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RESOLUTION OF SOLUBLE CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ISOENZYMES, FROM LIVER AND HEPATOCYTES, IDENTIFIES A NOVEL IBMX-INSENSITIVE FORM

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Abstract—DEAE chromatography of a high speed supernatant fraction from a homogenate of rat liver, prepared under isotonic conditions in the presence of protease inhibitors, yielded three peaks of cyclic nucleotide phosphodiesterase activity (PDE activity). The first peak could be resolved on Affi-gel Blue chromatography to yield a Ca^{2+}/calmodulin stimulated cyclic GMP specific PDE and a cyclic AMP and cyclic GMP hydrolysing PDE whose activity was insensitive to Ca^{2+}/calmodulin. These two activities could also be clearly resolved by Mono-Q chromatography of soluble extracts from both liver and hepatocytes. These had different molecular weights, kinetics of substrate utilization, thermostabilities, dependence on Mg^{2+} and inhibitor sensitivities. The cyclic AMP and cyclic GMP utilizing PDE resolved in these procedures appears to be a novel enzyme form (PDE-MQ-I) which is insensitive to inhibition by the so-called non-selective PDE inhibitor IBMX and displays catalytic activity in the absence of Mg^{2+}. None of the inhibitors tested were capable of inhibiting this form showing that the catalytic activity of this species could be distinguished from all the other soluble activities. This novel enzyme hydrolysed both cyclic AMP and cyclic GMP with K_m values of 25 μM and 237 μM, respectively. The V_{max} ratio of hydrolysis of cyclic GMP/cyclic AMP was above unity (1.4). It accounted for 30% of the soluble cyclic AMP PDE activity and 10% of the cyclic GMP PDE activity assessed at 1 μM substrate. Gel filtration of PDE-MQ-I indicated a size of 33,150 Da, in contrast to the size of 237,500 Da observed for the Ca^{2+}/calmodulin PDE-MQ-II. Thermal inactivation of PDE-MQ-I and PDE-MQ-II yielded single exponential decays with $t_{\frac{1}{2}}$ values of 6.33 min and 0.7 min at 60° respectively. In the presence of saturating Ca^{2+}, PDE-MQ-II was activated by calmodulin with an EC_{50} of *ca.* 30 ng/ml. In the presence of calmodulin, PDE-MQ-II was activated by Ca^{2+} with an EC_{50} of *ca.* 20 μM. Chromatography of homogenates on Mono-Q also identified a cyclic GMP-activated cyclic nucleotide PDE (PDE-MQ-III) and two cyclic AMP specific activities (PDE-MQ-IV and PDE-MQ-V). These exhibited very different inhibitor sensitivities and could be readily distinguished using the compound Ro-20-1724 which yielded IC_{50} values for inhibition of >500 μM, 13 μM and 1.5 μM, respectively, for the hepatocyte enzymes. PDE-MQ-III to -V exhibited K_m values for the hydrolysis of cyclic AMP of 37.7 μM, 0.62 μM and 0.62 μM and Hill coefficients of 1.62, 0.69 and 1.01, respectively. Rolipram was found to potently inhibit both the cyclic AMP specific forms. The compound ICI 118233 failed to inhibit any of the isolated soluble phosphodiesterases. A similar multiplicity of phosphodiesterase species was observed in the soluble fraction from both rat liver homogenates and isolated hepatocytes. We suggest that care be taken in interpreting experiments performed to assess adenylate cyclase activity in intact cells by monitoring intracellular cyclic AMP accumulation in the presence of IBMX due to the possible presence of an IBMX-insensitive phosphodiesterase.

Multiple forms of cyclic nucleotide phosphodiesterase have been demonstrated in a wide range of tissues, including heart [1], adipose tissue [2] and brain [3]. Various techniques have been employed in the separation of phosphodiesterase isoenzymes, with ion exchange chromatography being the most widely used of these. Resolved peaks of cyclic nucleotide phosphodiesterase activity have been characterized on the basis of substrate specificity, sensitivity to agents such as calcium and calmodulin, kinetic parameters and response to various phosphodiesterase inhibitors [4]. Studies of this nature have provided strong evidence for the existence of functionally distinct enzyme forms and this, in turn, raises the possibility that each form may have a unique role in the regulation of intracellular cyclic AMP levels. However, in no single tissue has a thorough investigation been made in order to define in full the range of phosphodiesterase isoenzymes associated with both the soluble cytosol and membrane fractions.

In liver, we have attempted to analyse the phosphodiesterase activities associated with the various membrane fractions. Using Percoll fractionation [5], we were able to show that phosphodiesterase activity was associated with all membrane fractions except lysosomes and the nucleus. The activities in the plasma membrane fraction [6], the mitochondrial fraction [7] and the endoplasmic reticulum subfractions [8, 9] have been identified and characterized. A number of these particulate phosphodiesterase iso-enzymes have been studied in some detail. These include two cyclic AMP-specific forms: the plasma membrane phosphodiesterase [10, 11] and the 'dense vesicle' phosphodiesterase [11–14], both of which are stimulated and regulated by hormones [5]. In comparison to such analyses the cyclic nucleotide

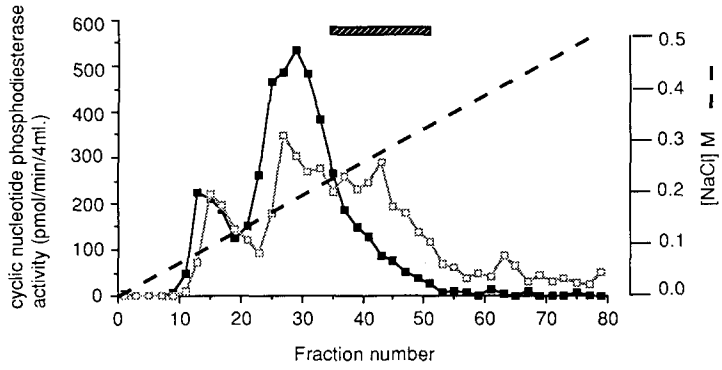

Fig. 1. DE-52 elution profile of rat liver soluble PDE activity. High speed supernatant of rat liver (250 mg of protein) was chromatographed on a DE-52 column as described in Materials and Methods and 4 ml fractions were collected. Fractions were assayed for cyclic nucleotide PDE activity as described in Materials and Methods. One micromolar cyclic AMP substrate is represented by open symbols (□) while closed symbols (■) depict a substrate of 1 μM cyclic GMP. The linear 0–0.5 M NaCl gradient is shown as a broken line. The horizontal hatched rectangle delineates the elution peak of endogenous calmodulin. Yield of protein in the peaks was as follows, I = 29 mg, II = 37 mg, and III = 11 mg.

phosphodiesterase activity of the soluble fraction of liver has received little attention. A crude separation of the phosphodiesterase activity of rat liver soluble fractions has been performed using DEAE chromatography which identified three peaks of activity (labelled PDE-I*, PDE-II and PDE-III, based upon the order of their elution [15]). However, recent studies on soluble enzymes in cardiac and adrenal tissues [4, 16] have implied that the complement of enzymes in the soluble fractions may be more complex than originally surmised from crude separations performed on DEAE chromatography. Furthermore, earlier investigators used hypotonic extraction conditions and sonication (see e.g. Refs 17–19), which have been shown to release membrane-bound phosphodiesterases from liver [6, 12, 13]. Also, protease inhibitors, which are essential to prevent modification of phosphodiesterases [9, 19–21], were not added. With this in mind we have undertaken to resolve and characterize the cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities from the soluble fraction of rat liver under isotonic conditions and in the presence of protease inhibitors.

MATERIALS AND METHODS

Alcohol dehydrogenase, benzamidine hydrochloride, bovine serum albumin, carbonic anhydrase, cyclic AMP, cyclic GMP, cytochrome *c*, Dowex 1X8-400 (chloride form, 200–400 mesh), EGTA, ferritin, 3-isobutyl 1-methylxanthine, ovalbumin, PMSF, Sephacryl S-200 and S-300, snake venom (*Ophiophagus hannah*), bovine brain calmodulin, soybean trypsin inhibitor (Type II-S) and Tris were from Sigma Chemical Co. (Poole, U.K.). Collagenase (150 mU/mg) was from Worthington (distributed by Cambridge BioScience, Cambridge, U.K.). [^{3}H]-cyclic AMP and [^{3}H]-cyclic GMP were from Amersham International (Amersham, U.K.). DEAE-cellulose DE-52 was from Whatman Biochemicals (Maidstone, U.K.). Mono-Q-HR 5/5 column was from Pharmacia A.B. Biotechnology (Uppsala, Sweden). Affi-gel Blue was from Bio-Rad (Watford, U.K.). Leupeptin was from Peptide Research Foundation (distributed by Scientific Marketing Associates, London, U.K.). Dithiothreitol was from Boehringer (U.K.) Ltd (Lewes, U.K.). Dimethyl sulphoxide was from Koch-Light Ltd (Haverhill, U.K.). Milrinone was a gift from Stirling-Winthrop Ltd (Guildford, U.K.), ICI 118233 was a kind gift from Dr M. Collis, ICI Pharmaceuticals (Alderley Park, Macclesfield, U.K.) and Ro 20-1724 was a gift from Roche Products Ltd (Welwyn Garden City, U.K.).

Preparation of high speed supernatant from rat liver homogenates. Liver was excised from one male Sprague–Dawley rat (250–275 g), rinsed several times in ice cold homogenizing Buffer 'A' (50 mM Tris/HCl pH 7.5, 0.25 M sucrose, 5 mM benzamidine, 0.2 mM PMSF, 20 μM leupeptin, 0.1 mM EGTA, and 0.1 mM dithiothreitol). The tissue was diced and homogenized using five up/down strokes (setting 5) at approximately 1:4 (v/v) and passed through two layers of muslin. The final volume of the homogenate was 40–50 ml. All subsequent operations were carried out at 4°. The homogenate was centrifuged for 10 min at 706 g_{av} (Beckman JA-20 rotor) to obtain a low speed pellet and supernatant. The latter was recentrifuged at 163,570 g_{av} for 60 min to obtain the high speed supernatant and pellet. The supernatant was carefully decanted avoiding any fat deposits and the pellet discarded. Phosphodiesterase activity was fractionated either on DE-52 or Mono-Q ion exchange columns.

Preparation of high speed supernatant from hepatocytes. Hepatocytes were prepared from one male

* Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; Ro-20-1724, [4-(3-butoxy-4-methoxybenzyl)-2-imidazoline]; milrinone, 1,2-dihydro-6-methyl-oxo-5-(4-pyridyl)-nicotinonitrile; ICI 118233, 6-[*p*-(3-methylureido)phenyl]-3[2H]-pyridazinone; PDE, phosphodiesterase.

Table 1. Effects of agents on Mono-Q phosphodiesterase peaks

Peak	Enzyme source	Ca^{2+}/calmodulin on cAMP PDE	Cyclic GMP on cAMP PDE	Ca^{2+}/calmodulin on cGMP PDE
I	Liver	1.13 ± 0.06 (N = 4)	0.90 ± 0.09 (N = 4)	1.72 ± 0.16* (N = 3)
	Hepatocyte	0.91 ± 0.06 (N = 3)	1.09 ± 0.09 (N = 3)	1.28 ± 0.06* (N = 4)
II	Liver	4.77 ± 1.01* (N = 5)	1.10 ± 0.17 (N = 5)	2.16 ± 0.21* (N = 4)
	Hepatocyte	3.83 ± 0.35* (N = 3)	0.93 ± 0.09 (N = 3)	2.26 ± 0.20* (N = 3)
III	Liver	1.09 ± 0.15 (N = 4)	6.32 ± 1.86* (N = 3)	1.04 ± 0.07 (N = 3)
	Hepatocyte	1.05 ± 0.09 (N = 3)	5.92 ± 0.39* (N = 3)	1.09 ± 0.14 (N = 3)
IV	Liver	1.20 ± 0.13 (N = 6)	0.90 ± 0.08* (N = 5)	ND
	Hepatocyte	1.23 ± 0.13 (N = 3)	0.81 ± 0.09 (N = 3)	ND
V	Liver	0.98 ± 0.04 (N = 5)	0.83 ± 0.06* (N = 5)	ND
	Hepatocyte	1.22 ± 0.01* (N = 3)	0.77 ± 0.03* (N = 3)	ND

Results are the means ± SE, with N being the number of experiments. Values shown are fold-effects relative to control in the absence of agent. Phosphodiesterase activity of pooled peaks was measured as described in Materials and Methods, at substrate concentrations of 1 μM cyclic nucleotide. Final assay concentrations of Ca^{2+} and calmodulin were 50 μM and 2 μg/ml respectively, while the cyclic GMP concentration used was 2 μM. An asterisk (*) indicates that the effect of the agent was significantly different from control, with $P < 0.05$. ND indicates that while enzyme activity was too low for an accurate detection of small effects, no gross effects of Ca^{2+} and calmodulin were detectable.

Sprague–Dawley rate (240–260 g) essentially as described by us previously [5] except that Ca^{2+}-free Krebs buffer was used throughout the perfusion and trypsin inhibitor (0.01% w/v) was included during collagenase digestion. Cell viability was determined by the Trypan Blue exclusion method and was >90%.

Cells were washed three times in Ca^{2+}-free Krebs buffer by repeated resuspension and pelleting at 600 rpm (MSE Centaur) for 2 min (three 80 ml washes per 10 ml of packed cells). Washed cells were homogenized in ice cold Buffer 'A' (1:4 v/v) using 15 up/down strokes in a tight-fitting pestle. The final volume of homogenate was approximately 40 ml. A high speed supernatant was prepared exactly as for rat liver supernatants.

Fractionation of hepatocytes. Hepatocyte P1 and S1 fractions were prepared as for rat liver. The P1 fraction was washed to release any trapped soluble activity by resuspending in ice cold Buffer 'A' (1:4 v/v) using five up and down strokes in a tight-fitting pestle. This was centrifuged to produce a further S1 and P1 fraction. This procedure was repeated once more to yield a washed P1 fraction and three S1 fractions. These were spun to yield S2 and P2 fractions.

DEAE-cellulose ion exchange chromatography. Approximately 35 ml of high speed supernatant prepared from rat liver was loaded onto a column of DE-52 (3.0 cm × 1.4 cm) which had been previously equilibrated in Buffer 'B' (50 mM Tris/HCl at final pH 7.5, 5 mM benzamidine, 0.2 mM PMSF, 2 μM leupeptin, 0.1 mM EGTA and 0.1 mM dithiothreitol). The column was loaded with this supernatant fraction at 1.5 ml/min and was then washed with approximately 100 ml of Buffer 'B' at 1 ml/min. Under these conditions >95% of the phosphodiesterase activity (assessed at 1 μM cyclic AMP or cyclic GMP) bound to the column. Phosphodiesterase activity was subsequently eluted at 1 ml/min using a 400 ml linear salt gradient between 0 and 0.5 M NaCl contained in Buffer 'B' at pH 7.5.

Phosphodiesterase assay. Cyclic nucleotide phosphodiesterase activity was assayed by a modification of the two-step procedure of Thompson and Appleman [3] and of Rutten *et al.* [22] as described by us previously [6]. In all cases we used a freshly prepared slurry of Dowex:H_2O:ethanol (1:1:1) for determination of activities. All assays were done at 30° and initial rates were taken from linear time courses.

Assay of endogenous calmodulin. Fractions of column eluate were boiled for 5 min in order to destroy any endogenous phosphodiesterase activity, whilst retaining heat-stable calmodulin. Aliquots (10 μl) were assayed for their ability to stimulate calmodulin-deficient phosphodiesterase activity (PDE-MQ-II) in the presence of 100 μM Ca^{2+}.

Chromatography of Peak I (DE-52) on Affi-gel Blue. Pooled fractions from Peak I of the DE-52 chromatography step were made up to 5 mM $MgCl_2$ and loaded at 0.5 ml/min onto a column of Affi-gel Blue (4 cm × 0.5 cm) which had been previously equilibrated in Buffer 'B' containing 5 mM $MgCl_2$ and 0.15 M NaCl. Total unbound protein was collected and the column was washed in Buffer 'B' (8 bed volumes) and the total wash collected. The phosphodiesterase activity of the unbound and washed fractions was determined.

Mono-Q ion exchange chromatography. High speed supernatants from either rat liver (20 ml) or hepatocytes (30 ml) were loaded at 1 ml/min onto a Mono-Q ion exchange system which had been previously been equilibrated with Buffer 'B'. The column was washed in Buffer 'B' (approximately 1 bed volume). Under these conditions greater than 95% of the phosphodiesterase activity (assessed at 1 μM cyclic AMP or cyclic GMP) bound to the column. Phosphodiesterase activity was then eluted at 1 ml/min using a 95 ml gradient of NaCl (0–0.5 M) in Buffer 'B'. Fractions of 1 ml were collected on

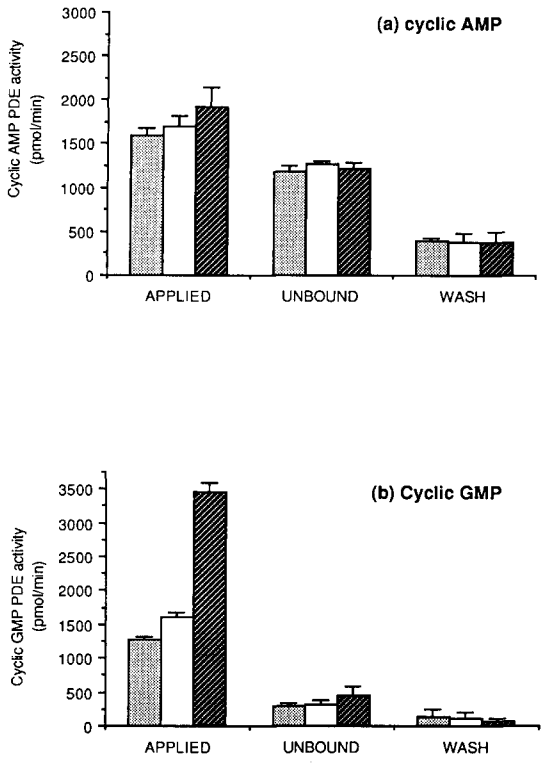

Fig. 2. Chromatography of DE-52 Peak I on Affi-gel Blue. DE-52 Peak I from rat liver was chromatographed on an Affi-gel Blue column as described in Materials and Methods. Samples of the applied peak, the unbound 'flow-through' and wash were assayed for cyclic nucleotide phosphodiesterase activity as described in Materials and Methods using either (a) 1 μM cyclic AMP or (b) 1 μM cyclic GMP as substrate. The activities shown are the means ± SD of triplicate determinations from a single representative experiment. The shaded bars (▨) represent control activity, open bars (□) represent PDE activity measured in the presence of 50 μM $CaCl_2$ and hatched bars (▧) the activity in the presence of 50 μM $CaCl_2$ and 2 μg/ml calmodulin.

ice and assayed for phosphodiesterase activity. The column was immersed in an ice jacket for the duration of the experiment.

Binding of Mono-Q peaks I *and* II *to Affi-gel Blue.* Pooled fractions from each of the first two peaks (PDE-MQ-I and PDE-MQ-II) of the Mono-Q colume step were each made up to 5 mM $MgCl_2$ and individually incubated with portions of Affi-gel Blue. This was done by mixing 0.75 ml of each peak with 100 μl (packed volume) of Affi-gel Blue gel matrix for 30 min at 4° on a rotary mixer. The gel was pelleted using a microcentrifuge at maximum speed (14,000 g_{av}) and the supernatant containing unbound material removed. The pelleted gel was washed by adding 1 ml of Buffer 'B' containing 5 mM $MgCl_2$ and mixing for a further 10 min and pelleting as before. This was repeated once more and the two washes were pooled to give a combined wash (2 ml). Phosphodiesterase activity of both the unbound and the wash fractions was determined. Total unbound activity was determined by summing the activity from the binding step with the activity retrieved by washing.

Effect of Ca^{2+}*/calmodulin and cyclic GMP on phosphodiesterase activity.* Fractions were carefully pooled from the mid-point of peaks to minimize any cross contamination from neighbouring activities. Generally three or four fractions (3 or 4 ml) were chosen for study and these were diluted where necessary in Buffer 'B'. Activities were determined in the presence of 50 μM $CaCl_2$ and 2 μg/ml calmodulin or 2 μM cGMP. In all cases the final concentration of EGTA in the assay was 25 μM.

Determination of IC_{50} *values for phosphodiesterase inhibitors on resolved activities.* Phosphodiesterase inhibitors were dissolved in DMSO at a stock concentration of 25 mM and subsequently diluted in Buffer 'B' to provide a range of drug concentrations. DMSO was found to have either no or a small inhibitory effect (<15%) on the resolved phosphodiesterase activities. In instances where small inhibitory effects were noted then appropriate corrections were made to allow assessment of the inhibitory potency of the drugs. It was assumed that the effects of DMSO and the drugs were additive. IC_{50} values were determined at 1 μM cyclic AMP from dose–response curves over a range of 0.1 μM to 500 μM of the compounds using a minimum of three drug concentrations for insensitive forms and five to seven for sensitive forms. The phosphodiesterase assays were performed in duplicate.

Determination of kinetic parameters for rat hepatocyte Peaks I–V. Peak I from the Mono-Q column was subjected to a further chromatographic step to minimize any possible residual contaminating activity from Peak II. Pooled fractions from the ion exchange step were made up to 5 mM $MgCl_2$ and applied at 40 ml/hr to a column of Affi-gel Blue (2.2 cm × 0.76 cm) previously equilibrated in Buffer 'B' containing 5 mM $MgCl_2$ and 0.15 M NaCl. Total unbound activity was collected and the column washed with four 2 ml washes of Buffer 'B'/5 mM $MgCl_2$/0.15 M NaCl. Washes containing the majority of the PDE activity were pooled with the unbound activity and used in the kinetic experiments. Peaks II–V, from the Mono-Q step, were used without any further purification. The final Mg^{2+} concentration in the assay was 10 mM for all kinetic experiments. Kinetic parameters were determined using nineteen concentrations of cyclic nucleotide from 30 nM to 100 μM (Peaks IV and V) and 0.1 μM to 1 mM (Peaks I–III). All assays were done in triplicate.

Velocities were determined from linear time courses (up to 10 min) and no more than 20% of substrate was hydrolysed. Kinetic data was obtained from Hill plots and, in instances where limiting K_m values are quoted, also from Lineweaver–Burk plots and parameters were determined after subjecting the data to least-squares analysis.

Determination of molecular weights by gel filtration. The molecular weight of rat hepatocyte Peaks I and II were determined using gel filtration on either Sephacryl S-200 for Peak I or S-300 for Peak II. Samples of pooled Peaks I and II were applied to S-200 (51 cm × 1.5 cm) and S-300 (83 cm × 1.4 cm) columns respectively, previously equilibrated in 50 mM Tris/HCl pH 7.4, 0.1 M NaCl and 0.1 mM EGTA. Columns were run at 8 ml/hr

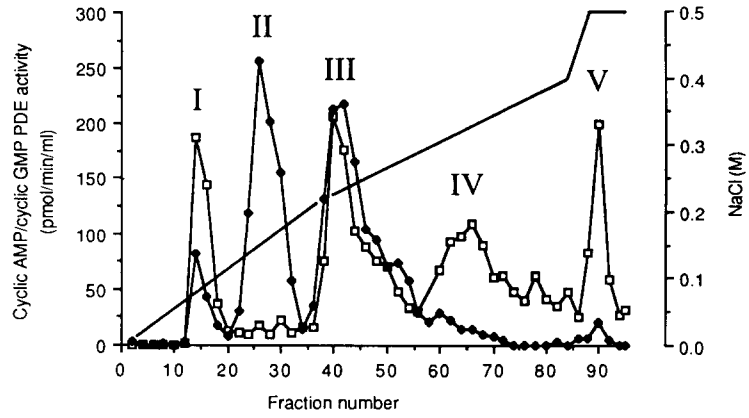

Fig. 3. Mono-Q ion exchange profile of rat liver soluble PDE activity. High speed supernatant of rat liver (120 mg of protein) was chromatographed on a Mono-Q column as described in Materials and Methods and 1 ml fractions were collected. Fractions were assayed for cyclic nucleotide PDE activity as described in Materials and Methods. One micromolar cyclic AMP substrate is represented by open symbols (□) while closed symbols (■) depict a substrate of 1 μM cyclic GMP. The 0–0.5 M NaCl gradient is shown as a solid line. The letters indicate the occurrence of peaks PDE-MQ-I to -V. The yield of protein was as follows, MQ-I = 1.62 mg, MQ-II = 1.14 mg, MQ-III = 0.525 mg, MQ-IV = 0.098 mg and MQ-V = 0.110 mg.

in this buffer and fractions were collected and assayed for phosphodiesterase activity. Columns were calibrated using proteins of known molecular weight, i.e. cytochrome *c* (12,400), carbonic anhydrase (29,000), ovalbumin (45,000), bovine serum albumin (66,000), alcohol dehydrogenase (150,000), catalase (232,000) and ferritin (440,000).

RESULTS

Distribution of cyclic nucleotide phosphodiesterase activity in rat hepatocytes

Fractionation of rat hepatocytes into a washed P1 fraction and S2 and P2 fractions followed by an analysis of the phosphodiesterase activities contained in these fractions indicated that 50% of the cyclic AMP (7576 pmol/min out of 15,183 pmol/min) and 45% of the cyclic GMP PDE activity (11,749 pmol/min out of 26,619 pmol/min) of the homogenate was contained in the soluble fraction. The yield of cyclic AMP PDE activity during the fractionation was 108% and for cyclic GMP PDE was 89%. Calmodulin activated cGMP PDE activity was only detected in the soluble fraction (1.4-fold stimulation) whilst cyclic GMP activated cyclic AMP hydrolysis could be detected in all of the fractions prepared.

Chromatography of rat liver soluble fraction on DE-52 column

Three peaks of cyclic nucleotide hydrolysing activity were eluted from a DE-52 column by a 0 to 0.5 M linear NaCl gradient (Fig. 1). The first peak hydrolysed both cyclic AMP and cyclic GMP. Phosphodiesterase activity expressed by the first peak assayed with either cyclic AMP or cyclic GMP as substrate, was stimulated upon the addition of Ca^{2+} and calmodulin together, but not when Ca^{2+} was added alone (Fig. 2). Similarly, the addition of calmodulin alone had no effect upon activity of this enzyme. The stimulation of cyclic AMP phosphodiesterase activity by Ca^{2+}/calmodulin (at 1 μM substrate concentration) was small (Fig. 2) in contrast with the marked stimulation of cyclic GMP phosphodiesterase (1 μM substrate) seen with saturating Ca^{2+}/calmodulin.

The second peak of phosphodiesterase activity which eluted from the column also hydrolysed both cyclic nucleotides. In this case the cyclic AMP hydrolysing activity, assayed at 1 μM substrate, was stimulated approximately two-fold by 2 μM cyclic GMP. This peak was not stimulated by Ca^{2+}/calmodulin.

The third peak was cyclic AMP-specific in that no detectable activity was observed using cyclic GMP as a substrate. Its activity was unaffected by either the addition of Ca^{2+}/calmodulin or low concentrations (2 μM) of cyclic GMP.

These eluted activities accounted for over 95% of the applied cyclic nucleotide phosphodiesterase activity and increasing the NaCl concentration to 0.8 M did not result in the elution of further activity.

Chromatography of the first peak, eluting from the DE-52 column, on Affi-gel Blue resulted in the separation of two distinct phosphodiesterase activities, one of which was highly specific for cyclic GMP as a substrate and the other of which accounted for the majority of the cyclic AMP phosphodiesterase activity in the first fraction form the DE-52 column. In a representative experiment, whilst only 3% of the cyclic AMP phosphodiesterase remained bound to the Affi-gel Blue column, over 70% of the cyclic GMP phosphodiesterase activity was retained. It was evident, however, that >95% of the Ca^{2+}/calmodulin-stimulated cyclic AMP and cyclic GMP phosphodiesterase activity was bound to this Affi-gel Blue column (Fig. 2).

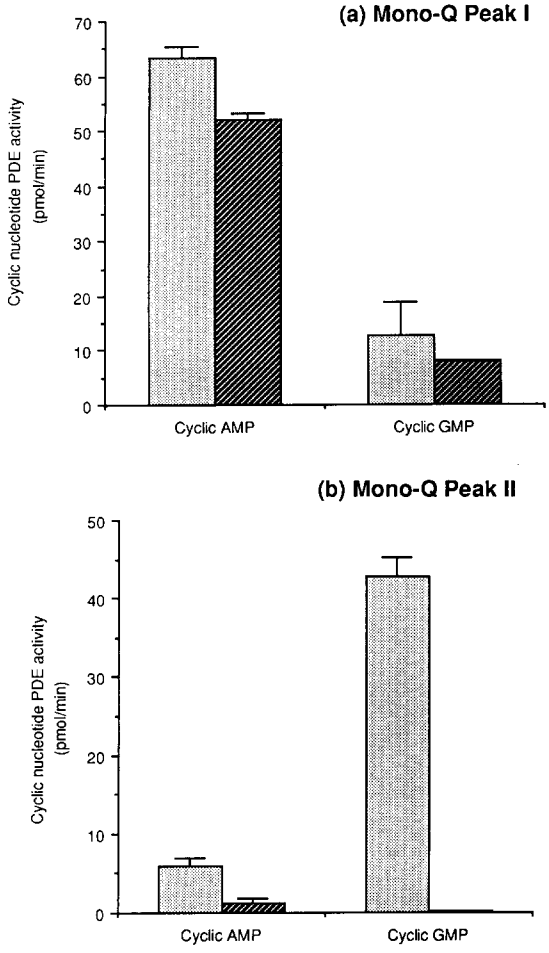

Fig. 4. Chromatography of Mono-Q Peaks I and II on Affi-gel Blue. Mono-Q Peaks I and II were chromatographed on an Affi-gel Blue column, with the applied and unbound samples being assayed for cyclic nucleotide PDE activity (both procedures as described in Materials and Methods). Shaded bars (▨) represent the total PDE activity applied to the column while hatched bars (▧) represent the unbound activity. The activities shown are the means ± SD of triplicate determinations from a single representative experiment.

Chromatography of rat liver soluble fraction on Mono-Q column

The soluble fraction from rat liver, prepared under identical conditions to the DE-52 experiments, was subjected to chromatography on a Mono-Q anion exchange column. Greater than 95% of the cyclic AMP phosphodiesterase and the cyclic GMP phosphodiesterase activities of the supernatant fraction (assayed at 1 μM substrate concentration) routinely bound to this column.

A sodium chloride gradient of 0 to 0.5 M, contained in Buffer 'B', was used to elute the bound phosphodiesterase activity, with greater than 95% of the total activity being retrieved in the various fractions. A number of different experiments were carried out to optimize the shape of the gradient which gave the clearest separation of peaks and Fig. 3 shows a typical profile assayed with both 1 μM cyclic AMP and 1 μM cyclic GMP.

Five major peaks of activity were eluted, called PDE-MQ I–V inclusive. These represented >95% of the applied activities and we noted that no further phosphodiesterase activity was eluted upon increasing the NaCl concentration to 1 M in the elution buffer.

The ability of Ca^{2+}/calmodulin to alter the activity of these enzymes was assessed as was the effect of cyclic GMP on their ability to hydrolyse cyclic AMP (Table 1).

The first peak (PDE-MQ-I) was a predominantly cyclic AMP-hydrolysing activity and constituted a significant proportion of the total activity of the high speed supernatant. The cyclic GMP phosphodiesterase activity of this peak was stimulated on average some 0.7-fold by Ca^{2+}/calmodulin.

The second peak (PDE-MQ-II) hydrolysed cyclic AMP very poorly, but the cyclic GMP phosphodiesterase activity of this peak accounted for a substantial proportion of the total cyclic GMP phosphodiesterase activity of the high speed supernatant. Ca^{2+}/calmodulin caused a two-fold stimulation of the cyclic GMP phosphodiesterase activity of Peak II, and greater than four-fold stimulation of the cyclic AMP activity. However, even following Ca^{2+}/calmodulin stimulation Peak II still only represented a small fraction (*ca.* 5%) of the total cyclic AMP activity of rat liver high speed supernatant.

The third peak (PDE-MQ-III) hydrolysed both cyclic AMP and cyclic GMP; the cyclic AMP phosphodiesterase activity was stimulated some six-fold by 2 μM cyclic GMP.

The fourth (PDE-MQ-IV) and fifth (PDE-MQ-V) peaks were specific for cyclic AMP as substrate and their activities were insensitive to Ca^{2+}/calmodulin. Two micromolar cyclic GMP caused a small (10–17%), statistically significant ($P < 0.05$) inhibition of the cyclic AMP phosphodiesterase activity of both peaks when assayed with 1 μM cyclic AMP as substrate. Nevertheless, cyclic GMP did not appear to act as a substrate, with no detectable activity being observed.

Chromatography of rat liver Mono-Q Peaks I *and* II *on Affi-gel Blue*

PDE-MQ-I from the Mono-Q column behaved very differently from PDE-MQ-II when it was chromatographed on Affi-gel Blue (Fig. 4). Thus, the majority of the cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activity of PDE-MQ-I was not bound to this gel. In contrast, virtually all of the cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activity of PDE-MQ-II bound to Affi-gel Blue. Greater than 95% of the Ca^{2+}/calmodulin-stimulated cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activity of PDE-MQ-II remained bound to the column.

Chromatography of rat hepatocyte soluble fraction on Mono-Q column

Chromatography of rat hepatocyte high speed supernatant of a Mono-Q column, using experimental conditions identical to those used for rat liver supernatants, resulted in a broadly similar elution profile to that seen with rat liver supernatant (Fig. 5). Again, five major peaks of activity were

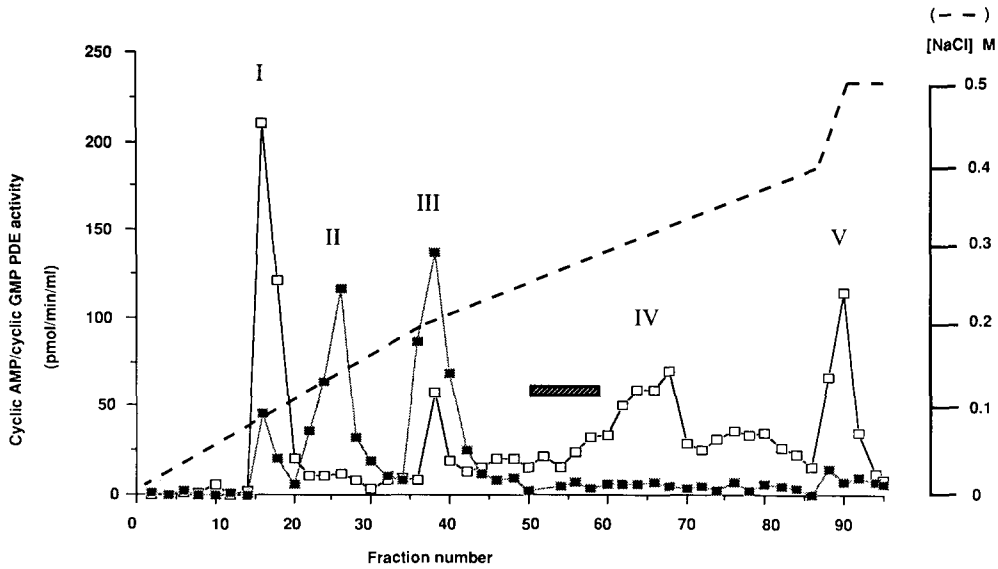

Fig. 5. Mono-Q ion exchange profile of rat hepatocyte soluble phosphodiesterase activity. High speed supernatant of rat hepatocyte (80 mg of protein) was chromatographed on a Mono-Q column as described in Materials and Methods and 1 ml fractions were collected. Fractions were assayed for cyclic nucleotide PDE activity as described in Materials and Methods. One micromolar cyclic AMP substrate is represented by open symbols (□) while closed symbols (■) depict a substrate of 1 μM cyclic GMP. The 0–0.5 M NaCl gradient is shown as a broken line. The horizontal hatched rectangle delineates the elution peak of endogenous calmodulin. The yield of protein for the various peaks was as follows, MQ-I = 0.744 mg, MQ-II = 1.944 mg, MQ-III = 0.9 mg, MQ-IV = 0.923 mg and MQ-V = 0.123 mg

observed. The major difference between liver and hepatocyte profiles was that the cyclic AMP phosphodiesterase activity of PDE-MQ-III was substantially reduced in hepatocyte profiles compared to those from liver. The ratios of cyclic GMP phosphodiesterase to cyclic AMP phosphodiesterase activities for PDE-MQ-III were 2.2 ± 0.4 (mean ± SE, N = 4) for liver and 4.7 ± 0.7 (mean ± SE, N = 4) for hepatocyte.

As with the activities resolved by Mono-Q chromatography of rat liver soluble fractions, the activity from the soluble fraction from hepatocytes (Table 1; Fig. 5) showed that PDE-MQ-II was the Ca^{2+}/calmodulin-stimulated enzyme, PDE-MQ-III was the GMP-stimulated enzyme and PDE-MQ-IV and PDE-MQ-V the cyclic AMP-specific enzymes. Importantly, the PDE-MQ-1 enzyme was present indicating that it was not from a cell type distinct from hepatocytes. This activity accounted for 30 ± 5% (N = 3) of the total soluble cAMP PDE activity and 10 ± 3% (N = 3) of the cyclic GMP PDE activity from hepatocytes.

Using cyclic AMP as a substrate it was evident that PDE-MQ-III could be activated by low concentrations of cyclic GMP in the assay (Table 1; Fig. 6), with an EC_{50} value of 0.47 ± 0.01 μM for this process. Higher concentrations of cyclic GMP, however, lead to the dose-dependent inhibition of this enzyme activity, with an IC_{50} of 53 ± 10 μM (Table 3; Fig. 6).

Characterization of rat hepatocyte peaks PDE-MQ-I and PDE-MQ-II

An analysis of substrate utilization (Table 2) by hepatocyte peaks PDE-MQ-I and PDE-MQ-II was

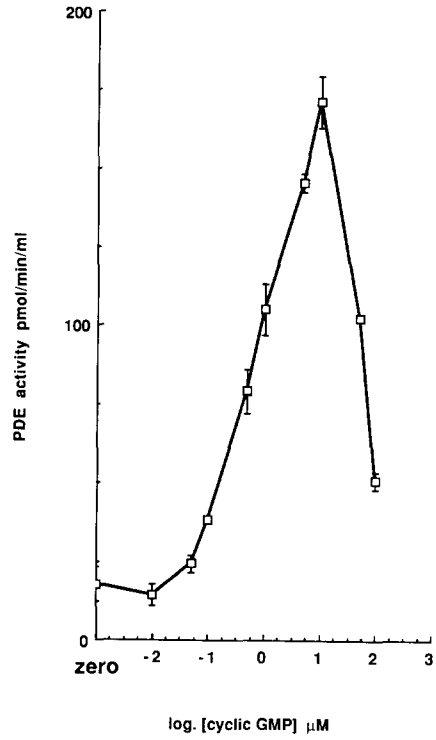

Fig. 6. The response of the hydrolysis of cyclic AMP by hepatocyte PDE-MQ-III to cyclic GMP. High speed supernatant of rat hepatocyte was chromatographed on a Mono-Q column as described in Materials and Methods and the PDE-MQ-III fraction isolated. Assays were done at 1 μM cyclic AMP in the presence of increasing concentrations of unlabeled cyclic GMP.

Table 2. Kinetic properties of rat hepatocyte PDE-MQ-I, II, III, IV and V

	Constants derived from Hill plots*				Limiting values of K_m†				
	cAMP		cGMP		cAMP		cGMP		
Peak	K_m (μM)	h	K_m (μM)	h	K_{m1} (μM)	K_{m2} (μM)	K_{m1} (μM)	K_{m2} (μM)	Observations
I	24.9 ± 5.9	0.97 ± 0.09	237 ± 56	1.04 ± 0.04	NA	NA	NA	NA	3
II	a	a	5.1 ± 1.7	0.72 ± 0.02	a	a	3.11 ± 0.36	12.87 ± 1.17	3
III	37.7 ± 6.7	1.62 ± 0.20	35.5 ± 6.9	1.22 ± 0.06	NA	NA	NA	NA	4
IV	0.62 ± 0.34	0.69 ± 0.05	a	a	0.36 ± 0.14	2.57 ± 1.6	a	a	3
V	0.62 ± 0.07	1.01 ± 0.13	a	a	NA	NA	a	a	4

Phosphodiesterase activities were resolved from rat hepatocyte high speed supernatants using a Mono-Q column. All values quoted for PDE-MQ-I were determined using an enzyme preparation which had been further chromatographed on Affi-gel Blue (as described in Materials and Methods). Kinetic parameters were determined as described in Materials and Methods. K_m values and Hill constants were calculated from linear Hill plots* or Lineweaver–Burk plots†. In instances where Lineweaver–Burk plots were downwardly curving, and thus indicative of apparent negative co-operativity, then limiting values for K_{m1} and K_{m2} were obtained from their linear extremae. In all instances triplicate determinations of phosphodiesterase activity were performed at 19 substrate concentrations over the ranges specified in Materials and Methods.

h, Hill coefficient; a, extremely low activity with this cyclic nucleotide as substrate (<4% with the other), thus analysis not done; NA, not applicable.

carried out. PDE-MQ-I displayed linear kinetics for both cyclic AMP and cyclic GMP hydrolysis with K_m values of 25 μM and 237 μM for cyclic AMP and cyclic GMP respectively. The V_{max} ratio for cyclic GMP/cyclic AMP indicated that the activity had a slightly higher V_{max} value for cyclic GMP compared to cyclic AMP. Since the preparations used were impure, absolute V_{max} values could not be determined. PDE-MQ-II, the Ca^{2+}/calmodulin-stimulated enzyme, displayed non-linear kinetics. Evaluation of kinetic data by Hill plots indicated a K_m for cyclic GMP of 5 μM and a Hill coefficient of 0.72. The limiting values (K_{m1} and K_{m2}) determined from Lineweaver–Burk plots indicated values of 3.1 μM and 12.9 μM, respectively. The K_m for cyclic AMP could not be determined accurately due to the low cyclic AMP hydrolysing capacity of this peak (<4% of that seen with cyclic AMP).

Both peaks exhibited linear temperature inactivation profiles but displayed different $t_{\frac{1}{2}}$ values at 60°. Peak I had a $t_{\frac{1}{2}}$ of 6.3 min for decay of activity whilst Peak II had a $t_{\frac{1}{2}}$ of 0.7 min for decay of activity.

Determination of the molecular weight of PDE-MQ-I and PDE-MQ-II by gel filtration chromatography indicated that PDE-MQ-I had a molecular weight of 33,150 Da which differed markedly from that of PDE-MQ-II, 237,500 Da. Both PDE-MQ-I and PDE-MQ-II chromatographed as single activity peaks on gel filtration although losses in activity were routinely observed for both enzymes during this procedure.

Dose effect studies showed that the phosphodiesterase activity of PDE-MQ-I was insensitive to the addition of Mg^2 to the assay. This was in marked contrast to PDE-MQ-II and the other resolved presence of saturating Ca^{2+}, and Ca^{2+} activated this enzyme with an EC_{50} of 20 ± 2 μM at saturating calmodulin concentrations (Fig. 8).

The activity of hepatocyte PDE-MQ-I was insensitive to activation by Ca^{2+}, calmodulin or Ca^{2+}/calmodulin, whereas hepatocyte PDE-MQ-II was stimulated in a dose-dependent fashion by these two agents together (Fig. 8).

These results indicate that the activities residing in PDE-MQ-I and PDE-MQ-II contain single phosphodiesterase activities which have markedly different characteristics.

Sensitivity of soluble phosphodiesterase peaks to phosphodiesterase inhibitors

Further characterization of the phosphodiesterase activity peaks were carried out using six phosphodiesterase inhibitors IBMX, Ro 20-1724, ICI 118233, Milrinone, Rolipram and Zaprinast. IBMX is generally considered to be a non-specific inhibitor of phosphodiesterase activity whilst Ro 20-1724 shows some selectivity towards the cyclic AMP-specific enzyme forms [1, 23]. Milrinone is also found to be selective for cyclic AMP specific forms whilst ICI 118233 has been shown [11] to inhibit only the 'dense-vesicle' enzyme of all the membrane-bound cyclic AMP phosphodiesterases which occur in rat hepatocytes. Zaprinast has been shown to be selective for the cGMP phosphodiesterase in human lung [24] and Rolipram also shows selectivity for the cyclic AMP specific class of PDE [25]. Table 4 shows the concentrations of drug required for half-maximal inhibition (IC_{50}) of the cycle AMP phosphodiesterase activity of each peak from high speed supernatants of rat liver and rat hepatocyte.

PDE-MQ-I, was found to be insensitive to inhibition by any of the six agents. PDE-MQ-II was most sensitive to inhibition by Zaprinast and IBMX; the enzymes which required the presence of Mg^{2+} to elicit phosphodiesterase activity in a dose dependent fashion (Table 3; Fig. 7). Calmodulin activated this enzyme with an EC_{50} of 31.5 ± 0.85 ng/ml, in the least effective inhibitory agents being Ro 20-1724 and Rolipram. IBMX elicited the half-maximal inhibition of PDE-MQ-III at 97 μM when liver was the enzyme source and at 323 μM when hepatocytes were

Table 3. Effect of cGMP and Mg^{2+} on rat hepatocyte PDE MQ-I, II, III, IV, and V

Peak	EC_{50} Mg^{2+} (μM)	IC_{50} cGMP (μM)	EC_{50} cGMP (μM)	Observations
I	b	ND	NA	3
II	20 ± 2	NA	NA	3
III	193 ± 18	43.0 ± 9.8	0.466 ± 0.098	4
IV	50 ± 7	>500	NA	3
V	40 ± 6	>500	NA	4

Phosphodiesterase activities were resolved from rat hepatocyte high speed supernatants using a Mono-Q column. All values for PDE-MQ-I were determined using an enzyme preparation which had been further chromatographed on Affi-gel Blue (as described in Material and Methods). EC values for Mg^{2+} effects were determined using either 1 μM cyclic AMP (enzymes I, III, IV and V) or 1 μM cyclic GMP (enzyme II) as substrates.

ND, not determined; b, no effect on activity; NA, not applicable.

used as the source. However, PDE-MQ-III was very insensitive to inhibition by any of the other agents tested. PDE-MQ-IV and PDE-MQ-V, both being cyclic AMP-specific activities, each were potently inhibited by IBMX, Milrinone, and Rolipram. The most potent of these was rolipram, which exhibited IC_{50} values of 0.5 μM and 0.095 μM for PDE-MQ-IV and PDE-MQ-V, respectively. In contrast, Ro 20-1724 was a much more potent inhibitor of PDE-MQ-V than PDE-MQ-IV. This was especially evident when liver was used as the enzyme source (Table 4). ICI 118233 failed to inhibit any of these enzyme fractions.

DISCUSSION

In a number of tissues and cells including adipose [26], heart [1], liver [27] and 3T3-L1 adipocytes [28] it has been shown that the soluble fraction of homogenates can contribute a significant proportion of total phosphodiesterase activity. Indeed in rat hepatocytes approximately 50% of the total cyclic AMP phosphodiesterase activity is soluble (this study and Ref. 5).

DEAE-cellulose fractionation of a high speed soluble fraction prepared from rat liver revealed the presence of three major peaks of cyclic nucleotide phospodiesterase activity, as has been observed previously [15]. The first peak of activity eluting from DEAE columns has been termed PDE I in a number of investigations done on other tissues and is assumed to be the Ca^{2+}/calmodulin stimulated phosphodiesterase activity. Such an activity has been noted to occur in rat liver soluble fractions previously (see Ref. 29). However, we make here the novel observation that the cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities of the first peak could be separated from each other using chromatography on Affi-gel Blue. This implies the presence of two distinct enzymic activities in this peak: one predominantly hydrolysing cyclic AMP and the other cyclic GMP. Of these two activities it is the species which preferentially hydrolyses cyclic GMP which is the Ca^{2+}/calmodulin-stimulated enzyme.

In order to assess this by an independent methodology we used chromatographic fractionation on an FPLC system. The rat liver soluble fraction was applied to a Mono-Q column; this allowed for the separation of five peaks of activity (PDE-MQ-I to V), presumably because of the higher resolving ability of Mono-Q compared to DE-52.

PDE-MQ-I was found to hydrolyse cyclic AMP predominantly and bound very poorly to Affi-gel Blue. In contrast, PDE-MQ-II, which preferentially hydrolysed cyclic GMP, bound to the Affi-gel Blue column and was stimulated by Ca^{2+}/calmodulin. The behaviour of these two Mono-Q resolved peaks was analogous to the behaviour of the cyclic AMP and cyclic GMP activities of the first DE-52 peak during Affi-gel Blue chromatography. This behaviour strongly suggests that the first DE-52 peak is comprised of two distinct enzyme activities. The PDE-MQ-II enzyme contributes a significant proportion of the total soluble cyclic GMP phosphodiesterase activity (see Fig. 3) and is increased upon stimulation by Ca^{2+}/calmodulin. Although five-fold activation was obtained with cyclic AMP as substrate Ca^{2+}/calmodulin, this stimulation is not likely to increase significantly the total soluble cyclic AMP phosphodiesterase activity since the basal activity is very low.

PDE-MQ-I expressed a cyclic AMP phosphodiesterase activity which was insensitive to stimulation by Ca^{2+}/calmodulin or by low concentrations of cyclic GMP and was independent of Mg^{2+}. In the majority of instances, however, we noted a small stimulation of cyclic GMP phosphodiesterase activity of PDE-MQ-I by Ca^{2+}/calmodulin. This was found to represent a cross-contamination of PDE-MQ-I with PDE-MQ-II, occurring in some preparations as it could be removed by chromatography on Affi-gel Blue, a procedure which was employed prior to all our studies with this fraction. It should be noted that the cyclic GMP phosphodiesterase activity of Affi-gel Blue purified PDE-MQ-I has a markedly different K_m for cyclic GMP than for PDE-MQ-II (i.e. 268 μM for PDE-MQ-I compared with 5 μM for PDE-MQ-II). This would strongly suggest that the cyclic GMP hydrolysing ability of PDE-MQ-I is distinct from that of PDE-MQ-II both as regards its kinetic behaviour, sensitivity to stimulation by Ca^{2+}/calmodulin and Mg^{2+} dependency.

PDE-MQ-I was very insensitive to inhibition by

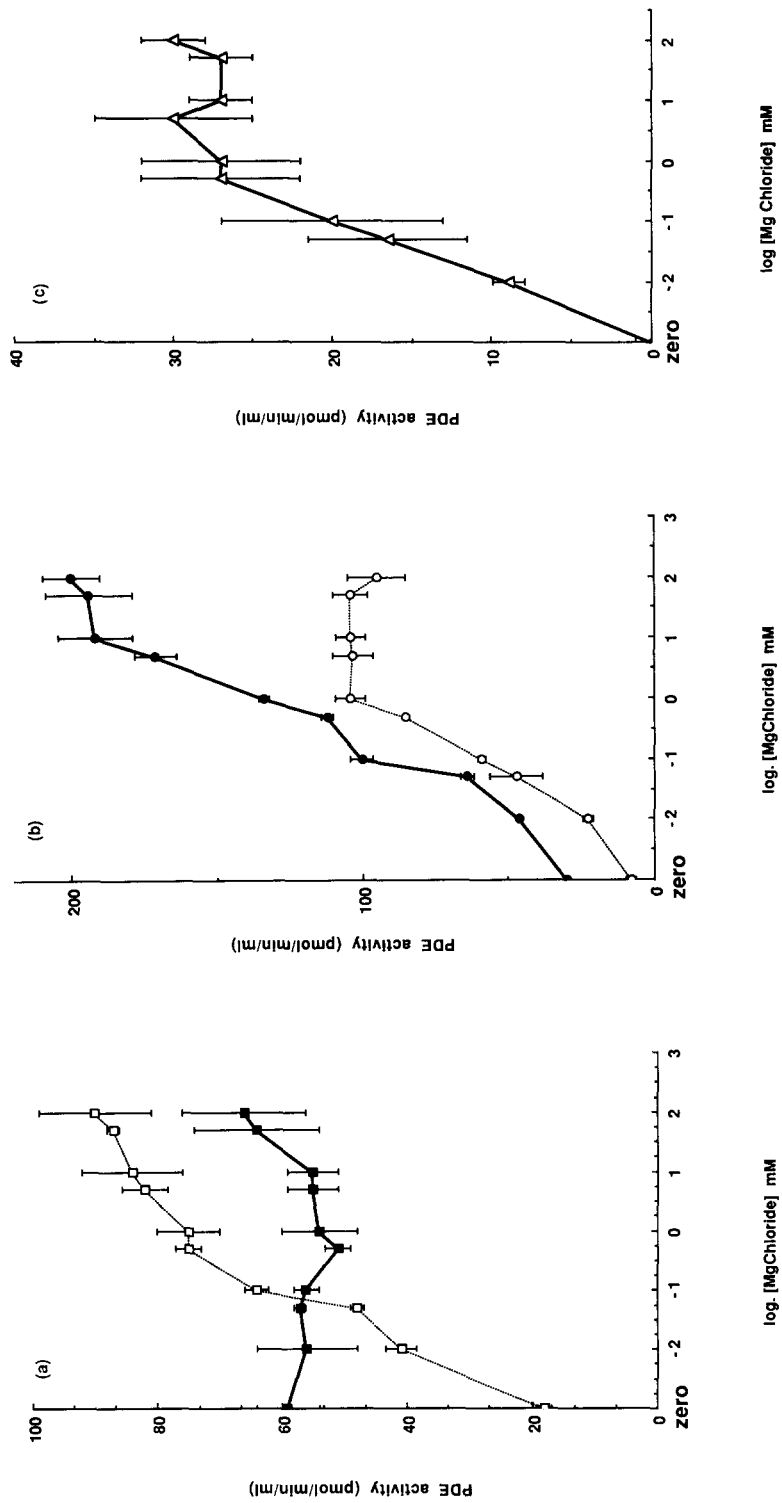

Fig. 7. Action of Mg^{2+} on rat hepatocyte soluble phosphodiesterases. High speed supernatant of rat hepatocyte was chromatographed on a Mono-Q column as described in Materials and Methods and 1 ml fractions were collected. Fractions were assayed for cyclic nucleotide PDE activity as described in Materials and Methods. PDE-MQ-I, -IV and -V were assayed using cyclic AMP as a substrate and PDE-MQ-II and -III with cyclic GMP as substrate. All assays were done under identical conditions and the enzymes were in an identical buffer. Mg^{2+} was added to assays at the indicated concentrations (Panel a) PDE-MQ-I (■) and PDE-MQ-II (□); (panel b) PDE-MQ-III (●) and PDE-MQ-V (○); (panel c) PDE-MQ-IV (△).

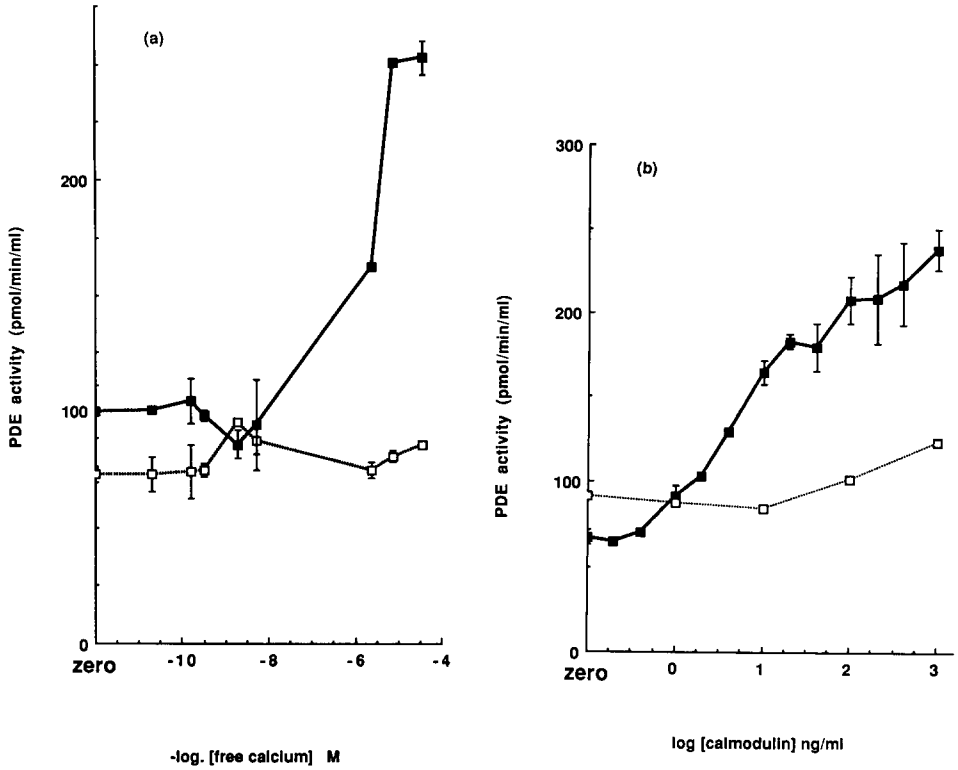

Fig. 8. The regulation of hepatocyte PDE-MQ-III by Ca^{2+} and calmodulin. High speed supernatant of rat hepatocyte was chromatographed on a Mono-Q column as described in Materials and Methods and the PDE-MQ-II fraction isolated. Assays were done with 1 μM cyclic GMP as substrate. In (a) we show the stimulatory effect of Ca^{2+} assessed in the absence (□) and in the presence (■) of 2 μg/ml calmodulin and in (b) the stimulatory effect of calmodulin assessed in the absence (□) and in the presence (■) of 50 μM Ca^{2+}.

the phosphodiesterase inhibitors tested. This included the most commonly used so-called non-specific inhibitor, IBMX. A phosphodiesterase showing such insensitivity to IBMX is highly unusual. This enzyme is also rather novel in that, unlike most other phosphodiesterases studied [13, 14, 30; for PDE-MQ-II as seen in this study] it is unable to bind to Affi-gel Blue, even in the presence of Mg^{2+}. This may indicate that PDE-MQ-I does not have the appropriate 'dinucleotide fold' structure, required for a protein to bind to Affi-gel Blue [31] or it may be related to the lack of an interacting divalent cation binding site.

In view of the differences in the properties of PDE-MQ-I and PDE-MQ-II, namely kinetic parameters, molecular weights, thermal stabilities, Mg^{2+}-dependency, binding to Affi-gel Blue and inhibitor sensitivities it would seem likely that these two activities are distinct iso-enzymic forms and that PDE-MQ-I is a novel, hitherto unreported species. Furthermore, the properties of this enzyme is also highly distinct from the other phosphodiesterase activities reported here.

PDE-MQ-III contributes the majority of the soluble cyclic GMP phosphodiesterase activity at 1 μM substrate and also accounts for a large proportion of the soluble cyclic AMP phosphodiesterase activity of rat liver. For the hepatocyte derived PDE-MQ-III the ratio of cyclic AMP PDE:cyclic GMP PDE activity is lower when compared to the liver derived form. It may be that this difference arises as a result of the presence of a slightly different form of phosphodiesterase iso-enzyme derived from another cell type in rat liver homogenates. Certainly, in the presence of micromolar concentrations of cyclic GMP it seems likely that, even in hepatocytes, the cyclic AMP phosophodiesterase activity of PDE-MQ-III would make a major contribution to the total soluble cyclic AMP hydrolysis.

Subclasses of cyclic AMP specific phosphodiesterase, namely membrane-bound and cytosolic species, have been observed in a number of tissues, for example, adipose [2], ventricular muscle [1] and liver [11, 13, 14, 32]. In a number of studies on cardiac tissue [1, 33, 34] it has been suggested that the third peak of activity eluting from a DEAE column (PDE-III) is a cyclic AMP specific phosphodiesterase which forms the target for action of agents which exert positive inotropic actions. Recently, this interpretation has been questioned as Reeves *et al.* [16] have shown that the so-called PDE-III fraction of cardiac tissue can in fact be resolved into two fractions of high affinity cyclic AMP phosphodiesterase activity showing different sensitivities to inhibitors. Here we show that two peaks of cyclic AMP-specific phosphodiesterase activity can be identified in the soluble fractions from both liver and hepatocytes, by resolution using Mono-Q ion exchange chroma-

Table 4. IC_{50} values for Mono-Q cyclic AMP phosphodiesterase peaks

Drug	Enzyme source	I	II	III	IV	V
IBMX	Liver	N.I.* (N = 5)	5.6 ± 1.4 (N = 5)	97 ± 22 (N = 3)	14.4 ± 5.3 (N = 3)	10.9 ± 1.1 (N = 3)
	Hepatocyte	N.I.* (N = 4)	6.7 ± 1.5 (N = 5)	323 ± 69 (N = 4)	5.2 ± 0.9 (N = 4)	9.8 ± 5.2 (N = 4)
Milrinone	Liver	N.I.* (N = 5)	145 ± 2 (N = 4)	N.I.* (N = 3)	10.3 ± 5.3 (N = 4)	17 ± 2.6 (N = 3)
	Hepatocyte	N.I.* (N = 3)	61 ± 25 (N = 3)	N.I.* (N = 3)	7.3 ± 1.5 (N = 3)	20 ± 5 (N = 3)
Ro 20-1724	Liver	N.I.* (N = 3)	N.I.* (N = 3)	N.I.* (N = 3)	190 ± 30 (N = 3)	1.6 ± 0.8 (N = 3)
	Hepatocyte	N.I.* (N = 3)	303 ± 107 (N = 3)	N.I.* (N = 3)	13 ± 4 (N = 3)	1.5 ± 0.8 (N = 4)
ICI 118233	Hepatocyte	N.I.* (N = 3)	N.I.* (N = 3)	N.I.* (N = 3)	N.I.* (N = 3)	N.I.† (N = 3)
Zaprinast	Hepatocyte	N.I.* (N = 3)	4.5 ± 1.1 (N = 3)	N.I.* (N = 3)	300 ± 141 (N = 3)	N.I.* (N = 3)
Rolipram	Hepatocyte	N.I.* (N = 3)	N.I.* (N = 3)	N.I.* (N = 3)	0.5 ± 0.07 (N = 3)	0.095 ± 0.005 (N = 3)

N.I., not inhibited at concentrations up to either 500 μM* or 100 μM† of the agent. Results are the means ±SE, with N being number of experiments. Values shown are the concentrations of drug required for 50% inhibition of enzyme activity (IC_{50} values) for activity peaks prepared from rat liver and hepatocyte. Phosphodiesterase activity of pooled peaks was measured as described in Materials and Methods, at a substrate concentration of 1 μM cyclic AMP. All drugs effect were corrected for the effects of solvent (DMSO) before calculation of IC_{50} values.

tography, as opposed to simple DEAE-cellulose chromatography which resolves a single activity peak (PDE-III). This would provide strong support for their contention that there is more than one cyclic AMP-specific phosphodiesterase in the soluble fraction. It might also imply that such a multiplicity will also be seen in other tissues.

Both of these cyclic AMP-specific species were potently inhibited by Rolipram with PDE-MQ-V showing a slightly greater susceptibility. Both were similarly inhibited by IBMX, showed small differences in their susceptibility to inhibition by Milrinone but differed markedly in the ability of Ro 20-1724 to elicit inhibition. PDE-MQ-V was inhibited by Ro 20-1724 at much lower concentrations than those required to inhibit PDE-MQ-IV. This was noted using fractions derived from either rat liver or hepatocytes. This would imply that Ro 20-1724 may provide a useful compound to distinguish enzymes in this subclass. Indeed, the use of Ro 20-1724, milrinone and IBMX may thus provide useful tools for discriminating between both soluble and membrane-bound [13] forms of cyclic AMP-specific phosphodiesterases. Kinetic analysis shows that they both express low K_m values for cyclic AMP, although the kinetics of cyclic AMP hydrolysis by PDE-M-IV show apparent negative co-operativity.

Two high affinity, membrane-bound cyclic AMP phosphodiesterases have been identified and characterized in rat liver and rat hepatocytes. These are the 'dense-vesicle' and peripheral plasma membrane phosphodiesterases [10–14]. However, whilst cyclic GMP can exert a potent inhibitory effect on the hydrolysis of cyclic AMP by the particulate rat liver 'dense-vesicle' cycle AMP specific phosphodiesterase, the cyclic AMP phosphodiesterase activities of PDE-MQ-IV and PDE-MQ-V were relatively insensitive to inhibition by cyclic GMP. Furthermore, the 'dense-vesicle' enzyme is also very potently inhibited by Milrinone [13] whereas PDE-MQ-IV and PDE-MQ-V have IC_{50} values which are some 10-fold lower (Table 3). The compound, ICI 118233 exerted but a weak effect on PDE-MQ-IV and PDE-MQ-V yet is an extremely potent and selective inhibitor of the 'dense-vesicle' enzyme [11]. Western blotting analysis, using an antibody directed against the 'dense-vesible' enzyme [11, 13], did not detect any such enzyme in any of these soluble fractions whilst it was found in the membrane fraction (results not shown). From such studies it is clear that the 'dense-vesicle' phosphodiesterase had not been released from its membrane environment. As this enzyme can be very readily solubilized if endogenous proteases are related in an active state [13, 35] this would seem to suggest that our use of isotonic extraction conditions, gentle homogenization and the presence of protease inhibitors prevented the release of membrane phosphodiesterase species. This study, together with our earlier observations [11] also shows that ICI 118233 appears to be a unique agent in that it expresses a specificity for but a single phosphodiesterase species, namly the 'dense-vesicle' enzyme.

We have identified here five soluble phosphodiesterase activities, found in both rat liver and hepatocytes, which appear to be distinguishable by a number of criteria. In particular we have shown the

presence of a novel phosphodiesterase (PDE-MQ-I) which has the highly unusual property of being extremely insensitive to a number of phosphodiesterase inhibitors including the most commonly used non-selective inhibitor IBMX. This suggests that caution should be applied in interpreting whole cell cyclic AMP accumulation studies where such a compound is routinely used to block the hydrolysis of cyclic AMP. Indeed, this explains our [36] observations, in hepatocytes, that even at high concentrations of IBMX, glucagon-mediated increases in intracellular cyclic AMP can still be degraded. That PDE-MQ-I is resistant to inhibition by IBMX does, however, exclude the possibility that this enzyme can be activated by insulin, as the ability of insulin to decrease intracellular cyclic AMP concentrations by activating phosphodiesterase activity can be completely obliterated by IBMX [33].

The ability to resolve rapidly phosphodiesterase activities from liver and hepatocytes should aid in determining the functional role of these isoenzyme species and is a necessary prelude to a full characterization of the range of species identified here. The identification of a novel phosphodiesterase in liver which is insensitive to IBMX and other common inhibitors, indicates that care be taken in experiments used to monitor intracellular cyclic AMP accumulation where blockade of phosphodiesterase activity has been assumed to be achieved using such inhibitors. The development of selective inhibitors against this novel species should provide information on the physiological role that this enzyme plays and may determine whether such compounds can be of possible therapeutic use.

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