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A Potent and Selective Inhibitor of Cyclic AMP Phosphodiesterase with Potential Cardiotonic and Antithrombotic Properties

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SUMMARY

Some biochemical and pharmacological properties of a novel, potent inhibitor of cyclic AMP phosphodiesterase, *N*-cyclohexyl-*N*-methyl-4-(7-oxy-1,2,3,5-tetrahydroimidazo[2,1-*b*]quinazolin-2-one) butyramide (RS-82856), were investigated. RS-82856 selectively inhibits the high affinity form of cyclic AMP phosphodiesterase (type IV) isolated from human platelets (K_i = 0.5 nM) with only weak effects on both the nonspecific and cyclic GMP-sensitive phosphodiesterases. The inhibitor reduces both maximum velocity and substrate affinity of the type IV enzyme. This mixed pattern of partial competitive and noncompetitive inhibition was also obtained with one of the two high affinity forms of phosphodiesterase found in dog heart (K_i = 0.75 nM). Of several human and dog tissues examined, RS-82856 exhibits marked selectivity for the platelet high affinity enzyme. It also has significant inhibitory effects on cardiac membrane-bound phosphodiesterase. RS-82856 inhibits the aggregation of human platelets in response to adenosine 5′-diphosphate (IC_{50} = 0.11 μM) *in vitro* and is active *ex vivo* for at least 2 hr following oral administration (10 mg/kg) to rhesus monkeys. Administration of RS-82856 to instrumented, anesthetized dogs by either intravenous or intraduodenal routes increases cardiac contractile force and reduces afterload. These data suggest that RS-82856 may be useful as an agent to increase cardiac output in the treatment of congestive heart failure.

Some inhibitors of cyclic 3′:5′-nucleotide phosphodiesterase (EC 3.1.4.17) elicit positive inotropic and afterload reduction effects and have potential utility in the treatment of congestive heart failure (1–3). Phosphodiesterase inhibitors have also been described that prevent the aggregation of blood platelets in response to a variety of physiological inducers (3–6) and dilate blood vessels (7, 8). From a therapeutic perspective, it may be advantageous to administer a cardiotonic agent that also has antithrombotic properties to those patients with a history of myocardial infarction and an increased risk of coronary or pulmonary thrombosis.

Platelets, cardiac muscle, vascular, and other tissues contain multiple molecular forms of cyclic AMP phosphodiesterase (3, 9–13). These enzymes have been recently grouped into distinct types based on their substrate affinity and regulatory properties (14). Types I and II are sensitive to stimulation by calmodulin and cyclic GMP, respectively (14), and hydrolyze both cyclic AMP and cyclic GMP.[2] In contrast, the type IV enzyme selectively hydrolyzes cyclic AMP and has a high affinity for this substrate (14). The relative abundance of these phosphodiesterases can vary in different tissues (3).

Two structurally dissimilar compounds, anagrelide (4) and cilostamide (5), have been described as potent inhibitors of the type IV cyclic AMP phosphodiesterase in human platelets. This study was designed to examine some biochemical and pharmacological properties of a new phosphodiesterase inhibitor (RS-82856) which contains structural elements of these two compounds.

Materials and Methods

[G-^{3}H] or [^{32}P]adenosine 3′:5′-monophosphate (10–50 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). RS-82856,*N*-cyclohexyl-*N*-methyl-4-(7-oxy-1,2,3,5-tetrahydroimidazo-[2,1-b]quinazolin-2-one) butyramide (Fig. 1), was synthesized in the Institute of Bio-Organic Chemistry, Syntex Research.[3]

Aggregation Studies

Human platelets. Blood was collected by venipuncture into evacuated tubes containing sodium citrate (30 mM final concentration).

[1] Contribution Number 234 from the Institute of Bio-Organic Chemistry.

[2] The type III enzyme is a rhodopsin-sensitive cyclic GMP phosphodiesterase found in the retinal rod outer segment of vertebrates.

[3] R. Alvarez, J. J. Bruno, G. H. Jones, A. M. Strosberg, and M. C. Venuti, manuscript in preparation.

ABBREVIATIONS: PRP, platelet-rich plasma; EDTA, ethylenediaminetetraacetate.

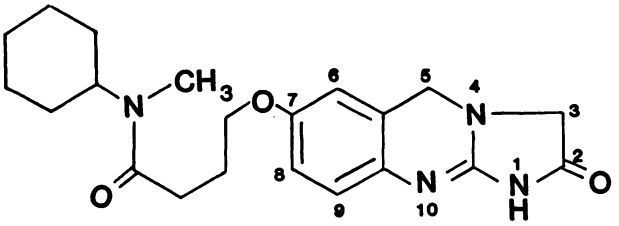

Fig. 1. Chemical structure of *N*-cyclohexyl-*N*-methyl-4-(7-oxy-1,2,3,5-tetrahydroimidazo[2,1-b]quinazolin-2-one) butyramide (RS-82856).

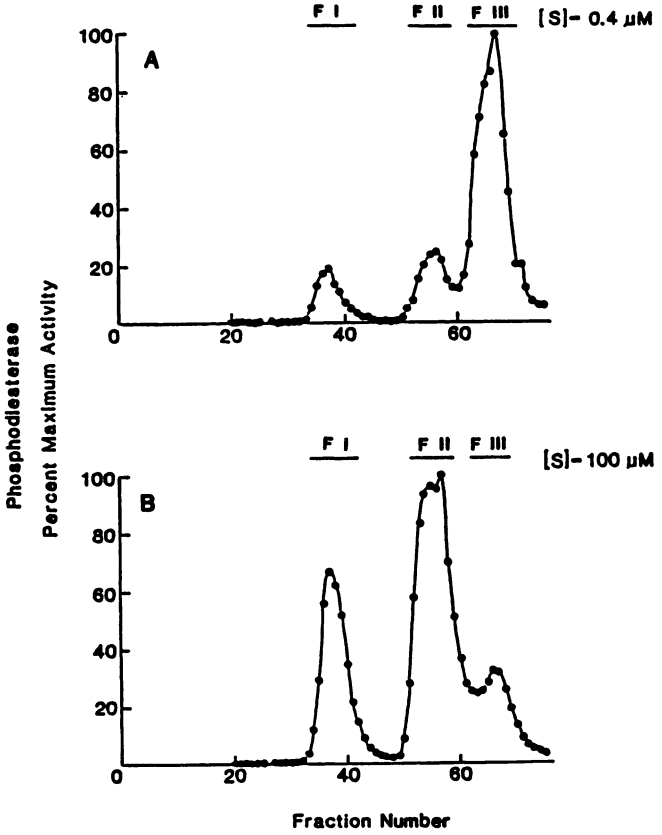

Fig. 2. Elution pattern for human platelet cyclic AMP phosphodiesterase following DEAE-cellulose column chromatography. The sodium acetate gradient was from fractions 20–75. Aliquots from each tube were assayed at 0.4 (A) and 100 μM (B) cyclic AMP. Enzyme assayed in the presence of 100 μM cyclic AMP contained 1 unit of calmodulin (Sigma) and 0.4 mM $CaCl_2$. *F*, fraction.

PRP was collected after centrifugation for 15 min at 200 × *g* at room temperature. Platelet concentration was determined with a Royco cell counter (Cell-Crit 921). Siliconized glassware or plastic test tubes were used in all procedures. Aggregation was followed by the turbidimetric procedure of Born (15) using a Payton aggregation module. Test compounds were added to stirred PRP (450 rpm) at 37° and incubated for 5 min prior to induction of aggregation by ADP (5 μM). The total volume was 1 ml. The degree of inhibition was determined by measuring the change in percentage transmission of the primary phase of ADP-induced aggregation after 5 min of incubation. Experiments were repeated at least twice with platelets obtained from different donors. The results presented are representative data.

Rhesus monkey platelets. For *in vitro* studies, blood was collected by venipuncture from conscious, female rhesus monkeys into plastic syringes containing sodium citrate (0.38% final concentration). PRP was prepared by centrifugation (200 × *g*) for 15 min. Platelets were adjusted to 700,000/μl using autologous platelet-poor plasma. Full aggregation was induced by 20 μM ADP. RS-82856 was dissolved in 10% DMF and incubated with PRP for 5 min before the induction of

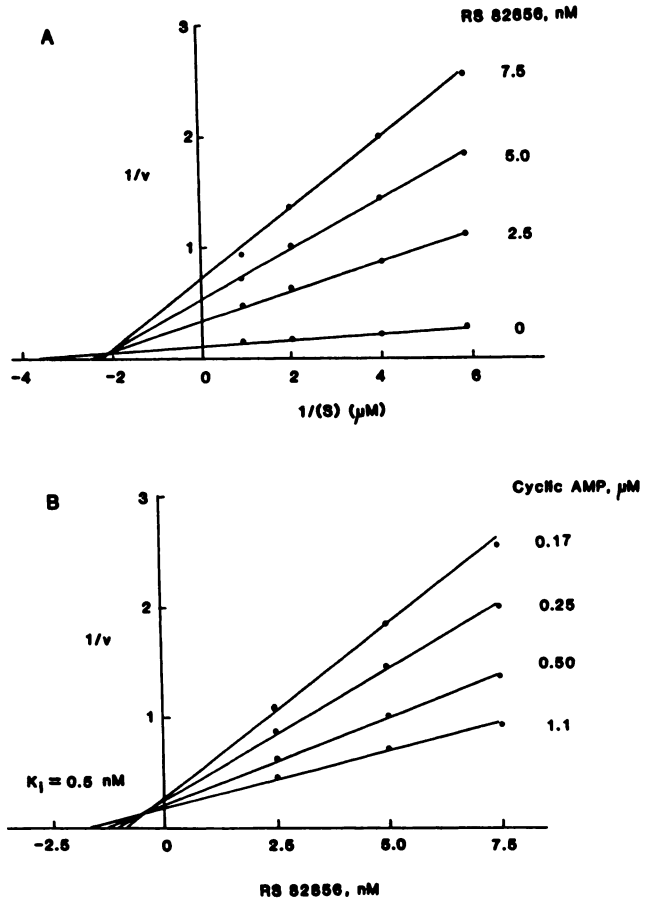

Fig. 3. Reciprocal plot (1/*v* vs. 1/[*S*] for human platelet type IV cyclic AMP phosphodiesterase in the presence of 0, 2.5, 5.0, and 7.5 nM RS-82856. B. Dixon plot (1/*v* vs. [RS-82856]) in the presence of 0.17, 0.25, 0.50, and 1.1 μM cyclic AMP. Velocity is expressed as nmol of cyclic AMP hydrolyzed/min/mg of protein.

aggregation. For *ex vivo* studies, rhesus monkeys were dosed via gastric intubation with 10 mg/kg of RS-82856 suspended in carboxy methyl cellulose (6 mg/ml) or with carboxy methyl cellulose alone after a 12-hr fasting period. Ten-ml blood samples were collected into citrate (0.38% final concentration) at times 0, 1, and 2 hr post-dosing through a polyethylene catheter. After drawing blood, the catheter was flushed with 1 ml of citrate and platelet aggregation studies were performed as described above for the *in vitro* studies.

Phosphodiesterase Preparations

Human platelet. Blood was obtained from donors who had not taken aspirin or similar medications for at least 2 weeks and was collected by venipuncture into evacuated glass tubes (Vacutainer, Becton, Dickinson, Rutherford, NJ) containing EDTA (7.7 mM final concentration). PRP was obtained by centrifuging the blood in polycarbonate tubes at 200 × *g* for 15 min at 4°. All subsequent steps were performed at 4°. A platelet pellet was obtained by further centrifugation of the PRP at 1,000 × *g* for 15 min. The pellet was resuspended in a volume of buffer A (0.137 M NaCl, 12.3 mM Tris-HCl buffer, pH 7.4, at 37°, 1.54 mM EDTA, and 20 mM glucose) equal to the original PRP volume. The suspension was centrifuged at 1,100 × *g* for 15 min and the pellet was resuspended in buffer A. The pellet was centrifuged at 1,100 × *g* and the pellets were resuspended in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.7, containing 1 mM $MgCl_2$. The hypotonically lysed platelet suspension was centrifuged at 48,000 × *g* for 15 min and the supernatant was saved. The pellets were frozen on dry ice and briefly thawed at 22°. The supernatant was combined with the pellet fraction and the resulting suspension was centrifuged at 48,000 × *g* for 30 min.

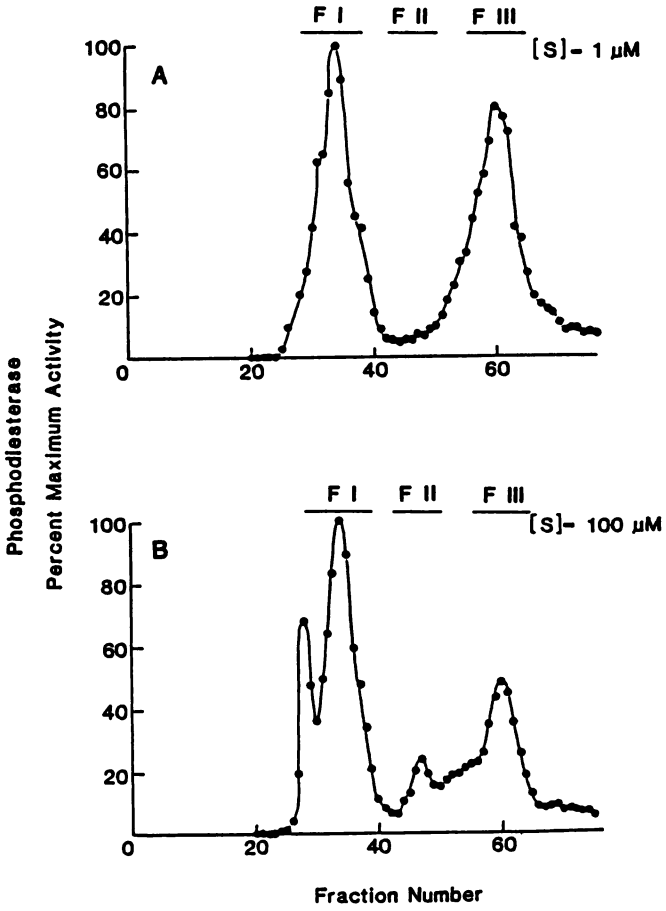

Fig. 4. Elution pattern for dog heart cyclic AMP phosphodiesterase following DEAE-cellulose column chromatography. The sodium acetate gradient was from fractions 20–75. Aliquots from each tube were assayed at 1.0 (A) and 100 μM (B) cyclic AMP. Enzyme assayed in the presence of 100 μM cyclic AMP contained 1 unit of calmodulin and 0.4 mM $CaCl_2$. The shoulder on fraction (*F*) I which appeared in this elution pattern was not reproducible in three other column separations.

The pellet and supernatant fractions were used as the crude membrane-bound and soluble enzyme preparations. An aliquot of the supernatant (5 ml) was applied to a DEAE-cellulose (DE52, Whatman Chemical Separation Ltd.) column (1.5 × 20 cm) and chromatographed using a linear gradient of 0–1 M sodium acetate at a flow rate of 0.5 ml/min as described by Hidaka and Asano (9). For kinetic studies the enzyme from each major peak was pooled and stored at −20°.

Dog heart. The left ventricle was dissected, minced, washed free of blood, and homogenized for 1 min in a Waring Blendor in 10 volumes of cold 0.01 M Tris-HCl buffer, pH 7.7. The homogenate was passed through two layers of cheesecloth and centrifuged at 12,000 × *g* for 20 min. All steps were performed at 4°. The supernatant was used as a source of enzyme. An aliquot of the dog heart supernatant (5 ml) was applied to a DEAE-cellulose (DE52) column. Separation of the soluble dog heart phosphodiesterase forms was performed as described above for the platelet preparation. Membrane-bound phosphodiesterase activity found in the pellet fraction was stored at −20°.

Other tissues. Crude, soluble and membrane-bound phosphodiesterases from various human and dog tissues were similarly prepared up to the column step for the cardiac enzyme and stored at −20°. Prior to assay the crude, soluble enzyme was thawed and passed through a 0.45-μm filter to remove particulate material. The filtrate was used as the soluble enzyme. Human cardiac tissue (septum) from a patient with idiopathic cardiomyopathy was provided by Dr. Michael R. Bristow of the Department of Cardiovascular Surgery and Medicine, Stanford University Medical Center, Stanford, CA.

Cyclic AMP phosphodiesterase assay. The phosphodiesterase incubation medium contained 12 mM Tris-HCl buffer, pH 7.7, 0.5 mM $MgCl_2$, 0.137 M NaCl, 20 mM glucose, and appropriate concentrations of cyclic [^{3}H]AMP (0.2 μCi) in a total volume of 1.0 ml. Following addition of the enzyme the contents were mixed and incubated for 10 min at 30°. The reaction was terminated by adding 10 μl of 0.1 M EDTA, pH 7.0, mixing, and immediately immersing the tubes in a boiling water bath for 90 sec. After the tubes were cooled in an ice water bath, 0.1 ml (100 μg) of 5′-nucleotidase from snake venom (*Crotalus atrox*, Sigma V-7000) was added to each tube. The contents were mixed and incubated for 30 min at 30°. The nucleotidase reaction was terminated by immersing the tubes in a boiling water bath for 60 sec. Labeled adenosine was isolated from alumina columns according to the method of Filburn and Karn (16). Assays were performed in triplicate. Hydrolysis of cyclic AMP was 10–20% after 10 min of incubation. DEAE-cellulose (DE52) column eluates were assayed by the method of Hidaka and Asano (9). Solutions of RS-82856 were prepared in dimethylsulfoxide. The final concentration of dimethylsulfoxide in the phosphodiesterase assay was 1%.

Cardiovascular Studies

General studies. Mongrel dogs were anesthetized intravenously with 35 mg/kg of sodium pentobarbital. Anesthesia was maintained with an intravenous infusion of pentobarbital (~5 mg/kg/hr). Blood pressure was measured with a Statham pressure transducer via a cannula inserted from a femoral artery into the abdominal aorta. Heart rate was recorded by a cardiotachometer from a limb lead II electrocardiogram. Right ventricular contractile force was recorded from a Walton-Brodie strain gauge sutured to the right ventricle following a midsternal thoracotomy. A Harvard respirator was used to ventilate the dogs with room air through an endotracheal tube. A femoral vein was cannulated for the intravenous administration of compounds. In select studies, a midline laparotomy was performed and a cannula sutured into the duodenum for intraduodenal administration of test compound.

In studies conducted to determine the involvement of catecholamines in the inotropic responses to RS-82856, dogs were pretreated with reserpine, 2.5 mg/kg/day subcutaneously, for 2 days and subsequently instrumented as above.

Vasodilatory studies. Mongrel dogs were anesthetized as in the general studies. Following exposure of the abdominal aorta and right external iliac artery, an external perfusion circuit including a Sigma-motor pump interposed between the proximal external iliac artery and the distal external iliac artery was set up. The aorta was ligated below the origin of the external iliac arteries to minimize collateral blood flow to the hind limb. The rate of blood perfusion to the hind limb was adjusted to produce a perfusion pressure which was approximately equivalent to mean arterial blood pressure as measured from the brachial artery and maintained at this level by the pump. Thus, any change in perfusion pressure represented a change in hind limb vascular resistance. Compounds were administered into the perfusion circuit between the pump and the hind limb. All data were recorded on Beckman R611 or Type R dynographs.

Results and Discussion

Platelet cyclic AMP phosphodiesterase. Cyclic AMP phosphodiesterases from human platelets were separated by DEAE-cellulose column chromatography into three major fractions (Fig. 2). Fractions I, II, and III are numbered in order of their elution. Fraction I has a low K_m (1.1 μM) for cyclic GMP and a high K_m (0.5 mM) for cyclic AMP hydrolysis. Cyclic AMP phosphodiesterase activity was stimulated by low concentrations of cyclic GMP (K_a = 1.2 μM). These observations are in reasonable agreement with those of Hidaka and Asano (9). Thus, the fraction I activity in human platelets corresponds to

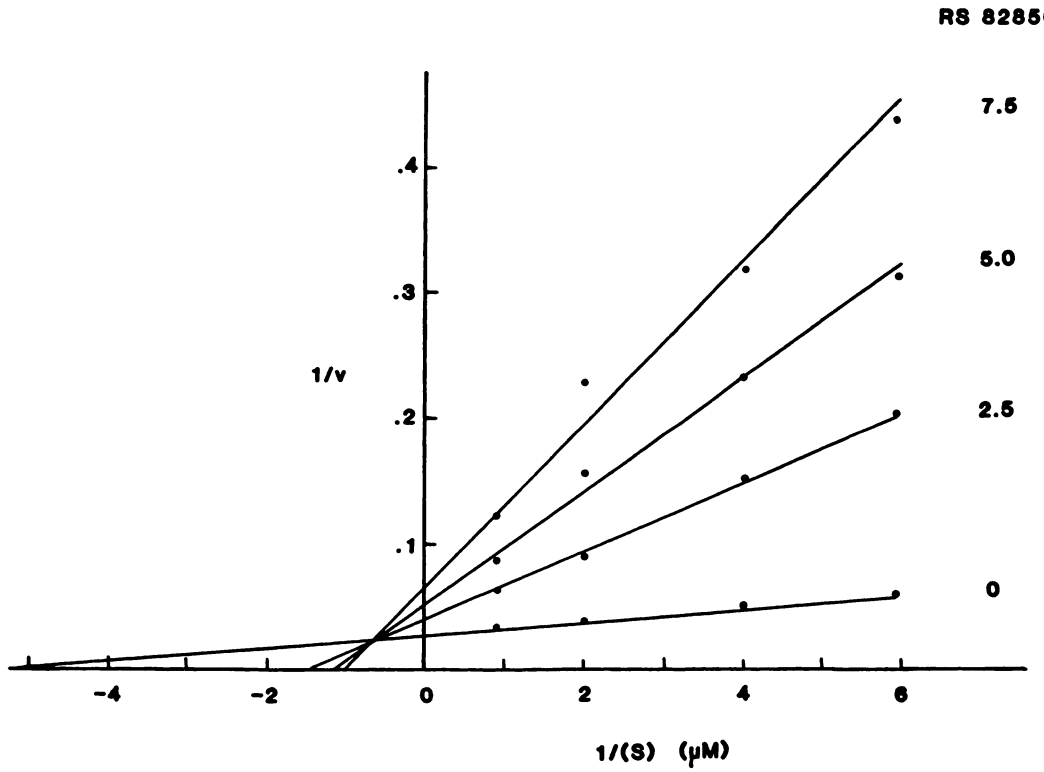

Fig. 5. Reciprocal plot (1/*v* vs. 1/[*S*]) for dog heart cyclic AMP phosphodiesterase (fraction III) in the presence of 0, 2.5, 5.0, and 7.5 nM RS-82856. Velocity is expressed as nmol of cyclic AMP hydrolyzed/min/mg of protein. From the Dixon plot of these data the K_i for RS-82856 was 0.75 nM. Inhibition of enzyme activity by a saturating concentration of RS-82856 (1 μM) was determined in the presence of each concentration of the substrate. To reveal more clearly the pattern of inhibition, phosphodiesterase activity not inhibited by 1 μM RS-82856 was subtracted from the velocity data. The values subtracted were 2.29, 2.87, 4.31, and 5.93 nmol/min/mg of protein in the presence of 0.17, 0.25, 0.50, and 1.1 μM cyclic AMP, respectively. Thus, a mixed-type inhibitory pattern was obtained for that fraction of the enzyme susceptible to inhibition by RS-82856.

the type II enzyme (14). Fraction II has a high K_m for both cyclic AMP and cyclic GMP (50 μM). The enzyme, however, was not stimulated by either calmodulin or cyclic GMP. Therefore, this enzyme does not fit the criteria for any of the four enzyme types as recently described (14). In accord with previous observations (9), fraction III is a cyclic AMP-selective phosphodiesterase (K_m = 0.3 μM) with only low specific activity using cyclic GMP as the substrate. Fraction III corresponds to the type IV enzyme.

RS-82856 strongly inhibits the fraction III (type IV) cyclic AMP phosphodiesterase (IC_{50} = 1.2 nM) with relatively weak effects on fraction II (IC_{50} = 1.8 μM) and fraction I (IC_{50} = 60 μM). RS-82856 reduces both maximum velocity and affinity for substrate of the type IV enzyme (Fig. 3). This pattern of partial competitive and noncompetitive inhibition was also obtained for both anagrelide and cilostamide. RS-82856 (K_i = 0.5 nM) is more potent than either anagrelide (K_i = 8.8 nM) or cilostamide (K_i = 12 nM).

Positional isomers of RS-82856 were also examined as inhibitors of the high affinity enzyme. Maximum inhibitory activity was obtained with the *N*-cyclohexyl-*N*-methyl-4-butyramidyl(oxy) side-chain attached to C-7 (Fig. 1). When attached to C-8 or C-6, potency decreased by 9 and 99%, respectively. Activity was virtually abolished with the side-chain at C-9. These results indicate that the inhibitory binding site for RS-82856 contains a sterically defined, bulk-tolerant, lipophilic region. Attachment to this domain significantly enhances potency of the compound.

Replacement of the *N*-methyl group of RS-82856 with several bulky, lipophilic substitutions also enhanced inhibitory potency.[4] For example, the N-2-benzoyloxyethyl derivative exhibits more than 10 times the potency of RS-82856 (K_i = 0.04 nM). Structure-activity relationships obtained from other analogs revealed that potent inhibition required an intact, relatively planar, tricylic ring structure with a carbonyl group at C-2.[4]

Cardiac cyclic AMP phosphodiesterase. Chromatographic separation of the soluble phosphodiesterase activities from a dog heart homogenate revealed the presence of two high affinity forms (Fig. 4), whereas only one form predominates in platelets (Fig. 2). In agreement with the observations of Kariya *et al.* (17), fraction I is a low K_m (0.5 μM) cyclic AMP phosphodieserase which is sensitive to calmodulin stimulation (type I). Fraction II has a high K_m (50 μM) for cyclic AMP and is stimulated by cyclic GMP (type II). Fraction III is a cyclic AMP-selective phosphodiesterase (type IV) with a high affinity for this substrate (K_m = 0.58 μM).

RS-82856 inhibits the fraction III (type IV) cardiac enzyme (IC_{25} = 1.9 nM) with a very weak effect on fraction II (type II) (IC_{25} = 0.1 mM) and no significant inhibitory effect on fraction I (type I) in the concentration range of 0.1 nM–10 μM. Inhibition of the fraction III enzyme by a saturating concentration (1 μM) of RS-82856 was only partial. The percentage of total enzyme activity inhibited by 1 μM RS-82856 decreased from 65 to 55% as the substrate concentration increased from 0.17 to 1.1 μM.

[4] R. Alvarez, J. J. Bruno, G. H. Jones, A. M. Strosberg, and M. C. Venuti, manuscript in preparation.

TABLE 1

Inhibition of crude soluble and membrane-bound cyclic AMP phosphodiesterase from various human and dog tissues by RS-82856

RS-82856 was tested in log order increments in the range of 0.1 nM—0.1 mM. Except for the platelet enzyme, only partial inhibition was obtained with these crude preparations. Concentration-dependent inhibition of enzyme activity was observed at low concentrations of RS-82856 (1–10 nM) which approached apparent saturation in the range of 10 nM–1 μM, generally followed by another concentration-dependent inhibition in the presence of 1 μM–0.1 mM RS-82856. Human lymphocytes do not contain a membrane-bound phosphodiesterase. The substrate concentration was 1 μM.

	Membrane-bound		Soluble	
	IC_{25}	% Inhibition at 1 μM	IC_{25}	% Inhibition at 1 μM
	nM		nM	
A. Human Tissue				
Platelet	0.6	91	0.9	89
Heart	6.4	35	1,100	24
Lymphocyte			150,000	1
B. Dog Tissue				
Platelet	0.7	89	2.4	80
Heart	1.9	41	1,000	25
Liver	18	33	11	36
Spleen	130	28	56	30
Large intestine	310	26	3,700	17
Stomach	5,800	18	7,600	18
Lung	6,000	21	10,000	18
Kidney	27,000	12	13,000	11
Small intestine	47,000	12	4,100	15
Skeletal muscle	56,000	8	54,000	4

Additional studies will be required to determine whether fraction III contains more than one enzyme form. At present, we have no convincing explanation for partial inhibition by RS-82856. The kinetic pattern of inhibition by RS-82856 with the cardiac fraction III enzyme is typical of a mixed-type inhibitor with a K_i of 0.75 nM (Fig. 5).

Tissue selectivity. Plasma membranes from various tissues contain a high affinity form of cylic AMP phosphodiesterase (18). For this reason, we examined the effect of RS-82856 on both membrane-bound and soluble forms of phosphodiesterase isolated from several human and dog tissues. Remarkable selectivity was observed for the platelet membrane-bound and soluble enzymes (Table 1). Although RS-82856 is a potent inhibitor of the membrane-bound cardiac enzyme, only partial inhibition was obtained. Similarly, the compound partially inhibits crude enzyme activity from all of the other tissues examined (Table 1). This partial inhibition pattern can be explained by the presence of multiple molecular forms of phosphodiesterase with different sensitivity to inhibition by RS-82856 as observed in the separated platelet enzymes. Thus, differences in the relative abundance of an inhibitor-sensitive phosphodiesterase can account for the apparent tissue selectivity.

Platelet aggregation. RS-82856 inhibits the aggregation of human platelets induced by 5 μM ADP (IC_{50} = 0.11 μM)[5] and is more potent than either anagrelide (2.6 μM) or cilostamide (1.9 μM). Similarly, RS-82856 inhibits the aggregation of rhesus monkey platelets *in vitro* in response to 20 μM ADP (IC_{50} = 0.09 μM). Platelet aggregation (*ex vivo*) in response to 20 μM ADP was also inhibited by 45–59%, 1 hr after oral administration of 10 mg/kg of RS-82856 to two rhesus monkeys. Significant inhibition (31–52%) was also evident 2 hr after the oral dose. Platelets from a monkey receiving a vehicle control were not inhibited.

[5] Cyclic AMP accumulation was examined in washed human platelets as previously described (19). After 10 min incubation, 1 μM RS-82856 and 1 μM prostaglandin E_1 increased intracellular cyclic AMP 3.6- and 12.2-fold, respectively. Strong synergism was obtained when both compounds were combined (28.3-fold).

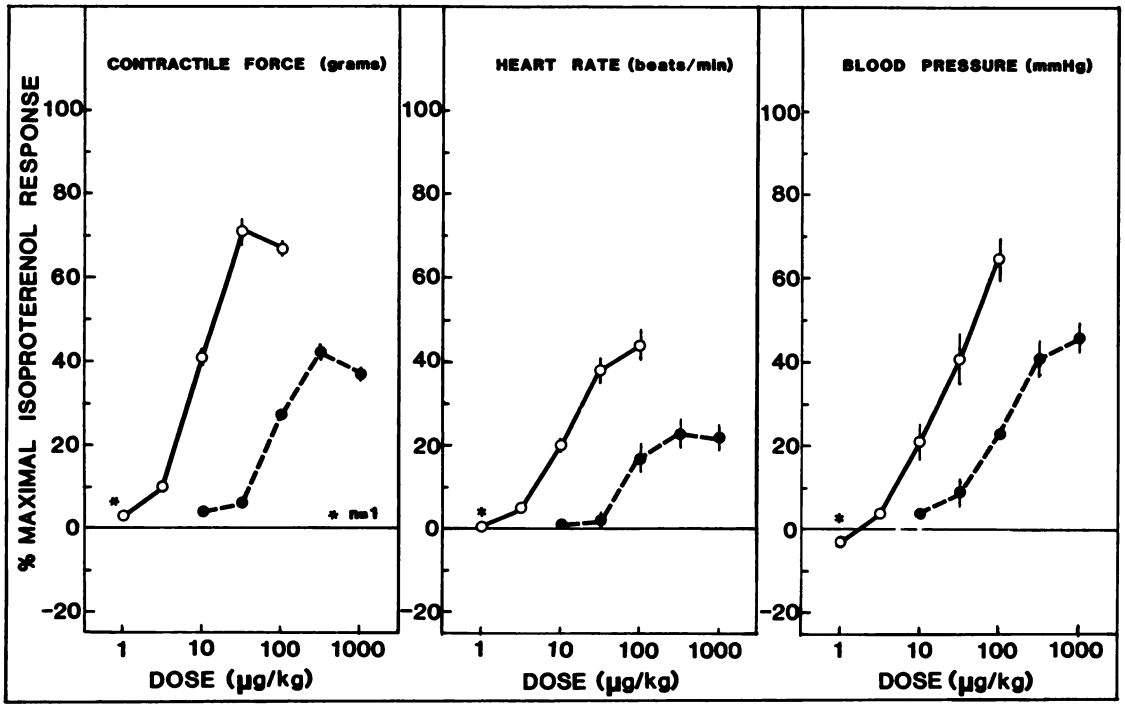

Fig. 6. Effects of RS-82856 following intravenous (○—○, n = 4) and intraduodenal (●- -●, n = 3) administration to anesthetized dogs. Responses are expressed in terms of the maximal responses to isoproterenol in the same preparations. The blood pressure responses are decreases in mean arterial pressure, whereas the contractile force and heart rate responses are increases.

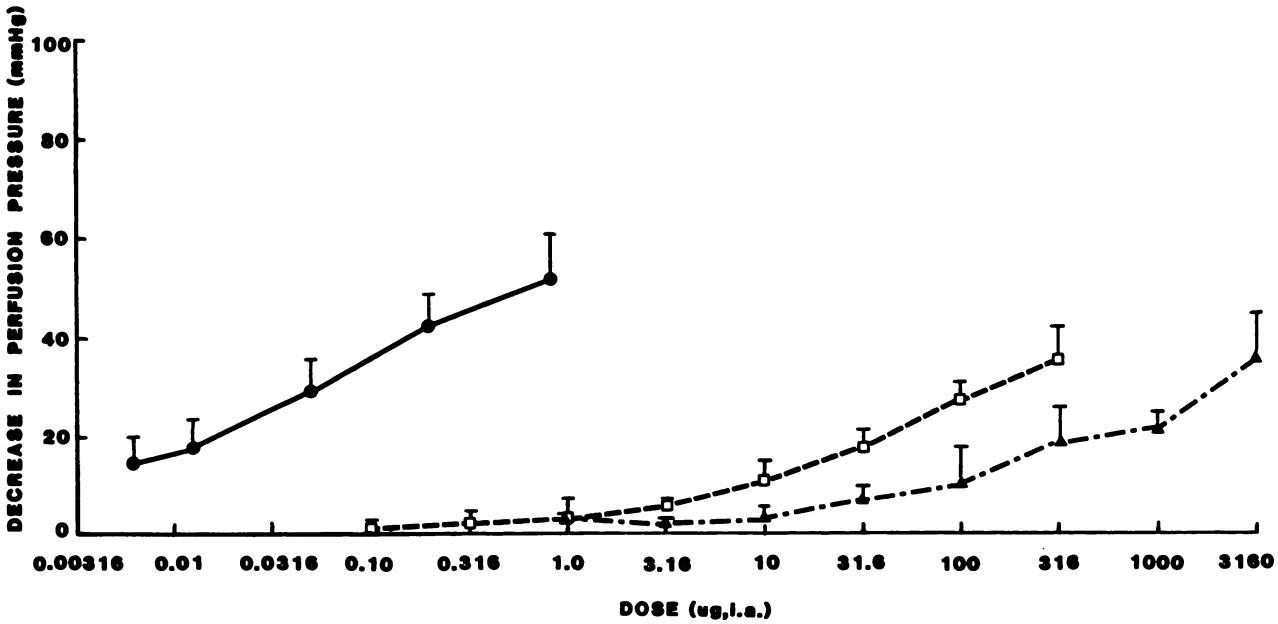

Fig. 7. Effects of isoproterenol (●—●, n = 8), RS-82856 (□- -□, n = 4), and milrinone (▲- - -▲, n = 4) on perfusion pressure of the hind limb in anesthetized dogs.

Cardiovascular effects. Intravenous administration of RS-82856 to anesthetized dogs resulted in increases in ventricular contractile force, heart rate, and decreases in mean arterial pressure (Fig. 6). These inotropic and afterload reduction effects were qualitatively similar to those induced by milrinone (20). RS-82856 was also active following intraduodenal administration. However, it was approximately 10 times less potent by this route than via intravenous administration (Fig. 6). It was also less efficacious on cardiac force, achieving approximately 40% of the maximal isoproterenol response, in contrast to greater than 70% of the maximal isoproterenol response achieved following intravenous administration. These observations indicate lower oral bioavailability than expected. Nevertheless, following intraduodenal administration, RS-82856 exhibited a duration of action greater than 8 hr.[6]

RS-82856 exhibited vasodilatory activity in the canine perfused hind limb preparation (Fig. 7). Although nearly 1000 times less potent than isoproterenol, it was 18 times more potent than milrinone on a molar basis at decreasing perfusion pressure following intra-arterial administration. Catecholamines do not appear to be involved in the inotropic response to RS-82856 since the dose-response curve was unaltered in reserpinized dogs.[6]

The results reported here indicate that RS-82856 is a potent and selective inhibitor of type IV cyclic AMP phosphodiesterase in human platelets. The compound inhibits the aggregation of human platelets *in vitro* and exhibits oral activity in monkeys. Administration of the compound to anesthetized dogs increases cardiac contractile force and decreases blood pressure, indicating the presence of both inotropic and afterload reduction properties. Thus, the compound may be useful as an agent to increase cardiac output in the treatment of congestive heart failure. The potent inhibition of platelet aggregation and apparent tissue selectivity suggest that the compound may also possess useful antithrombotic properties and exhibit a reduced incidence of adverse effects.

[6] Unpublished observations.

Acknowledgments

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