Specific antibodies and the selective inhibitor ICI 118233 demonstrate that the hormonally stimulated ‘dense-vesicle’ and peripheral-plasma-membrane cyclic AMP phosphodiesterases display distinct tissue distributions in the rat

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Polyclonal-antibody preparations DV1 and PM1, raised against purified preparations of rat liver insulin-stimulated ‘dense-vesicle’ and peripheral-plasma-membrane cyclic AMP phosphodiesterases, were used to analyse rat liver homogenates by Western-blotting techniques. The antibody DV1 identified only the 63 kDa native subunit of the ‘dense-vesicle’ enzyme, and the antibody PM1 only the 52 kDa subunit of the plasma-membrane enzyme. These antibodies also detected the subunits of these two enzymes in homogenates of kidney, heart and white adipose tissue from rat. Quantitative immunoblotting demonstrated that the amount of these enzymes (by wt.) varied in these different tissues, as did the expression of these two enzymes, relative to each other, by a factor of as much as 7-fold. The ratio of the dense-vesicle enzyme to the peripheral-plasma-membrane enzyme was lowest in liver and kidney and highest in heart and white adipose tissue. ICI 118233 was shown to inhibit selectively the ‘dense-vesicle’ cyclic AMP phosphodiesterase in liver. It did this in a competitive fashion, with a K_i value of $3.5 \mu\text{M}$. Inhibition of tissue-homogenate cyclic AMP phosphodiesterase activity by ICI 118233 was used as an index of the contribution to activity by the ‘dense-vesicle’ enzyme. By this method, a tissue distribution of the ‘dense-vesicle’ enzyme was obtained which was similar to that found by using the immunoblotting technique. The differential expression of isoenzymes of cyclic AMP phosphodiesterase activity in various tissues might reflect a functional adaptation, and may provide the basis for the different physiological actions of compounds which act as selective inhibitors.

INTRODUCTION

Cyclic AMP phosphodiesterases provide the sole means of degrading the key second messenger cyclic AMP in mammalian cells. As such, their activities play an important role in controlling intracellular cyclic AMP concentrations and hence cellular functioning (Wells & Hardman, 1977; Thompson & Strada, 1978; Beavo *et al.*, 1982; Houslay *et al.*, 1983; Houslay, 1986). It is known that mammalian cells exhibit multiple forms of cyclic AMP phosphodiesterase activity, although the functional relevance of such multiplicity has yet to be established (see Houslay *et al.*, 1983). Indeed, relatively few cyclic AMP phosphodiesterases have been purified to homogeneity, and no attempts have been made to assess distribution of specific phosphodiesterase isoenzymes in various tissues of a particular animal species.

Challenge of cells with either insulin or hormones which activate adenylate cyclase has been shown to enhance cyclic AMP phosphodiesterase activity in a variety of tissues (Thompson & Strada, 1978; Francis & Kono, 1982; Houslay *et al.*, 1983; Houslay, 1986). We have shown that there are at least three specific high-affinity cyclic AMP phosphodiesterases in rat liver whose activities are under rapid reversible regulation by hormones (Heyworth *et al.*, 1983; Houslay, 1986). We have identified, purified and characterized two of these. They differ in their physical properties, thermal stability, kinetics of degradation of cyclic AMP and cyclic GMP, inhibitor sensitivities, ^{125}I -labelled tryptic-peptide maps,

and in the ability of cyclic GMP to inhibit the hydrolysis of cyclic AMP (Marchmont *et al.*, 1981; Houslay *et al.*, 1987; Pyne *et al.*, 1987). One of these is the peripheral-plasma-membrane enzyme, whose activity can be stimulated by insulin through a process which involves its phosphorylation (Marchmont & Houslay, 1980*a,b*, 1981) and where activation by insulin can be blocked by pre-treatment of hepatocytes with glucagon (Heyworth *et al.*, 1983; Wilson *et al.*, 1983). The other enzyme is the ‘dense-vesicle’ cyclic AMP phosphodiesterase (Pyne *et al.*, 1987), whose activity is rapidly increased when hepatocytes are treated with either insulin or glucagon (Heyworth *et al.*, 1983). Glucagon exerts its stimulatory effect on this enzyme by a cyclic AMP-mediated process (Heyworth *et al.*, 1983), which is quite distinct from the mechanism, as yet unidentified, whereby insulin exerts its stimulatory effect on this enzyme (Heyworth *et al.*, 1984). Indeed, in contrast with results with the plasma-membrane enzyme (Heyworth *et al.*, 1983; Wilson *et al.*, 1983), exposure of hepatocytes to both insulin and glucagon leads to the synergistic activation of the ‘dense-vesicle’ enzyme (Heyworth *et al.*, 1983). The different mechanisms of hormonal regulation of these two enzymes implies that they may have specific functional roles in particular tissues.

In the present study, using both polyclonal-antibody preparations specific for each of these enzyme preparations and a highly selective compound, ICI 118233, which specifically inhibits the ‘dense-vesicle’ phosphodiesterase, we demonstrate that both of these enzymes are

Fig. 1. Structure of ICI 118233 {6-[*p*-(3-methylureido)phenyl]-3[2H]-pyridazinone}

expressed in rat tissues other than liver. However, the ratios of their extents of expression were very different in the various tissues examined.

MATERIALS AND METHODS

ICI 118233 {6-[*p*-(3-methylureido)phenyl]-3[2H]-pyridazinone} (Fig. 1) was kindly given by Dr. M. Collis, ICI Pharmaceuticals, Alderley Edge, Macclesfield, Cheshire, U.K. Peroxidase-linked antibody was kindly given by the Scottish Antibody Production Unit (Low Hospital, Carlisle, Lanarkshire, Scotland). 125 I-Protein A was obtained from New England Nuclear. Other radioisotopes were from Amersham International, Amersham, Bucks., U.K. Nitrocellulose was from Schleicher & Schuell, Dassel, Germany. All biochemicals were from Boehringer Corp. (U.K.).

Tissues were isolated from male Sprague-Dawley rats and immediately diced and homogenized with a Teflon/glass homogenizer at 250 rev./min in ice-cold iso-osmotic 0.25 M-sucrose containing 10 mM-Tris/HCl, 1 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride and 2 mM-benzamidine, final pH 7.4. Samples were then removed and immediately boiled in electrophoresis buffer as described by Laemmli (1970).

Protein was determined as described by Bradford (1976).

Cyclic AMP phosphodiesterase activity was assayed by a modification of the two-step procedure of Thompson & Appleman (1971) and of Rutten *et al.* (1973) as described previously (Marchmont & Houslay, 1980b). All assays were done at 30 °C and initial rates were taken from linear time courses.

The 'dense-vesicle' and peripheral-plasma-membrane cyclic AMP phosphodiesterases were purified as described previously (Pyne *et al.*, 1987; Houslay *et al.*, 1987). The production and specificity of the polyclonal antibodies DV1 and PM1 to the 'dense-vesicle' and peripheral-plasma-membrane enzymes, respectively, has been described previously (Houslay *et al.*, 1987; Pyne *et al.*, 1987).

SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970), with a 5%-acrylamide stacking gel and a 10% separating gel on an LKB slab-gel electrophoresis apparatus. Gels were run at 300 V for 2.5 h. After electrophoresis was completed, the separated proteins were transferred to nitrocellulose and immunoblotted with either the antibody DV1 or the antibody PM1, by procedures detailed by Milligan *et al.* (1986). Labelled bands were identified by using either peroxidase-linked anti-rabbit IgG or 125 I-labelled Protein A. Quantification of 125 I-labelled enzymes was performed by locating bands by autoradiography and subsequently excising them for radioactivity counting with an LKB gamma counter. The amount of enzyme present was determined by constructing a standard curve with various amounts of the purified enzymes which were subjected to electrophoresis and immunoblotting. When the peroxidase-linked second antibody was used for detec-

Fig. 2. Polyclonal antibodies DV1 and PM detect single enzyme species in homogenates of liver, heart, kidney and white adipose tissue

Data are given with the antiserum DV1 against the 'dense-vesicle' enzyme (*a-d*) and the antiserum PM1 against the peripheral-plasma-membrane enzyme (*e-h*) used to immunoblot homogenates of white fat (*a, e*), heart (*b, f*), liver (*c, g*) and kidney (*d, h*) tissue from rat. This was done as described in the Materials and methods section, with equal quantities of protein (50 µg) being loaded on to each track. Detection used a peroxidase-linked anti-rabbit IgG as second antibody, with *o*-dianisidine as substrate. A digitized image of the two gels is shown here.

tion, the blots were scanned to produce images by using an Abaton Scan 300 high-resolution optical digitizer operated by an Apple Macintosh computer using Abaton C-scan (V1.0) software.

The plasma-membrane and cytosol forms of cyclic GMP-stimulated cyclic nucleotide phosphodiesterases were purified from rat liver as described previously (Pyne *et al.*, 1986). Ca^{2+} /calmodulin-activated cyclic nucleotide phosphodiesterase was purified from rat liver 100 000 *g* supernatant by the procedure of Sharma *et al.* (1980). The 'dense-vesicle' enzyme was released from a membrane pellet, obtained by centrifuging a rat liver homogenate for 20 min at 100 000 *g*, by the hypo-osmotic-lysis procedure of Loten *et al.* (1980).

RESULTS

We have shown previously (Pyne *et al.*, 1987; Houslay *et al.*, 1987) that the antisera DV1 and PM1 caused the specific immunoprecipitation of the 'dense-vesicle' and peripheral-plasma-membrane cyclic AMP phosphodiesterase respectively. Furthermore, they did not interact with the purified cyclic GMP-stimulated cyclic AMP phosphodiesterase (Pyne *et al.*, 1987) or the Ca^{2+} /calmodulin-stimulated cyclic nucleotide phosphodiesterase (results not shown) from rat liver.

Immunoblot analysis of homogenates of liver, heart, kidney and white adipose tissue identified a single major labelled band at 52 kDa with antiserum PM1 and at 63 kDa with antiserum DV1 (Fig. 2). It was apparent from the different degrees of staining that were observed in such experiments, which used identical amounts of protein, that the distribution of these two enzymes in the various tissues was not the same. The amounts of each enzyme in each tissue were measured by using the specific antibodies and 125 I-Protein A (Table 1).

The compound ICI 118233 specifically inhibited the 'dense-vesicle' phosphodiesterase and did not appear to

Table 1. Use of antibodies DV1 and PM1 to assess the distribution of the 'dense-vesicle' (DV) and peripheral-plasma-membrane (PM) cyclic AMP phosphodiesterases (PDE) in rat tissues

Results are presented as averages $\pm$ S.D. of three separate experiments. TD ratio is the tissue distribution ratio relative to that in liver (1). Ratio of DV PDE/PM PDE is taken with respect to that in liver (1).

Tissue	DV PDE		PM PDE		Ratio DV PDE/PM PDE*	Ratio DV PDE/PM PDE†
	($\mu\text{g}/\text{mg}$ of homogenate protein)*	TD ratio	($\mu\text{g}/\text{mg}$ of homogenate protein)*	TD ratio		
Liver	0.15 ± 0.02	(1)	0.15 ± 0.03	(1)	(1)	(1)
Kidney	0.14 ± 0.02	0.9	0.12 ± 0.02	(0.8)	1.1	1.3
Heart	0.75 ± 0.08	5	0.11 ± 0.03	(0.7)	7.1	7.4
White adipose tissue	0.48 ± 0.06	3.2	0.11 ± 0.02	(0.7)	4.6	4.6

* Assessed by quantitative immunoblotting by using ^{125}I -Protein A as described in the Materials and methods section.

† Assessed by densitometric scanning of peroxidase-identified reaction.

Fig. 3. Inhibition of the 'dense-vesicle' cyclic AMP phosphodiesterase by ICI 118233

(a) Lineweaver-Burk plot of the reciprocal of the rate of cyclic AMP hydrolysis against the reciprocal of cyclic AMP substrate concentration. Assays were performed in the absence of inhibitor ($\square$), with $1 \mu\text{M}$ -ICI 118233 ($\blacklozenge$) and with $10 \mu\text{M}$ -ICI 118233 ($\blacksquare$). (b) Replots of slope against inhibitor concentration of the data presented above yielded a straight line of correlation coefficient 0.99.

Table 2. Use of ICI 118233 to measure the distribution of the 'dense-vesicle' cyclic AMP phosphodiesterase (DV PDE) in rat tissues

Tissue homogenates were assayed with $0.1 \mu\text{M}$ -cyclic AMP as substrate and in the presence and absence of $100 \mu\text{M}$ ICI 118233. Data are given as averages $\pm$ S.D. of three separate experiments. Activity of DV PDE is calculated relative to the specific activity of the tissue homogenate, assuming that activity inhibited by ICI 118233 represents the activity of the DV PDE. At these concentrations of cyclic AMP substrate and inhibitor, the activity of the purified DV PDE was completely inhibited (greater than 98%). Tissue distribution ratio is calculated with respect to that in liver.

Tissue	Inhibition of homogenate (%)	Activity of DV PDE (pmol/min per mg of protein)	Tissue distribution ratio of presumptive DV PDE activity
Liver	24 ± 10	0.8	(1)
Kidney	12 ± 5	1.1	1.4
Heart	44 ± 3	2.6	3.3
White adipose tissue	59 ± 7	1.9	2.4

inhibit any other cyclic AMP phosphodiesterase activity in liver. Thus, at a concentration of $100 \mu\text{M}$ -ICI 118233 and $0.1 \mu\text{M}$ -cyclic AMP it had no inhibitory action (less than 5%) on the plasma-membrane phosphodiesterase, both the plasma-membrane and cytosol forms of cyclic GMP-stimulated phosphodiesterase, Ca^{2+} /calmodulin-activated phosphodiesterase activity, total cytosol ($100\,000 \text{ g}$ supernatant) phosphodiesterase activity and phosphodiesterase activity in a total membrane fraction from which the 'dense-vesicle' enzyme had been released by hypo-osmotic lysis (Loten *et al.*, 1980).

Assayed over a highly extended substrate concentration range, the dense-vesicle enzyme displays evidence of apparent negative co-operativity (Pyne *et al.*, 1987). Analysis of inhibition was done at low substrate concentrations (0.1 – $1.0 \mu\text{M}$ -cyclic AMP), which allowed

us to follow essentially the 'high-affinity' component of its activity. This yielded an apparently linear Lineweaver-Burk plot (Fig. 3). Under such conditions ICI 118233 inhibited the 'dense-vesicle' enzyme in a competitive fashion (Fig. 3), yielding a K_i value of $3.6 \pm 0.2 \mu\text{M}$ (Fig. 3). At a substrate concentration of $0.1 \mu\text{M}$ -cyclic AMP, the concentration of ICI 118233 that gave 50% inhibition of activity (IC_{50}) was $3.8 \pm 1.2 \mu\text{M}$ ($n = 4$).

ICI 118233 was used to assess the degree of inhibition of cyclic AMP phosphodiesterase activity in homogenates from the various tissues at a concentration ($100 \mu\text{M}$; Table 2) where it was shown to inhibit over 98% of the activity of the 'dense-vesicle' enzyme purified from liver. An assessment of the tissue distribution of the 'dense-vesicle' enzyme was made, assuming that the inhibited fraction of activity represented solely that of the 'dense-vesicle' enzyme (Table 2).

DISCUSSION

Cyclic AMP phosphodiesterases are known to exist in multiple forms in mammalian tissues (Wells & Hardman, 1977; Thompson & Strada, 1978; Beavo *et al.*, 1982; Houslay *et al.*, 1983). Immunological and peptide-mapping studies have now indicated that this multiplicity is due to the expression of distinct isoenzymes (Takemoto *et al.*, 1982; Mumby *et al.*, 1982; Pyne *et al.*, 1986, 1987; Harrison *et al.*, 1986). The observation that the activity of specific isoenzymes is under rapid control by hormones suggests that various forms have particular functional roles within the cell (Houslay, 1986; Houslay *et al.*, 1983). As such, one might expect that each tissue will exhibit a particular complement of cyclic AMP phosphodiesterase isoenzymes. Indeed, elution profiles of crude tissue extracts on ion-exchange chromatography have implied that there may be differences in the tissue distribution of multiple forms (Appleman *et al.*, 1973; Thompson & Strada, 1978). However, such conclusions have been qualified by concerns about differential extraction of various enzymes and their susceptibility to degradation by proteolysis during extraction and purification procedures (Beavo *et al.*, 1982; Houslay *et al.*, 1983). In order to approach this problem, we have used a technique where tissues are disrupted rapidly in a medium containing proteinase inhibitors, then boiled immediately in SDS sample buffer, and subjected to SDS/polyacrylamide-gel electrophoresis with detection of specific enzymes by quantitative immunoblotting. This avoids problems of differential extraction and of proteolysis.

In this study we have focused on two high-affinity cyclic AMP phosphodiesterases, whose activities are regulated by hormones (Heyworth *et al.*, 1983) and which we have purified to apparent homogeneity from rat liver (Marchmont *et al.*, 1981; Pyne *et al.*, 1987; Houslay *et al.*, 1987). These are the peripheral-plasma-membrane enzyme and the 'dense-vesicle' enzyme.

The peripheral-plasma-membrane enzyme is a 52 kDa monomeric species which can be detected specifically with the polyclonal antibody PM1 (Pyne *et al.*, 1987; Fig. 2). The 'dense-vesicle' enzyme exhibits a single 63 kDa subunit, which can be detected specifically with the antibody DV1 (Pyne *et al.*, 1987; Fig. 2). Proteinase action can, however, convert both enzymes into lower-molecular-size forms (Pyne *et al.*, 1987; N. J. Pyne & M. D. Houslay, unpublished work). Indeed, solubiliza-

tion of the 'dense-vesicle' enzyme from the membrane vesicle where it is located can be achieved by a 'hypo-osmotic shock' procedure, in which endogenous proteinases (Loten *et al.*, 1980; Pyne *et al.*, 1987) cleave an anchoring peptide, releasing a soluble 57 kDa species (Pyne *et al.*, 1987). Analysis of a total liver homogenate (Fig. 2) shows quite clearly, however, that the antibodies PM1 and DV1 detected the native 52 kDa and 63 kDa subunits of the plasma-membrane and 'dense-vesicle' enzymes respectively. Indeed, that single stained bands were obtained shows that these antibodies do not cross-react with any of the other phosphodiesterase enzymes that are found in liver. The single band at 63 kDa detected by immunoblotting analysis of a liver homogenate indicates that the high-molecular-mass species that was immunoprecipitated by treatment of a liver-membrane detergent extract with antibody DV1 was indeed an aggregated species, as indicated by our tryptic-peptide analysis (Pyne *et al.*, 1987).

These two phosphodiesterases are not, however, found exclusively in liver tissue. Analysis of homogenates of heart, kidney and white (epididymal) fat tissue showed that both of these enzymes appeared to be expressed in all of these tissues. However, it was apparent (Fig. 3) that the amounts of these two enzymes, expressed per mg of protein extract, varied between the tissues examined (Table 1). Estimates of the amount of each phosphodiesterase in liver are in close agreement with previous work (Pyne *et al.*, 1987; Houslay *et al.*, 1987). Similarly, combining values for the 'dense-vesicle' enzyme catalytic activity (Table 2) and protein content (Table 1), the resulting specific activity also closely agrees with the specific activity previously presented for the purified enzyme (Pyne *et al.*, 1987). It is important to note that the ratio of the two phosphodiesterases in a specific cell type may differ from values reported in this study, which represent tissue averages. Immunohistochemical techniques may allow the distribution of the enzymes among different cell types to be studied.

ICI 118233 caused the selective inhibition of the 'dense-vesicle' enzyme, and did not appear to inhibit any of the other cyclic AMP phosphodiesterase activities found in liver. Thus the amount of inhibition of cyclic AMP phosphodiesterase activity elicited by ICI 118233 might be assumed to reflect that of the 'dense-vesicle' enzyme in liver. Making the assumption that this compound acted similarly in the other tissues examined, its relative effectiveness at inhibiting the cyclic AMP phosphodiesterase activity of other tissues can be used to estimate the amount of the 'dense-vesicle' enzyme in these tissues. By this method, a very similar distribution of the 'dense-vesicle' phosphodiesterase was found (Table 2) to that obtained with the specific antibody DV1 (Table 1). Although there are potent inhibitors of the plasma-membrane enzyme, e.g. ICI 63917 (Houslay *et al.*, 1987), such compounds are also effective inhibitors of the 'dense-vesicle' and other cyclic AMP phosphodiesterases in the cell (Pyne *et al.*, 1987; Houslay *et al.*, 1987; N. J. Pyne & M. D. Houslay, unpublished work), which precludes their use in a comparable analysis.

The 'dense-vesicle' enzyme thus appears to account for a major fraction of the total cyclic AMP phosphodiesterase activity in heart and in epididymal white adipose tissue (Tables 1 and 2). Part of insulin's major anti-lipolytic action on white adipose tissue is believed to

be exerted through actions on cyclic AMP metabolism (Fain, 1980; Londos *et al.*, 1985), and thus the 'dense-vesicle' enzyme may provide a major physiological target for insulin's action in this tissue. Indeed, our observation that the 'dense-vesicle' enzyme provides a major fraction of the phosphodiesterase activity in adipose tissue may account for the reports by various investigators that insulin elicits a very potent stimulation of cyclic AMP phosphodiesterase activity in this tissue. The enzyme that we have identified with the antibody DV1 and by inhibition with ICI 118233, in adipose tissue, undoubtedly reflects the native, membrane-bound, form of the proteinase-solubilized species purified by Saltiel & Steingewalt (1986). This enzyme was shown to be a dimeric species consisting of two subunits, each of around 60 kDa.

Interestingly, the 'dense-vesicle' enzyme and that isolated from bovine cardiac tissue can both be potently inhibited by compounds which exert positive inotropic actions and, furthermore, their ability to hydrolyse cyclic AMP is extremely sensitive to inhibition by cyclic GMP, which itself provides a poor substrate for these enzymes (Harrison *et al.*, 1986; Pyne *et al.*, 1987). In this regard ICI 118233 is a highly potent inotropic agent. Thus the observation that the 'dense-vesicle' enzyme provides a major proportion of the high-affinity cyclic AMP phosphodiesterase activity in cardiac tissues lends support to the contention (Harrison *et al.*, 1986; Houslay *et al.*, 1987; Pyne *et al.*, 1987) that this enzyme might act as a major target for phosphodiesterase inhibitors which exert positive inotropic actions.

Our data thus demonstrate that a variety of rat tissues express both of these distinct insulin-stimulated cyclic AMP phosphodiesterases. However, their relative expression varies markedly between the tissues. As insulin activates these two enzymes by different routes, and as increases in the intracellular concentrations of cyclic AMP elicited by either glucagon or adrenaline (β -adrenoceptor) activate the 'dense-vesicle' enzyme (Houslay *et al.*, 1983; Wilson *et al.*, 1983; Heyworth *et al.*, 1984), it is likely that their differential expression has a functional significance which is peculiar to particular tissues. The present study thus provides the first clear evidence of differential expression of specific phosphodiesterase isoenzymes in the tissues of a particular animal. It gives a rational basis to the current interest (see, e.g., Bristol & Evans 1983; Farah *et al.*, 1984; Weishaar *et al.*, 1985) in the development of inhibitors against specific phosphodiesterase isoenzymes for various therapeutic uses.

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