pp 139–188, Elsevier/North-Holland, Amsterdam, New York, and Oxford.
Nagai, Y., & Iwamori, M. (1980) *Mol. Cell. Biochem. 29*, 81–90.
Ottolenghi, P. (1979) *Eur. J. Biochem. 99*, 113–131.
Polnaszek, C. F., Marsh, D., & Smith, I. C. P. (1981) *J. Magn. Reson. 43*, 54–64.
Schwarzmann, G., & Sandhoff, K. (1987) *Methods Enzymol. 138*, 319–341.
Schwarzmann, G., Hoffmann-Bleihauer, P., Schubert, J., Sandhoff, K., & Marsh, D. (1983) *Biochemistry 22*, 5041–5048.
Schwarzmann, G., Sonderfeld, S., Conzelmann, E., Marsh, D., & Sandhoff, K. (1984) *Inst. Natl. Sante Rech. Med.*, [*Colloq.*] *126*, 195–210.
Skou, J. C. (1965) *Physiol. Rev. 3*, 596–617.
Skou, J. C., & Esmann, M. (1979) *Biochim. Biophys. Acta 567*, 436–444.
Yamakawa, T., & Nagai, Y. (1978) *Trends Biochem. Sci.* (*Pers. Ed.*) *3*, 128–131.

Inhibition of Sodium–Calcium Exchange in Cardiac Sarcolemmal Membrane Vesicles. 1. Mechanism of Inhibition by Amiloride Analogues

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ABSTRACT: The mechanism by which terminal guanidino nitrogen substituted analogues of amiloride inhibit Na–Ca exchange in purified cardiac sarcolemmal membrane vesicles has been investigated. These inhibitors block both Na_i-dependent Ca^{2+} uptake and Na_o-dependent Ca^{2+} efflux. Inhibition of Na–Ca exchange monitored in K^+ is noncompetitive vs Ca^{2+} but competitive vs Na^+. Substitution of sucrose for K^+ results in mixed kinetics of inhibition vs Ca^{2+}, suggesting a complex interaction between inhibitor and carrier under this condition. Amiloride derivatives also block two other modes of carrier action: Na–Na exchange is inhibited in a competitive fashion with Na^+ and kinetics of Ca–Ca exchange inhibition are mixed vs Ca^{2+} in either sucrose or K^+. However, Ca–Ca exchange inhibition can be alleviated by increasing K^+ concentration. Dixon analyses of Na–Ca exchange block with mixtures of inhibitors suggest that these agents are interacting at more than one site. In addition, Hill plots of inhibition are biphasic with Hill coefficients of 1 and 2 at low and high inhibitor concentrations, respectively. These results indicate that amiloride derivatives are mechanism-based inhibitors that interact at two classes of substrate-binding sites on the carrier; at low concentration they bind preferentially to a site that is exclusive for Na^+, while at higher concentration they also interact at a site that is common for Na^+, Ca^{2+}, and K^+.

Na–Ca exchange has been postulated to play a key role in Ca^{2+} homeostasis of electrically excitable cells [for reviews see Baker and DiPolo (1984), Reeves (1985), and Kaczorowski (1985)]. This process transports Ca^{2+} across the plasma membrane coupled to Na^+ movement in the opposite direction and is controlled by transmembrane electrical and Na^+ concentration gradients. By use of cardiac sarcolemmal membrane vesicles, the Na–Ca exchange reaction has been demonstrated to be electrogenic (Reeves & Sutko, 1980), to possess a stoichiometry of $3Na^+:Ca^{2+}$ (Reeves & Hale, 1984), and to be bidirectional (Philipson & Nishimoto, 1982). Moreover, the kinetics of transport have been extensively characterized in this preparation (Kadoma et al., 1982; Reeves & Sutko, 1983; Philipson, 1985). The V_{max} of Na–Ca exchange in vesicles is very high, and operation of the transporter is kinetically symmetrical in most respects. The carrier possesses multiple classes of ion-binding sites, and Na^+ appears to interact in a highly cooperative manner. In addition to Na–Ca exchange, this system will also function in nonproductive Ca–Ca (Slaughter et al., 1983) and Na–Na (Reeves, 1985) exchange modes, studies of which have provided insight into the mechanism of carrier action.

A model based on flux measurements has been proposed to describe the operation of the cardiac transporter (Reeves et al., 1984; Reeves, 1985). In this scheme, two classes of ion-binding sites exist: a common site at which either one or two Na^+ or a single Ca^{2+} binds (A-site) and a distinct site (B-site) at which the third transported Na^+ binds. Occupation of the A-site by two Na^+ and the B-site by a single Na^+ promotes transport of Ca^{2+} bound to an A-site on the opposite face of the carrier. Binding of Ca^{2+} at the A-site allows broader substrate specificity at the B-site, where it has been postulated that alkali metal ions which stimulate Ca–Ca exchange interact. This mechanism is similar in many respects to the one proposed for Na–Ca exchange action in squid axon (Blaustein, 1977).

To test putative models of Na–Ca exchange and elucidate the physiological role of this transport reaction, specific mechanism-based inhibitors will be required. To date, few inhibitors of Na–Ca exchange have been identified [for a review see Kaczorowski et al. (1988)]. Recently, inhibition

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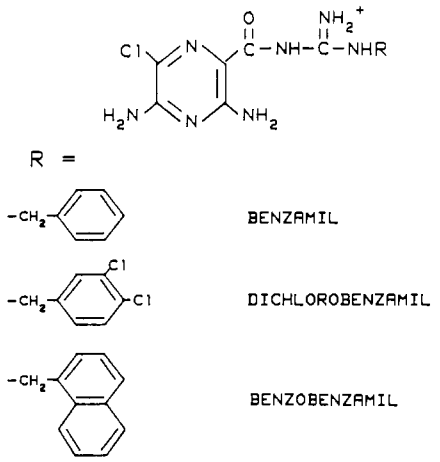
Chart I: Amiloride Analogues



of Na–Ca exchange has been described in plasma membrane vesicles derived from pituitary, heart, and brain by derivatives of amiloride (Siegl et al., 1984; Kaczorowski et al., 1985). One group of analogues that are potent blockers of Na–Ca exchange possess aryl substituents on a terminal guanidino nitrogen atom (Chart I). Preliminary evidence suggested that the protonated form of amiloride inhibits Na–Ca exchange by functioning as a Na^+ mimic, thereby reversibly blocking carrier activity by acting as a nontransportable substrate. Similar proposals have been made regarding amiloride inhibition of other Na-dependent transport reactions (Benos, 1982; Lazdunski et al., 1985). The purpose of the present investigation has been to elucidate the mechanism of Na–Ca exchange inhibition by guanidino nitrogen substituted amiloride derivatives in heart. The results indicate that these agents can interact with the cardiac transporter at both classes of Na^+-binding sites but that they have a preference for the B-site. This is in marked contrast to the mechanism of Na–Ca exchange inhibition by bepridil, a structurally dissimilar inhibitor (Garcia et al., 1988). Preliminary reports of this work have appeared in abstract form (Slaughter et al., 1984; Garcia et al., 1986).

Experimental Procedures

Materials

$^{45}CaCl_2$ (800–1200 mCi/mmol) was purchased from the Radiochemical Center, Amersham Inc., while $^{22}NaCl$ (carrier free, 200 μCi/mL) was bought from New England Nuclear. Analogues of amiloride were synthesized as previously described by Cragoe et al. (1967). All other reagents were purchased from commercial sources and were of the highest purity commercially available. GF/C glass-fiber filters were obtained from Whatman.

Methods

Preparation of Cardiac Sarcolemmal Membrane Vesicles. Samples of cardiac tissue were obtained from fresh porcine or bovine left ventricle. Purified sarcolemmal membrane vesicles were prepared by a modification of the procedure of Kuwayama and Kanazawa (1982) as described (Slaughter et al., 1983). Vesicles were resuspended in 160 mM NaCl and 20 mM Mops–Tris,[1] pH 7.4, at a concentration of 2 mg of protein/mL, rapidly frozen in liquid nitrogen, and stored at −80 °C until use. For those experiments in which the internal composition of membrane vesicles was changed, vesicles were thawed and diluted 20-fold into appropriate medium. Membranes were then collected by centrifugation (150000*g* for 60 min) and resuspended in corresponding media at a protein concentration of 2 mg of protein/mL. For equilibration of Na^+, a 20-min incubation at room temperature was sufficient, while a 4-h incubation at 0 °C was required for Ca^{2+}. Identical results were obtained whether bovine- or porcine-derived membrane vesicles were employed.

Transport Assays. Na–Ca exchange activity was assayed as previously described (Reeves & Sutko, 1983; Slaughter et al., 1983). Briefly, a 2–5 μL aliquot of vesicles was placed on the side of a 12 × 75 mm polystyrene tube containing incubation media. The reaction was started by vortexing the tube and stopped by addition of 4 mL of ice-cold quench buffer containing 160 mM KCl, 1 mM EGTA, and 20 mM Mops–Tris, pH 7.4. Vesicles were immediately filtered through GF/C filters, and filters were washed twice with 4 mL of the cold quench buffer. Timing was done with a GraLab 625 timer set to beat once per second. Vesicle-associated $^{45}Ca^{2+}$ trapped by the filters was determined by liquid scintillation techniques. Triplicate assays were routinely performed under each experimental condition, and the data were averaged. The standard error of the mean of these determinations was typically less than 5%. Because many of the kinetic analyses in this investigation depend on an accurate assessment of initial transport rates, 1-s time points were routinely measured (unless otherwise indicated in the text). Control experiments indicate that data obtained at this time point satisfy the criteria of initial velocity conditions.

Stock solutions of all drugs were prepared daily in Me_2SO. The final concentration of Me_2SO in the transport assay was never allowed to exceed 0.5%. Control experiments showed no effect of these Me_2SO concentrations on the different transport reactions.

Protein Determination. Protein was determined according to the method of Lowry (Lowry et al., 1951) using bovine serum albumin as standard.

Results

Inhibition of Na–Ca Exchange by Amiloride Analogues. Recently, several guanidino nitrogen substituted derivatives of amiloride have been identified as effective inhibitors of Na–Ca exchange in heart, brain, and pituitary plasma membrane vesicles (Kaczorowski et al., 1985). In this study, the mechanism by which these compounds block Na–Ca exchange in cardiac sarcolemmal membrane vesicles has been explored. Three amiloride analogues that inhibit Na_i-dependent Ca^{2+} uptake with different potencies were chosen for investigation (Kaczorowski et al., 1985; Chart I): benzamil (BNZ), K_i = 100 μM; 3′,4′-dichlorobenzamil (DCB), K_i = 20 μM; and 2′,3′-benzobenzamil (BZB), K_i = 10 μM. Inhibitors that display a range of potencies were selected for study because some aspects of the mechanism of block are better illustrated with the weaker agents (see below). It has previously been shown that all these compounds completely block cardiac Na–Ca exchange in a concentration-dependent and reversible manner (Siegl et al, 1984; Kaczorowski et al., 1985).

To determine the interaction between these inhibitors and substrates of Na–Ca exchange, inhibition patterns have been assessed as a function of either Ca^{2+} or Na^+ concentration. Under standard conditions for assay of Na–Ca exchange (i.e., 160 mM Na^+_{in} and 160 mM K^+_{out}), Ca^{2+} uptake is a saturable function of extravesicular Ca^{2+}. As shown by the Eadie–

[1] Abbreviations: BNZ, benzamil or *N*-benzylamiloride; DCB, 3′,4′-dichlorobenzamil or *N*-(3,4-dichlorobenzyl)amiloride; BZB, 2′,3′-benzobenzamil or *N*-(naphthylmethyl)amiloride; Mops, 3-(*N*-morpholino)-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

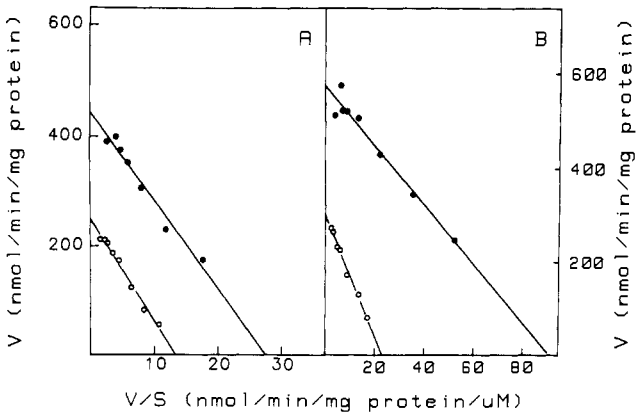


FIGURE 1: Kinetic analysis of Na–Ca exchange inhibition by DCB. Aliquots (2 μL) of cardiac sarcolemmal membrane vesicles equilibrated in 160 mM NaCl were diluted 100-fold into either 160 mM KCl (A) or 320 mM sucrose (B) media containing increasing concentrations of $^{45}CaCl_2$ (5–150 μM) at 37 °C. All solutions were buffered with 20 mM Mops–Tris, pH 7.4. This was done in the absence (●) or prsence (○) of 20 μM DCB. Reactions were terminated after 1 s as described under Methods. Transport activity was determined from differences in Ca^{2+} uptake with and without an outwardly directed Na^+ gradient present. Results are presented in an Eadie–Hofstee representation.

Hofstee plot of Figure 1A, transport in this vesicle preparation displays a K_m for Ca^{2+} of 17 μM and a V_{max} of 463 nmol of Ca^{2+} accumulated min^{-1} (mg of protein)$^{-1}$. The effect of a K_i concentration of DCB is shown in Figure 1A. The parallel line behavior indicates that inhibition is noncompetitive with respect to Ca^{2+}, since only the V_{max} of the transport reaction is altered. Essentially identical results were obtained with BZB. BNZ, a weaker inhibitor of Na–Ca exchange, exerts a mixed-type inhibition pattern (a K_i concentration of this inhibitor reduced V_{max} by 50% and raised the K_m for Ca^{2+} from 14 to 22 μM). In addition, high concentrations of DCB and BZB appear to produce slightly mixed kinetic patterns of inhibition (see discussion of Hill coefficients below).

Substitution of sucrose for KCl in the dilution buffer alters the kinetics of Na–Ca exchange in cardiac sarcolemmal vesicles. As shown by the data of Figure 1B, both the slope and intercept of an Eadie–Hofstee plot are affected when compared to the plot presented in Figure 1A. These reults indicate an increase in V_{max} [from 460 to 600 nmol of Ca^{2+} min^{-1} (mg of protein)$^{-1}$] and decrease in the K_m for Ca^{2+} (from 17 to 7 μM) in sucrose medium. Indeed, the K_m for Ca^{2+} in Na_i-dependent Ca^{2+} uptake varies linearly as a function of extravesicular K^+ (J. P. Reeves, unpublished observations). This could reflect a competitive interaction between K^+ and Ca^{2+} at the Ca^{2+}-binding site of the carrier. Observations consistent with this are (1) rates of Na_o-dependent Ca^{2+} efflux are higher in sucrose than in K^+ media for equivalent Na^+ concentrations (Garcia et al., 1988) and (2) the K_m for Na^+ in Na–Ca exchange is reduced 4-fold (from 20 to 5 mM) in sucrose medium (data not shown). Since Na^+ and Ca^{2+} compete for a common binding site on the carrier (Reeves & Sutko, 1983), these observations suggest that K^+ also interacts at this site. For these reasons, Na–Ca exchange inhibition has also been monitored in sucrose. The data shown in Figure 1B are obtained with DCB and display obvious mixed kinetics of inhibition vs Ca^{2+}, ca. a 50% decrease in V_{max} and a 2-fold increase in the K_m for Ca^{2+} (from 7 to 14 μM). Similar mixed inhibitory patterns have been obtained with BNZ and BZB. These results demonstrate that both competitive and noncompetitive interactions are possible with these agents, which indicates that amiloride inhibitors may interact at multiple sites on the transporter (see below).

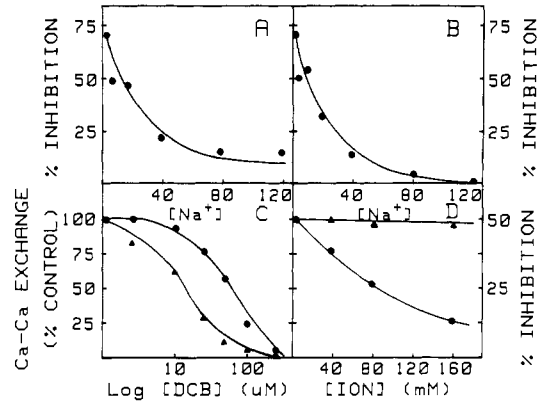


FIGURE 2: Inhibition of Na–Ca, Na–Na, and Ca–Ca exchange by amiloride analogues. (A) Effect of BNZ on Na_o-dependent Ca efflux. Aliquots (5 μL) of membrane vesicles equilibrated in 160 mM KCl, 100 μM $^{45}CaCl_2$, and 20 mM Mops–Tris, pH 7.4, were diluted 20-fold into media containing mixtures of KCl and NaCl to yield the indicated concentrations of NaCl in a final concentration of 160 mM salt. This was done in the absence or presence of 100 μM BNZ. All efflux solutions contained 0.1 mM EGTA and were buffered to pH 7.4 with 20 mM Mops–Tris. Reactions were carried out at 25 °C and terminated after 10 s as described under Methods. Rates of efflux were determined by the difference between the $^{45}Ca^{2+}$ content of vesicles diluted into either 160 mM KCl or into NaCl-containing solutions, and the percent inhibition of $^{45}Ca^{2+}$ efflux elicited by 100 μM BNZ is represented as a function of the Na^+ concentration in the efflux media. (B) Effect of BNZ on Na–Na exchange. Aliquots (2 μL) of membrane vesicles equilibrated in either 160 mM NaCl or 160 mM KCl were diluted 50-fold into solutions containing mixtures of KCl and NaCl to yield the indicated Na^+ concentrations in a final concentration of 160 mM salt. This was done with 1.4 μCi $^{22}NaCl$ (carrier free) present, in the absence or presence of 200 μM BNZ. Reactions were carried out at 37 °C for 6 min. The percent inhibition of Na–Na exchange by 200 μM BNZ determined from the differences in $^{22}Na^+$ uptake between Na^+- and K^+-loaded vesicles is plotted versus the Na^+ concentration in the dilution media. (C and D) Effect of amiloride analogues on Ca–Ca exchange. (C) Aliquots (2 μL) of membrane vesicles equilibrated in either 320 mM sucrose and 100 μM $CaCl_2$ (▲) or 160 mM KCl and 100 μM $CaCl_2$ (●) were diluted 100-fold into media of the same composition, respectively, except containing 10 μM $^{45}CaCl_2$. This was done in the absence or presence of increasing concentrations of DCB. (D) Aliquots (2 μL) of membrane vesicles equilibrated in 320 mM sucrose and 40 μM $CaCl_2$ were diluted 50-fold into solutions containing increasing concentrations of KCl (●) or choline Cl (▲) with 10 μM $^{45}CaCl_2$ and 100 μM BNZ present. The osmolarity of the dilution media was kept constant by balancing the solutions with sucrose. In both (C) and (D), transport experiments were carried out at 37 °C and terminated after 1 s. Data are presented as either percent control Ca–Ca exchange activity (C) or percent inhibition of Ca–Ca exchange (D) with respect to an untreated control.

Na_o-dependent Ca^{2+} efflux was monitored to investigate the interaction between amiloride derivatives and Na^+. Typical data have been obtained with BNZ. For example, at a K_m concentration of Na^+ (20 mM), BNZ (200 μM) inhibits the initial rate of Ca^{2+} efflux by ca. 50% without affecting rates of non-carrier-mediated Ca^{2+} movement. This result, when compared with data on Ca^{2+} uptake (Siegl et al., 1984), indicates that BNZ blocks both influx and efflux modes of Na–Ca exchange with similar potencies. Furthermore, it suggests that these inhibitors function directly at the level of the carrier, rather than acting in an indirect fashion (e.g., by nonspecifically affecting membrane permeability or by influencing carrier activity secondarily by decreasing the intravesicular Ca^{2+}-binding capacity of the vesicles). When the initial rate of Na_o-dependent Ca^{2+} efflux is monitored as a function of Na^+ concentration with or without 100 μM BNZ present, inhibition by BNZ is most pronounced at the lowest Na^+ concentration and becomes progressively less as the Na^+ level is elevated. The data shown in Figure 2A represent the

percent inhibition of Na_o-dependent Ca^{2+} efflux under these conditions. At 5 mM Na^+, transport is inhibited 70%, but inhibition is reduced to 15% as Na^+ concentration is increased to 80–120 mM. Reversal of inhibition clearly occurs in a range of Na^+ concentrations corresponding to saturation of the carrier by Na^+ and suggests competition between BNZ and Na^+ at a Na^+-binding site(s). This conclusion has been supported additionally by Na_i-dependent Ca^{2+} uptake studies, where it is observed that elevation of intravesicular Na^+ will relieve DCB block of Ca^{2+} transport (data not shown).

Notably, these results contrast markedly with those obtained by using other inhibitors of Na–Ca exchange that appear to function by disrupting the membrane environment surrounding the transport protein. For example, dodecylamine, a highly lipophilic cation, has been shown to inhibit Na–Ca exchange in cardiac membrane vesicles (K_i = 20 μM), and inhibition is noncompetitive with respect to Ca^{2+} (Philipson, 1984). However, when dodecylamine block of Na_o-dependent Ca^{2+} efflux is monitored with varying Na^+, elevating Na^+ concentration only partially reverses inhibition, the concentration dependence for Na^+ is very broad, and the concentration range over which reversal of inhibition occurs does not correspond to saturation of the carrier with Na^+ (e.g., at 20 μM dodecylamine, 80, 60, 50, and 40% inhibition are found at 20, 40, 80, and 160 mM Na^+, respectively). Therefore, there is a fundamental difference in the mode of action of these two classes of Na–Ca exchange inhibitors, with the amiloride series being mechanism based since they interact directly at substrate-binding sites on the transport protein.

Effect of Amiloride Analogues on Na–Na and Ca–Ca Exchange. It has been well documented that the cardiac transporter can carry out Na–Na (Reeves, 1985) and Ca–Ca (Slaughter et al., 1983) exchange reactions. Therefore, the effects of inhibitors on these other modes of carrier action have been determined. In order to monitor Na–Na exchange activity, vesicles were loaded with either 160 mM NaCl or 160 mM KCl and diluted into a medium containing varying concentrations of $^{22}NaCl$, and accumulation of $^{22}Na^+$ was measured at 6 min. At each extravesicular Na^+ concentration, $^{22}Na^+$ uptake is greater in Na-loaded than in K-loaded vesicles, a reflection of Na–Na exchange activity. Net $^{22}Na^+$ accumulation achieves a maximal value at concentrations greater than ca. 20 mM extravesicular Na^+. This concentration dependence is similar to that observed for Na^+ in Na_o-dependent Ca^{2+} efflux, as expected. BNZ (200 μM) inhibits Na–Na exchange markedly at low Na^+ concentrations, but as the concentration of this ion is increased, inhibition is alleviated. The Na^+ reversal of BNZ-mediated Na–Na exchange inhibition is depicted in graphic form in Figure 2B and is consistent with the observation that amiloride inhibitors are competitive with Na^+ (see above). The results shown in Figure 2B further indicate that these inhibitors interact directly at a Na^+-binding site(s) on the transporter and that this site(s) is involved in both Na–Ca and Na–Na exchange reactions.

The effects of amiloride derivatives on Ca–Ca exchange have been studied under two conditions, with or without alkali metal ions present that stimulate this activity (Slaughter et al., 1983). For these experiments, vesicles were washed, resuspended in a sucrose-containing medium, and preincubated overnight at 0 °C with 20 μM $^{45}CaCl_2$. The ^{45}Ca-loaded vesicles were then diluted into sucrose medium containing 20 μM $^{40}Ca^{2+}$, which promotes $^{45}Ca^{2+}$ efflux via Ca–Ca exchange. A parallel series of experiments was performed by using KCl in place of sucrose in the dilution buffer. In each case, passive efflux of $^{45}Ca^{2+}$ was monitored by replacing Ca^{2+} in the dilution buffer with 0.1 mM EGTA. Inclusion of extravesicular $^{40}Ca^{2+}$ markedly stimulates rates of $^{45}Ca^{2+}$ efflux over passive efflux rates, and the rate of Ca^{2+} loss is more rapid in the presence of K^+ than in sucrose, in agreement with previous findings (Slaughter et al., 1983). When these experiments are repeated in the presence of BNZ (200 μM), this agent blocks Ca–Ca exchange in both sucrose- and K^+-containing media, although the passive rate of Ca^{2+} efflux is not affected. However, block is more complete in sucrose than in K^+ media at equivalent inhibitor concentrations (e.g., 100% vs 50% inhibition at 200 μM BNZ). Thus, amiloride derivatives can block all three modes of carrier action: Na–Ca, Na–Na, and Ca–Ca exchange.

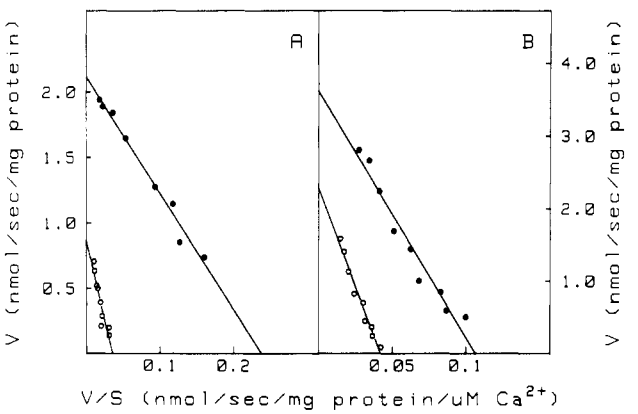


FIGURE 3: Kinetic analysis of Ca–Ca exchange inhibition by BNZ. Aliquots (2 μL) of membrane vesicles equilibrated in 320 mM sucrose and 50 μM $CaCl_2$ were diluted 50-fold into either 320 mM sucrose (A) or 160 mM KCl (B) media containing increasing concentrations of $^{45}CaCl_2$ (5–150 μM) at 37 °C. This was done in the absence (●) or presence (○) of 200 μM BNZ. All solutions were buffered to pH 7.4 with 20 mM Mops–Tris, and transport reactions were terminated after 1 s. Results are presented in an Eadie–Hofstee representation.

To explore the effects of K^+ on these patterns of Ca–Ca exchange inhibition, vesicles were loaded with $^{40}Ca^{2+}$ and then Ca_i-dependent $^{45}Ca^{2+}$ uptake was monitored in sucrose or K^+ media as a function of DCB concentration (Figure 2C). Under these conditions, initial rates of Ca–Ca exchange are progressively inhibited as the concentration of DCB is elevated. However, DCB is ca. 5-fold more effective in sucrose than in K^+ media. This suggests a competitive interaction between amiloride derivatives and K^+. Indeed, data obtained with BNZ (100 μM) and varying K^+ reveal that inhibition of Ca–Ca exchange can be completely alleviated by raising K^+ concentration (Figure 2D). This effect is not due to an increase in ionic strength of the dilution medium since choline, which does not stimulate Ca–Ca exchange activity (Slaughter et al., 1983), had absolutely no effect on BNZ inhibition of transport (Figure 2D). Thus, the presence of a stimulatory alkali metal ion ameliorates the inhibitory effects of amiloride derivatives on Ca–Ca exchange.

The effects of BNZ on the kinetics of Ca–Ca exchange are illustrated in the Eadie–Hofstee plots of Figure 3, done in either sucrose (A) or K^+ (B) media. In agreement with previous findings (Slaughter et al., 1983), K^+ increases the V_{max} of Ca–Ca exchange almost 2-fold in control experiments. Inclusion of K^+ also increases the K_m for Ca^{2+} from 9 μM in sucrose to 34 μM in 160 mM KCl. This latter effect is similar to the action of K^+ on the kinetics of Na–Ca exchange and correlates with the observation that the K_m for Ca^{2+} in Ca–Ca exchange increases linearly with increasing K^+ (J. P. Reeves, unpublished observation). In sucrose as well as in K^+, BNZ produces mixed kinetic patterns of inhibition. In sucrose, 200 μM BNZ reduced the V_{max} of Ca–Ca exchange by 60% and

increased the K_m for Ca^{2+} 2.7-fold (from 9 to 24 μM), while in K^+ the V_{max} was reduced 36% and the K_m was increased 1.7-fold (from 34 to 57 μM) by this inhibitor. Thus, at equivalent BNZ concentrations, the kinetics of inhibition vs Ca^{2+} are markedly mixed in sucrose but become more noncompetitive in K^+. Similar results have been obtained with DCB. In addition, kinetic plots indicate that K^+ lowers the maximal extent of inhibition produced by equivalent inhibitor concentrations.

It has been suggested that stimulation of Ca–Ca exchange by alkali metal ions might reflect an interaction of these ions at the B-site of the carrier (Slaughter et al., 1983; Reeves et al., 1984). Competition between K^+ and amiloride derivatives in the Ca–Ca experiments described above is consistent with this hypothesis, given data from Na–Ca exchange studies that suggest an interaction of inhibitor at the B-site (i.e., amiloride inhibition is primarily noncompetitive vs Ca^{2+} but competitive vs Na^+). However, when the complex interaction between K^+ and the carrier is considered as the latter functions in the Ca–Ca exchange mode (Figure 3), these data are not sufficient to prove the hypothesis that the monovalent cation regulatory site and the B-site of the carrier are the same. Moreover, when the effects of a series of monovalent stimulatory cations (e.g., K^+, Li^+, Rb^+, and Na^+) are compared in sucrose media, both for their ability to stimulate Ca–Ca exchange and to relieve block of transport by BNZ, there is no clear correlation (data not shown). This latter observation would suggest that the binding site(s) for alkali metal ions and the site at which the third transported Na^+ interacts may be distinct.

Interaction of Amiloride Derivatives with the Na–Ca Exchange Carrier. The data presented above indicate that amiloride analogues interact directly with substrate-binding sites on the Na–Ca exchange transporter. To investigate this in greater detail, inhibition of Na–Ca exchange has been monitored with mixtures of inhibitors present. Given that the three compounds used in this study are structurally similar, it was expected that Dixon analyses of inhibitor action with two amiloride derivatives present would yield parallel line kinetics, typical of behavior displayed by mutually exclusive inhibitors interacting at a unique site (Segel, 1975). For this experiment, initial rates of Na_i-dependent Ca^{2+} uptake were monitored as a function of increasing DCB concentration, in the absence or presence of fixed amounts of BZB. As shown by the plot in Figure 4A, inhibition due to DCB alone yields linear data until ca. 60–70% block of transport occurs. At this point, further increases in the concentration of DCB result in the plot curving upward. This kinetic pattern is not expected for an interaction of inhibitor at a single site on the transport protein. Moreover, including increasing concentrations of BZB during repetition of the experiment results in the generation of a family of curves that do not display parallel line behavior in a Dixon plot (Figure 4A). At low DCB concentrations, plots of these data are linear and intersecting, while at higher concentrations, the graphs deviate significantly from linearity. However, when the data of Figure 4A are replotted as a function of the square of DCB concentration, the resulting graphs become linear. The same results are obtained when the concentration of BZB is varied in the presence of fixed concentrations of DCB (data not shown). These results indicate that the interaction of amiloride derivatives with the Na–Ca exchange protein may occur at two different sites.

That amiloride derivatives interact at two sites on the transport protein has been confirmed by analyzing the ability of different analogues to inhibit Na_i-dependent Ca^{2+} uptake in Hill plots. Typical data obtained with DCB are shown in

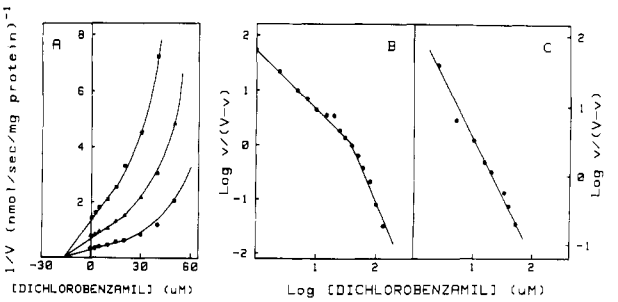


FIGURE 4: Characterization of Na–Ca exchange inhibition by amiloride analogues. (A) Effect of BZB on inhibition of Na–Ca exchange by DCB. Aliquots (2 μL) of membrane vesicles in 160 mM NaCl and 20 mM Mops–Tris, pH 7.4, were diluted 100-fold into a solution of 160 mM KCl and 20 mM Mops–Tris, pH 7.4, containing 10 μM $^{45}CaCl_2$ and increasing concentrations of DCB. This was done in the absence (●) or presence of either 20 (▲) or 40 μM (■) BZB. The reaction was carried out at 37 °C and terminated after 1 s. Data are presented in the form of a Dixon plot. (B and C) Hill analyses of Na–Ca exchange inhibition by DCB and BZB. Aliquots (2 μL) of membrane vesicles in 160 mM NaCl were diluted 100-fold into 160 mM KCl and 40 μM $^{45}CaCl_2$ in the presence of increasing concentrations of DCB without (B) or with (C) 20 μM BZB present. Transport reactions were carried out at 37 °C, and incubations were terminated after 1 s. Data are presented in the form of Hill plots.

Figure 4B,C. In Figure 4B, inhibition of Na–Ca exchange by DCB alone displays a biphasic Hill plot with Hill coefficients of 1 and 2 in the low and high range of inhibitor concentrations, respectively. The break in the Hill plot occurs at a concentration of 36 μM DCB. In further experiments, it has been observed that the inhibitor concentration corresponding to the break in the graph could be shifted depending on the concentration of Ca^{2+} used in this experiment; at low (5 μM) Ca^{2+} and high (100 μM) Ca^{2+} the break occurs at 30 and 50 μM DCB, respectively, indicating apparent competition between Ca^{2+} and inhibitor at the latter's second site of interaction. When the experiment is repeated in the presence of a fixed concentration of BZB (20 μM) and varying DCB, the resulting Hill plot appears monophasic with a Hill coefficient of 2 (Figure 4C). These data suggest that DCB interacts at two different sites on the carrier but that the interaction is directed primarily to one site at low inhibitor concentration. Consistent with this idea are profiles of inhibition observed in the presence of either BNZ or BZB alone. In both cases, these agents also produce biphasic Hill plots with Hill coefficients of 1 and 2. Therefore, these results, together with the primarily noncompetitive patterns of Na–Ca exchange inhibition vs Ca^{2+} described above, indicate that amiloride derivatives can bind at both classes of Na^+-binding sites on the transport protein. However, they have a preference for the B-site, which binds Na^+ but not Ca^{2+}. As inhibitor concentration is raised, a second interaction can occur at the A-site, which is the common binding site for Na^+ and Ca^{2+}.

Discussion

The results of this study strongly suggest that guanidino nitrogen substituted analogues of amiloride are mechanism-based inhibitors of Na–Ca exchange in cardiac sarcolemmal membrane vesicles. These inhibitors block all modes of carrier action, Na–Ca, Na–Na and Ca–Ca exchange, in a complete and reversible manner. Inhibition of Na–Ca exchange under ionic conditions (i.e., with 160 mM Na^+ and K^+ present on opposite sides of the membrane) that approximate "physiological conditions" is noncompetitive vs Ca^{2+} but competitive vs Na^+. In addition, block of Na–Na exchange is also competitive vs Na^+. This profile suggests that the protonated acylguanidinium moiety of amiloride functions as

a Na^+ mimic and that inhibition occurs by an interaction of this group at a Na^+-binding site(s) on the carrier protein to tie up the transporter in a nonproductive complex. Similar proposals have been advanced for the mechanism by which amiloride and amiloride analogues inhibit other Na^+-transporting systems (Benos, 1982; Mahnensmith & Aronson, 1985; Lazdunski et al., 1985). This mode of action is in marked contrast to that of other structural classes of Na–Ca exchange inhibitors (e.g., dodecylamine) which appear to influence carrier activity predominantly by modifying the membrane environment (Philipson, 1984), rather than by competing directly with carrier substrates.

Although amiloride analogues and Na^+ interact competitively with the transporter, this process appears to occur at more than one type of Na^+-binding site. Dixon analyses of Na–Ca exchange inhibition by individual amiloride analogues and analyses of inhibition with various mixtures of analogues present are consistent with an interaction of these agents at two separate sites. Moreover, Hill analyses of inhibition reveal that amiloride analogues block Na–Ca exchange with Hill coefficients of 1 and 2 at low and high inhibitor concentrations, respectively. These results suggest that amiloride inhibitors interact at two different types of Na^+-binding sites but that they have a preference for one class of sites at low concentration.

Additional data supporting these ideas were obtained from several different kinetic studies. First, substitution of sucrose for K^+ in Na–Ca exchange experiments results in mixed rather than noncompetitive inhibitory patterns vs Ca^{2+}. Since K^+ competes with Ca^{2+} for the common site shared by Na^+ and Ca^{2+} (see below), removal of K^+ apparently allows greater access of inhibitor to this site. As a result, there is a clear competitive component of inhibition observed with respect to Ca^{2+} under these conditions. Other data consistent with an interaction of inhibitor at this common Na^+, Ca^{2+} site are the observations that BNZ, a relatively weak inhibitor of Na–Ca exchange, produces mixed kinetics of inhibition vs Ca^{2+} in K^+ and that very high concentrations of DCB and BZB also cause mixed kinetic patterns vs Ca^{2+} in this medium. Furthermore, all inhibitors produce mixed kinetics of Ca–Ca exchange inhibition in sucrose medium, but this pattern is more noncompetitive in nature when K^+ is present. Given these results, it would appear most likely that amiloride analogues interact first at a site on the carrier protein that is exclusive for Na^+, with a further interaction occurring at another site shared by Na^+ and Ca^{2+}. The interaction of inhibitor at this second site can be modulated by the ionic composition of the medium in such a way that removal of K^+ results in enhanced inhibitor binding at the common Na^+, Ca^{2+} site. This behavior is clearly manifested by degeneration of strictly noncompetitive inhibitory profiles with the appearance of a competitive component in the kinetics of inhibition vs Ca^{2+}.

The inhibitory effects of amiloride analogues on Ca–Ca exchange can also be explained by the scheme outlined above. However, such a description is complicated somewhat by uncertainty as to which site on the carrier is the locus of interaction of stimulatory alkali metal ions. On the one hand, mixed kinetic patterns of Ca–Ca exchange inhibition in sucrose are consistent with inhibitor interacting at both a Ca^{2+}-binding site and a separate site on the transporter. Inclusion of K^+ would be expected to interfere with inhibitor binding at the Ca^{2+} site, resulting in Ca^{2+} kinetics that are now more noncompetitive in nature and perhaps in some relief of inhibition. Both of these results are observed with K^+. Moreover, it has been postulated that stimulatory cations increase rates of Ca–Ca exchange by interacting at the site that exclusively binds Na^+ during Na–Ca exchange (Slaughter et al., 1983; Reeves et al., 1984; Reeves, 1985). Therefore, stimulatory cations would be expected to completely alleviate block of Ca–Ca exchange by competing with inhibitor in its primary interaction at that binding site. This behavior has been observed with K^+ also. Yet, no clear correlation was detected between the ability of different stimulatory cations to relieve inhibition of Ca–Ca exchange and to stimulate the V_{max} of the exchange reaction. Such a finding might have a number of explanations. Perhaps various stimulatory cations interact to different extents with the Ca^{2+}-binding site and the cation regulatory site, or the stimulatory cation-binding site could be distinct from the site that is specific for Na^+ and amiloride analogues, or possibly multiple cation-binding sites are involved in stimulation of Ca–Ca exchange activity. Any of these phenomena would effectively obscure a correlation between relief of inhibition and stimulation of carrier activity by inorganic cations. Given that amiloride analogues interact with at least two sites on the transporter in a manner that is dependent on the ionic composition of the medium, it is not clear that the present data can differentiate between these or other possibilities.

The results presented in this study support and extend several aspects of the model proposed for the cardiac Na–Ca exchange reaction (Reeves, 1985). Clearly, data indicating a preferential interaction of inhibitor with various Na^+ sites on the carrier are consistent with the description of two different substrate-binding sites, the A- and B-sites, as has been envisioned in the hypothetical transport mechanism. Other data supporting this model have been derived from experiments demonstating electrogenicity of transport (Reeves & Sutko, 1980), a stoichiometry of $3Na^+$:Ca^{2+} (Reeves & Hale, 1984), cooperativity in the transport reaction with respect to Na^+ (i.e., a Hill coefficient of 3; Kadoma et al., 1982), and a competitive interaction between Na^+ and Ca^{2+} on rates of Ca^{2+} movement (i.e., Hill coefficients for inhibition of Na–Ca exchange by Na^+ of 1 and 2 at low and high Na^+ concentrations, respectively; Reeves & Sutko, 1983). Because the B-site appears more accessible to amiloride inhibitors than the A-site, it may be located in proximity to the membrane or in a hydrophobic pocket on the protein, while the A-site is probably exposed to the aqueous environment. This would account for the structure–activity relationship observed for Na–Ca exchange inhibition where potency of inhibitors increases with increasing hydrophobicity of the guanidino nitrogen substituent (Kaczorowski et al., 1985). Furthermore, block by some less hydrophobic amiloride derivatives (e.g., BNZ or even amiloride, itself) exhibits more significantly mixed kinetics vs Ca^{2+} than found with other inhibitors (e.g., DCB), suggesting that decreasing hydrophobicity may lessen an interaction at the B-site. Perhaps potent amiloride analogues are effective because they easily partition into the membrane, are anchored by their hydrophobic guanidino nitrogen substituent, and diffuse along the surface of the bilayer into the B-site, resulting in a high local concentration of cationic inhibitor that effectively competes with Na^+ for binding to the protein.

The conclusions of this study support the idea that amiloride analogues can be used to probe the mechanism of cardiac Na–Ca exchange. Indeed, these compounds have been used to investigate block of this transport reaction in intact cardiac myocytes by both flux (Kim & Smith, 1986) and electrophysiological techniques (Bielefeld et al., 1986), as well as to detect Na–Ca exchange activity in isolated cardiac muscle (Siegl et al., 1984). Although amiloride analogues are

mechanism-based inhibitors of Na–Ca exchange, they can also interfere with other plasmalemmal ion-transporting systems. Recently, it has been demonstrated that these molecules possess significant Ca^{2+} entry blocker activity in cardiac preparations (Bielefeld et al., 1986; Garcia et al., 1987). Because amiloride derivatives can inhibit voltage-dependent Ca^{2+} channels, their use in investigating the physiological role of Na–Ca exchange in intact systems should be viewed with caution. More selective inhibitors must be found to clarify the function of Na–Ca exchange in intact systems.

To test the proposed mechanism of Na–Ca exchange inhibition by amiloride analogues, it would be desirable to perform comparable studies with other structurally distinct inhibitors. Few such inhibitors have been described (Kaczorowski et al., 1988). Recently, it has been found that the antiarrhythmic agent bepridil will inhibit cardiac Na–Ca exchange and that the mechanism of block is different from that of amiloride analogues (Garcia et al., 1988). Data obtained with this inhibitor have been used to verify the mechanistic proposals derived from study of amiloride inhibitors.

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References

Baker, P. F., & DiPolo, R. (1984) *Curr. Top. Membr. Transp. 22*, 195–247.

Benos, D. J. (1982) *Am. J. Physiol. 242*, C131–C145.

Bielefeld, D. R., Hadley, R. W., Vassilev, P. M., & Hume, J. R. (1986) *Circ. Res. 59*, 381–389.

Blaustein, M. P. (1977) *Biophys. J. 20*, 79–111.

Cragoe, E. J., Woltersdorf, O. W., Bicking, J. B., Kwong, S. F., & Jones, J. H. (1967) *J. Med. Chem. 10*, 66–75.

Garcia, M. L., King, V. F., & Kaczorowski, G. J. (1986) *Biophys. J. 49*, 545a.

Garcia, M. L., King, V. F., Cragoe, E. J., & Kaczorowski, G. J. (1987) *Biophys. J. 51*, 428a.

Garcia, M. L., Slaughter, R. S., King, V. F., & Kaczorowski, G. J. (1988) *Biochemisry* (following paper in this issue).

Kaczorowski, G. J. (1985) *Annu. Rep. Med. Chem. 20*, 215–226.

Kaczorowski, G. J., Barros, F., Dethmers, J. K., Trumble, M. J., & Cragoe, E. J. (1985) *Biochemistry 24*, 1394–1403.

Kaczorowski, G. J., Garcia, M. L., King, V. F., & Slaughter, R. S. (1988) in *Handbook of Experimental Pharmacology; Calcium in Drug Actions* (Baker, P. F., Ed.) pp 163–183, Springer-Verlag, Berlin.

Kadoma, M., Froehlich, J., Reeves, J. P., & Sutko, J. (1982) *Biochemistry 21*, 1914–1918.

Kim, D., & Smith, T. W. (1986) *Mol. Pharmacol. 30*, 164–170.

Kuwayama, H., & Kanazawa, T. (1982) *J. Biochem. (Tokyo) 91*, 1419–1426.

Lazdunski, M., Frelin, C., & Vigne, P. (1985) *J. Mol. Cell. Cardiol. 17*, 1029–1042.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mahnensmith, R. L., & Aronson, P. S. (1985) *J. Biol. Chem. 260*, 12586–12592.

Philipson, K. D. (1984) *J. Biol. Chem. 259*, 13999–14002.

Philipson, K. D. (1985) *Biochim. Biophys. Acta 821*, 367–376.

Philipson, K. D., & Nishimoto, A. Y. (1982) *J. Biol. Chem. 257*, 5111–5117.

Reeves, J. P. (1985) *Curr. Top. Membr. Transp. 25*, 77–127.

Reeves, J. P., & Sutko, J. L. (1980) *Science (Washington, D.C.) 208*, 1461–1464.

Reeves, J. P., & Sutko, J. L. (1983) *J. Biol. Chem. 258*, 3178–3182.

Reeves, J. P., & Hale, C. C. (1984) *J. Biol. Chem. 259*, 7733–7739.

Reeves, J. P., Hale, C. C., de la Pena, P., Slaughter, R. S., Boulware, T., Froehlich, J., Kaczorowski, G. J., & Cragoe, E. J. (1984) in *Epithelial Calcium and Phosphate Transport: Molecular and Cellular Aspects* (Bronner, F., & Peterlik, M., Eds.) pp 77–82, Alan Liss, New York.

Segel, I. H. (1975) in *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*, pp 474–479, Wiley-Interscience, New York.

Siegl, P. K. S., Cragoe, E. J., Trumble, M. J., & Kaczorowski, G. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3238–3242.

Slaughter, R. S., Sutko, J. L., & Reeves, J. P. (1983) *J. Biol. Chem. 258*, 3183–3190.

Slaughter, R. S., de la Pena, P., Reeves, J. P., Cragoe, E. J., & Kaczorowski, G. J. (1984) *Biophys. J. 45*, 81a.