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Effect of Inorganic Calcium Channel Blockers on Dihydropyridine Binding to Cardiac Sarcolemma

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SUMMARY

The effects of the inorganic Ca^{2+} channel blockers Cd^{2+} and La^{3+} on dihydropyridine (DHP) binding in highly enriched cardiac sarcolemma preparations has been examined. Cd^{2+} produced an apparent competitive inhibition of DHP binding with a K_i of 60 μM. DHP binding in the presence of La^{3+} produced nonlinear Scatchard plots when performed in intact membrane vesicle preparations. Evaluation of DHP binding in saponin-permeabilized vesicles or in the presence of the ionophore A23187 yielded linear Scatchard profiles in the presence of La^{3+}. Under these conditions, La^{3+} produced a mixed-type inhibition, with effects on both K_d and B_{max}. These results suggest that La^{3+} must have access to the interior of sealed vesicles for expression of full inhibitory activity and that La^{3+} may produce inhibition of DHP binding by interaction with only one surface of the membrane. In order to evaluate the sidedness of the La^{3+} interaction, membrane preparations consisting of 74% right side out and 26% leaky vesicles were isolated. In the absence of saponin, La^{3+} decreased maximum DHP binding in this preparation approximately 25%, with no significant change in K_d. When binding was performed in saponin-permeabilized preparations, however, La^{3+} produced dramatic decreases in both DHP binding affinity and capacity. These results are consistent with the hypothesis that La^{3+} produces inhibition of DHP binding by interaction with sites accessible only from the cytoplasmic membrane surface. To obtain additional support for this hypothesis, DHP binding was examined in rat ventricular myocytes grown in culture. La^{3+} and Cd^{2+}, at concentrations in the extracellular buffer that substantially inhibited K^+ depolarization-induced $^{45}Ca^{2+}$ influx, had little or no effect on DHP binding.

The Ca^{2+} channel of the myocardial cell is responsible for the triggered influx of Ca^{2+} during the cardiac action potential. Although Ca^{2+} is the physiological ion of interest, this channel is known to allow Sr^{2+}, Ba^{2+}, and, to a much lesser extent, Mn^{2+} to enter the cell (for review see, Ref. 1). Currents carried by Ba^{2+} are generally greater in magnitude, when compared with Ca^{2+} currents. However, currents obtained in ion mixtures of Ba^{2+} and Ca^{2+} are smaller than currents observed in solutions containing a comparable concentration of either ion alone (2, 3). This anomalous mole-fraction effect has led to a model in which the channel is envisioned as a multi-ion pore with at least two high affinity Ca^{2+} binding sites (translocation sites) (2, 3). These sites impart channel selectivity for divalent cations. In the complete absence of divalent cations, the channel becomes relatively nonselective, allowing passage of monovalent cations such as Na^+, Li^+, and K^+ (for review of the evidence in support of this model, see Ref. 4).

Several inorganic divalent cations are known to be blockers of Ca^{2+} channel current. These include Co^{2+}, Ni^{2+}, Mn^{2+}, Cd^{2+}, and, to a much lesser extent, Mg^{2+}. The trivalent cations of the lanthanide series are also potent inhibitors of the Ca^{2+} channel. The inorganic channel blockers may inhibit current flow by interaction with the translocation sites within the aqueous pore structure (4, 5) or by interaction with some external site (3, 6). Several organic Ca^{2+} channel blockers are also known to diminish both Ca^{2+} and Ba^{2+} currents. These include compounds in the DHP, phenylalkylamine, and benzothiazepine classes.

It has been known for some time that the DHP Ca^{2+} channel agonists and antagonists bind with high affinity to sites in nerve and muscle. Inorganic di- and trivalent cations appear to have multiple effects on DHP binding measured in crude membrane preparations from these tissues. Most appear to stimulate binding at low concentrations whereas several, including Cd^{2+} and La^{3+}, produce inhibition above 10 μM (7–10). The inhibition of DHP binding produced by La^{3+} and Co^{2+} in rat cortical membranes appears to result from a decrease in the maximal binding capacity (B_{max}) rather than an effect on the affinity, because little change in the equilibrium dissociation constant (K_d) was observed (8). A similar characterization of the effect of inorganic Ca^{2+} channel blockers on DHP binding parameters in heart has not been performed.

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ABBREVIATIONS: DHP, dihydropyridine; RO, right side out; QNB, quinuclidinyl benzilate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PG, pregradient; IO, inside out.

In a recent study, the effect of divalent cation chelation on DHP binding was examined in highly enriched sarcolemma preparations isolated from canine ventricle (11). DHP binding in the presence of EDTA was only partially inhibited, when measured in intact membrane vesicles. However, permeabilization of the vesicles with saponin or by a freeze/thaw procedure resulted in greater than 90% loss of binding in the presence of EDTA. Ouabain binding, which under most conditions requires the presence of Mg^{2+} [or some other divalent cation (12)] at the cytoplasmic membrane surface, exhibited similar inhibitory profiles when measured in the presence and absence of EDTA, suggesting that divalent cations trapped within sealed vesicles of RO orientation support both ouabain and DHP binding. These results suggested that divalent cations stimulate DHP binding in isolated cardiac sarcolemma preparations by interaction with sites accessible from only the cytoplasmic membrane surface. The location of the site(s) at which inorganic cations produce inhibition of DHP binding and the identity of these sites with those responsible for inhibition of channel current has not been established.

The purpose of the present study was to evaluate the effects of two Ca^{2+} channel blockers, Cd^{2+} and La^{3+}, on high affinity DHP binding in highly enriched cardiac sarcolemma preparations in an effort to 1) determine whether these blockers interact with sites on the normal cytoplasmic membrane surface or with the extracellular membrane face and 2) determine whether these ions produce inhibition of DHP binding in heart by effects on B_{max} or K_d.

Experimental Procedures

Materials. [^{3}H]Nitrendipine, (+)-[^{3}H]PN200-110, [^{3}H]QNB, and [^{3}H]ouabain were obtained from New England Nuclear (Boston, MA). Unlabeled nitrendipine was generously supplied by Dr. Alexander Scriabine (Miles Laboratories, New Haven, CT). Saponin and A23187 were obtained from Calbiochem (San Diego, CA). Pyruvate kinase and lactate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Isolation of sarcolemma-enriched preparation from canine ventricle. Membrane preparations were isolated from canine ventricle by two different procedures. In *procedure A*, sarcolemma-enriched preparations were isolated according to the procedure of VanAlstyne *et al.* (13) with the modifications described by Frankis and Lindenmayer (14). Following the isolation procedure, the sarcolemma preparation was suspended in 10 mM Tris·HCl, pH 7.4, stored at 2°, and used within 24 hr. This preparation is highly enriched in surface membrane markers (13) and has been shown to exhibit high affinity nitrendipine binding with a K_d of 0.09 nM a B_{max} of approximately 1 pmol/mg of protein at 22° (11, 15). Membrane preparations isolated by procedure A were employed for all DHP binding studies reported except those shown in Fig. 6. In *procedure B*, membrane preparations were isolated according to the procedure of VanAlstyne *et al.*.[1] Briefly, canine ventricular tissue (80–120 g of wet weight) was minced in a Cuisinart food processor and homogenized (single pass of a motor-driven Teflon pestle in a 200-ml Potter Elvehjem glass tube) in 5 volumes of 250 mM sucrose, 15 mM $NaHCO_3$, pH 7.0, at 2° (medium A). The homogenate was centrifuged at 1600 × *g* for 10 min. The supernatant was discarded and the pellet was resuspended in 5 volumes of medium A, homogenized, and centrifuged as above. The resulting pellet was resuspended in 5 volumes of 10 mM Tris·HCl, pH 7.2, at 2° (medium B), with four passes of a motor-driven Teflon pestle. The homogenate was centrifuged at 1600 × *g* for 10 min and the resulting supernatant was removed and saved. This step was repeated four times and the pooled supernatants were subjected to centrifugation at 40,000 × *g* for 20 min. The resulting pellets were resuspended in 80 ml of 10 mM Tris·HCl, pH 7.0, at 2° (medium C). This fraction is designated as the PG fraction. Aliquots of this suspension (10 ml) were layered over 24% sucrose (14 ml) in 10 mM Tris·HCl, pH 7.4, at 2°, and centrifuged at 73,400 × *g* for 30 min. A sarcolemma-enriched fraction (L1) was obtained at the interface between the buffer and the sucrose layer, and a pellet (P1) was obtained at the bottom of the tube. The L1 fraction was harvested, diluted 5- to 10-fold with 10 mM Tris·HCl, pH 7.4, at 2° (medium D), and centrifuged at 74,300 × *g* for 20 min. The resulting pellet was resuspended in 7 ml of medium D and stored overnight on ice.

The L1 fraction (3 ml) was layered over 20 ml of a solution containing 250 mM sucrose, 10 mM Tris·HCl, pH 7.4, at 2°, and Percoll (6.95 g/100 ml). Centrifugation at 73,400 × *g* for 12 min yielded upper, middle, and bottom layers (L2, L3, and L4), which were harvested from the percoll gradient, diluted with medium D, and centrifuged for 60 min at 73,400 × *g*. Membranes from these three fractions were diluted approximately 5-fold and centrifuged at 230,000 × *g* for 30 min. At this stage, the membranes form a thin sheet over a small Percoll pellet at the bottom of the tube. The membranes from the L3 and L4 fractions were resuspended without disturbing the Percoll pellet, in a small volume of 10 mM Tris·HCl, pH 7.4, at 22° (medium E) and were stored on ice. The membranes of the L2 layer were distributed over the Percoll pellet in two distinct layers, an upper white layer and a bottom amber-colored layer. These two layers were separated by washing the pellet with a small volume of medium E, using a Pasteur pipet. These two fractions are designated L2a and L2b for the white and amber membranes, respectively. Any membranes not used within 24 hr following isolation were kept frozen at −80° until use. Membrane protein was determined by the method of Lowry *et al.* (16), using bovine serum albumin as the standard.

Measurement of specific ouabain binding. Total [^{3}H]ouabain binding was determined according to the method of Inagaki *et al.* (17), in the presence of 5 mM Tris·H_3PO_4, 5 mM $MgCl_2$, 50 mM Tris·HCl, pH 7.4, and 2×10^{-6} M ouabain, with [^{3}H]ouabain (specific activity, 100 cpm/pmol). The binding reactions were terminated after 30 min at 37° by filtration through Millipore nitrocellulose filters (Type HA; 0.45μm pore size) on a Hoefer filtration apparatus. The filters were washed five times with 5-ml aliquots of ice-cold distilled water and the radioactivity associated with each filter was determined by standard liquid scintillation technique. Nonspecific binding was determined by including 1 mM unlabeled ouabain in the binding reaction medium. Specific binding was defined as total minus the nonspecific.

Measurement of specific QNB binding. Aliquots of membrane preparation in 10 mM Tris·HCl, pH 7.4, were added to phosphate buffer containing 19.5 mM KH_2PO_4, 40.5 mM Na_2HPO_4, pH 7.4, and 1.5 nM [^{3}H]QNB (100 cpm/fmol). After 1 hr at 22°, the binding was terminated by filtration through Whatman Type A/E glass fiber filters. The filters were washed five times with 5-ml aliquots of ice-cold distilled water and the radioactivity associated with the filters was determined by the liquid scintillation technique. Nonspecific binding was determined by including 1 μM atropine in the binding reaction medium. Specific binding was defined as total minus nonspecific.

Measurement of equilibrium DHP binding. DHP binding was measured according to methods previously described (15). Aliquots of the sarcolemma preparation were salt-"loaded" by incubation of the preparation with buffered salt solution containing 140 mM KCl, 10 mM Tris·HCl, pH 7.4 (DHP binding buffer), for 15–18 hr at 2°. Aliquots of loaded preparation were added to binding buffer containing [^{3}H]nitrendipine or (+)-[^{3}H]PN200-110 (specific activity, 80–100 cpm/fmol). The reactions at 22° were terminated after 120 min by filtration through glass fiber filters. The filters were washed seven times with 5-ml aliquots of ice-cold distilled water and the radioactivity trapped on the filter was determined by the liquid scintillation technique. Nonspecific binding was determined by including 100 nM unlabeled nitrendipine in the binding reaction medium.

Measurement of ouabain-sensitive Na^+,K^+-ATPase activity.

[1] E. VanAlstyne *et al.*, manuscript in preparation.

Na^+,K^+-ATPase activity was determined according to the method of Schwartz *et al.* (18), using the pyruvate kinase/lactate dehydrogenase linked enzyme system. Briefly, aliquots of sarcolemma preparation in 10 mM Tris·HCl, pH 7.4, were added to a cuvette with reaction buffer containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 25 mM Tris·HCl, pH 7.4, 2.5 mM phosphoenolpyruvate, 0.4 mM NADH, and 10 units each of pyruvate kinase and lactate dehydrogenase. Following incubation at 37° for 3 min, the reaction was initiated by addition of Na_2ATP (final concentration, 2.5 mM). The oxidation of NADH was monitored on a Beckman DU-40 spectrophotometer at a wavelength of 340 nm. Activity was determined in the absence and presence of 1 mM ouabain. All assays were performed in duplicate on each membrane preparation examined.

Permeabilization of the membrane vesicles with saponin. In order to express maximum [^{3}H]ouabain binding and Na^+,K^+-ATPase activity, sarcolemma preparations were pretreated for 15–18 hr at 2° with the detergent-like compound saponin, at a final concentration of 0.3 mg/ml. Protein concentration during the incubation with saponin was 0.3 mg/ml. These conditions were found to be optimum in this preparation for stimulation of both ouabain binding and ouabain-sensitive Na^+,K^+-ATPase activity by saponin. Aliquots of saponin-treated preparations were subsequently employed for the binding and enzyme studies, as described above.

Cell Culture. Neonatal rat heart ventricular myocytes were prepared by a modification of the method of Mark and Strasser (19). Briefly, ventricular cells were obtained for primary culture by trypsin digestion of 1- to 3-day-old neonatal ventricular tissue cut into 1- to 2-mm cubes. Cells were cultured for 2 days in 35-mm Falcon culture dishes in a 1:1 mixture of Dulbecco's modified Eagle's medium and Hank's F12 medium (GIBCO Laboratories, Grand Island, NY), with HEPES buffer and 10% fetal calf serum, in a water-saturated air atmosphere. Media also contained 50 μg/ml 5-bromo-2′-deoxyuridine to inhibit fibroblast proliferation.

DHP binding to intact ventricular myocytes. Following the above culture procedure, the cells were removed from the incubator and their culture medium was aspirated and replaced with 1 ml of binding buffer containing 95 mM NaCl, 50.4 mM KCl, 1 mM $MgCl_2$, 0.02 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES/Tris, pH 7.4, and various concentrations of (+)-[^{3}H]PN200-110. Nonspecific binding was determined in duplicate dishes by inclusion of 10^{-6} M nifedipine. Binding reactions were terminated after 75 min at room temperature (22°) by rapid aspiration of the binding buffer followed by washing of the cells three times with 1.5 ml of ice-cold binding buffer. Cells were digested with 0.5% NaOH and the radioactivity was determined by liquid scintillation counting.

Uptake of $^{45}Ca^{2+}$ in ventricular myocytes. $^{45}Ca^{2+}$ uptake was determined as previously described (20, 21). Briefly, cells were removed from the incubator and their culture medium was aspirated and replaced by 1 ml of an EGTA solution containing 110 mM NaCl, 5.4 mM KCl, 0.2 mM EGTA, 25 mM glucose, and 20 mM HEPES/Tris, pH 7.4. After 5 min, this solution was aspirated and replaced by either 1 ml of a low K^+ solution containing 110 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 20 mM HEPES/Tris pH 7.4, or a high K^+ solution containing 80 mM KCl in equimolar substitution with NaCl. Both solutions contained 10 μCi of $^{45}Ca^{2+}$ ml. The reaction was terminated after 5 sec by rapid aspiration and washing of the cells three times with 1.5 ml of ice-cold EGTA solution. Radioactivity was determined on NaOH digests of the cells. For both the binding and Ca^{2+} uptake studies, protein was determined on parallel dishes of cells using the method of Lowry *et al.* (16).

Data analysis. Curve-fitting computer programs that employed the Marquardt algorithm for fitting nonlinear models were used for analysis of equilibrium binding data. Specific details of the fitting procedure have been described elsewhere (15). Significant differences between mean values for K_d and B_{max} under the various experimental conditions was determined using the Student *t* test, with Bonferroni's correction for multiple comparisons where appropriate.

Results

Effect of inorganic Ca^{2+} channel blockers on nitrendipine binding. A number of inorganic metal cations are known to be inhibitors of Ca^{2+} channel current in cardiac myocytes. In addition, several of these cations are known to inhibit nitrendipine binding in crude microsomal preparations from heart. Fig. 1 shows the effect of three of these inorganic metal cations on nitrendipine binding in the isolated cardiac sarcolemma preparation. Mn^{2+} did not inhibit nitrendipine binding under these conditions but produced a slight increase in binding between 0.003 and 1 mM. Similar profiles have been obtained for Mg^{2+}, Ba^{2+}, and Ca^{2+} ions (data not shown). However, Cd^{2+} and La^{3+} are potent inhibitors of nitrendipine binding. As seen in Fig. 1, Cd^{2+} is a more effective inhibitor of nitrendipine binding when compared with La^{3+}. In all preparations examined, La^{3+}, even at concentrations above 1 mM, never inhibited more than 60%, whereas Cd^{2+} at 1 mM inhibited greater than 90%. The inhibition of nitrendipine binding by Cd^{2+} was examined in greater detail in paired equilibrium binding experiments (Fig. 2). Cd^{2+} produced an apparent competitive inhibition of nitrendipine binding, with a K_i of 60 μM. At the highest concentration tested, Cd^{2+} apparently decreased B_{max} from 1.064 ± 0.036 to 0.878 ± 0.108 pmol/mg of protein. This decrease, however, was not significant.

Effect of La^{3+} on DHP binding. In contrast to the effect of Cd^{2+} on DHP binding, equilibrium nitrendipine binding experiments revealed nonlinear Scatchard plots in the presence of La^{3+} (Figs. 3, A and B). In five paired experiments, DHP binding in the presence of La^{3+} was better fit in all cases by a

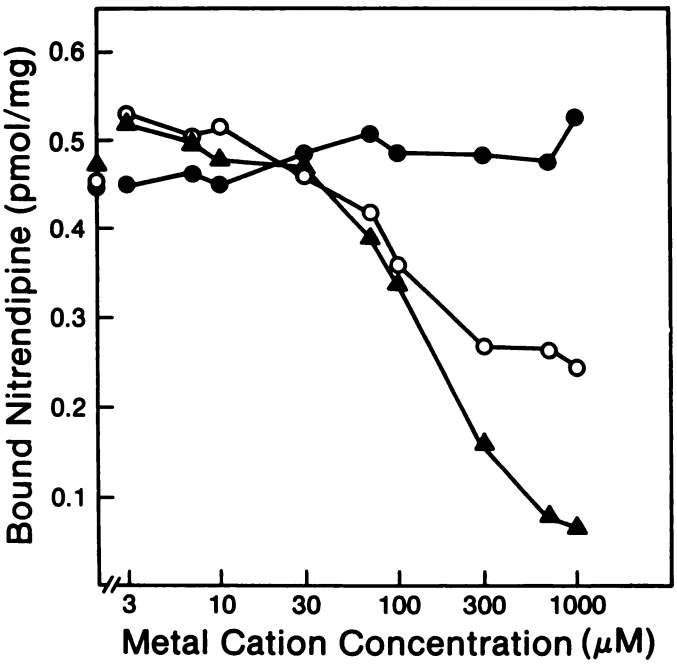

Fig. 1. Effect of inorganic Ca^{2+} channel blockers on nitrendipine binding. Sarcolemma preparation was salt-loaded by incubation at 2° for 15–18 hr with a solution containing 140 mM KCl and 10 mM Tris·HCl, pH 7.4 at 22° (DHP binding buffer). Aliquots of loaded preparation (~25 μg of protein) were added to reaction medium, at 22° containing salts identical to those in the loading medium, [^{3}H]nitrendipine at a free concentration of 0.2 nM, and the indicated concentration of $MnCl_2$ (●), $LaCl_3$ (○), or $CdCl_2$ (▲). Specific binding was determined as described in Experimental Procedures. Each *point* represents the average of experiments performed in duplicate on two sarcolemma preparations.

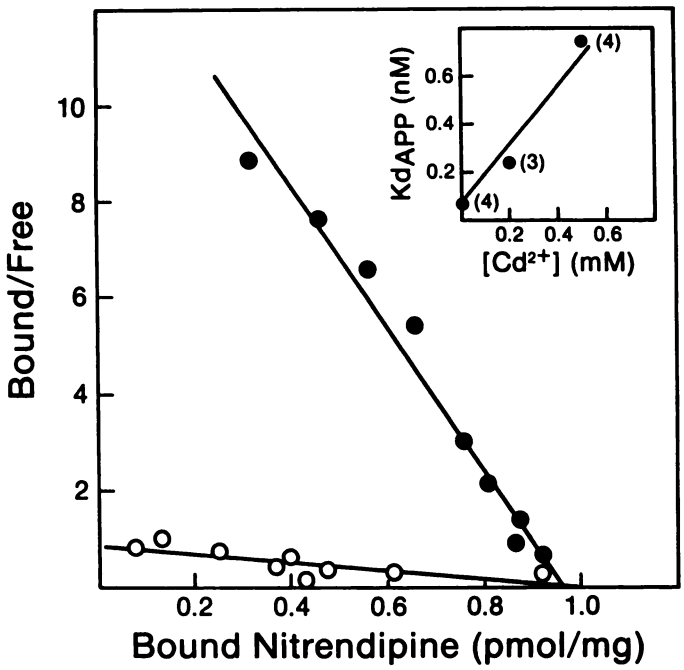

Fig. 2. Effect of Cd^{2+} on equilibrium nitrendipine binding. Aliquots of salt-loaded sarcolemma preparation were added to DHP binding buffer containing [^{3}H]nitrendipine ranging from 0.036 to 1.24 nM for control (●) or binding buffer containing 0.5 mM $CdCl_2$ and [^{3}H]nitrendipine ranging in concentration from 0.086 to 3.1 nM (○). Specific binding ranged from 93 to 78% of total binding for control and from 67 to 56% of total binding for assays performed in the presence of Cd^{2+}. *Inset*, replot of the apparent equilibrium dissociation constant (K_d) versus Cd^{2+} concentration. *Values in parentheses*, number of sarcolemma preparations examined, each in duplicate. In this and all subsequent figures, the units of Bound/Free are pmol/mg of protein/nM.

two-site model, based on residual sum of squares values. In two of five, the improved fit was significant at $p < 0.05$ and in one at $p < 0.06$, based on the F statistic calculated as previously described (15). A Scatchard plot that was linear under control conditions and nonlinear in the presence of La^{3+} would suggest the presence of multiple DHP binding sites with differential sensitivity to inhibition by La^{3+} or possibly a La^{3+}-induced negative cooperativity. However, because these preparations consist of sealed RO, IO, and leaky vesicles (14), an alternative explanation for the curvilinear nature of the Scatchard plot is that La^{3+} inhibits in a sidedness fashion, i.e., by interaction with either the normal cytoplasmic or extracellular membrane surface, but not with both. The inability of La^{3+} to cross the vesicle membrane would limit the inhibition of nitrendipine binding by La^{3+} to a single population of vesicles, either RO or IO vesicles, depending on the sidedness of the La^{3+} interaction. To test this possibility, the effect of La^{3+} on nitrendipine binding was examined in the absence and presence of the ionophore A23187 (Fig. 3B). This ionophore, which is commonly used to transport Ca^{2+} and Mg^{2+} across biological membranes, will bind a variety of divalent cations and forms a strong complex with La^{3+} (22). Additionally, it has been shown that A23187 will transport lanthanides across cell membranes (23). Nitrendipine binding in the presence of La^{3+} produced nonlinear Scatchard profiles in the absence of A23187. However, nitrendipine binding in the presence of A23187 was linear, with a dramatic change in K_d reminiscent of the effects seen with Cd^{2+}. A similar profile was observed when binding of the pure enantiomer (+)-PN200-110 was performed in the absence and presence of the permeabilizing agent saponin (Fig. 4A). When performed in intact membrane vesicles, the binding of (+)-PN200-110 produced nonlinear Scatchard profiles in the presence of La^{3+}. However, binding in saponin-pretreated preparations was linear in the presence of La^{3+}. In the absence of La^{3+}, neither saponin nor A23187 were found to have any effect on DHP binding parameters. When the vesicles were permeabilized with saponin, La^{3+} dose dependently inhibited (+)-PN200-110 binding, demonstrating significant increases in K_d and reductions in B_{max} at each concentration of La^{3+} examined (Fig. 4B; Table 1). These results are consistent with the hypothesis that La^{3+} must have access to the interior of sealed vesicles for expression of maximum inhibitory activity and suggest that La^{3+} interacts with the DHP-binding protein in a sidedness fashion.

Effect of Ca^{2+} on the inhibition of DHP binding produced by Cd^{2+} and La^{3+}. The ability of Ca^{2+} to overcome the inhibition of DHP binding produced by Cd^{2+} and La^{3+} was examined in paired equilibrium binding experiments. Initial experiments revealed that the inhibition produced by 0.2–0.3 mM Cd^{2+} was unaffected by the presence of 0.01 to 10 mM Ca^{2+}. It was, however, possible to partially recover binding in the presence of 0.03 mM La^{3+} by elevation of the Ca^{2+} concentration in the binding buffer to 10 mM (Fig. 5). In three paired experiments, the mean ± SE K_d for (+)-PN200-110 was 16.4 ± 0.26, 53.9 ± 9.4, and 30.6 ± 1.3 pM for binding in the presence of 10 μM $CaCl_2$ (a), 10 μM $CaCl_2$ plus 30 μM $LaCl_3$ (b), and 10 mM $CaCl_2$ plus 30 μM $LaCl_3$ (c), respectively. Thus, whereas high concentrations of Ca^{2+} can partially reverse the effect of low

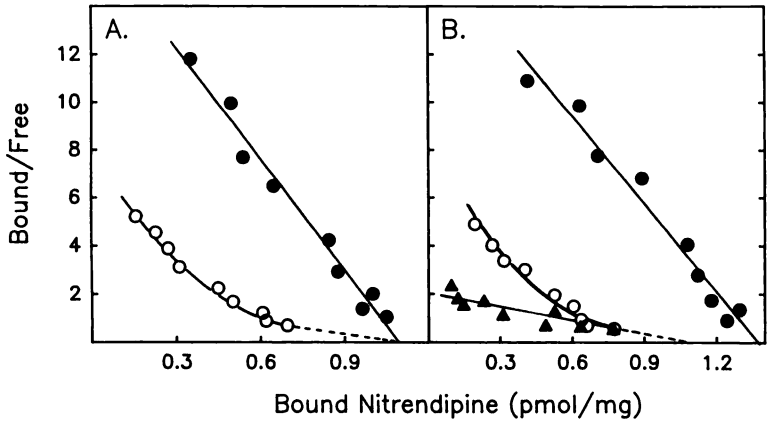

Fig. 3. A, Effect of La^{3+} on equilibrium nitrendipine binding. Specific [^{3}H]nitrendipine binding was determined in binding buffer containing 10 μM $CaCl_2$ (●) or 10 μM $CaCl_2$ and 150 μM $LaCl_3$ (○). B, Effect of La^{3+} on nitrendipine binding in the absence and presence of the ionophore A23187. Specific [^{3}H]nitrendipine binding was determined in binding buffer containing 10 μM $CaCl_2$ (●) or in binding buffer containing 10 μM $CaCl_2$ and 100 μM $LaCl_3$ in the absence (○) or presence of 0.1 μg/ml A23187 (▲). The individual binding parameters obtained from the curve-fitting procedure were as follows. A, ●, K_d = 64.7 pM, B_{max1} = 1093 fmol/mg; ○, K_{d_1} = 90.5 pM, K_{d_2} = 1.68 nM, B_{max1} = 583 fmol/mg, B_{max_2} = 414 fmol/mg. B, ●, K_d = 82.1 pM, B_{max} = 1372 fmol/mg; ○, K_{d_1} = 76.0 pM, K_{d_2} = 0.3 nM, B_{max_1} = 598 fmol/mg, B_{max_2} = 395 fmol/mg; ▲, K_d = 0.45 nM, B_{max} = 964 fmol/mg.

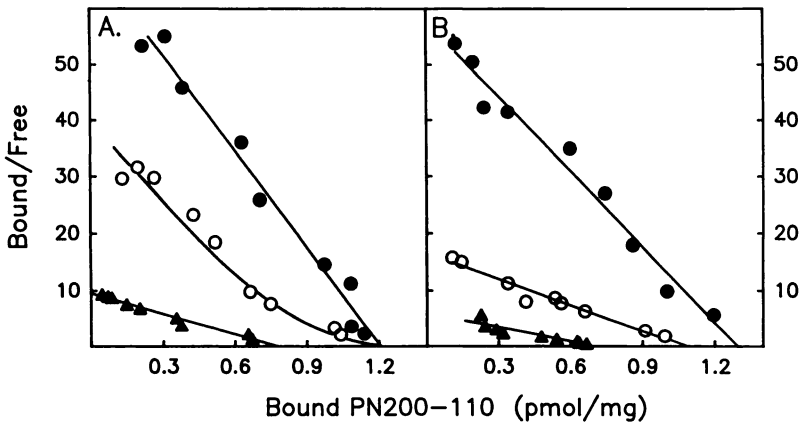

Fig. 4. A, Effect of La^{3+} on equilibrium (+)-PN200-110 binding in the absence and presence of saponin. Specific (+)-[^{3}H]PN200-110 binding was determined in DHP binding buffer containing 10 μM $CaCl_2$ (●) or 10 μM $CaCl_2$ and 30 μM $LaCl_3$ (○). Binding was also determined in sarcolemma preparation pretreated with saponin, as described in Experimental Procedures, in the presence of 10 μM $CaCl_2$ and 30 μM $LaCl_3$ (▲). The (+)-[^{3}H]PN200-110 concentration ranged from 4.1 to 500 pM; specific binding ranged from 94 to 81% of total binding for control and from 91 to 70% of total binding for assays performed in the presence of La^{3+}. B, Effect of La^{3+} on (+)-PN200-110 binding in the presence of saponin. Specific (+)-[^{3}H]PN200-110 binding was determined in saponin-pretreated preparations in binding buffer with 10 μM $CaCl_2$ and 3 μM $LaCl_3$ (●), 30 μM $LaCl_3$ (○), or 100 μM $LaCl_3$ (▲). The individual binding parameters obtained from the curve-fitting procedure were as follows. A, ●, K_d = 17 pM, B_{max} = 1207 fmol/mg; ○, K_{d_1} = 14.5 pM, K_{d_2} = 115 pM, B_{max_1} = 572 fmol/mg, B_{max_2} = 605 fmol/mg; ▲, K_d = 82.0 pM, B_{max} = 780 fmol/mg. B, ●, K_d = 22.5 pM, B_{max} = 1300 fmol/mg; ○, K_d = 64.7 pM, B_{max} = 1077 fmol/mg; ▲, K_d = 115 pM, B_{max} = 704 fmol/mg.

TABLE 1

Summary of effect of La^{3+} on (+)-PN200-110 binding in saponin-treated sarcolemma preparations

Specific (+)-[^{3}H] PN200-110 binding was determined in the presence and absence of La^{3+} as described in the legend to Fig. 4B. Values represent the mean ± standard error of duplicate binding experiments performed on six sarcolemma preparations.

Condition	K_d	B_{max}
	pM	fmol/mg
Control	15.9 ± 1.9	877 ± 102
0.03 mM La^{3+}	48.6 ± 4.0	706 ± 85
0.1 mM La^{3+}	115 ± 7.2	557 ± 56

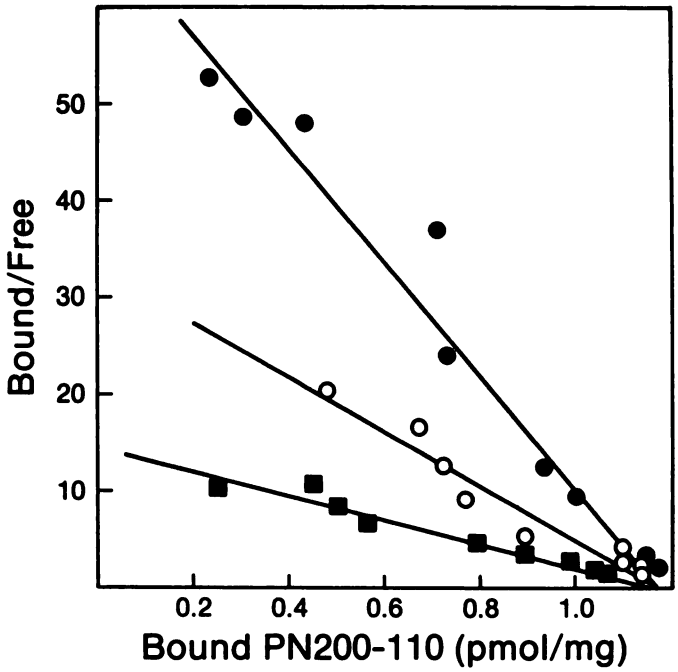

Fig. 5. Effect of Ca^{2+} on the inhibition of (+)-PN200-110 binding produced by La^{3+}. Specific (+)-[^{3}H]PN200-110 binding was determined in saponin-pretreated preparations in binding buffer with 10 μM $CaCl_2$ (●), 10 μM $CaCl_2$, and 30 μM $LaCl_3$ (■), or 10 mM $CaCl_2$ and 30 μM $LaCl_3$ (○).

concentrations of La^{3+} on DHP binding, inhibition of DHP binding by the inorganic channel blockers appears to be relatively insensitive to changes in Ca^{2+} concentration.

Isolation of RO Sarcolemma Vesicles. The results presented above suggest that La^{3+} inhibits DHP binding in a sidedness fashion. In order to localize the La^{3+} site of interaction to either the cytoplasmic face or the extracellular membrane surface, sarcolemma preparations consisting of sealed vesicles with predominately RO orientation were isolated from canine ventricle by the procedure of VanAlstyne et al. (procedure B; see Experimental Procedures). This isolation consists of a two-step enrichment procedure. First, a microsomal membrane fraction (PG) is layered over 24% sucrose. Following centrifugation, a sarcolemma-enriched fraction is isolated at the sucrose/buffer interface (L1) and a pellet (P1) is obtained at the bottom of the centrifuge tube. At this stage in the isolation procedure, the L1 fraction exhibits substantial enrichment in sarcolemma markers. When compared with the initial PG fraction, L1 was 17-, 22-, and 14-fold enriched in ouabain binding, Na^+,K^+-ATPase activity, and QNB binding, respectively (Tables 2 and 3). However, the L1 fraction is only 8.3-fold enriched in DHP binding sites relative to the PG fraction (Table 2). Thus, as shown previously (24, 25), DHP binding

TABLE 2

Comparison of ouabain, DHP, and QNB binding site densities of selected membrane fractions

Specific ouabain, (+)-PN200-110, and QNB binding were determined in saponin-treated membrane fractions, as described in Experimental Procedures. Values represent the mean ± standard error determined on fractions from four separate isolations; all values are paired. The numbers in parenthesis represent the values of ouabain and QNB binding relative to (+)-PN200-110 binding for each fraction.

Fraction	Binding		
	Ouabain	(+)-PN200-110	QNB
		pmol/mg	
PG	34.09 ± 3.4 (262)	0.130 ± 0.01 (1)	0.776 ± 0.10 (6)
P1	23.09 ± 3.4 (174)	0.133 ± 0.02 (1)	0.268 ± 0.06 (2)
L1	577.9 ± 25.0 (535)	1.08 ± 0.084 (1)	11.20 ± 1.5 (10)
L2a	866.8 ± 53.1 (500)	1.73 ± 0.18 (1)	16.36 ± 1.9 (9)
L2b	703.0 ± 64.7 (653)	1.08 ± 0.12 (1)	11.91 ± 2.0 (11)
L3	596.4 ± 27.0 (564)	1.06 ± 0.09 (1)	9.57 ± 1.5 (9)
L4	299.4 ± 8.4 (507)	0.590 ± 0.04 (1)	4.55 ± 0.89 (8)

TABLE 3

Evaluation of vesicle sidedness in the selected membrane fractions

Na^+, K^+-ATPase and ouabain binding were determined in the presence and absence of saponin, as described in Experimental Procedures. All values represent the mean ± standard error determined on each fraction from four separate isolation procedures; all values are paired. The values for percentage of RO, IO, and leaky (L) vesicles for each fraction were determined as described in the text.

Fraction	Na^+, K^+-ATPase		Ouabain binding		RO	IO	L
	− Saponin	+ Saponin	− Saponin	+ Saponin			
	μmol/mg/hr		pmol/mg			%	
PG	4.18 ± 0.81	12.44 ± 1.1	27.87 ± 3.3	34.09 ± 3.4	48.2	18.2	33.6
P1	3.10 ± 0.51	7.54 ± 0.64	13.83 ± 1.5	23.09 ± 3.4	18.8	40.1	41.1
L1	51.85 ± 3.8	272.7 ± 18.0	451.5 ± 20.8	577.8 ± 25.0	59.1	21.9	19.0
L2a	119.7 ± 9.6	446.6 ± 28.3	975.2 ± 68.4	866.8 ± 53.1	73.8		26.2
L2b	59.50 ± 16.3	321.5 ± 47.0	555.0 ± 71.2	703.0 ± 64.7	60.4	21.1	18.5
L3	55.53 ± 15.2	280.3 ± 24.6	426.9 ± 21.2	596.4 ± 27.0	51.8	28.4	19.8
L4	33.83 ± 5.7	135.7 ± 6.4	193.6 ± 10.2	299.4 ± 8.4	39.8	35.3	24.9

sites do not co-purify with other sarcolemma markers. This is clearly evident by the different ratio of ouabain:QNB: (+)-PN200-110 binding observed in the L1 fraction versus the P1 fraction (Table 2, numbers in parentheses). Furthermore, the P1 fraction has significantly greater amounts of DHP binding sites. The average yield of membrane protein in the P1 fraction was 586 ± 105 mg/100 g of ventricle (wet weight), whereas the yield of protein in the L1 fraction was 11.4 ± 2.9 mg/100 g of ventricle. Thus, the average total yield of DHP binding sites was 77.9 and 12.3 pmol for the P1 and L1 fractions, respectively. Clearly, a large number of DHP binding sites are associated with a membrane fraction that has a lower surface membrane marker enrichment.

The second step in this isolation procedure involves subfractionation of L1 on a Percoll gradient. This yielded four layers, with the highest surface membrane marker enrichment found near the top of the tube, i.e., in the lower buoyant density fraction (Table 2). Despite the fact that the L2a fraction exhibited a 3- to 4-fold higher surface membrane marker activity, compared with the L4 fraction, the ratio of ouabain:QNB:(+)-PN200-110 binding sites remained essentially constant and averaged 550:9:1 for each fraction, suggesting co-enrichment of DHP binding sites with sarcolemmal markers during the subfractionation on Percoll.

In order to determine the sidedness characteristics of the membrane fractions, Na^+,K^+-ATPase activity and ouabain binding were determined in the presence and absence of saponin (Table 3). Na^+,K^+-ATPase activity in the absence of saponin reflects the presence of leaky vesicles, whereas the activity in the presence of saponin represents the activity in all vesicles. Ouabain binding in the absence of saponin reflects binding to RO and leaky vesicles, whereas ouabain binding in the presence of saponin represents total binding in all vesicle populations. A summary of the sidedness characteristics of each fraction is given in Table 3. The results of this characterization indicate that the L2a fraction is devoid of IO vesicles, being composed of 74% RO and 26% leaky vesicles. The percentage of IO vesicles progressively increases from the L2a to the L4 fraction from the Percoll gradient. It is interesting to note that the fraction that has the highest percentage of IO vesicles, the P1 fraction, also has the greatest total yield of high affinity DHP binding sites (see above). This fraction also has the highest level of ouabain-insensitive ATPase activity, i.e., Mg^{2+}-ATPase (Table 4). In addition, the L2a fraction, which has the lowest IO percentage, also has the lowest Mg^{2+}-ATPase activity. The Mg^{2+}-ATPase activity observed in each fraction was essentially unaffected by saponin treatment, suggesting that this activity is associated with either leaky or IO vesicles. These characteristics (sidedness, DHP binding site number, and Mg^{2+}-ATPase activity) suggest that the P1 fraction may contain membranes of T-tubule origin (see Discussion).

TABLE 4

Evaluation of ouabain-insensitive ATPase activity

ATPase activity was determined, as described in Experimental Procedures, in the presence of 1 mM ouabain. All values represent the mean ± standard error determined on each fraction from four separate isolation procedures; all values are paired.

Fraction	Ouabain-insensitive ATPase activity	
	− Saponin	+ Saponin
	μmol/mg/hr	
PG	62.58 ± 8.6	59.33 ± 6.0
P1	63.99 ± 10.7	67.09 ± 5.8
L1	29.84 ± 4.0	31.65 ± 4.4
L2a	17.00 ± 2.9	14.24 ± 3.2
L2b	18.68 ± 4.2	19.34 ± 2.3
L3	28.96 ± 4.1	26.43 ± 4.7
L4	49.19 ± 8.6	49.24 ± 10.1

Effect of La^{3+} on DHP binding in RO vesicles. If La^{3+} inhibits DHP binding by interaction with the normal extracellular membrane surface, then binding of (+)-PN200-110 in the L2a fraction should be substantially inhibited by a high concentration of La^{3+} in the binding buffer without need for membrane permeabilization with saponin. If, on the other hand, La^{3+} produces inhibition of DHP binding by interaction with the cytoplasmic membrane surface, then binding in the L2a fraction should only be partially inhibited (because of the presence of 25% leaky vesicles in this fraction) and the effect of La^{3+} should be sensitive to the presence of saponin. To test these possibilities, binding of (+)-PN200-110 was determined in the absence of La^{3+}, in the presence of 0.2 mM La^{3+}, and in the presence of 0.2 mM La^{3+} and saponin (Fig. 6). The profile observed is clearly indicative of the profile expected for La^{3+} interaction with the cytoplasmic membrane surface. In the absence of saponin, La^{3+} decreased B_{max} to 73.6 ± 7.7% of control (Table 5), with no significant difference seen in K_d. This percentage is essentially identical to that of the RO vesicle population in this fraction (see Table 3). Thus, the presence of La^{3+} at the extracellular membrane surface has little effect on DHP binding in the isolated cardiac sarcolemma preparation. In the presence of saponin, La^{3+} produced a dramatic increase in K_d and decrease in B_{max}, suggesting that La^{3+} inhibits DHP

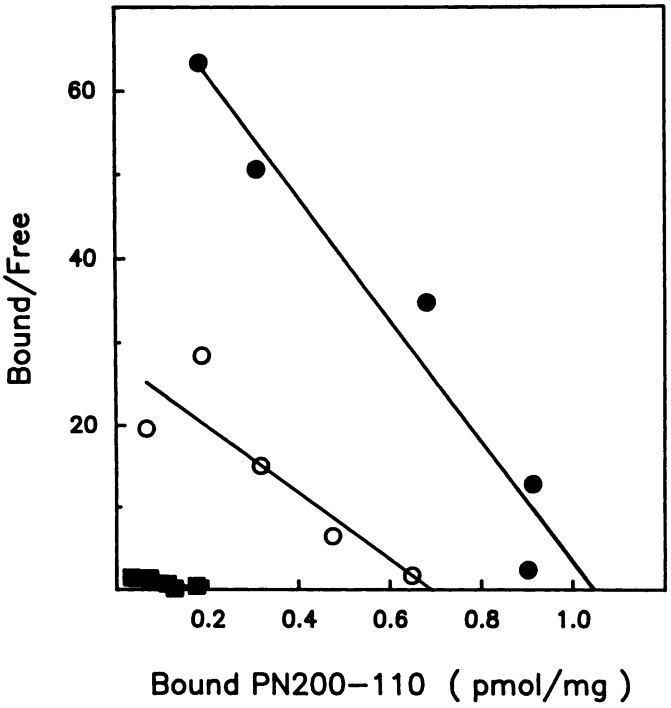

Fig. 6. Effect of La^{3+} on (+)-PN200-110 binding in sarcolemma preparation obtained from the L2a fraction from the Percoll gradient. Specific (+)-[^{3}H]PN200-110 binding was determined in binding buffer containing 10 μM $CaCl_2$ (●) or 10 μM $CaCl_2$ and 200 μM $LaCl_3$ (○). Binding was also determined in the presence of 10 μM $CaCl_2$ and 200 μM $LaCl_3$ in saponin-pretreated preparation from the same L2a fraction (■).

TABLE 5

Summary of (+)-PN200-110 binding parameters for the L2a sarcolemma fraction

Specific (+)-[^{3}H] PN200-110 binding was determined as described in the legend to Fig. 6. Values are paired and represent the mean ± standard error of binding experiments performed on three sarcolemma L2a fractions.

Condition	K_d	B_{max}
	pM	pmol/mg
Control	18.7 ± 4.0	1.406 ± 0.20
0.2 mM La^{3+}	27.3 ± 4.3	1.061 ± 0.24
0.2 mM La^{3+} in saponin-treated preparation	116 ± 38	0.456 ± 0.18

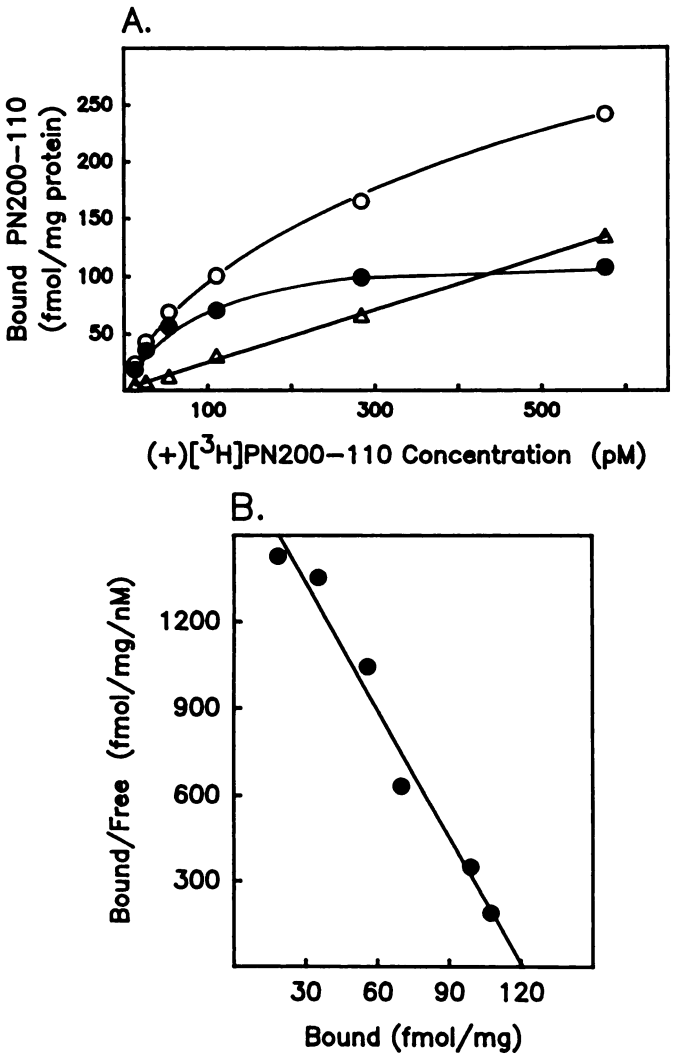

Fig. 7. A, Saturation isotherm for (+)-[^{3}H]PN200-110 binding to intact cardiac myocytes. Total (○), nonspecific (△), and specific (●) binding were determined in intact neonatal ventricular myocytes, as described in Experimental Procedures. B, Scatchard transformation of the specific binding from A showing a single class of binding sites with a K_d of 68.0 pM and a B_{max} of 120.9 fmol/mg of protein.

binding by interaction with sites accessible from the inside of sealed RO vesicles, i.e., the cytoplasmic membrane surface.

DHP binding and $^{45}Ca^{2+}$ uptake in ventricular myocytes. In order to obtain additional support for the hypothesis that inorganic Ca^{2+} channel blockers do not affect DHP binding via sites exposed to the extracellular solution, binding was examined in intact ventricular myocytes. Binding of (+)-PN200-110 to rat neonatal ventricular myocytes was specific and saturable over the concentration range studied (Fig. 7). The K_d and B_{max} values were 77.3 ± 4.9 pM and 113 ± 16 fmol/mg of protein, respectively (three experiments). Fig. 8 illustrates the effect of La^{3+} and Cd^{2+} on the binding of (+)-[^{3}H] PN200-110 to this myocyte preparation. La^{3+} slightly, although not significantly, stimulated binding over the concentration range of 5–200 μM. La^{3+} produced only slight inhibition of binding (20 ± 3%) at concentrations as high as 500 μM. Like La^{3+}, Cd^{2+} displayed little effect on DHP binding to intact cardiac myocytes, reducing binding by only 21 ± 5% at 500 μM. However, unlike their effects on (+)-[^{3}H]PN200-110 binding, both La^{3+} and Cd^{2+} were effective in reducing K^+-stimulated $^{45}Ca^{2+}$ uptake into the ventricular cells. This uptake has been previously shown to be abolished by 1 μM nifedipine and likely represents Ca^{2+} movement through L-type Ca^{2+} channels (20, 21). When La^{3+} (100 μM) or Cd^{2+} (500 μM) was present during the $^{45}Ca^{2+}$ uptake procedure (5-sec exposure), stimulated Ca^{2+} uptake was reduced by 75% in the presence of La^{3+} and was completely abolished by Cd^{2+} (Fig. 9). These results clearly indicate that, at concentrations that substantially inhibit $^{45}Ca^{2+}$ uptake, La^{3+} and Cd^{2+} in the extracellular solution have little or no effect on DHP binding to cardiac myocytes.

Discussion

The results of the present study demonstrate that the inorganic Ca^{2+} channel blockers Cd^{2+} and La^{3+} inhibit DHP binding in highly enriched cardiac sarcolemma preparations isolated from dog ventricle. At micromolar concentrations, both cations inhibit DHP binding by producing dramatic increases in the apparent K_d. However, La^{3+} clearly decreased B_{max} at all con-

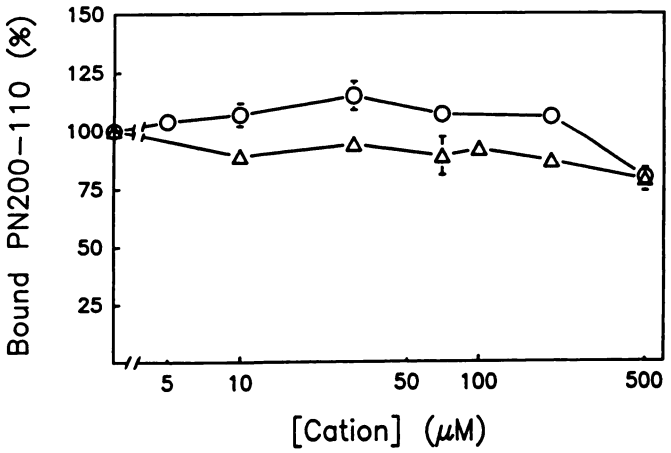

Fig. 8. Effect of La^{3+} and Cd^{2+} on (+)-PN200-110 binding to intact myocytes. Specific (+)-[^{3}H]PN200-110 binding to the ventricular myocytes was determined after 75 min in the absence or presence of various concentrations of La^{3+} (○) or Cd^{2+} (△). The free concentration of (+)-[^{3}H] PN200-110 in the binding assay was 40 pM. Values represent the mean ± standard error (three experiments). Where not shown, the standard error was less than the size of the *symbol* employed.

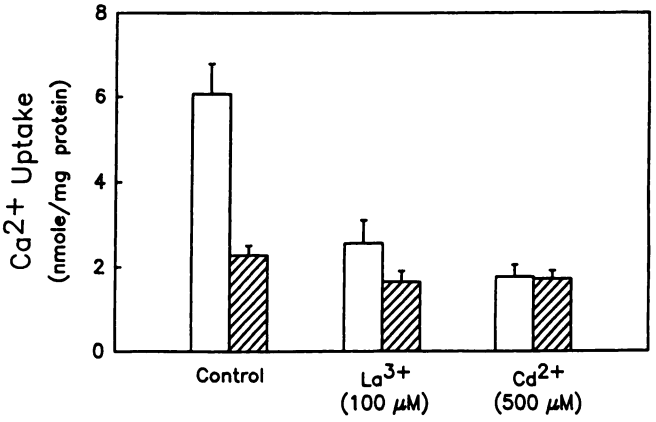

Fig. 9. Effect of La^{3+} and Cd^{2+} on $^{45}Ca^{2+}$ uptake in intact ventricular myocytes. La^{3+} (100 μM) or Cd^{2+} (500 μM) were added to the myocytes during the 5-sec measurement of $^{45}Ca^{2+}$ influx. □, Ca^{2+} uptake in 80 mM KCl buffer; ▨, Ca^{2+} uptake observed in 5.4 mM KCl buffer. Values represent the mean ± standard error (three experiments).

centrations tested and Cd^{2+} may decrease B_{max} at higher concentrations. These results, which represent the first description of the effects of inorganic Ca^{2+} channel blockers on DHP binding parameters in heart, differ from the findings of Gould *et al.* (8). These authors found that La^{3+} and Co^{2+} produce inhibition of nitrendipine binding in rat brain homogenates by affecting B_{max} without changing K_d. Although it is possible that tissue differences might explain this disparity, the sidedness of the La^{3+} interaction was not a consideration in previous studies (8).

Our initial studies revealed that Cd^{2+} could produce close to complete inhibition of DHP binding at a concentration of 1 mM, whereas La^{3+} inhibited only approximately 60% (Fig. 1). This finding was reminiscent of the inhibition produced by chelation of divalent cations with EDTA (11). EDTA produced a 60% inhibition of DHP binding when measured in intact vesicle preparations, but binding could be completely eliminated by EDTA if the vesicles were permeabilized with saponin. A detailed characterization of vesicle sidedness in that study revealed that low concentrations of divalent cations were required at the cytoplasmic membrane surface for expression of DHP binding (11). Clearly, a similar situation exists for inhibition of DHP binding by La^{3+}. The analysis, however, is complicated by the finding that Scatchard plots for the DHPs in intact vesicle preparations are curvilinear in the presence of La^{3+}. Two results suggest that the curvilinear nature of the Scatchard plot results from the sidedness of the La^{3+} interaction. First, La^{3+} produced linear Scatchard profiles with a dose-dependent decrease in DHP binding in saponin-treated preparations and, second, La^{3+} produced linear profiles in the presence of the ionophore A23187. Thus, it appears that La^{3+} access to the inside of sealed membrane vesicles is required for expression of full inhibitory activity.

Previous studies revealed that the vesicles in the sarcolemma preparation are essentially impermeable to divalent cations such as Mg^{2+} and Ca^{2+} (11). If, in fact, the inorganic channels blockers interact with only one side of the membrane to inhibit DHP binding, then similar inhibitory profiles should have been observed with both Cd^{2+} and La^{3+}. Clearly, however, there was little evidence of a permeability barrier with regard to the effect of Cd^{2+} on DHP binding. The most likely explanation for this finding is that Cd^{2+} can enter sealed vesicles via the Na^+/Ca^{2+} exchanger. It is well established that the cardiac Na^+/Ca^{2+} exchanger can promote Ca^{2+}/Ca^{2+} exchange in vesicular membrane preparations (26, 27). Furthermore, Trosper and Philipson (28) have reported stimulation of $^{45}Ca^{2+}$ efflux by extravesicular Cd^{2+} in cardiac membrane preparations, consistent with Ca^{2+}/Cd^{2+} exchange. Preliminary studies performed in the sarcolemma preparations employed here have also shown that vesicular $^{45}Ca^{2+}$ efflux is stimulated by the presence of extravesicular Cd^{2+} (but not La^{3+}), consistent with Ca^{2+}/Cd^{2+} exchange. Ca^{2+} trapped within sealed membrane vesicles during the isolation procedure (11, 26) could exchange for extravesicular Cd^{2+} during the time required for equilibration of the DHP binding reaction (90–120 min).

To confirm the sidedness of the La^{3+} interaction, it was necessary to evaluate the effect of La^{3+} on DHP binding in a sarcolemma preparation in which the predominate orientation of the vesicles was either RO or IO. To accomplish this goal, preparations were isolated by the procedure of VanAlstyne *et al.*[1] This procedure yields a highly enriched sarcolemma preparation in which all of the sealed vesicles are of RO orientation. In the present study, the sidedness characteristics and fold-enrichment of sarcolemma markers were confirmed before use in the La^{3+} studies. The results of this characterization revealed that a substantial number of DHP binding sites reside in a pellet fraction (P1) that has a relatively low enrichment with respect to sarcolemmal markers. Thus, at this stage in the isolation procedure, it would appear that DHP binding sites do not co-purify with surface membrane markers. Similar findings have been reported in both dog (24) and chick (25) heart preparations. Additionally, this pellet fraction has a high level of Mg^{2+}-ATPase activity and a high percentage of IO vesicles. Skeletal muscle T-tubule preparations also have a very high specific activity of DHP binding sites, the vesicles are predominately of IO orientation, and these preparations have characteristically high Mg^{2+}-ATPase activity (29, 30). These results suggest that the P1 membrane fraction may be a good starting material for the ultimate isolation of a T-tubule preparation from heart. Alternatively, the lack of co-purification of sarcolemma markers may reflect the presence of contaminating

surface membranes in the L1 fraction, which lack DHP binding sites. In this regard, sarcolemma preparations from heart have been reported to be substantially contaminated (up to 42%) with surface membrane from vascular endothelial cells (31). Recently, Colden-Stanfield *et al.* (32) showed that microsomal membrane preparations obtained from cultured vascular endothelial cells lack high affinity DHP binding sites and voltage-sensitive Ca^{2+} channels. These membrane preparations, however, exhibited a relatively high ouabain binding site density (32). Thus, the presence of endothelial cell plasmalemma in the sarcolemma preparation from dog heart could partially account for the relatively high ratio of ouabain:QNB:DHP binding observed in the L1 and Percoll fractions, relative to that seen in the P1 pellet.

Subfractionation of the membranes obtained in the L1 layer on Percoll yielded a membrane fraction that was extremely enriched in sarcolemma markers and that was composed of sealed vesicles of RO orientation. Maximum DHP binding in this fraction was inhibited approximately 25% by the presence of extravesicular La^{3+}, i.e., the percentage of leaky vesicles in this membrane fraction. This inhibition occurred without significant reduction in K_d. These results are consistent with the hypothesis that La^{3+} produces inhibition of DHP binding in isolated cardiac sarcolemma by interaction with sites accessible only from the cytoplasmic membrane surface. To further address this hypothesis, DHP binding was examined in intact rat ventricular myocytes grown in culture. Previous studies have demonstrated that both La^{3+} and Cd^{2+} inhibit DHP binding in isolated microsomal preparations from rat ventricle (7). However, the results of the present study clearly demonstrate that DHP binding to intact ventricular myocytes is little affected by La^{3+} or Cd^{2+} in the extracellular buffer at concentrations that dramatically inhibit Ca^{2+} influx via L-type Ca^{2+} channels.

The inability of La^{3+} and Cd^{2+} to inhibit DHP binding in intact myocytes raises an important question concerning the identity between the metal cation binding site responsible for inhibition of DHP binding and the binding site responsible for inhibition of current flow through the Ca^{2+} channel. Although the whole cell results suggest that the sites are not the same, two results obtained in the isolated sarcolemma preparation suggest that the two binding sites may be identical. First, the concentrations of Cd^{2+} and La^{3+} required to inhibit DHP binding are similar to those necessary to inhibit Ca^{2+} channel current measured electrophysiologically at either the whole-cell or single-channel level (1, 4, 5). Second, the inhibition of DHP binding by these cations is little affected by elevation of the Ca^{2+} concentration in the binding buffer (up to 10 mM), consistent with electrophysiological results that show that submillimolar concentrations of La^{3+} and Cd^{2+} can inhibit current carried by 3–100 mM Ba^{2+} or Ca^{2+} (1, 4, 5, 33). Thus, the affinity of the DHP-binding protein for Cd^{2+} and La^{3+}, relative to the affinity for Ca^{2+}, is in the range expected for interaction with the site responsible for inhibition of Ca^{2+} channel current. It is interesting to note, however, that Ba^{2+}, Ca^{2+}, and Mn^{2+} produce no inhibition of DHP binding at concentrations up to 3 mM. Thus, divalent cations that exhibit high affinity and rapid permeation through the channel, as described by Lansman *et al.* (5), have little effect on DHP binding, whereas cations such as La^{3+} and Cd^{2+} that are classified as having ultrahigh affinity and low permeation through the channel (5) produce potent inhibition of DHP binding. Clearly the affinity of a particular divalent cation for the DHP-binding protein is not the sole determinant responsible for inhibition of binding.

La^{3+} is thought to inhibit the Ca^{2+} channel by interaction with the translocation sites within the pore (4, 5) or with some external site on the channel (3, 6). If in fact La^{3+} binds in the sarcolemma preparation to the site responsible for inhibition of current flow, the results of the present study would favor the intrapore binding site. Ca^{2+} channels in the isolated cardiac sarcolemma preparation are probably in an inactivated state under the binding conditions employed in the present study. Likewise, DHP binding in the rat myocyte preparation was performed in high K^+ buffer, which would favor the inactivated state of the channel. Accessibility of the La^{3+} binding site from only the cytoplasmic surface would suggest that, in the inactivated state, the translocation sites are not accessible to ions in the extracellular space, i.e., the inactivation "gate" may shield the translocation sites from the extracellular solution. However, if the Ca^{2+} channel opens as it does during the measurement of K^+ depolarization-induced $^{45}Ca^{2+}$ influx, La^{3+} and Cd^{2+} gain access to sites within the pore structure, which in the isolated membranes may be accessible only via the cytoplasmic membrane surface because of channel inactivation. Alternatively, the inorganic cation binding sites responsible for inhibition of current flow and for inhibition of DHP binding may be unique sites on the channel structure.

Acknowledgments

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References

1. Hagiwara, S., and L. Byerly. The calcium channel. *Annu. Rev. Neurosci.* **4**:69–125 (1981).
2. Hess, P., and R. W. Tsien. Mechanism of ion permeation through calcium channels. *Nature (Lond.)* **309**:453–456 (1984).
3. Almers, W., and E. W. McCleskey. Non-selective conductance in calcium channels of frog muscle: calcium selectivity in a single-file pore. *Nature (Lond.)* **353**:585–608 (1984).
4. Tsien, R. W., P. Hess, E. W. McCleskey, and R. L. Rosenberg. Calcium channels: mechanism of selectivity, permeation and block. *Annu. Rev. Biophys. Biophys. Chem.* **16**:265–290 (1987).
5. Lansman, J. B., P. Hess, and R. W. Tsien. Blockade of current through single calcium channels by Cd^{2+}, Mg^{2+}, and Ca^{2+}. *J. Gen. Physiol.* **88**:321–347 (1986).
6. Kostyuk, P. G., S. L. Mironov, and Y. M. Shuba. Two ion-selecting filters in the calcium channel of the somatic membrane of mollusc neurons. *J. Membr. Biol.* **76**:83–93 (1983).
7. Ehlert, F. J., W. R. Roeske, E. Itoga, and H. I. Yamamura. The binding of [^{3}H]nitrendipine to receptors for calcium channel antagonists in the heart, cerebral cortex, and ileum of rats. *Life Sci.* **30**:2191–2202 (1982).
8. Gould, R. J., K. M. M. Murphy, and S. H. Snyder. [^{3}H]Nitrendipine-labeled calcium channels discriminate inorganic calcium agonists and antagonists. *Proc. Natl. Acad. Sci. USA* **79**:3656–3660 (1982).
9. Janis, R. A., J. G. Sarmiento, S. C. Maurer, G. T. Bolger, and D. J. Triggle. Characteristics of the binding of [^{3}H]nitrendipine to rabbit ventricular membranes: modification by other Ca^{++} channel antagonists and by the Ca^{++} channel agonist Bay K 8644. *J. Pharmacol. Exp. Ther.* **231**:8–15 (1984).
10. Luchowski, E. M., F. Yousif, D. J. Triggle, S. C. Maurer, J. G. Sarmiento, and R. A. Janis. Effects of metal cations and calmodulin antagonists on [^{3}H] nitrendipine binding in smooth and cardiac muscle. *J. Pharmacol. Exp. Ther.* **230**:607–613 (1984).
11. Schilling, W. P. Effect of divalent cation chelation on dihydropyridine binding in isolated cardiac sarcolemma vesicles. *Biochim. Biophys. Acta* **943**:220–230 (1988).
12. Schwartz, A., H. Matsui, and A. H. Laughter. Tritiated digoxin binding to ($Na^+ + K^+$)-activated adenosine triphosphate: possible allosteric site. *Science (Wash. D. C.)* **160**:323–325 (1968).
13. VanAlstyne, E., R. M. Burch, R. G. Knickelbein, R. T. Hungerford, E. J. Gower, J. G. Webb, S. L. Poe, and G. E. Lindenmayer. Isolation of sealed vesicles highly enriched with sarcolemma markers from canine ventricle. *Biochim. Biophys. Acta* **602**:131–143 (1980).
14. Frankis, M. B., and G. E. Lindenmayer. Sodium-sensitive calcium binding to sarcolemma-enriched preparations from canine ventricle. *Circ. Res.* **55**:676–688 (1984).
15. Schilling, W. P., and J. A. Drewe. Voltage-sensitive nitrendipine binding in

an isolated cardiac sarcolemma preparation. *J. Biol. Chem.* **261**:2750–2758 (1986).
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
17. Inagaki, C., G. E. Lindenmayer, and A. Schwartz. Effects of sodium and potassium on binding of ouabain to the transport adenosine triphosphatase. *J. Biol. Chem.* **249**:5135–5140 (1974).
18. Schwartz, A., K. Nagano, M. Nakao, G. E. Lindenmayer, and J. C. Allen. The sodium- and potassium-activated adenosine triphosphatase system. *Methods Pharmacol.* **1**:361–388 (1971).
19. Mark, G. E., and F. F. Strasser. Pacemaker activity and mitosis in cultures of newborn rat ventricular cells. *Exp. Cell Res.* **44**:217–233 (1966).
20. Lacerda, A. E., D. Rampe, and A. M. Brown. Effects of protein kinase C activators on cardiac Ca^{2+} channels. *Nature (Lond.)* **335**:249–251 (1988).
21. Rampe, D., A. Skattebol, D. J. Triggle, and A. M. Brown. Effects of McN-6186 on voltage-dependent Ca^{2+} channels in heart and pituitary cells. *J. Pharmacol. Exp. Ther.* **248**:164–170 (1989).
22. Pfeiffer, D. R., P. W. Reed, and H. A. Lardy. Ultraviolet and fluorescent spectral properties of divalent cation ionophore A23187 and its metal ion complexes. *Biochemistry* **13**:4007–4014 (1974).
23. Amellal, M., and Y. Landry. Lanthanides are transported by ionophore A23187 and mimic calcium in the histamine secretion process. *Br. J. Pharmacol.* **80**:365–370 (1983).
24. Williams, L. T., and L. R. Jones. Subcellular distribution of myocardial binding sites for a calcium antagonist, [^{3}H]nitrendipine. *J. Biol. Chem.* **258**:5344–5347 (1983).
25. Rengasamy, A., J. Ptasienski, and M. M. Hosey. Purification of the cardiac 1,4-dihydropyridine receptor/calcium channel complex. *Biochem. Biophys. Res. Commun.* **126**:1–7 (1985).
26. Bartschat, D. K., and G. E. Lindenmayer. Calcium movements promoted by vesicles in a highly enriched sarcolemma preparation from canine ventricle. *J. Biol. Chem.* **255**:9626–9634 (1980).
27. Slaughter, R. S., J. L. Sutko, and J. P. Reeves. Equilibrium calcium-calcium exchange in cardiac sarcolemmal vesicles. *J. Biol. Chem.* **258**:3183–3190 (1983).
28. Trosper, T. L., and K. D. Philipson. Effects of divalent and trivalent cations on Na^+-Ca^{2+} exchange in cardiac sarcolemma vesicles. *Biochim. Biophys. Acta* **731**:63–68 (1983).
29. Hidalgo, C., C. Parra, G. Riquelme, and E. Jaimovich. Transverse tubules from frog skeletal muscle: purification and properties of vesicles sealed with the inside-out orientation. *Biochim. Biophys. Acta* **855**:79–88 (1986).
30. Jaimovich, E., P. Donoso, J. L. Liberona, and C. Hidalgo. Ion pathways in transverse tubules: quantitation of receptors in membranes isolated from frog and rabbit skeletal muscle. *Biochim. Biophys. Acta* **855**:89–98 (1986).
31. Tomlins, B., S. E. Harding, M. S. Kirby, P. A. Poole-Wilson, and A. J. Williams. Contamination of a cardiac sarcolemma preparation with endothelial plasma membrane. *Biochim. Biophys. Acta* **856**:137–143 (1986).
32. Colden-Stanfield, M., W. P. Schilling, A. K. Ritchie, S. G. Eskin, L. T. Navarro, and D. L. Kunze. Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. *Circ. Res.* **61**:632–640 (1987).
33. Lee, K. S., and R. W. Tsien. Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature (Lond.)* **302**:790–794 (1983).

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