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Regulation by noradrenaline of the mitochondrial and microsomal forms of glycerol phosphate acyltransferase in rat adipocytes

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1. Incubation of rat adipocytes with 1 μM-noradrenaline caused a decrease in both the *N*-ethylmaleimide-sensitive (microsomal) and *N*-ethylmaleimide-insensitive (mitochondrial) glycerol phosphate acyltransferase activities measured in homogenates from freeze-stopped cells. 2. The effects of noradrenaline on glycerol phosphate acyltransferase activity were apparent over a wide range of concentrations of glycerol phosphate and palmitoyl-CoA. 3. The effect of noradrenaline was reversed within cells by the subsequent addition of insulin or propranolol. 4. Inclusion of albumin in homogenization buffers abolished the effect of noradrenaline on the *N*-ethylmaleimide-sensitive activity. The effect of noradrenaline on the *N*-ethylmaleimide-insensitive (mitochondrial) activity was, however, not abolished by inclusion of albumin in buffers for preparation of homogenates from freeze-stopped cells. 5. Inclusion of fluoride in homogenization buffers did not alter the observed effect of noradrenaline. 6. The inactivating effect of noradrenaline persisted through the subcellular fractionation procedures used to isolate adipocyte microsomes (microsomal fractions). The effect of noradrenaline on mitochondrial glycerol phosphate acyltransferase did not persist through subcellular fractionation. 7. Noradrenaline treatment of cells significantly decreased the $V_{max.}$ of glycerol phosphate acyltransferase in isolated microsomes without changing the activity of NADPH–cytochrome *c* reductase. Glycerol phosphate acyltransferase activity in microsomes from noradrenaline-treated cells is unstable, being rapidly lost on incubation at 30°C. Bivalent metal ions (Mg^{2+}, Ca^{2+}) or post-microsomal supernatant protected against this inactivation. 8. Glycerol phosphate acyltransferase activity in microsomes from noradrenaline-treated cells could not be re-activated by incubation with either alkaline phosphatase or phosphoprotein phosphatase-1. 9. Addition of cyclic AMP-dependent protein kinase catalytic subunits to adipocyte microsomes incubated with [γ-^{32}P]ATP considerably increased the incorporation of ^{32}P into microsomal protein, but did not cause inactivation of glycerol phosphate acyltransferase. 10. These findings provide no support for the proposal that inactivation of adipocyte microsomal glycerol phosphate acyltransferase by noradrenaline is through a phosphorylation type of covalent modification.

The biosynthesis of glycerolipids can be initiated by the acylation of either glycerol phosphate or dihydroxyacetone phosphate. When glycerol phosphate is used as the initiating molecule, the initial acyl transfer is catalysed by GPAT. Total GPAT activity in incubated rat adipocytes responds to hormonal stimulation of the cells. Adrenaline has been shown to cause a time- and dose-dependent decrease in GPAT activity (Sooranna & Saggerson, 1976*a*, 1978), which may be blocked by insulin or the β-adrenoceptor antagonist propranolol (Sooranna & Saggerson, 1978). Four other enzymes of the adipocyte triacylglycerol synthesis pathway show broadly similar regulation by catecholamine hormones (for summary see Saggerson *et al.*, 1979). The mechanisms mediating these effects of the catecholamines remain unclear. Nimmo & Houston (1978) treated microsomes (microsomal fractions)

Abbreviations used: NEM, *N*-ethylmaleimide; GPAT, glycerol phosphate acyltransferase (EC 2.3.1.15).

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from the epididymal fat-pads of starved rats with rabbit muscle cyclic AMP-dependent protein kinase and observed a substantial inactivation of GPAT. Subsequent treatment of these microsomes with alkaline phosphatase (Nimmo & Houston, 1978) or with rabbit muscle phosphoprotein phosphatase (Nimmo, 1980) reversed this inactivation. Further indirect evidence to suggest that adipose-tissue microsomal GPAT might be regulated by a phosphorylation/dephosphorylation has been provided by Nimmo (1981) who demonstrated, using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, that a protein band (M_r 54000), whose labelling with iodo[^{3}H]acetate is prevented by palmitoyl-CoA, can also be phosphorylated by using [γ-^{32}P]ATP and cyclic AMP-dependent protein kinase.

GPAT in liver and other mammalian tissues is found in both mitochondria and microsomes. These two forms of the enzyme differ in affinity for substrates, in acyl-group specificity and in sensitivity to inhibition by thiol-group reagents (for references see introduction to Saggerson *et al.*, 1980). The same pattern is observed in rat adipocytes, where it may be shown (Saggerson *et al.*, 1979, 1980) that mitochondrial GPAT is insensitive to *N*-ethylmaleimide, whereas the microsomal activity is inhibited by this reagent. In adipose tissue a higher proportion of the total GPAT appears to be in the microsomal fraction compared with the liver (Schlossman & Bell, 1976; Bates & Saggerson, 1979; Saggerson *et al.*, 1979, 1980), although this assessment is somewhat dependent on the assay conditions used (Saggerson *et al.*, 1979). In rat liver a number of short- or long-term changes in physiological state result in alterations in the activity of the mitochondrial rather than the microsomal form of GPAT (Bates & Saggerson, 1977, 1979; Bates *et al.*, 1977).

The objectives of this study were fourfold: firstly, to investigate the relative contributions of the mitochondrial and microsomal forms of GPAT in adipocytes to the decrease in total activity induced by catecholamine treatment; secondly, to investigate changes in GPAT kinetic properties resulting from catecholamine treatment of cells; thirdly, to investigate whether catecholamine-promoted inactivation of GPAT is reversible within cells; fourthly, to gain further insights into the possibility that effects of catecholamines on this enzyme might be due to modification by protein kinase-mediated phosphorylation.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 160–180g were used throughout. These were bred in the animal colony at University College, London and had constant access to water and GR3-EK cube diet (E. Dixon and Sons, Ware, Herts., U.K.).

Chemicals

These were obtained and treated as described by Fernandez & Saggerson (1978) and Bates & Saggerson (1979). In addition, DL-propranolol hydrochloride, histone (type IIA) and bovine plasma albumin were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Before use, albumin was subjected to a defatting procedure (Chen, 1967) with minor modifications (Saggerson, 1972). Alkaline phosphatase (from calf intestine) was from Boehringer (London) Ltd. (Lewes, East Sussex, U.K.). [γ-^{32}P]ATP was from Amersham International (Amersham, Bucks., U.K.). DEAE-cellulose DE-52 was obtained from Whatman Chemical Separation Ltd. (Maidstone, Kent, U.K.). Calcium phosphate gel was prepared by the method of Keilin & Hartree (1938). Phosphoprotein phosphatase-1 (from muscle) was generously given by Professor P. Cohen, Department of Biochemistry, University of Dundee. Cyclic AMP-dependent protein kinase holoenzyme was prepared from bovine heart essentially as described by Brostrom *et al.* (1969), except that the fractionation stage using Sepharose 6B was omitted. Protein kinase catalytic subunits were separated from regulatory subunits by elution of the catalytic subunits from a DEAE-cellulose column with cyclic AMP (Rubin *et al.*, 1974).

Isolation of adipocytes from epididymal adipose tissues

These were obtained by collagenase treatment of epididymal-fat-pad pieces (Rodbell, 1964) and washed twice with Krebs–Ringer bicarbonate medium (Krebs & Henseleit, 1932) containing fatty acid-poor albumin (10mg/ml). The cells were then suspended as a stock in the same medium (cells equivalent to two-thirds of a fat-pad per ml).

Incubation of adipocytes

Samples (1.0ml) of the stock cell suspension were added to 25ml silicone-treated flasks and uncubated with shaking (70 cycles/min) at 37°C in a final volume of 4ml of Krebs–Ringer bicarbonate containing 1.27mM-Ca^{2+}, fatty acid-poor albumin (40mg/ml), adenosine deaminase (4munits/ml) and 5mM-glucose. The flask contents were continuously gassed with O_2/CO_2 (19:1). Incubation times and other additions to flasks are indicated in the legends to individual Tables and Figures.

Preparation of adipocyte extracts

In most cases these were prepared in a medium consisting of 0.25M-sucrose, 10mM-Tris/HCl buffer, pH7.4, 1mM-EDTA and 1mM-dithiothreitol. This is

referred to as 'sucrose medium'. 'Sucrose/albumin medium' was sucrose medium containing fatty acid-poor albumin (10mg/ml). 'Sucrose/F^- medium' contained 0.19M-sucrose, 10mM-Tris/HCl buffer, pH 7.4, 50mM-NaF, 10mM-EDTA and 1mM-dithiothreitol, and 'sucrose/F^-/albumin medium' was sucrose/F^- medium containing fatty acid-poor albumin (10mg/ml).

After incubation, adipocytes were recovered by centrifugation at 200$g_{av.}$ for 20s, and the underlying medium was removed by aspiration. Fat-free homogenates were prepared after freeze-stopping of the cells with liquid N_2 by using an Ultra-Turrax tissue disintegrator (Janke und Künkel, Staufen, German Federal Republic) as described by Sooranna & Saggerson (1976*a*). Mitochondrial, microsomal and 100000*g* post-microsomal supernatant fractions were obtained by differential centrifugation (Saggerson *et al.*, 1980) of extracts obtained by breaking unfrozen adipocytes by agitation on a vortex mixer (Martin & Denton, 1970).

The 100000*g* supernatant fractions were concentrated by dialysis overnight at 4°C against 100vol. of sucrose (70%, w/v) containing 10mM-Tris/HCl buffer, pH 7.4, 1mM-EDTA and 1mM-dithiothreitol. The concentrated supernatants were then re-dialysed for 12h against 200vol. of 0.25M-sucrose/10mM-Tris/HCl buffer (pH 7.4)/1mM-EDTA/1mM-dithiothreitol. Volume expansion during this second dialysis was restricted. Finally, the extracts were stored at −20°C until use.

For study of protein phosphorylation in microsomes, the microsomes were isolated by the method of Nimmo & Houston (1978), except that adipocytes rather than fat-pads were used as the starting material.

Analytical methods

GPAT was assayed radiochemically at 30°C by following the incorporation of [^{14}C]glycerol 3-phosphate into butanol-soluble products (Saggerson *et al.*, 1980). Assays were performed in a final volume of 1.0ml containing 100mM-Tris/HCl buffer, pH 7.4, 0.7mM-dithiothreitol, fatty acid-poor albumin (1.75mg/ml) and 0.4–0.8μCi of [U-^{14}C]-glycerol 3-phosphate. Palmitoyl-CoA and unlabelled *sn*-glycerol 3-phosphate were also present at the concentrations indicated in the legends to individual Tables and Figures. Where indicated, 10mM-*N*-ethylmaleimide was also included in GPAT assays to inhibit microsomal activity, leaving the NEM-insensitive mitochondrial activity (Saggerson *et al.*, 1979, 1980). Reactions were initiated by addition of 0.1ml of fat-free homogenate or 10–20μg of mitochondrial or microsomal-fraction protein. GPAT assays were performed for 7min. GPAT activities in fat-free homogenates are expressed relative to lactate dehydrogenase activity (Sooranna & Saggerson, 1976*a*), where 1 unit of lactate dehydrogenase reduces 1μmol of pyruvate/min at 25°C. GPAT activities in subcellular fractions are expressed relative to mitochondrial or microsomal-fraction protein.

Lactate dehydrogenase (EC 1.1.1.27) was assayed spectrophotometrically at 25°C and 340nm (Saggerson, 1974).

The activity of protein kinase preparations was determined by monitoring the phosphorylation of histone IIA by [γ-^{32}P]ATP (Reimann *et al.*, 1971); 1 unit of protein kinase represents the amount of enzyme required to incorporate 1nmol of [^{32}P]P_i into histone per min at 30°C.

Before use in adipocyte incubations, adenosine deaminase (EC 3.5.4.4) was dialysed overnight at 4°C against 0.9% NaCl to remove $(NH_4)_2SO_4$. The dialysed enzyme was standardized spectrophotometrically at 265nm at 25°C by the method of Kalckar (1947) on the morning of the experiments. A unit of adenosine deaminase is the amount of enzyme needed to deaminate 1μmol of adenosine/min at 25°C in this assay.

[^{32}P]P_i bound to microsomal proteins by enzymic phosphorylation was measured by the method of Walsh *et al.* (1971). ATP was measured in deproteinized extracts obtained after incubation of microsomes by the method of Greengard (1963).

Glycerol was assayed enzymically in deproteinized samples of adipocyte incubation media by the method of Garland & Randle (1962). Non-esterified fatty acids in incubation media were measured as described by Cheng & Saggerson (1978).

Protein was measured by the method of Lowry *et al.* (1951), with fatty acid-poor bovine serum albumin as a standard.

Statistical methods

Statistical significance was determined by Student's *t* test for paired samples.

Results and discussion

Incubation of adipocytes with adrenaline for 1h has previously been shown to cause inactivation of total GPAT by approx. 50% (Sooranna & Saggerson, 1976*a*). This effect was only investigated at one concentration of each of the substrates glycerol phosphate and palmitoyl-CoA. The experiment shown in Fig. 1 was therefore performed to ascertain whether GPAT assay conditions could influence the catecholamine effect. Fig. 1 shows that inactivation by noradrenaline was seen over a 6-fold range of [glycerol phosphate], and with [palmitoyl-CoA] between 40 and 130μM (albumin was present at 1.75mg/ml). The effect of noradrenaline was only slight when [palmitoyl-CoA] was 10μM, but

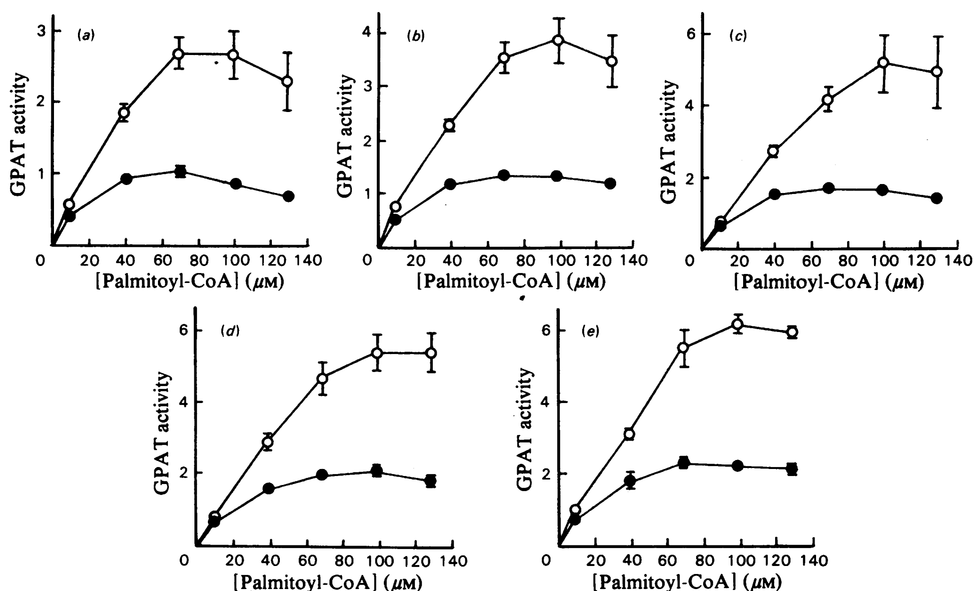

Fig. 1. *Effect of noradrenaline on total GPAT activity in fat-free homogenates*
Adipocytes were incubated for 1 h with or without 1 μM-noradrenaline, followed by freeze-stopping and preparation of homogenates in sucrose medium. Total GPAT was assayed with the indicated palmitoyl-CoA concentrations and with the following *sn*-glycerol 3-phosphate concentrations: (*a*) 0.25 mM, (*b*) 0.5 mM, (*c*) 0.75 mM, (*d*) 1.0 mM, (*e*) 1.5 mM. The values are the means from two separate experiments and are expressed as nmol/min per unit of lactate dehydrogenase activity. The bars indicate the range of values. Where not shown, these lie within the symbols. ○, Control cells; ●, noradrenaline-treated cells.

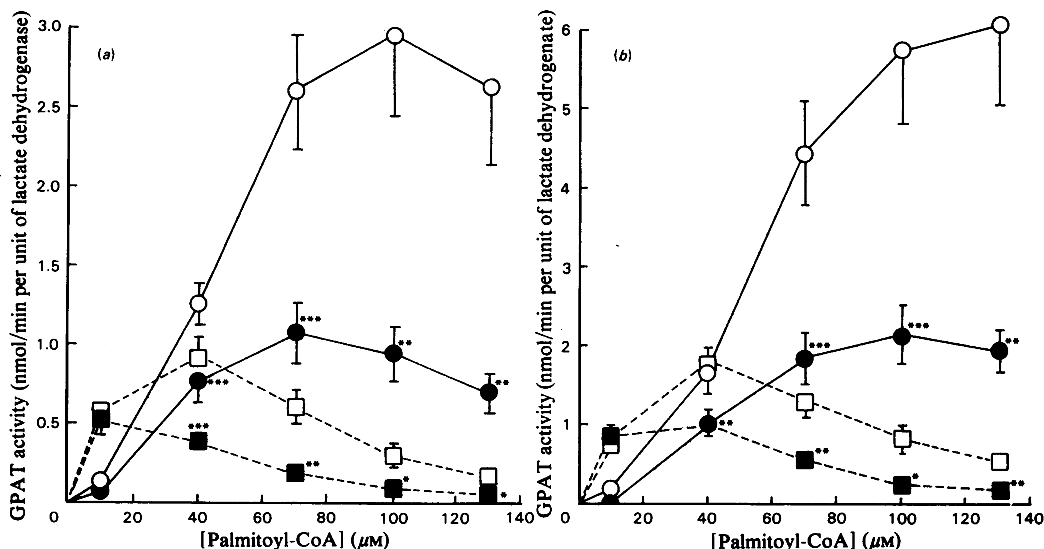

Fig. 2. *Effect of noradrenaline on NEM-insensitive and NEM-sensitive GPAT in fat-free homogenates*
Adipocytes were incubated for 1 h with or without 1 μM-noradrenaline, followed by freeze-stopping and preparation of homogenates in sucrose medium. GPAT activity was measured in the presence and absence of 10 mM-NEM with the indicated palmitoyl-CoA concentrations and with either (*a*) 0.25 mM- or (*b*) 1.0 mM-*sn*-glycerol 3-phosphate. The values are means ± S.E.M. for four separate experiments. Where not shown, the bars lie within the symbols. Significance of noradrenaline effects is indicated by *, **, *** for $P < 0.05$, <0.02, <0.01 respectively. Open symbols, control cells; closed symbols, noradrenaline-treated cells; ○, ●, NEM-sensitive GPAT; □, ■, NEM-insensitive GPAT.

inhibitory effects of 50–70% were seen with higher concentrations of this substrate.

This experiment was extended (Fig. 2) by making measurements of GPAT activity in the presence of NEM, thereby permitting assessment of the mitochondrial (NEM-insensitive) and microsomal (NEM-sensitive) forms (Saggerson *et al.*, 1979, 1980). These values are shown in Fig. 2 together with a statistical analysis of the effect of noradrenaline at two representative concentrations of glycerol phosphate (0.25 and 1 mM). Again, effects of noradrenaline were not apparent in assays with 10 μM-palmitoyl-CoA, but were seen with all the other tested [palmitoyl-CoA]. Fig. 2 shows that both microsomal and mitochondrial forms of GPAT were inactivated by noradrenaline. As shown by Saggerson *et al.* (1979), a lower concentration of palmitoyl-CoA was required to obtain maximal NEM-insensitive activity than for the NEM-sensitive (microsomal) GPAT. After exposure of cells to noradrenaline, both forms of the enzyme appeared to be slightly more susceptible to inhibition by higher concentrations of palmitoyl-CoA. For reasons that are explained elsewhere (Rider & Saggerson, 1983), at the lowest [palmitoyl-CoA] tested (10 μM), NEM-sensitive GPAT is an under-estimate of the true activity of the microsomal enzyme. However, this does not alter the fact that at 40 μM-palmitoyl-CoA, under the present assay conditions, adipocyte mitochondrial and microsomal GPAT activities are broadly comparable. Therefore, with a saturated acyl substrate such as palmitate, the mitochondria may play a significant role in the initiation of glycerolipid synthesis. It was only at relatively high [palmitoyl-CoA] that the microsomal enzyme became dominant (Fig. 2).

The inactivation of total GPAT could also be brought about by agents other than the catecholamine hormones. In a series of experiments in which 1 μM-noradrenaline decreased total GPAT

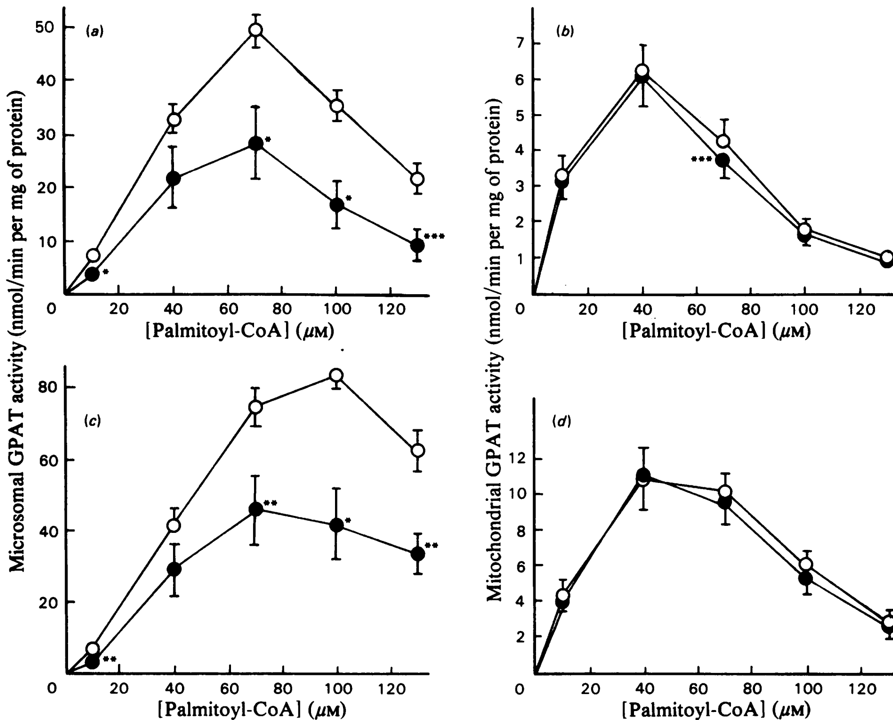

Fig. 3. *Effect of noradrenaline on microsomal and mitochondrial GPAT activities*
Adipocytes were incubated for 1 h with or without 1 μM-noradrenaline, followed by subcellular fractionation in sucrose medium to isolate mitochondria and microsomes. GPAT activity was measured in the presence of either (*a*, *b*) 0.25 mM- or (*c*, *d*) 1.0 mM-*sn*-glycerol 3-phosphate. GPAT activity in microsomes (*a*, *c*) was assayed in the absence of NEM, whereas mitochondria (*b*, *d*) were assayed for GPAT in the presence of 10 mM-NEM to minimize contamination by microsomal GPAT (Saggerson *et al.*, 1979, 1980). The values are means of four separate experiments. The bars indicate S.E.M. Significance of noradrenaline effects is indicated by *, **, *** for $P < 0.02$, < 0.01, < 0.001 respectively. ○, Control cells; ●, noradrenaline-treated cells.

activity by 37% (measured with 0.5mM-glycerol phosphate and 65 μM-palmitoyl-CoA), the activity was also decreased by 47% by corticotropin (100ng/ml) and by 38% by glucagon (100ng/ml) (results not shown).

The next experiment was undertaken to ascertain whether inactivation of GPAT by noradrenaline would persist through the isolation of mitochondria and microsomes from adipocytes. These procedures took approx. 20min and 90min respectively. Fig. 3 shows that the effect of noradrenaline on the mitochondrial enzyme is lost during the isolation of mitochondria. On the other hand, GPAT activity in microsomes isolated from noradrenaline-treated cells was found to be significantly decreased compared with the control activity. With isolated microsomes, the dependence of GPAT activity upon [glycerol phosphate] was hyperbolic. The K_m of the microsomal GPAT obtained from control incubations of adipocytes was 0.28 ± 0.07 mM (Fig. 4) and was similar to that reported for the liver microsomal GPAT (Nimmo, 1979). Fig. 4 shows that exposure of cells to noradrenaline significantly decreased the

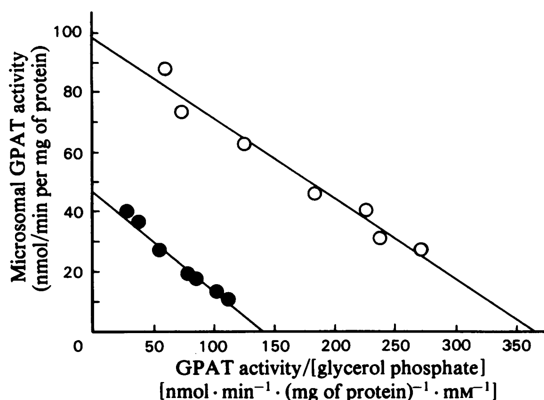

Fig. 4. *Eadie–Hofstee plots for the response of microsomal GPAT from control and noradrenaline-treated adipocytes to sn-glycerol 3-phosphate concentration*
Adipocytes were incubated for 1h with or without 1 μM-noradrenaline, followed by isolation of microsomes in sucrose medium. GPAT was assayed at 70 μM-palmitoyl-CoA and *sn*-glycerol 3-phosphate concentrations of 0.1, 0.13, 0.2, 0.25, 0.5, 1.0 and 1.5 mM. The values are means for four separate experiments. By linear regression analysis, *r* was found to be 0.98 and 0.97 without and with noradrenaline respectively. $V_{max.}$ and K_m for glycerol phosphate were evaluated from the four individual sets of data and were: $V_{max.}$ (control), 99.1 ± 23.1 nmol/min per mg of protein; $V_{max.}$ (noradrenaline), 53.5 ± 16.2 nmol/min per mg of protein ($P < 0.05$ compared with the control). K_m (control), 0.28 ± 0.07 mM; K_m (noradrenaline), 0.55 ± 0.17 mM. ○, Control; ●, with noradrenaline.

$V_{max.}$ relative to the microsomal protein content. Although some increase in the K_m was also generally seen, this was not statistically significant. The activity of the microsomal marker NADPH–cytochrome *c* reductase was also measured in these microsomal preparations and, by contrast, was found to be unaffected by noradrenaline treatment (results not shown). The noradrenaline-induced inactivation of microsomal GPAT was found to be quite stable provided that the preparation was kept in the cold. In fact, conveniently, microsomes could be frozen and kept for several days with no loss of the noradrenaline effect.

Fig. 5 and Table 1 summarize a series of experiments designed to test whether inactivation of GPAT by noradrenaline could be reversed within the cells. It has been shown previously (Sooranna & Saggerson, 1978) that propranolol or insulin added at the start of incubations can block the effect of catecholamines on GPAT. Fig. 5 shows that addition of either of these agents after a 40min period of exposure to noradrenaline resulted in restoration of GPAT activity to values that were not significantly different from either the zero-time activities or activities in control incubations without noradrenaline. As also shown by Sooranna & Saggerson (1978), inactivation of GPAT by catecholamine hormones was a relatively gradual process under the experimental conditions employed. This is also shown in Fig. 5. Similarly, restoration of activity by propranolol and insulin was not immediate, although the effect of insulin appeared to be more rapid than that of the β-blocker. Surprisingly, the addition of propranolol or insulin at 40min did not immediately arrest lipolysis as measured by glycerol release (Fig. 5). In a subsequent experiment (results not shown) it was found that addition of propranolol after 10min immediately arrested lipolysis. Other studies (Cheng & Saggerson, 1978; Sooranna & Saggerson, 1982) have also shown that addition of insulin or propranolol at this earlier time results in an immediate arrest of noradrenaline-stimulated lipolysis. The results shown in Fig. 5 tentatively suggest that, after a more prolonged period of exposure to noradrenaline (40min), lipolysis is more resistant to the effects of insulin or the β-blocker. The fact that GPAT is rapidly re-activated by insulin (Fig. 5*b*) under conditions where lipolysis is barely affected by the hormone is noteworthy, since it suggests that effects of insulin on GPAT and lipolysis may involve dissimilar mechanisms. In a previous study (Sooranna & Saggerson, 1976*b*) it was demonstrated that addition of high concentrations of non-esterified fatty acids to adipocyte incubations did not cause inactivation of GPAT and therefore did not mimic the effect of the catecholamine hormones. Table 1 shows that 40min of incubation

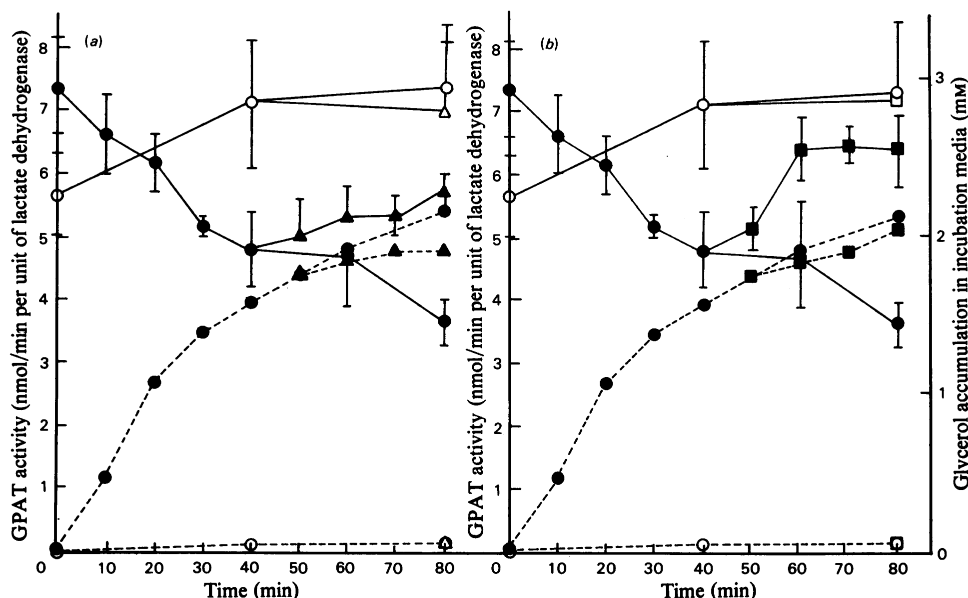

Fig. 5. *Reversal of noradrenaline-induced inactivation of total GPAT in fat-free homogenates by insulin and propranolol*
Adipocytes were incubated with or without 1 μM-noradrenaline. After 40 min, 10 μM-DL-propranolol (*a*) or 1.5 nM-insulin (*b*) was added as indicated to some flasks. At the indicated times cells were freeze-stopped, and fat-free homogenates were prepared in sucrose medium. Total GPAT was assayed with 1.0 mM-*sn*-glycerol 3-phosphate and 100 μM-palmitoyl-CoA. The values are the means ± S.E.M. for five separate experiments. Where not shown, the bars lie within the symbols. Continuous lines, GPAT activity; broken lines, glycerol accumulation. ○, No additions, △, +propranolol; □, +insulin; ●, +noradrenaline; ▲, +noradrenaline and propranolol; ■, +noradrenaline and insulin. Statistical analysis (GPAT activity): (*a*) versus zero-time activity, $P < 0.01$ at 40 and 80 min with noradrenaline alone and $P > 0.05$ at 80 min with noradrenaline + propranolol; versus 80 min value with noradrenaline + propranolol, $P < 0.01$ for 80 min value with noradrenaline alone; versus 80 min value with propranolol alone, $P > 0.1$ for 80 min value with noradrenaline + propranolol; (*b*) versus zero-time activity, $P > 0.05$ at 80 min with noradrenaline + insulin; versus 80 min value with noradrenaline + insulin, $P < 0.02$ for 80 min value with noradrenaline alone; versus 80 min with insulin alone, $P > 0.1$ for 80 min value with noradrenaline + insulin.

with noradrenaline resulted in substantial accumulation of non-esterified fatty acid. The restorative actions of propranolol and insulin on GPAT therefore occurred in the presence of high extracellular fatty acid concentrations. Addition of insulin resulted in a small decrease in non-esterified fatty acid over the 40–80 min period, presumably by promoting re-esterification. Non-esterified fatty acid concentration did not change over the same period in the presence of propranolol. Table 1 shows that insulin and propranolol acted to restore both the NEM-sensitive and NEM-insensitive forms of GPAT.

It has been suggested (Nimmo, 1980) that phosphorylation of GPAT by cyclic AMP-dependent protein kinase might be involved in the mechanism whereby catecholamine hormones inactivate the enzyme in adipocytes. If this is so, inclusion in homogenization media of fluoride might be expected to preserve the phosphorylation state of the enzyme and hence increase the measured magnitude of the noradrenaline effect. Fluoride inhibits various phosphoprotein phosphatase activities, such as phosphorylase *a* phosphatase (Danforth *et al.*, 1962) and glycogen synthase *b* phosphatase (Kato & Bishop, 1972), although others, such as myosin light-chain phosphatase, may be fluoride-insensitive (Morgan *et al.*, 1976). The Mg^{2+}-dependent phosphatidate phosphohydrolase in adipocytes also undergoes a relatively stable inactivation when the cells are exposed to noradrenaline (Cheng & Saggerson, 1978). In this instance the effect of noradrenaline can be abolished by inclusion of albumin homogenization buffers, and it has been suggested that the effect of noradrenaline is brought about by the tight binding of some (presumably hydrophobic) regulatory ligand(s) to the enzyme rather than by a phosphorylation/dephosphorylation type of mechanism (Cheng & Saggerson, 1980). Table 2 shows that inclusion of

Table 1. *Reversal of noradrenaline-induced inactivation of N-ethylmaleimide-sensitive GPAT and N-ethylmaleimide-insensitive GPAT by insulin and propranolol*

These measurements were made in the same experiments shown in Fig. 5. The numbers in parentheses in the second column indicate the times of addition of noradrenaline, insulin or propranolol. The values are means ± S.E.M. for five experiments. GPAT activities were assayed with 0.25mM-*sn*-glycerol 3-phosphate and 40μM-palmitoyl-CoA. NEM-insensitive GPAT was assayed in the presence of 10mM-NEM. NEM-sensitive activity was obtained by subtraction of the insensitive activity from the total GPAT activity. The *P* values in parentheses are for comparison of various treatments against the zero-time value. *, **, *** indicate $P < 0.5$, < 0.1, < 0.05 respectively for comparison at 80min of noradrenaline + propranolol or noradrenaline + insulin treatments versus the respective controls containing propranolol or insulin.

Sampling time (min)	Additions to incubation medium	GPAT activities (nmol/min per unit of lactate dehydrogenase)		Non-esterified fatty acid concn. in incubation media (mM)
		NEM-sensitive	NEM-insensitive	
0	None	2.5 ± 0.5	0.71 ± 0.03	0.09 ± 0.01
40	Noradrenaline (0)	1.2 ± 0.2 ($P < 0.05$)	0.44 ± 0.03 ($P < 0.01$)	5.73 ± 0.35
80	Noradrenaline (0)	0.9 ± 0.2 ($P < 0.02$)	0.41 ± 0.03 ($P < 0.01$)	6.96 ± 0.27
80	None	2.3 ± 0.4	0.79 ± 0.10	—
80	Propranolol (40)	2.9 ± 0.7	0.98 ± 0.12	—
80	Insulin (0)	2.2 ± 0.4	0.81 ± 0.11	—
80	Noradrenaline (0) + propranolol (40)	2.3 ± 0.4* ($P > 0.5$)	0.70 ± 0.04*** ($P > 0.5$)	5.92 ± 0.17
80	Noradrenaline (0) + insulin (40)	1.8 ± 0.3** ($P > 0.2$)	0.61 ± 0.05*** ($P > 0.5$)	5.00 ± 0.28

Table 2. *Effect of inclusion of albumin and fluoride in homogenization media on inactivation of GPAT by noradrenaline*

Adipocytes were incubated for 1h with or without noradrenaline, followed by freeze-stopping and preparation of fat-free homogenates in the indicated media. Total GPAT was assayed with 1.0mM-*sn*-glycerol 3-phosphate and 100μM-palmitoyl-CoA. NEM-insensitive GPAT was assayed with 0.25mM-*sn*-glycerol 3-phosphate and 40μM-palmitoyl-CoA in the presence of 10mM-NEM. Each value represents the mean ± S.E.M. for three separate experiments, and are expressed as nmol/min per unit of lactate dehydrogenase. Percentage changes caused by noradrenaline are shown in parentheses.

	Noradrenaline (1μM) added to cell incubation	Homogenization medium			
		Sucrose	Sucrose/albumin	Sucrose/F^-	Sucrose/F^-/albumin
Total GPAT	−	6.4 ± 0.6	14.4 ± 3.3	5.6 ± 0.8	7.9 ± 1.6
	+	2.2 ± 0.2 (−66%)	13.1 ± 3.8 (−9%)	2.2 ± 0.2 (−61%)	8.5 ± 1.5 (+8%)
NEM-insensitive GPAT	−	1.1 ± 0.3	3.3 ± 0.3	1.0 ± 0.2	2.6 ± 0.2
	+	0.3 ± 0.1 (−73%)	1.4 ± 0.2 (−58%)	0.3 ± 0.1 (−70%)	1.1 ± 0.1 (−58%)

fluoride in homogenization buffers did not alter the effect of noradrenaline on GPAT activities. On the other hand, homogenization in medium with albumin abolished the effect of noradrenaline on total GPAT activity. Since the concentration of albumin in the homogenization buffer was 10mg/ml and GPAT assays (1.0ml) were initiated with 0.1ml of homogenate, the carry-over of albumin increased its concentration in the assays from 1.75 to 2.75mg/ml. Under the conditions employed for the assay of total GPAT (100μM-palmitoyl-CoA; see Fig. 1), increasing the [albumin] would be expected to decrease the total GPAT activity by lowering the free concentration of palmitoyl-CoA. In fact the opposite was seen (Table 2); inclusion of albumin increased total GPAT activity. An increase in NEM-insensitive GPAT activity was also observed when homogenization was performed in the presence of albumin. Reference to Fig. 2(*a*) suggests that, with the assay conditions employed (40μM-palmitoyl-

CoA), the carry-over of albumin into the assay would again decrease the NEM-insensitive GPAT from control cells, but would slightly increase that measured in extracts from noradrenaline-treated cells. In most cases, therefore, besides abolishing the noradrenaline effect on total GPAT, albumin appeared to have a separate stimulatory effect on GPAT activities. Inclusion of albumin did not abolish the effect of noradrenaline on the NEM-insensitive (mitochondrial) GPAT. The effect of noradrenaline on total GPAT that is abolished by homogenization with albumin must therefore be confined to the NEM-sensitive GPAT. It is concluded that noradrenaline is unlikely to cause inactivation of microsomal GPAT by a covalent-modification type of mechanism, and it is suggested that albumin removes inhibitory factor(s) that may be present in control cells but are present in larger amount in noradrenaline-treated cells. A covalent-modification type of mechanism cannot be discounted as an explanation for the effect of noradrenaline on the NEM-insensitive activity, although fluoride does not alter the effect (Table 2) and the effect is relatively labile (Fig. 3). It was also noticed that fluoride addition resulted in decreased GPAT activity (Table 2). This was more noticeable with measurement of total GPAT than with assay of NEM-insensitive activity in crude extracts. Furthermore, the inhibitory effect of fluoride was greater when albumin was included in homogenization media. These effects are not understood. In other experiments (results not shown), inclusion of fluoride in sucrose medium used for subcellular fractionation resulted in a loss of 48% of microsomal GPAT relative to microsomal protein and a loss of 37% of mitochondrial GPAT. Fluoride did not affect the preservation through centrifugation procedures of the effect of noradrenaline on microsomal GPAT and did not prevent the loss of the noradrenaline effect when mitochondria were isolated (see Fig. 3).

As noted in the introduction, GPAT in fat-pad microsomes from starved rats is inactivated and re-activated under conditions favouring protein phosphorylation and dephosphorylation respectively (Nimmo & Houston, 1978; Nimmo, 1980). One of the initial objectives of the present study was to attempt to relate these findings to the inactivating effect of noradrenaline. Firstly, it was decided that the experimental approach of Nimmo & Houston (1978) should be repeated in an attempt to inactivate GPAT by treatment of microsomes with cyclic AMP-dependent protein kinase. It was intended that this preparation could then be compared with microsomes from noradrenaline-treated cells in attempts to re-activate the enzyme. Unexpectedly, in our hands, the protein kinase did not inactivate microsomal GPAT (Fig. 6). Microsomes were isolated from adipocytes of fed rats by the method of Nimmo & Houston (1978) and were then incubated exactly as described by Nimmo (1980), except that bovine cardiac protein kinase catalytic subunits rather than the rabbit muscle enzyme were used. In the absence of added protein kinase, ^{32}P from [γ-^{32}P]ATP was incorporated slowly into microsomal protein, reaching a plateau of approx. 3 nmol/mg of protein after 10 min. GPAT activity decreased by approx. 20% on incubation for 30 min in the absence of added protein kinase. Addition of protein kinase catalytic subunits considerably increased the initial rate of phosphorylation of microsomal protein and resulted in the incorporation of approx. 13 nmol of ^{32}P into protein over 30 min. Nimmo (1980) observed the incorporation of approx. 20 nmol of $^{32}P/\mu g$ of protein in microsomes from fat-pads of starved rats. Fig. 6 shows that protein kinase had no effect whatever on GPAT inactivation. This lack of effect could not be attributed to depletion of the added ATP, since approx. 60% was still remaining after 30 min (Fig. 6). Further experiments were performed in an attempt to explain the discrepancy between these findings and those of Nimmo & Houston (1978) and Nimmo (1980). These other workers used tissue from starved animals. However, incubation of adipocyte microsomes from starved rats with cyclic AMP, $MgATP^{2-}$ and cyclic AMP-dependent protein kinase for 30 min had no effect on GPAT activity (results not shown). It therefore seems highly unlikely that differences in the nutrition of the animals were the cause of the discrepancy. To test the possibility that factor(s) present in adipocyte microsomes might be inhibitory towards the added protein kinase, the phosphorylation of histone was studied in the presence and absence of microsomal protein at concentrations equivalent to those used in Fig. 6. The addition of microsomes was found to decrease the rate of histone phosphorylation only slightly (results not shown). Another possible reason for the lack of effect of the protein kinase on microsomal GPAT could be inaccessibility of the GPAT to the protein kinase. The catalytic site of GPAT is thought to be on the cytoplasmic side of the endoplasmic reticulum (Bell *et al.*, 1979); however, the orientation of potential phosphorylation sites is unknown. Sonication of adipocyte microsomes from fed rats did not render GPAT susceptible to inactivation by protein kinase (similar conditions to Fig. 6; results not shown). Bell & Coleman (1980) have suggested that the effects seen by Nimmo & Houston (1978) might have resulted from inactivation of carnitine palmitoyltransferase by protein kinase. The carnitine palmitoyltransferase was added to generate palmitoyl-CoA in a coupled assay for GPAT. This possibility seems highly unlikely, since the assays of Nimmo & Houston (1978) were linear with time. The specific activity of

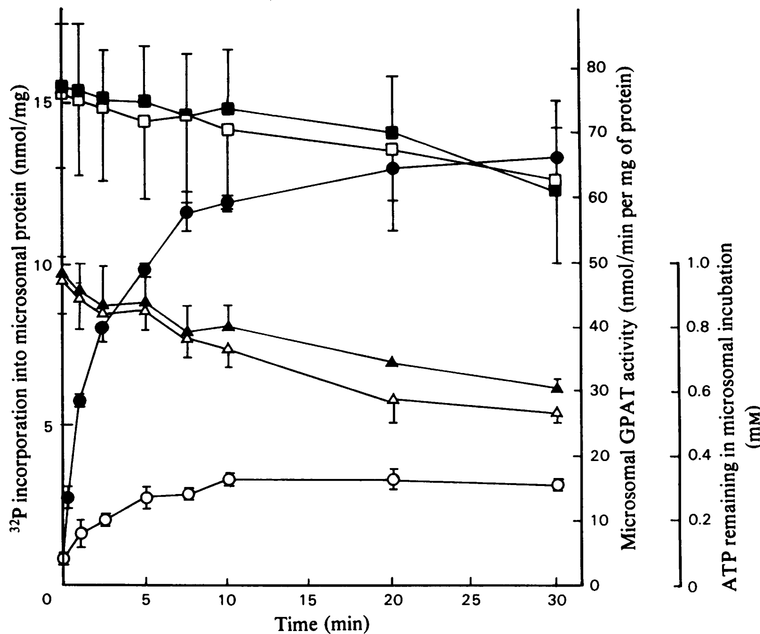

Fig. 6. *Effect of cyclic AMP-dependent protein kinase catalytic subunits on microsomal GPAT activity*
Adipocyte microsomes were prepared from the epididymal fat-pads of fed rats and incubated at a protein concentration of 0.3 mg/ml in 0.2 ml containing 0.25 M-sucrose, 10 mM-Tris/HCl (pH 7.4), 10 mM-$MgCl_2$, 1 mM-EDTA and 1 mM-dithiothreitol with (closed symbols) and without (open symbols) bovine cardiac protein kinase catalytic subunits (4 units/ml) at 30°C. The reactions were initiated by the addition of 1 mM-[γ-^{32}P]ATP (20–30 Ci/mol), and 20 μl samples were removed at the indicated times for measurement of protein-bound ^{32}P (○, ●), GPAT activity (□, ■) and ATP concentration (△, ▲). GPAT was assayed by using 0.5 mM-*sn*-glycerol 3-phosphate and 70 μM-palmitoyl-CoA. The values are means ± S.E.M. for three separate experiments.

GPAT relative to microsomal protein reported here (e.g. Fig. 6) is higher than that observed by Nimmo & Houston (1978). This difference is attributable to the use of whole fat-pads rather than adipocytes as the source of microsomes in the work of Nimmo & Houston (1978). The specific activity of GPAT in microsomes from whole fat-pads with the direct assay used here is similar to that found by Nimmo & Houston (1978) (results not shown). It is concluded that differences in GPAT assay procedures are unlikely to account for the observed discrepancies.

Despite this failure to inactivate microsomal GPAT with protein kinase, attempts were made to activate/re-activate GPAT by treatment of microsomes with phosphatases. Incubation of microsomes from noradrenaline-treated adipocytes (obtained as in Fig. 3) with 5 mM-$MgCl_2$ and either calf intestinal alkaline phosphatase (1.3 EC units/ml) or phosphoprotein phosphatase-1 (3 EC munits/ml), 0.12 mg/ml, did not result in any re-activation of GPAT. These concentrations of the respective phosphatases have been shown rapidly to re-activate GPAT previously inactivated by treatment with protein kinase (Nimmo & Houston, 1978; Nimmo, 1980). An interesting phenomenon was observed in the course of these experiments and in others attempting to detect phosphoprotein phosphatase activity in adipocyte high-speed supernatants. The findings from these experiments are shown in Table 3. Incubation of microsomes from either control or noradrenaline-treated cells in sucrose medium at pH 7.4 for 30 min resulted in inactivation of GPAT. This decrease in activity was greater in microsomes from noradrenaline-treated cells (a 72% decrease) than in control microsomes (29% decrease). The apparent effect of noradrenaline was therefore accentuated by this incubation. Addition of 100000 *g* post-microsomal supernatant had a protective effect, particularly with microsomes from noradrenaline-treated cells. This finding is interesting in view of the observation by Sooranna & Saggerson (1978) that GPAT in crude homogenates prepared from control or catecholamine-treated cells is stable for at least 1 h at either 0–4°C or 30°C. Addition of $MgCl_2$ (5 mM) or Ca^{2+}/EGTA to give a free [Ca^{2+}] of approx. 1 μM also protected the microsomal

Table 3. *Effect of metal ions and concentrated 100000g supernatant on GPAT activity in microsomes isolated from control and noradrenaline-treated adipocytes*

Adipocytes were incubated with or without noradrenaline for 1 h, followed by isolation of microsomes and, for the control cells, 100000*g* supernatant also, which was then concentrated as described in the Materials and methods section. Microsomes were incubated at 30°C at a protein concentration of 0.3 mg/ml in a final volume of 0.1 ml containing 0.25 M-sucrose, 10 mM-Tris/HCl, pH 7.4, 1 mM-EDTA, 1 mM-dithiothreitol and metal ions as indicated, with or without 100000*g* supernatant (5 mg of protein/ml); 1 μM free Ca^{2+} (approx.) was obtained with 4.15 mM-$CaCl_2$ + 5 mM-EGTA. Samples (30 μl) were removed at 0 and 30 min for assay of GPAT with 1.0 mM-*sn*-glycerol 3-phosphate and 100 μM-palmitoyl-CoA. Each value is expressed as nmol/min per mg of microsomal protein and is the mean from two separate experiments. The percentage decrease in GPAT activity caused by noradrenaline is given in parentheses.

	GPAT activity (nmol/min per mg of microsomal protein)			
Microsomal incubation time ...	0 min		30 min	
Additions to microsome incubation	Control cells	Noradrenaline-treated cells	Control cells	Noradrenaline-treated cells
None	52	26 (50)	37	7 (81)
100000*g* supernatant	59	31 (47)	52	23 (56)
5 mM-$MgCl_2$	66	35 (47)	58	25 (57)
5 mM-$MgCl_2$ + 100000*g* supernatant	62	42 (32)	64	35 (45)
1 μM-free Ca^{2+}	51	26 (49)	52	18 (65)
1 μM-free Ca^{2+} + 100000*g* supernatant	58	32 (45)	60	31 (48)
5 mM-$MgCl_2$ + 1 μM-free Ca^{2+}	66	35 (47)	63	33 (48)
5 mM-$MgCl_2$ + 1 μM-free Ca^{2+} + 100000*g* supernatant	71	40 (44)	66	37 (44)

GPAT against inactivation. The reason why GPAT activity is lost on incubation of microsomes alone and why noradrenaline accelerates this loss is not understood. It should be stressed that, although addition of 100000*g* supernatant, metal ions or combinations thereof protected against loss of GPAT activity on incubation, none of these agents caused any reversal of the effect of noradrenaline, as evidenced by the fact that the percentage decrease in GPAT activity by noradrenaline was never lessened.

There are a number of conclusions from this study. Previously reported inactivation of GPAT after catecholamine treatment of adipocytes is now seen to be due to decreased activity in both the mitochondria and the microsomes. The hormonal effect can be detected in assays covering a wide range of substrate concentrations and is shown to be reversible within cells. No evidence is forthcoming to suggest that the effect of noradrenaline on microsomal GPAT activity is directly mediated through a phosphorylation/dephosphorylation mechanism, since the effect is abolished by albumin and is unaffected by fluoride. Furthermore, under conditions where protein kinase results in quite extensive phosphorylation of microsomal proteins, GPAT is not inactivated. Attempts to reverse the effect of noradrenaline by protein dephosphylation were facilitated by the fact that noradrenaline-promoted inactivation of microsomal GPAT persisted through the isolation of microsomes. Again, no evidence was forthcoming to suggest that the effect of the hormone on microsomal GPAT was directly attributable to protein phosphorylation. These conclusions do not disprove the possibility that GPAT might undergo phosphorylation without alteration in catalytic activity or the possibility that phosphorylation or dephosphorylation might render the enzyme more or less susceptible, respectively, to the effects of regulatory ligands.

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