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MULTIPLE MOLECULAR FORMS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN CARDIAC AND SMOOTH MUSCLE AND IN PLATELETS

ISOLATION, CHARACTERIZATION, AND EFFECTS OF VARIOUS REFERENCE PHOSPHODIESTERASE INHIBITORS AND CARDIOTONIC AGENTS

RONALD E. WEISHAAR,* STEPHEN D. BURROWS, DIANNE C. KOBYLARZ, MARY M. QUADE and DALE B. EVANS

Department of Pharmacology, Warner-Lambert/Parke-Davis Pharmaceutical Research, Ann Arbor, MI 48105, U.S.A.

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Abstract—Multiple molecular forms of cyclic nucleotide phosphodiesterase have been identified previously in several tissues and cell types using a variety of different isolation methods. In the present study, the different molecular forms of phosphodiesterase (PDE) were isolated from cardiac muscle (guinea pig left ventricle), vascular smooth muscle (bovine coronary arteries) and human platelets using the same isolation procedure in each instance. These enzymes were then characterized kinetically, and the effects of various reference PDE inhibitors and cardiotonic agents on each form were examined. A low K_m, low V_{max} form of phosphodiesterase (PDE I) was found in all three tissue/cell types. PDE I activity was stimulated by calmodulin in cardiac and smooth muscle, but not in platelets. In smooth muscle and platelets, PDE I preferentially hydrolyzed cyclic GMP, whereas cardiac muscle PDE I hydrolyzed cyclic AMP and cyclic GMP equally. A high K_m, high V_{max} form of phosphodiesterase (PDE II) was found in cardiac muscle and platelets, but not in smooth muscle. PDE II activity was not stimulated by calmodulin and there was no substrate specificity. A low K_m, low V_{max} cyclic AMP-specific form of phosphodiesterase (PDE III) was found in all three tissue/cell types. The activity of PDE III was not stimulated by calmodulin. The reference inhibitors theophylline and papaverine exerted nonspecific inhibitory effects on all forms of phosphodiesterase. Other reference inhibitors (M & B 22,948 and dipyridamole) and several cardiotonic agents (AR-L 57, CI-914, CI-930, amrinone, and MDL 17,043) exerted selective inhibitory effects on only one molecular form of phosphodiesterase. The degree of selectivity was often dependent upon the tissue or cell from which the molecular form of phosphodiesterase was isolated. These studies demonstrate that (i) there is heterogeneity regarding the number of phosphodiesterases present in various tissue/cell types, as well as their substrate specificity and their ability to be stimulated by calmodulin, and (ii) these different molecular forms of phosphodiesterase can be selectively inhibited by different pharmacological agents. The possibility exists that such selective inhibitors may produce discrete changes in cyclic AMP or cyclic GMP levels, and that these changes may be produced in specific tissues and/or cells.

In 1971 Thompson and Appleman [1] identified three forms of phosphodiesterase in rat brain cortex. Since that time, multiple molecular forms of phosphodiesterase have been identified in a number of other tissues, including cardiac muscle [2], vascular smooth muscle [3], liver [4], lung [5], and platelets [6]. A variety of methods have been used to isolate these different molecular forms of phosphodiesterase, including centrifugation [7], gel electrophoresis [8], isoelectric focusing [9] and anion-exchange chromatography [10]. This variety of isolation procedures has resulted in conflicting information regarding both the number of phosphodiesterases present in a tissue/cell type, as well as the kinetic properties and substrate specificity of the different molecular forms of the enzyme. For example, using differential centrifugation techniques, Amer and Mayol [7] isolated two forms of phosphodiesterase from human platelets. A later study by Hidaka and Asano [6], using diethylaminoethyl ether (DEAE)-cellulose anion exchange chromatography to isolate phosphodiesterases, showed that human platelets actually contain three molecular forms of phosphodiesterase. Similar discrepancies have been observed for myocardial tissue [10–12] and tracheal smooth muscle [13, 14]. These difficulties have largely been overcome by the increasing use of DEAE-cellulose anion-exchange chromatography for isolating the different molecular forms of phosphodiesterase. However, no study has been undertaken in which this single method of isolation has been used to systematically compare the number and kinetic characteristics of the various phosphodiesterases present in several

* Address for reprints: Ronald E. Weishaar, Warner-Lambert/Parke-Davis Co., 2800 Plymouth Rd., Ann Arbor, MI 48105.

different tissue and/or cell types, and the effects of reference phosphodiesterase inhibitors on these different enzymes.

In the present study, the multiple molecular forms of phosphodiesterase were isolated from cardiac muscle, coronary arteries and platelets. K_m and V_{max} values were determined for each molecular form, as well as substrate specificity (cyclic AMP vs cyclic GMP), and the ability of calmodulin to increase enzymatic activity. In addition, the effects of a variety of reference phosphodiesterase inhibitors (theophylline, papaverine, M & B 22,948 and dipyridamole), and cardiotonic agents (CI-914, CI-930, MDL 17,043, amrinone, carbazeran and AR-L 57) on each molecular form of phosphodiesterase were also examined.

MATERIALS AND METHODS

Isolation of multiple molecular forms of phosphodiesterase (PDE)

The method of Thompson *et al.* [10], with minor modifications, was used to isolate phosphodiesterases from cardiac muscle, vascular smooth muscle and platelets.

Cardiac phosphodiesterases. Guinea pig left ventricular and septal muscle was used for these studies. Male guinea pigs weighing 300–500 g were anesthetized with diethyl ether, after which the heart was rapidly excised and placed in ice-cold saline (three to four hearts were used for each isolation). The left ventricle and septum were then dissected and minced with a single-edge razor blade, and the mince was placed in 10 vol. of "PDE isolation buffer" (2 mM $MgCl_2$, 1 mM dithiothreitol and 10 mM Tris–HCl, pH 7.5). The mince was then homogenized using a Brinkmann PT-20 Polytron (three bursts of 10-sec duration at a rheostat setting of 5.0). This and all subsequent procedures were performed at 4°. The homogenate was sonicated (30 sec/ml homogenate) and then centrifuged at 30,000 *g* for 20 min. The resulting supernatant fraction was filtered through four layers of gauze and applied to a DEAE-cellulose column (40 × 1.6 cm), prepared as described by Cheung [15], and equilibrated with freshly prepared 70 mM sodium acetate/5 mM 2-mercaptoethanol (pH 6.5). The column was then washed with 2–3 bed volumes of sodium acetate/2-mercaptoethanol, after which the phosphodiesterases were eluted from the column using a continuous 70–1000 mM sodium acetate gradient (pH 6.5, containing 5 mM 2-mercaptoethanol; total volume 400 ml). The flow rate was approximately 25 ml/hr. Fractions (8 ml) were collected and assayed for cyclic AMP- and cyclic GMP-phosphodiesterase activity (substrate concentration = 1.0 μM) in the presence and absence of 0.1 units of calmodulin and 10 μM $CaCl_2$. Appropriate fractions were pooled and dialyzed against 70 mM sodium acetate/5 mM 2-mercaptoethanol for 20 hr. Cross-contamination was observed with peak II (PDE II) and peak III (PDE III) phosphodiesterases, which could be eliminated by rechromatography [2, 10, 12]. Following complete separation, the combined phosphodiesterase fractions were concentrated to 14% of the original volume using an Amicon ultrafiltration cell fitted with a UM-10 membrane, according to the method of Wells *et al.* [3]. Following concentration, the protein was then diluted to 65% with ethylene glycol monoethyl ether, and stored at −20°. No significant change in hydrolytic activity was observed with storage up to 6 weeks.

Vascular smooth muscle phosphodiesterases. Bovine coronary arteries (right, left anterior descending and left circumflex arteries) from hearts obtained from a local slaughterhouse were used for these studies. Arteries from two hearts were used for each isolation. Hearts were kept in an ice-saline (0.9% NaCl) slurry, and the arteries were dissected within 2 hr after the animals were killed. After removing all fat and connective tissue, the arteries were everted, cut into 1 mm cubes with a single edge razor blade, and homogenized with a Brinkmann Polytron (three bursts of 10-sec duration at a rheostat setting of 10.0). The resulting homogenate was treated as previously described for the cardiac homogenate.

Platelet phosphodiesterases. Human blood was obtained from normal volunteer donors and citrated. Platelets were then isolated according to the method described by Garcia-Sevilla *et al.* [16], with slight modifications. Briefly, the blood was centrifuged at 100 *g* for 15 min (25°), and the resulting platelet-rich plasma was titrated to pH 6.5 with acid–citrate–dextrose solution (0.8% citric acid, 2.2% trisodium citrate and 2.5% dextrose). The platelet-rich plasma was then centrifuged at 5100 *g* for 15 min (25°) to sediment the platelets. The resulting platelet pellet was then washed twice with 5 ml of PDE isolation buffer using a Dounce homogenizer and recentrifuged at 5100 *g* for 15 min (25°). The final pellet was homogenized using a glass homogenizer tube and a close-fitting serrated teflon pestle (40 strokes). This latter procedure was performed at 4°, and the homogenate thus obtained was thereafter treated as previously described for the cardiac homogenate.

Microscopic evaluation of the platelet-rich plasma, as well as the platelet pellets, demonstrated that the final pellet was rich in platelets and contained very few red blood cells. Plastic beakers and pipettes were used throughout the platelet isolation to prevent adhesion of platelets to glass.

Measuring phosphodiesterase activity

Phosphodiesterase activity was measured as described previously [10, 17], in a reaction medium containing 40 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$ and 4 mM 2-mercaptoethanol. Unless otherwise noted, the concentration of substrate ([^{3}H]cyclic AMP or [^{3}H]cyclic GMP) was 1.0 μM. All reference phosphodiesterase inhibitors and cardiotonic agents examined were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the reaction medium was either 2.5% (cardiac and platelet studies) or 1.25% (smooth muscle studies). This concentration of DMSO inhibited enzyme activity by approximately 10%. The IC_{50} values (concentration which produced 50% inhibition of substrate hydrolysis) for the various agents examined were determined from concentration–response curves, in which concentrations ranged from 10^{-7} to 10^{-4} M for the more potent inhibitors and 10^{-5} to 10^{-3} M for the less potent inhibitors (half-log increments). Three to four such concentration–response curves were generated

for each agent, typically using different enzyme preparations for each concentration–response.

Reagents

All reagents used were of the highest obtainable commercial purity. [2,8-^{3}H]-Labeled cyclic AMP (30–50 Ci/mmole) and [8-^{3}H(N)]-labeled cyclic GMP (10–25 Ci/mmole) were obtained from New England Nuclear. Calmodulin (from bovine heart), *Ophiophagus Hannah* snake venom, and theophylline were purchased from the Sigma Chemical Co. Diethylaminoethyl ether (DEAE)-cellulose was purchased from Matheson, Coleman & Bell. Y-590 was obtained from the Yoshitomi Pharmaceutical Co. Papaverine was obtained from the Merck Sharp & Dohme Pharmaceutical Co. Dipyridamole was obtained from the Ciba-Geigy Co. Carbazeran was obtained from the Pfizer Pharmaceutical Co. CI-914, CI-930, M & B 22,948, amrinone, milrinone, amipazone, AR-L 57 and sulmazole (AR-L 115BS) were prepared by the Warner-Lambert/Parke-Davis Chemistry Department.

Statistical evaluation

IC_{50} values were calculated using the method of Hubert [18], by employing the following equation:

$$\frac{P_i = e^{\alpha} + BX_i}{1 + e^{\alpha} + BX_i}$$

in which P_i = probability of response, α = estimate of the intercept (IC_{50} if intercept is determined to be at 50% of control response), and B = estimate of the slope. This procedure also provides 95% confidence limits for the IC_{50} value. The application of the methodology to the analysis of dose–response relationships has been described in detail by Ward [19].

RESULTS

Isolation of multiple forms of phosphodiesterase

The different molecular forms of phosphodiesterase from cardiac muscle, vascular smooth muscle and platelets were resolved by DEAE-cellulose chromatography of the 30,000 *g* supernatant fraction (Fig. 1). For cardiac muscle (Fig. 1, A and B), these forms varied according to substrate specificity in that peak I (PDE I) and peak II (PDE II) hydrolyzed cyclic AMP and cyclic GMP with apparent equal affinity, whereas peak III (PDE III) hydrolyzed only cyclic AMP. In addition, the activity of PDE I from cardiac muscle could be stimulated by calmodulin, whereas no such stimulation could be observed with PDE II or PDE III. Cross-contamination of the three forms of PDE could be eliminated by combining those fractions containing the highest enzymatic activity, followed by dialysis and rechromatography. This procedure resulted in virtually the complete elimination of cross-contamination [2, 10, 12].

Separation of the different molecular forms of phosphodiesterase in platelets and coronary arteries is illustrated in Fig. 1, C–F. As with cardiac muscle, platelets contain three molecular forms of phosphodiesterase (Fig. 1, C and D), whereas only two forms of PDE could be identified in coronary arteries (Fig. 1, E and F). Unlike cardiac muscle, PDE I in platelets and in smooth muscle displayed a clear preference for cyclic GMP as substrate. In addition, although the activity of cardiac and smooth muscle PDE I could be stimulated by calmodulin, no such stimulation of platelet type I PDE activity by calmodulin was observed (Fig. 1, A–F).

The possibility that contaminating amounts of calmodulin in the platelet PDE I preparation could be responsible for the inability of exogenous calmodulin and calcium to stimulate enzyme activity was evaluated by comparing the effects of the calcium chelator ethyleneglycol-bis-(β-amino ethyl ether)*N*,*N*′-tetraacetic acid (EGTA) on the hydrolytic activity of PDE I. As Table 1 shows, 20 μM EGTA had no effect on the basal activity of PDE I from cardiac muscle, smooth muscle or platelets. This concentration of EGTA, however, was sufficient to completely abolish the stimulatory effect which exogenous calmodulin and calcium exerted on cardiac and smooth muscle PDE I.

A second form of phosphodiesterase (PDE II), which hydrolyzed cyclic AMP and cyclic GMP with apparent equal affinity, was identified in both cardiac muscle and platelets but not coronary arteries. As can be seen in Fig. 1, panels A–D, calmodulin had

Table 1. Effect of EGTA on basal and calmodulin-stimulated peak I phosphodiesterase activity

Condition	Activity (pmoles cGMP hydrolyzed · min^{-1} · μg^{-1})		
	Guinea pig left ventricle	Bovine coronary artery	Human platelet
Basal activity	1.29	0.52	1.55
+20 μM EGTA	1.21	0.48	1.61
+0.1 units Calmodulin +10 μM $CaCl_2$	5.28	1.04	1.22
+0.1 units Calmodulin +10 μM $CaCl_2$ +20 μM EGTA	1.70	0.48	1.51

Basal enzyme activity was measured as described in Materials and Methods using 1.0 μM [^{3}H]cyclic GMP as substrate. Each value represents the mean of two to three separate determinations.

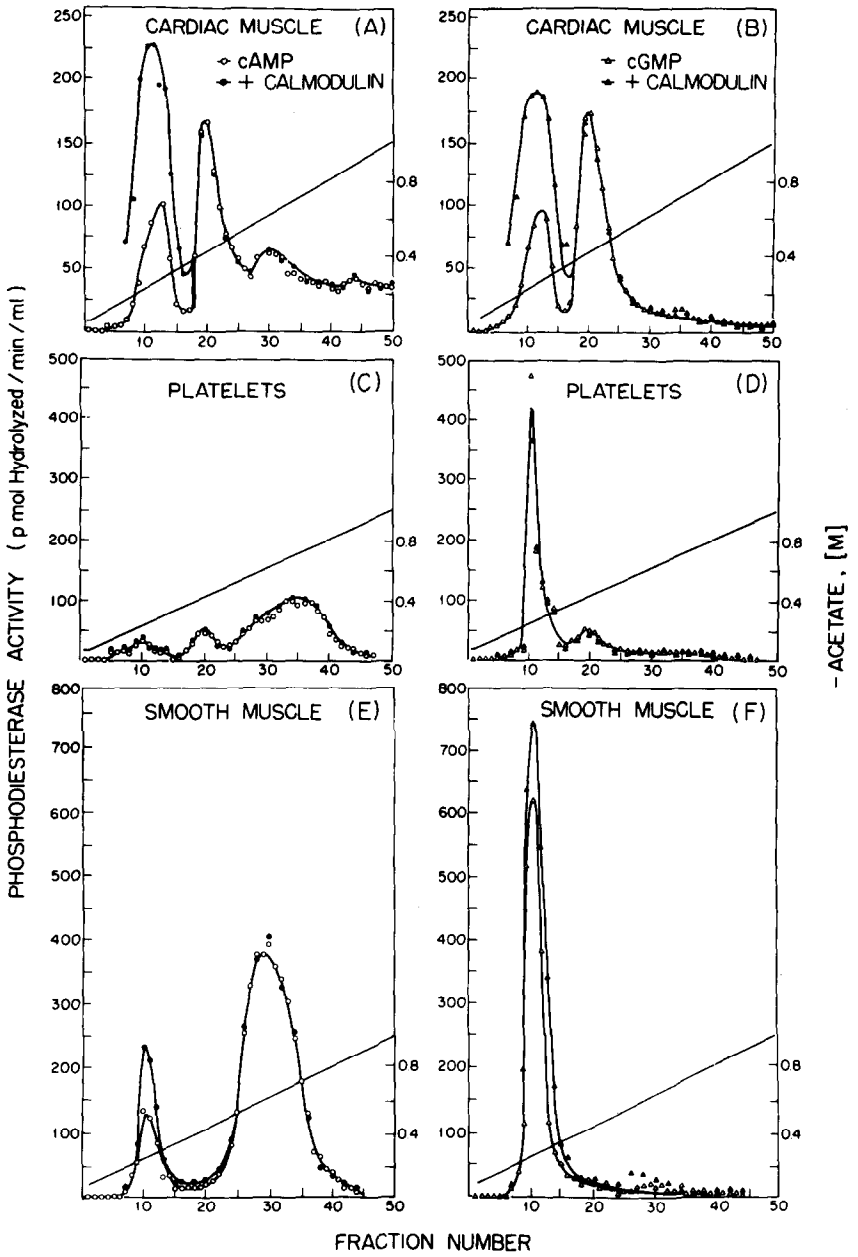


Fig. 1. Cyclic AMP (○) and cyclic GMP (△) phosphodiesterase activity in 8.0-ml fractions eluted from DEAE-cellulose by a 0.07 to 1.00 M sodium acetate gradient. Each figure is representative of five to seven comparable isolations. Substrate concentration was 1.0 μM for both cyclic AMP and cyclic GMP. Filled symbols refer to enzyme activity in the presence of 0.1 units calmodulin and 10 μM $CaCl_2$. Enzyme activity was measured as described in Materials and Methods.

no stimulatory effect on this molecular form of the enzyme in either tissue/cell type.

Cardiac muscle, smooth muscle and platelets were all found to contain a third molecular form of phosphodiesterase (peak PDE III), which hydrolyzes only cyclic AMP. The activity of PDE III was not stimulated by calmodulin. Inclusion of 20 μM EGTA had no effect on basal PDE II or PDE III activity from cardiac muscle, smooth muscle, or platelets (data not shown).

Kinetic characterization of the different phosphodiesterases

The method of Hofstee [20] was used to determine the K_m (substrate concentration at which half-maximal reaction velocity is observed) and V_{max} (maximal velocity of the reaction) for each molecular form of phosphodiesterase in the different tissue/cell types examined.

PDE I. In cardiac muscle, high- and low-affinity

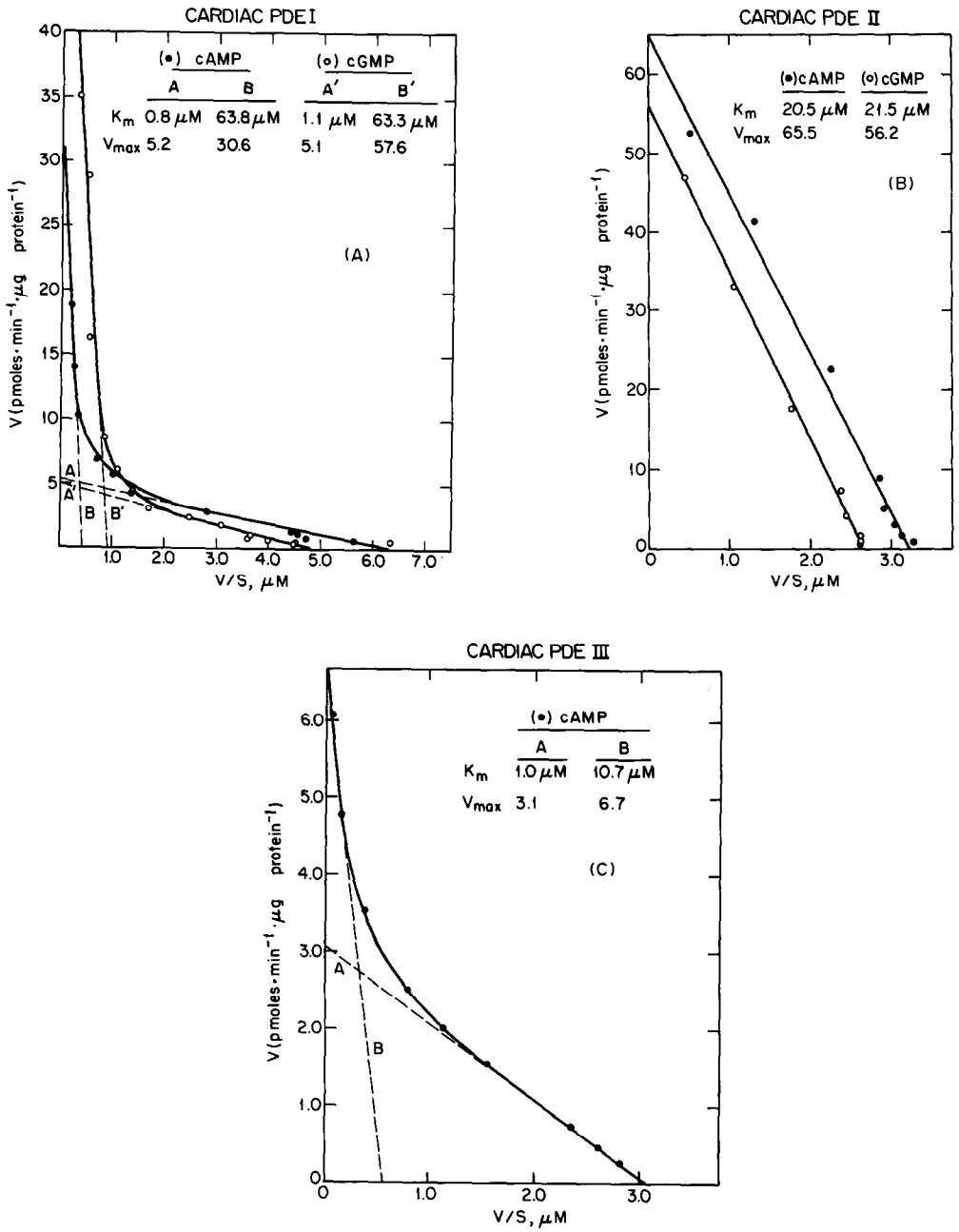


Fig. 2. Determination of K_m and V_{max} values for cyclic AMP (●) and cyclic GMP (○) hydrolysis by (A) peak I phosphodiesterase, (B) peak II phosphodiesterase, and (C) peak III phosphodiesterase from guinea pig left ventricular muscle, using Hofstee analysis [20]. For these evaluations phosphodiesterase activity was measured at substrate concentrations ranging from 0.1 to 100 μM. Values are means of duplicate determinations and are representative of two to three experiments with different preparations. Enzyme activity was measured as described in Materials and Methods.

sites of hydrolysis by PDE I were observed for both cyclic AMP and cyclic GMP (Fig. 2A, Table 2). The K_m of the high-affinity site was comparable for each substrate (~1.0 μM), as were the values for V_{max}. In platelets, high- and low-affinity hydrolytic sites were also observed for both cyclic AMP and cyclic GMP (Table 2). However, the K_m for the high-affinity hydrolytic site for cyclic AMP was greater than the K_m for cyclic GMP (~ 10 vs 1.0 μM). In addition, V_{max} values for cyclic GMP hydrolysis by platelet PDE I were considerably greater than for hydrolysis of cyclic AMP (Table 2). In coronary arteries, K_m values for cyclic AMP and cyclic GMP hydrolysis by PDE I were comparable (~3 μM). As was the case with platelet PDE I, the value of V_{max} was considerably greater for cyclic GMP than for cyclic AMP

Table 2. Kinetic characterization of phosphodiesterases from cardiac and smooth muscle and from platelets

Tissue/Cell type	PDE type	Substrate	K_m (μM)		V_{max} (pmoles·min^{-1}·μg^{-1})		Effect of calmodulin
			High affinity	Low affinity	High affinity	Low affinity	
Cardiac muscle (guinea pig left ventricle)	I	Cyclic AMP	0.8 ± 0.1	43 ± 11	4.3 ± 0.3	22 ± 9	Calmodulin stimulates
		Cyclic GMP	0.9 ± 0.1	53 ± 10	3.7 ± 0.5	34 ± 24	Calmodulin stimulates
	II	Cyclic AMP	—	20 ± 1	—	64 ± 1	No effect
		Cyclic GMP	—	22 ± 1	—	56 ± 1	No effect
	III	Cyclic AMP	0.9 ± 0.1	8.3 ± 0.4	2.3 ± 0.7	5.3 ± 1.3	No effect
		Cyclic GMP*	—	—	—	—	
Platelets (human)	I	Cyclic AMP†	~10	>1000	~0.1	~5.0	
		Cyclic GMP	1.0 ± 0.1	67 ± 7	1.6 ± 1.4	4.8 ± 3.7	No effect
	II	Cyclic AMP	—	24 ± 9	—	2.0 ± 0.8	No effect
		Cyclic GMP	—	25 ± 4	—	2.3 ± 1.1	No effect
	III	Cyclic AMP	0.4 ± 0.02	7.5 ± 1.9	1.7 ± 0.5	1.5 ± 1.4	No effect
		Cyclic GMP*	—	—	—	—	
Smooth muscle‡ (bovine coronary arteries)	I	Cyclic AMP	2.5 ± 0.4	140 ± 32	0.9 ± 0.3	30 ± 14	Calmodulin stimulates
		Cyclic GMP	3.0 ± 0.8	17 ± 8	5.0 ± 0.8	—	Calmodulin stimulates
	III	Cyclic AMP	0.9 ± 0.1	14 ± 5	1.2 ± 0.3	3.4 ± 0.3	No effect
		Cyclic GMP*	—	—	—	—	

K_m (substrate concentration at which half-maximal reaction velocity is observed) and V_{max} (maximal velocity of the reaction) values were determined using the method of Hofstee [20]. Enzyme activity was measured as described in Materials and Methods. For these evaluations enzyme activity was measured at substrate concentrations ranging from 0.1 to 100 μM. Values are mean ± S.E. of duplicate determinations and are representative of two to four experiments with different preparations.

* PDE III does not hydrolyze cyclic GMP to an appreciable degree.

† PDE I hydrolysis of cyclic AMP is not sufficient for accurate determination of K_m and V_{max}.

‡ There is no PDE II in bovine coronary arteries.

(Table 2). These observations likely explain the preferential hydrolysis of cyclic GMP by coronary artery and platelet PDE I observed in Fig. 1, B and C.

PDE II. In cardiac muscle, only a single site was observed for both cyclic AMP and cyclic GMP hydrolysis by type II phosphodiesterase (Fig. 2B, Table 2). The K_m value for hydrolysis was comparable for either substrate as was the V_{max} value, which indicates that, as with cardiac PDE I, there is no substrate specificity (Table 2). The two enzymes differ, however, in that the K_m for cyclic AMP and cyclic GMP hydrolysis was considerably higher for PDE II than for PDE I (~20 vs ~1.0 μM).

As with cardiac PDE II, only a single low-affinity hydrolytic site was observed for both cyclic AMP and cyclic GMP hydrolysis by platelet PDE II (K_m = ~25 μM). V_{max} values for cyclic AMP and cyclic GMP hydrolysis by platelet PDE II were comparable as well (Table 2). No type II PDE was observed in coronary arteries (Fig. 1, E and F).

PDE III. As previously described, PDE III hydrolyzes only cyclic AMP. This molecular form of the enzyme displays anomalous kinetic behavior, however, as is illustrated for cardiac PDE III in Fig. 2C. Although Hofstee analysis apparently reveals the presence of both a low K_m (~1.0 μM) and a high K_m (~10.0 μM) hydrolytic site for cyclic AMP, these sites are typically described by a hyperbola rather than by two straight lines. Similar anomalous kinetic behavior was also observed with smooth muscle PDE III and platelet PDE III (data not shown). The K_m and V_{max} values for cardiac, coronary artery and platelet PDE III are given in Table 2. Potential explanations for such anomalous kinetic behavior will be addressed in the Discussion.

Effects of reference phosphodiesterase inhibitors and cardiotonic agents

To further characterize the differences between the various molecular forms of phosphodiesterase present in cardiac and smooth muscle and in platelets, the inhibitory effects of a variety of phosphodiesterase inhibitors and cardiotonic agents on each of the different forms of phosphodiesterase isolated from these three tissue/cell types were evaluated (the effects of several other agents on phosphodiesterases from a particular tissue or cell were also examined). The agents chosen for evaluation varied considerably in structure and include those agents that have been characterized previously as non-specific inhibitors (e.g. theophylline) [21], inhibitors of cyclic GMP-phosphodiesterase (e.g. M & B 22,948) [5], and inhibitors of cyclic AMP phosphodiesterase (e.g. CI-914) [22]. The effects of this core of reference inhibitors and cardiotonic agents on the different

Table 3. Effects of reference phosphodiesterase inhibitors, cardiotonics and other agents on cardiac phosphodiesterases

	IC_{50} (μM)					
	PDE I		PDE II		PDE III	
Agent	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP*
Carbazeran	99 (91–110)†	110 (70–140)	34 (32–36)	31 (27–35)	6.9 (6.0–7.7)	—
Amrinone	>1000	>1000	720 (560–880)	580 (400–770)	46 (38–55)	—
MDL 17,043	>1000	>1000	860 (250–1500)	450 (330–570)	14 (11–16)	—
CI-914	>1000	>1000	750 (630–870)	530 (420–640)	8.2 (5.9–10)	—
CI-930	820 (660–980)	>1000	400 (310–500)	360 (240–480)	2.9 (2.3–3.5)	—
Milrinone	310 (190–430)	340 (170–510)	220 (140–300)	200 (120–290)	2.5 (1.9–3.1)	—
Papaverine	24 (21–27)	27 (22–32)	6.0 (5.3–6.6)	5.1 (4.3–6.0)	3.8 (3.6–4.1)	—
Sulmazole (AR-L 115BS)	>1000	>1000	240 (190–280)	180 (120–230)	480 (370–590)	
AR-L 57	360 (290–440)	320 (210–430)	60 (50–69)	49 (38–60)	750 (590–920)	—
Dipyridamole	54 (29–78)	44 (22–65)	7.9 (4.3–12)	10 (1.1–19)	40 (33–47)	—
M & B 22,948	16 (12–20)	38 (12–65)	47 (38–57)	55 (44–66)	700 (450–940)	—
Theophylline	230 (140–310)	310 (250–340)	210 (200–220)	170 (150–190)	340 (310–370)	—

The IC_{50} values (concentration which inhibits substrate hydrolysis by 50%) were determined from concentration–response curves, in which concentrations ranged from 10^{-7} to 10^{-4} M for the more potent inhibitors and from 10^{-5} to 10^{-3} M for the less potent inhibitors (half-log increments). Enzyme activity was measured as described in Materials and Methods. Three to four such concentration–response curves were generated for each agent, typically using different enzyme preparations for each concentration–response.

* PDE III does not hydrolyze cyclic GMP to an appreciable degree.

† Values in parentheses represent 95% confidence limits, as determined by the method of Hubert [18].

Table 4. Effects of reference phosphodiesterase inhibitors, cardiotonics and other agents on platelet phosphodiesterases

	IC_{50} (μM) [or % inhibition at 1.0×10^{-5} M ± S.E.M.]						
	PDE I			PDE II		PDE III	
Agent	Cyclic AMP	Cyclic GMP		Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP*
Carbazeran	[14 ± 3%]	76 (51–100)†	[10 ± 4%]	[34 ± 7%]	[32 ± 14%]	1.9 (1.3–2.5)	[78 ± 3%]	—
Amrinone	[5 ± 1%]	240 (86–387)	[15 ± 6%]	[7 ± 3%]	[23 ± 5%]	9.5 (6.5–13)	[36 ± 1%]	—
MDL 17,043	[2 ± 0%]	>1000	[2 ± 2%]	[6 ± 2%]	[4 ± 3%]	6.6 (5.9–7.3)	[57 ± 1%]	—
CI-914	[6 ± 1%]	220 (190–250)	[8 ± 1%]	[6 ± 1%]	[11 ± 5%]	4.5 (3.1–5.9)	[67 ± 5%]	—
CI-930	[13 ± 3%]	91 (74–110)	[15 ± 1%]	[1 ± 1%]	[2 ± 0%]	0.6 (0.4–0.8)	[85±3%]	—
Amipazone	[11 ± 2%]	130 (110–140)	[5 ± 3%]	[5 ± 6%]	[6 ± 6%]	0.3 (0.2–0.3)	[92 ± 4%]	—
Y-590	[33 ± 4%]	41 (13–68)	[30 ± 1%]	[18 ± 2%]	[21 ± 6%]	0.018 (0.010–0.020)	[96 ± 3%]	—
Milrinone	[16 ± 4%]	110 (92–130)	[8 ± 3%]	[16 ± 3%]	[24 ± 0%]	1.0 (0.8–1.1)	[87 ± 2%]	—
Papaverine	[34 ± 8%]	11 (9.5–12)	[47 ± 2%]	[58 ± 4%]	[63 ± 9%]	1.1 (0.7–1.5)	[79 ± 4%]	—
AR-L 57	[32 ± 3%]	26 (24–27)	[31 ± 1%]	[18 ± 4%]	[33 ± 6%]	500 (300–710)	[6 ± 2%]	—
Dipyridamole	[80 ± 5%]	0.9 (0.8–1.1)	[95 ± 5%]	[66 ± 1%]	[89 ± 1%]	36 (22–99)	[26 ± 6%]	—
M & B 22,948	[72 ± 6%]	1.2 (1.0–1.3)	[88 ± 1%]	[24 ± 4%]	[44 ± 1%]	1000 (700–1400)	[7 ± 2%]	—
Theophylline	[4 ± 1%]	1000 (660–1400)	[−1 ± 0%]	[8 ± 4%]	[9 ± 0%]	330 (170–490)	[6 ± 3%]	—

The IC_{50} values (concentration which inhibits substrate hydrolysis by 50%) were determined from concentration–response curves, in which concentrations ranged from 10^{-7} to 10^{-4} M for the more potent inhibitors (10^{-9} to 10^{-5} M for Y-590), and from 10^{-5} to 10^{-3} M for the less potent inhibitors (half-log increments). Enzyme activity was measured as described in Materials and Methods. Three to four such concentration–response curves were generated for each agent (or three to four measurements were made at 10^{-5} M), typically using different enzyme preparations.

* PDE III does not hydrolyze cyclic GMP to an appreciable degree.

† Values in parentheses represent 95% confidence limits, as determined by the method of Hubert [18].

molecular forms of cardiac phosphodiesterase are shown in Table 3. The reference inhibitor theophylline was found to exert weak non-selective inhibitory effects on all forms of phosphodiesterase (Tables 3–5). Papaverine also exerted a relatively non-selective inhibitory effect on all forms of phosphodiesterase, although its inhibitory effect on cardiac and platelet PDE I and PDE II was less than that on PDE III (Tables 3–5). A similar degree of "selectivity" was also observed with carbazeran and milrinone (Tables 3 and 4). None of these agents, however, displayed the selectivity for inhibiting PDE III which was observed with CI-914, CI-930, amrinone or MDL 17,043. All of these latter agents exerted an inhibitory effect on cardiac PDE III which was several orders of magnitude greater than their inhibitory effects on PDE I or PDE II (Table 3). Of these agents, CI-930 was the most potent inhibitor of PDE III ($IC_{50} = 2.9\ \mu M$). By contrast, the IC_{50} values for inhibition of cyclic AMP hydrolysis by cardiac PDE I and cardiac PDE II by CI-930 were 820 and 400 μM respectively.

Selective inhibitory effects on the different molecular forms of cardiac phosphodiesterase were also observed with M & B 22,948, AR-L 57, sulmazole (AR-L 115BS) and dipyridamole, although the "selectivity" of these agents was not as great as that which was observed with CI-914 and CI-930 (Table 3). M & B 22,948 exerted a potent inhibitory effect on cardiac PDE I (IC_{50} for cyclic AMP hydrolysis = 16 μM), while exerting modest-to-minimal inhibitory effects on cyclic AMP hydrolysis by cardiac PDE II and cardiac PDE III ($IC_{50} = 47$ and 700 μM respectively). AR-L 57, sulmazole and dipyridamole all exerted somewhat greater inhibitory effects on cardiac PDE II than on PDE I or PDE III. Of these three agents, dipyridamole was the most potent and also the most "selective" inhibitor of cardiac PDE II (Table 3).

The effects of the various reference phosphodiesterase inhibitors and cardiotonic agents on the different molecular forms of platelet phosphodiesterase are shown in Table 4. In several cases, percent inhibition at 1.0×10^{-5} M rather than IC_{50} values were determined. This compromise was necessitated by the fact that platelet PDE I hydrolyzes very little cyclic AMP, and also because only relatively small quantities of PDE II could be isolated

Table 5. Effects of reference phosphodiesterase inhibitors, cardiotonics and other agents on coronary artery phosphodiesterases*

	IC_{50} (μM)			
	PDE I		PDE III	
Agent	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP†
Carbazeran	130 (100–160)‡	98 (81–120)	3.7 (3.2–4.2)	—
Amrinone	>1000	>1000	410§	—
MDL 17,043	>1000	>1000	115§	—
CI-914	800 (280–1300)	>1000	90§	—
CI-930	395 (290–500)	87 (46–130)	85§	—
Milrinone	170 (130–222)	110 (87–130)	5.1 (4.5–5.7)	—
Papaverine	40 (33–46)	16 (14–17)	2.6 (2.4–2.8)	—
AR-L 57	210 (170–260)	63 (54–73)	150 (90–210)	—
Dipyridamole	32 (20–45)	3.6 (2.2–5.0)	17 (16–19)	—
M & B 22,948	10 (8.0–12)	2.4 (0.9–3.9)	340 (230–460)	—
Theophylline	330 (270–380)	500 (400–600)	350 (300–400)	—

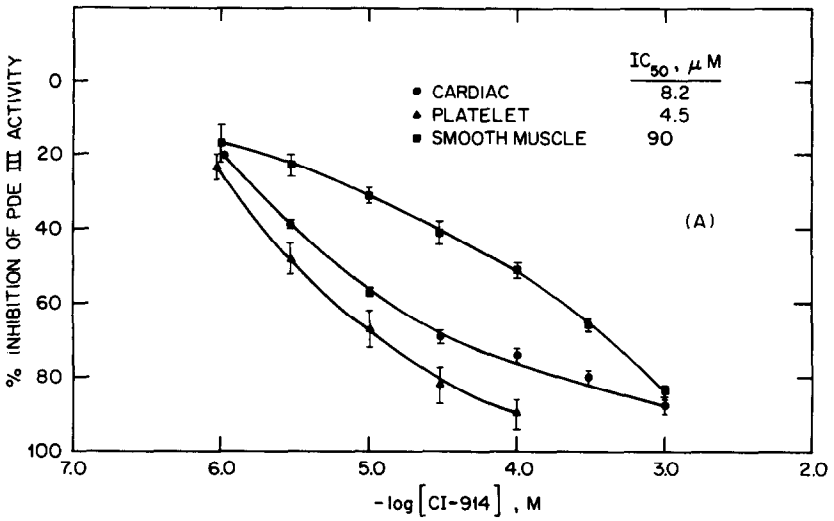
The IC_{50} values (concentration which inhibits substrate hydrolysis by 50%) were determined from concentration–response curves, in which concentrations ranged from 10^{-7} to 10^{-4} M for the more potent inhibitors, and from 10^{-5} to 10^{-3} M for the less potent inhibitors (half-log increments). Enzyme activity was measured as described in Materials and Methods. Three to four such concentration–response curves were generated for each agent, typically using different enzyme preparations for each concentration–response.

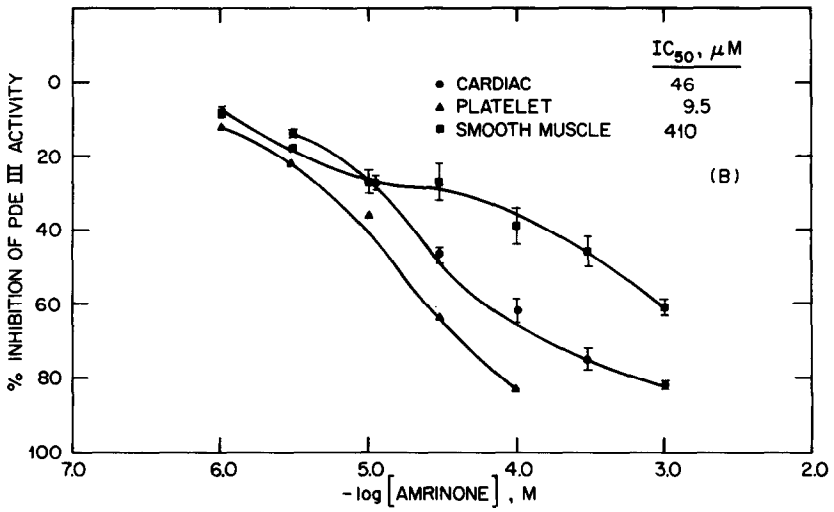
* There is no PDE II in bovine coronary arteries.

† PDE III does not hydrolyze cyclic GMP to an appreciable degree.

‡ Values in parentheses represent 95% confidence limits, as determined by the method of Hubert [18].

§ Ninety-five percent confidence limits could not be obtained due to the non-sigmoidal shape of the concentration–response curve (see Fig. 2).


IC50, μM
• CARDIAC 8.2
▲ PLATELET 4.5
■ SMOOTH MUSCLE 90
(A)
% INHIBITION OF PDE III ACTIVITY
0
20
40
60
80
100
7.0
6.0
5.0
4.0
3.0
2.0
-log [CI-914] , M


IC50, μM
• CARDIAC 46
▲ PLATELET 9.5
■ SMOOTH MUSCLE 410
(B)
% INHIBITION OF PDE III ACTIVITY
0
20
40
60
80
100
7.0
6.0
5.0
4.0
3.0
2.0
-log [AMRINONE] , M

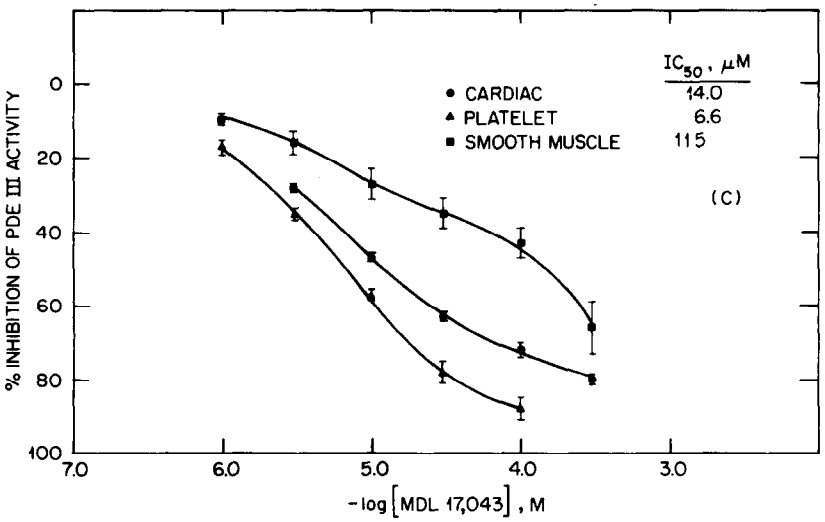

IC50, μM
• CARDIAC 14.0
▲ PLATELET 6.6
■ SMOOTH MUSCLE 115
(C)
% INHIBITION OF PDE III ACTIVITY
0
20
40
60
80
100
7.0
6.0
5.0
4.0
3.0
-log [MDL 17,043] , M

Fig. 3 (A–C).

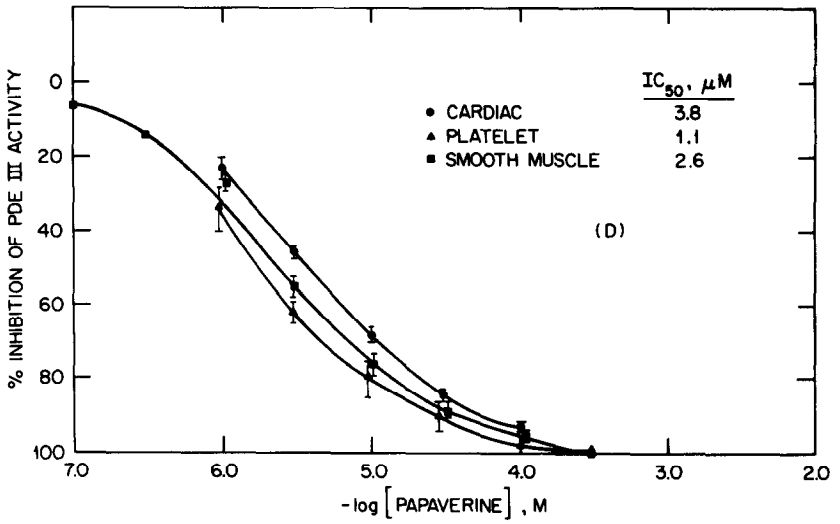


Fig. 3. Inhibitory effects of (A) CI-914, (B) amrinone, (C) MDL 17,043, and (D) papaverine on cyclic AMP hydrolysis by cardiac type III phosphodiesterase (●), platelet type III phosphodiesterase (▲), and bovine coronary artery type III phosphodiesterase (■). Each symbol represents three to four duplicate determinations of percent inhibition of phosphodiesterase activity using different enzyme preparations. Substrate concentration for these experiments was 1.0 μM cyclic AMP. Enzyme activity was measured as described in Materials and Methods.

from platelets. Few differences were observed between the effects which the compounds exerted on cardiac phosphodiesterases and their effects on platelet phosphodiesterases, with the exceptions that (i) in most instances, the inhibitory effects of the selective PDE III inhibitors, e.g. CI-914, were slightly greater on the platelet PDE III than on the cardiac PDE III, and (ii) whereas dipyridamole and AR-L 57 exerted a "selective" inhibitory effect on cardiac PDE II, these agents were found to exert comparable inhibitory effects on both platelet PDE I and PDE II.

In addition to the core compounds, the effects of two reference platelet aggregation inhibitors, Y-590 and amipazone, on platelet phosphodiesterases were also examined. Both agents were found to be selective inhibitors of platelet PDE III (Table 4). Amipazone exerted an inhibitory effect comparable to that of CI-930, whereas Y-590 exerted an inhibitory effect roughly 1.5 orders of magnitude greater than that of CI-930 (IC_{50} = 0.018 μM).

Table 5 shows the effects of the core group of compounds on the two different molecular forms of coronary artery phosphodiesterase (coronary artery PDE I and PDE III). As can be seen, agents which exert selective inhibitory effects on cardiac and platelet PDE I (e.g. M & B 22,948) or PDE III (e.g. CI-914 and CI-930) likewise exert a selective inhibitory effect on coronary artery PDE I or PDE III. The selective PDE III inhibitors such as CI-914, however, all exerted a less potent inhibitory effect on smooth muscle PDE III than on cardiac or platelet PDE III (Tables 3–5). For example, whereas the IC_{50} values for CI-914 for inhibiting cardiac and platelet PDE III were 8.2 and 4.5 μM, respectively, the IC_{50} value for inhibition of smooth muscle PDE III by CI-914 was 90 μM. These differences between the effects of CI-914 and the other selective PDE III inhibitors on cardiac muscle and platelet PDE III and on smooth muscle PDE III are best illustrated when the concentration–response curves to these agents are examined (Fig. 3, A–D). For comparative purposes, the effect of the non-selective inhibitor papaverine on the different PDE IIIs is included. As can be seen, all four inhibitors exerted a progressively increasing inhibitory effect on cardiac and platelet PDE III at concentrations ranging from 10^{-7} to 10^{-3} M. However, CI-914, amrinone and MDL 17,043, but not papaverine, exerted a biphasic inhibitory effect on smooth muscle PDE III (Fig. 3, A–D). The IC_{50} values obtained from such concentration–response curves are thus artificially elevated. Potential explanations for these biphasic responses will be addressed in greater detail in the Discussion.

DISCUSSION

Since the existence of multiple molecular forms of phosphodiesterase was initially reported by Thompson and Appleman in 1971 [1], other laboratories have shown that multiple forms of phosphodiesterase are present in a variety of other tissues and cells [2–6]. Conflicting reports have appeared regarding the number of phosphodiesterases present in these different tissue/cell types, as well as the kinetic characteristics of each [6, 7, 10–14], which may be related to the different methods by which the enzymes were isolated. The demonstration of multiple molecular forms of phosphodiesterase has also prompted a reevaluation of the inhibitory effects which reference phosphodiesterase inhibitors such as the methylxanthines, papaverine, and dipyridamole exert on the different types of PDE. While many reference inhibitors have been shown to exert rather non-selective inhibitory effects on all forms of phosphodiesterase [5, 22–24], recent reports have

shown that certain inhibitors, including M & B 22,948, ICI 74,917, CI-914 and MDL 17,043, exert selective inhibitory effects on specific molecular forms of phosphodiesterase [5, 22–25].

The goals of the present study were (i) to evaluate the different molecular forms of phosphodiesterase present in several tissue/cell types using the same isolation procedure, and compare both the number of phosphodiesterases present in each tissue/cell type, as well as the kinetic characteristics of each, and (ii) to evaluate the effect of various reference phosphodiesterase inhibitors, cardiotonic agents, and other relevant agents on each molecular form of phosphodiesterase present in the different tissue/cell types examined.

Variation was noted regarding the number of phosphodiesterases present in cardiac muscle, smooth muscle, and platelets, as well as the kinetic properties of each molecular form. Thus, whereas three distinct molecular forms of phosphodiesterase are present in guinea pig ventricular muscle and human platelets, only two forms of PDE were identified in bovine coronary arteries. In addition, differences between particular molecular forms of phosphodiesterase in the various tissue/cell types were also noted. For example, although PDE I from guinea pig ventricular muscle hydrolyzes cyclic AMP and cyclic GMP to comparable degrees, PDE I from coronary arteries and from platelets preferentially hydrolyzes cyclic GMP. For platelet PDE I, this preferential hydrolysis is apparently the result of differences in the K_m of PDE I for cyclic AMP and cyclic GMP. In the case of coronary artery PDE I, however, the K_m values for cyclic AMP and cyclic GMP are comparable, and the observed substrate specificity is apparently due to a much greater value of V_{max} for cyclic GMP hydrolysis as compared to cyclic AMP hydrolysis.

Terasaki and Appleman [2] have shown previously that in rat ventricular muscle, as is the case with coronary artery smooth muscle and platelets, PDE I preferentially hydrolyzes cyclic GMP. Preliminary experiments in this laboratory confirm this finding (data not shown). The molecular basis for this difference between the substrate specificities of guinea pig ventricular PDE I and other types of PDE I is not known at the present time. It is tempting to speculate that this difference may reflect a modification of the intracellular function which PDE I plays in the guinea pig ventricle as compared to the rat ventricle.

Differences in the response to calmodulin by cardiac, smooth muscle and platelet PDE I were also observed in the present study. The activities of both cardiac and smooth muscle PDE I were stimulated by calmodulin (in the presence of Ca^{2+}), whereas calmodulin had no effect on the activity of platelet PDE I. These differences are apparently not the result of contaminating amounts of calmodulin in any of the PDE I preparations. The possibility that a subunit of platelet PDE I which imparts calmodulin sensitivity was removed during the isolation of the platelets, due to the activity of proteolytic enzymes, cannot be excluded. However, in preliminary studies in which crude platelet phosphodiesterase activity was measured early in the isolation procedure (immediately following disruption of the platelets via homogenization, and prior to sonication), hydrolytic activity was comparable in the presence and absence of calmodulin (data not shown).

PDE II, in contrast to PDE I or PDE III, has a high K_m for both cyclic AMP and cyclic GMP (~20–30 μM as opposed to ~1.0 μM). This difference likely indicates that, in cardiac muscle and platelets, PDE II plays a different role in regulating intracellular cyclic nucleotide homeostasis than does PDE I or PDE III. The latter two forms of phosphodiesterase may, for example, be responsible for maintaining cyclic nucleotide concentrations at normal levels during basal metabolic conditions, whereas PDE II may be responsible for returning cyclic nucleotide levels to normal following a stress condition, e.g. subsequent to stimulation of beta- or histamine-receptors, when cyclic nucleotide levels within the cell, particularly cyclic AMP, would be expected to rise considerably, perhaps approaching the K_m for PDE II.

The observation that coronary artery smooth muscle contains no PDE II, as well as the observation that the V_{max} of cardiac PDE II is greater than the V_{max} of platelet PDE II, are also of interest and imply additional variations in the manner by which cyclic nucleotide homeostasis is maintained in cardiac and smooth muscle and in platelets.

In contrast to PDE I and PDE II, PDE III hydrolyzes only cyclic AMP. Although both a high- and a low-affinity site for hydrolysis of cyclic AMP by PDE III were observed, Hofstee analysis revealed that PDE III displays anomalous kinetic behavior toward cyclic AMP, in that the two sites for hydrolysis can be described by a hyperbola rather than by two straight lines, as is the case for PDE I. This anomalous kinetic behavior was observed for PDE III for all three tissue/cell types from which the enzyme was isolated. Wells *et al.* [3] have reported previously that the low K_m, cyclic AMP-specific form of phosphodiesterase present in porcine coronary arteries displays comparable anomalous kinetic behavior toward cyclic AMP. Russell and coworkers [26] have postulated that such anomalous behavior may be due either to negative cooperativity, as defined by Koshland *et al.* [27], or else to the combined action of two Michaelis–Menten enzymes. These authors used a computer modelling approach to distinguish between these two possibilities. The possibility that two enzymes were responsible for the anomalous kinetic behavior was discounted since it would imply contamination in excess of that which was detectable physically or by activity measurements [26]. The authors thus conclude that negative cooperativity between two cyclic AMP binding sites is the most reasonable explanation to account for the observed anomalous behavior of type III phosphodiesterase.

While displaying characteristics distinct from the other forms of phosphodiesterase isolated, the different PDE IIIs isolated in the present study also differed from each other in that, whereas each form of PDE III was inhibited to comparable degrees by non-selective phosphodiesterase inhibitors such as theophylline and papaverine, the IC_{50} values for the selective PDE III inhibitors such as CI-914 and amrinone were much lower for cardiac and platelet PDE

III than for coronary artery PDE III. This difference is apparently due to an unusual biphasic effect which these latter agents exert on smooth muscle PDE III. Preliminary studies have shown that at low concentrations CI-914 and several other selective PDE III inhibitors exert a competitive inhibitory effect on cyclic AMP hydrolysis by coronary artery PDE III. At higher concentrations, however, inhibition is non-competitive. The basis for the unusual effects which the selective PDE III inhibitors exert on smooth muscle PDE III at higher concentrations is not known and is currently under investigation.

Recently, Yamamoto *et al.* [28] identified two types of low K_m cyclic AMP-selective phosphodiesterases (PDE III) in calf liver. These two enzymes, which are not interconvertible, differ in their apparent K_m for cyclic AMP and also the degree to which they are inhibited by various selective phosphodiesterase inhibitors such as cilostamide and Ro 20-1724. Non-selective phosphodiesterase inhibitors such as papaverine and theophylline exert comparable inhibitory effects on both subtypes [28]. The observation of "subtypes" of PDE III may be relevant to the present study, since an altered tissue or cellular distribution of such subtypes may explain the observation that selective inhibitors such as CI-914, MDL 17,043 and amrinone are more potent inhibitors of cardiac and platelet PDE III than PDE III from coronary arteries.

The second goal of this study was to evaluate the effects of various reference phosphodiesterase inhibitors and other agents on each molecular form of phosphodiesterase present in the different tissue/cell types examined. The results of this study clearly illustrate the heterogeneity of actions by which these various agents inhibit phosphodiesterase activity. Theophylline and papaverine both exert relatively nonselective inhibitory effects on all forms of phosphodiesterase, regardless of the tissue or cell type from which the phosphodiesterases were isolated. M & B 22,948, as has been reported previously by Bergstrand *et al.* [5], exerted a potent inhibitory effect on each of the type I phosphodiesterases, while exerting lesser inhibitory effects on PDE II and PDE III. Several of the cardiotonic agents examined (CI-914, CI-930, amrinone, and MDL 17,043), as well as the platelet aggregation inhibitors amipazone and Y-590 [29], exerted a potent inhibitory effect on PDE III, while exerting little inhibitory effect on PDE I or PDE II. The selectivity observed with these agents for PDE III was somewhat greater than that observed for PDE I with M & B 22,948. Of the different selective PDE III inhibitors evaluated, Y-590 was the most potent and amrinone the least.

The reference platelet aggregation inhibitor dipyridamole, as well as the cardiotonic agents AR-L 57 and AR-L 115BS, exerted "selective" inhibitory effects on cardiac PDE II, as compared to cardiac PDE I and cardiac PDE III. Interestingly, both dipyridamole and AR-L 57 exerted a marked inhibitory effect on platelet PDE I as well as platelet PDE II (the effect of AR-L 115BS on platelet phosphodiesterases was not evaluated). The basis for this difference is not known at the present time, but may reflect the previously described differences between cardiac and platelet PDE I.

The relevance of these selective inhibitory effects on phosphodiesterase activity to the actual cellular response to these various agents has been addressed recently. Weishaar *et al.* have shown that in isolated guinea pig left atrial muscle, the selective PDE III inhibitor CI-914 exerts a selective effect on tissue levels of cyclic nucleotides—increasing cyclic AMP levels in a concentration-dependent manner, while having no effect on cyclic GMP levels [30]. The non-selective phosphodiesterase inhibitor theophylline, however, increases tissue levels of both cyclic AMP and cyclic GMP [30]. Kukovetz *et al.* [31] have also shown that in bovine coronary arteries the vasorelaxant effects of nitroglycerin and sodium nitroprusside, both of which stimulate guanylate cyclase [32], as well as the relaxant effect of 8-bromocyclic GMP, are potentiated by the selective PDE I inhibitor M & B 22,948.

In conclusion, the present study confirms earlier reports that multiple molecular forms of phosphodiesterase exist within the cell, which vary according to substrate specificity and kinetic characteristics, as well as to their response to calmodulin and to various pharmacological agents. The use of a uniform procedure to isolate phosphodiesterases from the different tissues and cells examined in the present study removes an additional variable which has previously made it difficult to compare results obtained from different laboratories. This study also demonstrates that particular molecular forms of phosphodiesterase may vary in different tissue/cell types. Thus, whereas PDE I hydrolyzes cyclic AMP and cyclic GMP to comparable degrees in guinea pig ventricular muscle, in bovine coronary arteries and human platelets PDE I preferentially hydrolyzes cyclic GMP.

Finally, the inhibitory effects of a large number of agents which have been shown previously to inhibit the activity of crude or only partially purified phosphodiesterase on each of the different molecular forms of phosphodiesterase isolated were also evaluated. On the basis of this latter investigation, these various agents were discretely characterized as to their selective and/or non-selective inhibitory effects. Such information will likely prove useful in subsequent studies aimed at identifying the intracellular responsibilities of the different molecular forms of phosphodiesterase, as well as the various metabolic responses to cyclic AMP and cyclic GMP.

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