Cyclic AMP-dependent protein kinase in mammary tissue of the lactating rat

Activity ratio and responsiveness of the target enzymes acetyl-CoA carboxylase and glycogen phosphorylase to β -adrenergic stimulation

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The role of cyclic AMP in acute regulation of the metabolism of mammary tissue in the lactating rat was examined by measuring the activity ratio of cyclic AMP-dependent protein kinase (A-kinase) and by examining the properties of this enzyme in its two major isoenzymic forms. Isoenzyme II is the major form in soluble extracts of rat mammary tissue. A-kinase activity ratio in such extracts is unaffected by starvation of the lactating rat. Treatment of the intact rat with isoprenaline, or addition of isoprenaline to incubations *in vitro* of mammary acini, resulted in a major increase in the activity ratio of A-kinase. These treatments equally affected isoenzymes I and II. The treatment *in vitro* led to a rapid depletion of A-kinase as subsequently measured in extracts of acini. The degree of activation of the enzymes acetyl-CoA carboxylase and glycogen phosphorylase in extracts of mammary tissue and of acini was assessed as a function of these treatments. The increased activation of A-kinase induced by isoprenaline was unaccompanied by significant changes in the activity of acetyl-CoA carboxylase in acini, although we previously showed that this agent activates acetyl-CoA carboxylase in intact mammary tissue. Contrastingly, isoprenaline-induced enhancement of A-kinase activity was accompanied by an increase in the activity ratio of phosphorylase in acini. These results indicate that: (a) a normal response of expressed A-kinase activity to cyclic AMP operates in mammary acini and mammary tissue from lactating rats; (b) rapid modulation of the total amount of soluble A-kinase is mediated in mammary epithelial cells by cyclic AMP; (c) phosphorylase, an ultimate target of the protein phosphorylation cascade initiated by A-kinase, is activated in acini under conditions where A-kinase activity is enhanced; and (d) mechanisms other than that of the A-kinase phosphorylation/inhibition model for acetyl-CoA carboxylase regulation must operate in mammary tissue preparations and *in vivo* to account for the response of this enzyme to enhanced A-kinase activity.

INTRODUCTION

Several previous reports from this (Clegg & Mullaney, 1985; Clegg *et al.*, 1986, 1987; Clegg, 1988) and other laboratories (Wilde & Kuhn, 1981; Plucinski & Baldwin, 1982; Williamson *et al.*, 1983; Bussmann *et al.*, 1984) have addressed the question of the role of cyclic AMP-mediated mechanisms in the acute regulation of aspects of mammary tissue metabolism. The conclusion to which they point is that this tissue, in the lactating animal, is refractory to the usual cascade of events initiated by an increase in intracellular cyclic AMP. Thus neither cytosolic protein phosphorylation (Clegg *et al.*, 1987) nor the activities of several enzymes, including lactose synthase and acetyl-CoA carboxylase, are influenced by raising the intracellular concentration of cyclic AMP in various preparations *in vitro* derived from rat mammary tissue (Wilde & Kuhn, 1981; Clegg *et al.*, 1986).

Measurement of the activity ratio of cyclic AMP-dependent protein kinase (A-kinase) in principle offers the possibility of a more precise determination of intracellular cyclic AMP status than can be made by the direct measurement of cellular cyclic AMP content (Corbin, 1983). This is particularly the case for mammary

tissue in lactating rats, where measurement of possibly small changes in the intracellular concentration of cyclic AMP is confounded by the presence of a high concentration of this nucleotide in milk; thus a rather cumbersome estimate must be made firstly of the milk content of the tissue, and then, based on the cyclic AMP content of expressed milk, of the contribution to total tissue cyclic AMP from that milk (Sapag-Hagar & Greenbaum, 1974). Furthermore, an assessment can also be made, by separation of the two A-kinase isoenzymes (I and II) combined with measurement of A-kinase activity ratios, of the extent to which each of the two isoenzymes ‘senses’ and responds to intracellular cyclic AMP under given circumstances, processes that can vary independently between the two isoenzymes (Livsey *et al.*, 1984).

In the present study, these techniques have been exploited to examine further the role of cyclic AMP in intracellular signalling in mammary epithelial cells, after first defining the properties of A-kinase in these cells. In addition, previous results have indirectly suggested that the enzyme phosphorylase in rat mammary tissue may not share with acetyl-CoA carboxylase the property of resistance to cyclic AMP-mediated increase in its

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phosphorylation (Clegg *et al.*, 1987). Here we report experiments in which phosphorylase *a* activity of mammary epithelial cells has been measured, as an index of phosphorylation of the enzyme protein, under conditions known to result in an increase in the intracellular concentration of cyclic AMP. In liver and muscle, the activity of phosphorylase kinase in the phosphorylation of phosphorylase (leading to activation of the latter) is stimulated by cyclic AMP and by Ca^{2+} . Ca^{2+} causes activation by binding to the δ subunit (calmodulin) of phosphorylase kinase, whereas cyclic AMP, by enhancing the activity of A-kinase, provokes phosphorylation primarily of the β subunit of phosphorylase kinase and its consequent activation (Cohen, 1987). Results in the present paper establish that, for cyclic AMP, the final consequence of this particular cascade is also achieved in mammary tissue.

EXPERIMENTAL

Lactating female Wistar rats, housed and fed as previously described (Robson *et al.*, 1984), were used 10–13 days after the birth of their first litter, as a source of mammary tissue. Tissue was sampled from anaesthetized animals either by freeze-clamping or for the preparation of acini exactly as described elsewhere (McNeillie & Zammit, 1982; Robson *et al.*, 1984). When animals were treated with isoprenaline, this was administered to the anaesthetized rat by intraperitoneal injection (0.5 mg/kg body wt.) 30 min before mammary tissue was freeze-clamped. Acini were prepared and incubated as described previously (Clegg & Mullaney, 1985). Isoprenaline (1 μM) was added to incubations as indicated; its addition was always accompanied by that of the phosphodiesterase inhibitor Ro 7-2956 (1 mM). After 15 min, acini were rapidly harvested by centrifugation and immediately used for the preparation of extracts. Acetyl-CoA carboxylase was assayed as described by Clegg *et al.* (1986).

Extracts for the assay of A-kinase were prepared [the precautions outlined by Corbin (1983) and Honor *et al.* (1985) being observed] by homogenizing frozen pulverized tissue or freshly sedimented acini at 4 °C in 4 vol. of buffer (10 mM- KH_2PO_4 , 10 mM-EDTA, 1 mM-3-isobutyl-1-methylxanthine, adjusted to pH 7.4 at room temperature with Tris base) at 0 °C, by using a Polytron homogenizer operating with a PTA10S probe at 18000 rev./min for 10 s (acini) or 20 s (mammary tissue). Bovine serum albumin was immediately added to give a final concentration of 10 mg/ml. After centrifugation for 15 min at 8800 g, the supernatant fraction of this homogenate was further diluted (usually 200-fold; see the Results and discussion section) with the homogenizing buffer containing bovine serum albumin (10 mg/ml). For some experiments, a different buffer was used (10 mM-Mes/NaOH, 0.2 mM-EDTA, 0.1 mM-dithiothreitol, pH 6.8, containing 1 mM-benzamidine/HCl, 0.1 mM-phenylmethane sulphonyl fluoride, 2 μg of soya-bean trypsin inhibitor/ml and 0.5 mM-3-isobutyl-1-methylxanthine), without effect on the results obtained. A series of control experiments was done (results not shown) to validate the extraction conditions employed for the determination of A-kinase activity ratio. Authentic A-kinase isoenzymes [I or II, purified by the method of Palmer *et al.* (1980) from rat liver] were added to either tissue or acini before homogenization. The activity ratio

of these preparations alone was assayed, without first subjecting them to homogenization. The activity ratio of endogenous mammary A-kinase was also measured. These results were compared with the measured activity ratio of the homogenates of mammary tissue prepared in the presence of each exogenous A-kinase. This comparison, as discussed in detail by Palmer *et al.* (1980) and Zajac *et al.* (1986), allowed the conclusion that no artifactual alteration in A-kinase activity had been induced by the conditions of sample preparation. When isoenzymes I and II of A-kinase were resolved by DEAE-cellulose chromatography (Livsey & Martin, 1988), extracts were prepared as described above but without the addition of bovine serum albumin (see the legend to Fig. 2). Fractions eluted from DEAE-cellulose were collected into tubes containing bovine serum albumin such that its final concentration was 10 mg/ml; this was necessary to avoid loss of activity in these fractions.

A-kinase activity was measured at 30 °C as described by Roskoski (1983), with the synthetic peptide Kemptide (120 $\mu\text{g}/\text{ml}$) or histone (300 $\mu\text{g}/\text{ml}$; Sigma type IIS) as a phosphate acceptor and 66 μM -ATP in a final assay volume of 60 μl . Under these conditions, phosphorylation of either substrate was linear with respect to time for at least 20 min. A fixed duration of incubation of 15 min was routinely used in these studies. When present, the concentration of bovine heart A-kinase inhibitor was 0.6 mg of protein/ml. Kemptide was used as phosphate acceptor in determinations of A-kinase activity ratio; the measured phosphate incorporation was corrected for that obtained in its absence, representing phosphorylation of proteins endogenous to the mammary extract. Basal A-kinase activity was assessed in the absence of cyclic AMP as the difference between the phosphorylation measured in the presence and absence of the bovine heart A-kinase inhibitor. The cyclic AMP-stimulated activity was assessed as the difference between activity in the presence of a maximally effective concentration of cyclic AMP and activity in the absence of cyclic AMP but with the bovine heart inhibitor present.

Catalytic subunit of A-kinase was quantified by the e.l.i.s.a. assay described by Schwoch & Hamann (1982). The antiserum used in this assay was generously given by Dr. G. Schwoch; the assay was standardized against bovine heart catalytic subunit of A-kinase.

Extracts for the assay of phosphorylase *a* and total phosphorylase (*a* plus *b*) were prepared from acini or mammary tissue as described for liver by Hue *et al.* (1975). These activities were measured over 10 min at 37 °C by methods described by Blackmore & Exton (1985). The DNA content of homogenates of mammary tissue and acini was determined by the method of Labarca & Paigen (1980).

Sources of chemicals and materials were as follows: [γ - ^{32}P]ATP and [U - ^{14}C]glucose 1-phosphate from Amersham International; bis-benzimide H 33258 fluorochrome from Fluka; 1,2-dimethoxyethane from Aldrich Chemical Co.; collagenase CLS III from Worthington; essentially fatty-acid-free bovine serum albumin (fraction V) from BCL; other biochemicals from Sigma Chemical Co. Catalytic subunit of A-kinase from bovine heart (Sigma product no. P2645) was 10–20 % pure (based on Coomassie Blue staining after SDS/polyacrylamide-gel electrophoresis), depending on the batch. Bovine heart inhibitor of A-kinase was Sigma product P8140. Ro 7-2956 was generously given by Roche Products.

Fig. 1. A-kinase activity in mammary tissue: dependence on cyclic AMP

Mammary-tissue homogenate (diluted in buffer containing 10 mg of bovine serum albumin/ml to a concentration equivalent to 1 part of tissue by wt. in 1000 parts of buffer by vol.; 10 μ l containing 41 ng of DNA) was used at each of the concentrations of cyclic AMP tested. The 100% activity was 36.6 nmol/min per mg of DNA in this experiment, representative of eight that have been performed. Similar results were found when mammary acini were homogenized in place of intact tissue.

RESULTS AND DISCUSSION

Cyclic AMP-dependence of mammary A-kinase

It was necessary to use considerable dilutions of mammary tissue and acini homogenates in order that a linear response was obtained between activity and quantity of sample when assaying over 15 min. To avoid the inactivation of A-kinase catalytic subunit that occurs in very dilute solutions of protein, the sample-preparation procedure was such that bovine serum albumin was always present at 10 mg/ml. This requirement was imposed by the ATPase activity of the sample extracts. The dilutions were such that the volume of the diluted sample (usually 10 μ l) added to assays contained non-sedimentable extract derived from approx. 10 μ g wet wt. of acini or tissue containing 30–60 ng of DNA. Under these conditions A-kinase activity was linear with respect to time over 30 min; the basal kinase activity (in the absence of added cyclic AMP) was stimulated by additions of cyclic AMP up to a final concentration of 0.6 μ M, but was then progressively inhibited by higher concentrations of this effector, as shown in Fig. 1. Inhibition of A-kinase by cyclic AMP is commonly observed in extracts of many tissues, but usually at much higher concentrations of the cyclic nucleotide than is the case with mammary tissue. The phenomenon reported here may be related to the ability, described below, of mammary-tissue extracts to mediate another change in the properties of A-kinase catalytic subunit in a cyclic AMP-dependent manner; in any event its physiological significance is currently not clear. A similar dose/response to cyclic AMP was seen if histone replaced Kemptide as the phosphate acceptor; although the activity ratio was unaffected, in this case the basal activity (measured in the absence of both cyclic AMP and the A-kinase inhibitor protein) was a much greater fraction of that achieved in

the presence of a maximally effective concentration of cyclic AMP, owing to the ability of histone to act as phosphate acceptor for a much wider variety of protein kinases than those recognizing Kemptide as a substrate.

The data in Fig. 1 were obtained with tissue from fed rats. Maximal A-kinase activities were not significantly different when tissue was from rats starved for 24 h or treated with isoprenaline. Concentrations of cyclic AMP giving 50% of maximal activity (determined from plots of mean percentage of maximal response versus log of concentration of cyclic AMP for five preparations in each treatment category) showed only small differences: for fed rats, 54 nM; for starved, 100 nM; for isoprenaline-treated, 32 nM.

Stimulation of protein kinase activity in the presence of mammary-tissue extracts and exogenous A-kinase

Tissue extracts in general may contain inhibitors and/or activators of protein kinase activity. Experiments were done with mammary extracts to test for the presence of such effectors. Pure A-kinase catalytic subunit was added to mammary extracts, and the combined protein kinase activity compared with the sum of the individually measured activities. These experiments showed that extracts from mammary tissue and acini enhanced the activity of bovine heart A-kinase (or, perhaps, vice versa); in 19 experiments, the mean enhancement ($\pm$ S.E.M.) was $184 \pm 4\%$ of the sum of individual activities. However, since these experiments measured net phosphorylation of Kemptide, it was possible that the effect observed was an expression of altered protein phosphatase activity in the assay incubations. Direct assay of the dephosphorylation of 32 P-labelled Kemptide (results not shown) by mammary extracts demonstrated that this was negligible over the normal 15 min time course of protein kinase assays, regardless of the presence or absence of added exogenous A-kinase (cf. Clegg & Annan, 1986). Thus we concluded that the observed effects resulted from authentic stimulation of protein kinase activity in the joint presence of A-kinase catalytic subunit and mammary tissue extracts. Stimulation was not found if the mammary extract was boiled for 10 min, but was not lost on dialysis of the extract (results not shown).

These findings are open to several interpretations. It may be that mammary extracts contain an agent that stimulates the activity of A-kinase against Kemptide. Alternatively, added A-kinase may stimulate a Kemptide kinase activity intrinsic to the mammary extract. It has been reported that an acetyl-CoA carboxylase kinase activity (acetyl-CoA carboxylase kinase 3) in mammary tissue, similar to that described by Carling *et al.* (1987) in liver, requires conditions favouring protein phosphorylation to maximize its activity (Ottey *et al.*, 1989). However, it is unlikely that this activity is involved in the phenomenon considered here, since all the stimulated Kemptide kinase activity is inhibited by the bovine heart protein inhibitor of A-kinase (results not shown).

Activity ratio of mammary A-kinase

The activity ratio of A-kinase in extracts of freeze-clamped mammary tissue was 0.071 (Table 1). This did not change significantly in tissue from animals that had been starved for 24 h before tissue sampling (activity ratio 0.077) or from those that had been starved for 24 h and then re-fed for 2 h (activity ratio 0.079).

Two forms of the A-kinase holoenzyme (I and II) are

Table 1. Isoprenaline treatment leads to activation of A-kinase in mammary tissue and isolated mammary acini

When intact tissue was the experimental subject, isoprenaline treatment was administered to the whole animal as an intraperitoneal dose (0.5 mg/kg body wt.) 30 min before freeze-clamping the tissue. Acini were treated *in vitro* with 1 μ M-isoprenaline in the presence of 1 mM-Ro 7-2956. Statistically significant differences relative to the corresponding untreated control were assessed by Student's *t* test (for paired samples in experiments with acini): **P* < 0.05; ***P* < 0.025; ****P* < 0.001. † One-way analysis of variance, by using $\log_{10}$ -transformed values to normalize the data, demonstrated this value to be significantly different (*P* < 0.025 according to *t*-test criteria) from that of a pooled population of untreated acini and tissue samples.

Preparation	Treatment	No. of observations	A-kinase activity (nmol of 32 P transferred/min per mg of homogenate DNA)		A-kinase activity ratio
			Without added cyclic AMP	With 0.7 μ M cyclic AMP	
Acini	None	9	1.28 $\pm$ 0.23	11.23 $\pm$ 1.02	0.114 $\pm$ 0.030
Acini	Isoprenaline	9	2.43 $\pm$ 0.46*	4.80 $\pm$ 0.69**	0.471 $\pm$ 0.056***
Tissue	None	9	2.18 $\pm$ 1.44	33.84 $\pm$ 5.22	0.071 $\pm$ 0.024
Tissue	Isoprenaline	8	4.89 $\pm$ 1.43†	21.10 $\pm$ 1.53**	0.280 $\pm$ 0.067**

widespread in a variety of tissues. They share a common catalytic subunit, but have different regulatory subunits, and may be separated from one another by ion-exchange chromatography on DEAE-cellulose. This technique also resolves both holoenzyme forms from the free catalytic subunit, which is acidic and therefore does not bind to the anion-exchanger. Such separations revealed that isoenzyme II was the predominant form in these extracts (see Fig. 2). In quantitative terms, of the total A-kinase activity in mammary-tissue extracts prepared from normally fed control rats that was recovered from DEAE-cellulose columns, 32 $\pm$ 3 % was eluted as isoenzyme I (mean $\pm$ S.E.M. of nine separate experiments). These extracts were the same ones that yielded an average activity ratio of 0.071 (Table 1). The recovery of activity from DEAE-cellulose was 149 $\pm$ 17 % of that loaded.

Evidently the extracts contained an inhibitor that was removed by ion-exchange chromatography.

Treatment of lactating rats with isoprenaline resulted in a major increase in the activity ratio of mammary tissue A-kinase, to 0.280. This was brought about by an increase in basal activity as well as by a substantial decline in the maximally cyclic AMP-stimulated rate (Table 1). As with control preparations from fed rats, the recovery of total A-kinase activity from DEAE-cellulose column separations of isoenzymes I and II in mammary extracts from isoprenaline-treated rats was greater than 100 %: in this case, 138 $\pm$ 10 % of the activity loaded. Some 34 $\pm$ 5 % of the activity recovered was in the form of isoenzyme I. This result indicates that both isoenzyme forms responded by additionally dissociating to a similar extent when experiencing an increased concentration of

Fig. 2. Separation of A-kinase mammary tissue isoenzymes by ion-exchange chromatography on DEAE-cellulose

A 1 ml portion of a 10000 *g* supernatant fraction (total A-kinase activity of 1.42 nmol/min) prepared from mammary-tissue homogenate was loaded on to a column (8 mm $\times$ 140 mm) of DEAE-cellulose equilibrated with 10 mM-Mes/NaOH/0.2 mM-EDTA/0.1 mM-dithiothreitol, pH 6.8. After washing with this buffer until no further protein (monitored as A_{280} ; ·····) was eluted, the column was developed with a linear gradient of NaCl (0–0.4 M) in the same buffer, at 3 ml/min. Recovery of activity in the separation illustrated (representative of six experiments) was 78 %, and fraction volume was 3 ml.

Table 2. Isoprenaline is without effect on acetyl-CoA carboxylase activity in mammary acini

Acini were incubated for 15 min either without additions or in the presence of 1 μ M-isoprenaline and 1 mM-Ro 7-2956. Values relating to tissue were obtained for extracts of freeze-clamped tissue sampled from normally fed animals. Statistically significant differences, relative to the corresponding value in untreated acini, were assessed by Student's *t* test: ****P* < 0.001. Activity ratio is the ratio of 'Initial' to 'Total' activity, both measured at 20 mM-citrate.

Preparation	Treatment	No. of observations	Acetyl-CoA carboxylase activity (μ mol/min per mg of homogenate DNA)			
			'Initial' activity (measured at 0 mM-citrate)	'Initial' activity (measured at 20 mM-citrate)	'Total' activity (measured at 20 mM-citrate)	Acetyl-CoA carboxylase activity ratio
Acini	None	7	0.092 $\pm$ 0.018	0.157 $\pm$ 0.029	0.273 $\pm$ 0.046	0.56 $\pm$ 0.02
Acini	Isoprenaline	7	0.095 $\pm$ 0.017	0.165 $\pm$ 0.026	0.263 $\pm$ 0.033	0.61 $\pm$ 0.02
Tissue	None	23	0.059 $\pm$ 0.008	0.145 $\pm$ 0.016	0.516 $\pm$ 0.017***	0.29 $\pm$ 0.04***

intracellular cyclic AMP; thus in both the control and treated extracts, a similar percentage of the undissociated holoenzyme complement was accounted for as isoenzyme I. Because of the difficulties, discussed above, encountered in attempting to measure accurately the intracellular cyclic AMP concentration in mammary tissue of lactating animals, this effect of isoprenaline on the cyclic AMP status of mammary tissue in the intact lactating animal has not hitherto been demonstrated directly, although its likelihood has been inferred from the known responses of isolated mammary epithelial-cell membranes (Ladha *et al.*, 1985) and acini (Clegg & Mullaney, 1985) to this adrenergic agonist.

Essentially similar results were obtained with acini. Their incubation in the presence of isoprenaline and Ro 7-2956 (a treatment known to cause a 20-fold increase in the intracellular concentration of cyclic AMP; Clegg & Mullaney, 1985) resulted in an increase in A-kinase activity ratio to 0.471 from its value in control acini of 0.114. As with isoprenaline treatment in the intact animal, the increase in this ratio arose from both an enhancement of basal activity and a depression of cyclic AMP-stimulated activity (Table 1). The decline in total A-kinase activity in soluble extracts of acini exposed to isoprenaline was matched by a decline in the amount of catalytic subunit as assessed by e.l.i.s.a. from 212 $\pm$ 77 to 105 $\pm$ 33 ng of protein/mg wet wt. of acini (mean $\pm$ S.E.M. of five paired observations). The mean content of catalytic subunit in isoprenaline-treated extracts, as a percentage of the paired control value, was 54 $\pm$ 7%. The corresponding value for A-kinase activity in the same group of extracts was 64 $\pm$ 6%.

Response of acetyl-CoA carboxylase activity to increased A-kinase activation in mammary tissue and acini

It has previously been shown that acetyl-CoA carboxylase, affinity-purified from acini incubated with isoprenaline and Ro 7-2956, was not significantly different, in respect of its $V_{\max}$, K_a for citrate, or of the occupancy and peptide mapping of phosphorylation sites within the protein molecule, from enzyme purified from acini incubated under control conditions without the adrenergic agonist and phosphodiesterase inhibitor (Clegg *et al.*, 1986, 1987). The results in Table 2 further support the conclusion (Clegg *et al.*, 1987) that this

enzyme is unresponsive, in mammary acini, to increased intracellular concentrations of cyclic AMP; they show no significant change in expressed ('initial') activity (measured at 20 mM-citrate) or 'total' activity in crude extracts of isoprenaline/Ro 7-2956-treated acini. The ratio of these two activities (activity ratio) is increased, but not significantly so, in the presence of the cyclic AMP-elevating agents. This ratio reflects the activation status [and usually therefore, by inference, phosphorylation status) of acetyl-CoA carboxylase (Hardie *et al.*, 1984) (but see also Haystead & Hardie, 1986)]. We have also shown previously that treatment of intact lactating rats with isoprenaline increased the activity ratio (as defined above) of acetyl-CoA carboxylase as well as both the expressed and 'total' activities of the enzyme (Mullaney *et al.*, 1987). Similar effects have also been reported after infusion of isoprenaline into perfused mammary tissue of the lactating rat (Clegg & Calvert, 1988). This response of acetyl-CoA carboxylase towards cyclic AMP-elevating agents is paradoxical in that it takes place in the opposite direction to that expected, i.e. the activity ratio increases under conditions where A-kinase becomes more active. This appears to contradict studies *in vitro* in which the purified enzyme has frequently been shown to be inactivated as a result of phosphorylation by A-kinase (Hardie & Guy, 1980) and other protein kinases (Hardie *et al.*, 1986). However, it has been previously noted in several independent studies by ourselves and others that acetyl-CoA carboxylase activity in mammary tissue may respond in this anomalous way to agents that increase the intracellular concentration of cyclic AMP (Plucinski & Baldwin, 1982; Williamson *et al.*, 1983; Clegg *et al.*, 1986; Munday & Williamson, 1987).

Response of glycogen phosphorylase activity to increased A-kinase activation in mammary tissue and acini

Similar experiments to those described above were performed for the measurement of glycogen phosphorylase activity in acini and mammary tissue in defined states of A-kinase activation. The results are shown in Table 3. The degree of activation (expressed as the ratio of phosphorylase *a* to total phosphorylase activity) of this enzyme was greater in control tissue than in control acini (significance tested by Student's *t* test, *P* < 0.001), and in both cases was increased by treatment with isoprenaline,

Table 3. Phosphorylase activity in mammary tissue and acini: response to isoprenaline

Incubation and sampling were as in Table 2. Isoprenaline was administered to anaesthetized rats (see the Experimental section) 30 min before freeze-clamping of isoprenaline-treated mammary tissue. Statistically significant differences (assessed as in Table 1) were as follows: ** $P < 0.01$. All values for tissue samples were significantly greater (unpaired t test; $P < 0.01$) than their counterparts in untreated acini.

Preparation	Treatment	No. of observations	Phosphorylase activity ($\mu\text{mol}/\text{min}$ per mg of homogenate DNA)		Phosphorylase activity ratio
			Phosphorylase a	Phosphorylase $a + b$	
Acini	None	13	0.139 ± 0.018	0.340 ± 0.034	0.40 ± 0.03
Acini	Isoprenaline	13	0.173 ± 0.029	0.277 ± 0.038	$0.60 \pm 0.04^{**}$
Tissue	None	6	0.470 ± 0.045	0.615 ± 0.050	0.76 ± 0.03
Tissue	Isoprenaline	5	0.558 ± 0.024	0.688 ± 0.020	0.81 ± 0.03

although the increase only achieved statistical significance in acini. A tendency was noted from these results for the total phosphorylase activity to be lower in isoprenaline-treated samples, although this was not a statistically significant effect. We performed experiments to test whether isoprenaline was causing the accumulation, in extracts, of a substance(s) interfering with the assay of phosphorylase activity. One of these is shown in Table 4. A solution of purified phosphorylases a and b was made in the same buffer as that used to prepare extracts of mammary tissue and acini for the determination of phosphorylase activity. Extracts of acini were then prepared in this buffer as described in the Experimental section. Before the homogenization stage, a known volume of the solution of purified phosphorylases a and b was added to a portion of acini suspension; control experiments were performed in which buffer alone replaced either the suspension of acini or the solution of pure enzymes. Phosphorylase activities (a and $a + b$) were then determined in each of the three resulting preparations. This procedure was repeated with acini that had been exposed to isoprenaline plus Ro 7-2956. In principle, the measured activity both of phosphorylase a and

of total phosphorylase in the acini extract prepared in the presence of purified phosphorylases should equal the sum of the respective activities in the two control extracts prepared as described above. In practice, the combined assay of pure enzymes and untreated acini extracts gave results for phosphorylase a of $66 \pm 4\%$ of the expected sum total and for total phosphorylase, $71 \pm 5\%$. The corresponding values for extracts from isoprenaline-treated acini were $67 \pm 8\%$ and $66 \pm 6\%$ (mean values $\pm$ S.E.M. for three preparations each of untreated and isoprenaline-treated acini). These results show that isoprenaline treatment of acini does not selectively lead to the accumulation of substances interfering with the subsequent assay of phosphorylase activity. Therefore the apparent decline in total phosphorylase activity of isoprenaline-treated acini is not an assay-based artefact induced by the treatment procedure. These results also demonstrate that the assay of phosphorylase in acini extracts, under the conditions described in the Experimental section, leads to a systematic under-estimation of its activity by around 30%. In the present study, phosphorylase activity in mammary acini was found to respond to β -adrenergic stimulation in accordance with

Table 4. Effect on phosphorylase activity of isoprenaline treatment of mammary acini: diminution of total activity is not an assay artefact

Assays were performed at 37°C for 10 min in a final volume of $80\ \mu\text{l}$. Activities were expressed as m-units per assay, where 1 unit of activity catalyses the incorporation of $1\ \mu\text{mol}$ of glucose 1-phosphate into glycogen under these conditions. Specific radioactivity of the [^{14}C]glucose 1-phosphate substrate was $67\ \text{mCi}/\text{mol}$. Approx. $1\text{--}2\ \mu\text{g}$ of the purified phosphorylase a and b preparations (from rabbit muscle) were present when indicated. Samples ($20\ \mu\text{l}$) of acini extract, equivalent to 8.0 and $6.1\ \mu\text{g}$ of DNA in the preparations from which they were derived (control and treated respectively), were used as appropriate. Further details of experimental design are given in the text. The results shown are from one comparison, representative of three that have been performed. When phosphorylase activities in a mixture of phosphorylase a and phosphorylase b were assayed and compared with the sum of activities measured in each sample separately, assay performance, as expressed in the right-hand column, was 97% for phosphorylase a and 89% for total phosphorylase.

Experimental treatment	Phosphorylase activity detected by assay	Composition of sample assayed...	Phosphorylase activity measured (m-units)			Assay performance using mixed samples [(3) as % of the arithmetic sum of (1) plus (2)]
			(1) Purified phosphorylases a and b	(2) Extract of mammary acini	(3) Mixture of (1) + (2)	
Control	a		11.21	1.38	8.45	67
Control	$a + b$		25.17	5.75	22.00	71
Isoprenaline	a		9.17	1.95	8.52	77
Isoprenaline	$a + b$		17.32	3.50	15.51	74

the model established for muscle (Cohen, 1987), whereas A-kinase-induced activation of phosphorylase could not be demonstrated in intact mammary tissue, perhaps because the level of activation was already so high (activity ratio 0.76) in control samples.

General discussion

These findings represent the first direct demonstration of A-kinase activation in mammary tissue by cyclic-AMP-increasing treatments administered either to intact animals or to mammary acinar preparations *in vitro*. The soluble forms of both isoenzymes I and II respond coordinately to an increase in the intracellular concentration of cyclic AMP. This conclusion is supported by direct measurement, by immunochemical techniques, of total and dissociated forms of R_I and R_{II} (the regulatory subunits of isoenzymes I and II respectively) in mammary cytosols (R. A. Clegg, unpublished work).

A further novel finding is that A-kinase is rapidly depleted in the soluble compartment of mammary epithelial cells after their treatment *in vitro* with isoprenaline. A similar phenomenon is shown by an A-kinase in brush-border membranes of renal and intestinal epithelia. In this system, exposure to cyclic AMP results in rapid degradation of the catalytic subunit by a protease of exceptionally high substrate specificity (Alhanaty *et al.*, 1985); association between catalytic and regulatory subunits protects the former against the action of this protease. In contrast, the mechanisms underlying the rapid changes in mammary-cell A-kinase after exposure to isoprenaline do not involve proteolytic loss of catalytic subunit (R. A. Clegg, unpublished work).

The response of glycogen phosphorylase in acini after exposure to isoprenaline demonstrates that mammary cells share an ability with many other cell types to react to an increase in intracellular cyclic AMP with an increased level of phosphorylation of those proteins that are acceptor substrates for A-kinase. This demonstration is reinforced by the finding that all the individual enzymic components of this response system are present in mammary cells (Clegg, 1988).

Mammary tissue has been shown (Ottey *et al.*, 1989) to contain a protein kinase activity that is activated by AMP, for which acetyl-CoA carboxylase is one of a small number of preferred substrates (Carling *et al.*, 1987), and we have found that mammary tissue in the fed lactating rat contains rather high concentrations of AMP (0.044 $\mu\text{mol/mg}$ of DNA; Clegg & Calvert, 1988), whereas acini *in vitro* contain considerably less ($< 0.005 \mu\text{mol/mg}$ of DNA; R. A. Clegg, unpublished work). It is therefore likely that this protein kinase will express a diminished activity in acini compared with intact mammary tissue, and this could account for the more activated state of acetyl-CoA carboxylase in untreated acini than in intact tissue from untreated animals.

It remains unclear what counter-regulatory mechanisms ensure that acetyl-CoA carboxylase activity is unchanged or increased in response to an enhancement in mammary cellular A-kinase activity.

We are grateful to Dr. Michael Munday for discussion throughout this work, and to Kate Roy, Kevin Connor and Aileen Campbell for skilled technical assistance. K.A.O.

acknowledges support from the S.E.R.C. in the form of a C.A.S.E. Studentship.

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