Expression of Protein Kinase-A in the Rat Mammary Gland

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Declaration

All the data were collected by myself between 1992 and 1995, with the exception of the Northern blotting studies (Chapter 4) which were performed by Dr M.Travers and Dr M.Barber. With these exceptions I declare that the work contained in this thesis is my own, undertaken under the supervision of Dr R.A.Clegg and Professor W.R.Miller. No part of this work has been submitted for conbsideration for any other degree or award.

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Summary

Throughout the course of pregnancy, lactation and involution, mammary tissue embarks on a programme of proliferation, differentiation and de-differentiation; the terminal differentiation events enable expression of the tissue's physiological functions of biosynthesis and secretion, while involution leads to reductive remodelling process in which the tissue returns to a less differentiated state. The following study describes how PK-A (cAMP dependent protein kinase) activity and the expression of generic C- and R-subunits are developmentally regulated in the rat mammary gland throughout the pregnancy/lactation/involution cycle.

A polyclonal antibody was raised against a synthetic peptide corresponding to a juxta-C terminal region of the catalytic subunit of PK-A. Immunotitration-inhibition studies were performed, which showed no discernible difference in its potency or titre against mammary tissue C-subunit preparations regardless of the physiological state of the donor animal. Immunoinhibition studies were also performed against mammary tissue sampled throughout pregnancy and lactation. A small proportion of mammary tissue's PK-A activity was found to be non-immunoinhibitable; this appeared to be proportional to the level of total PK-A activity present and independent of the tissues physiological state. Non-immunoinhibitable/non-immunoprecipitable PK-A activity was also demonstrated in preparations of C-subunit purified from pregnant and lactating sheep mammary tissue. It therefore appeared that mammary tissue contained a subpopulation of C-subunit molecules not recognised by the antibody. It was further demonstrated that PK-A C-subunit was susceptible to deactivation by a factor present

within soluble mammary tissue extracts. Deactivation was linear with respect to time, required PK-A dissociation and was temperature sensitive. Deactivation also appeared to be partially inhibited by moderate concentrations of MgATP.

"Expressed" and "total" activities of 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 Cα and Cβ 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 also estimated immunochemically and related to levels of C-subunit catalytic activity and of mRNAs. It was found that C-subunit isoform mRNAs are expressed coordinately during mammary development and that a marked decline in expression, per cell, at around parturition is parallelled by a fall in total PK-A activity. The expressed activity of PK-A underwent characteristic changes throughout pregnancy and lactation, reaching a peak late into pregnancy. The PK-A activity ratio reached a peak in early lactation. C-subunit protein mass closely parallelled total PK-A activity throughout pregnancy and lactation, thereby demonstrating the constancy of C-subunit specific activity during these developmental events. Regulatory subunits (R-subunits) were probed with the photoaffinity label 8-azido-[^{32}P]cAMP; both particulate and cytosolic fractions of mammary tissue were examined. The abundance of RII as a proportion of total R-subunit increased throughout pregnancy and lactation in both cellular compartments. Litter removal from mid-lactating rats abruptly interrupts the expression of differentiated function in mammary tissue. Accompanying this, total and expressed PK-A activities were diminished after 24 and 48h; C-subunit mass also decreased but not in proportion to total activity such that specific activity was reduced

in comparison with nursing rats. These effects were apparently not transcriptionally driven, since the levels of Cα mRNA remained unaltered. Furthermore, these changes were accompanied by a decline in the amount of R-subunit which was proportionally greater than that of the C-subunit. A cAMP-binding protein of 37-38kDa, thought to be derived from the proteolytic cleavage of RI or RII, became more prominent in both tissue fractions following litter removal. The relative abundance of R-subunit isoforms remained unaltered by litter removal in soluble cytosolic tissue extracts; both RI and RII isoforms were coordinately down-regulated. However in the particulate fraction there was an apparent preferential down-regulation of the RI isoform, leading to a noticeable increase in the RII:RI ratio possibly reflecting the association of the RII isoform with AKAPs (A-kinase anchoring proteins).

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List of abbreviations

The abbreviations used throughout this thesis are those described as "accepted" in the instructions to authors of the Biochemical Journal (Biochem.J. (1994) **297**, 1-15.). Exceptions, together with abbreviations used commonly throughout this thesis are as follows:

ATP Adenosine 5' triphosphate

cAMP Adenosine 3', 5'-cyclic monophosphate

BSA Bovine serum albumin

cpm Counts per minute

C-subunit Catalytic subunit of PK-A (see below)

DEAE Diethylaminoethyl

DTT Dithiothreitol

ECL Enhanced chemiluminescence

EDTA Ethylene diamine tetra acetic acid

EGTA Ethylene glycol-bis (β-amino-ethyl ether) N,N,N',N',-tetra acetic acid

g Gram

GDP Guanosine 5'-diphosphate

GTP Guanosine 5'-triphosphate

h Hour

HAP Hydroxy apatite

IBMX 3-isobutyl-methyl xanthine

IgG Immune gamma globulins

K_i Equilibrium dissociation constant of a inhibitory compound

KLH Keyhole limpet haemocyanin

M Molar

MES 2-[N-morpholino] ethane-sulphonic acid

μ (prefix) Micro (10^{-6} x)

m (prefix) Milli (10^{-3} x)

min Minute

MOPS 3-[N-morpholino] propane-sulphonic acid

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate buffered saline

PK-A Cyclic-AMP dependent protein kinase (also known as protein kinase A)

PMSF Phenylmethylsulphonyl fluoride

RI Regulatory subunit of PK-A (see above), type I isoform

RII Regulatory subunit of PK-A (see above), type II isoform

R-subunit Regulatory subunit of PK-A (see above)

SDS Sodium dodecyl sulphate

SEM Standard error of the mean

TBS Tris buffered saline

TCA Trichloroacetic acid

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Chapter 1

Literature review

1.1 Mammary gland development

1.1.1 Introductory comments

The mammary gland is a unique organ, not only in its secretory products but in its ability to complete an entire cycle of growth and differentiation each time it is called upon to provide nutrition for a new set of offspring. In its most fully differentiated state, that of full lactation, the gland possesses vast numbers of lobulo-alveolar structures, which consist principally of specialised secretory epithelial cells, committed to milk formation and secretion and an extracellular matrix (ECM) which maintains the structures in a functional conformation (Walker et al, 1989 and Talhouk et al, 1992). In a single day it may produce considerably more than its own weight of secretion; in energetic terms, as many joules per day as it contains in its own mass (Knight & Peaker, 1982a). A satisfactory lactation, vital to reproductive and in the case of the dairy cow, economic success, can only be expected when the mammary gland has reached a complete state of development, both in terms of the number of cells and in their ability to synthesize and secrete milk.

The mammary gland grows slowly during embryonic and juvenile life and does not mature until the reproductive system begins to function at puberty. Therefore unlike most organs which have completed morphogenesis by the time of birth and whose subsequent development is mainly the enlargement or replication of pre-existing structures, the mammary gland undergoes most of its morphogenesis in the sub-adult and adult animal.

The structural and functional development of the mammary gland may be divided into several stages:

(1) foetal development, development of a rudimentary ductal system at birth.

(2) Growth during puberty, ductal growth and elongation.

(3) Mammogenesis, the growth and differentiation of mammary ductal and alveolar tissue.

(4) Lactogenesis the onset of copious milk secretion around parturition.

(5) Lactation, the continuing production of milk.

(6) Involution, the return of the gland to the less differentiated state at cessation of lactation.

In this chapter the hormonal control of mammary development during pregnancy, lactation and involution will be considered in coordination with other events in the female reproductive cycle. The focus will be upon the hormonal controls as they are understood in the rodent, although comparative studies in other species may also be discussed.

1.1.2. Foetal development

Development begins in the foetus, in which parenchymal structures are derived from the ectoderm (which also gives rise to the skin). This early stage of development leading to the formation of a rudimentary system of ducts at birth, is independent of hormonal influences, although in the male rat, androgens have been shown to suppress ductal growth during the later stages of foetal development. However development does require the presence of stromal tissues, which may provide stimulatory growth factors as well as physical support for the developing parenchymal structures.

1.1.3 Development during puberty

From birth to the beginning of sexual maturity, in most mammalian species the mammary gland consists of a nipple and a rudimentary ductal system. At the onset of puberty in the female, enhanced oestrogen secretion causes further developments and refinement to the ductal system and stromal elements. The critical role of oestrogen in regulating mammary development is demonstrated by the observation that in rodents ovariectomy prior to puberty suppresses mammary development and that this inhibition is reversed by administration of oestrogen (Forsyth, 1989 and 1991). In some species including humans, puberty is also associated with an accumulation of lipids in the fat cells, such that eventually 85% of the mammary gland is fat tissue.

1.1.4 Development during pregnancy

The mammary gland also undergoes cycles of proliferation, differentiation, function and regression during the adult reproductive cycle, with the final stages of secretory lobular development taking place during pregnancy and lactation. This final maturation of the lobulo-alveolar elements, sometimes called terminal differentiation is brought about by the hormonal milieu of pregnancy. Underlining the importance of development during pregnancy, normally referred to as mammogenesis, it has been estimated that 60% of all mammary growth takes place during first pregnancy in the rat (Griffith & Turner, 1961) 78% in the mouse (Brookreson & Turner, 1959) and sheep (Anderson, 1975) and 98% in the hamster (Sinha et al, 1970). The total DNA content of the mammary gland shows an increase throughout pregnancy, indicating growth which continues until day 10 or 12 of lactation (Munford, 1963).

During the first half of pregnancy the gland grows by branching of the ductal system and

budding of the alveoli (reviewed by Daniel & Silberstein, 1987). Thus the parenchyma successively replaces the fat pad or stroma which becomes more and more reduced and disappears completely by the end of pregnancy. Where the first lobuloalveoli form, their lumina are devoid of secretory proteins and remain flattened. Close inspection of the glandular cells during early pregnancy shows poor cytoplasmic organisation. Cells contain a few protein granules enclosed by vacuoles and lipid droplets of various sizes scattered throughout the cytoplasm indicating the onset of secretory activity. Differentiation continues throughout pregnancy with lobulo-alveolar development completed by the second half of pregnancy. The cytoplasm of the secretory cells shows increased activity; enlarged it contains numerous fat globules and Golgi filled with protein granules. The alveolar lumina remain narrow until parturition although filled with protein granules.

1.1.5 Development during lactation

At this stage mammary epithelial cells, having acquired the full complement of intracellular enzymes and proteins, begin to meet the prodigious demands of milk synthesis and secretion. The onset of copious milk secretion or lactogenesis is a gradual process which begins at parturition and, in the rat, normally continues for 20-25 days. Differentiated milk-secreting cells can be histologically identified by their large size and by the polarisation of membrane organelles. They also contain abundant endoplasmic reticulum located basally and Golgi-derived vesicles in the apex of the cell. Evidence of secretory activity is seen as a highly folded apical membrane, fat droplets in the cytosol and casein micelles in secretory vesicles (review by Pitelka & Hamamoto, 1983).

As already discussed above, most mammary gland growth occurs during pregnancy.

However the degree to which growth is complete at parturition and the relative contributions of cell number and the secretory activity to milk yield throughout lactation varies between species (Knight & Peaker, 1982a). In rats, cell proliferation may account for as much as three quarters of the increase in daily milk yield leading up to peak lactation with the remainder being accounted for by greater cellular activity (Knight et al, 1984). Following peak lactation, the volume of milk produced by the mother gradually declines until milk removal ceases and the offspring are finally weaned. The maintenance of milk secretion during the later stages of lactation depends on the number of secretory cells lost, the extent of replacement (if any) and the retention of synthetic capacity of the cells. In the rat decreasing cellular activity is entirely responsible for the decline in milk yield (Knight et al, 1984).

The population of cells responsible for the mammary growth during lactation have not yet been fully defined. Mammary growth may occur as a result of proliferation of all differentiated cells (discussed by Knight & Peaker, 1982a), in which case the dogma that proliferation and differentiation are mutually exclusive may be incorrect. It is possible that cells can de-differentiate in order to proliferate as histological evidence exists, showing the presence of secretory products in mitotic cells (Franke & Keenan, 1979 and Traurig, 1967). Alternatively proliferation may arise from the presence of a stem-cell population (Medina & Smith, 1990), although this latter possibility has not yet been conclusively demonstrated.

1.1.6 Mammary involution

At weaning the mammary gland undergoes a reductive remodelling process termed involution, during which it gradually returns to a less differentiated state. Milk synthesis

ceases and there is a significant collapse of lobulo-alveolar structures (Walker et al, 1992). The process of involution is dominated by two major events: a loss of epithelial cells by programmed cell death and a proteolytic degradation of the extracellular matrix, which ordinarily maintains the alveolar structures in a functional conformation (Talhouk et al, 1992).

The process is rapid in the rat, in which formation of new milk stops within 8-10h of pup removal. The morphological changes that characterise involution are elegantly described by Helminen & Ericson (1968); within 24h of the onset of involution changes in the rough endoplasmic reticulum, Golgi vesicles and secretory vesicles are observed, plus a marked decline in the activity of enzymes associated with milk synthesis. There also follows a marked increase in the activity of free lysosomal enzymes and metalloproteinases (Jones, 1967 and 1968). By 48h there is extensive morphological evidence of autophagic activity by the lysosomes within the epithelial cells accompanied by leucocytic infiltration of the tissue and a substantial decrease in both casein and mRNA and its transcription (Levy, 1963, Greenbaum et al, 1963 and Teyssot & Houdebine, 1981). After 3 days macrophages take over the task of removing fragmented epithelial cells and involution is well on its way to completion.

1.2 Control of mammary growth during pregnancy

The control of mammary gland growth is a complex process involving factors intrinsic to the gland (local factors) and the whole animal (systemic control) as well as external influences such as environment, climate and diet. In vivo mammogenesis requires sex steroids, a lactogenic hormone (either prolactin or placental lactogen) and probably one or more unknown growth factors. In addition interactions between the mammary

epithelial elements and the stroma in which they are situated are clearly of importance although poorly understood. Precisely how all these hormones and growth factors function in a concerted manner to regulate mammary development during pregnancy, remains the subject of continuing investigation.

1.2.1 Hormonal controls

Pregnancy creates a hormonal milieu that triggers extensive development of mammary gland in preparation for lactation. Evidence for multiple hormonal involvement in the induction of mammary growth became available from the classical ablation and replacement experiments performed in the 1950's (Lyons, 1958., Lyons et al, 1958., and Nandi, 1958). These early *in vivo* studies demonstrated the effect of a combination of the hormones oestrogen, progesterone, adrenal steroids, prolactin and growth hormone in inducing lobulo-alveolar development of the mammary gland in triply operated virgin rats (hypophysectomised-ovariectomized-adrenalectomised). Both Nandi and Lyons were able to stimulate ductal proliferation by injection of oestrogen, deoxycorticosterone acetate and growth hormone. However addition of progesterone, prolactin and prednisalone to this regime were required in order to produce full lobuloalveolar development typical of the late pregnant animal. Only prolactin and adrenal steroids were apparently required for milk secretion. The effect of these hormones in combination with insulin, upon the ultrastructure and differentiation of mammary epithelial cells has been confirmed *in vitro* (review by Topper & Freeman, 1980).

The injection of oestrogen and progesterone into experimental animals causes developmental changes in the mammary gland comparable to those observed during pregnancy. However both oestrogen and progesterone are without effect upon

hypophysectomised animals (Lyons et al, 1958) calling into question the direct mitogenic role of oestrogen upon mammary epithelial cells. Furthermore, oestradiol failed to stimulate proliferation when added to the medium of primary cell cultures (Yang et al, 1980 and Imagawa et al, 1982) or whole-gland cultures (DuBois & Elias, 1984). In contrast more recent reports have indicated that in mixed cultures of mammary epithelial and stromal cells, epithelial proliferation may be enhanced by oestradiol (McGath, 1983 and Haslam, 1986). Further work using plastic implants capable of delivering bioactive oestradiol (17-β) molecules *in situ* have allowed investigation into the possible direct effects of this classical ductal mitogen. When implanted into the mammary tissue of ovariectomized rats, oestradiol stimulated local end bud formation, while contra lateral gland were unaffected (Silberstein & Daniel, 1982). The authors concluded that oestrogen may act directly as a ductal mammogen, however since the animals were not hypophysectomised, it is possible that oestrogen stimulated the release of a pituitary factor such as growth hormone. If so, oestradiol must also be acting to sensitise the gland locally, since contra lateral glands were unaffected, and could therefore be said to have a direct (non-systemic) action on the mammary gland. Alternatively oestrogen may be acting to stimulate the release of a local agent promoting synthesis of an unidentified ductal mitogen.

There is some evidence that GH and prolactin could act independently of oestrogen at the level of the growing gland. Both growth hormone and prolactin can stimulate mammary epithelial cell growth *in vivo* (Yang et al, 1980a and DuBois & Elias, 1984). Using unprimed, ovariectomized animals it has been demonstrated that bovine and mouse GH placed in slow release implants, stimulates local end bud formation. Contra lateral glands were unaffected, indicating GH did not act systemically. Prolactin was similar to GH in

its ability to stimulate end bud growth.

In addition to the above, placental lactogen is secreted in appreciable quantities during pregnancy and is the major contributor to lactogenic activity during gestation (Forsyth, 1986). Placental lactogen, has both GH and prolactin like biological activities and levels are higher in animals carrying a large number of foetuses, resulting in a greater degree of mammary growth, appropriate to the needs of the young (Flint et al, 1985 and Knight & Peaker, 1982b). In rodents, coitus results in bi-daily prolactin surges which are replaced by rising placental lactogen levels. Both the mouse and the rat show different forms of placental lactogen between early (up to day 12) and late pregnancy (Robertson & Friesen, 1981).

1.2.2 Growth factors

The inability of classical mammogens to stimulate morphogenesis or even normal levels of DNA synthesis in end buds *in vitro* indicates that other factors may act in conjunction with these hormones. Studies indicate that a variety of growth factors are also involved in the hormonal regulation of mammary development. These include the insulin-like growth factor (IGF-1), epidermal growth factor (EGF), mesenchyme-derived growth factor (MDGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), pituitary-derived mammary growth factor (PMGF) and the transforming growth factors (TGF-α and TGF-β). These growth factors may facilitate developmental processes of the parenchymal and stromal elements of the mammary tissues in development of the parenchyma.

One growth factor which has received particular attention is EGF, with the discovery that EGF receptor levels, in whole mouse mammary gland, vary according to their

physiological state (Edery et al, 1985). Receptor levels are relatively high in virgin animals but rise during early pregnancy; levels then steadily decline throughout the remainder of pregnancy and into lactation. Such observations are consistent with a role for EGF in ductal development, but do not establish a causal relationship. This is made somewhat difficult by the fact that EGF has no focal source. However valuable information has been gleaned from the use of primary cell cultures and slow release implants. Using serum supplemented media in collagen gel cultures Yang and co-workers (1980) demonstrated that EGF and agents which raise intracellular cAMP levels, strongly stimulated mammary epithelial proliferation. Using the same culture system but employing serum free media Nandi and co-workers (1984) demonstrated that a combination of insulin, EGF, transferrin, BSA and cholera toxin stimulated cellular proliferation up to 10 fold. Deletion experiments showed that insulin and EGF were absolute requirements for cellular proliferation. Later studies (Imagawa et al, 1985) have shown that EGF was able to synergise with prolactin and progesterone at low concentrations (1ng/ml) to stimulate growth. Slow release implants have also been used to demonstrate the *in vitro* effects of EGF on ductal growth in the regressed glands of ovariectomized virgin mice (Daniel & Silversein, 1985). It was found that EGF stimulated end bud growth in a dose dependent manner. By comparison insulin , PDGF, transferrin and TGF-β had no effect.

However when EGF receptor activity was examined by autoradiographic analysis in 5 week old mice most receptor activity was found in the stroma adjacent to the end buds. Few if any receptors were seen in the luminal epithelium. These observations are supported by the finding that receptor levels measured in a mammary fibroblast cell line were 100 fold greater than those measured in a mammary epithelial cell line COMMA-

1D (Danielson et al, 1984). The presence of relatively high levels of EGF receptors on stroma suggests that it is the primary target site. However since it is well established that stimulation of EGF receptors leads to their rapid down regulation, the absence of epithelial receptors in the parenchyma may reflect stimulation by EGF. Therefore EGF or an EGF-like peptide may be a natural ductal mitogen.

1.2.3 The role of cyclic AMP

It is now widely accepted that regulation of mammary growth involves a complete interplay between systemic and locally produced modulators, and that classical ductal mammogens alone cannot support/stimulate ductal growth. Therefore attention is now focused on identifying other possible, locally synthesized growth regulators and the receptor-mediated pathways through which they operate. Levels of cAMP in both rat and mouse mammary gland are known to increase during the intense cellular proliferation of pregnancy, an observation consistent with it having a mitogenic role (Greenbaum et al, 1971). Agents which raise intracellular cAMP levels are known to stimulate mammary epithelial proliferation *in vitro* (Yang et al, 1980). Cholera toxin implants irreversibly activate receptor associated adenyl cyclase, strongly stimulate end bud growth and so demonstrate that a recognised intracellular effector can orchestrate all events for ductal growth (Silberstein et al, 1984). Cyclic AMP may activate the pathway through which ductal growth is stimulated, but since the classical ductal mammogens such as oestrogen do not act through an adenyl cyclase-coupled receptor pathway this may be indicative of an as yet unidentified cAMP mediated pathway.

1.2.4 Stromal interactions

The requirement for a stromal matrix for maximal ductal development is apparent from the inability of mammary epithelia to form ducts and end buds *in vitro*. Even when cultured in a floating collagen gel, which optimises the potential for spatial organisation (Yang et al, 1980a) epithelial cells form thin walled tubes and spiked cul-de-sacs. Fatty stroma appears to be necessary for organotropic growth, as demonstrated by studies in which mammary fat pad was artificially enriched with type I collagen (Daniel et al, 1984). Into these fragments of mammary duct were transplanted which grew as described above in collagen gels, but on reaching the fatty stroma, normal end buds appeared. However the need for specific mammary stroma has been largely discounted with the demonstration that adult ducts can grow in ectopic locations as well as in the mammary fat pad (Horhino, 1967 and 1971).

1.2.5 The extracellular matrix (ECM)

Culture models of mammary epithelial function indicate that cell-matrix interactions are necessary for lactation and survival of alveolar cells *in vivo.* Details of these studies are discussed in section 1.3.3.

1.3 Control of mammary tissue development during lactation

As discussed above, pregnancy is important in nurturing the necessary growth and structural development of the gland in preparation for lactation. Although growth of the gland continues beyond pregnancy in some species, including the rat (see Knight & Peaker, 1982a for review) the terminal purpose of mammary development remains the synthesis and secretion of milk, and is the subject of this section. Lactation is a complex

physiological process involving the manufacture and secretion of several hundred milk components. To date, the production of only a few of them has been examined experimentally. Species differences in the hormonal requirements add to the complexity of the problem.

1.3.1 Control of milk synthesis

The function of the fully differentiated mammary epithelial cell is the synthesis and secretion of milk. The major components of milk, apart from water, are lactose, fats and milk proteins. In most species, lactose is the predominant milk carbohydrate and is synthesised within the Golgi complex by lactose synthase, which consists of galactosyl transferase and the specifier protein α-lactalbumin (see Kuhn, 1983b and Leong, 1990 for review of lactose synthesis). Although lactose, and other milk solids appear in the gland well before the onset of lactation (Fleet et al, 1975) the major increase in the rate of lactose synthesis occurs around the time of parturition when copious milk secretion is triggered by a combination of progesterone withdrawal and increasing levels of circulating prolactin (see Kuhn, 1983a for review). During lactation, the prime regulator of lactose synthesis is the concentration of glucose in the Golgi, rather than the activity of lactose synthase (Faulkner & Peaker, 1987). The mechanism of milk protein synthesis is essentially identical to that of all secreted proteins in eukaryotic cells. Milk protein genes are under transcriptional, translation and post-translational control by prolactin and other hormones. The majority of studies have centred on casein, the most abundant protein produced by the mammary gland; although the induction of milk proteins is not coordinately regulated and casein mRNA expression can occur in the absence of α-lactalbumin and whey acidic protein (WAP) mRNA expression *in vitro* (Lee et al, 1984

and 1985). Effects on gene expression are chronically, rather than acutely, regulated, for although casein mRNA induction occurs within one hour of prolactin addition *in vitro*, marked increases in mRNA levels do not occur until several hours later (Matusik & Rosen, 1978). This induction is the consequence of an enhanced rate of gene transcription, with prolactin also acting to stabilise the synthesised transcript and increase the mRNA half life (Teyssot & Houdebine, 1980 and 1981). Increased levels of mRNA tend to result in an increased rate of protein synthesis (Houdebine & Gaye, 1975), although there are exceptions to this rule such as in the pseudopregnant rabbit (Houdebine, 1976). A level of post-translational control has been demonstrated in lactating mice expressing foreign milk protein genes where increased milk protein synthesis *in vitro* was not matched *in vivo* (Wilde et al, 1992). Caseins once synthesised are also susceptible to intracellular degradation during secretion (Wilde et al, 1992) although this process appears to be inversely related to the differentiative state of the tissue (Wilde et al, 1989) and may be more important in tempering net protein secretion in mammary tissue from pregnant animals.

Fatty acids are synthesised in the cytoplasm of milk secreting cells by acetyl-CoA carboxylase and fatty acid synthase, with palmitate being the predominant fatty acid in milk. However, it is triglycerides which make up nearly all milk fat. In the rat, half the mammary glands requirement is met by circulating lipoproteins, the remaining half from *de novo* synthesis within the gland. Glucose is the major precursor of fatty acid in the lactating rat mammary gland; while acetate is the primary precursor in the ruminant mammary gland (Laarveld et al, 1985). At peak lactation (10-15 days *post partum*) the mammary gland of the rat utilises 30mmol of glucose per day (Williamson & Robinson, 1977) of which about 20mmol is used for lipogenesis, primarily for fatty acid and

triglyceride synthesis (Williamson, 1980). The rate of mammary lipogenesis is stringently regulated according to the level and type of substrate available to the gland (Munday & Hardie, 1987). Prolactin is involved in the long-term control of lipogenic enzymes, acting on sheep mammary explants to activate and increase the total level of acetyl-CoA carboxylase (Barber et al, 1991), the key regulatory enzyme which catalyses the first reaction committed to fatty acid synthesis. Prolactin is also required for the induction of fatty acid synthase (Speake et al, 1975). Pyruvate dehydrogenase (PDH) is another important regulatory enzyme in the lactating rat mammary gland. The PDH complex is a multienzyme complex which catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA and is located in the mitochondrial matrix space. The overall reaction is essentially irreversible and regulates the supply of acetyl-CoA which is available for oxidation or fatty acid synthesis. Short term regulation of fatty acid synthesis is achieved by Insulin, which changes the activities of (PDH), acetyl-CoA carboxylase and glucose uptake.

In addition to hormonal regulation of lipogenesis, medium-chain fatty acids (and ketone bodies), which are present in rat milk, inhibit lipogenesis, at least *in vitro*, as a consequence of a direct inhibition of glycolysis (Heesom et al, 1992). Furthermore, fatty acids may equally modulate the activity of acetyl-CoA. This acute regulation of milk fat synthesis might offer an additional mechanism of modulating the activities of lipogenic enzymes.

1.3.2 Hormonal requirements of lactation

Parturition results in a marked change in the hormonal milieu and is characterised by falling levels of progesterone as a result of placental loss, and in some species, including

the rat, placental lactogen. At the same time there is an increase in the circulating levels of prolactin, oestrogens, oxytocin and adrenal corticoids, these factors taken together stimulate the onset of copious milk secretion.

In addition, insulin is a very basic hormonal requirement of lactation. The response of *in vitro* systems to lactogenic agents is often dependent upon or enhanced by insulin. For example Topper & Freeman (1980) found insulin to be essential for the stimulation of milk protein synthesis by prolactin and cortisol in mammary explants from mid-pregnant mice. Circulating levels of insulin decrease during lactation, however mammary tissue is able to function at lower insulin concentrations by increasing its receptor concentration (Flint, 1982) and remains a highly insulin-sensitive and insulin-responsive tissue (Burnol et al, 1983 and Jones et al, 1984a, b). In some tissues insulin can produce profound decreases in cAMP concentrations (and thus PK-A activities) through activation of a phosphodiesterase. For example, insulin is able to antagonize hormone-activated lipolysis in adipose tissue, via its activation of a membrane-associated cGMP-inhibited phosphodiesterase (PDE 3), which leads to a reduction in cAMP, a decrease in PK-A activity, and a lowering of the phosphorylation and thereby activity of the hormone-sensitive lipase, the rate-limiting enzyme in the regulation of lipolysis (Elks et al, 1985; Beebe et al, 1985 and Manganiello et al, 1995).

The importance of prolactin has been demonstrated by studies in which prolactin secretion was specifically inhibited by administration of ergot-alkaloid bromocriptine (Cowie et al, 1980). Hormones other than prolactin are also important in maintaining lactation, in ruminants growth hormone is a potent lactogenic hormone (Cowie et al, 1980), however the effect upon rodents is much more modest.

In the rat, prolactin is the predominant galactopoietic hormone, with its effects being

modified by growth hormone; treatment with both bromocriptine and anti-growth hormone serum being required to fully inhibit milk secretion (Madon et al, 1986). Established lactation, or galactopoiesis requires insulin, glucocorticoid, prolactin and thyroid hormones. In addition oxytocin is required for milk let-down. This hormone is released from the posterior pituitary lobe by suckling of the young via a neuroendocrine reflex. Although both prolactin and oxytocin are secreted in response to suckling young the physiological roles of these two hormones are quite different. Prolactin acts on the mammary epithelial cells to stimulate the production and secretion of milk into the alveolar lumina. Oxytocin acts on the myoepithelial cells facilitating ejection of the milk from alveoli into ducts and mammary sinuses, thus becoming available to the young.

1.3.3 The extracellular matrix

Culture models of mammary epithelial function indicate that cell-matrix interactions are necessary for lactation and for the survival of alveolar epithelial cells *in vivo*. Since the alveolus is present in the pregnant and lactating animal only, it is possible that the major function of basement membrane in this tissue is to contribute to differentiation and to maintain survival, but only until the cessation of lactation.

Evidence that the basement membrane is important in maintaining cell phenotype has largely been provided by cell culture models in which mammary epithelial cells were removed from mid- to late pregnant animals and placed in conventional cell culture. Cells cultured in this way grew in monolayers and quickly lost their ability to lactate, unable to synthesize milk protein in response to lactogenic hormones (Emermann et al, 1977 and Lee et al, 1984). However some epithelial characteristics are retained such as prolactin receptors (Streuli, 1994).

The introduction of the EHS matrix has significantly advanced the lactational culture model (Li, et al, 1987). This is a reconstituted basement membrane isolated from the murine Engelbreth-Holm-Swarm tumour (Kleinman et al, 1986) and allows cells isolated as whole alveoli from pregnant mammary glands to retain their morphology and to secrete milk proteins. Even trypsinized cells from monolayer cultures will form alveolar-like structures which are capable of secreting milk into the central lumen (Streuli et al, 1991). Furthermore, these cells could be made to express β-casein after several days culture with prolactin, a function that was blocked by an anti-β_1-integrin antibody, indicating that direct cell-matrix interactions were essential for milk protein expression (Streuli et al, 1991). The basement membrane has therefore been shown to provide signals for differentiation that are absent when cells are plated out in monolayers. The EHS culture model has also been used to confirm the role of the basement membrane in mammary cell survival. In culture, mouse mammary cells grown on plastic or collagen I undergo apoptosis. Culture on EHS matrix, results in a 15-fold suppression of apoptosis, suggesting that mammary cell survival is controlled by the cell-matrix interaction, and that in the absence of a correct matrix the cells die by apoptosis (Edwards & Streuli, 1995).

1.3.4 Local control of milk secretion

In addition to systemic control by circulating hormones, the rate of milk secretion by the mammary gland is modulated locally by changes in the frequency and completeness of milk removal. More frequent removal of milk from a gland produces an increased rate of milk secretion, as when hourly milk removal (Linzell & Peaker, 1971) is compared to twice daily milk removal (Henderson et al, 1983). These effects are rapidly reversed

when the gland is returned to twice daily milking (Henderson et al, 1983). The increase in milk secretion has been shown to result from the removal of a milk constituent, which acts upon secretory epithelial cells limiting milk secretion. The feedback inhibitor of lactation of FIL (Addey et al, 1991) has been identified in a fraction of goat whey proteins of molecular weight 10-30kDa (Wilde et al, 1987). This fraction inhibited lactose and casein synthesis in rabbit mammary explants and inhibited milk accumulation in lactating rabbits when introduced into the gland (Wilde et al, 1987).

As well as systemic and local controls, mammary development and lactation are also subject to external influences such as environment, climate, diet and the number of suckling young (see Knight & Peaker, 1982a for review).

1.3.5 The role of cAMP

The mitogenic role of cAMP in mammary development has been discussed (section 1.2.3). Several reports have addressed the question of the role of cAMP mediated mechanisms in acute regulation of aspects of mammary tissue metabolism (Wilde & Kuhn, 1981., Clegg, 1988 and Clegg & Ottey, 1990). The conclusion to which they point is that this tissue is refractory to the usual cascade of events initiated by an increase in cAMP. Thus neither cytosolic phosphorylation events nor the activities of several enzymes, including lactose synthase and acetyl-CoA carboxylase, are influenced by raising the concentration of cAMP in mammary tissue preparations (Wilde & Kuhn, 1981 and Clegg et al, 1986). One possibility to explain these altered responses is that the cAMP-dependent protein kinase (PK-A) itself has distinctive properties in mammary tissue, some evidence to this effect has already been reported (Takhar & Munday, 1992).

1.4 Control of mammary gland involution

Involution of the mammary gland and the accompanying loss of epithelial cells is a consequence of events triggered by the cessation of lactation. However the nature of the cellular signals induced by these events which eventually trigger the epithelial cell death and the involution remain to be elucidated.

1.4.1 Hormonal controls

The phases of mammary gland development and function are under stringent hormonal control, some of these hormones also have a critical role in maintenance of lactation and retard involution. In most experimental studies, mammary involution is initiated when animals are in the height of their lactation, usually by removal of the litter. In this way the role of the suckling stimulus and lactogenic hormones in maintaining lactation can be demonstrated.

The role of suckling was elegantly demonstrated by (Selye, 1931) who showed that mammary gland of a lactating rat could be maintained in a secretory state after ligating galactophores, provided suckling was allowed to continue. Further studies showed that prolactin, administered daily to lactating rats, segregated from their litters, retarded mammary involution (Hooker & Williams, 1940). More recently Richards & Benson (1971) examined in a comprehensive fashion the involution inhibitory effects of oxytocin and the anterior pituitary hormones administered singly and in combination. In doing so they demonstrated that both oxytocin and prolactin would retard mammary involution in the rat (Richards & Benson, 1971). However, close inspection of the secretory cells showed that despite the maintenance of lobuloalveolar structure, cell organelles did go through some involutionary changes when oxytocin alone was administered. More recent

experiments show that a combination of GH, prolactin and ACTH were needed in order to maintain both structure and function of secretory alveoli (Richards & Benson, 1971). Others have also shown that hydrocortisone and prolactin inhibit mammary gland regression when injected in high doses into rodents (Ossowski et al, 1979).

1.4.2 Apoptosis and programmed cell death (PCD)

It has been shown that during involution the majority of secretory epithelial cells die by apoptosis, a form of programmed cell death (Walker et al, 1989., Strange et al, 1992., Bielke et al, 1995). Programmed cell death has been recognised in a number of developmental and remodelling events in tissues and to tumour regression in response to treatment with chemotherapeutic agents (for review Fesus et al, 1991). Furthermore it has been recently recognised that similar gene products which were previously associated with proliferation may also be involved in regulation of programmed cell death. For instance, c-myc (Even et al, 1992) and p53 (Lowe et al, 1993 and Symonds et al, 1994) which have been identified as regulators of the G1 phase of the cell cycle are also important regulators of cell death, both *in vitro* and *in vivo*.

1.4.3 The role of cyclic AMP

Apoptosis may also be triggered by cellular signal transducers such as cAMP. Cyclic AMP can be substituted for glucocorticoid in triggering apoptosis in lymphoid cells (Dowd & Miesfeld, 1992), and is a major second messenger molecule that regulates the activity of protein kinase A (PK-A). In the nucleus the catalytic subunit of PK-A exerts its action at least in part, by modulating the activity of transcription factors such as CREB (Montminy & Bilezikjian, 1987) CREM (Foulkes et al, 1991) and AP-1 (Abate et al,

1991). AP-1 is a classical G1 marker of proliferating cells. It is a transcription factor and may consist of dimeric complexes between different Fos and Jun family members as well as ATF/CREB family members (Hai & Curran, 1991). The synthesis of AP-1 or its activity in cells can be induced by a wide variety of growth factors and intracellular signals mediated by protein kinase C, protein kinase A and many oncogene products. In mouse mammary gland a transient activation of nuclear PK-A occurs very early after weaning (24h) and peaks at day 1 and 2 of involution (Marti et al, 1994 and 1995). This coincides with a rise of c-fos, junB, junD and to a lesser extent c-jun mRNA levels and a transient induction of AP-1 (cfos/junD) DNA binding activity are apparent within 4 days of involution (Marti et al, 1994 and 1995).

Table 1.1 Regulators of the mammary development, lactation and involution

Development		
Fetal	**Puberty**	**Pregnancy**
Androgens (inhibit)	oestrogen growth hormone cortisol T_3 insulin growth factors	oestrogen progesterone growth hormone prolactin cortisol T_3 insulin growth factors
Lactation		
Initiation	**Maintenance**	**Milk let down**
oestrogen progesterone prolactin cortisol	Prolactin insulin cortisol T_3	oxytocin
Involution		
prolactin (removal) oxytocin (removal)		

1.5 Abnormal mammary growth (breast cancer)

1.5.1 Introductory comments

Breast carcinoma is the most common malignancy in women in North America and Western Europe, and in the UK carries a higher rate of mortality than any other malignant disease (Forrest, 1986). Despite advances in treatment and screening, only modest increases in survival have been achieved. One major problem in trying to reduce the morbidity and mortality from breast cancer is the lack of a clear understanding of the natural history and molecular pathology of the disease.

It is now acknowledged that both normal proliferative and tumorigenic processes have many molecular features in common including expression of growth factors (Varmus, 1989 and Cross & Dexter, 1991), extracellular matrix components and proteinases (Liotta, 1991 and Matisian, 1990), intracellular oncogene and protooncogene products (Cantley et al, 1991), tumour suppressor gene products (Marshall, 1991) and genes associated with proliferation.

Furthermore the normal ontogenic growth so far described in the branching morphogenesis of the mammary gland is relevant to cancer biology. The growth of the end bud through adipose stroma provides a useful model for tissue invasion, while ductal elongation must be seen as rapid and aggressive even when compared with highly malignant tissue, its growth is tightly regulated by tissue-level homeostatic factors. Even more directly, in the rat the mammary end bud is the target tissue for certain carcinogens.

1.5.2 Mitogenic control

Many of the mammary mitogens and mammogens so far described in the development of mammary tissue are also involved in mitogenic signal transduction in human breast

cancer cells. In this context they are discussed here. The steroid hormone 17β-oestradiol has been established as a preeminent breast epithelial cell mitogen and an etiological factor in the development of human breast cancer (Davidson & Lippmann, 1989). The oestrogen receptor directly controls transcription of genes required for proliferation, such as thymidine kinase and myc. In addition oestrogen is able to stimulate proliferation through autocrine and paracrine loops, inducing synthesis and secretion of polypeptide growth factors TGF-α, IGF-1 and PDGF (Lippmann & Dickson, 1990). Oestrogens accelerate the rate of progression of breast cancer cells through the G1 phase of the cell cycle. Increased transit rate might result from increased degradation of the mitotic cyclin, cyclin B1 (Thomas & Thomas, 1994), or increasing the expression of calmodulin (Rasmussen & Means, 1987) and/or cyclin D1. Cyclin D1 is a critical determinant of the GS transit time in human breast cancer cells (Musgrove et al, 1994). Aberrant expression of cyclin D1 is observed in many human breast cancer cell lines, and may contribute to neoplastic growth (Keyomarsi & Pardee, 1993). Progestins, IGF-1 and insulin also stimulate cyclin D1 expression in breast cancer cells and accelerate progression through the cell cycle (Musgrove et al, 1993).

The polypeptide growth factors EGF, TGF-α and IGF-I and II, basic fibroblast growth factor (bFGF) and insulin also stimulate breast cancer growth in tissue culture (Lippmann & Dickson, 1990). Gene related to the EGF receptor include the c-erbB2 (neu or HER2), c-erbB3 and c-erbB4, which are expressed in breast cancer tissues and associated with poor prognosis.

The ubiquitous calmodulin protein regulates many calcium-sensitive cellular processes via the formation of a calcium-calmodulin complex. Essential to cell proliferation it serves to activate an extensive number of calmodulin sensitive enzymes involved in

growth regulation. Intracellular cAMP and calcium, cell cycle progression, DNA replication and mitosis are all subject to regulation by calmodulin.

Finally, with the finding that prolactin receptors are present in both normal and neoplastic tissue (Field et al, 1993), prolactin has recently been proposed to act as a local growth factor stimulating mammary tumours (Mershon et al, 1995).

1.5.3 Cross-Talk

The extent to which interactions occur between steroid hormones, polypeptide growth factors and cAMP responsive pathways in breast cancer cells remains to be fully understood. The induction of progesterone receptor synthesis by oestrogen is well characterised. However another important clinical observation is the inverse relationship which exists between oestrogen and EGF receptor levels found in most tumours, indicating that mitogenic signals may be initiated either by intracellular oestrogen or by the transmembrane EGF receptor pathway. Tumours which express mainly EGF receptor activity are associated with poor differentiation, metastasis and low survival rates. The two pathways were therefore thought to be independent. However there is evidence that EGF activates some mitogenic signals through the oestrogen receptor pathway (Ignar-Trowbridge et al, 1992).

Receptor phosphorylation provides a mechanism for crosstalk between responses initiated by steroid hormones, polypeptide growth factors and cAMP, protein kinase C or calmodulin. The oestrogen receptor is phosphorylated on at least 6 serine residues (LeGoff et al, 1994), of which some are catalysed by PK-A and PK-C. However both oestrogen and progesterone receptors have been found to exist as phosphoproteins *in vivo* (Moudgil,1990). Furthermore ligand binding has been shown to increase receptor

phosphorylation (Denner et al, 1990). It is possible that a change in phosphorylation state might alter its ability to activate gene transcription. Some reports have even suggested that phosphorylation of steroid hormone receptors can increase their binding to specific DNA sequences (Denner et al, 1989). Treatment of immature rat uterine cells grown in primary culture with estradiol, IGF-1, or agents which raise cAMP, resulted in a significant increase in intracellular progesterone receptor (Aronica & Katzenellenbogen, 1991. and Cho et al, 1994). More recent studies using transient transfection of uterine cells with a simple oestrogen-responsive reporter gene, have shown that agents which raise cAMP are able to stimulate oestrogen receptor-mediated transcription (Aronica & Katzenellenbogen, 1993).

Multiple interrelated processes initiate growth in human breast cancer cells. A more complete understanding of these mitogenic signal transduction pathways might provide the basis for the development of therapeutic strategies for intervention of human tumour growth *in vivo*.

1.5.4 The role of cyclic AMP

There has recently been a profusion of papers describing how cAMP might influence breast cancer proliferation through a variety of mechanisms (see section 1.7 for greater detail). Cyclic AMP is reported to regulate cell growth via the cAMP dependent protein kinase or PK-A (Krebs, 1972), which depending upon the cell type can inhibit or activate the MAP kinase cascade through certain phosphorylation events (see section 1.7.1). However the regulatory subunits (R-subunit) of this kinase have also been the focus of much investigation. There are two major types of PK-A, type I and type II which are distinguished by their R-subunits, RI and RII (see section 1.6.2). Human breast cancer

cell lines express predominantly type I PK-A and this differential pattern of expression has also been correlated with neoplastic growth. The cAMP analogue, 8-Cl cAMP, as been used in numerous studies to inhibit the growth of human breast cancer cell lines in culture, associated with down regulation of the type I PK-A and decreased ras proto-oncogene protein expression. In mouse mammary cells, inhibition of cell growth was correlated with the ability of 8-Cl cAMP to decrease the amount of type I PK-A mRNA. The reduction in the level of type I PK-A with respect to type II PK-A by 8-Cl cAMP reduced growth rate in these cells and antagonised the proliferation induced by the polypeptide growth factor TGF-α (Ciardiello et al, 1990).

Cyclic AMP also plays a critical role in breast cancer cell proliferation by modulating the activity of oestrogen and progesterone receptors. The cyclic nucleotide can also induce expression of prolactin receptors, an effect that might be mediated through cAMP dependent activation of the oestrogen receptor pathway (Aronica & Katzenellenbogen, 1993 and Cho et al, 1994). Hence the steroid hormone and polypeptide growth factors mitogenic signalling pathways are influenced by intracellular cAMP in human breast cancer cells.

1.6 The cAMP dependent protein kinase or protein kinase A (PK-A)

1.6.1 Introduction

The second messenger cAMP was first observed as a heat stable factor which could be generated by treatment of a particulate subcellular fraction obtained from rat liver with adrenaline. Production required the presence of ATP and the factor could induce activation of the cytosolic enzyme glycogen phosphorylase. Subsequently the active principle was identified as cAMP. Since the Nobel prize winning work of Sutherland and

co-workers, who first developed the concept of cAMP as a intracellular second messenger of hormone action, many of the signal transduction mechanisms surrounding cAMP have been characterised.

The discovery of cAMP was quickly followed by the demonstration that most eukaryotic cells possess a plasma-membrane associated enzyme adenylate cyclase, which can produce cAMP from ATP and that activity of this enzyme could be regulated by various hormones.

With the discovery of the cAMP dependent protein kinase (PK-A) it was quickly realised that, at least in mammalian cells, PK-A was the major if not only, intracellular receptor for cAMP. In holoenzyme form (R_2C_2), PK-A exists as a tetrameric structure composed of two catalytic subunits (C-subunit) bound to, and inhibited by a regulatory subunit dimer (R-subunit). PK-A is activated and inactivated as follows:

$$\mathbf{R_2C_2 + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C}$$

Inactive **Active**

Cyclic AMP binds to the R-subunits of the protein kinase and the equilibrium outlined above, shifts to the right hand side, releasing free C-subunit both physically and functionally from inhibition by the R-subunits. In its free state active C-subunit mediates protein phosphorylation which is the primary mechanism of cAMP action in mammalian cells. Cyclic AMP is hydrolysed by cAMP phosphodiesterases allowing the equilibrium to shift back to the left hand side, and C-subunit to reassociate with the R-subunits. The cAMP-specific (Type IV) phosphodiesterases (PDEs) are a diverse family of proteins, the regulation of which is beyond the scope of this thesis (see Bolger, 1994 for review).

Cyclic AMP is synthesised by adenylyl cyclases which convert ATP to cAMP and are controlled dynamically by a variety of hormones, neurotransmitters and other regulatory molecules (see Tang & Gilman, 1992 for review). Adenylyl cyclase catalytic subunit is activated or inhibited by the heterotrimeric GTP-binding proteins G_s and G_i respectively. G-proteins are a diverse family of proteins, the α-subunit of which is responsible for activation or inhibition of adenylyl cyclases (see Gilman, 1995 for review). Therefore PK-A is responsive to hormones which bind to and activate the β-adrenergic class of receptor which are physically coupled to G_s, causing activation of adenylyl cyclase and increased synthesis of cAMP.

In order that the unmodified form of the protein substrate can be regenerated, a phosphoprotein phosphatase catalyses a dephosphorylation reaction (see Cohen, 1993 for review). Thus the steady state, phosphorylation-dephosphorylation equilibrium between an active and inactive form of a protein is dynamically regulated by protein kinases, phosphoprotein phosphatases and their respective effectors. Together these constitute a cyclic cascade system and can provide the cell with an efficient and sensitive control mechanism that has potential for amplification and cooperativity of response (Figure 1.1).

One of the superficially puzzling aspects of the use of a second messenger system such as cAMP is that, while many cells possess the components of the system, each responds in a unique way to a rise in intracellular cAMP; such that in liver cells it promotes gluconeogenesis and glycogen breakdown, while in the adrenal cortex it promotes increased steroid release. It appears that many different effector hormones can induce different responses, all via PK-A.

These varied effects may reflect differences in intracellular responses to cAMP and, since all the actions are mediated by PK-A, it follows that PK-A must act upon different target

substrates in each cell type. Different patterns of expression of target proteins for PK-A phosphorylation may help explain how different tissues are able to respond uniquely to increases in intracellular cAMP. This hypothesis is dependent upon the broad substrate specificity of PK-A, as exemplified by the promiscuous phosphorylation of numerous proteins in tissue homogenates. Thus the way in which a cell responds to a rise in intracellular cAMP will be determined by available substrates within that cell. Despite the apparent wide range of potential protein substrates in cells, it is clear that, in general there are only a small number of true target proteins in any given hormone sensitive tissue. Some restriction is obviously achieved by appropriate compartmentalisation of cellular proteins, but there are also some structural criteria which are also important. The enzyme will only phosphorylate serine or threonine residues present within a random coil i.e. those present in a β-sheet, β-turn or α-helix are not phosphorylated. A consensus sequence has also been identified as **RRXS/TY** or **RXXRXXS/TY** where Y is a hydrophobic residue (Zetterqvist et al, 1990. and Taylor et al, 1993).

1.6.2. Modulation of PK-A activity

Cyclic AMP itself can influence PK-A expression; negative feedback of PK-A activity can be achieved by down-regulation of the level of C-subunit (Hemmings, 1986 and Schwoch, 1987) presumably by a protease with particular efficiency against free C-subunit (Alhanaty & Shaltiel, 1979). It can also be achieved by increasing the level of R-subunit relative to C-subunit as confirmed by the identification of RIα as the extinguisher of cAMP-induced gene responses (Boshart et al, 1991 and Jones et al, 1991). A consensus cAMP responsive element has been identified within the RIα promoter (Nowak et al, 1987) and may explain cAMP stimulated transcription (Tasken et al, 1991)

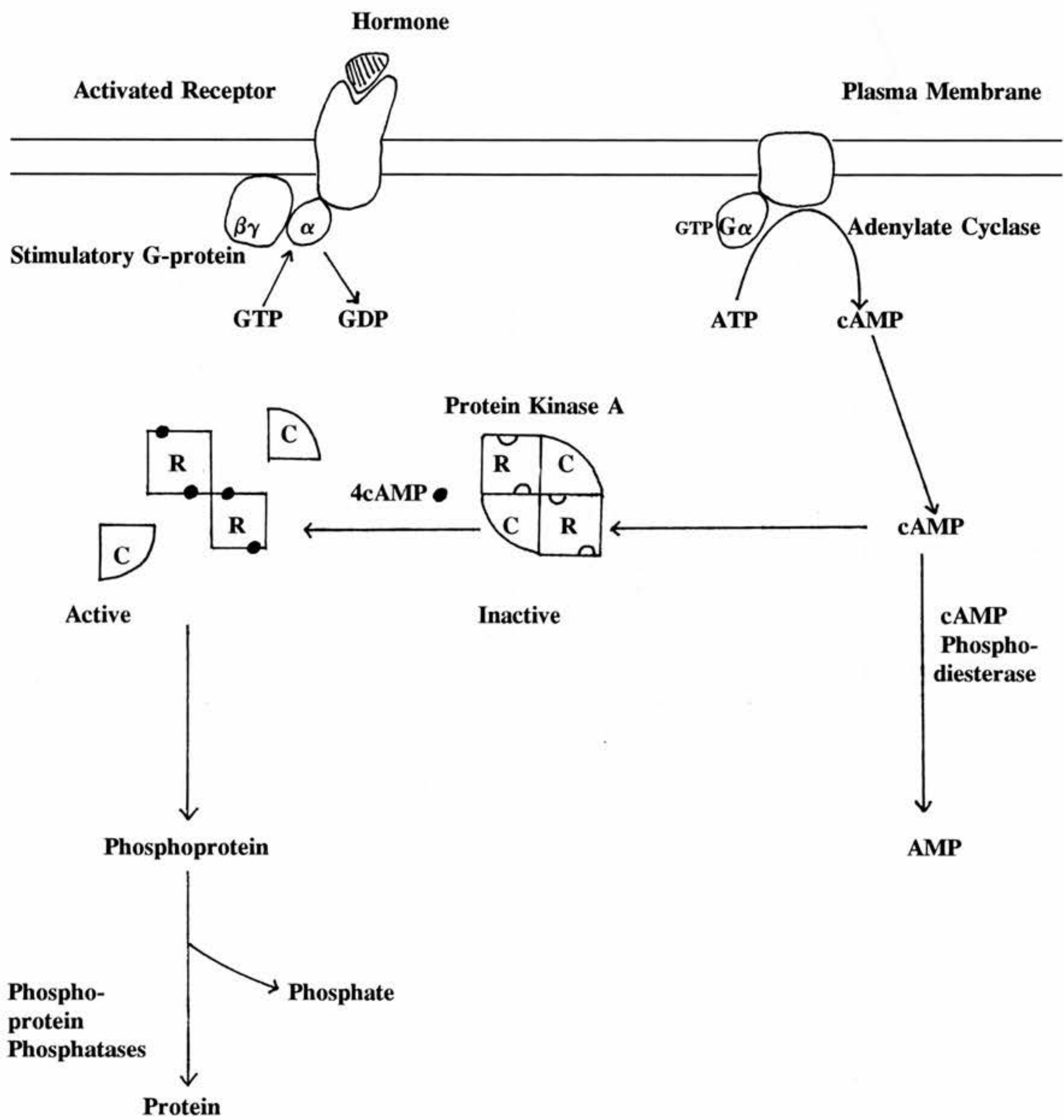


Figure 1.1 Schematic view of PK-A activation and protein phosphorylation

as well as the compensatory increase in RI in cells over expressing C-subunit (Uhler & Mcknight, 1987).

The ratio of R-subunits to C-subunit in terminally differentiated tissues has been reported to be approximately 1.0. This has been demonstrated in a number of species of heart (Corbin et al, 1977) several tissues from adult rabbits (Hofmann et al, 1977) and in the postnatal developing rat liver (Wittaack et al, 1983) and rat brain (Lohmann et al, 1978). This suggests that the two subunits are coordinately regulated. However Uno et al (1984) analysing yeast mutants with altered cAMP metabolism, determined that the subunits of protein kinase A were not encoded by the same gene locus, suggesting the possibility that the levels of regulatory and catalytic subunits could be independently regulated. There are several reports in the literature indicating that at least under certain circumstances in some cells there are greater amounts of R-subunit than C-subunit. Richards & Rolfes (1980) reported that ovarian granulosa cells from immature, hypophysectomised rats could be stimulated by oestrogen and follicle-stimulating hormones to express a 10-20 fold increase in the level of type II R-subunit with little or no increase in the levels of either type I R-subunit or C-subunit activity. Conversely, treatment of neuroblastoma-glioma hybrid cells with dibutyryl cAMP or addition of reagents that elevate intracellular cAMP have been shown to result in selective increase in the type I R-subunit with no change in type II or C-subunit levels (Walker et al, 1979 and Lohmann et al, 1983). More recently Ekanger et al (1989) demonstrated that R-subunit transiently exceeds that of C-subunit before a period of intense cellular proliferation in the regenerating rat liver. In this instance both RIα and RIIα were over expressed.

In addition to the R-subunits, another class of endogenous proteins that inhibit the C-subunit are the protein kinase inhibitors (PKI's). PKI's are small extremely potent

inhibitors of C-subunit, possessing nanomolar K_i values (White-House & Walsh, 1983 and Van Patten et al, 1991). The inhibition of the C-subunit is very specific, as other protein kinases are not inhibited by PKI's (Kemp et al, 1988 and Van Patten et al, 1992). Three PKI isoforms are known to exist, PKIα (Olsen & Uhler 1991a., 1991b and Van Patten et al, 1991) PKIβ1 (Van Patten et al, 1991 and Scarpetta & Uhler, 1993) and PKIβ2 (Scarpetta and Uhler, 1993). The α and β isoforms are products of distinct genes (Olsen & Uhler, 1991a., 1991b., Van Patten et al, 1991 and 1992) whereas PKIβ1 and β2 are splice varients of the same genes (Scarpetta & Uhler, 1993). Both PKIα and PKIβ are able to inhibit C-subunit with equal efficiency (Van Patten et al, 1992). The PKI protein and related peptides, containing the pseudosubstrate sequence RRNAI (Scott et al, 1985) have been used to study phosphorylation by PK-A C-subunit as described else where in this thesis (Olsen & Uhler, 1989). However, despite a wealth of structural and biochemical studies a clear physiological role of the PKI's in the cAMP signal transduction pathway has not yet been elucidated.

1.6.3 PK-A isoenzymes and structural variation

The earliest recognition of PK-A diversity was based on the elution characteristics of the enzyme during ion-exchange chromatography on columns of DEAE cellulose (Corbin et al, 1975). It is now well recognised that two readily distinguishable forms of the regulatory subunits exist (RI and RII) and that these associate with C-subunit to constitute the two major isoenzymes of PK-A, namely type I and type II. Tissue distribution studies have shown that most types of mammalian cells contain both isoenzymes, although the relative proportions of each vary markedly between tissues. RI and RII differ in their molecular size (Taylor et al, 1990) and in the autophosphorylation

site present on RII (ser 95). The phosphorylated protein has a lower affinity for the C-subunit and hence dissociation will occur at a lower concentration of cAMP (Granot et al, 1980). Once dissociated, this reaction can be reversed by the action of phosphoprotein phosphatases, thus raising the possibility that protein phosphatases may regulate protein kinase activity selectively at the type II isoform.

In place of the phosphorylation site on RII the RI subunit has a pseudophosphorylation site, capable of binding MgATP. In the presence of ATP, the rate of association of cAMP saturated RI with the C-subunit is increased, as is dissociation of the cAMP (Chou et al, 1980). Under normal conditions, in healthy cells RI will be saturated with ATP (Hofmann et al, 1975).

These differences may explain, at least in part the ability of PK-A isoenzymes to respond differently to given agonists, thus in certain cells where both isozymes are present, selective activation of one isoform may be provoked by agonists acting via specific receptors (Livsey et al, 1984). This is of particular interest to the signalling biochemistry in mammary tissue where it has been shown that certain cAMP signalling pathways operate as normal, whereas at least one, that communicating with acetyl CoA Carboxylase is inoperative (Clegg, 1988., Clegg & Calvert, 1988 and Clegg & Ottey, 1990).

Further diversity is produced by molecular heterogeneity among the R-subunits, at least two forms of each R-subunit type exist (RIα, RIβ ,RIIα and RII β) encoded by separate genes. Details of their tissue and cellular distribution are beginning to emerge; RIα and RIIα are expressed in most cell types, whereas RIβ and RIIβ are predominantly expressed in the brain (for review see Taylor et al, 1990a). The C-subunits from both isoenzymes are indistinguishable by a large number of criteria, i.e. similar chromatographic,

chemical, physical, immunological, catalytic properties and are able to reassociate with both type I and type II R-subunits (Uhler & McKnight, 1987). There are different isoelectric forms (Sugden et al, 1976) but these are not specifically tied to one R-subunit isoform. However using molecular genetic techniques, C-subunit was also found to consist of a family of closely related gene products Cα, Cβ and Cγ. The isoforms Cα and Cβ share greater than 90% homology and are present in all tissues examined, where Cα is usually the major isoform. Cγ shares approximately 80% homology with Cα and Cβ and has different enzymological properties including an insensitivity to the protein kinase inhibitor peptide (PKI) as a result of a single amino acid substitution at (Beebe et al, 1992). However this isoform is highly restricted in its distribution, only detected in the human testis (Beebe et al, 1990 and Taylor et al, 1990b). The biological significance of C-subunit isoforms has yet to be demonstrated; there is no apparent restriction in the distribution of C-subunit isoforms between type I and type II holoenzyme forms. However, the apparent conservation between species would argue that this heterogeneity is not simply adventitious. In addition, various novel C-subunit molecules have been cloned including a C-terminal splice variant from C.elegans (Gross et al, 1990). Aplasia neurones have also been shown to contain a collection of structurally and functionally diverse C- and R-subunits (Beushausen et al, 1992), which are able to target different substrates (Panchal et al, 1994). A bovine variant has also been identified, Cβ2 (Wiemann et al, 1991) which lacks the usual myristoylation site and contains in its place a putative amphiphilic α-helix, such a moiety is usually associated with membrane and mitochondrial targeting.

C-subunit is also one of a growing number of proteins found to be acylated (McIlhinney, 1990). C-subunit is subject to co-translational modification in which a fatty myristoyl

group is covalently attached to its N-terminal glycine (Carr et al, 1982). This is a relatively stable modification, however there may be some scope for the co-existence of both myristoylated and non-myristoylated forms of this protein within a single cell, increasing heterogeneity of PK-A still further.

The functional significance of this modification is not apparent, however in some cases myristoylation has served to target and anchor proteins to a membrane site (Grand et al, 1989). There is also a growing body of evidence that C-subunit may undergo translocation to a membrane site following dissociation from the R-subunits (Meinkoth et al, 1990). However mutation of the N-terminal glycine, thus preventing myristoylation, does not appear to effect either catalytic activity or the C-subunit's ability to form holoenzyme, neither does it effect the enzyme's ability to regulate biological processes occurring in distinct subcellular compartments (Clegg et al, 1989). Other possible functions of this covalent modification may be related to the stability and turnover of the protein and therefore the lifetime of the protein *in vivo* (Yonemoto et al, 1993).

1.6.4 A-Kinase anchoring proteins (AKAPs)

As described above PK-A is a multifunctional enzyme with a broad substrate specificity. Therefore the question still remains as to how the kinase is able to phosphorylate the correct target proteins rapidly and preferentially in response to activation by individual hormones. The discovery that there are two major PK-A isoforms (type I and type II) has raised the speculation that they may provide additional specificity to the cAMP system by being preferentially located in different cellular compartments. The traditional view that the enzyme is free to diffuse throughout the cytoplasm, would lead to an indiscriminate burst of phosphorylation following hormone activation and this is clearly

not the case with only a few target proteins being phosphorylated. Thus a preferred hypothesis has arisen in which PK-A is compartmentalised and that individual hormones preferentially activate specific pools of kinase that are co-localised with substrate proteins. Support for PK-A compartmentalisation is provided by three lines of evidence. Firstly different hormones activate specific PK-A subtypes (Harper et al, 1985); secondly cAMP accumulates in different cell compartments in response to different hormones (Barsony et al, 1990); finally PK-A subunits are detected in different cellular compartments (Litvin et al, 1984 and Livesey et al, 1982).

A growing body of evidence has shown that type II PK-A holoenzyme can be tethered at specific subcellular locations through interaction of its R-subunits (RII) with A-kinase anchoring proteins (AKAPS). Biochemical and immunological studies have shown that PK-A localisation is directed through the R-subunit (Scott, 1991). Initially PK-A anchoring proteins were identified as contaminants that co-purified with RII after affinity chromatography on cAMP agarose. Further detailed studies were made possible by the development of the overlay assay, essentially a modification of the western blotting procedure, in which proteins fractionated from cell extracts, potentially containing AKAPS, were immobilised on nitrocellulose (Lohman et al, 1984 and Carr et al, 1992a). The majority of RII-anchoring activity is detected in particulate, cellular fraction, implying attachment to membrane and cytoskeletal components (Scott, 1991). Furthermore tissue-specific patterns of RII anchoring proteins have also been found (see Scott & Carr, 1992 for review).

Most recently the functional significance of the RIIβ isoform and PK-A localisation has been elucidated in white and brown fat cells, where it is the principle R-subunit isoform. Cummings et al (1996) described the features of gene knockout mice specifically bred

to lack the gene encoding RIIβ. Loss of the RIIβ gene was compensated for by an increase in RIα, PK-A activity in brown adipocytes was stepped-up, giving increased energy expenditure, leanness and a marked resistance to diet-induced obesity. Although the AKAP and compartmentalization of PK-A in brown adipocytes is unknown, the isoform switch from type I to type II in RIIβ deficient brown fat cells is likely to cause subcellular redistribution of PK-A, possibly contributing to the disregulation of PK-A activity.

Utilising recombinant DNA and protein chemistry techniques, a family of RII deletion mutants have been screened in order to map the AKAP-binding region on RII (Scott et al, 1990 and Taylor et al, 1990). This was narrowed down to the N-terminal region of RII, a region which has also been shown to be important for the dimerisation of RII (Scott et al, 1990). In order to define more closely the AKAP-binding and dimerisation domains, amino-terminal deletion mutants were constructed lacking the first five or ten amino acids of RIIα. Both mutants were unable to bind AKAPs (as assessed by overlay) and only the five amino deletion RIIα retained the ability to dimerise. Thus it appeared that the AKAP-binding determinants were within the first five amino acid residues of the RIIα protomer, whereas determinants of dimerisation were located closer to the C-terminal. To identify their individual contribution to AKAP-binding, residues were systematically replaced by alanines; in this way residues 3 and 5 (both isoleucines) were shown to be of critical importance in RIIα interaction with AKAPs (Hausken et al, 1994). Complementary studies have examined the regions within AKAPS that interact with RII. However a comparison of several primary AKAP sequences has showed no striking homology between them, suggesting that the RII binding site is a conserved secondary motif such as a amphipathic α-helix. Using computer aided structure analysis, an area

predicted to be amphipathic helix was identified and shown to be shared by all AKAPS examined (Scott & Carr, 1992., Carr et al, 1991., Carr et al, 1992a., Carr et al, 1992b., Coghlan et al, 1994 and McCartney et al, 1995). Disruption of this structure by the introduction of a proline residue proved to inhibit AKAP/RII interaction (Carr et al, 1991 and Carr et al, 1992a). Furthermore, a synthetic peptide (anchoring inhibitor peptide) encompassing the putative helical region blocked all RII-AKAP interaction (as measured by overlay) and bound RII or the type II holoenzyme with nanomolar affinity (Carr et al, 1992a).

The anchoring inhibitor peptide has been used in certain critical experiments to determine whether disruption of the anchoring could antagonise certain cAMP responsive events. Using whole cell electrophysiology recording techniques it has been shown that anchoring of PK-A by AKAPs is essential for the modulation of α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) kainate responsive glutamate currents. This was achieved by using a microelectrode as a delivery system to introduce the anchoring inhibitor peptides into cultured hippocampal neurones (Rosenmund et al, 1994). The anchoring inhibitor peptide blocked AMPA/kainate responsive currents; this effect was mimicked by the PK-A inhibitor peptide (PKI), however the effect of both peptides was not additive. Inhibition by the former was overcome by addition of C-subunit protein, suggesting the anchoring inhibitor peptide interfered with PK-A dependent phosphorylation but did not directly inhibit kinase activity. Control peptides unable to block RII/AKAP interaction had no effect. These observations are supported by similar work carried out using the L-type Ca^{2+} channels in skeletal muscle cells (Johnson et al, 1994). These observations provide evidence that PK-A anchoring is a physiologically relevant regulatory mechanism, ensuring rapid and preferential phosphorylation of key

substrates. Until recently the AKAPs were thought to function exclusively in the localisation of PK-A. However, this view has now been modified with the discovery that certain AKAPs also function to localise the phosphatase 2B, calcineurin (CaN). The yeast two hybrid system has been used (Durfee et al, 1993) to identify AKAP target proteins in mouse. It was found that AKAP79 interacted with calcineurin, but not with the RII alone. This suggested the existence of a ternary complex between CaN, the AKAP and PK-A. Such a complex had been identified in bovine brain by two separate fractionation methods (Coghlan et al, 1995a) Firstly, CaN was immunoprecipitated from purified bovine brain extracts and was shown to co-precipitated with both AKAP75 (bovine homologue of AKAP79) and PK-A. Secondly, AKAP75 and the CaN holoenzyme were co-purified (on cAMP-agarose) with R-subunit and specifically eluted using the PK-A anchoring inhibitor peptide. Co-localisation of kinases and phosphatases may account for the exquisite modulation of certain phosphorylation events that are necessary for maintaining cellular homeostasis.

1.6.5 Nuclear translocation of PK-A and gene transcription

Transcriptional regulation of eukaryotic genes by cAMP requires the presence of the cAMP dependent protein kinase, of which both R- and C-subunits have been postulated to play a role in gene transcription (Constantinou et al, 1985., Cho-Chung, 1980 and 1993). The R-subunits which bind cAMP have also been shown to bind DNA (Jungmann et al, 1986 and Wu & Wang, 1989) and share structural homology with the bacterial catabolite activator protein (CAP) which can directly stimulate gene expression. However there is some difference of opinion as to whether R-subunits enter the nucleus. The cAMP analogue 8-Cl cAMP is reported to induce rapid nuclear translocation of RIIβ

(Ally et al, 1988). While more recent studies, involving microinjection of fluorescently labelled subunits and holoenzyme have reported rapid and reversible translocation of the C-subunit to the nucleus while type I holoenzyme and RI remained in the cytoplasm (Meinkoth et al, 1990 and Adams et al, 1991). There is also growing evidence that the free C-subunit may be all that is required for gene transcription (Riabowol et al, 1988 and Grove et al, 1987). Therefore it remains uncertain as to whether R-subunits serve any function other than to anchor and inhibit C-subunit.

Nuclear localisation of the C-subunit in stimulated cells has been suggested by immunological studies using antibodies directed against the C-subunit (Nigg et al, 1990), immunofluorescence studies (Byus & Fletcher, 1982) and studies using the thermostable PK-A inhibitor (Whitehouse & Walsh, 1983). It has been further postulated that free C-subunit possesses a potential nuclear localisation signal which is blocked by binding of the R-subunits or the thermostable PK-A inhibitor peptide, PKI (Wen et al, 1995., Meinkoth et al, 1990 and Fantozzi et al, 1992). Once in the nucleus C-subunit can affect the expression of a number of gene products. Cyclic AMP regulated gene expression frequently involves a DNA element known as the cAMP regulated enhancer or cAMP response element (CRE). Many transcription factors bind to this element including the protein CREB (Montminy & Bilezikjian, 1987), which becomes activated as a result of phosphorylation by PK-A (Gonzalez & Montminy, 1989). This modification stimulates the interaction with one or more of the general transcription factors or allows the recruitment of a co-activator such as CBP, CREB binding protein (Chrivia et al, 1993).

1.7 Cyclic AMP dependent protein kinase and cellular proliferation and growth

Cyclic AMP has long been implicated in the regulation of growth in both normal and malignant cells (Cho-Chung et al, 1991) including breast cancer cells (Houge et al, 1992 and Miller et al, 1985). This second messenger is also implicated in apoptopic cell death in lymphoid cells and recently, activation of PK-A was found to be associated with the induction of programmed cell death during involution of the lactating mammary gland (Marti et al, 1994).

1.7.1 Cross talk with the MAP kinase cascade

In mammalian tissues the mitogen activated protein kinase (MAP kinase) is rapidly phosphorylated and activated in response to many extracellular stimuli including growth factors, hormones and neurotransmitters. The various stimuli which can activate the MAP kinase cascade employ distinct initial signal transduction pathways. Some such as EGF and insulin induce receptor tyrosine kinase (Ahn et al, 1991 and Sturgill et al, 1988); or non-receptor tyrosine kinases (Klauser & Samelson, 1992 and Gupta et al, 1992). Others stimulate G-protein coupled receptors, generating the second messengers diacylglycerol, inositol 1,4,5 trisphosphate, calcium or activating ion channels (Bading & Greenberg, 1991., Ely et al, 1990., Vouret-Crariari et al, 1993 and Winitz et al, 1993).
It is likely that the MAP kinase cascade serves specific functions in different cell types. In PC12 cells nerve growth factor (NGF) induces neuronal differentiation, via the MAP kinase cascade (Cowley et al, 1994). However in other cell/tissue models MAP kinase activation is associated with growth and proliferation. Activation of MAP kinases has been closely correlated with the control of cell cycle progression in fgfg cells (Maller, 1991). For receptor tyrosine kinases a sequence of events leading to MAP kinase

activation is emerging. Activated receptor tyrosine kinase increase GTP binding to c-Ras through guanine nucleotide exchange factors and adapter proteins leading to its activation. Activated c-Ras may then activate c-Raf kinase by direct binding (Warne et al, 1993., Marshall et al 1994 and 1995), c-Raf can phosphorylate and activate MAP kinase kinase (Dent et al, 1992), which in turn phosphorylates and activates MAP kinase (Gomez & Cohen, 1991). Many transforming oncogenes encode mutant proteins whose corresponding proto-oncogene products are components of this signal transduction network and are activated in response to growth factor receptor stimulation (Nakielny et al, 1992).

The effect of cAMP upon the MAP kinase cascade has been delineated in several cell types including Rat-1 and NIH 3T3 fibroblasts, rat adipocytes and human arterial smooth muscle cells (Burgering et al, 1993., Cook and McCormick, 1993., Graves et al, 1993., Sevetson et al, 1993 and Wu et al, 1993). In these cells cAMP inhibits the activation of Raf-1, resulting in the inhibition of both growth factor stimulated cell proliferation and MAP kinase activation. It has been further shown that Raf-1 is phosphorylated by PK-A at a consensus sequence within the Raf-1 regulatory domain (Wu et al, 1993) reducing the affinity of Raf-1 for Ras.

While growth factors and cAMP have antagonistic effects on fibroblast growth, both NGF and cAMP have been shown to induce neuronal differentiation of PC12 cells (Green & Tischler, 1976., Schubert et al, 1978., Gunning et al, 1981 and Richter-Lansberg & Jastorff, 1986). Moreover the treatment of NGF plus cAMP has been shown to have a synergistic effect on neurite formation (Gunning et al, 1981 and Richter-Lansberg & Jastorff, 1986). The way in which cAMP induces differentiation is unclear, however such findings have led to speculation that cAMP might actually stimulate the MAP kinase

cascade in PC12 cells. Some reports have disagreed with this hypothesis (Frodin et el, 1994 and Vaillancourt et al, 1994). However, more recent work has shown that PK-A probably interacts by phosphorylation of Raf-1 (Erhardt et al, 1995 and Kikuchi et al, 1996). One of the major differences between the Ras/Raf/MEK/MAP kinase cascades in PC12 and rat fibroblast cells is the presence of B-Raf in PC12 cells, in which it appears to be the major MEK activator (Jaiswal et al, 1994 and Moodie et al, 1994). The PK-A consensus sequence that is present on Raf-1 is not present on the B-Raf moiety (Sithanandam et al, 1990). There is some evidence that B-Raf is resistant to inhibition by PK-A and that this might in some way contribute to the cAMP stimulation of the MAP kinase cascade in PC12 cells (Erhardt et al, 1995).

1.7.2 RI as a positive regulator of cell growth

As already discussed PK-A exists as type I and type II isoenzymes which are distinguished by their different R-subunits, RI and RII. The ratio of the two isoenzymes has been shown to vary according to the phase of growth of the cell cycle. An enhanced expression of RI has been correlated with active cell growth and cell transformation, or the early stages of differentiation, while a decrease in RI and/or increase in RII has been correlated with growth inhibition and differentiation/maturation (Lohmann & Walter, 1984). Furthermore, differential expression of RI and RII subunits and their mRNA has also been correlated with neoplastic transformation and tumour growth. RI is the major if not sole R-subunit of PK-A detected in a variety of human cancer cell lines (Cho-Chung et al, 1989), including those derived from hormone-dependent and hormone independent breast cancers. However, in primary breast tumours the picture is less clear; significant increases in the RI/RII ratio (Handschin & Eppenberger, 1979), increases in

RI (Weber et al, 1981) or no change at all in the RI/RII ratio (Handschin et al, 1983) have all been reported. Changes in the pattern of PK-A isoenzyme expression have been commonly observed accompanying *in vitro* cell transformation (for review see Cho-Chung, 1993). Alteration in PK-A isoenzyme expression has also been found during carcinogenesis. During dimethyl-benz(α) anthracene (DMBA)-induced mammary carcinogenesis in rats, there was a transient increase in RI which coincided with the action of the carcinogen in the mammary tissue (Cho-Chung et al, 1987). An increase in RI also appears to be an early response to a variety of growth stimulatory agents, such as TGF-α in normal rat fibroblasts (Tortora et al, 1989), and oestrogen in rat uterus and DMBA-induced rat mammary carcinoma (Houge et al, 1992). Thus, RI appears to be a positive effector of cell growth responding to a variety of types of growth (both ontogenic and neoplastic) and stimulatory signals. One obvious difficulty with this concept derives from the fact that R-subunits do not possess catalytic activity. There has been some speculation that the R-subunits have a separate functional role which is unrelated to kinase activity and this remains open to question.

The use of RIα retroviral vectors provided the first direct evidence for a role of RI in cell proliferation (Tortora & Cho-Chung, 1990). Human mammary MCF-10A, rat thyroid FRTL5 and Chinese hamster CHO cells were all infected with the RIα retroviral vector. These cells showed morphological changes and were able to proliferate in a hormone independent manner, exhibiting a cell cycle distribution similar to that of the parent MCF-10A cells growing in the presence of hormones and serum (Tortora et al, 1992).

The use of the synthetic cAMP analogues has been one favoured approach to test the biological effects of PK-A. One such analogue, 8-Cl cAMP has been reported to have unique growth-inhibiting effects under physiological concentrations (μM), in a number

of different cell lines, without causing cytotoxicity (Katsaros et al, 1987 and Tagliaferri et al, 1988). It appears to inhibit growth while allowing cells to progress through their normal cell cycle, albeit at a slower rate (Pepe et al, 1991). Thus, 8-Cl cAMP does not prevent mitosis like cytotoxic drugs. Its mechanism of action has been ascribed to its ability to selectively modulate the RI and RII isoforms, by down regulating the RI and up regulating the RII isoform, thus restoring the normal balance of R-subunits (Rohlff et al, 1993). The analogue does not affect the catalytic subunit of PK-A (Roger et al, 1988 and Vintermyr et al, 1993). Furthermore, its effects in neoplastic mouse lung epithelial cells have been shown to be independent of the PK-A activity (Lange-Carter et al, 1993). Growth inhibition of a number of cell lines by 8-Cl cAMP, has also been shown to be accompanied by suppression of myc and ras proto-oncogenes and TGF-α, morphological changes, differentiation and reversal of transformation (Cho-Chung et al, 1989). This is of immediate relevance to clinical oncology as 8-Cl cAMP has been included in preclinical trials for the treatment of human breast cancer (Cho-Chung, 1992a). However, recent reports have suggested that the growth inhibitory effects of 8-Cl cAMP in CHO cells and lymphoblasts were caused by biological active metabolites of 8-Cl cAMP rather than the intact analogue (VanLookeren Campagne et al, 1991). This finding was subsequently disputed with the report that non-hydrolysable forms of this analogue also cause growth inhibition in HL-60 cells (Yokozaki et al, 1992).

The possibility that the type I R-subunit is a positive regulator of cell growth, essential for cancer cell growth, has been explored with the use of antisense oligodeoxynucleotides, which are complimentary to RI mRNA. It was found that depletion of RIα using this strategy led to growth arrest in human cancer cells of mammary or epithelial origin, including breast (MCF-7), colon (LS174T) and gastric

(TMK-1) carcinoma, with no sign of cytotoxicity (Yokosaki et al, 1993). The effect of RI antisense was further correlated to a decrease in RI and a concomitant increase in RII levels. However the coordinated expression of RI and RII was without effect upon C-subunit levels.

The increase in RII might be responsible for the observed differentiation of cells treated this way. Evidence for this is provided by the finding that exposure of cells to RII antisence oligonucleotides results in a blockage in cAMP induced growth inhibition and differentiation, at least in HL-60 cells (Tortora et al, 1990).

In conclusion, it has been proposed that the two isoforms of the PK-A regulatory subunit, RI and RII, are positive and negative regulators, respectively of cell proliferation, a unifying hypothesis to explain cAMP stimulation versus inhibition of cell growth. Thus, cancer cells can acquire differentiation and stop growing upon the restoration of a normal balance of RI/RII via the use of antisence oligonucleotides.

By comparing the morphological and biochemical effects of 8Cl cAMP as compared with those induced by okadaic acid (a ser/thr phosphatase inhibitor) which is known to induce apoptopic cell death, it was shown that 8-Cl cAMP induces apoptopic rather than necrotic cell death in a human mammary carcinoma cell line (MCF-7) (Boe et al, 1995). These effects of 8-Cl cAMP were mimicked by the metabolite 8-Cl adenosine, while stable cAMP analogues such as 8-CPT cAMP and N^6-benzoyl cAMP, or foskolin failed to induce cell death. Thus the effects of 8-Cl cAMP in inducing apoptopic cell death seem to be independent of PK-A activation, occurring through conversion to potent cytotoxic metabolites.

1.8 Aims of this study

The aim of the present study was to gain insight into the modulation of PK-A activity throughout the normal pregnancy/lactation/involution cycle of the rat mammary gland. Primary objectives were (i) to assess whether expression of PK-A generic C-subunit and of the individual regulatory subunits (R-subunits: RI and RII) were developmentally regulated in rat mammary tissue; (ii) to evaluate the possibility that catalytic activity of C-subunit is acutely modulated in mammary tissue throughout the pregnancy/lactation/involution cycle. Three distinct isoenzymes of C-subunit (α, β and γ, see section 1.6.2) have been described; since no functional significance has been suggested for this diversity it was hoped to determine whether selective expression of the isoforms occurred between pregnancy and lactation. Therefore the expression of Cα and Cβ genes were probed in rat mammary tissue by northern-blotting of their mRNA transcripts. This also enabled correlation of the steady-state level of these mRNAs and of total PK-A activity with C-subunit protein mass, allowing the molecular level of PK-A regulation and consistency of C-subunit specific activity to be determined. C-subunit protein mass was determined by an immunochemical method, customized to deal with the problems inherent in measuring this protein at low concentrations in rat mammary tissue.

Aberrant regulation of acetyl-CoA carboxylase has been reported in mammary tissue from lactating animals (Clegg, 1988 and Clegg & Calvert, 1988). Since the regulation of this enzyme is initiated via a cAMP-dependent mechanism, there have been some suggestions that mammary PK-A has distinctive properties; some evidence has been reported in support of this (Takhar & Munday, 1992). In order to identify any distinguishing characteristics of mammary tissue C-subunit, the present study also aimed

to examine the immunochemical properties of the PK-A C-subunit throughout pregnancy and lactation. Of particular interest was the maintenance of the specific catalytic activity of the C-subunit which was determined in conjunction with total PK-A activity and C-subunit protein mass, as described above.

The regulatory subunits of PK-A have been the subject of much investigation (see section 1.5.4) in which the enhanced expression of the RI isoform has been linked to neoplastic growth, cell transformation, or the early stages of differentiation; most of the published work relating specifically to the mammary gland has focused upon neoplastic cell growth. R-subunit levels were determined using the photoaffinity label 8-azido-[^{32}P]cAMP to examine mammary tissue extracts for their content of R-subunit isoforms and other cAMP binding proteins. The work described here hoped to examine the relationship between expression of R-subunit isoforms during normal ontogenic growth and development of the mammary gland which occurs throughout pregnancy and lactation.

One further objective was the measurement of PK-A activity in mammary tissue during pregnancy, lactation and involution in order to relate functional changes in mammary PK-A to the developmental state of the tissue. Both "total", measured in the presence of excess cAMP, and its "expressed" activity, representing dissociated free C-subunit present within the tissue at the time of sampling to measure expressed activity of mammary tissue, were measured.

With the discovery that cAMP may be a signal transducer for involutionary processes in other tissues (Dowd and Miesfield, 1992); the study further aimed to determine the pattern of PK-A expression in rat mammary tissue during involution. PK-A activity, C-subunit protein mass, C-subunit mRNA and expression of R-subunit isoforms were all

examined in animals whose litters had been removed and comparisons were made with corresponding measurements made, as described above in pregnant and lactating rat mammary tissue.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Rats

Female Wistar rats were studied at various, defined stages of pregnancy and lactation. Animals were given food and water *ad libitum,* and were fed on the C.R.N (E, expanded) animal diet from S.D.S, Witham, Essex. All animals were maintained on a constant lighting schedule (lights on between 08:00-20:00h), all experiments were started between 09:00 and 10:00h. For studies involving pregnant animals, only those carrying 8 or more fetuses were used, while for lactating rats, litter size was adjusted to 10 pups after birth.

Litter removal

Pups were removed from day 10 lactating rats, day 1 being defined as the first day to immediately follow the birth of the pups. Rats were then sacrificed 24h (LR24) and 48h (LR48) following removal of the litter.

Tissue

Prior to tissue sampling rats were anaesthetized with a non-recovery dose of sodium pentobarbital (60mg/kg body weight), delivered intraperitoneally. Dissection commenced once deep anaesthesia was attained. The mammary tissue was first freed from skin and abdominal wall, without cutting through major blood vessels before being freeze-clamped between aluminium tongs cooled in liquid nitrogen, excised and cryopulverized. Thus the gland was sampled with the circulation intact. Pre-weighed aliquots of powdered tissue were routinely stored at -70°C prior to any usage.

2.1.2 Sheep

Sheep were Finn-Dorset Horn cross-breds, between 2 and 5 years of age. Mammary samples were taken from approximately 100 day pregnant ewes and ewes into their 15-25th day of lactation. Animals were fed on hay *ad libitum,* plus a cereal mix (425g/day) fed as two meals at approximately 07:00 and 16:00h. On the day of sampling animals were deeply anaesthetized by intrajugular infusion of sodium pentobarbital (30mg/kg body weight), delivered via a cannula which had been inserted the previous day. Subsequently, animals were exsanguinated and tissue quickly excised and cryopulverized. Tissue was stored at -70°C in pre-weighed aliquots prior to use.

2.1.3 Chemicals

All chemicals used were of analar grade or above, from the following suppliers:-

BDH Chemicals Ltd, Poole, Dorset.

$CaCl_2$ ($6H_2O$)
EDTA
Glycerol
$MgCl_2$ ($6H_2O$)
2-Mercaptoethanol
$NaHCO_3$
NaOH
NaH_3
Orthophosphoric acid
Sucrose
Tris (base)

Calbiochem Novabiochem, Nottingham, Nottinghamshire.

Bistris propane

Fisons Scientific Equipment, Loughborough, Leicestershire.

$(COOH)_2$ Mg ($4H_2O$)
HCl
H_2O (HPLC grade high purity)
KCl

KF
K_2HPO_4 ($3H_2O$)
KH_2PO_4 Anhydrous
NaCl
NaF
NaH_2PO_4 ($2H_2O$)
Na_2HPO_4 ($2H_2O$)
$Na_4P_2O_7$ ($10H_2O$)
SDS and Urea

Fluka Biochemika Ltd, Glossop, Derbyshire.

Bisbenzimide (H33258)
EGTA

Premier Brands UK Ltd, Stafford, Staffordshire.

Marvel (Skimmed milk powder)

Sigma Chemical Company Poole, Dorset.

Ammonium Persulphate
Benzamidine-Hydrochloride
DTT
MES
MOPS
PMSF

2.1.4 Electrophoresis, Western blotting and immunodetection chemicals

Amersham International Plc, Amersham, Buckinghamshire.

ECL reagents 1 and 2 (RPN 2106)
HyBond-C extra

Biometra Ltd, Maidstone, Kent.

Bis-2 solution (2% ultra pure grade bis-acrylamide)
Acryl-40 solution (40% w/v ultra pure grade acrylamide)
Acryl/Bis 37.5:1 solution (38.98% w/v ultra pure grade acrylamide and 1.04% w/v bis-acrylamide, final concentration ratio 37.5:1, final overall acrylamide concentration 40% w/v)

2.1.5 Fine chemicals

Biorad, Hemel Hempstead, Hertfordshire.

Dye reagent concentrate (Bradford protein assay)
Hydroxyapetite (HAP)

Bohringer Mannheim UK (Diagnostics and Biochemicals) Ltd, Lewes, East Sussex.
Fatty acid free BSA

Pharmacia Biotech, St Albans, Hertfordshire.

Protein G sepharose
Sephadex G10

Pierce, Chester, Cheshire.

Iodogen

Sigma Chemical Company Ltd, Poole, Dorset.

Activated charcoal (untreated powder)
5'AMP (sodium salt, type II from yeast)
ATP (disodium salt)
Bovine Serum Albumin (Fraction V)
Coomassie brilliant blue G
3',5'cAMP (free acid)
DNA (salmon)
IBMX
Kemptide Synthetic Peptide
Molecular weight standards
Prestained molecular weight markers
Soyabean Trypsin Inhibitor (Type II-S)
Specific PK-A inhibitor peptide (synthetic peptide corresponding to the active site of the heat stable inhibitor protein of the cAMP dependent protein kinase, Cheng et al, 1986).

Whatman International Ltd, Maidstone, Kent.

DE-52 (DEAE cellulose)

Other

PK-A C-subunit peptides 322-332 and 1-12, synthesized as described in section 2.3.1

2.1.6. Radiolabelled chemicals

Amersham International Plc, Amersham, Buckinghamshire.

[γ-^{32}P] ATP ($\geq$4500 Ci/mmol)

ICN Pharmaceuticals Inc

8-azido adenosine 3',5'cyclic [^{32}P] monophosphate (50 Ci/mmol)
Sodium ^{125}I (17Ci/mg I)

2.1.7 Enzymes

Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd, Lewes, East Sussex.

Endoproteinase Lys-C sequencing grade
Endoproteinase Glu-C sequencing grade

In-house

Recombinant PK-A C-subunit (unmyristoylated)

2.1.8 Drugs and hormones

Calbiochem Novabiochem (UK) Ltd, Beeston, Nottingham.

Forskolin

Sigma Chemical Company Ltd, Poole, Dorset.

Sodium pentobarbital

2.1.9 Antibodies

SAPU (Scottish Antibody Production Unit) Carluke, Lanarkshire.

Anti sheep/goat IgG serum (donkey)
AP-linked anti sheep IgG serum (donkey)
HRP-linked anti sheep IgG serum (donkey)
Non-immune sheep serum

In- house antibodies

As-365, anti peptide 322-332/C-subunit serum (sheep), as described in section 2.3.
ab-365, anti C-subunit IgG, affinity purified from As-365, as described in section 2.3.5.
As-201, anti peptide 1-12/C-subunit serum (sheep)

2.2 Preparation and synthesis of materials

2.2.1 Purification of PK-A catalytic subunit

PK-A C-subunit was purified from sheep heart and mammary tissue (both pregnant and lactating) by the method described by (Flockhart & Corbin, 1984). All operations were carried out at 0-4°C. After excision tissue was quickly trimmed, diced and rinsed in normal saline before being frozen at -70°C until required. Approximately 200g of tissue was used per purification, and this was homogenised in 3 volumes of buffer (10mM potassium phosphate, 1mM EDTA and 0.1mM DTT pH 6.8 containing protease inhibitors 0.1mM PMSF, 1mM benzamidine-HCl, 0.2μg/ml soyabean trypsin inhibitor and 25μg/ml leupeptin) using a Waring blender at top speed, for 1min. The resulting homogenate was then centrifuged at 10,000 rpm, for 30min. Supernatant was saved and the pellet resuspended in the original volume of homogenisation buffer, repeating homogenisation and centrifugation steps. The supernatants from both spins were combined, filtered through glass wool and added to DEAE cellulose (55g) equilibrated in homogenisation buffer. This mixture was then incubated at 4°C for 3h, stirring every 15min. The DEAE cellulose was batch-washed with 4 x 750ml homogenisation buffer, and the resulting slurry poured into a column (6cm x 4.5cm, 130ml packed bed volume). The column was washed overnight, using a total volume of 750ml (55mM potassium phosphate, 1mM EDTA and 0.1mM DTT pH 6.8) and was eluted with a cAMP containing buffer (45mM potassium phosphate pH 6.8, 0.1mM DTT and 0.1mM cAMP added immediately before use), at a flow rate of 2.72ml/min. Fractions (6.8ml) were

collected at 2.5min intervals; 64 fractions being collected in total. UV absorbance (280nm) was measured throughout the procedure, in order to monitor protein elution. A small aliquot was taken from each fraction, diluted 1:100 in homogenisation buffer containing 0.075% w/v BSA (fraction V) and assayed for PK-A activity (as described in section 2.6.2). Those fractions representing the activity peak, (typically 20-55) were pooled and loaded onto a HAP-column (1.5cm x 12cm) equilibrated in buffer (40mM potassium phosphate, 0.1mM DTT pH 6.8) at 2.5ml/min. The column was then washed in this same buffer, and at the same flow rate for approximately 30min, or until the UV absorbance monitor had returned back to base line indicating no further elution of protein. Bound protein was eluted from the column in 2ml fractions by application of a linear phosphate gradient (40-350mM potassium phosphate, 0.1mM DTT pH 6.8) at a flow rate of 1ml/min. The column was then eluted further with 350mM potassium phosphate, 0.1mM DTT pH 6.8 (60ml). Forty (2ml) fractions were collected in total, for assay of PK-A activity aliquots of each fraction were first diluted 1:100 in BSA containing buffer as described above. Active fractions were pooled (usually 20-30), and diluted into 3 vols distilled water. In a final concentration procedure, the diluted pool was pumped at a flow rate of 2ml/min onto a small HAP-column (1 x 1cm), equilibriated in 40mM potassium phosphate and 0.1mM DTT pH6.8. The column was eluted manually with buffer (350mM potassium phosphate, 0.1mM DTT pH 6.8), from which 7 x 10 drop fractions were collected, of which number 7 was ultimately discarded, its primary purpose being to define the volume of the 10 drop fractions (approximately 0.5ml). A sample of each remaining fraction was diluted 1:1000 and assayed for PK-A activity. Protein was also measured as described in section 2.5.1. Less active fractions were discarded, glycerol was added to retained fractions (final concentration of 50% v/v)

which were then stored at -20°C. Glycerolised fractions were subjected to SDS-PAGE analysis on 10% Laemmli gels (as described in section 2.5.4 and 2.5.5), sufficient protein being loaded so as to give a "readable" result after staining with Coomassie blue (approximately 2-3μg). A pure preparation produced a single band of predicted molecular weight (40kDa).

2.2.2 Autophosphorylation of PK-A C-subunit

^{32}P-labelled C-subunit was prepared for use as an internal standard, enabling the recovery of C-subunit to be monitored throughout the various stages of the immuno-quantification experiments (details in section 2.7). On the day of the autophosphorylation the following solution was prepared 26.7mM MOPS/KOH pH 7.0, 21.3mM magnesium acetate, 5.3mM DTT. This solution was then used in the formulation of the following reaction mixture, inside a screw top Eppendorf tube; 13mM MOPS, 10.7mM magnesium acetate, 2.7mM DTT, 10% v/v Triton X-100, 180μCi [γ-^{32}P] ATP and 20μg C-subunit, total volume 1090μl. The reaction mixture was incubated at 30°C for 30min before being quenched by the addition of 200μl phosphatase inhibitor mix (200mM NaF, 20mM EDTA, 20mM EGTA, 5mM sodium pyrophosphate, 0.2mM sodium orthovanadate pH 7.0 with NaOH). The following were then added to the mixture; 5mg/ml cytochrome c (50μl), 100mM ATP (100μl) and 4.23ml bistris propane buffer (20mM bistris propane, 0.1mM DTT, 0.1% w/v NaN_3, 10% v/v glycerol and 0.01% w/v BSA (fatty acid free), pH 6.8 with HCl). The solution was mixed and loaded onto a CM-sepharose column (1cm x 1cm) pre-equilibrated in the same bistris propane buffer. The column was washed with 5 x 0.5ml buffer and eluted with 5 x 0.5ml bistris propane buffer (additionally containing 300mM NaCl). Fractions (approximately 0.5ml) were collected and glycerol

(0.5ml) added to the most radioactive fractions, which were then stored at -20°C. All fractions and washes were subject to TCA precipitation analysis (as described in section 2.5.3). However fractions containing the putative phosphorylated C-subunit were also subjected to SDS-PAGE analysis, followed by autoradiography (as described in section 2.5.4 and 2.5.10) in order to assess the degree of the autophosphorylation and association of radioactivity with the C-subunit.

2.2.3 Iodination of Protein G

This reagent was prepared as a means of detecting IgG molecules bound indirectly to nitrocellulose blots via their cognate antigens. The following reagents were dispensed into Iodogen coated tubes and incubated at room temperature for 20min; 0.5M Na-phosphate buffer pH 7.4 (10μl), 0.5mg/ml Protein G in 1M Na-phosphate buffer (10μl) and 500μCi ^{125}I (10μl). To terminate the reaction 0.5M Na-phosphate buffer pH 7.5 (100μl) was added to the incubation followed by 2% w/v KI (200μl). Tube contents were then transferred to a G10 Sephadex column (1cm x 1cm), pre-equilibrated in RIA buffer (60mM NaH_2PO_4, 154mM NaCl, 0.5% w/v BSA (fatty acid free), 0.1% w/v NaN_3 pH 7.4 with H_3PO_4). Radioactive fractions emerging close to the void volume of the column were pooled and subjected to TCA precipitation and SDS-PAGE analysis in order to check the extent of contamination of protein bound radioactivity with free ^{125}I [iodide] which elutes

later from a G10 Sephadex column. Before being used in the detection of IgG, the pooled fractions were diluted into TBS buffer (0.9% w/v NaCl, 5mM Tris/HCl pH 7.6 containing 1% w/v fatty acid free BSA, 0.01% NaN_3, and 0.02% w/v KI). The resulting solution (62.5ng/ml, 1000cpm/ng) was stored at 4°C prior to use.

2.3 Raising of an anti PK-A C-subunit antibody

A peptide corresponding to the amino acid residues 322-332 of the catalytic subunit of PK-A was synthesized and used to raise the polyclonal antiserum (As-365) in sheep, initially as a conjugate with keyhole limpet haemocyanin (KLH). Such residues were predicted and subsequently confirmed to reside on the surface of the intact C-subunit (Knighton et al, 1991).

2.3.1 Peptide synthesis

The peptide PK-AC 322-332-cys (G-D-T-S-N-F-D-D-Y-E-E-C subsequently abbreviated to 322-332) was synthesized by conventional F-MOC chemistry using a commercial automated synthesizer, and further purified by gel filtration on a G-10 Sephadex column (28 x 900) in 2.5% acetic acid, and preparative hplc on a reversed phase column (25 x 150mm). Final purity was checked by analytical reversed phase hplc and found to be greater than 95%. Peptide identity was confirmed by mass spectroscopy.

2.3.2 Cross-linking peptide (322-332) to KLH

The heterobifunctional reagent MBS was employed to cross link the peptide 322-332 to KLH. This contains a succinimido-ester functionality to react with $-NH_2$ groups (pH tolerance is broad with its optimum at pH 7.0), while also containing a maleimido functionality to react with -SH groups (pH tolerance range is narrow, pH 6.5-7.5, inactive at pH 8.0). In this way the MBS moiety was able to act as a "link" between KLH and the peptide antigen 322-332 and its C-terminal cysteine residue. The KLH (5mg) was dissolved in 50mM Na-phosphate buffer pH 8.0 (1ml), contained in a glass Reacti-vial (Pierce); MBS was dissolved in DMSO at a final concentration of 5mg/ml. This solution

(10μl) was then pipetted into the Reacti-vial while vigorously stirring the KLH solution. Stirring continued for 30min at room temperature. The final concentration of MBS was 160μM representing a 100 fold excess over KLH. The reaction mixture was cooled on ice and loaded onto a Sephacryl S200 HR column (13 x 210mm) equilibrated in 50mM-Na phosphate buffer pH 8.0 and held at 4°C. The column was run using the same buffer at approximately 0.75ml/min and 1ml fractions were collected. The absorbance of these fractions was continuously monitored and the excluded absorbance peak (fractions 17-22, 4.5ml total volume) pooled. The pooled fractions were transferred to a 16mm tube. Peptide 322-332 (5mg) was dissolved in 1.125ml 1M Na phosphate buffer pH 7.0 and added quantitatively to the stirred "activated" solution of KLH. Empirically determined, this volume of buffer was sufficient to achieve a pH of 7.2; however the pH was checked and any necessary adjustment made accordingly with 1M NaH_2PO_4. The reaction was allowed to continue for 3h at room temperature. Aliquots of 0.68ml were then snap frozen in liquid nitrogen and stored at -20°C.

2.3.3 Immunisation strategy

Antibodies to the KLH-peptide conjugate were raised in Finn-Dorset cross bred sheep, aged approximately 1 year. Immunogen was prepared by diluting a freshly thawed aliquot of the peptide-KLH conjugate (containing approximately 0.6mg each of peptide and KLH) into 1.25ml saline before emulsification in Freunds complete adjuvant (2.5ml). The resulting emulsion was then administered to the animal as 4 x 0.5ml subcutaneous injections at sites close to the major nodes of the lymphatic drainage of the limbs, and as 2 intramuscular sites in the hind limbs. Further inoculations were formulated in Freunds incomplete adjuvant and given as described above a further 4 times at 10 day intervals.

These were followed by three booster injections of unconjugated peptide (100μg, per dose, divided between 6 sites of injection as described above) prepared as above and administered monthly.

2.3.4 Antiserum preparation

The animal was bled via the jugular vein, seven days after the final injection. Whole blood was allowed to clot by incubation at 37°C for 1h and a further 6h at 4°C. Serum was then prepared by centrifugation at 18,000g, 4°C for 1h; and complement inactivated serum produced by a further incubation at 56°C for 30min. Antiserum was stored in 200μl aliquots and stored at -20°C.

2.3.5 Affinity purification of the anti PK-A C-subunit antiserum (As-365)

From a polyclonal antiserum such as As-365 it was possible to extract selectively those IgG molecules which recognised the intact C-subunit of PK-A. A purified preparation of bovine C-subunit (100μg/ml) was spotted onto 1cm x 1cm squares (20μl/square) of nitrocellulose (0.45μ HyBond-C extra), supported on slightly undersized Whatman 3mm blotting pads. Six squares were used per preparation; protein was allowed to adsorb for 1.5min before rinsing the squares in TBS (0.9% w/v NaCl, 5mM Tris/HCl pH 7.6, containing 0.1% w/v NaN_3 and 0.1mM PMSF). These were then blocked with 2% w/v BSA (fatty acid free) in TBS, at room temperature for 1.5h. the antiserum was diluted 1:10 in this same blocking solution. After a brief rinse in fresh TBS the squares were incubated with diluted antiserum (10ml) at room temperature for 2-3h. Squares were then intensively washed with 3 changes of the BSA block solution over a 45min period, followed by 2 x 15min washes in TBS alone. Squares were then incubated with 3ml 0.5M

glycine/HCl pH 2.5 at room temperature for 1h. Finally, nitrocellulose squares were removed from the acidic buffer which was then neutralized with 2M Tris/HCl pH 8.1 (0.591ml per 3ml glycine/HCl buffer) to give a final pH of 7.4. An aliquot (0.19ml) of this solution was saved for protein determination (as described in section 2.5.1); to the remaining (3.4ml), soyabean trypsin inhibitor was added to a final concentration of 0.1mg/ml and BSA (fatty acid free) to 0.02%. This solution was then dialysed overnight against 300ml TBS, aliquoted, snap frozen in liquid nitrogen and stored at -20°C until required.

2.4 Characterisation and uses of As-365

2.4.1 ELISA (Enzyme Linked Immunosorbent Assay)

This technique was used as a means of testing putative sheep antisera. The assay was of the direct type, that is the antigen, in this case KLH-322-332 conjugate (0.5mg/ml) or the peptide 322-332 (0.2mg/ml), were first plated out (50μl/well) into a 96 well ELISA plate (Immulon-2 plates were used to efficiently adsorb peptide antigen). This was then covered and incubated at room temperature, overnight. Any remaining non-specific protein binding sites were then blocked by washing with a Tween-20 containing buffer (PBS (7.2mM Na_2HPO_4, 1mM NaH_2PO_4 and 144mM NaCl)/0.05% v/v Tween-20). The putative antiserum was serially diluted in PBS/BSA 1% w/v solution, in the range of 1:5 through to 1:1000. Similar dilutions were also made for non-immune sheep serum. An aliquot (200μl) of the diluted antiserum, or non-immune serum control was then added to the immobilised peptide antigen in each well and incubated at room temperature for 2h. After intensive washing with PBS/0.05% Tween-20 the presence of any gamma-globulins was revealed by incubating the plates with a suitable second antibody, in this

case, Alkaline Phosphatase linked anti-sheep IgG serum, raised in donkeys. This was diluted 1:1000 in PBS/BSA 1% w/v and incubated (100μl/well) at room temperature for 2h; the wells were then thoroughly washed with PBS/0.05% Tween-20. A coloured product, proportional to the amount of gamma globulin bound was then generated by incubating each well, in the dark, at room temperature with 200μl of a 1mg/ml solution of the chromogenic substrate p-nitrophenol in phosphate buffer for 1.5h. Plates were then read on a Microtitre plate reader (absorbance 450nm).

2.4.2 Peptide competition

Since As-365 was raised against a synthetic peptide it was expected that treatment of the antiserum with its peptide antigen 322-332 would lead to a loss of immune reactions seen in Western blotting experiments. Peptide "competition" experiments were therefore performed using purified PK-A C-subunit and rat mammary tissue extracts in order to identify specific immune reactions of the antiserum. The immune reaction of most interest was that of the putative PK-A C-subunit, running at approximately 40kDa.

Peptide was dissolved in PBS/BSA 1% w/v to give a 1mg/ml solution, 20μl of which was incubated at 4°C, overnight with an equal volume of As-365 or non-immune sheep serum. The antiserum and non-immune serum was then used to probe Western blots of purified C-subunit or rat mammary tissue extracts (the latter prepared as for assay of PK-A activity, section 2.6.1). Western blots were first blocked with 4% w/v Marvel in TBS (0.9% w/v NaCl, 5mM Tris/HCl pH 7.6), blots were physically divided into lanes using a Miniblotter (Biorad) and probed with the peptide treated antiserum (diluted a further 1:500 in block solution). Control lanes were probed with diluted antiserum, or non-immune sheep serum which had been incubated, as described above, with the peptide

solvent alone. The Miniblotter was then placed on a rocking table and incubated at room temperature for 2-3h. After extensively washing blots with TBS, blots were finally treated with a HRP-linked (horseradish peroxidase) anti sheep IgG antiserum, at a final dilution of 1/5000 for 1h at room temperature followed by ECL detection and autoradiography (as described in sections 2.5.9 and 2.5.10).

2.4.3 Cleveland mapping

Using this technique PK-A C-subunit was cut into specific fragments using one of two proteinase enzymes, following the general procedures described by (Cleveland et al, 1977). Two proteinases were employed; Endoproteinase Lys-C from Lysobacter enzymogenes, which cleaves at the C-terminal side of lysine residues and Endoproteinase Glu-C (V8), from Staphylococcus aureus which specifically cleaves at the C-terminal side of glutamic acid residues. An aliquot of purified bovine heart C-subunit (20μl) was diluted with an equal volume of Laemmli disaggregation buffer (62.5mM Tris/HCl, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 5% w/v 2-mercaptoethanol and 0.05mg/ml bromophenol blue) and boiled for 2min. An aliquot of this mixture (10μl, equivalent to 5μg of protein) was then loaded onto the stacking gel of a 12% SDS-PAGE gel (which additionally contained 1mM EDTA). Stock solutions (100μg/ml) of the proteinase enzymes were routinely stored in 50% glycerol at -20°C. On the day of the experiment this solution was diluted 1:20 in a solution containing 62.5mM Tris/HCl, 0.1% w/v SDS, 1mM EDTA and 10% glycerol pH 6.8. An aliquot (20μl) of dilute enzyme was then overlaid onto the C-subunit containing sample already within the sample well. The proteins were run into the stacking gel, until the marker dye was just about to enter the separating gel. The current was switched off and the gel allowed to stand for 30min.

Electrophoresis was continued thereafter as described in section 2.5.4. Gels were then blotted onto nitrocellulose, "ghost" gels were stained with Coomassie blue and dried in order to visualise protein fragmentation. Blots were blocked in 4% w/v Marvel in TBS and probed with As-365 (diluted 1:1000 in block solution) followed by anti-sheep IgG (diluted 1:1000 in block solution) as described in section 2.5.7. Detection was made by incubation of the blots with ^{125}I-protein G, as described in section 2.5.8.

2.4.4 Immunoprecipitation

These experiments were designed to assess the ability of As-365, and derived affinity purified preparations (ab-365), to recognise both the free C-subunit of PK-A, and that associated with the R-subunits as holoenzyme. Lactating rat mammary cytosolic extracts (day 12) were prepared as for the assay of PK-A activity and described in section 2.6.1. Cytosols were diluted 1:10 in homogenisation buffer containing 10mg/ml BSA (fatty acid free). Neat antiserum and non-immune sheep serum, or solutions of affinity purified ab-365 and non-immune sheep gamma globulins (both at 30μg IgG/ml), were all diluted 1:10 in this same BSA containing buffer. A panel of immuno-precipitations were then performed in which fixed quantities of the dilute mammary tissue extract (50μl) were incubated with various quantities of the As-365/ab-365, in the presence of 0.67μM cAMP (final volume 120μl).

A parallel set of incubations were also carried out in the absence of cAMP, in order to examine the dependency of antibody/antigen recognition upon holoenzyme dissociation. Samples were incubated on ice for 15min before addition of a 50:50 slurry of Protein G Sepharose prepared in TBS containing 1% w/v BSA (fatty acid free), 0.02% w/v $NaN_{3;}$; 60μl was added to incubations containing As-365 and 10μl to incubations containing the

affinity purified ab-365. Samples were then spun at 8,800g for 2min. The supernatant was collected, transferred to fresh tubes and assayed for total PK-A activity as described in section 2.6.2.

2.4.5 Immunoinhibition

Since As-365 was raised against a peptide corresponding to residues 322-332 of the PK-A catalytic subunit, which are located at the surface of the protein, close to the binding sites for substrate peptide and for adenine nucleotides, the possibility that the antiserum would inhibit the catalytic activity of C-subunit was investigated.

Immunotitration inhibition analysis of purified C-subunit

Titration-like experiments were performed in which various quantities of As-365 were incubated with purified PK-A C-subunit (100μunits). C-subunit preparations from several sources were used, including bovine heart, pregnant and lactating sheep mammary tissue and finally a recombinant source. The antiserum was first diluted 1:10 in PBS/ 1% w/v BSA (fraction V). Purified preparations of C-subunit were also diluted in 1% w/v BSA/PBS to give a solution containing approximately 4munits/ml.

A parallel series of control tubes was also set up, containing non-immune sheep serum in place of As-365. All tubes were incubated at 37°C for 30min, after which time 10μl of the reaction mixture was assayed for total PK-A activity, as described in section 2.6.2. From the calculation of total PK-A activity, titration curves were constructed from which titre values of the antiserum for different preparations of C-subunit could be derived.

Immunoinhibition analysis of rat mammary extracts

The first of these experiments were designed to assess the ability of As-365 to immunoinhibit both activated/dissociated and inactive/associated PK-A activity in rat mammary tissue extracts. Lactating rat mammary tissue extracts (day 12) were prepared, as for the assay of PK-A activity (section 2.6.1), and diluted 1:10 in homogenisation buffer containing 10mg/ml BSA (fraction V) and the following protease inhibitors: 0.1mM benzamidine, 0.1mM PMSF and 1μg/ml soya bean trypsin inhibitor. As-365 and non-immune sheep serum were also diluted 1:10, in the same buffer. Aliquots (50μl) of diluted mammary extract (containing approximately 120μunits of PK-A activity) were pipetted into a series of Eppendorf tubes together with 4μM cAMP or H_2O (20μl); 50μl of the dilute antiserum or non-immune serum was then added to each tube. All tubes were mixed and incubated at 37°C for 30min before being assayed in triplicate for total PK-A activity as described in section 2.6.2.

In similar experiments, mammary extracts prepared from tissue sampled at different stages of pregnancy and lactation, were incubated with an excess quantity of As-365. In this way the quantity of immunoinhibitable PK-A activity could be assessed throughout rat mammary tissue development. Cytosolic, mammary tissue extracts, prepared as for PK-A assay (section 2.6.1), were diluted 1:10 in homogenisation buffer containing 10mg/ml BSA (fraction V) and protease inhibitors as described above. As-365 and non-immune sheep serum were also diluted 1:10, in the same buffer. Aliquots (50μl) of diluted mammary extract (containing a maximum 120μunits of PK-A activity) were pipetted into a series of Eppendorf tubes together with 4μM cAMP (20μl); 50μl of the dilute antiserum (theoretically capable of immunoneutralising 250μunits of C-subunit activity), or non-immune serum was then added to each tube. All tubes were mixed and

incubated at 30°C for 30min before being assayed in triplicate for total PK-A activity as described in section 2.6.2.

2.4.6 Immunodetection of PK-A C-subunit in Western blots

As-365 was used to detect and quantify C-subunit in purified preparations of this protein, and in mammary tissue extracts, as discussed in sections 2.5.7 and 2.7.3.

2.5 Assays and analytical methods

2.5.1 Protein assay

The assay was commonly used to measure the protein content of tissue extracts and solutions, as described by (Bradford, 1979). The assay is based upon the observation that the maximum absorbance A_{max}, of an acidic solution of Coomassie brilliant blue G, shifts from 465nm to 595nm when binding to protein occurs. The dye interacts principally with basic and aromatic amino acids. A standard curve was constructed by dilution, in water, of a BSA standard (kept frozen in aliquots at -20°C), in the range of 2-15μg/ml. Samples were also diluted to fall within this range.

2.5.2 DNA assay

The DNA content of tissue homogenates was measured via the enhancement of fluorescence which occurs when the fluorochrome Bisbenzimide binds to DNA. RNA does not interfere with this assay. Such determinations give a measure of cellularity and so allowed results to be standardised on a per mg DNA basis.

The assay is based on that described by (Labarca & Paigen, 1980). Standards of salmon sperm DNA were stored at -20°C as a stock solution in 2mM EDTA. Tissue homogenates

were diluted into 0.5ml assay buffer (2M NaCl, 50mM Na-phosphate, pH 7.4). A standard curve of salmon DNA (0-2μg/ml) was also constructed in a final volume of 0.5ml assay buffer To these, a further 1ml of assay buffer was added followed by 1.5ml bisbenzimide reagent (200μg/ml in DNA assay buffer). Final assay volume was 3ml; all solutions were then incubated at room temperature for 1h. Fluorescence was read on a Perkin-Elmer 3000 spectrofluorometer, with an excitation wavelengh set at 335nm and emission set at 445nm and expressed in arbitrary units.

Most samples for DNA analysis were whole mammary homogenates, these were generally diluted to a final "in assay" dilution of 1:3000 to 1:6000 with respect to wet tissue weight. At lesser dilutions turbidity of the homogenate samples hindered the attainment of stable readings.

2.5.3 TCA precipitation analysis

The object of this procedure was to precipitate proteins out of solution enabling the partitioning of radioactivity between protein bound and unbound states to be determined. An aliquot (50μl) of the radioactive solution was counted (conventional γ-counting for ^{125}I and Cerenkov for ^{32}P); PBS/BSA 1% w/v (fraction V), (50μl) was then added as a carrier protein. The mixture was then double diluted with an equal volume of ice cold 10% w/v TCA and incubated on ice for 15min. After spinning on a bench top centrifuge for 2min (8,800g) the supernatant was removed and the pellet washed briefly by resuspending in 1ml of the TCA solution and recentrifuging as above. The tube was then blotted dry and counted for radioactivity. Results were normally expressed as per cent TCA precipitable counts (cpm).

2.5.4 Polyacrylamide gel electrophoresis (PAGE)

The sodium dodecyl sulphate (SDS) Tris-glycine discontinuous system was used as described by (Laemmli & Favre, 1973). Slab gels, 14 x 16 x 0.15cm were used. Protein solutions were solubilised by boiling for 2min in Laemmli sample buffer; 62.5mM Tris/HCl pH 6.8, 2% w/v SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 2mM PMSF and 0.05mg/ml bromophenol blue marker. Gels were usually run in pairs at 200V for 3h.

2.5.5 Coomassie blue dye staining

Staining of gels was applied to detect proteins following SDS-PAGE. When electrophoresis was complete, gels were soaked for at least 1h in a fixer solution (25% isopropanol, 10% acetic acid) and then stained by soaking the gels for 10-20min in 0.1% Coomassie Brilliant blue R prepared in fixer. Protein bands were visualised by washing the gel, over several hours in destainer (7% acetic acid, 1% glycerol), until the background cleared.

2.5.6 Western blotting

Electrophoretic blotting of proteins from SDS-PAGE gels onto nitrocellulose membranes was as described by (Towbin et al, 1979). Transfer buffer contained 25mM Tris, 192mM glycine and 20% v/v methanol. Gels were typically blotted at room temperature for 2.5h at 350mA or overnight at 60mA.

2.5.7 Immunodetection of proteins on nitrocellulose

Blots were washed in TBS (0.9% w/v NaCl, 5mM Tris/HCl pH 7.6) for approximately

1h at room temperature in order to remove contaminating methanol which is a major constituent of transblot buffer. Blots were then incubated for a minimum of 1h, at room temperature, in a "block" solution consisting of 4% w/v Marvel (skimmed milk powder) in TBS. After a brief 10min wash in fresh TBS the blots were then probed with the first antibody; this was specific for the protein of interest e.g As-365 was immunospecific for PK-A C-subunit. Neat antisera such as As-365 were generally diluted 1:1000 in block solution; affinity purified antibodies such as ab-365, of lower IgG concentration were diluted to a lesser extent e.g 1:100. Blots were incubated at room temperature with the first antibody for 2-3h followed by an extensive washing period of 1h, with a minimum of 5 changes of TBS during this period. A second antibody was used in order to amplify the detection of the first; this was diluted in block solution as for the primary antibody and incubated with the blot at room temperature for 1h. The blot was then vigorously washed in TBS before being subjected to detection procedures such as ECL or further incubation with ^{125}I-protein G.

2.5.8 ^{125}I-Protein G detection

^{125}I-protein G was used as the detection system in experimental blots which had been probed with a sheep antibody (As-365, ab-365 or As-201) followed by anti sheep IgG antiserum, raised in donkeys. Prior to use the ^{125}I-protein G was diluted in TBS solution containing 1% w/v BSA (fatty acid free), 0.01% w/v NaN_3, 0.02% w/v KI; (final concentration of 62.5ng/ml, approximately 1000 cpm/ng). Blots were incubated in this solution for 2-3h, washed for 1h (with a minimum of 5 changes of TBS, 0.01% w/v NaN_3, 0.02% w/v KI), dried in air and exposed to X-ray film.

2.5.9 ECL detection

ECL (Enhanced Chemiluminescence) is a light emitting, non-radioactive method of detection of specific immobilized antigens, conjugated indirectly with a HRP-labelled (Horse Radish Peroxidase) antibody. Blots were blocked in 4% w/v Marvel/TBS and then treated with the specific first antibody as described in section 2.5.7. Blots were then incubated with a HRP-linked IgG diluted 1:1000 in blocking solution for 1h at room temperature. Incubation with the HRP-linked IgG, was followed by vigorous washing in TBS, excess liquid was drained off and the blot was then incubated for 1min with a 1:1 mixture of ECL reagents 1 and 2 (Amersham RPN 2106). Excess mixture was drained off the blot which was placed between sheets of Saran Wrap. In order to avoid the problem of light scattering throughout the blot, it was important at this stage to remove all excess ECL mixture by applying sufficient pressure to the wrapped blot with a paper towel. The blot was then exposed to Fuji X-ray film for approximately 1min (although both shorter and longer periods were often required depending upon the strength of signal obtained) in a Kodak X-Omatic cassette, with card for extra compression.

2.5.10 Autoradiography

Autoradiography was used to detect proteins (a) labelled either directly or indirectly with the radioactive isotopes ^{32}P or ^{125}I; or (b) linked indirectly to a source of chemiluminescence, such as during ECL detection (2.5.9). The radioactive blot or dried radioactive gel was exposed to Fuji X-ray film in a Kodak X-Omatic cassette at -70°C. For ECL detection, blots were exposed to film at room temperature. The film was then developed for 2min with Kodak GBX developer and fixed for 5min using Kodak GBX fixer. The resulting autoradiographs were then analysed by densitometry in order to

quantify individual protein bands. Alternatively, the autoradiograph was used as a reference, to locate radioactive protein bands on gels which were then excised and counted for radioactivity.

2.6 Assay of PK-A activity

2.6.1 Mammary extract preparation

Extracts for the assay of PK-A activity were prepared by homogenization of frozen, pulverized tissue, at 4°C in 4 volumes of ice cold buffer (10mM MES/NaOH, 0.2mM EDTA, 0.1mM DTT and 0.5mM IBMX, pH 6.8 containing the following protease inhibitors: 0.1mM benzamidine-HCl, 0.1mM PMSF and 1μg/ml soyabean trypsin inhibitor) by using a Polytron homogenizer operating on setting 4 giving 22,000 rev/min for 2 x 10s. A sample of the resulting homogenate was withdrawn for DNA estimation (as described in section 2.5.2). Homogenisation buffer containing 11.1 mg/ml BSA (fraction V) was added to aliquots of the resulting homogenate, diluting the samples a further 10-fold and giving a final BSA concentration of 10mg/ml. Gross particulate material was removed by centrifugation for 15min at 8,800g in a bench top Eppendorf. Aliquots (200μl) of the resulting supernatant were then snap frozen and stored at -70°C for a maximum period of 1 month prior to assay. Immediately before assay, extracts were thawed and diluted a further 20-fold in homogenization buffer, containing 10mg/ml BSA (fraction V). Validation experiments showed that for mammary tissue extracts, a dilution of 1:1000 (with respect to wet tissue weight), resulted in a range of activities over which the assay was linear with respect to amount of sample added (results not shown).

2.6.2 Activity measurement

The assay is based upon the incorporation of a ^{32}P from [γ-^{32}P] ATP into a peptide substrate (Kemptide), as described by (Roskoski, 1983). Tissue extracts were incubated with radiolabelled ATP, in triplicate under three distinct conditions: in the absence of cAMP and the presence of the specific PK-A inhibitor peptide, the absence of both cAMP and inhibitor and finally in the presence of cAMP and absence of inhibitor. Such measurements allowed the calculation of phosphorylation not attributable to PK-A. Total activity was taken as that measured in the presence of a maximally stimulating concentration of cAMP (0.6μM, Clegg & Ottey, 1990). Basal activity was that measured in the absence of additional cAMP. An activity ratio was derived from these two values as follows:-

$$\textbf{Activity Ratio} = \frac{\textbf{Basal Activity}}{\textbf{Total Activity}}$$

PK-A activity was expressed in units as follows:-

Unit definition: 1μmol phosphate transfered/min

In principle the measurement of PK-A activity ratio offers the possibility of a more precise determination of the intracellular cAMP status of tissue than can be made by the direct measurement of tissue cAMP (Corbin, 1983 and Clegg & Ottey, 1990). This is especially true for mammary tissue derived from lactating animals, where this nucleotide is to be found at relatively high levels in milk (approximately 2-5μM, Sapag-Hagar & Greenbaum 1974). Furthermore, since all known physiological responses to cAMP occur through the PK-A system it is useful to know the levels of dissociation and therefore the

consequential activation of this enzyme.

On the day of assay, a radioactive ATP/Kemptide mixture was made up at 4°C containing the following:- 750μl(40mM MOPS/KOH pH 7.0, 32mM magnesium acetate)

15μl 10mM ATP

15μl 400mM DTT

150μl 2mg/ml Kemptide

7.5μl 10mCi/ml [γ-^{32}P] ATP*

Final Volume 1.5ml with distilled water

*(volume adjusted as isotope decayed to give a mixture containing 5μCi/ml)

An aliquot (40μl) of this mixture was pipetted into Eppendorf tubes, held on ice, which contained either 10μl PK-A inhibitor peptide (0.6mg/ml), distilled water or cAMP (4μM). Samples were thawed, and if necessary, diluted further (within the range 0.1-5munits/ml) in homogenisation buffer plus 10mg/ml BSA (fraction V). Tubes were then pre-warmed for 3min by transfer, in a timed sequence to a dry block held at 30°C. The reaction was started by the addition, again in a timed sequence, of 10μl of diluted sample. Tubes were then mixed and incubated for 15min. Under these conditions phosphorylation of Kemptide was linear with respect to time for at least 20min (results not shown), a fixed duration of 15min was therefore routinely used throughout these studies. The reaction was stopped by spotting 30μl of the reaction mix onto labelled squares (2cm x 2cm) of Whatman P81 cation exchange cellulose paper. These were then dropped into a stirred beaker containing 300ml of 75mM phosphoric acid. Papers were washed until the end of all incubations and then for a further 5min before being transferred to a fresh beaker of phosphoric acid for the second 5min wash. The 5min washes were repeated twice more before removing the papers and blotting them dry with paper towelling. Papers were

placed in scintillation vials and scintillant (5ml, Emulsifier-Safe) added, before samples were counted for 4min on a Packard 1600 TR Liquid Scintillation Analyser, from which a cpm value was obtained. Two sets of blanks were additionally run, consisting of ATP/Kemptide mix (20μl) spotted onto papers and added at the washing stage, both before and after the experimental series. These allowed the assessment of background contamination, indicating whether or not the washing procedure was sufficient. Finally in order to determine the specific radioactivity of the ATP/Kemptide mix, a further aliquot (40μl) of this mixture, equivalent to the input to each assay tube was spotted onto filter papers and placed directly into vials for counting.

2.7 Immuno-quantification of C-subunit

2.7.1 Immuno-quantification of C-subunit in rat mammary tissue extracts

In these experiments mammary tissue extracts were prepared from animals sacrificed at different stages of pregnancy and lactation together with tissue from lactating animals whose litters had been removed. Attempts to immunoquantify C-subunit in mammary extracts prepared for assay of PK-A activity proved unsatisfactory; problems arose from the low concentration of tissue proteins, large quantities of BSA and high levels of non-immune reactions. Therefore less dilute cytosols were prepared and a strategy of cytosol "clean-up" and affinity purification of As-365 implemented.

2.7.2 Preparation of mammary tissue extracts

All operations were carried out at 0-4°C. One part by weight of cryopulverised tissue was homogenised with a Polytron for 2 x 10s at setting 4 (2,200 rev/min) in 4 volumes of homogenisation buffer (10mM MES/NaOH pH 6.8, 1.2mM EDTA, 1mM EGTA, 0.1mM

DTT, 0.5mM IBMX with the following protease inhibitors: 0.1mM benzamidine/HCl, 0.1mM PMSF, 1μg/ml soyabean trypsin inhibitor and phosphatase inhibitors: 10mM NaF, 0.25mM sodium pyrophosphate, 10mM sodium orthovanadate) which was supplemented with 4μM cAMP immediately before use. Gross particulate material was removed by centrifugation at 8,800g, 4^{0}C for 15min. The following reagents were then added to 0.5ml of the supernatant; ^{32}P labelled C-subunit (10μl, 0.043μg), 50mg/ml cytochrome c (20μl) and PBS/BSA 15% w/v (fatty acid free) (35μl). The ^{32}P labelled C-subunit (prepared as described in section 2.2.2), was present as an internal standard, reporting C-subunit recovery throughout subsequent stages of the immuno-quantification process (see below). The above mixture was first filtered through 5μm and 0.45μm Acrodiscs before loading onto a 1ml CM Sepharose column, equilibrated in 10mM MES/NaOH pH 6.8, 0.2mM EDTA, 0.1mM DTT and 0.5mM IBMX. The column was washed in buffer (5 x 0.5ml), which were bulk collected and subjected to TCA precipitation analysis. Elution was carried out with 5 x 0.5ml 8M urea, 2% SDS, 15% glycerol, in 125mM Tris/HCl pH 6.8. Fractions were monitored for radioactivity; the most radioactive fraction (usually the second) was retained and replicate aliquots (200μl) of this solution disaggregated by adding 2-mercaptoethanol (2.5μl) and by boiling for 2min.

2.7.3 Immuno-quantification procedure

Samples of 180μl were then loaded and run on duplicate SDS-PAGE (8% gels, 14 x 16cm) in the Laemmli buffer system (as described in section 2.5.4). At least one lane on each gel was reserved for loading with disaggregated ^{32}P-labelled C-subunit (0.043μg) and prestained M_r standards (Sigma). On completion of electrophoresis, separated

proteins were electroblotted onto nitrocellulose sheets (section 2.5.6). After suitable washing and blocking, the electroblots were probed with affinity purified ab-365 (0.2μg/ml) or non-immune sheep IgG of the same concentration (as described in section 2.5.7). Treatment with second antibody (antisheep IgG, raised in donkeys and diluted 1:1000) was followed by exposure of blots to ^{125}I labelled Protein G (62.5 ng/ml, approximately 1000 cpm/ng) and exposed to X-ray film (see sections 2.5.7, 2.5.8 and 2.5.10). The position on the nitrocellulose of radioactive bands was determined by reference to the autoradiograph and excised. Control "bands" of the same dimensions and from the same lanes as those of the experimental series were also excised and counted for ^{32}P and for ^{125}I. Detection of ^{32}P was made by Cerenkov counting (in the absence of scintillant), on a Packard 1600 TR Liquid Scintillation analyser. ^{125}I was detected by conventional γ-counting on a Packard, Cobra Autogamma machine. A third gel was also loaded with 4 x aliquots (0.043μg) of the disaggregated ^{32}P labelled C-subunit, equivalent to that added to mammary tissue samples as internal standard. However, this gel was not electroblotted but fixed and dried; C-subunit bands were located by autoradiography and counted by Cerenkov in order to obtain the number of cpm associated with the labelled C-subunit (see below).

2.7.4 Calculation of C-subunit protein mass

The ^{32}P labelled C-subunit was employed as a reporter of C-subunit recovery (internal standard) and enabled an estimation of the endogenous C-subunit protein mass to be made for experimental samples. Labelled C-subunit was detected in both experimental samples and controls by Cerenkov counting. By measuring those counts associated with a known quantity of labelled C-subunit, an estimate of that remaining in each

experimental sample could be made. Since all experimental samples received the same quantity of internal standard, any differences between them in this recovery value could be related to different conditions experienced by C-subunit in the homogenates, or during experimental procedures. Thus, a correction value could be applied to γ-counts for the same samples, which represented total C-subunit mass (both endogenous and internal standard). A final estimate of C-subunit protein mass could be made for each sample by reference to the γ-counts associated with a known quantity of ^{32}P C-subunit, which had been run separately on the original SDS-PAGE gel (see above). C-subunit mass representing the internal standard was deducted from this value and following further corrections for dilutions, an estimate of tissue C-subunit was made. Such data was used, in conjunction with total PK-A activity data to estimate the specific catalytic activity of C-subunit in rat mammary tissue.

2.8 Northern blotting

2.8.1 RNA preparation from rat mammary tissue

RNA was prepared from rat mammary tissue by the method previously described by (Travers & Barber, 1993). Mammary tissue stored at -80°C (0.5g) was pulverised under liquid nitrogen to produce a fine powder before being allowed to thaw into 3.5ml GIT (4M guanidine isothiocyanate, 50mM Tris, 20mM EDTA, 2 % v/v sarkosyl, 1% v/v 2-mercaptoethanol). The resulting slurry was then passed through a series of fine needles (18 and 21 gage) in order to shear the DNA present. The homogenate was weighed and solid CsCl added to it (1g CsCl: 2.5g homogenate), mixing well. Homogenate was then layered onto a 1.2ml cushion of 5.7M CsCl and 0.1M EDTA, contained in a 5ml Beckman polyallomer tube, using a sterile Pasteur pipette. Tubes were balanced and filled to capacity with GIT prior to spinning at 800rpm, 20°C for 18h in a Beckman T7

(SW55 rotor). The supernatant was then removed with a sterile pasteur pipette, down to the level of the CsCl cushion and discarded. The CsCl cushion was also discarded and the sides of the tube wiped dry before quantitatively resuspending the pellet in 1ml water (deionised HPLC grade). To this a 1:1 v/v mixture of chloroform/butanol was added (1ml), mixed thoroughly with the sample and spun at 2750rpm, room temperature for 10min. The top aqueous phase was then added to a sterile Corex tube. The organic phase was re-extracted, by adding an equal volume of water (deionised) and spinning as described above. The aqueous phase from this extraction was then removed and combined with that of the first.

RNA was precipitated by addition of 0.1 volumes 3M sodium acetate pH 6.0 and 2.5 volumes absolute alcohol. This was then mixed thoroughly and incubated overnight at -20°C before being spun at 11,000rpm, °4 C for 30min. Discarding the supernatant, samples were freeze dried to remove all traces of solvent and redissolved in water (deionised, 400μl per 0.5g original weight mammary tissue). The RNA concentration of the solution was then determined by measuring the absorbance ratio of 1μl (diluted into 1ml deionised water) read at 260nm (and 280nm); water (deionised) was used as a blank. A standard curve was constructed by dilution, in water (deionised), of an RNA standard of known concentration; thus the absorbance of the sample could be converted into μg/μl. The absorbance ratio of the sample 260nm/280nm was used to determine the purity of the RNA sample i.e. a ratio of 1.6-1.8 equalled a sample free from DNA and protein.

2.8.2 Northern blotting

Northern blots were performed using RNA prepared from rat mammary tissue and by the method previously described by (Travers & Barber, 1993). RNA samples were prepared

by heating the equivalent of 2μg RNA (in approximately 2.5μl) at 65°C for 5min, after addition of 7.5μl LDB (0.03mM MOPS, 1.3mM EDTA, 10.6mM sodium acetate, 66% v/v formamide and 20% v/v formaldehyde). Samples were then cooled on ice, tracking dye added (5μl) and pulse spun directly before being loaded onto a MOPS gel. Samples were then separated on a MOPS gel (1.2% agarose, 5% v/v formaldehyde and 0.005% v/v ethidium bromide) run at 70V for 2h. Gels were photographed (under UV) in order to visualise the RNA before being given two 15min washes with 10 x SSC buffer (0.3M sodium citrate buffer pH 7.0, containing 3M NaCl). Gels were then placed "wells down" on a piece of Whatman 3mm paper, which had been soaked in water, before addition of the nylon membrane (ICN Biotrans cut to size). A further two sheets of Whatman paper were placed over the membrane; care was taken at all stages to ensure maximum contact between membrane and gel. To these a large wadge of paper towels was added (6cm in height) and a glass plate, on top of which a 1kg weight was placed. The whole was then placed above a reservoir containing 10 x SSC, with which the lower layer of Whatman paper made contact to form a salt bridge. The gel was then left to blot overnight (approximately 16h). The blot was fixed by drying at 37°C for 30min and stored at 4°C until ready to probe.

2.8.3 Detection of Cα and Cβ mRNA

The cDNA probe for the α-isoform of the rat PK-A C-subunit and the riboprobe for the rat β-isoform and rat α-casein cDNA were as described by (Massa et al, 1991). 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 loading of 28s rRNA in each electrophoretic track, as

described by (Travers and Barber, 1993). After assay of tissue DNA content (described in section 2.5.2), the resulting corrected levels of C-subunit mRNAs were then calculated on a per mg DNA basis.

2.9 Photoaffinity labelling

Photoaffinity labelling has been extensively used to study binding proteins for the cyclic nucleotides cAMP and cGMP. The most widely used photoaffinity analogue of cAMP is

8-N_3[^{32}P] cAMP, which was used here to detect cAMP binding proteins in a variety of mammary tissue extracts as described by (Walter & Greengard, 1983).

2.9.1 Mammary extract preparation

Extracts were prepared by homogenising frozen, pulverized tissue in 10 volumes of ice cold buffer (10mM MES/NaOH, 0.2mM EDTA, 0.1mM DTT and 0.5mM IBMX, pH 6.8 containing the following protease inhibitors: 0.1mM benzamidine-HCl, 0.1mM PMSF and 1μg/ml soyabean trypsin inhibitor) using a Polytron homogenizer, setting 4, giving approximately 22,000 rev/min for 2 x 10s. Cytosolic extracts were made by spinning the resulting homogenate at 40,000 rpm, 4°C for 1h. The resulting supernatant was then removed, and snap frozen in aliquots (200μl), before being stored at -70°C for a maximum 1 month period.

Particulate extracts were made by resuspending the pellet fractions using a semi-micro, hand operated, Potter-type, all glass homogeniser, in 1ml of homogenisation buffer, and respinning as above. The supernatant was then discarded and the pellet resuspended in homogenisation buffer, such that the final protein concentration was within the range of

2.0-5.0mg/ml.

2.9.2 Photoaffinity labelling of tissue extracts

Samples in a grouped experiment were equalized with respect to their protein concentrations, by dilution in homogenisation buffer supplemented with protease inhibitors. Samples were generally used at a protein concentration between 0.5-2.0mg/ml. 8-Azido [^{32}P] cAMP was diluted in homogenisation buffer, to a working concentration of 3μM (approximately 150μCi/ml) immediately before use. The reaction was carried out in a 96 well polystyrene microtitre plate, into which the standardised protein sample (50μl), diluted 8-azido [^{32}P] cAMP (15μl) and 0.37M MES/0.053M $MgCl_2$ solution (15μl), were pipetted and incubated at room temperature in darkness for 1h. The plate was then irradiated for 60s using a 15watt long wavelengh UV lamp, at approximately 20cm from the samples. The reaction was stopped by the addition of 3 x Laemmli sample buffer (40μl) to each well. Samples were transferred to screw-top Eppendorf tubes, disaggregated by boiling for 3min, loaded onto 12% acrylamide gels and subjected to SDS-PAGE. (Samples from experiments of paired design were run on the same gel). Dye fronts were removed and discarded prior to fixation in order to diminish the burden of unbound radioactivity. Gels were fixed overnight, or for a minimum 8h period in gel fixer solution (10% v/v glacial acetic acid, 10% v/v glycerol and 40% v/v methanol) and dried, under vacuum at 60°C for 1h, before being subject to autoradiography. Radiolabelled proteins were quantified by densitometric analysis of autoradiographs. Alternatively the position on the nitrocellulose of radioactive bands was determined by reference to the autoradiograph and excised. Control bands of the same dimensions and from the same lanes as those of the experimental series were also excised and added to scintillation vials

together with scintillant (5ml, Emulsifier-Safe), samples were then counted for 4min on a Packard 1600 TR Liquid Scintillation Analyser, from which a cpm value was obtained.

2.9.3 Photoaffinity cAMP competition

In order to show that the labelled protein bands were authentic cAMP binding proteins, competition experiments were performed using radio-inert cAMP. Protein samples were diluted in homogenisation buffer with and without non-radioactive cAMP (136μM) and taken through the photoaffinity labelling procedure described above. Bands seen in the experimental lanes but not in the presence of non-radioactive cAMP were deemed to be cAMP binding proteins. Further competition experiments, using cGMP and 5'AMP in place of cAMP, were also performed under these same conditions in order to define the properties of these proteins still further.

2.10 DEAE-cellulose resolution of PK-A holoenzyme isoenzymes from rat mammary tissue

2.10.1 Mammary extract preparation

One part, by weight, of rat mammary tissue from day 10-12 lactating animals was homogenized in 4 volumes of buffer (10mM MES/NaOH, 0.2mM EDTA and 0.1mM DTT pH 6.8 plus protease inhibitors: 0.1mM benzamidine-HCl, 0.1mM PMSF and 1μg/ml soyabean trypsin inhibitor). Aliquots (1ml) were then centrifuged at, 8,800g for 15min at 4°C. Supernatant was collected and spun for a further 15min. The supernatant from this centrifugation was aliquoted (1ml), snap frozen and held at -70°C for a maximum period of 1 month.

2.10.2 Preparation of DEAE-cellulose

The DEAE cellulose (50g) was suspended in 250ml 100mM MES (pH approximately 4.5); under these conditions, bicarbonate adsorbed onto the cellulose is converted into carbonic acid. The slurry was then transferred to a stoppered 1 litre Buchner flask and a vacuum applied. Gentle swirling promoted the non-catastrophic "boiling off" of CO_2, until no more gas emerged. The slurry was then transferred to a plastic beaker and the pH adjusted to 6.8 with NaOH. After allowing for settling, the supernatant was aspirated off and replaced with 250ml of 10mM MES/NaOH, 0.2mM EDTA, and 0.1 mM DTT pH 6.8. The slurry was swirled and allowed to settle several times over a 15min period after which the pH was again adjusted to 6.8. The supernatant was replaced with fresh MES buffer and the process repeated before resuspending the equilibrated cellulose as a 1:1 slurry (settled bed:supernatant buffer volume). NaN_3 was added to a final concentration of 0.2% and the slurry transferred to a plastic screw-top bottle for storage at 4°C. Omnifit columns (15mm x 150mm) were poured in advance of isoform separation, and stored at 4°C prior to use.

2.10.3 Isoform resolution

The isoenzymes I and II of PK-A were resolved from rat mammary tissue as described by (Livsey & Martin, 1988). On the day of the experiment, cytosolic extracts (see 2.10.1) were thawed and filtered through 5μm and 0.45μm Acrodiscs. A small aliquot of filtered extract was saved for assay of PK-A activity, (as described in section 2.6.2). The remainder was loaded onto a DEAE-cellulose column (15mm x 100mm, 22.5ml packed bed volume) equilibrated in MES homogenisation buffer described above, at a flow rate of 3ml/min. This flow rate was maintained for about 1h in order to wash the column

before elution of bound protein with a salt gradient. A small aliquot (0.5ml) of this bulk-wash was retained for assay of PK-A activity. Resolution of PK-A isoforms was achieved by the application of a NaCl salt gradient (0-400mM in MES buffer). Fractions were collected at 1min intervals (3ml) and assayed for PK-A activity under 2 conditions: plus cAMP/ minus PK-A inhibitor peptide and minus cAMP/ plus inhibitor.

Chapter 3

Immunochemical properties of PK-A catalytic subunit in mammary tissue

Introduction

The following describes the characterisation of an anti C-subunit antiserum and its use in examining the biochemical properties of PK-A catalytic subunit derived from mammary tissue. As-365 was raised in sheep against a peptide corresponding to residues 322-332, a region predicted and subsequently shown to reside on the surface of murine C-subunit. This region is conserved in both α and β isoforms of the C-subunit and is present in all species examined (Knighton et al, 1991). The antiserum was primarily raised for immuno-quantification of C-subunit. Long term objectives existed; to examine the expression of PK-A catalytic subunit throughout the normal pregnancy/lactation/involution cycle of rat mammary tissue. Therefore, it was required that the antiserum be able to recognise the intact C-subunit protein in rat mammary tissue extracts, as well as the purified protein and the peptide antigen, 322-332. Hence a succession of experiments were carried out, designed to investigate the properties of this antiserum, and to assess its suitability for C-subunit immuno-quantification purposes.

Since residues 322-332 reside close to the substrate peptide-binding and adenine nucleotide-binding sites on the intact C-subunit, the possibility that the antiserum could

immunoinhibit C-subunit catalytic activity was also investigated. Inhibition of C-subunit activity, by As-365 was initially studied using purified preparations of the PK-A C-subunit. Additional experiments defined the ability of the antiserum to immunoinhibit PK-A activity present within rat mammary tissue extracts.

3.1 Recognition of the peptide antigen (ELISA)

The immunisation strategy, described in section 2.3.3, was used to raise the anti-peptide antiserum, As-365 from a single sheep (365). A number of bleeds were generated from which putative antisera were produced, as described in section 2.3.4. ELISA assays, of the "direct" type were performed upon putative sheep antiserum, as described in section 2.4.1. In order to assess the success of the immunisation strategy in provoking an immune response, antisera generated from initial bleeds were tested using the keyhole limpet haemocyanin-peptide conjugate (KLH-322-332) as antigen. ELISA assays indicated the presence of antibodies to this large conjugate (results not shown), however it was not clear whether the antisera possessed antibodies that would also recognise the "target" peptide sequence 322-332. Therefore, subsequent assays employed the unconjugated peptide 322-332 or KLH alone. Antisera generated from initial bleeds were strongly reactive towards the KLH but poorly reactive to the peptide 322-332 (results not shown). Following boosting of the animal with the free peptide, antisera were generated which reacted strongly in ELISA assays towards the peptide 322-332. Furthermore for dilutions between 1/100 and 1/10,000, a linear relationship was observed between the antiserum concentration and measured absorbance at 450nm when peptide 322-332 was used as antigen (Figure 3.1). In all

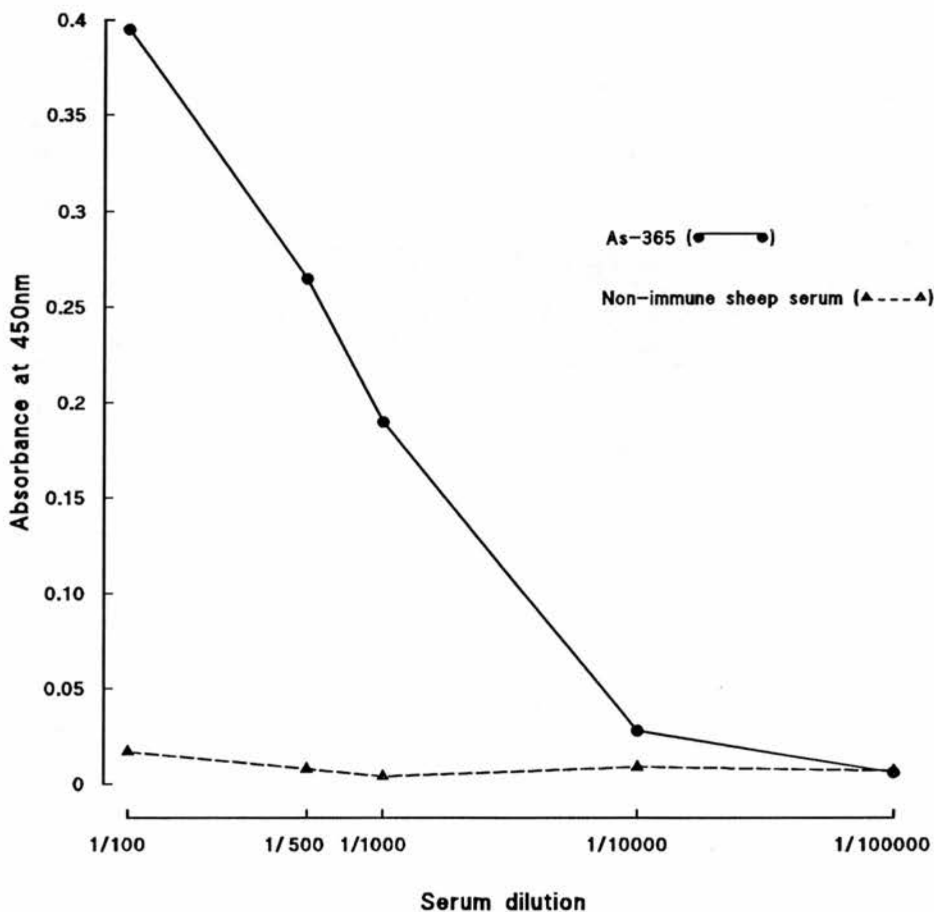


Figure 3.1 Linearity of As-365 dilution and antigen recognition
The above Figure is representative of several ELISA assays which were carried out, as described in section 2.4.1, using peptide 322-332 as antigen. A number of As-365 dilutions were used, to determine the assays linearity, with respect to concentration of serum and consequently IgG concentration.

four major bleeds were taken from a single animal (sheep 365). Antisera generated from bleeds three and four were used in this and subsequent results chapters. ELISA assays indicated very similar IgG titres with respect to both peptide 322-332 and C-subunit moieties (results not shown), therefore the antisera are commonly referred to as As-365 throughout.

3.2 Recognition of PK-A C-subunit in Western blots

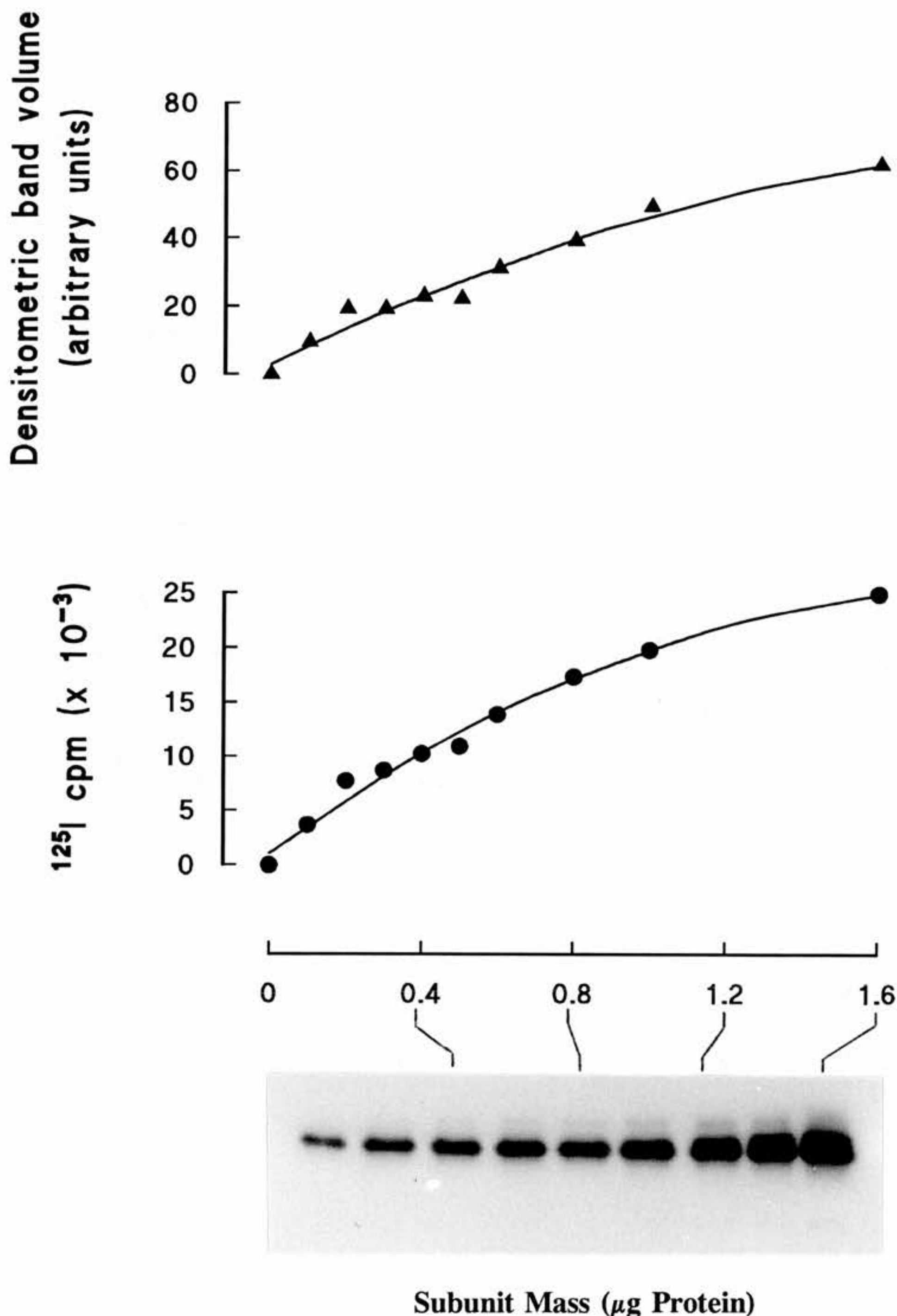
Having shown that As-365 could recognise the C-subunit peptide 322-332 (section 3.1), it was expected that the antiserum would also recognise the intact C-subunit protein. In order to investigate this further, Western blots were prepared using a purified C-subunit preparation (derived from sheep heart). Further studies examined the ability of the antiserum to recognise proteolytic fragments derived from C-subunit. For the purpose of comparison, a second anti C-subunit antiserum, As-201, was also used throughout this section. Like As-365, As-201 was raised in sheep against a peptide antigen. However the peptide used to raise As-201 corresponded to residues 1-12, of the N-terminal sequence of the intact Cα-subunit; plus an additional tyrosine moiety (GNAAAAKKGSEQY). It was also desirable that the antiserum be able to recognise C-subunit in a variety of cell and tissue extracts, and in particular rat mammary extracts. Therefore a number of Western blots were prepared, and As-365 used to detect C-subunit within soluble rat mammary tissue extracts for which PK-A activity data already existed.

3.2.1 Quantitative recognition of the intact C-subunit in Western blots by As-365

Purified C-subunit, prepared from sheep heart (as described in section 2.2.1), was disaggregated and between 0.1 and 1.6μg C-subunit protein loaded onto polyacrylamide gels before being subjected to SDS-PAGE and Western blotting, as described in sections 2.5.4 and 2.5.6. Electroblots were then blocked and probed with the diluted As-365, as described in section 2.5.7. Detection was made using ^{125}I-protein G and autoradiography, as described in section 2.5.8. Quantification of C-subunit was made by densitometric analysis of autoradiographs, or alternatively, by excision of protein bands and conventional γ-counting, as described in section 2.5.10.

The antiserum quantitatively recognised the intact C-subunit in Western blots (Figure 3.2); in that it gave a Western blot signal related in intensity to mass of C-subunit protein in the electrophoresed sample. This was independent of the quantification system involved, both quantitative densitometric analysis and γ-counting of radioactive protein bands revealed an immunological signal which was proportional to the quantity of C-subunit protein present.

In order to examine whether recognition of the C-subunit protein by As-365 could be selectively competed out by the peptide antigen (322-332), antiserum was first treated with the peptide (as described in section 2.4.2). Also used in this experiment was a second anti C-subunit antiserum, As-201. A purified preparation of C-subunit, derived from sheep heart (10 x 0.1μg) was loaded onto 8% polyacrylamide gels and subjected to SDS-page and Western blotting (as described in sections 2.5.4. and 2.5.6). As-365 and As-201, were first incubated with their respective peptide antigens, or peptide solvent (as described in section 2.4.2.). Electroblots were mechanically divided into



Subunit Mass (μg Protein)

Figure 3.2 As-365 recognition of the intact C-subunit
The above data represents a single experiment. Various quantities of a purified preparation of C-subunit (sheep heart) were loaded onto a 8% polyacrylamide gel, subjected to SDS-PAGE and Western blotted onto nitrocellulose before being probed with As-365 (as described in sections 2.5.4 and 2.5.6). Detection was made using ^{125}I-protein G (as described in sections 2.5.7 and 2.5.8). Quantification of C-subunit protein was exacted by both densitometric analysis of the resulting autoradiographs, and by radioactivity counting of excised bands from the electroblots (as described in section 2.5.10).

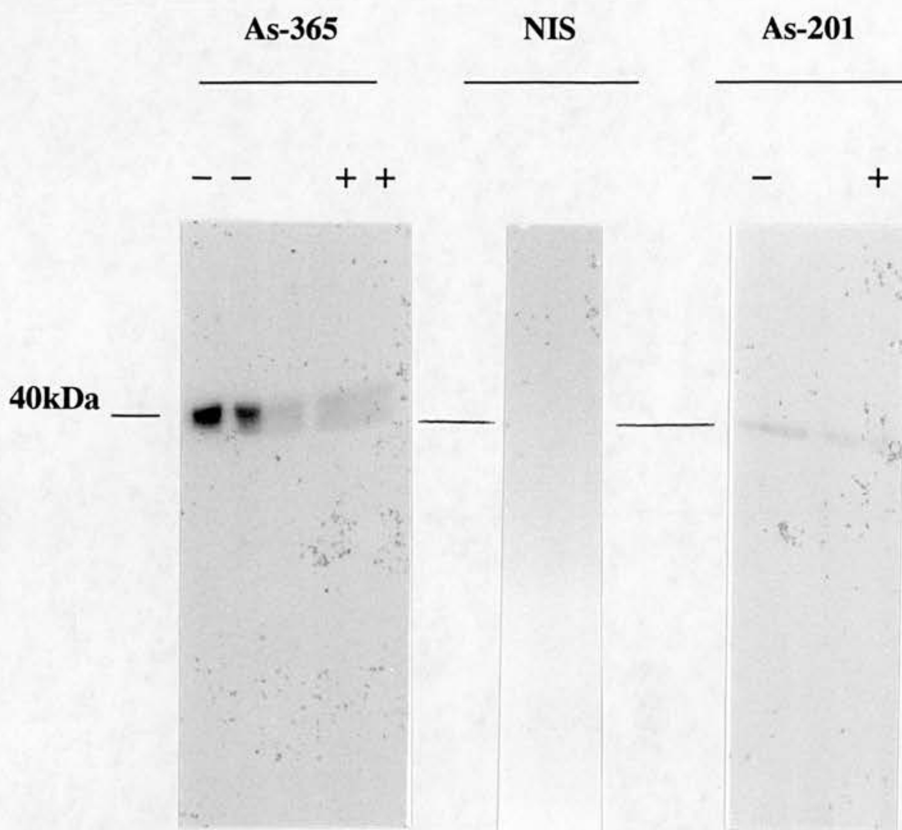


Figure 3.3 Competitive action of peptide 322-332 in Western blots of purified C-subunit

The above data represents a single experiment. Purified C-subunit (10 x 0.1μg from sheep heart) was loaded onto a 8% polyacrylamide gel and subjected to SDS-PAGE and Western blotting as described in sections 2.5.4. and 2.5.6. As-365 and As-201, were first incubated with their respective peptide antigens, or peptide solvent as described in section 2.4.2 and indicated on the Figure by "+" and "−" signs respectively. Electroblots were mechanically divided into lanes; duplicate lanes were then probed with the above antisera (final dilution 1/1000 with respect to neat antisera). Control lanes were probed with untreated non-immune sheep serum (NIS), also diluted 1/1000. Detection was made using ^{125}I-protein G as described in sections 2.5.7, 2.5.8 and 2.5.10.

lanes, enabling a single blot to be probed with treated/control antisera and non-immune sheep serum (NIS). Both antisera and NIS were diluted 1/1000 with respect to the neat serum. Detection was made using ^{125}I-protein G and autoradiography of dried electroblots, as described in sections 2.5.7, 2.5.8 and 2.5.10. As expected, following treatment of the two antisera with their respective peptide antigens, recognition of the intact C-subunit was substantially diminished (Figure 3.3). However treatment of As-365 or As-201 with peptides 1-12 and 322-332 respectively had no effect upon C-subunit recognition (results not shown). Hence the peptides 322-332 and 1-12 could act as selective competitors in antisera recognition of C-subunit. It was also of interest that the intensity of signal produced via As-365, under identical conditions and dilutions, was much more intense than that generated via As-201.

3.2.2 Recognition of C-subunit fragments

Since As-365 appeared to contain populations of IgG molecules which recognised the intact C-subunit and the C-subunit peptide 322-332, it was expected that the antiserum would also recognise degraded fragments of C-subunit protein, as long as they contained the 322-332 motif. The ability of the As-365 to recognise such fragments, was investigated by the generation of proteolytic fragments and by adaptation of those methods first described by (Cleveland et al, 1977). Purified C-subunit (bovine heart) was first digested by one of two proteinases, Endo-glu-C (V8) or Endo-lys-C, followed by SDS-PAGE and Western blotting as described in sections 2.4.3. Proteolytic degradation was then detected by probing the electroblots with either As-365 or another anti-peptide antiserum, As-201. Both antisera were diluted 1:1000 prior to probing of

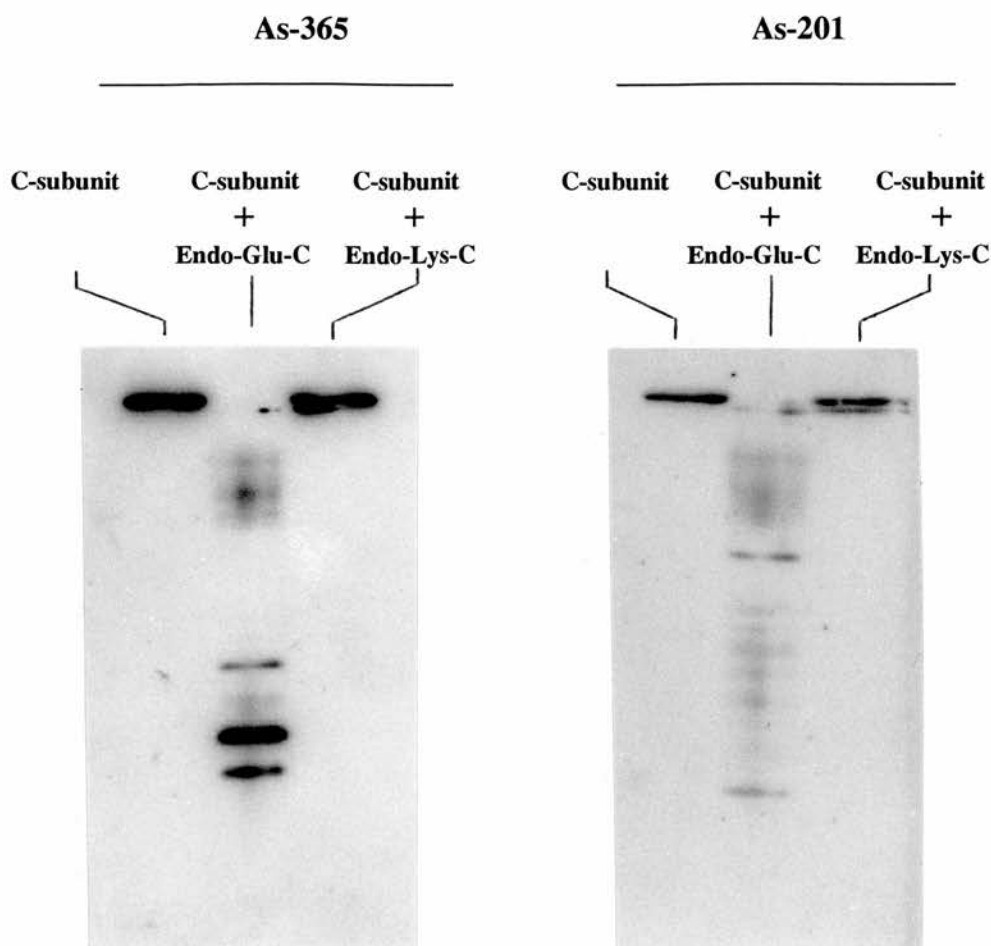


Figure 3.4 As-365 recognition of C-subunit proteolytic fragments
Purified C-subunit, derived from bovine heart (5μg per track) was loaded on to the stacking gel of a 15% polyacrylamide gel and digested using the analytical proteases Endo-lys-C or Endo-glu-C, as described in section 2.4.3. The resulting digest was then subject to SDS-PAGE and Western blotting, as described in section 2.5.4 and 2.5.6. Electroblots were then probed with either As-365 or a second anti C-subunit antibody, As-201, both diluted 1/1000. Detection was made using ^{125}I protein G and autoradiography, as described in section 2.5.8.

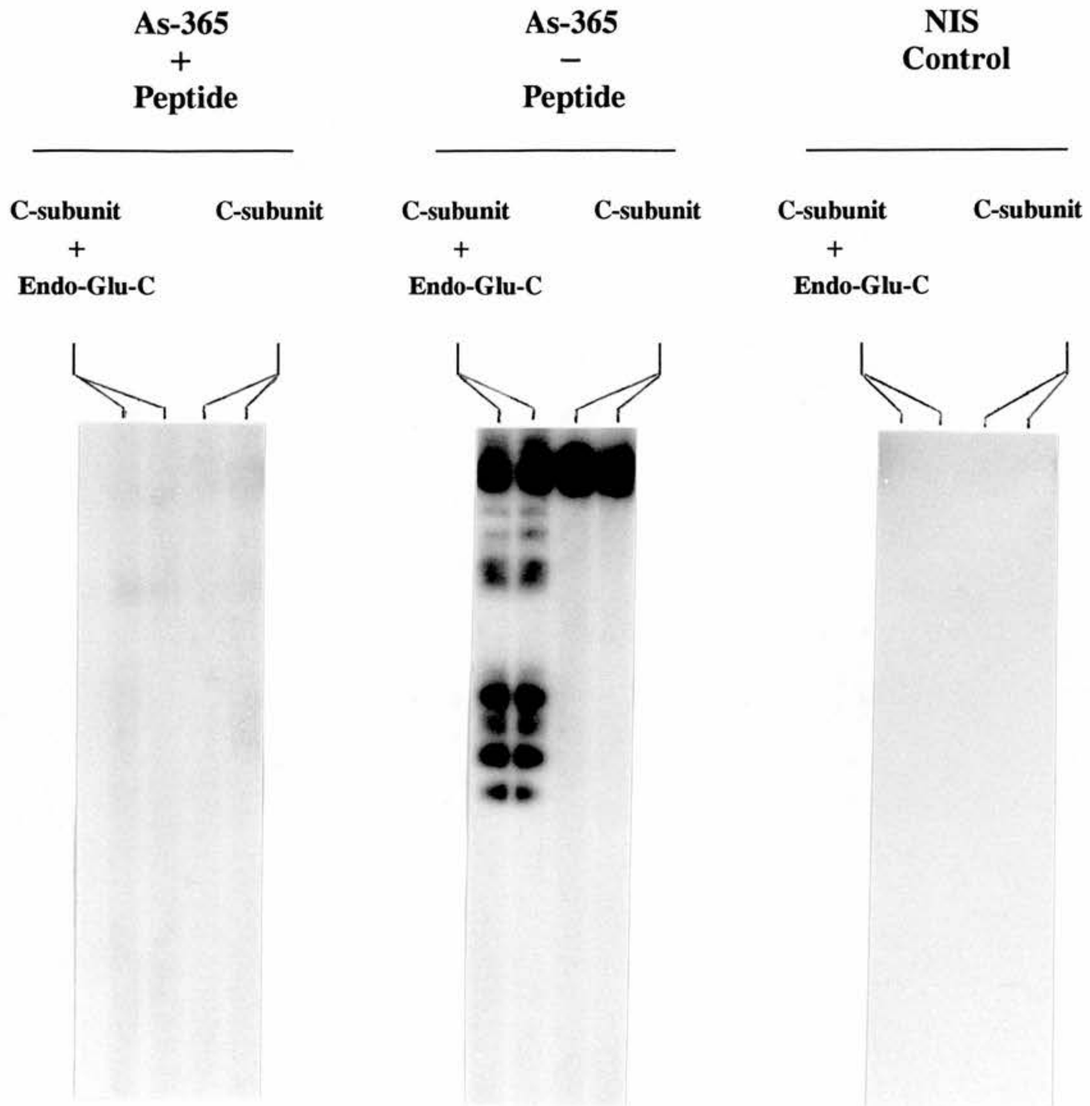


Figure 3.5 Peptide competition of 322-332 in As-365 recognition of C-subunit proteolytic fragments

Purified C-subunit, derived from bovine heart (2μg track) was digested with Endo-Glu-C and subjected to SDS-PAGE followed by Western blotting, as described in section 2.4.3. Control lanes on the gel contained the intact C-subunit preparation alone. Electroblots were mechanically divided into lanes using a Miniblotter. As-365 was incubated overnight with the peptide 322-332 before being used to probe the blot, as described in section 2.4.2. Control lanes were probed with the untreated As-365 or with non-immune sheep serum (NIS).

the electroblots, as described in section 2.5.7. Detection was made using ^{125}I protein G and autoradiography, as described in section 2.5.8.

Of the two endoproteinases employed in these experiments, the Endo-glu-C enzyme proved the most effective in digesting the C-subunit protein. Endo-lys-C generated a single protein fragment, which migrated close to the intact C-subunit and was detected by both As-365 and As-201 (Figure 3.4), and coomassie blue dye staining (results not shown). Endo-glu-C produced a succession of protein fragments, and those fragments recognised by the two antisera were quite dissimilar in pattern from each other (Figure 3.4). Peptide competition experiments were performed in order to check the specificity of these immune reactions (as described in section 2.4.2). Recognition of As-365 for both the intact C-subunit and proteolytic fragments was almost completely competed out by pretreatment of the antiserum with its respective peptide antigen (Figure 3.5).

3.2.3 Recognition of C-subunit in rat mammary extracts

The ability of the antiserum to recognise the C-subunit in mammary tissue extracts was investigated. A series of Western blots were prepared, using soluble rat mammary extracts, identical to those prepared for assay of PK-A activity, as described in section 2.6.1. Extracts derived from both pregnant and lactating rats were subsequently disaggregated, and quantities representing approximately 4mg tissue loaded onto gels, subjected to SDS-PAGE and Western blotted, as described in section 2.5.4 and 2.5.6. Electroblots were then probed with As-365, at a dilution of 1:1000 as described in section 2.5.7. Detection was made using a HRP-linked second antibody followed by ECL as described in section 2.5.9.

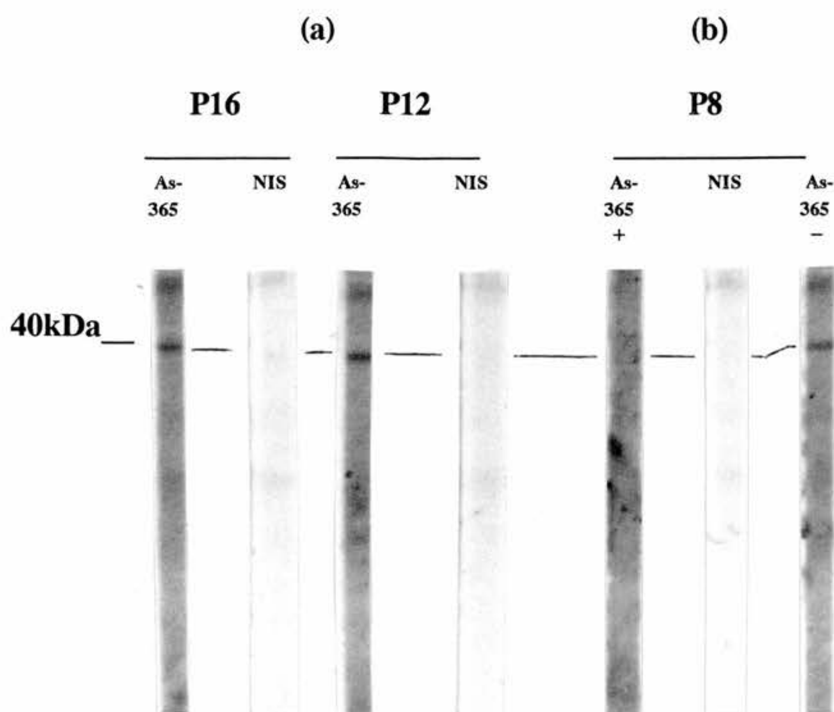


Figure 3.6 Specific recognition of C-subunit in pregnant rat mammary tissue extracts

Rat mammary tissue extracts were prepared as for assay of PK-A activity (section 2.6.1). Samples equivalent to 4mg of mammary tissue were then loaded onto 8% gels and subjected to SDS-PAGE followed by Western blotting. Electroblots were transferred to a Miniblotter and alternate lanes probed with either As-365 or non-immune sheep serum (NIS), as shown in (a). In (b) As-365 was first treated with peptide in a competition experiment (as indicated by "+" and "−" signs respectively and described in section 2.4.2), before being used to probe a similar electroblot. Detection was made using ECL, as described in section 2.5.9. P8, P12 and P16 refer to the number of elapsed days of pregnancy and lactation respectively.

In mammary tissue extracts derived from pregnant rats, As-365 recognised a protein of the same apparent molecular weight as authentic sheep heart C-subunit run in the same gels (40kDa). Many other protein bands were detected by ECL, however only the 40kDa protein was found to be specific, not being detected on Western blots treated with non-immune serum. Detection of this protein was also diminished following treatment of As-365 with peptide 322-332 in competition experiments (Figure 3.6), described in section 2.4.2. However, in mammary tissue extracts derived from lactating animals, non-immune reaction occurring at the same position as the authentic C-subunit made definition of C-subunit somewhat difficult (results not shown). Since these non-immune reactions at approximately 40kDa occurred only in tissue extracts derived from lactating animals it seemed probable that they arose from unspecific binding of antibodies to rodent α-casein molecules (M_r approximately 40kDa, Jones et al, 1985), as these proteins are only expressed during lactation. Conversely to C-subunit, α-casein molecules are highly acidic in nature, thus affording the opportunity to separate selectively these two proteins by a simple ion exchange mechanism (details of this methodology and its results are given in more detail in sections 2.7 and 4.4).

3.3 Immunoinhibition analysis using As-365

Since the As-365 was raised against a peptide sequence of the PK-A C-subunit, closely associated with both substrate peptide and ATP binding, it was expected that the antiserum should inhibit catalytic activity. In order to investigate this further, immunotitration-inhibition experiments were performed using purified C-subunit, prepared from various sources (preparation described in section 2.2.1).

Immunoinhibition studies were also carried out on a panel of rat mammary extracts, sampled throughout pregnancy and lactation.

3.3.1 Immunotitration inhibition analysis of purified C-subunit

Titration-like experiments were performed, as described in section 2.4.5, using As-365 against purified C-subunit obtained from several sources including: pregnant and lactating sheep mammary tissue and a recombinant source (murine Cα, Slice & Taylor, 1989). As-365 was incubated for 30min at 37°C together with a solution of C-subunit, containing approximately 4munits/ml PK-A activity, in a series of incubations as shown in Table 3.1 overleaf. PK-A activity remaining was then measured, as described in section 2.6.2. Curves were constructed from which titre values of the antiserum for the different preparations of C-subunit could be derived (Figure 3.7).

As predicted the antiserum was an effective inhibitor of C-subunit catalytic activity, independent of the enzymes source (Figure 3.7). No major differences were revealed in the titre values of As-365 between preparations of C-subunit derived from mammary tissue of pregnant (54μunits/μl As-365) and lactating sheep (58μunits/μl As-365) or of a recombinant, non-myristoylated C-subunit (77μunits/μl As-365). Such experiments therefore suggested that there were no major changes to the *intrinsic* specific catalytic activity of the C-subunit, as induced by differentiation in the mammary gland, or by manipulation of its acylation status using recombinant expression techniques.

It is also of interest that 100% inhibition of C-subunit catalytic activity was never achieved. A residual 15% of input activity consistently remained. It was not possible at this stage to determine whether this activity was due to the nature of the antiserum

As-365 (μl)	NIS (μl)	C-subunit solution (μl)	1% BSA/PBS
0	50	25	50
0	10	25	90
0.5	9.5	25	90
1.0	9.0	25	90
3.0	7.0	25	90
5.0	5.0	25	90
7.0	3.0	25	90
10.0	0	25	90
50.0	0	25	50

Table 3.1 Immunotitration inhibition analysis of purified C-subunit
Various quantities of As-365 (0-10μl) were incubated together with a fixed quantity of purified C-subunit (100μunits) derived from various sources including lactating and pregnant sheep mammary tissue and finally a recombinant source. The final serum concentration was 10μl; the final incubation volume was 125μl. All incubations were carried out at 37°C for 30min followed by assay for total PK-A activity as described in section 2.6.2.

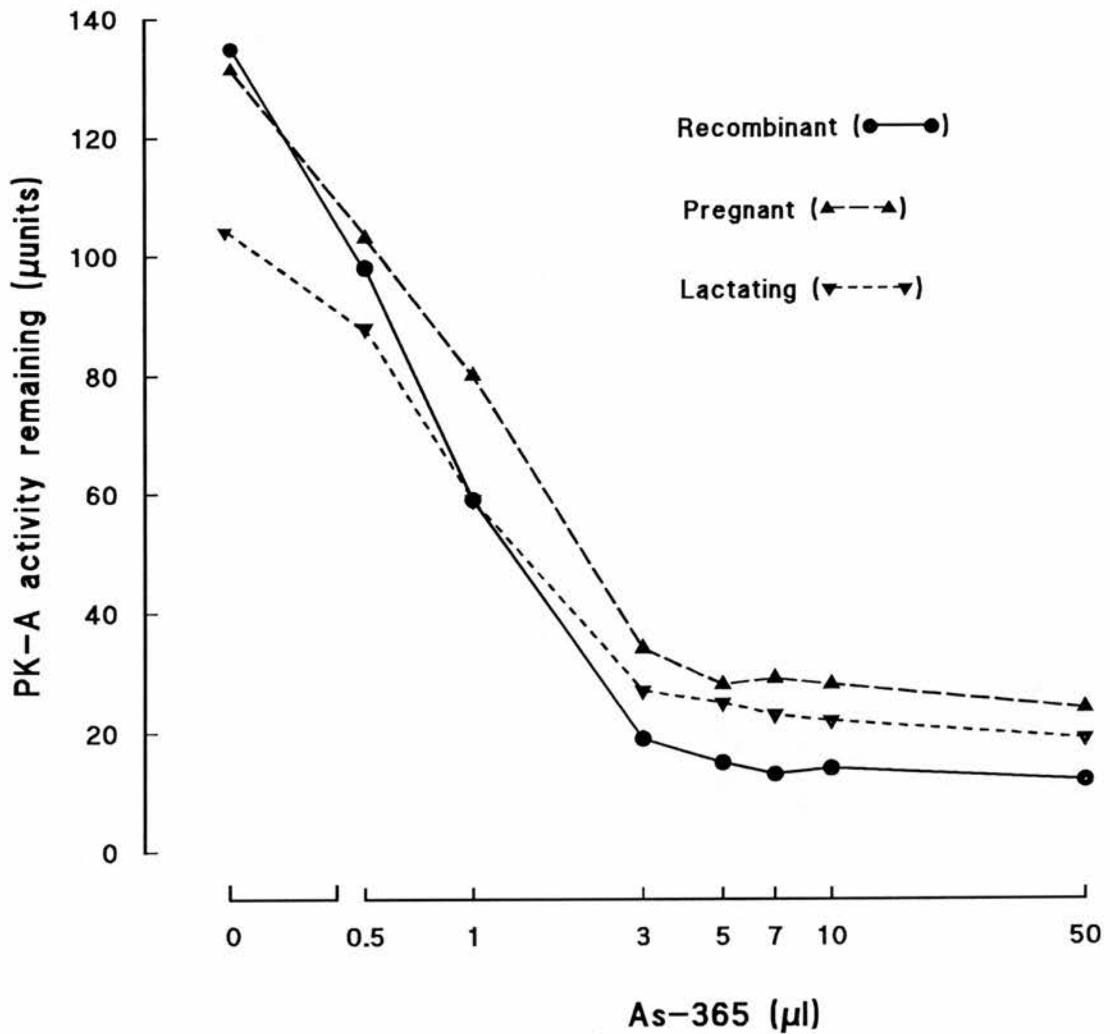


Figure 3.7 Immunotitration inhibition of the purified C-subunit
Titration experiments were performed in which purified C-subunit (100μunits) was incubated at 37°C, with a range of As-365 concentrations as shown in Table 3.1 and further described in section 2.4.5. Total activity was then measured, in the absence of cAMP as described in section 2.6.2. C-subunit prepared from different sources were used, including pregnant and lactating sheep mammary tissue and from a recombinant source (murine Cα, Slice & Taylor, 1989).

or to the resistance of certain C-subunit molecules to immunoinhibition. However, it was possible to attribute this residual kinase activity to PK-A, due to the design of the assay which incorporates the use of a specific, PK-A inhibitor peptide (as described in section 2.6.2). This phenomenon also appeared to be independent of the tissue, or other, source from which the C-subunit preparation was derived.

3.3.2 Immunoinhibition of PK-A in rat mammary extracts

The following experiments examine the ability of the antiserum to inhibit PK-A catalytic activity in crude rat mammary extracts. Rat mammary tissue was sampled from rats in their twelfth day of lactation (L12) and extracts were prepared from the tissue, as for the measurement of PK-A activity, described in section 2.6.1.

Initial experiments aimed to determine whether cAMP, and therefore dissociation of the holoenzyme complex, was first necessary before immunoinhibition by the antiserum. Extracts were incubated with As-365 at 37°C, both in the presence and absence of cAMP ($0.6\mu M$), as described in section 2.4.5. Non-immune sheep serum (NIS) was substituted for As-365, in controls which were designed to distinguish the effects of the antiserum from possible non-immunological effects induced by cAMP. As-365 was found to be an effective inhibitor of rat mammary PK-A activity, even when cAMP was absent during the initial incubation of antiserum and tissue extract (Figure 3.8); although this was possibly a reflection of immunoinhibition occurring during assay of total PK-A activity, when cAMP was present in the assay mixture. There was also a great deal of non-immune inhibition present in control samples in which cAMP was present. As illustrated in Figure 3.8, the additional non-immune inhibition arrising from the presence of cAMP ($\delta 1$) was greater than the increase in

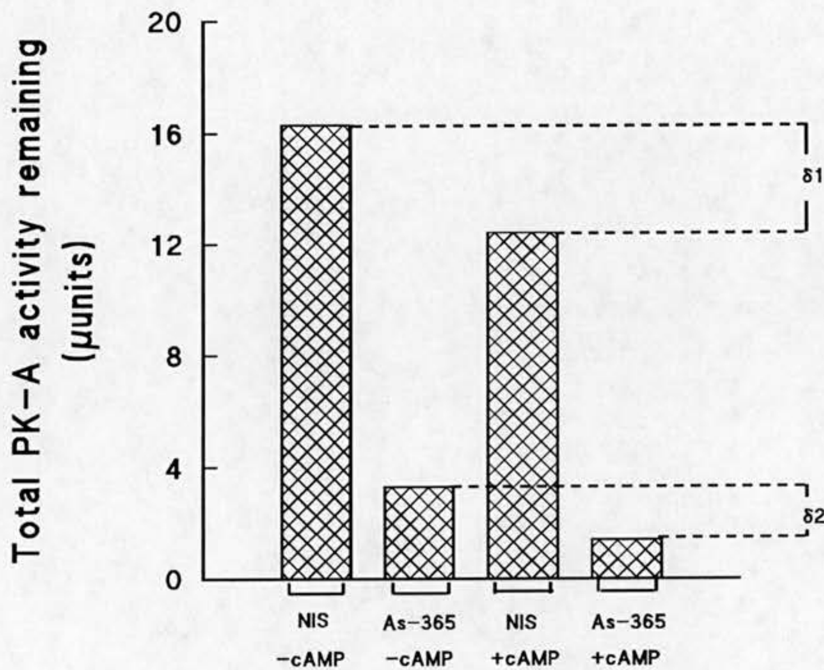


Figure 3.8 As-365 immunoinhibition of rat mammary tissue extracts
Immunoinhibition experiments were performed as described in section 2.4.5. L12 rat mammary tissue extracts (approximately 160μunits), were incubated with As-365 or NIS, at 37^{0}C for 30min, in both the presence and absence of 0.6μM cAMP. δ1 represents non-immune, deactivation of PK-A following cAMP induced dissociation in mammary tissue. δ2 represents further immunoinhibition occuring as a result of PK-A dissociation. The above histogram is representative of three similar experiments.

immunoinhibition (δ2). Therefore it was not possible to conclude from these experiments whether dissociation of the holoenzyme complex was necessary before antiserum recognition of the C-subunit.

3.3.3 Immunoinhibition analysis of rat mammary PK-A activity throughout pregnancy and lactation

Rat mammary extracts were prepared from tissue sampled at different stages (4 day increments) of pregnancy and lactation, as described in sections 2.1.1 and 2.6.1. Extracts were then incubated with excess As-365 (5μl sufficient to inhibit approximately 250μunits of PK-A activity) in the presence of 0.6μM cAMP, for 30min at 30°C, as described in section 2.4.5. Residual PK-A activity was then measured as described in section 2.6.2. In total, three developmental series of rat mammary tissue extracts were analysed in this way. In these studies, the total amount of tissue imput was constant, but PK-A activity input was variable, dependent upon the developmental stage of the tissue extract being examined; higher levels of activity were present in those tissue extracts derived from lactating animals than those from pregnant. Nevertheless, As-365 was found to be an effective inhibitor of PK-A activity, under the concentration used and in all extracts examined, regardless of the stage of pregnancy or lactation of the tissue of origin. As previously found, not all PK-A activity was inhibited, but a residual of approximately 15% remained in all samples examined (Figure 3.9). Hence the proportion of non-immunoinhibitable activity appeared to remain constant throughout pregnancy and lactation, apparently unaffected by the differentiation processes of the tissue (Figure 3.9).

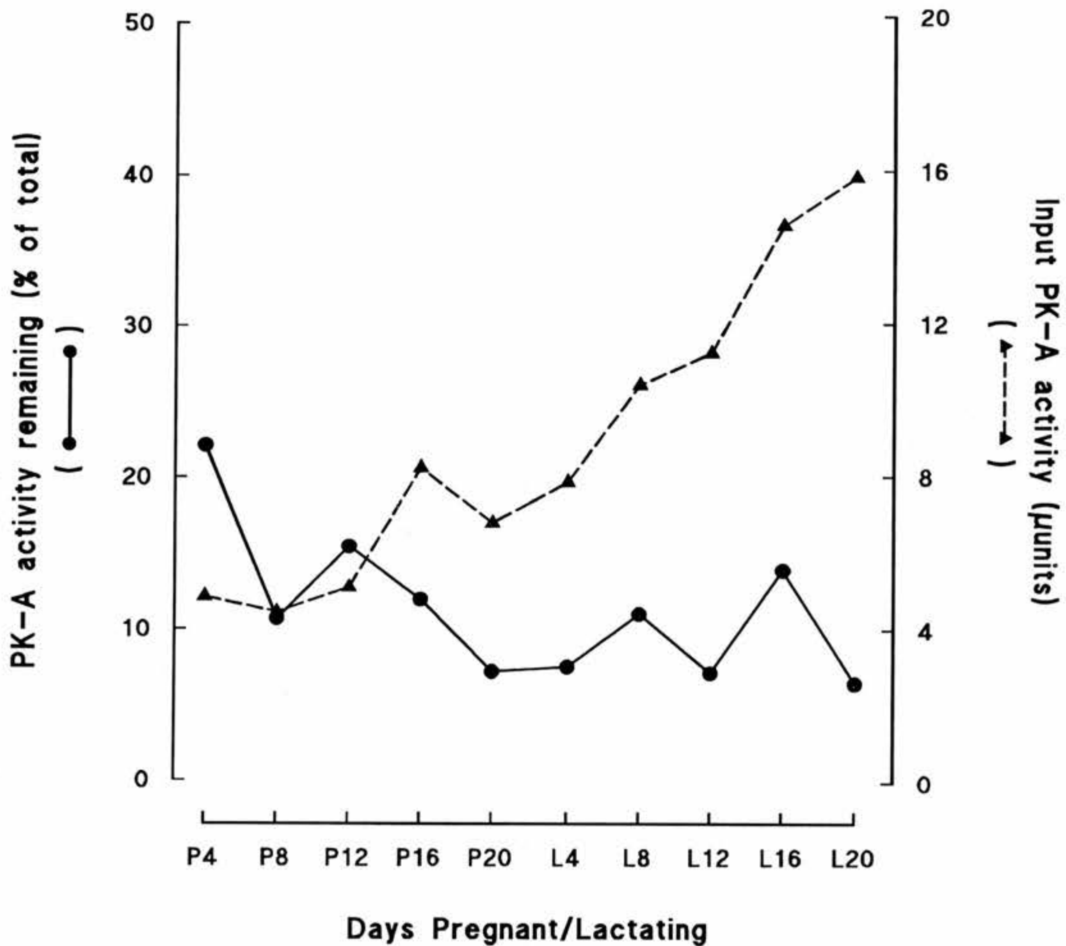


Figure 3.9 Immunoinhibition analysis of rat mammary tissue extracts
Extracts of rat mammary tissue were prepared as described in section 2.6.1 and incubated in the presence of 0.6μM cAMP with As-365 for 30min at 30°C. BSA was present to a final concentration of 1% to minimise spontaneous activity loss. Each incubation contained the equivalent of 0.1mg mammary tissue and 5μl As-365, capable of immunoinhibiting approximately 250μunits of C-subunit activity. Total PK-A activity was then measured in the presence of cAMP (as described in section 2.6.2). P4, L4 etc refer to elapsed days of pregnancy or lactation respectively. The results shown above are from a single experiment, representative of three carried out.

It is also of interest that under the lower incubation temperatures used here, non-immune inhibition of PK-A activity, first described in section 3.3.2 above, was significantly reduced. This finding prompted a more detailed investigation (described in section 3.4) into the nature of this apparently endogenous inhibition of rat mammary PK-A activity.

3.3.4 Immunoprecipitation of PK-A activity in rat mammary extracts by As-365 and ab-365

Immunoprecipitation experiments were performed in order to determine whether As-365 and affinity purified antiserum (ab-365), required holoenzyme dissociation prior to recognition of the C-subunit. Various quantities of either As-365 or ab-365 were incubated together with rat mammary tissue extracts (L12) both in the presence and absence of 0.6μM cAMP, as described in section 2.4.4. Thus a series of incubations were set up as shown in the Table 3.2, overleaf.

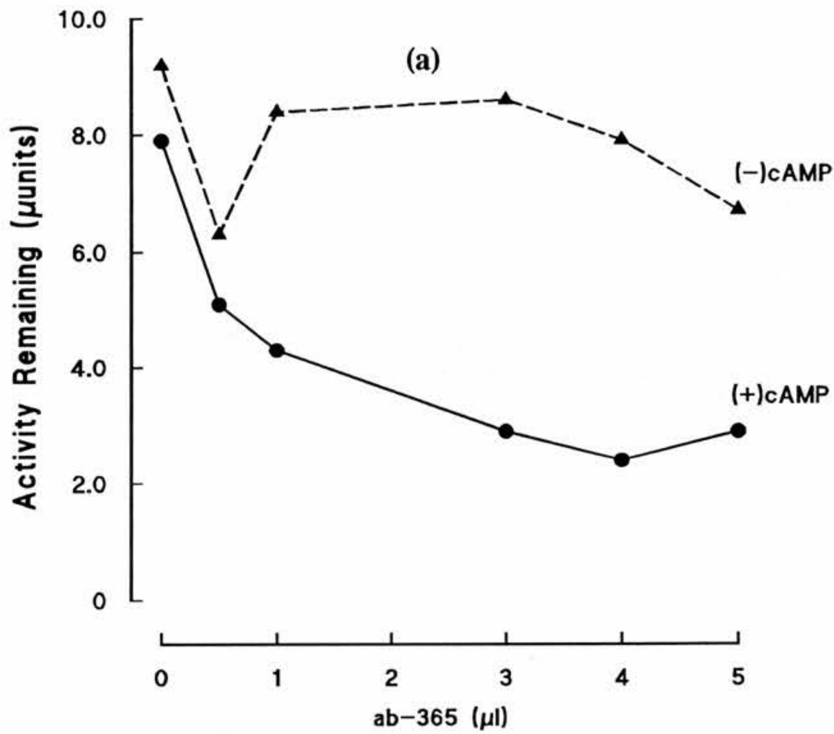
At lower concentrations of As-365 and under all concentrations of Ab-365 used, immunoprecipitation was found to be cAMP dependent (Figure 3.10). Only at higher concentrations of antiserum did any significant loss of PK-A activity occur in the absence of cAMP. It is also of interest that even under saturating concentrations of antibody, 100% immuno-precipitation was never achieved; a residual of approximately 15% of total PK-A activity consistently remained in these incubations.

3.4 Detection of a C-subunit deactivating factor in rat mammary tissue

Tissue extracts in general may contain inhibitors and/or activators of protein kinase

μl L12 cytosol (1:500 wrt to wet tissue wt.)	μl As-365 (1:10) or Ab-365 (3μg/ml IgG)	μl NIS (1:10) or Non-immune IgG (3μg/ml)	μl cAMP (4μM) or H_2O
50	-	50	20
50	50	-	20
50	40	10	20
50	30	20	20
50	10	40	20
50	5	45	20

Table 3.2 Immuno-precipitation of PK-A activity in rat mammary tissue extracts by As-365 and ab-365
Various quantities of As-365 (0-5μl), or the affinity purified ab-365 were incubated with soluble rat mammary tissue extract (L12, day 12 of lactation) in the presence of 0.6μM cAMP at 0°C for 5min. Protein G was added to all incubations which were held at 0°C for a further 15min followed by brief centrifugation (8,800g for 2min). The remaining PK-A activity was measured in supernatant as described in section 2.6.2.



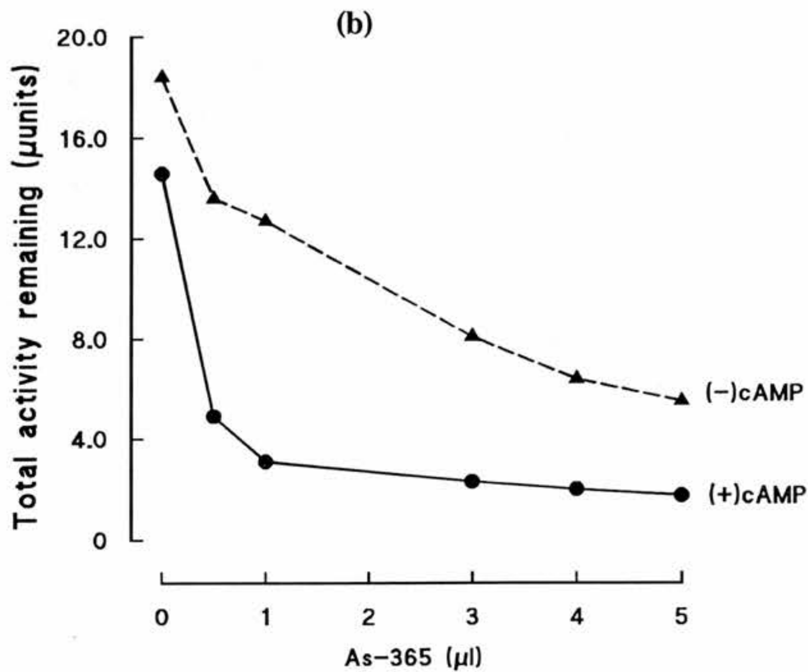


Figure 3.10 Cyclic AMP dependence of As-365 and ab-365 immunoprecipitation of PK-A activity in rat mammary tissue extracts

Immunoprecipitation experiments were performed as shown in Table 3.2 and further described in section 2.4.4. L12 rat mammary extracts were incubated with various quantities of affinity purified ab-365 (a) or As-365 (b), either in the presence or absence of 0.6μM cAMP. Immunocomplexes were precipitated using Protein G Sepharose, followed by brief centrifugation (8,800g/2min). The above Figure shows data from two individual experiments.

activity. The existence of a PK-A deactivating factor/s in rat mammary tissue was suggested from those studies outlined in sections 3.3.2 and 3.3.3. The following experiments were performed to test for their presence in a number of rat mammary tissue extracts, prepared as for assay of PK-A activity and described in section 2.6.1. By variation of the incubation conditions it was possible to characterise the nature of this activity loss; thus a number of experiments were carried out to investigate the effects of temperature, PK-A dissociation and a time scale for PK-A deactivation.

To determine the extent of spontaneous activity loss, through incubation alone, rat mammary tissue extract (L20, approximately 70μunits) and a purified preparation of PK-A C-subunit (lactating sheep mammary tissue, approximately 1munit) were incubated at 30 or 37°C for 30min, as shown in Table 3.3. Samples which had been diluted, but not subjected to the incubation procedure served as controls. All samples were assayed for total PK-A activity as described in section 2.6.2. Initial findings showed that incubation alone had no effect on total PK-A activity levels, measured for both the C-subunit preparation and rat mammary tissue extracts, and this remained true for both incubation temperatures used (Table 3.3).

To investigate whether PK-A dissociation was a mandatory condition of its deactivation in rat mammary tissue, tissue extracts from both pregnant and lactating animals (P20 and L20) were incubated at 37°C for 30min in the presence and absence of cAMP (0.67μM), as shown in Table 3.4. The presence of cAMP had a pronounced effect upon total PK-A activity levels, as measured in lactating and pregnant rat mammary samples (Table 3.4). However it was not possible to prove conclusively from such studies that the activity losses directed by cAMP were mechanistically caused by PK-A

Incubation temperature (°C)	**C-subunit (μl)**	**Tissue extract L20 (μl)**	**BSA 10mg/ml (μl)**	**Total munits remaining**
No pre-incubation	50	-	50	1.15
No pre-incubation	-	50	50	0.07
30	50	-	50	1.23
37	50	-	50	1.12
30	-	50	50	0.08
37	-	50	50	0.08

Table 3.3 Effect of incubation temperature upon PK-A C-subunit and PK-A activity within rat mammary tissue extracts

Purified C-subunit (lactating sheep mammary, approximately 1.0 munit) and lactating rat mammary tissue extract (L20, prepared as described in section 2.6.1 and diluted 1:10 in 10mg/ml BSA) were incubated at 30 and 37°C for 30min before being assayed for total PK-A activity as described in section 2.6.2. The results shown above are from a single experiment.

$4\mu M$ cAMP ($20\mu l$) or H_2O ($20\mu l$)	Tissue extract P20 (μl)	Tissue extract L20 (μl)	BSA 10mg/ml (μl)	Total μunits remaining
H_2O	50	-	50	39
H_2O	-	50	50	35
cAMP	50	-	50	0.0
cAMP	-	50	50	18

Table 3.4 Effect of cAMP upon PK-A activities within rat mammary tissue extracts
Lactating and pregnant rat mammary tissue extracts were prepared as described in section 2.6.1 and diluted 1:10 in 10mg/ml BSA. These were then incubated at 37°C for 30min, as shown above, in both the presence and absence of cAMP ($0.67\mu M$). Residual PK-A activity was then measured as described in section 2.6.2. The results shown above are from a single experiment.

Incubation temperature (°C)	C-subunit (μl)	Tissue extract L20 (μl)	BSA 10mg/ml (μl)	Total munits remaining
No pre-incubation	-	50	50	0.08
No pre-incubation	50	-	50	1.52
0	50	50	50	1.56
30	50	50	50	1.24
37	50	50	50	0.64

Table 3.5 The ability of mammary extract to promote the deactivation of exogenous C-subunit
Purified C-subunit (approximately 1.5munits) was incubated with a lactating rat mammary tissue extract (L20, prepared as described in section 2.6.1 and diluted 1:10 in 10mg/ml BSA) for 30min at 0, 30 and 37°C before being assayed for residual catalytic activity as described in section 2.6.2. The results shown above are from a single experiment, representative of three performed.

dissociation alone.

Further experiments were carried out in which purified C-subunit (lactating sheep mammary tissue) was incubated with rat mammary tissue extracts (L20), in the absence of cAMP. Kinase activity was then measured (section 2.6.2) and compared to the sum of the individually measured activities i.e. C-subunit alone and mammary extract alone. Initial results showed that in the absence of cAMP the free C-subunit was subject to deactivation by factor/s present within rat mammary tissue extracts (results not shown). The experiment was repeated at various temperatures (0,30 and 37°C), as shown in Table 3.5. Results indicated that the susceptibility of free C-subunit to deactivation in mammary tissue extracts was temperature sensitive, being moderate at 0-30°C but extreme at 37°C (Table 3.5).

In a similar experiment lactating sheep mammary C-subunit was incubated at 37°C for 30min with rat mammary tissue extract (L16) in the absence of cAMP. Samples were then dissagregated and subjected to SDS-PAGE and Western blotting, as described in sections 2.5.4 and 2.5.6. C-subunit was detected using As-365, ^{125}I-protein G and autoradiography, as described in sections 2.5.8. Autoradiographs showed no indications of C-subunit proteolytic degradation, however when radioactive C-subunit bands were located, excised and counted, a small but significant fall in total number of counts (cpm) was recorded (results not shown). In a single experiment, rat mammary extracts (L16) were incubated in the presence of cAMP (0.67μM) and +/- MgATP (0.2mM), at 37°C for 30min before being assayed for total PK-A activity. Results showed that MgATP provided partial protection to PK-A activity within rat mammary tissue (results not shown).

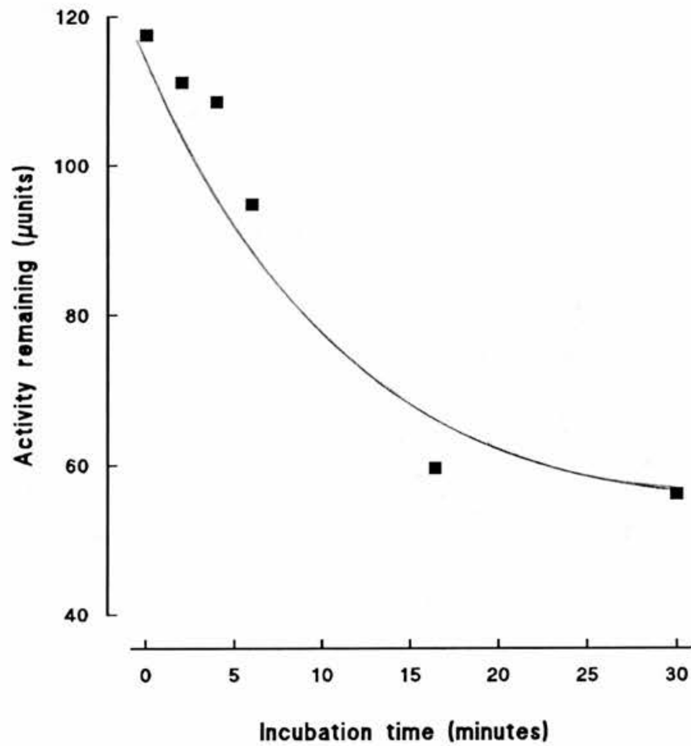


Figure 3.11 Time course of C-subunit deactivation of catalytic activity
Purified C-subunit was incubated with lactating rat mammary tissue extracts, prepared as described in section 2.6.1, for 0, 2, 4, 16 and 30 min respectively before being assayed for residual catalytic activity as described in section 2.6.2. The results are from a single experiment.

Finally a time course for the PK-A deactivation was measured. Purified C-subunit (lactating sheep mammary tissue) was incubated at 37°C with rat mammary tissue extract (L20) as described above, for 0,2,4,8,16 and 30 mins before being assayed for total PK-A activity as described in section 2.6.2. Results showed a time-dependent loss of PK-A activity under the conditions used (Figure 3.11).

3.5 Discussion

The results discussed in this chapter demonstrate the immunological properties and therefore the biochemical uses of the polyclonal antiserum, As-365. However, further to this, studies have also revealed some interesting immunochemical properties of C-subunit within rat mammary tissue. Using the technique of ELISA, it was shown that the antiserum recognised the C-subunit peptide antigen, corresponding to residues 322-332. Western blotting studies, using C-subunit purified from sheep heart, demonstrated the ability of the antiserum to recognise the intact protein. As-365 was also shown to give a Western blot signal related in intensity to the mass of C-subunit protein in the electrophoresed sample. Furthermore recognition of the intact C-subunit could be competed out by pre-treatment of the antiserum with the C-subunit peptide (322-332) suggesting that identical, or overlapping populations of IgG molecules were responsible for recognition of both.

The antiserum was also shown to detect multiple proteolytic fragments from bovine heart C-subunit, generated by the endoproteinase, Endo-glu-C. Digestion of the purified C-subunit with another endoproteinase, Endo-lys-C was more limited, generating only a single fragment which was detected by both As-365 and a second anti

C-subunit antiserum, As-201. No homology was identified between the pattern of protein fragments recognised by As-365, as compared with those recognised by As-201. This was not suprising since the two antisera were originally raised against peptide antigens which corresponded to amino acid sequences located at opposite ends of the intact C-subunit molecule; such that each antiserum could only recognise those fragments which contained residues 322-332/1-12 respectively. Recognition of both intact C-subunit and proteolytic fragments by As-365 was competed out by pre-treatment of the antiserum with the peptide 322-332.

As-365 detected a protein in pregnant rat mammary tissue extracts which had a similar mobility on SDS gels as authentic sheep heart C-subunit run in the experiments (40kDa ref). Estimates of the molecular weight of PK-A C-subunit have varied between 39-42kDa, depending upon tissue, species and experimental technique used. The present result is comparable to that of myristoylated bovine heart C-subunit, estimated at 40kDa, also by SDS-PAGE (Shoji et al, 1983 and Carlson et al, 1979). However, attempts to estimate the relative C-subunit mass in mammary tissue extracts derived from lactating animals was hampered by the presence of non-immune signals occurring at the same position as the authentic C-subunit. Such reactions probably occurred as a result of non-specific binding of antibodies to rodent α-casein molecules. High levels of BSA in the mammary extract samples, prepared for assay of PK-A activity, also contributed significantly to the high levels of background detected.

Preparing samples of greater tissue concentration, in the absence of BSA, and further passing the samples through individual CM-Sepharose columns, prior to SDS-PAGE, completely eliminated the appearance of non-immune signals at the 40kDa position for

all samples analysed. Immunoquantification studies also employed affinity purified As-365 (ab-365); a process designed to isolate those IgG molecules with the greatest affinity for C-subunit from those with lower to zero affinity for C-subunit. The use of a ^{32}P labelled C-subunit as internal standard, enabled corrections to be made for differential loading of gels and geometrical variation of CM-sepharose columns etc., thus enabling an estimation of C-subunit protein mass, including that from lactating animals to be made. C-subunit protein mass could now be examined throughout pregnancy and lactation, and compared to other measurements of PK-A expression, such as total activity. Such comparisons would be necessary in identifying developmental fluctuations in the specific catalytic activity of C-subunit and post transcriptional/translational regulation of C-subunit protein mass (see Chapter 4).

In addition to recognising the C-subunit in Western blots, As-365 was also shown to be an effective inhibitor of its catalytic activity, inhibiting both the purified protein and that PK-A activity intrinsic to rat mammary tissue extracts. As previously discussed, As-365 was raised against a peptide corresponding to residues 322-332 of the primary structure of PK-A catalytic subunit. From the determination of the 3D structure of C-subunit (Knighton et al, 1991), it is now known that approximately 70 residues, including amino acids 322-332, extend over a large portion of the surface of the small lobe of the C-subunit. Despite being outside the catalytic core, this area seems to participate in peptide/nucleotide recognition. Thus, As-365 is able to inhibit catalytic activity by binding to, and obstructing this important region of the C-subunit.

Studies, in which a fixed quantity of PK-A activity was titrated out by increasing levels antiserum, (immunotitration-inhibition analysis), revealed no major differences in the

titre of As-365, between purified C-subunit preparations from mammary tissue of pregnant and lactating sheep or of a recombinant non-myristoylated C-subunit. Such results provided evidence for a general lack of change in the intrinsic specific activity of C-subunit; as induced in mammary tissue by differentiation or by the manipulation of its acylation status using recombinant expression techniques. Similar conclusions were drawn from studies performed on a panel of rat mammary tissue extracts, taken from animals at various stages of pregnancy and lactation. No major differences were revealed in the inhibitory capacity of As-365 between pregnancy and lactation. However not all PK-A activity was neutralised by As-365 during these immuno-inhibition experiments. A residual 15% of input activity consistently remained following incubation of excess As-365 with purified preparations of C-subunit and rat mammary tissue extracts. This residual activity was wholly attributable to PK-A, due to the design of the assay which incorporates the use of the PK-A specific inhibitor peptide (as described in section 2.6.2). One explanation for this residual PK-A activity may lie in the polyclonal nature of As-365; if some IgG molecules were less effective at occluding substrate and adenine nucleotide-binding sites than others, then not all C-subunit molecules would be inhibited by IgG binding. Alternatively such findings may be indicative of a sub-population of C-subunit molecules which are resistant to immuno-inhibition by As-365.

The findings of immuno-precipitation experiments, appear to favour the latter of these two hypotheses. As for the immuno-inhibition studies described above, immunoprecipitation experiments revealed that even under saturating concentrations of antiserum, 100% immuno-precipitation of PK-A activity was never achieved. This was

true whether purified preparations of C-subunit were used or rat mammary tissue extracts. Unlike the immuno-inhibition experiments, described in section 3.3.1 and 3.3.2, the effectiveness of immuno-precipitation was not dependent upon the ability of the antiserum to inhibit C-subunit catalytic activity, only IgG binding was necessary to promote precipitation by protein G Sepharose. Therefore it must be concluded that residual PK-A activity was as a result of C-subunit not being recognised by the antiserum, supporting the idea an atypical population of C-subunit molecules exist within mammary tissue. Such molecules may have all or part of the epitope 322-332 occluded either by some small molecule, not removed by the purification process, or by some post translational/proteolytic modification.

Several independent lines of evidence have suggested that PK-A is atypical in its biochemical properties in mammary tissue (Clegg, 1988., Clegg & Ottey, 1990., Bussman et al, 1984. and Takhar & Munday, 1992). From the studies outlined above there is no indication of any significant alterations in the intrinsic catalytic capacity of the C-subunit, as brought about by differentiation and development of the mammary tissue throughout pregnancy and lactation. However possible hetergeneity was demonstrated with the identification of a subpopulation of C-subunit molecules, apparently not recognised by the As-365.

Immuno-precipitation techniques, also confirmed that recognition of the C-subunit by As-365 was dependent upon the dissociation of the holoenzyme complex. Only at higher concentrations of antiserum did any significant loss of PK-A activity occur in the absence of cAMP; probably as a result of a mass action-type effect in which the high concentration of IgG "pulls" the reversible PK-A subunit interaction in the

direction of dissociation, even in the absence of cAMP. However when immunotitration-precipitation like experiments were performed using the affinity purified Ab-365, which contained a lower IgG concentration, immuno-precipitation was found to be wholly cAMP/dissociation dependent.

From immunoinhibition experiments described in section 3.3.2, it became apparent that PK-A activity associated with rat mammary tissue extracts, was susceptible during incubation procedures, to inhibition by a factor/s other than As-365. Further experiments, described in section 3.4, clarified this position, showing that the activity losses were not due to incubation alone. Purified C-subunit could be deactivated by its co-incubation with rat mammary tissue extracts, while endogenous mammary activity losses occurred following incubation of the extracts with cAMP. Thus deactivation of PK-A C-subunit was dependent upon its dissociation from the regulatory subunits. The present finding that free C-subunit becomes deactivated following its combination with mammary tissue extracts does not contradict the work of (Clegg & Ottey, 1990) who made similar measurements but observed an increase in combined activity; since these measurements were effectively made at time zero, when the mammary inhibitor/deactivator had little chance to make an impact.

Activity losses were also shown to be temperature dependent, activity loss was greatest at 37°C, where it showed a slow linear progression with respect to time, deactivation became moderate at 30°C. It appeared then that rat mammary tissue contained a deactivator of PK-A activity; the thermo-susceptibility of PK-A activity and the time dependency of the deactivation seemed to connect with the involvement of a protein. In contrast no evidence of proteolytic degradation was found when extracts were

incubated under conditions that would lead to PK-A deactivation before being analysed by SDS-PAGE and Western blotting procedures. As-365 failed to detect any proteins other than the intact C-subunit, despite being previously shown to detect proteolytic fragments generated by the endoproteinase, Endo-Glu-C.

PK-A deactivation has been described in other tissues, some of the characteristics of the rat mammary PK-A deactivation are shared by the heat-stable inhibitor of PK-A, first described by Walsh and colleagues in rabbit skeletal muscle (Walsh et al, 1971). For example, both PK-A inhibitors interacted with free C-subunit but not the undissociated holoenzyme. However the kinetics characteristics of mammary PK-A deactivation were not shared by the Walsh inhibitor which has been shown to be a competitive inhibitor and therefore appears to be instantaneous in its recognition of free C-subunit either in purified systems or in crude extracts (Ashby & Walsh, 1973 and Whitehouse & Walsh, 1983). The time dependency of mammary PK-A deactivation seemed to rule out inhibition. Deactivation of mammary PK-A was inhibited by the presence of MgATP, which provided moderate but significant protection to the PK-A activity.

PK-A deactivation has been reported in other tissues, such as the identification of a proteinase, responsible for specific, restricted and limited proteolysis of the C-subunit in brush border membranes isolated from rat small intestine (Alhanaty & Shaltiel, 1979). This proteolytic activity specifically clipped the free C-subunit with the appearance of a 30kDa degradation product which was devoid of catalytic activity, no intermediatory degradation products were detected. The attack of C-subunit by the membranal proteinase was blocked by the presence of MgATP and did not affect the

undissociated (inactive) form of the holoenzyme. The proteinase appeared to be specific to C-subunit with other endogenous and exogenous proteins being unaffected; neither could the proteolytic degradation be simulated by other proteinases such as trypsin. The brush border extracts also inactivated pure exogenous C-subunit without addition of cAMP whatsoever. The fact that no such proteolytic products were detected in rat mammary tissue extracts may be due to hydrolysis of the epitope recognised by the antiserum, As-365; concordant with this observation is the finding that MgATP afforded C-subunit some protection from deactivation by the mammary inhibitor, with the ATP binding site lying close to the epitope and residues 322-332. MgATP has also been shown to prevent proteolysis by stabilizing the holoenzyme complex (Hofmann et al, 1975) which it binds with an affinity 100 times greater than C-subunit. Similar proteinases have also been described for rat renal brush border (Schmitz et al, 1973; and Hopfer et al, 1973).

The specific inactivation of C-subunit, by a proteinase or deactivator, together with the prevention of its action by substrate and R-subunits, raise the possibility it may have a distinct physiological (possibly regulatory) assignment. In principle it might be the first step initiating its biodegradation or constitute a safety device to prevent phosphorylation of proteins where and when such phosphorylation may have undesired consequences. Down regulation of total PK-A activity has been decribed previously following prolonged β-agonism of perfused mammary tissue or mammary acini (Clegg & Ottey, 1991).

Chapter 4

PK-A expression in the developing rat mammary gland

Introduction

The main objective of this study was to assess whether the expression of PK-A, both in terms of its activity and generic C- and R-subunits (R-subunits: RI and RII) were under developmental regulation in the rat mammary gland, throughout pregnancy and lactation. The structural and functional development which occurs during pregnancy and lactation is discussed more fully in the Introductory chapter, however to summarise here; upon pregnancy a dramatic phase of development occurs, under the influence of lactogenic hormones, ductal cells begin to proliferate and alveolar structures bud out from the ducts, filling the fat pad and its periphery. By late pregnancy, alveolar cells cease to proliferate and begin to synthesize and secrete milk components, differentiation of these cells is complete by lactation, immediately following parturition.

An early objective of the study was to measure mammary tissue PK-A activity throughout pregnancy and lactation and to relate functional changes in PK-A activity to that of the developmental or proliferational state of the tissue. In order to establish the level at which total PK-A activity is regulated, both C-subunit protein mass and C-subunit mRNA expression were measured.

C- and R-subunits are classically believed to be dynamically regulated in order to maintain the one to one ratio present in the holoenzyme complex. Studies were

therefore undertaken to measure the relative abundance of R-subunit protein mass within this same mammary tissue, by a photoaffinity labelling technique using 8-azido-[^{32}P]cAMP. Such an approach incidentally allowed the distinction to be made between the relative levels of the two major isoenzymes of PK-A, type I and type II, which are defined by their characteristic R-subunit isoforms, RI and RII. In conjunction with this, mammary PK-A isoenzymes were also separated and partially quantified using ion exchange chromatography on DEAE cellulose.

4.1 Developmental rat series

Whole mammary tissue was sampled from female Wister rats at various, defined stages of pregnancy and lactation (as described in section 2.1.1). Tissue was sampled at 4 day increments i.e. P4 to P20, referring to the number of elapsed days of pregnancy, and L4 to L20, referring to the number of elapsed days of lactation. Thus a series of tissue samples was collected representing rat mammary gland development throughout pregnancy and lactation. In total seven such rat series were collected; these were then used to study the effects of rat mammary development upon the expression of PK-A activity, C-subunit protein mass, Cα and Cβ mRNA abundance and the relative abundance of R-subunit isoforms (RI and RII).

4.2 Pregnant and lactating sheep mammary tissue

Whole mammary tissue was sampled from 4 x 100 day pregnant ewes and 6 x ewes in their 15 to 25th day of lactation, as described in section 2.1.2. Tissue was used to study the expression of PK-A activity and the relative abundance of R-subunit isoforms (RI

and RII) between pregnancy and lactation. Examination of ruminant tissue provided a contrast to those, more detailed studies carried out upon rat mammary tissue and assisted identification of important, non-species specific developmental trends in PK-A expression.

4.3 PK-A activity measurement

Soluble tissue extracts were made (as described in section 2.6.1) and assayed for both "total" PK-A activity i.e. that measured in the presence of excess cAMP and "expressed activity" i.e. that measured in the absence of added cAMP, as described in section 2.6.2. By measuring total PK-A activity, an index of the total amount of C-subunit protein present in the tissue, and also therefore, the cells capacity for cAMP-dependent protein phosphorylation, could be determined. This parameter does not however reflect immediate intracellular conditions, *in vivo*. These are more accurately reflected by expressed PK-A activity. Expressed activity provided a measure of the dissociated C-subunit, free from regulatory subunits, present in the tissue at the moment of sampling. Procedures were adopted to prevent association/dissociation of the C- and R-subunits of PK-A, (as described in section 2.6.1) and thus maintain the $R_2 + 2C \rightleftharpoons R_2C_2$ equilibrium.

4.3.1 PK-A activity throughout pregnancy and lactation in the rat mammary gland

Based on fresh tissue weight, total activity was seen to approximately double during the course of pregnancy and again during lactation. During pregnancy most of the increase occurred between P12 and P16, whereafter a plateau of activity was reached persisting

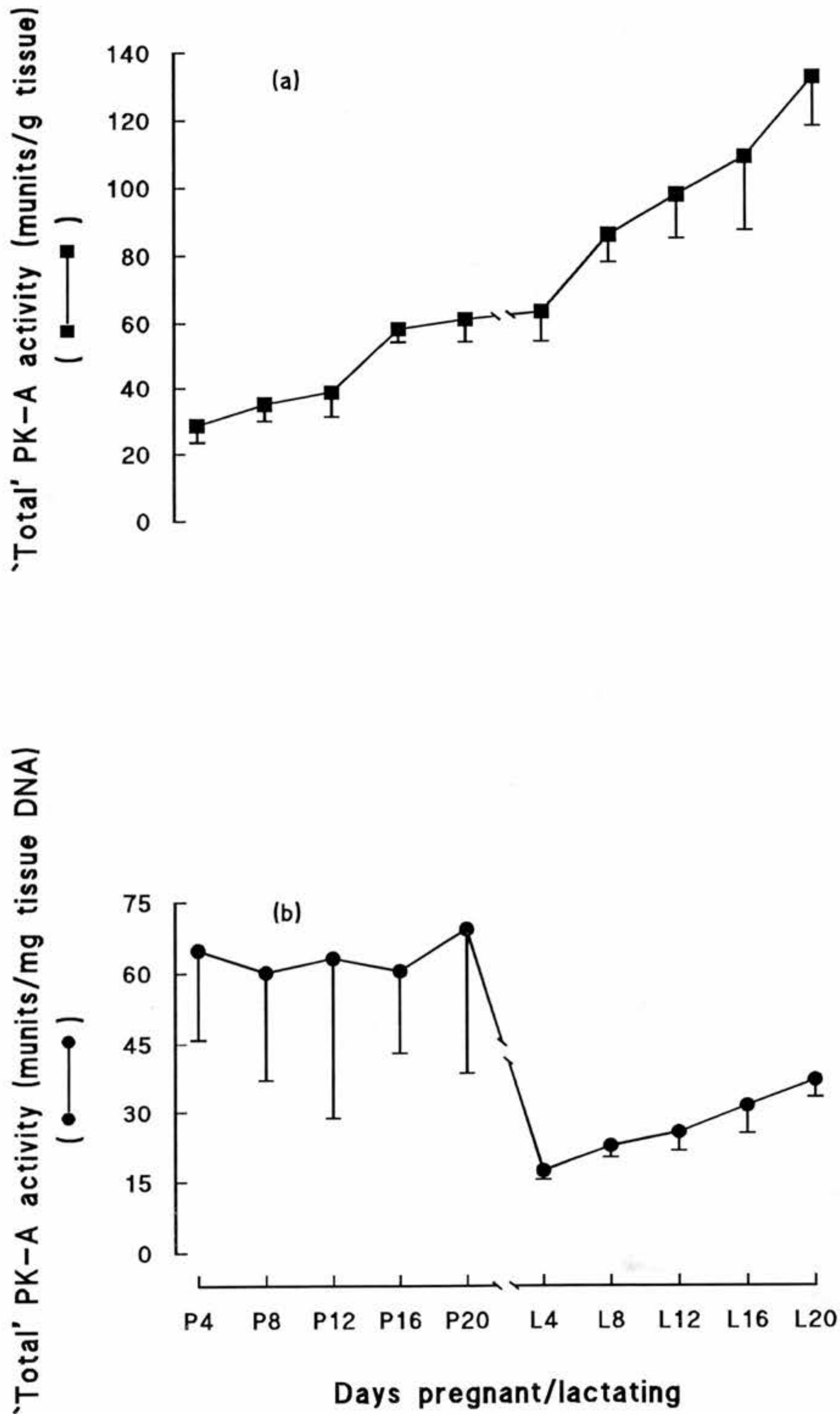


Figure 4.1 Total PK-A activity in rat mammary tissue throughout pregnancy and lactation

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 section 2.6). Results are means (± S.E.M.) of determination on 6 series of pregnant/lactating animals. Results are expressed on both wet tissue weight (a) and tissue DNA basis (b).

through parturition until L4 of lactation. From L4 onwards total activity increased in a more or less linear function with time until L20, the last *post partum* day on which measurements were made (Figure 4.1a).

Rat mammary tissue DNA (DNA/g tissue) increased progressively throughout pregnancy, rising sharply at parturition and early lactation, to remain nearly constant by mid-lactation (Figure 4.2). Levels of tissue protein also increased, following a similar progression to DNA, throughout both pregnancy and lactation (Figure 4.2). Therefore when total PK-A activity was expressed on a tissue DNA basis, the trends described above were reversed to give the developmental pattern shown in Figure 4.1b. Total activity was found to be greatest during pregnancy, falling dramatically at parturition before rising steadily throughout lactation; although prepartum values were never reached (Figure 4.1b).

The level of expressed PK-A activity (per mg of DNA), fell quickly with the onset of pregnancy to peak again in late pregnancy (Figure 4.3a), remaining at a low but constant level throughout lactation. Expressed activity did not remain in fixed proportion to total activity throughout the course of pregnancy and lactation, thereby giving rise to characteristic fluctuations in the PK-A activity ratio (expressed activity/unit of total activity), accompanying the growth and differentiation of the tissue as shown in (Figure 4.3b). It was expected that the activity ratio of PK-A would reflect the intracellular concentration of cAMP throughout pregnancy and lactation. The activity ratio was generally higher during lactation than pregnancy, reaching a peak at L4 (Figure 4.3b). Published figures for the concentration of cAMP in rat mammary tissue, corrected for milk content (Sapag-Hagar & Greenbaum, 1974), show a peak at

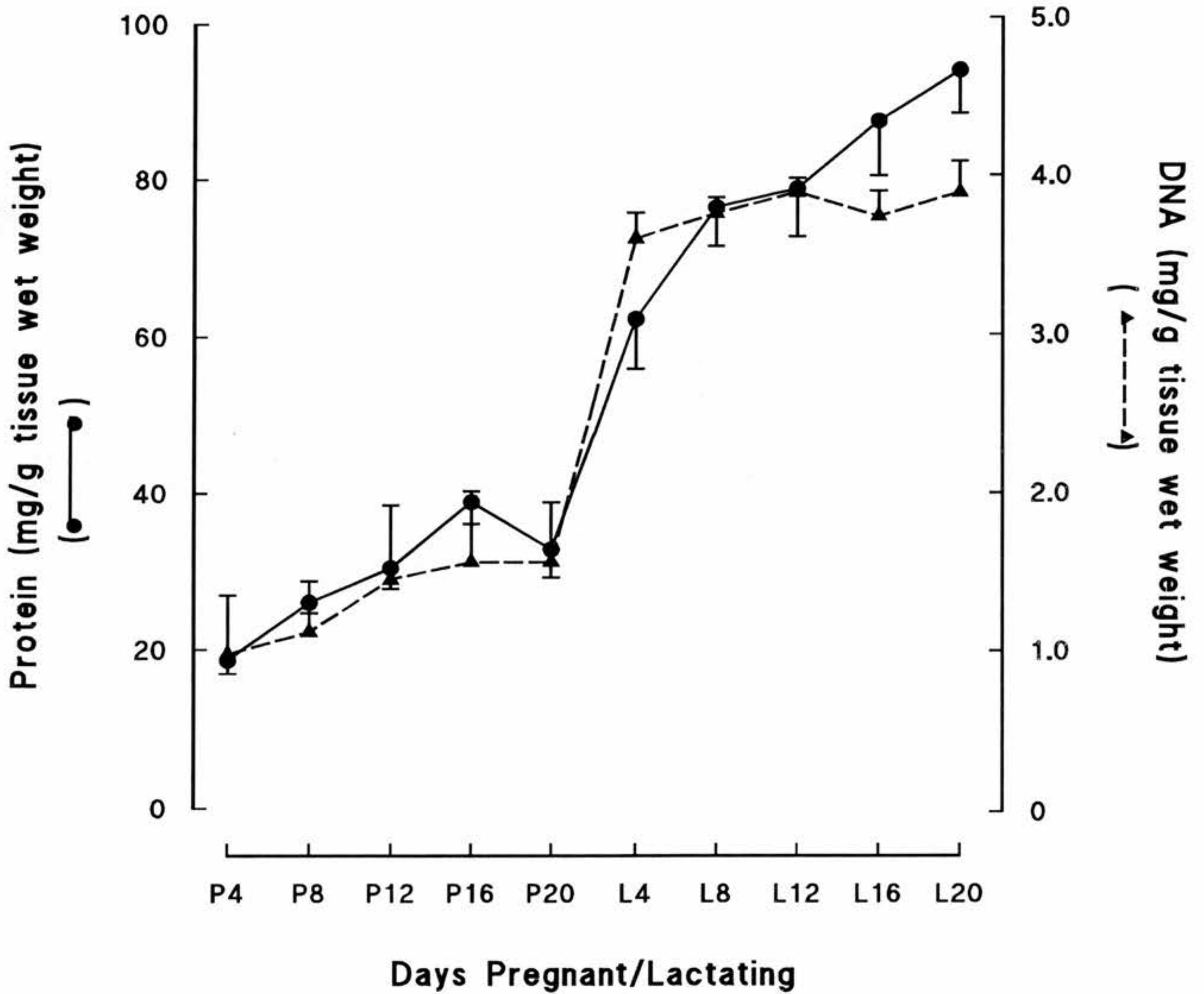


Figure 4.2 Composition of rat mammary tissue throughout pregnancy and lactation

Mammary tissue was sampled from rats at defined stages of pregnancy and lactation. Extracts were prepared and assayed for protein and DNA content (as described in sections 2.5.1 and 2.5.2). Results are means (± S.E.M.) of determinations on 6 series of pregnant and lactating animals.

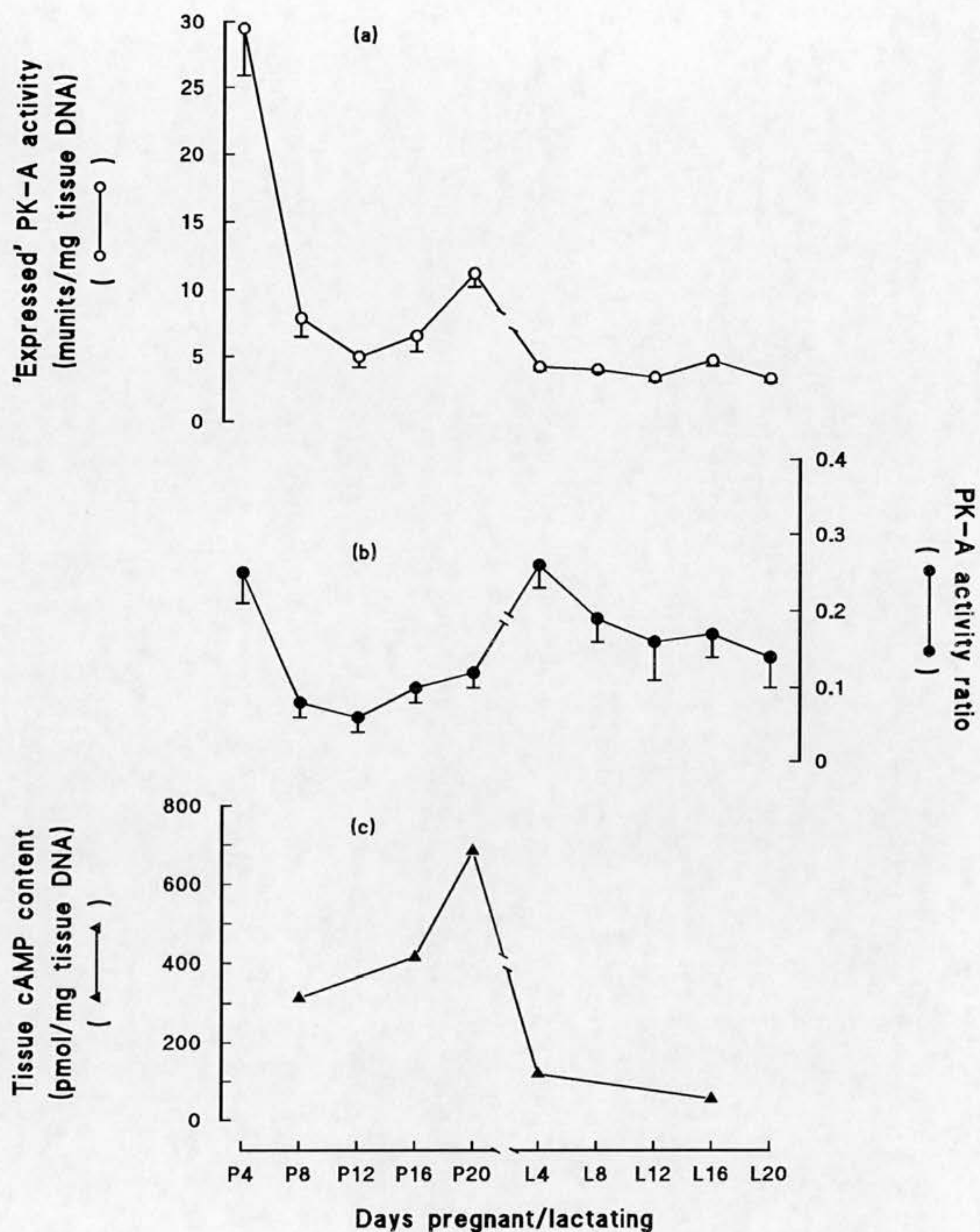


Figure 4.3 Expressed PK-A activity and activity ratio of rat mammary tissue throughout pregnancy and lactation

Mammary tissue was sampled from rats at defined stages of pregnancy and lactation. Extracts were prepared and assayed for expressed PK-A activity (as described in section 2.6). Results are means (± S.E.M.) of determination on 6 series of pregnant/lactating animals. The activity ratio equals expressed/total PK-A activity. Tissue cAMP content is that data originally described by Sapag-Hagar & Greenbaum (1974).

P20, with values being greatest during pregnancy and falling with the onset of lactation (Figure 4.3c). Therefore despite expectations, the activity ratio of PK-A did not reflect the apparent cAMP status of rat mammary tissue.

4.3.2 PK-A activity during pregnancy and lactation in the sheep mammary gland

To determine whether PK-A activity was under developmental regulation in the ruminant mammary gland, total and expressed PK-A activities were measured in pregnant and lactating sheep mammary tissue extracts. Whole mammary tissue was sampled from pregnant and lactating ewes (as described in section 2.1.2). Soluble extracts were made (as described in section 2.6.1) and assayed for total and expressed PK-A activity (as described in section 2.6.2).

When expressed on a wet tissue weight basis, total PK-A activities were found to be higher in tissue extracts derived from lactating animals (64.09 $\pm$ 5.8 munits/g tissue), compared with those from pregnant animals (52.31 $\pm$ 3.6 munits/g tissue), (Table 4.1). An increase in total PK-A activity between pregnancy and lactation was tested for using the Student's t test and found to be significant for $P<0.1$. Levels of DNA, per wet weight of sheep mammary tissue, remained relatively constant, figures were not significantly different between pregnancy and lactation (Table 4.1). Therefore, when total PK-A activity was expressed on the basis of tissue DNA, it was found to be higher in tissue extracts derived from lactating animals than in those from pregnant animals (18.6 $\pm$ 1.7 and 15.6 $\pm$ 1.7 munits/mg DNA respectively). Expressed PK-A activities showed a similar trend, mean values were higher for lactating tissue than pregnant (Table 4.1). An increase in expressed PK-A activity between pregnancy and lactation

Tissue source	DNA mg/g tissue	Protein mg/g tissue	Total PK-A activity munits/mg tissue	Total PK-A activity munits/mg DNA	Expressed PK-A activity munits/g tissue	Activity ratio
Pregnant sheep	3.5 ±0.5	34.9 ±5.4	52.3 ±3.6*	15.6 ±1.7	10.5 ±0.9**	0.2 ±0.03
Lactating sheep	3.5 ±0.2	48.9 ±1.9	64.1 ±5.8	18.6 ±1.7	16.2 ±1.1	0.262 ±0.04

Table 4.1 PK-A activity during pregnancy and lactation in the sheep mammary gland

Mammary tissue was sampled from pregnant and lactating sheep as described in section 2.1.2. Extracts were prepared and assayed for total and expressed activity as described in section 2.6. Protein and DNA contents were also measured as described in sections 2.5.1 and 2.5.2. Results are means (± S.E.M) for not less than 4 animals. Statistically significant increases between pregnancy and lactation were assessed by Student's t test (of randomized, 2 group design): $*P<0.1$ and $**P<0.005$.

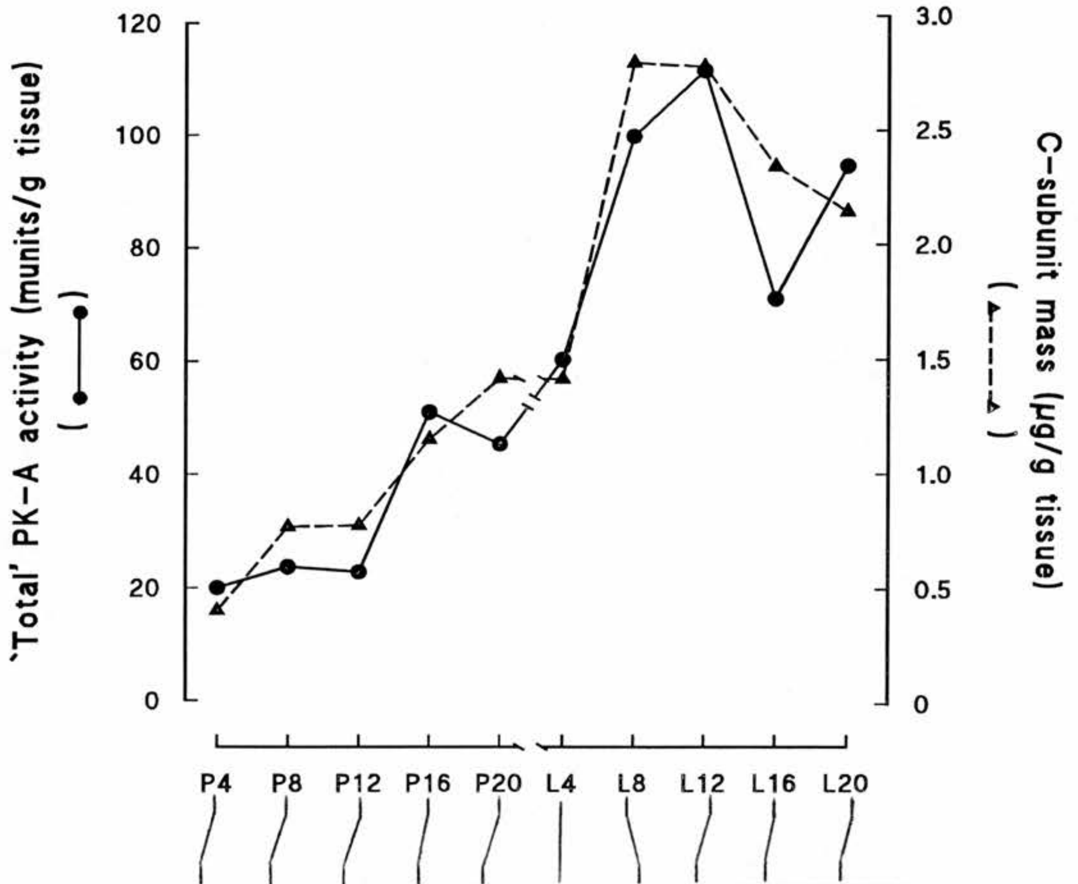
was tested for using the Student's *t* test and found to be significant for $P < 0.0.05$. The PK-A activity ratio also appeared to be elevated in lactating sheep mammary tissue, with respect to that from pregnant animals; however this failed to reach statistical significance in a Student's *t* test for $P < 0.1$.

4.4 C-subunit protein mass in the rat mammary gland throughout pregnancy and lactation

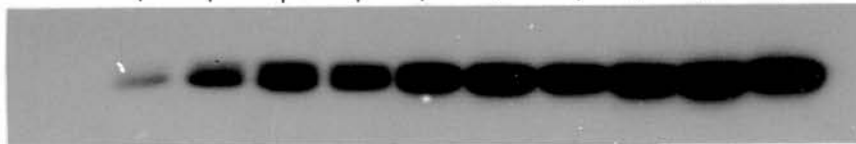
Given the characteristic pattern of total PK-A activity, previously described in rat mammary tissue extracts, over pregnancy and lactation (section 4.3.1), it was hoped to establish whether or not this activity was related to levels of C-subunit protein present within the tissue, or upon the presence of some uncharacterised intracellular modulator of kinase activity, differentially expressed throughout pregnancy and lactation.

Using the anti C-subunit antiserum (As-365), and the immunoquantification procedure outlined in section 2.7, C-subunit protein was measured in mammary tissue extracts prepared from a pregnant and lactating rat series (section 4.1). Results were compared with PK-A activity data, which already existed for these same tissue samples, to yield the developmental profile shown in Figure 4.4. The immunoquantification profile of C-subunit mirrored that of total PK-A activity, allowing the conclusion that the specific activity, intrinsic to C-subunit, remained constant throughout rat mammary development. The level of total PK-A activity in mammary tissue was apparently dependent upon the corresponding level of C-subunit protein within the tissue. The mean specific activity was ± S.E.M 37.8 ± 2.4 units/mg of C-subunit protein for rat

(a)



(b)



Days Pregnant/Lactating

Figure 4.4 Quantification of C-subunit protein mass in rat mammary tissue throughout pregnancy and lactation

The above Figure (a) shows the combined data from two separate experiments using the same tissue samples, and is representative of determinations carried out on two series of pregnant and lactating animals. PK-A activity was measured in rat mammary tissue extracts prepared and assayed as described in sections 2.6.1 and 2.6.2. C-subunit mass was measured in similar mammary tissue extracts as described in section 2.7. The autoradiograph (b) shows samples from a single immuno-quantification experiment.

Tissue source	Specific catalytic activity (units/mg protein)
Pregnant sheep	7.9 $\pm$ 2.5
Lactating sheep	5.3 $\pm$ 1.9
Sheep Heart	4.9 $\pm$ 1.0

Table 4.2 Specific activity of PK-A C-subunit
The PK-A C-subunit was purified from various sheep tissues as described in section 2.2.1. Preparations were assayed for protein, as described in section 2.5.1. Catalytic activity was assayed as described in section 2.6.2. The above figures are means ($\pm$S.E.M) for not less than three preparations.

mammary tissue. This value was higher than those previously reported for other rat tissues, and those estimates of specific activity made from purified C-subunit preparations derived from pregnant and lactating sheep mammary tissue (Table 4.2).

4.5 Cα and Cβ mRNA expression throughout pregnancy and lactation in the rat mammary gland

Given the characteristic pattern of PK-A total activity demonstrated throughout rat mammary development, and its apparent close correlation to C-subunit protein mass (sections 4.3.1 and 4.4), experiments were initiated to examine Cα and Cβ mRNA expression throughout pregnancy and lactation, and therefore determine if C-subunit protein mass was under transcriptional regulation. Studies were performed using six series of pregnant and lactating rat tissue samples, for which PK-A activity and C-subunit data already existed.

RNA was prepared from rat mammary tissue as described in section 2.8.1; Northern blotting was carried out as described in section 2.8.2. C-subunit mRNAs were detected in Northern-blot experiments with isoenzyme specific probes as shown in Figure 4.5 and further described in section 2.8.3. The abundance of mRNA for both isoforms (expressed per mg of tissue DNA) reflected the total PK-A activity throughout pregnancy and lactation (compare the lower panel of Figure 4.5 with Figure 4.1b). Although there was a decrease in the level of mRNA for both Cα and Cβ at the transition from pregnancy to lactation, there was no indication of any change in the ratio of Cα to Cβ mRNA during the mammary developmental programme. Although the method used was not appropriate for the determination of absolute amounts of

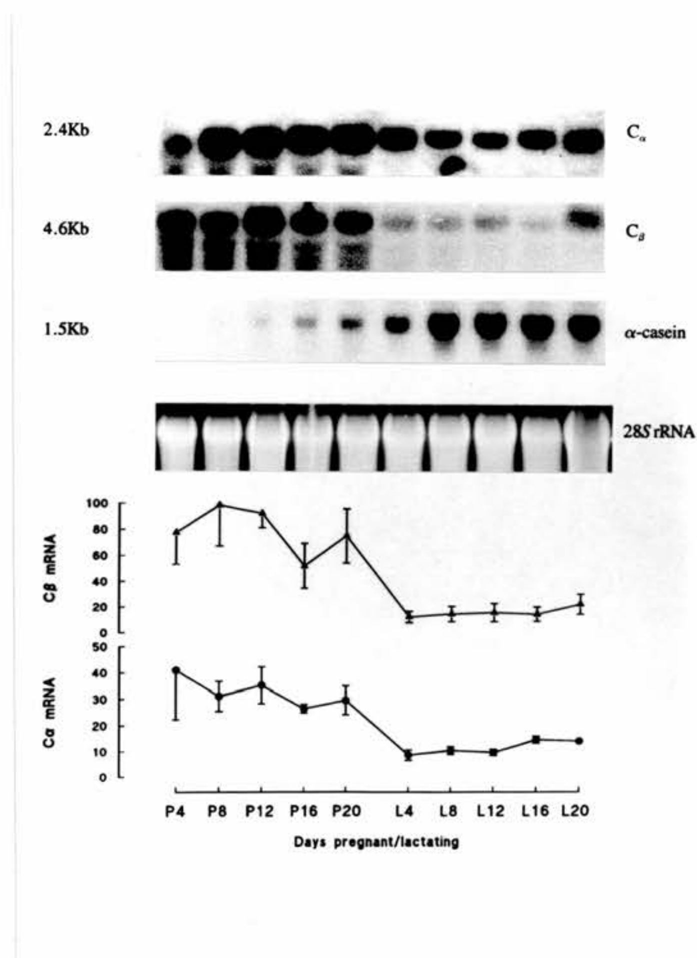


Figure 4.5 Northern blot analysis of mRNA encoding C-subunit isoform α and β in mammary tissue throughout pregnancy and lactation

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 the appropriate correction factors for minor variations in RNA loadings. Autoradiographs of blots hybridized with probes specific for Cα, Cβ and rat α-casein are shown above for a single representative series of lactating and pregnant mammary tissue samples. Densitometric analysis of the 3 such data sets gave the numerical values plotted (means $\pm$ S.E.M) in the lower part of the Figure.

mRNA, it is clear from the intensity of the autoradiographic signals elicited when cDNA probes of roughly equivalent specific radioactivity were used, that the mRNA for Cα is considerably more abundant than that for Cβ. The pattern of expression of α-casein throughout pregnancy and lactation (Figure 4.5) was in close agreement with the results of others (Hobbs et al, 1982).

4.6 Cyclic AMP-binding proteins of the mammary gland

As previously discussed, both total and expressed PK-A activities appear to be developmentally regulated throughout pregnancy and lactation (section 4.3). Furthermore, levels of total PK-A activity were shown to be in direct proportion to the level of C-subunit protein, which itself proved to be under transcriptional regulation (sections 4.4 and 4.5). To examine the possibility that R-subunit expression was also developmentally regulated, the cAMP analogue, 8-azido-[^{32}P]cAMP was used to detect, mammary cAMP-binding proteins as described in section 2.9. Photoaffinity labelling additionally allowed the distinction to be made between the levels of the two major PK-A isoenzymes, type I and type II, which are defined by their characteristic R-subunit isoforms RI and RII.

4.6.1 Cytosolic cAMP-binding proteins in the rat mammary tissue

Soluble, cytosolic extracts were prepared from each developmental series (as described in section 2.9.1) consisting of pregnant and lactating rat mammary tissue samples as described in section 4.1. In each experiment extracts were equalised for protein before being labelled with the cAMP analogue 8-azido[^{32}P]cAMP, as described in section

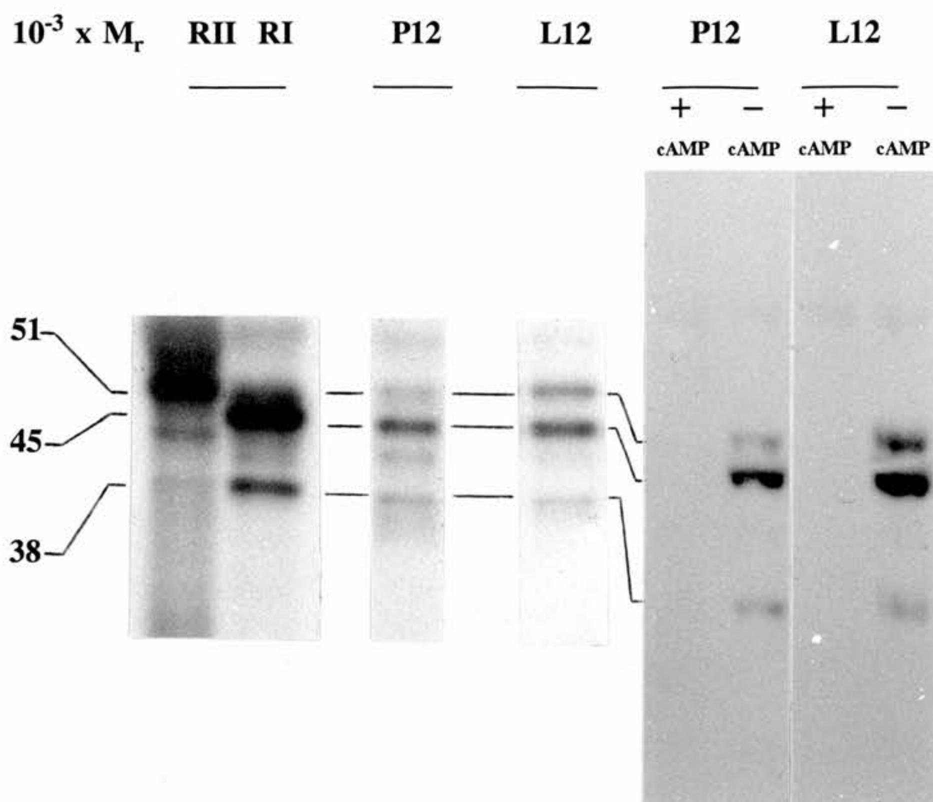


Figure 4.6 Cytosolic cAMP-binding proteins in rat mammary tissue
Cyclic AMP-binding proteins were identified by photoaffinity labelling as described in section 2.9. The autoradiograph shows from left to right: standard signals obtained from Human tumour biopsy samples (known to contain either predominantly RII or RI respectively); signals from P12 and L12 rat mammary extracts taken from the same autoradiograph and P12 and L12 samples taken from a separate experiment in which photoaffinity labelling was carried out both in the presence and absence of radio inert cAMP (as indicated by "+" and "-" respectively).

2.9.2. Samples from each series were run together in a single gel allowing comparison of the developmental expression of R-subunits and their isoforms. In total, four series of rat tissue samples were examined in this way. In order to identify high affinity, specific cAMP-binding proteins, cAMP competition experiments were also performed, as described in section 2.9.3.

The photo-activated ligand, 8-azido-[^{32}P]cAMP labelled two major proteins from the cytosolic fractions of mammary tissue from both pregnant and lactating animals. These migrated on SDS-PAGE at the same position as for RI and RII standards (M_r 45000-51000 respectively), which had been subjected to the same experimental procedure (Figure 4.6). A minor band of M_r 37-38000 was also detected in all cytosolic mammary samples examined. This protein most probably corresponded to one of a family of fragments derived from R-subunits by the proteolytic cleavage of the susceptible hinge region, and previously described by (Weldon & Taylor, 1985). Labelling of all three bands was successfully competed out by excess cAMP (Figure 4.6) and was absent when no u.v. irradiation was applied to the samples (results not shown).

The lower molecular weight R-subunit (RI) was apparently the major cAMP-binding protein within rat mammary tissue and this remained the case throughout both pregnancy and lactation. However the relative amounts of RI and RII varied throughout this period. There was a progressive increase in the levels of RII throughout pregnancy and lactation, which largely fostered the increase in the RII:RI ratio also observed. In early pregnancy RII accounted for just 12% of the total R-subunit, by late lactation it accounted for more than 38% (Figure 4.7). The RII:RI ratio was able to increase progressively throughout pregnancy and lactation despite an increase in the level of RI

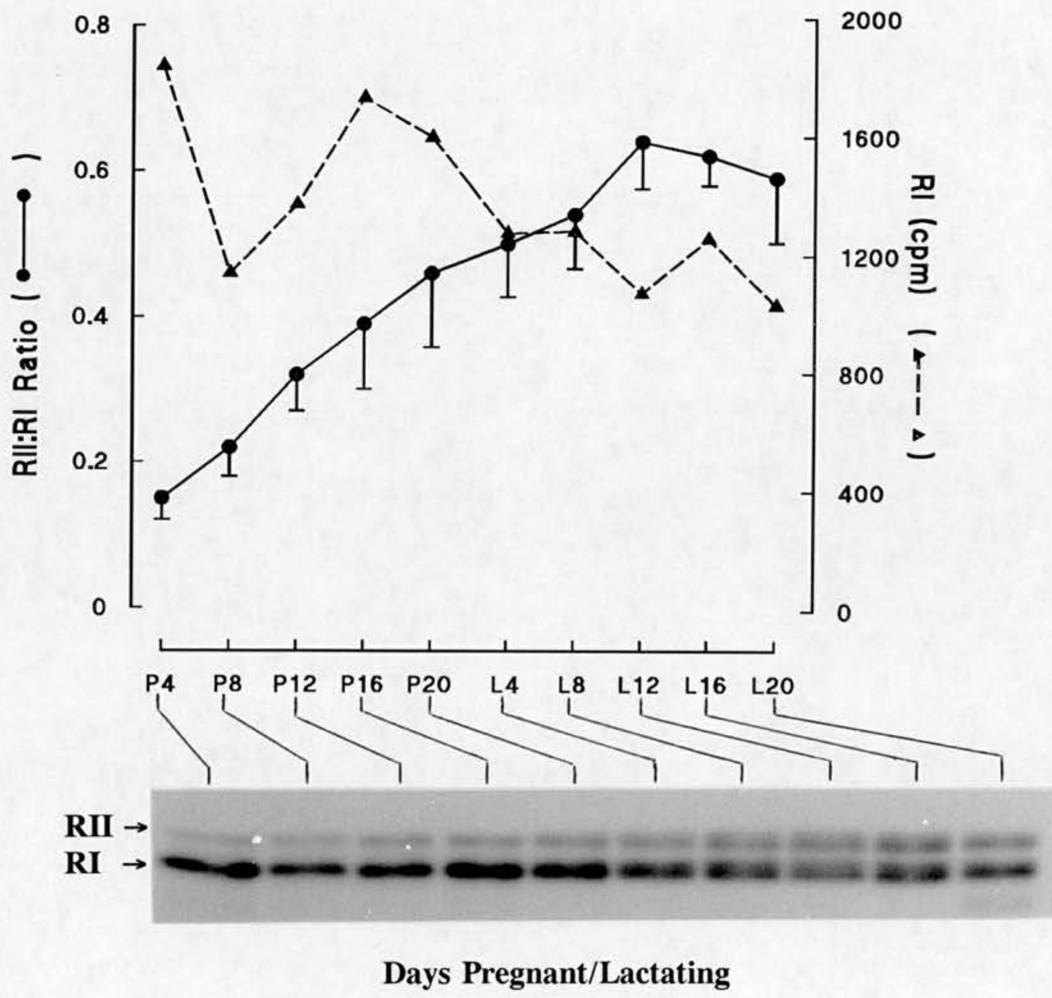


Figure 4.7 Cytosolic R-subunit composition in the rat mammary gland
The relative abundance of the RI and RII isoforms was determined in cytosolic rat mammary extracts as described in section 2.9. The autoradiograph shows the changing levels of the two major R-subunit isoforms throughout pregnancy and lactation in a single labelling experiment; the graph shows RI levels from the same experiment and the RII:RI ratio (± S.E.M.) from determinations made on four series of pregnant and lactating animals.

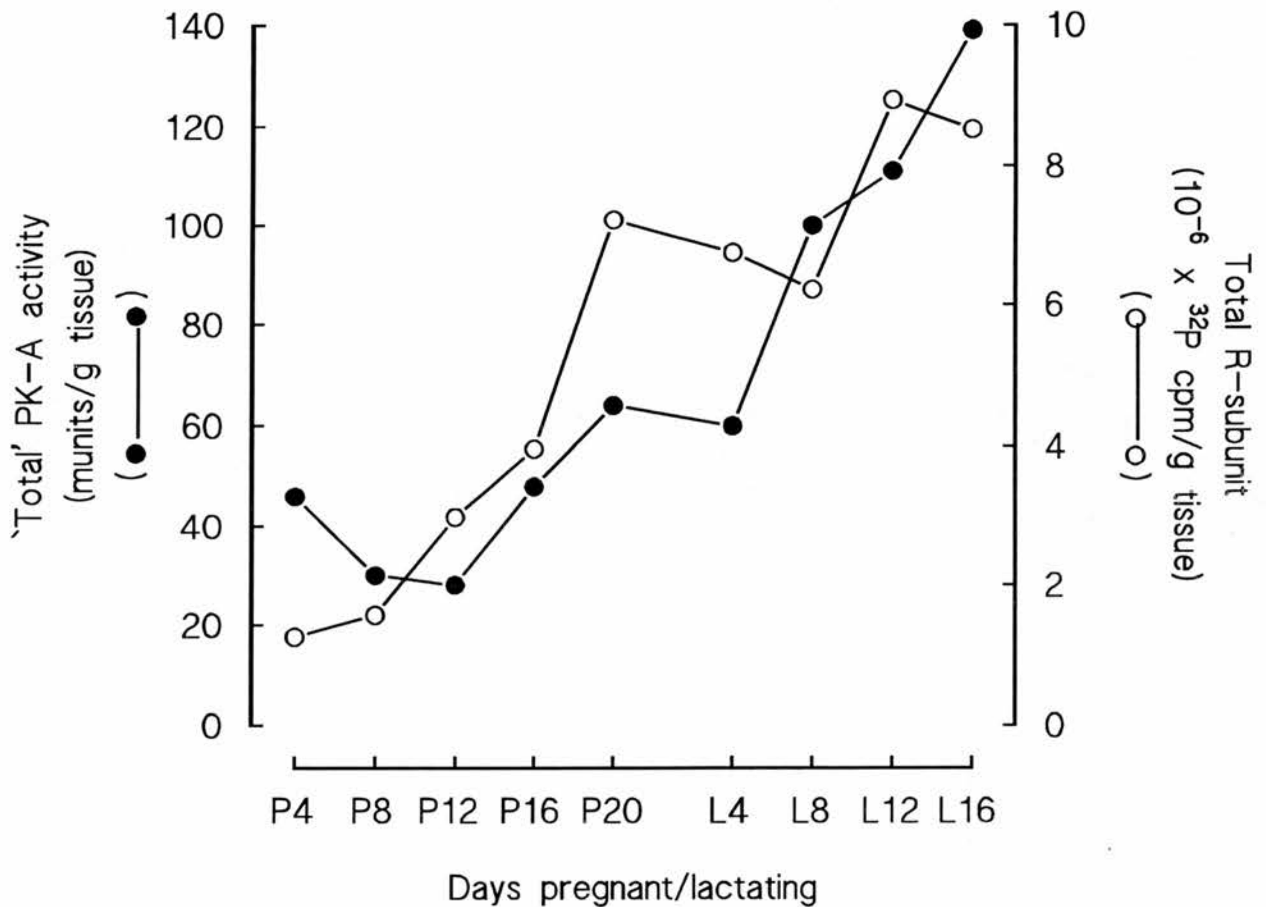


Figure 4.8 Comparison of R- and C-subunit levels in the rat mammary gland throughout pregnancy and lactation

Total C-subunit and R-subunits were compared throughout pregnancy and lactation for a single representative series of rats. At each stage, assay of C-subunit catalytic activity and photoaffinity labelling of R-subunits were performed on the same tissue extract, as described in Materials and methods, sections 2.6 and 2.9 respectively.

which peaked during mid-pregnancy; sustained at this time by increasing levels of RII. However, following parturition and into early lactation, levels of RI declined back to those seen during early pregnancy which further contributed to the increasing RII:RI ratio (Figure 4.7).

Total R-subunit levels (RI + RII), were also compared to C-subunit protein mass determined for the same set of tissue samples (section 4.4). The exact molecular ratio of the two PK-A subunits (normally expected to equal 1.0) could not be evaluated, since absolute levels R-subunit protein mass were not measured here. However, the ratio of R- to C-subunit was shown to remain relatively constant throughout much of pregnancy and lactation; although a transient over-expression of mammary R-subunit between late pregnancy and early lactation was suggested by the data presented (Figure 4.8).

4.6.2 Cytosolic cAMP-binding proteins in the sheep and goat mammary tissue

To determine whether R-subunit expression was under developmental regulation in the ruminant mammary gland, photoaffinity labelling experiments were performed on pregnant and lactating, sheep and goat mammary tissue extracts, prepared and labelled, as described for rat mammary tissue, in sections 2.9.1 and 2.9.2.

The photo-activated ligand, 8-azido[^{32}P]cAMP, labelled two major cAMP-binding proteins in cytosolic extracts derived from lactating and pregnant, sheep and goat mammary tissue (Figure 4.9). These migrated on SDS-PAGE close to the positions for rat mammary RI and RII; apparent molecular weights for the goat and pregnant sheep RI and RII were M_r 46000 and 51000 respectively. However, R-subunits isoforms

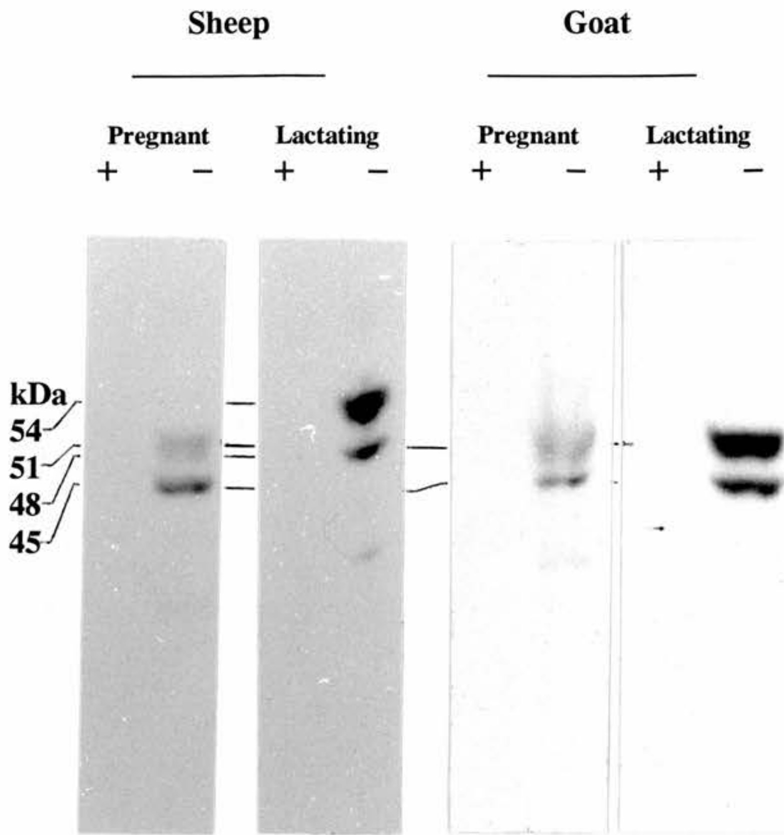


Figure 4.9 Cytosolic cAMP-binding proteins in sheep and goat mammary tissue The above autoradiographs represent cAMP-binding proteins identified in cytosolic, mammary tissue extracts, derived from (left to right) pregnant and lactating sheep and goat respectively. Such reactions were competed out using excess cAMP as described in section 2.9.3 ("+" and "−" indicate the presence and absence respectively of excess cAMP during photoaffinity labelling of the mammary tissue extracts).

Tissue source	Cytosolic RI (cpm)	Particulate RI densitometric band volume (arbitrary)	Cytosolic RII (cpm)	Particulate RII densitometric band volume (arbitrary)	Cytosolic RII:RI ratio	Particulate RII:RI ratio
Pregnant sheep	600 ±26	50.6 ±8.1	478 ±46	89.4 ±7.1	0.800 ±0.02	1.8 ±0.14
Lactating sheep	399 ±40	88.5 ±12.3	899 ±70	202.5 ±14.2	2.250 ±0.2	2.4 ±23

Table 4.3 Relative abundance of cytosolic and particulate R-subunit isoforms throughout pregnancy and lactation in the sheep mammary gland
Mammary tissue was sampled from pregnant and lactating sheep as described in section 2.1.2. Cytosolic and particulate mammary tissue extracts were made and labelled with 8-azido-[^{32}P]cAMP as described in sections 2.9.1 and 2.9.2. The above figures are taken from two separate labelling experiments (for cytosolic and particulate samples respectively) and are means (± S.E.M) for not less than 4 animals.

Tissue source	RI (cpm)	RII (cpm)	RII:RI ratio
Pregnant goat	161 ± 34	154 ± 10	1.03 ± 0.16
Lactating goat	194	350	1.81
Pregnant & lactating goat	223	301	1.35

Table 4.4 Relative abundance of cytosolic R-subunit isoforms throughout pregnancy and lactation in the goat mammary gland
The above figures are taken from a single experiment in which mammary tissue was sampled from three pregnant and two lactating goats, as described in section 2.1.2. Extracts were made and labelled with 8-azido-[^{32}P]cAMP as described in sections 2.9.1 and 2.9.2.

derived from lactating sheep mammary tissue exhibited higher molecular weights (M_r 48000 and 54000 for RI and RII respectively, this is best illustrated in Figure 4.12a). A minor band of M_r 39000 was also detected in all cytosolic sheep and goat mammary samples examined. Labelling of all three bands was successfully competed out by excess cAMP (Figure 4.9). The relative amount of RI and RII did not remain constant between pregnancy and lactation. The RI isoform was in slight excess over RII in samples from pregnant animals. However, in samples derived from lactating animals RII levels far exceeded those of the RI isoform. Thus the RII:RI ratio rose from 0.8 to >2.0 between pregnancy and lactation for sheep mammary tissue and increased from 1.0 to 1.85 for goat mammary tissue (Tables 4.3 and 4.4). Interestingly, it was possible to sample goat mammary tissue from animals which were simultaneously lactating and pregnant; when photoaffinity experiments were performed on such tissue, a level of RI and RII expression was found, intermediate between that in pregnant only or lactating only animals (Table 4.4).

4.6.3 Particulate cAMP-binding proteins in the rat mammary tissue

The cytosolic R-subunit isoforms, RI and RII, have been shown to be subject to dynamic regulation throughout pregnancy and lactation in rat mammary tissue (section 4.6.1). In order to examine the possibility that the particulate forms of these proteins were also under developmental regulation, photoaffinity labelling experiments were performed on mammary samples derived from two rat series. Cytosolic and particulate extracts were prepared simultaneously from each series of mammary tissue samples, by the methods described in section 2.9.1. Samples were equalised for protein before

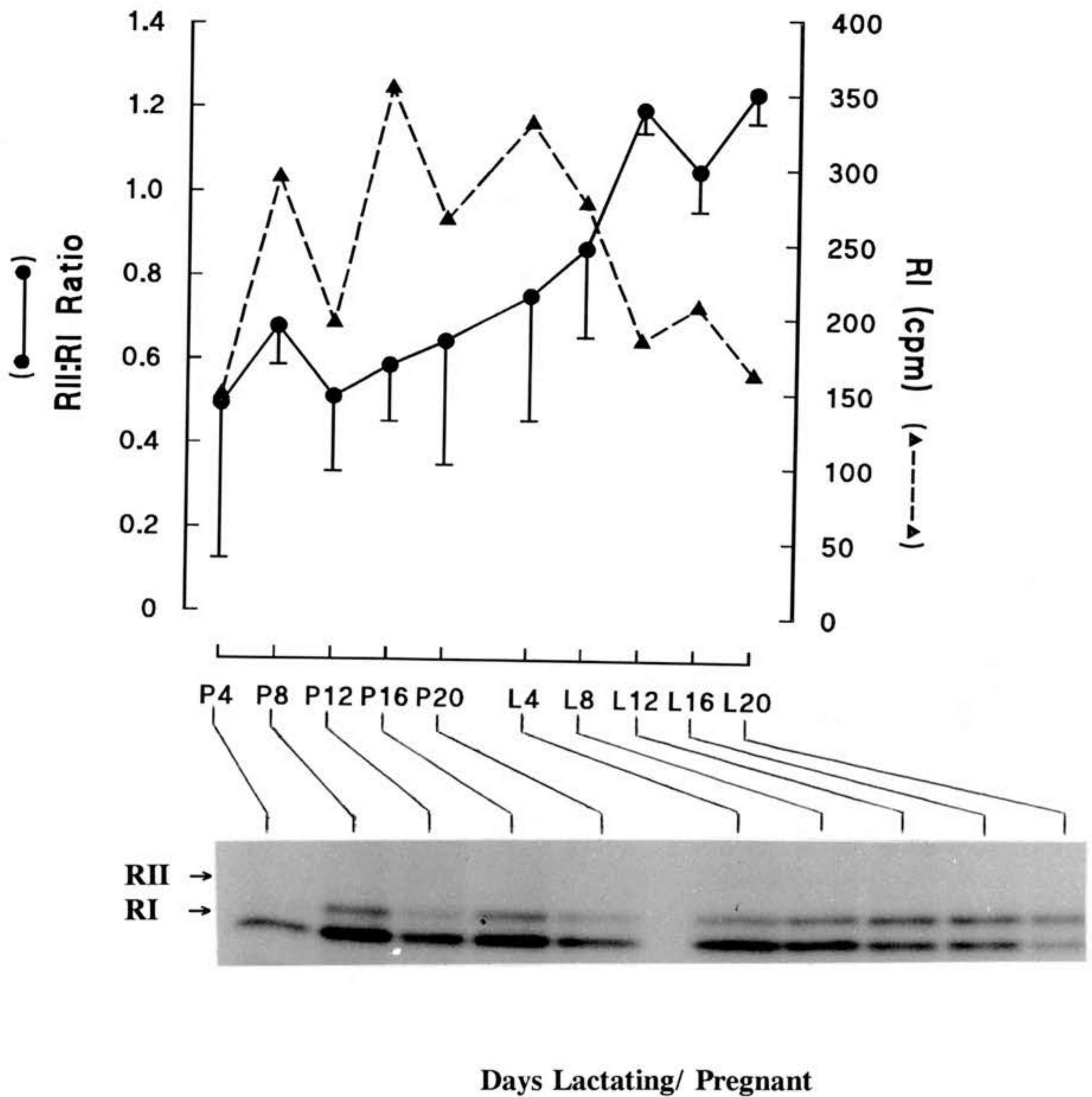


Figure 4.10 Particulate R-subunit composition of rat mammary tissue
The relative abundance of the RI and RII isoforms was determined in particulate rat mammary extracts as described in section 2.9. The autoradiograph shows the changing levels of the two major R-subunit isoforms throughout pregnancy and lactation in a single labelling experiment; the graph shows RI levels from the same experiment and the RII:RI ratio (± S.E.M.) from determinations made on two series of pregnant and lactating animals.

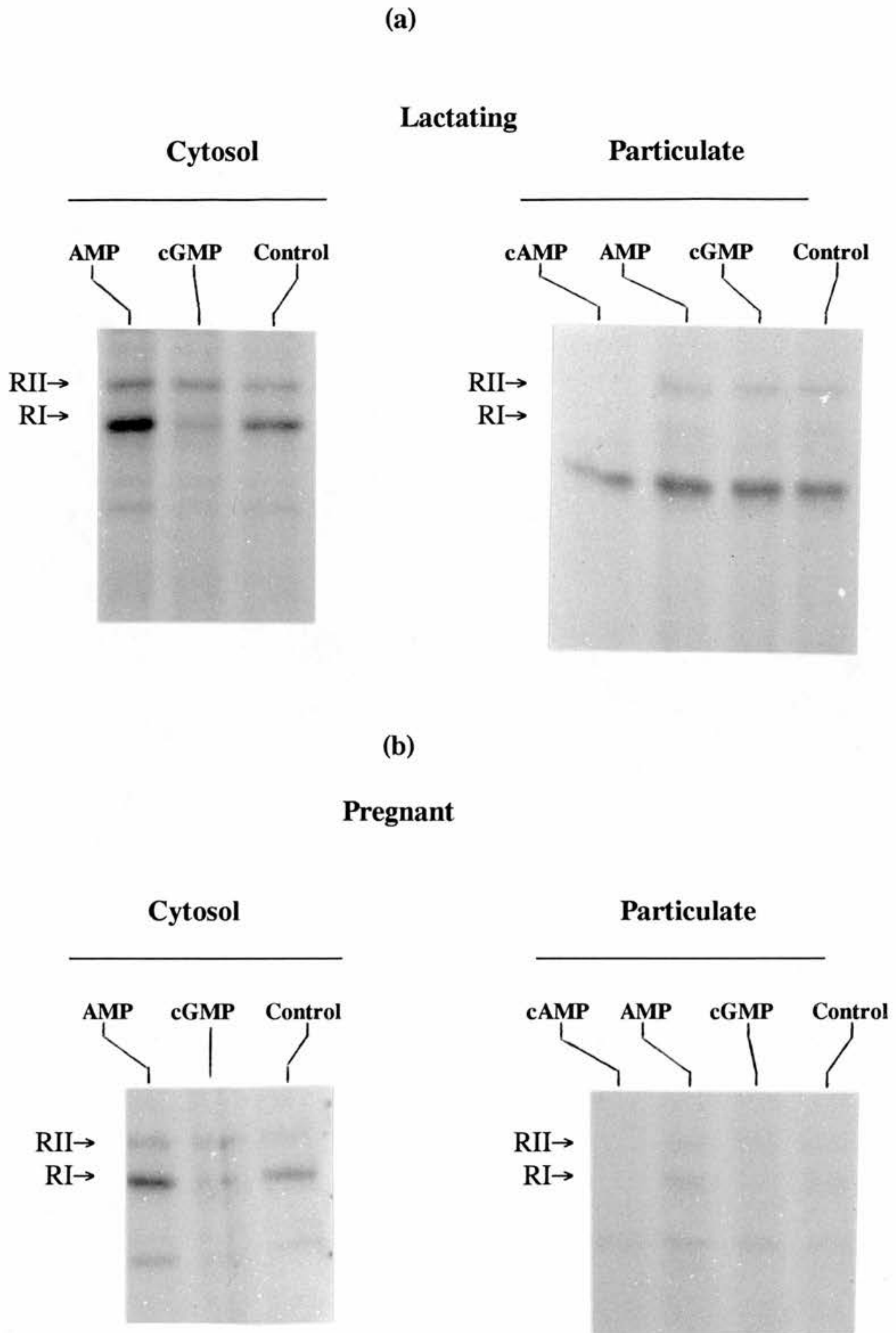


Figure 4.11 Specificity of cAMP-binding proteins in cytosolic and particulate rat mammary tissue

The above Figure shows data from a single competition experiment in which photoaffinity labelling was performed (as described in section 2.9.3.) in the presence and absence of cGMP and AMP, in addition to radioinert cAMP. All samples were equalised with respect to protein concentration prior to labelling. The above autoradiograph shows cytosolic and particulate fractions from (a) lactating rat mammary tissue (b) pregnant rat mammary tissue respectively.

being photoaffinity labelled, as described in section 2.9.2.

As for the cytosolic fraction, the photoaffinity ligand labelled two major proteins in the particulate fractions from rat mammary tissue (Figure 4.10). These ran at the same positions as those cytosolic bands, previously denoted RII and RI (section 4.6.1). The minor M_r 37-38000 cAMP-binding protein, previously described in cytosolic fractions, was not always seen in the particulate samples. Both particulate cAMP-binding proteins (RI and RII), could be successfully competed out by cAMP, as described in section 2.9.3, and shown in Figure 4.11. The total amount of cAMP-binding protein (i.e. RI + RII calculated on a per mg protein basis) was found to be greater in cytosolic mammary extracts, with respect to those of the particulate compartment (results not shown). The level of RI relative to RII did not remain constant between pregnancy and lactation (Figure 4.10). RI remained in excess of RII throughout most of pregnancy. However, following parturition, RI levels rapidly decreased, falling below those of RII; at the same time total levels of RII increased, reaching a peak at L12. This combination of events led to the increase in the RII:RI ratio observed throughout lactation (Figure 4.10).

4.6.4 Specificity of mammary tissue cAMP-binding proteins

In order to further characterise cAMP-binding proteins described in rat mammary tissue, additional competition experiments were performed in addition to those utilising cAMP. As already described, excess cAMP prevented any significant radio-labelling of those cytosolic and particulate proteins, denoted RI and RII, as well a M_r 37-38000 protein band, found in cytosolic mammary tissue extracts. Similar competition

experiments were performed using the related compounds, cGMP and AMP (136μM). AMP apparently increased labelling of the RI and RII bands, at least for cytosolic rat mammary tissue extracts; bands were too faint to determine the effect of AMP on labelling of particlate tissue extracts. Conversely, cGMP reduced the level of radio-labelling of the putative RI band, in all cytosolic samples examined by approximately 40-50% (Figure 4.11); once again bands for particulate fractions were too faint to ascertain whether cGMP had a similar effect on the labelling of these samples. A similar effect of cGMP has been documented before by (Handschin et al, 1983).

4.6.5 Particulate cAMP-binding proteins in the sheep mammary tissue

Common developmental trends between the cytosolic fractions of sheep and rat mammary tissue have been shown (section 4.6.1 and 4.6.2). Therefore cAMP-binding proteins were examined in the particulate fraction of mammary tissue extracts prepared from pregnant and lactating sheep. Both particulate and cytosolic extracts were prepared from the same tissue samples as described in section 2.9.1. All samples were equalised with respect to their protein concentration before being labelled with the photoaffinity ligand 8-azido [^{32}P]cAMP as described in section 2.9.2. Competition experiments using radio-inert cAMP were also carried out, as described in section 2.9.3.

The photoaffinity ligand detected two major cAMP-binding proteins in the particulate fraction of sheep mammary tissue. These ran at the same positions on SDS-PAGE as for Lactating sheep, cytosolic mammary proteins, previously denoted RI and RII (M_r 48000 and 54000 respectively, Figure 4.12). However unlike cytosolic extracts the

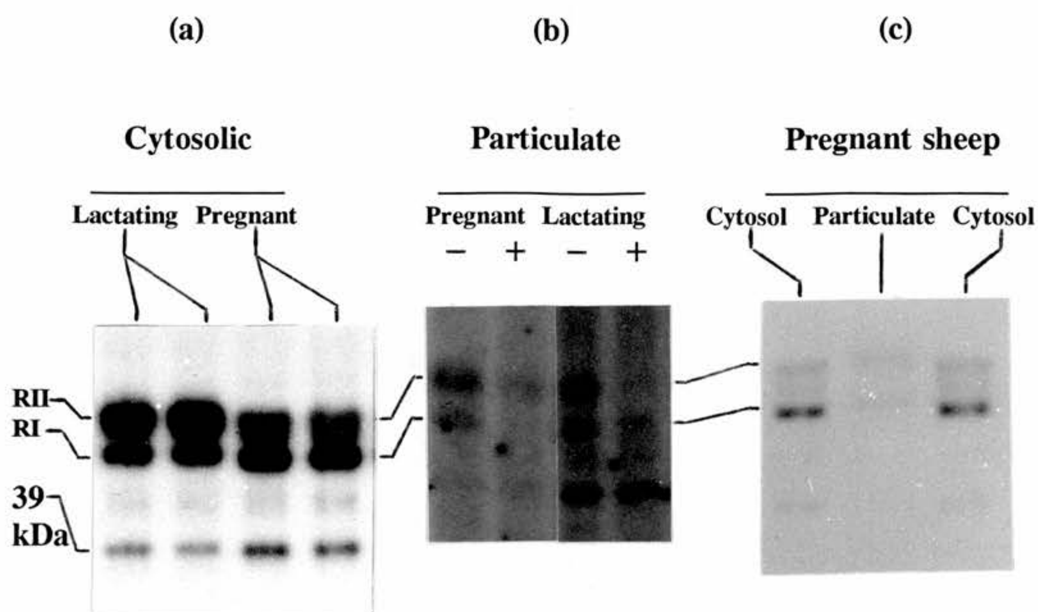


Figure 4.12 Comparison of sheep cAMP-binding proteins between pregnancy and lactation in both particulate and cytosolic compartments
Autoradiographs show: (a) Cyclic AMP-binding proteins labelled by 8-azido-[^{32}P]cAMP in cytosolic extracts of pregnant and lactating sheep mammary tissue. (b) Shows particulate cAMP-binding proteins labelled in extracts derived from the same pregnant and lactating sheep tissue ("+" and "−" refers to the presence and absence respectively, of radioinert cAMP during photoaffinity labelling, section 2.9.3). (c) Shows cAMP-binding proteins from pregnant sheep, cytosolic and particulate mammary tissue extracts which were equalised for protein, labelled and run side by side in the same gel.

apparent M_r of RI and RII remained constant between pregnancy and lactation (Figure 4.12). Binding of the ligand to both proteins was successfully competed out by excess cAMP (Figure 4.12(b)). The M_r 39000 protein observed in cytosolic mammary extracts was not present in particulate extracts examined here (Figure 4.12).

The relative amounts of RI and RII also varied between pregnancy and lactation. RI and RII were present in approximately equal quantities in samples derived from pregnant animals; levels of both isoforms decreased with lactation, falling below pregnant levels (results not shown). However R-subunit loss was greatest with respect to the RI isoform, thus an increase in the RII:RI ratio was observed in samples from lactating animals. As for the rat mammary gland, total levels of R-subunits were lower for the particulate fractions than in the corresponding cytosolic compartment (data not shown).

4.7 DEAE cellulose separation of type I and type II PK-A isoenzymes from rat mammary

PK-A is known to exist as two major isoenzymes, classically denoted type I and type II, which refers to the order in which they are eluted from a DEAE cellulose column in a salt gradient. These distinctive PK-A isoenzymes are further defined by their characteristic R-subunit isoforms, RI and RII (as previously described in section 4.6). It was therefore possible to assess the relative abundance of mammary tissue PK-A isoenzymes, following their resolution on DEAE cellulose, and subsequent assay of column fractions for total PK-A activity (i.e. that measured at a saturating concentration of cAMP, as described in section 2.6.2).

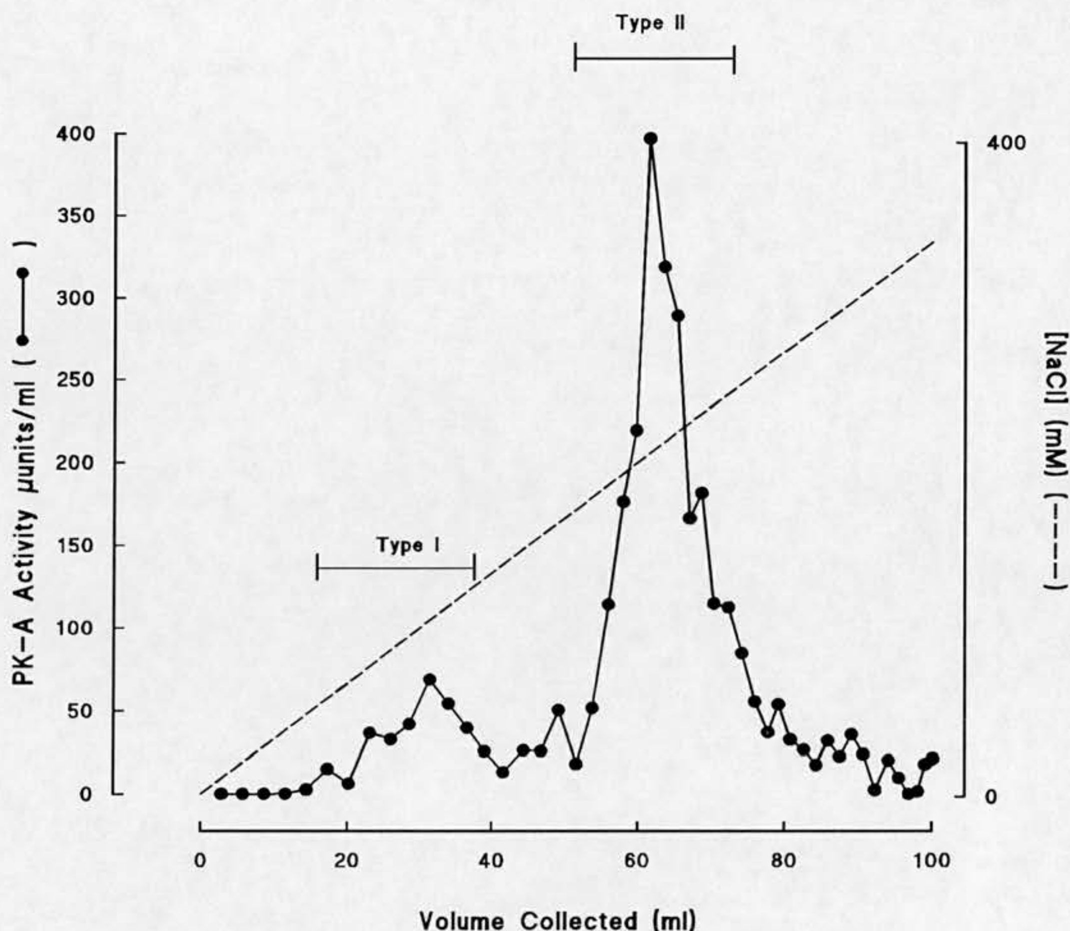


Figure 4.14 Resolution of PK-A isoenzymes from lactating rat mammary tissue by ion-exchange chromatography on DEAE cellulose
A 0.5ml portion of a 8,800g supernatant fraction (total PK-A activity 50μunits) prepared from lactating rat mammary tissue (as described in section 2.10.1) was loaded onto a column (15mm x 100mm, 22.5ml packed bed volume) of DEAE cellulose equilibrated with 10mM MES/NaOH, 0.2mM EDTA, 0.1mM DTT pH 6.8. After washing with this same buffer (100ml) until no further protein was eluted (monitored at 280nm) the column was developed with a linear gradient of NaCl (0-400mM) in the same buffer, at 3ml/min. Fraction volume was 3ml and these were assayed for total PK-A activity as described in section 2.6.2. Type-I and Type-II refer to the two major isoenzymes of PK-A.

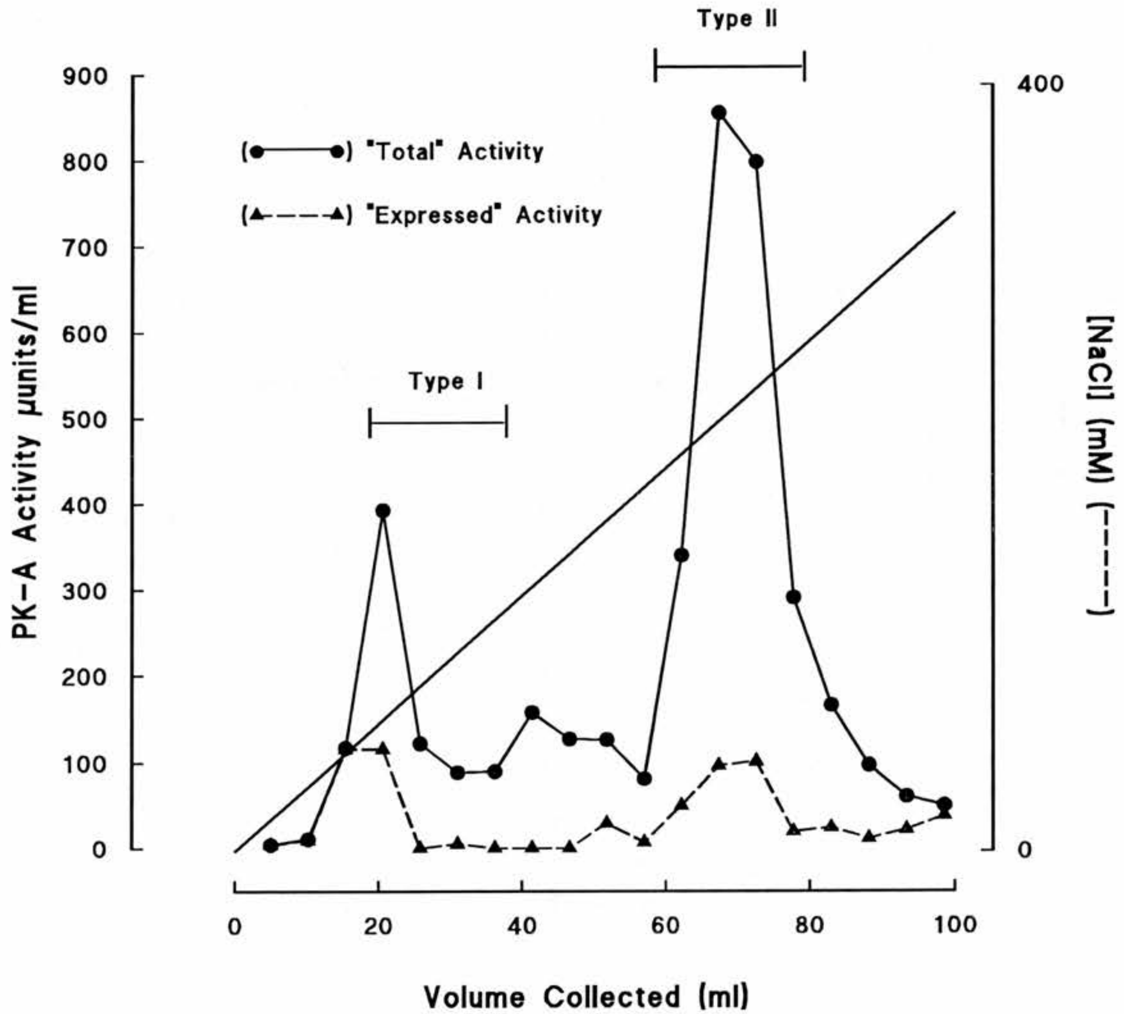


Figure 4.15 Separation of PK-A holoenzyme from lactating rat mammary tissue by ion-exchange chromatography on DEAE cellulose

The above Figure represents a single experiment in which both "total" and "expressed" PK-A activities were measured for isoenzyme fractions resolved from lactating rat mammary tissue extracts. Precedures were as described in the legend to Figure 4.14.

4.7.1 DEAE cellulose separation of lactating rat mammary PK-A isoenzymes

Experiments were performed on soluble mammary extracts, originating from between day 10 (L10) and L12 lactating rats, prepared as described in section 2.10.1. Isoenzymes were resolved by ion-exchange chromatography, in which samples were loaded onto a DEAE cellulose column and subsequently eluted by the application of a salt gradient, as described in section 2.10.3. Fractions were collected and assayed for total PK-A activity, in both the absence and presence of the PK-A inhibitor peptide, in order to distinguish PK-A activity from that of other mammary kemptide kinases which may also have been resolved by this technique. That activity not inhibited by the peptide, and therefore not associated with PK-A, was deducted before plotting the resolution profiles shown in Figures 4.14, 4.15 and 4.16. In a single experiment, expressed PK-A activity was also measured in order to validate the separation of holoenzyme from the free C-subunit (Figure 4.15). The recovery of activity from DEAE-cellulose columns was approximately 62% of that loaded. Such separations revealed that type II isoenzyme was the predominant form of PK-A in lactating rat mammary tissue; the type I isoenzyme accounted for only 30% of the total PK-A activity recovered from the column (Figure 4.14).

Peak positions and levels of both isoenzyme type I and type II agreed with the published findings of (Clegg & Ottey, 1990). However, results were of stark contrast to previous findings, generated through photoaffinity labelling of R-subunits (section 4.6.1), whereby RI was found to be the predominant R-subunit isoform.

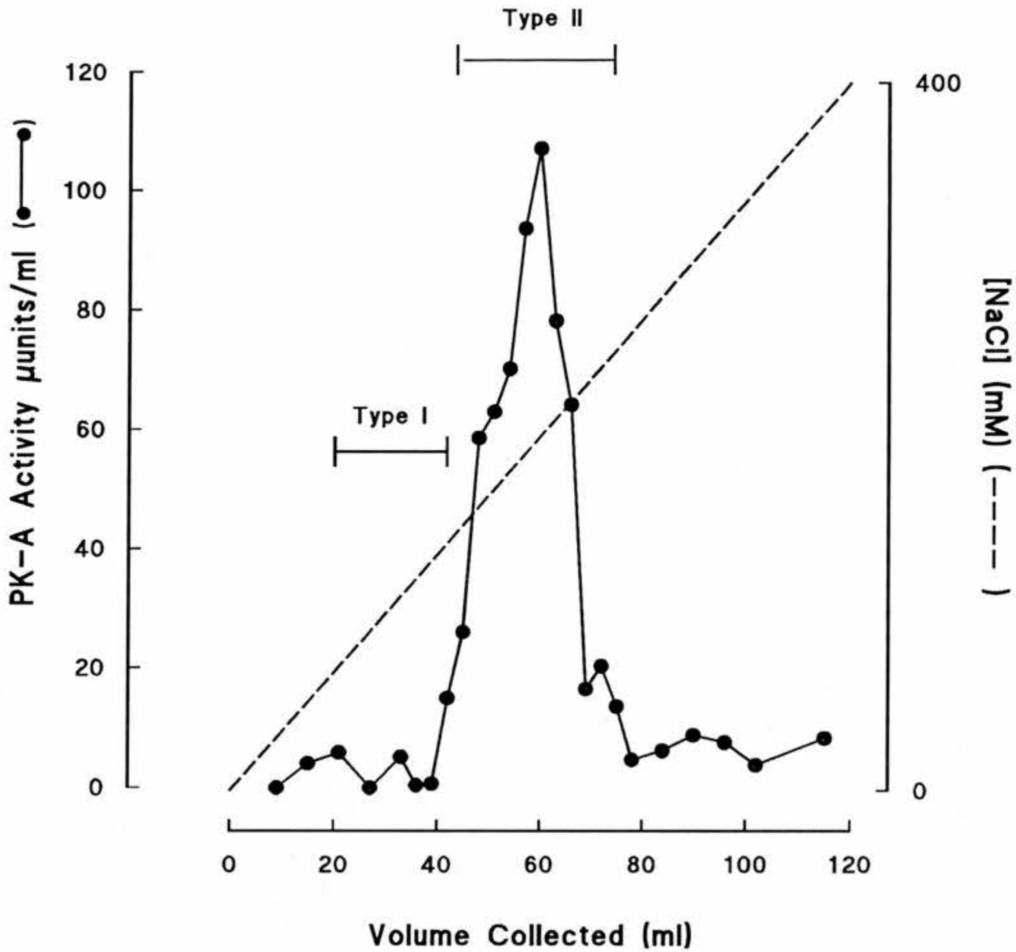


Figure 4.16 Separation of pregnant rat mammary PK-A isoenzymes by ion-exchange chromatography on DEAE cellulose

The above Figure represents a single experiment in which PK-A isoenzymes were resolved for a pregnant rat mammary tissue extract, prepared and subject to the same precedures as described in the legend to Figure 4.14.

4.7.2 DEAE separation of pregnant rat mammary PK-A isoenzymes

Experiments were performed on soluble mammary extracts, originating from between day 12 (P12) and P16 pregnant rats. Isoenzymes type I and II were resolved on a DEAE cellulose column (as described in section 2.10.3). As described for lactating rat mammary tissue, the type II enzyme was apparently the predominant isoenzyme (Figure 4.16), accounting for >90% of the total PK-A activity recovered. Once more, results were not in complete agreement with those findings made by photoaffinity labelling (section 4.6.1). The level of RII expression has previously been shown to be at its lowest during pregnancy, yet the results shown here clearly stated the opposite.

4.8 Discussion

With the onset of pregnancy there is a marked change in the cellular composition of mammary tissue (see Knight & Peaker, 1982b for review). As mammary development progresses adipocytes, which comprise a major proportion of the tissue weight before, and during early pregnancy, become progressively replaced by proliferating, secretory epithelial cells. The DNA content of rat mammary tissue (mg DNA/g tissue) changed markedly over pregnancy and lactation as a result of epithelial proliferation. The extent and pattern of changes reported here are largely in agreement with those changes previously reported by others (Tucker & Reece, 1963ab and Munford, 1963). Therefore, PK-A activity was described in terms of tissue DNA, as opposed to tissue wet weight; in this form the data was best able to describe that activity associated with proliferating (as during pregnancy) and differentiating (as during lactation) epithelial cells.

Total PK-A activity was shown to decline sharply following parturition, closely paralled by C-subunit protein mass allowing the conclusion that the overall specific activity of C-subunit was constant throughout rat mammary development. This conclusion was consistent with those findings discussed earlier (chapter 3, section 3.3.3) where the amount of an anti-C-subunit antiserum (As-365) required to maximally inhibit the PK-A activity in pregnant and lactating rat mammary extracts was found to be directly proportional to the total activity present. Immunotitration inhibition studies had also revealed no major differences in the titre of As-365 between mammary tissue C-subunit preparations purified from pregnant and lactating sheep (section 3.3.1).

The absolute level of mammary tissue C-subunit protein found in this study, approximately 2μg/g of tissue in lactating animals, does not differ significantly from that reported by others (Clegg & Connor, 1991). However when the specific activity of C-subunit in rat mammary tissue was determined (approximately 38 $\pm$2.4 units/mg C-subunit protein) and compared to measurements made for purified C-subunit preparations, including those from lactating and pregnant sheep mammary tissue, it was found that the C-subunit present in mammary extracts possessed a specific activity several-fold greater than the best values measured for the purified protein. Findings may be indicative of major losses of activity as a result of C-subunit purification or an enhancement of C-subunit activity within mammary tissue extracts. Enhancement of C-subunit activity has been described previously in lactating rat mammary tissue extracts (Clegg & Ottey, 1990).

Evidence for the deactivation of C-subunit in rat mammary tissue extracts has been put forward in the previous chapter (section 3.4). Deactivation of C-subunit in other tissues

has been previously described (Alhanaty & Shaltiel, 1979., Schmitz et al, 1973. and Hopfer et al, 1973) where losses were largely attributed to the activity of specific proteases. Certainly it is well established that C-subunit is extremely labile and susceptible to rapid deactivation in tissue extracts unless special care is taken, hence the rigorous conditions applied to the preparation of tissue extracts and measurement of PK-A activity (section 2.6).

Several independent lines of evidence have suggested that PK-A is atypical in its biochemical properties in mammary tissue. In particular there has been suggestion that the acute regulatory functions of PK-A are modified in mammary tissue from lactating animals (Bussmann et al, 1984., Clegg, 1988., Clegg & Ottey, 1990). Inhibitory effects on the activity of acetyl-CoA carboxylase, and the associated changes in the phosphorylation level of serine-79 have been shown to be absent. In other tissues inactivation of acetyl-CoA carboxylase was initiated by a cAMP-dependent process, although the actual phosphorylation event has been attributed to the AMP-activated kinase (Hardie, 1992). One explanation for these altered responses has been that PK-A itself has distinctive properties in mammary tissue. Some evidence for this has been reported by (Takhar & Munday,1992) who observed changes to C-subunit substrate specificity. Findings reported here indicate that these properties do not derive from changes to the intrinsic specific activity of C-subunit, with respect to the substrate, Kemptide, between pregnancy and lactation. However, it is possible that mammary tissue may contain a novel C-subunit, not recognised by Northern Cα and Cβ probes or by the C-subunit antibody (As-365/ab-365). In this respect it is of interest that 15% of C-subunit appears to be insensitive to immunotitration and immunoprecipitation by

As-365. Such C-subunit may exhibit abberrant sensitivity to inhibitors and deactivators, and/or intrinsic differences in C-subunit acylation or autophosphorylation. In relation to putative tonic influences of PK-A on mammary growth and development, it is likely that expressed activity is the most physiologically relevant of the catalytic parameters determined, being indicative of cAMP dependent protein phosphorylation events. It is therefore of interest that this remains relatively constant throughout mid-pregnancy but approximately doubles during late-pregnancy, immediately preceding the steep increase in the rate of DNA synthesis which persists throughout early/mid lactation. This enhancement in expressed PK-A activity does not persist correspondingly, but instead declines back to base line.

It was expected that the PK-A activity ratio should be a reflection of the cAMP concentration within the tissue. However, the present results, taken together with those published by others (Sapag-Hagar & Greenbaum, 1974), appear to show that this is not the case in developing rat mammary tissue. The apparent peak in the cAMP profile coincides with that in expressed PK-A activity and not that of activity ratio. The disparity between previously published data and that of the present study may be attributable to the gross technical problems inherent in deriving the intracellular cAMP content of mammary tissue; where high levels of the cyclic nucleotide in milk make accurate estimates of intracellular cAMP extremely difficult.

Alternatively intracellular sequestration of cAMP (other than by PK-A R-subunits) or changes in the specific activity of C-subunit might also have a role to play. The present study found no evidence of any intrinsic differences in the specific activity of C-subunit between pregnancy and lactation, in either rat mammary tissue extracts or purified

sheep preparations. However, there is some evidence in the current literature, for the existence of "mute" C-subunit molecules (Steinberg, 1991), or C-subunit with altered specific activities in small, distinct sub-cellular populations (Clegg & Connor, 1991). Further regulation of PK-A activity can be achieved by negative feedback; cAMP influences PK-A expression by initiating the down regulation of C-subunit (Hemmings, 1986 and Schwoch, 1987) presumably by a protease with a particular efficiency against free C-subunit (Alhanaty and Shaltiel, 1979). It can also be achieved by increasing the level of R-subunit relative to C-subunit; in the present study there is some evidence to suggest this occurs around parturition (see 4.6.1). The principle was recently confirmed by the identification of RIα (Boshart et al, 1991 and Jones et al, 1991) as the extinguisher of cAMP-induced gene responses, TSE (tissue specific extinguisher).

The expression of Cα- and Cβ-subunit mRNA, in the rat mammary gland, 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 the Cα subunit predominates over that of the Cβ subunit, is characteristic of the liver and kidney, but unlike that of the brain, where the two mRNAs are roughly equal (Showers & Maurer, 1986). Absolute levels of both Cα and Cβ are repressed during lactation. Furthermore, since Cα and Cβ mRNA and total C-subunit protein levels parallel each other, this suggests that transcriptional factors are the major determinants of Cα and Cβ activity. The mechanism for 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 the C-subunit expression is not yet known.

R-subunits were identified in rat mammary tissue extracts as those proteins which bound both 8-azido[^{32}P]cAMP and cAMP interchangeably. Related molecules such as AMP did not inhibit binding of 8-azido[^{32}P]cAMP to designated cAMP-binding proteins, however cGMP was shown to produce a 40-50% reduction in binding to the putative RI band, a similar effect of cGMP has been described previously for the purified protein (Handschin et al, 1983). These proteins ran at similar positions on SDS-PAGE as tissue standards known to over express either RI or RII respectively. The apparent molecular weights of RI and RII were calculated as 45 and 51kDa respectively. These values were slightly lower than the published molecular weights for RI and RII in other tissues and species; RI was determined by SDS-PAGE and given as 49kDa from porcine, bovine and rabbit skeletal muscle, and RII as 56-58kDa from bovine heart (Nimmo et al, 1977 and Hofmann et al, 1975). It has been shown that the apparent molecular weight of RII determined by SDS-PAGE can differ significantly between species and tissues (Robinson-Steiner et al, 1984). In addition, the extent of migration can differ for some forms of RII, depending on the presence of phosphate in the autophosphorylation site (Rubin & Rosen, 1975., Rosen et al, 1975 and Robinson-Steiner et al, 1984). RI does not undergo autophosphorylation and shows similar but slightly less pronounced discrepancies in molecular weight when determined by different methods (Nimmo et al, 1977 and Døskeland, 1978).

In addition to the two R-subunits a minor cAMP-binding protein of approximately 37-

38kDa was also detected in cytosolic mammary extracts. This may have been one of a family of protein fragments derived from the R-subunits by proteolysis at the susceptible hinge region as previously described by others (Weldon & Taylor, 1985). Total and relative levels of RI and RII isoforms did not remain constant throughout rat mammary development. Furthermore, both cytosolic and particulate forms of these proteins were apparently subject to developmental regulation. When measured in cytosolic mammary extracts, RI was found to be the most abundant R-subunit isoform throughout rat mammary development; as a fraction of total R-subunit RI levels peaked at day 12 of pregnancy, coinciding with the peak in cellular proliferation, declining throughout lactation where cell numbers remain approximately constant. Thus the present study showed a level of RI expression, throughout normal rat mammary development, which reflected the changes in the rate of cellular proliferation in the tissue. Such findings were consistent with the hypothesis that type-I is a general growth promoter. Type I and type II PK-A isoenzymes have been shown to be under separate hormonal control, at least in sex steroid-dependent tissues where increased expression of RI has been correlated positively with hormone induced growth (Døskeland et al, 1975, and Russel, 1978). Cho-Chung and co-workers have repeatedly demonstrated a predominance of the RI isoform in tumour cells derived from mammary tissue and have shown strong correlation between RI and cellular proliferation (Cho-Chung, 1992). Selective inhibition of the RI isoform using cAMP analogues or antisense oligonucleotides leads to cell growth inhibition in a wide variety of cell lines and tumours including those derived from mammary tissues (Yokozaki et al, 1993. and Ciardiello et al, 1993).

An alternative explanation for the early predominance of the RI isoform in rat mammary development might be that the RI gene generally responds more rapidly than that of RII in response to demand for increased R-subunit synthesis; as shown when tissues expand in response to anabolic hormones (Russel, 1978, and Døskeland et al, 1975) re-feeding (Ekanger et al, 1988) and regeneration (Ekanger et al, 1989).

In contrast levels of the RII isoform were seen to steadily increase throughout both pregnancy and lactation, thus the RII:RI ratio also increased over this same period to peak in late lactation. Preferential expression of type II PK-A has been shown to be induced by treatment of tumour cells with cAMP analogues or differentiating agents and is typical of terminally differentiated tissues (Cho-Chung et al, 1991).

Unlike the R-subunits, the 37-38kDa protein showed no discernible developmental pattern of expression; its abundance remained proportional to total R-subunit concentration throughout both pregnancy and lactation. Neither was there any evidence to suggest that the fragment was derived specifically from either R-subunit isoform (RI or RII).

The levels of R-subunits reported in the present results are also in agreement, at least for lactating tissue extracts, with those observations made by others (Clegg & Connor, 1991, using an antibody based technique previously described by Ekanger & Døskeland, 1988). Estimation of RI and RII levels were given as 97 ± 11 and 54 ± 5 pmol protein/g wet weight tissue respectively. However, data from an earlier report in which relative levels of PK-A isoenzymes, type I and type II were estimated using DEAE cellulose ion exchange chromatography, showed the type II isoenzyme to be the predominant form in cytosolic mammary tissue extracts derived from lactating rats

(Clegg & Ottey, 1990, using methods previously described by Livsey & Martin, 1988). This observation was duplicated in the present study, using a similar methodology which revealed that type II was the predominant isoenzyme in extracts derived from both pregnant and lactating rat mammary tissue. Photoaffinity labelling had consistently shown a predominance of the RI isoform throughout both pregnancy and lactation. Despite increasing levels of the RII isoform into lactation figures did not transcend those of RI for cytosolic rat mammary tissue extracts i.e. the RII:RI ratio did not exceed 1.0. Since PK-A isoenzymes are defined by their characteristic R-subunits, such high levels of RI would normally be expected to confer a predominance of the type I holoenzyme upon mammary tissue. There was therefore, a clear disparity between those findings made by ion-exchange resolution of isoenzymes and those made by photoaffinity labelling of R-subunits. One explanation may be that a reservoir of free RI existed within the tissue, such a reservoir had been described by others (Øgreid et al, 1987) in carcinomas derived from rat mammary tissue; while in the regenerating rat liver, expression of total R-subunit has been shown to transiently exceed that of the C-subunit before a period of most intense cellular proliferation (Ekanger et al, 1989). In the present study the ratio of R- to C-subunit remained relatively constant throughout much of pregnancy and lactation; although a transient over-expression of mammary R-subunit between late pregnancy and early lactation was suggested by the data presented. The exact molecular ratio of the two PK-A subunits (normally 1.0) could not be evaluated, since absolute levels R-subunit protein mass were not measured.

The disparity might also be explained by differences in the two technologies. DEAE cellulose ion-exchange can only be used to detect R-subunits associated with the

holoenzyme complex, since quantification of isoenzymes is achieved by measuring the activity of the associated C-subunit. Therefore, there is some scope for underestimation of R-subunits by this method. Any activity loss or aberration of the specific activity of associated C-subunit, for example by co-purification with inhibitory factors, might lead to underestimation of isoform abundance. Likewise, preferential activation of the type I isoenzyme within tissue extracts would reduce that RI detected. Differential activation of PK-A isoenzymes has been described in human breast cancer cells (Livsey et al, 1984). Several reports have also been published warning against the possible incorrect identification of PK-A isoenzymes when using DEAE-cellulose analysis alone. For example the elution behaviour of several type-II isoenzymes has been found to differ (Corbin et al, 1977 and Beebe et al, 1984), while murine adipose tissue type-I has been shown to elute at a higher salt concentration thus contaminating type-II isoenzyme (Malkinson et al, 1983). Similar reports have also been made for rat thyroid (Toru-Delbaufte et al, 1982). Other workers have found that a second fractionation on DEAE-cellulose is sometimes required to completely resolve the two isoenzymes, especially when the type-I to type-II ratio was high (Robinson-Steiner et al, 1984).

R-subunit levels were also measured for the particulate fraction of rat mammary tissue where they were found to be in lower abundance with respect to that of the cytosolic compartment. However the relative levels of RII (as a fraction of total R-subunit) were greater than those seen in cytosolic tissue extracts throughout both pregnancy and lactation. Levels of this isoform increased throughout rat mammary development, to become the most abundant R-subunit isoform by day 16 of pregnancy. Therefore, preferential localisation of the RII isoform to the particulate compartment of mammary

tissue seemed to be implicated by the data. The mechanism of type II PK-A localisation probably involves protein-protein interaction between RII and specific RII-anchoring proteins. The majority of A-kinase anchoring proteins or AKAPs have been detected in the insoluble, particulate fractions, implying attachment to membranes and the cytoskeleton (see Scott & Carr, 1992, for review). Biochemical and immunocytochemical studies have shown that PK-A localisation is directed through the R-subunit (Scott, 1991) and may be a key regulatory event in determining the intracellular sites of hormone action. Certain tissues including brain contain up to 75% of either RII isoform (RIIα and RIIβ) in particulate form, associated with plasma membrane, cytoskeletal component, secretory granules, or the nuclear membrane via AKAPs.

In most cells RI and type I PK-A appear to be unanchored, cytoplasmic proteins although some exceptions to this general rule have been reported. For example the bulk of the RI in human erythrocytes is tightly bound to the plasma membrane (Rubin et al, 1972) and in c.elegans the RI-like subunit is associated with the detergent-insoluble fraction of homogenates (Lu et al, 1990). A recent study carried out with normal human T-lymphocytes, demonstrated reversible targeting of PK-A type I to the cytoplasmic surface of the plasma membrane (Skålhegg et al, 1994) however, it is not yet known whether targeting was via R- or C-subunits.

Analysis of ruminant mammary tissue provided a contrast to those, more detailed studies discussed above and assisted in the identification of important, non-species specific developmental trends in PK-A expression. The increase in tissue DNA (mg/g tissue) which accompanied cellular proliferation in the rat mammary gland was not

apparent in that of the sheep, where values were not significantly different between pregnant and lactating animals. Neither was there a sudden fall in total PK-A activity (per mg tissue DNA) with the onset of lactation, as previously described for rat mammary tissue. Instead a small but significant increase in total PK-A activity was observed between pregnancy and lactation. Expressed PK-A activity showed a similar pattern of expression to total activity between pregnancy and lactation.

PK-A activity ratio was in closer agreement to rat mammary tissue data; values were greater in tissue extracts derived from lactating with respect to pregnant animals. Such an outcome may be indicative of the greater significance of this parameter in sheep mammary development and function, with respect to total PK-A activity. No data is as yet available on C-subunit protein concentrations between pregnancy and lactation; however there was no indication of developmental changes to the specific catalytic activity of C-subunit, from determinations made upon C-subunit protein purified from pregnant and lactating sheep mammary tissue. At present no data is available to determine whether sheep mammary C-subunit is under transcriptional regulation.

Analysis of R-subunit isoforms within the cytosolic fraction of sheep mammary tissue revealed a pattern of expression not dissimilar to that already described for the rat mammary gland. However the relative abundance of the RII isoform was much greater than that exhibited in the rat. The RI isoform which showed a predominance in samples derived from pregnant sheep, was replaced by RII as the most abundant R-subunit isoform, in samples derived from lactating animals. In the mid-pregnant rat the RII:RI ratio was approximately 0.3 and this figure rose to 0.7 by mid-lactation; in contrast this ratio rose from 0.8 to >2.0 between pregnancy and lactation in sheep mammary tissue

measured. Therefore the doubling in the level of RII with respect to RI appeared to be a common developmental signal in both the rat and sheep mammary gland.

Also peculiar to sheep mammary extracts was a mobility shift of the RI and RII bands between pregnancy and lactation. The putative RI and RII protein bands, derived from lactating animals, exhibited apparent molecular weights 2-3kDa greater than those derived from lactating animals. Such mobility shifts may have arisen as a result of phosphorylation events. It is generally acknowledged that the RII isoform contains a PK-A recognition sequence which becomes readily autophosphorylated (Durgerian and Taylor, 1989); this site is replaced by a pseudophosphorylation site binding MgATP in the RI isoform. However, RI may be subject to phosphorylation by other kinases; the cGMP-dependent protein kinase has been shown to phosphorylate RI, with slow kinetics (Geahlen and Krebs, 1980).

The expression of the R-subunit isoforms in the particulate fractions of sheep mammary tissue showed a similar trend to those already discussed for the cytosolic fractions. However there was no shift in the apparent molecular weight of the R-subunit isoforms between pregnancy and lactation. R-subunits derived from pregnant and lactating sheep mammary tissue exhibited apparent molecular weights (as deduced from SDS-PAGE) previously shown to be characteristic of Lactating sheep mammary, cytosolic R-subunits. Total R-subunit levels were lower (on a protein basis) in these extracts than in those of the cytosolic fraction. The relative level of the RII isoform was also in much greater abundance throughout both pregnancy and lactation, probably as a consequence of the presence of AKAPs and their association with type II isoenzyme (Scott, 1991).

Chapter 5

Expression of PK-A in the rat mammary gland following litter removal

Introduction

After weaning the lactating mammary gland undergoes a reductive remodelling process termed involution, during which the tissue gradually returns to a less differentiated state, to resemble that observed prior to pregnancy. The biological changes which occur during mammary involution are discussed more fully in the Introductory chapter (section 1.6). However to summarise here: involution is accompanied by the apoptopic death of the majority of secretory epithelial, and myoepithelial cells, and by extensive fat infiltration.

Persistent expression of the differentiated function by mammary epithelial cells during lactation requires the continued stimulation of sensory nerve endings, by the suckling young. This ultimately stimulates prolactin production by neuronal feedback to the hypothalamus (Grosvenor & Mena, 1974). Interruption of this feedback mechanism leads to the abrupt cessation of milk synthesis and initiates involution of the mammary gland (Lascelles & Lee, 1987). Thus involution may be triggered and studied experimentally by removal of the litter from the nursing rat. Incidentally, this also leads to milk stasis, the accumulation of milk within the mammary tissue, which itself triggers involutional progression of the mammary epithelium (Flint et al, 1981).

The process of de-differentiation and involution entails the concerted modification of

the expression of large numbers of genes, such pleiotropic transcriptional regulation is a well documented function of PK-A (Montminy et al, 1990). Furthermore cAMP and PK-A have both been implicated in the initiation of involution and apoptopic cell death, in a number of cell and tissue models including mammary gland involution (Dowd & Miesfeld, 1992 and Marti et al, 1994). The present study therefore undertook to characterise the behaviour of PK-A, both in terms of its activity and generic C- and R-subunits (both RI and RII), in the mammary gland following litter removal.

5.1 Litter removal

Mammary tissue was sampled from lactating rats (LR24 and LR48), whose litters had been removed for 24 and 48h respectively, as described in section 2.1.1. Tissue was also harvested from day 12 lactating rats (L12), which were used as controls. In total, three sets of tissue samples were collected, 5 x LR24, 5 x LR48 and 5 x L12. These were then used to study the effects of litter removal upon the expression of PK-A activity, C-subunit protein mass, Cα mRNA abundance and the relative abundance of R-subunits (both RI and RII).

5.2 PK-A activity in the rat mammary gland following litter removal

Soluble extracts were prepared, as described in section 2.6.1, from the sets of rat mammary tissue described above (section 5.1). Samples were then assayed for both total PK-A activity i.e. that measured in the presence of excess cAMP, and expressed activity; that measured in the absence of added cAMP, as described in section 2.6.2. By measuring total PK-A activity, an index of the total amount of C-subunit protein

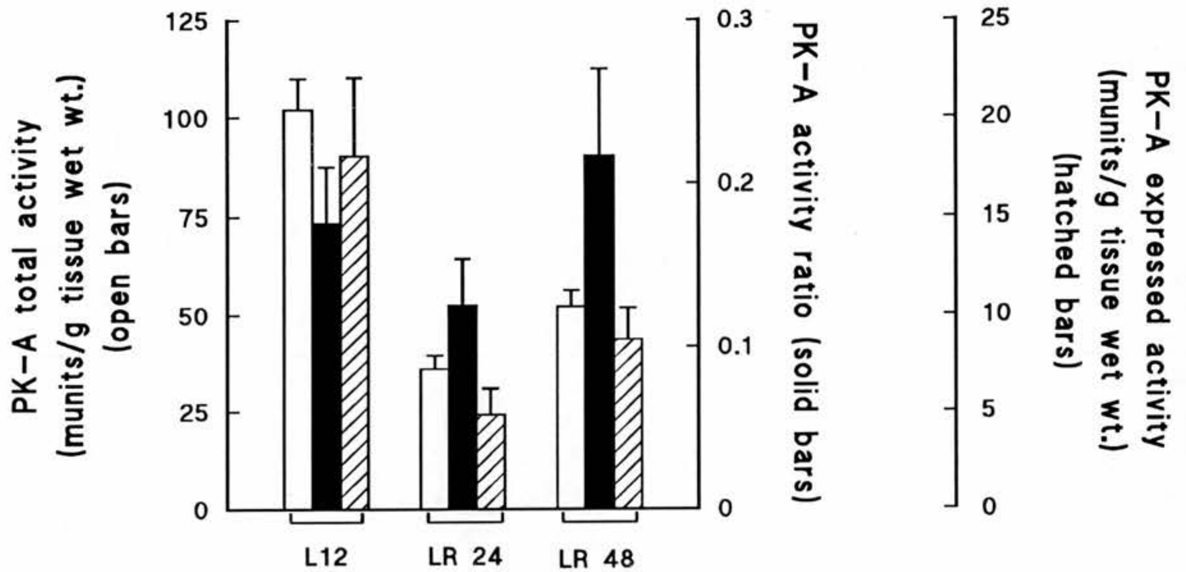


Figure 5.1 PK-A activity (total, expressed and activity ratio) in rat mammary tissue following litter removal

Mammary tissue was sampled from rats as follows: L12, at day 12 of lactation; LR24, 24h following removal of litter at day 10 of lactation; LR48, 48h following litter removal. Extracts were prepared and assayed for PK-A activity (as described in section 2.6). Total activity was that measured in the presence of $0.67\mu M$ cAMP, while expressed activity was that measured in the same extracts without added cAMP. Results are means ($\pm$ S.E.M) of determinations on not less than 5 animals.

present in the tissue, and therefore the cell's capacity for cAMP-dependent protein phosphorylation, could be determined. Based on wet tissue weight, total PK-A activity declined sharply following the first 24h of litter removal; activity declined from 102.0 $\pm$ 7.4 (for L12 control rats) to 36.0 $\pm$ 3.2 munits/g tissue (for LR24 rats). However total activity recovered somewhat following 48h litter removal (LR48) to 52.1 $\pm$ 4.3 munits/g tissue, (Figure 5.1). This recovery was analysed using the Student's *t* test and found to be statistically significant for $P < 0.005$.

The DNA and protein content of the tissue declined only slightly following litter removal (Table 5.1), therefore total PK-A activity also fell when calculated on the basis of tissue DNA. Total activity more than halved, falling from 35.6 $\pm$ 1.9 (L12 control) to 15.7 $\pm$ 1.1 munits/mg DNA (LR24). As for values calculated on the basis of wet tissue weight, total PK-A activity recovered slightly following 48h litter removal, reaching 17.0 $\pm$ 1.4 munits/ mg DNA.

By measuring expressed PK-A activity a measure of the free C-subunit, present within the tissue at the time of sampling was obtained. Procedures were adopted to prevent the association/dissociation of the C- and R-subunits (as described in section 2.6.1). Following 24h litter removal expressed activity underwent a similar, but somewhat exaggerated response to that of total PK-A activity. Based on wet tissue weight, figures fell from 14.5 $\pm$ 2.4 (for L12 controls) to 5.7 $\pm$ 1.2 munits/g tissue (LR24), giving rise to a transient decrease in activity ratio, although this was outside the error limits of the experiment (Figure 5.1). However, expressed activity recovered slightly over the next 24h of non-suckling to 7.3 $\pm$ 1.3 munits/g tissue (LR48), to give rise to an increase in the activity ratio (Figure 5.1). Expressed activity showed a comparable

Tissue Status	Protein (mg/g wet wt.)	DNA (mg/g wet wt.)
LR24	73.0 ± 6.6	2.81 ± 0.27
LR48	79.3 ± 4.2	3.06 ± 0.15
L12	80.7 ± 4.5	3.82 ± 0.03

Table 5.1 Composition of rat mammary tissue following litter removal
DNA and protein were determined for those mammary tissue samples described in section 5.1 (L12, LR24 and LR48). Extracts were prepared and assayed for protein and DNA content (as described in section 2.5.1 and 2.5.2 respectively). Values are (means ± S.E.M.) for groups of not less than 5 animals.

trend when data was expressed on a per mg DNA basis (results not shown).

5.3 Effects of litter removal upon C-subunit protein mass

Litter removal has been shown to expedite the down regulation of PK-A activity measured in rat mammary tissue extracts. Adopting the immunoquantification procedure described in section 2.7, tissue derived from LR24 and LR48 rats (for which PK-A activity data already existed) was analysed for C-subunit protein mass. Thus, allowing the effect of litter removal (if any), upon the specific catalytic activity of the C-subunit protein to be investigated. Soluble tissue extracts were made (as described in section 2.7.2) and C-subunit protein mass quantified (as described in section 2.7.3). Mammary extracts derived from nursing rats in their twelfth day of lactation (L12) were used as controls.

Following 24h litter removal, C-subunit protein mass declined from 2.51 ± 0.20 (for L12 controls) to 1.5 ± 0.27 μg/g tissue (LR24), as shown in Figure 5.2. Levels recovered slightly to 1.68 ± 0.18 μg/g tissue following a further 24h of non-suckling (LR48); however this failed to reach significance when tested using the Students t-test. Although C-subunit protein mass appeared to follow the general pattern of decline and recovery observed for total PK-A activity (section 5.2), the decline in C-subunit protein mass was less exaggerated, with the result that total PK-A activity fell more quickly than could be explained by loss of C-subunit mass alone. Thus litter removal appeared to be accompanied by a decline in the specific activity of C-subunit (Figure 5.2).

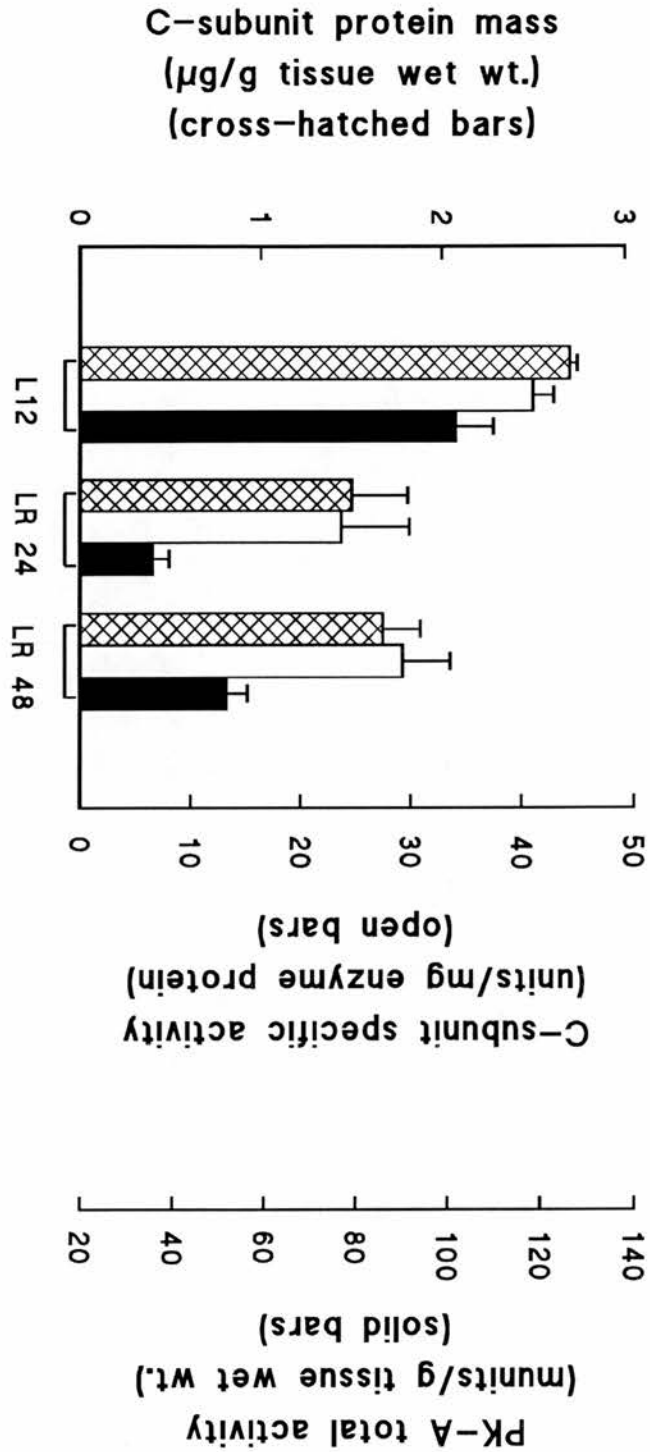


Figure 5.2 C-subunit protein mass, specific catalytic activity and PK-A total activity: effect of litter removal

C-subunit protein mass was quantified in rat mammary tissue samples for which PK-A activity data already existed (L12, LR24 and LR48 are as described in section 5.1). The quantification procedure was that described in section 2.7. PK-A activity data is that described in Figure 5.1. Results are (± S.E.M) for not less than 4 animals.

5.4 Cα mRNA levels in the rat mammary gland following litter removal

Initiation of involution by litter removal was found to be accompanied by a rapid decline in total PK-A activity, as described in section 5.2. Furthermore corresponding levels of C-subunit protein were also seen to fall, but not in fixed proportion to total PK-A activity. Thus the fall in total PK-A activity could not be wholly explained in terms of declining C-subunit protein mass. In order to determine the degree of transcriptional regulation of C-subunit protein mass, levels of Cα mRNA were measured by Northern blotting techniques, as described in section 2.8. Studies were performed on groups of 5 animals for which PK-A activity data already existed, as described in section 5.1.

The abundance of Cα mRNA (expressed mRNA/g of tissue wet weight) did not reflect PK-A protein mass, measured in both LR24 and LR48 mammary samples (Figure 5.3). In contrast to total PK-A activity and C-subunit protein mass, which were seen to fall following the first 24h of litter removal, levels of Cα mRNA showed a small increase, although this fell outside the error limits of the experiment (Figure 5.3). Forty eight hours after litter removal, levels of Cα mRNA decreased to those levels observed for control mid-lactating rats (L12); once again this was in contrast to total PK-A activity which recovered slightly between 24 and 48h litter removal, but did not attain those levels observed in L12 controls. Thus C-subunit protein mass and total PK-A activity did not appear to be transcriptionally regulated in the rat mammary gland following litter removal.

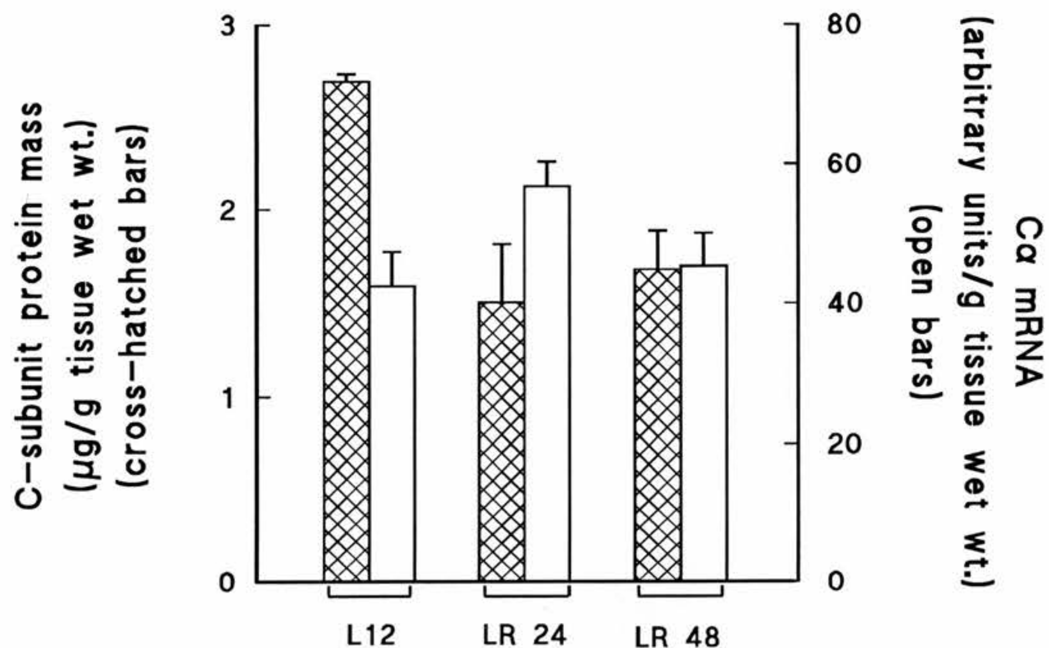


Figure 5.3 Abundance of Cα mRNA following litter removal: Comparison with C-subunit protein levels

Quantification of C-subunit protein mass was as described in section 2.7. Quantification of Cα mRNA was as described in section 2.8. Results are means (± S.E.M) for 5 animals. C-subunit protein mass is that data previously shown in Figure 5.2.

5.5 Cyclic AMP-binding proteins

The initiation of involution by litter removal was accompanied by a decline in both total and expressed PK-A activities. C-subunit protein levels also fell but not in proportion to total PK-A activity; neither did C-subunit protein levels appear to be transcriptionally regulated. It therefore followed, that unless R-subunit levels were similarly down-regulated following litter removal, the C-: R-subunit ratio, classically regarded as equalling 1.0 in terminally differentiated tissues (see Beebe & Corbin, 1986 for review), would not be maintained.

The relative abundance of cAMP-binding proteins in tissue extracts derived from LR24, LR48 and L12 rats were examined using the cAMP analogue, 8-azido [^{32}P]cAMP. Photoaffinity labelling also allowed the distinction to be made between the levels of the two major isoforms of PK-A, type I and type II, which are defined by their characteristic R-subunits. In order that a more dynamic picture of the subcellular localisation of PK-A isoenzymes could be obtained, both cytosolic and particulate mammary extracts were examined in this manner.

5.5.1 Cytosolic cAMP-binding proteins

Soluble extracts were prepared (as described in section 2.9.1) from tissue derived from LR24, LR48 and L12 rats, for which PK-A activity, C-subunit quantification and Cα mRNA data already existed. Tissue extracts derived from nursing rats (L12) were used as controls. Both control and litter removed tissue extracts were run together in grouped experiments, in which samples were equalised with respect to protein concentration prior to photoaffinity labelling. Labelling was carried out as described

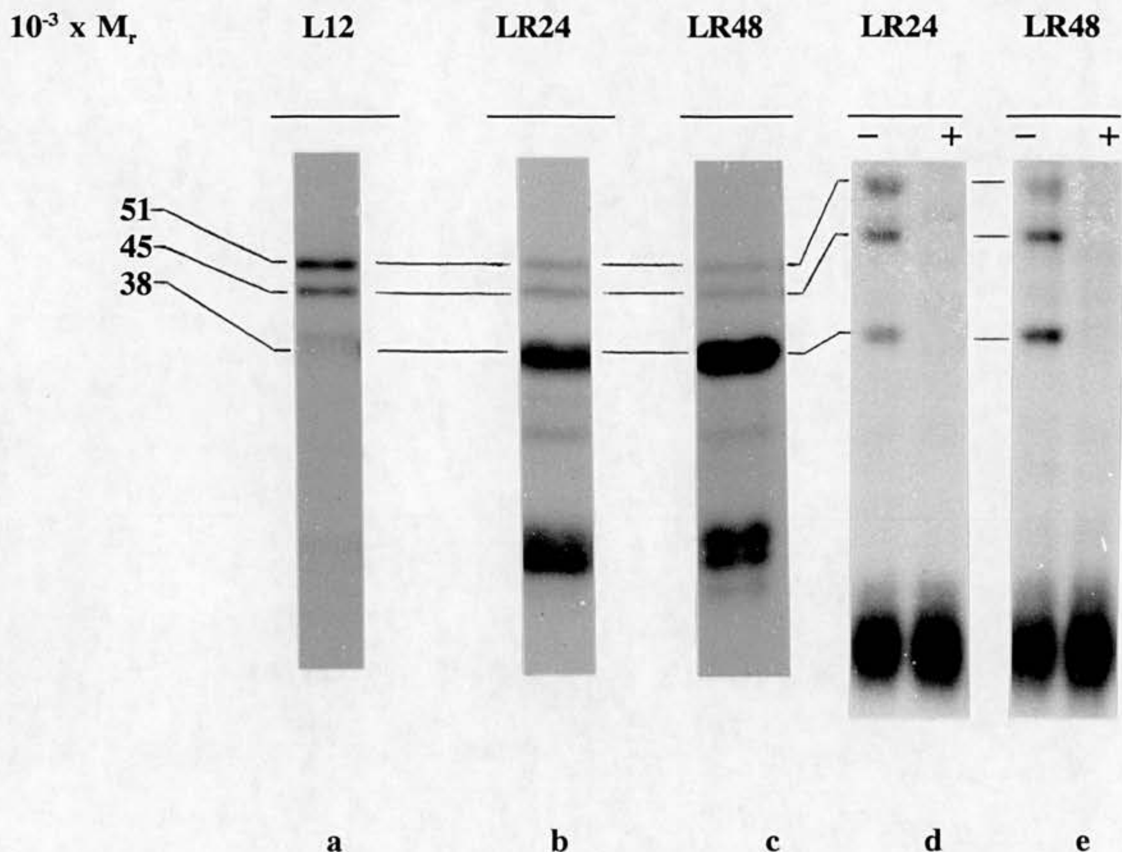


Figure 5.4 Cytosolic cAMP-binding proteins in mammary tissue following litter removal

Mammary tissue was sampled from rats as follows: L12, at day 12 of lactation; LR24, 24h following removal of litter at day 10 of lactation; LR48, 48h following litter removal. Extracts were prepared and labelled with the photoaffinity ligand 8-azido-[^{32}P]cAMP, as described in the section 2.9. Lanes a-c represent control and litter removed samples run in a single experiment. Lanes d and e represent a separate cAMP competition experiment in which samples were labelled both in the presence and absence of radio-inert cAMP. Results are representative of data for not less than 4 animals.

in section 2.9.2.

Three major proteins were labelled in the cytosolic fractions of mammary tissue sampled from LR24 and LR48 rats (Figure 5.4). These migrated on SDS-PAGE at the same positions as cAMP-binding proteins identified in the L12 control rats i.e. RI, RII and a 37-38kDa R-subunit degradation product (also described in section 4.6.1). Labelling of all three bands was successfully competed out by radio-inert cAMP as described in section 2.9.3 (Figure 5.4), and was absent when no u.v. irradiation was applied to the samples (results not shown). In addition to these major cAMP-binding proteins, numerous lower molecular weight bands were also labelled. However, labelling of these was not competed out by radio-inert cAMP, allowing the conclusion that they were not specific cAMP-binding proteins/peptides (Figure 5.4).

Litter removal appeared to have a marked effect upon the level of R-subunits in the cytosolic compartment of rat mammary tissue. Levels of both RI and RII were shown to be substantially diminished following 24h litter removal; levels were depleted by approximately 50-60% with respect to those levels measured in L12 controls (Figure 5.5). The depression in total R-subunit (RI + RII) persisted until 48h litter removal, although there was no further reduction in R-subunit levels (Figures 5.5 and 5.6). The RII:RI isoform ratio was only marginally depressed by litter removal (Figures 5.9), with the decline in total R-subunit apparently brought about by decreasing levels of both RI and RII, and there did not appear to be preferential down regulation of either isoform.

The apparent loss of R-subunit following litter removal was accompanied by a dramatic increase in the level of the 37-38kDa cAMP-binding protein, believed to be a product

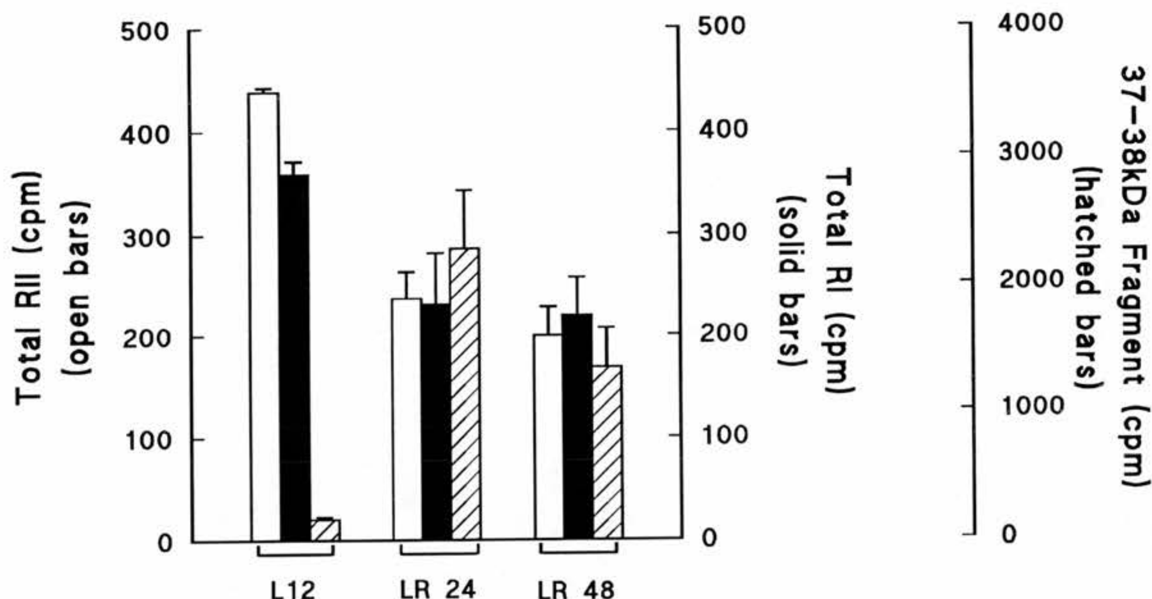


Figure 5.5. Cytosolic R-subunit composition of mammary tissue following litter removal

Mammary tissue samples were those described in section 5.1. Extracts were prepared and labelled with the photoaffinity ligand 8-azido-[^{32}P]cAMP, as described in the section 2.9. The above histograph shows data from a single experiment in which sets of five L12, LR24 and LR48 animals, were equalised with respect to protein concentration, photoaffinity labelled and run on the same SDS-PAGE gel. The relative levels of cytosolic cAMP-binding proteins in control and litter removed samples were determined after excision of radio-labelled bands and liquid scintillation counting to obtain a cpm value. The results are means (±S.E.M.) for five animals.

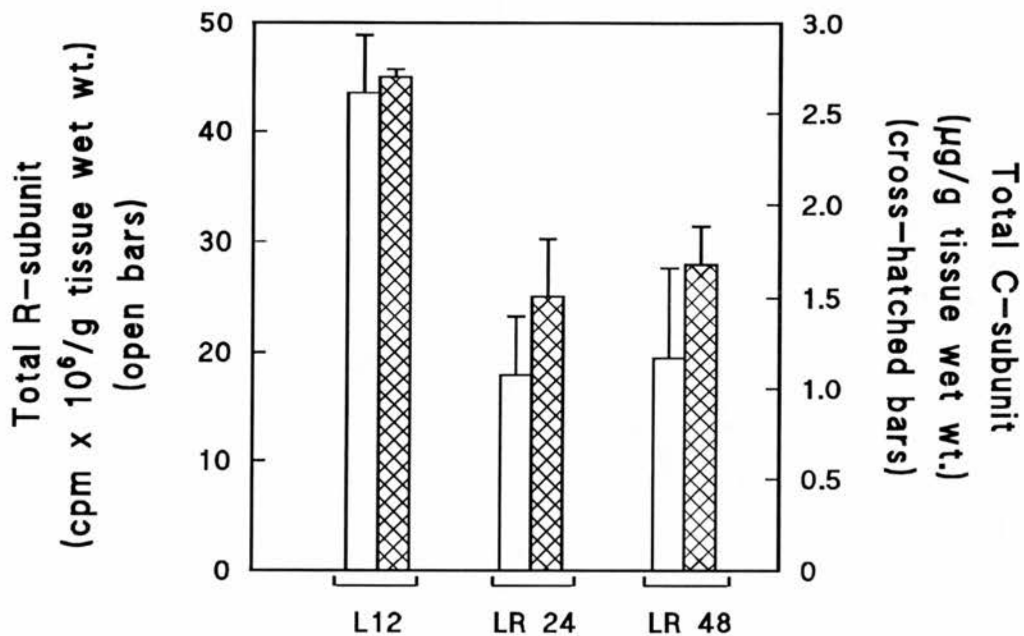


Figure 5.6 Cytosolic R- and C-subunit levels in rat mammary tissue following litter removal

The above Figure represents combined data from two separate experiments; immunoquantification of PK-A C-subunit, as described in section 5.3 and arbitrary levels of R-subunits (RI + RII) as described in section 5.5. Results are means (± S.E.M.) for not less than 4 animals.

of R-subunit degradation (Weldon & Taylor, 1985). The level of 8-azido-[^{32}P]cAMP binding associated with this protein exceeded that of the L12 control samples, run in the same experiment, by excess of ten fold, and levels of total R-subunit in the same samples, by more than six-fold (Figure 5.5). Similar levels of this protein were also apparent in samples from LR48 rats (Figure 5.5).

Therefore, as well as triggering C-subunit down-regulation (section 5.3), litter removal was also shown to stimulate the rapid degradation of R-subunits (both RI and RII isoforms); although there is some suggestion, comparing the two sets of data, that loss of C-subunit protein mass was less dramatic than R-subunit degradation (Figure 5.6).

5.5.2 Particulate cAMP-binding proteins

Particulate samples were prepared by the re-suspension of pellet fractions following the preparation of soluble, cytosolic extracts from mammary tissue samples, as described in section 2.9.1. Accordingly particulate tissue extracts were derived from LR24, LR48 and L12 rats, for which PK-A activity, C-subunit quantification, Cα mRNA and cytosolic R-subunit data already existed. Tissue extracts derived from nursing rats (L12) were used as controls. Both control and litter removed (LR24 and LR48) tissue extracts were run together in grouped experiments following equalisation for protein, as described in section 2.9.2. Photoaffinity labelling revealed the presence of three cAMP-binding proteins in the particulate compartment of LR24 and LR48 rat mammary tissue. These migrated on SDS-PAGE gels at identical positions to cAMP-binding proteins associated with control, cytosolic extracts run in the same experiment (Figure 5.7) and were identified as being RI, RII and the 37-38kDa degradation product

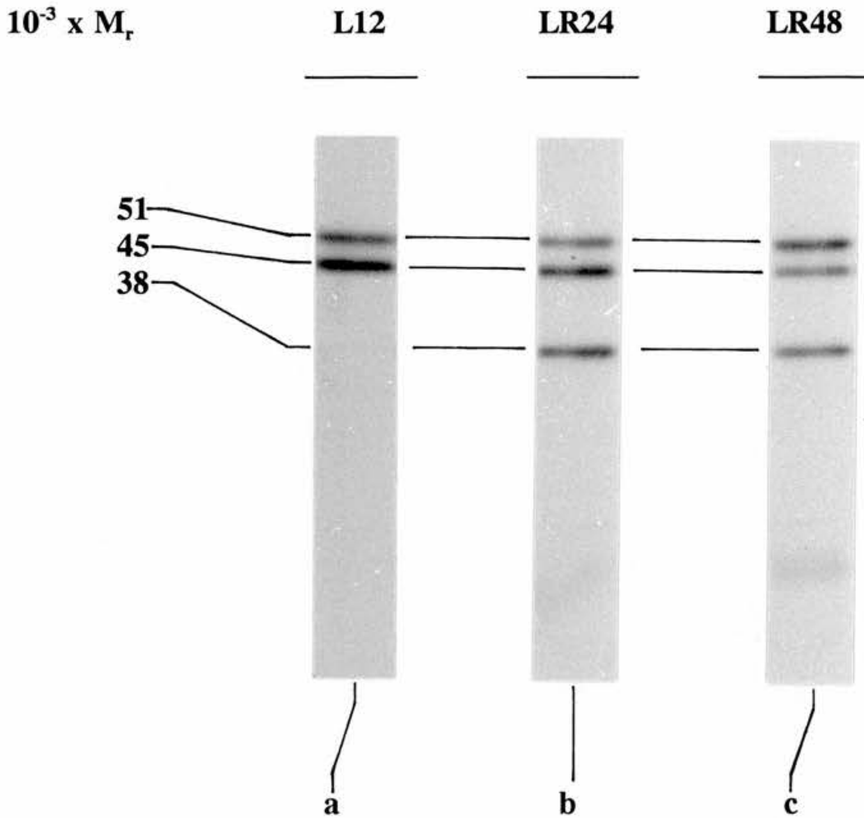


Figure 5.7 Particulate cAMP-binding proteins in rat mammary tissue following litter removal

Mammary tissue was sampled from mid-lactating rats as described in section 5.1. Extracts were prepared and labelled with the photoaffinity ligand 8-azido-[^{32}P]cAMP as described in the section 2.9. Results are representative of data for not less than 4 animals.

respectively. In competition experiments (described in section 2.9.3), all three protein bands were successfully competed out by radio-inert cAMP, (results not shown). The unspecific labelling of small proteins and peptides observed in cytosolic extracts was absent from all particulate samples examined here.

As for the cytosolic compartment, litter removal led to a deterioration in the total level of R-subunit (RI + RII); both LR24 and LR48 samples exhibited depleted levels of R-subunit as compared to L12 controls. However, unlike the cytosolic compartment, the decline in total R-subunit was brought about by the preferential down regulation of the RI isoform (Figure 5.8). There was no corresponding decline in the level of the RII isoform, thus litter removal was also shown to be accompanied by a substantial increase in the RII:RI ratio (Figure 5.9).

Accompanying the decline in RI, was a large increase in the level of the 37-38kDa cAMP-binding protein, which had previously been described (section 4.6.3) as absent or present at very low concentrations, in the particulate fractions derived from nursing rats, such as L12 controls examined in the same experiments (Figure 5.8). Levels of the 37-38kDa cAMP-binding protein, as a proportion of total 8-azido[^{32}P]cAMP binding activity, did not appear to be as great as previously described for cytosolic tissue extracts (section 5.5.1). The 37-38kDa protein represented approximately 5% of the total 8-azido[^{32}P]cAMP binding activity in the particulate fraction of L12 controls; this rose to 25 and 30% for LR24 and LR48 samples respectively. Corresponding values for cytosolic samples, derived from the same tissue, showed an increase from 22% for L12 controls to 83 and 76% for LR24 and LR48 samples respectively.

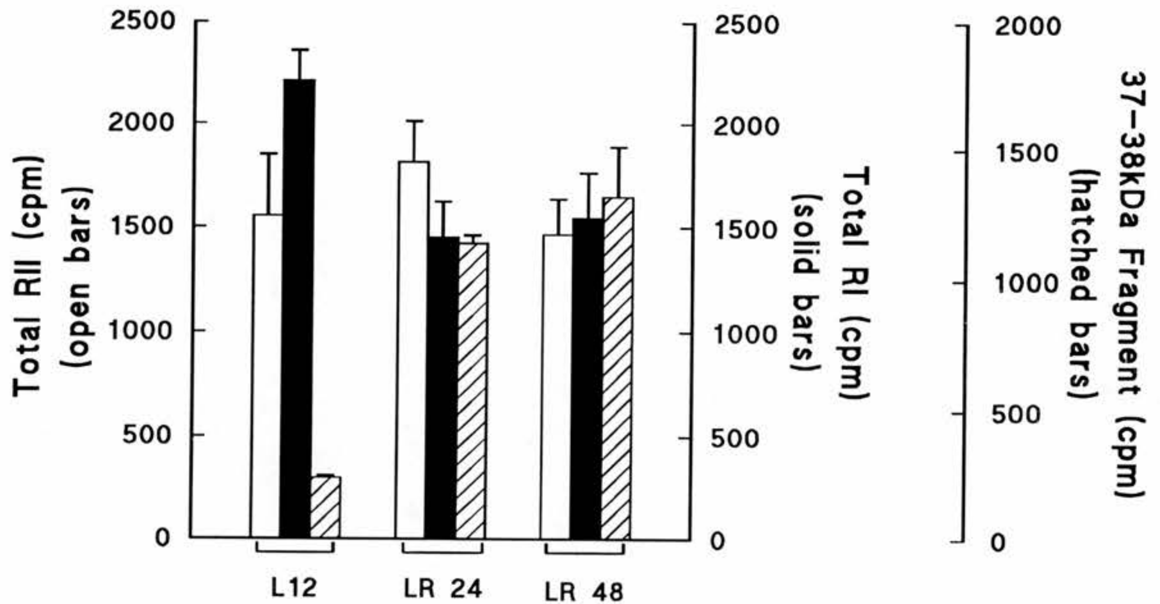


Figure 5.8 Particulate R-subunit composition of mammary tissue following litter removal

The above Figure shows data from a single experiment in which particulate mammary tissue extracts were prepared and labelled with the photoaffinity ligand 8-azido-[^{32}P]cAMP, as described in the section 2.9. The above histograph shows the relative levels of cAMP-binding proteins in the particulate fractions of control and litter removed samples. Values are means ($\pm$S.E.M.) for not less than four animals.

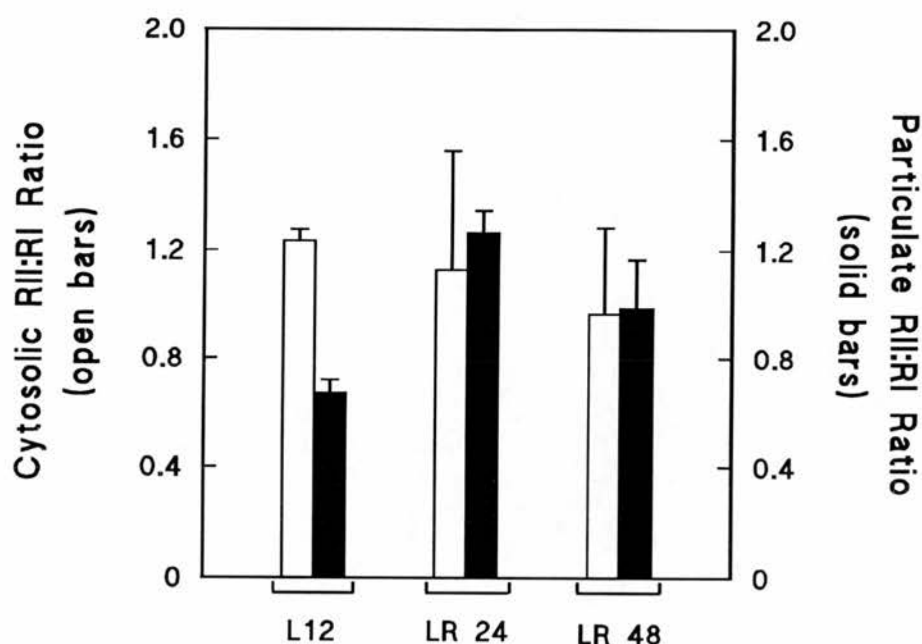


Figure 5.9 The relative abundance of cytosolic and particulate R-subunit isoforms

The above Figure represents a single experiment of paired design in which the relative levels of the R-subunit isoforms RI and RII were measured for both cytosolic and particulate fractions of the rat mammary tissue. Results are means (± S.E.M.) for not less than 4 animals.

5.6 Discussion

The significance of the suckling stimulus in maintaining the functional integrity of the mammary gland and lactation has been demonstrated by others (Selye, 1934 and Flint et al, 1981). Many studies have employed the technique of removing the litter at the height of lactation to prevent milk removal and initiate involution (see Lascelles & Lee, 1987 for review). Although such an event is uncommon in nature, being limited to the accidental loss of the offspring, this does allow involution to be initiated in a controlled manner. The effects of abrupt weaning upon the expression of PK-A in the rat mammary gland are discussed here.

Based upon tissue wet weight or DNA, 24h litter removal resulted in a rapid decline in both total and expressed PK-A activities. The decline in expressed activity was more marked, as compared to total activity with the result that the PK-A activity ratio also fell following litter removal. Activity recovered slightly during the next 24h litter removal, but did not return to those levels measured in nursing, control females.

Many independent studies have implicated the involvement of cAMP and PK-A in the mediation of involution and apoptopic cell death, in a number of cell and tissue models. The action of certain cAMP analogues, including 8-Cl cAMP have been found to trigger apoptopic cell death in lymphoid cells (Dowd & Miesfeld, 1992) and increased nuclear PK-A activity has been associated with early stages of involution in the mouse mammary gland (Marti et al, 1994). Furthermore, total PK-A activity (expressed on a tissue DNA basis) was found to be markedly suppressed during lactation in the rat mammary gland (Gardner et al, 1994 and section 4.3.1). It is therefore possible that suppression of PK-A activity is necessary in order to safeguard lactation. In the rat,

lactation may be rescued by return of the pups up to, and including 24h from litter removal (Flint et al, 1981). Therefore the initial suppression of PK-A activity (both total and expressed) 24h from litter removal may be indicative of some physiological mechanism, initiated to protect lactation following the temporary separation of mother and offspring. Beyond 24h litter removal involutionary processes become irreversible, at the same time pups fail to survive without their mother. It is of interest therefore, that following 48h from litter removal a partial recovery of total and expressed PK-A activity was observed; recovery of PK-A activity then, appeared to coincide with mammary gland commitment to involution.

C-subunit protein mass was also shown to fall following litter removal but not in fixed proportion to total PK-A activity, leading to a decline in the specific catalytic activity of the C-subunit. Neither did C-subunit protein mass appear to be transcriptionally regulated; levels of Cα mRNA remained relatively constant between lactating controls and animals whose litters had been removed for 24 and 48h respectively. Maintenance of C-subunit protein mass has previously been shown to come under transcriptional regulation in the rat mammary gland throughout pregnancy and lactation; where the specific catalytic activity of the C-subunit was also shown to remain constant (Gardner et al, 1994 and section 4.5). Therefore, litter removal appeared to initiate regulatory mechanisms other than those in place during mammary tissue development. The abatement of C-subunit protein mass following litter removal may be achieved by the stimulation of degradation pathways, specific for the PK-A C-subunit present within rat mammary tissue. Deactivation of free C-subunit in rat mammary tissue has already been described elsewhere in this thesis (section 3.4). Degradation of the PK-A C-

subunit has been reported in other tissues, such as the identification of a protease specific to C-subunit in brush border membranes isolated from rat small intestine (Alhanaty & Shaltiel, 1979). A similar protease has also been described for rat renal brush border (Schmitz et al, 1973; and Hopfer et al, 1973).

The apparent fall in C-subunit specific activity may be explained by the presence of a specific PK-A inhibitor present within the mammary tissue. Inhibition of PK-A activity has been described, in a variety of tissues, including the specific, heat-stable inhibitor protein of PK-A, described in rabbit muscle by Walsh and colleges (Walsh et al, 1971). There is also some evidence in the current literature, for the existence of "mute" C-subunit molecules such as those described by Reed et al (1983) and Steinberg (1991), or C-subunit with altered specific activities in small, distinct sub-cellular populations (Clegg & Connor, 1991). The present result is indicative of divergence in the regulation of C-subunit activity between lactating tissue and that undergoing involutionary processes. Work discussed earlier in this thesis has already shown the consistency of the intrinsic specific activity of mammary tissue C-subunit throughout pregnancy and lactation (sections 3.3 and 4.4).

Several studies have established that hormones or cAMP itself are capable of regulating the PK-A system, at least in part by altering the PK-A subunit concentration or by transcriptional or post-transcriptional changes in the production or degradation of PK-A subunits (Spaulding, 1993 and Garrel et al, 1995). Down-regulation of the C-subunit and reduced catalytic activity might be expected in tissue which has experienced elevated cAMP levels. *In vitro* cells exposed to high levels of cAMP for prolonged periods show increased turnover of C-subunit or elevated levels of R-subunits both

resulting in reduced catalytic activity (Spaulding, 1993).

PK-A deactivation may also be achieved by increasing the level of R-subunit relative to C-subunit, the principle was recently confirmed by the identification of RIα (Boshart et al, 1991 and Jones et al, 1991) as the extinguisher of cAMP-induced gene responses.

Three distinct cAMP-binding proteins were detected in soluble, cytosolic extracts derived from rat mammary tissue which had undergone litter removal for 24 and 48h. These migrated on SDS-PAGE gels at the same apparent molecular weights as the RI, RII and a 37-38kDa protein present in L12 control samples run in the same experiments.

Litter removal brought about a drastic decrease in the R-subunit levels measured in soluble, cytosolic tissue extracts. Both R-subunit isoforms (RI and RII) appeared to be equally effected. Greatest losses occurred between 0 and 24h litter removal, with moderate losses of R-subunit occurring between 24 and 48h litter removal. In contrast, levels of the 37-38kDa protein increased dramatically, between 0-24h; an observation consistent with this protein being a product of R-subunit degradation. A similar protein has been described by others (Handschin et al, 1983) and is thought to arise by the hydrolysis of R-subunits at the susceptible "Hinge region" (Takio et al, 1980) which is exposed only in the free subunits (Sugden et al, 1976., Potter & Taylor, 1979 and Weldon & Taylor, 1985). Further studies have revealed that both types of R-subunits are subject to proteolysis (Sugden et al, 1976., Weber & Hilz, 1978., 1979 and Corbin et al, 1978), with the generation of 35-39kDa cAMP-binding products of RI and RII respectively. These degradation products were unable to recombine with C-subunit to form holoenzymes, leading to a cessation of biological activity (Weber & Hilz, 1979).

Cleavage of released R-subunits at these protease sensitive sites might provide a signal for further degradation, which may eventually possibly lead to a loss of cAMP-binding activity; no smaller, high affinity cAMP-binding proteins/peptides were found within the mammary samples examined here.

The proteolytic cleavage of the R-subunit did not appear to be confined to mammary involution. The 37-38kDa protein was also observed in pregnant and lactating rat mammary tissue, albeit in smaller quantities, indicating that a basal level of R-subunit degradation was present during mammary development (as described in section 4.6.1). Increased degradation of R-subunit following litter removal may have been coincidental with the activation of non-specific proteases following the onset of involution. However, Northern blot studies have shown an inverse relationship between the expression of proteases and their inhibitors in the mouse mammary gland following litter removal (Strange et al, 1992); the first days of weaning were characterised by the expression of certain protease inhibitors, while proteases were expressed more latterly into involution. Therefore, it may also be possible that abrupt weaning was followed by the increased expression of a protease, specific for the PK-A regulatory subunit.

Prior to litter removal C- and R-subunits appeared to be coordinately down-regulated. However, 24 and 48h from litter removal the regulation of C- and R-subunits appeared to diverge; the data suggests an increase in the C-:R-subunit ratio between 0-24h and 24-48h litter removal which would be expected to lead to an increase in the population of free C-subunit within the tissue. This may account for the partial recovery of expressed PK-A activity following 48h litter removal, but presents a possible paradox between 0 and 24h litter removal where the C-:R-subunit ratio rises while the expressed

activity decreases. Most tissues examined have been shown to contain equimolar amounts of R- and C-subunits (Corbin et al, 1977; Hofmann et al, 1977). In the present study, the ratio of C- and R-subunits was shown to remain relatively constant throughout rat mammary development (see Chapter 4), although the molecular ratio of these two subunits was not determined since the absolute amount of R-subunit was not measured. Deviation from the usual 1:1 ratio has rarely been reported, and usually entails over expression of R-subunit relative to C-subunit as first reported by Prashad et al (1979) in neuroblastoma cells following prolonged treatment with dibutyryl cAMP. More recently the C-:R-subunit ratio has been shown to decrease during the pre-replicative phase of liver regeneration (Ekanger et al, 1989).

In the present study the particulate fraction of rat mammary tissue was also examined for cAMP-binding proteins and shown to contain the same inventory of cAMP-binding proteins as described previously, for soluble cytosolic extracts. Three cAMP-binding proteins were present including RI, RII and the 37-38kDa protein. As for the cytosolic compartment, there was a significant reduction in the level of total R-subunit (RI + RII) following litter removal; R-subunit loss was greatest during the initial 24h period of non-suckling, whereafter R-subunit levels remained low, but relatively stable. However, unlike the cytosolic compartment there was no significant loss of the RII isoform; the loss of the RI isoform was wholly responsible for the decrease observed in total R-subunit. Therefore litter removal also led to a significant increase in the RII:RI ratio.

Conversely, levels of the 37-38kDa protein increased following litter removal, most notably between 0 and 24h; control mammary tissue (L12) contained very small or

negligible quantities of this cAMP-binding protein. Hence, removal of the suckling stimulus stimulated the down-regulation of R-subunit protein in both the cytosolic and particulate compartments; and that down regulation was achieved, at least in part, by an increase in the rate of proteolytic cleavage of intact R-subunits to form 37-38kDa cAMP-binding protein fragments.

The extent of R-subunit degradation differed between cellular compartments. R-subunit degradation appeared greatest in the cytosolic compartment, which also contained higher levels of the 37-38kDa protein (expressed as a percentage of total 8-azido[^{32}P] cAMP-binding activity). There were further differences in relative degradation of the two R-subunit isoforms, RI and RII. In the cytosolic compartment both isoforms were equally down regulated with the resulting RII:RI ratio remaining approximately constant. Conversely there was no significant loss of RII from the particulate compartment. Divergent turnover of PK-A subunits has been reported in rat liver, brain and kidney (Weber & Hilz, 1986), in which type I holoenzyme was renewed much faster than protein kinase II. The authors described the basic molecular mechanism leading to this higher turnover of type I PK-A as being related to the greater instability of free subunits compared to that of the holoenzyme. Since other studies have indicated that RII is by far the most sensitive isoform to proteases, *in vitro* (Weber & Hilz, 1979), elevated turnover of RI may be an indicator of preferential activation of the type I holoenzyme. A growing body of evidence has also shown that the type II PK-A holoenzyme can be tethered at specific subcellular locations through interaction of its R-subunits with an AKAP, or A-Kinase Anchoring Protein (Scott, 1991). The majority of these proteins have been detected in particulate tissue fractions, implying attachment

to membrane and skeletal components (see Scott and Carr 1992 for review). Although specific mammary AKAPs have yet to be identified it remains a possibility that the RII isoform is associated with similar proteins in the particulate compartment of rat mammary tissue. Association with AKAPs may afford RII protection from the proteolytic degradation, and help explain the difference between the two cellular compartments discussed above. Evidence for similar proteins which will bind to, and anchor the RI isoform has been insufficient to the effect that RI is generally regarded as remaining "free" in solution.

If, as the data suggests, C-subunit was present in excess over R-subunit, then the mechanism that normally maintains the stoichiometry between these two subunits was disrupted following the onset of involution. Although the level of many proteins is maintained by transcriptional regulation (as for PK-A C-subunit throughout rat mammary development, section 4.5) regulation can also occur at the level of translation and/or degradation of mRNA and at the level of protein degradation. It is therefore possible that the two subunits were regulated coordinately at one level (e.g. transcription) but regulated differently at another level e.g. translation or degradation. C-subunit activity and therefore protein phosphorylation was also apparently subject to further regulation, in that the specific activity of the enzyme was reduced following the initiation of involution, possibly by the action of a specific kinase inhibitor.

Chapter 6

Summary and discussion

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. The end of lactation sees a reductive remodelling process termed involution during which the tissue returns to a less differentiated state such as that observed prior to pregnancy. Such processes entail concerted modification of expression of large numbers of genes. Since such pleiotropic transcriptional regulation is a well documented function of PK-A (Montminy et al, 1990) the behaviour of this kinase in mammary tissue was investigated here. The following discussion summarises how PK-A activity and the expression of PK-A generic C-subunit and R-subunits (RI and RII) were developmentally regulated throughout the pregnancy/lactation/involution cycle of the rat mammary gland.

The expression of the Cα- and Cβ-subunit mRNA appeared to be coordinately regulated throughout pregnancy and lactation, in that their ratio to one another did not change. Cα appears to be expressed constitutively in most cells, however the expression of Cβ is tissue specific (Showers & Maurer, 1986., Uhler et al, 1986 and Hedin et al, 1989). Cα mRNA was shown to predominate over that of Cβ in rat mammary tissue; this pattern of expression is characteristic of liver and kidney, but unlike brain, where the abundance of the two mRNAs is roughly equal (Showers and Maurer, 1986). Absolute levels of both the Cα and Cβ mRNAs were repressed during

lactation; a pattern mirrored by both C-subunit protein and total PK-A activity levels, suggesting that transcriptional processes were major determinants of Cα and Cβ activity. The mechanism of this repression during lactation is as yet unknown; however it is in sharp contrast with control of 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 (for review see Rillema, 19??). 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 the C-subunit expression remains open to further investigation.

In relation to putative tonic influences of PK-A on mammary growth and development, it is likely that expressed activity is the most physiologically relevant of the catalytic parameters determined, being indicative of cAMP dependent protein phosphorylation events. It is therefore of interest that this remains relatively constant throughout mid-pregnancy but approximately doubles during late-pregnancy, immediately preceding the steep increase in the rate of DNA synthesis which persists throughout early/mid lactation. This enhancement in expressed PK-A activity does not persist correspondingly, but instead declines back to base line.

Several independent lines of evidence have suggested that PK-A is atypical in its biochemical properties, at least in lactating rat mammary tissue (Clegg, 1988., Clegg and Ottey, 1990., Takhar and Munday, 1984 and Bussmann et al, 1984). The present work suggests that there are no changes to the intrinsic specific activity of C-subunit, with respect to the substrate, Kemptide, <u>between</u> pregnancy and lactation. C-subunit protein mass was shown to be closely paralled by total PK-A activity throughout

pregnancy and lactation, thereby demonstrating the consistency of C-subunit specific catalytic activity. However, it is possible that mammary tissue may contain a novel C-subunit, not recognised by Northern Cα and Cβ probes or by the C-subunit antibody (As-365/ab-365). In this respect it is of interest that 15% of C-subunit appears to be insensitive to immunotitration and immunoprecipitation by As-365 (see below). Such C-subunit may exhibit abberrant sensitivity to inhibitors and deactivators, and/or intrinsic differences in C-subunit acylation or autophosphorylation. For the purpose of C-subunit immunoquantification a polyclonal antibody was raised against a synthetic peptide representing residues 322-332 of the C-subunit of PK-A (denoted As-365). This epitope lies close to the binding sites both for substrates peptide and adenine nucleotide, explaining the ability of the antibody to inhibit the catalytic activity of PK-A C-subunit. As-365 showed no discernable difference in its potency titre against mammary tissue extracts or purified mammary C-subunit preparations derived from pregnant and lactating animals (as revealed by immunoinhibition studies). Such preparations differed markedly in their levels of C-subunit expression, supporting the above conclusion that C-subunit specific activity is unaffected by the differentiation of mammary tissue throughout pregnancy and lactation.

The absolute level of rat mammary tissue C-subunit protein found in the present study, of approximately 2μg/g tissue in lactating animals, is in agreement with that deduced by Clegg & Connor (1991). However mammary C-subunit also possessed a specific activity several-fold greater than the best values measured for the purified protein, including those from lactating and pregnant sheep mammary tissue. Such findings may be indicative of major activity losses as a result of C-subunit purification or an

enhancement of C-subunit activity within mammary tissue extracts. Enhancement of C-subunit activity has been described previously in lactating rat mammary tissue extracts (Clegg & Ottey, 1990).

Immunoinhibition and immunoprecipitation studies using mammary tissue extracts and purified C-subunit preparations (including a non-myristoylated recombinant C-subunit), also revealed a sub-population of C-subunit molecules not recognised by the As-365. The proportion of this residual activity appeared to be independent of the tissues physiological state or tissue of origin. Such molecules may have all or part of the epitope 322-332 occluded, either by some small molecule not removed by the purification process, or by some form of post-translational or proteolytic modification. Since both Cα nd Cβ isoenzymes are known to contain the amino acid sequence used to raise As-365, it is unlikely that the residual activity was a consequence of the C-subunit heterogeneity described above.

In the course of these, and further investigations mammary tissue was shown to contain a deactivator of PK-A activity which did not share the same characteristics of the heat stable inhibitor of PK-A (PKI) first described by Walsh and colleagues (Walsh et al, 1971). Deactivation of PK-A was found to be critically time and temperature dependent; modest but significant protection was also provided by the presence of MgATP. PK-A deactivation has been described in other rat tissues; a specific C-subunit proteinases has been identified in brush border membranes isolated from rat small intestine and kidney (Alhanaty & Shaltiel, 1979, Schmitz et al, 1973 and Hopfer et al, 1973). However, Western blotting studies using As-365, revealed no evidence for the degradation of the C-subunit, possibly due to proteolytic modification of residues

322-332. The specific deactivation of C-subunit, by a proteinases or deactivating factor, together with the prevention of its action by substrate and R-subunits, raise the possibility it may have a distinct physiological (possibly regulatory) assignment within rat mammary tissue.

Cho-Chung and co-workers have repeatedly demonstrated a predominance of the type I R-subunit (RI) in tumour cells and have established a strong correlation between RI and cellular proliferation (Cho-Chung, 1992). In contrast the preferential expression of the type II PK-A has been shown to be induced by treatment of tumour cells with cAMP analogues and is typical of terminally differentiated tissues (Cho-Chung et al, 1991). The present study revealed a pattern of RI expression, throughout normal rat mammary development, which reflected the changes in the rate of cellular proliferation in the tissue; levels increased throughout pregnancy to peak around parturition or early lactation, after which, cell numbers have been shown to remain relatively constant (Knight & Peaker, 1982a). RI therefore appeared to be a positive regulator of mammary cell growth, however, an alternative explanation may be that the RI gene generally responds more rapidly than that of RII in demand for increased R-subunit synthesis e.g. in rat liver regeneration (Ekanger et al, 1989).

RI remained the predominant isoform in rat mammary tissue throughout pregnancy and lactation, however, levels of the RII isoform (and consequently the RII:RI ratio) increased throughout mammary development. A growing body of evidence has shown that type II PK-A holoenzyme can be tethered at specific subcellular locations through interaction of its R-subunits (RII) with A-kinase anchoring proteins (AKAPs). The majority of AKAPs are located in the particulate, cellular fractions, implying

attachment to membrane and skeletal components (Scott, 1991). R-subunit expression was therefore examined in both cytosolic (described above) and particulate fractions of rat mammary tissue. Not surprisingly a greater proportion of particulate R-subunit was found to comprise of RII. As for the cytosolic compartment the RII:RI ratio also increased throughout both pregnancy and lactation. The vast majority of R-subunit was shown to be located in the cytosolic compartment of rat mammary tissue, this pattern of compartmentalisation has been found for most tissues examined, with the exception of the brain (Scott & Carr, 1992).

Also present in cytosolic extracts, but absent from all particulate samples examined, was a cAMP-binding protein of 37-38kDa. This may have been one of a family of protein fragments derived from R-subunits by proteolysis at the susceptible hinge-region, as previously described by others (Weldon & Taylor, 1985). Unlike the regulatory subunits this protein showed no discernable developmental trend in its expression, and indicated that a basal level of R-subunit degradation was in operation throughout rat mammary development.

The initiation of involution by removal of the suckling stimulus (litter removal) was shown to have quite dramatic effects upon the expression of PK-A in rat mammary tissue. Both total and expressed PK-A activities declined 24h after litter removal, recovering somewhat over the next 24h. C-subunit protein mass also declined markedly, however expression of Cα mRNA changed little over this same period suggesting that a mechanism other than transcriptional processes were the major determinants of Cα activity. Cα has already been shown to greatly exceed that of Cβ mRNA throughout pregnancy and lactation in the rat mammary gland, it is therefore

unlikely that a decrease in the level of Cβ mRNA was responsible for the decline in total C-subunit protein mass. The decrease in C-subunit protein mass may therefore be explained by a decrease in mRNA stability, translational events or by C-subunit degradation; the latter presumably by a proteinases with particular efficiency against C-subunit. C-subunit degradation has already been discussed above (Alhanaty & Shaltiel, 1979).

The decline in total PK-A activity was greater than could be explained by the decrease in C-subunit protein mass alone, suggesting that a decline in the specific activity of the C-subunit had occurred. The specific activity of mammary tissue C-subunit has previously been shown to remain consistent throughout pregnancy and lactation, therefore PK-A activity appeared to be subject to further regulation in tissue undergoing the process of involution. Deactivation of C-subunit within rat mammary tissue extracts has already been described above (also see sections 3.4 and 3.5). C-subunit may also have been subject to inhibition by a specific protein kinase inhibitor present within mammary tissue. C-subunit has been shown to be highly susceptible to a class of endogenous proteins the PKI's, as first described by Walsh and colleagues (Walsh et al, 1971). The presence of "mute" C-subunits (Steinberg, 1991) or molecules with altered specific catalytic activities such as those described by Clegg & Connor (1991) might provide an explanation for the altered specific activities.

Many independent studies have implicated the involvement of cAMP and PK-A in the mediation of involution and apoptopic cell death (Dowd & Miesfeld, 1992 and Marti et al, 1994). In the rat involution is initiated by removal of the pups and the suckling stimulus; however, lactation may still be rescued by return of the pups up to, and

including 24h from litter removal (Flint et al, 1981). Therefore the initial suppression of PK-A activity (both total and expressed) may be indicative of a physiological mechanism, initiated to protect lactation following the temporary separation of mother and offspring. The suppression of total PK-A activity (expressed on a tissue DNA basis) in the rat mammary gland during lactation (Gardner et al, 1994) appears to further imply a negative role for PK-A in lactational processes. Beyond 24h litter removal the process of involution becomes irreversible, at the same time a partial recovery of total and expressed PK-A activity was observed. Recovery of PK-A activity then, appeared to coincide with mammary gland commitment to involution.

Litter removal was also shown to induce a drastic decrease in the level of R-subunit in mammary tissue extracts. In contrast, the level of the 37-38kDa proteolytic fragment (Potter & Taylor, 1979) dramatically increased. Increased degradation of R-subunit may therefore be indicative of activation of a specific protease in mammary tissue as already described for the C-subunit. The extent of R-subunit degradation differed between cellular compartments; degradation appeared greatest in the cytosolic compartment, which also contained higher levels of the 37-38kDa proteolytic fragment. There were further differences in the relative degradation of R-subunit isoforms (RI and RII). Both isoforms were equally down-regulated in the cytosolic compartment; hence, the isoform ratio (RII:RI) remained constant. Conversely there was no significant loss of RII in the particulate compartment, presumably reflecting protection from proteolytic degradation by association with AKAPs.

Prior to litter removal C- and R-subunits appeared to be coordinately regulated, in that the ratio of C- and R-subunits remained relatively constant throughout rat mammary

development; although the molecular ratio of these two subunits was not determined since the absolute amount of R-subunit was not measured. However, following litter removal R-subunit degradation appeared to exceed that of the C-subunit. Most tissues examined have been shown to contain equimolar amounts of R- and C-subunits (Corbin et al, 1977; Hofmann et al, 1977). Deviation from the usual 1:1 ratio has rarely been reported, and usually entails over expression of R-subunit relative to C-subunit as that first reported in neuroblastoma cells by Prashad et al (1979). If, as the data suggests, C-subunit was present in excess over R-subunit, then the mechanism that normally maintains the stoichiometry between these two subunits was disrupted following the onset of involution.

Expression of PK-A activity, as well as generic C- and R-subunits appeared to be intimately regulated throughout the pregnancy/lactation/involution cycle of the rat mammary gland. In summary the above study showed that the level of C-subunit protein mass, and therefore total PK-A activity was maintained by transcriptional regulation throughout the course of pregnancy and lactation. Mammary tissue was also shown to contain a mechanism for deactivation/degradation of free C-subunit. However the specific catalytic activity of mammary C-subunit was unaffected by the differentiation of the tissue throughout pregnancy and lactation. C- and R-subunits appeared to be coordinately regulated throughout much of pregnancy and lactation (although R-subunit transiently exceeded C-subunit around parturition). It is therefore possible that the two subunits were regulated coordinately at one level (e.g. transcription) but regulated differently at another level e.g. translation or degradation. Levels of the two major R-subunit isoforms (RII and RI) were also developmentally

regulated; levels of the RII isoform gradually increased throughout pregnancy and lactation while RI levels were shown to peak round parturition or early lactation. The involution of mammary tissue brought about changes in the regulation of PK-A activity; C-subunit protein mass and therefore total PK-A activity were no longer subject to transcriptional regulation. Both C- and R-subunits were subject to degradation within involuting mammary tissue, however R-subunit degradation appeared much greater, thus the mechanism that normally maintains the stoichiometry between the C- and R-subunits was also disrupted. C-subunit activity was further regulated, in that the specific activity of the enzyme was reduced following the initiation of involution, possibly by the action of a specific kinase inhibitor.

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