Inhibition of Sodium–Calcium Exchange in Cardiac Sarcolemmal Membrane Vesicles. 2. Mechanism of Inhibition by Bepridil

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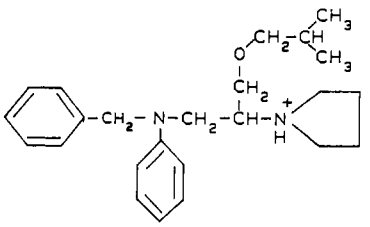
ABSTRACT: Bepridil, an antiarrhythmic agent, inhibits Na–Ca exchange in cardiac sarcolemmal membrane vesicles (K_i = 30 μM) by a novel mechanism, different from that determined for amiloride analogues [Slaughter, R. S., Garcia, M. L., Cragoe, E. J., Jr., Reeves, J. P., & Kaczorowski, G. J. (1988) *Biochemistry* (preceding paper in this issue)]. Bepridil causes partial inhibition of Na_i-dependent Ca^{2+} uptake but complete block of Na_o-dependent Ca^{2+} efflux. Inhibition of Na–Ca exchange is noncompetitive vs Ca^{2+} but competitive vs Na^+ in both K^+ and sucrose. Bepridil also blocks Ca–Ca exchange, with or without K^+ present. However, K^+ has two effects on inhibition: it reduces the potency of bepridil and causes inhibition to become partial. Inhibition of Ca–Ca exchange displays noncompetitive kinetics vs Ca^{2+} in either sucrose or K^+. Dixon analyses of Na–Ca exchange inhibition caused by mixtures of bepridil and amiloride analogues demonstrate that these compounds produce a competitive interaction at a common carrier site that is evident only at low concentrations of amiloride inhibitors. Hill plots of bepridil inhibition of Na–Ca and Ca–Ca exchange display unitary Hill coefficients. These results indicate that bepridil interacts at only one substrate-binding site, the site selective for Na^+, where amiloride analogues also preferentially interact. However, unlike amiloride, bepridil does not interact at the common Na^+, Ca^{2+}-binding site of the carrier. During stimulation of Ca–Ca exchange, K^+ may bind at other sites besides the site selective for Na^+, because in addition to being competitive with bepridil, it prevents complete abolition of Ca–Ca exchange, suggesting that this Na^+ site is not involved in carrier turnover. These findings indicate that bepridil is a mechanism-based Na–Ca exchange inhibitor that interacts at a transporter site which binds Na^+ but not Ca^{2+}.

Elucidation of the role of Na–Ca exchange in Ca^{2+} homeostasis of electrically excitable tissues has been hampered by a lack of potent selective inhibitors for this process. Although several agents block Na–Ca exchange [for a review see Kaczorowski et al. (1988)], their specificity is not great enough to be useful in physiological studies. However, it is important to understand the action of these agents and identify structural classes of compounds that function as mechanism-based inhibitors so that more selective blockers can be designed.

The mechanism by which terminal guanidino nitrogen derivatives of amiloride block Na–Ca exchange in cardiac sarcolemmal membrane vesicles has been defined (Slaughter et al., 1988). These inhibitors act as Na^+ mimics and bind at two different Na^+ sites on the transport protein, a site that is selective for Na^+ and a site that is shared by Na^+ and Ca^{2+}. However, amiloride analogues have a preference for the selective Na^+-binding site, and their primary interaction occurs at this locus.

Upon investigating drugs with cardiovascular actions, it was discovered that bepridil, a known Ca^{2+} entry blocker (Galizzi et al., 1986) and antiarrhythmic agent (Alpert et al., 1985; Flaim & Cummings, 1986), blocked Na–Ca exchange (Garcia et al., 1985). Because of structural and mechanistic differences between amiloride and bepridil, the mode of action of bepridil has now been characterized. Bepridil, unlike amiloride, was found to interact exclusively at a substrate-binding site on the carrier that is selective for Na^+. These findings have extended the model that was previously proposed for the transport mechanism of Na–Ca exchange in heart (Reeves et al., 1984) and further illustrate that bepridil is a novel mechanism-based inhibitor of Na–Ca exchange with a significantly different mechanism from that of amiloride. Preliminary reports of this work have appeared in abstract form (Garcia et al., 1985, 1986).



BEPRIDIL

Experimental Procedures

Materials

$^{45}CaCl_2$ (800–1200 mCi/mmol) was purchased from the Radiochemical Center, Amersham Inc. Bepridil, β-[(2-methylpropoxy)methyl]-*N*-phenyl-*N*-(phenylmethyl)-1-pyrrolidineethanamine, was a generous gift of McNeil Pharmaceuticals, Spring House, PA. Analogues of amiloride were obtained from the chemical collection of Merck Sharp and Dohme Research Laboratories. All other reagents were purchased from commercial sources.

Methods

Preparation of Cardiac Sarcolemmal Membrane Vesicles. Fresh samples of porcine left ventricle were used for the preparation of purified cardiac sarcolemmal membrane vesicles. Vesicles were prepared by a modification of the procedure of Kuwayama and Kanazawa (1982) as described by Slaughter et al. (1983). Vesicles were suspended in a medium

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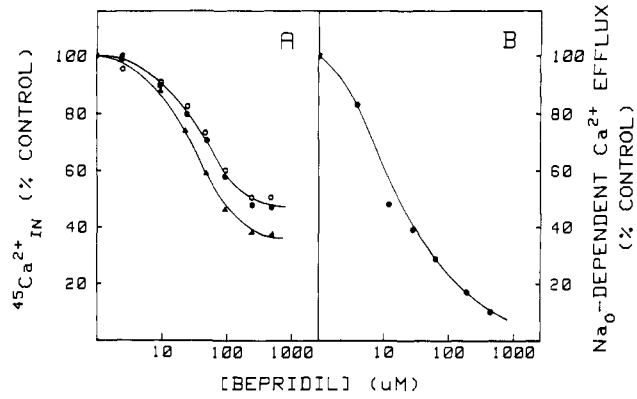

FIGURE 1: Inhibition of Na–Ca exchange by bepridil. (A) Effect of bepridil on Na_i-dependent Ca^{2+} uptake. Membrane vesicles equilibrated with 160 mM NaCl and 20 mM Mops–Tris, pH 7.4, were diluted 100-fold into 160 mM KCl (●, ○) or 320 mM sucrose (▲) buffered to pH 7.4 with 20 mM Mops–Tris, containing 10 μM $^{45}CaCl_2$ and appropriate concentrations of bepridil at 37 °C. Uptakes were determined after 1 (●,▲) or 10 s (○). (B) Effect of bepridil on Na_o-dependent Ca^{2+} efflux. Membrane vesicles equilibrated with 160 mM KCl, 20 mM Mops–Tris, pH 7.4, and 100 μM $^{45}CaCl_2$ were diluted into 10 mM NaCl, 150 mM KCl, 1 mM EGTA, and 20 mM Mops–Tris, pH 7.4, containing appropiate concentrations of bepridil at 25 °C. The reaction was terminated after 3 s. Inhibition of $^{45}Ca^{2+}$ influx (A) or efflux (B) is represented as a function of extravesicular bepridil concentration.

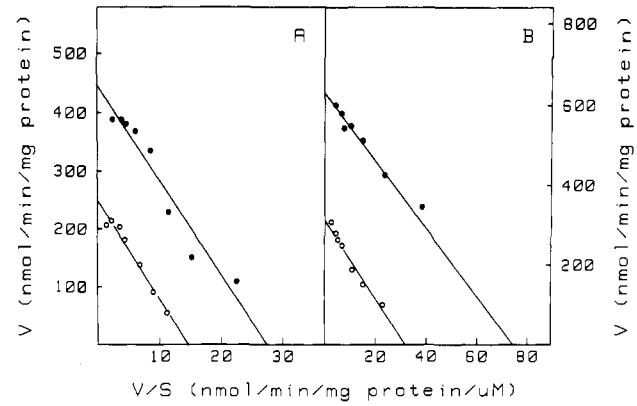

FIGURE 2: Effect of bepridil on the Ca^{2+} concentration dependence of Na–Ca exchange. Membrane vesicles equilibrated with 160 mM NaCl and 20 mM Mops–Tris, pH 7.4, were diluted into 160 mM KCl (A) or 320 sucrose (B) buffered to pH 7.4 with 20 mM Mops–Tris, containing increasing concentrations of $^{45}CaCl_2$ in the absence (●) or presence (○) of 100 μM bepridil at 37 °C. Uptakes were determined after 1 s. Data are presented as Eadie–Hofstee plots. (A) K_m = 17 μM, V_{max} = 457 nmol min^{-1} (mg of protein)$^{-1}$ (●); K_m = 17 μM, V_{max} = 250 nmol min^{-1} (mg of protein)$^{-1}$ (○). (B) K_m = 8.1 μM, V_{max} = 624 nmol min^{-1} (mg of protein)$^{-1}$ (●); K_m = 10 μM, V_{max} = 330 nmol min^{-1} (mg of protein)$^{-1}$ (○).

of 160 mM NaCl and 20 mM Mops–Tris,[1] pH 7.4, at a protein concentration of ca. 2 mg/mL and stored at –80 °C after rapid freezing in liquid nitrogen. Intravesicular buffer conditions were adjusted as described (Slaughter et al., 1988).

Transport Assays. Na–Ca exchange and Ca–Ca exchange were monitored as previously described (Slaughter et al., 1988). Triplicate assays were routinely performed for each time point, and the data were averaged. The standard error of the mean of these determinations was typically less than 5%. All transport measurements, unless otherwise indicated, were made at 1 s. Under these conditions, initial velocity conditions are maintained. Solutions of bepridil or amiloride analogues were made fresh daily in Me_2SO.

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

RESULTS

Inhibition of Na–Ca Exchange by Bepridil. Bepridil, like terminal guanidino nitrogen derivatives of amiloride, inhibits Na–Ca exchange activity in heart. To elucidate its mode of action and compare this with the mechanism determined for the amiloride series of inhibitors, the inhibitory properties of bepridil were characterized. Bepridil causes blockage of Na_i-dependent $^{45}Ca^{2+}$ uptake in cardiac sarcolemmal membrane vesicles with the concentration dependence shown in Figure 1A. Interestingly, under the conditions of these experiments, bepridil reproducibly produces incomplete inhibition of uptake with a maximal loss of 50% Na–Ca exchange activity. This contrasts with inhibition caused by amiloride, which is complete under identical conditions (Siegl et al., 1984; Kaczorowski et al., 1985). The IC_{50} for bepridil is ca. 30 μM, and concentrations of inhibitor 10-fold higher (e.g., 300 μM) produce no more than 50% inhibition. Although this finding is derived from rapid transport measurements (i.e., 1-s uptakes), incomplete blockade of transport is not due to slow onset of drug action since identical data have been obtained by using a 10-s measurement (Figure 1A). This residual Na–Ca exchange activity still displays electrogenic behavior since it can be stimulated with valinomycin and K^+ (data not shown), which rules out the possibility that bepridil alters the carrier to a form that now transports 2Na:Ca.

To determine what conditions affect the extent of inhibition, both ionic composition of the medium and orientation of inhibitor with respect to binding sites on the carrier were varied. Substitution of sucrose for K^+ changes the inhibitory properties of amiloride analogues (Slaughter et al., 1988). However, this substitution has little effect on bepridil inhibition; maximal inhibition is slightly increased, from 50% to 60%, but the IC_{50} for bepridil remains the same (Figure 1A). Alternatively, bepridil produces complete block of Na_o-dependent Ca^{2+} efflux in a concentration-dependent fashion (Figure 1B). For this experiment, vesicles preloaded with $^{45}Ca^{2+}$ were diluted, at zero time, into inhibitor, 10 mM Na^+, and EGTA to initiate the efflux reaction. As the concentration of bepridil is increased, loss of intravesicular Ca^{2+} is completely prevented. Importantly, bepridil (up to 500 μM) has no effect on passive Ca^{2+} permeability of the vesicle membrane. The K_i for bepridil in inhibiting efflux is 30 μM (Figure 1B). These results illustrate that partial inhibition of transport is not related to the inhibitor's physical properties (i.e., limited solubility in aqueous media) because either partial or complete block of transport activity can occur in media of identical composition. These results also suggest that when bepridil and Na^+ are simultaneously present at the same membrane surface, complete block of transport prevails but that when inhibitor and Na^+ are initially on opposite sides of the membrane (as in Na_i-dependent Ca^{2+} uptake), inhibition of Na–Ca exchange is partial. Thus, it is expected that if bepridil is allowed to gain access to the intravesicular space by preincubation with inhibitor before the transport experiment is performed, inhibition of Na–Ca exchange should be complete. This is the result obtained when Na-loaded membranes are incubated with 1–250 μM bepridil for 1 h before transport in the presence of equivalent extravesicular concentrations of inhibitor is measured. Such conditions result in complete block of Na–Ca exchange in a concentration-dependent fashion (K_i = 10 μM).

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

The kinetics of Na–Ca exchange were next examined to investigate how bepridil interacts with the transporter. As shown in Figure 2, bepridil inhibition was monitored vs Ca^{2+} in either K^+ (A) or sucrose (B). The Eadie–Hofstee plot of Figure 2A indicates that Ca^{2+} uptake in a control preparation displays a K_m for Ca^{2+} of 17 μM and a V_{max} of 457 nmol of Ca^{2+} accumulated min^{-1} (mg of protein)$^{-1}$. Addition of 100 μM bepridil to the K^+ medium (i.e., a nearly maximal inhibitory concentration) results in parallel line kinetics in an Eadie–Hofstee representation (Figure 2A). This indicates that bepridil inhibition is strictly noncompetitive vs Ca^{2+} concentration.

Substitution of sucrose for K^+ has been shown to alter both the kinetics of Na–Ca exchange and the kinetic patterns of Na–Ca exchange inhibition by amiloride analogues (Slaughter et al., 1988). As illustrated in Figure 2B, replacement of K^+ with sucrose results in both a decrease in the K_m for Ca^{2+} (from 17 to 8 μM) and an increase in the V_{max} for Ca^{2+} uptake (from 457 to 624 nmol min^{-1} (mg of protein)$^{-1}$ in a control preparation. Inclusion of 100 μM bepridil partially blocks Na_i-dependent Ca^{2+} uptake as expected, but the kinetics of inhibition vs Ca^{2+} remain noncompetitive (Figure 2B). This is in marked contrast to results obtained with amiloride inhibitors, where, under identical experimental conditions, primarily noncompetitive kinetic patterns of inhibition in the presence of K^+ change to mixed kinetic patterns vs Ca^{2+} in sucrose media.

Since there is no direct interaction between bepridil and Ca^{2+}, experiments were designed to test for an interaction between inhibitor and Na^+. When bepridil inhibition of Na_i-dependent Ca^{2+} uptake was monitored as a function of varying intravesicular Na^+, it was noted that the potency of bepridil increased as Na^+ was reduced (data not shown). This result suggests a possible competition between Na^+_i and $inhibitor_o$ for a binding site(s) on the transporter. To further clarify this interaction, Na_o-dependent Ca^{2+} efflux has been investigated. In these experiments, Ca^{2+} efflux was monitored from preloaded vesicles as a function of Na^+ concentration in either K^+ or sucrose, with or without bepridil present. Increasing Na^+ concentration increases the rate of Ca^{2+} efflux from vesicles in a manner that displays saturability. It is noteworthy that the effectiveness of Na^+ is enhanced in sucrose medium, consistent with previous data indicating a competitive interaction of K^+ at the common Na^+, Ca^{2+}-binding site of the carrier (Slaughter et al., 1988). When 250 μM bepridil is added to either efflux medium, rates of Ca^{2+} loss from vesicles are reduced. The percent inhibition of Ca^{2+} efflux at each Na^+ concentration is represented in Figure 3A for experiments carried out in both K^+ and sucrose. At 5 mM Na^+, efflux is approximately 90% inhibited, but as Na^+ is increased to 160 mM, inhibition of the rate of Ca^{2+} release is reduced to 20%. Importantly, results obtained in the two different dilution media are identical. These data indicate that a competitive interaction exists between bepridil and Na^+. Such a relationship is consistent with the ability of intravesicular Na^+ to modulate the potency of bepridil as it inhibits Na_i-dependent Ca^{2+} uptake. The interaction of bepridil could occur at either or both types of Na^+-binding sites on the transporter. However, inhibition by bepridil is noncompetitive vs Ca^{2+}. This observation, together with the finding that K^+ does not alter bepridil's effectiveness in blocking Na_o-dependent Ca^{2+} efflux, implies that this inhibitor does not interact at the common Na^+, Ca^{2+}-binding site. Rather, these results suggest that bepridil interacts exclusively at the carrier's selective Na^+-binding site. This is in counterdistinction to results

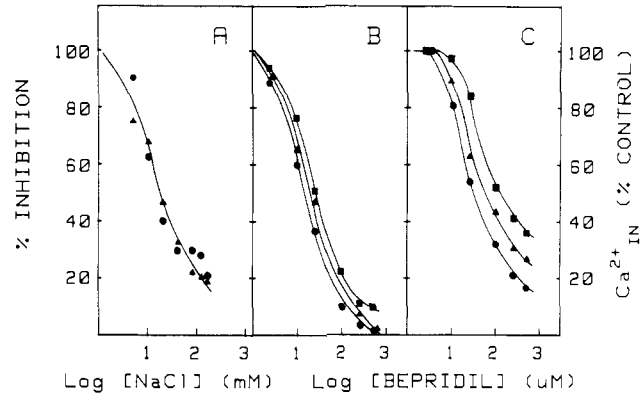

FIGURE 3: Effect of bepridil on Na–Ca and Ca–Ca exchange. (A) Effect of bepridil on Na_o-dependent Ca^{2+} efflux. Membrane vesicles equilibrated with 160 mM KCl, 20 mM Mops–Tris, pH 7.4, and 100 μM $^{45}CaCl_2$ were diluted into media containing different amounts of NaCl in the absence or presence of 250 μM bepridil at 25 °C. The osmolarity of the dilution medium was kept constant by balancing with either KCl (●) or sucrose (▲), and the medium was buffered with 20 mM Mops–Tris and 1 mM EGTA, pH 7.4. The reaction was terminated after 3 s. Data are plotted as percent Ca^{2+} efflux inhibition produced by bepridil vs Na^+ concentration in the medium. (B and C) Inhibition of Ca–Ca exchange by bepridil. Membrane vesicles were equilibrated with 320 mM sucrose, 20 mM Mops–Tris, pH 7.4, and 100 μM $CaCl_2$ (B) or 160 mM KCl, 20 mM Mops–Tris, pH 7.4, and 100 μM $CaCl_2$ (C) and diluted into 320 mM sucrose (●), 40 mM KCl and 240 mM sucrose (▲), or 160 mM KCl (■) containing 10 μM $^{45}CaCl_2$ and appropriate concentrations of bepridil at 37 °C. Uptakes were determined at 1 s. Data in (B) and (C) are presented as percent inhibition of Ca–Ca exchange with respect to an untreated control.

obtained with amiloride analogues, which have been found to function at both classes of ion-binding sites.

Effect of Bepridil on Ca–Ca Exchange. Ca–Ca exchange was monitored both in the absence and in the presence of K^+ (which acts to stimulate the rate of this reaction; Slaughter et al., 1983) in order to characterize further the properties of bepridil inhibition. Addition of increasing concentrations of bepridil to sucrose medium results in complete block of Ca–Ca exchange activity with a K_i of ca. 14 μM (Figure 3B). As K^+ concentration is increased, two effects are discernable in the action of bepridil on Ca–Ca exchange; the potency of bepridil is reduced, and inhibition becomes incomplete (Figure 3B). For example, with vesicles equilibrated in 320 mM sucrose and diluted into 160 mM KCl, the IC_{50} of bepridil is 24 μM and maximal inhibition is only 90%. The effects of K^+ are even more evident in the experiments of Figure 3C, where inhibitory efficacy is assessed by using vesicles preequilibrated in 160 mM KCl followed by dilution into media of varying K^+. As an illustration, when 160 mM K^+ is present on both sides of the membrane, bepridil causes at most 65% inhibition and displays an IC_{50} of 50 μM. Clearly, under these conditions, inhibition is partial, and the potency of bepridil is almost 4-fold less than the value determined in sucrose.

Kinetic experiments monitoring the effects of bepridil on Ca–Ca exchange were performed with either sucrose (Figure 4A) or K^+ (Figure 4B) media bathing both membrane surfaces of the sarcolemmal vesicles (i.e., the two extremes of conditions described in parts B and C of Figure 3). The control membrane preparation in sucrose displays a K_m for Ca^{2+} of ca. 3 μM and a V_{max} of 62 nmol of Ca^{2+} accumulated min^{-1} (mg of protein)$^{-1}$ for Ca–Ca exchange. Addition of 17 μM bepridil to the dilution buffer causes a reduction in the V_{max} of Ca^{2+} uptake to 28 nmol min^{-1} (mg of protein)$^{-1}$ without affecting the K_m for Ca^{2+}. Identical noncompetitive inhibition vs Ca^{2+} is found when the experiment is repeated in K^+ media (Figure 4B). While both the K_m and V_{max} of Ca^{2+} uptake are increased

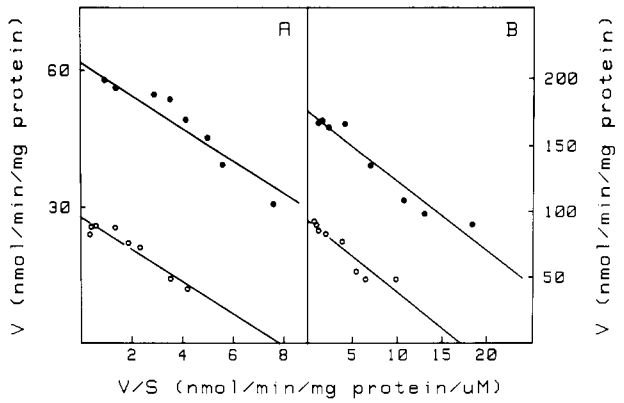

FIGURE 4: Effect of bepridil on the Ca^{2+} concentration dependence of Ca–Ca exchange. (A) Membrane vesicles equilibrated with 320 mM sucrose, 20 mM Mops–Tris, pH 7.4, and 100 μM $CaCl_2$ were diluted into 320 mM sucrose and 20 mM Mops–Tris, pH 7.4, containing increasing concentrations of $^{45}CaCl_2$ in the absence (●) or presence (○) of 17 μM bepridil. (B) Membrane vesicles equilibrated with 160 mM KCl, 20 mM Mops–Tris, pH 7.4, and 100 μM $CaCl_2$ were diluted into equivalent media containing increasing concentrations of $^{45}CaCl_2$ in the absence (●) or presence (○) of 100 μM bepridil. Uptakes were done at 37 °C and terminated after 1 s. Data in (A) and (B) are presented in the form of Eadie–Hofstee plots. (A) K_m = 3 μM, V_{max} = 62 nmol min^{-1} (mg of protein)$^{-1}$ (●); K_m = 3 μM, V_{max} = 28 nmol min^{-1} (mg of protein)$^{-1}$ (○). (B) K_m = 5.5 μM, V_{max} = 177 nmol min^{-1} (mg of protein)$^{-1}$ (●); K_m = 5.6 μM, V_{max} = 93 nmol min^{-1} (mg of protein)$^{-1}$ (○).

in the control preparation by the presence of K^+, a maximally effective concentration of bepridil decreases only the V_{max} of the reaction to approximately half that of the control, with no effect on K_m.

These findings are consistent with results obtained from studies of Na–Ca exchange inhibition (see above) that indicate no direct competition between bepridil and Ca^{2+}. In terms of the effects of K^+ on bepridil inhibition of Ca–Ca exchange, there are at least two possible interpretations. Since K^+ does not affect bepridil binding to the B-site of the carrier in the Na–Ca exchange mode, K^+ may not bind to the B-site during Ca–Ca exchange, but rather it could exert its effects allosterically to control potency and maximal extent of inhibition by bepridil. Alternatively, K^+ may bind to the B-site in the Ca–Ca but not the Na–Ca exchange mode and compete with bepridil at this site to reduce inhibitor potency. At the same time, K^+ could bind to yet another site on the carrier to modulate the maximal extent of inhibition that can occur. In both schemes, the remote K^+ site may be the stimulatory monovalent cation site for Ca–Ca exchange. With the present data, these models are indistinguishable. However, previous observations have suggested that monovalent stimulatory cations interact at an additional site on the transporter besides the selective Na^+-binding site (Slaughter et al., 1988).

Interaction of Bepridil with the Na–Ca Exchange Transporter. If the interaction of bepridil with the transporter is limited to one class of ion-binding sites but amiloride analogues interact at both classes of substrate-binding sites, with a preference for one site at low concentration [see Slaughter et al. (1988)], certain types of kinetic behavior are predictable. For example, Dixon analyses of Na_i-dependent Ca^{2+} uptake inhibition by bepridil in the presence of low concentrations of amiloride analogues should display patterns consistent with both agents interacting competitively. Alternatively, more complex behavior between inhibitors is expected when the concentration of amiloride is elevated (Segel, 1975). As a test of this idea, the results shown in Figure 5 have been obtained. A Dixon plot of bepridil inhibition is linear until high concentrations of bepridil are employed, at which point deviation

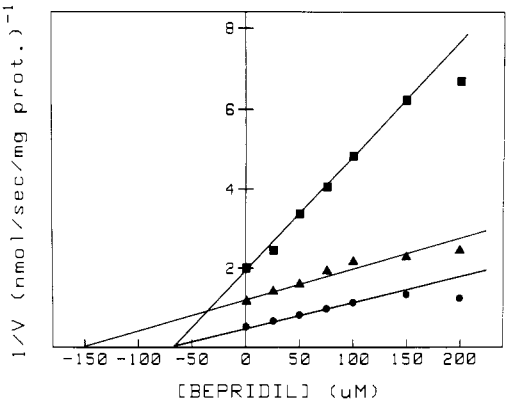

FIGURE 5: Effect of an amiloride analogue on inhibition of Na–Ca exchange by bepridil. Membrane vesicles equilibrated with 160 mM NaCl and 20 mM Mops–Tris, pH 7.4, were diluted into 160 mM KCl and 20 mM Mops–Tris, pH 7.4, containing 10 μM $^{45}CaCl_2$ and different concentrations of bepridil in the absence (●) or presence of 20 (▲) or 40 μM (■) 3′,4′-dichlorobenzamil at 37 °C. Uptakes were determined at 1 s. Data are presented in the form of Dixon plots.

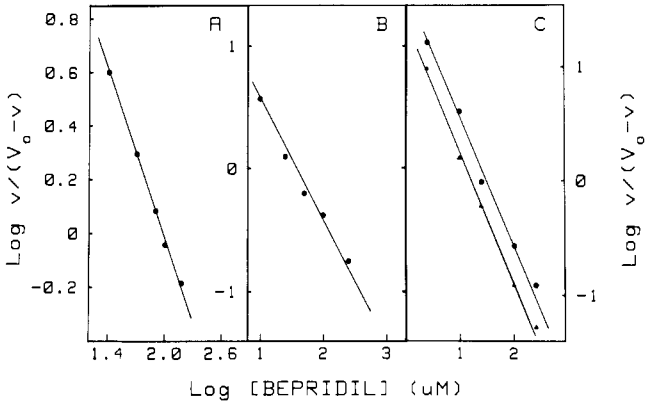

FIGURE 6: Hill analyses of Na–Ca exchange and Ca–Ca exchange inhibition by bepridil. (A) Na_i-dependent $^{45}Ca^{2+}$ uptake. Data obtained as in Figure 1A are represented in the form of a Hill plot. (B) Na_o-dependent $^{45}Ca^{2+}$ efflux. Data obtained by the procedures described in Figure 1B are plotted by Hill analysis. (C) Hill analyses of inhibition of Ca–Ca exchange by bepridil in either sucrose (▲) or KCl (●) media. Experimental data were obtained under the conditions described in Figure 3.

from linearity occurs because inhibition by bepridil is partial. Linear behavior is consistent with bepridil interacting at one site on the carrier. When the experiment is repeated in the presence of 20 μM 3′,4′-dichlorobenzamil, a line parallel to the original graph is obtained (Figure 5). This result indicates that bepridil and dichlorobenzamil interact competitively at a single site under these conditions. However, when the experiment is repeated in the presence of 40 μM dichlorobenzamil, the parallel line behavior degenerates (Figure 5), suggesting that the amiloride analogue is now interacting at an additional site(s). Similar results have been obtained in experiments using either fixed concentrations of benzamil or 2′,3′-benzobenzamil and varying bepridil concentrations (data not shown). These findings are consistent with the hypothesis that both bepridil and low concentrations of amiloride analogues competitively occupy the selective Na^+-binding site on the carrier, but that amiloride analogues bind to yet another site as their concentration is elevated.

As a final test of the hypothesis that bepridil interacts at a single site on the transporter, bepridil inhibition of Na_i-dependent Ca^{2+} uptake (Figure 6A), Na_o-dependent Ca^{2+} efflux (Figure 6B), and Ca–Ca exchange in both sucrose and K^+ (Figure 6C) has been subjected to Hill analyses. The data from these experiments yield, in every case, monophasic Hill

plots with Hill coefficients of 1. The unitary Hill coefficient confirms that bepridil interacts at only one site on the carrier protein to produce its inhibitory effect and is a further indication that bepridil acts in a specific fashion to inhibit the Na–Ca exchange transporter.

Discussion

The results presented in this study demonstrate that bepridil is an effective mechanism-based inhibitor of Na–Ca exchange in cardiac sarcolemmal membrane vesicles and that its mechanism of action is different from that of amiloride analogues [see Slaughter et al. (1988)]. Bepridil's mechanism has been analyzed in terms of a model proposed for cardiac Na–Ca exchange, where distinct substrate-binding sites are postulated to function in carrier turnover (Reeves et al., 1984; Reeves, 1985). This model has previously been used to define the action of amiloride analogues. Therefore, a direct comparison of the mechanisms by which these two agents function should be possible.

Several lines of evidence suggest that bepridil interacts at only one of the two classes of carrier Na^+-binding sites. Bepridil reversibly inhibits Na–Ca exchange in both influx and efflux modes. Inhibition under all conditions is strictly noncompetitive vs Ca^{2+}. Absence of any mixed kinetic patterns, as well as the fact that substitution of K^+ has no effect on inhibitor potency in the Na–Ca exchange mode, indicates that bepridil does not interact at the common Na^+, Ca^{2+}-binding site. Consistent with this are the noncompetitive kinetics of Ca–Ca exchange inhibition that are observed. However, bepridil inhibition of Na–Ca exchange is competitive with Na^+. In addition, Hill analyses of Na–Ca and Ca–Ca exchange inhibition display Hill coefficients of 1, indicating that bepridil interacts at a single site on the carrier. Moreover, bepridil interacts competitively at this site with either low concentrations of amiloride derivatives or with K^+ as it acts to stimulate Ca–Ca exchange activity. The only substrate-binding site displaying properties consistent with these characteristics is the selective Na^+-binding site (B-site), where the third Na^+ that is transported binds. Occupation of this site by bepridil prevents carrier turnover in either Na–Ca or Ca–Ca exchange modes. Amiloride analogues, on the other hand, have been found to interact at both this site and the common Na^+, Ca^{2+}-binding site, depending on inhibitor concentration (Slaughter et al., 1988). The data obtained with bepridil are completely consistent with the postulated mechanism of action of amiloride derivatives. A scheme summarizing the interaction of these two agents with the carrier in either of two different turnover modes is shown in Figure 7.

It has been postulated that the carrier's B-site is located close to the membrane surface, while the binding site shared by Na^+ and Ca^{2+} (A-site) is more exposed to the aqueous environment (Slaughter et el., 1988). This is based on the observations that amiloride derivatives have a preference for the B-site and that their potency correlates with the hydrophobicity of terminal guanidino nitrogen atom substitution. Consistent with this is the finding that bepridil interacts solely at the B-site. Bepridil is a very lipophilic agent and inhibits a number of other membrane-bound systems besides Na–Ca exchange, including voltage-dependent Ca^{2+} channels (Galizzi et al., 1986), the sarcolemmal Ca^{2+}-ATPase (via its interaction with a hydrophobic domain of calmodulin; Lamers et al., 1985), and K^+ channels (Reiser & Sullivan, 1986). Therefore, bepridil would be expected to partition easily into the membrane, causing a high local concentration to be present at substrate-binding sites located near the membrane surface. Despite this, there appears to be specificity to the interaction between bepridil and the

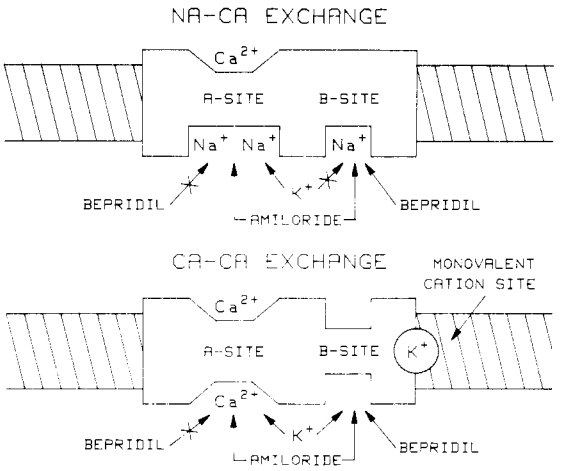

FIGURE 7: Schematic representation of the interactions of bepridil and amiloride with the Na–Ca exchange transporter. The two types of substrate-binding sites are represented by the A-site (the common Na^+ and Ca^{2+} binding site) and the B-site (the selective Na^+-binding site) as the carrier functions in either Na–Ca or Ca–Ca exchange reactions. The interactions of various agents with the transport protein are illustrated.

B-site because many hydrophobic cations do not inhibit Na–Ca exchange (unpublished observations), and other classes of membrane-active agents (e.g., ionic detergents) appear to block transport by different mechanisms (Philipson, 1984; Slaughter et al., 1988). Nonetheless, few structural similarities are apparent between bepridil and amiloride analogues, making it difficult to conceive a hypothetical structure for the B-site of the carrier.

One of the characteristics of bepridil inhibition that is clearly unique is the partial block of transport activity produced under certain conditions. For several reasons, this phenomenon cannot be a consequence of some spurious physical interaction, such as limited solubility of bepridil. Under similar ionic conditions, bepridil inhibits Na_i-dependent Ca^{2+} uptake and Na_o-dependent Ca^{2+} efflux with nearly equivalent potencies and identical Hill coefficients, yet block of the former is partial (i.e., 50%) while block of the latter is complete. If solubility of inhibitor were limiting, partial inhibition would be expected in both cases. Moreover, when experiments are performed in solutions of low ionic strength (i.e., sucrose), inhibition of Ca–Ca exchange is complete, even though block of Na_i-dependent Ca^{2+} uptake is partial at equivalent inhibitor concentrations. However, if vesicles are preincubated with bepridil before Ca^{2+} uptake is initiated, complete block of transport is observed. A major difference between these two experiments is that in the latter case inhibition is measured after an equilibrium has been established between Na^+ and bepridil and inhibitor is present at both sides of the membrane simultaneously. Therefore, this change in inhibitory pattern may be kinetically important. In addition, it has been noted that bepridil inhibition of Ca^{2+}-ATPase is complete (K_i = 40 μM; unpublished observations) under ionic conditions where Na–Ca exchange inhibition is partial, further indicating that solubility of bepridil is not limiting. Finally, inspection of the buffer solutions containing bepridil showed no physical evidence of a two-phase system. Taken together, these findings provide a compelling argument that the partial inhibitory profiles observed with bepridil are mechanistically meaningful.

The partial inhibition of Na_i-dependent Ca^{2+} uptake that is observed may provide a clue to the mechanism of this transport reaction. If it is assumed that bepridil functions under these conditions by interacting only with external carrier binding sites, maximal inhibition of 50% could indicate random

release of Na^+ from A- and B-sites. Upon turnover in the Ca^{2+}-uptake mode, the three Na^+ that occupy these sites must be released to solution in order to recycle the carrier and allow Ca^{2+} to bind at the external face of the protein. Since bepridil binds exclusively at the B-site, it can only interact when this site is unoccupied. During random release of substrates, the carrier would be accessible to inhibitor only 50% of the time during turnover. Because bepridil inhibition is completely reversible, a situation could be envisioned, with appropriate rate constants for inhibitor binding and transporter recycling, in which partitioning occurs between blocked and free carrier to yield 50% maximal inhibition of transport. Alternatively, if bepridil and Na^+ are initially present at time zero on the same surface of the membrane (e.g., as in Na_o-dependent Ca^{2+} efflux), equilibrium between blocked and free carrier can be driven completely to the blocked state by increasing inhibitor concentration. This would result in total inhibition of transport, as has been observed for Ca^{2+} efflux. Therefore, partial inhibition of Na–Ca exchange under certain conditions (e.g., when the transporter has Na^+ initially bound) may be a result of bepridil's ability to interact with only the B-site of this protein.

Partial inhibition of Ca–Ca exchange under certain conditions also has mechanistic importance. It has been postulated that monovalent cations which stimulate the rate of Ca–Ca exchange interact exclusively at the B-site (Slaughter et al., 1983; Reeves et al., 1984). Although some data obtained with amiloride derivatives are consistent with this idea, other data, especially a lack of correlation between different cations to stimulate Ca–Ca exchange rates and relieve amiloride block of transport activity, do not support this hypothesis (Slaughter et al., 1988). To examine this issue unambiguously, an inhibitor is required that will only interact at the B-site of the carrier. Since bepridil is such an agent, a competitive interaction between K^+ and bepridil would indeed suggest that monovalent stimulatory cations bind at the B-site. However, the observation that increasing K^+ concentration not only reduces the potency of bepridil but also reduces the maximal extent of inhibition that occurs implies that there is an additional monovalent cation binding site(s) that is not accessible to bepridil and that, when occupied, makes the carrier refractory to inhibition by this agent (Figure 7). It should be noted that the present data do not distinguish between whether the bepridil interaction can be controlled by this allosteric K^+ site acting alone or whether an additional interaction of K^+ at the B-site is required.

Of the various Ca^{2+} entry blockers that have been tested for the ability to inhibit Na–Ca exchange, only bepridil is an effective inhibitor at low concentrations. Whether this activity plays a role in bepridil's antiarrhythmic activity is presently not clear. There have been reports that other Ca^{2+} entry blockers (e.g., verapamil, diltiazem, and various dihydropyridines) will inhibit Na–Ca exchange in cardiac sarcolemmal membrane vesicles (Erdreich et al., 1983; Takeo et al., 1985a,b), but these compounds are only marginally active at very high concentration. In marked contrast, diltiazem is an effective inhibitor of the mitochondrial Na–Ca exchange reaction (Matlib et al., 1985). Interestingly, amiloride analogues that block Na–Ca exchange are also effective Ca^{2+} entry blockers and inhibit Ca^{2+} channels by interacting at a cation-binding site in the channel pore (Garcia et al., 1987). Unfortunately, one of the activities that prevents the use of currently available Na–Ca exchange inhibitors in physiological studies is this ability to block voltage-dependent Ca^{2+} channels at concentrations similar to that at which inhibition of Na–Ca exchange occurs. To remedy this situation, inhibitors must be discovered that will bind to the transport protein with high specificity at sites other than substrate-binding sites. Whether these agents are natural product toxins or antibody reagents, their use in deciphering the physiological role of Na–Ca exchange will be invaluable.

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