EVIDENCE FOR PHOSPHORYLATION AND ACTIVATION OF ACETYL CoA CARBOXYLASE BY A MEMBRANE-ASSOCIATED CYCLIC AMP-INDEPENDENT PROTEIN KINASE

Relationship to the activation of acetyl CoA carboxylase by insulin

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1. Introduction

Exposure of rat epididymal adipose tissue or fat cells to insulin for a few minutes results in a marked increase in the activity of acetyl CoA carboxylase assayed immediately upon extraction in the absence of added citrate [1–4]. In contrast, treatment of tissue or cells with adrenaline leads to a marked decrease in this initial activity [2–5]. The effects of adrenaline are probably brought about by increased phosphorylation of acetyl CoA carboxylase by cyclic AMP-dependent protein kinase. Acetyl CoA carboxylase from a number of sources has been shown to become less active following phosphorylation by cyclic AMP-dependent protein kinase [3,6–7] while significant increases in the overall level of phosphorylation have been observed under conditions where cellular cyclic AMP levels are raised in both fat and liver cells [3,5,8]. Other studies have indicated that acetyl CoA carboxylase is phosphorylated at multiple sites both with purified preparations [4,6,9,10] and within intact fat cells [11]. Analysis of tryptic phosphopeptides has indicated that adrenaline brings about the phosphorylation within fat cells of sites on acetyl CoA carboxylase which correspond closely with those phosphorylated on purified mammary gland enzyme with cyclic AMP-dependent protein kinase [11].

The activating effects of insulin are not simply the converse of the inhibitory actions of adrenaline [3]. In particular, adrenaline results in a decrease in the activity of the enzyme even after incubation of cell extracts with citrate to induce maximum polymerisation of the enzyme [3,5] whereas the effects of insulin are no longer apparent after incubation with citrate [2,3]. Recent studies in which tryptic phosphopeptide analysis has been carried out suggest that insulin causes the increased phosphorylation of a site different from those phosphorylated by cyclic AMP-dependent protein kinase (R. W. B., unpublished) this is in accord with the observation that insulin treatment leads to a small increase in overall phosphorylation of acetyl CoA carboxylase [3]. We therefore have begun to explore the hypothesis [3] that a cyclic AMP-independent protein kinase in fat cells may bring about the actions of insulin upon the activity of acetyl CoA carboxylase. This paper reports the finding of such a kinase associated with the plasma membranes of fat cells.

2. Materials and methods

2.1. *Materials*

[γ-^{32}P]ATP was obtained from NEN, 6072 Dreieich and protease inhibitors from the Peptide Research Institute, Osaka. Monospecific antiserum to acetyl CoA carboxylase was prepared as in [12], partially-purified beef heart protein kinase as in [13] and the protein inhibitor of cyclic AMP-dependent protein kinase as in [14]. Plasma membranes were freshly prepared each day using the Percoll procedure [15] and suspended in buffer (pH 7.4) containing KCl (150 mM), Tris (10 mM), MOPS (20 mM) and EGTA (1 mM).

2.2. *Preparations of acetyl CoA carboxylase*

Rat epididymal fat pads were incubated for 30 min in the absence of hormones [5] and extracted in medium (pH 7.4) containing sucrose (0.25 M) Tris (20 mM), EGTA (2 mM) and reduced glutathione (7.5 mM). A

high speed supernatant was prepared by centrifugation at 30 000 × *g* for 30 min at 4°C and acetyl CoA carboxylase precipitated with 35% saturated ammonium sulphate. After dialysis against two changes of buffer (as for membrane resuspension), the preparation was stored at −20°C for up to 4 days and then centrifuged at 10 000 × *g* for 1 min just prior to use. The specific activity of all the preparations used was 50–100 munits/mg protein, where 1 unit enzyme transforms 1 μmol substrate/min at 37°C.

In some experiments more purified preparations were used. These were prepared as above, then centrifuged at 100 000 × *g* for 10 min at 20°C (Beckman Airfuge). After incubation for 30 min at 30°C in the presence of sodium citrate (20 mM), the supernatant was again centrifuged (160 000 × *g* for 20 min at 20°C; Beckman Airfuge) to precipitate the polymerised acetyl CoA carboxylase. The pellet was resuspended in and dialysed against buffer to remove citrate. The specific activity of these preparations was 500–1000 munits/mg protein.

Protein determinations were carried out according to [16] with bovine albumin as standard.

2.3. *Incubation of acetyl CoA carboxylase before assay of activity and phosphorylation*

Incubations were carried out in 25 μl final vol. containing buffer (150 mM KCl, 10 mM Tris, 20 mM MOPS, 1 mM EGTA, 5 mM $MgCl_2$ (pH 7.4)) and as appropriate acetyl CoA carboxylase (100–150 munits/ml), plasma membranes (0.5–1.0 mg protein/ml) and other additions. The mixture was equilibrated at 37°C for 2–3 min prior to initiation of reaction with ATP or [γ-^{32}P]ATP (500–1000 dpm/pmol). After the incubation period, the activity of acetyl CoA carboxylase was assayed either immediately or after a further 20 min in the presence of 20 mM sodium citrate [5]. Alternatively, for incubations performed with [γ-^{32}P]-ATP, reactions were terminated by the addition of 0.5 ml ice-cold trichloroacetic acid (10%, w/v). The ^{32}P-labelled phosphoproteins in the pellet were resolved by SDS–polyacrylamide gel electrophoresis (5–6% gels) and quantified by densitometric scanning of autoradiograms as in [5,15].

3. Results

3.1. *Effects of incubation with plasma membranes and ATP on the activity of acetyl CoA carboxylase*

Incubation of the preparations of acetyl CoA carboxylase with or without ATP for 2 min at 37°C resulted in little change in activity (table 1). However, if incubations were carried out in the presence of both plasma membranes and ATP, the initial activity of acetyl CoA carboxylase increased markedly but the activity after citrate treatment remained unchanged. The plasma membrane preparations contained no detectable acetyl CoA carboxylase activity and had no effect on the activity of added acetyl CoA carboxylase in the absence of ATP.

The time-course of the activation is indicated in fig.1. Maximum activation was observed after 2 min and was constant for at least 10 min thereafter. Similar results were obtained using more purified preparations of acetyl CoA carboxylase (spec. act. 500–1000 munits/mg protein) and at lower concentrations of ATP (0.15–0.2 mM). No activation was observed if Mg^{2+} was not present. However, Ca^{2+} did not appear to be required as the addition of 1 mM EGTA or Ca-EGTA buffer to yield ~10 μM free Ca^{2+} did not affect activation appreciably.

The effect of the plasma membranes was not mimicked by the addition of 5′-AMP or ADP (0.5 mM) or by a heat-treated extract of the membranes (not shown). The extract was prepared by incubating membranes with ATP (1 mM) for 2 min at 37°C followed by heating for 5 min at 100°C and centrifugation at 10 000 × *g* for 5 min.

The effect of plasma membranes in the presence of ATP contrasted markedly with the effects of added cyclic AMP-dependent protein kinase. In the presence of cyclic AMP, incubation with this kinase resulted in a marked inhibition in both the initial activity of acetyl CoA carboxylase and the activity assayed after citrate treatment (table 1, fig.1). Moreover, while the effects of added cyclic AMP-dependent protein kinase were largely inhibited by the addition of a preparation of cyclic AMP-dependent protein kinase inhibitor protein, the addition of this preparation had no effect on the activation of acetyl CoA carboxylase observed with plasma membranes and ATP (table 1). The addition of defatted bovine serum albumin (10 mg/ml) or a mixture of protease inhibitors (pepstatin, antipain and leupeptin each to 0.5 μg/ml) did not affect the increase in acetyl CoA carboxylase activity.

3.2. *Phosphorylation of acetyl CoA carboxylase in the presence of plasma membranes and [γ-^{32}P]ATP*

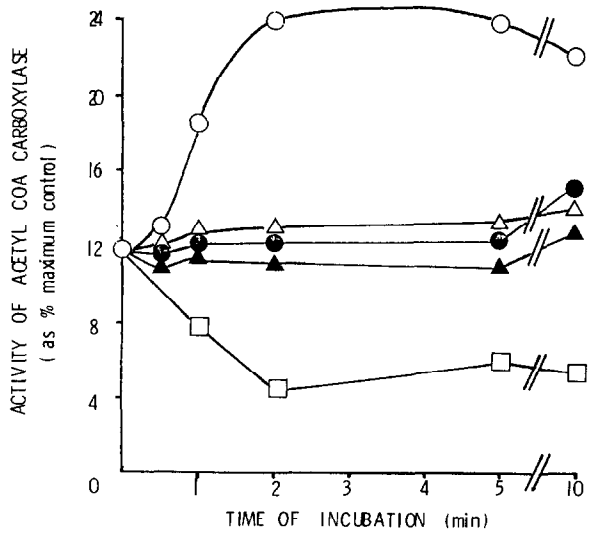
Incubation of either plasma membranes or acetyl CoA carboxylase preparations alone with [γ-^{32}P]ATP

Table 1
Effects of incubation with plasma membranes in the presence of ATP on the activity of acetyl CoA carboxylase

	Activity of acetyl CoA carboxylase (% max control)	
	Initial	After citrate treatment
Non-incubated enzyme (control)	10.5 ± 2.1 (8)	(100)
Plasma membranes	<0.5	<0.5
Enzyme after incubation for 2 min with:		
(i) no additions	10.7 ± 2.3 (8)	98.0 ± 0.8 (4)
(ii) ATP	11.3 ± 3.1 (8)	93.1 ± 3.9 (4)
(iii) plasma membrane preparation	12.3 ± 2.6 (8)	93.6 ± 7.3 (4)
(iv) as (iii) plus ATP	18.4 ± 2.9[a] (8)	92.0 ± 3.4 (4)
(v) as (iv) plus inhibitor protein	18.6 ± 2.0[a] (3)	97.3 ± 4.1 (3)
(vi) ATP plus cyclic AMP + cyclic AMP-dependent protein kinase	4.8 ± 1.0[a] (4)	80.6 ± 2.4[a] (4)
(vii) as (vi) plus inhibitor protein	9.1 ± 2.1 (3)	95.9 ± 6.1 (3)

[a] $P < 0.01$ *vs* appropriate control

Acetyl CoA carboxylase (spec. act. 50–100 munit/mg protein) was incubated with the additions indicated prior to assay immediately (initial activity) or after further 20 min in the presence of 20 mM sodium citrate. Concentrations of additions were as follows: ATP (1 mM); plasma membranes (0.5–1.0 mg protein/ml); cyclic AMP-dependent protein kinase preparation (0.25 mg protein/ml); cyclic AMP (50 μM); cyclic AMP-dependent protein kinase inhibitor protein preparation (0.07 mg protein/ml). Results are expressed as mean ± SEM of the no. independent obs. (using different batches of both plasma membranes and acetyl CoA carboxylase) in parentheses and are expressed as % of activity found in the non-incubated enzyme after citrate treatment (100–150 munit/ml)



resulted in the incorporation of ^{32}P into proteins (fig.2). However, under these conditions none of the major labelled proteins have a subunit M_r on the basis of SDS–polyacrylamide gel electrophoresis corresponding to that of acetyl CoA carboxylase, namely 230 000 [4,8,12]. The phosphorylated proteins associated with the plasma membrane preparation are probably two plasma membrane proteins of subunit M_r ~67 000 and 61 000, found to be phosphorylated in

Fig.1. Time-course of the ATP-dependent activation of acetyl CoA carboxylase in the presence of plasma membranes. Details of incubation and additions as in table 1 for enzyme alone (△) and in the presence of: ATP (●); plasma membranes (▲); plasma membranes + ATP (○); cyclic AMP-dependent protein kinase + ATP + cyclic AMP (□).

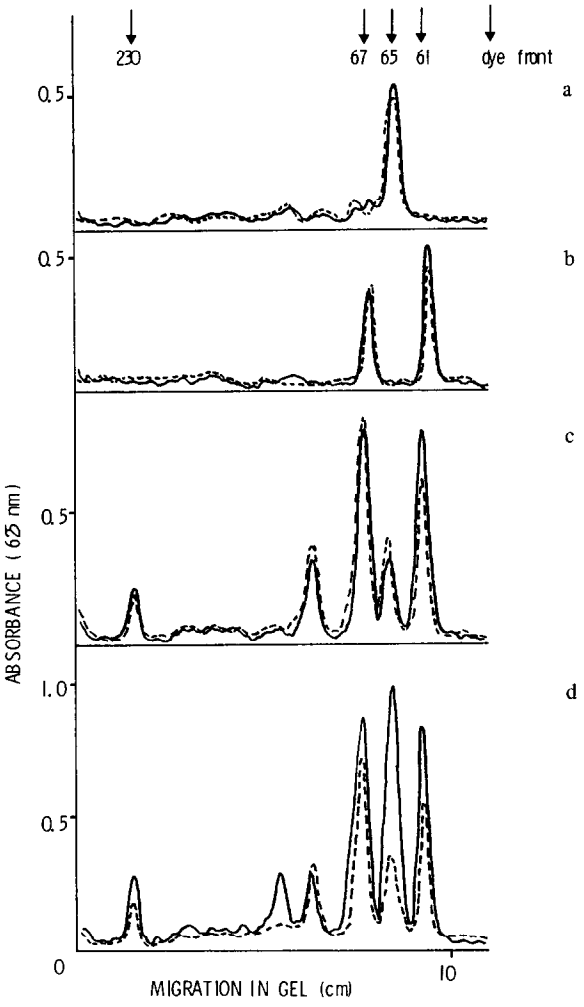


Fig.2. ^{32}P-Labelled phosphoproteins separated by SDS–polyacrylamide gel electrophoresis following incubation of acetyl CoA carboxylase (spec. act. 750 munit/mg protein) with plasma membranes and [γ-^{32}P]ATP. Incubations were carried out as in table 1 except that [γ-^{32}P]ATP (0.15 mM and 500 dpm/pmol) was present and incubations were terminated after 1 min by the addition of trichloroacetic acid. Solid lines are densitometric traces of autoradiographs locating the separated ^{32}P-labelled phosphoproteins following incubations of: (a) acetyl CoA carboxylase; (b) plasma membrane preparation; (c) as (b) + acetyl CoA carboxylase; (d) as (c) + cyclic AMP. Dashed traces show the ^{32}P-labelled phosphoproteins present after incubation under the same conditions + cyclic AMP-dependent protein kinase inhibitor protein. Subunit M_r-values were determined as in [5,15] and are indicated as $\times 10^{-3}$.

intact fat cells [15]. The identity of the phosphorylated protein in the acetyl CoA carboxylase preparations of subunit M_r ~65 000 is unknown but it appears to be a substrate for cyclic AMP-dependent protein kinase (see below) which suggests it may be a fat cell protein we had designated 4A [5].

In contrast, if acetyl CoA carboxylase is incubated with [γ-^{32}P]ATP in the presence of plasma membranes then a substantial increase in the incorporation of ^{32}P into a protein of subunit M_r 230 000 is apparent (fig.2, table 2). The migration of this protein on SDS–polyacrylamide gel electrophoresis was identical to that of fat cell acetyl-CoA carboxylase and the identity was confirmed by demonstrating that all the ^{32}P associated

Table 2
Phosphorylation of acetyl CoA carboxylase in the presence of plasma membranes and [γ-^{32}P]ATP

Other additions	Phosphorylation (as % control value)	
	Enzyme alone	Enzyme + plasma membrane preparation
(i) [γ-^{32}P]ATP	17 ± 3 (9)	(100, control value)
(ii) as (i) + inhibitor protein	17 ± 3 (4)	90 ± 7 (8)
(iii) as (i) + cyclic AMP	42 ± 10 (5)	257 ± 52 (8)
(iv) as (iii) + inhibitor protein	17 ± 1 (4)	152 ± 12 (5)

Concentrations of additions as given in table 1, other details as fig.2 except that acetyl CoA carboxylase preparations of spec. act. 50–100 munit/mg protein were used. Phosphorylation of acetyl CoA carboxylase calculated from the area of the peak representing acetyl CoA carboxylase in the densitometric scans of autoradiographs and expressed as % of that observed in the presence of [γ-^{32}P]ATP and plasma membranes. Results are mean ± SEM for no. obs. in parentheses (each from different batches of both plasma membranes and acetyl CoA carboxylase)

with the band was precipitated with antibody to acetyl-CoA carboxylase as described in [12].

The incorporation of ^{32}P from [γ-^{32}P]ATP into acetyl-CoA carboxylase in the presence of plasma membranes was rapid and quite similar to the time-course of the increase in activity of acetyl-CoA carboxylase. The incorporation of ^{32}P from [γ-^{32}]ATP into acetyl-CoA carboxylase in the presence of plasma membranes may be slightly faster than the time course of the increase in activity of acetyl-CoA carboxylase. Values for the incorporation of ^{32}P were 53 ± 16, 93 ± 7 and 98 ± 4% of the 2 min value at 0.5, 1 and 10 min, respectively (mean ± SEM for 3 obs.); corresponding values for the increase in acetyl-CoA carboxylase activity in parallel experiments were 25 ± 3, 60 ± 4 and 108 ± 6% of the 2 min value at 0.5, 1 and 5 min, respectively (mean ± SEM for 4 obs.). Incorporation was not altered by the presence of the protein inhibitor of cyclic AMP-dependent protein kinase (fig.2, table 2) indicating that the phosphorylation of acetyl-CoA carboxylase in the presence of plasma membranes was not brought about by the catalytic subunit of cyclic AMP-dependent protein kinase. However, if cyclic AMP was added, an increase in phosphorylation of both acetyl-CoA carboxylase and the 65 000 M_r component was observed in the presence of plasma membranes. These increases in phosphorylation were greatly diminished if the inhibitor protein of cyclic AMP-dependent protein kinase was added (fig.2, table 2). The results of these experiments are compatible with the plasma membranes containing cyclic AMP-dependent protein kinase activity being able to phosphorylate these proteins, but only in the presence of added cyclic AMP and in the absence of the inhibitor protein. The presence of 10 μM Ca^{2+} (using Ca-EGTA buffer) or the addition of excess EGTA (1 mM) did not affect the phosphorylation of acetyl-CoA carboxylase in the presence of plasma membranes.

An estimate of the extent of phosphorylation can be made based on the assumption that the maximum specific activity of fat cell acetyl-CoA carboxylase is 4–5 unit/mg protein. On this basis, the observed incorporation in the presence of membranes represents 0.2–0.5 mol phosphate/mol acetyl-CoA carboxylase subunit (M_r 230 000) in the absence of cyclic AMP and 0.5–1.2 mol/mol in the presence of cyclic AMP.

4. Discussion

These results are compatible with the plasma membranes prepared from fat cells containing an ATP-requiring protein kinase which is capable of phosphorylating acetyl-CoA carboxylase and that this phosphorylation is associated with an increase in acetyl-CoA carboxylase activity. This increase may involve polymerisation of the enzyme and could explain why the time course of the increase in activity appeared to lag slightly behind the time course of phosphorylation. The kinase appears to be insensitive to Ca^{2+} at least under the conditions of this study. Moreover, the kinase is quite distinct from cyclic AMP-dependent protein kinase which is also able to phosphorylate the enzyme but in this case, phosphorylation leads to a decrease in the enzyme activity. The change in activity brought about by the membrane-bound kinase is similar to that seen after exposure of fat cells to insulin; the initial activity of acetyl-CoA carboxylase is increased but no change is apparent after citrate treatment. In contrast, phosphorylation by cyclic AMP-dependent protein kinase brings about changes similar to those following incubation of fat cells with adrenaline since both initial activity and that observed after citrate treatment is diminished.

Two other interpretations of our observations must be considered:

(i) The observed increase in activity and phosphorylation could be the result of activation of a kinase associated with the enzyme preparation but which is completely inactive in the absence of some heat labile factor in the plasma membranes. This possibility is difficult to eliminate.

(ii) The increase in activity could be due to limited proteolysis quite independent of the phosphorylation, since ATP-dependent proteolytic activity has been reported [23,24].

However, possibility (ii) seems unlikely for the following reasons: The addition of proteolytic inhibitors and albumin were without effect; there was no alteration in the activity of the enzyme expressed after citrate treatment; there was no evidence of any loss of protein associated with the acetyl-CoA carboxylase subunit separated by SDS–polyacrylamide gel electrophoresis (M_r 230 000) or the appearance of any satelite bands of lower molecular weight. Nevertheless, complete elimination of possibility (ii) must await the demonstration that the increase in activity can be reversed upon dephosphorylation.

It seems reasonable to conclude that insulin may act on acetyl-CoA carboxylase through increasing the activity of the plasma membrane kinase. The fact that the kinase is associated with the cell membrane may mean that no 'second messenger' as such is involved in the effect of insulin on the activity of acetyl-CoA carboxylase. Other studies have demonstrated that increased phosphorylation of a number of other fat cell proteins occurs following exposure of fat cells to insulin. These include 3 intracellular proteins with subunit M_r: ~130 000, which is probably ATP citrate lyase [3,12,17–19]; ~35 000 which may be the ribosomal protein S6 [19–21]; ~22 000 which appears to be a protein related to the phosphoprotein phosphatase inhibitor-1 [19,22]; and in addition a plasma membrane-associated protein of M_r 61 000 [15]. Clearly future studies should be directed towards exploring the extent to which these proteins are also substrates for the plasma membrane-associated kinase. It must also be demonstrated that this kinase phosphorylates the same site on acetyl-CoA carboxylase as that observed to be phosphorylated to a greater extent following exposure of fat cells to insulin. Most importantly, the stimulation of kinase activity by insulin must be demonstrated. These studies are in progress.

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