Subcellular localization and hormone sensitivity of adipocyte cyclic AMP phosphodiesterase

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Treatment of intact adipocytes with either or both insulin and adrenaline stimulated membrane cyclic AMP phosphodiesterase activity only in the endoplasmic reticulum subfraction. The cyclic GMP-inhibited cyclic AMP phosphodiesterase activity was also found in this fraction. Quantitative Western blotting using a specific polyclonal antibody, raised against the homogeneous 'dense-vesicle' cyclic AMP phosphodiesterase from rat liver, identified a single 63 kDa species which was localized in the adipocyte endoplasmic reticulum fraction. The ability of adrenaline to stimulate adipocyte membrane cyclic AMP phosphodiesterase was shown to be mediated via β -adrenoceptors and not α_1 -adrenoceptors. Membrane cyclic AMP phosphodiesterase was stimulated by glucagon but not by vasopressin, A23187 or 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Treatment of adipocytes with either chloroquine or dansyl cadaverine failed to affect the ability of insulin to stimulate cyclic AMP phosphodiesterase activity. Treatment of an isolated adipocyte endoplasmic reticulum membrane fraction with purified protein kinase A increased its cyclic AMP phosphodiesterase activity some 2-fold. When this fraction was treated with purified protein kinase A and [³²P]ATP, label was incorporated into a 63 kDa protein which was specifically immunoprecipitated with the antiserum against the liver 'dense-vesicle' cyclic AMP phosphodiesterase.

INTRODUCTION

In adipocytes, the intracellular concentrations of cyclic AMP are controlled by the complex co-ordinate regulation of both adenylate cyclase and cyclic AMP phosphodiesterase activities. Changes in intracellular cyclic AMP concentrations profoundly affect the metabolic status of the cell. Thus, lipolysis is promoted by hormones such as catecholamines. These, through activation of adenylate cyclase, increase cyclic AMP content and activate cyclic AMP-dependent protein kinase which gives rise to the subsequent phosphorylation and activation of hormone-sensitive lipase (Belknap *et al.*, 1986). Conversely, an increase in cyclic AMP phosphodiesterase activity, with subsequent increased hydrolysis of cyclic AMP, leads to a reduction in the rate of lipolysis (Manganiello, 1987).

Many investigators have shown that treatment of intact adipocytes with either insulin or adrenaline leads to the activation of cyclic AMP phosphodiesterase activity associated with a crude membrane fraction. However, the identity of the target enzyme(s) and the mechanisms of hormonal activation are not clear. Nevertheless, as the ability of adrenaline to activate phosphodiesterase activity can apparently be mimicked by agents which increase intracellular cyclic AMP concentrations and also by the addition of dibutyryl cyclic AMP, this suggests that protein kinase A may play either a direct or an indirect role in mediating the activation of this enzyme. The mechanism by which insulin activates this enzyme is obscure (Zinman & Hollenberg, 1974; Kono *et al.*, 1975; Makino & Kono, 1980; Francis & Kono, 1982; Wilson *et al.*, 1983).

We showed in hepatocytes that insulin activated two distinct cyclic AMP phosphodiesterases (Heyworth *et al.*,

1983). One was associated with the plasma membrane and was not activated by agents that increased intracellular cyclic AMP concentrations. The other enzyme, which we called the 'dense-vesicle' species, was associated with the endoplasmic reticulum fraction and could be activated by two different mechanisms. One of these mechanisms was triggered by insulin and the other mechanism of activation was mediated by increases in the intracellular concentrations of cyclic AMP (Heyworth *et al.*, 1983; Wilson *et al.*, 1983).

Using specific polyclonal antibodies against these two enzymes, we have been able to show that rat adipocytes predominantly express the microsomal or 'dense-vesicle' phosphodiesterase (Pyne *et al.*, 1987b). In liver, we showed this to be a dimeric protein consisting of a native 63 kDa subunit which was anchored to the bilayer. However, in liver extracts, this species was highly susceptible to proteolysis and could be readily solubilized, occurring as predominantly a 57 kDa species and a minor 51 kDa species derived from it (Pyne *et al.*, 1987a). We (Heyworth *et al.*, 1983; Wilson *et al.*, 1983) and others (Francis & Kono, 1982) have shown that solubilization of this enzyme, from either hepatocyte or adipocyte membranes, neither alters the activity of this enzyme nor affects its degrees of activation by either insulin or agents which increase the intracellular concentration of cyclic AMP.

That the insulin-stimulated cyclic AMP phosphodiesterase in adipocytes is a dimeric protein of 63 kDa receives support from the work of Saltiel & Steigerwalt (1986) who partially purified such a species from adipocytes, and from the recent studies of Degerman *et al.* (1987) who appear to have purified an apparently similar enzyme from adipocytes.

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In this study we define the intracellular localization of the hormone-activated cyclic AMP phosphodiesterase, examine its regulation by hormones and its similarities to the so-called 'dense-vesicle' enzyme in hepatocytes, and show that it can be activated and phosphorylated by cyclic AMP-dependent protein kinase.

MATERIALS AND METHODS

Worthington collagenase was obtained from Cambridge Bioscience, Cambridge, U.K. Monocomponent porcine insulin was supplied by Du Pont (U.K.) Ltd., Stevenage, Herts, U.K. Leupeptin was from Scientific Marketing Associates, London, U.K., Percoll from Pharmacia, Milton Keynes, Bucks., U.K., Schleicher & Schuell nitrocellulose paper from Anderman & Co., Kingston-upon-Thames, Surrey, U.K. and Protein A (immunoprecipitin) from Gibco Ltd., Paisley, Scotland, U.K. All radiochemicals were purchased from Amersham International plc, Aylesbury, Bucks., U.K. All other fine chemicals including protein kinase inhibitor (rabbit muscle) were obtained from Sigma, Poole, Dorset, U.K.

Cell isolation

Adipocytes were isolated from the epididymal fat pads of male Sprague-Dawley rats (150–200 g) according to Honnor *et al.* (1985). Tissue was minced with scissors, rinsed, then digested for 40 min in a shaking water bath at 37 °C in Krebs–Ringer Hepes buffer (KRH) containing glucose (2 mM), adenosine (200 nM), defatted bovine serum albumin (10 mg/ml) and collagenase (1 mg/ml). Cells were then filtered through nylon mesh, washed by flotation, and finally resuspended in KRH containing glucose (2 mM), adenosine (200 nM) and defatted bovine serum albumin (30 mg/ml) to approx. 2×10^5 cells/ml. They (5 ml) were then pre-incubated for 20 min at 37 °C before the addition of hormones. After hormone addition, cells were incubated for 6 min and then centrifuged for 10 s at 800 rev./min (75 g_{av}) on an MSE benchtop centrifuge at room temperature. The incubation medium which formed the infranatant was aspirated and the cell plug was homogenized in 5 ml of ice-cold homogenization buffer and fractionated as below.

Preparation of subcellular fractions

In some experiments, cells from each incubation (approx. 10^6 cells) were homogenized in 5 ml of homogenization buffer containing Tris/HCl (20 mM, pH 7.4), EDTA (1 mM), sucrose (0.25 M), benzamidine (2 mM), phenylmethanesulphonyl fluoride (PMSF) (0.1 mM) and leupeptin (1 μ g/ml). The homogenate was then separated into crude particulate and cytosolic fractions by centrifugation at 100 000 g for 1 h.

To enable a more detailed study of the subcellular distribution of phosphodiesterase activity to be performed, a differential centrifugation procedure was used. This is based on that of Simpson *et al.* (1983), except that plasma membranes were prepared from the pellet (P1) fraction by centrifugation in iso-osmotic Percoll (15%). Typically, cells from six rats (approx. 1.2×10^7) were homogenized (10 strokes of a Teflon/glass homogenizer at 4 °C) in 20 ml of homogenization buffer followed by centrifugation at 16 000 g for 15 min to produce a pellet (P1) and supernatant (S1). P1 was resuspended in 0.5 ml of homogenization buffer and dispersed in 8 ml of iso-osmotic Percoll before centrifugation at 10 000 g for

10 min. The resulting 'band' of plasma membranes was removed and dispersed in 40 ml of homogenization buffer. Subsequent centrifugation at 48 000 g for 45 min produced a loose pellet of plasma membranes (fraction PM). The remaining material from the Percoll step was removed and washed once in homogenization buffer followed by centrifugation at 48 000 g to produce a pellet (P2). S1 was centrifuged at 48 000 g for 20 min to produce a pellet (P3) and supernatant S2 which was finally centrifuged at 212 000 g for 70 min to produce a pellet (P4) and membrane-free cytosol (fraction S). Further centrifugation (365 000 g , 1 h) did not produce a further membrane fraction. Each subfraction was washed once by suspension in homogenization buffer followed by recentrifugation and finally resuspended to give a protein concentration of approx. 0.2 mg/ml.

Enzyme assays

The following markers were used to assess the purity of the subfractions: succinate-cytochrome *c* reductase (Mackler *et al.*, 1962), NADPH-cytochrome *c* (Williams & Kamin, 1962), galactosyl transferase (Fleischer *et al.*, 1969) and 5'-nucleotidase (Avruch & Wallach, 1971). Cyclic AMP-phosphodiesterase activity was measured according to Marchmont & Houslay (1980) using substrate concentrations of 0.1 μ M. Protein was measured according to Peterson (1977).

Production of antiserum and Western blotting

Antisera were raised by immunizing New Zealand White rabbits with purified 'dense-vesicle' phosphodiesterase from rat liver as described by Kilgour *et al.* (1989). Antiserum DV4 was purified before use by ion-exchange chromatography on DEAE cellulose (DE52). Adipocyte subfractions were immunoblotted using the method of Milligan *et al.* (1986). Briefly, after polyacrylamide-gel electrophoresis, proteins were transferred to nitrocellulose paper. Unreacted sites on the paper were then blocked with 3% gelatin. The paper was incubated with DV4 (1:100 dilution) overnight followed by extensive washing. Protein bands cross-reacting with the antiserum were revealed using 125 I-linked second antibody.

Treatment of adipocyte membrane fractions with cyclic AMP-dependent protein kinase

The catalytic subunit of cyclic AMP-dependent protein kinase was prepared from bovine heart (Reimann & Beham, 1983). Membrane fractions were prepared as described above and finally resuspended in incubation buffer containing Tris/HCl (20 mM, pH 7.4), $MgCl_2$ (5 mM), sucrose (0.25 M), benzamidine (2 mM), PMSF (0.1 mM), leupeptin (1 μ g/ml), sodium fluoride (50 mM) and β -glycerophosphate (10 mM). Experiments were performed at 30 °C in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase (40 units/ml) and 0.1 mM-ATP. For investigation of the phosphorylation status of membrane proteins, [γ - 32 P]ATP (100 μ Ci/ml) was included during incubations. Full details of experiments are given in the legends to Fig. 2 and Table 4.

Immunoprecipitation from phosphorylated membranes

Phosphorylations were terminated by the addition of ice-cold 'detergent buffer' containing (final concentrations) Triton X-100 (1%), sodium deoxycholate (1%),

Table 1. Distribution of marker enzyme specific activities in fractions isolated from adipocytes

Enzyme specific activities were measured in each subfraction and are expressed as means from at least four separate cell preparations. Specific activities are in nmol/min per mg of protein. Figures in parentheses indicate the percentage of the recovered activity in each of the four membrane fractions. Protein and marker recoveries were > 89 %. ER, endoplasmic reticulum

Enzyme (organelle)	Fraction ...	Specific activity			
		P2	P3	P4	PM
Succinate dehydrogenase (mitochondria)		91.9 (96.6)	2.9 (0.9)	0.1 (0.1)	2.1 (2.5)
NADPH dehydrogenase (ER)		0.2 (0.5)	85.0 (67.5)	14.0 (18.8)	4.3 (13.1)
Galactosyltransferase (Golgi)		3.6 (13.2)	16.2 (17.2)	17.4 (31.3)	9.4 (38.3)
5'-Nucleotidase (plasma membrane)		5.4 (13.5)	12.0 (1.9)	2.5 (3.0)	29.5 (81.7)
Recovered protein (μ g)		198	58	97	220

SDS (0.1 %), NaCl (0.15 M) and EDTA (1 mM). DV4 was diluted in 10 mM-phosphate-buffered 0.15 M-saline and added to the above mixture. The following were also included to give (final concentrations) protein kinase inhibitor (100 μ g/ml), sodium pyrophosphate (2 mM) and gelatin (0.05 %). Immunoprecipitation was allowed to proceed at 4 °C for 18 h. The immune complex was precipitated by the addition of Protein A (0.5 % final concentration) for 90 min at 4 °C. The resulting precipitate was collected by centrifugation at 12000 g for 2 min and washed four times before addition of SDS sample buffer (Laemmli, 1970). Samples were then boiled

for 3 min and run on 10 % polyacrylamide gels followed by autoradiography.

RESULTS AND DISCUSSION

Simpson *et al.* (1983) described a methodology for the subcellular fractionation of adipocytes which they used to show that the glucose transporter had a dual localization. Using their procedure we obtained similar results for the distribution of marker enzymes (Table 1), which allowed us to define the endoplasmic reticulum fraction (fraction P3), mitochondrial + lysosomal + nuclear fraction (fraction P2) and plasma membrane fraction (fraction PM). We were not able, however, to resolve clearly the putative Golgi fraction (fraction P4) from that of the endoplasmic reticulum, which expressed a similar specific activity of the marker enzyme galactosyltransferase. Nevertheless, in Table 2 we see that cyclic AMP phosphodiesterase activity was found associated with all of these membrane fractions. This distribution is very similar to that observed in hepatocytes (Heyworth *et al.*, 1983; Houslay, 1985).

Challenge of intact adipocytes with insulin elicited a marked increase (approx. 70 %) in the cyclic AMP phosphodiesterase activity found associated with the endoplasmic reticulum subfraction only (Table 2). As no change in the activity of the Golgi fraction occurred, then it is likely that the insulin-stimulated cyclic AMP phosphodiesterase is located specifically in the endoplasmic reticulum, rather than being derived from any Golgi contamination of this fraction.

Similarly, challenge of intact adipocytes with the β -adrenoceptor agonist isoproterenol caused activation of the cyclic AMP phosphodiesterase specifically in the endoplasmic reticulum (P3) fraction (Table 2).

The endoplasmic reticulum (P3) fraction seems then to contain cyclic AMP phosphodiesterase activity which is activated by both insulin and isoproterenol. This may be due to two distinct hormone-stimulated enzymes being present. However, in hepatocytes we have described

Table 2. Hormonal activation of membrane-bound low- K_m phosphodiesterase activity and localization of the 'dense-vesicle' cyclic AMP phosphodiesterase in rat adipocytes

Adipocytes were isolated and then preincubated for 20 min before addition of hormones for 6 min. Concentrations of ligands were insulin (1 nM) and isoproterenol (1 μ M). After this time cells were harvested and subfractionated as described in the Materials and methods section. Phosphodiesterase activity was then measured in each fraction using 0.1 μ M-cyclic AMP as substrate (means $\pm$ S.E.M. from seven separate experiments). When unlabelled cyclic GMP was added to assays in order to test for inhibition, it was present at 2 μ M. Significant differences with respect to controls are indicated: * $P < 0.05$, ** $P < 0.01$ (Student's paired *t* test). Immunoreactivity is given as data from a typical experiment, where equivalent amounts of each membrane fraction were Western-blotted using the antiserum DV4 and data expressed as the amount of radioactivity (c.p.m.) associated with the 125 I-labelled second antibody used to reveal cross-reacting proteins. A 'specific localization' is given as c.p.m./ μ g of membrane protein loaded onto the gel for the separate experiments. n.d., not detected.

Fraction	Cyclic AMP phosphodiesterase activity (pmol/min per mg) (adipocyte pretreatment prior to membrane isolation)					Immunoreactivity	
	Untreated	Untreated + cGMP in assay	Insulin- treated	Isoproterenol- treated	Insulin/ isoproterenol- treated	c.p.m.	c.p.m./ μ g
H	11.2 $\pm$ 2.0	10.5 $\pm$ 0.9	18.8 $\pm$ 4.3	16.0 $\pm$ 3.0	19.1 $\pm$ 1.8	n.d.	n.d.
P2	16.7 $\pm$ 0.3	22.3 $\pm$ 4.1*	16.7 $\pm$ 0.3	17.0 $\pm$ 0.3	16.7 $\pm$ 0.3	n.d.	n.d.
P3	23.6 $\pm$ 5.4	3.2 $\pm$ 0.8**	40.1 $\pm$ 7.2**	39.3 $\pm$ 6.0**	61.4 $\pm$ 7.9**	4590	79.1 $\pm$ 7.5
P4	4.3 $\pm$ 1.1	1.8 $\pm$ 0.4**	6.7 $\pm$ 0.8	7.2 $\pm$ 0.9*	8.4 $\pm$ 1.1*	1027	10.6 $\pm$ 2.2
PM	29.2 $\pm$ 4.0	19.7 $\pm$ 3.5*	23.1 $\pm$ 3.9	25.3 $\pm$ 7.0	29.7 $\pm$ 4.1	1919	8.7 $\pm$ 1.2

Fig. 1. Use of the antiserum DV4 to probe adipocyte membrane fractions by Western blotting

Membrane fractions P3 (58 μ g; lane a), P4 (97 μ g; lane b) and PM (220 μ g; lane c) were subjected to polyacrylamide-gel electrophoresis before being probed with the antiserum DV4 by Western blot analysis. This interaction was revealed using an 125 I-labelled second antibody, and the Figure shows a typical experiment.

(Heyworth *et al.*, 1983; Houslay, 1985; Pyne *et al.*, 1987a,b) an enzyme, called the 'dense-vesicle' cyclic AMP phosphodiesterase, which is activated both by insulin and by agents which elevate the intracellular concentration of cyclic AMP. Using immunoblotting with a specific polyclonal antiserum to the 'dense-vesicle' cyclic AMP phosphodiesterase, we have shown that an immunoreacting species of identical subunit molecular size is expressed in adipocytes (Pyne *et al.*, 1987b). It is tempting then to suggest that insulin and isoproterenol activate a single cyclic AMP phosphodiesterase in the endoplasmic reticulum (P3) fraction, which is akin to the 'dense-vesicle' cyclic AMP phosphodiesterase described in hepatocytes.

In order to investigate whether the endoplasmic reticulum (P3) fraction contains a species which is akin to the 'dense-vesicle' cyclic AMP phosphodiesterase, we subjected the various membrane fractions to immunoblotting using the polyclonal antiserum DV4, which specifically interacts with this enzyme. The results of such studies are shown in Fig. 1 and in Table 2. It is clear that although immunoreactivity is found in the plasma membrane, Golgi and the endoplasmic reticulum fractions, analysis on a specific activity basis shows that this enzyme is

clearly localized in the endoplasmic reticulum (P3) fraction. We have shown in hepatocytes (Pyne *et al.*, 1987a,b) that a unique property of this enzyme is its particular susceptibility to inhibition by cyclic GMP. Using this as a criterion, we noted that low concentrations of cyclic GMP elicited an 86% inhibition of cyclic AMP phosphodiesterase in the endoplasmic reticulum (P3) fraction (Table 2), compared with only 58% in the Golgi fraction (P4) and 32% in the plasma membrane (PM) fraction. Indeed, in the P2 fraction we actually observed an augmentation of activity, presumably due to the presence of a membrane-bound cyclic GMP-stimulated cyclic AMP phosphodiesterase (Pyne *et al.*, 1986).

Such studies indicate that an enzyme akin to the 'dense-vesicle' cyclic AMP phosphodiesterase in hepatocytes is functionally expressed in adipocytes, where fully saturating concentrations (10-fold increases elicited no further stimulation; results not shown) of either insulin or isoproterenol yielded additive stimulation of activity when adipocytes were challenged with both ligands together. This is similar to that which we observed for the 'dense-vesicle' enzyme in hepatocytes challenged with insulin and glucagon, where either additive (Heyworth *et al.*, 1984) or even synergistic (Heyworth *et al.*, 1983) effects have been obtained. In the hepatocyte system we have employed a number of approaches to demonstrate that these two hormones activate the same enzyme by distinct routes (Wilson *et al.*, 1983).

The prime location of this enzyme appears to be the endoplasmic reticulum (P3) fraction, where it can be functionally stimulated by hormone treatment of adipocytes. This enzyme is also found contaminating the plasma membrane and Golgi fractions, although there was little detectable enhancement of activity in such fractions after hormone treatment of adipocytes. In the case of the plasma membrane this may be because the contaminating activity is such a small amount of the total cyclic AMP phosphodiesterase activity in that fraction (< 10%), that any hormonal stimulation would not be detectable (maximal change expected in approx. 7%). Indeed, it is interesting to note that insulin appeared to cause a small decrease in the activity of the plasma membrane fraction. However, this activity was not statistically different from the control values ($P < 0.05$). In the case of P4 (Golgi) fraction, it appears that some 13.6% of the total 'dense-vesicle phosphodiesterase' is found in this fraction, as determined by immunoblot analysis (Table 2). This is consistent with a similar contamination of the P4 fraction with NADPH dehydrogenase activity, the marker used for the P3 (endoplasmic reticulum) fraction. Assuming that all the cyclic GMP-inhibitable activity in the P3 fraction is due to the activity of the 'dense vesicle phosphodiesterase' and that this represents some 61% of the total activity, as derived from the distribution found using immunoblotting (Table 2), then the total 'dense-vesicle phosphodiesterase' activity is some 1.94 μ units. The fraction of this activity found in the P4 (Golgi) fraction would thus be equivalent to some 0.264 μ units, yielding a calculated specific activity of some 2.7 pmol/min per mg of protein. This value is similar to the cyclic GMP-inhibited component of the P4 fraction, which is 2.5 pmol/min per mg of protein (Table 2). Hormonal activation of the 'dense-vesicle phosphodiesterase' component of the P4 fraction, where both insulin and isoprenaline are assumed to give a roughly 2-fold increase in activity of this enzyme, can

Table 3. Effect of hormonal treatment of intact adipocytes upon particulate low- K_m cyclic AMP phosphodiesterase activity

Adipocytes were prepared as described in the Materials and methods section and preincubated for 20 min before addition of agents. In cells undergoing ligand pretreatment, this was performed for 20 min at 37 °C. Specific activity (pmol/min per mg) was measured in the 100 000 *g* particulate fraction and the increase in specific activity with respect to untreated adipocytes is expressed as a mean percentage $\pm$ S.E.M. with number of replicates in parentheses. * indicates that activity was significantly above that seen in membranes from control cells ($P < 0.05$; Student's *t* test).

Treatment	Activation (%)
10 nM-Glucagon (6 min)	51 $\pm$ 17 (4)*
1 μ M-Adrenaline (6 min)	49 $\pm$ 18 (4)*
1 μ M-Isoproterenol (6 min)	75 $\pm$ 15 (6)*
10 μ M-Propranolol (6 min) then 1 μ M-adrenaline (6 min)	4 $\pm$ 3 (3)
10 μ M-Phentolamine (6 min) then 1 μ M-adrenaline (6 min)	53 $\pm$ 12 (3)*
1 nM-Insulin (6 min)	73 $\pm$ 10 (12)*
1 μ M-A23187 (5 min)	0 $\pm$ 2 (4)
10 nM-TPA (15 min)	22 $\pm$ 20 (4)
10 nM-Vasopressin (5 min)	1 $\pm$ 1 (5)
1 nM-Insulin (6 min) with 1 mM-EGTA-pretreated cells	65 $\pm$ 12 (4)*
1 nM-Insulin (6 min) with 0.2 mM-chloroquine-pretreated cells	65 $\pm$ 17 (4)*
1 nM-Insulin (6 min) with 0.1 mM-dansyl cadaverine-pretreated cells	78 $\pm$ 20 (3)*

be calculated to give rise to specific activities of 7.2, 7.2 and 12.2 pmol/min per mg of protein for insulin, isoprenaline and both hormones together respectively. These values are not too dissimilar from those obtained in practice (Table 2), although the activation observed with both hormones added together was rather lower than might be expected.

The mechanism whereby insulin stimulates this enzyme is obscure (see Houslay, 1985). However, in hepatocytes it has been shown that lysosomotropic agents, dansyl cadaverine (Wilson *et al.*, 1983) and pertussis toxin treatment (Heyworth *et al.*, 1984) can prevent insulin activating the 'dense-vesicle' cyclic AMP phosphodiesterase. Here, however, we see that neither dansyl cadaverine nor the lysosomotropic agent chloroquine blocked the ability of insulin to stimulate the adipocyte enzyme (Table 3). Furthermore, we failed to note any significant decrease in cyclic AMP phosphodiesterase activity in any of the other membrane fractions after insulin challenge (Table 2), implying that translocation of this enzyme was not elicited by insulin. The inability of chloroquine and dansyl cadaverine to block insulin's activation of this enzyme in adipocytes is the first difference that we have observed in the regulation of this enzyme in these two tissues. The distinction, however, does not extend to pertussis toxin, which Elks *et al.* (1983) have shown to block insulin's activation of phosphodiesterase activity in adipocytes.

Activation of adipocyte membrane cyclic AMP

phosphodiesterase by adrenaline has been shown by various investigators to be mediated by increased intracellular cyclic AMP concentrations (Zinman & Hollenberg, 1974). We support this here by showing that the action of adrenaline in stimulating this enzyme can be blocked by using a β -adrenoceptor antagonist and not with an α_1 -adrenoceptor antagonist, and of course it can be mimicked by the β -adrenoceptor agonist isoproterenol (Table 3). For the first time we show (Table 3) that glucagon can also stimulate this enzyme, as it does in the liver (Heyworth *et al.*, 1983). Adrenaline can exert effects on adipocytes through α_1 -adrenoceptors, which stimulate inositol phospholipid metabolism, but this signalling system does not appear to offer a route for stimulation of adipocyte membrane phosphodiesterase activity. Consistent with this was our inability to activate this enzyme by exposing adipocytes to vasopressin, the Ca^{2+} ionophore A23187 or the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Table 3).

It has recently been shown that exposure of a crude adipocyte membrane fraction to protein kinase A could stimulate cyclic AMP phosphodiesterase activity associated with it (Gettys *et al.*, 1988). We show here that treatment of the endoplasmic reticulum fraction, but not the plasma membrane fraction, with protein kinase A can lead to a profound stimulation of cyclic AMP phosphodiesterase activity (Table 4). Furthermore, when our experiments were performed in the presence of [32 P]ATP, then we could specifically immunoprecipitate a 63 kDa phosphoprotein using the antiserum DV4 (Fig. 2). Such experiments indicate that the cyclic AMP phosphodiesterase recognized by this specific antiserum can be phosphorylated by protein kinase A in adipocyte membranes. We (Kilgour *et al.*, 1989) have recently been able to demonstrate that incubation of hepatocyte membranes with purified protein kinase A led to the phosphorylation and activation of the 'dense-vesicle' phosphodiesterase. However, using hepatocyte mem-

Table 4. Effect of cyclic AMP-dependent protein kinase on phosphodiesterase activity in endoplasmic reticulum and plasma membrane subfractions from adipocytes

Fractions P3 (endoplasmic reticulum) and PM (plasma membrane) were prepared from untreated adipocytes as described in the Materials and methods section. Membranes were incubated at 30 °C for 10 min in the presence or absence of the isolated catalytic unit of cyclic AMP-dependent protein kinase before being assayed for low- K_m cyclic AMP phosphodiesterase activity using 0.1 μ M-cyclic AMP as substrate. In experiments done without either ATP or protein kinase A (A-kinase), then incubation at 30 °C for 10 min did not lead to any change in enzyme activity ($< 5\%$). Errors are S.E.M. for $n = 3$ experiments done with different membrane preparations.

Fraction	Phosphodiesterase activity (pmol/min per mg)		Activation (fold)
	– A kinase	+ A kinase	
Endoplasmic reticulum (P3)	24.1 $\pm$ 1.5	49.4 $\pm$ 3.2	2.05
Plasma membrane (PM)	20.2 $\pm$ 0.7	22.2 $\pm$ 0.9	1.1

Fig. 2. Analysis of material from adipocyte P3 membranes treated with A-kinase and [γ - 32 P]ATP by immunoprecipitation with the antiserum DV4

A P3 membrane fraction was treated with [γ - 32 P]ATP in either the absence (lane a) or presence (lane b) of the isolated catalytic unit of cyclic AMP-dependent protein kinase, as described in detail in the Materials and methods section. Membranes were then solubilized and material immunoprecipitated with the antiserum DV4 subjected to SDS/polyacrylamide-gel electrophoresis with subsequent autoradiography. A typical experiment of one performed three times is shown.

branes, it was found necessary to dephosphorylate the membranes before exposure to protein kinase A as it appeared that another kinase, probably 5'-AMP kinase, can also phosphorylate the 'dense-vesicle' phosphodiesterase. The phosphorylation caused by this other kinase appeared, however, to block the action of protein kinase A without causing any activity change in the 'dense-vesicle' phosphodiesterase. This, however, does not appear to be the case in adipocytes.

Our experiments thus indicate that an enzyme which is equivalent to the 'dense-vesicle' cyclic AMP phosphodiesterase, described in hepatocytes, is expressed and hormonally regulated in adipocytes. This enzyme is phosphorylated and activated by protein kinase A. It may be this enzyme which accounts for the activity that Gettys *et al.* (1988) were able to activate by treating adipocyte membranes with cyclic AMP-dependent protein kinase. Activation of the 'dense-vesicle' type of high-affinity cyclic AMP phosphodiesterase is thus employed both by hormones which stimulate adenylate cyclase and by insulin to control the intracellular concentrations of cyclic AMP. Insulin exploits this route in order to lower the intracellular concentration of cyclic

AMP and glucagon and β -adrenoceptor agonists employ it to ensure that the elevation of cyclic AMP is transient.

We thank the M.R.C., A.F.R.C. and the California Metabolic Research foundation for support.

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