Specific Binding of $[^3]$HLY186126, an Analogue of Indolidan (LY195115), to Cardiac Membranes Enriched in Sarcoplasmic Reticulum Vesicles

Raymond F. Kauffman, Barbara G. Utterback, and David W. Robertson

LY186126 was found to be a potent inhibitor of type IV cyclic AMP phosphodiesterase located in the sarcoplasmic reticulum of canine cardiac muscle. This compound, a close structural analogue of indolidan (LY195115), was prepared in high specific activity, tritiated form to study the positive inotropic receptor(s) for cardiotonic phosphodiesterase inhibitors such as indolidan and milrinone. A high-affinity binding site for $[^3]$HLY186126 was observed ($K_d=4$ nM) in purified preparations of canine cardiac sarcoplasmic reticulum vesicles. Binding was proportional to vesicle protein, was inactivated by subjecting membranes to proteolysis or boiling, and was dependent on added Mg$^{2+}$. Scatchard analysis suggested the presence of a single class of binding sites in the membrane preparation. Indolidan, milrinone, and LY186126 (all at 1 $\mu$M) produced essentially complete displacement of bound $[^3]$HLY186126, while nifedipine, propranolol, and prazosin had little or no effect at this concentration. This represents the first reported use of a radioactive analogue to label the inotropic receptor for cardiotonic phosphodiesterase inhibitors. The results suggest that $[^3]$HLY186126 is a useful radiotag for examining the subcellular site(s) responsible for positive inotropic effects of these drugs. (Circulation Research 1989;64:1037-1040)

Indolidan (LY195115), a dihydropyridazinone cardiotonic (Figure 1), exhibits potent positive inotropic and vasodilator activities and is being tested clinically for treatment of congestive heart failure.1 This compound was recently shown to be a potent, selective inhibitor of cyclic AMP (cAMP) phosphodiesterase (PDE) located in sarcoplasmic reticulum membranes (SR-PDE).2 Based on high affinity for cAMP ($K_m=0.46$ $\mu$M) and lack of stimulation by calmodulin and cyclic GMP, SR-PDE is classified as a type IV PDE according to current nomenclature3 and appears to be identical to cyclic GMP-inhibited PDE, or PDE III, from cardiac muscle as described by others.4-5 Potencies for in vivo stimulation of cardiac contractility by indolidan and a series of cardiotonic PDE inhibitors (e.g., milrinone, enoximone, and imazodan) were highly correlated with $IC_{50}$ values versus SR-PDE, suggesting that inhibition of SR-PDE is involved in the positive inotropic effects of these drugs.2 The ability of these same agents to relax serotonin-contracted, isolated vascular smooth muscle was also highly correlated with $IC_{50}$ values versus cardiac SR-PDE,6 suggesting that a similar or identical PDE isozyme in the vasculature may be responsible for vasodilation. Thus, the pharmacological actions of indolidan appear to be explained by isozyme-selective inhibition of PDE.

A close structural analogue of indolidan, LY186126, was prepared in a high specific activity, tritiated form (Figure 1) to radiolabel the positive inotropic receptor(s) for this class of cardiotonics. These studies were designed to test further the hypothesis that inhibition of SR-PDE is the biochemical mechanism underlying direct cardiac effects of the dihydropyridazinones. The results demonstrate that $[^3]$HLY186126 binds specifically and with high affinity to a highly purified preparation of cardiac sarcoplasmic reticulum (SR) vesicles. Preliminary findings suggest a role for this site in mediating positive inotropic effects of these compounds.

Materials and Methods

Isolation of Canine Cardiac Membranes

Crude cardiac microsomes were prepared by standard techniques of differential centrifugation. Purpose-bred hounds (15–20 kg) were anesthetized...
with sodium pentobarbital (35 mg/kg i.v.), and their hearts were removed and placed in 154 mM NaCl (0-4°C). All subsequent operations were carried out at 0-4°C. Ventricular muscle was homogenized in 50 mM Tris-Cl, 5 mM MgCl₂, pH 7.4 (1:10, w/v vol) using a Polytron apparatus (Brinkmann Instruments, Westbury, New York) equipped with the PT20 generator. Following a low speed spin at 12,000g for 7 minutes, the supernatant fluid was centrifuged at 100,000g for 60 minutes. The resulting pellet was resuspended in the above buffer containing 0.01% bovine serum albumin (BSA) and was subsequently used in the binding assay.

Purified SR vesicles (fraction E) were isolated from canine ventricles by the procedure described by Jones and Cala. Vesicle protein was determined by the BCA protein assay using bovine serum albumin (BSA; fraction V) as the protein standard. Membrane vesicles were stored frozen at -80°C in 0.25 M sucrose, 10 mM histidine, pH 7.5, for up to 2 months with no detectable changes in properties of the binding site described below.

**Determination of Enzyme Activities**

PDE activity of cardiac SR vesicles was determined at 1 μM cAMP as described in a previous report. Dimethyl sulfoxide was utilized as solvent for stock solutions of LY186126. Solutions of the inhibitor were prepared on the day of an experiment, and controls were run to ensure that carryover solvent (2.5%, vol/vol) had no effect on assay results. Inhibition curves were constructed by varying the concentration of inhibitor over a range of 2.5 log units encompassing the IC₅₀ (concentration producing 50% inhibition of PDE activity). Ca²⁺-ATPase activity was quantitated at 37°C by appearance of inorganic phosphate as described previously.

**Determination of Membrane Binding**

Binding studies were carried out at 25°C in 1.0 ml of the following medium: 50 mM Tris-Cl, 5 mM MgCl₂, 0.01% BSA, pH 7.4, in the presence of varying concentrations of [³H]LY186126. Dimethyl sulfoxide (DMSO) was employed as solvent for displacing ligands; control studies demonstrated that DMSO had no detectable effect on binding. Binding reactions were allowed to proceed to completion (60 minutes), at which time the membrane suspension was filtered using a vacuum apparatus (Brandel cell harvester, Gaithersburg, Maryland) onto Whatman GF/C filters (Clifton, New Jersey) 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. 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.

**Analysis of Data**

IC₅₀ values for inhibition of PDE activity were determined graphically from inhibition curves (percent activity versus negative logarithm of inhibitor concentration). Kᵢ values for [³H]LY186126 binding isotherms were determined by nonlinear least-squares analysis using LUNDON I software (Lundon Software, Inc, Cleveland, Ohio).

**Materials**

The synthesis and characterization of [³H]LY186126 (1,3-dihydro-3,3-dimethyl-1-[³H₃]-methyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2H-indol-2-one; 79.2 Ci/mmol) has been described. The radio-labeled 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 LY186126 were as described in the literature (indicated compounds were designated as numbers 20 and 26, respectively, in this reference). Milrinone was kindly supplied by Sterling-Winthrop, New York, New York, and prazosin was a gift from Pfizer Laboratories, New York, New York. BCA protein reagent was obtained from Pierce Chemicals, Rockford, Illinois. BSA (fraction V), trypsin (bovine pancreatic), and propranolol were purchased from Sigma Chemical, St. Louis, Missouri. Nifedipine was obtained from Miofar, Milano, Italy. [³H]cAMP was purchased from New England Nuclear, Boston, Massachusetts.

**Results**

LY186126 was examined for inhibitory effects on SR-PDE. Concentration-dependent inhibition was observed with an IC₅₀ of 0.12 μM determined at 1 μM cAMP (data not shown). Assuming competitive inhibition, a Kᵢ value of approximately 40 nM was calculated based on the published Kᵢ value of 0.46 μM for cAMP.

Initial attempts to detect specific binding of [³H]LY186126 to SR vesicles were hampered by high levels of nonspecific binding. Soaking the glass fiber filter in buffer solution containing 0.05% BSA and inclusion of BSA in the binding medium (see "Materials and Methods") lowered nonspecific binding sufficiently to allow facile detection of specifically bound radiolabel. Specific binding of [³H]LY186126 was directly proportional to membrane vesicle protein, and control experiments dem-
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FIGURE 2. Saturation isotherm for [3H]LY186126 binding to canine cardiac sarcoplasmic reticulum vesicles. Binding was determined at 25°C in the presence of the indicated concentrations of [3H]LY186126 as described in the methods section. Data points represent the mean of triplicate determinations. Specific binding was defined as the portion of total binding displaced by 100 μM indolidan. Inset: Scatchard replot of the binding data.

... demonstrated that BSA had no effect on specific binding to SR vesicles (data not shown). Furthermore, specific binding was abolished by the following: removal of Mg<sup>2+</sup> from the binding medium, boiling the membranes for 1 minute, or subjecting membranes to proteolysis with trypsin (data not shown).

A representative binding isotherm depicting total, specific, and nonspecific binding is shown in Figure 2. Despite the use of BSA in the binding medium, nonspecific binding was unacceptably high at [3H]LY186126 concentrations greater than 80–100 nM; consequently, data in this range were unreliable and are not reported. Scatchard replots of the binding data were linear (Figure 2, inset), suggesting the presence of a single, saturable class of binding sites in the SR membrane preparation. Nonlinear least-squares analysis of the data yielded $K_d$ and $B_{max}$ values of 3.8±1.3 nM and 780±100 fmol/mg vesicle protein (mean±SEM; n=3). Binding site density was enriched approximately 10-fold in the purified SR vesicles relative to crude cardiac microsomes from canine left ventricles (data not shown).

The specificity of [3H]LY186126 binding to SR vesicles is demonstrated in Table 1. Essentially complete displacement of [3H]LY186126 was produced by 1 μM cold LY186126, fully consistent with the $K_d$ value reported above. Indolidan and milrinone (1 μM) also produced nearly complete displacement of bound radiolabel. By contrast, propranolol, prazosin, and nifedipine had little or no effect at the concentration of 1 μM.

**Discussion**

LY186126 was previously shown to be equipotent to indolidan as a positive inotropic agent in

**TABLE 1. Selective Displacement of [3H]LY186126 by Cardiotonic Phosphodiesterase Inhibitors**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>[3H]LY186126 bound (fmol/mg vesicle protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>514±18</td>
</tr>
<tr>
<td>+ LY186126</td>
<td>7±9</td>
</tr>
<tr>
<td>+ indolidan</td>
<td>33±13</td>
</tr>
<tr>
<td>+ milrinone</td>
<td>30±11</td>
</tr>
<tr>
<td>+ nifedipine</td>
<td>489±18</td>
</tr>
<tr>
<td>+ propranolol</td>
<td>440±24</td>
</tr>
<tr>
<td>+ prazosin</td>
<td>393±15</td>
</tr>
</tbody>
</table>

[3H]LY186126 binding was determined at 10 nM radioligand using canine cardiac sarcoplasmic reticulum vesicles as described in "Materials and Methods." Experiments were conducted in the presence or absence (Control) of the indicated compounds at a concentration of 1 μM. Data are presented as the mean±SEM of triplicate determinations.
pentobarbital-anesthetized dogs; 50% increases in contractility were produced by 4.4 or 6.8 μg/kg i.v. (bolus injection) of LY186126 or indolidan, respectively.10 In the present report, LY186126 was found to be a potent inhibitor of SR-PDE. Based on the observed IC₅₀ of 0.12 μM, LY186126 was equipotent to indolidan (IC₅₀=0.13 μM) as an inhibitor of SR-PDE. These findings, together with the chemical means for facile introduction of tritium into the indole methyl group, provided the rationale for selecting LY186126 as the target compound for synthesis of a potential radioligand for the cardiotonic receptor(s) of indolidan.

A specific, high-affinity (Kₐ=4 nM), saturable binding site for [³H]LY186126 was detected in highly purified preparations of cardiac SR vesicles. The protein nature of the site was indicated by sensitivity to boiling and proteolysis. Based on enrichment in SR vesicles relative to crude homogenates, the site would appear to be located within SR membranes. Nevertheless, additional studies are required to establish the precise subcellular location(s) of this binding site in cardiac membranes.

Selective displacement of the radiolabel by cardiotonic PDE inhibitors was observed. Indolidan, milrinone, and LY186126 were potent displacers of bound radiolabel, while ligands for α-adrenergic (prazosin), β-adrenergic (propranolol), and calcium channel (nifedipine) receptors had little or no effect upon binding at a concentration (1 μM) known to produce essentially complete displacement at these receptors.11-13 Consequently, the site labeled by [³H]LY186126 displayed both specificity and selectivity for cardiotonic PDE inhibitors. In future investigations, Kₐ values for binding by a variety of cardiotonic PDE inhibitors will be determined and compared with inotropic potencies to assess more rigorously the pharmacological relevance of this site.

Binding of [³H]LY186126 was dependent upon the presence of the divalent cation Mg²⁺. Since cyclic nucleotide phosphodiesterases are known to require divalent cations for activity,14 this finding is consistent with the hypothesis that [³H]LY186126 binds to a PDE isozyme, perhaps the membrane-bound type IV cAMP phosphodiesterase (SR-PDE) previously shown by us and others to be an important site of action for cardiotonic PDE inhibitors.2,4,15

The relation between the binding site for [³H]LY186126 and SR-PDE is currently under investigation in our laboratory, and results of this study will be presented in a future report.

In summary, [³H]LY186126 was shown to bind with high affinity to a site in purified preparations of canine cardiac SR vesicles. The specificity for binding by cardiotonic PDE inhibitors is consistent with the hypothesis that [³H]LY186126 labels a site that mediates or contributes to positive inotropic actions of these drugs. Thus, results obtained to date indicate that [³H]LY186126 is a useful probe for investigating the molecular pharmacology of these drugs. Future efforts will be devoted to determining the subcellular location, identity, and pharmacological relevance of the site labelled by [³H]LY186126.

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Key Words: [³H]LY186126  sarcoplasmic reticulum  binding site  indolidan (LY195115)  milrinone
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