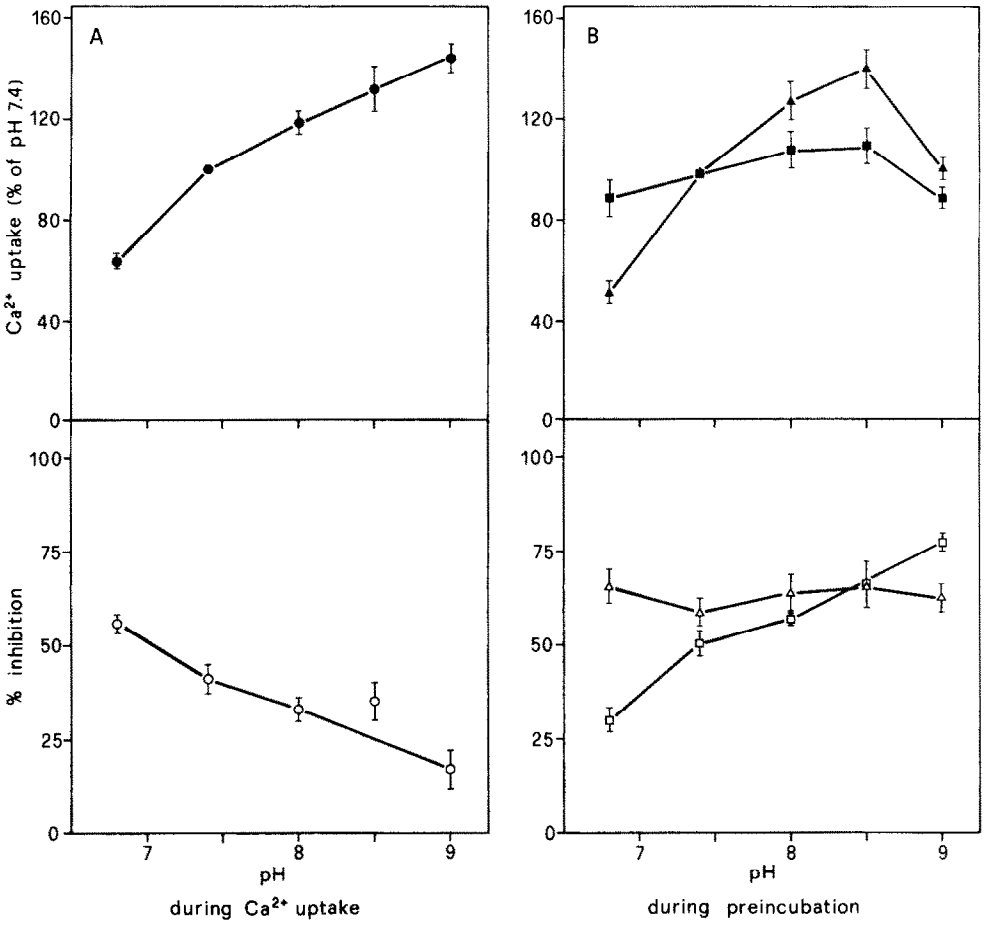

Fig. 2. Influence of pH on Na^+/Ca^{2+} exchange activity in absence and presence of amiloride. (A) Na^+-loaded vesicles were directly assayed for $^{45}Ca^{2+}$ uptake (30 sec) in KCl medium buffered at indicated pH values in absence (●—●) and presence (○—○) of 2.5 mM amiloride, as described under Materials and Methods. Upper panel: normalized (see text) Na^+/Ca^{2+} exchange activities of control vesicles as a function of pH during $^{45}Ca^{2+}$ uptake. At pH 7.4, actual value of Na_i^+-dependent Ca^{2+} uptake was 17.52 ± 1.38 nmol $^{45}Ca^{2+}$/mg protein/30 sec (N = 4). Lower panel: percentage inhibition of $^{45}Ca^{2+}$ uptake by 2.5 mM amiloride plotted vs pH values during $^{45}Ca^{2+}$ uptake. (B) Na^+-loaded vesicles were preincubated for 20 min at 37° at indicated pH values in absence (■—■, ▲—▲) and presence (□—□, △—△) of 8 mM amiloride. 3-μl aliquots of preincubated vesicles were then assayed for $^{45}Ca^{2+}$ uptake (30 sec) by means of dilution into KCl medium buffered either at pH 7.4 (■—■, □—□) or at the pH values used during vesicle preincubation (▲—▲, △—△), as described under Materials and Methods. Upper panel: normalized (see text) Na^+/Ca^{2+} exchange activities of control vesicles as a function of pH during vesicle preincubation. At pH 7.4, actual values of Na_i^+-dependent Ca^{2+} uptake for (■—■) and (▲—▲) were 26.84 ± 1.80 nmol $^{45}Ca^{2+}$/mg protein/30 sec (N = 4) and 19.14 ± 1.02 nmol $^{45}Ca^{2+}$/mg protein/30 sec (N = 4), respectively. Lower panel: percentage inhibitions of $^{45}Ca^{2+}$ uptake by 8 mM amiloride plotted vs pH values during vesicle preincubation. Final concentration of amiloride (74.8 μM) attained in uptake medium after dilution of preincubated vesicles had no effect on Na^+/Ca^{2+} exchange activity. All data points are means ± SE of 3 experiments.

pH values used during vesicle preincubation, the intravesicular amiloride levels remained fairly constant. However, in both experimental conditions, the intravesicular concentration of amiloride markedly dropped at low values at pH 8.5 and 9.0.

Effect of intravesicular Ca^{2+} *on inhibition of* Na^+/Ca^{2+} *exchange activity by amiloride*

Reeves and Poronnik [29] have recently reported that intravesicular Ca^{2+} induces activation of Na_i^+-dependent $^{45}Ca^{2+}$ uptake by cardiac sarcolemmal vesicles. We found that pretreatment of Na^+-loaded vesicles with 0.5 mM $CaCl_2$ produced an increase of approximately 40% in $^{45}Ca^{2+}$ uptake, compared with vesicles incubated without added $CaCl_2$ (Fig. 3A and B).

When present in the medium for $^{45}Ca^{2+}$-uptake assay, amiloride abolished the stimulation of exchange activity by intravesicular Ca^{2+}, so that no significant differences in exchange activities between Ca^{2+}-treated and -untreated vesicles could be evidenced in the presence of amiloride up to 4 mM (Fig 3A). Instead, when vesicles were preincubated with $CaCl_2$ in the presence of amiloride, stimulation of

Table 2. Intravesicular levels of amiloride in cardiac sarcolemmal vesicles preincubated with the drug at different pH values

pH during vesicle preincubation	uncharged* form of $amiloride_o$ (%)	$\neq pH_s$ $amiloride_i$ (μg amiloride/mg prot)	pH 7.4 $amiloride_i$ (μg amiloride/mg prot)
6.8	2.45	11.09 ± 0.69	8.30 ± 0.71
7.4	9.09	11.28 ± 0.62	11.28 ± 0.62
8.0	28.49	10.69 ± 1.10	15.58 ± 1.22
8.5	55.86	1.79 ± 1.60	8.04 ± 0.43
9.0	80.00	1.76 ± 0.80	3.87 ± 0.65

Na^+-loaded vesicles were preincubated with 8 mM amiloride at indicated pH values in the same experimental conditions of Fig. 2B. Aliquots of preincubated vesicles were then diluted into KCl medium buffered either at the pH values used during vesicle preincubation or at pH 7.4 and incubated for 30 sec at 37°. The amounts of amiloride associated with the vesicles ($amiloride_i$) were estimated as described under Materials and Methods and are means ± SE from at least 3 independent determinations, each in duplicate.

* Concentrations of the uncharged form of amiloride at indicated external pH values as percentage values of the total drug in the preincubation medium, calculated from the Henderson–Hasselbalch equation, assuming a pK'_a value of 8.4 for amiloride.

exchange activity by intravesicular Ca^{2+} was not affected by amiloride up to 6 mM (Fig. 3B). In fact, the data indicated that the percentage inhibitions for amiloride were identical, whether the vesicles were pretreated with $CaCl_2$ in the presence of increasing amounts of amiloride or not.

Influence of Na^+ on inhibition of Na^+/Ca^{2+} exchange activity by amiloride in cardiac sarcolemmal vesicles preincubated or otherwise with the drug

We have recently shown that the inhibition of Na^+/Ca^{2+} exchange activity by $amiloride_i$ is competitively overcome by an outwardly directed Na^+ gradient [8]. The pattern of interaction between amiloride and Na_o^+, which is both substrate and inhibitor of the exchanger, was further investigated by studying the effect of low concentrations of Na_o^+ (5–30 mM) at a fixed Na_i^+ concentration of 160 mM on the inhibition of Na^+/Ca^{2+} exchange activity by amiloride acting either from outside or inside sarcolemmal vesicles. Changes in Na_o^+ concentration affected the inhibition of Na_i^+-dependent Ca^{2+} uptake by amiloride in a

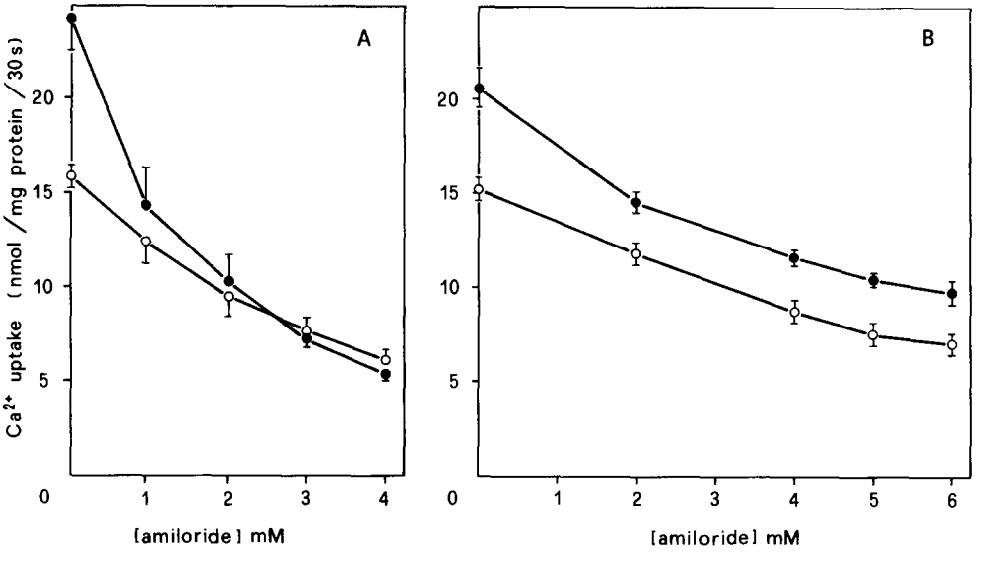

Fig. 3. Effect of pretreatment of cardiac sarcolemmal vesicles with $CaCl_2$ on inhibition of Na^+/Ca^{2+} exchange activity by amiloride. (A) Na^+-loaded vesicles were preincubated for 30 min at 37° in absence (○—○) and presence (●—●) of 0.5 mM $CaCl_2$. 3-μl aliquots of preincubated vesicles were then assayed for $^{45}Ca^{2+}$ uptake (30 sec) by dilution into KCl buffer (pH 7.4) containing increasing amounts of amiloride (see Materials and Methods). (B) Na^+-loaded vesicles were preincubated for 30 min at 37° with increasing amounts of amiloride in absence (○—○) and presence (●—●) of 0.5 mM $CaCl_2$. 3-μl aliquots of preincubated vesicles were then assayed for $^{45}Ca^{2+}$ uptake (30 sec) by dilution into KCl buffer (pH 7.4) (see Materials and Methods). Assay media for $^{45}Ca^{2+}$ uptake were adjusted so that final concentrations of Ca^{2+} were identical for vesicles preincubated with or without 0.5 mM $CaCl_2$. All data points are means ± SE of 3 experiments.

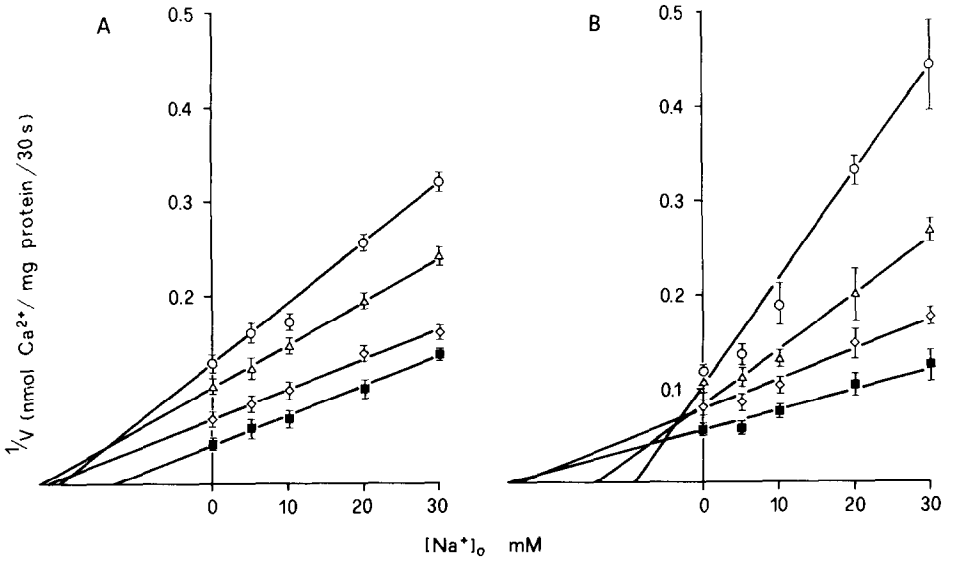

Fig. 4. Influence of Na_o^+ on inhibition of Na^+/Ca^{2+} exchange activity by amiloride in cardiac sarcolemmal vesicles preincubated or otherwise with the drug. (A) Dixon plot for Na^+/Ca^{2+} exchange activity of Na^+-loaded vesicles directly assayed for $^{45}Ca^{2+}$ uptake (30 sec) in mixtures of NaCl and KCl to generate concentrations of Na_o^+ (abscissa), containing 0 (■—■), 2 (◇—◇), 4 (△—△) and 5 (○—○) mM amiloride (see Materials and Methods). (B) Dixon plot for Na^+/Ca^{2+} exchange activity of Na^+-loaded vesicles preincubated for 20 min at 37° in absence (■—■) and presence of 2 (◇—◇) 4 (△—△) and 5 (○—○) mM amiloride. 3-μl aliquots of preincubated vesicles were then assayed for $^{45}Ca^{2+}$ uptake (30 sec) by dilution into mixtures of NaCl and KCl to generate concentrations of Na_o^+ (abscissa) (see Materials and Methods). All data points are means ± SE of 3 experiments. Least-squares regression lines are shown in panels A and B.

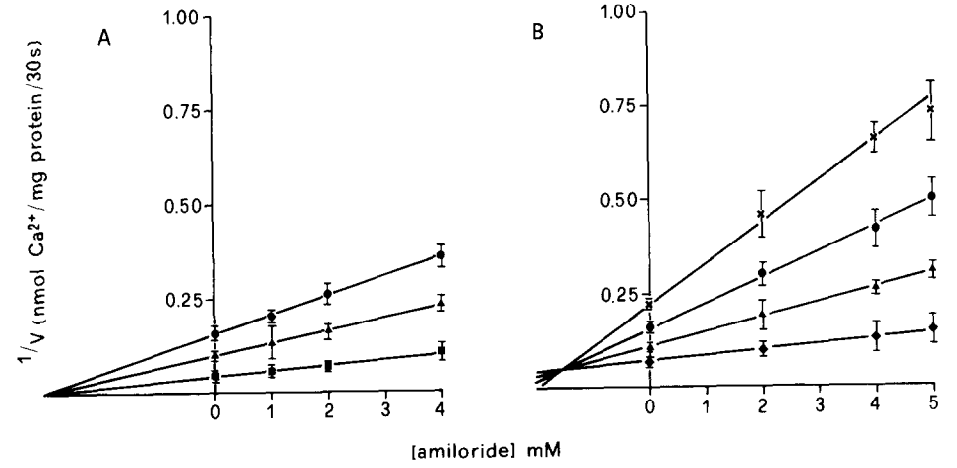

Fig. 5. Influence of Na_i^+ on inhibition of Na^+/Ca^{2+} exchange activity by amiloride in cardiac sarcolemmal vesicles preincubated or otherwise with the drug. In these experiments, initially Na^+ (160 mM)-loaded vesicles were diluted 1:150 into mixtures of 160 mM NaCl and 160 mM choline chloride, buffered to pH 7.4 with 20 mM MOPS/Tris, to generate the final Na_i^+ concentrations indicated and incubated overnight at 0°. Vesicles were then pelleted and resuspended at about 3 mg of protein/ml in buffers of the same NaCl and choline chloride concentrations. (A) Dixon plot for Na^+/Ca^{2+} exchange activity of vesicles loaded with 30 (●—●), 60 (▲—▲) and 160 (■—■) mM NaCl directly assayed for $^{45}Ca^{2+}$ uptake (30 sec) in presence of increasing amounts of amiloride, as described under Materials and Methods. (B) Dixon plot for Na^+/Ca^{2+} exchange activity of vesicles loaded with 20 (×—×), 30 (●—●), 60 (▲—▲) and 80 (◆—◆) mM NaCl preincubated for 20 min at 37° with increasing amounts of amiloride. 3-μl aliquots of preincubated vesicles were then assayed for $^{45}Ca^{2+}$ uptake (30 sec) as described under Materials and Methods. All data points are means ± SE of 3 experiments. Least-squares regression lines are shown in panels A and B.

different manner, depending on whether the drug acted on the same or opposite side of the sarcolemma as Na^+ (Fig. 4).

The double inhibition plot by Dixon in Fig. 4A shows that, with amiloride and Na^+ on the same side of the membrane, competitive inhibition was obtained at the lowest concentration (2 mM) of amiloride, the two lines of $^{45}Ca^{2+}$ uptake activity in the absence and presence of 2 mM amiloride being parallel. However, as $amiloride_o$ was increased to 4 and 5 mM, the data no longer yielded parallel lines, indicating mixed competitive-noncompetitive inhibition. By contrast (Fig. 4B), with amiloride and Na^+ on the opposite side of the sarcolemmal membrane, a synergic interaction between the two inhibitors was evident. In fact, the Dixon plot of $amiloride_i$ inhibition with different concentrations of Na_o^+ displayed lines intersecting the line of the control at points above the abscissa.

In addition, when the effect of Na^+ on amiloride inhibition was studied in vesicles loaded with increasing concentrations of Na^+ (Fig. 5), competitive and non-competitive inhibitions were obtained with amiloride acting on the same (Fig. 5B) and opposite (Fig. 5A) side of the vesicle sarcolemma as Na^+, respectively.

DISCUSSION

In this paper, we show that amiloride acts with similar apparent dissociation constants at the *cis* and *trans* sides of the sarcolemma, indicating that the drug interacts with the exchange system on both sides of the vesicle membrane with similar affinities. Furthermore, the lack of linearity in the Dixon plot for $amiloride_o$ inhibition of the exchanger over 4 mM amiloride (Fig. 1A) suggests that, at the highest concentrations used, the drug may inhibit Na_i^+-dependent Ca^{2+} uptake at more than one single class of sites on the carrier.

The pH-dependence of Ca^{2+} uptake by cardiac sarcolemmal vesicles shows stimulation of activity at alkaline pH and inhibition at acid pH, according to Wakabayashi and Goshima [27] and Philipson *et al.* [28], who suggest that the ionization state of a histidine residue on the Na^+/Ca^{2+} exchanger may be important in regulating exchange activity. On the other hand, 20-min preincubation of the vesicles at pH 9.0 decreases stimulation of exchange activity, regardless of the pH value used for the Ca^{2+}-uptake assay (see upper panel of Fig. 2B). Although passive Ca^{2+} flux is unaffected by pH changes, this alkaline treatment of the vesicles may have determined some alterations in charge distribution in the ionizable structural groups on the sarcolemma responsible for ionic permeability changes, leading to a fall in Na^+ gradient and consequently to a reduction in Na^+/Ca^{2+} exchange activity. It cannot be excluded that this reduction is also due to some structural modification of the exchanger. In any case, these alterations appear to be transient, since complete recovery of Na^+/Ca^{2+} exchange activity from preincubation at pH 9.0 can be obtained upon reincubation of the vesicles for 10 min at pH 7.4 (not shown).

Furthermore, only slight differences in exchange activities are found when control vesicles preincubated at various pH values are assayed for $^{45}Ca^{2+}$ uptake at pH 7.4. This finding can be explained taking into account that vesicles may equilibrate to the external pH value (7.4) within the 30 sec of Ca^{2+}-uptake assay, because of the rapidity with internal pH in small vesicles can change when external pH is altered [30]. This event is indeed suggested by the observation that no ΔpH is evidenced in vesicles preincubated either in absence or presence of amiloride, as determined by monitoring fluorescence of 9-aminoacridine (data not shown).

Amiloride inhibits exchange activity in non-preincubated vesicles at all tested pH values (6.8–9.0), producing its maximum effect at acid pH. Since amiloride is a weak base with a $pK'_a \sim 8.4$ [25], these findings are in agreement with the notion that protonation of amiloride's guanidino group and hence the cationic form of the molecule is required for inhibitory activity [7, 26]. By contrast, an alkaline pH during preincubation of cardiac sarcolemmal vesicles with amiloride apparently increases the inhibitory effect of the drug. This finding may suggest that, when amiloride acts as an inhibitor of the exchange system from the inner side of the vesicles, its effect is related to the lipophilicity of the drug. It has been shown that uptake of amiloride by cells occurs predominantly, if not exclusively, via nonionic diffusion of the unprotonated form of the compound [24, 25]. This implies that, as external pH is increased, the external concentration of the uncharged form of amiloride rises (see Table 2), as also one can expect the total amount of amiloride into the vesicle does. Our findings show that the intravesicular amiloride levels in vesicles preincubated at various pH values and then incubated for 30 sec at pH 7.4 increase as the external pH rises from 6.8 to 8.0. By contrast, intravesicular amiloride levels remain fairly constant when preincubated vesicles are incubated at the pH values used during vesicle preincubation.

These findings suggest that, as well as the pH during preincubation may influence the influx of amiloride into the vesicles, the pH during 30-sec incubation may influence the efflux of amiloride from the vesicles by changing the extent of intravesicular amiloride protonation.

The marked drop in intravesicular amiloride content, which occurs at pH 8.5 and 9.0 in both experimental conditions, suggests that the actual intravesicular concentrations of amiloride cannot be measured presumably due to a loss through fast free diffusion [25] of the uncharged form of the drug from the vesicles.

The pH-dependence of amiloride inhibition of Na^+/Ca^{2+} exchange activity in vesicles preincubated at pH 6.8–8.0 appears to be related to the intravesicular levels of the drug. An increase in the extent of amiloride inhibition is substained by an increase in amiloride content in vesicles incubated at pH 7.4, whereas no increase either in the extent of inhibition or intravesicular content is observed in vesicles incubated at the pH values used during preincubation.

On the whole, the data on pH-dependence of amiloride inhibition of Na^+/Ca^{2+} exchange activity indicate that protonated amiloride is the active form

under conditions in which the drug acts from outside the vesicle membrane. Conditions favouring the entry of the uncharged form of amiloride into the vesicles, such as alkaline pH during vesicle preincubation, consequently favour the inhibition of the exchanger when the drug acts from inside the vesicles. However, in the latter case too, the active form of amiloride appears to be the protonated one, since, once inside the vesicles, amiloride may associate with H^+ ions, as also suggested by Dubinsky and Frizzell [31] in isolated rabbit ileum brush border vesicles.

Concerning the kinetic characteristics of Na^+/Ca^{2+} exchange inhibition by amiloride, we have recently shown that amiloride has a non-competitive inhibitory effect with respect to extravesicular Ca^{2+} [8], since high concentrations of calcium do not reverse amiloride inhibition. The same pattern of amiloride inhibition towards extravesicular calcium was also obtained under conditions in which the drug acts from outside the vesicles (not shown), in agreement with the findings of Siegl *et al.* [4], Gill *et al.* [32] and Kaczorowsky *et al.* [33] in cardiac, synaptic and pituitary plasma membrane vesicles, respectively.

On the other hand, the pattern of interaction between amiloride and Na^+ on the Na^+/Ca^{2+} exchange system strictly depends on the sidedness of amiloride action. In fact, Na_o^+ protects against $amiloride_o$ inhibition, in agreement with the observations of Siegl *et al.* [4] in guinea-pig heart sarcolemmal vesicles and of Kaczorowski *et al.* [33] in pituitary plasma membrane vesicles. At low concentrations of $amiloride_o$ (2 mM), interaction appears to occur preferentially at one single site, while at higher concentrations (4 and 5 mM), amiloride may also bind to another site, as suggested by the mixed competitive non-competitive inhibition (see Fig. 4A). The finding that $amiloride_i$ behaves as a competitive inhibitor towards Na_i^+ when the exchange activity is measured in vesicles loaded with increasing concentrations of Na^+ (see Fig. 5B) confirms the pattern of interaction between Na^+ and amiloride when both act on the same side of the vesicle membrane.

In contrast, with amiloride and Na^+ at opposite sides of the sarcolemma, the inhibition by $amiloride_o$ is non-competitive with respect to Na_i^+ (see Fig. 5B) and the combined effects of $amiloride_i$ in the presence of Na_o^+ are greater than those expected on the basis of the individual actions of the two inhibitors (see Fig. 4B). The synergic interaction between $amiloride_i$ and Na_o^+ may be interpreted as the ability of the drug bound to the intravesicular side of the vesicle membrane to cause a change in protein conformation, favouring the interaction of Na^+ with the exchange system on the opposite side. A similar hypothesis has been made by Knauf [34] to explain the effect of increasing intracellular chloride on the ability of stilbenedisulfonates to block chloride exchange by band 3 in human erythrocytes.

The competition of amiloride towards Na^+ when both act on the same side of the vesicle membrane, the non-competitive behaviour when they act on the opposite side of the sarcolemma and the lack of competition of amiloride with respect to calcium strongly support the view that amiloride mainly interacts with a Na^+ binding site on the exchange system [18].

The side-dependent effect of amiloride on Na^+/Ca^{2+} exchanger is also suggested by the fact that the stimulatory effect of intravesicular Ca^{2+} on Na_i^+-dependent Ca^{2+} uptake is abolished by amiloride only when the drug is allowed to act from outside the vesicles. However, the mechanisms of different effects of $amiloride_o$ and $amiloride_i$ on exchanger stimulation by $CaCl_2$ cannot be explained on the basis of our present data. The stimulation of Na^+/Ca^{2+} exchange by treatment of the vesicles with $CaCl_2$ has been ascribed to the influence of intravesicular Ca^{2+} on exchange activity, involving a decrease in the apparent K_m of the exchange system for extravesicular Ca^{2+} [29].

In conclusion, although amiloride is not a specific inhibitor of Na^+/Ca^{2+} exchange, the elucidation of its pattern of interaction with the exchanger may be useful in determining the relative importance of this system to the mechanism of cardiac muscle contractility and in defining the role of the Na^+/Ca^{2+} exchange in physiological and pathological processes. In particular, the key role of the exchanger in cardiac muscle contraction makes it a possible target for inotropic drugs [13, 35].

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