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LY195115: A Potent, Selective Inhibitor of Cyclic Nucleotide Phosphodiesterase Located in the Sarcoplasmic Reticulum

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SUMMARY

LY195115 selectively inhibited the peak III isozyme of cardiac cyclic nucleotide phosphodiesterase (PDE) eluted from DEAE-cellulose columns. Inhibition curves were biphasic, suggesting heterogeneity within this preparation. Since peak III PDE is reported to be derived from membranes, effects of LY195115 upon PDE associated with cardiac membranes were examined. LY195115-sensitive PDE measured in the various membrane fractions correlated well with the sarcoplasmic reticulum marker Ca^{2+}-ATPase ($r = 0.94$; $p < 0.001$), but not with Na^+,K^+-ATPase or azide-sensitive ATPase. Membrane disruption failed to reveal latent LY195115-sensitive PDE in sarcolemmal vesicles known to be primarily right side out. The results suggest that LY195115-sensitive PDE is located within sarcoplasmic reticulum membranes with a distribution similar or identical to that of Ca^{2+}-ATPase. Accordingly, LY195115-sensitive PDE was referred to as SR-PDE. A subfraction of sarcoplasmic reticulum vesicles (free SR vesicles) was sufficiently homogeneous with respect to SR-PDE activity to carry out steady state kinetic studies. Double reciprocal plots of cAMP hydrolysis were linear, yielding K_m and V_{max} values of 0.46 ± 0.03 μM and 700 ± 90 pmol/min/mg of vesicle protein, respectively. LY195115 was a linear competitive inhibitor of SR-PDE with a K_i of 80 ± 10 nM. $-\log IC_{50}$ values for inhibition of SR-PDE by a series of structural analogues of LY195115 correlated highly with published $-\log ED_{50}$ values for stimulation of cardiac contractility *in vivo* ($r = 0.91$, $p < 0.001$). Consequently, *in vivo* effects of LY195115 upon the heart appear to result primarily from competitive inhibition of SR-PDE, or from binding to a site with a topography similar or identical to that of the catalytic site of SR-PDE.

LY195115 (Fig. 1) is a positive inotropic agent under investigation for treatment of congestive heart failure. Based on *in vivo* actions (1), LY195115 is classified as an inotropic-vasodilator, implying that the compound simultaneously increases contractility of the heart and decreases resistance to flow of blood through the vasculature. Such combined actions have been shown to be more effective than either inotropic or vasodilator therapy alone at improving cardiac performance of patients in heart failure (2).

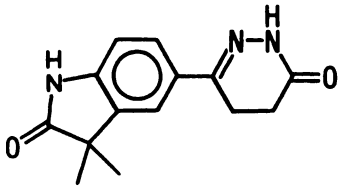
The biochemical basis for stimulation of cardiac contractility by LY195115 is unknown. Positive inotropic effects of this compound upon isolated, electrically driven cardiac muscle were not attenuated by prazosin or propranolol, demonstrating that α_1- or β-adrenergic receptors are not involved (1). In addition, high concentrations of LY195115 failed to inhibit Na^+,K^+-ATPase,[1] generally considered to be a site of action for positive inotropic effects of cardiac glycosides (see Ref. 3 for a review). LY195115, therefore, does not appear to interact with these classical receptors involved in the regulation of contractility.

Various compounds with *in vivo* profiles of action similar to that of LY195115 (see Fig. 1 for representative examples) have been shown to inhibit a specific fraction/isozyme of cardiac cAMP phosphodiesterase (4–6), generally referred to as PDE III (see below). Based on these findings, inhibition of PDE III was postulated to play a role in the mechanism of these agents (4–6). Consistent with the proposed mechanism, IC_{50} values for inhibition of PDE III appeared to correlate with *in vivo* inotropic potencies (6). Detailed biochemical and pharmacological studies carried out with certain of these agents support a role for increased levels of cAMP (presumably due to PDE inhibition) in the mechanism of positive inotropic actions (7–11). LY195115 is structurally related to certain of these compounds (Fig. 1). Consequently, it appeared relevant to examine the effects of LY195115 upon cardiac PDE isozymes.

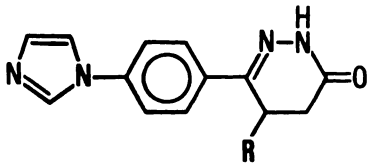
Cardiac PDE activity can be resolved into three major peaks of activity by anion exchange chromatography (4, 12). The resulting isozymes have been labeled in order of elution. PDE

[1] R. F. Kauffman, unpublished results.

ABBREVIATIONS: LY195115, 1,3-dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2*H*-indol-2-one; PDE, cyclic nucleotide phosphodiesterase; SR-PDE, cAMP phosphodiesterase located in sarcoplasmic reticulum; MV, crude myocardial vesicle; SLV, sarcolemmal vesicle; SR, sarcoplasmic reticulum; FSRV, free sarcoplasmic reticulum vesicle; JSRV, junctional sarcoplasmic reticulum vesicle; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.



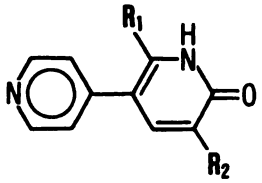
1. LY195115

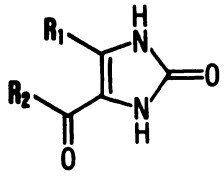


2. CI-914: R = H

3. CI-930: R = CH_3

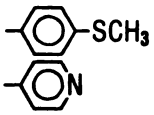
Fig. 1. Chemical structures of LY195115 and selective PDE III inhibitors.





4. amrinone: R_1 = H; R_2 = NH_2

5. milrinone: R_1 = CH_3; R_2 = CN



6. MDL 17043: R_1 = CH_3; R_2 = $-SCH_3$

7. MDL 19205: R_1 = C_2H_5; R_2 =

I is relatively specific for cGMP and is stimulated by Ca^{2+} and calmodulin. In contrast, PDE II is stimulated by low concentrations of cGMP. Both PDE I and PDE II are characterized as soluble enzymes with low affinity for cAMP (K_m = 20–50 μM). PDE III, however, is characterized by high affinity for cAMP (K_m ~ 10^{-6} M) and relative selectivity for cAMP as substrate. This latter isozyme has been characterized further as the solubilized form of a particulate enzyme, although the subcellular origin has not yet been determined (12).

For heart and a variety of other tissues, the steady state kinetics of cAMP hydrolysis by PDE III are complex, i.e., double reciprocal plots are curved downward (for a review, see Ref. 13). Evidence has been presented to support negative cooperativity as an explanation for nonlinear kinetics (14); however, catalytic site heterogeneity due to multiple enzymes, proteolytic degradation, or aggregation has not been ruled out. Indeed, previous studies of PDE isozymes from rat liver suggested that the appearance of negative cooperativity in fraction III depended upon proteolytic digestion (15). Because properties of the enzyme might be altered by solubilization and/or isolation, it would seem critical to characterize PDE III and its interaction with inhibitors while in the membrane-bound state. Furthermore, since inotropic-vasodilators in Fig. 1 have been reported to be selective inhibitors of PDE III, such an analysis may be crucial in assessing the mechanism of inhibition and its role in pharmacological actions of these drugs.

In the studies described herein, effects of LY195115 upon isozymes of cardiac PDE were examined. Based on the above considerations, particular attention was paid to effects upon PDE activity associated with cardiac membranes. LY195115-sensitive PDE was quantitated in various membranes and compared with activities of marker enzymes for sarcolemmal, sarcoplasmic reticular, and mitochondrial membranes. In addition, steady state kinetic studies were carried out with membrane fractions enriched in LY195115-sensitive PDE. The results indicate that LY195115 is a potent, competitive inhibitor of low K_m, cAMP PDE located in the SR, referred to as SR-PDE. Furthermore, correlation studies between SR-PDE inhibition and *in vivo* inotropic effects for a series of LY195115 analogues support a role for inhibition of SR-PDE in the biochemical mechanism for stimulation of cardiac contractility by these compounds.

Materials and Methods

Preparation of cardiac membranes. Highly purified SLVs were prepared from ventricles of pentobarbital-anesthetized dogs as described by Jones *et al.* (16), except that myocardial vesicles were subjected to a total of four 30-sec homogenizations with the Polytron apparatus at a setting of 7.5 (Brinkmann Instruments, PT20ST). In certain experiments the membrane barrier of SLVs was disrupted with the detergent SDS (16), the peptide ionophore alamethicin (17), or by repeated cycles of freezing and thawing (Ref. 18, a modification of Ref. 19) according to published methods. During the preparation of SLVs, membranes sedimenting at 17,000 × *g* for 20 min were harvested as a source of mitochondrial membranes (20).

MVs (Procedure I) and subfractions of SR vesicles, A–E, were prepared from ventricles of pentobarbital-anesthetized dogs as described (21, 22). Fraction A contained relatively pure sarcolemmal membranes, although contamination by SR membranes was greater than in the SLV preparation described above. Fractions B, C, and D are considered to be derived from JSRVs, whereas fraction E originates from FSRVs (23). In agreement with results obtained by others (22, 23), ATP-dependent Ca^{2+} uptake by JSRVs, but not FSRVs, was increased 4–8-fold by ruthenium red or high concentrations of ryanodine (data not shown). Aliquots of the various membrane fractions (2–4 mg of vesicle protein/ml) were stored frozen at −80° in 0.25 M sucrose, 10 mM histidine, pH 7.5, until used. Under these conditions no loss of PDE activity was detected after 6 months of storage. Vesicle protein was determined by the method of Lowry *et al.* (24) using bovine serum albumin (fraction V) as the protein standard.

Fractionation of cardiac isozymes of PDE. The three major isozymes of canine cardiac PDE were separated essentially as described previously (12). Homogenates (10%, w/v) were prepared from minced left ventricular muscle in ice-cold deionized H_2O using the Polytron apparatus at a setting of 6 for 7 sec. Homogenates were sonicated for 15 min with a Branson sonifier at a setting of 5 (model 185, standard tip). PDE activity was eluted stepwise from a DEAE-cellulose column

with sodium acetate buffers as described (12). Ethylene glycol was added to column fractions to a final concentration of 30% (v/v), and fractions were stored at −20°.

Enzyme assays. Na^+,K^+-ATPase, Ca^{2+}-ATPase, and azide-sensitive ATPase were determined at 37° by appearance of P_i using a colorimetric technique as described previously (17). Na^+,K^+-ATPase activity was measured in the following medium: 50 mM histidine, 3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, and 3 mM ATP (Na^+), pH 7.4. Na^+,K^+-ATPase activity was the portion of total ATPase activity inhibited by 1 mM ouabain, whereas azide-sensitive (mitochondrial) ATPase was that activity inhibited by 5 mM NaN_3. Ca^{2+}-ATPase activity was measured in 50 mM histidine, 3 mM $MgCl_2$, 10 mM KCl, and 50 μM $CaCl_2$, pH 7.4. Ca^{2+}-ATPase activity was that portion of activity inhibited by 1 mM EGTA/Tris. Ca^{2+}-ATPase activity of mitochondrial membranes was determined in the presence of 5 mM sodium azide in order to reduce background activity. PDE was assayed by the two-step method as described by Thompson *et al.* (12). Reactions were carried out in 10 mM Tris/Cl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 1 μM [^{3}H]cAMP (1.67×10^3 Bq) unless stated otherwise. PDE reactions were initiated by adding sufficient enzyme to hydrolyze less than 20% of the substrate in 60 min at room temperature (22 ± 2°). Reactions were terminated by placing tubes in boiling H_2O for 45 sec. PDE activity was linear versus time and protein concentration, and LY195115 had no effect upon the snake venom (*Ophiophagus hannah*) used to convert [^{3}H]AMP to [^{3}H]adenosine in the second step of the assay (data not shown). Dimethyl sulfoxide was utilized as solvent for PDE inhibitors. Solutions were prepared on the day of an experiment, and controls were run to ensure that carryover solvent (2.5%, v/v) had no effect upon assay results.

Analysis of data. IC_{50} values (concentration at which 50% inhibition of activity occurred) were determined graphically from plots of the percentage of PDE activity at 1 μM cAMP versus the negative logarithm of inhibitor concentration. PDE activity was determined in duplicate at 10 inhibitor concentrations spanning 5 log units (3×10^{-9} to 3×10^{-4} M) in order to generate inhibition curves. K_m and V_{max} values were obtained by weighted least squares linear regression analysis (Minitab computer program) of double reciprocal plots. A weighting factor of $1/v^4$ was employed, where v is the velocity of the reaction (25). K_i values were determined from the x intercept of slope replots. Statistical significance of linear correlations was assessed as in Ref. 26, and confidence intervals for slopes and intercepts of regression lines were determined as in Ref. 27. The Student's t test was employed to evaluate the significance of experimental values versus controls; p values less than 0.05 were taken to indicate statistical significance.

Materials. LY195115 and related structural analogues were synthesized in the laboratory of one of the authors (D. W. R.). Details of synthesis and characterization were described elsewhere (28). Amrinone (Sterling-Winthrop) and MDL 19205 (piroximone, Merrell Dow) were kindly provided by the sources indicated. CI-914, CI-930, MDL 17043 (fenoximone),[2] and milrinone were synthesized and characterized at Lilly Research Laboratories according to published techniques (29–31).

Alamethicin was generously supplied by Dr. J. E. Grady of The Upjohn Co. SDS was obtained from Calbiochem. The calcium ionophore A23187 was provided by Dr. R. L. Hamill of Eli Lilly and Company. [^{3}H]cAMP was obtained from New England Nuclear. DEAE-cellulose resin (coarse mesh), bovine cardiac calmodulin, and all other reagents were purchased from Sigma.

Results

Effects of LY195115 upon cardiac PDE isozymes resolved by DEAE-cellulose chromatography. The three major isozymes of canine cardiac PDE were separated, and the effects of LY195115 upon each fraction were examined using 1 μM cAMP as substrate. As shown in Fig. 2, LY195115 was a poor inhibitor of PDE isozymes I and II. At 300 μM, LY195115 inhibited both fractions by about 30%. Higher concentrations could not be examined because of precipitation. IC_{50} values of approximately 800 μM were obtained for both of these isozymes by extrapolation of the inhibition curves.

LY195115 inhibited PDE III in a biphasic manner—a distinct shoulder was evident in the inhibition curve (Fig. 2). Approximately 40% of PDE activity was characterized by high sensitivity to LY195115, inhibition occurring over the concentration range of 0.01–3 μM, whereas remaining activity was inhibited at concentrations greater than 10 μM. This observation suggested that the peak III isozyme, a solubilized form of "low K_m, membrane-bound PDE" (12), was not a homogeneous preparation. Assuming that functionally distinct enzymes were responsible for the two phases in Fig. 2, half-maximal inhibition for each phase was estimated to occur at 0.1 and 100 μM.

Inhibition curves for LY195115 versus PDE activity associated with myocardial membrane fractions. Cardiac membrane fractions enriched in sarcolemmal and SR membranes to varying degrees were prepared, and effects of LY195115 upon cAMP PDE activity associated with the membranes were examined. Inhibition curves in Fig. 3 are from a single preparation of cardiac membranes. The data are representative of results obtained with three independent membrane preparations. LY195115 inhibited PDE activity of crude myocardial vesicles, known to contain primarily SR and sarcolemmal membranes (21), over the concentration range of 0.003–10 μM (Fig. 3A). Half-maximal inhibition occurred at 0.1–0.2 μM. Although the inhibition curve appeared to be monophasic, 20–25% of PDE activity was resistant to inhibition by LY195115.

Similar to observations made with PDE III, inhibition curves for SLVs were biphasic (Fig. 3B). Approximately 50% of PDE activity was inhibited over the concentration range of 0.003–10 μM with half-maximal inhibition occurring at approximately

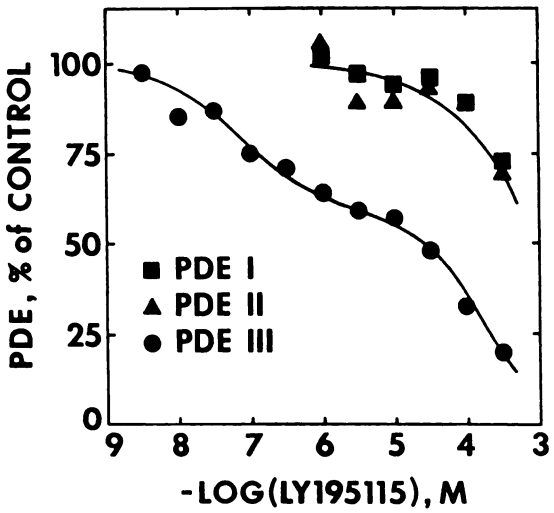


Fig. 2. Effects of LY195115 upon fractionated isozymes of cardiac PDE. PDE was determined at 1 μM cAMP in the presence of the indicated concentrations of LY195115 as described in Materials and Methods. The three major isozymes of cardiac PDE are labeled in order of elution from DEAE-cellulose columns (see text). Data points are the average of duplicate determinations. Results are from a single preparation of cardiac PDE isozymes, and are representative of findings from two independent preparations.

[2] MDL 17043 was previously referred to as fenoximone, but current nomenclature for the compound is enoximone (sources: *World Pharmaceutical Report* **999**:21, 1985; and Merrell Dow Research Laboratories).

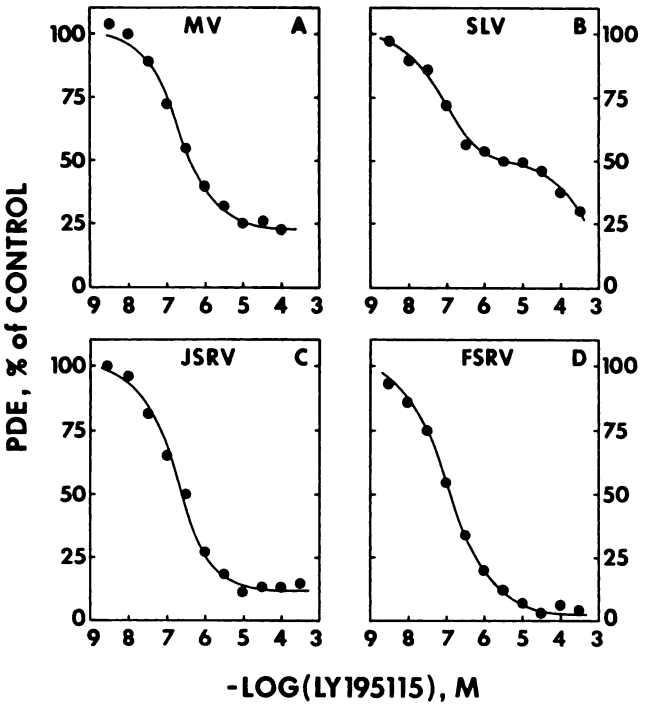


Fig. 3. Inhibition by LY195115 of PDE associated with cardiac membranes. PDE was determined at 1 μM cAMP in the presence of the indicated concentrations of LY195115. Data points are the average of duplicate determinations. C represents fraction D of JSRV.

0.1 μM. The second phase of PDE activity was inhibited at LY195115 concentrations greater than 10 μM. Half-maximal inhibition for this phase was estimated by extrapolation to occur at 800 μM.

Inhibition curves for JSRVs and FSRVs (Fig. 3, C and D) and for mitochondrial membranes (data not shown) were highly similar to those obtained with MVs. The only notable difference between the membranes was the maximal extent of inhibition which approached 100% for FSRVs. For this subfraction of SR vesicles, the IC_{50} for LY195115 was determined to be 0.13 ± 0.02 μM ($n = 5$).

Marker enzyme analysis of LY195115-sensitive PDE. For all membrane preparations examined in Fig. 3, a portion of PDE activity was characterized by high sensitivity to LY195115 (i.e., half-maximal inhibition at approximately 0.1 μM). Furthermore, in the case of FSRVs, nearly 100% of PDE activity was highly sensitive to LY195115. Based on these findings, LY195115 was postulated to be a usefl tool for resolving membrane-associated PDE into components of activity. One component, "LY195115-sensitive PDE," was characterized by high sensitivity to this inhibitor. The other component(s) was(were) characterized by low sensitivity to LY195115 (half-maximal inhibition at ≥800 μM). For subsequent analysis, therefore, LY195115-sensitive PDE was defined as that portion of total PDE inhibited by 10 μM LY195115.

If LY195115-sensitive PDE is an endogenous enzyme in one of the membranes examined, then activity of this PDE should copurify with marker enzymes for that membrane. Accordingly, activities of marker enzymes for the sarcolemma (Na^+,K^+-ATPase), SR (Ca^{2+}-ATPase), and mitochondria (azide-sensitive ATPase) were determined and compared with that of LY195115-sensitive PDE. Membrane vesicles characterized by intermediate degrees of purity (fractions A–C) (22, 23) and a mitochondrial membrane fraction were included in this study in order to improve the capability of detecting a significant correlation with one or more of the marker enzymes.

Three independent preparations of the various membranes were examined in this study. Although quantitative differences in enzyme activities were observed, an identical conclusion was reached in all cases regarding copurification with marker enzymes. The results from a representative preparation of cardiac membranes are presented in Table 1. Total PDE activity was highly correlated with Ca^{2+}-ATPase ($r = 0.94$, $p < 0.001$), demonstrating that the predominant form(s) of PDE in these membranes copurified with SR membranes. Inspection of the data in Table 1 revealed that LY195115-sensitive PDE failed to copurify with either Na^+,K^+-ATPase or azide-sensitive ATPase—correlation coefficients versus these enzymes were −0.56 and −0.46, respectively. Furthermore, PDE activity characterized by low sensitivity to LY195115 did not correlate significantly with any of the marker enzymes, regardless of whether or not SLVs were included in the analysis.

In contrast to the above findings with Na^+,K^+-ATPase and azide-sensitive ATPase, LY195115-sensitive PDE copurified well with Ca^{2+}-ATPase activity for all membrane fractions in Table 1. This point is examined in greater detail in Fig. 4. As can be seen, LY195115-sensitive PDE was highly correlated with and directly proportional to Ca^{2+}-ATPase ($r = 0.94$; $p < 0.001$). Moreover, this relationship was apparent despite the use of biochemically and functionally distinct subfractions of SR vesicles in this analysis (22, 23). The slope of the regression line in Fig. 4 was nearly identical to the value obtained when total PDE was examined; consequently, LY195115-sensitive PDE was the major form of PDE detected in these membranes.

Membrane fractions A–E in Table 1 contained variable amounts of Ca^{2+} oxalate as a result of the loading step prior to sucrose density gradient centrifugation (21, 22). During incubations in the absence of ATP (e.g., during PDE assays), this Ca^{2+} oxalate was released into the suspending medium (data not shown). Consequently, it was of interest to examine whether or not Ca^{2+} or oxalate had an effect upon LY195115-sensitive PDE activity. MVs were used in these experiments since this fraction did not contain Ca^{2+} oxalate. Concentrations of Ca^{2+} used in this experiment exceeded the amounts released by SR subfractions into the suspending medium (data not shown). The results, shown in Table 2, indicate that neither Ca^{2+} nor oxalate had a significant effect upon LY195115-sensitive PDE.

Effects of membrane disruption upon PDE activity associated with SLVs. SLV preparations used in this study consist of approximately 80% sealed, right side out vesicles (Ref. 18, and references therein). To test for the possibility of latent PDE activity, sarcolemmal membranes were treated with the peptide ionophore alamethicin (17) or were subjected to repeated cycles of freezing and thawing (18), in order to disrupt the membrane barrier. The activity of Na^+,K^+-ATPase, known to be a latent enzyme in SLV preparations (16, 17), was also measured to verify the effectiveness of membrane disruption.

The results of this series of experiments are presented in Table 3. Similar to results of others (17–19), membrane disruption produced 3–5-fold increases in Na^+,K^+-ATPase activity of SLVs. By contrast, freezing and thawing or treatment with alamethicin had no significant effect upon LY195115-sensitive PDE. Phosphodiesterase assays are conducted in a hypotonic

TABLE 1

Marker enzyme analysis: PDE associated with cardiac membranes

Canine cardiac membranes were prepared, and PDE and ATPase activities were determined as described in Materials and Methods. All membrane fractions were treated with SDS prior to assay of Na^+,K^+-ATPase. Data are presented as the mean of triplicate determinations. LY195115-sensitive PDE was quantitated as the portion of total PDE inhibited by 10 μM LY195115 (see text). Subfractions of MVs are labeled A–E according to nomenclature described in Ref. 21 and 22 and in Materials and Methods.

Membrane fraction	PDE		Na^+,K^+-ATPase	Ca^{2+}-ATPase	N_3-sensitive ATPase
	Total	LY195115-sensitive			
	pmol/min/mg protein			*μmol/hr/mg protein*	
SLV	32	16	170	0.4	19.1
MV	416	316	26.4	73.7	34.0
A	296	192	130	39.8	31.5
B	340	251	14.2	67.5	55.9
C	405	320	19.5	99.0	63.0
D	474	408	9.0	136	30.7
E	705	662	6.8	145	14.2
MITO[a]	83	59	2.8	25.6	236

[a] MITO, mitochondrial membrane fraction.

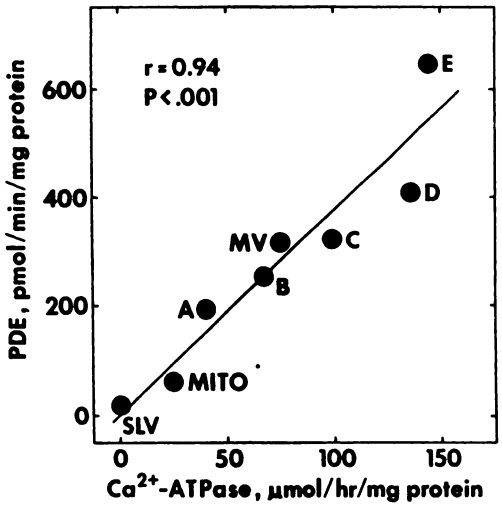


Fig. 4. Relationship between LY195115-sensitive PDE and Ca^{2+}-ATPase activity of cardiac membranes. Ca^{2+}-ATPase and PDE (assayed at 1 μM cAMP) were determined as described in Materials and Methods. LY195115-sensitive PDE was quantitated as the portion of total PDE inhibited by 10 μM LY195115 (see text). Data points represent the average of triplicate determinations. Subfractions of MVs are labeled *A–E* according to nomenclature described in Refs. 21 and 22, and in Materials and Methods. *MITO*, mitochondrial membrane fraction. The correlation coefficient, *r*, and the probability of the null hypothesis, *p*, are indicated in the figure.

TABLE 2

Lack of effect of Ca^{2+}, oxalate, or Ca^{2+} plus calmodulin upon LY195115-sensitive PDE of MVs

PDE activity of MVs was determined at 1 μM cAMP. Data are presented as the mean ± standard error of triplicate determinations. LY195115-sensitive PDE was quantitated as the portion of total PDE inhibited by 10 μM LY195115 (see text). When present, the concentration of added calmodulin was 1.6×10^{-7} M. None of the experimental conditions resulted in a significant change in LY195115-sensitive PDE versus the control rate.

Experiment	LY195115-sensitive PDE
	pmol/min/mg protein
I. Control	324 ± 38
II. 0.5 mM EGTA	326 ± 16
III. 100 μM $CaCl_2$	321 ± 20
IV. 100 μM Na^+ oxalate	355 ± 18
V. 100 μM $CaCl_2$ + 100 μM Na^+ oxalate	331 ± 26
VI. 100 μM $CaCl_2$ + calmodulin	354 ± 53

TABLE 3

Effects of membrane disruption upon Na^+,K^+-ATPase and LY195115-sensitive PDE activities of SLVs

Membrane disruption and enzyme assays were performed as described in Materials and Methods. Enzyme activities are presented as the mean ± standard error of triplicate determinations. LY195115-sensitive PDE was quantitated as the fraction of total PDE inhibited by 10 μM LY195115. Ca^{2+}-ATPase activity of the SLV preparation used in these experiments was 5.6 μmol/hr/mg of protein. ATPase and PDE activities were determined simultaneously using the same batch of control or treated vesicles. -Fold increases in activity were calculated as the ratio of activities for treated versus control vesicles.

Treatment	Na^+,K^+-ATPase		LY195115-sensitive PDE	
	μmol/hr/mg of Protein	-Fold increase	pmol/min/mg of Protein	-Fold increase
I. Control	34 ± 2		75 ± 9	
II. Alamethicin	175 ± 2[a]	5.15	76 ± 9	1.01
III. Freeze-thaw	106 ± 4[a]	3.12	72 ± 4	0.96

[a] $p < 0.05$ versus control.

medium. Consequently, the failure of membrane disruption to increase PDE activity could have been an artifact due to rupture of control vesicles in the assay medium. However, this did not appear to be the case, since essentially identical results were obtained when 100 mM NaCl and 10 mM KCl (equivalent to ionic conditions in the Na^+,K^+-ATPase assay) were included in the PDE assay medium (data not shown).

Inhibition of cAMP phosphodiesterase by LY195115: Steady state kinetic studies. cAMP phosphodiesterase activity of FSRVs was nearly homogeneous and characterized by high sensitivity to LY195115 (Fig. 3D); consequently, steady state kinetic studies were carried out with this membrane fraction. Double reciprocal plots were linear at cAMP concentrations up to 10 times the K_m, or 90% occupancy of the catalytic site (Fig. 5). K_m and V_{max} values were determined to be 0.46 ± 0.03 μM and 700 ± 90 pmol/min/mg of protein, respectively. An intersecting pattern was observed when double reciprocal plots were determined in the presence of variable concentrations of LY195115 (Fig. 5). A K_i value of 80 ± 10 nM was obtained from the slope replot (Fig. 5, *inset*).

Effects of PDE III inhibitors upon LY195115-sensitive PDE. The compounds depicted in Fig. 1 are selective inhibitors of PDE III (4–6, 32). It was of interest, therefore, to determine the effects of these compounds upon LY195115-sensitive PDE. The nonselective inhibitor theophylline was included as a reference compound. Except for theophylline, all of the compounds examined were relatively potent inhibitors of LY195115-sensitive PDE (Table 4). In general, IC_{50} values

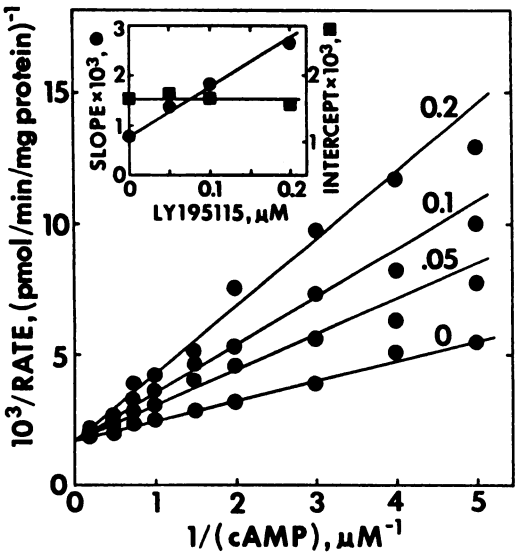


Fig. 5. Lineweaver-Burk plots for cAMP PDE activity of FSRVs in the absence or presence of varying concentrations of LY195115. PDE was determined at cAMP concentrations indicated in the figure according to procedures described in Materials and Methods. Data points represent the average of duplicate determinations. Micromolar concentrations of LY195115 included in the reaction mixture are indicated by the *numbers* adjacent to individual *lines*. *Lines* drawn through the data were determined by weighted least squares linear regression. *Inset:* Replots of slopes (●) and intercepts (■) as a function of the concentration of LY195115.

TABLE 4

IC_{50} values for inhibition of LY195115-sensitive PDE and PDE III by theophylline and selective PDE III inhibitors

IC_{50} values for inhibition of PDE activity of FSRVs were determined at 1 μM cAMP as described in Materials and Methods.

Compound	IC_{50}	
	LY195115-sensitive PDE	PDE III[a]
	μM	
CI-930	0.13	0.9
CI-914	0.46	6.1
Milrinone	1.1	2.5
MDL 17043	2.7	14
Amrinone	4.8	35
Theophylline	280	360

[a] IC_{50} values versus PDE III (guinea pig heart, 1 μM cAMP) were obtained from Ref. 6.

were lower than corresponding literature values (32) obtained with PDE III isolated from guinea pig (Table 4). The IC_{50} for theophylline, 280 μM, was similar to the value obtained with PDE III, confirming previous observations of nonselective PDE inhibition by this methylxanthine (32). A significant correlation was observed between $-\log IC_{50}$ values for the two PDE preparations ($r = 0.96$; $p < 0.01$), demonstrating that structural requirements for inhibiting PDE activity of these two preparations were similar.

Relationship between ED_{50} values for *in vivo* inotropic activity and IC_{50} values versus LY195115-sensitive PDE. In order to examine the relationship between inhibition of PDE and positive inotropic actions of LY195115, IC_{50} values versus PDE activity of FSRVs were determined for a series of structural congeners (including compounds from Fig. 1) and compared with inotropic ED_{50} values obtained from previous studies using pentobarbital-anesthetized dogs (1, 6, 28, 33).

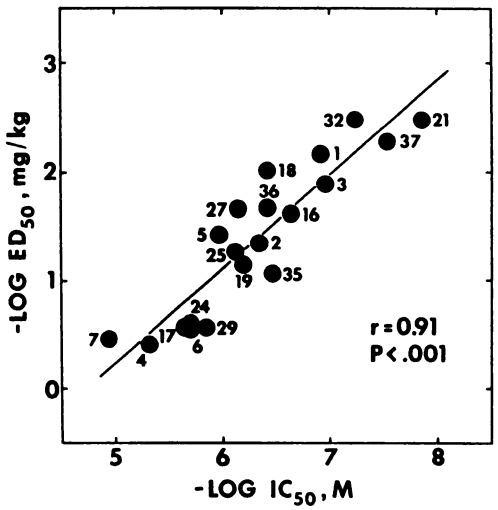


Fig. 6. Relationship between $-\log IC_{50}$ values for inhibition of cAMP PDE of FSRVs, and $-\log ED_{50}$ values for stimulation of cardiac contractility in anesthetized dogs for a series of analogues of LY195115. $-\log IC_{50}$ values were determined at 1 μM cAMP using FSRVs as described in Materials and Methods. $-\log ED_{50}$ values were taken from or calculated from published data (1, 6, 28, 33). The *line* drawn through the data points was determined by linear regression analysis. The correlation coefficient, *r*, and the probability of the null hypothesis, *p*, are indicated in the figure. The structures of compounds **1–7** are presented in Fig. 1. Other compounds are numbered as in Ref. 28, and chemical names are listed in a footnote below.[3]

ED_{50} values were defined as the intravenous dose required to produce a 50% increase in the rate of development of contractile tension as reported by a strain gauge sutured to the left ventricle.

As can be seen in Fig. 6, $-\log ED_{50}$ values and $-\log IC_{50}$ values were highly correlated over a range of 3 log units in the latter parameter ($r = 0.91$; $p < 0.001$). Most of the compounds in Fig. 6 were dihydropyridazinones; nevertheless, the relationship also appeared to hold for the bipyridines (amrinone, milrinone) and imidazolones (MDL 17043, MDL 19205) examined. The regression equation for the data was: $-\log ED_{50} = 0.867 (-\log IC_{50}) - 4.09$. The 95% confidence interval for the slope was ±0.190; thus, the slope was not significantly different from the theoretical value of 1.0 predicted for a functional relationship between these two parameters.

Discussion

LY195115 was shown to be a selective inhibitor of PDE III (Fig. 2), a solubilized preparation of low K_m, membrane-bound PDE from cardiac muscle (12). Inhibition curves were biphasic, raising the likelihood of heterogeneity within this peak of PDE

[3] Chemical names for compounds in Fig. 6 (except **1–7**) are as follows: **16,** 1,3-dihydro-5-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl-2*H*-indol-2-one; **17,** 1,3-dihydro-5-(1,4,5,6-tetrahydro-1-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one; **18,** 1,3-dihydro-3-methyl-5-(1,4,5,6,-tetrahydro-6-oxo-3-pyridazinyl)-2*H*-indol-2-one; **19,** 3-ethyl-1,3-dihydro-5-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2*H*-indol-2-one; **21,** 1,3-dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one; **25,** 1,3-dihydro-1,3,3-trimethyl-5-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2*H*-indol-2-one; **27,** 3-ethyl-1,3-dihydro-3-methyl-6-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2*H*-indol-2-one; **29,** 3,3-diethyl-1,3-dihydro-5-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2*H*-indol-2-one; **31,** 4,5-dihydro-6-(1,2,3,4-tetrahydro-6-quinolinyl)-3(2*H*)-pyridazinone; **32,** 3,4-dihydro-6-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2(1*H*)-quinolinone; **35,** 3,4-dihydro-4,4-dimethyl-6-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2(1*H*)-quinolinone; **36,** 3,4-dihydro-3,3-dimethyl-6-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2(1*H*)-quinolinone; **37,** 1,3,4,5-tetrahydro-7-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2*H*-1-benzazepin-2-one.

activity. Rather than attempting to purify the solubilized enzyme to homogeneity, the approach was taken to examine particulate PDE in the membrane-bound state. Accordingly, PDE activity associated with various cardiac membrane fractions was determined.

Cardiac membranes, enriched to varying degrees in marker enzymes for sarcolemmal, SR, and mitochondrial membranes, were utilized in this study. LY195115 inhibited PDE activity associated with all of the membrane fractions examined (Fig. 3, Table 1); however, characteristics of individual inhibition curves varied. In all cases, a portion of PDE activity was characterized by high sensitivity to LY195115 (i.e., half-maximal and maximal inhibition at approximately 0.1 and 10 μM, respectively). Since PDE activity of FSRVs was essentially homogeneous with respect to inhibition by LY195115, the conclusion was reached that complex inhibition curves for other membrane fractions were in all probability due to the presence of two or more enzymes, one of which was potently inhibited by LY195115.

Based on the above line of reasoning, LY195115-sensitive PDE was quantitated as the portion of total PDE inhibited by 10 μM LY195115. LY195115-sensitive PDE copurified with the SR marker, Ca^{2+}-ATPase, but not with sarcolemmal or mitochondrial membrane markers. The correlation with Ca^{2+}-ATPase was highly significant ($r = 0.94$; $p < 0.001$) and applied to both junctional and free SR vesicles (Fig. 4). Furthermore, this correlation was not due to an artifact related to variable extents of Ca^{2+} oxalate loading in the SR subfractions.

Although these data support the presence of LY195115-sensitive PDE in SR membranes, small amounts were always detected in SLV preparations. In order to address the question of whether this activity was due to an endogenous sarcolemmal protein or to a contaminant, the criterion of enzyme latency was employed. Successful application of latency analysis requires: 1) the availability of techniques for membrane disruption that do not alter the specific activity of the enzyme under study, and 2) low permeability of the membrane to the enzyme substrate. For the present analysis, two distinct methods of disrupting sarcolemmal membranes were employed, both of which have been characterized in detail (17, 18). Concerning the second requirement, permeability of SLV to cAMP has been shown to be very low (34).

Membrane disruption by alamethicin treatment or by repeated cycles of freezing and thawing failed to produce a significant increase in LY195115-sensitive PDE associated with SLVs. This finding indicated the absence of latent activity in SLVs, and, this, LY195115-sensitive PDE associated with these membranes was in all probability a contaminant. Furthermore, LY195115-sensitive PDE in SLVs may be accounted for by a previously postulated low-level contamination by SR membranes (16, 21). The alternative explanation for these results, that LY195115-sensitive PDE is an endogenous sarcolemmal protein with its catalytic site facing the extracellular surface, was considered extremely unlikely.

Taken together, the biochemical data support a location for LY195115-sensitive PDE within membranes of the SR with a distribution similar or identical to that of Ca^{2+}-ATPase. Accordingly, LY195115-sensitive PDE was referred to as SR-PDE. SR-PDE was the predominant form of PDE detected in the membranes examined. Although not all subcellular membranes of cardiac tissue were included in this study, the data suggest that SR-PDE may prove to be a useful marker enzyme for the SR. Definitive assignment to a single versus multiple subcellular location will require immunohistochemical studies with antibodies raised to the purified enzyme.

The results of this study suggest that at least a portion of activity in PDE III is derived from SR-PDE. This was supported by the following observations. 1) Both PDE III and SR-PDE are particulate enzymes displaying high affinity for cAMP. 2) LY195115 potently inhibited a portion of activity in the PDE preparation with half-maximal inhibition occurring at approximately the same concentration (0.1 μM) as observed with SR-PDE. 3) $-\text{Log}IC_{50}$ values for a series of selective PDE III inhibitors correlated significantly with corresponding values for SR-PDE.

In recent reports, selective PDE III inhibitors were shown to inhibit a form of PDE isolated by immunoprecipitation from hypotonic extracts of bovine cardiac muscle (CGI-PDE) (35, 36). The order of potency for inhibition of CGI-PDE was identical to that observed with SR-PDE (milrinone > MDL17043 > amrinone), suggesting that CGI-PDE may also be a solubilized form of SR-PDE. Relevant to this discussion, the conditions used to homogenize cardiac muscle in these studies (hypotonic media in the presence of sulfhydryl groups) have been shown to solubilize low K_m, membrane-bound PDE in adipocytes (37, 38). Studies aimed at solubilizing and characterizing SR-PDE activity are under way in this laboratory, and it will be of interest to determine the relationship, if any, between these enzymes.

Ca^{2+} plus calmodulin failed to produce a significant increase in SR-PDE activity (Table 3). Furthermore, in contrast to results obtained with PDE II, cGMP inhibited SR-PDE with an IC_{50} of 0.85 μM at 1 μM cAMP (data not shown). These findings, together with the subcellular location, low K_m, and sensitivity to selective PDE III inhibitors, provided additional evidence of a distinction between SR-PDE and soluble PDE isozymes (PDEs I and II).

LY195115 was a linear competitive inhibitor of cAMP hydrolysis by SR-PDE. This observation supported the hypothesis that LY195115 binds to the catalytic site of this enzyme. Based on the K_i of 80 nM, LY195115 appeared to bind more tightly than cAMP, since the K_m for the latter was 0.46 μM.

Several lines of evidence support a role for inhibition of SR-PDE in the cardiostimulant actions of LY195115. First, LY195115 was shown to be a highly potent inhibitor of SR-PDE. Second, inhibition by SR-PDE was selective—IC_{50} values versus PDE I and PDE II were 3–4 orders of magnitude greater than the corresponding value for SR-PDE. Additional evidence for specificity derived from the demonstration that LY195115 was without effect upon the following enzymes/proteins: Na^+,K^+-ATPase, calmodulin, myofibrillar Ca^{2+}-ATPase, and Ca^{2+}-ATPase of SR vesicles.[4] Finally, for a series of analogues of LY195115, a highly significant correlation was found between $-\text{log}IC_{50}$ values versus SR-PDE and $-\text{log}ED_{50}$ values for stimulation of cardiac function in anesthetized dogs. Evidently, the compounds examined in Fig. 6 interact primarily at a single binding site, the molecular topography of which is similar or identical to that of the catalytic site of SR-PDE, to produce increases in cardiac contractility *in vivo*.

Although the biochemical and correlative data support a role

[4] R. F. Kauffman, unpublished results.

for SR-PDE inhibition, they clearly do not constitute proof of a mechanism for LY195115. Detailed biochemical and pharmacological studies with isolated cardiac muscle and myocytes are required to evaluate the precise role of SR-PDE inhibition and/or other potential inotropic mechanisms. In preliminary studies, for example, positive inotropic effects of LY195115 in isolated cardiac muscle were associated with increases in cAMP (data not shown). The concentration and time dependence for this effect and its relationship to inotropic actions are currently being examined.

In summary, the positive inotropic agent LY195115 was shown to be a potent, selective inhibitor of SR-PDE. The available data suggest that at least a portion of PDE III, the solubilized version of low K_m, membrane-bound PDE, is derived from SR-PDE. Finally, the ability of IC_{50} values versus SR-PDE to predict *in vivo* inotropic activity for a series of LY195115 congeners suggests a role for inhibition of SR-PDE in the biochemical mechanism of this compound.

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