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Analysis of the Binding Sites for the Cardiotonic Phosphodiesterase Inhibitor [^{3}H]LY186126 in Ventricular Myocardium

MICHAEL ARTMAN, DAVID W. ROBERTSON, LYNN MAHONY, and W. JOSEPH THOMPSON

Departments of Pharmacology (M.A., W.J.T.) and Pediatrics (M.A.), University of South Alabama College of Medicine, Mobile, Alabama 36688; Lilly Research Laboratories, Indianapolis, Indiana 46285 (D.W.R.); and the Department of Pediatrics, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235 (L.M.)

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SUMMARY

The positive inotropic action of the newer cardiotonic phosphodiesterase inhibitors such as indolidan, milrinone, and imazodan has been previously attributed to selective inhibition of cGMP-inhibitable Type IV (high affinity) cAMP phosphodiesterase activity. However, the subcellular binding site(s) for this class of compounds has not been defined. We have characterized the binding of [^{3}H]LY186126, an analogue of indolidan, in subcellular fractions prepared from rabbit and sheep ventricular myocardium. Binding required magnesium ion and exhibited rapid association and dissociation kinetics. Specific binding (defined by ligand displacement with 5 μM indolidan) to enriched rabbit sarcoplasmic reticulum (SR) membrane vesicles was saturable (B_{max} = 714 ± 77 fmol/mg of protein) and of high affinity (K_d = 6.2 ± 1.4 nM). Linear and nonlinear analyses of the binding isotherms fit a single-site model. Mixed SR preparations from sheep myocardium exhibited binding characteristics (B_{max} = 944 ± 115 fmol/mg; K_d = 8.5 ± 2.3 nM) comparable to those of rabbit cardiac SR. Further subfractionation of sheep SR indicated that the binding sites were equally distributed between free (B_{max} = 630 fmol/mg; K_d = 4.4 nM) and junctional SR (B_{max} = 569 fmol/mg; K_d = 10.9 nM). Specific binding of [^{3}H]LY186126 was also demonstrated in the cytosolic subfraction of rabbit myocardium that contained Type IV phosphodiesterase activity (Peak III from anion exchange chromatography). Competition for [^{3}H]LY186126 binding studied in rabbit SR showed that, of the compounds tested, lixazinone (RS 82856) competed most effectively (IC_{50} = 0.030 ± 0.008 nM), followed by indolidan (0.14 ± 0.05 nM), cGMP (17.8 ± 2.6 nM), milrinone (39.3 ± 13.2 nM), and imazodan (192 ± 73 nM). In contrast, rolipram, which does not inhibit SR-associated Type IV phosphodiesterase activity, was not effective at competing for [^{3}H]LY186126 binding (IC_{50} > 30 μM). These results indicate that [^{3}H]LY186126 has specific binding sites in myocardial subcellular fractions that contain cGMP-inhibitable Type IV (high affinity) cAMP phosphodiesterase activity.

A new class of cardiotonic drugs with combined inotropic and vasodilator activities has been under development during the past few years (1). The positive inotropic action of these drugs is postulated to result from the relatively selective inhibition of myocardial Type IV, or high affinity, PDE activity (E.C. 3.1.4.17) (2–8). Agents such as indolidan, milrinone, imazodan, and lixazinone (RS 82856) are representative examples of the newer cardiotonic PDE inhibitors.

Type IV PDE activity can be found in both cytosolic and particulate subcellular fractions, with the relative distribution being dependent upon the species of animal under study and conditions of tissue processing (5–10). Recent work suggests that inhibition of activity associated with the SR is a major determinant of the magnitude of the cardiotonic response (7–9). It has been postulated that this PDE activity is the pharmacological receptor for these newer cardiotonic drugs (11). However, little is known regarding the characteristics of this potential drug-receptor interaction.

[^{3}H]LY186126, a radiolabeled derivative of indolidan, was prepared to permit detailed biochemical studies regarding the interaction of the cardiotonic PDE inhibitors with their pharmacological receptor. Preliminary studies demonstrated that this radioligand bound with high affinity to purified free SR vesicles isolated from canine myocardium (12). We have now shown that [^{3}H]LY186126 binding sites exist in ventricular myocardium prepared from rabbits and sheep and we have characterized some of the biochemical properties of this interaction.

Materials and Methods

Chemicals. [^{3}H]LY186126 [1,3-dihydro-3,3-dimethyl-1-[^{3}H$_3$]methyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-

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ABBREVIATIONS: PDE, phosphodiesterase; SR, sarcoplasmic reticulum; BSA, bovine serum albumin.

2-one] was prepared by custom synthesis (Amersham Corporation). The chemical structure of [^{3}H]LY186126 is provided in Fig. 1. Radiochemical purity was 98%, as judged by reverse phase HPLC on a 5-μm Ultrasphere ODS 9.25 cm × 4.6 mm column, eluted isocratically with acetonitrile/tetrahydrofuran/water (100:25:375). The specific activity was 79.2 Ci/mmol and was determined by UV. The radioligand was supplied in ethanol and was diluted with glass-distilled water immediately before use.

Nontritiated LY186126 has been demonstrated to exert a dose-dependent positive inotropic effect following intravenous bolus administration to pentobarbital-anesthetized dogs. The dose of LY186126 required to increase contractility by 50% was 4 μg/kg in this model. Contractility was indexed using the first derivative of the left ventricular pressure curve at 60 mmHg (LV dP/dt_{60}). This dose of LY186126 compares favorably with results from similar studies using the parent cardiotonic compound, indolidan (LY195115). The dose of indolidan required to increase LV dp/dt_{60} by 50% was 7 μg/kg (13).

Other drugs were generously supplied as follows: milrinone (WIN 47203), 1,6-dihydro-2-methyl-6-oxo(3,4′-bipyridine)-5-carbonitrile (Sterling-Winthrop); imazodan (CI-914), 4,5-dihydro-6-[4-(1*H*-imidazol-1-yl)phenyl]-3-(2*H*)-pyridazinone (Warner-Lambert); lixazinone, *N*-cyclohexyl-*N*-methyl-4-(7-oxo-1,2,3,5-tetrahydroimidazo-[2,1-6] quinazolin-2-one) butyramide (RS 82856; Dr. Robert Alvarez, Syntex); and rolipram, 4-[3-(cyclopentoxy)-4-methoxyphenyl]-2-pyrrolidinone (Dr. Ian Williams, Pfizer). All chemicals were reagent grade. Histidine and sucrose were purchased from Sigma Chemical Company, KCl from J. T. Baker, and $NaHCO_3$ from Fisher Scientific. Glass-distilled water was used throughout the experiments.

Myocardial subfractions. Enriched SR vesicles were prepared from maternal sheep or adult rabbit ventricular myocardium, according to the method of Mahony and Jones (14). Either a single sheep heart or three rabbit hearts were used as starting tissue for each SR preparation. Briefly, ventricular muscle was trimmed of atria, great vessels, and gross epicardial fat and was homogenized three times for 30 sec in 4 volumes of $NaHCO_3$, using a Brinkman Polytron PT-20 (50% maximal setting). The homogenate was centrifuged twice at 14,000 × g (R_{max}) for 20 min, in a JA-17 rotor with a Beckman J2-21 centrifuge. The final supernatant was centrifuged at 45,000 × g (R_{max}) for 30 min, in a JA-20 rotor (Beckman J2-21). The pellet contains enriched SR that is a mixture of free and junctional SR. The vesicles were suspended in 0.6 M KCl/30 mM histidine and recentrifuged as described above (45,000 × g). The washed SR was resuspended (syringe and cannula) in 0.25 M sucrose/10 mM histidine and stored at −70°.

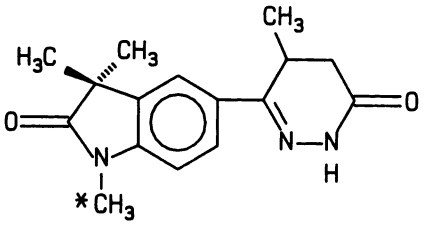


Fig. 1. Chemical structure of [^{3}H]LY186126. The parent compound, indolidan (LY 195115), lacks the two methyl groups on the indolone and dihydropyridazinone rings. Both compounds are potent inhibitors of the Type IV PDE located in myocardial SR from dog and rabbit. In the dog SR, K_i values for indolidan and LY186126 were 80 and 40 nM, respectively (5). In the rabbit SR, IC_{50} values (0.25 μM cAMP as substrate) were 200 nM for indolidan and 50 nM for LY186126. * indicates the position of the tritium label.

TABLE 1

Marker enzyme activities in the crude myocardial homogenate and enriched SR subfraction prepared from ventricular myocardium of rabbits

The average enrichment of the SR Ca^{2+}-K^+ ATPase was 6-fold. Values represent the mean ± standard error of six different preparations.

Preparation	Na^+-K^+ ATPase	Cytochrome *c* oxidase	Ca^{2+}-K^+ ATPase
	μmol/mg/hr	μmol/mg/min	μmol/mg/hr
Homogenate	5.0 ± 2.1	0.40 ± 0.02	5.1 ± 2.0
Enriched SR	5.8 ± 2.7	0.72 ± 0.06	29.6 ± 2.5

Ca^{2+}-K^+ ATPase activity was used to determine the degree of enrichment of the SR preparations (14). Ouabain-inhibitable NA^+-K^+ ATPase (determined in the presence of 0.75 μg of alamethacin/μg of protein) and cytochrome *c* oxidase activities were measured to confirm minimal contamination by sarcolemmal membranes (15) and mitochondria (16), respectively. For some experiments, mixed SR vesicles prepared from sheep myocardium were further subfractionated into free and junctional SR by differential centrifugation (17). These subfractions were characterized by Ca^{2+}-K^+ ATPase activity and stimulation of ATP-dependent calcium uptake by 300 μM ryanodine (17).

Cytosolic subfractions were prepared from rabbit ventricular myocardium using DEAE-cellulose (Whatman DE-52) anion exchange chromatography, according to methods published previously from our laboratory (8, 18, 19). Pooled Peak III fractions that contained Type IV PDE activity (4, 7–9, 19) were used for the binding studies reported here.

Protein was measured by the method of Bradford (20), using BSA as the standard.

Binding assays. Unless stated otherwise, equilibrium binding of [^{3}H]LY186126 was determined at 25°, with an assay volume of 0.1 ml, using 25–50 μg of SR protein. The standard assay contained 50 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, and 0.01% BSA. After a 30-min incubation period, a 0.075-ml aliquot was transferred to a Whatman GF/C filter that was soaked in Tris/BSA overnight, filtered under vacuum (300 mmHg; Gast vacuum pump), and rinsed twice with 5 ml of ice-cold Tris/Mg/BSA. Appropriate control experiments demonstrated that, under these conditions, Type IV PDE activity was retained by the filters. As expected, binding of PDE to the filter reduced basal activity (in the absence of vacuum filtration/washing), but cGMP-inhibitable Type IV PDE activity could be recovered from the filter even after vacuum filtration and washing.

Individual filters were placed into 22-ml polyethylene vials with 8 ml of scintillation fluid (Budget-Solve; Research Products International Corporation) and radioactivity was determined. For calculations of bound drug, a 40% efficiency factor was used. Specific [^{3}H]LY186126 binding was determined by subtracting the binding remaining in the presence of 5 μM indolidan from the total binding.

Competition experiments were performed by incubating 10 nM [^{3}H] LY186126 (25°, 30 min) in the presence of varying concentrations of the unlabeled test agent. Cardiotonic agents were solubilized in either dimethyl sulfoxide or ethanol and were then diluted with the standard binding buffer. Vehicle controls (equivalent dilutions) were performed to confirm the lack of effect of dimethyl sulfoxide or ethanol on [^{3}H] LY186126 binding.

Determination of the association time course was performed using 10 nM [^{3}H]LY186126 (176,000 cpm) and 50–100 μg of protein for each time point. Binding was initiated by the addition of protein and then terminated at timed intervals by filtration and washing. The dissociation time course was characterized using 30 nM [^{3}H]LY186126 and 100 μg of protein for each time point. After equilibrium was reached (30 min), excess indolidan was added and binding was quantitated by filtration at timed intervals.

Data analyses. Data shown are the mean of duplicate or triplicate determinations for each point and, unless otherwise indicated, are representative of results from experiments using two to four different SR preparations. Binding site analyses were performed using LUNDON-1 computer software (Lundon Software, Inc.). This program uses a nonlinear least squares solution of ligand binding parameters without requiring user-supplied initial estimates of binding parameters. One-site model estimates were made using linear methods of Scatchard/Rosenthal (21, 22). One-site model estimates were also made using nonlinear methods (LUNDON-1), as were two-site model estimates.

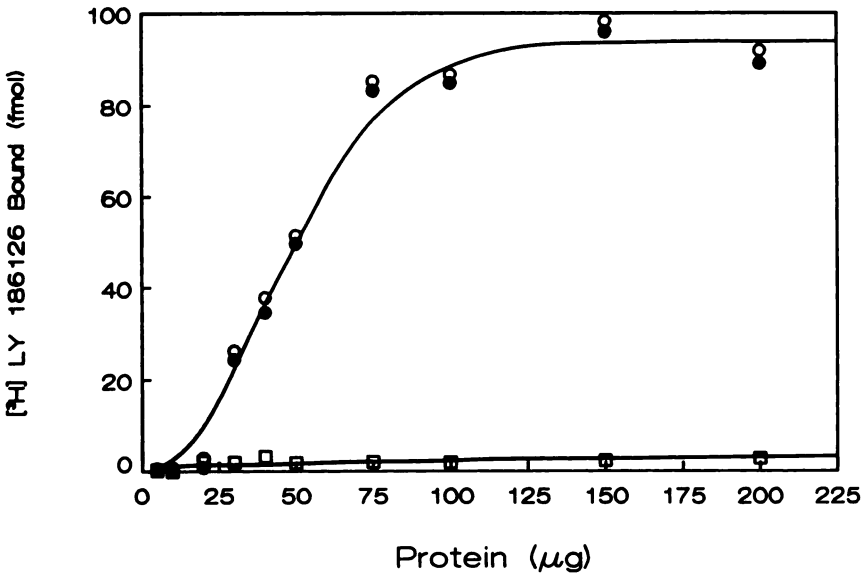


Fig. 2. Effect of varying concentration of rabbit SR membrane protein on total (○), specific (●), and nonspecific (□) binding of 10 nM [^{3}H]LY186126. The total reaction volume was 0.10 ml. At membrane protein concentrations greater than approximately 1 mg/ml, specific binding reached a plateau.

TABLE 2

Effects of $MgCl_2$ and EDTA on specific binding of 10 nM [^{3}H] LY186126 in enriched SR from rabbit myocardium

The standard buffer contained 50 mM Tris, 5 mM $MgCl_2$, and 0.01% BSA. $MgCl_2$ was omitted and/or EDTA (0.5 mM) was included as indicated. [^{3}H]LY186126 (10 nM) and 50 μg of SR membrane protein were used in all assays (30 min; 25°). Values represent the mean of duplicate determinations.

Buffer	Specific binding
	fmol/mg
+ $MgCl_2$ (5 mM)	1190
− $MgCl_2$	118
+ EDTA, − $MgCl_2$	77

Competition experiments were analyzed using the IBM PC version of Curve Fit (M. L. Jaffe Associates, Silver Springs, MD). This program calculates values for the four-parameter logistic function $Y = A-D/1+(X/C)^B + D$, wherein X = dose, Y = percentage of inhibition, A = Y at zero dose, D = Y at infinite dose, $C = IC_{50}$ (Y halfway between A and D), and B = slope factor. This analysis utilizes the 2+2 linear regression approach described by Rodbard and Hutt (23). The IC_{50} values are reported with the 95% confidence limits derived from the full concentration-response curve (12–14 competitor concentrations).

Results

Marker enzyme analyses confirm that the rabbit myocardial SR membrane vesicles under study were enriched in Ca^{2+}-K^+ ATPase activity, with relatively little coenrichment of the sarcolemmal Na^+-K^+ ATPase or mitochondrial cytochrome *c* oxidase (Table 1). These membranes contain almost exclusively cGMP-inhibitable Type IV PDE activity (7, 8).

Specific binding of 10 nM [^{3}H]LY186126 to rabbit mixed SR vesicles was linear between approximately 25 and 100 μg of membrane protein (Fig. 2). Saturation of binding sites was reached at approximately 850 fmol of [^{3}H]LY186126/mg of SR protein. Also demonstrated (Fig. 2) is that approximately 95–98% of the total binding is specific binding if the SR protein concentration is less than 2 mg/ml. When samples were boiled for 5 min before addition of the ligand to the assay mixture, specific binding was less than 5% that of non-boiled samples. Binding of [^{3}H]LY186126 was determined at 25°; experiments using higher temperatures showed an increase in nonspecific binding and those using lower temperatures were unsuccessful because indolidan (5 μM) appeared to precipitate. The effects of omitting magnesium ion and/or adding EDTA are presented in Table 2. These results demonstrate that specific binding is dependent upon the presence of magnesium ion.

[^{3}H]LY186126 demonstrated a rapid rate of association to rabbit cardiac SR membranes (Fig. 3A). Specific binding reached a plateau after 30 sec and remained constant for 120 min. Specific binding was also readily reversible following the addition of excess indolidan (Fig. 3B). It should be noted that the filtration method may be inadequate to characterize very rapid initial events during association and dissociation. The dissociation rate constant (k_{-1}) was 0.036 sec^{-1}, as determined from the slope of the $\ln[B_e/B]$ versus time plot (Fig. 4A) (24). The association rate constant (k_1) was derived by first determining the observed rate constant (k_{obs}) from the slope of the $\ln[B_e/(B_e-B)]$ versus time plot (Fig. 4B) (24), without any consideration of dissociation. The association rate constant was 6.22 liter·μmol^{-1}·sec^{-1}, as calculated from equality to $(k_{obs}-k_{-1})/[LY186126]$. The equilibrium dissociation constant (K_d) of [^{3}H]LY186126 binding derived from these kinetic constants (k_{-1}/k_1) was 5.8 nM.

Binding of [^{3}H]LY186126 to mixed SR membranes from rabbit was saturable and showed high affinity (Fig. 5). Binding isotherm analysis using nonlinear methods (LUNDON-1; see Materials and Methods) indicated that the best fit of the data was to a single-site model. The maximum binding site density (B_{max}) was 714 ± 77 fmol/mg of protein and the equilibrium dissociation constant (K_d) was 6.2 ± 1.4 nM. Linear analysis by the Scatchard/Rosenthal plot (Fig. 5A) yielded similar values (B_{max} = 698 fmol/mg; K_d = 5.8 nM). The K_d derived from equilibrium binding analysis was comparable to the value obtained from the kinetic analysis (5.8 nM).

Soluble or cytosolic myocardial Type IV cAMP PDE activity has been shown previously to elute as the third peak of activity obtained by anion exchange chromatography (Peak III) (2–9, 19). This fraction contains both cGMP-inhibitable and cGMP-insensitive Type IV activity (8). [^{3}H]LY186126 binding was also observed in the Peak III cytosolic fraction prepared from rabbit myocardium (Fig. 6). Specific [^{3}H]LY186126 binding fit a single site, with B_{max} = 890 ± 147 fmol/mg and K_d = 13.6 ±

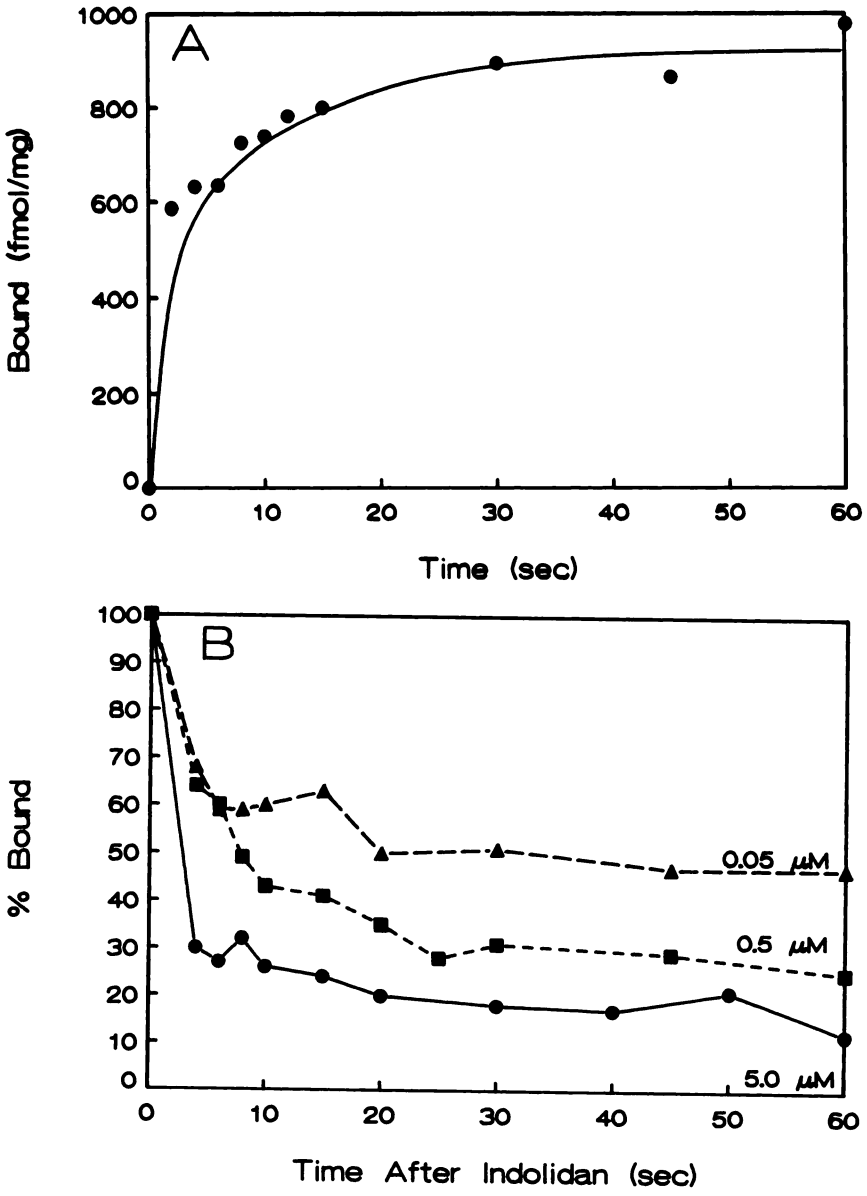


Fig. 3. A, Association time course for binding of 10 nM [³H]LY186126 to rabbit SR vesicles. Binding was conducted at 25°, initiated by the addition of protein (80 μg/0.10 ml), and terminated by rapid filtration. Association was rapid and reached equilibrium by approximately 30 sec. In other experiments, binding remained constant through 120 min. B, Dissociation time course for 30 nM [³H]LY186126, using 100 μg of protein/0.10 ml. Binding (25°) was allowed to reach equilibrium (30 min), after which an excess of unlabeled indolidan was added. Binding was terminated at the indicated time points by membrane filtration, as described in Materials and Methods. Three different concentrations of indolidan were used; 5.0 μM (●), 0.5 μM (■), and 0.05 μM (▲).

4.0 nM. One difference between binding to soluble versus membrane fractions was a higher degree of nonspecific binding by soluble PDE fractions. Specific binding could not be demonstrated in rabbit crude myocardial homogenates.

Sheep myocardium was studied to provide a species comparison and a larger ventricular mass to facilitate further subfractionation of mixed SR into vesicle populations selectively enriched in either free or junctional SR (Table 3). Results obtained for [³H]LY186126 binding to mixed SR vesicles prepared from sheep ventricular myocardium were similar to those of rabbit mixed SR. [³H]LY186126 binding to sheep mixed SR fit a single-site model with B_{max} = 944 ± 115 fmol/mg and K_d = 8.5 ± 2.3 nM. Furthermore, the results obtained with free SR (Fig. 7A) were comparable to those from junctional SR (Fig. 7B) (B_{max} = 630 and 569 fmol/mg, respectively; K_d = 4.4 and 10.9 nM, respectively).

Displacement of [³H]LY186126 binding was characterized using mixed SR prepared from rabbit ventricular myocardium (Fig. 8). A selective inhibitor of cGMP-inhibitable Type IV PDE activity, lixazinone (RS 82856), was the most potent compound tested, showing half-maximal displacement of [³H]LY186126 binding (IC_{50}) at 0.030 ± 0.008 nM. The parent compound indolidan ranked next in order of potency, with an IC_{50} of 0.14 ± 0.05 nM. Milrinone and imazodan, the other cardiotonic Type IV PDE inhibitors tested, exhibited IC_{50} values of 39.3 ± 13.2 and 192 ± 73 nM, respectively. Rolipram is also a selective Type IV PDE inhibitor, but it differs from the other drugs tested in that rolipram is relatively ineffective as an inhibitor of the cGMP-inhibitable Type IV PDE activity (7, 10) found in rabbit myocardial SR (8). However, rolipram inhibits the cGMP-insensitive Type IV PDE forms found predominately in brain and kidney tissue (10, 25). Rolipram did not compete for [³H]LY186126 binding over the range of concentrations tested (IC_{50} > 30 μM; data not shown). Cyclic GMP, an inhibitor of rabbit SR Type IV PDE activity (8), displaced [³H]LY186126 with an IC_{50} of 17.8 ± 2.6 nM.

The 50% displacement values of [³H]LY186126 binding by rabbit SR for lixazinone (RS 82856), indolidan, cGMP, milrinone, and imazodan were compared with their 50% inhibition values for rabbit SR Type IV PDE activity (Fig. 9). Competition

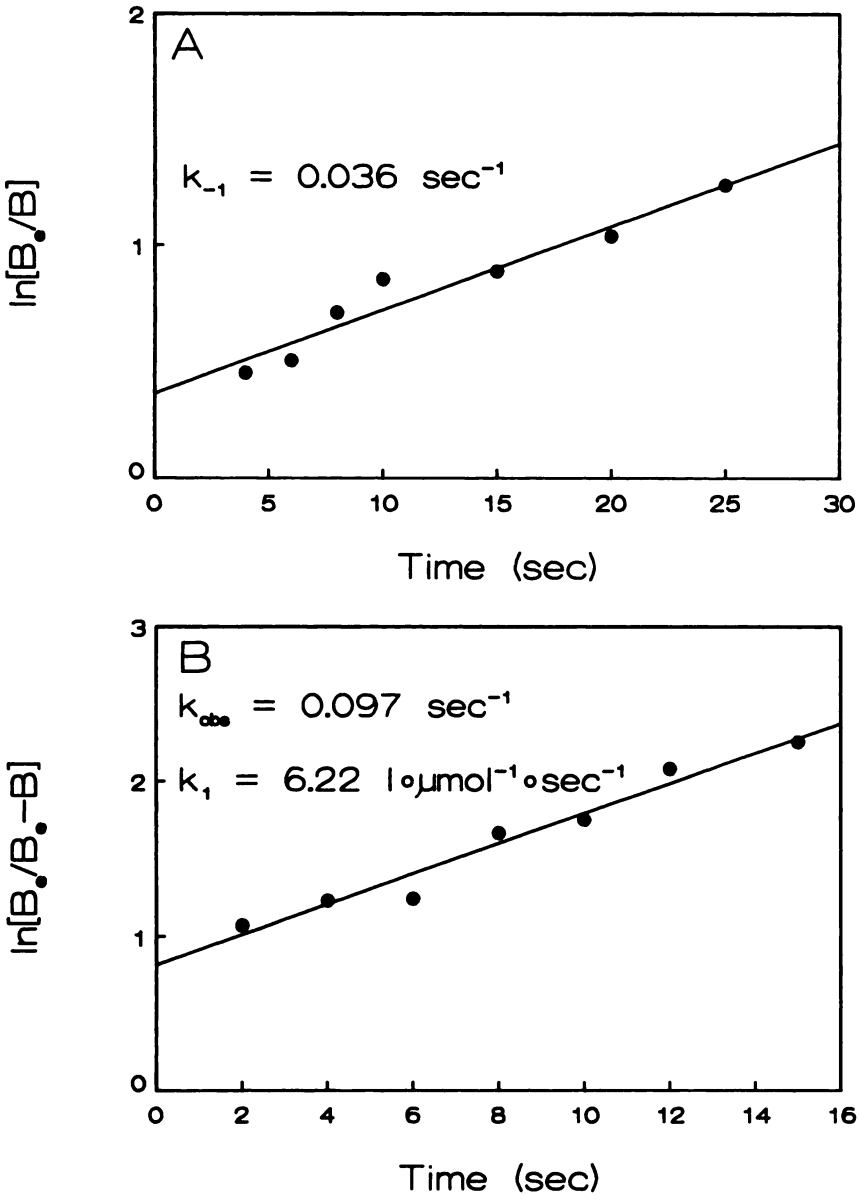


Fig. 4. A, Fractional binding of [^{3}H]LY186126 versus time during the initial 25 sec following displacement by 0.5 µM indolidan (data from Fig. 3B). $B_•$ represents the quantity of bound ligand at equilibrium and B represents the amount bound at each time point after displacement by indolidan. The slope of the relationship ln[$B_•/B$] versus time represents the dissociation rate constant (k_{-1}) (see text for additional details). B, Fractional binding of [^{3}H]LY186126 versus time during the initial 15 sec following initiation of binding (data from Fig. 3A). $B_•$ represents the quantity of bound ligand at equilibrium and B represents binding at each time point. The slope of the relationship ln[$B_•/B_•-B$] versus time represents the observed association rate constant (k_{obs}) without consideration of ligand dissociation.

for [^{3}H]LY186126 binding and inhibition of Type IV PDE activity showed a linear correlation ($r = 0.99$; $p < 0.05$).

Discussion

Substantial progress has been made recently in our understanding of the mechanism of action of a relatively new group of cardiotonic agents (e.g., indolidan, milrinone, and imazodan). These drugs appear to selectively inhibit the cGMP-inhibitable form of Type IV (high affinity) cAMP PDE within the myocardium. The hypothesis has been developed that selective inhibition of this enzyme accounts for the positive inotropic effects of these cardiotonic drugs (5–11). Our findings in rabbit heart indicated that the contractile effects of several newer cardiotonic agents may be mediated by changes in cAMP metabolism (8). Further studies have shown that the subcellular distribution of this enzyme is important to the mechanism of action of the quinazoline (e.g., lixazinone; RS 82856), bipyridine (e.g., milrinone), and dihydropyridazinone (e.g., indolidan and imazodan) derivatives (5–9). Weishaar *et al.* (7, 9) have emphasized the correlation between inhibition of the membrane-associated Type IV PDE activity and differences in inotropic responsiveness among different species. In the rabbit, cGMP-inhibitable Type IV PDE has been found in both cytosolic and particulate (SR) subcellular fractions (8). Moreover, inhibition of the SR-bound enzyme correlated much better with cardiotonic activity than did inhibition of cytosolic activity (8).

The results of the present study provide a partial characterization of the site(s) to which these compounds bind within the myocardium. The selective Type IV PDE inhibitor [^{3}H] LY186126 binds to myocardial fractions that we and others have shown to contain almost exclusively cGMP-inhibitable Type IV PDE activity (7, 8). Specific, high affinity, saturable, and reversible binding of [^{3}H]LY186126 was demonstrated in mixed SR, free SR, junctional SR, and the cytosolic fraction obtained by anion exchange chromatography. Approximately 95% of the total binding to the membrane fractions was displaced with excess indolidan, the parent congener of the labeled binding ligand. The cytosolic DE-52 Peak III fraction showed

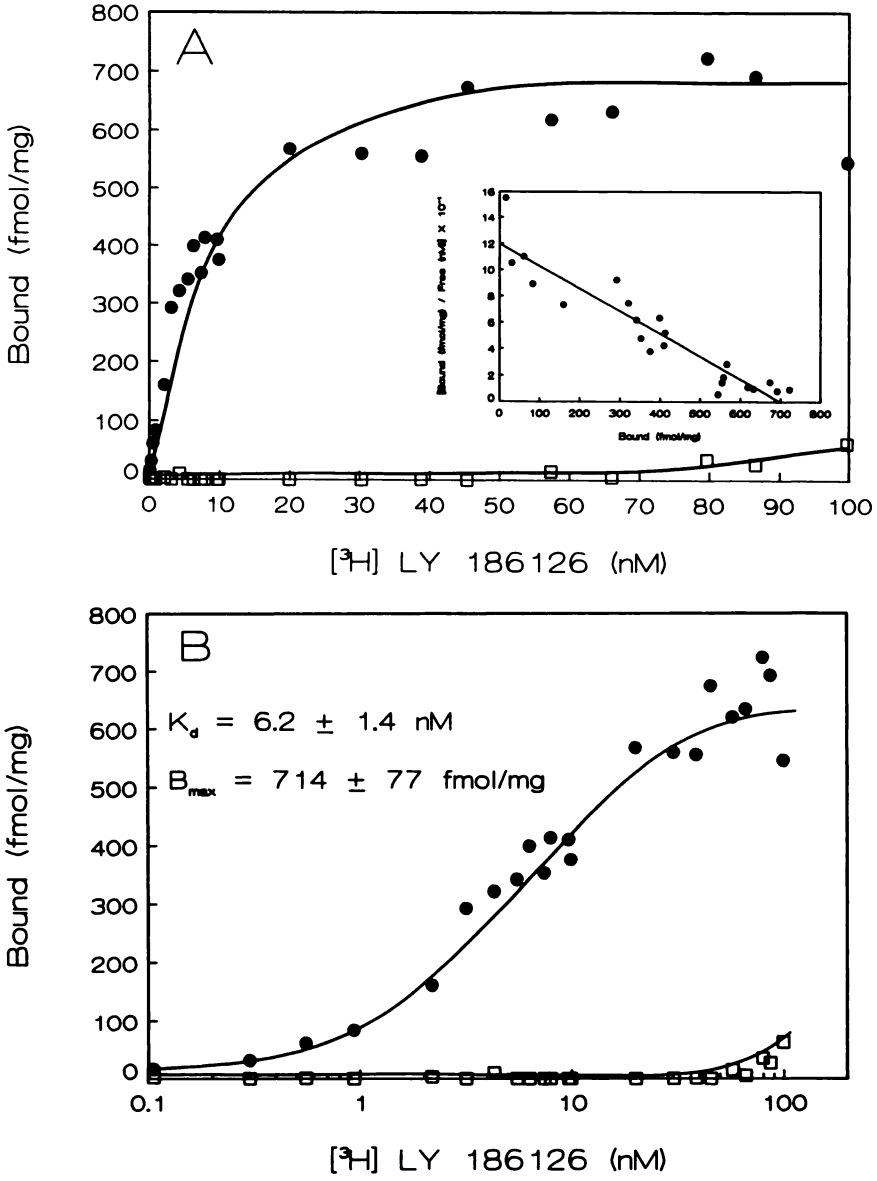


Fig. 5. Equilibrium binding of [³H]LY186126 to mixed SR prepared from rabbit ventricular myocardium. Binding was performed at 25° for 30 min using 50 μg of SR protein in 0.10 ml of assay mixture that contained 50 mM Tris-HCl, 5 mM $MgCl_2$, and 0.01% BSA (pH = 7.4). ●, Specific binding (defined as that displaced by 5 μM indolidan); □, nonspecific binding. Each *point* represents the mean of triplicate determinations. Nonlinear analysis (LUNDON-1; see Materials and Methods) indicated the best fit of the data was to a single-site model with B_{max} = 714 ± 77 fmol/mg and K_d = 6.2 ± 1.4 nM. *Inset*, a Scatchard/Rosenthal plot of bound/free versus bound ligand.

much higher nonspecific binding and approximately 50% reduced ligand affinity, as compared with the SR membranes.

The cardiotonic acceptor sites required magnesium ion for drug binding, and specific binding of [³H]LY186126 was observed best at room temperature. Further characterization of the binding showed that the rates of ligand association and dissociation were rapid at room temperature and that equilibrium was attained after 30–60 sec. Events during the initial seconds of association and dissociation are difficult to analyze precisely using the filtration method. For this reason, our results cannot exclude an additional more rapid component during the earliest phases of association and dissociation. However, the dissociation binding constant calculated from the kinetic data (5.8 nM) was in good agreement with the value obtained under equilibrium conditions (6.2 nM).

Computer-assisted analyses of the binding isotherms showed that the data best fit a single-site model. Thus, it is likely that a single class of acceptor sites for these drugs is present in rabbit and sheep cardiac SR. The number of binding sites in the SR preparations of both species was near 1000 fmol/mg of membrane protein. Unfortunately, the lack of kinetic parameters for pure Type IV PDE and SR precludes a direct estimation of the number of [³H]LY186126 binding sites that would be expected in rabbit SR if the drug were binding only to Type IV PDE molecules. However, using the data of Harrison *et al.* (11, 26) for the highly purified cytosolic Type IV PDE from bovine heart (apparent V_{max} = 6 μmol/min/mg; subunit M_r 110,000) and with the assumptions that the soluble enzyme approximates the particulate enzyme and each mol of enzyme subunit binds 1 mol of ligand, approximately 1200 fmol of PDE binding sites would be expected/mg in the rabbit mixed SR preparation (apparent V_{max} = 2.5 nmol/min/mg) (8). Thus, using these assumptions, the number of ligand binding sites observed in mixed SR was of a reasonable magnitude if the majority of the sites are Type IV PDE. Specific binding of [³H]LY186126 was not observed in cruder cardiac tissue fractions, reflecting a much lower specific activity of this enzyme than in the enriched SR.

The results from the competition experiments support the concept that [³H]LY186126 binding in subcellular fractions

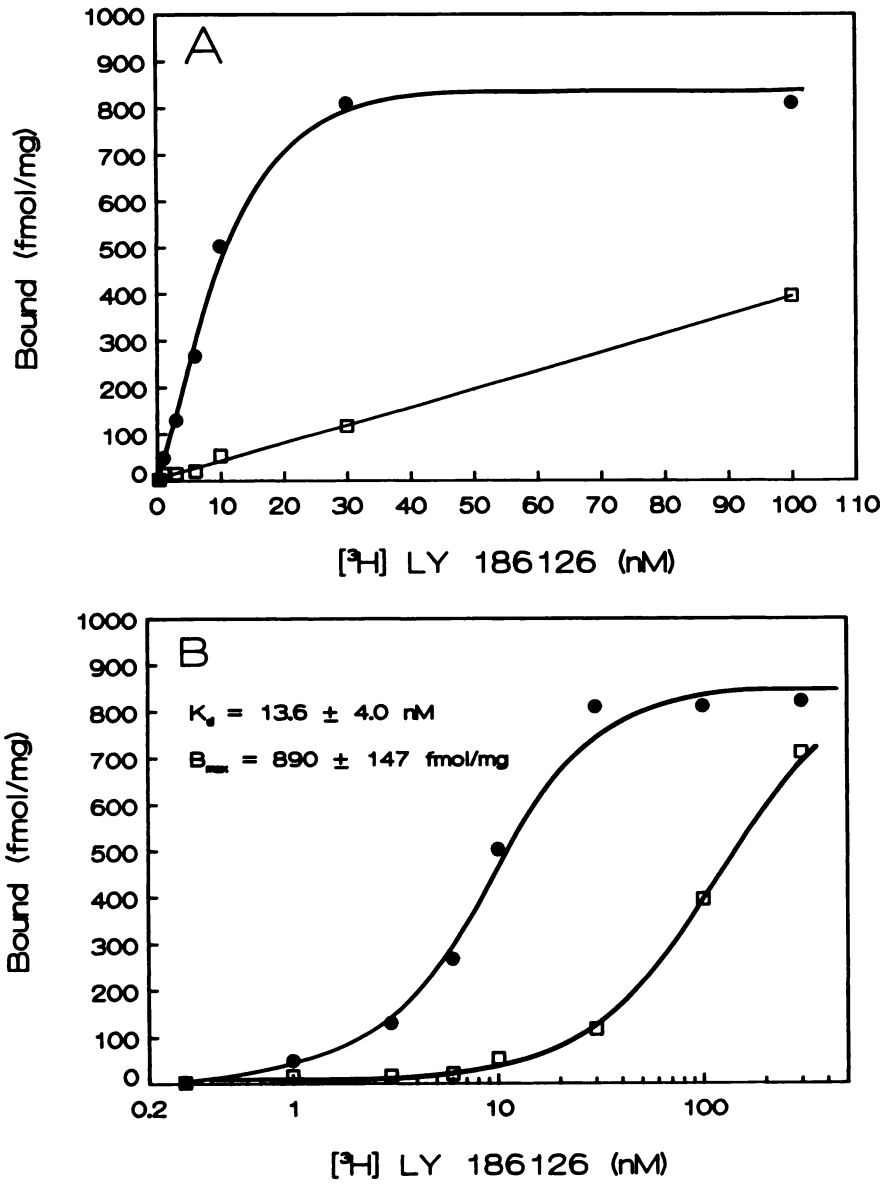


Fig. 6. Equilibrium binding of [^{3}H]LY186126 to a cytosolic Peak III fraction prepared by anion exchange chromatography from rabbit ventricular myocardium. DE-52 Peak III is enriched in both forms of Type IV (high affinity) cAMP PDE activity. Binding conditions were the same as those described in the legend to Fig. 5, except that 25 μg of protein was used. ●, Specific binding; □, nonspecific binding. The data best fit a one-site model with B_{max} = 890 ± 147 fmol/mg and K_d = 13.6 ± 4.0 nM (see Materials and Methods).

TABLE 3

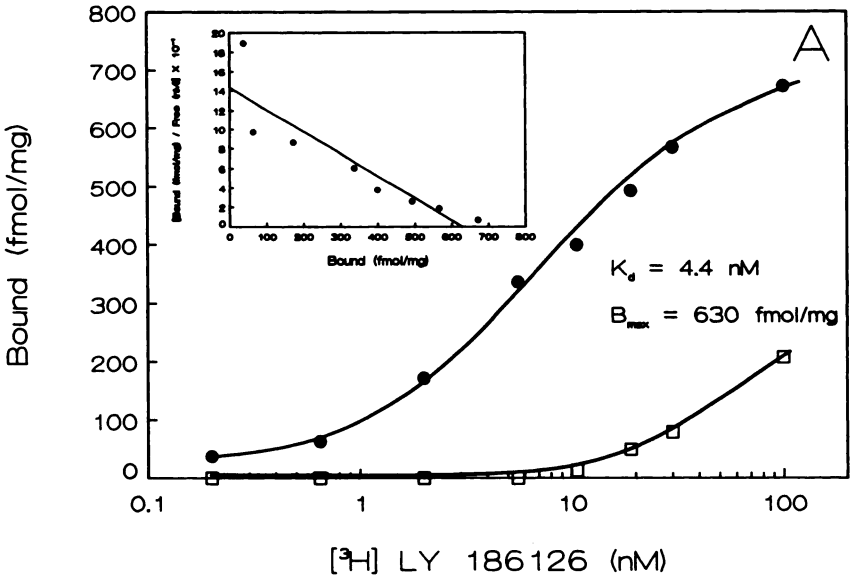
Characterization of SR subfractions prepared from sheep ventricular myocardium

Maximal calcium uptake was determined using 50 μM free calcium in the absence or presence of 300 μM ryanodine.

Subfraction	Ca^{2+}-K^+ ATPase	Maximal Ca^{2+} uptake	
		−Ryanodine	+Ryanodine
	μmol/mg/hr	nmol/mg	
Free	134.5	5239	6112
Junctional	48.6	1875	3225

that contain cGMP-inhibitable Type IV PDE is to a site either on the enzyme or on a SR protein closely associated with the enzyme. Compounds that are potent and selective competitive inhibitors of this form of enzyme activity were also effective at competing for [^{3}H]LY186126 binding sites. Furthermore, the inhibitory potency toward Type IV PDE enzyme activity in rabbit SR correlated with the relative potency of these agents for displacement of [^{3}H]LY186126 from SR. Displacement of [^{3}H]LY186126 by cGMP and cardiotonic PDE inhibitors from rabbit SR was comparable to that observed using canine myocardium (12). Interestingly, the displacement curves for lixazinone and indolidan were more shallow than those of the other compounds tested. Although analyses of the binding isotherms fit a single-site model, these differences in slope suggest site heterogeneity or changes in site conformation.

Our studies of [^{3}H]LY186126 binding in sheep myocardium demonstrated that the binding sites are relatively evenly distributed between the free and junctional SR. Although incomplete separation of SR subpopulations might partially contribute to these findings, the relative Ca^{2+}-K^+ ATPase activity, calcium uptake, and ryanodine sensitivity are consistent with adequate separation of free and junctional SR. In addition, preliminary results from [^{3}H]LY186126 binding studies using free SR prepared from canine myocardium (12) were similar to results obtained using rabbit and sheep myocardium. Moreover, previous studies with canine myocardium indicate that indolidan-sensitive PDE activity is located in both junctional and



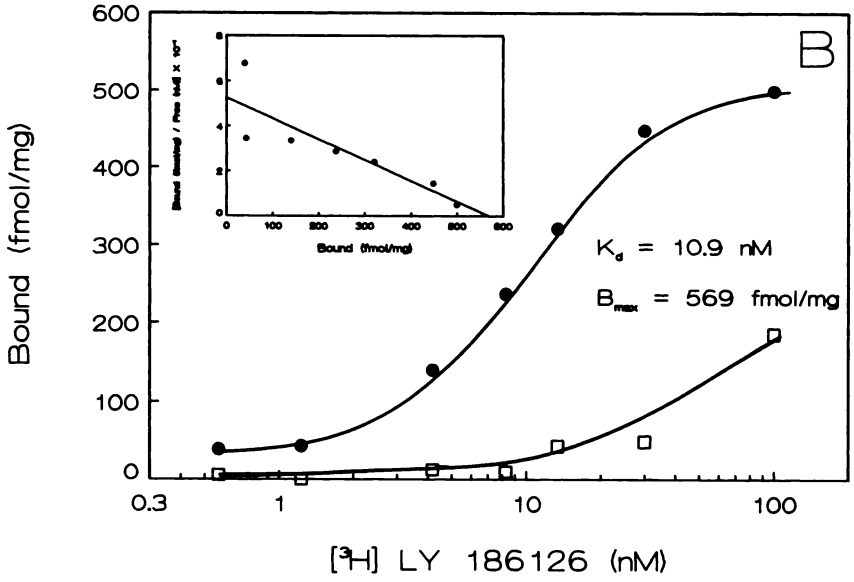


Fig. 7. Equilibrium binding of [³H]LY186126 to free SR (A) and junctional SR (B) prepared from sheep ventricular myocardium. Binding conditions were identical to those described in the legend for Fig. 5, except that 25 μg of protein was used. ●, Specific binding; □, nonspecific binding. *Insets*, Scatchard/Rosenthal plots of bound/free versus bound ligand. Free SR, B_{max} = 630 fmol/mg; K_d = 4.4 nM. Junctional SR, B_{max} = 569 fmol/mg; K_d = 10.9 nM.

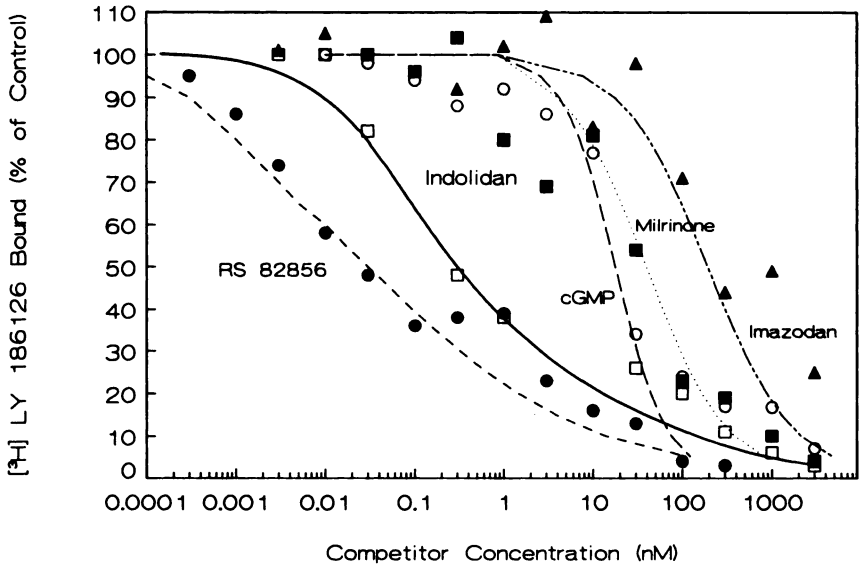


Fig. 8. Competition for [³H]LY186126 binding to mixed SR prepared from rabbit ventricular myocardium. Equilibrium binding conditions were as described above (Fig. 5 and Materials and Methods), except for the addition of varying amounts of one of the compounds tested to the incubation medium. Each *data point* displayed on the *curves* is the average of two to four experimental determinations for each given competitor concentration. The *lines* indicate computer-generated curves that were fit to the data using a linear least squares method over the full concentration-response range (12–14 different competitor concentrations). This iterative program (Curvefit) uses 2+2 linear regression to calculate values for the four-parameter logistic function defined in Materials and Methods (23). The resultant IC_{50} value is expressed along with the 95% confidence limits, in order to provide an estimate of the variance for each IC_{50} value. The experimentally determined IC_{50} values (±95% confidence limits) were 0.030 ± 0.008 nM for RS 82856 (lixazinone) (●), 0.14 ± 0.05 nM for indolidan (□), 17.8 ± 2.6 nM for cGMP (○), 39.3 ± 13.2 nM for milrinone (■), and 192 ± 73 nM for imazodan (▲).

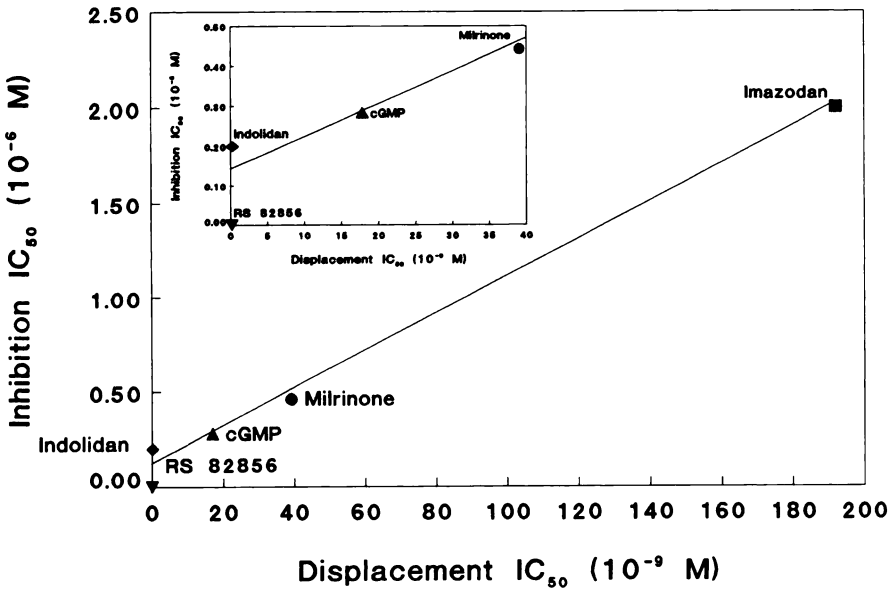


Fig. 9. Correlation of rabbit SR Type IV PDE inhibition and [^{3}H]LY186126 binding displacement. Displacement IC_{50} values were from the experiments described in Fig. 8 and inhibition IC_{50} values were from results reported previously from our laboratory (8), except that the IC_{50} values for RS 82856 (lixazinone; 0.50 ± 0.03 nM) and indolidan (0.20 ± 0.02 μM) were determined recently.

free SR subfractions (5). Given the central importance of cAMP in the regulation of intracellular calcium concentration and the distinct functional roles of free and junctional SR, these findings suggest that control of cAMP hydrolysis by Type IV PDE may be involved in the modulation of calcium uptake (free SR) and release (junctional SR). However, this potential regulatory role of Type IV PDE is speculative, at present, and considerable additional research will be required in order to understand the precise mechanistic relationship between inhibition of Type IV PDE and positive inotropy.

The finding that specific binding of [^{3}H]LY186126 also occurs in cytosolic Peak III fractions (containing both cGMP-inhibitable and cGMP-insensitive Type IV PDE activities) further supports the association of binding sites with enzyme activity. As with Type IV PDE, it is conceivable that a portion of the soluble binding sites may have been released from membranes by tissue fractionation. Alternatively, however, both particulate and soluble binding sites may be present in intact myocardial tissue.

In summary, our data demonstrate that high affinity, saturable, binding sites for [^{3}H]LY186126 exist in the myocardium of rabbits and sheep. Because these sites also exist in canine myocardium (12), they appear to exist in a variety of species that are responsive to cardiotonic PDE inhibitors. Moreover, our results support the concept that the myocardial acceptor site for indolidan (and related cardiotonic PDE inhibitors) is closely associated with the cGMP-inhibitable Type IV (high affinity) cAMP PDE. Although it is probable that the inhibitor binds either to the enzyme or to a closely associated protein, the specific molecular site remains to be determined. Full characterization of the cardiotonic binding site will likely require purification of the cardiotonic binding site-enzyme complex.

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Send reprint requests to: Michael Artman, M.D., University of South Alabama College of Medicine, Department of Pharmacology, 3130 Medical Sciences Building, Mobile, AL 36688.