Biochem. J. (1986) 240, 99–106 (Printed in Great Britain)

Evidence that activation of acetyl-CoA carboxylase by insulin in adipocytes is mediated by a low-M_r effector and not by increased phosphorylation

Timothy A. J. HAYSTEAD and D. Grahame HARDIE*
M.R.C. Protein Phosphorylation Group, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

1. The activation of acetyl-CoA carboxylase (measured in a crude supernatant fraction) caused by insulin treatment of adipocytes was completely unaffected by the addition of a large amount of highly purified protein phosphatase to the supernatant fraction. Under the same conditions the inhibition of acetyl-CoA carboxylase by adrenaline was totally reversed. 2. Experiments with ^{32}P-labelled adipocytes showed that insulin increased the total phosphorylation of acetyl-CoA carboxylase from 2.7 to 3.5 molecules of phosphate/240 kDa subunit, and confirmed that this increase was partially accounted for by phosphorylation within a specific peptide (the 'I-site' peptide). Protein phosphatase treatment of the crude supernatant fractions removed over 80% of the ^{32}P radioactivity from the enzyme and removed all detectable radioactivity from the I-site peptide. 3. The effect of insulin on acetyl-CoA carboxylase activity, but not the effect on phosphorylation, was lost on purification of the enzyme on avidin–Sepharose. The effect on enzyme activity was also lost if crude supernatant fractions were subjected to rapid gel filtration after treatment under conditions of high ionic strength, similar to those used in the avidin–Sepharose procedure. 4. These results show that, although insulin does increase the phosphorylation of acetyl-CoA carboxylase at a specific site, this does not cause enzyme activation. They suggest instead that activation of the enzyme by insulin is mediated by a tightly bound low-M_r effector which dissociates from the enzyme at high ionic strength.

INTRODUCTION

Acetyl-CoA carboxylase catalyses the first step committed to fatty acid synthesis, and is generally believed to be an important regulatory enzyme in the pathway. The purified enzyme has been shown to be regulated both by allosteric effectors (activation by citrate and inhibition by fatty acyl-CoA) and by reversible phosphorylation (Hardie, 1980; Hardie *et al.*, 1984). Acetyl-CoA carboxylase purified from mammary gland (Hardie & Guy, 1980; Munday & Hardie, 1984), liver (Tipper & Witters, 1982) and adipose tissue (Brownsey *et al.*, 1981) is inactivated by phosphorylation by cyclic AMP-dependent protein kinase, and re-activated by treatment with protein phosphatase (Hardie & Guy, 1980; Tipper & Witters, 1982; Munday & Hardie, 1984). Exposure of isolated cells to hormones that increase cyclic AMP, i.e. glucagon in hepatocytes (Holland *et al.*, 1984) and adrenaline or glucagon in adipocytes (Brownsey & Hardie, 1980; Holland *et al.*, 1985), leads to inactivation of acetyl-CoA carboxylase, associated with increased phosphorylation within the same tryptic peptides that are phosphorylated on the purified enzyme by cyclic AMP-dependent protein kinase. Thus there is good evidence that these hormones inhibit fatty acid synthesis, at least in part, via direct phosphorylation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase.

Several other cyclic AMP-independent protein kinases which phosphorylate acetyl-CoA carboxylase have been purified (Shiao *et al.*, 1981; Lent & Kim, 1982; Munday & Hardie, 1984; Hardie *et al.*, 1986). These protein kinases either inactivated or had no effect on enzyme activity. In no case has a purified protein kinase been reported to activate acetyl-CoA carboxylase, although incubation of the enzyme from adipose tissue with a plasma-membrane-enriched fraction from the same tissue was reported to produce an activation that was dependent on the presence of ATP (Brownsey *et al.*, 1981). Although phosphorylation of acetyl-CoA carboxylase did occur under these conditions, it preceded activation, and there was no direct evidence that phosphorylation caused the activation.

Insulin and epidermal growth factor stimulate fatty acid synthesis in both hepatocytes (Geelen *et al.*, 1978; Holland & Hardie, 1985) and adipocytes (Haystead & Hardie, 1986), and in adipocytes this is accompanied by an activation of acetyl-CoA carboxylase, which is readily detectable in crude cell extracts (Halestrap & Denton, 1973; Witters *et al.*, 1983; Haystead & Hardie, 1986). Since this is opposite to the effect of all of the well-characterized phosphorylation reactions cited above, one might expect insulin (and epidermal growth factor) to act via dephosphorylation of acetyl-CoA carboxylase, as insulin does for pyruvate dehydrogenase in adipocytes (Hughes *et al.*, 1980). However, insulin has been reported to stimulate the phosphorylation of acetyl-CoA carboxylase in both adipocytes (Brownsey & Denton, 1982; Witters *et al.*, 1983) and hepatocytes (Witters, 1981; Holland & Hardie, 1985). Insulin and epidermal growth factor also stimulate the phosphorylation of several other cytosolic proteins in intact cells, including ATP citrate lyase (Alexander *et al.*, 1979; Ramakrishna & Benjamin, 1979; Holland & Hardie, 1985), ribosomal protein S6 (Smith *et al.*, 1979; Thomas

* To whom reprint requests should be addressed.

et al., 1982) and unidentified polypeptides of 22 kDa (Blackshear *et al.*, 1983) and 46 kDa (Le Cam, 1982; Holland & Hardie, 1985). However, in no case is the function of the increased phosphorylation clearly established.

Although different methods of peptide analysis have been used in the existing studies, insulin has been clearly shown to increase phosphorylation of acetyl-CoA carboxylase at site(s) distinct from those phosphorylated by cyclic AMP-dependent protein kinase (Brownsey & Denton, 1982; Witters *et al.*, 1983; Holland & Hardie, 1985). After insulin treatment of adipocytes, increased Ca^{2+}- and cyclic AMP-independent acetyl-CoA carboxylase kinase activity can be measured in crude cell supernatant fractions, leading to the proposal that binding of insulin to its cell-surface receptor activates a soluble protein (serine) kinase which phosphorylates and activates acetyl-CoA carboxylase (Brownsey *et al.*, 1984).

In contrast with the effects of cyclic AMP-increasing hormones in adipocytes or hepatocytes (Holland *et al.*, 1984, 1985), effects of insulin on acetyl-CoA carboxylase activity do not persist during purification of the enzyme on avidin–Sepharose (Witters *et al.*, 1983; Holland & Hardie, 1985). These findings cast doubt on the hypothesis that insulin-induced phosphorylation of acetyl-CoA carboxylase causes the associated enzyme activation. In the present paper we report further evidence against this hypothesis and in favour of the idea that the effects of insulin on acetyl-CoA carboxylase activity are mediated by a tightly bound low-M_r effector.

EXPERIMENTAL

Animals

Maintenance of rats was as in Haystead & Hardie (1986).

Materials

Protein phosphatase-2A catalytic subunit was purified up to and including the polylysine–Sepharose step, and assayed with [^{32}P]phosphorylase *a*, as described by Resink *et al.* (1983). [^{32}P]Phosphate was from Amersham International, Amersham, Bucks., U.K. Avidin–Sepharose was synthesized as described by Tipper & Witters (1982). Trypsin (treated with tosylphenylalanylchloromethane, 'TPCK') was from Worthington/Millipore Corp. (Freehold, NJ, U.S.A.).

Sources of other materials have been described previously (Haystead & Hardie, 1986).

Isolation and incubation of adipocytes

Adipocytes were isolated and incubated as described previously (Haystead & Hardie, 1986), except that the NaCl concentration in buffer A was 120 mM (quoted incorrectly as 590 mM in Haystead & Hardie, 1986). [^{32}P]Phosphate, where added, was used at 80 μCi/ml and cells were preincubated for 90 min before addition of hormone. Hormone treatments were for 15 min in all cases, with insulin at 0.9 nM and adrenaline at 1 μM.

Homogenization of cells and isolation of acetyl-CoA carboxylase

In experiments where acetyl-CoA carboxylase was not purified, tissue from six rats was used, and cells were broken with a Polytron homogenizer in the presence of 50 mM-NaF as described previously, with a 3–5 s burst at setting 3 and without prior freezing of cells (Haystead & Hardie, 1986). In experiments where acetyl-CoA carboxylase was purified, tissue from 40 rats was used, and cells were homogenized in the same medium by vortex-mixing for 2 min in a stoppered glass tube. These two methods of homogenization gave very similar yields of enzyme activity in the crude extracts. In experiments where polyacrylamide-gel electrophoresis was to be carried out on crude cell fractions, cells were washed twice in medium lacking serum albumin before homogenization, otherwise the large quantities of this protein derived from the medium interfered with electrophoresis.

Acetyl-CoA carboxylase was purified as described by Holland *et al.* (1985). Specific radioactivity of the purified enzyme was measured by precipitating samples of known protein concentration in 25% (w/v) trichloroacetic acid and determining radioactivity in the pellet by Čerenkov counting.

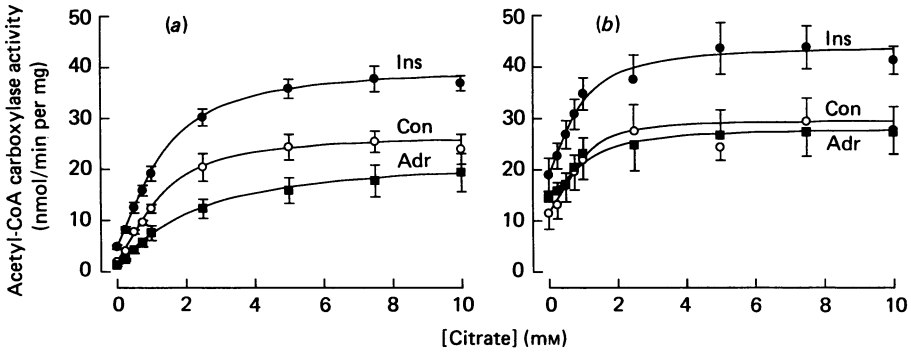

Fig. 1. Acetyl-CoA carboxylase activity in crude extracts prepared from control (Con), insulin (Ins)- or adrenaline (Adr)-treated adipocytes

Initial velocity was measured as a function of citrate concentration, and the results shown are means ± S.E.M. for five separate cell preparations. The continuous lines are theoretical curves based on the kinetic parameters shown in Table 1. (*a*) Cells homogenized in 50 mM-NaF and extracts incubated for 5 min at 37 °C before assay: the effects of both hormones were significant ($P < 0.05$), except for the effect of adrenaline at zero citrate concentration. (*b*) Cells homogenized without NaF and incubated with protein phosphatase-2A for 5 min at 37 °C before assay: the effect of insulin was significant ($P < 0.05$) at all citrate concentrations, whereas the effects of adrenaline were not significant.

Table 1. Kinetic parameters of acetyl-CoA carboxylase in crude postmitochondrial supernatants from control, insulin- or adrenaline-treated adipocytes

Supernatant fractions were incubated with or without protein phosphatase as described in the Experimental section. Mean values of initial velocity (v) from the five separate experiments shown in Fig. 1 were fitted to the equation:

$$v = V_0 + \frac{(V_{max.} - V_0)[C]^h}{K_a + [C]^h}$$

where V_0 is the velocity without added citrate, [C] is the citrate concentration, K_a is the apparent dissociation constant for citrate, and h the Hill coefficient. $A_{0.5}$ (concentration of citrate giving half-maximal activation) is given by $K_a = (A_{0.5})^h$. Values for V_0 are shown as means ± S.E.M. ($n = 5$).

Treatment	V_0 (nmol/min per mg)	$V_{max.}$ (nmol/min per mg)	$A_{0.5}$ (citrate) (mM)	h
(*a*) No protein phosphatase				
Control	2.0±0.2	26.6	1.18	1.53
Insulin	4.9±0.4	39.9	1.24	1.43
Adrenaline	1.4±0.2	22.2	2.22	1.18
(*b*) +protein phosphatase-2A				
Control	11.7±3.2	29.5	0.81	1.95
Insulin	19.1±3.3	44.0	0.80	1.41
Adrenaline	14.5±1.5	28.0	0.99	1.54

Protein phosphatase treatment

In experiments where protein phosphatase was used, NaF was omitted from the homogenization medium and cell supernatant fractions were incubated with protein phosphatase-2A [16 units (nmol/min)/ml final concn.] for 5 min at 37 °C. Dephosphorylation was stopped by addition of 0.5 M-NaF (final concn. 50 mM). Controls were treated in the same way, except that the protein phosphatase was omitted.

Measurement of specific radioactivity of ATP

Samples (0.5 ml) of ^{32}P-labelled cell suspension were added to 50 μl of 55% (v/v) $HClO_4$ at the same time as cells were homogenized for acetyl-CoA carboxylase purification. The specific radioactivity of the γ-phosphate of ATP was determined as described previously (Holland *et al.*, 1985). Insulin treatment of cells did not affect this parameter.

Centrifuge-desalting experiments

Cell supernatant fractions were prepared as in Haystead & Hardie (1986), and 0.1 vol. of water or 5 M-NaCl was added. Samples (0.4 ml) were immediately centrifuged (3000 *g*; 5 min) through plugs of Sephadex G-25 (packed volume approx. 1 ml) in 2 ml disposable syringes as described by McCarthy & Hardie (1982). The Sephadex G-25 had been pre-equilibrated in homogenization medium without NaCl and pre-centrifuged at 3000 *g* for 5 min before addition of sample (bed volume before initial centrifugation = 2 ml).

Analysis of ^{32}P-labelled tryptic peptides

Purified ^{32}P-labelled acetyl-CoA carboxylase was precipitated with trichloroacetic acid, digested with trypsin and analysed by two-dimensional electrophoresis (pH 3.6) and chromatography as in Brownsey & Hardie (1980). Radioactive peptides were quantified by scraping off the cellulose corresponding to radioactive spots after dampening the surface with a fine spray of water. Peptides were eluted in 50% (v/v) pyridine and radioactivity was determined by Čerenkov counting.

Other analytical procedures

Acetyl-CoA carboxylase was assayed as described previously (Haystead & Hardie, 1986). SDS/polyacrylamide-gel electrophoresis was carried out in 5–15%-acrylamide gradient gels in the buffer system of Laemmli (1970). Autoradiography was carried out with Kodak X-Omat S film in X-Omatic intensifying cassettes at −70 °C. Protein concentrations were determined by the dye-binding method of Bradford (1976).

Expression of results and statistical significance

Unless stated otherwise, results are expressed as means ± S.E.M., with the numbers of observations shown in parentheses, and the significances of differences from control values were determined by the paired t test.

RESULTS

Treatment of adipocyte extracts with protein phosphatase-2A reverses the effects of adrenaline, but not the effects of insulin

As reported previously (see the Introduction), insulin or adrenaline treatment of isolated adipocytes results in an increase or decrease respectively in acetyl-CoA carboxylase activity which is measurable in a crude post-mitochondrial supernatant fraction prepared from the cells (Fig. 1*a*). If the supernatant fractions were pretreated with large amounts of the catalytic subunit of protein phosphatase-2A before assay, the effect of adrenaline was completely abolished, but the effect of insulin was still clearly evident (Fig. 1*b*). Protein phosphatase pretreatment very dramatically decreased the dependence of acetyl-CoA carboxylase activity on the allosteric activator, citrate, irrespective of the hormone treatment of the cells (Fig. 1). This was reflected in a large decrease in the concentration of citrate giving half-

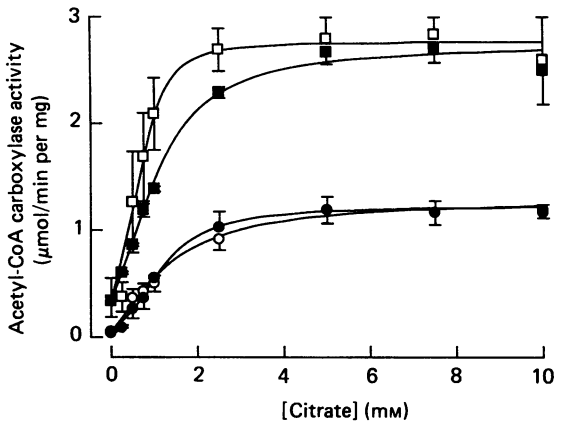

Fig. 2. Activity of acetyl-CoA carboxylase purified by avidin–Sepharose chromatography from control (○, □) or insulin-treated (●, ■) adipocytes

Crude extracts had been treated with (□, ■) or without (○, ●) protein phosphatase-2A before purification. Values shown are means ± S.E.M. from experiments with three separate cell preparations. There were no significant differences between values for enzyme from control and insulin-treated cells either with or without protein phosphatase treatment; however, the effect of the protein phosphatase treatment itself was significant ($P < 0.05$) at all citrate concentrations for both control and insulin-treated cells. Continuous lines are theoretical curves based on the kinetic parameters shown in Table 2.

maximal activation ($A_{0.5}$) and a large increase in the activity measured in the absence of added citrate (V_0) (Table 1). However, the effect of insulin is entirely on the $V_{max.}$, rather than the citrate dependence, of acetyl-CoA carboxylase (Haystead & Hardie, 1986; Fig. 1*a*), and the elevation of $V_{max.}$ by insulin was not affected by protein phosphatase treatment (Table 1).

Effects of protein phosphatase treatment of adipocyte extracts on the kinetic parameters of acetyl-CoA carboxylase measured after avidin–Sepharose purification

Supernatant fractions from control, insulin- or adrenaline-treated adipocytes were treated with or without protein phosphatase-2A exactly as described above, and acetyl-CoA carboxylase was purified on avidin–Sepharose in the presence of 50 mM-NaF. As reported previously (Witters *et al.*, 1983), the effect of insulin on acetyl-CoA carboxylase activity completely disappears on purification (Fig. 2). This is in marked contrast with the effect of adrenaline, which persists during purification (Holland *et al.*, 1985). Once again, protein phosphatase treatment of the crude supernatant fraction dramatically decreased the dependence of the purified enzyme on citrate. However, protein phosphatase treatment also elevated the $V_{max.}$ of the enzyme (Table 2), an effect that was not apparent in the crude-extract measurements (Table 1).

Effects of protein phosphatase treatment on the phosphorylation state of acetyl-CoA carboxylase

To monitor the dephosphorylation of acetyl-CoA carboxylase by protein phosphatase-2A, experiments identical with those described above were carried out with extracts of ^{32}P-labelled adipocytes. Fig. 3 (tracks 1 and 2) shows SDS/polyacrylamide-gel analysis of the crude supernatant fractions and demonstrates that insulin stimulates the phosphorylation of a 240 kDa polypeptide. This polypeptide bound quantitatively to avidin–Sepharose, and co-migrated with purified acetyl-CoA carboxylase (results not shown). If the supernatant fractions were treated with protein phosphatase-2A as described above, the radioactivity associated with the 240 kDa polypeptide in the crude extracts was largely removed (tracks 3 and 4). Fig. 3 also demonstrates that insulin stimulated the phosphorylation of a prominent phosphopeptide of 116 kDa, which has been identified previously as ATP citrate lyase (Ramakrishna & Benjamin, 1979).

SDS/polyacrylamide-gel electrophoresis of acetyl-CoA carboxylase purified from ^{32}P-labelled cells showed that the preparations contained a single radioactive polypeptide of 240 kDa, as reported previously (Holland *et al.*, 1985). To estimate the stoichiometry of phosphorylation of the enzyme, we measured the specific radioactivity of the γ-phosphate of cellular ATP by an h.p.l.c. method, so that we could convert the radioactivity per mol of purified enzyme into molecules of phosphate per subunit. The results (Table 3) showed that insulin produced a 33% stimulation of total phosphorylation that was statistically significant and corresponded to an increase of 0.7 molecule/subunit. If the crude supernatants were treated with protein phosphatase before

Table 2. Kinetic parameters of acetyl-CoA purified by avidin–Sepharose affinity chromatography from control or insulin-treated adipocytes

The crude postmitochondrial-supernatant fractions were incubated with or without protein phosphatase prior to purification. Kinetic parameters were estimated from the data shown in Fig. 2 as described in the legend to Table 1. Values for V_0 are means ± S.E.M. ($n = 3$).

Treatment	V_0 (μmol/min per mg)	V_{max} (μmol/min per mg)	$A_{0.5}$ (citrate) (mM)	h
(*a*) No protein phosphatase				
Control	0.06 ± 0.02	1.27	1.29	1.46
Insulin	0.06 ± 0.01	1.19	1.08	2.21
(*b*) + protein phosphatase-2A				
Control	0.35 ± 0.21	2.77	0.64	2.32
Insulin	0.35 ± 0.15	2.83	1.07	1.87

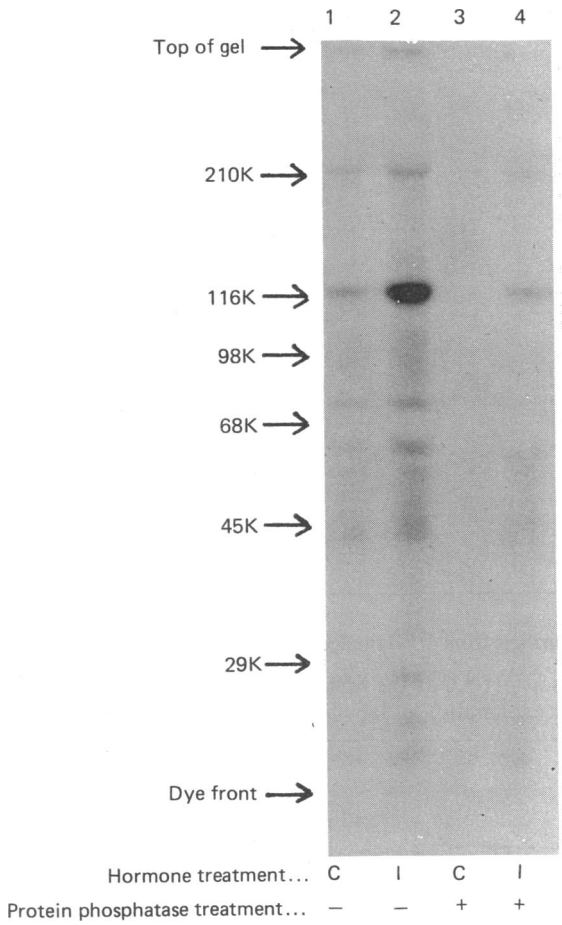

Fig. 3. Analysis of crude supernatant fractions from ^{32}P-labelled adipocytes by SDS/polyacrylamide-gel electrophoresis

Postmitochondrial supernatants were prepared from (C) control or (I) insulin-treated ^{32}P-labelled adipocytes and were incubated for 5 min at 37 °C either with (+) or without (−) the purified catalytic subunit of protein phosphatase-2A. Samples were analysed by electrophoresis on 5–15% acrylamide gradient gels. The photograph shows an autoradiogram of the dried gel. Arrows indicate the migration of marker proteins (K = kDa): myosin heavy chain (210 K); β-galactosidase (116 K); phosphorylase (98 K); serum albumin (68 K); ovalbumin (45 K); carbonic anhydrase (29 K).

enzyme purification, the estimated phosphate content was decreased by 82% and 81% for the enzyme from control and insulin-treated cells respectively. After protein phosphatase treatment, the phosphate contents of the enzymes from control and insulin-treated cells were not significantly different.

In order to examine the sites dephosphorylated during protein phosphatase treatment, enzyme isolated from control cells or insulin-treated cells was digested exhaustively with trypsin and analysed by two-dimensional electrophoresis/chromatography. Fig. 4 shows that insulin stimulates phosphorylation of acetyl-CoA carboxylase at a site within a tryptic peptide with mobility similar to the 'I-site' peptide described by Brownsey & Denton (1982). When an identical experiment was carried out, but the crude supernatant fractions were treated with protein phosphatase before enzyme purification, none of the marked peptides (O, A, I) were detectable after exposure of autoradiograms for the same time (48 h) as for Fig. 4 (results not shown). After exposure for 7 days, the O and A peptides were detectable, but we could still not detect radioactivity at the position of the I-site peptide.

When the radioactive spots shown in Fig. 4 (insulin) were scraped from the plates, eluted, and their radioactivity determined, the I-site peptide accounted for 6.5% of the radioactivity loaded, or 8.8% of the radioactivity recovered in spots O, A and I (means of two experiments).

Evidence that the effect of insulin on acetyl-CoA carboxylase activity is mediated by a dissociable effector

The results discussed above strongly suggested that the effect of insulin on enzyme activity was not due to increased phosphorylation, since dephosphorylation of acetyl-CoA carboxylase, which occurred at all sites (including the I site), did not reverse the insulin effect. The simplest alternative was that insulin was acting by changing the concentration of an effector molecule which bound tightly to acetyl-CoA carboxylase (and was thus still effective even after the large dilution involved in homogenization and assay of adipocyte extracts). We therefore examined the effects of gel filtration of the crude supernatant fractions.

Since the effect of insulin on enzyme activity is

Table 3. Phosphate content of acetyl-CoA carboxylase isolated from control and insulin-treated adipocytes

The protein phosphatase inhibitor NaF was added to the homogenization medium, or, for the protein phosphatase incubations, immediately after the phosphatase treatment. Phosphate contents were estimated from the specific radioactivities of purified acetyl-CoA carboxylase and of extracted adenine nucleotides as described in the Experimental section. Results are means ± S.E.M.

Treatment		Phosphate content (molecules/ 240 kDa subunit)
Cells	Extract	
Control	Incubated without addition	2.72 ± 0.10 (5)
Insulin	Incubated without addition	3.46 ± 0.13 (5)*
Control	Incubated with protein phosphatase	0.49 ± 0.01 (2)
Insulin	Incubated with protein phosphatase	0.63 ± 0.03 (2)

* Significantly different from control ($P < 0.02$).

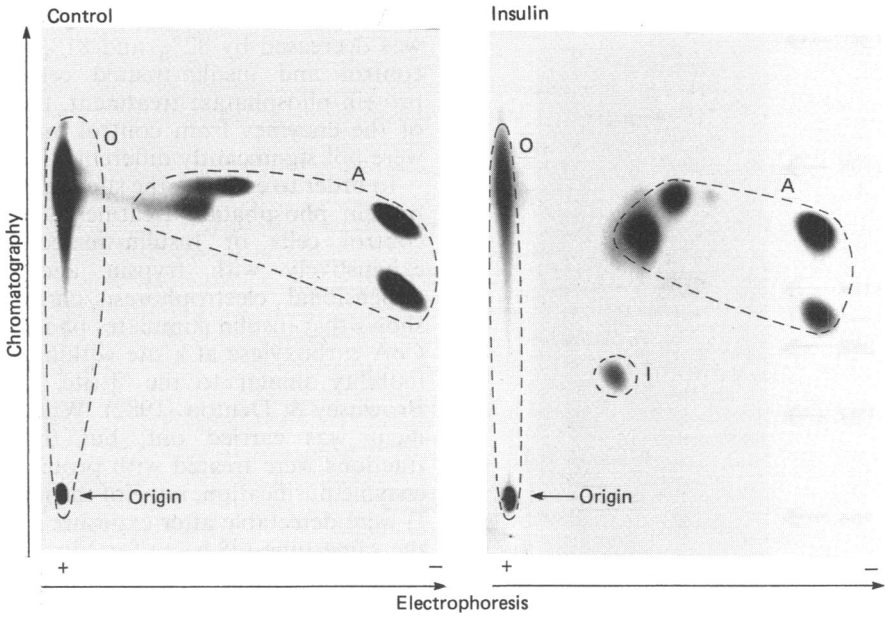

Fig. 4. Analysis of tryptic peptides derived from acetyl-CoA carboxylase purified from ^{32}P-labelled adipocytes in the presence of NaF

Peptides were analysed on thin-layer cellulose plates by electrophoresis at pH 3.6 as the first dimension and chromatography as the second dimension, followed by autoradiography. See the text for explanation of labelling of radioactive peptides.

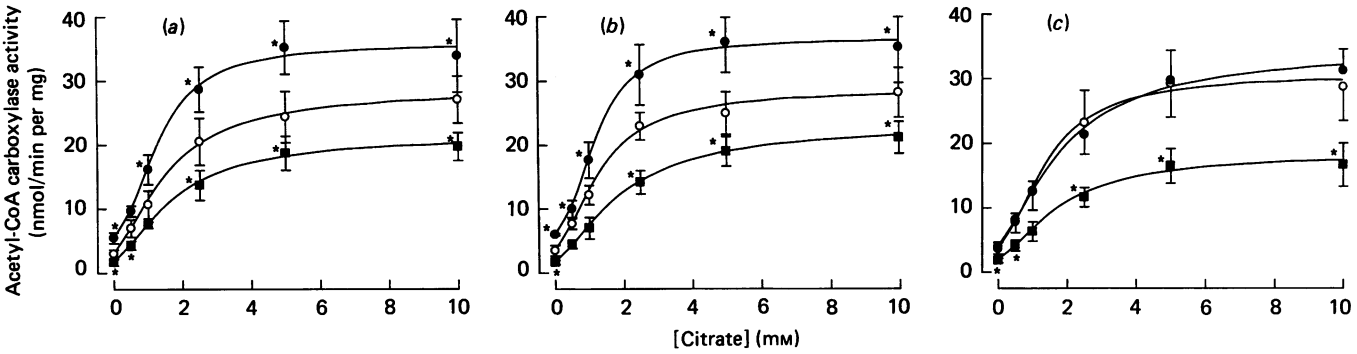

Fig. 5. Acetyl-CoA carboxylase activity in crude extracts prepared from control (○), insulin- (●) or adrenaline- (■) treated adipocytes

(*a*) Untreated extracts kept at 4 °C; (*b*) extracts centrifuge-desalted without high-salt treatment; (*c*) extracts made 0.5 M in NaCl and then centrifuge-desalted into low-ionic-strength medium. All extracts were assayed at the same time after homogenization. Results shown are mean values (±S.E.M.) from nine (*a*, *c*) or five (*b*) separate experiments: values significantly ($P < 0.05$) different from respective controls are indicated by asterisks. Continuous lines are theoretical curves obtained by fitting of these mean values to the Hill equation (see legend to Table 1).

reported to disappear slowly on storage of the extract (Halestrap & Denton, 1973), we used the technique of centrifuge-desalting, in which the extract is centrifuged through a plug of Sephadex G-25 in a disposable syringe (McCarthy & Hardie, 1982). By this method, small dissociable molecules can be removed from protein within 30 s. Control experiments in which [^{14}C]aspartate was added to the crude supernatant fraction showed that after centrifuging through Sephadex G-25, 95% of the protein but < 1% of the radioactivity was recovered.

Crude supernatant fractions from control or insulin-treated cells were diluted with 0.1 vol. of water or NaCl (final concn. 0.5 M) and them immediately centrifuge-desalted through Sephadex G-25 equilibrated in low-ionic-strength medium. If the samples were centrifuge-desalted without the high-salt treatment, the effects of insulin and adrenaline were preserved (Fig. 5). This treatment appeared to produce a slight activation of the enzyme irrespective of the hormonal treatment (cf. Figs. 5*a* and 5*b*), although this effect was not statistically significant in the present series. If the crude fractions were treated with NaCl (final concn. 0.5 M) before centrifugation, the activities of enzyme from control and insulin-treated cells were not significantly different (cf. Figs. 5*b* and 5*c*), mainly owing to a loss of activity on centrifuge-desalting of the insulin sample. If the extract was treated with 0.5 M-NaCl and assayed directly without gel filtration (NaCl concn. in assay also 0.5 M), the effect of insulin was still evident, although the salt treatment caused some inhibition in both control- and

insulin-treated samples (results not shown). The inhibition of acetyl-CoA carboxylase by adrenaline was not significantly affected by any of these treatments.

DISCUSSION

This study confirms two previous contrasting observations, i.e. that insulin treatment of adipocytes results in increased phosphorylation of acetyl-CoA carboxylase at a specific site (Brownsey & Denton, 1982), but that the effects of insulin on enzyme activity, unlike the effects on phosphorylation, do not persistent during purification on avidin–Sepharose (Witters *et al.*, 1983). We considered three different hypotheses that could explain these findings:

(1) activation of acetyl-CoA carboxylase by insulin is unrelated to increased phosphorylation;

(2) activation of acetyl-CoA carboxylase by insulin is caused by increased phosphorylation, but is only expressed in the presence of additional factor(s) which are removed by purification on avidin–Sepharose;

(3) acetyl-CoA carboxylase activation by insulin is caused by increased phosphorylation, but the avidin–Sepharose treatment selects against, or partially inactivates, the insulin-activated enzyme.

Our present results strongly support the first hypothesis. The second and third hypotheses are effectively ruled out by the experiments with protein phosphatase-2A, which does not reverse the effects of insulin treatment of cells on acetyl-CoA carboxylase activity when added to crude supernatant fractions. The control experiments with ^{32}P-labelled cells showed that > 80% of the phosphate incorporated into the enzyme during the 105 min labelling period was removed by phosphatase treatment. After protein phosphatase treatment, the acetyl-CoA carboxylase was estimated to contain only approx. 0.5 molecule of phosphate/subunit, and there was no detectable radioactivity in the insulin-sensitive I site. The almost total dephosphorylation of acetyl-CoA carboxylase by the protein phosphatase treatment was also shown by polyacrylamide-gel electrophoresis of crude supernatant fractions (Fig. 3), which rules out the possibility that some enzyme remains phosphorylated in the I site but that it is not recovered by the avidin–Sepharose procedure. In contrast with the complete failure of the protein phosphatase to reverse the effects of insulin, the effects of adrenaline were rapidly reversed by this treatment, which is entirely consistent with the proposal that inhibition by adrenaline is caused by phosphorylation by cyclic AMP-dependent protein kinase (Brownsey *et al.*, 1979; Brownsey & Hardie, 1980; Holland *et al.*, 1985).

Our data cannot rule out the possibility that increased phosphorylation changes the affinity of acetyl-CoA carboxylase for the low-M_r effector, but that under the conditions of our assay the enzyme is saturated with the effector. However, if that is the case, it would seem inevitable that the enzyme should also be saturated with the effector *in vivo*, so that the phosphorylation would have no physiological effect.

One unexplained feature of the protein phosphatase experiments was the finding that treatment of the crude supernatant fractions both decreased the dependence of acetyl-CoA carboxylase on citrate and increased $V_{max.}$, but that the latter effect was only evident after purification of the enzyme (cf. Tables 1 and 2). Control experiments showed that the amount of ^{14}C fixed was proportional to the enzyme added under all conditions, so this anomaly was not an artefact of the enzyme assay. The explanation of these findings is not clear, and we can only suggest that some dissociable factor(s) present in the crude supernatant fractions prevent the expression of the change in V_{max}.

Although our results argue against a role of phosphorylation in the activation of acetyl-CoA carboxylase by insulin, they do confirm the previous observations that insulin brings about increased phosphorylation. Our study is the first in which the effect of insulin on phosphorylation has been quantified by measuring the specific radioactivities of acetyl-CoA carboxylase and ATP purified from the cells. We find that insulin increases the total phosphorylation of the enzyme by 33%, which is somewhat larger than the effects measured previously by densitometric scanning of autoradiograms of crude extracts subjected to SDS/polyacrylamide-gel electrophoresis (Brownsey & Denton, 1982). According to our estimates of the total degree of phosphorylation, this increase represents 0.7 molecule of phosphate/subunit, i.e. it is near-stoichiometric. Quantification of the radioactivity in each group of peptides after two-dimensional electrophoresis/chromatography showed that the 'I-site' peptide accounted for 6–9% of the radioactivity present in the enzyme from insulin-treated cells, corresponding to only 0.2–0.3 molecule of phosphate/subunit. However, it is possible that some of the radioactive peptides which remained on the origin during electrophoresis (Fig. 4, spots 'O'; 41% of radioactivity recovered) contained the I site in partially digested form.

Although our results appear to rule out increased phosphorylation as the mechanism by which insulin activates acetyl-CoA carboxylase in adipocytes, they provide only suggestive evidence as to the actual mechanism of action. After addition of NaCl to the supernatant fraction and rapid gel filtration, the specific activity of the extract from insulin-treated cells was decreased to that of extract from control cells, indicating that an activator is being removed. This treatment also caused a slight activation of extracts from control cells, but this effect was also observed on gel filtration without prior addition of NaCl, i.e. conditions where the effect of insulin persists. The putative activator must bind tightly to acetyl-CoA carboxylase, since it remains effective during homogenization, dilution, and rapid gel filtration at low ionic strength. Brownsey & Denton (1982) also found that the insulin effect persisted through partial purification of acetyl-CoA carboxylase by $(NH_4)_2SO_4$ and citrate precipitations, although the effect was greatly decreased by these procedures. We found that the treatment with NaCl and the gel filtration were both required to remove the insulin effect, suggesting that the putative activator binds to acetyl-CoA carboxylase through ionic interactions which can be disrupted at high ionic strength. We can only speculate as to the identity of this putative activator, although we have found (Munday & Hardie, 1984) that acetyl-CoA carboxylase is activated by spermine, a compound that has been found to mimic some other effects of insulin (Damuni *et al.*, 1984; Tung *et al.*, 1985). Saltiel *et al.* (1983) have also reported the partial purification of a low-M_r compound which activated acetyl-CoA carboxylase, and which increased in activity when a rat liver plasma-membrane

fraction was incubated with insulin. Direct proof that insulin activates acetyl-CoA carboxylase by increasing the concentration of a low-M_r activator can only come from isolation and identification of the factor and the demonstration that insulin increases its concentration in the relevant cell compartment *in vivo*.

This study was funded by Research Group and Project Grant support from the Medical Research Council and a Project Grant from the British Heart Foundation.

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Received 14 May 1986/30 June 1986; accepted 24 July 1986