PROTEOLYSIS OF CYCLIC AMP PHOSPHODIESTERASE-II ATTENUATES ITS ABILITY TO BE INHIBITED BY COMPOUNDS WHICH EXERT POSITIVE INOTROPIC ACTIONS IN CARDIAC TISSUE

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Abstract—Extraction of frozen canine cardiac muscle rendered soluble over 90% of the cyclic AMP phosphodiesterase activity. The residual activity was membrane-bound. Ion exchange chromatography of the soluble activity on DE-52 allowed for the resolution of three distinct cyclic AMP phosphodiesterase fractions termed PDE-I, PDE-II and PDE-III in order of elution from the column by a linear NaCl gradient. The relative ratio of cyclic AMP phosphodiesterase activity exhibited by these three peaks was $1:0.65:0.82$ and of cyclic GMP phosphodiesterase activity was $1:0.52:0.05$ for PDE-I, PDE-II and PDE-III respectively. PDE-II and PDE-III were further purified by re-chromatography on DE-52. Fractions PDE-II and PDE-III were thermolabile at 50°, decaying as single exponentials with half lives of 180 sec and 77 sec respectively. All three species exhibited non-linear Lineweaver–Burke plots for the hydrolysis of cyclic AMP, exhibiting both high and low affinity components. Hydrolysis of cyclic GMP by all three components obeyed normal kinetics, yielding linear plots. PDE-I was a $Ca^{2+}/$calmodulin-activated species which exhibited a low K_m for both cyclic AMP and cyclic GMP but hydrolysed cyclic GMP with a higher V_{max} than for cyclic AMP. PDE-II exhibited a much lower K_m for cyclic AMP than for cyclic GMP and a much higher V_{max} for the hydrolysis of cyclic AMP. PDE-III exhibited a low K_m for both cyclic AMP and cyclic GMP, however, its V_{max} for cyclic AMP was about 40-fold higher than for cyclic GMP. Cyclic GMP acted as a potent inhibitor ($IC_{50} = 6.3 \mu M$) of cyclic AMP hydrolysis catalysed by PDE-III but not of the hydrolysis of cyclic AMP by PDE-II ($IC_{50} = 33.2 \mu M$). The phosphodiesterase inhibitors milrinone, CI-930, UK-35,493, carbazeran and buquineran acted as potent inhibitors of cyclic AMP hydrolysis catalysed by both PDE-II and PDE-III enzymes. They did not inhibit PDE-I activity. PDE-II, when prepared in the absence of protease inhibitors exhibited a reduced potency to inhibition by these compounds. Treatment of purified PDE-II with trypsin caused a reduction in enzyme activity and reduced dramatically the sensitivity of PDE-II activity to inhibition by these various compounds. The action of proteolysis in attenuating the inhibitory effect of these compounds on PDE-II was most dramatic with CI-930, milrinone, amrinone, buquineran and UK35,493 and least dramatic with carbazeran and IBMX. It is suggested that "selective" phosphodiesterase inhibitors, which exert positive inotropic actions, may do so by inhibiting the activity of both PDE-II and PDE-III enzyme forms and that endogenous proteolysis may attenuate the action of such inhibitors.

Cyclic AMP phosphodiesterases (PDE) are ubiquitous enzymes which provide the sole route for the degradation of the intracellular second messenger, cyclic AMP [1–5]. As such an appreciation of the functioning of this system is of some importance. All tissues examined to date exhibit multiple forms of PDE activity [1–5]. Isolation and subsequent characterization of specific PDE species using peptide mapping [6–8] and immunological [8, 9] techniques indicate that these multiple forms are distinct gene products. The major activities that can be identified in tissues are a $Ca^{2+}/$calmodulin-stimulated enzyme, species of cyclic GMP-stimulated cyclic nucleotide phosphodiesterases and multiple species of high affinity cyclic AMP phosphodiesterases [1–5]. These enzymes have very different physical properties, vary in intracellular localization, exhibit different kinetics of hydrolysis of cyclic AMP and cyclic GMP and have different inhibitor selectivities. This latter observation has given rise to considerable interest in the development of specific inhibitors for these

various cyclic AMP phosphodiesterases [10–14]. Inhibitors arising from such studies have been demonstrated to affect a variety of physiological processes including cardiac contractility, platelet aggregation, vascular relaxation, cell proliferation and secretory exocytosis. Of particular interest have been inhibitors which exert positive inotropic actions on the heart and hence have a potential therapeutic use in the treatment of congestive heart failure [10–14].

Tissue extracts of soluble cyclic AMP phosphodiesterase activity have been separated, by ion-exchange chromatography [5, 10, 15], into three distinct forms PDE-I, PDE-II and PDE-III. PDE-I appears to be the $Ca^{2+}/$calmodulin-stimulated phosphodiesterase, PDE-II a high affinity enzyme capable of degrading both cyclic AMP and cyclic GMP and PDE-III phosphodiesterase activity which exhibits little activity towards cyclic GMP. A number of investigators [10, 16, 18] have demonstrated that agents which exert positive inotropic effects exert a

much more potent inhibitory effect on PDE-III than on the other two cyclic AMP phosphodiesterase forms.

We demonstrate here that the susceptibility of PDE-II activity, isolated from a soluble extract of canine heart, to inhibition by agents known to exert positive inotropic effects in the heart is markedly attenuated by proteolysis of the enzyme.

MATERIALS AND METHODS

Isolation of soluble phosphodiesterase activity. Hearts were removed from anaesthetized dogs, following sacrifice by exsanguination. The ventricles were diced into 1 cm cubes, frozen in liquid nitrogen and stored at $-80°$ prior to use. Approximately 15 g of this tissue was used for each enzyme preparation. After thawing, the tissue was chopped into small pieces using scissors and then homogenized in 100 ml of buffer A (250 mM sucrose, 10 mM Tris, 0.1 mM

PMSF and 0.3 mM benzamidine, final pH 7.4) at 4° in a motor driven homogenizer. The homogenate was then filtered through muslin cloth and centrifuged at $1400\,g$ for 15 min. The pellet (P_1) was retained and the supernatant (S_1) centrifuged at $30{,}000\,g$ for 15 min. The pellet (P_2), which formed, was retained and the supernatant (S_2) re-centrifuged at $30{,}000\,g$ for 15 min to give a pellet (P_3) and supernatant (S_3). This supernatant (S_3) was applied to a column of DE-52 cellulose (2.5 × 10 cm) which had been pre-equilibrated with buffer B (20 mM Tris, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.3 mM benzamidine, 0.1 mM PMSF final pH 7.4). The supernatant (S_3) was applied at a flow rate of $2\,ml\,.\,min^{-1}$ and then the column was washed with three bed volumes of buffer B. After this the phosphodiesterase PDE activity was eluted at a flow rate of $2\,ml\,.\,min^{-1}$ with 300 ml of buffer B containing a linear gradient of 0–0.35 M NaCl at a final pH 7.4. Three-millilitre fractions were collected and assayed
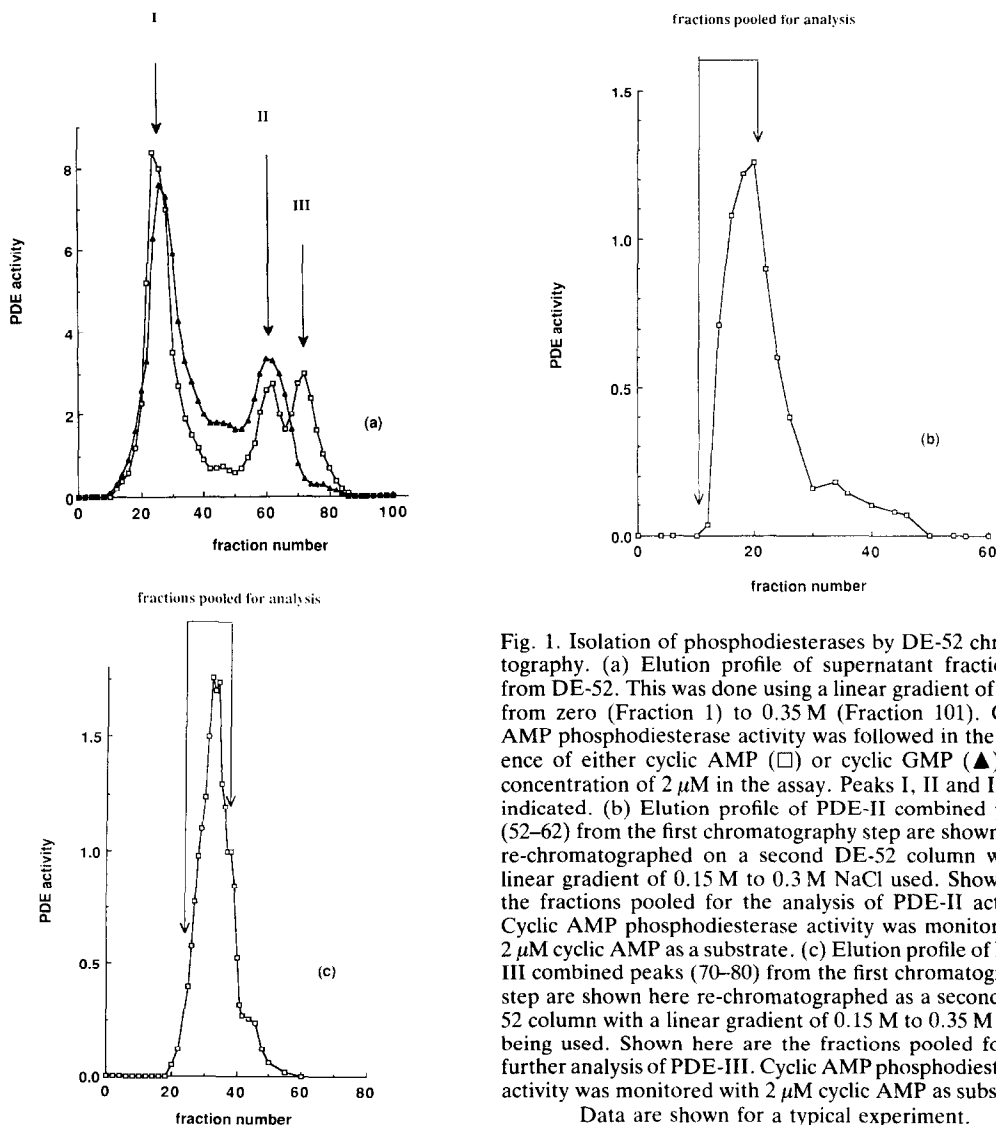

Fig. 1. Isolation of phosphodiesterases by DE-52 chromatography. (a) Elution profile of supernatant fraction S_3 from DE-52. This was done using a linear gradient of NaCl from zero (Fraction 1) to 0.35 M (Fraction 101). Cyclic AMP phosphodiesterase activity was followed in the presence of either cyclic AMP (□) or cyclic GMP (▲) at a concentration of 2 μM in the assay. Peaks I, II and III are indicated. (b) Elution profile of PDE-II combined peaks (52–62) from the first chromatography step are shown here re-chromatographed on a second DE-52 column with a linear gradient of 0.15 M to 0.3 M NaCl used. Shown are the fractions pooled for the analysis of PDE-II activity. Cyclic AMP phosphodiesterase activity was monitored at 2 μM cyclic AMP as a substrate. (c) Elution profile of PDE-III combined peaks (70–80) from the first chromatography step are shown here re-chromatographed as a second DE-52 column with a linear gradient of 0.15 M to 0.35 M NaCl being used. Shown here are the fractions pooled for the further analysis of PDE-III. Cyclic AMP phosphodiesterase activity was monitored with 2 μM cyclic AMP as substrate. Data are shown for a typical experiment.

for cyclic AMP and cyclic GMP phosphodiesterase activity. Fractions (52–62) at the leading edge (Fig. 1a) of the second peak of cyclic AMP phosphodiesterase (PDE-II) were combined and diluted (1:3) with buffer B. They were then applied (2 ml . min^{-1}) to a DE-52 column (10 × 2.5 cm) that had been pre-equilibrated in buffer B. The column, with bound phosphodiesterase activity was washed with three column volumes of buffer B containing 0.15 M NaCl at a final pH 7.4. Activity was then eluted with a 100 ml gradient of 0.15 M–0.35 M NaCl in buffer B, final pH 7.4, collecting 2 ml fractions. The leading edge (fractions 10–20) were combined and used in this study (see Fig. 1b).

Isolation of PDE-III was performed by taking the fractions (70–80) which formed the third peak of cyclic AMP phosphodiesterase activity eluting from the first DE-52 column (see Fig. 1a). These were treated exactly as specified above for the peak 2 (PDE-II) fractions, with re-chromatography yielding a single symmetrical peak of cyclic AMP phosphodiesterase activity. Fractions 26–42 were pooled as shown in Fig. 1c yielding PDE-III.

Studies on PDE-I were performed using combined fractions 20–35 from chromatography of S_3 on DE-52 (Fig. 1a). Enzyme fractions that had been prepared fresh each day were used in all of the studies described herein. Enzyme fractions prepared using fresh rather than frozen tissue had identical properties to those described herein.

In some experiments these procedures were performed in the absence of the protease inhibitors PMSF and benzamidine.

Extraction of membrane pellets. The pellets P_1 and P_2 were subjected to a variety of treatments in an attempt to release residual phosphodiesterase activity. Thus the pellets P_1 and P_2 were treated with either the detergent Triton X-100 (1% v/v) or the detergent Nadeoxycholate (1% v/v) in buffer A at 4° for 45 min prior to centrifugation at 30,000 g for 45 min. Samples (5 ml) of the supernatant were subjected to chromatography on a 60 ml bed volume column of Sephadex G-50, which had been pre-equilibrated in buffer A, in order to remove detergent.

Portions of the pellet were also treated with either a hypotonic buffer (10 mM Tris–HCl, pH 7.4) for 40 min or with buffer A containing 0.5 M NaCl. After a 40 min period at 4°, these samples were centrifuged at 30,000 g for 20 min and the supernatants taken for assay of phosphodiesterase activity.

Treatment of PDE-II with trypsin. Benzamidine and PMSF were removed from the purified PDE-II preparation by either dialysis against buffer B (20 mM Tris–HCl, 7 mM 2-mercaptoethanol) or re-chromatography on DE-52 with elution using an NaCl gradient (0.15 M–0.3 M) in buffer B. Samples (1.9 µg protein) of purified PDE-II were incubated with specified amounts of trypsin in a total volume of 100 µl for 10 min at 30°. Reactions were terminated with the addition (10 µl) of trypsin inhibitor at twice the amount of trypsin added. Samples were removed to ice prior to removal of a sample (20 µl) for assay of cyclic AMP phosphodiesterase activity. Treatment of PDE-II with trypsin inhibitor alone did not exert any of the effects noted. Unless stated otherwise,

PDE-II samples (1.9 µg) were treated with 200 ng trypsin for 10 min at 30°.

Thermal denaturation of phosphodiesterases. Samples of PDE-II and PDE-III were incubated for periods of up to 10 min at 50° prior to removal for immediate assay of cyclic AMP phosphodiesterase activity at 30° as described below.

Assay of cyclic AMP phosphodiesterase activity. Assays were performed routinely by a modification [19] of the two-step procedure of Thompson and Applemann [20]. However, when assays were performed on homogenates then the Dowex-1 resin was resuspended in EtOH : H$_2$O in order not to underestimate phosphodiesterase activity due to the presence of contaminating adenosine deaminase activity [21]. We added 400,000 cpm of labelled cyclic nucleotide to each assay.

The assay mixture contained 5 mM MgCl$_2$ and 10 mM Tris–HCl buffer pH 7.4 together with either [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP. All assays were done at 30° and initial rates were taken from linear timecourses. Concentrations of substrates are given in the appropriate legends.

Stock solutions of inhibitors were dissolved in DMSO and then diluted into the assay so that the final [DMSO] was always less than 0.1% in the assay. This concentration of DMSO had no detectable effect on phosphodiesterase activity of any of the fractions.

Protein determinations were done using a modified [22] microbiuret [23] method using bovine albumin as standard.

[^{3}H]-cyclic AMP and [^{3}H]-cyclic GMP were from Amersham International UK. Dowex-1 resin, PMSF, benzamidine, isobutylmethylxanthine trypsin, bovine trypsin inhibitor and *Ophiophagus hannah* venom were from Sigma Chemicals. All other biochemicals were from Boehringer Corp. (U.K.). All general chemicals were of AR grade from BDH Chemicals, Poole, U.K. All inhibitors were synthesized and given as gifts from Pfizer Central Research, Sandwich, Kent, U.K., as were the canine hearts.

RESULTS

Extraction of cyclic AMP phosphodiesterase activity showed that less than 4% of the total activity remained in pellet P_1 and less than 2% of the total activity in pellets P_2 and P_3. Thus, extraction of frozen cardiac muscle with a buffer A released over 90% of the total cyclic AMP phosphodiesterase activity in this tissue.

Treatment of pellets P_1, P_2 and P_3 with either hypotonic buffer or buffer A containing 0.5 M NaCl failed to release any of the residual activity. Treatment with the detergents, under the conditions tested, inhibited the residual activity.

Application of the high speed supernatant (S_2), which contained over 90% of total cardiac cyclic nucleotide phosphodiesterase activity, to the DE-52 column resulted in the binding of all of the cyclic AMP and cyclic GMP phosphodiesterase activity to the column. Treatment of the column with a linear gradient (0–0.35 M) of NaCl in a buffer A effects the release of all of the applied activity (over 90%). When cyclic AMP phosphodiesterase activity was
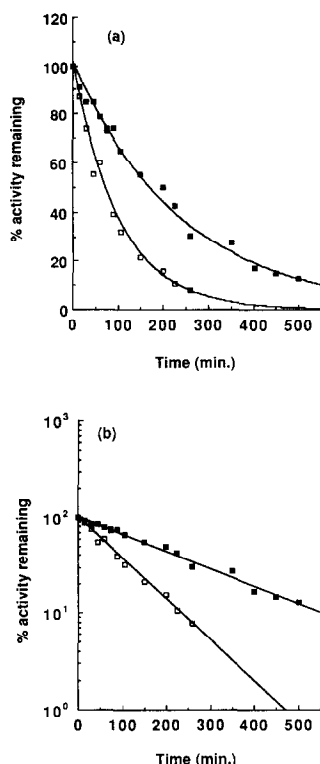
Fig. 2. Thermal inactivation of PDE-II and PDE-III activity. This was performed using the re-chromatographed enzyme samples as described in Materials and Methods. Assays of cyclic AMP phosphodiesterase activity were done with $2\,\mu M$ cyclic AMP as substrate. Data is shown for changes in PDE-II (■) and PDE-III (□) activity expressed as loss (%) of activity against time (a) and also (b) in semi-log form as log % activity remaining against time for a typical experiment performed at least three times. In (a) the curve drawn is a single exponential and, like in (b) the correlation coefficients are 0.99 for both enzymes.

monitored, then three distinct peaks of activity (PDE-I, PDE-II and PDE-III) were eluted (Fig. 1a). When cyclic GMP phosphodiesterase activity was

monitored then two major peaks of activity were noted. These co-migrated with the cyclic AMP phosphodiesterase activity found in peaks PDE-I and PDE-II, with only residual cyclic GMP phospho-diesterase activity found in peak PDE-III (Fig. 1a). The ratio of cyclic AMP phosphodiesterase activity found in these three peaks (PDE-I:PDE-II:PDE-III) was 1:0.65:0.82 and for cyclic GMP phospho-diesterase activity it was 1:0.52:0.05.

Fractions at the leading edge of peak PDE-II were combined and subjected to re-chromatography on DE-52 (Fig. 1b), in order to isolate a purified PDE-II fraction. Fractions forming peak PDE-III were similarly combined and re-chromatography on DE-52 (Fig. 1c) to give a purified PDE-III fraction.

The activities of both PDE-II and PDE-III decayed as single exponentials (Fig. 2a) and gave linear semi-log decay plots (Fig. 2b) when subjected to thermal denaturation at 50°. They had very different half-lives of 180 ± 9 sec and 77 ± 6 sec for the denaturation of PDE-II and PDE-III respectively.

The decay of activity in these fractions as a single exponential indicates that only a single phospho-diesterase activity formed the major (greater than 90%) fraction of the activity in the PDE-II and PDE-III fractions. Indeed, as these two phospho-diesterases exhibited very different half-lives then any cross contamination would have yielded a biphasic plot. These experiments support the con-tention that re-chromatography yielded an effective separation of the two activities as has been pointed out by Weishaar and colleagues using a guinea-pig cardiac tissue extract [16].

The cyclic nucleotide phosphodiesterase activity of the fractions was analysed by Lineweaver–Burke plots. All three species exhibited non-linear plots when cyclic AMP phosphodiesterase activity was followed, yielding high and low affinity components (Table 1). Linear plots were, however, observed for the degradation of cyclic GMP by these three fractions, yielding single values for K_m and V_{max} (Table 1). All three species exhibited high affinity components for the hydrolysis of cyclic AMP. Whilst PDE-II was capable of degrading cyclic GMP with a

Table 1. Kinetic constants for isolated cardiac phosphodiesterase activities

| Species | Substrate | K_m $(\mu M)^*$ | | V_{max} (pmol . min/μg protein) | |
		high affinity component	low affinity component	high affinity component	low affinity component
PDE-I	cyclic AMP	0.2	10.0	8.1	41.7
	cyclic GMP	1.8	—	54.1	—
PDE-II	cyclic AMP	2.8	20.0	32.1	57.8
	cyclic GMP	—	31.0	—	38.33
PDE-III	cyclic AMP	0.8	17.0	19.5	67.0
	cyclic GMP	5.0	—	0.5	—

Enzyme activity was determined over a substrate range of 0.2–50 μM cyclic nucleotide. PDE-II and PDE-III activity was determined on the re-chromatographed preparations. All assays were done at pH 7.4 and at 30° using 0.2 μg protein/assay.

* High and low affinity components were determined as limiting values as described previously by us [7, 8, 19].

Table 2. Inhibition of PDE-II and PDE-III activity

Enzyme: Presence of protease inhibitor*:	PDE-II			PDE-III			K_i-values‡	
	Yes	No	(ratio)†	Yes	No	(ratio)†	PDE-II	PDE-III
Inhibitor	IC$_{50}$ values (μM)							
IBMX	4.1 ± 1.3	8.2 ± 1.2	(2.1)	4.5 ± 0.5	4.4 ± 0.4	(0.98)	3.8	3.6
Milrinone	1.1 ± 0.1	5.2 ± 0.8	(4.7)	1.5 ± 0.9	2.1 ± 0.6	(1.4)	1.0	1.2
CI-930	0.8 ± 0.3	4.9 ± 0.8	(6.1)	1.7 ± 0.6	0.7 ± 0.4	(0.4)	0.7	1.4
UK-35493	0.6 ± 0.1	1.3 ± 0.3	(2.2)	0.9 ± 0.1	0.8 ± 0.2	(0.9)	0.6	0.7
Amrinone	10.1 ± 3.4	N.D.		31.2 ± 6.3	N.D.		9.4	2.5
Carbazeran	4.0 ± 0.7	10.5 ± 1.2	(2.6)	6.2 ± 2.2	2.4 ± 0.5	(0.4)	3.7	5.0
Buquineran	7.6 ± 1.5	15.2 ± 1.8	(2.0)	8.6 ± 0.8	7.9 ± 1.2	(0.9)	7.1	6.9

All assays were performed at 30° using 0.2 μM cyclic AMP as substrate. Re-chromatography PDE-II and PDE-III fractions were used.

* Extraction and isolation of PDE fractions was done as described in the Materials and Methods section either using (Yes) or not using (No) the protease inhibitors PMSF and benzamidine throughout these procedures.

† This is the ratio of IC$_{50}$ values exhibited by enzyme preparations done in the absence (No) and presence (Yes) of protease inhibitors. Errors are SD for 5 separate experiments. Assays were performed with 0.2 μM cyclic AMP as substrate and treatment with increasing concentrations of the various compounds was performed in order to assess the concentration (EL$_{50}$) at which 50% inhibition of enzyme activity ensued.

‡ The compounds tested inhibited PDE-II and PDE-III in a competitive fashion as regards the high affinity (low K_m) component. K_i values were calculated assuming the kinetic constants given for the low K_m component of each PDE activity in Table 2. K_i values are given for the enzymes prepared in the presence of protease inhibitors based upon the experimentally determined IC$_{50}$ values.

N.D.—not determined.

comparable V_{max} to that seen for PDE-I, it showed a relatively low affinity for cyclic GMP. In contrast PDE-III showed a high affinity component for cyclic GMP but was only capable of hydrolysing cyclic GMP extremely poorly.

Employing cyclic AMP (0.2 μM) as substrate, cyclic GMP inhibited the activities of PDE-I, PDE-II and PDE-III with IC$_{50}$ values (concentrations effecting 50% inhibition) of 3.6 μM, 33.2 μM and 6.2 μM respectively. Thus, in contrast to PDE-II, the ability of both PPDE-I and PDE-III to hydrolyse cyclic AMP could be potently inhibited by cyclic GMP. However, PDE-I could also hydrolyse cyclic GMP effectively, whereas PDE-II could only hydrolyse cyclic GMP extremely weakly. Low concentrations (0.2–2 μM) of cyclic GMP did not stimulate the cyclic AMP phosphodiesterase activity of the PDE-II enzyme.

When Ca^{2+} (500 μM) and calmodulin (10 μg/ml) were added to the phosphodiesterase assays then only the activity of PDE-I was increased. Thus for PDE-I, the addition of Ca^{2+}/calmodulin enhanced the V_{max} for cyclic AMP hydrolysis some 3.2-fold and that for cyclic GMP hydrolysis some 1.8-fold. The action of Ca^{2+}/calmodulin was on the V_{max} of the reaction only, as the K_m values for both cyclic AMP and cyclic GMP hydrolysis by PDE-I were unchanged.

The compounds milrinone, CI-930, UK-35493, amrinone, buquineran and carbazeran failed to exert any inhibitory effect (at 100 μM) on the cyclic AMP phosphodiesterase activity of PDE-I, determined in either the presence or absence of Ca$^+$/calmodulin at a cyclic AMP substrate concentration of 0.2 μM. However, these compounds acted as potent inhibi-

tors of the cyclic AMP phosphodiesterase activity exhibited by both PDE-II and PDE-III enzymes (Table 2). We did note, however, that if the preparation of the enzyme fractions had been performed in the absence of the protease inhibitors PMSF and benzamidine then the ability of these compounds to inhibit the activity of the PDE-II enzyme was markedly attenuated. Thus the IC$_{50}$ values for inhibition of PDE-II by these various compounds increased some 2–6-fold, depending upon the compound when using enzymes prepared in the absence of protease inhibitors (Table 2). K_i values for these various inhibitors are also given in Table 2 for both PDE-II and PDE-III enzymes prepared in the presence of protease inhibitors. These data clearly demonstrate that these compounds inhibit both of these phosphodiesterases equally effectively and very potently. In contrast, IC$_{50}$ values obtained for the inhibition of PDE-III by these compounds were not increased using enzyme fractions prepared in the absence of protease inhibitors (Table 2).

Incubation of PDE-II with the protease trypsin caused an irreversible loss of cyclic AMP phosphodiesterase activity. The proportion of activity lost during a 10 min incubation at 30° dependent upon the amount of trypsin added to the incubation mixture (Fig. 3). However, treatment of PDE-II with trypsin afforded a dramatic reduction in the ability of milrinone to inhibit this enzyme (Fig. 4). Indeed, the trypsin-mediated loss of the inhibitory action of milrinone was far more acute than the reduction in cyclic AMP phosphodiesterase activity ensuing from the trypsin-mediated proteolysis of PDE-II (Figs 3 and 4). Thus trypsin effected a 50% reduction of PDE-II activity at around 450 mg, whereas a
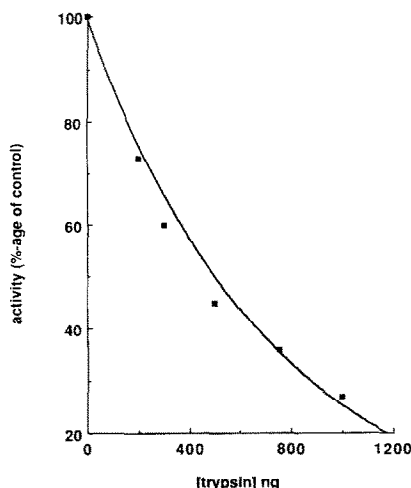
Fig. 3. Inhibition of PDE-II activity by treatment with trypsin. Aliquots of purified PDE-II activity were treated with increasing concentrations of trypsin before the reaction was stopped with trypsin inhibitor prior to assay with cyclic AMP (0.2 μM) as substrate. Details are given in Materials and Methods. This data is of a typical experiment of one performed three times.
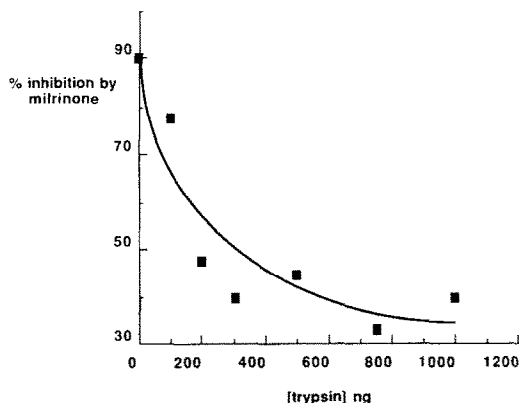

Fig. 4. Loss of sensitivity of PDE-II to inhibition by milrinone after treatment with trypsin. Purified PDE-II was treated, as described in Materials and Methods, with increasing concentrations of trypsin before reaction was stopped with trypsin inhibitor. The percentage inhibition, caused by milrinone (10 μM) of cyclic AMP phosphodiesterase activity assessed with 0.2 μM cyclic AMP as substrate is given. This data is typical of an experiment performed three times.

* Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; IBMX, 3-isobutyl-l-methylxanthine; milrinone, 1,6-dihydro-2-methyl-6-oxo-[3,4'-bipyridinel]-5-carbonitrile; amrinone, 5-amino-]3-4'-bipyridinel]-6(IH)-one; buquineran (UK14275); 6,7-dimethoxy-4-[4-(3-n-butylureido)piperidino]quinazoline; carbazeran (UK31557), 6,7-dimethoxyl-1-[4-(ethylcarbanoyoxy)piperidino]phthalazinc; CI-930, 4,5-dihydro-6-[4-(1H-imidazol-1-yl)phenyl]-5-methyl-3(2H) pyridazinone; UK-35493, 6,7-dimethoxy-4-[4-(2-(-isothiazolidnyl)ethyl)-1-piperidinyl] quinazoline S,S dioxide.
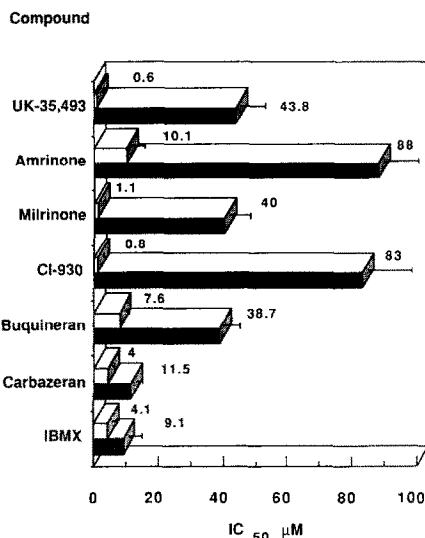

Fig. 5. Trypsin treatment of PDE-II reduces its ability to be inhibited by various drugs. PDE-II, that had been purified in the presence of protease inhibitors, was treated to remove protease inhibitors as described in Materials and Methods and then used directly (open bars) or treated (solid bars) with trypsin (200 ng) for 10 min at 30° with the addition of 400 ng of trypsin inhibitor to block trypsin action. These enzyme preparations were then assayed in the presence of 0.2 μM cyclic AMP as substrate together with increasing concentrations of the various inhibitors. The concentration of compounds which caused 50% inhibition (IC_{50}) was determined for the "native" and "trypsin-treated" enzymes. These values are shown. Errors are SD for three experiments using different enzyme preparations.

reduction of 50% in the effectiveness of milrinone to act as an inhibitor of PDE-II activity occurred at only 240 ng trypsin. The ability of trypsin-catalysed proteolysis to attenuate the inhibitory action of various compounds on PDE-II activity was not confined to milrinone (Fig. 5). Thus the ability of a wide variety of inhibitors to act on PDE-II was reduced after subjecting this enzyme to the action of trypsin. Trypsin-treatment had relatively small effects on the inhibitory actions elicited by IBMX* and carbazeran compared to the very dramatic effects which were seen with buquineran, milrinone, amrinone, UK35,493 and, in particular, CI-930 (Fig. 5). Treatment of PDE-II with trypsin under the conditions employed in Fig. 4 attenuated the activity exhibited by PDE-II assayed with 0.2 μM cyclic AMP by some 20–25% range. The Lineweaver–Burke plots for the hydrolysis of cyclic AMP by fraction of PDE-II that had been pre-treated with 200-ng of trypsin (see Methods) were non-linear with limiting values of K_m for the high and low affinity components of 12.5 μM and 22 μM respectively and for V_{max} of 20.4 pmol/min/μg protein and 38.6 pmol/min/μg protein respectively. The values for the Hill coefficient for the hydrolysis of cyclic AMP by PDE-II were $h = 0.5$ and $h = 0.57$ for the native and trypsin-treated enzymes.

DISCUSSION

All tissues examined to date [1–5] exhibit multiple

forms of cyclic AMP phosphodiesterase activity although the functional importance of individual species remains to be elucidated [4]. Certainly that the activity of specific species can be activated rapidly through the action of hormones [2, 4] suggests unique functional roles for particular species.

In a number of tissues [2, 5, 10, 15, 16], resolution of soluble cyclic AMP phosphodiesterase activity into three distinct forms has been achieved using ion-exchange chromatography. These have been called PDE-I, PDE-II and PDE-III after the order in which they can be eluted from the ion-exchange (DEAE) column [15]. Studies on liver [5] indicated that PDE-I was a Ca^{2+}/calmodulin activity enzyme exhibiting a preference for hydrolysis of cyclic GMP; PDE-II an enzyme capable of acting with equal effectiveness on both cyclic AMP and cyclic GMP and PDE-III an enzyme with a marked preference for action on cyclic AMP. That PDE-III appears to be a cyclic AMP-specific enzyme has aroused considerable interest [9, 10, 16–18] in the possibility that it might provide a target for the action of those cyclic AMP phosphodiesterase inhibitors which can exert a positive inotropic action on the heart. This concept arose from an observation using canine cardiac tissue enzymes that the cardiotonic agent amrinone appeared to inhibit PDE-III with an IC_{50} of around $20 \mu M$ whilst having no effect on PDE-I and only a small inhibitory action on PDE-II. However, no kinetic characterization of the PDE activities in this tissue was performed.

Here we show that the major fraction of cyclic nucleotide phosphodiesterase activity in canine cardiac tissue is soluble. Its fractionation on ion-exchange chromatography reveals three distinct forms which, in accord with previous investigators, we have termed PDE-I, PDE-II and PDE-III. We show here that, as with liver, PDE-I is the Ca^{2+}/calmodulin-stimulated enzyme. In agreement with other investigators [9, 16, 18] employing this enzyme from cardiac tissue, we found that the activity of PDE-I was not inhibited by either milrinone or amrinone. Furthermore, we shown here that various other "selective" phosphodiesterase inhibitors did not inhibit PDE-I. However, although PDE-I is often regarded as cyclic GMP-selective as, indeed, it exhibits a higher V_{max} for cyclic GMP than for AMP, it should be noted that in canine cardiac tissue PDE-I expresses a low K_m for cyclic AMP (Table 1) and a capacity to hydrolyse cyclic AMP which is certainly the equal of that of PDE-II and PDE-III (Fig. 1a, Table 1). Thus one should not underestimate the possible importance of the role of PDE-I in modulating cyclic AMP metabolism.

PDE-II appeared to hydrolyse both cyclic GMP and cyclic AMP with a similar maximum velocity whereas PDE-III showed relatively little activity towards cyclic GMP (Table 1). This implies that PDE-III is a cyclic AMP selective enzyme.

A species reflecting the properties of PDE-III has recently been purified to apparent homogeneity from both bovine cardiac tissue [9] and from rat liver [8]. A key feature of this enzyme is that whilst it hydrolyses cyclic GMP rather weakly, it expresses a low K_m value for this cyclic nucleotide indicating that cyclic AMP can bind to it with high affinity. Thus

cyclic GMP can act as a potent inhibitor of the hydrolysis of cyclic AMP by PDE-III. This is in marked contrast to PDE-II, which exhibits a relatively high K_m for cyclic GMP (Table 1) and thus cyclic GMP is a relatively ineffective inhibitor of cyclic AMP hydrolysis by PDE-II.

It has been shown recently that not only the Ca^{2+}/calmodulin-activated phosphodiesterase [1, 24] but also various other phosphodiesterases [8, 9, 25] can be affected by proteolysis during the isolation and purification procedures. Indeed, 2-mercaptoethanol which is added routinely by the majority of investigators can itself stimulate thiol-activated proteases which have been shown to act on phosphodiesterases [25]. Unlike other studies which have compared the actions of selective phosphodiesterase inhibitors on the three phosphodiesterase enzymes from cardiac tissues, we performed this study on enzymes prepared in the presence of the protease inhibitors PMSF and benzamide. As with others [16, 18] we found that the activity of PDE-I was not inhibited by selective inhibitors. However, in contrast to these other investigators we found that the selective inhibitors, including milrinone and CI-930, were just as effective at inhibiting the activity of PDE-II as they were at inhibiting the activity of PDE-II. However, if we prepared our enzymes over a similar timescale, but omitting protease inhibitors during enzyme preparation, then we observed that the ability of the selective inhibitors to inhibit PDE-II activity was markedly reduced, with IC_{50} values increasing some 2–6-fold (Table 2). In contrast, their ability to inhibit PDE-III was unchanged (Table 2). In order to assess whether the altered inhibitory potency towards PDE-II was due to proteolytic action we investigated the effect of treating the PDE-II enzyme, which has been prepared in the presence of protease inhibitors, with the protease trypsin. Such a treatment not only caused a loss of enzyme activity (Fig. 3) but also markedly affected the ability of milrinone to inhibit the protease-treated enzyme (Fig. 4). Indeed, treatment with trypsin was about twice as effective at reducing the inhibitory potency of milrinone than of abolishing enzyme activity. The proteolytic attenuation of inhibitor action was not confined to milrinone, as we noted that trypsin treatment could elicit dramatic reductions in the inhibitory potency of a wide variety of "selective" phosphodiesterase inhibitors (Fig. 5). We suggest that such results indicate that the reduced inhibitory sensitivity of PDE-II, isolated in the absence of protease inhibitors, is due to the partial proteolysis of this enzyme during its purification. As shown with our experiments on milrinone (Fig. 4), the extent of proteolysis will determine the degree of attenuation of action of such inhibitors. Thus, one might expect that if preparations are performed in the absence of protease inhibitors then results will vary depending upon the precise method and time of preparation. Indeed, if instead of completing isolation of the enzymes within a morning, a whole day was taken then, then in the absence of protease inhibitors we noted that the IC_{50} for inhibition of PDE-II amrinone could rise to a value of ca. $107 \mu M$ whereas that for PDE-III was unchanged. Proteolysis of PDE-II may thus account for the fact that Kariya and co-workers [18]

observed, using cardiac tissue, that cyclic AMP phosphodiesterase activity of PDE-II was only some 10% of that of PDE-III and that amrinone exerted little inhibitory effect on this, presumably residual, activity.

We suggest, therefore, that the examination of cyclic AMP phosphodiesterases should be performed on enzymes isolated using protease inhibitors. Our results indicate that the "selective" cyclic AMP phosphodiesterase inhibitors can potently inhibit the activities of PDE-II and PDE-III with similar efficiency. It may be that such compounds can modulate physiological processes not only by inhibiting the activity of PDE-III but also by inhibiting the activity of PDE-II. In contrast to the canine cardiac PDE-II activity described here, PDE-II in rat liver [7] and bovine cardiac tissue [26] appears to be stimulated by low concentrations of cyclic GMP and displays kinetics indicative of positive co-operativity. This suggests that there may be distinct species differences between PDE-II enzymes. Indeed, this contention is supported by the demonstration that both the physical properties and kinetics of hydrolysis of cyclic GMP for the rat and bovine PDE-II enzyme are very different [7, 26]. In marked contrast to this, the kinetic properties of the PDE-III enzymes from rat, bovine and canine sources appear to be very similar. As it has been noted [10] that distinct species-dependent effects occur using various compounds which can exert positive inotropic actions, it is tempting to suggest that PDE-II may be an important site of action of such compounds.

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