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Effect of divalent cation chelation on dihydropyridine binding in isolated cardiac sarcolemma vesicles

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The effect of divalent cation chelation on specific nitrendipine and ouabain binding has been determined in a highly enriched sarcolemma preparation isolated from canine ventricle. Maximal high-affinity nitrendipine binding measured in the absence of added calcium or magnesium was 997 ± 103 fmol/mg protein. Nitrendipine binding in the presence of EDTA significantly decreased to 419 ± 42 fmol/mg protein ($P < 0.001$) which equates to 42.0% of control. The simultaneous presence of EDTA and A23187 in the binding buffer resulted in a decrease in nitrendipine binding to below detectable levels. These results suggest that divalent cations trapped within vesicles can support high affinity nitrendipine binding. Evaluation of dihydropyridine binding at various pH values suggested that the loss of binding below pH 7.0 and above pH 8.0 may result indirectly from a change in divalent cation binding rather than a direct effect on dihydropyridine binding per se. The maximal binding of ouabain determined in the presence of magnesium and inorganic phosphate averaged 340 ± 7.4 pmol/mg protein. Pre-treatment of the preparation with sodium dodecyl sulfate (SDS) in order to express binding in sealed inside-out (IO) vesicles, increased ouabain binding to 471 ± 27 pmol/mg protein. Thus, these preparations averaged 27.8% sealed IO vesicles. Addition of EDTA in the absence of magnesium in the binding buffer reduced ouabain binding to 204 ± 7.7 and 11.7 ± 3.5 pmol/mg protein in control and SDS-treated preparations, respectively. These findings suggest that this sarcolemma preparation consists of 43.6% sealed right-side-out (RO) vesicles which contain sufficient endogenous divalent cation trapped in the intravesicular space, to support ouabain binding. The correspondence between the percentage of ouabain binding that remains in the presence of EDTA and the percentage of nitrendipine binding observed under the same conditions is consistent with the hypothesis that divalent cations support nitrendipine binding by interaction with a site or sites accessible only from the cytoplasmic membrane surface and that nitrendipine and ouabain binding sites occur in the same vesicles (i.e., the nitrendipine binding site is of sarcolemma origin).

Introduction

Calcium channels present in the surface membrane of the myocardial cell are responsible for the triggered influx of calcium ions during the cardiac action potential. Based on voltage-sensitivity and pharmacological responsiveness there now appear to be at least two different types of cardiac calcium channels [1]. The most widely studied calcium channel and the channel responsible for the majority of the calcium influx during the cardiac action potential is sensitive to the 1,4-dihydropyridine calcium channel modulator drugs.

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These drugs bind with high affinity to sarcolemma preparations from heart [2].

That the high-affinity dihydropyridine binding site found in isolated membrane preparations from nerve and muscle may be associated with calcium channels was suggested by the observed sensitivity of the binding to divalent cations [3]. With the exception of magnesium, a good correlation between the ability of a particular cation to pass through the channel and its ability to stimulate dihydropyridine binding has been a consistent finding [3–7]. In addition, early studies revealed that the inorganic cation blockers of calcium channel current (e.g., La^{3+}, Cd^{2+}) could inhibit dihydropyridine binding in isolated membrane preparations. However, studies on the sensitivity of nitrendipine binding to divalent cations in membrane preparations from guinea pig nerve and muscle showed that removal of divalent cations from these preparations with EDTA resulted in only a partial loss of binding. At maximum, a 60% loss of binding was observed with EDTA in membranes from cerebral cortex and ileum and only a 25% loss of binding occurred in membranes isolated from heart [5]. Dihydropyridine binding could be restored by addition of calcium to membranes previously treated with EDTA. These results suggest that isolated membranes may possess both divalent cation sensitive and insensitive dihydropyridine binding sites. Results from another study [6] revealed similar effects of EDTA on nitrendipine binding in microsomal preparations from guinea pig ileal and aortic smooth muscle and cardiac muscle. However, while binding was reduced only 25% in ileal muscle microsomes by 1 mM EDTA, a 90–95% loss of binding was observed if the membranes were exposed to EDTA during the isolation procedure [6]. Thus, while it is possible that the differential effect of EDTA on dihydropyridine binding may reflect the presence of divalent cation-insensitive binding sites, an alternative possibility is that EDTA cannot effectively chelate all of the contaminating divalent cations present in these preparations.

The purpose of the present study was to evaluate the effect of divalent cation chelation on high affinity dihydropyridine binding in a highly enriched sarcolemma preparation obtained from canine ventricle. The results of this study revealed that divalent cations trapped within sealed, right-side-out (RO) vesicles can support dihydropyridine binding suggesting that divalent cations are required at the cytoplasmic membrane surface. No evidence was obtained to suggest that divalent cation insensitive sites were present in these preparations. Kinetic binding studies suggested that the divalent cation binding site and the dihydropyridine binding site may be allosterically linked. Finally, evaluation of dihydropyridine binding at various pH values revealed that the loss of dihydropyridine binding below pH 7.0 and above pH 8.0 results indirectly from a change in divalent cation binding rather than a direct effect of pH on dihydropyridine binding per se.

Materials and Methods

Materials

[^{3}H]Nitrendipine, (+)-[^{3}H]PN200-110 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). A23187 and saponin were obtained from Calbiochem (San Diego, CA). All other chemicals were of reagent grade.

Isolation of sarcolemma-enriched preparation

Membrane preparations were isolated from canine ventricle according to the procedure of VanAlstyne [8] with the following modifications [9]: (1) medium A was 15 mM sodium bicarbonate (pH 7.0) and (2) the heart (approx. 100 g wet weight) was minced in a Cuisinart tissue processor in approx. 25 ml of medium A; one 20-s pulse followed by a 10-s pulse. The remainder of the procedure was identical to that previously reported. The final pellet was resuspended in 10 mM Tris-HCl (pH 7.4 for 22°C). The preparation was stored at 5°C and used within 24 h following isolation. This sarcolemma preparation has been shown to be 27–40-fold enriched in the following surface membrane markers: ouabain binding, Na^+/K^+-ATPase activity, beta-receptor-coupled adenylate cyclase activity and dihydroalprenolol binding [8]. In addition, this preparation consists of osmotically responsive membrane vesicles which exhibit calcium flux consistent with the presence

of sodium/calcium exchange and calcium/calcium exchange mechanisms [10,11]. Estimates of endogenous calcium associated with the sarcolemma preparation following isolation, determined by atomic absorption spectroscopy suggest that the calcium concentrations within the intravesicular space could be as high as 200 μM [11]. This preparation has been shown to exhibit specific high affinity nitrendipine binding with a dissociation constant of 0.09 nM and a maximum binding capacity of 800–1000 fmol/mg protein at 22°C [2].

Measurement of specific ouabain binding

Total [^{3}H]ouabain binding was determined according to Inagaki et al. [12] in the presence of 5 mM H_3PO_4 adjusted to pH 7.4 with Tris-base, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4) and $2 \cdot 10^{-6}$ M ouabain with [^{3}H]ouabain (specific activity ≅ 100 cpm/pmol) or in the presence of 50 mM Tris-HCl (pH 7.4) without Mg^{2+} or inorganic phosphate. In some experiments EDTA adjusted to pH 7.4 with Tris-base, was added to the binding buffer without Mg^{2+} or inorganic phosphate. The binding reactions were terminated after 30 min at 37°C by filtration through Millipore nitrocellulose filters (Type HA; pore size 0.45 μm) on a Hoefer filtration apparatus. The filters were washed five times with 5 ml aliquots of ice-cold distilled H_2O. Radioactivity associated with the filter was determined by standard liquid scintillation technique. Non-specific binding was determined by addition of 1 mM unlabeled ouabain to the binding reaction medium. Specific binding was defined as total minus the non-specific. Aliquots of some preparations were pre-treated for 90 minutes at 20°C with sodium dodecyl sulfate (SDS) at a final concentration of 0.3 mg/ml. Protein concentrations during incubation with SDS was 0.3 mg/ml. These conditions were found to be optimum in this preparation for stimulation of ouabain binding and ouabain-sensitive Na^+/K^+-ATPase activity by SDS [2]. Aliquots of the SDS-treated preparations were subsequently employed for measurement of specific [^{3}H]ouabain binding as described above.

Measurement of equilibrium dihydropyridine binding

Dihydropyridine binding was measured according to methods described previously [2]. Aliquots of the sarcolemma preparation were salt-'loaded' by incubation of the preparation with buffered salt solutions of various ionic compositions for 15–18 h at 2°C. Binding buffer consisted of 140 mM KCl, 10 mM Tris-HCl (pH 7.4 for 22°C). Aliquots of loaded preparation (approx. 25 μg protein) were added to binding buffer (4 ml) in glass tubes containing [^{3}H]nitrendipine or (+)-[^{3}H]PN200-110 (specific activity 80–100 cpm/fmol). The reactions at 22°C were terminated after 75–120 min (i.e., at equilibrium) by filtration through glass fiber filters (Gelman type A/E) on a Hoefer filtration apparatus. The filters were washed seven times with 5-ml aliquots of ice cold H_2O. The filters were removed from the filtration manifold immediately after washing, placed in scintillation vials, and counted by standard liquid scintillation technique. Non-specific binding was determined by including 100 nM unlabeled nitrendipine in the binding buffer. All experiments were performed in duplicate. *N* equals the number of preparations examined.

Measurement of nitrendipine dissociation rate

Dissociation of nitrendipine was determined as previously described [2]. Briefly, aliquots of preparation were pre-labeled to equilibrium with 5.4 nM [^{3}H]nitrendipine. Dissociation was initiated by making a 220-fold dilution of the pre-labeled preparation into binding buffer containing 100 nM unlabeled nitrendipine. Duplicate samples were removed at various times and the radioactivity associated with the preparation was determined as described above for equilibrium binding.

Measurement of dihydropyridine binding versus pH

Equilibrium (+)-[^{3}H]PN200-110 binding was determined as described above at various pH values. The (+)-[^{3}H]PN200-110 concentration employed was close to the K_d value (20 pM). Total buffer concentration was 20 mM; 10 mM Tris, 10 mM Mes, was used for pH 6.0 to pH 8.0 and 10 mM Tris, 10 mM CAPS was used for pH 8.0 to 10.0. The pH of each buffer was adjusted to the appropriate value with either HCl or Tris base. All assays were terminated after 2 h at 22°C.

Data analysis

Curve fitting computer programs which employ the Marquardt algorithm for fitting non-linear models were used for analysis of equilibrium and kinetic binding data. Specific details of the fitting procedure have been described [2]. Data presentation is in the form of Scatchard plots.

Results

Effect of divalent cation chelation on nitrendipine and (+)-PN200-110 binding

Dihydropyridine binding in the isolated cardiac sarcolemma preparation is only partially inhibited by EDTA (Figs. 1–3). Assay of nitrendipine binding in the presence of EDTA revealed a significant ($P < 0.001$) decrease in the apparent B_{max} from 997 ± 103 to 419 ± 42 fmol/mg protein (mean ± S.E.; $n = 5$). This represents a 58% decrease in nitrendipine binding upon chelation of divalent cations accessible to EDTA. Binding in the presence of EGTA was either unaffected or partially inhibited (Fig. 2) suggesting that an ion other than calcium supports the majority of binding in these experiments. Studies by Ptasienski et al. [13] have suggested that divalent cation chelation in isolated membranes from chick heart results in conversion between high and low affinity forms of the nitrendipine binding site with dissociation constant of 0.1 and 2–5 nM, respectively. Only a small, but significant change ($P < 0.025$) in K_d from 0.0779 ± 0.009 to 0.132 ± 0.017 nM was observed upon chelation of divalent cations with EDTA in the isolated sarcolemma preparation from dog heart.

The nitrendipine binding which remains in the presence of EDTA could reflect the presence of divalent cation insensitive binding sites or the inability of EDTA to effectively chelate all of the endogenous divalent cations associated with these preparations. An obvious location for divalent cations, inaccessible to EDTA, would be trapped within the intravesicular space of 'sealed' sarcolemma vesicles. To test this hypothesis, nitrendipine binding was examined in the presence of both EDTA and the divalent ionophore, A23187

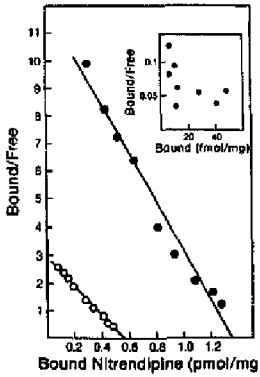

Fig. 1. Effect of EDTA on high-affinity nitrendipine binding in the presence and absence of the calcium ionophore, A23187. Aliquots of sarcolemma preparation were salt loaded by incubation at 5°C for 15–18 h with a solution containing 140 mM KCl, and 10 mM Tris-HCl (pH 7.4 for 20°C) (●; binding buffer) or binding buffer plus 1 mM EDTA (○). Aliquots of loaded preparation were added to reaction media at 22°C containing salts identical to those in the loading media and concentrations of [^{3}H]nitrendipine ranging from 0.03 to 1.0 nM. Specific binding was determined as described in Materials and Methods. Each point represents the average of duplicate determinations at each concentration of nitrendipine. The inset shows the results obtained for preparation loaded with binding buffer plus 1 mM EDTA and assayed for nitrendipine binding in reaction media containing binding buffer plus 1 μg/ml A23187. In this, and all subsequent figures, units of Bound/Free are pmol/mg protein per nM.

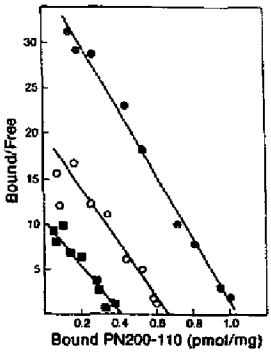

Fig. 2. Comparison of the effects of EDTA with EGTA on (+)-PN200-110 binding. Specific (+)-[^{3}H]PN200-110 binding was determined as described in the legend to Fig. 1 in the presence of 1 mM EGTA (○), 1 mM EDTA (■) or in the absence of chelator (●). The (+)-[^{3}H]PN200-110 concentration ranged from 3 to 500 pM.

(inset Fig. 1). Under these conditions, nitrendipine binding was very low and at the limit of detection for the amount of protein employed in this assay. In the presence of excess added divalent cations (1 mM $MgCl_2$), binding was unaffected by the presence of A23187.

In previous studies, results were obtained which suggested that consecutive freeze-thaw cycles could render the sarcolemma vesicles transiently leaky thus allowing the access of EGTA to the vesicle interior [14]. To further test the hypothesis that divalent cations trapped within the intravesicular space of sealed vesicles could support dihydropyridine binding in the absence of extravesicular divalent cations, nitrendipine binding was measured before and after freezing and thawing in the presence and absence of EDTA. Five freeze-thaw cycles in the presence of EDTA was found to have the same effect on nitrendipine binding as A23187

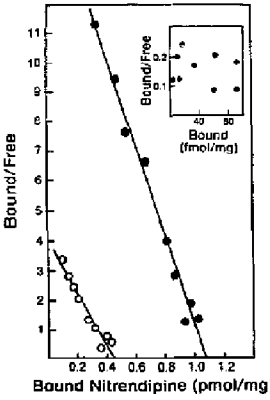

Fig. 3. Effect of EDTA on high affinity [^{3}H]nitrendipine binding before and after five consecutive freeze-thaw cycles. Aliquots of sarcolemma preparation were salt-loaded and assayed for specific nitrendipine binding as described in the legend to Fig. 1; control (●) and in the presence of EDTA (○). The inset shows the results obtained after five consecutive freeze-thaw cycles in the presence of EDTA. This was accomplished as follows: an aliquot of the sarcolemma preparation loaded with binding buffer plus 1 mM EDTA was frozen in a glass tube in a solid CO_2/acetone bath. The preparation was then immediately thawed by placing the tube in a water bath at 20°C. This freeze-thaw procedure was performed five times. Aliquots of this preparation were then assayed for nitrendipine binding.

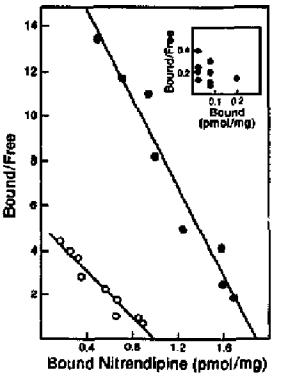

Fig. 4. Effect of EDTA on nitrendipine binding in the presence and absence of saponin. Specific [^{3}H]nitrendipine binding was determined as described in the legend to Fig. 1 in the presence of 10 μM $CaCl_2$ (●) or 1 mM EDTA (○). The inset shows the results obtained for sarcolemma preparation salt-loaded by incubation in binding buffer with 1 mg/ml saponin and assayed for nitrendipine binding in the presence of 1 mM EDTA. Protein concentration during the saponin pre-treatment was 1 mg/ml.

(fig. 3). Freezing and thawing in the absence of EDTA reduced nitrendipine binding approx. 16% (data not shown). Recent studies by Jaimovich et al. [15] showed that membrane vesicle preparations from skeletal muscle could be permeabilized with the detergent-like compound, saponin. Saponin had no effect on nitrendipine binding. In cardiac sarcolemma preparations made permeable by pretreatment with saponin, nitrendipine binding profiles were similar to those described above in the presence and absence of EDTA and divalent cations (i.e., no binding in the absence of divalent cations; Fig. 4).

In preparations treated with EDTA, binding could be restored by addition of Ca^{2+} or Mg^{2+} with ED_{50} values of 0.2 and 4 μM, respectively (Fig. 5). Thus the effect of EDTA is reversible and results from the chelation of divalent cations associated with the preparation.

In order to further characterize the effect of divalent cation chelation on dihydropyridine binding, the effect of EDTA on dissociation rate was determined in paired kinetic experiments (Fig. 6). Dissociation induced by dilution of prelabeled

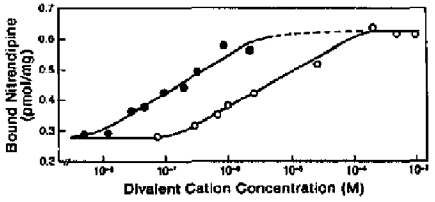

Fig. 5. Effect of Ca^{2+} and Mg^{2+} on nitrendipine binding in sarcolemma preparation pre-treated with EDTA. Sarcolemma preparation was salt-loaded by incubation in binding buffer containing 1 mM EDTA. Specific [^{3}H]nitrendipine binding was determined at a free nitrendipine concentration of 0.2 nM in binding buffer containing 1 mM EDTA and sufficient Ca^{2+} (●) or Mg^{2+} (○) to yield the indicated free divalent cation concentration. The free divalent cation concentration was calculated assuming EDTA dissociation constants of 0.005 μM and 0.63 μM for Ca^{2+} and Mg^{2+}, respectively [25]. All solutions were buffered to pH 7.4.

preparation into buffer containing excess unlabeled nitrendipine was monoexponential with time and yielded an average dissociate rate constant of $7.3 \cdot 10^{-4}$ s^{-1}. Dilution of pre-labeled preparation into buffer containing excess unlabeled nitrendipine and EDTA resulted in a 2.6-fold increase in the dissociation rate to $1.9 \cdot 10^{-3}$ s^{-1}. In a second set of paired kinetic experiments, saponin-treated preparation was pre-labeled to equilibrium in the presence of 0.2 nM [^{3}H]nitrendipine. Dissociation was initiated by addition of (a) unlabeled nitrendipine, (b) unlabeled nitrendipine plus EDTA (2 mM), or (c) EDTA only. The mean dissociation rate constants (± S.E.) were $9.6 \cdot 10^{-4} \pm 6.3 \cdot 10^{-5}$, $2.4 \cdot 10^{-3} \pm 1.7 \cdot 10^{-4}$, and $3.1 \cdot 10^{-3} \pm 5.4 \cdot 10^{-4}$ s^{-1} for each condition; respectively. In the presence of EDTA (both conditions), the dissociation rate was significantly ($P < 0.01$) increase versus the nitrendipine only condition. However, there was no significant difference between the EDTA only and the nitrendipine plus EDTA conditions. Thus, chelation of divalent cations by EDTA instantaneously increases the dissociation rate of drug from its receptor suggesting that divalent cation binding alosterically regulates the dihydropyridine binding site.

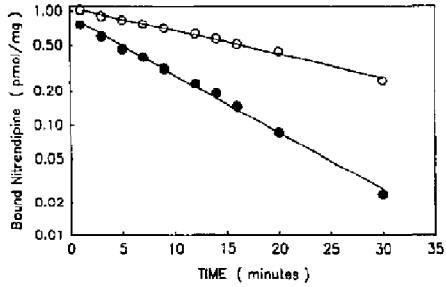

Fig. 6. Effect of EDTA on the dissociation rate of nitrendipine. Sarcolemma preparation (1 mg/ml) was salt-loaded by incubation with binding buffer containing 1 mg/ml saponin. After 15–18 h at 2°C, 40 μl of 0.5 μM [^{3}H]nitrendipine was added and the preparation was incubated at 22°C for 2 h. Dissociation was initiated by addition of the pre-labeled preparation (220-fold dilution) to binding buffer containing either 100 nM unlabeled nitrendipine (○) or 100 nM unlabeled nitrendipine plus 2 mM EDTA (●). Duplicate aliquots of the reaction mixture were removed at the indicated times and the bound nitrendipine determined as described in Materials and Methods. Each point represents the average of duplicate experiments performed on two sarcolemma preparations.

Effect of divalent cation chelation on ouabain binding

The results presented thus far clearly demonstrate that contaminating divalent cations (presumably Ca^{2+} or Mg^{2+}) trapped in the intravesicular space of approx. 42% of the total vesicle population can support dihydropyridine binding. Furthermore, these results support the conclusion that divalent cations interact with the binding protein in a sidedness fashion (i.e., with either the normal cytoplasmic surface or the normal extracellular surface, but not both). If we could document that divalent cations were trapped within vesicles of a particular orientation (i.e. either RO or IO vesicles), the localization of this divalent cation interaction to a particular membrane surface might be possible.

Previous studies revealed that a substantial amount of ouabain binding was observed in the complete absence of Mg^{2+} (i.e., in the presence of 1 or 10 mM EDTA) [2,16]. Since Mg^{2+} is required at sites on the cytoplasmic membrane surface for the expression of ouabain binding, Mg^{2+} trapped within RO vesicles may support both ouabain and dihydropyridine binding. If this is true, ouabain binding should be inhibited by EDTA to the same extent as dihydropyridine binding (approx. 60%)

and should be completely absent in the presence of both EDTA and A23187. Ouabain binding was measured in the presence and absence of A23187 and before and after treatment of the preparation with SDS (Table I). The ionophore, A23187, had no effect on ouabain binding in the absence or presence of SDS when Mg^{2+} was present in the assay medium. In the absence of added Mg^{2+}, a substantial amount of ouabain binding was observed, even in the presence of added EDTA. However, the presence of A23187 under these conditions produced a decrease in ouabain binding to a level comparable to that obtained with SDS. This effect was more pronounced if EDTA was added to the assay medium to chelate the divalent cations transported out of the vesicles by the ionophore. These findings suggest that ouabain binding in buffer only (i.e., in the absence of extravesicular divalent cations) occurs in RO vesicles that have a sufficient intravesicular concentration of Mg^{2+} to support ouabain binding. As noted above, ouabain binding was essentially the same in buffer only plus or minus EDTA. This result suggests that the RO vesicles containing intravesicular divalent cations are essentially impermeable to these divalent cations (i.e., sufficient divalent cation remains to support full ouabain binding even after incubation at 37 °C for 30 min in the presence of EDTA). In parallel experiments it was determined that full ouabain binding was obtained in the presence of excess Mg^{2+} even in the absence of added inorganic phosphate. Thus, the results obtained relate to the vesicle permeability for Mg^{2+} and not inorganic phosphate.

The values of ouabain binding in Table I can be used to calculate the percentage of leaky versus sealed vesicles in the preparation and the sidedness characteristics of the vesicles. A summary of these calculations is given in the bottom half of Table I. The amount of ouabain binding in the presence of Mg^{2+} and inorganic phosphate, in either the presence or absence of EDTA represents RO plus leaky vesicles. The average value for the preparations tested was 340 pmol/mg protein. The binding under these conditions after treatment of the preparations with SDS represents total binding (i.e., RO plus IO plus leaky). Total binding for these preparations averaged 468 pmol/mg protein. The difference between these two values represents IO vesicles (128 pmol/mg protein). The value of binding without SDS, in buffer only, in the presence of EDTA, represents

TABLE I

OUABAIN BINDING IN THE ISOLATED CARDIAC SARCOLEMMA PREPARATION

Ouabain binding was determined at 37 °C as described in Materials and Methods, under four different assay conditions: (a) 5 mM $MgCl_2$, 5 mM Tris-H_3PO_4 and 50 mM Tris-HCl (pH 7.4); (b) 50 mM Tris-HCl (pH 7.4); (c) 5 mM $MgCl_2$, 5 Tris-H_3PO_4, 1 mM Tris-EDTA and 50 mM Tris-HCl (pH 7.4); and (d) 1 mM Tris-EDTA and 50 mM Tris-HCl (pH 7.4). Assays were performed in the absence and presence of 1 μg/ml A23187 and on control preparations or preparations treated with 0.3 mg/ml SDS for 90 min at 20 °C. Total and non-specific binding were determined in duplicate on each preparation tested and for each condition. The values represent the mean ± S.E. determined on three preparations.

	Specific ouabain bound (pmol/mg protein)			
	Basal		SDS-treated	
A23187	−	+	−	+
(a) Mg, P_i	340 ± 7.4	349 ±14	471 ±27	467 ±40
(b) Buffer only	236 ± 10	71.3 ± 18	78.3 ± 6.6	63.3 ± 7.2
(c) Mg, P_i, EDTA	332 ± 17	337 ±12	473 ±30	461 ±23
(d) Buffer only, EDTA	204 ± 7.7	13.3 ± 3.2	11.7 ± 3.5	4.0 ± 0.6

IO + RO + leaky vesicles = (471 + 467 + 473 + 461) ÷ 4 = 468 = 100%
RO vesicles + leaky vesicles = (340 + 349 + 332 + 337) ÷ 4 = 340
RO vesicles = 204 ÷ 468 × 100 = 43.6%
Leaky vesicles = ((340 − 204) ÷ 468) × 100 = 29%
IO vesicles = ((468 − 340) ÷ 468) × 100 = 27.4%

binding to sealed RO vesicles (204 pmol/mg protein) and the difference between the binding in buffer only plus EDTA and that observed with Mg^{2+} and phosphate represents leaky vesicles (136 pmol/mg protein). Thus these preparations contain 43.6% RO, 27.4% IO and 29% leaky vesicles.

The correspondence between the percentage of ouabain binding that remains in the presence of EDTA (43.6%) and the percentage of dihydropyridine binding that remains under the same conditions (42.0%) suggests that (1) dihydropyridine and ouabain binding sites reside in the same vesicles and (2) divalent cations interact with sites on the cytoplasmic membrane surface to support dihydropyridine binding.

Effect of pH on dihydropyridine binding

To examine the possibility that pH could alter the effect of calcium on dihydropyridine binding, pH was varied from 6.0 to 10.0 at two different $CaCl_2$ concentrations. In the presence of 10 μM $CaCl_2$, the binding of (+)-PN200-110 increased from pH 6.0 to 7.3 and then decreased as pH was increased from pH 8.0 to 10.0 (Fig. 7). However, in the presence of 10 mM $CaCl_2$, essentially no effect of pH on (+)-PN200-110 binding was observed. Inhibition of binding at pH 6.0 and 10.0 in the presence of 10 μM $CaCl_2$ could be reversed by addition of 10 mM $CaCl_2$ indicating that the loss of binding at the pH extremes did not reflect degradation of the protein or of the ligand. The results of paired equilibrium binding experiments performed at pH 6.0, 7.4, and 10.0 revealed that the change in binding with pH reflects a change in B_{max} rather than K_d (data not shown).

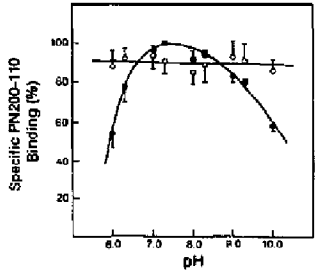

Fig. 7. Effect of pH on (+)-PN200-110 binding at two $CaCl_2$ concentration. Specific (+)-[^{3}H]PN200-110 binding was determined in saponin pre-treated preparation as described in the legend to Fig. 4 and in Materials and Methods, at various pH values in the presence of 10 μM $CaCl_2$ (●) or 10 mM $CaCl_2$ (○). All values represent the mean ± S.E. of duplicate experiments performed on four sarcolemma preparations.

Discussion

The results of the present study confirm that divalent cations are required for dihydropyridine binding in highly enriched sarcolemma preparations. Both calcium and magnesium at micromolar concentrations can support high affinity dihydropyridine binding. Since intracellular free magnesium concentration is in the millimolar range [17,18], the physiologically relevant ion is probably magnesium. Chelation of divalent cations with EDTA resulted in a large and highly significant change in B_{max} with only a small but significant change in K_d. This suggests that the dihydropyridine binding site is either absent in divalent cation depleted solutions or of very low affinity. No evidence was obtained to suggest that divalent cation chelation results in a conversion of high-affinity binding sites to sites with affinity in the 2–5 nM range as has been suggested for chick heart membranes [13]. The magnitude of bound nitrendipine at 1 nM free concentration (the maximum concentration used in the present study) was always > 90% of B_{max} and binding was best fit by a single site model. Curvilinear Scatchard plots would be expected over the concentration range employed if a population of sites with K_d on the order of 3 nM were present as observed by Ptasienski et al [13]. Yet as can be seen in Figs. 1, 3 and 4 (open circles) Scatchard plots were always linear in the presence of EDTA. The study of Ptasienski et al. [13] employed chick heart membranes isolated from sucrose gradient fractions at the 32%/40% interface. These membranes were less enriched in surface membrane markers compared to a lighter fraction [19] and thus may contain membranes of both sarcoplasmic reticulum (SR) and sarcolemma origin. Membrane fractions enriched in SR have been shown to have a high specific dihydropyridine binding when compared to sarcolemma-enriched fractions [20]. Thus the difference observed between the present study in highly enriched canine sarcolemma and that in

chick heart may reflect differences in the binding characteristics of the dihydropyridine binding protein associated with these different membrane fractions.

It has been demonstrated that certain divalent cations are required for dihydropyridine binding in isolated membrane preparations from brain and muscle [3–7]. Most reports revealed only a partial reduction of binding in the presence of EDTA. The maximum reduction in nitrendipine binding upon divalent cation chelation appeared to be tissue dependent [5]. Using three different approaches the results of the present study clearly demonstrate that divalent cations trapped within sealed vesicles support dihydropyridine binding in the presence of EDTA. First, the divalent cation ionophore, A23187, was employed to transport calcium and magnesium out of sealed vesicles. Second, a freeze-thaw procedure was used to allow EDTA to equilibrate with the vesicle interior. And third, the vesicles were permeabilized with saponin. Under all three conditions dihydropyridine binding in the presence of EDTA was reduced to less than 10% of control. Each procedure had little or no effect on dihydropyridine binding in the absence of EDTA (i.e., in the presence of excess divalent cations). Thus, the binding that remains in the presence of EDTA without these treatments reflects the impermeability of the sarcolemma vesicles to divalent cations and to EDTA rather than the presence of divalent cation insensitive dihydropyridine binding sites. Furthermore, these results suggest that divalent cations interact with the dihydropyridine binding protein in a sidedness fashion, i.e., with either the cytoplasmic or the extracellular membrane surface but not both.

In an effort to determine if these divalent cations were trapped within sealed RO or sealed IO vesicles, advantage was made of the finding that high-affinity ouabain binding to the Na^+/K^+-ATPase pump requires divalent cations at the cytoplasmic membrane surface. As shown in Table I, ouabain binding in the sarcolemma preparation in only partially inhibited by EDTA confirming previous reports [2,16]. The binding of ouabain could be essentially eliminated in the presence of EDTA with A23187 and/or detergent to permeabilize the membrane. Neither treatment had any effect in the absence of EDTA and presence of divalent cations. This result clearly demonstrates that divalent cations trapped within sealed RO vesicles support ouabain binding. The percentage of sealed RO vesicles determined in the presence of EDTA (43%; Table I) was essentially identical to the percentage of dihydropyridine binding site obtained in the presence of EDTA (42%). This result suggests that (1) divalent cations interact with the cytoplasmic membrane surface to support dihydropyridine binding and (2) the dihydropyridine binding protein is associated with vesicles that also contain the Na^+/K^+-ATPase pump. Thus, the dihydropyridine binding protein is of sarcolemma origin.

To explore the interaction between the divalent cation binding site and that for the dihydropyridine, dissociation of nitrendipine was examined in the presence and absence of divalent cations. Chelation of divalent cations with EDTA increased the dissociation rate of nitrendipine 2–3-fold. One possible explanation for this result is that the binding of divalent cations to the dihydropyridine binding protein allosterically regulates binding at the drug site. It is interesting that EDTA can initiate dissociation in the absence of excess unlabeled nitrendipine. This would suggest that two conformational changes take place during the dissociation experiment. The first conformational change occurs when divalent cations are initially chelated by EDTA. This results in the increase in dissociation rate constant. A second conformational change occurs following nitrendipine dissociation. This later conformational change must result in a decrease in the association rate constant essentially to zero, since binding can be completely displaced by the addition of EDTA alone.

In an attempt to understand the functional significance of the divalent cation binding site to calcium channel function, the effect of pH on dihydropyridine binding in high and low calcium was examined. In a recent patch clamp study of calcium channels in single ventricular myocytes from guinea pig, Irisawa and Sato [21] found that peak calcium current was inhibited by intracellular acidification with half-maximal inhibition occurring at pH 6.5 and complete block at pH 6.0. The threshold potential, peak current potential and the time course of inactivation were unchanged by lowering cytosolic pH suggesting that

protons do not affect the channel kinetics. In *Paramecium*, results were obtained suggesting that open calcium channels could be blocked by protonation of a single titratable site with a pK of 6.2 [22]. The location of the titratable site appeared to be at the cytoplasmic mouth of the channel and it was speculated that this site may interact with calcium to produce inactivation of the channel. In the present study it is clear that the effect of pH on dihydropyridine binding is dependent on the divalent cation concentration. At low calcium (10 μM) dihydropyridine binding was maximal at pH 7–8; binding decreased below pH 7 and above pH 8 consistent with a previous report [23]. Since the pK_a of the amine group of nitrendipine and PN200-110 is thought to be less than 3.5 [24], the pH dependence of binding probably reflect titration of some residue associated with the binding protein and not titration of the ligand. In the presence of high calcium (10 mM) very little effect of pH was observed. There are at least two possible interpretations of this result. First, high calcium may favor a conformation of the dihydropyridine binding protein which prevents titration of the site responsible for inhibition of binding. This however, would have to occur without significant alteration in the dihydropyridine binding site since little affect of high calcium on K_d or B_{max} was observed at pH 7.4. Second, it is possible that pH alters the affinity of the divalent cation binding site which then, through an allosteric mechanism, alters dihydropyridine binding. In favor of this hypothesis is the finding that pH alters B_{max} and not K_d, a result similar to the effects of divalent cation chelation with EDTA.

In summary, divalent cations interact with the cytoplasmic membrane surface of the myocardial cell and through an allosteric mechanism regulate the availability and/or affinity of the dihydropyridine binding site of the voltage-sensitive calcium channel. Affinity of the divalent cation binding site may be affected by pH and thus cyclic variations in cytosolic calcium and/or pH during the cardiac action potential or under conditions such as ischemia-induced cellular acidosis could influence the function of the channel thereby affecting contractile force and the sensitivity to drugs. While additional experimentation is needed to understand the complex interaction between pH, divalent cation concentration and dihydropyridine binding, it is clear from the present study that the sidedness characteristics and permeability of isolated membrane preparations for divalent cations and protons must be considered in future experimental design and interpretations.

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