Characterization and Pharmacological Relevance of High Affinity Binding Sites for \[^3\text{H}\]LY186126, a Cardiotonic Phosphodiesterase Inhibitor, in Canine Cardiac Membranes

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\[^3\text{H}\]LY186126, an analogue of the cardiotonic agent indolidan, was shown to bind reversibly and with high affinity (K_d=4 nM) to a single class of binding sites within canine myocardial vesicles. Binding site density measured in various cardiac membrane fractions correlated well with Ca^{2+}-ATPase activity (r=0.94; p<0.01), but not with Na^+\text{,K}^+-ATPase or azide sensitive ATPase, indicating a localization of these sites within sarcoplasmic reticulum membranes. Divalent cations were required for binding and displayed the following order of activation: Za^{2+}>Mn^{2+}>Mg^{2+}>Ca^{2+}. Differential activation of \[^3\text{H}\]LY186126 binding by various divalent cations was due to alterations in binding site density, rather than affinity. cGMP and selective inhibitors of type IV membrane-bound phosphodiesterase (SR-PDE), for example, indolidan, milrinone, imazodan, and enoximone, selectively displaced bound \[^3\text{H}\]LY186126; caffeine, theophylline, and rolipram were relatively impotent as inhibitors of radiolabel binding. K_a values from displacement curves were highly correlated with IC_{50} values for inhibition of SR-PDE (r=0.92; p<0.001). In addition, K_a values correlated well with published ED_{50} values for increases in cardiac contractility in pentobarbital-anesthetized dogs (r=0.94; p<0.001). The results support the hypothesis that \[^3\text{H}\]LY186126 labels the pharmacological receptor for the class of positive inotropic agents characterized as isozyme-selective phosphodiesterase inhibitors. Furthermore, the data suggest that the identity of the site labeled by \[^3\text{H}\]LY186126 is SR-PDE, the type IV isozyme of cardiac phosphodiesterase located in the sarcoplasmic reticulum. (Circulation Research 1989;65:154-163)

Indolidan (LY195115), a dihydropyridazinoquinoline cardiotonic, produces potent positive inotropic and vasodilatory effects of long duration in vivo and is currently undergoing clinical evaluation for treatment of congestive heart failure. This compound is a potent, selective, and competitive inhibitor of cyclic AMP (cAMP) phosphodiesterase (PDE) located in sarcoplasmic reticulum membranes (i.e., SR-PDE). Available data support a role for inhibition of SR-PDE in both positive inotropy and vasodilation produced by indolidan.

Based on high affinity for cAMP and lack of stimulation by calmodulin or cyclic GMP (cGMP), SR-PDE is classified as a type IV PDE isozyme and appears to be identical to cGMP-inhibited PDE isolated from bovine cardiac muscle by immunoprecipitation techniques. Although this class of PDE has been referred to as PDE III or FIII (based on elution profile of the solubilized enzyme from ion exchange columns), this nomenclature is now known to be inappropriate because the third peak of eluted activity in fact consists of a heterogeneous mixture of isozymes. Selective inhibition of type IV, cGMP-inhibited PDE is thought to mediate inotropic effects of several cardiotonic PDE inhibitors including amrinone, milrinone, enoximone, and imazodan.

Recently a radiolabeled congener of indolidan, \[^3\text{H}\]LY186126, was prepared in high specific activity form and shown to bind with high affinity to an apparent single class of sites within preparations of sarcoplasmic reticulum (SR) vesicles. Selective displacement of bound \[^3\text{H}\]LY186126 by indolidan...
and milrinone provided preliminary evidence of a role for this site in mediating positive inotropy. The present studies were conducted to characterize the properties of this site, including subcellular location, identity, and pharmacological relevance. Particular emphasis was placed on examining further the role of SR-PDE in mediating positive inotropic effects of indolidan and other cardiotonic PDE inhibitors. A preliminary report on a portion of these findings has appeared in the literature.

Materials and Methods
Isolation of Canine Cardiac Membranes
Highly purified sarcolemmal vesicles were prepared from ventricles of purpose-bred dogs anesthetized with sodium pentobarbital (35 mg/kg i.v.) as described by Jones et al., except that myocardial vesicles were subjected to a total of four 30-second homogenizations with the Polytron apparatus at a setting of 7.5 (PT20ST, Brinkmann Instruments, Westbury, New York). During the preparation of sarcolemmal vesicles, membranes sedimenting at 17,000g for 20 minutes were harvested as a source of mitochondrial membranes.

Myocardial vesicles (Procedure I) and subtraction of SR vesicles (fractions A through E) were isolated from canine ventricles as described. Fraction A contained relatively pure sarcolemmal membranes, although SR contamination was greater than 20% of the substrate in 60 minutes at room temperature (22 ± 2°C). Reactions were initiated by adding sufficient enzyme to hydroryze less than 20% of the substrate in 60 minutes at room temperature.

Preparation of sarcolemmal vesicles, membranes sedimenting at 17,000g for 20 minutes were harvested as a source of mitochondrial membranes.

Determination of Enzyme Activities
Cyclic nucleotide PDE activity of myocardial vesicles was determined at 1 μM cAMP by the two-step method essentially as described by Thompson et al. Reactions were carried out in 30 mM Tris/Cl, pH 8.0, 0.1 mM dithiothreitol, 5 mM MgCl₂, and 1 μM [³H]cAMP (1.67 × 10⁶ Bq). In certain experiments the concentration and identity of divalent metal cations included in the reaction medium were varied (Figure 12). PDE reactions were initiated by adding sufficient enzyme to hydrolyze less than 20% of the substrate in 60 minutes at room temperature (22 ± 2°C). Reactions were terminated by placing tubes in boiling water for 45 seconds. PDE activity was linear versus time and protein concentration (data not shown). Inhibition curves were generated by determining PDE activity in duplicate at 10 inhibitor concentrations (5 log units) spanning the IC₅₀ (concentration at which 50% inhibition of activity occurred). Dimethyl sulfoxide was used as a solvent for PDE inhibitors. Inhibitor solutions were prepared on the day of an experiment, and controls were run to ensure that carry-over solvent (2.5%, vol/vol) had no effect on assay results.

Ca²⁺-ATPase activity was quantitated at 37°C by appearance of inorganic phosphate as described previously. Reactions were carried out in 50 mM histidine, pH 7.4, 3 mM MgCl₂, 3 mM ATP (Na⁺), 100 mM KCl, and 50 μM CaCl₂. Ca²⁺-ATPase activity was that portion of total activity that was inhibited by 1 mM EGTA/Tris. In the case of mitochondrial membranes, Ca²⁺-ATPase was determined in the presence of 5 mM sodium azide in order to reduce background activity.

Determination of Membrane Binding
Binding studies were routinely carried out at 25°C in 1.0 ml of the following standard medium: 50 mM Tris-Cl, 5 mM MgCl₂, 0.01% BSA, pH 7.4, in the presence of varying concentrations of [³H]LY186126. Binding reactions were initiated by the addition of membrane vesicles (15–100 μg protein) to the medium and allowed to proceed to completion (60 minutes), at which time the membrane suspension was filtered using a vacuum apparatus (Brandel cell harvester) onto Whatman GF/C filters pretreated with 50 mM Tris-Cl, 0.05% BSA, pH 7.4, to minimize nonspecific binding. Filters were washed rapidly four times with cold (0–4°C) 50 mM sodium phosphate, pH 7.4. Total radioactivity retained by the filter was determined by scintillation counting filters in 10 ml Beckman Ready Protein® cocktail (Beckman Instruments, Fullerton, California). Specific binding was defined as the portion of total bound radioactivity displaced by 100 μM indolidan. Binding was determined in triplicate at each concentration of [³H]LY186126 unless stated otherwise. When the effect of pH on binding was examined (Figure 2), 50 mM histidine was included in the standard medium to provide effective buffering down to pH 5.0. In certain experiments, the identity and concentration of the divergent cation included in the binding medium was varied (see “Results”).

The time course for dissociation of bound [³H]LY186126 was determined in the following manner. Binding in the presence of 10 nM [³H]LY186126 was initially allowed to proceed to completion (in the standard medium) during a 60-minute incubation. On completion of this period, dissociation of bound radiolabel was induced by the addition of 100 μM indolidan. Bound [³H]LY186126 was subsequently determined by filtration of the suspension at various times after the addition of indolidan.

Displacement curves were generated using a procedure similar to that described above for binding experiments. A fixed concentration of 10 nM [³H]LY186126 was used in these experiments. Dimethyl sulfoxide was used as a solvent for displacing ligands; control studies demonstrated that carry-
over dimethyl sulfoxide in the binding medium (1%, vol/vol) had no detectable effect on binding. Displacement curves were constructed by varying the concentration of displacing ligand (0.5 log increments) over a range of 4 log units encompassing the IC₅₀ (concentration at which 50% of bound radioligand is displaced). Solutions of displacing ligands were prepared fresh on the day of an experiment.

Analysis of Data

IC₅₀ values versus SR-PDE were determined graphically from plots of the percentage of control PDE activity (in the absence of inhibitor) versus the negative logarithm of inhibitor concentration. Kᵢ values for [³H]LY186126 binding isotherms were determined by nonlinear least squares analysis using LIGAND and LUNDON software (Lundon Software, Cleveland, Ohio). Correlation coefficients were obtained from linear regression analysis, and statistical significance of linear correlations was assessed as described in the literature. Values of p<0.05 were taken to indicate statistical significance.

Materials

The synthesis and characterization of [³H]LY186126 (1,3-dihydro-3,3-dimethyl-1-[³H]methyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2H-indol-2-one; 79.2 Ci/mmol) is described in a separate report (D.W. Robertson et al, submitted manuscript). The radiolabeled drug was highly stable and the radiochemical purity was 99.8% after 1 year of storage at −20°C in ethanol (data not shown). The synthesis and characterization of indolidan and unlabeled LY186126 were as described in the literature (indicated compounds were designated as numbers 20 and 26, respectively, in this reference). Amrinone (Sterling-Winthrop, New York, New York) and piroximone (MDL 19205; Merrell Dow Pharmaceuticals, Cincinnati, Ohio) were kindly supplied by the sources indicated. Enoximone (MDL 17,043), imazodan (CI-914), CI-930, and milrinone were synthesized and characterized at Lilly Research Laboratories (Indianapolis, Indiana) according to published techniques.

Results

Experiments were conducted to determine whether specific binding of [³H]LY186126 to less purified membrane fractions was analogous to that recently demonstrated in preparations of highly purified SR vesicles. A representative saturation isotherm for binding of [³H]LY186126 to myocardial vesicles, a cardiac microsomal fraction, is shown in Figure 1. As can be seen, specific binding of [³H]LY186126 was concentration-dependent and saturable over the range examined. Furthermore, Scatchard replots of the binding data were linear (Figure 1; inset). Similar to results obtained with purified SR vesicles, high amounts of nonspecific binding hampered determination of binding at radioligand concentrations in excess of approximately 50 nM (data not shown). Binding curves were subjected to
FIGURE 2. Effects of pH on [3H]LY186126 binding to canine myocardial vesicles. Binding experiments were carried out in the presence of 10 nM [3H]LY186126 as described in “Materials and Methods.” Histidine (50 mM) was included in the standard binding medium to provide effective buffering of H+ down to pH 5. Data represent the mean±SEM of triplicate determinations.

analysis using nonlinear least-squares regression. The data were best fit to a single site model with $K_d$ and $B_{max}$ values of 4.1±1.3 nM and 383±61 fmol/mg vesicle protein, respectively. The Hill coefficient for binding was calculated to be 0.98±0.03. In separate studies, the $K_d$ for displacement by indolidan of [3H]LY186126 bound to myocardial vesicles (5.4±1.0 nM) was not significantly different from the value obtained with purified SR vesicles (5.2±1.7 nM). Thus, the only detectable difference between [3H]LY186126 binding to purified SR vesicles versus myocardial vesicles was the approximate twofold reduction in site density observed in the latter preparation.

To optimize specific binding, the effect of pH on [3H]LY186126 binding was determined. A single, relatively broad peak of binding was observed as a function of pH (Figure 2). Binding activity was maximal at a pH value of 7.4. The data suggest that the protonation state of amino acids having apparent $pK_a$ values of approximately 6.5 and 9.5 is critical to binding of [3H]LY186126.

The time course for dissociation of [3H]LY186126 from its binding site in cardiac sarcoplasmic reticulum vesicles is shown in Figure 3. The rate of dissociation was rapid; at the earliest time point sampled (1 minute), approximately 80% of bound label had already dissociated from the binding site. A $t_{1/2}$ of ±15–30 seconds was estimated from these data. This observation demonstrated the reversible nature of [3H]LY186126 binding. The filtration apparatus used in these studies (Brandel Cell Harvester) is not suitable for making binding measurements over short intervals (<60 seconds). Consequently, no attempt was made to determine precisely the kinetic constant for this rapid dissociation.

The binding of [3H]LY186126 (10 nM) to a variety of cardiac membrane fractions was examined and compared with the activity of marker enzymes for various cardiac membranes. In this analysis, marker enzymes (indicated in parentheses) for the following membranes were determined: sarcolemma ($Na^+,K^+-ATPase$), SR ($Ca^{2+}-ATPase$), and inner mitochondrial membrane (azide-sensitive ATPase). [3H]LY186126 binding failed to correlate with either $Na^+,K^+-ATPase$ or azide-sensitive ATPase activities (data not shown). In contrast to these findings, [3H]LY186126 binding correlated significantly with $Ca^{2+}-ATPase$ activity (Figure 4; $r=0.90, p<0.01$).

FIGURE 3. Time course for dissociation of bound [3H]LY186126 from canine cardiac SR vesicles (fraction E). Binding data were generated as described in “Materials and Methods.” Following completion of a 60-minute incubation of 10 nM [3H]LY186126 with canine cardiac SR vesicles, displacement of bound radiolabel was initiated by addition of 100 µM indolidan at $t=0$ minutes and remaining bound radioligand was determined at the times indicated. Data points represent the average of duplicate determinations.
Moreover, this relationship was apparent despite the use of biochemically and functionally distinct subfractions of SR vesicles in this analysis. In a previous communication, [3H]LY186126 binding to SR membranes was shown to be dependent on the presence of MgCl₂ in the medium. The ability of various divalent cations to support binding is examined in Figure 5. At a fixed concentration of 5 mM divalent cation, the amount of [3H]LY186126 bound at 10 nM of the radioligand was greatest with Zn²⁺, followed by Mn²⁺, Mg²⁺, and Ca²⁺. The amount of radioligand bound in the presence of Zn²⁺ was approximately four times that observed with Mg²⁺, while Ca²⁺ supported binding of only about 20% of the amount seen with Mg²⁺. Monovalent cations (e.g., Na⁺) were relatively ineffective at activating [3H]LY186126 binding. Even at a concentration of 118 mM NaCl, the amount of radiolabel bound was less than 10% of that observed with 5mM MgCl₂ (data not shown).

Saturation isotherms for [3H]LY186126 binding to myocardial vesicles were generated in the presence of either Zn²⁺ or Mg²⁺ at 5 mM (Figure 6). Kᵦ values for [3H]LY186126 were not significantly different using the two divalent cations (4.1±1.3 nM and 4.9±0.7 nM for Mg²⁺ and Zn²⁺, respectively); however, Bₘₐₓ was approximately fourfold greater in the presence of Zn²⁺ (383±61 and 1,771±140 fmol/mg vesicle protein for Mg²⁺ and Zn²⁺, respectively). The concentration dependence for activation of [3H]LY186126 binding by Mg²⁺ and Zn²⁺ was examined, and the results are shown in Figure 7. Zn²⁺ was a more potent activator of binding than Mg²⁺, with half-maximal activation for the divalent cations occurring at 50 μM and greater than 250 μM, respectively. Consistent with the findings in Figure 6, the data in Figure 7 confirm that Zn²⁺ supported the binding of [3H]LY186126 to a greater number of sites than did Mg²⁺.

Displacement curves were generated at 10 nM [3H]LY186126 for a structurally diverse set of PDE inhibitors to examine the identity and pharmacological relevance of the site labeled by this radioligand. The results, shown in Figure 8, demonstrated that cGMP and positive inotropic PDE inhibitors including indolidan, milrinone, imazodan, enoximone, and amrinone were potent inhibitors of radiolabel binding. In contrast, rolipram and the nonselective PDE inhibitors caffeine and theophylline were impotent at displacing [3H]LY186126 (IC₅₀ > 100 μM). Hill coefficients for displacement curves ranged from 0.85 to 1.10 and were not significantly different from 1.
a value of 1.0. Displacement of $[^3H]LY186126$ by cAMP was not attempted because of rapid hydrolysis of the cyclic nucleotide by SR-PDE under these experimental conditions.2

The relation between binding $K_d$ values obtained from displacement curves and $IC_{50}$ values versus canine cardiac SR-PDE is depicted in Figure 9. $IC_{50}$ values were obtained from inhibition curves determined at $1 \mu M$ cAMP (caffeine and rolipram; data not shown) or from published reports.2,13 A highly significant correlation was found between these two parameters ($r=0.95; p<0.001$). The relation, however, was not one of identity since the slope of the curve was significantly different from a value of 1.0.

A subset of the compounds tested in Figure 8, that is, the positive inotropic PDE inhibitors, was chosen for analyzing the pharmacological relevance of the $[^3H]LY186126$ binding site. Binding $K_d$ values were compared with published ED$_{50}$ values (milligrams per kilogram, intravenous administration) for stimulation of myocardial contractility by 50% in pentobarbital-anesthetized dogs.1-10,25,27 As shown in Figure 10, ED$_{50}$ values were highly correlated with $K_d$ values for binding to the site labeled by $[^3H]LY186126$ ($r=0.94; p<0.001$). The slope of the curve in Figure 10 was significantly less than 1.0, demonstrating that the relation was not one of identity.

Displacement curves for indolidan and cGMP, two structurally distinct ligands interacting at the $[^3H]LY186126$ binding site, were generated in the presence of either Mg$^{2+}$ or Zn$^{2+}$. In good agreement with the findings in Figure 6, there was only a slight (approximately twofold) difference between the $K_d$ values for indolidan determined in the presence of the two divalent cations (Figure 11A). By contrast, the displacement curve for cGMP was shifted 60-fold to the right ($K_d=0.4 \mu M$) when Zn$^{2+}$ was used in place of Mg$^{2+}$ (Figure 11B).

The ability of Mg$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ to activate cAMP PDE associated with myocardial vesicles was examined, and the results are shown in Figure 12. In a previous report, it was shown that SR-PDE accounted for approximately 80% of total PDE activity associated with this membrane preparation.2 Mg$^{2+}$ and Mn$^{2+}$ were potent activators of PDE activity; half-maximal activation occurred at less than 5 $\mu M$ of these cations. PDE activity in the presence of Mn$^{2+}$ was approximately 45% greater than that in the presence of Mg$^{2+}$. Although low concentrations of Zn$^{2+}$ (≤10 $\mu M$) produced a slight...
activation of PDE, complete inhibition was observed at concentrations ≥50 μM. The observation that SR-PDE was inactive in the presence of Zn²⁺ was used to examine the interaction of cAMP with the [³H]LY186126 binding site. Accordingly, cAMP was shown to displace [³H]LY186126 with a K_d of 13 μM when experiments were conducted in the presence of 5 mM Zn²⁺ (data not shown).

Discussion

[³H]LY186126 was previously shown to bind specifically and with high affinity (K_d=3.8 nM) to an apparent single class of binding sites in highly purified preparations of canine cardiac SR vesicles. In the present report, we demonstrated the presence of an apparently identical binding site (Figure 1) in a less purified membrane preparation (myocardial vesicles). Importantly, additional classes of binding sites were not detected in this membrane preparation. This finding was useful because myocardial vesicles can be obtained more readily and in much greater yield than purified SR vesicles (fraction E) with only a slight (approximately twofold) decrease in [³H]LY186126 binding site density. Con-
sequently, myocardial vesicles could be used interchangeably with SR vesicles in studies described herein to characterize the binding site.

[3H]LY186126 binding sites copurified with the SR marker, Ca\(^{2+}\)-ATPase, but not with sarcolemmal or mitochondrial membrane markers. The correlation between site density and Ca\(^{2+}\)-ATPase was significant \((r=0.90; p<0.01)\) and applied to both junctional and free SR vesicles (Figure 3). The biochemical data support a subcellular location of [3H]LY186126 binding sites within SR membranes with a distribution similar or identical to that of Ca\(^{2+}\)-ATPase. Furthermore, the results suggest that [3H]LY186126 binding may prove to be a useful marker for cardiac SR membranes. An interesting topic for future investigations will be the distribution of [3H]LY186126 binding sites in other tissues and species.

A structurally diverse set of PDE inhibitors was found to displace [3H]LY186126 bound specifically to the high affinity site in SR membranes. Relatively selective inhibitors of cGMP-sensitive type IV PDE, for example, indolidan, CI-914, and milrinone, displayed the highest affinities for this site. Furthermore, the binding site displayed selectivity with respect to subclasses of type IV PDE inhibitors since rolipram, a potent inhibitor of cGMP-insensitive type IV PDE, only weakly displaced [3H]LY186126. Binding \(K_d\) values obtained from displacement curves were highly correlated with IC\(_{50}\) values versus SR-PDE for this series of compounds. These results support the hypothesis that [3H]LY186126 labels SR-PDE, the cGMP-sensitive type IV isozyme of PDE located in cardiac SR membranes. Additional support for this conclusion was obtained from 1) the previous demonstration that dihydropyridazinones such as indolidan and LY186126 are potent, competitive inhibitors of SR-PDE \(^{213}\) to SR-PDE \(^{213}\); 2) location of [3H]LY186126 binding sites within SR membranes; 3) the demonstration that cAMP and cGMP, substrates for SR-PDE, \(^2\) bind to this site; and 4) the requirement of Mg\(^{2+}\), known to be essential for PDE activity, \(^{28}\) for binding. Nevertheless, additional studies are required to substantiate this hypothesis, particularly since the relation between binding \(K_d\) and IC\(_{50}\) was not one of identity.

Binding \(K_d\) values were furthermore shown to correlate significantly with ED\(_{50}\) values for in vivo positive inotropic effects for a structurally diverse set of positive inotropic agents, all of which are known to be relatively potent inhibitors of SR-PDE. \(^2\) Thus, the results support a role for the high affinity [3H]LY186126 binding site in mediating pharmacological effects of these agents in cardiac tissue. Taken together, our findings support the hypothesis that inhibition of SR-PDE is an important biochemical mechanism underlying direct positive inotropic effects of these compounds. Our data confirm and extend conclusions of others concerning an important role for isozyme-selective PDE inhibition in mediating cardiac effects of these drugs. \(^3,7,10\) Relevant to this discussion, recent studies by Weishaar et al and Kithas et al have clarified further the role of membrane-bound type IV PDE (SR-PDE), in contrast to soluble type IV isozymes, as the phar-
macological target of these compounds. These studies notwithstanding, additional or alternative mechanisms have been proposed for certain of these compounds.

Differential activation of [3H]LY186126 binding by Zn$^{2+}$ and Mg$^{2+}$ was shown to be due to an increase in the number of sites labeled in the presence of Zn$^{2+}$ (Figures 6 and 7). An identical conclusion was reached when activation of binding by Mn$^{2+}$ was examined (data not shown). These findings suggest a model in which binding of divalent cations to an activation site produces a conformational change in one or more membrane-bound proteins, thereby revealing functional binding sites for [3H]LY186126. Based on this model, variable binding site densities for [3H]LY186126 with the various divalent cations could be accounted for by differential extents of conversion to the active binding conformation for the radiolabel. An intriguing possibility is that equilibrium between active and inactive conformations of this site might be influenced by physiological regulatory events such as phosphorylation.

Although the data suggest that cGMP competes for the same site as the dihydropyridizazines (indolidan and LY186126), the question arose whether or not binding determinants for these two types of ligands were identical. To examine this point, use was made of the fact that Zn$^{2+}$ increased the $B_{max}$ for [3H]LY186126 binding without altering the $K_d$ (Figure 6). If both substrate (cGMP) and inhibitors were bound to an identical site, then Zn$^{2+}$ would not be expected to have an effect on the $K_d$ for cGMP. The results in Figure 11 show that Zn$^{2+}$ produced a substantial decrease in affinity for cGMP, indicating that binding determinants for this cyclic nucleotide are not identical to those of the dihydropyridizazines. Rather, these two classes of ligands probably interact with some common binding site amino acid residues, possibly at the catalytic site of SR-PDE, while possessing certain distinct binding interactions of their own. Although our data do not support the possibility of strong negative allosteric coupling between two distinct sites for these compounds, they nevertheless do not rule out this alternative explanation.

Because both Mn$^{2+}$ and Zn$^{2+}$ activated [3H]LY186126 binding to a greater extent than did Mg$^{2+}$, it was anticipated that these divalent cations would also activate SR-PDE to a greater extent than Mg$^{2+}$. In good agreement with the binding data in Figure 5, Mn$^{2+}$ did in fact support an enhanced rate of cAMP hydrolysis relative to Mg$^{2+}$ (Figure 12). By contrast, however, Zn$^{2+}$ completely inhibited PDE activity at concentrations $\geq 50 \mu M$. A further lack of correspondence between binding and PDE activity was seen in the dramatic difference in potencies for activation of these two parameters by Mg$^{2+}$ (Figures 7 and 12). These data point out the lack of a one to one correspondence between binding and enzyme activity, raising the possibility that the [3H]LY186126 binding site is not identical to the catalytic site of SR-PDE. However, inhibition of catalytic activity of SR-PDE by Zn$^{2+}$ is not surprising since this divalent cation produced a substantial decrease in affinity for cGMP (Figure 11), probably by altering topography at the catalytic site of SR-PDE. Additional binding studies, especially using purified SR-PDE, are required to more completely understand the precise relations among the [3H]LY186126 binding site, the catalytic site of SR-PDE, and the divalent metal ion activation site.

In summary, a single class of high affinity binding sites for [3H]LY186126 was shown by marker enzyme analysis to be located in SR membranes. A series of PDE inhibitors was demonstrated to be effective at displacing bound radiolabel, with selective SR-PDE inhibitors, including cGMP, having the highest affinities for this site. The results support the hypothesis that [3H]LY186126 labels the pharmacological receptor for the class of positive inotropic agents that are characterized as isozyme-selective PDE inhibitors, for example, indolidan, milrinone, imazodan, and enoximone. Furthermore, based on correlative data and other lines of biochemical evidence, the identity of this site is in all likelihood SR-PDE, the cGMP-sensitive type IV PDE located in SR membranes.

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