Cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities of rat mammary tissue

Ian MULLANEY and Roger A. CLEGG
Hannah Research Institute, Ayr KA6 5HL, Scotland, U.K.

(Received 24 October 1983/Accepted 5 January 1984)

Cyclic nucleotide phosphodiesterase activity in mammary tissue from rats in mid-lactation was resolved by DEAE-cellulose chromatography into three functionally distinct fractions: a Ca^{2+} /calmodulin-stimulated cyclic GMP phosphodiesterase, a cyclic GMP-stimulated low-affinity cyclic nucleotide phosphodiesterase, and a high-affinity cyclic AMP-specific phosphodiesterase. The absolute activities and relative proportions of high- and low-affinity enzymes resemble those found, for example, in liver, as distinct from those in excitable tissues. Three functional characteristics are described which are peculiar to mammary-tissue phosphodiesterases. Firstly, the concentration of free Ca^{2+} required to achieve half-maximal activation of the Ca^{2+} /calmodulin-stimulated phosphodiesterase is somewhat higher than for the analogous enzyme in other tissues; secondly, the activity of this enzyme towards cyclic AMP relative to that towards cyclic GMP is unusually low, and thirdly, the low-affinity cyclic nucleotide phosphodiesterase is inhibited by low concentrations of free Ca^{2+} .

Mammary tissue undergoes rapid changes in metabolic activity around parturition, leading to large increases in its capacity to synthesize protein, lipid and lactose (Baldwin & Yang, 1974). These changes are largely the result of increases in the amounts of key enzymes of the various pathways involved (Mayer, 1978). However, as has been re-emphasized for lipid-biosynthetic pathways (McNeillie & Zammit, 1982; Robinson *et al.*, 1978), lactating mammary tissue retains the ability to adjust the rate of its metabolism in response to acute physiological (or non-physiological) changes in the whole animal.

In most mammalian tissues, cyclic AMP plays a key role, along with the intracellular concentration of Ca^{2+} , in the transduction of extracellular physiological signals into intracellular metabolic responses (see, e.g., Nimmo & Cohen, 1977) during such acute transitions. Analyses of mammary-tissue cyclic AMP concentrations have been reported (Sapag-Hagar & Greenbaum, 1974a; Louis & Baldwin, 1975), but only slight consideration has been given to those enzyme activities in this tissue that jointly determine cyclic AMP concentration, i.e. adenylate cyclase (EC 4.6.1.1) and cyclic AMP phosphodiesterase (EC 3.1.4.17). The latter has been measured in rat mammary tissue (Douša & Rychlík, 1968; Sapag-Hagar &

Greenbaum, 1974b; Louis & Baldwin, 1975; Pizarro *et al.*, 1981), but the enzymes involved were not thoroughly characterized.

Evidence of the involvement of cyclic AMP in acute regulation of lipogenesis in mammary tissue is equivocal (Majumder & Turkington, 1971; Brownsey *et al.*, 1979; Brownsey & Hardie, 1980; Hardie & Guy, 1980; McNeillie *et al.*, 1981; McNeillie & Zammit, 1982; Munday & Williamson, 1982). Theophylline markedly inhibited lipogenesis in isolated rat mammary acini (Robson *et al.*, 1984), although in the same preparation no corresponding inhibition by either adrenaline (N. A. Robson, R. A. Clegg & V. A. Zammit, unpublished work) or glucagon (Robson *et al.*, 1984) was found.

It appears therefore that mammary tissue possesses at least some elements of the enzymic machinery required to enable it to respond to cyclic AMP-increasing hormones. Notwithstanding this, no responses to such hormones have been convincingly measured (Wilde & Kuhn, 1981; Plucinski & Baldwin, 1982; Munday & Williamson, 1982; Robson *et al.*, 1984). In contrast, a stimulation of lipogenesis in mammary acini by insulin has been reported (Robinson & Williamson, 1977; Agius & Williamson, 1980; Robson *et al.*, 1984), and it has been further shown (Robson *et al.*, 1984) that

insulin, added together with theophylline, not only abolishes theophylline's inhibitory effects but also provokes a stimulation of lipogenesis as great as that which it alone (i.e. in the absence of theophylline) achieves. These observations suggest that mammary cells are competent to effect an insulin-mediated decrease in cyclic AMP concentration, comparable with that described for adipocytes (Manganiello *et al.*, 1971) and hepatocytes (Pilkis *et al.*, 1975).

A more complete understanding of the role of cyclic AMP in acute metabolic responses of mammary tissue requires, among other things, further characterization of the enzymes of cyclic AMP metabolism. The present work describes such a characterization of rat mammary-tissue cyclic nucleotide phosphodiesterases.

Materials and methods

Materials

Wistar rats were maintained on a pelleted rat-breeding diet (Oxoid) and water *ad libitum*; they were housed under conditions of alternating 12h light and dark periods, the latter beginning at 20:00h. Litter size was adjusted to eight pups within 24h of parturition: animals giving birth to less than eight pups were not used. Mothers and pups were not separated until immediately before the mothers were killed.

Cyclic [8-³H]AMP (sp. radioactivity 28Ci/mmol) and cyclic [8-³H]GMP (sp. radioactivity 18.3Ci/mmol) were purchased from Amersham International plc (Amersham, Bucks., U.K.).

The following materials were purchased from Sigma (London) Chemical Co. (Poole, Dorset, U.K.): Dowex 1X8-400 (chloride form), cyclic AMP and cyclic GMP (both as sodium salts), phenylmethanesulphonyl fluoride, snake venom from *Ophiophagus hannah* and activator-deficient cyclic AMP phosphodiesterase from bovine heart.

Fluka EGTA (puriss grade) was obtained from Fluorochem, Glossop, Derbyshire, U.K., and DEAE-cellulose was Whatman DE52.

Preparation of phosphodiesterases

Methods for the initial stages of the preparation were adapted from Thompson & Appleman (1971), by using a modification of the homogenizing buffer described by Thompson *et al.* (1979a). The DEAE-cellulose column procedures were from Thompson *et al.* (1979b).

Inguinal and abdominal mammary tissue (3–4g) was removed from 10–12-day lactating rats, killed by cervical dislocation between 09:00 and 10:00h. The tissue was chopped with scissors, rinsed in two washes of 0.9% (w/v) NaCl and homogenized for 60s in 10vol. of a buffer composed of 10mM-Tris/

HCl/50mM-KF/20μM-phenylmethanesulphonyl fluoride, pH8.0, with a Polytron fitted with a PTA10S probe, at setting no. 4. All operations were done at 4°C. The homogenate was centrifuged at 1000g for 10min and filtered through glass wool. The resulting partially clarified homogenate was then ultrasonicated with the 9mm-diameter probe of a Soniprep 50 instrument (MSE Scientific Instruments, Manor Royal, Crawley, Sussex, U.K.) at full power for 15min/30ml of homogenate, in 1min bursts with 1min intervals for cooling, in an ice/salt bath. The preparation was then carefully adjusted to pH6.0 with 10% (v/v) acetic acid (if pH falls below 6, the enzyme precipitates with irreversible loss of up to 80% of its activity) and centrifuged at 20000g for 20min.

A 10ml portion of the acid supernatant was added to a DEAE-cellulose column (16mm diam. × 150mm) equilibrated with 70mM-sodium acetate/acetic acid buffer (pH6.5) containing 5mM-2-mercaptoethanol (free Ca²⁺ concn. ≤ 10μM). The column, with sample applied, was then washed with 5 column vol. of the same buffer; this wash was normally devoid of phosphodiesterase activity. Elution was with a linear gradient (400ml) of buffer (pH6.5) containing between 70 and 1000mM-sodium acetate/acetic acid/5mM-2-mercaptoethanol at a flow rate of 40ml/h. Column eluate was collected in 7ml fractions. These fractions were assayed for phosphodiesterase activity and the active fractions pooled.

Determination of the low concentration of protein in these pooled fractions posed problems attributable to interference by components of the buffer and/or samples with those methods suitable for the high-sensitivity determination of protein (as discussed by Thompson *et al.*, 1979a). No entirely satisfactory solutions to these problems were found. For this reason, their activities are expressed per ml, since these difficulties precluded determination of absolute specific (i.e. per mg of protein) activities. Comparison of rates between experiments is hampered by this shortcoming: such comparisons are therefore made only on a relative (%) basis. Otherwise, results from representative experiments (Fig. 4) or individual experiments (Table 2) are shown. Values of $K_{0.5}$ (concentration of substrate at which the measured velocity is 50% of the maximum velocity achieved under defined circumstances) and rate values for homogenates and pH 6 supernatants are unaffected by this restriction, and are given as means ± S.E.M. for the numbers of determinations in parentheses.

Mammary phosphodiesterase pools did not lose activity when stored at 4°C for up to 4weeks. In this respect they were similar to rat testis cyclic AMP phosphodiesterase (Beale *et al.*, 1977) and unlike phosphodiesterases of many other tissues,

e.g. rat brain (Thompson & Appleman, 1971), which need to be concentrated in some way to maintain stability. As a result of this, it was found unnecessary to add a cryoprotective agent to these pools; indeed, in early experiments, the use of an elution buffer containing 30% (v/v) ethylene glycol resulted in the loss of up to 80% of the original activity.

Purification of calmodulin from sheep brain

Frozen brains were allowed to thaw at room temperature, after which they were washed in 0.9% NaCl. All operations were performed at 4°C unless stated otherwise.

Brain tissue (200 g) was homogenized in 600 ml of 20 mM-Tris-HCl/1 mM-MgSO₄, pH 7.5, in a Waring blender for 60 s at maximum speed. Purification steps were then identical with those described by Lin *et al.* (1974). Ability to stimulate activator-deficient cyclic AMP phosphodiesterase was used as an index of calmodulin activity.

Assay of phosphodiesterase activity

Phosphodiesterase activity was determined by the two-step procedure of Thompson *et al.* (1974). The assay mixture (0.4 ml) contained 10 mM-Tris/HCl, 1 μ M- or 100 μ M-cyclic [³H]AMP or -cyclic [³H]GMP (approx. 200 000 c.p.m./assay), 1.1 mM-2-mercaptoethanol, 12.1 mM-MgCl, pH 8.0, and an appropriate dilution of the sample. The reaction was initiated by addition of sample, and the mixture was incubated for 10 min at 30°C and then stopped by firstly dipping the tubes containing the incubated mixture into liquid N₂ for 5 s and then transferring them to a boiling-water bath for 45 s. After cooling the tubes on ice, the second incubation was started by the addition of 100 μ l of a 1 mg/ml solution of snake venom and continued for 10 min at 30°C. The reaction was stopped by placing the tubes on ice.

Labelled product was separated from substrate on small columns (7 mm diam. $\times$ 10 mm) of Dowex 1X8-400 and quantified by using liquid-scintillation counting. One unit of activity results in substrate hydrolysis at a rate of 1 μ mol/min.

Protein determinations

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results and discussion

Kinetic heterogeneity of mammary-tissue cyclic nucleotide phosphodiesterase

As has been reported for many other tissues (see, e.g., Beavo *et al.*, 1982), the kinetics of cyclic AMP hydrolysis by mammary-tissue extracts did not

conform to a simple Michaelis-Menten model. Following the technique of Arch & Newsholme (1976), substrate-concentration-versus-velocity data were analysed by the graphical method of Eadie and Hofstee (Hofstee, 1952) and found to approximate to a two-component model (Fig. 1). The two kinetically distinguishable cyclic AMP phosphodiesterase activities had $K_{0.5}$ values of 21.6 and 1.3 μ M (Table 1) and V_{max} values of 1200 ± 93 (4) and 217 ± 44 (3) μ units/mg of protein respectively. Kinetic heterogeneity of cyclic AMP phosphodiesterase has been noted previously in mammary tissue, but not fully documented: Sapag-Hagar & Greenbaum (1974b) reported the presence of two components of $K_{0.5}$ 6 μ M and 70 μ M, and Pizarro *et al.* (1981) remarked on multiple kinetic forms without specifying $K_{0.5}$ values.

Similar analyses to that shown in Fig. 1 were performed with homogenates of mammary acini; they showed no significant differences from those with whole tissue homogenates (results not shown), indicating that adipocytes, myoepithelial cells and other cellular elements of mammary tissue made no significant contribution to cyclic AMP phosphodiesterase activity in the unfractionated tissue. Acini isolated from mammary tissue of late-pregnant rats gave similar $K_{0.5}$ and V_{max} values to their counterparts from lactating tissue (results not shown). Thus the different concentrations of cyclic AMP reported in pregnant- and lactating-rat mammary tissue (Sapag-Hagar & Greenbaum, 1974a) do not arise from gross changes in cyclic

Fig. 1. Cyclic AMP phosphodiesterase activity of lactating rat mammary tissue: two kinetically distinct forms. Phosphodiesterase activity in a homogenate (final protein concn. 0.47 mg/ml) of rat mammary tissue was assayed at cyclic AMP concentrations between 0.1 and 100 μ M. Results of a single representative experiment are presented as an Eadie-Hofstee plot after manipulation of the raw kinetic data as suggested by Arch & Newsholme (1976).

Table 1. *Cyclic nucleotide phosphodiesterase activity in rat mammary-tissue extracts*

Values given for phosphodiesterase activity and recovery are means $\pm$ S.E.M. for the numbers of determinations shown in parentheses. $K_{0.5}$ values were determined from Eadie-Hofstee plots after first manipulating the raw kinetic data, as described by Arch & Newsholme (1976), on the assumption that they resulted from the summation of a high-affinity and a low-affinity component with widely separated $K_{0.5}$ values. A representative plot is shown in Fig. 1. Abbreviation: n.d., not determined.

Substrate	Concn. (μ M)	Phosphodiesterase activity (munits/g of tissue)		Recovery in acid supernatant (% of activity in total homogenate)	$K_{0.5}$ (μ M)
		Homogenate	Acid supernatant		
Cyclic AMP	100	73.0 $\pm$ 1.5 (9)	36.2 $\pm$ 2.0 (7)	50 $\pm$ 4 (7)	21.6 $\pm$ 1.5 (3)
	1	6.4 $\pm$ 0.2 (9)	4.0 $\pm$ 0.3 (7)	62 $\pm$ 1 (7)	1.3 $\pm$ 0.3 (3)
Cyclic GMP	100	13.2 $\pm$ 0.7 (6)	4.5 $\pm$ 0.8 (3)	34 $\pm$ 6 (3)	n.d.
	1	1.6 $\pm$ 0.2 (5)	0.8 $\pm$ 0.1 (7)	51 $\pm$ 3 (5)	n.d.

AMP phosphodiesterase activity, although, of course, compartmentation effects are not evaluated by this technique. The increase in mammary-tissue cyclic AMP phosphodiesterase throughout pregnancy and lactation observed by Sapag-Hagar & Greenbaum (1974b) did not correlate with fluctuations which they also observed in tissue cyclic AMP content; it is possible that their phosphodiesterase measurements (expressed on a tissue-fresh-weight basis) reflect a progressive increase in the proportion of alveolar epithelial cells per unit weight of tissue as lactation proceeded.

Homogenates of mammary tissue are known to contain calmodulin (West & Clegg, 1981; Riss & Baumrucker, 1982), and the presence, in boiled extracts of rat mammary tissue, of an activator (presumed to be calmodulin) of 'calmodulin-deficient' mammary-tissue cyclic AMP phosphodiesterase has also been reported (Pizarro *et al.*, 1981). In order to make an initial assessment of the quantitative contribution of this putative Ca^{2+} /calmodulin-dependent phosphodiesterase to cyclic AMP phosphodiesterase activity in unfractionated mammary-tissue extracts, the hydrolysis of 100 μ M-cyclic AMP in control and EGTA-treated homogenates was compared. Contrary to expectation, the sequestration of Ca^{2+} by EGTA resulted in a stimulation [from 73.02 $\pm$ 1.54 (6) to 82.05 $\pm$ 2.9 (5) munits/g of tissue] rather than a decrease in cyclic AMP phosphodiesterase activity. This response is explicable in terms of the properties of individual mammary-tissue phosphodiesterases, examined below.

Resolution of multiple cyclic nucleotide phosphodiesterases

When mammary tissue from mid-lactating rats was homogenized and processed in ways reported to maximize the recoveries of phosphodiesterases in soluble form (Thompson *et al.*, 1979b; see the Materials and methods section), the recovery of

total homogenate cyclic AMP phosphodiesterase activity in the final pH6 supernatant was 50% measured at 100 μ M-cyclic AMP and 62% measured at 1 μ M-cyclic AMP (Table 1). DEAE-cellulose column chromatography of this supernatant resolved three peaks of cyclic AMP phosphodiesterase activity, as shown in Fig. 2. The first two peaks eluted with the ionic-strength gradient (labelled I and II, Fig. 2) also hydrolysed cyclic GMP; the third (peak III) hydrolysed only cyclic AMP.

High-affinity cyclic AMP-specific phosphodiesterase

The $K_{0.5}$ determined for cyclic AMP with peak-III phosphodiesterase was 1.06 μ M (mean of two determinations from Eadie-Hofstee plots). This value is in the range of those found for high-affinity forms of cyclic AMP phosphodiesterase in a variety of tissues (Beavo *et al.*, 1982), and this is clearly comparable with that of the high-affinity cyclic AMP phosphodiesterase activity found in unfractionated mammary homogenates. There was no evidence from kinetic data of the positive co-operativity reported (see, e.g., Russell *et al.*, 1972) for some phosphodiesterases of this general type.

Ca^{2+} /calmodulin-dependent phosphodiesterase

Peak-I phosphodiesterase was 5.8-fold ($\pm$ 0.7, $n = 3$) more active (at $V_{\max}$) with cyclic GMP than with cyclic AMP as substrate. The half-saturation constant for cyclic GMP was 2.1 $\pm$ 0.42 μ M (5); that for cyclic AMP was similar under the standard conditions of assay. Ca^{2+} and calmodulin affected this activity by increasing $V_{\max}$. 4-fold (average of four observations) without changing the $K_{0.5}$ for cyclic GMP [2.2 $\pm$ 0.65 μ M (4)] in the presence of Ca^{2+} and calmodulin: activity with cyclic AMP as substrate was too low to allow a detailed examination of the effects of Ca^{2+} and calmodulin.

In the presence of a 2000-fold excess of calmodulin, the Ca^{2+} -dependence of peak-I cyclic

Fig. 2. DEAE-cellulose chromatography of cyclic nucleotide phosphodiesterases in rat mammary-tissue extract. A sample (10 ml; 24.5 mg of protein) of the pH 6 supernatant fraction prepared from rat mammary-tissue homogenate was chromatographed on a column (16 mm diam. $\times$ 150 mm) of DEAE-cellulose as described in the Materials and methods section. Fractions were assayed for phosphodiesterase activities with, as substrates, 1 μ M-cyclic GMP ($\blacktriangle$), 1 μ M-cyclic AMP ($\bullet$) or 100 μ M-cyclic AMP ($\circ$). The concentration gradient of sodium acetate is indicated by the broken line and A_{280} by the continuous line. Fractions were pooled, as shown, into peaks I, II and III.

GMP phosphodiesterase was as shown in Fig. 3: the Ca^{2+} concentration required to effect half-maximal stimulation of activity was 6.3 μ M (mean of three determinations). This is at the upper extremity of the range of values reported (see, e.g., Cox *et al.*, 1981) for mammalian Ca^{2+} /calmodulin-dependent phosphodiesterases.

No marked changes in the cyclic AMP phosphodiesterase activities of peak II or peak III (to either $K_{0.5}$ or $V_{\max}$) were brought about by calmodulin in the presence of Ca^{2+} .

The general properties of this Ca^{2+} /calmodulin-dependent phosphodiesterase suggest a similarity to the 'high-affinity' forms of this enzyme reportedly present in liver plasma membranes (Smoake *et al.*, 1981) and immature testis cytosol (Purvis *et al.*, 1981), although the relative $V_{\max}$ values of these enzymes for hydrolysis of cyclic AMP and cyclic GMP were not reported. It is clearly distinct from both 'heart-type' and 'brain-type' enzymes

(Beavo *et al.*, 1982) on account of its higher apparent affinity for both cyclic AMP and cyclic GMP, and its greater maximum activity with cyclic GMP relative to cyclic AMP.

Peak-I phosphodiesterase appears to be unrelated to the 'activator-deficient' preparation from rat mammary tissue described by Pizarro *et al.* (1981). This latter preparation had much lower affinities for cyclic AMP and cyclic GMP than did peak-I enzyme, responded to calmodulin by increasing both its $V_{\max}$ and its $K_{0.5}$ for cyclic GMP, and was apparently capable of up to 50-fold activation by calmodulin. The magnitude of this response to calmodulin, taken together with the observed effect (this study; see above) of EGTA on the total cyclic nucleotide phosphodiesterase activity in mammary homogenates, suggest that the activity investigated by Pizarro *et al.* (1981) made only a very minor quantitative contribution to total mammary tissue phosphodiesterase: this conclu-

Fig. 3. Cyclic GMP phosphodiesterase activity of peak I: dependence on concentration of Ca^{2+}

Peak I was assayed for phosphodiesterase activity with $1 \mu\text{M}$ -cyclic GMP at various concentrations of free Ca^{2+} which were buffered with EGTA (final concn. 5 mM), and calculated by using an iterative computer program and dissociation constants of 3.23×10^{10} and 1.62×10^5 for CaEGTA and MgEGTA (modified from Smith & Martell, 1976) respectively. Data points are mean values (n = number of determinations); error bars indicate S.E.M. Calmodulin stimulation is expressed as a percentage of the activity in the absence of calmodulin at each concentration of free Ca^{2+} . The 'minus calmodulin' activities were, relative to 100% at 1 nM free Ca^{2+} : 100% (4) at $0.106 \mu\text{M}$ - Ca^{2+} , $96 \pm 2\%$ (4) at $1.2 \mu\text{M}$ - Ca^{2+} , $95 \pm 3\%$ (3) at $3.2 \mu\text{M}$ - Ca^{2+} , $108 \pm 13\%$ (3) at $5.9 \mu\text{M}$ - Ca^{2+} , $124 \pm 16\%$ (4) at $9.1 \mu\text{M}$ - Ca^{2+} and $50 \pm 25\%$ (4) at $100 \mu\text{M}$ - Ca^{2+} .

sion is supported by our inability to identify any such activity in the eluate of DEAE-cellulose columns such as that in Fig. 2.

Cyclic GMP-stimulated cyclic AMP phosphodiesterase

Apart from a minor high-affinity cyclic AMP phosphodiesterase component in peak II, the predominant activity in these fractions had a lower apparent affinity for cyclic AMP [$K_{0.5}$ $18.7 \pm 0.9 \mu\text{M}$ (5)] and an affinity for cyclic GMP ($K_{0.5}$) of $19 \mu\text{M}$ (mean of three determinations). V_{max} values for cyclic GMP hydrolysis relative to cyclic AMP were variable between preparations, as discussed below.

There was no evidence, from kinetic data, of a second 'high-affinity' cyclic GMP phosphodiesterase component in peak II, suggesting that the high-affinity cyclic AMP phosphodiesterase activity in these fractions was due to incomplete resolution between peaks II and III (see Fig. 2) resulting in some spill-over into peak-II fractions of peak-III activity, although alternative explana-

Fig. 4. Cyclic AMP phosphodiesterase activity of peak II: effect of cyclic GMP

Peak II was assayed for phosphodiesterase activity with a range of concentrations (5 – $100 \mu\text{M}$) of cyclic AMP in the absence ($\bullet$) and in the presence ($\circ$) of $1 \mu\text{M}$ -cyclic GMP. Activity was expressed relative to that measured, in each series of determinations, at $100 \mu\text{M}$ -cyclic AMP in the absence of cyclic GMP. The points are means of two determinations in two complete paired experiments. More determinations were available at selected substrate concentrations: e.g. at $10 \mu\text{M}$ -cyclic AMP, the relative activities in the absence and presence of $1 \mu\text{M}$ -cyclic GMP were $29.5 \pm 2.4\%$ (5) and $49.1 \pm 1.8\%$ (3) respectively.

tions, based either on the existence of multiple 'high-affinity' cyclic AMP phosphodiesterases, or on co-operative behaviour (see below), may be applicable.

The rate of hydrolysis of cyclic AMP by peak-II phosphodiesterase was unchanged by the presence of $1 \mu\text{M}$ -cyclic GMP when measured at $100 \mu\text{M}$ -cyclic AMP. However, hydrolysis of $10 \mu\text{M}$ -cyclic AMP was markedly stimulated by $1 \mu\text{M}$ -cyclic GMP, and a more complete analysis yielded a linear double-reciprocal plot in the presence of $1 \mu\text{M}$ -cyclic GMP ($K_{0.5}$ for cyclic AMP $14.7 \mu\text{M}$; mean of two determinations), but a non-linear plot in its absence. In Fig. 4 the data from two such analyses are presented as velocity-versus-substrate-concentration plots: in the presence of $1 \mu\text{M}$ -cyclic GMP, an apparently hyperbolic curve results, whereas in its absence a sigmoid character is evident. Such kinetic behaviour has been observed previously for cyclic GMP-stimulated cyclic nucleotide phosphodiesterases (Terasaki & Appleman, 1975; Martins *et al.*, 1982). In these cases it was attributed to positive co-operative interaction between catalytic sites (relatively non-selective for either cyclic AMP or cyclic GMP) and to the abolition of co-operativity on selective binding of cyclic GMP to a high-affinity allosteric site.

Because of the non-linear character of reciprocal

plots for cyclic AMP hydrolysis by peak-II enzyme in the absence of cyclic GMP, the $K_{0.5}$ values quoted above were derived not as elsewhere in the present work by extrapolation of double-reciprocal or Eadie-Hofstee plots, but by estimating, from plots of v versus $[S]$ (as in Fig. 4), the substrate concentration resulting in a velocity that was one-half that achieved at 100 μM -cyclic AMP.

A further property of peak-II phosphodiesterase was that cyclic nucleotide hydrolysis was stimulated by EGTA and inhibited by Ca^{2+} (Table 2). EGTA stimulation of cyclic GMP hydrolysis was marked both at saturating (100 μM) and sub-saturating (1 μM) concentrations of substrate. In contrast, the effect was present (but smaller in extent) at 100 μM -cyclic AMP but absent at 1 μM -cyclic AMP. The two experiments documented in Table 2 exemplify the extremes that we have encountered among preparations of peak-II phosphodiesterase of what appears to be a gradation in the ratio between basal rates of hydrolysis of cyclic AMP and cyclic GMP. For the preparation used in Expt. 1, this ratio was 9:1 at 100 μM cyclic nucleotide and 3:1 at 1 μM cyclic nucleotide, whereas for the preparation in Expt. 2, both corresponding values were close to unity. The extent by which EGTA was able to stimulate cyclic GMP hydrolysis increased roughly in proportion to this ratio; the experiments tabulated illustrate this effect. For this reason, the mean percentage increases recorded for the EGTA-treated samples hydrolysing cyclic GMP (Table 2) are subject to much wider variability, and hence much larger

standard errors, than the other mean percentage values given.

The data in Table 2 show that the inhibitory response of peak-II phosphodiesterase to Ca^{2+} was already saturated in the preparations obtained after DEAE-cellulose chromatography; no further inhibition of cyclic GMP activity was achieved by the addition of 100 μM - CaCl_2 . Although no Ca^{2+} was added at any stage to the preparation, no steps were taken to effect its rigorous removal. The inevitable contamination of the initial homogenate with traces of milk ensures that some Ca^{2+} was present at this stage, and its presence may have persisted through to later stages in the preparation. Preliminary experiments to assess the dependence of inhibition of peak-II enzyme on the concentration of free Ca^{2+} indicate that a half-maximal effect is achieved at a buffered free Ca^{2+} concentration around 10 μM . Mixing experiments using peak-I enzyme, Ca^{2+} and boiled samples of fractions from DEAE-cellulose column separations such as that in Fig. 2 have demonstrated the presence of a calmodulin-like activator in those fractions comprising peak II (results not shown), although free calmodulin probably is eluted only at much higher salt concentrations under these conditions: this finding is similar to that by others (Coquil, 1983; Donnelly, 1978). Since calmodulin is likely to alter the elution position from DEAE-cellulose of calmodulin-dependent enzymes, the possibility cannot be excluded that some calmodulin-dependent phosphodiesterase was present in peak II associated with calmodulin, as well as in

Table 2. *Effects of EGTA and Ca^{2+} on the cyclic nucleotide phosphodiesterase activity of peak II*
Activity was measured at 100 μM and 1 μM concentrations of cyclic AMP and cyclic GMP. EGTA (5 mM) or CaCl_2 (100 μM) was included in the assays where indicated. The results of two complete experiments are given both as absolute rates of activity and as percentages of the rate, for each substrate and each concentration, under unsupplemented assay conditions. Additionally, mean percentages $\pm$ s.e.m. (number of determinations) are given: these are pooled from values obtained in the experiments tabulated plus those from several additional experiments. Control experiments (not shown), in which both 5 mM-EGTA and 100 μM - CaCl_2 were added (free Ca^{2+} concn. approx. 1 nM), gave the same results as with EGTA alone.

		Cyclic nucleotide hydrolysis							
		Cyclic AMP				Cyclic GMP			
		100 μM		1 μM		100 μM		1 μM	
Additions	to assay	($\mu\text{units/ml}$)	(%)	($\mu\text{units/ml}$)	(%)	($\mu\text{units/ml}$)	(%)	($\mu\text{units/ml}$)	(%)
Expt. 1	None	511	100	10.9	100	56	100	3.5	100
	EGTA	650	127	7.1	65	271	481	13.1	374
	CaCl_2	546	107	11.9	109	58	103	4.7	134
Expt. 2	None	309	100	12.5	100	362	100	13.8	100
	EGTA	862	279	11.3	90	759	210	25.5	185
	CaCl_2	557	180	12.1	97	361	100	21.2	154
Pooled values	EGTA	179 $\pm$ 25 (5)		76 $\pm$ 6 (3)		362 $\pm$ 101 (5)		306 $\pm$ 63 (4)	
	CaCl_2	133 $\pm$ 19 (3)		103 $\pm$ 4 (2)		102 $\pm$ 1 (2)		144 $\pm$ 7 (2)	

the calmodulin-free form in peak I (see results above). Thus the phosphodiesterase activity of peak II, as documented above, was necessarily always measured in the presence of this endogenous calmodulin. It therefore remains to be determined whether the effects of Ca^{2+} on peak-II enzyme are calmodulin-mediated. These effects do, however, provide an explanation for the observed stimulation by EGTA of cyclic AMP ($100\text{ }\mu\text{M}$) hydrolysis in mammary-tissue homogenates. Although Ca^{2+} chelation will cause an inhibition of peak-I activity in crude homogenates, a net stimulation will nevertheless result, owing to the much greater relative contribution from peak-II than from peak-I enzyme (see Fig. 2) to hydrolysis of $100\text{ }\mu\text{M}$ -cyclic AMP in these unfractionated preparations.

It is noteworthy that an EGTA stimulation of peak-I cyclic GMP phosphodiesterase activity was found when this was measured in the absence of calmodulin (see Fig. 3). The magnitude of this effect between free Ca^{2+} concentrations of $100\text{ }\mu\text{M}$ and approx. 1 nM (5 mM -EGTA) was comparable (approx. 2-fold) with that observed, between the same limits of Ca^{2+} concentration, for peak-II activity (Table 2). Despite the possible presence of some peak-I-type enzyme in peak-II (discussed above), it seems unlikely that this would explain the effects of EGTA on peak-II phosphodiesterase activity that we have described, since these were observed in the presence of calmodulin, under which conditions EGTA caused a decline in peak-I-type cyclic AMP phosphodiesterase activity relative to that measured at $100\text{ }\mu\text{M}$ - Ca^{2+} (Fig. 3).

Physiological significance

The general features of the lactating-mammary-tissue profile of cyclic nucleotide phosphodiesterase activities resemble those of the liver and are, perhaps not surprisingly, dissimilar to those of excitable tissues (Arch & Newsholme, 1976). The three widespread phosphodiesterases (Beavo *et al.*, 1982), namely Ca^{2+} /calmodulin-dependent cyclic GMP phosphodiesterase, cyclic GMP-binding cyclic nucleotide phosphodiesterase and high-affinity cyclic AMP phosphodiesterase, clearly correspond broadly to peak-I, -II and -III activities characterized above.

The possible roles of high- and low-affinity forms of cyclic nucleotide phosphodiesterase in regulating intracellular cyclic AMP have been considered by Fell (1980). It is implicit in his treatment that a detailed knowledge of the subcellular distribution of the various cyclic nucleotide phosphodiesterases is a prerequisite for understanding their biological roles. No information is currently available regarding the subcellular localization of the phosphodiesterases characterized

here; thus detailed conclusions about their possible physiological significance would be premature.

The concentrations of cyclic AMP and cyclic GMP in mammary tissue of 10-day-lactating rats have been determined (Sapag-Hagar & Greenbaum, 1974a) to be 0.45 and $0.25\text{ }\mu\text{M}$ respectively. Thus none of the enzyme activities characterized here would be saturated with their substrates within the cell (disregarding compartmentation of substrate) and all would therefore increase their rate of catalysis in response to increases in substrate concentration. Similarly, changes in $K_{0.5}$ (and also, inevitably, of V_{max}) could effect changes in the rate of cyclic nucleotide hydrolysis *in vivo* and hence be of potential regulatory significance.

The responses of peak-I and peak-II enzymes to Ca^{2+} have the effect of antagonizing one another at different Ca^{2+} concentrations. Thus, at around $5\text{ }\mu\text{M}$ - Ca^{2+} , peak-I enzyme is stimulated, leading to a decrease in intracellular cyclic GMP. At higher concentrations of Ca^{2+} , peak-II cyclic GMP phosphodiesterase is inhibited, leading to an increase in intracellular cyclic GMP. Depending on the binding affinity of the allosteric site on the peak-II enzyme for cyclic GMP (which we have not measured), these changes in cyclic GMP concentration may lead to reciprocal changes in the concentration of cyclic AMP, mediated by the stimulation by cyclic GMP of cyclic AMP hydrolysis by peak-II enzyme. Such reciprocal changes in cyclic nucleotide concentrations have been observed during pregnancy and lactation in rat mammary tissue (Sapag-Hagar & Greenbaum, 1974a). It remains to be determined whether similar changes occur rapidly in mammary tissue in response to acute stimuli and also whether changes in the concentration of cytosolic Ca^{2+} in the range covering the Ca^{2+} sensitivities of peak-I and peak-II phosphodiesterase accompany such stimuli.

References

- Agius, L. & Williamson, D. H. (1980) *Biochem. J.* **192**, 361–364
- Arch, J. R. S. & Newsholme, E. A. (1976) *Biochem. J.* **158**, 603–622
- Baldwin, R. L. & Yang, Y. T. (1974) in *Lactation: A Comprehensive Treatise* (Larson, B. L. & Smith, V. R., eds.), vol. 1, pp. 349–411, Academic Press, New York and London
- Beale, E. G., Dedman, J. R. & Means, A. R. (1977) *J. Biol. Chem.* **252**, 6322–6327
- Beavo, J. A., Hansen, R. S., Harrison, S. A., Hurwitz, R. L., Martins, T. J. & Mumby, M. C. (1982) *Mol. Cell. Endocrinol.* **28**, 387–410
- Brownsey, R. W. & Hardie, D. G. (1980) *FEBS Lett.* **120**, 67–70
- Brownsey, R. W., Hughes, W. A. & Denton, R. M. (1979) *Biochem. J.* **184**, 23–32

- Coquil, J. F. (1983) *Biochim. Biophys. Acta* **743**, 359–369
- Cox, J. A., Malnoš, A. & Stein, E. A. (1981) *J. Biol. Chem.* **256**, 3218–3222
- Donnelly, T. E., Jr. (1978) *Biochem. Biophys. Res. Commun.* **82**, 964–970
- Dousa, T. & Rychlik, I. (1968) *Life Sci.* **7**, 1039–1044
- Fell, D. A. (1980) *J. Theor. Biol.* **84**, 361–385
- Hardie, D. G. & Guy, P. S. (1980) *Eur. J. Biochem.* **110**, 167–177
- Hofstee, B. H. J. (1952) *Science* **116**, 329–331
- Lin, Y. M., Piu, Y. P. & Cheung, W. Y. (1974) *J. Biol. Chem.* **249**, 4943–4954
- Louis, S. L. & Baldwin, R. L. (1975) *J. Dairy Sci.* **58**, 861–869
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Majumder, G. C. & Turkington, R. W. (1971) *J. Biol. Chem.* **246**, 5545–5554
- Manganiello, V. C., Murad, F. & Vaughan, M. (1971) *J. Biol. Chem.* **246**, 2195–2202
- Martins, T. J., Mumby, M. C. & Beavo, J. A. (1982) *J. Biol. Chem.* **257**, 1973–1979
- Mayer, R. J. (1978) *Vitam. Horm. (N.Y.)* **36**, 101–163
- McNeillie, E. M. & Zammit, V. A. (1982) *Biochem. J.* **204**, 273–280
- McNeillie, E. M., Clegg, R. A. & Zammit, V. A. (1981) *Biochem. J.* **200**, 639–644
- Munday, M. R. & Williamson, D. H. (1982) *FEBS Lett.* **138**, 285–288
- Nimmo, H. G. & Cohen, P. (1977) *Adv. Cyclic Nucleotide Res.* **8**, 145–266
- Pilkis, S. J., Claus, T. M., Johnson, R. A. & Park, C. R. (1975) *J. Biol. Chem.* **250**, 6328–6336
- Pizarro, M., Puente, J. & Sapag-Hagar, M. (1981) *FEBS Lett.* **136**, 127–130
- Plucinski, T. M. & Baldwin, R. L. (1982) *Endocrinology (Baltimore)* **111**, 2062–2065
- Purvis, K., Olsen, A. & Hansson, V. (1981) *J. Biol. Chem.* **256**, 11434–11441
- Riss, T. L. & Baumrucker, C. R. (1982) *J. Dairy Sci.* **65**, 1722–1728
- Robinson, A. M. & Williamson, D. H. (1977) *Biochem. J.* **168**, 465–474
- Robinson, A. M., Girard, J. R. & Williamson, D. H. (1978) *Biochem. J.* **176**, 343–346
- Robson, N. A., Clegg, R. A. & Zammit, V. A. (1984) *Biochem. J.* **217**, 743–749
- Russell, T. R., Thompson, W. J., Schneider, F. W. & Appleman, M. M. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1791–1795
- Sapag-Hagar, M. & Greenbaum, A. L. (1974a) *FEBS Lett.* **46**, 180–183
- Sapag-Hagar, M. & Greenbaum, A. L. (1974b) *Eur. J. Biochem.* **47**, 303–312
- Smith, R. M. & Martell, A. E. (1976) *Critical Stability Constants*, vols. 1–4, Plenum Press, New York
- Smoake, J. A., Johnson, L. S. & Peake, G. T. (1981) *Arch. Biochem. Biophys.* **206**, 331–335
- Terasaki, W. L. & Appleman, M. M. (1975) *Metab. Clin. Exp.* **24**, 311–319
- Thompson, W. J. & Appleman, M. M. (1971) *Biochemistry* **10**, 311–316
- Thompson, W. J., Brooker, G. & Appleman, M. M. (1974) *Methods Enzymol.* **38**, 205–212
- Thompson, W. J., Epstein, P. M. & Strada, S. J. (1979a) *Biochemistry* **18**, 5228–5237
- Thompson, W. J., Terasaki, W. L., Epstein, P. M. & Strada, S. J. (1979b) *Adv. Cyclic Nucleotide Res.* **10**, 69–92
- West, D. W. & Clegg, R. A. (1981) *Biochem. Soc. Trans.* **9**, 137P
- Wilde, C. J. & Kuhn, N. J. (1981) *Int. J. Biochem.* **13**, 311–316