A Peripheral and an Intrinsic Enzyme Constitute the Cyclic AMP Phosphodiesterase Activity of Rat Liver Plasma Membranes

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1. Approx. 10% of the rat liver cellular cyclic AMP phosphodiesterase activity was associated with a plasma-membrane fraction. 2. Lineweaver–Burk plots of this activity were clearly non-linear, yielding extrapolated K_m values of 0.7 and $60.6\mu\text{M}$. 3. Treatment of these membranes with high-ionic-strength NaCl solutions apparently released 80% of this activity assayed at $0.4\mu\text{M}$ -cyclic AMP, and 15% of the activity assayed at 1mM -cyclic AMP. 4. The high-salt-solubilized enzyme gave a non-linear Lineweaver–Burk plot. 5. The cyclic AMP phosphodiesterase activity of the washed high-salt-treated membranes exhibited a linear Lineweaver–Burk plot, yielding a K_m of $60\mu\text{M}$. 6. The high-salt-solubilized enzyme exhibited a single peak of activity upon polyacrylamide-gel electrophoresis, a single peak upon sucrose-density-gradient centrifugation (3.9 S) and decayed as a single exponential upon heat-treatment (half-life 1 min at 55°C). 7. The activity of washed high-salt-treated membranes decayed as a single exponential upon heat-treatment (half-life 42 min at 55°C), and was solubilized in the detergent Triton X-100. 8. Cytosol-derived cyclic AMP phosphodiesterase activity could bind to washed high-salt-treated plasma membranes, but was totally eluted by washing with 1mM - KHCO_3 , unlike the high-salt-solubilized enzyme, which required high salt concentrations to elute it. 9. We suggest that the cyclic AMP phosphodiesterase activity of rat liver plasma membranes can be resolved into two components: a single peripheral protein exhibiting apparent negative co-operativity, that is distinct from cytosol forms, and an intrinsic protein exhibiting normal Michaelis kinetics.

Cyclic AMP has been identified as the intracellular mediator of the action of a number of hormones. Cyclic nucleotide concentrations within a tissue will depend not only on the rate of its formation, catalysed by hormone-sensitive adenylate cyclase (EC 4.6.1.1), but also on the rate of degradation. Since the hydrolysis of cyclic AMP to 5'-AMP catalysed by the cyclic AMP phosphodiesterase (EC 3.1.4.17) is the only physiological mechanism known to terminate the action of the cyclic nucleotide, it represents a potential site for the regulation of tissue cyclic AMP concentration.

There is an apparently complex pattern of cyclic AMP phosphodiesterase activity associated with rat liver. Kinetic analysis of fresh homogenates implies the existence of two enzyme forms (Spears *et al.*, 1973; Arch & Newsholme, 1976), DEAE-

cellulose chromatography of a $100\,000g \times 60\text{min}$ supernatant derived from a sonicated homogenate resolves three forms, and treatment of the $100\,000g \times 60\text{min}$ pellet from an homogenate with low-ionic-strength buffer releases another form (Loten *et al.*, 1978). The derived patterns of activity in homogenates and $100\,000g$ supernatants may well be complicated by the presence of both soluble activator and inhibitor proteins (see, e.g., Lyn *et al.*, 1974, 1975; Teo & Wang, 1973; Kakiuchi *et al.*, 1973; Wang & Desai, 1977).

As the site of the glucagon-stimulated synthesis of cyclic AMP occurs at the plasma membrane and because insulin binding to cell-surface receptors may decrease intracellular cyclic AMP concentration by interaction with cyclic AMP phosphodiesterase (Sakai *et al.*, 1974; Kono *et al.*, 1975), we have

carried out a study of the cyclic AMP phosphodiesterase activity associated with the liver plasma membrane.

Materials and Methods

Materials

Ophiophagus hannah venom and Dowex 1 anion-exchange resin were from Sigma, Kingston upon Thames, Surrey, U.K. Cyclic AMP, adenosine, triethanolamine hydrochloride and all enzymes were from Boehringer (U.K.), Lewes, East Sussex, U.K. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of AR quality from BDH Chemicals, Poole, Dorset, U.K.

Plasma-membrane preparation

Rat liver plasma membranes were prepared from male Sprague-Dawley rats, weighing 200–300 g, by a modified (Houslay *et al.*, 1976) method of Pilkis *et al.* (1974). Membranes were either used fresh or after storage in liquid N₂ in 1 mM-KHCO₃ buffer, pH 7.2, at protein concentrations of 5–20 mg/ml. No differences in results were noted.

Distribution of cyclic AMP phosphodiesterase activity between total particulate and plasma-membrane fractions

Three rat livers were homogenized in 1 mM-KHCO₃, pH 7.2, as described by Pilkis *et al.* (1974). Half of this homogenate was taken for the preparation of a plasma-membrane fraction (Pilkis *et al.*, 1974) and the rest was centrifuged for 1 h at 100 000 *g*_{av.} to yield a supernatant and total particulate fraction.

Cyclic AMP phosphodiesterase assay

A two-step radioassay described in detail by Rutten *et al.* (1973) was used to determine activity in the liver homogenate, 100 000 *g* × 60 min supernatant and total particulate fraction. For routine assay of the activity associated with the liver plasma membrane, and for the activity of the high-salt-solubilized enzyme, a two-step radioassay procedure based upon that described by Thompson & Appleman (1971) was used. In the first stage, 100 μ l of a reaction mixture containing (final concentrations) 5 mM-MgCl₂, 3.75 mM-2-mercaptoethanol, cyclic [³H]AMP (approx. 100 000 c.p.m.), 40 mM-Tris/HCl buffer, final pH 7.4, and an appropriate amount of enzyme was used. During kinetic analysis the substrate concentration range was from 4×10^{-7} to 1×10^{-3} M. The reaction mixture was 'whirlmixed' and incubated at 30°C for an appropriate time interval, usually 30 min before termination by boiling for 2 min. After the reaction mixture had cooled to 4°C, 25 μ g of *Ophiophagus hannah* venom (1 mg/ml) was added, and incubation

of this mixture was carried out for 10 min at 30°C to attain complete conversion of 5'-AMP to adenosine. After this stage, 0.4 ml of a resin slurry [Dowex 1; 200–400 mesh; Cl⁻ form as resin/water (1:2, v/v)] was added to the reaction mixture. This mixture was vortex-mixed several times over a 15 min period before being centrifuged at 14 000 *g*_{av.} in a Jobling 320 microfuge for 4 min to sediment the resin thoroughly. Samples (150 μ l) of the supernatant were removed for counting to determine the [³H]adenosine in solution after removal of cyclic [³H]AMP by the resin. Corrections were made for the cyclic [³H]AMP not removed by the resin by using a boiled enzyme preparation as a blank.

Standard deviations for assays are given with (*n* – 1) degrees of freedom.

Dowex resin preparation

Dowex 1 (200–400 mesh; Cl⁻ form) was prepared as described by Pichard & Cheung (1976), except that 1 M-NaOH and 1 M-HCl were used.

Treatment of plasma membranes with NaCl solutions

To 1.5 ml of a 'cocktail' containing final concentrations of 20 mM-2-mercaptoethanol, various NaCl concentrations and 40 mM-Tris/HCl, final pH 7.4, was added 1.5 ml of the plasma-membrane preparation (4 mg/ml in 1 mM-KHCO₃, pH 7.2). This mixture was incubated for 45 min before 1.0 ml portions were loaded on to 3 ml of 60% sucrose in 10 mM-Tris/HCl buffer, pH 7.4. Centrifugation was carried out for 30 min at 300 000 *g*_{av.} at 4°C. The clear supernatant fractions were collected and tested for cyclic AMP phosphodiesterase activity (high-salt-solubilized enzyme). The membranes collecting at the interface were removed and washed twice with 1 mM-KHCO₃, pH 7.2, by centrifugation at 14 000 *g*_{av.} for 5 min at 4°C, before final resuspension in the same buffer (washed high-salt-treated membranes). Both the high-salt-treated enzyme and the high-salt-treated membranes could be stored in liquid N₂ without affecting the results, although normally these fractions were prepared fresh for immediate use.

Routinely, 0.4 M-NaCl was used in these experiments.

Treatment of washed high-salt-treated membranes with detergent

Plasma membranes after treatment with 0.4 M-NaCl and subsequent washing as described above were resuspended at 10 mg of protein/ml. Then 1.5 ml of this preparation was rapidly mixed with an equal volume of a solution containing 1% (v/v) Triton X-100, 10 mM-2-mercaptoethanol and 80 mM-Tris/HCl, pH 7.4. The mixture was incubated on ice for 30 min before 1 ml portions were taken and

centrifuged as described above for the treatment of plasma membranes with NaCl solutions. The supernatant fractions were taken as a soluble Triton X-100 extract of washed high-salt-treated plasma membranes.

Polyacrylamide-gel electrophoresis

This was carried out on 3 or 5% gels in a continuous buffer system of 0.0746 M-glycine/10 mM-Tris/KOH, final pH 8, as described by Davis (1964). Gels were run at 1 mA per tube for 90–120 min at 4°C.

Some gels were stained for protein with Amido Black [1% (w/v) in 7% (v/v) acetic acid], and excess stain was removed by soaking in 7% (v/v) acetic acid. Other gels (7.5 cm long) were cut into 1.5 mm slices and left for 24 h at 4°C in 100 μ l of 5 mM-2-mercaptoethanol/40 mM-Tris/HCl buffer, pH 7.4, to elute proteins from the polyacrylamide-gel matrix before samples were taken for assay of cyclic AMP phosphodiesterase activity.

Sucrose-density-gradient centrifugation

To 100 μ l of a marker-enzyme system containing 100 μ g of cytochrome *c* (horse heart), 20 μ g of malate dehydrogenase (EC 1.1.1.37; pig heart), 70 μ g of lactate dehydrogenase (EC 1.1.1.27; rabbit skeletal muscle), 12 μ g of fumarase (EC 4.2.1.2; pig heart) and 350 μ g of catalase (EC 1.11.1.6; bovine liver) was added 100 μ l of the cyclic AMP phosphodiesterase preparation (100 μ g of protein of the high-salt-soluble enzyme or 1 mg of protein of the 100 000 $g \times 60$ min supernatant from a rat liver homogenate). This mixture was carefully layered on to 3.6 ml of a continuous linear sucrose density gradient (15–30%). The gradients were prepared from equal volumes of 15 and 30% (w/v) sucrose solutions in either 10 mM-Tris/HCl buffer, final pH 7.4, or 0.4 M-NaCl/10 mM-Tris/HCl buffer final pH 7.4. After centrifugation in an MSE 6 $\times$ 4.2 ml swingout rotor at 58 000 rev./min (g_{av} , 450 000) for 20 h at 4°C, samples (150 μ l) were collected from the bottom of the tube. Portions (40 μ l) were taken for cyclic AMP phosphodiesterase assay. Assays for the marker enzymes were carried out as described in full previously (Houslay & Tipton, 1973). The marker proteins did not interfere with the sedimentation of the cyclic AMP phosphodiesterase activity. The $s_{20,w}$ values of the marker proteins used were those published previously (see Haga *et al.*, 1977) and were: cytochrome *c*, 1.91S; malate dehydrogenase, 4.3S; lactate dehydrogenase, 7.3S; fumarase, 9.1S; catalase, 11.3S.

Protein determination

Protein determinations were carried out by the microbiuret method of Goa (1953), with bovine

serum albumin as standard, as modified by Houslay & Palmer (1978).

Heat-stability

Portions (100 μ l) of enzyme containing either 100 μ g of protein of high-salt-solubilized enzyme or 1 mg of protein of either native plasma membranes or washed high-salt-treated membranes or the 100 000 $g \times 60$ min supernatant of a rat liver homogenate were used. These were incubated in a thermostatically controlled water bath for various time intervals at various temperatures before removal into an ice/water mixture at 0–4°C. The fractions were subsequently assayed for cyclic AMP phosphodiesterase activity.

Parenchymal-cell preparation

Isolated intact hepatocytes were prepared from 24 h-starved 200–300 g male Sprague–Dawley rats by the method of Elliott *et al.* (1976).

Computer-aided curve-fitting procedures

Estimates of K_m and V_{max} for the intrinsic enzyme and K_m , V_{max} , and n (the Hill coefficient) for the peripheral enzyme activity in isolated rat liver plasma membranes were achieved by a curve-fitting procedure. A Nottingham Algorithm Group Library program (NAGFLIB: 1326/427; Mk5; Dec. 75) designated EO4 GA/AF was used. This found a least-squares solution of M non-linear equations in N variables by minimizing the sum of the squares of:

$$F(X) = \sum_{i=1}^M Ri(X)^2, \quad M \geq N$$

where $X = (X_1, X_2, \dots, X_N)^T$ by a procedure (Fletcher, 1971) based on the method of Marquardt (1963). Subprograms for calculation of the residuals were prepared and the appropriate partial differentials calculated.

Binding of cyclic AMP phosphodiesterase activity to washed high-salt-treated plasma membranes

Washed high-salt-treated membranes and high-salt-solubilized enzyme from native plasma membranes (6 mg of protein) and a high-speed supernatant of an homogenate (100 000 $g \times 60$ min) were prepared as described above.

Washed high-salt-treated membranes (derived from 6 mg of native membrane protein) were divided into two portions (100 μ l). To one portion was added 100 000 $g \times 60$ min supernatant (6 mg of protein in 400 μ l containing 6 munits of enzyme activity at 1 mM-cyclic AMP substrate concentration). To the other portion was added high-salt-solubilized enzyme (0.5 mg of protein) and a sufficient volume of 1 mM-KHCO₃, pH 7.2, to achieve an overall 4-fold dilution in ionic strength (from 0.4 to 0.1 M-NaCl) (400 μ l overall). There was no apparent loss in the

enzyme activity of the high-salt-solubilized protein on reduction of ionic strength, and no activity sedimented under the centrifugation conditions used. Both membranes plus soluble protein mixtures were vortex-mixed and incubated for 1 h at 4°C before centrifugation at 14 000 *g* for 10 min at 4°C and the supernatant and membrane pellet fractions collected. The pellet was resuspended with 100 μ l of 1 mM-KHCO₃, pH 7.2, and samples from both fractions (25 μ l) were assayed for cyclic AMP phosphodiesterase activity.

Membrane fractions from this stage were divided into two further portions (approx 1.5 mg of protein each). One portion was incubated in a high-salt (0.4 M-NaCl)-solubilization 'cocktail' and centrifuged at 300 000 *g*_{av} for 30 min at 4°C as described above. The second portion was incubated in 1 mM-KHCO₃, pH 7.2, and centrifuged in an identical manner.

The supernatant and membrane fractions resulting from centrifugation were collected and assayed for cyclic AMP phosphodiesterase activity at 0.4 μ M-cyclic AMP. The activity of the endogenous integral (high-*K_m*) enzyme of the membranes was subtracted in all cases, and was more than 95% of the initial activity at the end of the experiment after removal of the absorbed activities.

All these fractions were prepared fresh before use. One unit of enzyme activity is defined as the amount hydrolysing 1 μ mol of cyclic AMP/min.

Results

Assay of cyclic AMP phosphodiesterase

It has been demonstrated that the [³H]adenosine produced in the first step of the assay by the action of 5'-nucleotidase can become bound to the Dowex 1 resin used in the second step, causing an underestimation of enzyme activity (Lynch & Cheung, 1975). We can demonstrate that the amount of [³H]adenosine bound by the resin is significantly affected by the concentration of cyclic AMP used in the assay. For the range of cyclic AMP concentrations normally used in kinetic studies (4×10^{-7} M– 1×10^{-3} M), the fraction of [³H]adenosine bound can vary from 50 to 41% (Fig. 1). If one fails to correct for this occurrence, then rates at low cyclic AMP concentrations would be apparently greater than reality, which could lead to a diagnosis of apparent negative co-operativity.

Furthermore it has been demonstrated (Rutten *et al.*, 1973) that, if tissue preparations are contaminated with adenosine deaminase, then the adenosine produced in the assay system can be converted to inosine, which can become absorbed to the resin, leading to serious underestimates of the rates of reaction. To prevent the resin binding of inosine, the assay method of Rutten *et al.* (1973)

Fig. 1. Effect of cyclic AMP on the binding of adenosine by Dowex anion-exchange resin

'Cocktails' were prepared as for the assay of cyclic AMP phosphodiesterase, except that unlabelled cyclic AMP was added instead of labelled material, and [³H]adenosine was added. Namely to 100 μ l portions containing (final concentrations) 5 mM-MgCl₂, 3.75 mM-2-mercaptoethanol, 40 mM-Tris/HCl pH 7.4, cyclic AMP (various concentrations similar to those used for enzyme assay), [³H]adenosine (50 nM; specific radioactivity 31 Ci/mmol) were added 25 μ l of snake venom (*Ophiophagus hannah*; 1 mg/ml), 0.4 ml of Dowex resin slurry (water/resin 2:1, v/v). After centrifugation at 1400 *g* for 4 min, 150 μ l of supernatant was removed and the radioactivity counted. The data demonstrate the percentage of [³H]adenosine bound by the resin.

was used to assess the cyclic AMP phosphodiesterase activity of the homogenate, total particulate fraction and 100 000 *g* $\times$ 60 min supernatant. Plasma membranes contained no detectable adenosine deaminase activity (<0.02 μ -unit/mg of protein), and the two-step procedure of Thompson & Appleman (1971) was used routinely; this compares with an activity of 8.2 munits/mg in the 100 000 *g* $\times$ 60 min supernatant.

In all of the experiments detailed, great care was taken to assess that the rates of reaction were linear over the times of incubation. Routine incubations were carried out so that less than 10% of the cyclic AMP was degraded, and under such circumstances enzyme activity was linear for as long as 2 h at 30°C.

Liver plasma membranes

Preparation of a liver plasma-membrane fraction shows that there was cyclic AMP phosphodiesterase associated with it. This comprised some 8% of the total cell cyclic AMP phosphodiesterase activity when assayed at 0.4 μ M-cyclic AMP and 5% at 1 mM-cyclic AMP (Table 1). Although our plasma-membrane fraction appeared to be free of endoplasmic reticulum (glucose 6-phosphatase activity $<0.5\%$ of the total) and mitochondria (succin-

Table 1. *Distribution of cyclic AMP phosphodiesterase activity between plasma-membrane and total particulate fractions of a rat liver homogenate*For details see the Materials and Methods section. Results are means $\pm$ s.d. for three determinations.

Cyclic AMP assay concentration	Specific activity (nmol/min per mg of protein)			Recovery (% of total)	
	Homogenate	Particulate	Plasma membrane	Particulate	Plasma membrane
1 mM	2.92 $\pm$ 0.09	2.42 $\pm$ 0.08	2.04 $\pm$ 0.19	29	5
1 μ M	0.062 $\pm$ 0.004	0.056 $\pm$ 0.006	0.062 $\pm$ 0.009	44	8

Table 2. *Kinetic parameters of the cyclic AMP phosphodiesterase activity of rat liver*

Fitting methods are: E, extrapolated lines from linear portions of the curved Lineweaver–Burk plots; CF1, computer curve fitting to the two enzyme species (see Materials and Methods section); H, Hill plot; W, method of Wilkinson (1961). Errors are $\pm$ s.d. on the averages of the calculated values for the preparations. (1) and (2) refer to the 'low- K_m ' and 'high- K_m ' enzymes respectively.

Preparation	Fitting method	No. of determinations	K_m (1) (μ M)	K_m (2) (μ M)	V_{max} (1) (pmol/min per mg)	V_{max} (2) (pmol/min per mg)	Hill coefficient (1) (n)
Native plasma membranes	E	8	0.9 $\pm$ 0.1	68.6 $\pm$ 5.2	108 $\pm$ 6.3	2542 $\pm$ 161	—
	CF1		0.7 $\pm$ 0.08	60.6 $\pm$ 4.3	108 $\pm$ 6.3	2648 $\pm$ 182	0.48 $\pm$ 0.12
Hepatocyte plasma membranes	E	4	0.8 $\pm$ 0.1	70.2 $\pm$ 6.8	120 $\pm$ 10.6	2640 $\pm$ 132	—
	CF1		0.8 $\pm$ 0.1	61.8 $\pm$ 5.3	126 $\pm$ 18.2	2755 $\pm$ 121	0.50 $\pm$ 0.05
High-salt-solubilized enzyme	E	6	0.9 $\pm$ 0.1	15.0 $\pm$ 0.6	50.1 $\pm$ 10.2	1100 $\pm$ 169	—
	H		0.7 $\pm$ 0.05	—	956 $\pm$ 62	—	0.59 $\pm$ 0.05
Washed high-salt-treated membranes	W	6	—	58 $\pm$ 6.2	—	1321 $\pm$ 102	—
Triton-solubilized enzyme from washed-high-salt-treated membranes	W	4	—	72 $\pm$ 8.3	—	1133 $\pm$ 210	—

ate dehydrogenase activity $<0.5\%$ of the total), it is undoubtedly true that our yield of plasma membranes was not 100% (yield of 5'-nucleotidase was 35%).

Cyclic AMP phosphodiesterase activity was proportional to protein concentration, and Lineweaver–Burk plots of this activity were clearly biphasic (Fig. 2), yielding extrapolated K_m values of 0.7 and 60.6 μ M. A similar result was obtained with a plasma-membrane fraction derived from isolated hepatocytes (Table 2).

Treatment of liver plasma membranes with high-salt solutions

Liver plasma membranes were treated with NaCl solutions of various ionic strengths (0.02–0.4 M) as described in the Materials and Methods section. This treatment effected the release of a fraction of the total cyclic AMP phosphodiesterase activity of the membranes. At any particular salt concentration this release was time-dependent, exhibiting a plateau at around 30–40 min. Routinely we carried out the incubations for 45 min, and Fig. 3(a) demonstrates the percentage release of activity over this time period at various concentrations of NaCl. The amount of cyclic AMP phosphodiesterase activity

Fig. 2. *Cyclic AMP hydrolysis by a purified rat liver plasma-membrane fraction*

A Lineweaver–Burk plot of the cyclic AMP phosphodiesterase activity is given over a substrate concentration range 0.4 μ M–1.0 mM. The points represent averages of data obtained using three plasma-membrane preparations with duplicate assays $\pm$ s.d. ($n = 6$).

released reached a plateau at $I > 2.5$. Clearly the apparent fractional activity released depends upon the assay concentration of cyclic AMP used, being 80% at 0.4 μ M-cyclic AMP and only 12% at 1 mM-

Fig. 3. Release of cyclic AMP phosphodiesterase activity from plasma membranes by high-ionic-strength treatment (a) and effect of Ca^{2+} concentration on the release of cyclic AMP phosphodiesterase activity (b)

(a) A purified plasma-membrane fraction (2 mg of protein/ml) was treated with various NaCl concentrations, and after centrifugation the released activity was analysed (see the Materials and Methods section). The cyclic AMP assay concentration was either $0.4 \mu\text{M}$ (▲) or 1.0 mM (●). (b) $I = 0.15$ and the assay concentration of cyclic AMP was $0.4 \mu\text{M}$. Δ, Activity released into $100000 \times 60 \text{ min}$ supernatant; ▲, activity retained in plasma membranes; ●, total activity recovered. Errors are given $\pm$ S.D. for $n = 6$ with three different membrane preparations.

Fig. 4. Lineweaver-Burk plots of the cyclic AMP phosphodiesterase activity of both the washed high-salt-treated plasma membranes and the high-salt-solubilized enzyme

For the washed high-salt-treated plasma membranes (●) the protein concentration was 1 mg/ml; three plasma-membrane preparations were used, and assay were performed in duplicate (data points are means $\pm$ S.D., $n = 6$). For the high-salt-solubilized enzyme (○) the protein concentration was 0.3 mg/ml; three plasma-membrane preparations were used, and assays were done in duplicate (data points are means $\pm$ S.D., $n = 6$).

cyclic AMP, suggesting the resolution of kinetically distinct forms.

Interestingly, the addition of Ca^{2+} to these incubations inhibited the release of this cyclic AMP phosphodiesterase activity. This is shown in detail for one particular ionic strength in Fig. 3(b).

Lineweaver-Burk plots of the cyclic AMP phosphodiesterase activity of the washed high-salt-treated membranes were clearly linear over a substrate range 4×10^{-7} – $1.0 \times 10^{-3} \text{ M}$ -cyclic AMP (Fig. 4), yielding a single K_m of $60 \mu\text{M}$ (Table 2). The enzyme activity was proportional to protein con-

centration, and change in protein concentration had no effect on the form of the Lineweaver–Burk plot.

The cyclic AMP phosphodiesterase activity released from the plasma membrane by high-ionic-strength treatment yielded a non-linear Lineweaver–Burk plot (Fig. 4) indicative of either two enzyme forms or apparent negative co-operativity. Extrapolated K_m values of 0.8 and 16 μM could be obtained (Table 2). As with the total activity in the plasma membrane, the activity of the high-salt-solubilized enzyme was proportional to protein concentration, and change in protein concentration had no effect on the form of the Lineweaver–Burk plot. The presence of 0.1 M-NaCl (after dilution into the assay) did not appear to alter the kinetics of the high-salt-solubilized enzyme, since dialysis had negligible effect on enzyme activity, and no effect on the form of Lineweaver–Burk plots and the kinetic constants obtained.

With hepatocyte plasma membranes as the enzyme source, similar results could be obtained for release of enzyme by high salt concentrations, the forms of the Lineweaver–Burk plots and the kinetic

parameters for the various activities (Table 2), indicating that both of the enzymes are found in this single cell type.

This high-salt-solubilized enzyme was not released or leached from the membranes during the three washings with 1 mM- KHCO_3 , involving 30 min incubations on ice, used in their preparation.

The 100 000 g $\times$ 60 min supernatant from a rat liver homogenate

The activity of the cyclic AMP phosphodiesterase in this fraction was clearly not linear with respect to protein concentration, and the form of the plot depended upon the cyclic AMP concentration used in the assay. At high protein concentrations (200 $\mu\text{g}/\text{ml}$), Lineweaver–Burk plots were clearly non-linear (concave down; results not shown), producing extrapolated apparent K_m values of 2.7 and 44 μM (Table 2). At low protein concentrations (10 $\mu\text{g}/\text{ml}$), a linear plot resulted (results not shown), exhibiting a single K_m of 44 μM (Table 2). In all cases the initial rates were obtained from linear time courses.

Fig. 5. *Sucrose-density-gradient centrifugation of the high-salt-solubilized enzyme*

Cyclic AMP phosphodiesterase activity was assayed at 0.4 μM (Δ) and 1 mM ($\bullet$) cyclic AMP. The experiment was also performed with 0.4 M-NaCl in the gradient ($\blacktriangle$) and assayed at 0.4 μM -cyclic AMP. The inset demonstrates the marker-enzyme distribution: a, cytochrome c; b, malate dehydrogenase; c, lactate dehydrogenase; d, fumarase; e, catalase; the arrow marks the position of the cyclic AMP phosphodiesterase activity. Fraction numbers were normalized against marker-protein distribution for direct comparison of runs with NaCl present and those without.

Fig. 6. Polyacrylamide-gel electrophoresis of the high-salt-solubilized enzyme

Cyclic AMP phosphodiesterase activity at 1 mM (●) and 0.4 μ M- (▲) cyclic AMP concentrations from individual gel slices is shown.

Sucrose-density-gradient centrifugation

The activity profile of the high-salt-solubilized plasma-membrane enzyme after sucrose-density-gradient centrifugation showed a single symmetrical peak of activity when fractions were assayed at either 0.4 μ M- or 1.0 mM-cyclic AMP (Fig. 5). As demonstrated in the inset, the sedimentation coefficients of our known standards plotted against fraction number gave a linear result, allowing us to estimate the sedimentation coefficient of the high-salt-solubilized enzyme as 3.9 S (± 0.15 , s.d., $n = 4$). In case some degree of aggregation had occurred because of the low-ionic-strength conditions, we ran a similar experiment with 0.4 M-NaCl included, obtaining an identical result.

When a fraction of the 100 000 $g \times 60$ min super-

Fig. 7. Heat-treatment of cyclic AMP phosphodiesterase

These were carried out as described in the Materials and Methods section, using cyclic AMP concentrations of 0.4 μ M (▲, Δ) or 1 mM (●, ○) in the assays. (a) Native plasma-membrane fraction at 55°C; (b) high-salt-solubilized enzyme at 55°C (▲, ●) and 45°C (Δ, ○); (c) washed high-salt-treated membranes at 55°C (Δ, ○) and 60°C (▲, ●); (d) 100 000 $g \times 60$ min supernatant of a rat liver homogenate at 55°C. Errors are given as $\pm$ s.d. ($n = 6$) for three different membrane preparations.

nantant of a rat liver homogenate was run in the same manner, two peaks of enzyme activity were found.

These had sedimentation coefficients of 3.55S (± 0.16 , s.d., $n = 5$) and 6.8S (± 0.1 , s.d., $n = 5$). Both activity peaks were observed at low (0.4 μ M) or high (1.0 mM) cyclic AMP assay concentrations, although the relative proportions of activity were dependent on the substrate concentrations employed.

The rates of reaction of cyclic AMP phosphodiesterase activity were linear with respect to time for all of these experiments.

Polyacrylamide-gel electrophoresis of high-salt-solubilized enzyme

Treatment of this enzyme by polyacrylamide-gel electrophoresis and elution of the enzyme activity from gel segments, clearly demonstrated a single symmetrical peak of activity whether the enzyme was assayed with 0.4 μ M- or 1.0 mM-cyclic AMP (Fig. 6). In all cases rates were taken from linear time courses.

Heat inactivation of cyclic AMP phosphodiesterase activity

Semi-logarithmic plots of percentage of enzyme activity against time for native membranes incubated at 55°C are shown in Fig. 7(a). These plots were clearly biphasic whether 0.4 μ M- or 1.0 mM-cyclic AMP was used as the assay substrate. At the low substrate concentration there was an initial rapid loss of 85–90% of the total activity, followed by a slow decay of the remainder (Fig. 7a). With 1 mM-cyclic AMP, the initial rapid loss of activity accounted for only 10–15% of the total, the remainder decaying at a slower rate (Fig. 7a). From both plots one could estimate the half-life of the first

component to be about 1 min and that of the second component to be 44 min (Table 3).

The cyclic AMP phosphodiesterase activity of the washed salt-treated membranes (Fig. 7c) decayed exponentially with time, irrespective of the substrate concentration used. At 55°C the half-life obtained was 42.5 min.

When the high-salt-solubilized enzyme was heat-treated at either 55°C or 45°C, the semi-logarithmic plots were linear (Fig. 7b), even when more than 90% of the activity was destroyed, yielding half-lives of 1.0 min and 6.1 min respectively. These results were identical irrespective of whether the assay cyclic AMP concentration was 1.0 mM or 0.4 μ M (Fig. 7b and Table 3).

With the 100 000 $g_{av} \times 60$ min supernatant of rat liver homogenate as the source of enzyme, heat-treatment at 55°C indicated two forms of the enzyme, with half-lives of 3.2 min and 10.4 min (Fig. 7d) with 1 mM-cyclic AMP.

Triton X-100 treatment of washed high-salt-treated plasma membranes

Exposure of the washed high-salt-treated plasma membranes with increasing concentrations of the non-ionic detergent Triton X-100 clearly resulted in the liberation of the residual cyclic AMP phosphodiesterase activity (Fig. 8). This solubilized enzyme yielded linear Lineweaver–Burk plots with K_m 72 μ M, a slight increase over that of the membrane-bound form (Table 2).

Rebinding of cyclic AMP phosphodiesterase activity

Table 4 shows the results of a typical rebinding experiment of cyclic AMP phosphodiesterase activity from solutions of either the high-salt-solubilized enzyme or the 100 000 $g \times 60$ min super-

Table 3. *Heat-stability of the cyclic AMP phosphodiesterase*

Errors are given as $\pm$ s.d. obtained on average slopes of six decay plots derived from three different enzyme preparations.

Enzyme preparation	Incubation temperature (°C)	Assay concentration of cyclic AMP	Half lives of components (min)	
			(1)	(2)
Native plasma-membrane fraction	55	0.4 μ M	1.1 $\pm$ 0.1	44 $\pm$ 2
	55	1.0 mM	1.1 $\pm$ 0.15	44 $\pm$ 3
High-salt-solubilized enzyme	55	0.4 μ M	1.0 $\pm$ 0.1	—
	55	1.0 mM	1.0 $\pm$ 0.15	—
	45	0.4 μ M	6.1 $\pm$ 0.2	—
	45	1.0 mM	6.1 $\pm$ 0.3	—
Washed high-salt-treated membranes	55	0.4 μ M	42.5 $\pm$ 4.0	—
	55	1.0 mM	42.5 $\pm$ 4.0	—
	60	0.4 μ M	12 $\pm$ 1.0	—
	60	1.0 mM	12 $\pm$ 0.5	—
100 000 $g \times 60$ min supernatant of an homogenate	55	1.0 mM	3.1 $\pm$ 0.3	10.2 $\pm$ 0.3

nant of a rat liver homogenate (cytosol-derived enzyme). Substantial portions of the activity added became bound to the membranes during the in-

cubation. The recoveries of 90% indicated that little activity was lost during the procedure.

Upon washing the membrane pellet containing the bound enzyme activity with 1 mM-KHCO₃, pH 7.2, which was part of our routine in plasma-membrane preparation (Houslay *et al.*, 1976), all of the cytosol-derived enzyme was eluted from the membrane, whereas little if any of the high-salt-solubilized enzyme was released.

Under conditions where both membrane pellets were washed with the high-salt-(0.4 M-NaCl) solubilization 'cocktail', all of the bound cytosol-derived enzyme and all of the bound high-salt-solubilized enzyme was eluted.

In performing these experiments with the high-salt-solubilized enzyme, the conditions used were not optimized for binding. We used a final NaCl concentration of 0.1 M, conditions under which we could only have expected a maximum of 44% of the enzyme to be bound (see Fig. 3a) compared with the 30% obtained, and also we used a dilute protein solution. The purpose of this was to avoid any self-aggregation phenomena that could lead to artifacts. Under these conditions, less than 1% of the enzyme activity could be sedimented by centrifugation at 100 000g × 60 min at 4°C (in the absence of added membranes). We noted, however, that dialysis overnight against 1 mM-KHCO₃, pH 7.2, did cause some aggregation of our high-salt-solubilized enzyme in concentrated solutions. That rebinding did occur under the conditions used and to such a high

Fig. 8. Treatment of washed high-salt-treated membranes with detergent

A washed high-salt-treated plasma-membrane fraction (1 mg of protein/ml) was treated with various Triton X-100 concentrations, and, after centrifugation, the released and residual activities were analysed. The cyclic AMP assay concentration was 1 mM. Protein concentration was 5 mg/ml. ●, Activity remaining in membranes; ○, activity solubilized.

Table 4. Binding of cyclic AMP phosphodiesterase activities to washed high-salt-treated plasma membranes
For details see the Materials and Methods section.

		Assay concentration of cyclic AMP	High-salt-solubilized enzyme	100 000g × 60 min supernatant of an homogenate
Incubation of soluble enzymes with washed high-salt-treated membranes	Bound (%)	{ 1 μM 1 mM	30 27	21 19
	Remaining in solution (%)	{ 1 μM 1 mM	60.2 60.5	76.4 74.2
	Recovery (%)	{ 1 μM 1 mM	90.6 87.1	89 92
	% of bound remaining bound	{ 1 μM 1 mM	96 100	≤1.5 ≤1.5
	% of bound being released	{ 1 μM 1 mM	<1.5 2.3	≥98 ≥98
	Recovery (%)	{ 1 μM 1 mM	87 88	89 90
Membranes washed in 1 mM-KHCO ₃ buffer, pH 7.2	% of bound remaining bound	{ 1 μM 1 mM	<1.5 <2.0	<1.0 ≤1.0
	% of bound being released	{ 1 μM 1 mM	>98 >98	>98 >98
	Recovery (%)	{ 1 μM 1 mM	86 85	85 88
Membranes washed in high-salt-solubilization 'cocktail'	% of bound remaining bound	{ 1 μM 1 mM	<1.5 <2.0	<1.0 ≤1.0
	% of bound being released	{ 1 μM 1 mM	>98 >98	>98 >98
	Recovery (%)	{ 1 μM 1 mM	86 85	85 88

extent (68% of the theoretical) indicates a high affinity of this membrane for this enzyme.

Discussion

We have demonstrated that in a purified plasma-membrane preparation there are two forms of cyclic AMP phosphodiesterase activity. These are an intrinsic membrane enzyme, which exhibits normal Michaelis kinetics and requires detergent to solubilize it, and a peripheral enzyme, which exhibits kinetics indicative of negative co-operativity and is solubilized under conditions of high ionic strength. The peripheral enzyme is apparently a single enzyme species, as it sedimented as a single peak upon continuous-sucrose-density-gradient centrifugation, migrated as a single species upon polyacrylamide-gel electrophoresis and decayed as a single exponential upon thermal denaturation. The form of the Lineweaver–Burk plots may then indicate apparent negative co-operativity, although, considering the small size of the enzyme (3.9S), it is possible that it may turn out to be a monomeric enzyme exhibiting a mnemonic mechanism (see e.g. Ricard *et al.*, 1974; Storer & Cornish-Bowden, 1977).

The peripheral enzyme is not an adsorbed species of the cytosol enzyme, for our rebinding studies demonstrate quite clearly that, although cytosol enzyme can be adsorbed to membranes treated with high-ionic-strength solutions, it was efficiently removed after a low-ionic-strength wash similar to that used in the preparation of plasma membranes, whereas the resorbed high-salt-solubilized enzyme was not. Furthermore, the cytosol enzymes exhibited heat sensitivities and *s* values unlike those of the peripheral enzyme. This peripheral enzyme is also distinct from the EDTA-released enzyme from a $100\,000g \times 60\text{ min}$ pellet characterized by Loten *et al.* (1978), which exhibited very different K_m and *s* values and sensitivity to thermal denaturation. Presumably the enzyme of Loten *et al.* (1978) was either an adsorbed cytosol species or one associated with other intracellular membranes.

Interestingly the Arrhenius-plot behaviour of the plasma-membrane cyclic AMP phosphodiesterase activity assayed under conditions in which more than 90% of the activity reflected that of the intrinsic protein has suggested that, although this enzyme is firmly bound to the liver plasma membrane in both hamsters and rats, it is exclusively localized on the inner half of the bilayer (Houslay & Palmer, 1978; Houslay, 1979). Its activity senses lipid-phase separations occurring in the cytosol facing half of the lipid bilayer only, and is modified by changes in bilayer fluidity that can be effected with the neutral local anaesthetic benzyl alcohol (Gordon *et al.*, 1979).

Having resolved the cyclic AMP phosphodiesterase activity of the rat liver plasma-membrane fraction into these two forms, we can derive a K_m and V_{max} for both species and a Hill coefficient for the peripheral enzyme. By using a curve-fitting procedure, it is possible to calculate the kinetic parameters for the K_m and V_{max} of the intrinsic enzyme and the K_m , V_{max} and Hill coefficient for the peripheral enzyme from Lineweaver–Burk plots of the cyclic AMP phosphodiesterase activity of the native plasma membranes. These data are given in Table 2, together with the estimated K_m and V_{max} values that can be obtained by extrapolation from 'linear' portions of the graphs. These are given purely for comparison with the calculated ones, as such a method is usually used by workers in this field (see e.g. Appleman & Terasaki, 1975).

We see also that attachment of the peripheral enzyme to the membrane has little effect on its kinetic behaviour. The reason for the attachment of this enzyme to the plasma membrane may well be to localize it or perhaps to modulate its activity by interaction with the insulin receptor.

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