Protein phosphorylation in rat mammary acini and in cytosol preparations *in vitro*

Phosphorylation of acetyl-CoA carboxylase is unaffected by cyclic AMP

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Phosphorylation of soluble proteins in rat mammary acinar cells was investigated. When phosphorylation proceeded in intact cells, in the presence of [^{32}P]P_i, the major non-casein phosphoproteins, including acetyl-CoA carboxylase, were unresponsive to incubation conditions that caused major increases in the intracellular concentration of cyclic AMP. The overall ^{32}P specific radioactivity (c.p.m./ μg of protein) of acetyl-CoA carboxylase, assessed after affinity purification of the enzyme with avidin–Sephadex, was unchanged by incubation under such conditions. Furthermore, the distribution of ^{32}P among tryptic phosphopeptides of the enzyme, resolved by reversed-phase h.p.l.c., was not altered by cyclic AMP-increasing treatments of the acinar cells. When cytosol fractions were incubated with [γ - ^{32}P]ATP, some phosphoproteins responded to the addition of micromolar concentrations of dibutyl cyclic AMP or cyclic AMP by undergoing an enhancement of phosphate incorporation. In these experiments *in vitro*, protein phosphatase activity did not make a major contribution to the net phosphorylation of individual phosphoproteins, and acetyl-CoA carboxylase was not prominent among the phosphoproteins identified after short (< 1 min) incubations of cytosols with [γ - ^{32}P]ATP. The resistance of protein phosphorylation to variations in the cyclic AMP concentration in intact mammary epithelial cells, demonstrated by this work, is one of several mechanisms that ensure the pleiotropic refractoriness of those cells to agents which normally cause a stimulation of adenylate cyclase activity in hormone-sensitive cells.

INTRODUCTION

Conflicting results have been reported from experiments probing the ability of mammary tissue in the lactating rat to respond metabolically to catecholamine hormones, or to cyclic AMP, the second messenger through which β -adrenergic stimuli are coupled to intracellular enzymic responses. The expected result of adding cyclic AMP to a highly lipogenic tissue, or to challenging it with β -adrenergic agonists, is that the rate of lipogenesis would be diminished. This result has frequently been recorded, for example in adipose tissue and adipocytes (see Saggerson, 1980). In the liver of the adult male rat, α - rather than β -receptors are predominantly involved in mediation of the adrenergic response (Sherline *et al.*, 1972; Exton, 1980): glucagon is the principal stimulatory hormone of adenylate cyclase, and this is markedly anti-lipogenic in the intact tissue and in isolated hepatocytes (Cook *et al.*, 1977; Geelen *et al.*, 1978; Holland *et al.*, 1984). The inhibition of lipogenesis after challenge of hepatocytes with glucagon and of adipocytes by either glucagon or β -adrenergic agonists is accomplished through the cyclic AMP-mediated phosphorylation and consequent inhibition of the enzyme acetyl-CoA carboxylase (EC 6.4.1.2; Holland *et al.*, 1984, 1985). In contrast, rat mammary acini and tissue-slice preparations when exposed to adrenaline or glucagon were found not to respond with any inhibition of lipogenesis (Williamson *et al.*, 1983; Plucinski & Baldwin, 1982), although injection of adrenaline into the unconscious lactating rat has been reported by Bussmann

et al. (1984) to result in a 50% inhibition of lipogenesis from glucose in mammary tissue. Glucagon receptors are absent from rat mammary epithelial cells (Robson *et al.*, 1984), but β -adrenergic receptors are abundant (Clegg & Mullaney, 1985; Lavandro *et al.*, 1985) and are competently coupled to adenylate cyclase in cell membrane preparations derived from mammary tissue (Bar, 1973) and rat mammary acini (Ladha *et al.*, 1985). Nevertheless, their occupation by physiological agonists in intact cellular preparations results in little or no increase in the intracellular content of cyclic AMP (Clegg & Mullaney, 1985), presumably as a result of the capacity and kinetics of the phosphodiesterases (characterized by Mullaney & Clegg, 1984) that hydrolyse this second messenger. Substantial increases in intracellular cyclic AMP do, however, result when mammary acini are treated with β -adrenergic agonists in the presence of the phosphodiesterase inhibitor Ro 7-2956, or with forskolin (Clegg & Mullaney, 1985). It has been established that cyclic AMP-dependent protein kinase is present in rat mammary tissue (Burchell *et al.*, 1978; Munday & Hardie, 1984) and is capable of phosphorylating and inactivating purified acetyl-CoA carboxylase, itself prepared from rat mammary tissue (Hardie & Guy, 1980). In spite of this, those treatments of acini that increase cyclic AMP up to 20-fold were ineffective in inhibiting lipogenesis in acini from normally fed lactating rats (Clegg *et al.*, 1986).

The work described here was undertaken in the light of these paradoxical findings. Our aim was firstly to make a general assessment of the responses of major non-casein phosphoproteins in mammary epithelial cells to increased

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intracellular concentrations of cyclic AMP. Secondly, we proposed to compare these phosphoproteins with those identifiable in rat mammary cytosols incubated *in vitro* with [γ - 32 P]ATP and to examine their responses to added cyclic AMP and to other potential effectors of protein kinases and phosphatases. A third objective was to investigate in greater detail in these systems the effects of cyclic AMP on the phosphorylation of acetyl-CoA carboxylase and on the distribution of phosphate groups among the tryptic phosphopeptides of this enzyme. The overall purpose of these investigations was to attempt to describe the extent of the apparent metabolic 'insulation' of rat mammary epithelial cells against changes in intracellular cyclic AMP, and to clarify the mechanism(s) by which this is achieved.

EXPERIMENTAL

Chemicals and materials

Sodium [32 P]phosphate (carrier-free; 10mCi/ml in dilute HCl) was from Amersham International. All other chemicals and materials used in this work were obtained from the sources previously given (Robson *et al.*, 1984; Clegg & Mullaney, 1985; Holland *et al.*, 1985; Clegg *et al.*, 1986).

Animals

The source and maintenance conditions of the lactating female Wistar rats used in this work have been described (Robson *et al.*, 1984; Clegg & Mullaney, 1985). All animals were allowed access to food and water *ad libitum*. Lactating females between 10 and 14 days *post partum* were used for all experiments.

32 P incorporation into mammary acinar cells incubated *in vitro*

Acini were prepared from mammary tissue by the method of Robson *et al.* (1984). They were incubated at a concentration of 25–40 mg wet wt./ml (final volume 5 ml) in 32 P-containing (0.2–0.5 mCi/ml) Krebs bicarbonate-buffered Ringer (Krebs & Henseleit, 1932) for 75 min, exactly as described previously for adipocytes (Holland *et al.*, 1985), with the additional inclusion of 2% (w/v) Ficoll as well as 5 mM-glucose and 4% (w/v) bovine serum albumin in the incubation medium. When effectors of the cyclic AMP system were present (as indicated in the text), they were added after 60 min of incubation. At the end of the incubation, the contents of each flask were transferred to an ice-cold centrifuge tube, and acini isolated by centrifugation for 15 s at 1500 *g*. After the cell-free supernatant was discarded, 5 ml of ice-cold buffered solution containing 250 mM-sucrose, 100 mM-KF, 20 mM-Tris/HCl, 2 mM-EGTA, 2 mM-EDTA and 7.5 mM-GSH, pH 7.4, was added to the pelleted acini, and the tube contents were homogenized at 0 °C with a Polytron homogenizer fitted with a PTA 10S probe, operating at 20000 rev./min for 20 s. A post-microsomal-supernatant fraction was prepared by centrifuging the resulting homogenate at 105000 *g* for 45 min at 0 °C. Phosphoproteins in this fraction were operationally designated as cytosol proteins.

[32 P]Phosphorylation of soluble proteins in a cytosol fraction from mammary acinar cells

After their isolation, by low-speed centrifugation, from the final wash medium of 2% (w/v) Ficoll and

5 mM-glucose in Krebs bicarbonate-buffered Ringer, acini were washed twice at 0 °C in a buffer (pH 7.4) containing 250 mM-sucrose, 100 mM-KCl and 10 mM-Tris/HCl, then homogenized in 2.5 vol. of this buffer with a Polytron homogenizer fitted with a PTA 10S probe operating for 20 s at 20000 rev./min. A post-microsomal supernatant was prepared as described above. The protein concentration of this fraction was adjusted to 10 mg/ml with the above buffer. Samples (5 μ l) were dispensed into incubations containing 1.25 μ mol of β -glycerophosphate/KOH, pH 7.4, 0.5 μ mol of MgCl₂, 0.05 μ mol of dithiothreitol and 0.1 μ mol of EGTA, as indicated in the text, at 37 °C. Phosphorylation was initiated by the addition of 3 nmol (10 μ Ci) of [γ - 32 P]ATP: the final incubation volume was 50 μ l. 32 P incorporation was stopped (usually after 30 s) by the addition of 100 μ l ice-cold 20% (w/v) trichloroacetic acid. Acid-insoluble material was collected by centrifugation for 2 min at 10000 *g*, and washed twice by resuspension in ice-cold 10% trichloroacetic acid and centrifugation, then once in aq. 90% (v/v) acetone.

Affinity isolation of acetyl-CoA carboxylase with avidin–Sepharose

This was achieved by the method of Witters *et al.* (1983) as described in Holland *et al.* (1985). The preparation of avidin–Sepharose was as detailed in Clegg *et al.* (1986).

Other methods

Protein was determined by the method of Bradford (1976). Disaggregation of trichloroacetic acid- and acetone-washed protein samples in SDS-containing buffer, and their electrophoresis in polyacrylamide gels, were by the Laemmli procedure (Laemmli & Favre, 1973). Caseins were prepared from rat milk by the methods we have described previously (West & Clegg, 1983): antibodies against each of the three major caseins and against a mixture of equal parts (by weight) of all three were raised in sheep, as was antibody against acetyl-CoA carboxylase (Clegg *et al.*, 1986).

The preparation and analysis by h.p.l.c. of limit tryptic digests of acetyl-CoA carboxylase was carried out as described by Holland *et al.* (1985), except that a C₈ (MOS Hypersil) rather than a C₁₈ reversed-phase column was used.

RESULTS

Phosphorylation of cytosol proteins in isolated acini incubated with [32 P]P_i

Under the conditions of radioactive labelling described in the Experimental section, the predominant [32 P]phosphoproteins in mammary acini cytosols were of M_r 42000 and 27000. A family of proteins of M_r between 23000 and 25000 were also highly labelled (Fig. 1). In further experiments (results not shown), these were all specifically precipitated by a mixture of antisera against the three major caseins of rat milk (M_r 42000, 27000 and 23000; West & Clegg, 1983). The additional phosphoproteins of M_r between 23000 and 25000 are probably isoforms of the M_r -23000 casein; minor components of similar apparent M_r appear in Coomassie-Blue-stained SDS/polyacrylamide-gel-electrophoretic analyses of this smallest rat casein as prepared from rat milk (Kaetzel &

Fig. 1. Soluble phosphoproteins of rat mammary acini

Samples were run on Laemmli 8%-polyacrylamide/SDS gels as follows: (1), rat α -casein; (2), rat β -casein; (3), rat γ -casein; (4), rat mammary acinar-cell cytosol fraction prepared as described in the Experimental section (note the densely stained band of bovine serum albumin); (5) as (4), after incubation of acini with [32 P] P_i . Tracks (1)–(4) are of Coomassie-Blue-stained gels; track (5) is an autoradiograph.

Ray, 1984; see also Fig. 1); evidently they are more highly phosphorylated than the major M_r -23000 form. In the intact mammary epithelial cell, caseins are confined to the lumen of Golgi and related vesicles. It is not unexpected that the relatively harsh homogenization procedures used to obtain the cytosols characterized here from mammary acini should have disrupted the Golgi vesicles and released their contents into the cytosol fraction. Much of the released casein, initially in micellar form, would have been rendered non-sedimentable by the sequestration of Ca^{2+} achieved by the Ca^{2+} -chelating agents present in the homogenization buffer. This accounts for the presence of caseins in these cytosol fractions.

In addition to caseins, major phosphoproteins of M_r 240000, 116000, 100000 and 57000 were present (Fig. 1). The M_r -240000 component was identified as acetyl-CoA carboxylase on the basis of its subunit M_r (Hardie & Guy, 1980), binding to avidin–Sephadex (see below) and precipitation with specific anti-(acetyl-CoA carboxylase) antibody, as in the report by McNeillie *et al.* (1981). Tentative identification of the M_r -116000 and -100000 components as ATP citrate lyase (EC 4.1.3.8) and glycogen phosphorylase (EC 2.4.1.1) respectively was made initially on the basis of subunit M_r alone. The identity of the M_r -57000 subunit is unknown. Coomassie-Blue-stained SDS/polyacrylamide-gel separations of these acini cytosol fractions revealed that the putative ATP citrate lyase and acetyl-CoA carboxylase subunits were the most abundant of the proteins of high M_r (> 80000). Overall, however, bovine serum albumin carried over from the incubation medium was their major protein component. This precluded the use of protein concentration as an index of cytosol concentration and thus necessitated the quantitative expression of protein-phosphorylation data on other bases for comparison between samples (see below).

Treatment of acini with isoprenaline in the presence of Ro 7-2956 and with forskolin increases the intracellular concentration of cyclic AMP by 2048% and 971% respectively (Clegg & Mullaney, 1985). The effect of these treatments on the extent of phosphorylation of the major non-casein phosphoproteins, relative to paired control incubations, was examined. Although cyclic AMP has been shown to cause a small increase in the rate of phosphorylation of caseins in an intact Golgi-vesicle system *in vitro* (West & Clegg, 1980), it is well established that activity of the protein kinase responsible for casein phosphorylation in the intact cell is independent of cyclic AMP. Therefore the incorporation of 32 P into the phosphoproteins in question was expressed relative to that into the M_r -42000 casein in the same gel track, the latter acting in this instance as an internal standard. Alternatively, phosphorylation was expressed per unit of lactate dehydrogenase (EC 1.1.1.27) activity in the 32 P-labelled cytosol fractions before disaggregation in SDS-containing buffer; Table 1 shows results expressed

Table 1. Phosphorylation of selected proteins in mammary cytosol is not enhanced by incubation in the presence of dibutyl cyclic AMP or of agents that increase intracellular cyclic AMP

Acini were incubated in the presence of [32 P] P_i for 75 min as described in the Experimental section; agents were added after 60 min, with control incubations receiving no additions. Acini were harvested, and cytosol fractions prepared. Phosphoproteins were separated by SDS/polyacrylamide-gel electrophoresis, detected by radioautography and quantified by excision from the dried gel and radioactivity counting. 32 P radioactivity of each band was expressed per unit of lactate dehydrogenase activity in the corresponding cytosol fraction, to normalize differences in concentration among these samples. Normalized values were then compared between control and experimental samples and expressed as a percentage of the former. The radioactivity incorporated into the phosphoprotein tabulated was in the approximate proportion (in descending order of M_r) 2:2:1:5:5. Values given are means $\pm$ S.E.M. for five determinations.

Phosphoprotein		32 P incorporation relative to control incubation (%)		
$10^{-3} \times M_r$	Identity	1mM-Dibutyl cyclic AMP	1 μ M-Isoprenaline plus 1mM-Ro 7-2956	100 μ M-Forskolin
240	Acetyl-CoA carboxylase	94.7 $\pm$ 6.6	101.0 $\pm$ 2.6	101.2 $\pm$ 4.7
116	ATP citrate lyase	100.2 $\pm$ 4.8	108.8 $\pm$ 9.9	100.1 $\pm$ 7.6
55	Unknown	104.0 $\pm$ 8.2	96.3 $\pm$ 8.1	96.5 $\pm$ 7.4
42	Casein	108.1 $\pm$ 12.2	112.9 $\pm$ 14.7	102.3 $\pm$ 13.3
27	Casein	89.9 $\pm$ 11.1	107.4 $\pm$ 5.2	94.8 $\pm$ 9.1

Fig. 2. Phosphorylation of mammary acinar proteins during incubation of a cytosol fraction with [γ - 32 P]ATP

Cytosol fractions (50 μ g of protein) were incubated with [γ - 32 P]ATP and analysed on 8%-polyacrylamide/SDS gels as described in the Experimental section. Panel (a) shows a typical autoradiograph from a sample incubated with 1 μ M-dibutyryl cyclic AMP. In panel (b) phosphate incorporation into the phosphoprotein of M_r 57300 is shown as a function of time in a single experiment, representative of six that have been performed. After incubation for 30 s in the absence of effectors of phosphorylation state ($\bullet$), 1 mM-sodium orthovanadate (Δ), 100 mM-KF ($\blacktriangle$) or 0.6 mM non-radioactive ATP ($\circ$) was added, and incubation was continued for a further 30 s; a control incubation ($\bullet$) received no additions. The 32 P content of the phosphoprotein of M_r 57300 was quantified as described in the legend to Table 1.

in this way. They indicate that neither experimental treatment resulted in an enhancement of the steady-state 32 P incorporation into the proteins examined. The same conclusion emerged when cytosol protein phosphorylation was quantified relative to casein, as described

above (results not shown). Although these quantitative analyses were not applied to other mammary cytosol phosphoproteins, inspection and comparison of control and experimental autoradiograms did not reveal any components characterized by a markedly cyclic AMP-dependent phosphorylation.

Phosphorylation of proteins in cytosol fractions prepared from mammary acini, incubated with [γ - 32 P]ATP

Several cytosolic proteins rapidly became phosphorylated at 37 $^{\circ}$ C. Fig. 2(a) shows an autoradiogram of these proteins, separated by SDS/polyacrylamide-gel electrophoresis after incubation of cytosol fraction with [γ - 32 P]ATP *in vitro* (conditions described in the Experimental section). The accompanying graph (Fig. 2b) is the time course of incorporation of 32 P into one of the most intensely labelled of these phosphoproteins, having an apparent subunit M_r of 57300 (average of ten determinations). Three other phosphoproteins, of M_r 26000, 47000 and 100000 [indicated, together with the M_r -57300 component, in Fig. 2(a) by arrows and M_r estimates which are averages for between seven and ten determinations], were also chosen for detailed examination; they behaved in a qualitatively similar manner, having an approximately linear net rate of phosphate incorporation for the first 1 min of incubation (results not shown). In subsequent experiments, 32 P incorporation after 30 s was therefore measured to determine the net rates of phosphorylation of these phosphoproteins. The effects of adding excess non-radioactive ATP or of inhibitors of protein phosphatase activity (fluoride, orthovanadate), in addition to the β -glycerophosphate already present, are shown in Fig. 2(b). Evidently little or no protein phosphatase activity was expressed under these conditions, and thus the measured rates of protein phosphorylation reflect the rates of phosphotransferase reactions catalysed by protein kinase(s) acting on the individual proteins examined.

The contribution of Ca^{2+} -dependent and cyclic AMP-dependent protein kinases towards phosphorylation of the four selected phosphoproteins was examined. When cytosol fractions were incubated in the presence of the endogenous concentration of free Ca^{2+} without EGTA, a markedly diminished background 32 P incorporation resulted throughout the SDS/polyacrylamide-gel separations when comparison was made with Ca^{2+} -free incubations (i.e. in the presence of 2 mM-EGTA). This hampered direct comparison between individual components when electrophoretic separations from incubations with and without EGTA were examined. This difficulty was circumvented by choosing a reference phosphoprotein (the M_r -57300 component) that was common to both conditions and was unaffected (as judged by inspection) by the presence or absence of Ca^{2+} . The 32 P content of other components in the same SDS/polyacrylamide gel track was then expressed relative to it. When results were analysed in this way, the phosphorylation of only one component (M_r 100000; average of five determinations) among the major phosphoproteins was stimulated by Ca^{2+} . The mean enhancement by Ca^{2+} of the 32 P incorporation relative to that into the M_r -57300 component was $324 \pm 62\%$ (mean $\pm$ S.E.M.; four experiments). Increasing the free Ca^{2+} concentration by the addition of 10 μ M- CaCl_2 to EGTA-free incubations led to a small additional increase (< 2 -fold) in phosphorylation of the M_r -100000 com-

Fig. 3. Cyclic AMP enhances phosphorylation of the cytosol protein of M_r 47000, and Ca^{2+} influences this effect

Incubations of cytosols and subsequent separation and quantification of phosphoproteins were carried out as in Fig. 2, in the presence of various concentrations of dibutyryl cyclic AMP. Endogenous Ca^{2+} (about $1 \mu\text{M}$) was either present (○) or sequestered by the addition of 2 mM-EGTA (●). The values plotted are averages from three experiments. Phosphoproteins of M_r 57300 and 26000 behaved in a similar manner.

ponent without altering the extent of phosphorylation of any of the other major phosphoproteins and without the complications of background variation encountered in the experiments with and without EGTA as described above. The relative phosphorylation enhancement of 4-fold in the presence of $10 \mu\text{M}$ - CaCl_2 , compared with Ca^{2+} -free incubations, was further increased to 9.3-fold in the presence of $10 \mu\text{M}$ - CaCl_2 plus 2 mg of calmodulin/ml. As with Ca^{2+} alone, Ca^{2+} plus calmodulin addition affected no other phosphoprotein except the M_r -100000 subunit. The degree of incorporation into this phosphoprotein in mammary cytosols incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and in cytosols prepared from mammary acini that had been incubated with $[\text{P}^{32}]\text{P}_i$ was more variable between experiments than that into the other phosphoproteins characterized.

The overall responses of cytosol phosphoproteins in dephosphorylation experiments (results not shown) similar to those illustrated in Fig. 2 were not changed by the presence of Ca^{2+} (or Ca^{2+} plus calmodulin) in the incubation medium.

In order to identify cyclic AMP-dependent cytosol protein substrates of kinase activity endogenous to the

preparation, control incubations in the presence of $10 \mu\text{M}$ - CaCl_2 were compared with incubations also containing $1 \mu\text{M}$ -dibutyryl cyclic AMP. Significant increases were observed in ^{32}P incorporation into the phosphoproteins of M_r 47000 and 57300 ($P < 0.005$; Student's paired-sample t test): the enhancement of phosphorylation, relative to control samples, found in the presence of cyclic nucleotide was $232 \pm 42\%$ and $167 \pm 9\%$ (five determinations) respectively. These changes were also induced by cyclic AMP and were abolished in the presence of the specific protein inhibitor of cyclic AMP-dependent protein kinase from heart muscle (results not shown). The dose-response relationship between dibutyryl cyclic AMP concentration and extent of ^{32}P incorporation into the M_r -47000 protein, and also the effect on this relationship of removing Ca^{2+} by the addition of 2 mM-EGTA, are shown in Fig. 3. Similar results (not shown) were seen with the protein of M_r 57300. The dose-response curves did not reach a convincing maximum, and consequently half-maximally effective concentrations of dibutyryl cyclic AMP could not be estimated. The steepest response of phosphorylation to changing dibutyryl cyclic AMP concentration occurred in the 0.1 – $1.0 \mu\text{M}$ range. The effects of Ca^{2+} were themselves dependent on the concentration of the cyclic AMP analogue. At a dibutyryl cyclic AMP concentration of $0.01 \mu\text{M}$, Ca^{2+} prevented the small cyclic AMP-dependent increase in incorporation that occurred in its absence. At $0.1 \mu\text{M}$ -dibutyryl cyclic AMP, Ca^{2+} was without effect, and at $> 0.1 \mu\text{M}$ -dibutyryl cyclic AMP Ca^{2+} enhanced the incorporation of ^{32}P .

Phosphorylation of the M_r -100000 subunit was unaffected by cyclic AMP regardless of the presence or absence of Ca^{2+} . In contrast with the phosphorylation pattern observed in cytosols from mammary acini incubated with $[\text{P}^{32}]\text{P}_i$, caseins and acetyl-CoA carboxylase were not major phosphoproteins in mammary cell cytosol incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, although a band corresponding in M_r to ATP citrate lyase was. Neither of these lipogenic enzymes was more highly phosphorylated in the presence of dibutyryl cyclic AMP (or cyclic AMP) than in its absence. This parallels their lack of response to forskolin and isoprenaline/Ro 7-2956 treatment in acini.

Phosphorylation of affinity-isolated acetyl-CoA carboxylase in mammary acini *in vitro*

Quantification of ^{32}P in acetyl-CoA carboxylase as reported above (see Table 1) was achieved by radioactive counting of the M_r -240000 protein subunit separated in polyacrylamide gels. A technique more selective for acetyl-CoA carboxylase was used in a further series of experiments, in which the enzyme was isolated by affinity chromatography on avidin-Sepharose from cytosols prepared from acini that had been incubated in $[\text{P}^{32}]\text{P}_i$ -containing medium. The products of a typical pair of preparations (from control and isoprenaline/Ro 7-2956-treated acini) when separated on SDS/5%-polyacrylamide gels were indistinguishable from one another both when the gels were stained with Coomassie Blue and when they were exposed to X-ray film to produce autoradiographs (results not shown); they were identical in these respects with the preparation illustrated in Fig. 1 of Holland *et al.* (1985). The ^{32}P specific radioactivities (c.p.m./ μg of protein) of the preparations from five such paired experiments are given in Table 2.

Table 2. Treatments that raise the intracellular concentration of cyclic AMP are not accompanied by hyperphosphorylation of acetyl-CoA carboxylase in mammary acini

Acini were incubated with the agent(s) shown and, in paired experiments, in their absence. [^{32}P] P_i was present throughout as described in the Experimental section. Acetyl-CoA carboxylase was isolated by using avidin–Sepharose, and the specific radioactivity of 10%–trichloroacetic acid-insoluble ^{32}P was determined. In Expts. 1 and 3, the specific radioactivity (c.p.m./pmol) of cellular ATP was determined in control and treated acini as described for hepatocytes by Holland *et al.* (1984). In each case, identical values for control and treated acini were found, which corresponded to phosphate contents of 2.89 (Expt. 1) and 3.01 (Expt. 3) mol/mol of acetyl-CoA carboxylase in control samples.

Experiment no.	^{32}P specific radioactivity of affinity-purified acetyl-CoA carboxylase (^{32}P c.p.m./ μg of protein)		
	Control	+ 100 μM -Forskolin	+ 1 μM -Isoprenaline + 1 mM-Ro 7-2956
1	5885	5221	—
2	1897	1930	—
3	3242	—	3264
4	2778	—	2810
5	4001	—	3899

Fig. 4. Reversed-phase h.p.l.c. analysis of tryptic phosphopeptides of acetyl-CoA carboxylase

The digests analysed in panels (a) and (b) were from purified acetyl-CoA carboxylase that had been incubated with [γ - ^{32}P]ATP (a) in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase, as described by Hardie & Guy (1980), and (b) in its absence. Approximately equal amounts of radioactivity (c.p.m.) were loaded for each analysis; recovery was 89% in (a), 81% in (b). The digests analysed in panels (c) and (d) were of acetyl-CoA carboxylases that had been purified from acini incubated with [^{32}P] P_i (c) in the presence of 1 μM -isoprenaline and 1 mM-Ro 7-2956 and (d) in their absence, as described in the Experimental section. These preparations had specific radioactivities of 3899 and 4001 c.p.m./ μg of protein respectively; 6000 c.p.m. was loaded for each analysis, and recovery in each case was 59%. The flow rates were 1 ml/min, and 1 ml fractions were collected. Other methods were as described in the Experimental section. The profiles shown are representative of at least three analyses of material arising from separate incubations using different preparations of acetyl-CoA carboxylase or mammary acini.

These show quite distinctly that neither of the cyclic AMP-elevating treatments applied to the acini caused any change in the degree of phosphorylation of acetyl-CoA carboxylase.

The distribution of ^{32}P among tryptic peptides of

acetyl-CoA carboxylase was analysed by reversed-phase h.p.l.c. (Fig. 4, panels c and d). As with the total ^{32}P content, no difference was seen between the tryptic-phosphopeptide profiles of control or treated acini. Fig. 4 also shows the ^{32}P -labelled phosphopeptide profiles

that were obtained from acetyl-CoA carboxylase purified from rat mammary tissue by avidin-Sephadex affinity chromatography and then phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence and absence of the catalytic subunit of cyclic AMP-dependent protein kinase before re-isolation with avidin-Sephadex. Incorporation of ^{32}P was 9-fold greater (per mg of enzyme protein) when the catalytic subunit of cyclic AMP-dependent protein kinase was present together with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, but the pattern of incorporation into individual sites in the acetyl-CoA carboxylase molecule (see Fig. 4, panels *a* and *b*) was very similar whether catalysed by this added protein kinase or by that which co-purified with acetyl-CoA carboxylase itself (cf. Munday & Hardie, 1984).

DISCUSSION

Incubation of acinar epithelial cells from lactating-rat mammary tissue with $[\text{P}^{32}]\text{P}_i$ led to incorporation of radioactivity into several proteins; not surprisingly, the greatest extent of labelling was into molecules identifiable as the three caseins of rat milk. Apart from these, two major phosphoproteins occurred in the cytosol fraction, which could be identified as acetyl-CoA carboxylase and (provisionally) ATP citrate lyase: a third, less intensely labelled, soluble phosphoprotein was probably phosphorylase. The degree of phosphorylation of all three of these proteins is known to be subject to modification by hormones in a number of different cell types, including adipocytes and hepatocytes (Benjamin & Singer, 1975; Avruch *et al.*, 1976, 1978; Garrison, 1978). Hormones and other agents that increase the intracellular concentration of cyclic AMP cause additional phosphorylation of these enzymes, relative to their state in untreated cells.

For acetyl-CoA carboxylase, this increased degree of phosphorylation results in a decrease in the V_{max} , and an increase in the K_a for citrate (Hardie & Guy, 1980), and these changes are sufficient to account, in hepatocytes and adipocytes, for the anti-lipogenic effects of the hormonal agents (glucagon in hepatocytes and glucagon or adrenaline in adipocytes) triggering the regulatory cascades (Holland *et al.*, 1984, 1985). It has further been established that these hormones cause additional phosphate incorporation into one particular tryptic peptide of acetyl-CoA carboxylase among those that can be separated by h.p.l.c. (Witters *et al.*, 1983; Holland *et al.*, 1984, 1985) or by t.l.c. (Brownsey & Denton, 1982), and that this is the same site into which phosphate incorporation is directed by cyclic AMP-dependent protein kinase when the catalytic subunit of this enzyme is incubated with purified acetyl-CoA carboxylase (Munday & Hardie, 1984). The results presented here show that these events do not ensue when mammary epithelial cells are treated in such a way as to increase the intracellular concentration of cyclic AMP by up to 20-fold: no change either in the degree of overall phosphorylation of acetyl-CoA carboxylase or in the distribution of phosphate residues within the molecule was caused by treatment of acini with isoprenaline in the presence of Ro 7-2956 or with forskolin. In particular, the extent of phosphorylation of the site preferred by cyclic AMP-dependent protein kinase was not affected by these cyclic AMP-elevating treatments.

Resistance of acetyl-CoA carboxylase to cyclic AMP-induced phosphorylation was also observed in a cell-free mammary tissue system when a cytosol fraction was

incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and cyclic AMP or its less readily hydrolysable analogue, dibutyryl cyclic AMP. Adipocyte cytosols, under similar conditions, show a marked enhancement in the phosphorylation of acetyl-CoA carboxylase by cyclic AMP and its analogues (Benjamin & Singer, 1975; Avruch *et al.*, 1976; Forn & Greengard, 1976; Brownsey *et al.*, 1979). In the isolated mammary cytosols of the present study, however, some other proteins, identified only by their electrophoretic mobility, exhibited cyclic AMP-enhanced phosphorylation. Cyclic AMP was most effective in provoking these responses at concentrations that are within the physiological range for the interior of mammary epithelial cells, as determined by Clegg & Mullaney (1985).

These results imply that more than one mechanism is responsible, in mammary epithelial cells of fed lactating rats, for maintaining a state of cyclic AMP resistance. One mechanism is dependent only on cell integrity; when it is inoperative, during incubations of mammary cytosol fractions *in vitro*, cyclic AMP-enhanced phosphorylation of several proteins becomes evident. However, the emergence of cyclic AMP-stimulated phosphorylation of acetyl-CoA carboxylase, and probably of other proteins also, requires its further purification from remaining cytosolic protein. The mechanism underlying this resistance to cyclic AMP is currently unknown. It may be that the concentration of cyclic AMP required to stimulate the cyclic AMP-dependent protein kinase is not reached within mammary acini under the experimental conditions used, or that the enzyme is already maximally stimulated in non-treated control acini. Determination of the activity ratio (Corbin, 1983) of cyclic AMP-dependent protein kinase in mammary tissue extracts would illuminate these possibilities. The phenomenon of resistance of cyclic AMP-dependent protein kinase-mediated phosphorylation of cellular proteins to cyclic AMP is not unique to mammary cells (see, e.g., Zajac *et al.*, 1986), and in this case is one of several mechanisms (see Robson *et al.*, 1984; Clegg & Mullaney, 1985; Clegg *et al.*, 1986) by which it is ensured that these cells remain unresponsive to agents that normally function to raise the intracellular concentration of cyclic AMP. This lack of responsiveness may be of physiological significance, allowing mammary-cell lipogenesis to proceed without interruption from episodic stress responses in the intact animal; however, it leaves undiscovered the identity of the agent that, in the intact lactating animal, is responsible for the rapid, extensive and reversible inhibition of the lipogenic pathway during starvation and fat-feeding.

R.E.D.A. was supported by an A.F.R.C. postgraduate studentship.

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Received 3 July 1986/3 September 1986; accepted 23 September 1986