Heterogeneity of cyclic nucleotide phosphodiesterases in liver endoplasmic reticulum

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A microsomal fraction from rat liver was subfractionated into three rough endoplasmic reticulum fractions RIII, RII and RI, together with a smooth endoplasmic reticulum plus Golgi fraction. Cyclic nucleotide phosphodiesterase activity was found in all fractions. Subsequently it was shown that Golgi fractions were essentially devoid of cyclic AMP phosphodiesterase activity and the activity resided in the smooth endoplasmic reticulum fraction. The activity of the endoplasmic reticulum constituted some 20% of the homogenate activity, with the major fraction of this being associated with the RII fraction and the least with the RI fraction. With the exception of the activity of the RI fraction, which was a peripheral enzyme, all of the other enzyme activities were integral, requiring detergent or repeated freeze–thawing to effect solubilization. All of the activities appeared to be exposed at the external surface of the endoplasmic reticulum, as they were inactivated by trypsin under conditions where glucose 6-phosphatase was not. All of these activities displayed distinct sensitivities to both thermal and trypsin inactivation, yielding activity decays consistent with a single enzyme species being present in each case. The freeze–thaw–solubilized enzymes yielded single symmetrical peaks on sucrose–density–gradient centrifugation and polyacrylamide–gel electrophoresis. The sedimentation coefficients for the enzymes in the smooth-endoplasmic-reticulum-plus-Golgi, RIII, RII and RI fractions were 3.2S, 4.2S, 4.5S and 4.5S respectively. Whereas the activity in the smooth-endoplasmic-reticulum-plus-Golgi fraction exhibited normal Michaelis kinetics, those in the other fractions yielded kinetics indicative of apparent negative co-operativity. All of the enzymes exhibited low K_m values towards cyclic AMP. The enzymes did not appear to be regulated by Ca^{2+} or calmodulin. ZnCl_2 was found to be a potent non-competitive inhibitor of the enzyme in all fractions. NaF was a weak non-competitive inhibitor. The bilayer fluidizing agent benzyl alcohol exerted dissimilar effects on the enzyme activities. It is concluded that the endoplasmic reticulum displays lateral heterogeneity, with single, rather distinct, cyclic AMP phosphodiesterases being found in the different fractions.

Homogenates of all tissues studied so far exhibit multiple forms of enzymes capable of degrading cyclic AMP and cyclic GMP. These enzymes are found in both soluble and particulate fractions and have been shown to differ as to their physical properties, kinetics, susceptibility to activation by Ca^{2+} –calmodulin and inhibition by methylxanthines and other agents (Appleman *et al.*, 1973; Wells & Hardman, 1977; Thompson & Strada, 1978). As yet, relatively few of these enzymes have been purified to homogeneity. Those that have reflect the heterogeneity of species found within any particular

cell type, namely a low-affinity calmodulin-activated soluble enzyme (Klee *et al.*, 1979; Morrill *et al.*, 1979); a high-affinity cyclic GMP-activated soluble cyclic AMP phosphodiesterase (Martins *et al.*, 1982); a high-affinity soluble cyclic AMP phosphodiesterase (Thompson *et al.*, 1979) and a high-affinity membrane-bound cyclic AMP phosphodiesterase (Marchmont *et al.*, 1981).

In liver, as in other tissues, the location, properties and regulation of cyclic AMP phosphodiesterases are ill-understood (Wells & Hardman, 1977; Thompson & Strada, 1978). Theoretical

studies have suggested that enzymes in specific intracellular locations may play important roles in regulating local cyclic AMP concentrations (Fell, 1980; Reynolds, 1982). Indeed in liver and fat-cells insulin and glucagon have been demonstrated to determine the activation of specific membrane-bound phosphodiesterases (Francis & Kono, 1982; Houslay *et al.*, 1983a,b), which in turn appear to regulate intracellular cyclic AMP concentrations (Houslay *et al.*, 1983a,b). Membrane-bound phosphodiesterases are responsible for a large fraction of the total cellular activity (Marchmont & Houslay, 1980). We have previously shown that two distinct species are associated with the plasma membrane. One is a low-affinity integral enzyme and the other a high-affinity, insulin-stimulated peripheral enzyme (Marchmont & Houslay, 1980; Houslay, 1981). Two species are associated with the mitochondrion, one located at the external surface of the inner membrane and the other at the external surface of the outer membrane (Cercek & Houslay, 1982). Here we examine the phosphodiesterase activities associated with the endoplasmic reticulum.

Materials and methods

Cyclic AMP, cyclic GMP, triethanolamine hydrochloride, NAD⁺ and NADH were all obtained from Boehringer (U.K.), Lewes, East Sussex, U.K. Snake (*Ophiophagus hannah*) venom, Dowex-1 anion-exchange resin, trypsin inhibitor (soya bean; type 1-S), trypsin (bovine pancreas; type 1), antipain dihydrochloride and all marker enzymes and assay substrates were from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Bovine brain calmodulin was given by Dr. R. Grand, University of Birmingham, Birmingham, U.K., and trifluoperazine dihydrochloride was donated by Smith, Kline and French, Welwyn Garden City, Herts., U.K. Cyclic [8-³H]AMP and cyclic [8-³H]GMP were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of AnalaR quality from BDH Chemicals, Poole, Dorset, U.K.

Cyclic nucleotide phosphodiesterase assay

Cyclic nucleotide phosphodiesterase activity of rat liver smooth and rough endoplasmic reticulum subfractions was determined by using a modification (Marchmont & Houslay, 1980) of an established procedure (Thompson & Appleman, 1971). Appropriate corrections were made for the binding of [³H]adenosine and [³H]guanosine to the resin under various assay conditions (Marchmont & Houslay, 1980; Cercek & Houslay, 1982). In order to obviate the difficulty due to contaminating adenosine deaminase, the phosphodiesterase activity of a crude liver homogenate was assayed as

described by Rutten *et al.* (1973). A modified microbiuret method, with bovine serum albumin as standard, was used to determine the protein concentration of the membrane fractions (Houslay & Palmer, 1978).

Preparation of membrane fractions

Male Sprague-Dawley rats weighing 200–300 g were used in all these studies. A crude microsomal fraction, prepared from a liver homogenate, was subjected to discontinuous-sucrose-density-gradient centrifugation as described by Andersson *et al.* (1978), yielding four distinct subfractions. These were designated by Andersson *et al.* (1978) as a 'smooth-plus-Golgi' (smooth endoplasmic reticulum plus Golgi membranes) fraction together with three rough endoplasmic reticulum fractions, R-I, R-II and R-III, in decreasing order of density. To prevent any possible contamination between the fractions during preparation, the regions of near contact of the fractions on the gradient were discarded when preparing the fractions for detailed studies. The subfractions obtained were resuspended in 0.25 M-sucrose/20 mM-Tris/HCl, pH 7.2, and washed by centrifugation at 300 000 *g*_{av} for 30 min. In such experiments the bottom of the tubes were layered with 60% sucrose/10 mM-Tris/HCl, pH 7.2, to prevent the membrane pellet 'annealing' itself to the tube. The membrane pellet was resuspended in 0.25 M-sucrose/20 mM-Tris-HCl, pH 7.2, for use in each case.

The smooth-plus-Golgi fraction was separated by a procedure described in detail previously (Andersson *et al.*, 1978) to yield Golgi subfractions, which were pooled, and a distinct smooth endoplasmic reticulum fraction. These subfractions were washed by centrifugation in a fashion identical with that described above, with the pellets being resuspended in 0.25 M-sucrose/20 mM-Tris/HCl, pH 7.2, for use in each case.

High-ionic-strength treatment

This was performed as described previously (Marchmont & Houslay, 1980).

Detergent treatment of endoplasmic reticulum subfractions

Subfractions were treated with 0.5% (w/v) Triton X-100 in 80 mM-Tris/HCl buffer, pH 7.4. The membrane suspension was incubated on ice in the presence of this detergent solution for 30 min. It was then centrifuged at 300 000 *g* for 30 min. The phosphodiesterase activity in the supernatant was determined.

Thermal denaturation procedure

Portions (100–200 μ l) of the smooth-plus-Golgi subfraction (3.8 mg of protein $\cdot$ ml⁻¹) and rough

subfractions RIII (3.2 mg of protein · ml⁻¹), RII (5.3 mg of protein · ml⁻¹) and RI (1.5 mg of protein · ml⁻¹) were incubated at the stated temperature over a range of time intervals. The samples were then immediately cooled to 4°C before being assayed for phosphodiesterase activity.

Sucrose density gradients

These were performed as described previously (Marchmont & Houslay, 1980) with 15–30% (w/v) continuous linear sucrose gradients in 50 mM-Tris/HCl buffer, pH 7.2. The phosphodiesterase enzymes were solubilized using four freeze–thawing cycles.

Polyacrylamide-gel electrophoresis

This was carried out under non-denaturing conditions as detailed previously (Marchmont & Houslay, 1980). The phosphodiesterase enzymes were solubilized by four cycles of freeze–thawing.

Trypsin and deoxycholate treatment

Freshly prepared subfractions of the smooth and rough endoplasmic reticulum were used (protein concentrations being those listed for the thermal-denaturation experiments). The microsomes were incubated for various time intervals up to 10 min at 30°C with either 50 or 100 µg of trypsin per mg of protein of the microsomal fraction, by using the procedures described by Nilsson & Dallner (1977) and Kreibich *et al.* (1973). The microsomal fractions were continually stirred during the incubation. At the end of the incubation period portions (70 µl) of the incubation mixture were removed and added to ice-cold trypsin inhibitor (10 µg per µg of trypsin final concentration). After vortex-mixing the samples, they were assayed for cyclic AMP phosphodiesterase and glucose 6-phosphatase activity. Trypsin and trypsin-inhibitor control blanks were used. No change in activity occurred in the control samples. To increase the permeability of the microsomal vesicles to trypsin, the vesicles were pre-treated for 5 min at 30°C with sodium deoxycholate (0.3 mg/mg of protein in the fraction). The effect of trypsin (50 µg of trypsin/mg of protein in the

fraction) on the glucose 6-phosphatase and cyclic AMP phosphodiesterase activities was assessed.

Other enzyme assays

Glucose 6-phosphatase and 5'-nucleotidase was assayed as described previously (Houslay & Palmer, 1978), adenosine deaminase as detailed by Marchmont & Houslay (1980), succinate dehydrogenase by the modified method of Bachmann *et al.* (1966) and UDP-galactosyltransferase by the modified method of Palmiter (1969).

Results

In all the experiments the reaction rates, in the presence of cyclic AMP and cyclic GMP substrates, were determined from linear initial rates. The reaction velocity was linear with respect to protein concentration. All the results are averages of duplicate or triplicate determinations.

An underestimation of the phosphodiesterase reaction rate can be made if adenosine deaminase is present in the enzyme preparations (Rutten *et al.*, 1973). However, no detectable adenosine deaminase activity (<0.02 µunits/mg of protein) was observed in the endoplasmic reticulum subfractions compared with a maximal activity of 10.2 m-units/mg of protein in the 100 000 g × 60 min liver supernatant fraction. Cytosolic lactate dehydrogenase contamination was not detectable in the endoplasmic reticulum fractions (<0.2 n-units/mg of protein). The plasma-membrane, mitochondrial and lysosomal contaminations (percentage of total) were estimated by 5'-nucleotidase (<6%), succinate dehydrogenase (<1%) and β-galactosidase (<3%) marker-enzyme assays respectively.

Distribution of cyclic AMP phosphodiesterase activity in the endoplasmic reticulum subfractions

The specific activity and total activity distribution of cyclic AMP phosphodiesterase activity is given in Table 1. Assayed at 1 µM-cyclic AMP substrate concentration the endoplasmic reticulum subfractions appear to account for around 20% of the

Table 1. *Distribution of phosphodiesterase activity in endoplasmic-reticulum subfractions*
Assays were performed at 30°C with 1 µM-cyclic AMP as substrate.

Fraction	Phosphodiesterase activity		Glucose-6-phosphatase activity		UDP-galactosyltransferase activity	
	Distribution* (%)	Specific activity (pmol/min per mg of protein)	Distribution* (%)	Specific activity (µmol/min per mg of protein)	Distribution* (%)	Specific activity (nmol/min per mg of protein)
Smooth-plus-Golgi	19	39.6	13	0.21	29	4
RIII	17	45.9	16	0.26	25	3
RII	56	42.4	66	0.34	23	0.9
RI	8	35.9	5	0.20	3	1.0

* Activity relative to total microsomal activity.

total homogenate phosphodiesterase activity. However, because of our fractionation procedure, where we purposely did not take the regions on the gradient where the fractions make close contact, then losses of material were inevitable, making this value a lower estimate of the total activity contributed by this fraction.

The smooth-plus-Golgi subfractions were separated and the cyclic AMP phosphodiesterase activity assessed, as well as the UDP-galactosyltransferase activity (Golgi marker) and the glucose 6-phosphatase activity (an endoplasmic-reticulum marker). The Golgi fraction was enriched with UDP-galactosyltransferase, having a specific activity of $9.9 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ compared with $0.8 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ in the smooth endoplasmic reticulum fraction, even though only around 60% of the total activity was found in the Golgi fraction. On the other hand, around 98% of the glucose 6-phosphatase activity was found in the endoplasmic reticulum fraction, having a specific activity of $0.29 \text{ units} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ compared with $0.02 \text{ units} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ in the Golgi fraction. The distribution of cyclic AMP phosphodiesterase paralleled that of glucose 6-phosphatase, where around 99% of the activity collected in the smooth endoplasmic reticulum subfraction, having a specific activity of $48 \text{ pmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ compared with $4.5 \text{ pmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ in the Golgi fraction.

Effect of detergent, freeze-thawing and low- and high-ionic-strength treatment on the phosphodiesterase activity of the subfractions

Subfractions were treated with low- and high-ionic strength solutions as well as with the detergent Triton X-100. After low-ionic-strength treatment >97% of the phosphodiesterase activity, assayed at $1 \mu\text{M}$ -cyclic AMP, remained associated with the membranes of the endoplasmic reticulum subfractions. Treatment with a range of high-ionic-strength (NaCl) solutions released the phosphodiesterase activity of the RI subfraction only, in a dose-dependent fashion (results not shown). A maximum release of approx. 98% was reached at 0.4 M -NaCl. Detergent treatment of the smooth and rough RIII and RII subfractions at a final concentration of 0.5% (w/v) Triton X-100 released 70–100% of the phosphodiesterase activities. Four freeze-thawing cycles, in liquid N_2 , released 40–60% of the smooth-plus-Golgi RIII and RII phosphodiesterase activities and 65–80% of the RI phosphodiesterase activity.

Treatment of subfractions with trypsin

Under the conditions used, incubation of freshly prepared fractions for 10–20 min with trypsin had

no effect on the glucose 6-phosphatase activity associated with them (>98% original activity in all). However, in the presence of deoxycholate, which permeabilized the vesicles, around 80% of the total activity was lost in about 10 min. The half-life for trypsin denaturation of glucose 6-phosphatase activity, in all of the fractions, was about 6 min using $50 \mu\text{g}$ of trypsin/mg of membrane protein.

In contrast with these results, incubation of freshly-prepared native fractions with either $50 \mu\text{g}$ or $100 \mu\text{g}$ of trypsin/mg of membrane protein resulted in a loss of cyclic AMP phosphodiesterase activity in all of the fractions (Fig. 1). There was an apparent first-order loss of activity in all fractions. However the half-lives for decay of activity were rather different in each case (Table 2).

Thermal inactivation

The cyclic AMP phosphodiesterase activities in each fraction exhibited very different sensitivities to thermal inactivation (Table 3). Thermal denaturation elicited an apparent first-order loss of activity. Linear plots of the logarithm of percentage activity versus time, covering up to 95% loss of activity, were obtained (results not shown). These yielded the half-lives for decay listed in Table 3. The activity in RI was most thermolabile and that in the smooth endoplasmic reticulum fraction was most thermostable.

After solubilization of the phosphodiesterase activity from these membrane fractions by freeze-thawing, these activities all exhibited a marked decrease in their thermostability (Table 3). At 45°C they all exhibited first-order decay patterns, again with the activity of the smooth endoplasmic reticulum fraction being most thermostable. At 55°C all of the activities decayed very rapidly with half-lives of less than 1 min.

Analysis of the phosphodiesterase activities by sucrose-density-gradient centrifugation and polyacrylamide-gel electrophoresis

Solubilized cyclic AMP phosphodiesterase activity released from the subfractions by repeated freeze-thawing was subjected to centrifugation on a linear 15–30% sucrose density gradient. Single symmetrical peaks of cyclic AMP phosphodiesterase activity were obtained (results not shown), yielding sedimentation coefficients of $3.2 \pm 0.1 \text{ S}$ for the enzyme from the smooth fraction, $4.2 \pm 0.1 \text{ S}$ for the RIII enzyme, $4.5 \pm 0.1 \text{ S}$ for the RII enzyme and $4.5 \pm 0.1 \text{ S}$ for the RI enzyme (means $\pm$ s.d.; $n = 4$).

Non-denaturing polyacrylamide-gel electrophoresis of the solubilized enzyme activities yielded single symmetrical peaks of enzyme activities for all of the fractions. These had R_F values of 0.37 (smooth), 0.34 (RIII), 0.34 (RII) and 0.32 (RI).

Fig. 1. *Trypsin inactivation of cyclic AMP phosphodiesterase activity in endoplasmic reticulum subfractions* Freshly prepared membrane subfractions were treated as described in the Materials and methods section using either 50 µg (▲) or 100 µg (●) of trypsin/mg of membrane protein. Fractions were: (a) smooth plus Golgi; (b) RIII; (c) RII; and (d) RI. Assays are the averages of duplicate determinations.

Table 2. *Trypsin sensitivity of cyclic AMP phosphodiesterase*

Full details are given in the Materials and methods section. Experiments were carried out at either 50 (a) or 100 (b) µg of trypsin/mg of protein in the fraction. Results are means ± s.d. (n = 3). Abbreviation used: *t*_{0.5}, half-life for decay of enzyme activity.

Fraction	<i>t</i> _{0.5} (min)	
	(a)	(b)
Smooth plus Golgi	4.0 ± 0.2	1.8 ± 0.2
RIII	7.3 ± 0.1	3.5 ± 0.3
RII	5.0 ± 0.1	2.3 ± 0.1
RI	12.8 ± 0.3	6.0 ± 0.2

Kinetic analysis

The results of the kinetic analysis carried out on the phosphodiesterase activity in each of the membrane fractions, using either cyclic AMP or cyclic GMP as substrates, are summarized in Table 4. The activity associated with the smooth endoplasmic reticulum appeared to exhibit a relatively low affinity for both substrates and to display normal Michaelis kinetics (Fig. 2). On the other hand, the activities in all of the rough endoplasmic reticulum fractions exhibited non-linear (downwardly curving) Lineweaver-Burk plots (Fig. 2). The limiting values for the kinetic constants are given in Table 3. All of these enzymes exhibited much lower *K*_m values for cyclic AMP than for cyclic GMP. The high-affinity component for the RI

enzyme displayed the lowest *K*_m value observed in all of the fractions.

No significant effect (<8% change) in the cyclic AMP phosphodiesterase activity of the various subfractions was observed in the presence of 10 µM-trifluoperazine, 10 µM-calmodulin, 1–100 µM-Ca²⁺, 10 µM-calmodulin + Ca²⁺, insulin (10 nM), tolbutamide (10 mM), concanavalin A (20 µg/ml) or pre-incubation with EGTA (1 mM) or EDTA (2 mM). Cyclic GMP did not augment the activity of cyclic AMP hydrolysis.

Action of ZnCl₂ and NaF

ZnCl₂ was a potent, reversible inhibitor of the cyclic AMP phosphodiesterase activity in all of the fractions. It appeared to exhibit linear non-competitive kinetics towards the enzyme from the smooth endoplasmic reticulum and towards both the high- and low-affinity components of the enzyme activities in the rough endoplasmic reticulum fractions. *K*_i values obtained were 60 µM for the smooth-plus-Golgi fraction, 48 µM and 45 µM for the high- and low-affinity components of RIII, 64 µM and 65 µM for the high- and low-affinity components of RII and 36 µM and 38 µM for the high- and low-affinity components of RI.

NaF exhibited a similar reversible non-competitive inhibition of the enzymes in all of these fractions. *K*_i values obtained were 34 mM for the smooth-plus-Golgi fraction, 45 mM and 40 mM for the high- and low-affinity components of RIII, 50 mM and 55 mM for the high- and low-affinity components of RII and 20 mM and 18 mM for the

Fig. 2. Kinetics of cyclic AMP hydrolysis by endoplasmic reticulum subfractions

Assays were carried out at 30°C using: (a) smooth-plus-Golgi membranes; (b) RIII; (c) RII; and (d) RI fractions. Units of v are $nmol \cdot min^{-1} \cdot (mg \text{ of protein})^{-1}$. The insets define hydrolysis of cyclic AMP at extended (high) substrate concentrations. Units for the inset axes are the same as those for the main Figures in (a)–(d).

high- and low-affinity components of RI. These were not ionic-strength effects, as no change in activity ensued when the fractions were treated with 50 mM-NaCl (pH was kept at 7.4). In all of these

experiments three different concentrations of inhibitor were added to the assay and limiting kinetic constants were evaluated from plots, with data comparable with those observed in Fig. 2.

Action of benzyl alcohol on cyclic AMP phosphodiesterase activity

The activity of the enzymes in the various fractions, expressed as a percentage of control (100%) activity, in the presence of 50 mM-benzyl alcohol was: smooth-plus-Golgi fraction, $100 \pm 3\%$; RIII fraction, $102 \pm 2\%$; RII, $112 \pm 1\%$; RI, $67 \pm 6\%$ (means $\pm$ s.d., $n = 3$). No further changes ($< 5\%$) in activity were observed using 100 mM-benzyl alcohol.

Discussion

The cyclic AMP phosphodiesterase activity associated with rat liver endoplasmic-reticulum forms a significant fraction of the total cellular activity. The endoplasmic reticulum exhibits a marked lateral asymmetry (for reviews, see DePierre & Ernster, 1977; Morre *et al.*, 1979) and on homogenization, is disrupted, forming heterogeneous vesicles that can be separated by a variety

of techniques including centrifugation. Employing a discontinuous-sucrose-density-gradient procedure it has been shown (Eriksson, 1973; Andersson *et al.*, 1978) to be quite simple to separate smooth endoplasmic reticulum plus Golgi vesicles from three well-defined rough endoplasmic reticulum vesicle fractions. These rough fractions are a ribosome-rich fraction (RI) and two ribosome-poor fractions, denoted as RII and RIII (Andersson *et al.*, 1978). We show here that these fractions all exhibited cyclic nucleotide phosphodiesterase activity and were essentially devoid of contaminants from lysosomes, mitochondria, plasma membranes and cytosol. The phosphodiesterase activity of the RI fraction was associated with the membrane in a rather different fraction than the other phosphodiesterase activities in that it was released by high-ionic strength treatment. It would appear to be a peripheral enzyme bound to the membrane predominantly by electrostatic interactions. On the other hand, the non-ionic detergent Triton X-100 was necessary to solubilize the other enzymes, implying that they interacted with the hydrophobic core of the bilayer and hence might be classed as integral enzymes. Freeze-thawing of the membranes was, however, sufficient to solubilize all of the enzyme species. This might suggest that even the integral enzymes were not deeply embedded in the bilayer (Wilson & Houslay, 1983), but perhaps merely anchored there by a hydrophobic pedicle (see Houslay, 1981). Many enzymes that have their globular functional regions deeply embedded in the bilayer are activated by increasing membrane fluidity (see Kimelberg, 1977; Gordon *et al.*, 1980). However, only the RII enzyme shows indications of being activated by the addition of benzyl alcohol at a concentration where it elicits a marked increase in bilayer fluidity (Gordon *et al.*, 1980).

Glucose 6-phosphatase activity is localized at the luminal surface of the endoplasmic reticulum (Lewis & Tata, 1973; DePierre & Dallner, 1975). Treat-

Table 3. *Thermostability of cyclic AMP phosphodiesterase*

Experiments were carried out as described previously (Marchmont & Houslay, 1980). Results are means $\pm$ s.d. ($n = 3$). Abbreviations used: N.D., not determined; $t_{0.5}$, half-life for decay of enzyme activity.

Fraction	Incubation temperature (°C)	$t_{0.5}$ (min)	
		Membrane-bound enzyme	Solubilized enzyme
Smooth plus Golgi	45	N.D.	12.0 ± 0.9
	60	6.5 ± 0.2	< 1
	65	1.3 ± 0.2	
RIII	45	N.D.	7.5 ± 0.7
	60	6.7 ± 0.4	< 1
RII	45	N.D.	6.0 ± 0.5
	60	3.8 ± 0.6	± 1
RI	45	N.D.	5.5 ± 0.3
	60	1.3 ± 0.6	< 1

Table 4. *Kinetic parameters of cyclic nucleotide phosphodiesterase activity in endoplasmic reticulum subfractions*

Parameters reflect the average of four experiments using a substrate concentration range of $0.3 \mu\text{M}$ – 2 mM with determinations at about 28 different concentrations. Assays were done at 30°C . Limiting kinetic constants were obtained by extrapolation of 'linear' regions at the extremities (see Fig. 2).

Substrate	Fraction	K_m^1 (μM)	K_m^2 (μM)	Maximum velocity [$\text{nmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$]	
				V_{max}^1	V_{max}^2
Cyclic AMP	Smooth plus Golgi	44	—	2.2	—
	RIII	2.6	56	0.21	1.3
	RII	3.8	200	0.25	5.4
	RI	0.8	63	0.59	7.8
Cyclic GMP	Smooth plus Golgi	60	—	4.6	—
	RIII	42	3980	4.2	51
	RII	94	1990	10.3	42
	RI	37	1780	12.8	67

ment with trypsin, which does not pass across the microsomal membrane, fails to affect the activity of this enzyme unless deoxycholate is present to render the membrane permeable to the proteinase (Kreibich *et al.*, 1973; DePierre & Dallner, 1975). We show in all fractions that, under conditions where glucose 6-phosphatase is only inactivated by trypsin in the presence of deoxycholate, the cyclic AMP phosphodiesterase activity is susceptible to trypsin in the intact membranes. This indicates that, in all fractions, the cyclic AMP phosphodiesterase activities are localised at the external, cytosol-facing surface of the endoplasmic reticulum.

The predominant cyclic AMP phosphodiesterase activities in each fraction are rather dissimilar, with each fraction appearing to exhibit a distinct species of activity. This can be concluded from a number of rather different studies. Trypsin inactivation (Table 2) of the phosphodiesterase activity in each fraction results in first-order decay plots, implying that primarily a single enzyme species is present with rather different half-lives in each case. First-order decay curves, with different half-lives exhibited by the various fractions, were obtained from heat-inactivation studies (Table 3). Interestingly, freeze-thaw solubilization of the enzymes rendered all the phosphodiesterase activities more thermolabile. The activity of the smooth endoplasmic reticulum fraction was more thermostable than those associated with the rough fractions, which now all exhibited fairly similar half-lives (Table 3). Further evidence that predominantly a single cyclic AMP phosphodiesterase activity was associated with each fraction came from our observations that the freeze-thawed solubilized enzymes each exhibited single peaks of activity on non-denaturing polyacrylamide-gel electrophoresis and sucrose-density-gradient centrifugation. Thus on the basis of size, charge, thermostability and trypsin denaturation, our results indicate rather distinct phosphodiesterases predominate in each of our separated fractions. Kinetic analysis again highlights the differences between the phosphodiesterase activities in each of these fractions (Table 4).

ZnCl₂ and NaF are often added to homogenates to inhibit the action of phosphatases (Belsham *et al.*, 1980; Cooper *et al.*, 1983). It has been suggested that certain intracellular phosphodiesterases may be regulated by phosphorylation reactions (Loten *et al.*, 1978; Galvagno *et al.*, 1979; Ball *et al.*, 1980; Boyes *et al.*, 1981) and this has been demonstrated by the liver peripheral plasma-membrane enzyme (Houslay, 1981). Here we show that the presence of these compounds leads to an inhibition of enzyme activity. This inhibition would appear to be exerted directly on the enzyme as, in both cases, fully reversible, linear, pure non-competitive inhibition ensued. ZnCl₂ was a potent inhibitor, yielding K_i

values of the order of 40 μ M, with the activity of the RI fraction being most susceptible to inhibition. NaF was a poor inhibitor, yielding K_i values of the order of 20–60 mM, again with the activity of the RI fraction being most susceptible to inhibition.

It is possible that the RI enzyme is a species distinct from the RII and RIII enzymes owing to its different association with the bilayer, decreased sensitivity to trypsin, decreased thermostability, its inhibition by benzyl alcohol and increased sensitivity to inhibition either by NaF or ZnCl₂. We presume that this peripheral enzyme (RI) must bind specifically to an integral membrane protein found uniquely in the RI fraction. For if this enzyme was merely associated with the membrane via an interaction with the phospholipid headgroup it should be found non-specifically binding to all of the fractions (see Houslay, 1981); this is clearly not so. In this respect it is reminiscent of the peripheral plasma-membrane phosphodiesterase, which is localized to the plasma membrane by binding to a single, unique class of protein sites on this membrane (Houslay & Marchmont, 1981).

All of these enzymes have very different properties from the two peripheral enzymes associated with the mitochondrion (Cercek & Houslay, 1982) and the two enzymes associated with the plasma membrane (Marchmont & Houslay, 1980). Clearly there are a number of rather specific cyclic AMP phosphodiesterases associated with cellular membranes. Their function, except for the hormone-modulated ones (Houslay *et al.*, 1983a,b), is, however, far from clear. It may be, as has been suggested by a number of investigators (Erneux *et al.*, 1980; Fell, 1980; Reynolds, 1982), that their role is to regulate local cyclic AMP concentrations. Perhaps by defining these activities clearly then we might begin to design experiments whereby we can appreciate the significance of these enzymes.

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