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COMPARISON OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ISOFORMS FROM RAT HEART AND BOVINE AORTA

SEPARATION AND INHIBITION BY SELECTIVE REFERENCE PHOSPHODIESTERASE INHIBITORS

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Abstract—The resolution as well as the biochemical properties of the multiple molecular forms of cyclic nucleotide phosphodiesterase, in a given tissue, may be strongly dependent upon experimental conditions of preparation (extraction of crude enzyme from tissues and fractionation procedures). In the present study, we compare the different molecular forms of cardiac (rat heart ventricle) and vascular (bovine aorta) phosphodiesterase isolated from crude extracts prepared either in sucrose medium or in hypotonic medium (in the presence of protease inhibitors and ion chelators) using two different fractionation procedures: isoelectric focusing on flat gel bed and DEAE–Trisacryl anion exchange chromatography.

Both the calmodulin-dependent and the cAMP-specific forms exhibited close IEF and chromatographic patterns and showed similar sensitivities towards reference inhibitors regardless of the tissue of origin. In marked contrast, the cGMP-specific isoform notably differed from one to another tissue with respect to its biochemical properties (only the cardiac tissue being capable of stimulation by cGMP) and sensitivities to xenobiotics. Thus the possibility exists that pharmacological agents may modulate phosphodiesterase activity differently in cardiac and vascular target tissues.

Since the pioneer work of Thompson and Appleman in 1971 [1], it is now well established that cyclic nucleotide phosphodiesterase exists under multiple forms in almost all tissues or cell types so far examined. These findings have prompted a renewal of interest in the area of modulators of cyclic nucleotide metabolism as therapeutic agents, since the different molecular forms of phosphodiesterase which vary from one organ or cell type to another can be selectively inhibited by various families of drugs. In the cardiovascular research area, these working hypotheses have proved to be specially fruitful. Indeed, numerous potent inotropic agents which selectively inhibit myocardial cAMP-specific phosphodiesterase (type IV) have been discovered (for reviews, see Refs 2–5). In addition, some of these new inotropic inhibitors of cardiac phosphodiesterase also share beneficial vasodilating properties probably related to the simultaneous inhibition of vascular phosphodiesterase. Thus, an accurate knowledge of the various vascular and myocardial isoenzymes may be helpful in designing more selective agents with either inotropic or vasodilative properties or both.

Three major forms of phosphodiesterase are currently found in the myocardial tissue from most mammalian species including rat [6, 7], guinea pig [8], dog [9], cow [10] and man [11]. However, conflicting information regarding the number of phosphodiesterase forms present in vascular smooth muscle has emerged. Thus, two forms have been isolated from porcine coronary arteries by Wells *et al.* [12], whereas one additional form specific for cGMP hydrolysis but insensitive to calmodulin has been pointed out by Lugnier *et al.* [13] in human, bovine and rat aorta. As emphasized by Wells and Hardman [14], the number as well as the biochemical properties of phosphodiesterase forms separated from a given tissue are strongly influenced by the isolation procedure employed. Thus, it is important to compare the isoenzymatic profiles obtained, from one tissue, by several methods of fractionation and also to compare phosphodiesterase profiles from different tissues using the same isolation procedure. Furthermore, extraction procedures of crude enzyme from tissues (the use of isotonic or hypotonic homogenization buffer, the presence or absence of protease inhibitors and cation chelators) may also influence the resolution of the different forms of cyclic nucleotide phosphodiesterase.

In the present study, we compare the various phosphodiesterase forms present in rat heart and bovine aorta from crude extracts prepared either in sucrose medium or in hypotonic medium, using two different methods of isolation: DEAE–Trisacryl anion exchange chromatography and isoelectric focusing on flat gel bed. Among the three major forms pointed out in both rat heart and bovine aorta, only the cGMP-specific one seems to notably differ from one tissue to another with respect to its bio-

chemical properties and sensitivities towards reference inhibitors.

MATERIALS AND METHODS

Phosphodiesterase preparations. During tissue homogenization, all the manipulations were conducted at +4°.

Isotonic medium. Fresh rat hearts washed out from contaminating blood and bovine aorta were rapidly minced and homogenized with a glass/glass Potter homogenizer in 3 vol. (v/w) of 0.32 M saccharose in 10 mM Tris–HCl buffer (pH 7.5). The homogenates were centrifuged at 105,000 *g* for 60 min.

Hypotonic medium. Tissues were homogenized as described above in 6 vol. (v/w) of 20 mM Tris–HCl buffer containing 2 mM Mg-acetate, 1 mM dithiothreitol, 5 mM EDTA, 2000 U/ml aprotinin (pH 7.5), then centrifuged at 105,000 *g* for 60 min.

Supernatants were either immediately fractionated or stored at −80° until use.

Isoelectrofocusing on preparative gel plates. Sephadex G75 gel plates were prepared as described in [7]. Isotonic or hypotonic cytosolic preparations from hearts and aorta (25–30 mg proteins) were applicated onto the gel, after 1 hr prefocusing. Focusing runs were performed for 16 hr at 4° with the following limiting conditions: 25 mA, 9 W, 1000 V. At the end of the experiment, the gel was sliced into 8 mm-wide bands and phosphodiesterase activity was eluted from each band with 3 ml of 160 mM Tris–HCl buffer (pH 8) containing 0.1 mM $CaCl_2$, 5 mM $MgCl_2$ and 1 mg/ml BSA. The eluates were stored at +4° and used as enzyme source for characterization studies within 2 weeks, or stored at −80° without substantial loss of enzyme activity within 2 months.

DEAE–Trisacryl anion exchange chromatography. Isotonic or hypotonic cytosolic preparations from hearts and aorta were applied to a DEAE–Trisacryl (IBF) column and eluted as previously described [13] with a linear 0–0.4 M NaCl gradient in 20 mM Tris–HCl buffer containing 2 mM Mg acetate, 1 mM dithiothreitol, pH 7.5. The different fractions from each peak were pooled and dialysed overnight against elution buffer without NaCl. Aliquots were stored at −80° with or without BSA (1 mg/ml) until use for phosphodiesterase characterization and for focusing experiments.

Phosphodiesterase assay. Cyclic nucleotide phosphodiesterase activity was assayed by a two-step radioisotopic procedure. Unreacted cyclic nucleotides and nucleosides were separated either according to Wells *et al.* [12] or with a batch resin method according to Boudreau and Drummond [15]. In the second case, nucleoside recoveries were determined by means of [$U^{14}C$] nucleosides systematically added in the second step of the enzymatic reaction. Isoelectrofocusing and DEAE elution profiles were assayed with either 0.25 or 1 μM cAMP and cGMP in the presence of calcium and saturating amount of calmodulin, in the presence of 1 mM EGTA or without exogeneous addition. Both incubation time and enzyme concentration were adjusted so that no more than 20% of the substrate was hydrolyzed under the assay conditions. In experiments designed to evaluate the activation of cAMP hydrolysis by cGMP, assays were performed with 5 μM cAMP in the presence of 1 μM cGMP without calmodulin or EGTA. The IC_{50} (concentration of a drug which inhibited 50% of the enzymatic activity) of several reference phosphodiesterase inhibitors were calculated by plotting the percentage of enzymatic activity determined at 0.25 or 1 μM substrate concentration vs the logarithmic concentration of the inhibitor. The tested compounds were dissolved either in Tris–HCl buffer, water or 1% DMSO, as indicated in the legend of Table 2, in concentrations ranging from 10^{-7} M to 10^{-3} M. Controls with vehicle were performed in each case. Results are the means of two to three determinations.

Materials. 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) was a gift of Hoffman-Laroche (Basel, Switzerland); 4-(3′-cyclopentyloxy-4′-methoxyphenyl)-2-pyrrolidone (Rolipram) was a gift of Schering (Berlin, F.R.G.). *N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinoyloxy) butyramide (Cilostamide) and *N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinoyloxy) valeramide (AALO5) were synthesized by C. Lugnier [16]. 3′,4′,3,5,7-Penta-*O*-ethylquercetin was synthesized by M. Picq [17]. 9,10-Dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2H-pyrimido (6,1-a) isoquinolin-4-one hydrochloride (Trequinsin) was a gift of Hoechst (Frankfurt, F.R.G.); 2-*O*-propoxyphenyl-8-azapurin-6-one (M&B 22,948) was a gift of May & Baker (Dagenham, U.K.); 2-(1-succinoyloxyethyl)-3-methyl-5-(2-oxo-2,5-dihydro-4-furyl)benzo(*b*)furanne morpholinium salt (Benfurodyl hemisuccinate, Eucilat) was a gift of Clin-Midy (Toulouse, France). Papaverine, 3-isobutyl-1-methyl xanthine (IBMX) were from Sigma (St. Louis, MO); Dipyridamole, Calmidazolium (R 24571) were from Boehringer (Mannheim, F.R.G.) and quercetin dihydrate from Fluka (Buchs, Switzerland).

RESULTS

Separation of the different phosphodiesterase forms from rat heart and bovine aorta by isoelectrofocusing

Fractionation from isotonic preparations. Phosphodiesterase activity of the 105,000 *g* supernatant from rat heart, prepared in isotonic medium, was currently resolved into three peaks by isoelectrofocusing [7] (Fig. 1A).

The pI 4.9 peak preferentially hydrolyzed cGMP at low substrate concentrations. Its calmodulin-stimulated activity was inhibited (70–75%) by 1 mM EGTA. When assayed in the presence of 0.25 μM cGMP without addition of exogenous calmodulin, the activity of this peak was close to (−10%) the calmodulin-stimulated level (Table 1).

The pI 5.55 peak hydrolyzed both cyclic nucleotides with a marked preference for cGMP at low substrate concentrations and proved to be quite insensitive to EGTA inhibition. Furthermore, as illustrated in Fig. 2, the hydrolysis of cAMP by this peak (fractions A) was drastically enhanced by cGMP in a 0.5–10 μM range of concentrations. Maximal stimulation of cAMP hydrolysis was obtained with 10 μM cAMP in the presence of 2.5 μM cGMP.

Several overlapping peaks focusing between pH

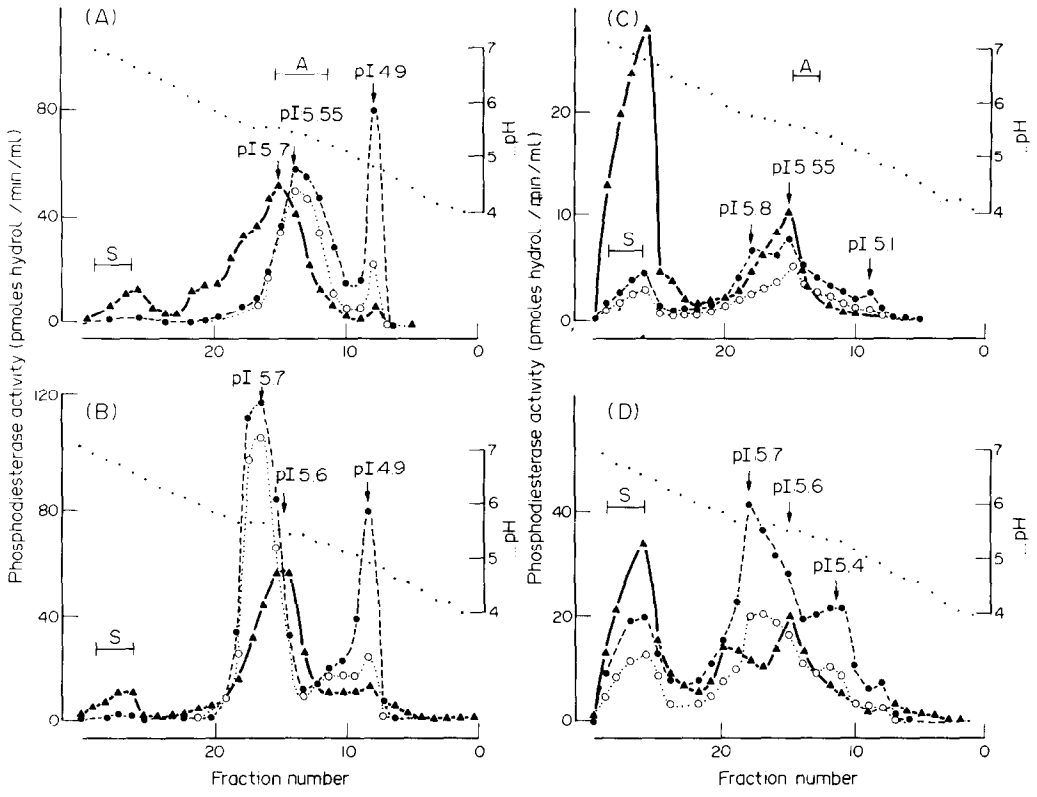

Fig. 1. Typical isoelectric-focusing profiles of cyclic nucleotide phosphodiesterase from rat heart and bovine aorta on Sephadex flat gel bed. (A) Isotonic rat heart preparation, (B) isotonic bovine aorta preparation, (C) hypotonic rat heart preparation, (D) hypotonic bovine aorta preparation. Each figure is representative of several comparable isolations. Phosphodiesterase activity was measured with 0.25 μM cyclic nucleotides in an incubation medium containing 50 μM $CaCl_2$: (▲—▲) cAMP without exogenous addition; (●---●) cGMP in the presence of 100 U calmodulin per assay; (○···○) cGMP in the presence of 1 mM EGTA; S, sample application area; A, cGMP-activable region.

5.55–6 specifically hydrolyzed cAMP and were totally insensitive to EGTA inhibition (not shown). The recovery in phosphodiesterase activity measured at 0.25 μM substrate concentration was currently about 60 and 70% for cAMP and cGMP phosphodiesterases, respectively.

Isoelectrofocusing patterns of 105,000 g supernatant from bovine aorta media layer prepared in

Table 1. Sensitivity of the calmodulin dependent type I phosphodiesterase isolated either by isoelectrofocusing or by DEAE–Trisacryl chromatography from isotonic and hypotonic rat heart and bovine aorta preparations to exogenous effectors

	Isoelectrofocusing			DEAE–Trisacryl chromatography		
	pI	CaM 100 U/assay	EGTA 1 mM	Ionic strength NaCl M	CaM 24 nM	EGTA 1 mM
Isotonic medium						
Heart	4.9	+10	−70	0.11	+88	−26
				0.12	+88	−26
Aorta	4.9	+15	−66	0.11	+124	−38
				0.12	+124	−38
Hypotonic medium						
Heart	5.0	+11	−54	0.10	+203	−20
	5.8	+56	−46			
Aorta	5.4	+31	−37	0.12	+797	−14
	5.7	+72	−18			

Phosphodiesterase activity was measured with 0.25 μM (isoelectrofocusing) or 1 μM (DEAE–Trisacryl chromatography) cGMP in the presence of calcium + calmodulin or in the presence of 1 mM EGTA and in the absence of exogenous effector (basal activity = 100).

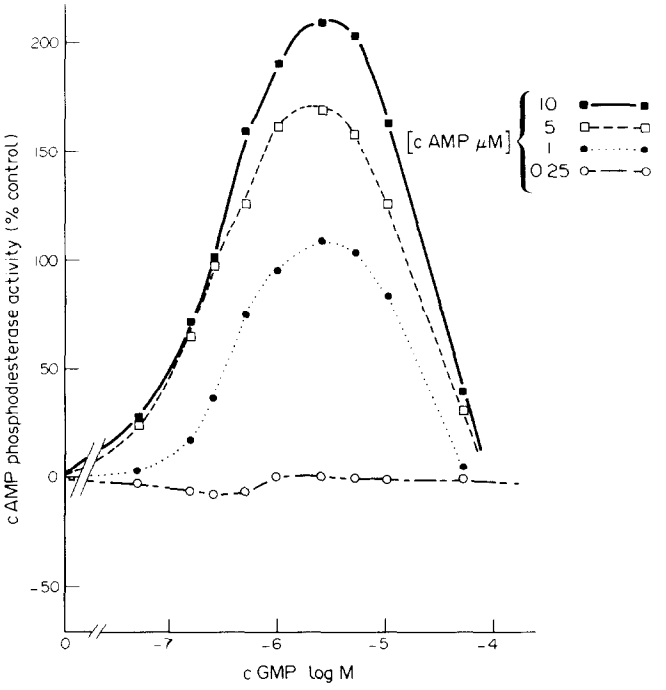

Fig. 2. cGMP-stimulation of cAMP hydrolysis by the cardiac pI 5.55 phosphodiesterase isoform. cAMP phosphodiesterase activity of the pooled fractions noted A in Fig. 1(A) was measured at ○—○, 0.25; ●—●, 1; □—□, 5 and ■—■, 10 μM substrate level in the presence of increasing amounts of cGMP. Results (triplicate) are expressed as a percentage increase with respect to control activity measured without cGMP.

isotonic medium were roughly similar to those obtained from rat heart isotonic cytosols (Fig. 1B).

The aorta pI 4.9 peak exhibited the same substrate specificity and EGTA sensitivity as the equivalent peak from rat heart (Table 1).

The two other peaks closely focused between pH 5.6–5.7. None of them were sensible either to calmodulin activation or to EGTA inhibition (not shown on profile 1B). Their respective affinity toward cAMP or cGMP was difficult to ascertain due to the important cross contamination. Nevertheless, the peak of pI 5.6 preferentially hydrolyzed cAMP while the pI 5.7 peak exhibited a marked preference for cGMP, at low substrate concentration. The cAMP hydrolyzing activity of this peak was not stimulated but rather inhibited (13%) by cGMP (not shown). The vascular phosphodiesterase activity was recovered in good yields (70 and 80% for cAMP and cGMP phosphodiesterase activities, respectively) as it was found for heart preparations.

Fractionation from hypotonic preparations

Isoelectrofocusing profile of heart 105,000 *g* supernatant prepared in hypotonic medium in the presence of a calcium chelator and a protease inhibitor, markedly differed from that obtained for isotonic preparations. The comparison of the two profiles illustrated in Fig. 1A (isotonic medium) and Fig. 1C (hypotonic medium) highlighted several major differences.

First of all, the appearance of an important amount of non-focused material which preferentially hydrolyzed cAMP coincided with an almost total disappearance of the cAMP hydrolyzing peaks between pI 5.55 and pI 6. Indeed, about 70% of the recovered cAMP phosphodiesterase activity measured at 0.25 μM cAMP (40.4% of the applied activity) was found in the area of sample application, whereas 28% only of the recovered cGMP phosphodiesterase activity (9.9% of the applied activity) did not migrate. This result indicates that non-specific hydrophobic interactions of the crude enzyme with Sephadex gel are higher in hypotonic-chelated than in sucrose medium, and that the cAMP specific form of phosphodiesterase is more prone to interact with the gel than the two other forms of the enzyme. Furthermore, these hydrophobic interactions proved to be highly irreversible since refocusing of the non-migrating material after resuspension of the gel in sucrose medium did not allow the migration of additional enzyme activity. Less than 3% of the reapplied cAMP phosphodiesterase activity focused in the 5.55–6 pH range (not shown). On the other hand, the calmodulin-sensitive form, with an acidic pI, was very substantially decreased while an additional cGMP-specific peak appeared at pH 5.8. Its calmodulin-stimulated activity was inhibited (65%) by 1 mM EGTA (Fig. 1C). In addition, the basal cGMP-hydrolyzing activity of this peak was stimulated (56%) by the adjunction of exogenous calmodulin and inhibited (46%) by EGTA addition (Table 1).

cAMP hydrolysis by the pI 5.55 peak was stimulated by 1 μM cGMP (76%). However, the extent

of the stimulation was markedly lower than that observed for the pI 5.55 peak separated from isotonic supernatant, on account of the important contamination by the adjacent cGMP-specific peak at pH 5.8. At last, the phosphodiesterase activity of heart cytosols prepared in hypotonic conditions proved to be rather labile during IEF fractionation. Especially, the cGMP phosphodiesterase activity was recovered in low yields (about 37%) whereas 70% recoveries were systematically obtained when IEF procedure was applied to isotonic material.

Isoelectrofocusing profiles of bovine aorta 105,000 *g* supernatant prepared in hypotonic medium (Fig. 1D) were roughly similar to that exhibited by rat heart supernatant obtained in identical hypotonic conditions. Furthermore, profiles 1B and 1D from aorta preparations showed the same kind of differences as that observed between profiles 1A and 1C from heart cytosols, namely an important amount of non-migrating material in the zone of sample application (44 and 22.6% of the recovered cAMP and cGMP phosphodiesterase activities) and a drastically reduced acidic calmodulin-sensitive form of pI 4.9. Contrary to the heart cAMP-specific form which precipitated almost entirely in hypotonic medium, 56% of the aorta cAMP-specific form effectively migrated. Beside the pI 5.6 peak already obtained in profile 1B (isotonic medium) an additional peak appeared in the pI 6.0 area.

Moreover, the calmodulin sensitive form with an acidic pI (4.9) gave rise to two well-separated cGMP-hydrolysing peaks of pI 5.4 and 5.7, both of them being similarly inhibited by 1 mM EGTA (52% with respect to the calmodulin-stimulated level Fig. 1D). In addition, the basal cGMP hydrolyzing activity (measured with 0.25 μM cGMP) of the pI 5.4 and 5.7 peaks was stimulated (31 and 72%, respectively) by the addition of exogenous calmodulin, and inhibited (37 and 18%, respectively) by 1 mM EGTA (Table 1). In the same experimental conditions, the equivalent fraction from rat heart gave a single peak of pI 5.8 (Fig. 1C). The aorta cGMP-specific form, insensitive to calmodulin activation and EGTA inhibition, which migrated at pH 5.7 in isotonic medium (Fig. 1B) was entirely masked by the prominent calmodulin-sensitive form of pI 5.7 with hypotonic preparations.

Separation of the different phosphodiesterase forms from rat heart and bovine aorta by DEAE-Trisacryl anion exchange chromatography

Fractionation from isotonic preparations. DEAE-Trisacryl anion exchange chromatography as well as isoelectrofocusing procedure resolved isotonic rat heart 105,000 *g* supernatant into three well-separated peaks of phosphodiesterase activity corresponding to the three main well-characterized forms of the enzyme [6, 7] (Fig. 3A).

The first eluted peak (0.11–0.12 M NaCl) very specifically hydrolyzed cGMP at low substrate

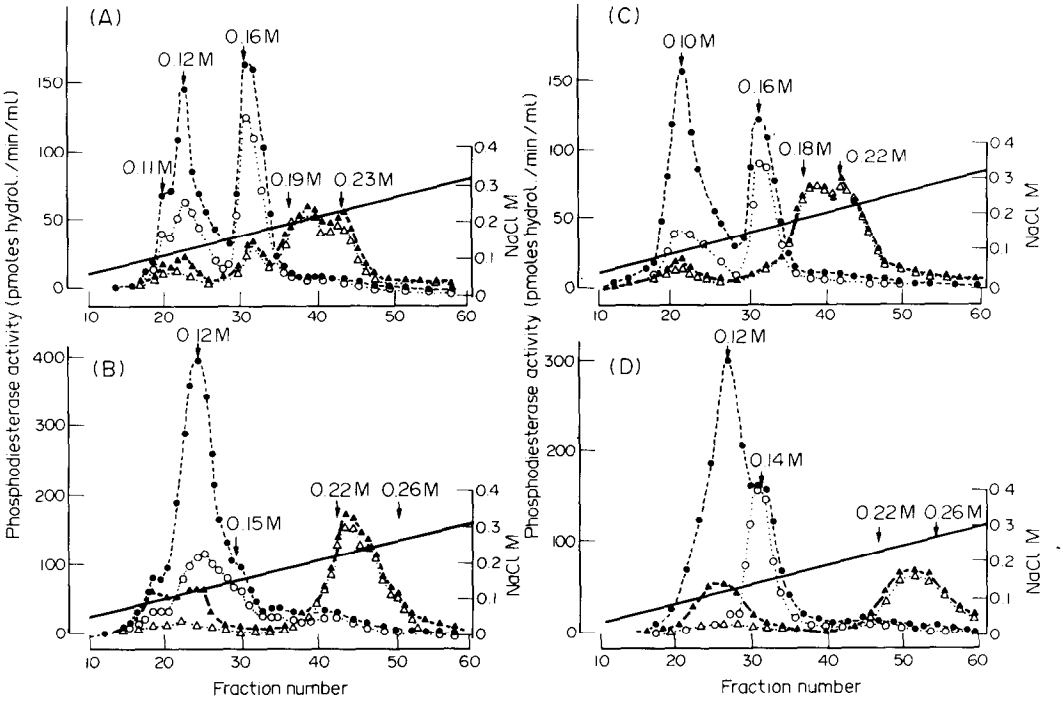

Fig. 3. Representative isolations of cardiac and aortic phosphodiesterase isoforms by DEAE-Trisacryl chromatography. (A) Isotonic rat heart preparation, (B) isotonic bovine aorta preparation; (C) hypotonic rat heart preparation; (D) hypotonic bovine aorta preparation. Each figure is representative of several comparable isolations. Phosphodiesterase activity was measured with 1 μM cyclic nucleotides: (▲—▲) cAMP in the presence of 100 U calmodulin per assay; (△ ··· △) cAMP in the presence of 1 mM EGTA; (●---●) cGMP in the presence of 100 U calmodulin per assay; (○···○) cGMP in the presence of 1 mM EGTA.

concentration. Its calmodulin-stimulated cGMP hydrolyzing activity was inhibited by 1 mM EGTA (60%). Its basal cGMP hydrolyzing activity was markedly enhanced by exogenous calmodulin addition (88%) and slightly inhibited by 1 mM EGTA (26%) (Table 1). Thus this isoenzymatic form corresponds quite well to the calmodulin-sensitive enzyme which focuses at pI 4.9.

The second peak eluted at 0.16 M NaCl exhibited the same substrate selectivity and the same weak sensitivity toward addition of EGTA or calmodulin as the pI 5.55 form separated by IEF. The cAMP hydrolyzing activity of this peak was only slightly stimulated (34%) by 1 μM cGMP (not shown).

Finally, as it was also revealed by IEF patterns, the cAMP specific phosphodiesterase form eluted between 0.19 and 0.23 M NaCl appeared rather heterogeneous.

The DEAE–Trisacryl elution profile (Fig. 3B) from bovine aorta 105,000 g supernatant prepared in isotonic medium, seemed quite different from that obtained with heart supernatant (Fig. 3A). Apparently, only two forms were separated, a heterogeneous calmodulin-dependent cGMP-hydrolyzing form and a heterogeneous cAMP phosphodiesterase form.

The major difference with isotonic rat heart preparation concerned the poor separation of the calmodulin-sensitive and the cGMP-specific, calmodulin-insensitive forms, the latter being totally unlighted in aorta profile as compared with heart profile.

Fractionation from hypotonic preparations. Elution patterns of hypotonic rat heart 105,000 g supernatant from the DEAE–Trisacryl columns were quite similar to that obtained from isotonic preparations (Fig. 3C). No major difference was found between profiles 3A and 3C. Both the calmodulin-sensitive and the cAMP-specific forms which appeared rather heterogeneous in profile 3A were eluted under quite homogeneous peaks at 0.10 M NaCl and between 0.18 and 0.22 M NaCl, respectively. Moreover, the calmodulin-stimulated activity was markedly inhibited by EGTA in hypo-

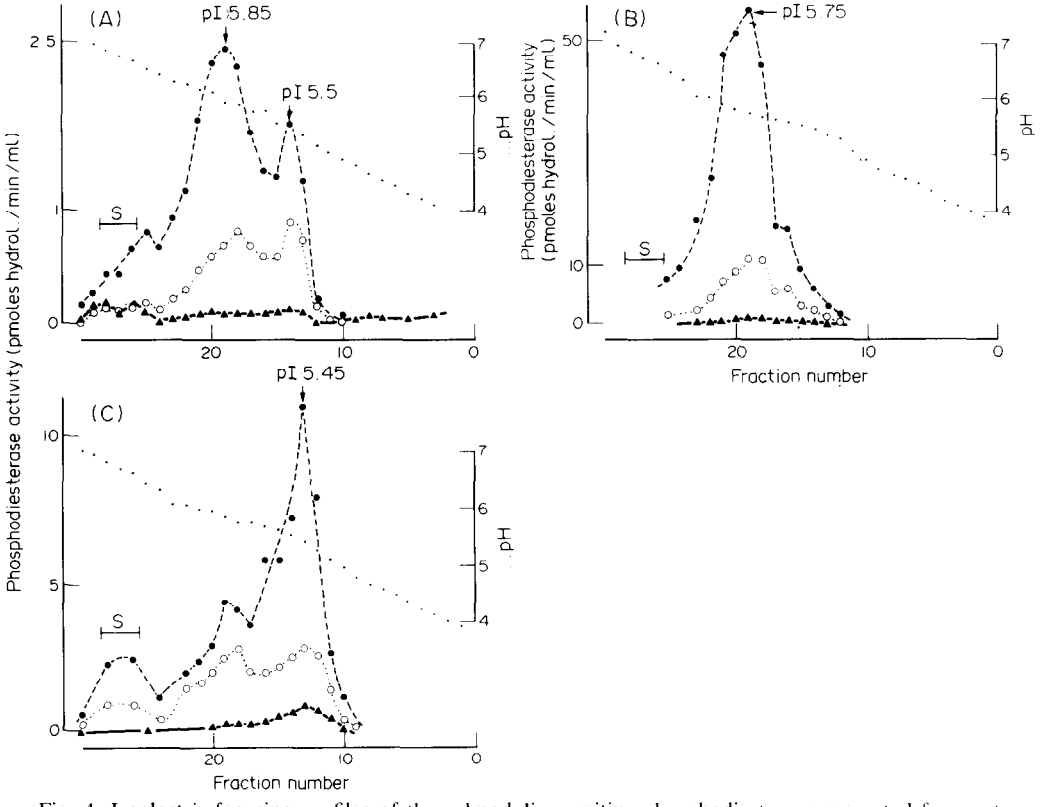

Fig. 4. Isoelectric-focusing profiles of the calmodulin-sensitive phosphodiesterase separated from rat heart (A) and bovine aorta (B, C) hypotonic cytosols by DEAE–Trisacryl chromatography. (A) An aliquot of the pooled fractions from the heart calmodulin-sensitive peak eluted by 0.10 M NaCl in Fig. 3(C) was used. Total activity (pmoles/min) of the applicated sample measured with 0.25 μM cGMP in the presence of calmodulin: 132, in the presence of EGTA: 44, without addition: 80. (B) An aliquot of the pooled fractions from the aorta calmodulin-sensitive peak eluted by 0.12 M NaCl in Fig. 3(D) was applicated. Total activity (pmoles/min) of the applicated sample measured with 0.25 μM cGMP in the presence of calmodulin: 758, in the presence of EGTA: 211, without addition: 248. (C) An aliquot of the above-mentioned aorta peak (total activity: 86 pmoles/min measured with 0.25 μM cGMP without addition) was incubated with calmodulin (1000 U) for 10 min at 20° prior to isoelectrofocusing. Phosphodiesterase activity was measured with 0.25 μM cyclic nucleotides in an incubation medium containing 50 μM $CaCl_2$. (▲—▲) cAMP without exogenous addition; (●– – –●) cGMP in the presence of 100 U calmodulin per assay; (○···○) cGMP in the presence of 1 mM EGTA; S, sample application area.

tonic medium (75% compared to 60% in Fig. 3A). The basal cGMP hydrolyzing activity of this peak was only slightly inhibited by 1 mM EGTA (20%) and strongly activated by the addition of exogenous calmodulin (203%).

The cAMP-hydrolyzing activity of the second peak eluted at 0.16 M NaCl was substantially increased by 1 μM cGMP (193% vs 34% for the equivalent peak obtained in isotonic medium). The whole recovery of cAMP-hydrolyzing activity was higher in hypotonic medium than in an isotonic one.

DEAE–Trisacryl elution patterns of hypotonic bovine aorta supernatant (Fig. 3D) were different from that obtained with isotonic preparation. Three isoforms were clearly resolved by this procedure, as well as by isoelectrofocusing of isotonic bovine aorta preparation (Fig. 1B).

The calmodulin-dependent cGMP phosphodiesterase activity was strongly inhibited by EGTA (90% compared to 72% obtained in Fig. 3B). Its basal cGMP-hydrolyzing activity was only slightly inhibited by 1 mM EGTA (14%) and drastically stimulated by the addition of exogenous calmodulin (797%) (Table 1).

The cGMP-specific form was more acutely resolved from the prominent calmodulin-sensitive enzyme than in isotonic preparation submitted to DEAE–Trisacryl chromatography (Fig. 3B). As previously shown [13], rechromatography of the pooled fractions eluted between 0.13 and 0.15 M NaCl gave rise to two well-separated calmodulin-sensitive and cGMP-specific peaks without cross-contamination (not shown).

Isoelectrofocusing of the different phosphodiesterase forms separated from rat heart and bovine aorta hypotonic cytosols by DEAE–Trisacryl anion exchange chromatography

In order to establish more firmly the relationships

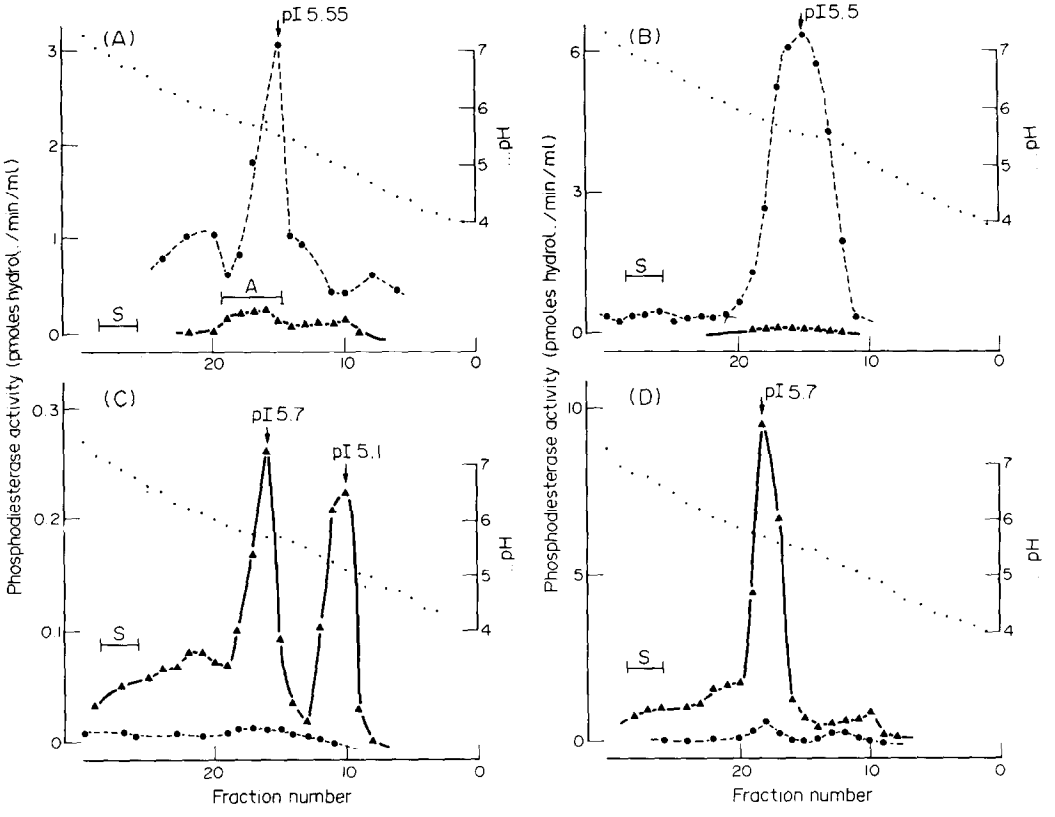

Fig. 5. Isoelectric-focusing profiles of the cGMP-specific (A, B) and cyclic AMP-specific (C, D) phosphodiesterases separated from rat heart (A, C) and bovine aorta (B, D) hypotonic cytosols by DEAE–Trisacryl chromatography. (A) An aliquot of the pooled fractions from the heart cGMP-specific peak eluted by 0.16 M NaCl in Fig. 3(C) was applicated. Total activity (pmoles/min) of the applicated sample measured with 0.25 μM cGMP without addition: 61. (B) An aliquot of the pooled fractions from the aorta cGMP-specific peak eluted by 0.14 M NaCl in Fig. 3(D) was used. Total activity (pmoles/min) of the applicated sample measured with 0.25 μM cGMP without addition: 321. (C) An aliquot of the pooled fractions from the heart cAMP-specific peak eluted between 0.18 and 0.22 M NaCl in Fig. 3(C) was used. Total activity (pmoles/min) of the applied sample measured with 0.25 μM cAMP without addition: 70. (D) An aliquot of the pooled fractions from the aorta cAMP-specific peak eluted between 0.22 and 0.26 M NaCl in Fig. 3(D) was applied. Total activity (pmoles/min) of the applicated sample measured with 0.25 μM cAMP without addition: 569. Phosphodiesterase activity was measured with 0.25 μM cyclic nucleotides in an incubation medium containing 50 μM $CaCl_2$. (▲—▲) cAMP without exogenous addition; (●---●) cGMP without exogenous addition; S, sample application area.

Table 2. Inhibition (IC_{50} μM) of the separated phosphodiesterase isoforms from bovine aorta and rat heart by reference phosphodiesterase inhibitors

	Bovine aorta			Rat heart		
Compounds	CaM-PDE cGMP 1 μM CaM	cGMP-PDE cGMP 1 μM EGTA	cAMP-PDE cAMP 1 μM EGTA	CaM-PDE cGMP 0.25 μM	cGMP-PDE cGMP 0.25 μM	cAMP-PDE cAMP 0.25 μM
Trequinsin*	13 (5.8)	5 (0.77)	0.4 (0.12)	4 (3.6)	2 (19)	0.2 (0.15)
Papaverine	62 (28)	6 (0.93)	3 (0.90)	11 (9.8)	4 (3.9)	2 (1.5)
Rolipram	2700 (1200)	246 (53)	5 (1.5)	493 (438)	281 (273)	3 (2.3)
Penta-*O*-ethyl Quercetin	125 (56)	121 (21)	7 (2.1)	189 (168)	13 (12.6)	8 (6.2)
IBMX	6 (2.7)	5 (0.86)	9 (2.7)	2 (1.8)	1 (0.97)	9 (7.0)
AAL O5	122 (54)	4 (0.68)	12 (3.6)	22 (19.6)	5 (4.9)	12 (9.3)
Dipyridamole	103 (46)	0.3 (0.07)	13 (3.9)	36 (32)	7 (6.8)	3 (2.3)
Cilostamide	256 (114)	28 (4.8)	19 (5.7)	38 (34)	12 (11.7)	13 (10.0)
Ro 201724	940 (418)	384 (73)	18 (5.4)	394 (350)	422 (410)	16 (12.4)
Calmidazolium	0.4 (0.18)	50 (8.7)	32 (9.6)	9 (8)	13 (12.6)	28 (21.7)
Eucilat	450 (200)	6 (1.05)	36 (10.8)	132 (117)	4 (3.9)	48 (37)
M&B 22948	21 (9.3)	0.4 (0.11)	247 (74)	15 (13.3)	1 (0.97)	58 (45)
Vardax†	200 (89)	13 (2.3)	117 (53)	321 (285)	151 (147)	103 (80)
Quercetin	200 (89)	2000 (349)	2000 (600)	57 (51)	20 (19.4)	23 (18)

IC_{50} values were determined from concentration–response curves as described in Materials and Methods. Results represent the means of two to three determinations. 95% confidence limits are about 15% of the given values. For clarity purposes they were not included in the table.

Data in parentheses indicate apparent K_i. With the exception of the "bovine aorta cGMP-PDE" column where experimentally determined apparent K_i are given, K_i were calculated according to Cheng and Prussof assuming competitive inhibitions ($K_i = IC_{50}/1 + S/K_m$). S was 1 μM for bovine aorta and 0.25 μM for rat heart isoforms. The following Michaelis constants determined for low substrate concentrations, as reported in [7] and [13], were used: vascular CaM PDE and cAMP PDE: 0.8 and 0.4 μM, respectively; cardiac CaM PDE, cGMP PDE and cAMP PDE: 2, 8.4 and 0.86, respectively.

* Dissolved in water.

† Dissolved in Tris–HCl 40 mM pH 7.5 (bovine aorta) or pH 8 (rat heart). All the other compounds were dissolved in DMSO as stock solutions. After appropriate dilutions with Tris buffer, the final concentration of DMSO in the assay was 1%.

between the forms obtained by the two fractionation procedures, the different phosphodiesterase forms separated by a first DEAE–Trisacryl chromatography were then subjected to isoelectrofocusing.

Pooled fractions of the first eluted peak (0.10–0.12 M NaCl) from both rat heart (Fig. 3C) and bovine aorta (Fig. 3D) gave a major peak of phosphodiesterase activity in the pI 5.75–5.85 region (Figs 4A and 4B). The calmodulin-stimulated cGMP hydrolyzing activity of both peaks was similarly inhibited by 1 mM EGTA (75% Fig. 4A; 80% Fig. 4B). Both peaks exhibited a marked specificity for the hydrolysis of cGMP (G/A ratio of 30 and 120, respectively). Moreover the heart fraction gave an additional peak at pH 5.50 which proved to be less sensitive to EGTA inhibition (50% with respect to the calmodulin stimulated level).

When an aliquot of the aorta fraction was briefly incubated in the presence of exogenous calmodulin before isoelectrofocusing (Fig. 4C), the major part of the calmodulin-sensitive phosphodiesterase activity focused in a more acidic pH range (compare Figs 4B and 4C).

The second eluted peaks from the DEAE–Trisacryl column (0.16 M NaCl for heart preparation, 0.14 M NaCl for aorta preparation) focused in the same pH range: 5.45–5.55 (Figs 5A and 5B). Whereas the cAMP-hydrolyzing activity of the heart peak was still stimulated (45%) by 1 μM cGMP (not shown), the activity of the aorta peak was not affected by exogeneous cGMP.

The cAMP-specific phosphodiesterase forms eluted at 0.18–0.22 M NaCl for heart preparations and 0.22–0.26 M NaCl for aorta preparations focused at pH 5.7. Furthermore isoelectrofocusing pattern of the heart peak showed an additional acidic peak (pI 5.1) which was not apparent on profiles 1A and 1C obtained from crude cytosols. However we have already obtained such acidic peak after an extensive purification of the heart cAMP-specific phosphodiesterase by affinity chromatography [18]. None of the cardiac peaks (pI 5.1, 5.7) was sensitive to cGMP inhibition.

Comparison of the sensitivity of the cardiac and vascular isolated phosphodiesterase forms to reference inhibitors (Table 2)

The various molecular forms of phosphodiesterase present in cardiac and vascular muscle were characterized further by their sensitivity toward various structurally distinct phosphodiesterase inhibitors. The cardiac phosphodiesterase forms used in this study were separated by IEF procedure from isotonic heart cytosols and the vascular ones were isolated from hypotonic preparations by DEAE–Trisacryl chromatography. These experimental conditions were shown to give optimal resolution of the different enzymatic forms in each tissue. In preliminary

experiments, we verified that IC_{50} values of the cAMP-specific phosphodiesterase inhibitor Rolipram towards the cardiac forms were not dependent on the isolation conditions: homogenization medium, procedure, pH of buffer.

Several reference inhibitors were found to exert non-selective inhibitory effects on the various phosphodiesterase isoforms. Thus, IBMX strongly inhibited all the forms of both cardiac and vascular phosphodiesterases as it was already reported for most of xanthine derivatives [3, 19]. Similarly, papaverine also exerted a relatively non-specific inhibitory effect although the inhibition of the vascular calmodulin-sensitive form appeared weaker than that of the other vascular and cardiac forms. Sulmazole (Vardax, ARL 115) manifested a modest (10^{-4} M range) and non-selective inhibitory effect toward each cardiac isoenzymes whereas it proved to be quite specific for the aorta cGMP-specific form. None of the drugs, except calmidazolium, exerted a selective inhibitory effect on type I calmodulin-sensitive phosphodiesterase. The selective effect of calmidazolium toward the type I enzyme, under its calmodulin-activated form, was higher in vascular than in cardiac tissue where this drug also markedly inhibited the cGMP-sensitive and the cAMP-specific phosphodiesterase.

Among reference compounds which specifically inhibited type IV cAMP-specific phosphodiesterase, Trequinsin (HL 725) was the most potent in both cardiac and aortic tissues with IC_{50} values in the 10^{-7} M range. Although its inhibitory potency was far less than that reported in human platelets (IC_{50} = 250 pM) [20], it was however one or two orders of magnitude higher than that observed for the other cAMP-specific inhibitors Rolipram and Ro 20-1724. Among all the tested compounds, Rolipram and to a lesser extent Ro 20-1724, exhibited the highest selectivity for the inhibition of the cAMP-specific form of phosphodiesterase, with IC_{50} values two orders of magnitude lower than that observed for the two other forms, in both cardiac and vascular tissues. Such high selective inhibitory effects of Rolipram and Ro 20-1724 towards cAMP-specific phosphodiesterase were also reported in various brain preparations [21–24] where it might be physiologically relevant and explain the behavioral alterations observed in animals [24] and, possibly, the antidepressive effects of Rolipram in man [25]. In addition, the aortic and the cardiac cAMP-specific, type IV, phosphodiesterases showed quite comparable sensitivity to reference inhibitors (Table 2). Indeed, a close and significant correlation was found between IC_{50} values of the 13 inhibitors listed in Table 2 for aorta enzyme and their IC_{50} values for cardiac enzyme (r, 0.832, $P < 0.001$).

Among the various compounds which preferentially inhibited the cGMP-sensitive cardiac phosphodiesterase, M&B 22,948 and Eucilat showed the highest inhibitory potency and selectivity. They likewise exerted a potent and selective effect on the cGMP-specific vascular enzyme. AAL 05, structurally related to Cilostamide, exerted a similar inhibitory profile on both the cardiac and the vascular enzyme, but with a lower selectivity than M&B 22,948 and Eucilat, whereas Cilostamide inhibited quite equally the cAMP-specific and the cGMP-specific forms in both tissues. In marked contrast, the flavonoid compound penta-*O*-ethyl quercetin which effectually inhibited the cardiac cGMP-sensitive phosphodiesterase was far less effective toward the aortic cGMP-specific phosphodiesterase. Similar results were found for quercetin (IC_{50} cardiac form: 20 μM/IC_{50} vascular form: 2000 μM). These results clearly highlighted a differential susceptibility to inhibitors between the cardiac cGMP-sensitive and the aorta cGMP-specific phosphodiesterases. If we excluded Rolipram and compound Ro 20-1724 which poorly inhibited these enzymatic forms, no correlation existed between IC_{50} values of the 11 remaining inhibitors for aorta enzyme and their IC_{50} values for cardiac enzyme (r, 0.003, not significant).

DISCUSSION

Although the existence of multiple molecular forms of phosphodiesterase is now well-agreed, problems regarding the number of phosphodiesterase forms present in a given tissue as well as their biochemical characteristics and physiological significance are still a matter of debate. Since discrepant results are classically attributed to the use of different methods of enzyme extraction and fractionation, one main goal of the present study was to evaluate the different molecular forms of phosphodiesterase in a given tissue (rat heart and bovine aorta) using two different media for tissue homogenization and two different techniques of fractionation. As is apparent on profiles shown in Figs 1, 3, 4 and 5 and from data recapitulated in Table 1, both the calmodulin-sensitive and the cAMP-specific forms exhibit close IEF and chromatographic patterns whatever the nature of tissue: rat heart or bovine aorta.

Thus, only minor differences were found between cardiac and vascular calmodulin-sensitive isoenzymes. In particular, the vascular form proved to be more markedly activated by exogenous calmodulin (+797%) than the cardiac one (+203%) when homogenates were performed in hypotonic medium and separated by DEAE–Trisacryl chromatography. However, the resolution of both cardiac and vascular enzymes is closely dependent on extraction and fractionation procedures. Thus, extraction in sucrose medium without calcium chelator, and isoelectrofocusing do not separate calmodulin from the calmodulin-sensitive enzyme. In these conditions, both heart and aorta enzymes focused at pI 4.9 under a quite saturated state as indicated by the lack of activation by a supplementary adjunction of calmodulin and the marked inhibition by EGTA (Table 1).

In marked contrast, enzyme extraction in hypotonic medium in the presence of calcium chelator, and DEAE–Trisacryl chromatography dissociate, at least partly, the endogenous calmodulin from the sensitive isoenzyme. Thus, when isoelectrofocusing was performed from hypotonic cytosols, both cardiac and vascular isoenzymes which were partly depleted in acidic calmodulin, focused within a more basic pH range (5.7–5.8) than the fully saturated fraction. The identity of these peaks was clearly demonstrated by

additional refocusing experiments. Indeed, after a first separation by DEAE–Trisacryl chromatography, a technique known to dissociate calmodulin from the isoenzyme, the cardiac and the vascular enzymes focused in the pH 5.75–5.85 range with comparable sensitivity to exogenous calmodulin activation and EGTA inhibition. If an aliquot of the same DEAE–Trisacryl separated fraction was briefly treated by exogenous calmodulin before isoelectrofocusing, the calmodulin-sensitive isoenzyme migrated then within an intermediate pH zone ($5.8 < 5.4 < 4.9$). Thus, the pI of the cardiac and vascular calmodulin-sensitive isoenzymes and, as a consequence, their migration in isoelectrofocusing profiles, depends on their calmodulin saturation level. On the other hand, similar Michaelian kinetics for the hydrolysis of cGMP in the presence of saturating amount of calmodulin have been reported for the cardiac [7] and the vascular [13] enzymes with K_m values of 2 and 0.8 μM, respectively.

In the same way, only minor detail points distinguish the cardiac cAMP-specific isoenzyme from the vascular one. The cardiac cAMP-specific enzyme appeared more heterogeneous, following both isoelectrofocusing and DEAE–Trisacryl fractionations, than the vascular form and also more prone to give hydrophobic interactions with solid supports than the vascular one, especially when homogenates were prepared in hypotonic conditions. The heterogeneity of the cardiac cyclic AMP-specific isoenzyme was also apparent after refocusing of the DEAE–Trisacryl separated fraction, whereas the vascular form appeared quite homogeneous whatever the technique of fractionation used. Both isoenzymes exhibit quite comparable sensitivity to reference inhibitors and prove to be insensitive to cGMP inhibition. They are thus clearly distinct from the "cGMP-inhibited phosphodiesterase" recently identified in bovine cardiac muscle [26] and platelets [27], which proved to be immunologically distinct from the cytosolic cAMP-specific enzyme from bovine lung and canine kidney [10]. After extensive purification, the cAMP-specific rat heart enzyme shares most of the biochemical characteristics of the canine kidney enzyme [18, 28]. In addition, the cardiac and vascular isoenzymes exhibited non-Michaelian kinetics (concave-downward Lineweaver–Burk plots) with low and high K_m values in the same range of magnitude: 0.86 and 86 μM for the cardiac form [7], 0.40 and 50 μM for the vascular form [13].

In marked contrast with the above observations, the cGMP-specific, calmodulin-insensitive form notably differs from one tissue to the other. Whereas the presence of such a form has been clearly established in myocardial tissue [3, 6–8, 19], its existence in vascular muscle is still a matter of debate [12, 13, 19]. Confrontation of the results given by isoelectrofocusing and DEAE–Trisacryl chromatography firmly confirms the presence of a cGMP-specific enzyme form in aorta tissue. Indeed, at the end of DEAE–Trisacryl chromatography of hypotonic cytosol, this form is contaminated by the calmodulin-sensitive enzyme and might be interpreted as a sub-fraction of the latter, but isoelectrofocusing of isotonic cytosols unambiguously separates these two forms by 1.8 pH unit. One of the main differences that distinguish the cardiac and the vascular isoenzymes concerns the stimulation of cAMP hydrolysis by cGMP. Indeed, the cAMP hydrolyzing activity of the cardiac isoenzyme was substantially increased in the presence of 1–5 μM cGMP whereas the activity of the vascular enzyme was unaffected or rather inhibited by cGMP concentrations higher than 1 μM.

Thus the cardiac pI 5.55 form which exhibits a marked preference for cGMP as substrate at low concentration is very likely identical to the so-called "cGMP-sensitive phosphodiesterase" found in some tissues to hydrolyze more efficiently cAMP when stimulated by cGMP [21–31]. The corresponding vascular cGMP-specific form clearly does not belong to this family. It might share some similitude with the "cGMP inhibited enzyme" recently identified in the bovine heart [26]. Although the physiological implication of these two enzyme species in their respective tissue is not clearly understood, their differential sensitivity to reference inhibitors suggests a possibility of selective pharmacological modulation of phosphodiesterase activity in cardiac and vascular target tissues.

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