Regulation of acetyl-CoA carboxylase in rat mammary gland

Effects of incubation with Ca^{2+} , Mg^{2+} and ATP on enzyme activity in tissue extracts

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1. The effect of preincubation of extracts of lactating rat mammary gland with ATP, Mg^{2+} and micromolar concentrations of Ca^{2+} on the activity of acetyl-CoA carboxylase was studied. 2. Both Mg^{2+} and Ca^{2+} activated the enzyme. Activation with Mg^{2+} (5 mM) was larger than that with Ca^{2+} (calculated free Ca^{2+} concentration = 20–50 μM), but the activity decreased after reaching a peak. The activation obtained with Ca^{2+} was stable for up to 180 min. 3. Incubation with Ca^{2+} and Mg^{2+} together resulted in an activation that was slightly higher than that with Mg^{2+} only and was stable (compare the results for Ca^{2+} alone). 4. Preincubation in the absence of Mg^{2+} , but not in the absence of Ca^{2+} , resulted in the impairment of subsequent activation with either Mg^{2+} (when preincubation was with Ca^{2+} alone) or Mg^{2+} plus Ca^{2+} . 5. KF (50 mM) prevented the activation of acetyl-CoA carboxylase by Ca^{2+} and Mg^{2+} . 6. MgATP^{2-} reversed (Mg^{2+} + Ca^{2+})-mediated activation and decreased the activity of acetyl-CoA carboxylase to about 10% of initial activity. Inhibition by ATP was unaffected by addition of cyclic AMP or cyclic AMP-dependent protein kinase inhibitor. 7. ^{32}P was incorporated into acetyl-CoA carboxylase when incubations were carried out in the presence of [γ - ^{32}P]ATP. Subsequent removal of ATP from the incubation medium resulted in rapid loss of ^{32}P from acetyl-CoA carboxylase. 8. It is suggested that extracts of rat mammary gland contain endogenous protein kinase and phosphatase activities that modulate acetyl-CoA carboxylase activity through reversible phosphorylation and dephosphorylation. The phosphatase activity is sensitive to both Mg^{2+} and micromolar concentrations of Ca^{2+} , whereas the kinase does not appear to be cyclic AMP-dependent.

The activity of acetyl-CoA carboxylase (EC 6.4.1.2) is regulated through the action of effector molecules on the enzyme [e.g. citrate (Miller & Levy, 1969), long-chain acyl-CoA (Denton *et al.*, 1977) and CoA (Yeh *et al.*, 1981)], and through covalent modification of the enzyme molecule (i.e. phosphorylation and dephosphorylation) (see Kim, 1979). The purified enzyme from mammary gland has been studied *in vitro* and has been shown to be sensitive to both types of regulation. In particular, Hardie and his co-workers have shown that acetyl-CoA carboxylase purified from lactating rat mammary gland could be phosphorylated *in vitro* by cyclic AMP-dependent protein kinase in the presence of Mg^{2+} ; this phosphorylation was accompanied by deactivation of the enzyme and was reversible by the action of protein phosphatase I

(Hardie & Cohen, 1979; Hardie & Guy, 1980). Therefore the activity of acetyl-CoA carboxylase in mammary gland *in vivo* may be regulated via changes in the phosphorylation state of the enzyme, as previously suggested for the enzyme from liver and adipose tissue (see Kim, 1979; Brownsey *et al.*, 1979). However, although acetyl-CoA carboxylase is thought to play a major regulatory role in the control of lipogenesis in rat mammary gland (see Williamson, 1980; Munday & Williamson, 1981), there is no direct evidence either that hormonal effects, which can be demonstrated on the gland *in vivo* and *in vitro*, can be attributable to changes in the phosphorylation state of the enzyme, or that the gland possesses endogenous protein kinase and phosphatase activities that can modulate acetyl-CoA carboxylase activity.

In extracts of rat adipocytes, endogenous phosphatase activity has been demonstrated; this activity

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required Mg^{2+} and micromolar concentrations of Ca^{2+} (Brownsey *et al.*, 1979). We have performed studies to find out whether the activity of acetyl-CoA carboxylase in extracts of rat mammary gland can be reversibly modulated by phosphorylation and dephosphorylation and to investigate the effects on dephosphorylation of Mg^{2+} and Ca^{2+} . Our results suggest that mammary extracts contain endogenous phosphatase and kinase activities and that Ca^{2+} and Mg^{2+} have different but complementary effects on phosphatase-mediated activation of acetyl-CoA carboxylase. A preliminary account of some of this work has been presented previously (McNeillie & Zammit, 1981).

Materials and methods

Animals

Female Wistar rats weighing 180–200 g were obtained from A. Tuck and Son, Battlesbridge, Essex, U.K. They were mated and the number of pups per litter was restricted to eight at birth. They were fed on Oxoid breeding diet (Oxoid, Basingstoke, Hants., U.K.).

Materials

Acetyl-CoA, ATP and proteolysis inhibitors were obtained from Sigma Chemical Co., Poole, Dorset, U.K. $NaH^{14}CO_3$ and $[\gamma\text{-}^{32}P]ATP$ were obtained from Amersham International, Amersham, Bucks., U.K. Inorganic chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K.

Preparation of homogenates

Fed animals at peak lactation (10–14 days after parturition) were anaesthetized with diethyl ether and the abdominal mammary glands were quickly dissected out and frozen in liquid N_2 . They were subsequently stored frozen at $-30^\circ C$. When required, the glands were powdered in a stainless-steel mortar, cooled in liquid N_2 and 1 g portions were homogenized (for two periods of 10 s with a Polytron tissue disintegrator fitted with probe OD10 and operated at setting 5) in 5 vol. of ice-cold extraction medium containing 0.25 M-sucrose, 20 mM-Tris, 20 mM-Mops (4-morpholinepropanesulphonic acid), 1 mM-dithiothreitol and 2 mM-EGTA, pH 7.4. Albumin (10 mg/ml) was added to the homogenate, which was immediately centrifuged at 10000 g for 60 s in an Eppendorff 5412 centrifuge. A portion (1 ml) of supernatant was applied to a column (1 cm $\times$ 30 cm) of Sephadex G-25 equilibrated with extraction medium and kept at $4^\circ C$. The eluate was used for assay or preincubation within 10 min of the preparation of the homogenate. The dilution of the extracts after this step was assessed by measuring lactate dehydrogenase activity. It was found to be always about 10-fold, so that the final

dilution of the tissue extract was 50-fold and the protein concentration was 0.7–1.0 mg/ml. It was shown to contain no detectable free Ca^{2+} by using the Ca^{2+} -sensitive arsenazo III dye.

Preincubation of tissue extracts

The eluate from the gel-filtration step (extract) was incubated for various periods of time (see the Figures) at $30^\circ C$ in the presence of combinations of the following: 5 mM-magnesium salt (see below), $CaCl_2$ /EGTA mixture to give a calculated free Ca^{2+} concentration of 20–50 μM (see below) and 5 mM-ATP, or with an equivalent volume of extraction medium. In experiments designed to minimize proteolysis, the following inhibitors were also included: phenylmethanesulphonyl fluoride (2 mM); soya-bean trypsin inhibitor, antipain, pepstatin and leupeptin (all at 0.1 mg/ml).

Assay of acetyl-CoA carboxylase activity

Acetyl-CoA carboxylase activity was assayed at $30^\circ C$ as described by Brownsey *et al.* (1979). The assay medium contained 100 mM-Tris/acetate, 0.5 mM-EDTA, 10 mM-magnesium acetate, 1 mM-dithiothreitol, 150 μM -acetyl-CoA, 7.5 mM-ATP, 20 mM-magnesium citrate, 15 mM- $KH^{14}CO_3$ (0.5 Ci/mol) and 10 mg of albumin/ml in a total volume of 0.45 ml contained in stoppered plastic tubes. The final pH was 7.4. The reaction was started by addition of 50 μl of extract. To avoid variation in the composition of the assay medium owing to addition of extracts preincubated with different concentrations of Mg^{2+} , Ca^{2+} and ATP, allowance was made in the preparation of assay medium for each individual reaction performed such that the final conditions of assay were identical irrespective of the previous treatment of extracts. It was ascertained that citrate included in the assay medium did not affect the linearity of the activity measured. Reactions were linear for at least 60 s. Initial activities were routinely measured by stopping the reaction at intervals during the first 30 s. Radioactivity incorporated into malonyl-CoA was measured as described previously (Brownsey *et al.*, 1979), with a Packard scintillation counter.

Control of free Ca^{2+} concentration

The concentration of free Ca^{2+} in preincubation medium was controlled as described previously (Denton *et al.*, 1978) by mixing appropriate volumes of stock solutions of 1 M- $CaCl_2$ and 1 M-EGTA. The total concentration of $CaCl_2$ was 12 mM and the calculated concentration of free Ca^{2+} (after allowance was made for EGTA added in the extract) was 20–50 μM (see the legend to Fig. 1).

Incorporation of ^{32}P into acetyl-CoA carboxylase

Tissue extracts (1–2 mg of protein/ml) were

incubated as described above in the presence of 5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (250 $\mu\text{Ci/ml}$) and 5 mM-magnesium acetate. Before measuring the incorporation of ^{32}P into acetyl-CoA carboxylase, samples (containing about 200 μg of protein) were first treated in one of two ways to prevent post-sampling changes in phosphorylation status of the enzyme. Either KF (final concn. 50 mM) and glucose (10 mM) plus hexokinase (10 units/ml) were added and the samples immediately cooled and kept at 4°C (Expt. 1, Table 1) or KF and sodium dodecyl sulphate (final concn. 1%, w/v) were added and the samples boiled for 5 min (Expts. 2 and 3, Table 1). Both treatments were preceded by addition of antipain, pepstatin and leupeptin (each at a final concn. of 0.1 mg/ml).

Each sample was then divided into two equal parts. One half was disaggregated and subjected to polyacrylamide-gel electrophoresis on 4%-acrylamide gel slabs by using the solutions and conditions recommended by Laemmli (1970). Acetyl-CoA carboxylase was immunoprecipitated from the other half by using the γ -globulin fraction and the procedure described by Brownsey *et al.* (1979). For samples that had been treated with sodium dodecyl sulphate, Triton X-100 was added (final concn. of 3%, v/v) before the addition of the antibody fraction. The immunoprecipitates were disaggregated and run on 4%-acrylamide gels as above. Gels were fixed and stained with Coomassie Brilliant Blue R. The ^{32}P content of the acetyl-CoA carboxylase band in stained gels was determined by one of two methods. In the first, the band was excised directly, dissolved (Tishler & Epstein, 1968), and the radioactivity of the resulting solution was measured with a liquid-scintillation counter. In the second method, gels were dried and autoradiographed; the relative ^{32}P content of the acetyl-CoA carboxylase band was determined by quantitative densitometry of the autoradiogram by using a Gilford 250 spectrophotometer fitted with a gel-scanning attachment.

Expression of results

Activity of acetyl-CoA carboxylase is expressed as a percentage of that measured at the beginning of preincubation (i.e. at zero time). Unless otherwise indicated, the results presented in the Figures are representative of those obtained from three to five similar experiments.

Results

Preliminary experiments established that there was no significant change in activity (about 1.5 $\mu\text{mol/min per g}$ fresh wt. at 30°C) during the time taken to prepare the eluate of the Sephadex G-25 column. Incubation of extracts at 30°C without further additions (i.e. in the presence of 2 mM-

EGTA) for up to 180 min resulted in no significant change in activity (Fig. 1).

Effect of preincubation with Mg^{2+} and/or Ca^{2+}

Preincubation of extracts with Mg^{2+} (5 mM-magnesium salt; see below) resulted in a rapid increase in activity of acetyl-CoA carboxylase (Fig. 1), reaching a maximum after 40–50 min. Thereafter there was a more gradual decrease in activity. This decrease, although always observed, was variable. These effects did not depend on the identity of the anion of the magnesium salt used; the acetate, chloride and sulphate salts gave similar results.

Incubation of extracts with Ca^{2+} -EGTA (calculated free Ca^{2+} concentration 20–50 μM ; see the legend to Fig. 1) also resulted in the activation of acetyl-CoA carboxylase. However, activation with Ca^{2+} was slower than that induced by preincubation with Mg^{2+} , and the maximal activity achieved with Ca^{2+} was only about half that observed in the presence of Mg^{2+} (Fig. 1). Moreover, when maximal activity was obtained it was stable for the rest of the incubation period (up to 180 min).

Fig. 1. Activation of acetyl-CoA carboxylase by Mg^{2+} and Ca^{2+}

Extracts of rat mammary gland were incubated at 30°C in the absence of added metal ions (●) and after addition of 5 mM-magnesium acetate (■), 5 mM-magnesium acetate, 12 mM- CaCl_2 and 10 mM-EGTA (○) or 12 mM- CaCl_2 and 10 mM-EGTA (□). The calculated free Ca^{2+} concentrations were 20–50 μM when allowance was made for EGTA already present in the extraction medium.

Incubation of extracts with Mg^{2+} plus Ca^{2+} resulted in rapid activation of acetyl-CoA carboxylase similar to that observed in the presence of Mg^{2+} alone (Fig. 1), but the maximal activity was slightly higher and was stable (cf. Ca^{2+} alone). These results suggested that Mg^{2+} and Ca^{2+} had different and complementary roles in the activation of acetyl-CoA carboxylase in mammary-gland extracts. The presence of Mg^{2+} was essential for maximal activation, whereas Ca^{2+} was required for stabilization of this activation.

When Ca^{2+} and Mg^{2+} were added after a period of preincubation in the absence of both metal ions (i.e. in the presence of 2 mM-EGTA), the degree of activation became progressively smaller the longer preincubation was continued (results not shown). This suggested that in the absence of Mg^{2+} and/or Ca^{2+} a component of the acetyl-CoA carboxylase-activating system was lost. Sequential addition of Mg^{2+} and Ca^{2+} to the preincubation medium indicated that incubation of the extracts in the absence of Mg^{2+} , but not of Ca^{2+} , was the cause of the loss of a component of the activation system of acetyl-CoA carboxylase. Thus incubation of extracts with Ca^{2+} alone for timed intervals before addition of Mg^{2+} resulted in a progressively smaller degree of activation the longer the preincubation without Mg^{2+} was continued (Fig. 2). However,

Fig. 2. Effect of preincubation in the absence of Ca^{2+} or Mg^{2+} on subsequent activation by metal ions

Extracts were preincubated in the presence of Mg^{2+} (○) or Ca^{2+} (□). After 30 and 70 min Mg^{2+} was added to a portion of the incubation mixture containing Ca^{2+} (■ and ▲) and at 75 min Ca^{2+} was added to a portion of the incubation mixture containing Mg^{2+} (●).

when extracts were preincubated with Mg^{2+} alone, the ability of subsequent addition of Ca^{2+} in stabilizing the activation achieved after 60 min of preincubation was unimpaired (Fig. 2).

Acetyl-CoA carboxylase may be activated by limited proteolysis (Swanson *et al.*, 1967). To investigate whether bivalent cation-activated proteinases, endogenous to the mammary preparation, were responsible for the activity changes described above, we repeated the above experiments in the presence of a mixture of proteolysis inhibitors (see the Materials and methods section). Inclusion of the inhibitors did not alter the pattern of the results obtained (results not given). Furthermore, no evidence of degradation of the acetyl-CoA carboxylase subunit (mol.wt. 240 000) was apparent when quantitative densitometry was performed on Coomassie Blue-stained polyacrylamide gels (results not shown) of samples withdrawn at intervals throughout incubations of mammary-tissue extract with Ca^{2+} and Mg^{2+} . The sensitivity of this method, however, was not expected to be high, since, even when electrophoresed under optimum conditions for separating acetyl-CoA carboxylase and fatty acid synthase protomers, the former appear as a small shoulder on the trailing edge of the peak of the latter in densitometric traces.

Activation by citrate before and after preincubation with Mg^{2+} and Ca^{2+}

Incubation (30 min at 30°C) of tissue extracts with 20 mM-citrate before and after preincubation (60 min) with Mg^{2+} and Ca^{2+} resulted in similar maximal activities. However, because after treatment with Mg^{2+} plus Ca^{2+} the enzyme was already partially activated, the degree of activation by citrate after incubation with Mg^{2+} and Ca^{2+} was smaller than that achieved before activation by metal ions (Fig. 3).

Effect of incubation with KF and ATP

Incubation of extracts with 50 mM-KF resulted in no change of acetyl-CoA carboxylase activity, nor did KF added directly to the assay medium affect enzyme activity. However, 50 mM-KF prevented activation by Ca^{2+} and Mg^{2+} (results not given). Substitution of KCl for KF had no effect on activation by Ca^{2+} and Mg^{2+} . These observations suggested that activation of acetyl-CoA carboxylase was due to phosphatase-mediated dephosphorylation of the enzyme.

Incubation of extracts with 5 mM-ATP and 5 mM-magnesium acetate resulted in a rapid inactivation of acetyl-CoA carboxylase to less than 10% of the initial activity. The ATP-induced inactivation was not sensitive to addition of cyclic AMP or of cyclic AMP-dependent protein kinase inhibitor (results not shown). Incubation of extracts with $MgATP^{2-}$ after

Fig. 3. Effect of preincubation with citrate on acetyl-CoA carboxylase activity before and after activation by Mg^{2+} and Ca^{2+}

Extracts were incubated with 20 mM-potassium citrate (▲) added at zero time or with 20 mM-magnesium citrate (■) added after preincubation for 60 min with Mg^{2+} and Ca^{2+} (●).

Fig. 4. Effect of incubation with ATP on the activity of acetyl-CoA carboxylase

Extracts were incubated with 5 mM-ATP and 5 mM-magnesium acetate (●). After 10 min portions were taken and mixed with 10 mM-glucose, hexokinase (10 units/ml) and either 10 mM-EDTA plus 50 mM-KF (□) or 12 mM- $CaCl_2$ plus 10 mM-EGTA (○).

Table 1. Relative incorporation of ^{32}P into acetyl-CoA carboxylase during incubation of tissue extracts with $[\gamma\text{-}^{32}P]ATP$

Incubations were carried out as described in the Materials and methods section. The samples designated '30 + additions' in the Table were obtained as follows. After 10 min of incubation, a sample was withdrawn and placed in a second tube at 30°C; to this was immediately added glucose (final concn. 10 mM), hexokinase (final concn. 10 units/ml) and a mixture of $CaCl_2$ and EGTA (see the legend to Fig. 1); incubation was then continued for a further 20 min. The methods used to stop incubations at the times indicated and to process samples for the determination of ^{32}P in acetyl-CoA carboxylase are described in the Materials and methods section. The results from three separate experiments are given. Direct measurement of ^{32}P incorporation into acetyl-CoA carboxylase was used in Expt. 1: 100% incorporation was equivalent to 0.18 nmol of ^{32}P /ml of incubation. In Expts. 2 and 3, relative ^{32}P incorporation into acetyl-CoA carboxylase was assessed by densitometric scanning of autoradiograms. Values are percentage ^{32}P incorporation relative to that after 30 min. Abbreviation used: n.t., not tested.

Time of incubation (min)	^{32}P incorporation into acetyl-CoA carboxylase (%)		
	Expt. 1	Expt. 2	Expt. 3
5	38	63	32
10	76	88	72
20	89	121	n.t.
30	100	100	100
30 + additions	48	36	39

activation with Mg^{2+} and Ca^{2+} resulted in reversal of this activation (results not shown). Depletion of ATP (with glucose and hexokinase; see the Materials and methods section) after inactivation of acetyl-CoA carboxylase resulted in immediate and rapid re-activation of the enzyme (Fig. 4).

Incorporation of ^{32}P into acetyl-CoA carboxylase

Incubation of tissue extracts with $[\gamma\text{-}^{32}P]ATP$ and magnesium acetate (each at 5 mM) resulted in rapid incorporation of ^{32}P into acetyl-CoA carboxylase. Subsequent removal of ATP from the incubation medium (see the Materials and methods section) and addition of Ca^{2+} resulted in rapid loss of ^{32}P from the enzyme (Table 1).

Discussion

The experiments reported in the present paper were performed on tissue homogenates from which small molecules were excluded by gel filtration. This enabled the study of the effects of Mg^{2+} , Ca^{2+} and

ATP on enzyme activity in the absence of interference from effectors of acetyl-CoA carboxylase activity (e.g. citrate, CoA).

The pattern of activation of acetyl-CoA carboxylase by Ca^{2+} or Mg^{2+} individually was different in several respects from that obtained in the presence of both cations, suggesting that Mg^{2+} and Ca^{2+} have different but complementary roles in activation of the enzyme. Brownsey *et al.* (1979) also reported that both Ca^{2+} and Mg^{2+} were required for activation of acetyl-CoA carboxylase in adipose-tissue extracts by endogenous phosphatase activity. The present results suggest that, whereas Mg^{2+} is required for maximal activation, Ca^{2+} is required to stabilize this activation. The observation that micromolar concentrations of Ca^{2+} are sufficient for the latter effect suggests that it may be of physiological importance. However, from the present results it is not possible to suggest mechanisms for the stabilizing effect of the addition of Ca^{2+} on the activity of acetyl-CoA carboxylase after preincubation with Mg^{2+} . Since the extracts contained no ATP, the decline in activity observed during the later stages of incubation with Mg^{2+} could not have been due to re-phosphorylation of the enzyme. One possibility is that after dephosphorylation (and subsequent polymerization) the enzyme tended to depolymerize spontaneously under the conditions of incubation. The effect of Ca^{2+} could then be due to stabilization of the polymeric form of the enzyme.

Both the experimental approaches used to investigate the possible contribution of proteolysis to the observed changes in acetyl-CoA carboxylase activity (use of proteinase inhibitors and quantification of the enzyme peak in sodium dodecyl sulphate/polyacrylamide gels) indicated that proteolytic degradation of acetyl-CoA carboxylase did not contribute significantly to its activation/deactivation behaviour under the experimental conditions.

Several observations suggested that the activation of the enzyme in the presence of Ca^{2+} and/or Mg^{2+} was due to dephosphorylation. Fluoride prevented the activation, which could be completely reversed on addition of ATP. Incubation of extracts with MgATP^{2-} resulted in a rapid and almost total loss of activity. Experiments carried out in parallel in which $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was used indicated that ATP-induced inhibition was accompanied by incorporation of ^{32}P into acetyl-CoA carboxylase, and sub-

sequent activation on removal of ATP was accompanied by a decrease in the ^{32}P content of the enzyme. Therefore it is suggested that mammary-gland extracts contain endogenous phosphatase and kinase activities, which determine the degree of phosphorylation of acetyl-CoA carboxylase. The identification of these activities requires further studies. However, the observation that ATP-induced inhibition of acetyl-CoA carboxylase activity was unaffected by addition of cyclic AMP or of the inhibitor of cyclic AMP-dependent protein kinase suggests that the endogenous kinase activity was not cyclic AMP-dependent.

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