Modulation of intracellular cyclic AMP content and rate of lipogenesis in mammary acini *in vitro*

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Relationships between the cyclic AMP content, the rate of lipogenesis and the activity of acetyl-CoA carboxylase in acini prepared from lactating rat mammary tissue were investigated by exposing them to agents that increase their cyclic AMP content in the presence or absence of insulin. (1) The dose-dependent inhibition of lipogenesis by theophylline in acini isolated from fed rats was highly correlated with the induced increases in acinar cyclic AMP content. Cyclic AMP of acini from 24 h-starved lactating rats was more sensitive in its response to theophylline than that in acini from fed animals. (2) Neither forskolin nor a mixture of isoprenaline and Ro 7-2956 were able significantly to change either the rate of lipogenesis or the activity of acetyl-CoA carboxylase in acini from fed rats when added to incubations *in vitro*, in spite of the large increases in cyclic AMP concentration produced by these agents. (3) Insulin was without effect on the activity of acetyl-CoA carboxylase and on either the basal or isoprenaline-stimulated cyclic AMP content of acini. (4) These results are discussed in terms of the possibility that the rate of lipogenesis and the cyclic AMP content in mammary acini can vary independently of one another and of the activity of acetyl-CoA carboxylase.

INTRODUCTION

It is well documented that changes in the basal (non-stimulated) concentration of cyclic AMP occur during the course of pregnancy and lactation in rat mammary tissue (Sapag-Hagar & Greenbaum, 1974; Louis & Baldwin, 1975). Results from experiments with explant cultures of mammary tissue from pregnant or pseudopregnant animals (Sapag-Hagar *et al.*, 1974; Cameron & Rillema, 1983) led to the expectation that alteration in cyclic AMP content of secretory cells may be involved in the regulation of mammary lipogenesis throughout lactation. In a previous study we found that lipogenesis in mammary acini from fed lactating rats is progressively inhibited *in vitro* by increasing concentrations of theophylline (Robson *et al.*, 1984). This effect may occur as a result of changes in the intracellular concentration of cyclic AMP that would be expected to accompany methylxanthine treatment, as, for example, in adipocytes (Fain & Malbon, 1979). Other possibilities exist, however, for the mechanism of action of theophylline and other methylxanthines in cellular metabolism that are unrelated to its effects on intracellular cyclic AMP concentrations. Thus, these compounds have been reported to inhibit glucose transport (Wilde & Kuhn, 1981), to increase intracellular Ca^{2+} flux (see, e.g., Kopf *et al.*, 1984) and to alter the affinity of insulin receptors in adipocytes (Joost & Steinfeldt, 1983).

The reaction catalysed by acetyl-CoA carboxylase is an important site in the control of flux through the pathway of fatty acid synthesis in liver and adipose tissue, its regulation being effected, at least in part (Lane *et al.*, 1979), by reversible phosphorylation (Kim, 1979; Brownsey & Denton, 1979; Holland *et al.*, 1984, 1985).

In the light of the findings by Hardie & Guy (1980) that purified acetyl-CoA carboxylase from rat mammary tissue was phosphorylated and inactivated *in vitro* in the presence of ATP and cyclic AMP-dependent protein kinase, it seemed likely that such phosphorylation-mediated inactivation would also occur in the intact epithelial cells of rat mammary tissue in response to agents that caused an increase in the intracellular concentration of cyclic AMP. The plausibility of this supposition was further strengthened by the demonstration that these consequences did indeed follow β -adrenergic challenge of adipocytes (Brownsey & Hardie, 1980; Witters *et al.*, 1983) and glucagon challenge of hepatocytes (Holland *et al.*, 1984) and of adipocytes (Holland *et al.*, 1985).

Despite this, it is evident that mammary epithelial cells *in vitro* are insensitive to glucagon (because they lack receptors for this hormone; Robson *et al.*, 1984) and do not increase their cyclic AMP content significantly in response to β -adrenergic stimulation except when simultaneously exposed to phosphodiesterase inhibitors (Clegg & Mullaney, 1985). Consequently, previous attempts to demonstrate effects of these hormones on lipogenic rate in acini may owe their lack of success (Robson *et al.*, 1984; Williamson *et al.*, 1983; Wilde & Kuhn, 1981) to an insufficient increase in the intracellular concentration of cyclic AMP. In addition, however, lipogenesis in this preparation appears to be resistant to increase in cyclic AMP directly by incubation with cyclic AMP and to inhibition by dibutyryl cyclic AMP. Using mammary tissue slices, Plucinski & Baldwin (1982) reported a stimulation of lipogenesis in response to prostaglandin E_1 , β -agonists and methylxanthine-mediated increases in cyclic AMP concentration. Appar-

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ently contrasting findings from experiments *in vivo* have been reported by Bussman *et al.* (1984), who observed a decrease in the rate of mammary tissue lipogenesis after subcutaneous injection of adrenaline into lactating rats. It is apparent, then, that the described effects of cyclic AMP on lipogenesis in mammary epithelial cells are inconsistent and ill-defined. The work reported here was undertaken to clarify this situation by examining the relationships in mammary acini between the ability of different effectors to alter the concentration of cyclic AMP (Clegg & Mullaney, 1985), the rate of lipogenesis and the activity of acetyl-CoA carboxylase.

MATERIALS AND METHODS

Rats

The source and maintenance of rats was as described previously (Robson *et al.*, 1984). When appropriate, animals were starved by the removal of food at 09:30 h on the day before their experimental use.

Preparation and incubation of acini

The methods used were those described by Robson *et al.* (1984) and Clegg & Mullaney (1985). Acini were incubated in a bicarbonate-buffered salts medium (Krebs & Henseleit, 1932) containing 5 mM-glucose, 2% (w/v) Ficoll and 4% (w/v) bovine serum albumin.

Other methods

The cyclic AMP content of acini was measured as described by Clegg & Mullaney (1985), and the rate of lipogenesis as described by Robson *et al.* (1984). Activity of acetyl-CoA carboxylase was measured with [^{14} C]bicarbonate in extracts of acini by the method of McNeillie & Zammit (1982). Extracts were prepared from acini that were rapidly harvested from incubations by centrifugation for 15 s at 800 *g*. The supernatant was discarded and the pellet frozen by immediate immersion in liquid N₂. Thereafter, the frozen acini pellets were processed exactly as described by McNeillie & Zammit (1982) for freeze-clamped mammary tissue pieces.

For affinity purification of acetyl-CoA carboxylase, acini pellets were not frozen but immediately homogenized in ice-cold buffer (pH 7.4; 25 ml/g wet wt. of acini) containing 20 mM-Tris/HCl, 250 mM-sucrose, 100 mM-KF, 2 mM-EDTA, 2 mM-EGTA, 7.5 mM-GSH, 2 mM-phenylmethanesulphonyl fluoride, and leupeptin, pepstatin and antipain (50 µg/ml each). Homogenization was for 30 s at 20000 rev./min with the PTA10S probe of a Polytron instrument. The homogenate was centrifuged at 8000 *g* for 10 min at 4 °C. After discarding the pellet, the supernatant was filtered through a plug of glass wool and further centrifuged, at 105000 *g* for 45 min at 4 °C. The resulting high-speed-supernatant fraction was treated with 0.64 vol. of a solution (475 g/l of water, adjusted to pH 7.0) of (NH₄)₂SO₄ at 4 °C. The mixture was stirred on ice for 30 min, and then the precipitated protein was collected by centrifugation at 10000 *g* for 20 min. Pellets from this procedure were dissolved each in 2 ml of avidin-Sepharose column buffer [0.1 M-Tris/HCl, 0.5 M-NaCl, 0.1 M-NaF, 1 mM-EDTA, 0.02% (w/v) NaN₃, 0.1% (v/v) 2-mercaptoethanol, pH 7.5] and loaded at a flow rate of 0.8 ml/min on to columns (100 mm × 6 mm) of avidin-Sepharose equilibrated with the same buffer. The subsequent purification

of acetyl-CoA carboxylase, conducted at 4 °C, was as described by Holland *et al.* (1984).

Acetyl-CoA carboxylase activity of purified preparations was measured at 30 °C in a final volume of 1.2 ml by the spectrophotometric assay linked to fatty acid synthase, as described by Hardie & Guy (1980), at a range of citrate concentrations between 0 and 20 mM. The total amount of magnesium acetate added was varied to maintain a concentration of 2 mM free Mg²⁺ at all citrate concentrations used. The reaction was started by adding the sample containing acetyl-CoA carboxylase (up to 200 µl), and the rate was determined after 6 min, when linearity was established. Kinetic data were fitted to the Hill equation for the derivation of K_a for citrate, V_{max} , and h , as described in Holland *et al.* (1985). Fatty acid synthase was purified from rabbit mammary tissue by the method of Hardie & Cohen (1978), but omitting the Sepharose 4B gel-filtration step. Residual traces of acetyl-CoA carboxylase activity contaminating this preparation were removed by passing it over a column (60 mm × 6 mm) of anti-(acetyl-CoA carboxylase) γ-globulin coupled to Sepharose 4B-CL. The acetyl-CoA carboxylase-free preparation was concentrated by precipitation with 0.5 vol. of 3.6 M-(NH₄)₂SO₄ and redissolution and dialysis in a solution (pH 7.0) containing 250 mM-potassium phosphate, 1 mM-EDTA, 10 mM-dithiothreitol and 30% (v/v) glycerol. Fatty acid synthase was stored in this solution (concn. 6 units/ml) at -20 °C.

Chemicals and materials

The sources of these were as given in Robson *et al.* (1984), McNeillie & Zammit (1982) and Clegg & Mullaney (1985).

Fig. 1. Theophylline-induced increase in intracellular cyclic AMP content of mammary acini: effect of duration of incubation

Acini from fed lactating rats were incubated at 37 °C for the times indicated, in the absence of theophylline (Δ) or in the presence of theophylline at a concentration of 5 mM (○) or 25 mM (●), before samples were rapidly quenched in HClO₄ and their content of cyclic AMP was measured. Bars indicate S.E.M. values around the means plotted, which are from five separate preparations incubated at each theophylline concentration.

Fig. 2. Dose-dependent increase in intracellular cyclic AMP content of mammary acini treated with theophylline: acini from starved rats are more theophylline-sensitive

Acini, prepared from either fed (●) or 24 h-starved lactating rats (○), were incubated at the indicated concentrations of theophylline and sampled as in Fig. 1. The mean cyclic AMP concentrations after incubation of acini (five separate preparations from rats in each state) for 15 min are plotted; bars indicate S.E.M. Values significantly different from those measured for the corresponding group in the absence of theophylline (tested by Student's *t* test for paired samples) are indicated as follows: †, *, *P* < 0.05; ††, **, *P* < 0.01; †††, ***, *P* < 0.001. Group mean values were significantly different (Student's *t* test, *P* < 0.05) between acini from fed and starved rats at 5 mM-, 10 mM- and 15 mM-theophylline.

Avidin-Sephadex was prepared as follows: 5 mg of avidin was coupled to 2 ml (settled volume) of CNBr-activated Sephadex 4B-CL; activation (28 mg of CNBr/ml of Sephadex) and coupling procedures were those recommended by Shaltiel (1974). The avidin-Sephadex was poured into columns and prepared for first use and subsequent re-use (conversion of avidin into monomeric form, and saturation of tight-binding sites with biotin) by the procedures described by Henrikson *et al.* (1979).

Antisera against rat mammary-gland acetyl-CoA carboxylase were raised in sheep: the antigen was generously provided by Dr. Grahame Hardie, Department of Biochemistry, University of Dundee. A γ -globulin

Fig. 3. Correlation between intracellular cyclic AMP content and inhibition of lipogenesis provoked by treatment of mammary acini with theophylline

Values for inhibition of lipogenesis are calculated from the data of Robson *et al.* (1984) on the effect of theophylline on rate of lipogenesis in rat mammary acini; in that work, lipogenesis was measured as an average rate between 15 and 60 min of incubation. Cyclic AMP content at corresponding theophylline concentrations (0, 5, 10, 15 and 20 mM) are therefore averages of values recorded from five separate acini preparations from fed lactating rats at 15, 30 and 60 min of incubation. Linear-regression analysis by the least-squares method gave the line shown, with a correlation coefficient of 0.9726.

fraction from one of these sera was coupled, as above, to Sephadex 4B-CL (2 mg of protein/ml settled volume).

Avidin and biotin were from Sigma Chemical Co. Sephadex 4B-CL is a Pharmacia product.

Expression of results

For comparison with earlier work, results are expressed per g wet wt., per 100 mg defatted dry wt. or per mg of DNA. For acini from fed mid-lactating rats, these are related as follows: 1 g wet wt. $\equiv$ 93 mg defatted dry wt. $\equiv$ 4.47 mg of DNA. When direct comparisons were made, the variability of results was similar when expressed on any of these bases. For statistical calculations, each observation was made on material derived from a separate preparation of acini.

RESULTS

Intracellular cyclic AMP content of theophylline-treated mammary acini

At all times when measurements were made, the concentration of cyclic AMP in mammary acini from fed rats was increased in the presence of 20 mM-theophylline, 2–3-fold over controls, during incubation *in vitro*. However, at the lowest concentration tested (5 mM), theophylline was without statistically significant effect on cyclic AMP content of acini (Fig. 1), as has been previously reported for 1 mM-theophylline (Clegg &

Table 1. Effects of insulin and cyclic AMP-increasing agents on lipogenesis and the intracellular cyclic AMP content of mammary acini *in vitro*

Acini were incubated at 37 °C in the presence of agents as indicated either for 15 min before the determination of cyclic AMP or for 45 min for determination of the rate of lipogenesis as described by Robson *et al.* (1984). In the latter case, $^3\text{H}_2\text{O}$ was added to all incubations after 15 min, and incorporation of ^3H into lipid was measured for up to a further 30 min. Under all conditions tabulated, incorporation was linear with time throughout this period. Values given are means $\pm$ S.E.M. (no. of determinations). Statistical significance of differences between values in treated and untreated acini was assessed by Student's *t* test for paired samples: *P < 0.05; **P < 0.01; ***P < 0.001. In addition, values sharing a common superscript letter were not significantly different from one another when tested as above. †Value from Clegg & Mullaney (1985); observations not paired with others recorded in this column. Abbreviation: n.t., not tested.

Additions to incubations	Rate of incorporation of ^3H from $^3\text{H}_2\text{O}$ into fatty acid ($\mu\text{mol}/\text{min}$ per mg of DNA)	Cyclic AMP content (pmol/g wet wt. of acini)
None	0.317 ± 0.037 (10)	215 ± 58 (7)
Insulin (1.68 munits/ml)	0.505 ± 0.120 (4) ^{a*}	246 ± 41 (7)
Isoprenaline (0.1 μM)	n.t.	487 ± 132 (5) ^{b***}
Isoprenaline (0.1 μM) plus insulin (1.68 munits/ml)	n.t.	330 ± 108 (5) ^{b***}
Isoprenaline (1 μM) plus Ro 7-2956 (1 mM)	0.402 ± 0.083 (3)	5810 ± 487 (4) [†]
Insulin (1.68 munits/ml) plus isoprenaline (1 μM) plus Ro 7-2956 (1 mM)	0.481 ± 0.209 (3) ^{a*}	n.t.
Forskolin (10 μM)	0.372 ± 0.37 (3)	1529 ± 178 (8) ^{c***}
Insulin (1.68 munits/ml) plus forskolin (10 μM)	0.469 ± 0.111 (3) ^{a*}	1821 ± 213 (8) ^{c***}

Table 2. Effect of insulin and cyclic AMP-increasing agents on acetyl-CoA carboxylase in isolated mammary acini

Acini were incubated at 37 °C for 45 min in the presence or absence of agents as indicated. Acetyl-CoA carboxylase activity was measured in crude extracts of acini and in purified preparations of the enzyme as described in the Materials and methods section. The activity of the crude extracts was measured as an 'initial' activity in the absence of added citrate. Values given are means $\pm$ S.E.M. or as simple means when replication was insufficient to permit meaningful error testing; the numbers of determinations are shown in parentheses. Statistical testing by Student's *t* test for paired samples revealed no significant differences between values from control and treated acini. Abbreviation: n.t., not tested.

Treatment of acini	Activity of acetyl-CoA carboxylase				
	In acini extracts ($\mu\text{mol}/\text{min}$ per 100 mg defatted dry wt.)	In avidin-Sephadex-purified preparations			
		V_{max} ($\mu\text{mol}/\text{min}$ per mg of protein)	K_a (citrate) (mM)	<i>h</i>	No. of determinations
Control (no additions)	1.306 ± 0.15 (5)	2.378 ± 0.286	1.0 ± 0.13	1.2 ± 0.09	(11)
Theophylline (20 mM)	1.211 ± 0.11 (5)	3.305	0.9	0.9	(2)
Isoprenaline (1 μM) plus Ro 7-2956	n.t.	2.887 ± 0.333	0.98 ± 0.13	1.02 ± 0.08	(6)
Insulin (1.68 munits/ml)	1.197 ± 0.10 (4)	1.945 ± 0.256	0.95 ± 0.45	1.18 ± 0.14	(5)

Mullaney, 1985). The sensitivity of the response of intracellular cyclic AMP to theophylline was increased in mammary acini isolated from lactating rats that had been starved for 24 h (Fig. 2).

Lipogenesis rate and intracellular cyclic AMP content of mammary acini

The correlation between the degree of inhibition of lipogenesis and the intracellular cyclic AMP concentra-

tion achieved by treatment of acini with various concentrations of theophylline between 0 and 20 mM is shown in Fig. 3. The results in Table 1, however, demonstrate that this correlation does not reflect a directly causal relationship, since neither forskolin nor the combination of isoprenaline and Ro 7-2956 (an inhibitor of cyclic AMP phosphodiesterase) caused any inhibition of lipogenesis in acini from fed rats under conditions where these agents have been shown (Clegg &

Mullaney, 1985) to elicit respectively an 8-fold and a 20-fold increase in cyclic AMP concentration over that in control acini. Table 1 also shows results of experiments in which agents that increase intracellular cyclic AMP were tested for their ability to antagonize the lipogenic effect of insulin in mammary acini *in vitro*. Neither forskolin nor the combination of isoprenaline and Ro 7-2956 was able significantly to diminish the insulin-stimulated rate of lipogenesis.

Effects of an increase in intracellular cyclic AMP on acetyl-CoA carboxylase activity of acini *in vitro*

The 'initial' activity of acetyl-CoA carboxylase, assayed without prior activation by protein phosphatase or citrate (McNeillie & Zammit, 1982), was measured in crude homogenates of theophylline-treated and isoprenaline/Ro 7-2956-treated acini. The results in Table 2 show that none of these effectors altered the initial activity of acetyl-CoA carboxylase. Furthermore, catalytic activity of avidin-Sepharose affinity-isolated enzyme, as reflected by the kinetic parameters $V_{\max}$, K_a for citrate and h , was unaffected (Table 2) by the presence of theophylline or of isoprenaline and Ro 7-2956 during incubation of acini before enzyme isolation.

Effects of insulin on acetyl-CoA carboxylase activity and intracellular cyclic AMP content of acini *in vitro*

Insulin is a competent effector of lipogenesis in mammary acini from fed lactating rats, causing an increase in the rate of fatty acid synthesis of around 60% (Table 1, and Robson *et al.*, 1984). Nevertheless, there was no effect on the activity either of acetyl-CoA carboxylase measured in extracts of insulin-treated acini or of affinity-purified acetyl-CoA carboxylase from such acini, compared with control untreated acini (Table 2). Insulin has been shown to stimulate a high-affinity cyclic AMP phosphodiesterase in rat mammary acini (Aitchison *et al.*, 1984). Despite this, insulin treatment of acini *in vitro* did not cause a statistically significant change in their unstimulated basal cyclic AMP content, nor was the hormone able to antagonize the small increase in acini cyclic AMP brought about by isoprenaline (see also Clegg & Mullaney, 1985) (Table 1).

DISCUSSION

The reciprocal correlation demonstrated here between the rate of lipogenesis and the concentration of cyclic AMP in mammary acini treated with a range of concentrations of theophylline is not a general one (although preliminary results suggest that it is upheld by other methylxanthines) and therefore cannot reflect a causal relationship between increase in cyclic AMP and depression of lipogenesis in this preparation. Two experimental observations reported here necessitate this conclusion. Firstly, the β -agonist/phosphodiesterase-inhibitor combination of isoprenaline/Ro 7-2956, although causing a 20-fold increase in acini cyclic AMP (Clegg & Mullaney, 1985), had no effect on either the rate of lipogenesis or acetyl-CoA carboxylase activity. Measurements of the activity of acetyl-CoA carboxylase purified from 10 μ M-forskolin-treated and from 20 mM-theophylline-treated acini showed that these agents likewise caused no change in the properties of this enzyme. Secondly, insulin treatment caused no change in the

cyclic AMP content of mammary acini from fed rats, but it increased the rate of lipogenesis substantially (Robson *et al.*, 1984). The present results suggest that, in mammary acini, β -agents themselves are not anti-lipogenic and, although they may provoke a small increase in intracellular cyclic AMP, this is not reversed by insulin. This contrasts with the effects of these compounds in adipocytes (Manganiello *et al.*, 1971).

These findings are in agreement with earlier reports (Williamson *et al.*, 1983; Robson *et al.*, 1984) of the inability of either cyclic AMP or its dibutyryl analogue, when applied to acini, to inhibit lipogenesis. The report by Plucinski & Baldwin (1982), that addition of cyclic AMP or treatment with isobutylmethylxanthine caused an increase in the rate of lipogenesis in rat mammary tissue slices, is not incompatible with the response reported here of acini to isoprenaline/Ro 7-2956 and to forskolin.

Despite the ability of insulin to stimulate lipogenesis in mammary acini from fed rats, the hormone had no effect on the activity or kinetic properties of acetyl-CoA carboxylase purified from insulin-treated acini or on the 'initial' activity of this enzyme measured in crude extracts of such acini. In insulin-treated adipocytes, the affinity-purified enzyme has unchanged kinetic properties (Witters *et al.*, 1983), although the molecule remains more highly phosphorylated (Brownsey *et al.*, 1979; Witters *et al.*, 1983). Comparable data on the phosphorylation state of acetyl-CoA carboxylase from mammary acini after treatment with insulin are not available. Nevertheless, circumstances have been described in which its phosphorylation state in rat mammary tissue undergoes change (McNeillie & Zammit, 1982; Munday & Hardie, 1985). Evidently there are multiple mechanisms through which insulin may affect lipogenesis and acetyl-CoA carboxylase in tissues committed to lipogenesis. Similarly, the effects of cyclic AMP on lipogenesis and acetyl-CoA carboxylase vary between tissues, as shown by comparison of the present work with, for example, that of Holland *et al.* (1984, 1985). Other examples of differential responses of this kind to cyclic AMP have been described (see, e.g., Hoyer *et al.*, 1984). The underlying mechanisms maintaining such differences are unknown at present, but may involve inhibitors of cyclic AMP-dependent protein kinase, selective activation of the type I and II isoenzymes of this enzyme (Livsey *et al.*, 1982), action of protein phosphatases, and modulation of the substrate competence of target proteins of protein kinase action. Further comparative investigations of β -adrenergic response and acetyl-CoA carboxylase regulation in adipose and mammary tissues should contribute towards the elucidation of which, among these at present theoretical possibilities, are of physiological importance.

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