Cyclic AMP-dependent protein kinase in rat mammary tissue: expression of catalytic and regulatory subunits throughout pregnancy and lactation

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‘Expressed’ and ‘total’ activities of cyclic AMP-dependent protein kinase (PK-A) were measured in extracts of rat mammary tissue sampled throughout pregnancy and lactation. Expression of the genes encoding the catalytic subunit (C-subunit) isoforms C_α and C_β was examined by Northern blotting, as a function of mammary development, to determine relative levels of their respective mRNAs. The content of C-subunit protein (all isoforms) was estimated immunochemically and related to levels of C-subunit catalytic activity and of mRNAs. It was found that C-subunit isoform mRNAs are expressed co-ordinately during mammary development and that a marked decline in expression, per cell, at around parturition is paralleled by a fall in ‘total’ PK-A activity. The ‘expressed’ activity of PK-A activity underwent

characteristic changes throughout pregnancy and lactation, reaching a peak late in pregnancy. The PK-A activity ratio reached a peak in early lactation. C-subunit protein mass closely paralleled ‘total’ PK-A activity throughout pregnancy and lactation, thereby demonstrating the constancy of C-subunit specific catalytic activity during these developmental events. Regulatory subunits (R-subunits) were probed with the photoaffinity label 8-azido-[32 P]cAMP. The abundance of R-II as a proportion of total R-subunit increased throughout pregnancy and lactation, and quantitative analysis of the photoaffinity labelling suggested inconstancy in the ratio of R:C subunits, with highest values occurring in late pregnancy/early lactation.

INTRODUCTION

Cyclic AMP (cAMP)-dependent protein kinase (PK-A) is ubiquitous in its distribution, but fulfils a variety of different functions within cells. Its role in acute regulation of metabolism via the phosphorylation of key rate-determining enzymes is abundantly documented (e.g. [1]). Further major roles in the regulation of growth and development in a variety of biological systems are suggested by diverse reports [2–6].

In mammals throughout the course of pregnancy, parturition and lactation, mammary tissue progresses through a programme of proliferation and differentiation; the terminal differentiation events enable expression of the tissue’s physiological functions of biosynthesis and secretion. This terminal differentiation occurs around parturition in the rat, and does not correlate with the proliferative expansion of mammary epithelial cell numbers [7].

Previous evidence from ourselves [8,9] and others [10,11] has indicated that the acute regulatory functions of PK-A are modified in the mammary tissue from lactating animals. Inhibitory effects on the activity of acetyl-CoA carboxylase and the associated changes in the phosphorylation level of serine-79 are absent. In other tissues, these changes to acetyl-CoA carboxylase are initiated via cAMP-dependent processes, with the phosphorylation state of serine-79 of acetyl-CoA carboxylase being ultimately determined by the balance of activities of AMP-activated kinase and the protein phosphatase responsible for its dephosphorylation, probably PrP 2A [12]. It is not known whether this aberrant regulation of mammary tissue acetyl-CoA carboxylase is also characteristic of pregnant animals, because the level of expression of the enzyme is so low that the necessary analytical data cannot be obtained. One possibility to explain

these altered responses is that PK-A itself has distinctive properties in mammary tissue; some evidence has been reported in support of this [11].

We have previously shown that PK-A, and other elements of the cAMP signalling system, are functionally intact in mammary tissue [8,9]; furthermore, AMP-activated protein kinase has been characterized and partially purified from rat mammary tissue [13], lending no support to the possibility that changes in its properties account for the resistance of mammary acetyl-CoA carboxylase to cAMP-initiated phosphorylation. We have also described the sequestration of a small fraction of PK-A catalytic subunit (C-subunit) molecules to sedimentable intracellular structures in rat mammary epithelial cells following elevation of intracellular cAMP, and the concomitant enhancement of specific activity of this sedimentable fraction of C-subunit [14], thereby demonstrating a capacity for novel regulatory properties of this enzyme.

In the present study our objectives were, firstly, to assess whether expression of PK-A generic C-subunit and of individual regulatory subunits (R-subunits: R-I and R-II) is developmentally regulated in rat mammary tissue. Three distinct isoforms of C-subunit (α , β and γ) have been described [15–17], although the functional significance of this diversity is not understood. Our second objective was to determine whether selective expression of C-subunit isoforms occurs during pregnancy or lactation. Thirdly, we wished to measure expressed activity of mammary tissue PK-A during pregnancy and lactation and to relate functional changes in mammary PK-A to the developmental/proliferational state of the tissue.

Both the ‘total’ PK-A activity, measurable in the presence of excess cAMP, and its ‘expressed’ activity, representing dis-

Abbreviations used: cAMP, cyclic AMP; PK-A, cAMP-dependent protein kinase; C-subunit, catalytic subunit of PK-A; R-subunit, regulatory subunit of PK-A; P4 etc., pregnant, 4 days after conception; L4 etc., lactating, 4 days after parturition.

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sociated free C-subunit molecules in the tissue at the time of sampling [18], have been measured throughout pregnancy and lactation. We have also probed the expression of C_α and C_β genes in rat mammary tissue by Northern blotting of their mRNA transcripts, enabling correlation of the steady-state level of these mRNAs and of total C-subunit catalytic activity with C-subunit protein mass. The latter was determined by an immunochemical method, which we have customized to deal with the known propensity of free C-subunit, at low concentrations, to adhere to surfaces of tubes, pipette tips and other labware. Finally, we have used the photoaffinity label 8-azido-[^{32}P]cAMP to examine mammary tissue extracts for their content of R-subunits and other cAMP-binding proteins. A preliminary account of some of these experiments has been reported [19].

EXPERIMENTAL

Animals and biological preparations

Female Wistar rats in their first pregnancy or lactation were housed at a constant temperature (22 °C) and in an imposed diurnal cycle with the light period from 08:00 to 20:00 h. Food (CRM pellets) and water were available *ad libitum*. Mammary tissue was freeze-clamped at between 09:00 and 09:30 h and sampled by using techniques described previously [9,20]; extracts were prepared [9] for the assay of PK-A activity, DNA and protein. Total cellular RNA was isolated from mammary gland by using guanidinium thiocyanate and CsCl centrifugation [21].

C-subunit was purified from mammary tissue of pregnant or lactating sheep (Finn-Dorset Horn crossbreeds) by the method of Flockhart and Corbin [22]. Typically, 200 g of tissue yielded 450 μg of enzyme protein. ^{32}P labelling of C-subunit was achieved by autophosphorylation of the pure protein in the presence of [γ - ^{32}P]ATP; C-subunit was re-isolated and separated from other radioactive products by ion-exchange chromatography on CM-Sepharose.

Assays and analytical methods

PK-A activity was measured with Kemptide as substrate [23]; 1 unit of activity is that which catalyses the transfer of 1 μmol of phosphate from the γ -position of ATP to Kemptide/min under conditions defined in [9]. Details of the Northern blotting and hybridization procedures have been previously described [24]. For the present experiments, the cDNA probe for the α -isoform of rat PK-A C-subunit and the riboprobe for the rat β -isoform (both described in [25]) and rat α -casein cDNA were used. Autoradiographic signals from the Northern blots were integrated by scanning densitometry. Correction factors were applied to the densitometric data from a single blot to adjust for differences in the actual loading of 28 S rRNA in each electrophoretic track as previously described [24]. After assay of tissue DNA content by a sensitive fluorimetric method [26], the resulting corrected levels of C-subunit mRNAs were then calculated on per-mg-of-DNA basis.

The radiolabelled photoaffinity ligand 8-azido-[^{32}P]cAMP was used approximately as described by Walter and Greengard [27] to detect cAMP-binding proteins in the same mammary tissue extracts as were used for the C-subunit analyses. Samples (containing 50 μg of extract protein) were incubated at room temperature in the dark in buffer [27] containing 0.15 mM benzamidine/HCl, 0.15 mM phenylmethanesulphonyl fluoride, 0.15 $\mu\text{g}/\text{ml}$ soya-bean trypsin inhibitor and 0.6 μM 8-azido-[^{32}P]cAMP (50 Ci/mmol) for 60 min. Reaction mixtures were

then irradiated for 60 s at 20 cm from a 15 W long-wavelength u.v. lamp. After conventional disaggregation in SDS-containing buffer, samples with equal quantities of extract protein were loaded and electrophoresed on Laemmli SDS/8%-polyacrylamide gels. Radiolabelled bands were located by autoradiography of dried gels. The extent of ^{32}P -ligand binding was assessed by cutting out relevant bands from the dried gel and determining ^{32}P by scintillation counting.

Immunochemical methods

The polyclonal antibody (ab365) used for immunochemical recognition of C-subunit was raised in sheep against a synthetic peptide (see below) corresponding to residues 322–332 of the bovine C-subunit α -isoform (R. A. Clegg and J. Beattie, unpublished work). Since this sequence of predominantly polar amino acids is conserved in the α , β and γ isoforms of C-subunit [15–17], this antiserum is a generic anti-C-subunit, with no selectivity between individual isoforms.

Immunological quantification of C-subunit was by the following procedure; all operations were at 0–4 °C. Freeze-clamped cryopulverized mammary tissue (1 part by wt.) was homogenized with a Polytron for 2×10 s at setting 4 (22000 rev./min), in 4 parts by vol. of buffer (10 mM Mes/NaOH, 1.2 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol, 0.5 mM isobutylmethylxanthine, pH 6.8, containing protease inhibitors: benzamidine/HCl, 0.1 mM; phenylmethanesulphonyl fluoride, 0.1 mM; soya-bean trypsin inhibitor, 1 $\mu\text{g}/\text{ml}$; and phosphatase inhibitors: NaF, 10 mM; sodium pyrophosphate, 0.25 mM; sodium orthovanadate, 10 μM) which was supplemented with 4 μM cAMP immediately before use. Gross particulate material was removed by centrifugation at 8800 *g* for 15 min. To 0.5 ml of the resulting supernatant was added BSA (final concn. 1%, w/v) and 10 μl of a solution of ^{32}P -labelled C-subunit (0.043 μg of protein; 6100 c.p.m.) to act as an internal standard, reporting recovery throughout subsequent stages of the procedure. The sample was passed through 5 μm and then 0.45 μm filters and loaded on to a column (1 ml bed volume) of CM-Sepharose equilibrated in 10 mM Mes/NaOH/0.2 mM EDTA/0.1 mM dithiothreitol/0.5 mM isobutylmethylxanthine, pH 6.8. Unbound protein was washed from the column with 5×0.5 ml of the same buffer. Bound protein (including C-subunit) was eluted with 5×0.5 ml of 8 M urea/2% SDS/15% glycerol in 125 mM Tris/HCl, pH 6.8. Fractions were collected at all stages and monitored for ^{32}P radioactivity. The most radioactive eluate fraction (usually the second portion) was retained. Replicate 200 μl portions were reduced with 2.5 μl of 2-mercaptoethanol and boiled for 2 min, then loaded and run on SDS/PAGE (8% gels; 14 cm $\times$ 16 cm vertical slabs) in the Laemmli buffer system [28]. At least one lane on each gel was reserved for loading with a sample containing 10 μl of the internal standard ^{32}P -C-subunit; prestained M_r standards were also run on each gel. On completion of electrophoresis, separated proteins were electroblotted from gels on to sheets of nitrocellulose [29], and these were then probed with affinity-purified ab365 (0.2 μg of protein/ml), or non-immune sheep γ -globulin at the same concentration, after suitable blocking and washing. Treatment with second antibody (anti-sheep IgG; 1/1000 dilution of serum raised in donkeys) was followed by exposure of blots to ^{125}I -labelled Protein G (62.5 ng/ml, approx. 1000 c.p.m./ng) and exposed to X-ray film. The positions on the nitrocellulose of radioactive bands corresponding to C-subunit were identified by reference to the autoradiograph and excised (together with control bands of the same dimensions); these were counted for ^{32}P (by Čerenkov counting) and then for ^{125}I by conventional γ -radiation counting.

Sources

Biochemicals and related reagents were from Sigma or Boehringer Mannheim unless otherwise specified. Wistar rats were bred from stock originally from A. Tuck (Battlesbridge, Essex, U.K.). CRMX rodent diet was from Special Diet Services (Witham, Essex, U.K.). [γ - 32 P]ATPi (3000 Ci/mmol) was from NEN Research Products (DuPont U.K. Ltd, Stevenage, Herts, U.K.). Na 125 I, [α - 32 P]dCTP (3000 Ci/mmol), [α - 32 P]CTP (400 Ci/mmol) and 0.45 μ m HiBond-C blotting membrane were from Amersham International. 8-Azido-[32 P]cAMP was from ICN Biomedicals. Hydroxyapatite and DEAE-cellulose used in the purification of C-subunit were Bio-Gel HT from Bio-Rad and DE 52 from Whatman respectively. CM-Sephacrose Fast Flow was from Pharmacia LKB Biotechnology. Protein G (recombinant) was from Calbiochem Novabiochem, and was iodinated by the Iodogen (Pierce) method. Pierce also supplied keyhole-limpet haemocyanin and the bifunctional cross-linking reagent maleimidobenzoyl *N*-hydroxysuccinimide ester. Kempptide, PK-A inhibitor peptide and the peptide immunogen described above were prepared with an Applied Biosystems 431A peptide synthesizer by using conventional F-MOC chemistry from precursors supplied by Novabiochem. Peptides were at least 90% homogeneous as determined by reversed-phase h.p.l.c. Northern blot probes for the α and β isoforms of rat PK-A C-subunit were gifts from Professor R. Maurer. The rat α -casein cDNA was given by Professor J. Rosen.

RESULTS

PK-A activity during pregnancy and lactation

Total PK-A activity, expressed on the basis of fresh tissue weight, approximately doubled throughout lactation; much of this increase occurred in the latter part of pregnancy between days 12 and 20 (P12–P20). A plateau of activity was reached by P16 and persisted until L4 (day 4 of lactation after parturition), after which a second incremental phase occurred, resulting in a further doubling of activity between L4 and L20, as illustrated in Figure 1(a). Cellular proliferation and tissue hypertrophy in developing mammary gland proceed in such a way that the tissue DNA content (mg of DNA/g of tissue) increases progressively throughout pregnancy, then more sharply at parturition and into early lactation, before settling to a near-constant level in mid-late lactation. These changes, the pattern and extent of which have been previously reported by others [30–32], are shown in Table 1, together with the developmental changes in mammary tissue protein and RNA content. Combining the data of Figure 1(a) and Table 1 allows expression of total PK-A activity per mg of DNA, giving the profile shown in Figure 1(b). Total PK-A activity is an index of the tissue's total capacity for cAMP-dependent phosphorylation, but is not immediately relevant to intracellular conditions *in vivo*. These are more accurately reflected by the expressed PK-A activity.

Expressed PK-A activity of mammary tissue in pregnancy and lactation is shown in Figure 2(b). It provides a measure of the dissociated C-subunit in the tissue at the moment of sampling, so long as procedures are adopted to prevent post-sampling changes in the association/dissociation status of the R- and C-subunits of PK-A [18]. The methods used in this study have been previously validated in this respect [9]. The activity ratio of PK-A (expressed/total activity; Figure 2a) was generally higher during lactation than pregnancy and reached a peak at L4; it is expected that this pattern will reflect changes in the effective intracellular concentration of cAMP (see, e.g., [9]) throughout pregnancy and

lactation. Published values for the concentration of cAMP in rat mammary tissue (corrected for milk content [33]) show a peak at P20, with values during pregnancy generally higher than those during lactation; discrepancies between these established findings and the present activity ratios may reflect intracellular sequestration of cAMP other than by R-subunits of PK-A, or changes in the specific activity of C-subunit. Alternatively, they may highlight the technical problems inherent in deriving the intra-

Figure 1 'Total' PK-A activity in mammary tissue throughout pregnancy and lactation

Mammary tissue was sampled from rats at defined stages of pregnancy and lactation. Extracts were prepared and assayed for 'total' PK-A activity as described in the Experimental section. Results are means ($\pm$ S.E.M.) of determinations on 6 series of pregnant/lactating animals.

Table 1 Composition of rat mammary tissue throughout pregnancy and lactation

Values (means $\pm$ S.E.M.) are for the paired abdominal/inguinal tissue pads of groups of 6 animals. Determinations were made on the same tissue samples that were used for the remainder of the present study. Total dissected gross weights for this mammary tissue pad in Wistar rats, as used in the present study, do not differ significantly for those reported by Munford [30].

Days pregnant (P) or lactating (L)	Protein (mg/g wet wt.)	DNA (mg/g wet wt.)	RNA (mg/g wet wt.)
P4	18.7 $\pm$ 1.7	0.98 $\pm$ 0.37	0.58 $\pm$ 0.16
P8	26.0 $\pm$ 1.3	1.12 $\pm$ 0.32	0.78 $\pm$ 0.08
P12	30.4 $\pm$ 2.6	1.45 $\pm$ 0.47	1.06 $\pm$ 0.04
P16	38.9 $\pm$ 2.8	1.56 $\pm$ 0.45	1.35 $\pm$ 0.18
P20	32.8 $\pm$ 3.6	1.56 $\pm$ 0.38	1.65 $\pm$ 0.16
L4	62.3 $\pm$ 6.3	3.60 $\pm$ 0.16	4.61 $\pm$ 0.28
L8	76.5 $\pm$ 4.9	3.76 $\pm$ 0.10	6.64 $\pm$ 1.32
L12	78.9 $\pm$ 6.1	3.89 $\pm$ 0.09	6.95 $\pm$ 0.89
L16	87.7 $\pm$ 7.1	3.74 $\pm$ 0.16	8.01 $\pm$ 0.46
L20	94.3 $\pm$ 5.6	3.89 $\pm$ 0.20	6.80 $\pm$ 0.45

Figure 2 'Expressed' PK-A and activity ratio in mammary tissue throughout pregnancy and lactation

Activity was assayed in tissue extracts from the same 6 series of pregnant/lactating animals as were used for the data in Figure 1. Activity ratio is expressed/total activity.

cellular cAMP content of mammary tissue samples when the primary data contain a variable and, in lactation, near-overwhelming contribution from milk cAMP. In other experiments, no indication of a difference in the specific activities intrinsic to C-subunit purified from mammary tissue of pregnant and lactating sheep was found (R. A. Gardner and R. A. Clegg, unpublished work), although the concepts of 'mute' C-subunit in tissue extracts [34] and of altered C-subunit specific activities in distinct subcellular populations [14] have received support in the past. Thus it may be that one or more intracellular modulators of C-subunit activity is differentially active between pregnancy and lactation. Possible changes in the specific catalytic activity of C-subunit *in situ* in mammary tissue during development are assessed below to address this possibility.

The absolute level of expressed activity (per mg of DNA) fell quickly from an initially high value in early pregnancy, then subsequently passed through a peak in late pregnancy (Figure 2b). Whereas total activity (per mg of DNA) underwent a progressive increase from early to late lactation (Figure 1b), expressed activity remained approximately constant throughout this period, at a somewhat lower level than in pregnancy (Figure 2b).

C_{α} and C_{β} mRNA in mammary tissue

C-subunit mRNAs were detected in Northern-blot experiments with isoenzyme-specific radiolabelled probes as shown in Figure 3. The abundance of mRNA for both isoforms (expressed per mg of tissue DNA) reflected the total PK-A activity throughout

Figure 3 Northern-blot analysis of mRNAs encoding C-subunit isoforms α and β in mammary tissue throughout pregnancy and lactation

Samples estimated to contain equal amounts of RNA were loaded into each electrophoretic lane; the intensity of the fluorescent signal from 28 S rRNA in each lane of the ethidium bromide-stained gel (shown above) was used to apply appropriate correction factors for minor variations in RNA loadings. Autoradiographs of blots hybridized with probes specific for C_{α} , C_{β} and rat α -casein are shown above for a single representative series of pregnant/lactating mammary tissue samples. Densitometric analysis of 3 such data sets gave the numerical values (means $\pm$ S.E.M.) plotted in the lower part of the Figure.

Figure 4 Quantification of C-subunit protein mass in mammary tissue throughout pregnancy and lactation

The values plotted were determined in extracts of mammary tissue from a single representative series of pregnant/lactating animals; in each physiological state, 'total' PK-A activity and C-subunit mass were measured in the same extract, as described in the Experimental section.

pregnancy and lactation (compare the lower panel of Figure 3 with Figure 1b). Although there was a decrease in the level of mRNA for both C_{α} and C_{β} at the transition from pregnancy to

Figure 5 R-subunit composition of rat mammary tissue

cAMP-binding subunits were identified by photoaffinity labelling as described in the Experimental section. The autoradiographs in (a) show (from left to right) signals obtained from 'standard' preparations known to contain predominantly R-II and R-I; signals from P12 rat mammary extracts (with or without excess cAMP); and signals from L12 rat mammary extracts (with or without excess cAMP). The positions of M_r standards are indicated at the left-hand side of the panel. (b) Autoradiograph showing the relative abundance of R-I and R-II in extracts of mammary tissue (50 μ g of protein/track) from a representative series of rats throughout pregnancy and lactation; arrows at the left-hand side mark the positions of R-II (upper arrow) and R-I. (c) Quantitative display of the data in (b). In (d), total C-subunit and R-subunits of mammary PK-A are compared throughout pregnancy and lactation for a single representative series of rats. At each developmental stage, assay of C-subunit catalytic activity and photoaffinity labelling of R-subunits were performed on the same tissue extract.

lactation, there was no indication of any change in the ratio of C_α to C_β mRNA during the mammary developmental programme. Although the methods used are not appropriate for determination of the absolute amounts of the two mRNAs, it is clear from the intensity of autoradiographic signals elicited when cDNA probes of roughly equivalent specific radioactivity were used that the mRNA for C_α is considerably more abundant than that for C_β . The pattern of expression of α -casein throughout pregnancy and lactation (Figure 3) contrasted with that of C_α and of C_β and was in close agreement with the results of others [35].

C-subunit protein mass in mammary tissue

Immunological quantification of the C-subunit content in mammary tissue throughout pregnancy and lactation yielded a profile

reflecting that for total PK-A activity (Figure 4). This result allows the conclusion that the specific catalytic activity intrinsic to C-subunit does not change during mammary development. The data in Figure 4 give a mean value ($\pm$ S.E.M.) for specific activity of 37.8 ± 2.4 units/mg of C-subunit protein.

cAMP-binding proteins of mammary tissue

The photoaffinity ligand 8-azido-[³²P]cAMP labelled two major proteins in cytosol fractions from mammary tissue. These migrated on SDS/PAGE as protomers corresponding to the molecular sizes and migration positions of R-I and R-II standards (M_r 45000 and 51000 respectively) which had been subjected to the same experimental procedures (Figure 5a). A minor band at M_r 37000–38000 was also present in the rat mammary samples and in the R-II standard; this may be one of the family of fragments derived from R-subunits by proteolysis at the susceptible hinge region, as originally described by Weldon and Taylor [36]. Radiolabelling of these bands was competed out by excess cAMP (Figure 5a) and was absent when samples were not subjected to u.v. irradiation (results not shown). R-I and R-II are therefore the major cAMP-binding proteins in mammary cytosols. It is furthermore apparent from Figure 5(a) that the relative amounts of R-I and R-II differ markedly between pregnant and lactating rat mammary tissue.

R-subunit abundance during pregnancy and lactation

Figures 5(b) and 5(c) illustrate the progressive increase in the ratio R-II:R-I throughout pregnancy and lactation: in early pregnancy, R-II accounts for only 12% of total R-subunit, whereas in established lactation this rises to 38%. There are pitfalls inherent in inferring R-subunit quantities from these binding data [27], nevertheless, the incorporation of ³²P into R-I subunit reproducibly followed a biphasic pattern of intensity with respect to cytosolic protein, with peaks in mid-pregnancy and mid-lactation (Figure 5b). The combined effect of these changes was to increase ³²P binding to total R-subunit by 5-fold (per g tissue wt.) from early pregnancy to late lactation; correspondence between R-subunit (as reflected by 8-azido-[³²P]cAMP binding) and C-subunit (as reflected by catalytic activity) in mammary tissue extracts is shown in Figure 5(d). This comparison suggests a higher C:R subunit ratio in established lactation than in late pregnancy/early lactation.

DISCUSSION

The expression of C_α - and C_β -subunit mRNA appears to be coordinately regulated throughout pregnancy and lactation, in that their ratio to one another does not change. This pattern of expression, in which C_α -subunit mRNA predominates over that of the C_β -subunit, is characteristic of liver and kidney, but unlike brain, where the abundance of the two mRNAs is roughly equal [16]. Absolute levels of both the C_α - and C_β -subunit mRNAs are repressed during lactation. Furthermore, as C_α - and C_β -subunit mRNA and total C-subunit protein levels parallel each other, this suggests that transcriptional processes are major determinants of C_α and C_β activity.

The mechanism of this repression during lactation is at present unknown; however, it is in sharp contrast with the control of the expression of milk protein genes, which are thought to be positively regulated at the transition from pregnancy to lactation by decreasing progesterone and increasing serum prolactin concentrations.

Whether the factors involved in the induction of milk protein

gene expression from mid-pregnancy to mid-lactation are also responsible for the repression of C-subunit expression is the subject of continuing investigation.

The present finding that overall C-subunit specific activity is unchanged during mammary development does not contradict our earlier report [14] of a substantial increase in the specific activity of a small fraction of the total C-subunit mass associated with sedimentable structures in mammary epithelial cells after their treatment with isoprenaline. The increase in specific activity, documented in that paper, of approx. 10-fold, affecting around 2% of C-subunit molecules, would be 'lost' in the experimental error of the present results. The absolute level of mammary tissue C-subunit protein found in the present study, of approx. 2 µg/g of tissue in lactating animals, is in agreement with that deduced in our earlier work [14], suggesting that mammary tissue C-subunit has a specific activity closely similar to that from bovine heart. Several independent lines of evidence have suggested that PK-A is atypical in its biochemical properties in mammary tissue [8–11]. Our present findings indicate that these properties do not derive from intrinsically distinctive catalytic characteristics of mammary tissue C-subunit.

Cho-Chung and co-workers have repeatedly demonstrated a predominance of R-I in tumour cells derived from mammary tissue and have established a strong correlation between R-I and cellular proliferation [5]. The present data show a steady decrease, throughout normal mammary development, in R-I as a fraction of total R-subunit, which does not reflect changes in the rate of cellular proliferation in the tissue and which continues across established lactation while cell numbers remain approximately constant. In the regenerating rat liver, expression of total R-subunit protein transiently exceeds that of C-subunit before the period of most intense cellular proliferation [37]. A transient over-expression of mammary R-subunit between late pregnancy and early lactation is suggested by the data presented here. Two other major transients coincide at this time: a surge in cellular proliferation and a decline in the expression per cell of C-subunit. Further studies are required to determine whether there are mechanistic as well as temporal linkages between these events.

This work was partly funded by the Scottish Office Agriculture and Fisheries Department. R.A.G. acknowledges receipt of a Co-operative Research Studentship from AFRC. R.A.C. acknowledges receipt (jointly with Dr. M. R. Munday, School of Pharmacy, University of London) of a Linked Research Group award and (jointly with Professor W. R. Miller, Department of Clinical Oncology, University of Edinburgh) of a grant in the Intracellular Signalling Programme, both from AFRC. Kate Roy provided invaluable technical support. We are grateful to Dr. Jim Beattie for help with the synthesis of peptides. Professor Stanley McKnight generously provided riboprobe vectors for mouse C_{α} and C_{β} , which were important in the development of this study, although not used in the experiments reported here. We are grateful to Professor R.

Maurer and Professor J. Rosen for generous gifts of the rat cDNA and riboprobe vectors described in the text.

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