The insulin- and glucagon-stimulated 'dense-vesicle' high-affinity cyclic AMP phosphodiesterase from rat liver

Purification, characterization and inhibitor sensitivity

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The hormone-stimulated 'dense-vesicle' cyclic AMP phosphodiesterase was solubilized as a proteolytically 'clipped' species, and purified to apparent homogeneity from rat liver with a 2000–3000-fold purification and a 13–18% yield. It appeared to be a dimer (M_r 112000), of two M_r -57000 subunits. Solubilization of either a liver or a hepatocyte membrane fraction, with sodium cholate in the presence of the protein inhibitor benzamidine, identified three protein bands which could be immunoprecipitated by a polyclonal antibody raised against the pure enzyme. The major band at M_r 62000 is suggested to be the native 'dense-vesicle' enzyme, having a M_r -5000 extension which serves to anchor this enzyme to the membrane and which is cleaved off during proteolytic solubilization; the M_r -200000 band is an aggregate of the M_r -62000 species, and the M_r -63000 species is possibly a precursor. The purified 'clipped' enzyme hydrolysed cyclic AMP with kinetics indicative of apparent negative co-operativity, with a Hill coefficient (h) of 0.43 and limiting kinetic constants of $K_{m1} = 0.3 \pm 0.05 \mu\text{M}$, $K_{m2} = 29 \pm 6 \mu\text{M}$, $V_{\max,1} = 0.114 \pm 0.015$ unit/mg of protein and $V_{\max,2} = 0.633 \pm 0.054$ unit/mg of protein. It hydrolysed cyclic GMP with Michaelis kinetics, $K_m = 10 \pm 1 \mu\text{M}$ and $V_{\max} = 4.1 \pm 0.2$ munits/mg of protein. Cyclic GMP was a potent inhibitor of cyclic AMP hydrolysis, with an IC_{50} (concn. giving 50% inhibition) of $0.20 \pm 0.01 \mu\text{M}$ -cyclic GMP when assayed at $0.1 \mu\text{M}$ -cyclic AMP. This enzyme was inhibited potently by several drugs known to exert positive inotropic effects on the heart, was extremely thermolabile, with a half-life of 4.5 ± 0.5 min at 40°C , and was shown to be distinct from the rat liver insulin-stimulated peripheral-plasma-membrane cyclic AMP phosphodiesterase [Marchmont, Ayad & Houslay (1981) *Biochem. J.* **195**, 645–652].

INTRODUCTION

It has been shown (Heyworth *et al.*, 1983, 1984) that insulin can stimulate the activity of at least three distinct cyclic AMP phosphodiesterases in hepatocytes, two of which are membrane-bound. One is the high-affinity peripheral-plasma-membrane phosphodiesterase (Marchmont & Houslay, 1980*a,b*; Marchmont *et al.*, 1981), which is activated solely by insulin, and the other is the so-called (Heyworth *et al.*, 1983) 'dense-vesicle' enzyme, which can be activated by both insulin and glucagon (Loten *et al.*, 1978, 1980; Heyworth *et al.*, 1983; Houslay *et al.*, 1983). The mechanism whereby glucagon activates the 'dense-vesicle' enzyme is distinct from that employed by insulin and is mediated by cyclic AMP (Wilson *et al.*, 1983). Insulin, however, activates the 'dense-vesicle' and plasma-membrane phosphodiesterases by completely distinct routes (Wilson *et al.*, 1983; Heyworth *et al.*, 1983, 1986).

The 'dense-vesicle' enzyme is an integral membrane protein which can be released from its membrane environment by the 'hypotonic shock' procedure developed by Loten *et al.* (1978), and which is believed (Loten *et al.*, 1980) to result from the action of endogenous proteinases. This procedure liberates an active, soluble and proteolytically 'clipped' enzyme which still reflects any activation elicited by either insulin or glucagon treatment of liver or hepatocytes (Loten *et al.*, 1978).

Release of a spectrum of phosphodiesterases has hindered purification of the detergent-solubilized enzyme (Loten, 1983; Francis & Kono, 1982). We have taken advantage of the ability to release an identifiably hormone-activated enzyme to purify the proteolytically 'clipped' solubilized form of the 'dense-vesicle' cyclic AMP phosphodiesterase to apparent homogeneity, by two different purification procedures. This 'dense-vesicle' enzyme is shown to be quite distinct from the insulin-stimulated plasma-membrane cyclic AMP phosphodiesterase.

MATERIALS AND METHODS

All radiochemicals (^{125}I , cyclic [^3H]AMP and cyclic [^3H]GMP) were from Amersham International, Amersham, Bucks., U.K. All biochemicals, including dipyrindamole (see Weishaar *et al.*, 1985), unless stated otherwise, were from Sigma, Poole, Dorset, U.K. Cyclic AMP and cyclic GMP were from Boehringer Corp. (U.K.), Lewes, East Sussex, U.K. DEAE-cellulose DE-52 and ECTEOLA-cellulose were from Whatman Biochemicals, Maidstone, Kent, U.K. Sepharose CL6B, DEAE-Sepharose 6B, Sephacryl S-200, aminopentyl-agarose and CNBr-activated Sepharose 6B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Affi-gel Blue was from Bio-Rad, Watford, Herts., U.K. Immuno-precipitin was from Gibco-BRL Chemicals, Paisley,

Scotland, U.K. ICI 63197 (2-amino-6-methyl-5-oxo-4-n-propyl-4,5-dihydro-*S*-triazolo[1,5-*a*]pyrimidine) was kindly given by ICI Pharmaceuticals, Macclesfield, Cheshire, U.K. (see Davies, 1973). Amrinone and milrinone (see Bristol *et al.*, 1984) were kindly given by Sterling Winthrop, Guildford, Surrey, U.K. RO-1724 [DL-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was kindly given by Roche Products, Welwyn Garden City, Herts., U.K. Buquineran (UK 14,275) and Carbazeran (UK 31,557) were kindly given by Pfizer Central Research, Sandwich, Kent, U.K. (see Bristol & Evans, 1983). SQ-20009 was from Squibb Pharmaceuticals (see Hwang & Szentivanyi, 1979).

Cyclic AMP phosphodiesterase activity was assessed by a modification of the two-step procedure of Thompson & Appleman (1971) as described in detail by us previously (Marchmont & Houslay, 1980b). Initial rates were taken from linear time courses in all experiments. All assays were performed at 30 °C at final pH 7.4. Errors were expressed as s.d. with ($n-1$) degrees of freedom for individual experiments.

SDS/PAGE and 'non-denaturing' PAGE, with subsequent elution of enzyme activity, and the determination of M_r , were performed as described by Marchmont & Houslay (1980b).

Protein concentrations were determined with a Bio-Rad protein assay kit (Bradford, 1976; Spector, 1978) for the solubilized enzyme and by a modification of the micro-biuret method (Goa, 1953) for the particulate enzyme.

Solubilization of the insulin- and glucagon-stimulated 'dense-vesicle' cyclic AMP phosphodiesterase

This was performed by a modification of the 'hypotonic shock treatment' of Loten *et al.* (1978), in order to solubilize the enzyme. Three rat livers were excised and immediately rinsed in ice-cold 0.25 M-sucrose containing 10 mM-Tris/HCl, pH 7.4, and 1 mM-EDTA (sucrose buffer). The livers were blotted dry and weighed, and all subsequent steps were performed at 4 °C. The livers were cut into small pieces and homogenized in 8 vol of sucrose buffer with a Teflon pestle plus glass vessel with eight up-and-down strokes of the pestle at 250 rev./min.

The homogenate was filtered through cheese-cloth and the filtrate centrifuged for 10 min at 1000 rev./min in a MSE Minor centrifuge. The supernatant was removed and re-centrifuged at 30000 g_{av} for 20 min in a Beckman Ja20 rotor. The pellet was resuspended in 2–3 vol. of 1 mM-EDTA/10 mM-Tris/HCl, final pH 7.4, and left for 40 min at 4 °C before a final centrifugation at 48000 g_{av} for 30 min. The supernatant was decanted and used for subsequent purification studies.

The purity of the final preparations was checked by SDS/PAGE of the [125 I]iodinated enzyme as described by Takemoto *et al.* (1982). Briefly, a sample of protein (2–10 μ g) was freeze-dried and rehydrated in 1 M-Tris/HCl (pH 7.5)/8% SDS before addition of 125 I (100–500 μ Ci) and chloramine-T (0.4 mg/ml) in a total volume of 40 μ l. After 1 min at room temperature the reaction was terminated with 40 μ l of 0.1 M-Tris/HCl, pH 7.0, containing 2% SDS and 2% β -mercaptoethanol, and the samples were boiled for 2 min. After cooling, the solution was made 0.1% Bromophenol Blue/10% glycerol final concentration, before SDS/PAGE. Two-dimensional chromatographic analysis of tryptic peptides

of the 125 I-labelled enzyme was performed as described by Takemoto *et al.* (1982).

Preparation of the immune serum

The 'dense-vesicle' phosphodiesterase was purified to apparent homogeneity and used as the immunogen. The immunization procedure involved combining an equal volume of immunogen with Freund's complete adjuvant before injecting it (intra-muscularly) into an adult rabbit. Injections were repeated over a period of 3 months with incomplete adjuvant. The immune serum was obtained by bleeding the animal and collecting 30 ml of blood each time. Collected blood was left at 4 °C for 2 h to allow the formation of a clot, which was subsequently removed and the plasma centrifuged at 15000 g_{av} for 15 min at 4 °C. To the supernatant was added 20 g (wet wt.) of DEAE-cellulose, which had been pre-equilibrated with 10 mM-KH₂PO₄/K₂HPO₄ (equilibrating) buffer, pH 7.5. This was left at 4 °C for 30 min with occasional stirring. The DEAE-cellulose was then removed by filtration. The filtrate was diluted in equilibrating buffer (1:1, v/v) before addition of solid (NH₄)₂SO₄ to 45% saturation, with stirring. After gentle stirring (15 min, 4 °C) it was centrifuged for 15 min at 15000 g_{av} . The pellet was resuspended in equilibrating buffer (5 ml) and dialysed against two changes of 2 litres of equilibrating buffer before being divided into portions for freeze-drying or storage at –70 °C.

Preparation of hepatocyte extracts for immunoprecipitation

Hepatocytes were prepared, preincubated and incubated as described in some detail by us previously (Heyworth *et al.*, 1983). Cells were harvested by centrifugation at 400 rev./min in an MSE Minor centrifuge for 2 min at 4 °C and then resuspended in 3 ml of ice-cold 0.25 M-sucrose containing 10 mM-Tris/HCl, final pH 7.4, 0.1 mM-PMSF, 2 mM-benzamidinium and 1 mM-EDTA. Disruption of the cells was achieved by 30 up-and-down strokes of a Teflon pestle in glass homogenizer (Camlab), and the homogenate was centrifuged at 18000 g_{av} for 15 min at 4 °C. The resultant pellet was treated in one of two ways.

(i) 'Hypotonic shock treatment', by resuspending it in 1 mM-EDTA/10 mM-Tris/HCl, pH 7.4, and leaving it at 4 °C for 30 min before centrifugation as above. The supernatant was freeze-dried and iodinated as above, but with omission of SDS, and then taken for immunoprecipitation.

(ii) Resuspension in 0.25 M-sucrose containing 10 mM-Tris/HCl, pH 7.4, 1 mM-EDTA, 0.1 mM-PMSF, 2 mM-benzamidinium and 1% sodium cholate and leaving it at 4 °C for 45 min. Centrifugation was performed as above and the supernatant dialysed against 2 litres of 0.25 M-sucrose/10 mM Tris/HCl (pH 7.4)/1 mM-EDTA/2 mM-benzamidinium/0.2 mM-PMSF before freeze-drying and iodination again with the omission of SDS. Iodinated samples were then taken for immunoprecipitation reactions.

Immunoprecipitation

Iodinated samples from the above procedures were resuspended in 0.8 ml of 10 mM KH₂PO₄/K₂HPO₄ buffer, final pH 7.4, containing 0.9% NaCl. To this was added 200 μ l of Mixture A, containing (final concns.) 50 mM-Tris/HCl, pH 7.4, 1% (w/v) Triton X-100, 1%

(w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM-EDTA, 0.15 M-NaCl, and 25 μ l of 2% (w/v) gelatin. This was then incubated for 1 h at 30 °C before Immunoprecipitin (25 μ l; 10%, w/v) was added and incubated for 15 min at 30 °C before centrifugation at 14000 g_{av} for 1 min. The pellet was washed once with Mixture A alone, twice with Mixture A containing 0.5 M-NaCl and twice with 10 mM-Tris/HCl, final pH 7.4. The pellet was added to 100 μ l of electrophoresis sample buffer (Laemmli, 1970), and boiled for 2 min before SDS/PAGE.

Single-dimension analysis of tryptic peptides derived from immunoprecipitated 125 I-labelled proteins separated on SDS/PAGE, as above, was performed by a modification of the method of Cleveland *et al.* (1977). Briefly, after location of proteins by autoradiography, radioactive bands of gel were excised and re-hydrated at room temperature for 30 min in 0.1% SDS in 0.125 M-Tris/HCl buffer, pH 6.7. The gel chip was then mixed with 40 μ l of 0.01% Bromophenol Blue, 10% glycerol and 0.125 M-Tris/HCl buffer, pH 6.7, containing 200 ng of trypsin. This was loaded on a 15% -acrylamide gel with a 5% stacking gel and, after the dye front was about to enter the main gel, power was switched off for 30 min, before the run was continued to completion. The dried gel was subjected to autoradiography and the X-ray film scanned with a Bio-Rad model 620 gel scanner operated by an Olivetti M21 microcomputer.

Purification of the 'dense-vesicle' cyclic AMP phosphodiesterase

Method 1. Step 1: solubilization of cyclic AMP phosphodiesterase from rat liver as described above.

Step 2: ECTEOA-cellulose chromatography. A column (20 cm $\times$ 2 cm) of ECTEOA-cellulose was equilibrated in buffer A, containing 10 mM-Tris/HCl, pH 7.4, 1 mM-MgCl₂, 2 mM-benzamidine and 0.1 mM-PMSF. The hypo-osmotic extract (60 ml) was applied at a flow rate of 0.5 ml/min. The column was then washed with buffer A until no further protein (A_{280}) was eluted, and then with 100 ml of buffer A containing 0.15 M-NaCl and 20% ethylene glycol or until no further protein (A_{280}) was eluted. The bound 'dense-vesicle' phosphodiesterase was then eluted with buffer A containing 0.4 M-NaCl and 20% ethylene glycol. Fractions (3 ml) containing the highest specific activity (pmol/min per mg) were pooled.

Step 3: hydrophobic chromatography. The fractions were diluted 1:1 (v/v) with buffer A before application to a column (5 cm $\times$ 1 cm) of aminopentyl-agarose that had been previously equilibrated with buffer A at a flow rate of 0.4 ml/min. The column was then extensively washed with 40 ml of buffer A or until no further protein was eluted. It was then washed with buffer A containing 0.275 M-NaCl and 20% ethylene glycol (100 ml) until A_{280} was constant. The 'dense-vesicle' cyclic AMP phosphodiesterase activity was eluted with buffer A containing 0.5 M-NaCl and 20% ethylene glycol. Fractions (3 ml) containing the highest specific activity were pooled; the NaCl was removed by gel filtration on a Sephadex G-25 column equilibrated in buffer A.

Step 4: guanine-Sepharose chromatography. Guanine-Sepharose, prepared as in Whitson & Appleman (1982), was pre-equilibrated in buffer A before use. Fractions from the previous step were applied (flow rate 0.1 ml/min) to a column (3 cm $\times$ 0.3 cm) of guanine-Sepharose. It was then washed with buffer A until no

further protein was eluted. The 'dense-vesicle' phosphodiesterase was eluted with buffer A containing 0.4 M-NaCl and 20% ethylene glycol. Fractions (1 ml) of the highest specific activity were pooled.

Method 2. Step 1: solubilization of cyclic AMP phosphodiesterase. This was as described above.

Step 2: two-step Affi-gel Blue procedure. The 'hypotonic-shock' extract was adjusted to 10 mM-MgCl₂ and applied to a column (5 cm $\times$ 1 cm) of Affi-gel Blue which had previously been equilibrated in buffer B. Buffer B contained 2 mM-benzamidine, 0.1 mM-PMSF, 5 mM-MgCl₂, 2 mM- β -mercaptoethanol and 10 mM-Tris/HCl, final pH 7.4. The column was washed with buffer B until no further protein (A_{280}) was eluted. The 'dense-vesicle' phosphodiesterase activity was eluted with buffer B containing 1 mM-EDTA in place of 5 mM-MgCl₂, and fractions (3 ml) containing the highest specific activity were pooled. These were adjusted to 10 mM-MgCl₂ and applied to a second Affi-gel Blue column (1 cm $\times$ 1 cm) previously equilibrated with buffer B. The column was washed with buffer B, continuing with 30–50 ml of buffer B after constant A_{280} was achieved (zero protein). Such a thorough washing was found to be essential to ensure the complete purity of this enzyme species. The 'dense-vesicle' cyclic AMP phosphodiesterase activity was then eluted with buffer B containing 1 mM-cyclic AMP and collected as a single fraction (30 ml).

Step 3: removal of cyclic AMP. DEAE-Sepharose CL6B was pre-equilibrated in 10 mM-Tris/HCl, final pH 7.4, with 5 mM-MgCl₂ and 2 mM- β -mercaptoethanol before application of the fraction (flow rate 0.3 ml/min). The column was washed with equilibrating buffer containing 0.1 M-NaCl until A_{280} was zero, before enzyme elution with equilibrating buffer containing 0.3 M-NaCl and 2 mM- β -mercaptoethanol. Fractions (3 ml) containing the highest specific activity were pooled.

Enzyme fractions were stored routinely at –70 °C. The cyclic AMP phosphodiesterase activity was stable for 3–4 weeks when the protein concentration was of the order of 5 μ g/ml and the buffer composition was 5 mM-MgCl₂ (with or without β -mercaptoethanol) and 10 mM-Tris/HCl, final pH 7.4.

The cytosolic and membrane-bound forms of the cyclic GMP-activated cyclic nucleotide phosphodiesterase and the peripheral-plasma-membrane insulin-stimulated cyclic AMP phosphodiesterase were purified to apparent homogeneity as described previously by us, as was the preparation of a polyclonal antibody to the latter enzyme (Pyne *et al.*, 1986; Houslay *et al.*, 1986).

RESULTS

'Hypo-osmotic shock' treatment of either a rat liver particulate fraction or a hepatocyte 'high-speed' pellet released some 55% of the total cyclic AMP phosphodiesterase activity associated with this fraction when assessed at 0.1 μ M-cyclic AMP. Sucrose-density-gradient analysis of this 'solubilized' activity revealed three peaks, with sedimentation coefficients ($s_{20,w}$) of 3.9 ± 0.2 S, 5.4 ± 0.15 S and 7.2 ± 0.37 S (Fig. 1), the major activity being associated with the 5.4 S fraction, with minor peaks at 7.4 S and 3.9 S (Fig. 1). If these assays were performed with added unlabelled cyclic GMP (2 μ M),

Fig. 1. Identification of the insulin- and glucagon-stimulated 'dense-vesicle' enzyme as a 5.4 S species

Sucrose-density-gradient centrifugation of the 'hypotonic-shock' released supernatant was performed as described previously by us (Marchmont & Houslay, 1980a; Heyworth *et al.*, 1983). Assays were done in the presence of $0.1 \mu\text{M}$ -cyclic $[^3\text{H}]\text{AMP}$. In (a) experiments were performed on a 'hypotonic shock' extract of liver membranes. The black and dotted areas denote the differences in cyclic AMP phosphodiesterase activity identified when $2 \mu\text{M}$ unlabelled cyclic GMP was included in the assay: the black area denotes an increase in activity with a peak at 7.4 S, and the dotted area a decrease in activity with a peak at 5.4 S. Similar data could be obtained with an extract from hepatocyte membranes. In (b) hepatocytes were pretreated with insulin (10 nM) together with glucagon (10 nM) (see the text) before membrane isolation and analysis of the 'hypotonic shock' supernatant. The black area denotes an increase in activity, with a peak occurring at 5.4 S. Results are for a typical experiment of one performed at least three times.

then cyclic AMP hydrolysis by the 5.4 S species was potentially inhibited. However, hydrolysis of cyclic AMP by the 7.2 S form was activated 2–3-fold at $0.1 \mu\text{M}$ -cyclic GMP, and that by the 3.9 S form was relatively unaffected. If hepatocytes were pretreated with insulin (10 nM) for 5 min, glucagon (10 nM) for 5 min, or glucagon (10 nM) for 10 min followed by insulin (10 nM) for 5 min (Fig. 1), the cyclic AMP phosphodiesterase activity of the major activity peak only (at 5.4 S) was increased, as noted previously (Heyworth *et al.*, 1983). Treatment of hepatocytes with insulin plus glucagon increased solely the activity of the major cyclic AMP phosphodiesterase peak (R_F 0.48) seen on non-denaturing

PAGE. Such an activity was also inhibited by cyclic GMP (results not shown).

Assayed at $0.1 \mu\text{M}$ -cyclic AMP, cyclic GMP ($2 \mu\text{M}$) almost completely ($>95\%$) inhibited the cyclic AMP phosphodiesterase activity of the purified 'dense-vesicle' enzyme. Under such conditions $2 \mu\text{M}$ unlabelled cyclic GMP elicited 49% inhibition of the cyclic AMP phosphodiesterase activity (41 pmol/min per mg of protein) exhibited by a 'hypotonic shock' extract of hepatocyte membranes. However, if cells were pre-treated with insulin (10 nM) for 5 min, in order to activate this enzyme (34%; 55 pmol/min per mg of protein), inhibition by cyclic GMP was increased, to some 60%. This increase in the degree of inhibition would be expected if insulin solely caused the increase in activity of such a 'cyclic GMP-inhibited' enzyme. Assuming that this insulin-stimulated enzyme is similarly (totally) inhibited by cyclic GMP to the native enzyme under these conditions, one can predict that a 62% inhibition should have been observed in this experiment. That a value close to this was observed provides further support for our contention that the hormone-regulated and high-cyclic GMP-inhibited cyclic AMP phosphodiesterase activities are one and the same. Furthermore, an immobilized preparation of the polyclonal antibody (DV_1) immunoprecipitated this insulin-activated component (results not shown).

By purification Method 1 the 'dense-vesicle' cyclic AMP phosphodiesterase, released by the 'hypotonic-shock' procedure, was purified to apparent homogeneity with an 18% yield and a 2773-fold purification over the specific activity of the original homogenate (Table 1; Fig. 2). By Method 2 the 'dense-vesicle' enzyme was purified to apparent homogeneity with a 13% yield and a 2142-fold purification over the specific activity of the homogenate (Table 1; Fig. 2). The properties of the enzyme purified by both methods appeared to be identical on all occasions. The criteria taken for purification of this species were that hydrolysis of cyclic AMP ($0.1 \mu\text{M}$) was potently inhibited by $2 \mu\text{M}$ -cyclic GMP, that the species exhibited a sedimentation coefficient of 5.4 S on sucrose density gradients, and that material taken from (insulin plus glucagon)-treated hepatocytes showed enhanced (180–200%) activity in the appropriate fractions.

Sucrose-density-gradient analysis of the purified 'dense-vesicle' cyclic AMP phosphodiesterase from both procedures yielded a single symmetrical peak ($s_{20,w} = 5.2 \pm 0.15 \text{ S}$) of cyclic AMP phosphodiesterase activity ($0.1 \mu\text{M}$ substrate). A M_r of approx. 100 000 can be deduced from this result, in accord with estimations of a M_r of $112\,000 \pm 4\,000$ by Sephacryl S200 gel-exclusion chromatography. Both procedures provided a preparation showing a single protein band on 'non-denaturing/dissociating' PAGE, which migrated with an R_F value of 0.48 ± 0.20 and which co-migrated with the single peak of phosphodiesterase activity eluted from the gel (Fig. 3). A subunit M_r of 57 000 (Fig. 3) was obtained by SDS/PAGE, suggesting that this enzyme is a dimer of two subunits of apparently identical molecular mass. Both procedures yielded an apparently homogeneous enzyme preparation. However, if after the hypo-osmotic solubilization procedure purification was carried out in the absence of proteinase inhibitors, a minor protein band of M_r 51 000 was also found together with that of M_r 57 000.

Table 1. Purification of the 'dense-vesicle' cyclic AMP phosphodiesteraseAssays were done at 30 °C in the presence of 0.1 μ M-cyclic AMP.

	Protein (mg)	Specific activity (pmol/min per mg of protein)	Total activity (pmol/min)	Yield (%) [‡]	Purification (fold)
Method 1					
Homogenate	880	6.24	5491	—	(1)
Step 1: solubilization	170	18.9	3213	(100)	3.8
Step 2: ECTEOA-cellulose	10.5	217.5	2284	71	51
Step 3: hydrophobic chromatography	0.34	4529	1540	48	1068
Step 4: guanine-Sepharose	0.033	17503	578	18	2773
Method 2					
Homogenate	780	10	7800	—	
Step 1: solubilization	180	28	5000	(100)	2.8
Step 2: Affi-gel Blue (first column)	3	610	1830	35	62
Step 3: DEAE-Sepharose chromatography of second Affi-gel Blue eluate	0.03	21420	642	13	2142

Two-dimensional mapping of 125 I-labelled tryptic peptides of the purified (57000- M_r subunit) 'dense-vesicle' enzyme were performed (Fig. 4a). That of the 51000- M_r species found when proteinase inhibitors were omitted was very similar (results not shown).

The purified 'dense-vesicle' phosphodiesterase hydrolysed cyclic AMP with kinetics indicative of apparent negative co-operativity (Fig. 5), having an apparent Hill coefficient (h) of 0.43, with limiting values of kinetic constants of $K_{m1} = 0.3 \pm 0.05 \mu\text{M}$, $K_{m2} = 28 \pm 6 \mu\text{M}$, $V_{\max,1} = 0.114 \pm 0.015$ unit/mg of protein and $V_{\max,2} = 0.633 \pm 0.054$ unit/mg of protein. The enzyme also hydrolysed cyclic GMP, exhibiting Michaelis kinetics, with high affinity but with a much lower $V_{\max}$. Values for cyclic GMP hydrolysis were $K_m = 10 \pm 1 \mu\text{M}$ and $V_{\max} = 4.1 \pm 0.2$ munits/mg of protein. The hydrolysis of cyclic AMP was inhibited by unlabelled cyclic GMP with an IC_{50} (concn. yielding 50% inhibition) of $0.2 \pm 0.01 \mu\text{M}$ -cyclic GMP when assayed at 0.1 μM -cyclic AMP. The percentage inhibition of cyclic AMP hydrolysis elicited by a variety of drugs designed to act as 'selective' phosphodiesterase inhibitors is given in Table 2.

The 'dense-vesicle' cyclic AMP phosphodiesterase was very heat-labile and, when incubated at 40 °C, its activity decayed as a single exponential with a half-life, $t_{0.5}$, of 4.5 ± 0.5 min. This property paralleled that ($t_{0.5} = 4.7$ min) of the insulin-stimulated component seen in hypo-osmotic extracts.

The polyclonal antibody DV₁ both immunoprecipitated the purified 'dense-vesicle' cyclic AMP phosphodiesterase and recognized it in Western (immuno-) blotting (Fig. 6). No recognition, or immunoprecipitation, was observed when either pre-immune or non-immune serum was used (results not shown). Antibody DV₁ did not immunoprecipitate (or immunoblot) either

the cytosol or membrane-bound forms of the rat liver cyclic GMP-stimulated cyclic nucleotide phosphodiesterase or the peripheral-plasma-membrane insulin-stimulated cyclic AMP phosphodiesterase (Table 3). In addition, a polyclonal antibody raised against the latter enzyme showed no cross-reactivity with the purified 'dense-vesicle' enzyme (Table 3).

SDS/PAGE of the immunoprecipitated [125 I]iodinated purified 'dense-vesicle' enzyme revealed a single species of M_r 57000. The proteinase inhibitor benzamidine blocks the release of the 'dense-vesicle' enzyme from membrane fractions by endogenous proteinases (Loten *et al.*, 1980). The presumed 'native' 'unclipped' enzyme can be detergent-solubilized by sodium cholate treatment of liver membranes in the presence of benzamidine and PMSF. Treatment of the detergent-solubilized extract of [125 I]iodinated membranes with the antibody DV₁ led to immunoprecipitation of radioactive material, which yielded three bands on SDS/PAGE. The major band evident in all preparations had an M_r of 62000. Comparison of this species with the purified 'clipped' enzyme, which had been 125 I-labelled in the absence of SDS, by single-dimension tryptic-peptide mapping (Fig. 4b), showed them to be very similar indeed (approx. 80% co-relation), indicating that the M_r -62000 protein was the 'native' species. There was, however, a minor band migrating just above this, which had an M_r of about 63000 and an intensity at least one-quarter less of that of the band migrating at 62000. This band may reflect a precursor species possessing a signal peptide. The third band had an M_r in excess of 200000. A tryptic peptide map of this high- M_r band was indistinguishable from that of the M_r -62000 species, implying that the high- M_r band was an aggregated form. Indeed, it appears that this band was an 'aggregation' artifact caused by the immunoprecipitation method, as no such species was

Fig. 2. Purification of the 'dense-vesicle' enzyme

(a) By Method 1; (b) by Method 2. The steps referred to are identified in both the text and Table 1. Assays were done at $0.1 \mu\text{M}$ cyclic AMP. ●, A_{280} (protein), expressed in arbitrary units; ○, cyclic AMP phosphodiesterase specific activity. Results are for a typical experiment of one performed over 20 times. The arrows with concentrations above denote changes in buffer NaCl concentrations, as detailed in the Materials and methods section, and in one instance (Step 2 of b) for EDTA addition. Note that all of the cyclic AMP eluate of the second Affi-gel Blue column was collected as a single fraction. No determination of activity was made, owing to this high concentration of unlabelled material.

Fig. 3. PAGE analysis of the purified 'dense-vesicle' enzyme

(a) Scan of a non-denaturing gel (silver-stained) of the purified enzyme (continuous line), together with an activity profile of cyclic AMP phosphodiesterase eluted from the gel (○). (b) Autoradiograph of a typical purified preparation of the 'dense-vesicle' enzyme that was dissociated in SDS, [^{125}I]iodinated and then subjected to PAGE with subsequent autoradiography. The arrow signifies the [^{125}I]-labelled enzyme: note that this is the only band on the gel that could be detected by protein staining. The material at the leading edge is free iodine (^{125}I). Results are shown of a typical experiment.

Fig. 4. Tryptic-peptide analysis of iodinated proteins

(a) This was performed as described by Takemoto *et al.* (1982), with the purified enzyme iodinated after dissociation with SDS. The origin is shown by an arrow, as are the chromatography and electrophoresis dimensions. An autoradiograph of a typical experiment is shown. (b) This shows a gel scan of a typical autoradiograph of a single-dimension analysis (Cleveland *et al.*, 1977) of tryptic peptides of the M_r -57000 purified (ii) 'dense-vesicle' enzyme and the M_r 62000 'native' enzyme (i) immunoprecipitated with antibody DV₁. ^{125}I -labelling of both enzymes was done in the absence of SDS. Major co-migrating peaks are indicated (●) together with major differences shown as arrows.

Fig. 5. Kinetics of cyclic AMP hydrolysis for the purified 'dense-vesicle' enzyme

This was done at 30 °C with the fully purified enzyme and is typical of at least four plots performed on different enzyme preparations. The curve drawn was generated by using the limiting values of the kinetic constants given in the text and Table 3.

observed on Western blotting of SDS/PAGE-separated proteins of the entire particulate fraction. In such instances only the M_r -62000 and -63000 species were observed. Western-blot analysis of hepatocytes subjected to SDS/PAGE gave identical results, indicating that the M_r -62000 species was the 'native' form (results not shown).

Sodium cholate solubilization, in the presence of benzamidine and PMSF, of radioiodinated membranes which had been previously been 'hypotonic-shock'-treated to release the 'dense-vesicle' enzyme yielded no material that could be immunoprecipitated by the antibody DV₁. The 'hypotonic-shock'-solubilized material, however, gave on immunoprecipitation a single band at M_r 57000 (Fig. 6).

DISCUSSION

Exposure of hepatocytes to either insulin or glucagon causes the activation of membrane-bound high-affinity cyclic AMP phosphodiesterase activity [for reviews see

Table 2. Properties of the 'dense-vesicle' cyclic AMP phosphodiesterase compared with those of the insulin-stimulated cyclic AMP phosphodiesterase and the cytosolic cyclic GMP-stimulated cyclic AMP phosphodiesterase from rat liver

M_r , subunit M_r and the kinetics of cyclic AMP and cyclic GMP hydrolysis for the peripheral-plasma-membrane and cyclic GMP-stimulated plasma-membrane enzyme were taken from Marchmont *et al.* (1981) and Pyne *et al.* (1986) respectively. Abbreviations: N.A., not applicable; IC_{50} , concentration of the drug which elicited 50% inhibition of cyclic AMP phosphodiesterase activity assayed at $1 \mu M$ -cyclic AMP substrate concentration. V_{max} is expressed in units/mg of protein.

	'Dense-vesicle' enzyme	Peripheral-plasma-membrane enzyme	Cyclic GMP-stimulated enzyme
M_r	112000	52000	134000
Subunit M_r	57000	52000	67000
Cyclic AMP hydrolysis			
h	0.43	0.62	1.5
K_{m1}	$0.3 \mu M$	$0.7 \mu M$	$40 \mu M$
K_{m2}	$28 \mu M$	$39 \mu M$	—
$V_{max.1}$	114 munits/mg	1.2 units/mg	4.8 units/mg
$V_{max.2}$	633 munits/mg	9.2 units/mg	—
Cyclic GMP hydrolysis			
K_m	$10 \mu M$	$120 \mu M$	$25 \mu M$
V_{max}	4 munits/mg	0.4 unit/mg	1.5 units/mg
IC_{50} of cyclic AMP hydrolysis (μM)			
Cyclic GMP	2	180	N.A.
Amrinone	71	1000	1000
Milrinone	1	7.3	1000
ICI 63197	100	8	1000
RO-1724	50	7.2	210
SQ-20009	4.6	1000	91
Dipyridamole	450	29	22
Carbazeran	4.5	9	43
Buquineran	14.5	99	790

Thompson & Strada (1978), Beavo *et al.* (1982), Francis & Kono (1982) and Houslay *et al.* (1983)]. In hepatocytes, insulin increased the phosphodiesterase activity in both the plasma membrane and so-called 'dense-vesicle' fraction, whereas glucagon only activated the 'dense-vesicle' enzyme (Heyworth *et al.*, 1983). The 'dense-vesicle' enzyme cannot be extracted by high-ionic-strength treatment (Heyworth *et al.*, 1983), but, unlike the plasma-membrane enzyme (Marchmont & Houslay, 1980a,b; Heyworth *et al.*, 1983), can be released when membranes are put into hypo-osmotic buffer (Loten *et al.*, 1978). This 'hypotonic shock' procedure appears to involve the proteolytic 'clipping' of the enzyme, solubilizing it in an active form still able to reflect activation elicited by hormones.

We have exploited the 'hypotonic shock' method of releasing, in a soluble form, an identifiably insulin- and/or glucagon-activated high-affinity cyclic AMP phosphodiesterase in order to purify this enzyme to apparent homogeneity. This enzyme is clearly very different from the plasma-membrane insulin-stimulated species (Marchmont *et al.*, 1981) and cyclic GMP-activated species (Pyne *et al.*, 1986), isolated from liver, as regards molecular size, kinetics of hydrolysis of both cyclic AMP and cyclic GMP, sensitivity to a variety of phosphodiesterase inhibitors (Table 2), and the high potency of cyclic GMP to inhibit cyclic AMP hydrolysis by the 'dense-vesicle' enzyme. Indeed, a number of agents known to exert potent positive inotropic effects on

the heart, e.g. amrinone, milrinone, carbazeran and buquineran (see Bristol & Evans, 1983; Farah *et al.*, 1984) were found to be potent and selective inhibitors of this enzyme (Table 2). It is therefore possible that the uncharacterized cardiac 'fraction III' phosphodiesterase, which is purported to be the site of action of such drugs (Endoh *et al.*, 1982; Kariya *et al.*, 1982; Weishaar *et al.*, 1985), may be similar to the 'dense-vesicle' enzyme that we have isolated here.

It has been suggested (Major *et al.*, 1983) that the 'dense-vesicle' and insulin-stimulated peripheral plasma-membrane phosphodiesterases might be related to each other. However, the very different ways by which insulin activates these two enzymes (Heyworth *et al.*, 1983; Wilson *et al.*, 1983), their different molecular sizes, kinetics, inhibition by selective inhibitors, lack of immunological cross-reactivity, little co-relation of iodinated [^{125}I]tryptic peptides (less than 20%, Fig. 4; see also Takemoto *et al.*, 1982) and differences in their subcellular localization and attachment to membranes (Heyworth *et al.*, 1983) suggest strongly that they are distinct enzyme species.

Both purification procedures yielded a protein of M_r 112000, with subunit M_r 57000. Thus the solubilized proteolytically 'clipped' enzyme appears to be a dimer consisting of two subunits of similar size. The polyclonal antibody (DV₁) raised against the 'dense-vesicle' enzyme clearly identified the M_r -57000 species on Western blotting of a purified enzyme preparation (Fig. 6). This

Fig. 6. Immunological analysis of the 'dense-vesicle' enzyme

This was done with the polyclonal antibody (DV₁) raised against the 'dense-vesicle' enzyme. Track 1 is an immunoprecipitate of the 'hypotonic-shock' supernatant from intact membranes which had been [¹²⁵I]iodinated. Track 2 is an immunoprecipitate of the sodium cholate-solubilized extract, performed in the presence of benzamide and PMSF, of a [¹²⁵I]iodinated membrane fraction. Track 3 is a Western blot of the purified enzyme. The antibody DV₁ failed to produce a Western blot or immunoprecipitate both the peripheral-plasma-membrane enzyme and the cytosol cyclic GMP-stimulated enzyme. Pre-immune serum and non-immune serum failed to blot or precipitate the 'dense-vesicle' enzyme. Sodium cholate solubilization of membranes that had been previously 'hypotonic-shock' treated did not give any species that could be immunoprecipitated or detected by Western blotting. Letter a defines the aggregated material found on immunoprecipitating the sodium cholate-solubilized membranes as in Track 2. Letter b indicates the minor M_r 63000 band in Track 2 and letter c the band at M_r 62000 in Track 2. Letter d indicates the M_r -57000 band immunoprecipitated from the 'hypotonic-shock' solubilized extract. Positions of M_r markers are shown. The gels are typical ones of experiments done at least three times.

antibody could also be used to immunoprecipitate the enzyme from the 'hypotonic-shock' extract, yielding a M_r -57000 species. A [¹²⁵I]-labelled liver membrane fraction was treated with sodium cholate, in the presence of proteinase inhibitors, in order to solubilize the presumed 'native' 'dense-vesicle' phosphodiesterase and then resolve it by SDS/PAGE of the immunoprecipitated material (Fig. 6). This analysis identified a major band of M_r 62000, with a second band at M_r 200000 and a minor species at M_r 63000. The high- M_r band proved to be an aggregate of the major band occurring at M_r 62000. As the latter band appeared to be highly similar to the M_r -57000 species, we suggest that it may be the 'native' form of the 'dense-vesicle' phosphodiesterase. Presumably the main globular mass of this enzyme (M_r 57000) is found anchored to the bilayer by a small hydrophobic pedicle of M_r around 5000, as is seen for aminopeptidases (Kenny & Booth, 1978). During the preparation of the present paper, the purification of the equivalent insulin-stimulated 'dense-vesicle' enzyme from adipocytes (see Houslay *et al.*, 1983) was reported (Saltiel & Steigerwalt, 1986). SDS/PAGE of the purified proteolytically clipped preparation yielded a subunit of M_r 60000, with the enzyme forming a dimer as reported here for the liver species.

It is clear from the present and other (Cercek & Houslay, 1982; Houslay *et al.*, 1983; Wilson & Houslay, 1983; Pyne *et al.*, 1986) studies that there are a number of very different species of high-affinity cyclic AMP phosphodiesterases in a particular cell type, rather than a single 'high-affinity' form as has been implied by various workers. That two such species are under close and complex hormonal control (see, e.g., Heyworth *et al.*, 1983, 1984; Houslay *et al.*, 1983; Wallace *et al.*, 1984; Houslay, 1986) suggests that they perform important functional roles in regulating cyclic AMP concentrations within the hepatocyte. The ability to purify them, and to utilize selective inhibitors, should allow their role and mechanism(s) of activation to be elucidated.

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Table 3. Specific immunoprecipitation of high-affinity cyclic AMP phosphodiesterase from rat liver

Neither of the antibodies altered the activities of these enzymes to hydrolyse cyclic AMP (0.1 μ M). See the Materials and methods section for details.

Cyclic AMP phosphodiesterase	Antibody . . .	Percentage immunoprecipitated				
		Anti-'dense-vesicle' PDE		Anti-peripheral-plasma-membrane PDE		
	Dilution . . .	1:100	1:50	1:75	1:50	1:20
'Dense-vesicle'		50	100	< 2	< 2	< 2
Insulin-stimulated peripheral plasma membrane		< 2	< 2	20	36	50
Cyclic GMP-stimulated plasma membrane		< 2	< 2	< 2	< 2	< 2

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