Identification and characterization of both the cytosolic and particulate forms of cyclic GMP-stimulated cyclic AMP phosphodiesterase from rat liver

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Two enzymes displaying cyclic GMP-stimulated cyclic AMP phosphodiesterase activity were purified from rat liver to apparent homogeneity: a 'particulate enzyme' found as an integral membrane protein associated with the plasma membrane, and a 'soluble' enzyme found in the cytosol. The physical properties of these enzymes were very similar, being dimers of M_r 134000, composed in each instance of two subunits of M_r = 66000–67000. Both enzymes showed similar kinetics for cyclic AMP hydrolysis. They are both high-affinity enzymes, with kinetic constants for the particulate enzyme of K_m = 34 μ M and V_{max} = 4.0 units/mg of protein and for the cytosolic enzyme K_m = 40 μ M and V_{max} = 4.8 units/mg of protein. In both instances hydrolysis of cyclic AMP appeared to show apparent positive co-operativity, with Hill coefficients ($h_{app.}$) of 1.5 and 1.6 for the particulate and cytosolic enzymes respectively. However, in the presence of 2 μ M-cyclic GMP, the hydrolysis of cyclic AMP obeyed Michaelis kinetics ($h_{app.}$ = 1) for both enzymes. The addition of micromolar concentrations of cyclic GMP had little effect on the V_{max} for cyclic AMP hydrolysis, but lowered the K_m for cyclic AMP hydrolysis to around 20 μ M in both cases. However, at low cyclic AMP substrate concentrations, cyclic GMP was a more potent activator of the particulate enzyme than was the soluble enzyme. The activity of these enzymes could be selectively inhibited by *cis*-16-palmitoleic acid and by arachidonic acid. In each instance, however, the hydrolysis of cyclic AMP became markedly more sensitive to such inhibition when low concentrations of cyclic GMP were present. Tryptic peptide maps of iodinated preparations of these two purified enzyme species showed that there was considerable homology between these two enzyme forms.

INTRODUCTION

Many hormones exert their actions on target cells by elevating the intracellular concentration of cyclic AMP. They do this by increasing the activity of the synthetic enzyme adenylate cyclase (see, e.g., Ross & Gilman, 1980). The predominant mechanism for eliminating cyclic AMP is the action of cyclic AMP phosphodiesterase, which consists of a family of enzymes associated with the cytosol and distinct membrane fractions (see Wells & Hardman, 1977; Thompson & Strada, 1978; Beavo *et al.*, 1982).

Cyclic AMP phosphodiesterases exist in 'low-affinity' and 'high-affinity' categories, where hormones appear to regulate specific 'high-affinity' species. The high-affinity enzyme forms appear to fall into two sub-classes, those enzymes exhibiting apparent negative co-operativity and those showing apparent positive co-operativity. For enzymes of the latter category, low concentrations of cyclic GMP have been found to stimulate the hydrolysis of cyclic AMP (Beavo *et al.*, 1982). Cyclic GMP is believed to achieve this effect by interacting at a regulatory site, specific for cyclic GMP, which is distinct from the substrate-utilizing site (Martins *et al.*, 1982).

Cyclic AMP phosphodiesterase activity represents a potentially important site of control of cyclic AMP

metabolism within the cell and, indeed, a number of hormones have been shown to exert stimulatory effects on the activity of specific membrane-bound cyclic AMP phosphodiesterases in a variety of cell types (see, e.g., Thompson & Strada, 1978; Francis & Kono, 1982; Houslay *et al.*, 1983). In many instances particular species have been identified owing to an activation that persists during enzyme isolation. However, in some instances, a rapid reversible activation of cyclic AMP phosphodiesterase activity *in situ* in the cell has also been inferred (Heyworth *et al.*, 1984). One means of identifying the latter species of hormonally regulated enzymes is to characterize fully individual purified enzymes and to assess their susceptibility to various selective inhibitors. In this way a role for particular phosphodiesterase species in regulating specific physiological processes may be gained from using such selective inhibitors in intact cells. To date, relatively few cyclic AMP phosphodiesterases have been purified to apparent homogeneity (see Beavo *et al.*, 1982) and, with one exception, the insulin-regulated plasma-membrane cyclic AMP phosphodiesterase (Marchmont *et al.*, 1981), they are all soluble cytosolic species.

In hepatocytes, insulin causes a rapid decrease in cyclic AMP concentrations that have been elevated by glucagon. This action is primarily due to the increased

Abbreviation used: IBMX, 3-isobutyl-1-methylxanthine.

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activity of cyclic AMP phosphodiesterase within the cell (see Heyworth *et al.*, 1983). It has been suggested that the cyclic GMP-activated cyclic AMP phosphodiesterase is a potential target for stimulation by insulin (Heyworth *et al.*, 1984). This phosphodiesterase activity has been observed in the soluble and particulate fractions of a variety of cells, including liver (see, e.g., Beavo *et al.*, 1971; Russell *et al.*, 1973; Terasaki & Appleman, 1975; Erneux *et al.*, 1977; Moss *et al.*, 1977). Cytosolic forms of this enzyme have also been purified to apparent homogeneity from bovine heart and adrenal gland (Martins *et al.*, 1982) and from calf liver (Yamamoto *et al.*, 1983a). Here we describe the first purification to apparent homogeneity of a membrane-bound species of this enzyme and contrast it with the properties of a soluble species of this enzyme also purified from rat liver. These forms should allow for the development of novel selective inhibitors to such species in order to assess their function in the hepatocyte.

MATERIALS AND METHODS

Materials

All radiochemicals (^{125}I , cyclic $[^3\text{H}]\text{AMP}$, cyclic $[^3\text{H}]\text{GMP}$) were from Amersham International, Amersham, Bucks., U.K. All biochemicals, unless stated otherwise, were from Sigma (U.K.), Poole, Dorset, U.K. Cyclic AMP and cyclic GMP were from Boehringer Corp. (U.K.), Lewes, East Sussex, U.K. DE-52 DEAE-cellulose was from Whatman Biochemicals (U.K.), Maidstone, Kent, U.K. Sepharose 6B, DEAE-Sepharose 6B, epoxy-activated Sepharose 6B and Sephacryl S-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Inositol trisphosphate and inositol bisphosphate were kindly given by Dr. M. Wakelam, Department of Biochemistry, Imperial College, London, U.K.

Assay of cyclic AMP phosphodiesterase activity

Assays were performed by a modification of the two-step method of Thompson & Appleman (1971) as described in detail by us previously (Marchmont & Houslay, 1980). In all instances linear time courses of cyclic AMP degradation and of enzyme concentration against activity were noted. Routine incubations were carried out so that less than 10% of the total cyclic AMP was degraded during the assay. All assays were performed at pH 7.4 and at 30 °C unless stated otherwise.

Protein was determined with the Bio-Rad assay kit (Bradford, 1976; Spector, 1978) for the soluble enzyme and by a modification (Houslay & Palmer, 1978) of the micro-biuret method of Goa (1953) for the membrane-bound/particulate enzyme and membrane fractions.

Liver plasma membranes were purified as described previously by us (Houslay *et al.*, 1976; Marchmont *et al.*, 1981). A 'salt-washed' fraction, from which peripheral proteins have been removed (Marchmont & Houslay, 1980), was prepared by washing the membranes in buffer A containing 0.5 M-NaCl as described below for the low-speed pellets (P_1) in Step 1(a).

Polyacrylamide-gel electrophoresis

Non-denaturing polyacrylamide-gel electrophoresis, with subsequent elution of gel slices for the assessment of cyclic AMP phosphodiesterase activity, and SDS/polyacrylamide-gel electrophoresis using 9% -acrylamide

running gels and 5% stacking gels, for analysis of protein purity, were performed as described in some detail by Marchmont & Houslay (1980). The final purity of the enzyme preparation and subsequent peptide mapping were as described by Takemoto *et al.* (1982). This utilized ^{125}I -labelled phosphodiesterases and tryptic digestion at 37 °C to yield iodinated peptides for two-dimensional analysis.

Preparation of fatty acids and prostaglandins

Immediately before use, fatty acids were dissolved in a small volume (0.5 ml) of ethanol. This (0.5 ml) was added to 9.5 ml of 20 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂, followed by agitation and then sonication to optical clarity in a glass vial (20 ml) suspended in a bath-type sonicator (approx. 20–30 min). The pH of this solution was adjusted to 7.4 with KOH. Prostaglandins were dissolved in a small volume (0.5 ml) of ethanol and diluted as above in 20 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂. The final concentration of ethanol in the assays was less than 1.0%, which alone had no effect on cyclic AMP hydrolytic activity, the kinetic parameters or the ability of cyclic GMP to affect them.

Analytical sucrose-density-gradient centrifugation

This was performed as is described in detail by Marchmont & Houslay (1980). Assays of marker enzymes were carried out as described fully by Houslay & Tipton (1973). $s_{20,w}$ values of the marker proteins used were those published previously (see Haga *et al.*, 1977); cytochrome *c*, 1.91S; malate dehydrogenase, 4.3S; lactate dehydrogenase, 7.3S; fumarase, 9.1S; catalase, 11.3S.

M_r determination on Sephacryl S-200

The purified enzyme preparation was applied to a column (27 cm × 1.6 cm) of Sephacryl S-200, equilibrated in 20 mM-Tris/HCl buffer (pH 7.4)/5 mM-MgCl₂, at a flow rate of 15 ml/h. Fractions (3 ml) were collected and assayed for cyclic AMP phosphodiesterase activity. Marker enzymes employed for calibration were cytochrome *c* (M_r 12 500), malate dehydrogenase (M_r 60 000), lactate dehydrogenase (M_r 140 000) and fumarase (M_r 240 000).

Preparation of particulate and cytosolic fractions containing cyclic GMP-stimulated cyclic nucleotide phosphodiesterase

In a standard purification, four male Sprague-Dawley rats (200–300 g) were killed and their livers quickly excised and rinsed in homogenizing buffer (1 mM-KHCO₃, pH 7.2, containing 0.1 mM-phenylmethanesulphonyl fluoride at 4 °C). The tissue was minced and homogenized in 3 vol. of ice-cold homogenizing buffer with a Potter-Elvehjem homogenizer (Teflon pestle, glass vessel) with five or six up-and-down strokes at 2500 rev./min. The homogenate was then filtered through one layer of cheesecloth, in a cold-room, and centrifuged at 2000 g_{av} for 10 min at 4 °C. This yielded a pellet (P_1) and a supernatant fraction (S_1).

Step 1: Preparation of fractions. (a) Preparation of soluble supernatant fraction. The supernatant (S_1 ; 90 ml) fraction from the above low-speed-centrifugation step was diluted to 300 ml with ice-cold buffer A (20 mM-Tris/HCl, pH 7.4, 1 mM-EDTA, 7 mM- β -mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride).

Fig. 1. Purification of the two cyclic GMP-stimulated cyclic AMP phosphodiesterases from rat liver

Results are given for the cytosolic (a, b) and particulate (c, d) enzymes for the DEAE-cellulose step (a, c) and subsequent cyclic GMP-affinity column step (b, d). In the affinity-column step (b, d), profiles are given for regions where phosphodiesterase activity was eluted: elution profiles are given for three stages. Stage I is the buffer-B column wash of the affinity matrix after application of the DEAE-cellulose eluate. Then Stage II, which begins at the first arrow, is a single wash with buffer B containing 0.5 M-NaCl. After this, the cyclic GMP-stimulated enzyme was eluted from this column, with cyclic GMP (10 mM) in buffer B containing 0.1 M-NaCl, as a single pooled fraction. Thus the profile shown on this graph after the second arrow (Stage III) is in fact the elution profile of the pooled enzyme from the affinity column when run on a further DEAE-Sepharose column (Step 4). For clarity, the cyclic AMP phosphodiesterase activity measured in the presence of low cyclic GMP concentrations is only plotted when it actually differed from (unstimulated) cyclic AMP hydrolysis. Thus cyclic GMP-stimulated cyclic AMP phosphodiesterase activity ($\blacktriangle$), cyclic AMP phosphodiesterase activity ($\circ$) and protein ($\bullet$) elution patterns are shown. Protein was monitored as A_{280} .

This was centrifuged for 30 min at 50000 $g_{av.}$ at 4 °C in a Beckman J21 centrifuge. The resulting supernatant (S_2) was removed and immediately re-centrifuged under identical conditions to remove any remaining membrane material. The supernatant from this (S_3) was then used for isolation of the cytosolic enzyme. Although this 'supernatant' fraction is contaminated by any soluble contents from microsomes/endoplasmic reticulum, lysosomes and mitochondria, we have shown previously (Cercek *et al.*, 1983) that such entities do not contain any cyclic GMP-activated cyclic AMP phosphodiesterase activity in either soluble or particulate form.

(b) Preparation of particulate fraction and solubilization of phosphodiesterase activity. The low-speed pellet (P_1) from the liver homogenate was washed with 4–5 vol. of buffer A containing 0.5 M-NaCl. This was performed by leaving the resuspended pellet on ice before re-centrifugation at 50000 $g_{av.}$ for 20 min at 4 °C in a Beckman J21 centrifuge. This yielded a 'salt-washed'

pellet (P_2), and a supernatant fraction (SW-SN- P_2), which was not used further. The resulting pellet (P_2) was immediately resuspended (three or four up-and-down strokes, by hand) in buffer A containing 1% sodium cholate. This was left at 4 °C for 1 h and subsequently centrifuged at 50000 $g_{av.}$ for 20 min at 4 °C to obtain a supernatant (SP_2) containing solubilized enzyme(s). Cyclic AMP phosphodiesterase activity in such a detergent extract was, in fact, not sedimented by centrifugation at 100000 $g_{av.}$ for 1 h. This detergent extract was dialysed for 2 h against two changes of 2 l of buffer A before proceeding to Step 2.

Purification of the soluble and particulate cyclic GMP-stimulated cyclic nucleotide phosphodiesterases

Step 2: DEAE-cellulose ion-exchange chromatography. Unless stated otherwise, both the soluble and particulate enzymes were treated identically in the following stages.

The supernatant (S_2) fraction (250 ml) and detergent (SP_2) extract (60–80 ml) of the particulate fraction were applied separately to individual DE-52 DEAE-cellulose columns (10 cm $\times$ 3 cm) which had previously been equilibrated with buffer A at a flow rate of 2.5 ml/min at 4 °C in a cold-room. The DE-52 columns were then washed with 2 bed vol. of buffer A, and bound enzyme was subsequently eluted with a 300 ml linear gradient of 0–0.4 M-NaCl, in buffer A, and fractions (3 ml) were collected. The gradient was analysed for protein and cyclic AMP phosphodiesterase activity at a substrate concentration of 1 μ M-cyclic AMP in the absence and presence of 2.5 μ M-cyclic GMP. Fractions of highest specific activity of the cyclic GMP-activated cyclic AMP phosphodiesterase were pooled (Fig. 1).

Step 3: Cyclic GMP-epoxy-activated Sepharose 6B chromatography. (a) Preparation of the affinity matrix. This was prepared as described by Martins *et al.* (1982). Briefly, epoxy-activated Sepharose 6B was first washed with 10 vol. of deionized water and then allowed to react with 20 mM-cyclic GMP in 'coupling buffer', containing 100 mM-sodium borate and 100 mM-NaCl, final pH 10. The mixture was left gently shaking for 18 h at 45 °C. The coupled resin was subsequently washed with 30 vol. of 100 mM-sodium borate (pH 10)/100 mM-NaCl and then finally 'blocked' by incubation with 1.0 M-ethanolamine in coupling buffer for a further 18 h at 23 °C. The affinity matrix was then washed as above before use (Fig. 1).

(b) Procedure. A column (3 cm $\times$ 1 cm) of affinity matrix was equilibrated, at 4 °C, in buffer B, composed of 20 mM-Tris/HCl, pH 7.4, 1 mM-EDTA and 2 mM-dithiothreitol. In each instance, pooled fractions from the previous step (Step 2) were made 2 mM in dithiothreitol and applied directly to the affinity column at a flow rate of 0.3 ml/min at 4 °C. For both cytosolic and particulate enzymes, the affinity matrix was washed with more than 20 bed vol. of buffer B containing 0.5 M-NaCl, until no protein was eluted from the column, as determined by A_{280} . At this point the column was washed with 2–3 bed vol. of buffer B containing 0.1 M-NaCl. The column was then allowed to attain room temperature (20 °C) and eluted with 30 ml of buffer B containing 0.1 M-NaCl and 10 mM-cyclic GMP. The total eluate was collected as a single fraction in each instance, rapidly cooled on ice and kept at 4 °C.

Step 4: Removal of cyclic GMP from affinity-matrix eluate. A column (2 cm $\times$ 1 cm) of DEAE-Sepharose CL-6B was equilibrated with buffer B containing 0.1 M-NaCl at 4 °C. The final eluate from the previous step was applied to this column at a flow rate of 0.3 ml/min at 4 °C. The column was then washed with 30 vol. of buffer B containing 0.125 M-NaCl to remove bound cyclic GMP. The cyclic GMP-stimulated cyclic AMP phosphodiesterase activity was eluted from the column with buffer B containing 0.3 M-NaCl. Fractions (2–3 ml) containing the highest specific activity were pooled and dialysed overnight against buffer B at 4 °C (Fig. 1).

Enzymes could be stored in batches at –70 °C with a protein concentration greater than 10 μ g/ml. Under such circumstances about 50% of enzyme activity decayed in 2 weeks. If samples were made to 10% (v/v) glycerol plus 2 mg of bovine serum albumin/ml, only about 20% activity was lost over this period. For these studies, enzymes were used within 1 day of their purification to apparent homogeneity, because of the activity decay. Also, purification was routinely completed within 2 days for this reason.

RESULTS

The cyclic GMP-stimulated cyclic nucleotide phosphodiesterase was localised in both soluble (S_1) and particulate (P_2) fractions from rat liver homogenates (Table 1). Additionally we observed a similar distribution of this enzyme between cytosolic and pellet fractions of hepatocytes prepared under iso-osmotic conditions (results not shown). The enzyme associated with the particulate fraction was clearly not either an adsorbed species or a peripheral protein (see Marchmont & Houslay, 1980), as it was resistant to both extensive washing and treatment with high-ionic-strength solutions (Table 1). Thus no cyclic GMP-activated enzyme was released into a 'high-salt' supernatant (SW-SN- P_2) from the low-speed pellet (Table 1).

Purified plasma membranes that had been treated with 'high-salt' solutions to strip off peripheral proteins (Marchmont & Houslay, 1980) exhibited a cyclic GMP-stimulated cyclic AMP phosphodiesterase activity (Table 1). This is presumably the source of the particulate variety of this enzyme.

The cyclic GMP-stimulated cyclic AMP phosphodi-

Table 1. Cyclic GMP-activated cyclic AMP phosphodiesterase activity in soluble and particulate fractions from rat liver

All assays were performed at 1 μ M cyclic AMP substrate concentrations. Where stated, 2.0 μ M unlabelled cyclic GMP was also added to the assays. Fractions were prepared as stated in the text.

Fraction	Specific activity (pmol/min per mg of protein)		Activity for cyclic AMP hydrolysis ($\frac{+ \text{cyclic GMP}}{- \text{cyclic GMP}}$)
	In absence of cyclic GMP	In presence of cyclic GMP	
Low-speed supernatant (S_1)	35	71	2.03
'Salt-washed' low-speed pellet (P_2)	28	95.2	3.39
'Salt-washed' supernatant from low-speed pellet (SW-SN- P_2)	32	31	0.97
'Salt-washed' plasma-membrane fraction	33	151	4.58

Table 2. Purification of the cytosolic and particulate forms of the cyclic GMP-activated cyclic AMP phosphodiesterase from rat liver

All assays were performed at 1 μ M-cyclic AMP substrate concentration. 'Ratio' is defined as the ratio of the cyclic AMP phosphodiesterase activity observed in the presence of a low concentration (2 μ M) of cyclic GMP to that observed in its absence.

Fraction	Total protein (mg)	Total activity (munits)	Specific activity (munits/mg of protein)	Ratio	Yield (%)	Purification (fold)
Cytosolic enzyme						
High-speed supernatant, Step 1 (S ₂)	1200	36	0.03	1.4	(100)	(1)
DEAE-cellulose chromatography, Step 2	100	17	0.17	1.6	60	6.5
Cyclic GMP-epoxy-Sepharose, Step 4	0.045	6	134	2.3	17	4466
Particulate enzyme						
Detergent-solubilized, Step 1 (SP ₂)	750	11.25	0.015	2.5	(100)	1
DEAE-cellulose chromatography, Step 2	70	6.4	0.092	2.5	50	5.9
Cyclic GMP-epoxy-Sepharose, Step 4	0.03	3	100	3	27	6500

esterase activity in the particulate fraction obtained from rat liver could be solubilized with the ionic detergent sodium cholate in a form where cyclic AMP hydrolysis is still stimulated by low concentrations of cyclic GMP (Table 2). Such an activity was not released into a soluble fraction (SW-SN-P₂) by treatment of the membrane fraction with a high-ionic-strength solution (see the Materials and methods section).

We noted (results not shown) that treatment of either the P₂ fraction or isolated plasma membranes with the non-ionic detergent Triton X-100 completely abolished stimulation of this species of cyclic AMP phosphodiesterase by cyclic GMP. Thus the ionic detergent sodium cholate was used in all our studies for solubilizing the particulate activity.

Starting with 1.2 g of soluble cytosolic protein, the purification scheme typically yielded 45 μ g of apparently homogeneous cyclic GMP-stimulated cyclic AMP phosphodiesterase. The yield was 17%, with a 4466-fold purification (Table 2). Starting with 0.75 g of solubilized particulate protein, the purification scheme typically yielded 30 μ g of homogeneous enzyme with a 27% yield and a 6500-fold purification (Table 2). From the result we can assess that the cyclic GMP-stimulated cyclic AMP phosphodiesterase activity is approximately equally distributed between cytosolic and particulate fractions and constitutes 0.01 mg/g of rat liver protein. The presence or absence of proteinase inhibitors such as phenylmethanesulphonyl fluoride (0.1 mM) did not affect the distribution of this activity between the particulate and cytosol fractions (results not shown). Also preparation of membrane and cytosol fractions under iso-osmotic conditions from hepatocytes (Heyworth *et al.*, 1983) also yielded both cytosolic and membrane-bound species of this enzyme.

Both purified forms migrated similarly on non-denaturing polyacrylamide gels, with R_F values of 0.30 ± 0.02 and 0.29 ± 0.01 for the activities of the cytosolic and particulate enzymes respectively. In each instance cyclic GMP-stimulated cyclic AMP phosphodiesterase activity could be eluted from gel slices and shown to co-migrate with the single iodinated protein band.

SDS/polyacrylamide-gel electrophoresis routinely gave a single band of protein, whether stained with Coomassie Blue or whether the more sensitive ¹²⁵I-labelled protein was used. Apparent M_r values in the range

Fig. 2. SDS/polyacrylamide-gel electrophoresis of the purified particulate and cytosolic cyclic GMP-activated cyclic AMP phosphodiesterases from rat liver

Purified enzymes from the final stage of the purification procedure were radioiodinated with ¹²⁵I as described by Takemoto *et al.* (1982). They were then denatured in boiling sample buffer and electrophoresed under denaturing conditions as described by Marchmont *et al.* (1981). This Figure is an autoradiograph of the gel. Track (a) is the particulate enzyme and track (b) is the cytosol enzyme. The origin is marked by an arrow.

Table 3. Kinetic parameters of the purified cytosolic and particulate cyclic GMP-stimulated cyclic AMP phosphodiesterases from rat liver

IC₅₀ is the concentration of inhibitor yielding 50% inhibition. For determinations of IC₅₀ for cyclic AMP hydrolysis, the stimulating concentration of cyclic GMP used was 2 μ M in all instances. The cyclic AMP substrate concentrations used in the IC₅₀ experiments were 3 μ M for IBMX experiments and 0.5 μ M for the fatty acid experiments. Results are means $\pm$ S.D. for three separate experiments. *No inhibition observed up to 5 mM addition.

	Cytosolic	Particulate
K_m for cyclic AMP (μ M)	40 $\pm$ 2	34 $\pm$ 2
(+ presence of 2 μ M-cyclic GMP)	20 $\pm$ 2	20 $\pm$ 2
$V_{max.}$ for cyclic AMP (units/mg of protein)	4.8 $\pm$ 0.25	4.0 $\pm$ 0.2
(+ presence of 2 μ M-cyclic GMP)	4.2 $\pm$ 0.13	3.8 $\pm$ 0.1
h for cyclic AMP	1.6 $\pm$ 0.05	1.5 $\pm$ 0.05
(+ presence of 2 μ M-cyclic GMP)	1.0 $\pm$ 0.1	1.0 $\pm$ 0.05
K_m for cyclic GMP (μ M)	25 $\pm$ 2	31 $\pm$ 3
$V_{max.}$ for cyclic GMP (units/mg of protein)	1.6 $\pm$ 0.12	2.1 $\pm$ 0.15
h for cyclic GMP	1.0 $\pm$ 0.05	1.0 $\pm$ 0.05
IC ₅₀ for IBMX (μ M) (cyclic AMP hydrolysis)	44 $\pm$ 6	50 $\pm$ 2
IC ₅₀ for IBMX (μ M) (cyclic AMP hydrolysis in presence of cyclic GMP)	19 $\pm$ 5	13 $\pm$ 2
IC ₅₀ for arachidonic acid (mM) (cyclic AMP hydrolysis)	1.6 $\pm$ 0.13	0.18 $\pm$ 0.03
IC ₅₀ for arachidonic acid (mM) (cyclic AMP hydrolysis in presence of cyclic GMP)	0.6 $\pm$ 0.03	0.078 $\pm$ 0.005
IC ₅₀ for (<i>cis</i> -16)-palmitoleic acid (mM) (cyclic AMP hydrolysis)	1.0 $\pm$ 0.15	Not observed*
IC ₅₀ for (<i>cis</i> -16)-palmitoleic acid (mM)	0.45 $\pm$ 0.05	1.6 $\pm$ 0.31

66000–67000 were routinely obtained for both the cytosolic and particulate forms (Fig. 2). M_r determination by Sephacryl S-200 chromatography yielded M_r values of 134000 and 135000 for the cytosol and solubilized particulate forms respectively. This was in agreement with $s_{20,w}$ of 7.4S and 7.6S obtained for the cytosol and solubilized membrane forms by sedimentation analysis in sucrose density gradients. We noted that high concentrations of either enzyme (greater than approx. 10 μ g/ml) led to aggregation, as has been described for other cyclic GMP-stimulated cyclic AMP phosphodiesterases (Yamamoto *et al.*, 1983a; Martins *et al.*, 1982). However, enzyme stability at -70°C was enhanced at such elevated protein concentrations.

Thermal denaturation of both forms of the enzyme occurred on incubation at 40°C . An exponential decay of activity ensued, yielding linear (first-order) plots of log activity versus time of incubation (results not shown). Half lives of 10 ± 1.2 min and 12.5 ± 1.7 min were obtained for the particulate and cytosolic forms of the enzyme respectively.

Kinetic properties of the purified enzymes

The kinetic properties of both of these enzymes were very similar (Table 3) as regards the hydrolysis of both cyclic AMP and cyclic GMP. Although the K_m values were similar for hydrolysis of both cyclic AMP and cyclic GMP, the $V_{max.}$ values observed for cyclic AMP hydrolysis were over twice those for cyclic GMP hydrolysis (Table 3). Furthermore, hydrolysis of cyclic GMP, by both enzymes, obeyed Michaelis kinetics with linear Lineweaver–Burke plots, yielding Hill plots with a coefficient ($h_{app.}$) of 1.0 (Table 3). On the other hand, hydrolysis of cyclic AMP exhibited kinetics indicative of apparent positive co-operativity (Fig. 3), yielding Hill

plots with coefficients ($h_{app.}$) in the range of 1.5–1.6 for both enzymes (Table 3). However, the presence of low concentrations (2 μ M) of cyclic GMP caused the kinetics of cyclic AMP hydrolysis to alter markedly (Fig. 3), with this activity now obeying Michaelis–Menten kinetics with a Hill coefficient ($h_{app.}$) of 1.0. There was also a decrease in the apparent K_m for cyclic AMP (Table 3).

At physiological concentration (0.2–20 μ M) of cyclic AMP (Heyworth *et al.*, 1983), the ability of low concentrations of cyclic GMP to augment cyclic AMP hydrolysis was observed (Figs. 3 and 4). Under conditions of low cyclic AMP substrate concentrations, it appeared that cyclic GMP could exert a greater stimulatory effect on cyclic AMP hydrolysis by the particulate enzyme than by the cytosolic enzyme (Fig. 4). However, cyclic GMP activated the two enzymes at very similar concentrations. The apparent K_a values for activation were 0.28 ± 0.05 μ M for the cytosolic enzyme and 0.23 ± 0.04 μ M for the particulate enzyme (Fig. 4).

IBMX similarly inhibited cyclic AMP hydrolysis by both purified forms (Table 3, Fig. 5). However, very low concentrations of IBMX were found actually to stimulate (Fig. 5) cyclic AMP hydrolysis, with a maximum activation of $25 \pm 3\%$ being observed for the cytosolic enzyme and $15 \pm 4\%$ being observed for the particulate enzyme at 1.5 μ M-IBMX. When cyclic AMP (3 μ M) hydrolysis was measured in the presence of cyclic GMP (2 μ M), not only was there an enhanced sensitivity to inhibition by IBMX, but also low concentrations of IBMX failed to elicit any stimulatory effect (Fig. 5).

Effect of fatty acids, prostaglandins and other agents on catalytic activity

Oleic acid, palmitic acid, tocopherol and prostaglandins A₁, B₁, E₁ and E₂, all at concentrations up to 0.1 mM,

Fig. 3. Cyclic AMP hydrolysis by the purified phosphodiesterases

Data are given for the purified particulate (a) and cytosolic (b) forms of the enzyme. In each case observations were made in the absence (○) and presence (▲) of (unlabelled) cyclic GMP (2 μM). A representative experiment of one performed three times is shown. The inset is of a Hill plot over the linear ($v = 10-90\% V_{\max}$) region, where $\log [(v/V_{\max})/(1-v/V_{\max})]$ is plotted against $-\log \{[\text{cyclic AMP}](\text{M})\}$.

Fig. 4. Low concentrations of cyclic GMP exert selective stimulatory effects on cyclic AMP hydrolysis by the particulate and cytosolic enzymes

At low cyclic AMP substrate concentrations (0.25 μM), the stimulatory effect of low cyclic GMP concentrations is shown for the cytosolic (●) and particulate (○) enzymes. A representative experiment of one performed at least three times is shown. Activation is shown as the percentage elevation of the control activity.

assayed at 0.5 μM-cyclic AMP, in either the presence or the absence of cyclic GMP (2 μM). Putrescine, spermine and spermidine at 1 mM elicited 18–26% inhibition of cyclic AMP (0.5 μM) phosphodiesterase activity whether or not cyclic GMP (2 μM) was present (results not shown).

However, the cyclic AMP phosphodiesterase activity of both the purified cytosolic and particulate forms was inhibited by both arachidonic acid itself as well as its analogue *cis*-16-palmitoleic acid (Table 3; Fig. 5). For both enzymes the cyclic GMP-stimulated activity was more susceptible to inhibition by these fatty acids. These fatty acids, however, exerted selective actions on the two purified enzymes, as the particulate species was considerably more sensitive to inhibition by arachidonic acid than was the cytosolic enzyme. The converse was true for *cis*-16-palmitoleic acid. Indeed, no inhibition of the particulate enzyme by palmitoleic acid was observed unless the enzyme was in its cyclic GMP-stimulated state (Table 3; Fig. 6).

Iodinated-peptide analysis

Tryptic peptides of ^{125}I -labelled forms of both of the purified enzyme forms were analysed as described by Takemoto *et al.* (1982). These displayed peptide maps that appeared to be highly co-related (greater than 80%). However, at least three peptides (marked by arrows) appeared to be distinct and specific for these two forms (Fig. 7).

DISCUSSION

Beavo *et al.* (1971) were the first to show that cyclic GMP-stimulated cyclic nucleotide phosphodiesterase

as well as inositol trisphosphate (5–50 μM), inositol biphosphate (5–50 μM) and fructose 2,6-bisphosphate (up to 1 mM) had little effect (less than 15%) on the phosphodiesterase activity of both purified forms when

Fig. 5. Response of cyclic AMP hydrolysis by the two enzymes to IBMX

The cyclic AMP phosphodiesterase activity of both the particulate (b) and cytosolic (a) enzymes were assayed in the absence (○) and presence (▲) of a stimulatory concentration (2 μ M) of cyclic GMP. The cyclic AMP substrate concentration was 3 μ M in all cases. A representative experiment of one performed three times is shown. Activity is shown as a percentage of that of the control (100%).

Fig. 6. Effect of fatty acids on cyclic AMP phosphodiesterase activity

Data are given for the particulate (○, △) and cytosolic (●, ▲) enzymes with (*cis*-16)-palmitoleic acid (a) and arachidonic acid (b) in the presence (△, ▲) and absence (○, ●) of stimulatory concentrations (2 μ M) of cyclic GMP. The cyclic AMP substrate concentration was 0.5 μ M. These data are representative of an experiment performed three times with different enzyme preparations. Activity is shown as a percentage of that of the control (100%).

activity could be found in both cytosolic and particulate fractions of rat liver. We show here that the particulate form appears to be localized to the liver plasma membrane and provide the first observation that such an enzyme can be solubilized in a state capable of still being activated by low cyclic GMP concentrations. The need to use detergents rather than high ionic strength to extract the particulate enzyme from the membrane indicates that it is an integral enzyme, interacting through hydrophobic interactions with the membrane, rather than either an adsorbed or a peripheral enzyme. Indeed, its localization to the plasma membrane, the site of cyclic AMP synthesis, might imply an important regulatory role for the particulate enzyme. The cytosol enzyme is unlikely to be derived from the plasma-membrane species by, for example, proteolysis, as the amount of cytosol enzyme

obtained was unaffected by homogenization in iso-osmotic buffers and was not affected by including proteinase inhibitors in the homogenization buffer.

To date, soluble fractions from bovine tissues have provided the only source of cyclic GMP-stimulated cyclic AMP phosphodiesterase to be purified. The enzymes from bovine adrenals, heart (Martins *et al.*, 1982) and bovine liver (Yamamoto *et al.*, 1983a) appear to be very similar, being dimers consisting of two subunits of M_r 102000–107000. In this regard they are distinct from the rat liver enzymes which we have isolated as dimers having subunits of M_r 67000.

Both the cytosolic and particulate forms purified here displayed similar thermostability and exhibited positive homotropic responses to cyclic AMP, with very similar kinetic constants. Importantly, when the kinetics of cyclic

Fig. 7. Iodinated-tryptic-peptide maps of the two phosphodiesterases

Two-dimensional iodinated-tryptic-peptide maps of the two enzymes were performed as described by Takemoto *et al.* (1982). The two enzymes showed approx. 80% homology by analysis as described by Takemoto *et al.* (1982). There were, however, peptides unique to each species, which are marked with arrows. The particulate (a) and cytosolic (b) forms are shown. Representative gels are shown here. The point of origin is shown (O), as is the limit of electrophoresis (+) assessed by the movement of the dye pylonin-y. Electrophoresis is the horizontal direction (+ ve to -ve) and chromatography in the vertical direction.

AMP hydrolysis were followed in the presence of stimulatory concentrations of cyclic GMP, then, for both purified forms, a marked decrease in the K_m and $h_{app.}$ was noted. Thus the loss of such co-operativity would allow these enzymic forms to respond to lower concentrations of cyclic AMP more rapidly. Although the K_a values for cyclic GMP stimulation of cyclic AMP hydrolysis at low cyclic AMP concentrations were very similar for both forms, we consistently observed that cyclic GMP exerted a larger stimulatory effect on the particulate form (Fig. 4). Indeed, membrane attachment of this form appeared to give an even more pronounced stimulatory action by cyclic GMP (Table 1), which was decreased somewhat on solubilization (Table 2).

Assessment of a K_m value as that concentration of cyclic nucleotide eliciting half-maximal velocity yields for cyclic AMP values of K_m for the particulate (34 mM) and cytosolic enzymes (40 μ M) which are very similar to those observed by the enzymes from bovine tissues (30–33 μ M; Martins *et al.*, 1982; Yamamoto *et al.*, 1983a). The same is true from the Hill coefficients ($h_{app.}$), being 1.5–1.6 in the present study and 1.8–1.9 for the bovine enzymes. There are, however, two particular points of difference.

Firstly the $V_{max.}$ values for cyclic AMP hydrolysis by the apparently homogeneous rat liver enzymes (4–4.8 units/mg of protein) are noticeably lower than those for the homogeneous bovine enzymes (120–170 units/mg of protein). Secondly, although K_m values for the hydrolysis of cyclic GMP are similar for all of the enzymes, hydrolysis of cyclic GMP by the rat liver enzymes in this study obeyed Michaelis–Menten kinetics ($h_{app.} = 1$), whereas positive homotropic effects were seen with the bovine enzymes. We have, however, made the novel observation that the addition of stimulatory concentrations of cyclic GMP, presumably by binding to its regulatory site, caused a marked change in the kinetics of cyclic AMP hydrolysis by both the rat liver enzymes (Table 3; Fig. 3), owing to the loss of positive homotropic effects. This observation might then explain why the hydrolysis of cyclic GMP by the rat liver enzyme obeys Michaelis–Menten kinetics, as both the regulatory and substrate sites would be expected to be occupied by cyclic GMP.

The degree of stimulation of cyclic AMP hydrolysis elicited by cyclic GMP was of a similar magnitude to that observed for the bovine heart enzyme (Martins *et al.*, 1982). It was, however, considerably less than the 32-fold stimulatory effect seen with the bovine liver enzyme. Nevertheless, the K_m for activation of the bovine liver enzyme (0.5 μ M) was remarkably similar (Yamamoto *et al.*, 1983a) to that we observed here with the rat liver enzymes (Fig. 4).

IBMX is a well-established inhibitor of cyclic AMP phosphodiesterases. However, it has been shown to interact with both substrate and regulatory sites of cyclic GMP-activated cyclic nucleotide phosphodiesterases. Indeed, low concentrations of IBMX have even been noted as causing activation of cyclic AMP hydrolysis for the bovine liver enzyme (Yamamoto *et al.*, 1983b) and crude preparations from rat liver (Erneux *et al.*, 1981, 1982). We observed here with both of the purified rat liver enzymes that low concentrations of IBMX stimulated cyclic AMP hydrolysis, in the absence of cyclic GMP, with the cytosolic form being more sensitive to stimulation than the particulate form. The effect was, however, abolished when experiments were performed in the presence of low, stimulatory, concentrations of cyclic GMP (Fig. 5). High concentrations of IBMX inhibited both forms of the enzyme similarly, although the presence of stimulatory concentrations of cyclic GMP considerably enhanced the sensitivity of cyclic AMP hydrolysis to inhibition by IBMX for both enzyme forms (Table 3; Fig. 4). In this instance the decrease in IC_{50} for inhibition by IBMX was greater for the particulate enzyme than for the cytosolic species (Table 3).

Yamamoto *et al.* (1983c) have shown that low concentrations (0.1 mM) of certain unsaturated fatty acids, such as *cis*-palmitoleic acid, could stimulate cyclic AMP hydrolysis by the purified bovine liver enzyme. We observed only marginal stimulations of the purified rat liver species with low concentrations of this unsaturated fatty acid. In fact, we found that higher concentrations of these agents actually inhibited the enzyme (Fig. 5). Intriguingly, both palmitoleic acid and arachidonic acid exerted inhibitory effects on the cyclic AMP phosphodiesterase activity of the purified cytosolic form, with markedly different IC_{50} values compared with those found for the purified particulate form (Table 3; Fig. 5). This implies that these purified forms have somewhat

different hydrophobic domains that can alter catalytic function towards cyclic AMP. Furthermore, the sensitivity of cyclic AMP hydrolysis to inhibition by these fatty acids was markedly increased when stimulatory concentrations of cyclic GMP were present (Table 3).

Tryptic-peptide maps of the purified cytosolic and particulate forms indicated that there was very close homology between these two forms. However, we observed that at least two peptides appeared to be specific to the particulate form, and one peptide was apparently specific to the cytosolic form. The similarity in M_r , thermostability and kinetics implies that the particulate species might be anchored to the plasma membrane by a relatively short transmembrane strand of peptide in a similar fashion to the aminopeptidases found in microvillar brush-border membranes (Kenny & Booth, 1978). It would be of interest to have knowledge of the protein sequence and genes coding for these two highly co-related species in order to ascertain why one enzyme is directed to the plasma membrane and the other to the cytosol.

Our studies indicate that there are two cyclic GMP-activated cyclic nucleotide phosphodiesterases in rat liver which are closely related but nevertheless distinct species. Their different sensitivity to inhibition by fatty acids suggests that there may be a hydrophobic domain which might usefully be exploited to identify selective inhibitors of these enzymes. Such agents will be of use in attempting to assess the physiological role of each of these enzymes and to determine why there should be both cytosolic and membrane-bound forms of such a similar enzyme. Our results also imply that there are distinct species differences between the cyclic GMP-stimulated cyclic nucleotide phosphodiesterases as regards physical and kinetic properties as well as inhibitor sensitivities. This is an important caveat which should be noted, in the development of potentially therapeutic drugs, when attempting to extrapolate from animal studies to man.

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