THE EFFECT OF CELL VOLUME ON MAMMARY GLAND METABOLISM

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SUMMARY

1. It is becoming increasingly apparent that cell volume, otherwise termed the cellular hydration state, regulates processes such as protein synthesis, lipogenesis and membrane transport. In this study, the effect of cell volume perturbations on mammary cell metabolism has been investigated. Thus, the effect of cell swelling and shrinking on protein synthesis and lipogenesis in rat mammary tissue has been examined. In addition, the effect of cell volume changes on the free cytosolic calcium concentration ($[Ca^{2+}]_i$) in rat mammary acinar cells has been studied.

2. Mammary tissue (explants and acini) isolated from lactating rats during peak lactation was used in this study. Protein synthesis was studied by measuring the incorporation of radiolabelled amino acids into trichloroacetic acid precipitable material. Cell swelling, induced by a hyposmotic shock markedly increased protein synthesis in mammary tissue explants and acini. Conversely, cell shrinking, induced by a hyperosmotic shock markedly inhibited protein synthesis in mammary explants and acini. The effect of cell swelling and shrinking on protein synthesis was a) dependent upon the extent of the osmotic challenge and b) reversible. Swelling-induced mammary protein synthesis was dependent upon the presence of extracellular calcium. In this connection, thapsigargin and tBHQ markedly inhibited volume-sensitive protein synthesis suggesting that protein synthesis is dependent upon luminal calcium stores. Isosmotic cell swelling, induced by using buffers containing urea significantly increased mammary protein synthesis.

3. The effect of cell volume perturbations on the $[Ca^{2+}]_i$ in mammary acinar cells isolated from rats during peak lactation was examined. The $[Ca^{2+}]_i$ was measured using the fura-2 dye technique. Cell swelling, induced by a hyposmotic challenge, increased the $[Ca^{2+}]_i$ in a fashion that was transient. The effect of a hyposmotic shock on the $[Ca^{2+}]_i$ was dependent upon the extent of the osmotic perturbation. The hyposmotically-induced increase in the $[Ca^{2+}]_i$ could not be attributed to a) a change in the *trans*-membrane sodium gradient or b) a change in the ionic strength of the incubation buffer. Removing extracellular Ca^{2+} (using EGTA) inhibited the effect of a hyposmotic shock on the $[Ca^{2+}]_i$ in rat mammary acinar cells, suggesting that a hyposmotic challenge increases the influx of Ca^{2+} rather than a release of Ca^{2+} from

intracellular stores. Thapsigargin increased the size of the volume-sensitive increase in the $[Ca^{2+}]_i$. This suggests that the activity of calcium pumps in the membranes of intracellular organelles is acting to limit the increase in the $[Ca^{2+}]_i$ following an osmotic shock. Isosmotic swelling had no significant effect on the $[Ca^{2+}]_i$. Similarly, a hyperosmotic challenge had no effect on the $[Ca^{2+}]_i$ in rat mammary acinar cells.

4. The effect of cell swelling and shrinking on lipogenesis by mammary tissue isolated from peak lactating rats was examined. Lipogenesis was studied by measuring the incorporation of radiolabelled acetate into mammary lipid. Cell swelling induced by a hyposmotic challenge increased mammary lipogenesis. In contrast, cell shrinking, induced by a hyperosmotic shock markedly inhibited mammary lipogenesis. The effects of cell swelling and shrinking on lipogenesis were dependent upon the extent of the osmotic challenge. Mammary lipogenesis was also stimulated by isosmotic cell swelling. Removing extracellular calcium (using EGTA) had no effect on lipogenesis under isosmotic and hyposmotic conditions.

5. The findings suggest that altering cell volume has marked effects on mammary cell metabolism. It is conceivable that the hydration state of mammary cells is an important regulator of milk synthesis. It is possible that factors such as substrate uptake control protein and fat synthesis by modulating mammary cell volume.

DECLARATION

This thesis has been compiled by myself and has not been offered in any previous application for a degree. I declare that all the work contained in this thesis is my own, undertaken under the supervision and guidance of Dr D.B. Shennan.

Candidate _ (Alastair Charles Graeme Grant)

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
DIDS	4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
ER	Endoplasmic reticulum
GIT	Guanidinium Isothiocyanate
GLUT	Facilitative glucose transporter
mRNA	Messenger ribonucleic acid
NMDG	N-methyl-D-glucamine
PCA	Perchloric acid
PMCA	Plasma membrane Ca^{2+}-ATPase
RVD	Regulatory volume decrease
RVI	Regulatory volume increase
SERCA	Sarcoplasmic (endoplasmic) reticulum Ca^{2+}-ATPase
SGLT	Sodium-glucose symporter
tBHQ	2,5-di-(t-butyl)-1,4-benzohydroquinone.
TCA	Trichloroacetic acid

CHAPTER ONE

GENERAL INTRODUCTION

1.1. ORIGIN OF THE STUDY

There are interspecies differences in the composition of milk (Jenness, 1974), meaning that cross-utilisation of milk may not provide the necessary nutritional gains. Currently, ruminant milk is processed to make it more suitable for human consumption. For example, the fat content of milk is the most commonly altered, through the process of skimming. Bovine milk is also processed to produce formula milks for human babies. The cost of processing milk for human consumption is considerable, so it would be advantageous for ruminants to secret milk with a composition similar to that of human milk. In addition, the dairy industries would benefit from milks with altered composition. From a health perspective, milk with a higher protein, calcium and phosphate content would be welcomed. However, a thorough knowledge of mammary gland biology is required if we are to be in a position to markedly alter the content of milk.

The present study was undertaken to investigate the control of mammary gland metabolism by mammary cell volume. It is becoming apparent that cell volume, otherwise termed the cellular hydration state, is an important modulator of various metabolic pathways in a variety of cells (Haussinger *et al.*, 1994a, 1994b; Haussinger and Schliess, 1995; Haussinger, 1996; Lang *et al.*, 1998a). For example, protein synthesis, lipogenesis, and glycogen synthesis are affected by a change to the cellular hydration state (Lang *et al.*, 1998a). It is apparent that several hormones (e.g. insulin, glucagon) may exert their

action through changing the cellular hydration state (Haussinger and Schiess, 1995; Haussinger, 1996). In addition, many membrane transport processes which supply substrates for metabolic pathways are markedly affected by a change to cell volume. In view of this it was postulated that milk synthesis could be controlled by the volume of mammary secretory cells. Indeed, a preliminary report has shown that milk protein synthesis can be respectively stimulated and inhibited by cell swelling and shrinking (Millar *et al.*, 1997). The major aims of the project were to 1) confirm and extend the observation that mammary protein synthesis is affected by cell volume 2) study the effect of cell volume perturbations on mammary lipogenesis and 3) examine the effect of changing cell volume on the transport of calcium by mammary secretory cells given that mammary protein synthesis is dependent upon calcium (Wilde *et al.*, 1981; Smith *et al.*, 1982; Duncan and Burgoyne, 1996). However, at this point it is appropriate to outline some basic features of mammary gland biology together with some of the most salient features of the control of metabolism by cell volume. In addition, the regulation of cell volume will also be covered in this chapter.

1.2. MAMMARY GLAND ANATOMY

1.2.1. Structure of the mammary gland

The macroscopic morphology of the mammary gland shows significant differences between mammalian species. Variation in the number, size and positioning of mammary glands are the easiest to identify. Humans have two mammary glands located pectorally, cows have four situated inguinally and rats have 12 situated all along the abdominal

surface (Mepham, 1987). Despite these very obvious differences in morphology, the histological structure of the mammary gland retains a remarkable degree of homology between species (Hollman, 1974).

The secretory epithelial cells (parenchyma) of the mammary gland are arranged in pear shaped structures known as alveoli. These comprise a single layer of epithelial cells surrounding a hollow lumen into which milk is vectorially secreted. The spatial arrangement of the epithelial cells means that there are two distinct sides to the alveolar structure; the basolateral (blood-facing) and apical (lumenal) aspects. During feeding of the neonate, milk is expelled from the alveolar lumen through small ductules into a common duct. This process is facilitated by the contraction of myoepithelial cells that surround each alveoli. Groups of several different alveoli may drain into a single common duct and these clusters are termed lobules (a schematic representation of this structure is shown in figure 1.1). The epithelial cells of each alveolus secrete a basement membrane from the basolateral aspect, which forms the basal lamina. This acts as a barrier between the epithelial cells and connective tissue.

The connective tissue makes up part of the stromal mammary tissue which also incorporates the skin, adipose tissue, blood and lymph vessels, and nerve tissue (Mepham, 1987). Substrates for synthesis of milk constituents are obtained from blood supplied by arteries local to the mammary gland (Linzell, 1974), e.g. inguinal glands are supplied by the pudic artery and pectoral glands are supplied by the thoracic or pectoral arteries. The arteries branch into a fine network of capillaries, which surround each alveolus, and these

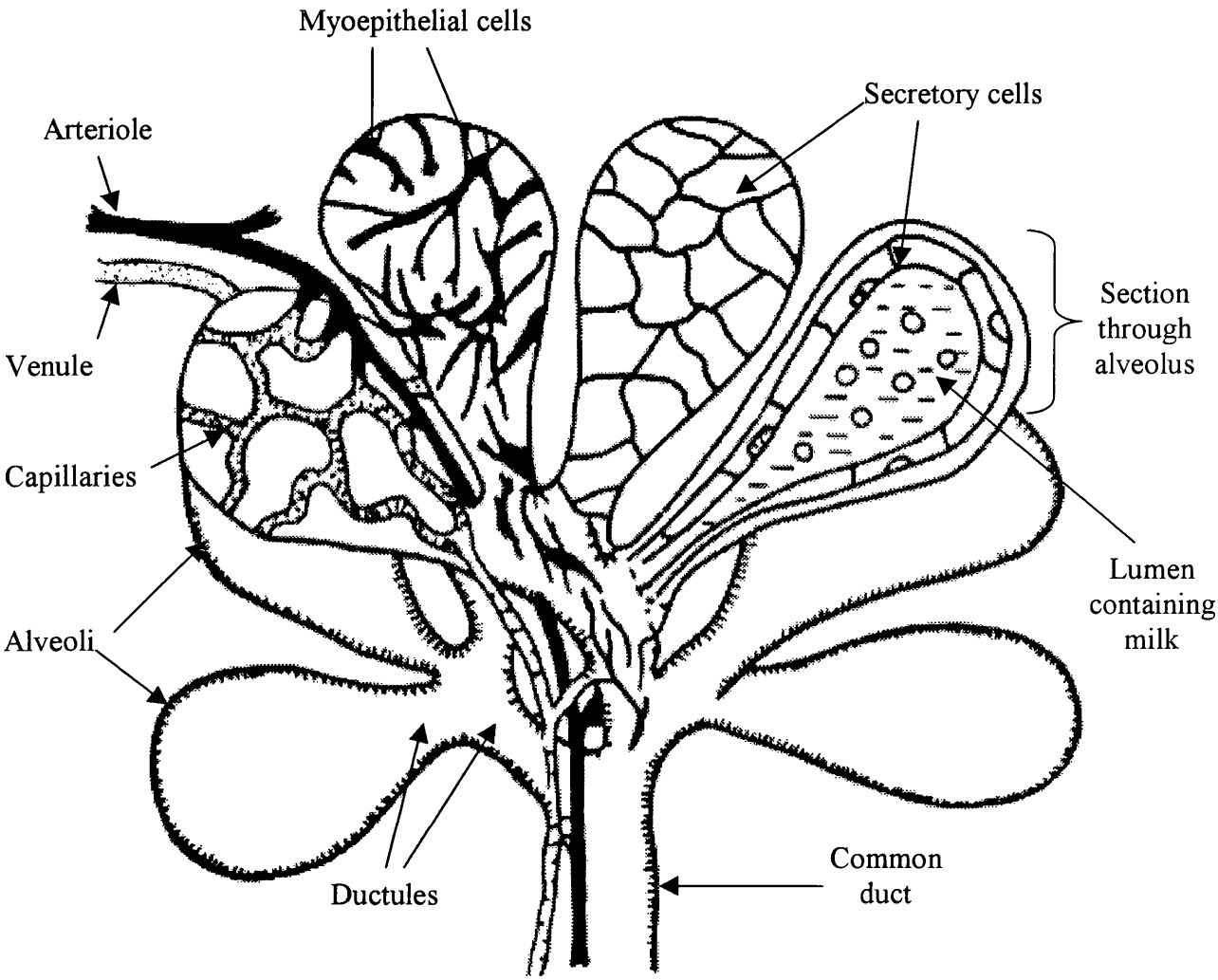

Fig 1.1: Schematic representation of mammary lobuloalveolar structure. Adapted from Mepham (1987).

in turn feed the veins that carry blood away from the gland. It is not uncommon to find an arteriovenous shunt that bypasses the capillary bed, joining arteries directly to veins (Linzell, 1974). Each lobuloalveolar structure is separated from the next by connective tissue. Situated within the connective tissue are adipocytes, which account for the majority of mammary gland volume during non-lactating periods (Knight and Peaker, 1982). However, during lactation adipocyte volume is dramatically reduced.

The lobuloalveolar morphology of the gland is common to all mammalian species. Some interspecies differences become apparent when looking at milk storage in the gland and in the structural arrangement of the teat. Bovine mammary glands have large hollow cisterns fed by numerous common ducts, which can store massive quantities of milk prior to secretion (Mepham, 1987). Rats on the other hand posses no such receptacle and therefore milk is retained within the alveolar lumen until required (Mepham, 1987). Also, the number of pores in the mammary gland teats are variable between species. In rats and cows, despite their differences in milk storage, both have a single orifice in each teat. Humans are different in that they have a number of orifices at each nipple, fed by several common ducts known as galactophores. This is a similar situation to monotremes (egg-laying mammals, e.g. duck-billed platypus), except that they do not have teats, only simple openings at the base of hairs on their abdomen (Mepham, 1987).

1.2.2. Development of the mammary gland

Development of the mammary gland is perhaps the most complex of any organ in the body. It appears to be the only organ that is not fully developed at birth and still requires

much differentiation and proliferation before it is capable of carrying out its function as a milk secreting unit (Mepham, 1987; Daniel and Silberstein, 1987). As with the morphology of the gland, it should be noted that there are significant differences between species in the rate of development. However, it is generally accepted that the most significant changes to mammary morphology occur during pregnancy and lactation (mammogenesis; Knight and Peaker, 1982). The importance of the earlier stages of development should not be undermined though, as they contribute a great deal to the successful development in later stages of the reproductive cycle. Prenatal development of the gland constitutes the laying down of the complex network of ductules through the mammary fat pad that determines positioning of the gland and will later allow precise formation of the lobuloalveolar structures (Knight and Peaker, 1982). Juvenile and pre-adult development of the gland involves continued development of the ductal network and an increase in mass of the mammary fat pad, although this is not representative of increased cellular proliferation but rather an increase in cell size (hypertrophy)(Knight and Peaker, 1982).

Up to and including the pre-adult stage of the organism, development of the mammary gland occurs at a rate comparable to the overall rate of tissue development and proliferation. Further development of the gland takes place during pregnancy and lactation. In rats it appears that 60% of mammary development takes place in these stages of reproduction (Anderson, 1974). Mammogenesis, as this stage of the development is termed, involves the elaboration of the ductal network and sees the first signs of lobuloalveolar structural development (Dembinski and Shiu, 1987). At the time of parturition the gland is virtually fully formed and capable of secreting milk. However,

there is evidence that further proliferation and differentiation occurs during the early stages of lactation in some species (Anderson, 1974).

Involution is the term used to describe the regression of the mammary gland towards the rudimentary structures formed in the stages leading up to pregnancy. Three types of involution have been defined: gradual involution, initiated involution and senile involution. The first refers to the gradual regression of the gland following peak lactation; initiated involution describes regression of the gland when milking is abruptly stopped, for instance in the death of the neonate; senile involution is caused by old age (Mepham, 1987; Russo and Russo, 1987). In each case, regression of the gland is not always complete, with some evidence of the lobuloalveolar structures remaining (Knight and Peaker, 1982).

1.3. MAMMARY CELL METABOLISM

The composition of milk is such that the neonatal infant is able to sustain growth and development in the early stages of life with nothing else in their diet. During lactation, the rate of metabolism within mammary tissue is therefore altered to favour the production of the various milk constituents. However, turnover of cellular components required for normal cell function continues irrespective of lactation. The precise composition of milk is highly species dependent (table 1.1 details some interspecies differences). Further interspecies differences can be observed when studying the timeframe over which milk composition changes. The rate of lactation and the composition of milk is comparable to the rate of development and thus the requirements of the neonate.

Table 1.1: Average composition of milk from various different species (adapted from Mepham, 1987)

Species	*Fat (g/l)*	*Casein (g/l)*	*Milk serum protein (g/l)*	*Lactose (mM)*	*Calcium (mM)*
Cow	37	28	6	133	30
Goat	45	25	4	114	22
Sheep	74	46	9	133	58
Pig	68	28	20	153	104
Horse	19	13	12	172	17
Man	38	4	6	192	7
Rat	103	64	20	90	80
Guinea-pig	39	66	15	83	41
Rabbit	183	104	32	60	214

1.3.1 Milk proteins

The majority of proteins found in milk are synthesised by the mammary gland itself. However, there are a number of different proteins contributed from sources outside the gland. The main proteins synthesised within the gland are the caseins and the whey proteins, which include whey acidic protein (WAP), α-lactalbumin and β-lactoglobulin. Synthesis of milk proteins in the mammary gland is consistent with the general pattern for protein synthesis in eukaryotic cells. Following transcription of the genome in the nucleus, the mRNA of the protein to be produced is transported to the cytosol where it is translated to a polypeptide sequence by ribosomes. Translation can be divided into three different phases; initiation, elongation and termination. The first phase involves the formation of the ribosomal unit at the 5' end of the mRNA molecule. The elongation phase involves the movement of the ribosome along the mRNA coding sequence, forming peptide bonds between amino acids provided by the tRNA anticodon. As one ribosome

vacates the start site, additional ribosomes are free to undergo initiation on the same mRNA sequence, thus forming a polysomal complex. Termination of translation occurs when the stop codon is reached at the 3' end of the molecule. At this stage the ribosome subunits dissociate and the polypeptide sequence is released. Secretory proteins such as the caseins must exit the cell via secretory vesicles which bud off from the *trans*-Golgi network (TGN). To advance to this structure within the cell, the protein must be targeted to the ER lumen during translation so that it may progress to the Golgi apparatus. This specific targeting is determined by the existence of peptide signal sequences that are translated at the start of the peptide sequence. As the protein is produced the signal sequence is recognised by specific receptors on the ER membrane so that the ribosomes become closely associated with the membrane. The emerging protein is directed through a channel into the ER lumen where it undergoes folding by chaperone proteins. Assuming correct folding is achieved, the protein may then proceed to the Golgi apparatus where it associates with other secretory proteins, in the case of the caseins forming micelles. Prior to secretion, secretory vesicles bud off from the trans-Golgi network and accumulate at the apical membrane of the epithelial cell. At this point the presence of proteins synthesised outside the mammary gland becomes apparent. The movement of these proteins across the epithelial cell is known as transcytosis and involves a complex progression through various organelles, following endocytosis at the basolateral aspect of the epithelial cell (Shennan and Peaker, 2000).

1.3.1.1. Caseins

Caseins are synthesised within the mammary gland and represent the largest component of the milk proteins, accounting for approximately 80% (Thompson and Farrell, 1974; Mepham, 1987). The caseins are a relatively hydrophobic family of phosphoproteins that form stable calcium-dependent micellar structures for secretion into milk. The casein component of milk is now recognised as a family of proteins consisting of a number of different isoforms: α_s-, β-, γ- and κ-caseins are the four main groups identifiable in bovine milk. The α_s-caseins can be further classified into either α_{s1}- or α_{s2}- subfamilies, and thereafter 4 genetic variants of α_{s1}-casein have been identified. Variation in κ-caseins has also been identified as a genetically controlled process. A number of different characteristics have been used to classify the caseins including relative mobilities on gel electrophoresis, amino acid composition, sensitivity to Ca^{2+}, phosphorus content and association behaviour (Thompson and Farrell, 1974).

Since the caseins are generally hydrophobic there has to be a component that can prevent dissociation of the micellar structure in the aqueous component of milk. κ-casein has been identified as a stabilising molecule that maintains the structure of the casein micelle while in the colloidal environment of milk (Thompson and Farrell, 1974). Micelles are conglomerates of smaller sub-micellar structures, held together by calcium phosphate structures that form strong interactions with phosphorylated serine residues on the constituent casein particles. κ-casein molecules are incorporated into submicelles situated on the periphery of the micellar conglomerate, adding to the stability of this essentially hydrophobic structure in the aqueous environment of milk (Mepham, 1987).

1.3.1.2. Whey proteins

Other proteins that have been identified in milk have been given the classification of whey (or milk serum) proteins (Mepham, 1987). β-Lactoglobulin is present in bovine, caprine and ovine milk but is absent from most other species and thus appears to be peculiar to ruminants (Mepham, 1987). It provides a good source of amino acids and is also seen to be associated with vitamins in milk. α-lactalbumin is extremely important in the catalysis of lactose formation and is thus almost universal as a component of milk (Mepham, 1987). It aids galactosyltransferase in the catalysis of lactose production by increasing the enzymes' affinity for glucose. It is situated on the luminal aspect of the Golgi and thus is secreted along with the lactose that it has aided the production of. WAP was primarily identified in rodent milk and appears to be absent from ruminant milk (Rosen, 1987).

β-lactoglobulin, WAP and α-lactalbumin are synthesised in the mammary gland, but this is not the case for all of the milk serum proteins. A significant complement of immunoglobulins (types IgA, IgG and IgM) are secreted into milk, conferring some degree of protection to the new-born. The ability of the neonate to counter infectious or toxic agents is limited and thus dependent on the maternally supplied defence in the early stages of life. In addition to the whey proteins mentioned above, albumin (as found in blood serum), lactoferrin and transferrin (iron binding milk proteins), proteases and their resulting peptide fragments (γ-casein is formed by this process), and numerous other proteins have been found in milk (Mepham, 1987). Although it is likely that some of these are essential enzymes involved in the maturation of milk within the lumen, their precise nature and function have yet to be clarified.

1.3.1.3. Transport of amino acids for milk protein synthesis

The milk proteins synthesised within the mammary gland are formed from the amino acids supplied to the gland by capillaries surrounding each individual alveoli. The lactating mammary gland has a large demand for amino acids to meet the requirements for milk protein synthesis. For example, the mammary tissue of a lactating cow secreting 35 litres of milk per day with a protein content of 32g/l needs over 1 kg of amino acids to sustain milk protein synthesis. The mammary gland is able to generate considerable aterio-venous amino acid concentration differences suggesting the presence of amino acid transport systems in the basolateral (i.e. blood-facing) aspect of the mammary epithelium (Hanigan *et al.*, 1992; Linzell and Peaker, 1971; Mepham, 1977; Metcalf *et al.*, 1991; Vina *et al.*, 1981a, 1981b). Furthermore, the finding that some amino acids are concentrated within mammary tissue with respect to plasma (Shennan *et al.*, 1997) suggests that an input of free energy is required to transport some amino acids into mammary epithelial cells. It appears that there are many amino acid transporters present in epithelial cells of the mammary gland (Baumrucker, 1985; Shennan and Peaker, 2000; Kansal and Sharma, 2001). There are two classes of amino acid uptake system within mammary tissue, Na^+-dependent and -independent. There are a number of different transporters in each class with varying degrees of substrate specificity. The Na^+-dependent mechanisms include system A, system ASC, system X^-_{AG} and system β and several other systems with broad substrate specificity which remain to be classified (Shennan and Peaker, 2000; Kansal and Sharma, 2001). System A is one of the predominant transporters for the uptake of neutral amino acids and has been identified in rat (Shennan and McNeillie, 1994a; Tovar *et al.*, 2000), mouse (Neville *et al.*, 1980; Sharma and Kansal, 1999) and bovine mammary tissue

(Baumrucker, 1985). This is by no means a universal transporter as no evidence of it has been found in guinea pig mammary tissue (Mepham *et al.*, 1985). However, system ASC, an alternative system for transport of neutral amino acids, has been identified in both guinea pig and bovine mammary tissue (Mepham *et al.*, 1985; Baumrucker, 1985).

System X^-_{AG} appears to be responsible for the transport of anionic amino acids in rat and mouse mammary tissue (Millar *et al.*, 1996; Millar *et al.*, 1997; Kansal *et al.*, 2000). Two transport proteins, namely GLAST and GLT-1, which could account for system X^-_{AG} activity have been found in rat mammary tissue (Martinez-Lopez *et al.*, 1998). Taurine is a β-amino acid and is an important organic osmolyte in the regulation of cell volume (Huxtable, 1992). Rat and pig mammary tissue have been shown to concentrate taurine in a Na^+-dependent manner that matches the characteristics of the high affinity system β amino acid transporter (Shennan and McNeillie, 1994b; Bryson *et al.*, 2001). Two Na^+-dependent amino acid transport mechanisms which operate with a broad substrate specificity have been identified in mouse mammary gland (Sharma and Kansal, 1999; Rehan *et al.*, 2000).

Investigations into Na^+-independent amino acid transport in mouse (Neville *et al.*, 1980; Verma and Kansal, 1993), rat (Shennan and McNeillie, 1994a), guinea pig (Mepham *et al.*, 1985) and bovine mammary gland (Baumrucker, 1985) have uncovered a system with functional characteristics similar to system L. This transport system has a wide substrate specificity and may represent the single most important amino acid carrier mechanism for neutral amino acids in the mammary gland (Shennan, 1998; Shennan and Peaker, 2000). In addition to system L there is also evidence in mouse mammary tissue for the presence

of system T; this mechanism prefers large aromatic neutral amino acids (Kansal and Kansal, 1996).

Several Na^+-independent amino acid transporters which accept cationic amino acids as substrates have been identified in lactating mammary tissue. Thus systems y^+ and y^+L have been identified in mouse and rat mammary tissue (Baumrucker, 1984; Calvert *et al.*, 1996; Sharma and Kansal, 2000). System y^+ is a mechanism which only accepts lysine, arginine and ornithine as substrates whereas system y^+L accepts both cationic and some neutral amino acids (Deves and Boyd, 1998).

Further studies will undoubtedly reveal the presence of other amino acid transport mechanisms in lactating mammary tissue and future work will focus on the regulation of the transport systems and how they co-ordinate their actions to provide the right mixture of amino acids required for protein synthesis.

Studies aimed at comparing the rate of amino acid uptake and their subsequent appearance in milk protein have indicated that the extraction of some amino acids from the circulation is insufficient to account for their output into milk protein (Backwell *et al.*, 1994, 1996). It has been suggested that the uptake of peptides by the mammary gland may account for the deficit (Backwell *et al.*, 1994). Indeed, a study by Blackwell and others (1994) has shown that the mammary gland is capable of utilizing dipeptides administered into the systemic circulation for casein synthesis. However, it was not clear if the mammary gland transported the peptides intact or whether the peptides were hydrolysed extracellularly followed by uptake of the individual amino acids. Recently, several studies have shown

that the rat mammary gland is capable of transporting dipeptides via a low affinity pathway (Shennan *et al.*, 1998a, 1999). Furthermore, the rat mammary gland is able to hydrolyse dipeptides extracellularly followed by uptake of the free amino acids (Shennan *et al.*, 1998a, 1999).

1.3.1.4. Regulation of mammary protein synthesis

Before considering the molecular aspects of protein synthesis it is important to take into account the diet of the lactating mother. Changes in either the quantity or quality of protein in the diet can have marked effects on both the quantity and composition of the protein component of milk. The importance of maternal diet was highlighted in a study by Geursen and others (1987), which showed that feeding rats on a low protein diet caused a marked decrease in the rates of α-lactalbumin synthesis and secretion, without changing these rates with respect to the caseins. This suggests that the composition of the mammary-synthesised proteins found in milk is partially controlled by the metabolic substrates encountered post-partum.

It is now established that mammary protein synthesis is dependent upon calcium. Thus removing calcium from the incubation medium decreases mammary protein synthesis (Wilde *et al.*, 1981; Smith *et al.*, 1982; Duncan and Burgoyne, 1996). Furthermore, when intracellular stores of Ca^{2+} were depleted there was a significant decrease in protein synthesis and a concomitant reduction in the number of polysomal translation units (Duncan and Burgoyne, 1996). An earlier investigation into the effects of calcium on translational initiation in eukaryotic cells illustrated a similar reduction in polysomes and

an increase in 80s monosomes (Chin *et al.*, 1987). Results from this study identified the inhibition as occuring during the initiation phase of translation, rather than during elongation or termination. It has been proposed that initiation of protein synthesis in eukaryotic cells is regulated by phosphorylation of 2 polypeptides known as eukaryotic initiation factors 2α and 4E (eIF2α, eIF4E) (Pain, 1986; Rhoads, 1993). When eIF2α binds the 40s ribosomal subunit it prevents the association of the 60s ribosomal subunit. This inibition can be reversed by eIF2B which sequesters eIF2α allowing translation to proceed. However, phosphorylation of eIF2α increases its affinity for eIF2B and therefore the less abundant eIF2B molecules are effectively inactivated, allowing eIF2α to bind and inhibit initiation of protein synthesis. In this connection, release of calcium from intracellular stores by vasopressin, which emulates IP_3 activity, led to a reduction in protein synthesis by the phosphorylation of eIF2α and subsequent repression of eIF2B activity (Kimball and Jefferson, 1990).

Cell volume has recently been implicated as a controlling factor in the translation of proteins in rat mammary tissue (Millar *et al.*, 1997). Increasing cell volume by a hyposmotic challenge increased protein synthesis whereas decreasing cell volume by a hyperosmotic challenge resulted in a marked inhibition of protein synthesis in rat mammary explants. Importantly, the study showed that the quantity of casein mRNA remained unchanged, despite significant differences in the amount of casein produced, suggesting that over the short time-frame measured, control of protein synthesis was happening at the translational stage, rather than at transcription.

1.3.2. Lactose synthesis by the mammary gland

Lactose is a disaccharide molecule with glucose and galactose moieties, and represents the simplest of the major milk constituents. Lactose is synthesised within the lumen of the Golgi apparatus by an enzyme complex known as lactose synthetase, which consists of galactosyltransferase (GT) and the milk whey protein α-lactalbumin (Ebner and Schanbacher, 1974; Mepham, 1987; Faulkner and Peaker, 1987). The general reaction catalysed by this enzyme is described by equation 1.1.

$$UDP\text{-}galactose + glucose \rightarrow lactose + UDP \qquad (1.1)$$

The concentration of lactose within milk is species dependent, however in most species it is relatively high. For example, the concentration of the disaccharide in human and bovine milk is respectively 204 and 140 mM (Peaker, 1977). However it should be noted that in some cases, notably species within the suborder pinnipedia (e.g. Californian sea lion, hooded seal), the milk contains no lactose at all (Jenness, 1974). The Golgi and apical membranes of mammary secretory cells are impermeable to lactose which means that the secretion of lactose (together with the milk ions) determines the volume of milk.

1.3.2.1. Glucose transport by the mammary gland

The lactating mammary gland has a large demand for glucose because it is the obligate precursor for lactose synthesis. Therefore the supply and uptake of glucose from the blood is a crucial step in the process of lactose synthesis. Glucose transport across both

the basolateral and Golgi membranes is therefore an important process in milk secretion.

Both Na^+-dependent and Na^+-independent mechanisms of glucose transport have been identified in the mammary gland. The two families of glucose transporter so far identified in mammalian cells are SGLT and GLUT. Transport of glucose via SGLT is Na^+-dependent whereas GLUT transporters mediate Na^+-independent facilitated diffusion. There is evidence for the presence of SGLT1 in rat (Shennan and Beechey, 1995), ovine (Shillingford *et al.*, 1996), bovine (Zhoa *et al.*, 1999) and human (Obermeier *et al.*, 2000) mammary tissue. However, in most cases the precise location of the transporter has yet to be identified. The finding that the efflux of a radiolabelled glucose analogue (3-O-methyl-D-glucose) from rat mammary tissue could be stimulated by reversing the Na^+-gradient suggests that SGLT1 exists on the plasma membrane of mammary cells (Shennan and Beechey, 1995). However, an intracellular location for SGLT1 cannot at this stage be ruled out.

There is good evidence that GLUT1 is expressed in lactating mammary tissue. GLUT1 is a saturable Na^+-independent glucose transporter. Studies in rat (Madon *et al.*, 1989, Burnol *et al.*, 1990), mouse (Prosser and Topper, 1986) and bovine tissue (Zhao *et al.*, 1996) suggest that GLUT1 is present in mammary epithelial cells during lactation. Other members of the GLUT family (GLUT3,-4, and -5) may also be present in mammary tissue of certain species. GLUT3 and GLUT5 mRNA have been found in bovine mammary tissue, although like SGLT1 the location of the transporters has yet to be precisely identified (Shennan and Peaker, 2000). GLUT4 is expressed in virgin and early pregnant rats but not during lactation which suggests that it is more likely involved in glucose

uptake by mammary adipocytes rather than epithelial cells (Shennan and Peaker, 2000).

Once inside the cell, glucose must get into the luminal space of the Golgi apparatus for lactose synthesis. So far, the mechanism of glucose transport in Golgi membranes has not been elucidated (Shennan and Peaker, 2000). However, there is evidence to suggest that GLUT1 may be expressed in a Golgi-enriched membrane fraction isolated from the lactating rat mammary gland (Madon *et al.*, 1990). Therefore, it is possible that GLUT1 allows glucose to reach the site of lactose synthesis within the Golgi lumen. However, there is a possibility that glucose-6-phosphate rather than glucose is transported across the Golgi/ER membrane to reach the site of lactose synthesis (see Leuzzi *et al.*, 2000).

1.3.2.2. Regulation of lactose synthesis

The two main areas of control of lactose synthesis appear to be in the supply of glucose and α-lactalbumin to the Golgi lumen. It is apparent from the above section that the provision of glucose to the mammary gland is very much dependent upon the rate of uptake from plasma. Thus, the control of mammary glucose uptake by mammary epithelial cells will be an important point of regulation. In this connection it has been shown that glucose transport is affected by a variety of factors including a) the stage of lactation b) by prolactin and c) the nutritional state of the lactating dam (Prosser and Topper 1986; Fawcett *et al.*, 1991; Threadgold and Kuhn, 1984). The second possible point of control is the supply of α-lactalbumin to the Golgi lumen. The presence of this milk whey protein is essential in increasing the affinity of GT for glucose. In the presence of μM concentrations of α-lactalbumin, the K_m for glucose is reduced from 1.4M to 5mM

(Ebner and Schanbacher, 1974). This is of course determined by the rate of protein synthesis within the gland, and therefore is subject to many controls.

1.3.3. Milk fat

Lipids are required by the neonate for normal growth and development. Given that the newborn of some species are incapable of synthesising their own complement of lipid it follows that the fat has to be supplied via milk. However, the requirement is species dependent. This means that there is enormous species diversity in both the composition and quantity of fat in milk; extreme examples of this are rhinoceros milk which has little or no fat, compared to certain seal species whose milk contains over 50% fat (Jenness, 1974). Fat occurs in milk in the form of globules enclosed in a milk fat globule membrane (MFGM); the precise composition of the fat contained in these globules is highly variable. However, in the majority of cases, triacylglycerols (TGs) make up the largest fraction of fat within milk; in rats they constitute 87.5% of total lipid and this increases to above 97% in bovine and human milk (Mepham, 1987). In addition to TGs, other lipid classes identified in milk are diacylglycerols, monoacylglycerols, cholesterol esters, cholesterol, free fatty acids and phospholipids.

The lactating mammary gland is able to a) synthesise fatty acids intracellularly from a supply of substrates extracted from the circulation and b) extract fatty acids from blood plasma. The relative importance of fatty acid uptake compared to intracellular synthesis depends upon species, stage of lactation and diet (Baumen and Davis, 1974; Linzell, 1974; Neville *et al.*, 1983). Synthesised fatty acids are generally shorter in chain length ($C_{8:0}$-

$C_{12:0}$) than their dietary counterparts.

1.3.3.1. Carbon sources for milk fat synthesis

The precise composition of the fat component in milk is determined by both de novo synthesis of fatty acids in the mammary gland and uptake of fatty acids derived from diet. The synthesis of fatty acids in mammals is fuelled by 3 main carbon sources. In ruminants the primary sources of carbon for intracellular fatty acid synthesis are acetate or β-hydroxybutyrate; non-ruminants generally use glucose as their primary source of carbon (Mepham, 1987). This difference can be accounted for by the prevalence of each substrate in the organisms. In ruminants, acetate is formed in the rumen as a result of fermentation and is therefore in greater abundance than glucose (Bauman and Davis, 1974). In non-ruminants however fermentation does not occur in the gut and therefore acetate is in short supply. Therefore, glucose has become the primary source of carbon for non-ruminating organisms.

Essential fatty acids that cannot be synthesised *de novo* must be obtained from dietary sources. The lipid fraction of milk is reflective of the dietary complement of fatty acids. There is a reduction in *de novo* synthesised fatty acids when there is a high proportion of fat in the diet whereas a low fat diet leads to an increase in synthesis, to compensate for the change in supply (Munday and Hardie, 1986). Indeed the activity of the fatty acid synthesising enzymes can be reduced allosterically by the presence of fatty acids gleaned from the diet.

1.3.3.2. Uptake of precursors for lipid synthesis

To date, there has been no reported studies of acetate uptake by mammary epithelial cells. Therefore, it is not known if specific carriers are involved in acetate uptake by mammary epithelial cells or whether acetate crosses the phospholipid bilayer (as acetic acid) by simple diffusion. Glucose, as was described in section 1.3.2.1 above, can enter mammary cells via specific membrane proteins.

The ability of the mammary gland to remove fatty acids from plasma, where they exist as triacylglycerol-rich lipoproteins (chylomicrons and very low density lipoproteins or VLDL), is dependent on the activity of lipoprotein lipase (LPL) (Barber *et al.*, 1997). This is due to the fact that the size of these circulating lipid fractions is too great to allow their free movement into the interstitial fluid (Barber *et al.*, 1997). LPL cleaves the fatty acid from position *sn* 1(3) of these molecules in the luminal aspect of the vascular endothelium (Barber *et al.*, 1997). This then frees the fatty acid to move into the interstitium and uptake by the mammary cells can then take place. Questions are still being asked about the method of fatty acid uptake. It is postulated that the movement of fatty acids is facilitated by a transporter known as fatty acid translocase (FAT) (Barber *et al.*, 1997). However, the existence of FAT on mammary epithelial cells, where it would be required to feed the milk fat synthesis system, is still in question (Barber *et al.*, 1997; Shennan and Peaker, 2000).

1.3.3.3. Fatty acid synthesis

Together, free fatty acids and those incorporated into triacylglycerols, diacylglycerols and monoacylglycerols account for over 90% of the milk fat globule (Mepham, 1987). Although fatty acids can be obtained from the mothers' diet, this only accounts for around 50% of fatty acids found in milk, so the requirement of *de novo* fatty acid synthesis is self-evident. The rate determining enzyme in fatty acid synthesis is acetyl-CoA carboxylase (ACC), which catalyses the formation of malonyl CoA from acetyl-CoA and a carbon donor, the first committed step in fatty acid synthesis (equation 1.2) (Wakil *et al.*, 1983).

$$Acetyl\text{-}CoA + CO_2 + ATP \leftrightharpoons Malonyl\text{-}CoA + ADP + P_i \qquad (1.2)$$

The next step in the synthesis of fatty acids is the formation of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH2 (equation 1.3), and is catalysed by fatty acid synthetase (FAS) (Wakil *et al.*, 1983).

$$Acetyl\text{-}CoA + Malonyl\text{-}CoA + NADPH_2 \leftrightharpoons Palmitate + NADP^+ + CoA + H_2O \qquad (1.3)$$

1.3.3.4. Regulation of mammary tissue fatty acid synthesis

It appears that the major point of regulation of fatty acid synthesis occurs at the committing step, catalysed by ACC. Changes in the activity of this enzyme by either allosteric effects or covalent modification results in a concomitant change in the rate of fatty acid synthesis. Allosteric modulators of ACC activity include citrate, which activates

the enzyme, and long-chain fatty acyl-CoA, which inhibits ACC activity (Hardie and Guy, 1980). In this way, the dietary complement of fats and glucose delivered to the gland can affect the rate of synthesis. Regulation of fatty acids synthesis also occurs by the reversible phosphorylation of ACC; when phosphorylated, ACC is inactive and when de-phosphorylated it is active (Kim *et al.*, 1989). There are known to be 8 serine residues on the enzyme recognised as sites susceptible to this covalent modification, but not all of them are involved in the regulation of ACC activity. Only the central Ser 77/79, and C-terminal 1200 and 1215 residues appear to change the activity of the enzyme when in different phosphorylation states (Davies *et al.*, 1990; Ha *et al.*, 1994). In addition to multiple phosphorylation sites there are six known enzymes that can carry out this modification; acetyl-CoA carboxylase kinase-2, protein kinase C, casein kinase-2, calmodulin-activated multiprotein kinase, cyclic AMP-dependent protein kinase and 5'AMP-dependent protein kinase. Early evidence also suggested a role for the phospholipid-dependent protein kinase (Hardie *et al.*, 1986). However, subsequent findings illustrated that phosphorylation by the two nucleotide-dependent enzymes was the most important in reducing ACC activity (Ha *et al.*, 1994). In each of these cases the phosphorylation and therefore deactivation of ACC was found to be reversible by a protein phosphatase. Recent investigations in rat mammary tissue indicate that protein phosphatase 2A (PP2A) is likely to be the enzyme involved in this reactivating process (Baquet *et al.*, 1993). It has been shown to dephosphorylate residues which inactivate the enzyme, and has also been shown to reactivate the enzyme when added following inhibition by kinases.

1.4. CELL VOLUME CHANGE AS A MODULATOR OF CELL FUNCTION

1.4.1. Cell volume changes affect metabolic processes

Although cells have to regulate their volume within relatively narrow limits it is evident that a change in cell volume can effect many metabolic processes (Haussinger, 1996; Lang *et al.*, 1998a). Indeed, it is becoming apparent that cell volume may be a very important signalling system. The general observation is that an increase in cell volume results in the activation of anabolic processes and a decrease in the cellular hydration state activates catabolic processes. For example, cell swelling has been shown, under certain experimental conditions, to increase protein synthesis in hepatocytes (Stoll *et al.*, 1992). Thus cell swelling, induced by a hyposmotic challenge or by isosmotic swelling, increases the incorporation of radiolabelled amino acids into trichloroacetic acid (T.C.A.)-precipitable material. Interestingly, at the same time, cell swelling inhibits proteolysis (Haussinger and Lang, 1991; vom Dahl *et al.*, 1995, 1996). A preliminary report also shows that mammary protein synthesis can be stimulated by a hyposmotic shock (Millar *et al.*, 1997). Cell swelling also stimulates lipogenesis in hepatocytes (Baquet *et al.*, 1991a, 1991b); a hyposmotic challenge increases the incorporation of radiolabelled acetate into lipid. Cell swelling has also been shown to stimulate glycogen synthesis in hepatocytes (Bauqet *et al.*, 1990). On the other hand cell shrinking decreases protein synthesis in hepatocytes and mammary tissue (Stoll *et al.*, 1992).

1.4.2. Challenges to cell volume

The hydration state of cells can be influenced by a variety of factors including anisomotic conditions, cellular metabolism and substrate uptake. In each case it is a change in the balance of osmotically active solutes across the plasma membrane (described by equation 1.4) and the associated movement of water to compensate for that change that results in either an increase or decrease in cell volume.

$$\Delta\Pi = RT\Sigma\theta\,(c_i - c_o) \qquad (1.4)$$

$\Delta\Pi$ is the change in effective osmotic gradient across the cell membrane, c_i and c_o are the respective concentrations of solutes inside and outside the cell, T is the temperature (°Kelvin), R is the gas constant at that temperature and θ is the reflection coefficient of each solute (Haussinger and Lang, 1991).

1.4.2.1. Anisosmotic conditions affect cell volume

Due to the maintenance of homeostasis, only a few cells types, under physiological conditions, will be exposed to anisosmotic conditions *in vivo*. Cells which do experience large changes to the external osmolarity include enterocytes, cells of the kidney medulla and endothelial cells of the hepatic portal vein. However, cells can experience large changes in osmolality under pathophysiological conditions which can have profound effects on the hydration state of the cells.

Indeed plasma fluid osmolalities have been shown to diverge from the normal (~285 mosmol/kg water) to anywhere between 220 and 350 mosmol/kg water (Hoffmann and Simonsen, 1989). Anisosmotic conditions can be due to antidiuretic hormone (ADH), including insensitivity, over-sensitivity and hyper- or reduced secretion. This in turn can lead to significant differences in the amount of water retained in plasma leading to a hypo- or hyperosmotic state. Also during trauma, sepsis and chronic illness there is likely to be changes in the production of catabolic hormones, reductions in muscle glutamine concentrations and irregularities in amino acid metabolism (Haussinger *et al.*, 1993a) all of which can lead to a change in the osmolality of plasma. The use of anisosmotic conditions is, however, an excellent experimental tool which can be used to manipulate cell volume *in vitro*. Thus, a hyposmotic challenge leads to cell swelling whereas a hyperosmotic challenge results in cell shrinking.

1.4.2.2. Substrate accumulation modulates cell volume

Accumulation of osmotically active solutes leads to a change in the hydration state of cells. In particular, uptake via Na^+-dependent transport systems such as (Na^+-K^+-Cl^-) cotransport, Na^+-dependent amino acid carriers and Na^+-dependent sugar transport systems can have a marked effect on cell volume. Sodium-dependent transport systems are able to generate large chemical gradients across cell membranes due to the significant trans-membrane electrochemical Na^+-gradient. Thus, substrate accumulation will increase the intracellular osmotic pressure which in turn will lead to the movement of osmotically obliged water into the cell (Haussinger and Lang, 1991).

The volume of the perfused liver has been shown to increase significantly when the perfusate was supplemented with glutamine (Baquet *et al.*, 1991a, 1991b). The addition of glutamine to the extracellular compartment raised intracellular glutamine (>30 mM) to such an extent that the cellular hydration state was increased. It appears that glutamine uptake via system N, a Na^+-dependent carrier, was responsible for the increase in the intracellular levels of glutamine (Kilberg *et al.*, 1980). It has been demonstrated that the transport of other amino acids (e.g. taurine) can lead to an increase in cell volume (Plomp *et al.*, 1990; Baquet *et al.*, 1991a; Hallbrucker *et al.*, 1991a).

1.4.2.3. The rate of metabolism affects cell volume

The rate of cellular metabolism can affect the cellular hydration state. It is clear that as larger particles, such as proteins, are being synthesised the pool of small, more osmotically active particles is depleted. Thus, if the rate of protein synthesis is high there must be a similarly high rate of uptake of amino acids to maintain the osmotic gradient, or water will tend to move out of the cell towards the higher concentration of solutes. Similarly, if the rate of protein synthesis is low, amino acids will accumulate and increase the osmotic pressure within the cell.

1.4.2.4. Hormones can influence cell volume

The influence of hormones on the steady state volume of hepatocytes has been investigated in numerous studies (vom Dahl *et al.*, 1991a, 1991b; Hallbrucker *et al.*, 1991b). In these studies it has been shown that insulin has the net effect of increasing cell

volume. Conversely glucagon appears to reduce the volume of isolated hepatocytes. Speculation as to the cause of these changes in volume led to the discovery that a number of ionic transporters normally involved in the regulation of cell volume are affected by insulin (Haussinger and Lang, 1991). Thus, systems reported to be activated in response to insulin include (Na^+-K^+-Cl^-)-cotransport, Na^+/H^+ exchange and activation of the Na^+/K^+ ATPase. The most important effect of these different mechanisms is the net accumulation of K^+. This accumulation has been seen to increase the steady state volume by approximately 10% after 20-30 min from the onset of insulin treatment (Zhande and Brownsey, 1996). Glucagon decreases cell volume due to the production of cAMP which in turn activates Cl^- channels and quinidine/Ba^{2+}-sensitive K^+ channels (Haussinger and Lang, 1991). The net effect is a decrease in $[K^+]_i$ and a concomitant decrease in intracellular water. The change in volume stimulated by the addition of glucagon is approximately -10%. Both effects have been measured at levels of hormone encountered under physiological conditions.

1.4.2.5. Oxidative stress affects cell volume

It is evident that oxidative stress exerted by hydroperoxides induces cell shrinkage in hepatocytes as a consequence of activating K^+ channels (Saha *et al.*, 1993). It has been reported that the production of hydrogen peroxide during the oxidation of monoamines in hepatocytes leads to K^+ efflux and hence cell shrinking.

1.4.3. Regulation of cell volume

As noted above, cells must maintain their volume within relatively narrow limits in order to survive. Therefore, in the face of many challenges to cell volume (see sections above), it is not surprising to find that cells have developed methods to allow them to regulate their volume. If cells are placed in a hyposmotic buffer they initially behave as perfect osmometers and swell, but subsequently reduce their volume. This process is termed a regulatory volume decrease (RVD), which depends on a reduction in the total quantity of intracellular osmolytes. On the other hand, cells also act like perfect osmometers when they are bathed in a hyperosmotic medium, but following the initial shrinking they increase their volume towards normal. This response is called a regulatory volume increase (RVI) and relies upon an increase in the cellular uptake of solutes and osmotically obliged water.

1.4.3.1. Regulatory volume decrease (RVD)

RVD is facilitated by a number of different mechanisms which ultimately result in the efflux of osmolytes from the cell. The main systems for the correction of volume are opening of K^+ and anion channels and/or the activation of (K^+-Cl^-)cotransport (O'Neill, 1999). There is a wealth of data relating to the parallel activation of K^+ and Cl^- channels (Lang *et al.*, 1998b), indicating the importance of these mechanisms in the correction of cell volume perturbations, in a large number of cell types. In addition, there is evidence for the parallel activation of K^+/H^+ exchange and Cl^-/HCO^-_3 exchange following cell swelling (Cala, 1983).

Organic osmolytes, such as amino acids, are also believed to play a role in cell volume regulation following swelling. Thus, if cells are swollen by a hyposmotic challenge the intracellular free amino acid pool, which significantly contributes to the total internal osmolality, decreases. This is a result of amino acids leaving the cell rather than a change in amino acid metabolism (Hoffmann and Lambert, 1983). For example, if Ehrlich ascites tumour cells are incubated in hypotonic medium the decrease in the cellular content of amino acids is accompanied by an equivalent increase in the extracellular amino acid content (Hoffmann and Lambert, 1983). Volume-sensitive amino acid efflux appears to be a widespread phenomenon (Kirk, 1997). The amino acids most affected by cell swelling are non-essential amino acids such as taurine, glycine and the anionic amino acids glutamate and aspartate.

It has been suggested that volume-activated amino acid efflux is mediated by volume-activated anion channels. This suggestion was originally based on the finding that swelling-induced amino acid transport could be attenuated by inhibitors (i.e. DIDS, NPPB, niflumate) of volume-sensitive anion channels (Kirk *et al.*, 1992). However, it must be borne in mind that channel inhibitors are notoriously non-specific (Cabantchik and Greger, 1992; Banderali and Roy, 1992; Boese *et al.*, 1996; Jackson and Strange, 1993). There is, however, convincing evidence from patch clamp studies of MDCK and C-6 glioma cells that amino acids such as taurine and glutamate are able to permeate volume-sensitive ion channels. However, there is now a considerable body of evidence to suggest that volume-sensitive amino acid efflux is a separate pathway from volume-activated anion channels (Davis-Amaral *et al.*, 1996; Lambert and Hoffmann, 1994; Roman *et al.*, 1996; Shennan *et al.*, 1994; Shennan *et al.*, 1997).

Swelling-induced solute transport pathways tend to be activated very quickly following a perturbation to the cellular hydration state. For example, volume-activated amino acid transport systems are activated within several minutes following cell swelling (Kirk, 1997). It has been suggested that cell swelling activates pathways which are already present in the membrane which are quiescent under isosmotic conditions (Kirk, 1997).

1.4.3.2. Regulatory volume increase (RVI)

RVI depends on the accumulation of osmotically active solutes into the cell. In particular, cells accumulate ions and organic osmolytes following cell shrinking. Ion transport processes which have been implicated in RVI include (Na^+-K^+-Cl^-) cotransport and Na^+/H^+ exchange functionally coupled to Cl^-/HCO_3^- exchange (Hoffmann and Simonsen, 1989).

Cells, when faced with a hypertonic environment accumulate certain small organic osmolytes such as amino acids, betaine and myo-inositol (Lang et al., 1998a). The transport of these molecules is coupled to Na^+ which means, under certain circumstances, that the intracellular/extracellular concentration ratio of the compounds can exceed 500 (Kwon and Handler, 1995). Transport systems which are activated by hyperosmotic conditions include the Na^+-dependent betaine transporter BGT1, the Na^+-inositol cotransporter SMIT and the Na^+-dependent taurine transporter NCT1 (Kwon and Handler, 1995).

It should be noted that the activation of the sodium-dependent organic osmolyte

transporters occurs several hours following a hyperosmotic challenge. Accordingly, the transcription of mRNA for the transport systems is affected by cell shrinking (Kwon and Handler, 1995).

1.4.3.3. Volume-activated transport processes in mammary tissue

Cell swelling, induced by a hyposmotic shock, increases the efflux of radiolabelled taurine from rat mammary tissue explants (Shennan *et al.*, 1994). This volume activated efflux of taurine is a) independent from the Na^+-gradient b) inhibited by anion transport inhibitors such as DIDS and NPPB and c) relatively temperature-insensitive. Volume-activated taurine efflux is a rapid process; taurine efflux increases within 30 sec following an osmotic challenge. Moreover, taurine efflux induced by a hyposmotic shock is a fully reversible process. Volume-sensitive taurine efflux has been demonstrated in the *in situ* perfused lactating gland suggesting that the pathway is present in the basolateral (i.e. blood-facing) aspect of the mammary epithelium (Calvert and Shennan, 1998). Evidence suggests that taurine efflux from the rat mammary gland does not utilise volume-activated anion channels (Shennan *et al.*, 1994).

It has been recently shown that $K^+(Rb^+)$ efflux from rat mammary tissue can be stimulated by a cell swelling. A hyposmotic shock increased the efflux of $K^+(Rb^+)$ from rat mammary tissue explants via a pathway which was not inhibited by the loop diuretic bumetanide suggesting that (Na^+-K^+-Cl^-) cotransport is not involved (Shennan and Gow, 2000). Moreover, volume-sensitive $K^+(Rb^+)$ efflux from mammary tissue was not inhibited when Cl^- was replaced by NO_3^- ruling out a role for (K^+-Cl^-) cotransport. Instead the results

from the study suggest that cell swelling activates $K^+(Rb^+)$ efflux via a channel (Shennan and Gow, 2000). To date, no reports of transport pathways activated by shrinking mammary cells have been published.

1.5. SPECIFIC AIMS AND OBJECTIVES

Preliminary investigations from this laboratory have shown that rat mammary tissue responds to an increase or decrease in cell volume by respectively increasing or decreasing the rate of protein synthesis (Millar *et al.*, 1997). The first stage of the current investigation was to confirm these initial findings and thereafter to further characterise the effects of cell volume on mammary protein synthesis. Thus, the sensitivity, reversibility, and the calcium dependence of volume-sensitive protein synthesis in rat mammary tissue was examined. In addition the effect of isosmotic cell swelling was examined on mammary protein synthesis. The effect of various pharmacological inhibitors on the rate of volume sensitive protein synthesis was also investigated. The effect of altering the cellular hydration status on cytosolic calcium was also investigated given the relationship between calcium and mammary protein synthesis. Finally, the effects of cell volume changes on mammary lipogenesis were studied.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1. Radiochemicals

L-[4,5-^{3}H]-leucine (61 Ci/mmol) and L-[^{35}S]-methionine (1000 Ci/mmol) were obtained from Amersham Life Science Ltd, UK. [1-^{14}C]-acetic acid (65 mCi / mmol) was purchased from ICN (Thame, Oxfordshire, UK).

2.1.2. Chemicals

Collagenase Type 2 (EC 3.4.24.3) was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). 'Ultima Gold' scintillation agent and 'Toluene Scintillator' were purchased from Packard Instrument B.V. (Pangbourne, Berks., U.K.). Guanidinium isothiocyanate was obtained from Melford Laboratories (Ipswich, Suffolk, UK). ATP standards and Luciferase reagents were part of an ATP assay kit supplied by Calbiochem (Beeston, Nottinghamshire, UK). All other chemicals were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

2.1.3. Rats

Primiparous female rats of the Wistar strain (A. Tuck and Son, Rayleigh, Essex, U.K.) were

fed on standard rat chow (CRM Irradiated Diet, Labsure, Cambridge, U.K.) *ad libitum* and allowed free access to water. They were housed at a constant temperature of 17°C and maintained on a 12 hour light/dark cycle. Animals were housed in groups of up to three individuals on wood shavings from mating and then singly prior to parturition. After parturition, animals were given shredded paper as nesting material and the pup numbers were adjusted to 10 per mother, where possible. Experiments were conducted on females at day 9 to 13 of lactation.

2.2. PREPARATION OF MAMMARY TISSUE

2.2.1. Explants

Rats were stunned with a blow to the head and were then killed by cervical dislocation. The right inguinal mammary gland was immediately removed and placed in ice-cold buffer consisting of (mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 or 20 Tris-MOPS, pH 7.4. The tissue was gassed for 5-10 minutes with 100% O_2 to remove excess blood and milk. The gland was removed from the buffer and all surface connective tissue removed. It was then cut into strips, less than 1mm in width that were further dissected to pieces between 0.1 and 1mg in weight. Once prepared, the explants were kept in ice cold buffer, similar in composition to that described above. The dissection process took no more than 3 hours from the death of the rat to completion of explants.

2.2.2. Acini

Rats were killed as described in section 2.2.1. Both inguinal mammary glands were removed and placed in ice cold extraction buffer consisting of: (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4, and were gassed with 100% O_2 to remove excess blood and milk. Both glands were stripped of superficial connective tissue and weighed. A total weight of approximately 5g was required for each acini preparation. The tissue was chopped in 10ml of buffer (detailed above), using a pair of scissors. The tissue was then sieved and washed with 10ml of fresh buffer. The tissue was transferred to a 250ml conical flask containing 30ml of collagenase digestion medium (buffer described as above but supplemented with 5% Ficol type 400 (w:v), 600mg bovine serum albumin and 30mg collagenase type 2). This was gassed for 5 minutes with 100% O_2, shaken vigorously and then placed in a shaker water bath (Grant OLS200) set at 37°C and 180 cycles/min.

After a forty-minute incubation the suspension was shaken vigorously and then poured through a sieve into a 30ml centrifuge tube, to remove any large pieces of undigested tissue. The digestate was centrifuged for 15 seconds at 550 x g (1800rpm; Centaur 1, MSE Scientific Instruments, Sussex, U.K.). The supernatant was decanted off and the acini were re-suspended in 15ml of wash buffer (buffer as above with 2% Ficol type 400 (w:v) with gentle agitation. The re-suspended acini were again centrifuged at 550 x g for 15 seconds. This wash process was repeated twice more before the pellet was re-suspended in a buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. The volume of buffer used to re-suspend the acini was dependent on the type of experiment (the precise composition of the buffers is given in the figure legends). On completion of the wash

process a small sample of the acini were removed and tested for cell viability using 'Trypan Blue' dye. A picture of the prepared acini is shown in figure 2.1.

2.3. MEASUREMENT OF PROTEIN SYNTHESIS IN MAMMARY EXPLANTS

Protein synthesis by mammary explants was measured using a modification of the technique described by Millar et al (1997).

Explants were prepared as described in section 2.2.1. The explants were placed in 20ml glass scintillation vials containing medium (see figure legends for details), maintained at 37°C. Each incubation medium contained amino acids at concentrations similar to that found in rat plasma (table 2.1).

There was a pre-incubation period of fifteen minutes prior to the addition of a radio-labelled amino acid (L-[4,5-^{3}H]-leucine or L-[^{35}S]-methionine). Tracer was added to give an activity of 1μCi/ml. Explants were gassed throughout with 100%O_2. At specific time points, a sample of the explants (5-25mg) was removed from the incubation vial, washed with ice cold buffer (containing (mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4), blotted on a piece of filter paper (Whatman No. 1) and then placed in a pre-weighed 1.5ml Eppendorf tube, containing 1ml of 10% trichloroacetic acid (TCA). The tubes were immediately vortexed and then left for at least 30 minutes.

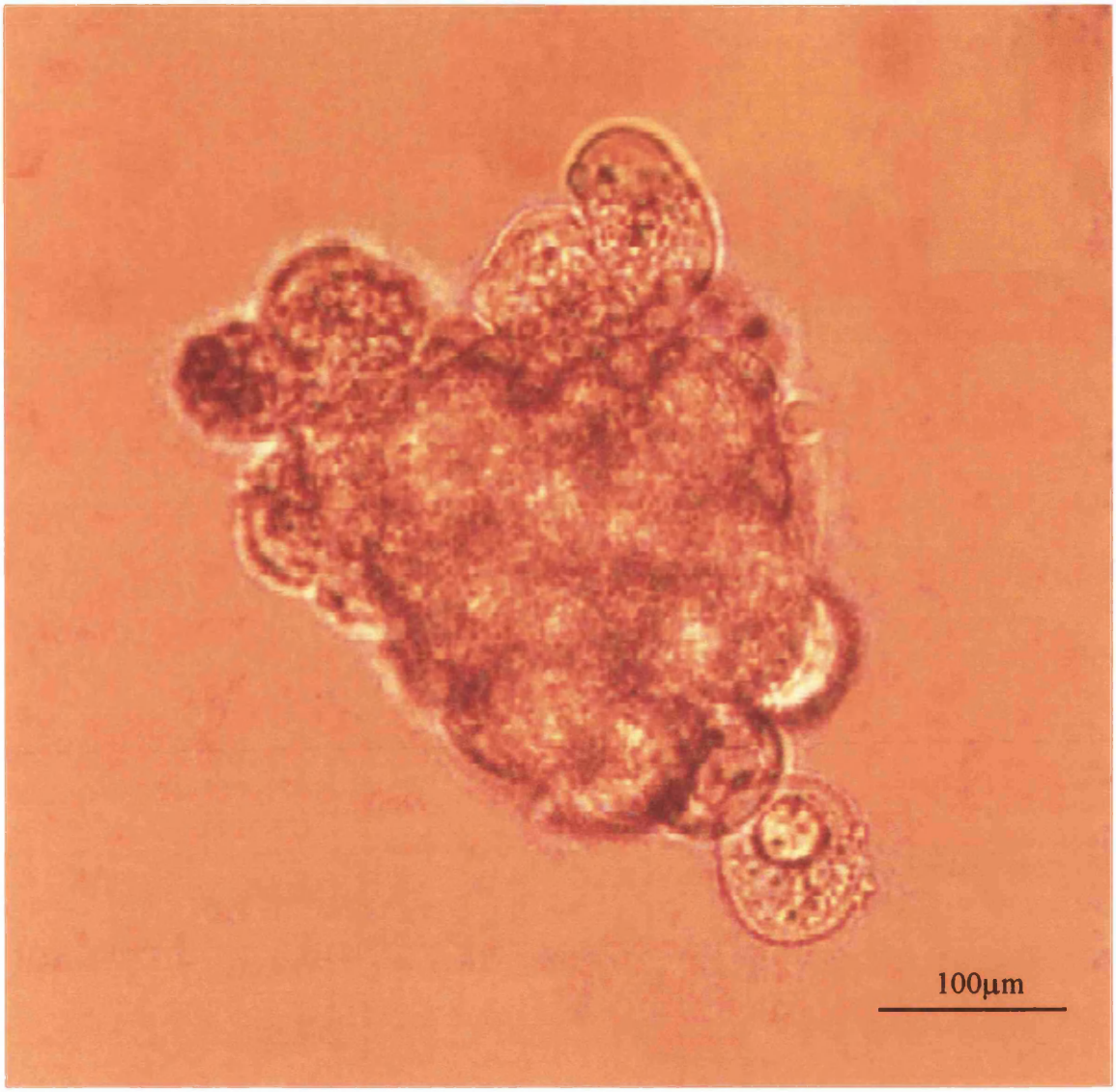

Figure 2.1: Photograph of rat mammary acini, x400 magnification (courtesy of Dr. Iain Gow).

Table 2.1: Concentrations of amino acids used in protein synthesis experiments. Amino acid concentrations were taken from Millar et al. (1997).

Amino Acid	Concentration (mM)
Alanine	0.680
Arginine	0.130
Asparagine	0.045
Aspartate	0.050
Cysteine	0.160
Glutamate	0.100
Glutamine	0.680
Glycine	0.350
Histidine	0.245
Isoleucine	0.145
Leucine	0.225
Lysine	0.245
Methionine	0.115
Phenylalanine	0.115
Proline	0.250
Serine	0.445
Threonine	0.390
Tryptophan	0.086
Tyrosine	0.150
Valine	0.240

The samples were spun at 13,000 x g for 2 minutes and the T.C.A. removed. 1ml of fresh T.C.A. was added and the tubes vortexed again for a minimum of a minute. This washing process was repeated thrice after which there was a minimal amount of free tracer observed in the supernatant, illustrated in figure 2.2. After the last wash, the T.C.A. supernatant was discarded and the samples were dried using blotting paper. The samples were weighed and 200µl of 90% formic acid was added. Each tube was vortexed and then left for a minimum of 16 hours for the tissue to dissolve. Once the tissue samples had completely dissolved they were removed using plastic pastettes and were placed in a 20ml plastic scintillation vial. The

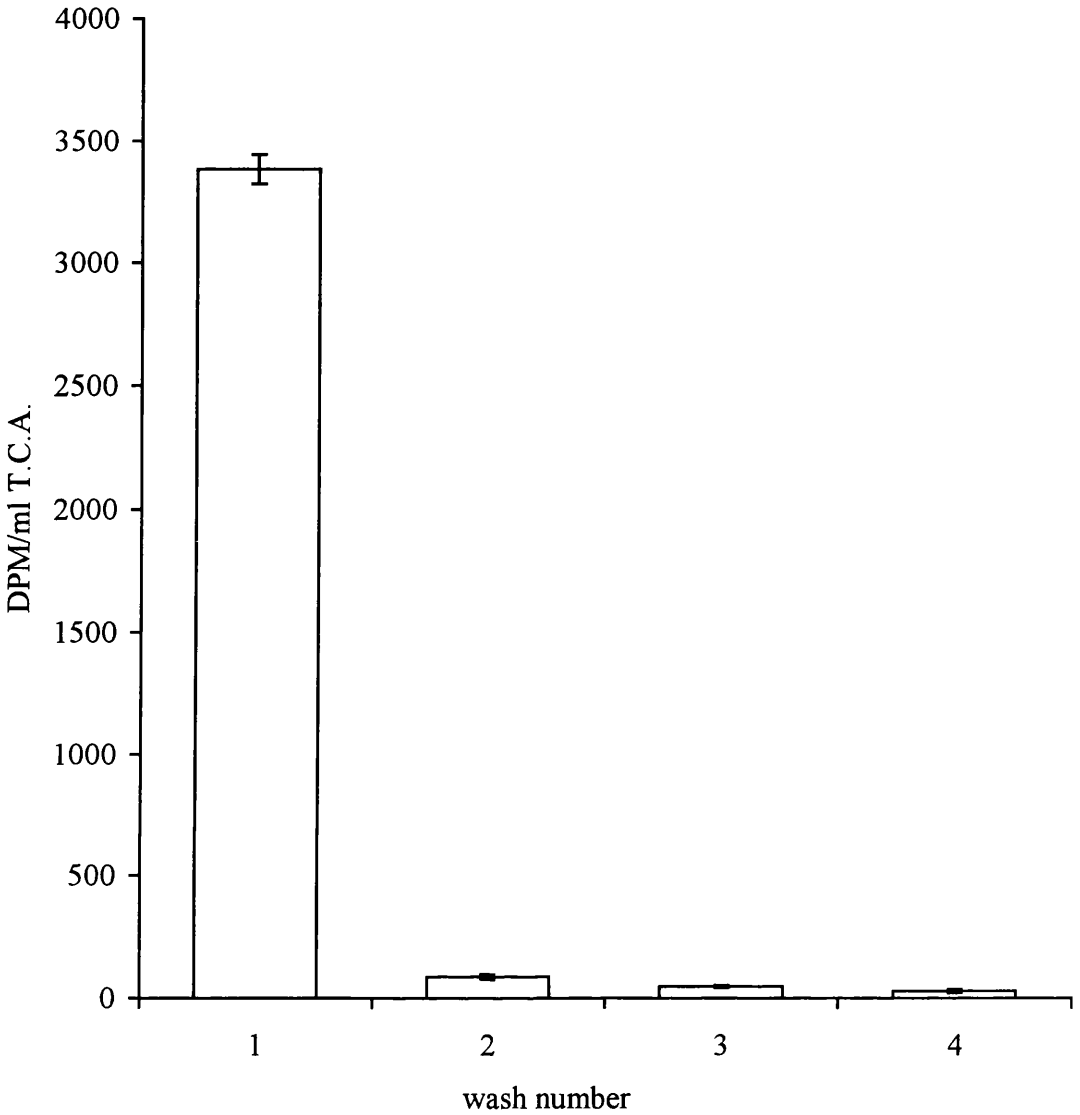

Figure 2.2: Appearance of free [^{3}H]-leucine in T.C.A. used to wash mammary explants after incubation. After each addition of T.C.A. the explants were vortexed and then centrifuged. The supernatant was removed and then placed in a scintillation vial. 20ml of 'Ultima Gold' scintillation cocktail was added and then counted for activity. Data are means of two sample vials ±SD.

Eppendorfs were washed using 900µl of dH_2O which was then added to the formic acid extract. 20ml of 'Ultima Gold' scintillant was added to the vials which were then shaken vigorously.

In parallel experiments the wet/dry ratios of mammary explants was determined. Approximately 10mg of the explants were removed and placed in pre-weighed glass scintillation vials. They were weighed and placed in an oven at 110°C for a minimum of 16 hours. After the drying process they were re-weighed and the dry/wet ratios were calculated. This was then used to calculate the dry weight of the tissue in the protein samples. The specific activity of the tracer was determined by taking 100µl samples of medium, in duplicate, from each incubation vial. These were supplemented with 20ml 'Ultima Gold' scintillant and counted. Results were calculated using equation 2.1.

$$\mu mole/g\ dry\ tissue\ weight = (dpm_t \div (W \times d)) \div N_o \qquad (2.1)$$

where W is the wet weight of the tissue sample (g), d is the dry/wet weight ratio, N_o is the specific activity of the tracer (dpm/µmole amino acid) and dpm_t is the activity associated with the tissue sample.

2.4. Measurement of Protein Synthesis in Mammary Acini

The protocol for measuring protein synthesis in mammary acini was adapted from the technique used by Millar et al. (1997). Acini were prepared as described in section 2.2.2. The acini were re-suspended in a buffer of which both type and volume were determined by the

type of experiment. The acini were then transferred to glass vials maintained at 37°C. Prior to addition of tracer there was a 10 minute pre-incubation. Addition of [^{3}H]-leucine (to 1μCi/ml) was considered time zero and at predetermined time points, 1.5ml aliquots of acini were transferred from the incubation vials to pre-weighed 2ml Eppendorfs. They were immediately centrifuged at 13,000 rpm on pulse (MSE Micro Centaur), after which the supernatant was removed and replaced with 1ml 10% T.C.A. The tubes were vortexed and left for a minimum 30 minutes.

After this, tubes were centrifuged at 13,000 rpm for 2mins and the supernatants were removed using glass pastettes. 1ml of fresh 10% T.C.A. was added to each tube and the pellets were re-suspended by vortexing. This washing process was repeated twice for each sample after which the level of free label in the supernatants was comparable to background. The supernatants were discarded and the pellets dried using blotting paper (Whatman No. 1). Each Eppendorf tube was re-weighed to determine the weight of the T.C.A. precipitate prior to the addition of 200μl of 90% formic acid. The samples were vortexed and left for a minimum of 1 hour, to solubilize the T.C.A. precipitate. Following this, the samples were transferred to 20ml plastic scintillation vials using plastic pastettes. The Eppendorfs were washed using 900μl aliquots of dH_2O, which were then also transferred to the scintillation vials. The samples were prepared for counting by adding 20ml of 'Ultima Gold' scintillation agent. Specific activity of the tracer was determined as described in section 2.3.2. Results were calculated using equation 2.2.

$$\mu mole/g\ dry\ tissue\ weight = (dpm_p \div Wp) \div N_o \qquad (2.2)$$

where W_p is the precipitate sample weight (g), N_o is the specific activity of leucine in the incubation buffer (dpm/μmole leucine) and dpm_P is the activity associated with the precipitate pellet

2.5. MEASUREMENT OF CELLULAR ATP LEVELS IN MAMMARY ACINI

The technique used to extract the ATP and isolate it was modified from the technique used by Clegg and Calvert (1988).

2.5.1. Preparation, extraction and isolation of adenine nucleotides

Acini were prepared as described in section 2.2.2. After the final wash the acini were re-suspended in buffers depending on the type of experiment (the precise composition of the buffers are given in the figure legends). The acini were then transferred to incubation vials maintained at 37°C. The acini were incubated for a total of 30 minutes, incorporating a 10 minute pre-incubation (as used in the acini protein studies). Acini were constantly gassed with 100% O_2, and frequently agitated by light swirling. At the end of each incubation 450μl of the acini suspension were removed and transferred to a pre-weighed 2ml Eppendorf containing an equal volume of 12% perchloric acid v/v (P.C.A.). The samples were mixed vigorously and left for a minimum of one hour. They were then mixed for a further 2 minutes on a vortex mixer and centrifuged for 2 minutes at 13,000rpm (MSE MicroCentaur). The supernatant was removed and mixed at a 1:1.1 ratio with an organic amine solution (equal volumes of tri-n-octylamine and 1,1,2-trichlorotrifluoroethane/ "Freon") in a 2ml Eppendorf, on a vortex mixer for 1 minute. The mixture was then centrifuged for 2 minutes in an MSE MicroCentaur at

3000 rpm. This caused the mixture to form 3 distinct layers. The top layer was removed and retained for analysis of ATP content, the rest was discarded. The P.C.A. pellet was dried using blotting paper and then weighed.

2.5.2. Determination of ATP concentration

The concentration of ATP in each extract was determined using an LKB L1250 Luminometer (Bromma, Sweden). Each ATP extract was diluted 20-fold using distilled water. The reaction vessel prior to addition of the sample contained 400µl of Hepes buffer and 100µl of the luciferase reagent. A 5µl sample of each extract was added to the reaction vessel and the luminescence recorded for ~30seconds. Readings were compared against a 5µl standard of 10µM ATP. Results were calculated using equation 2.3.

$$\mu moles/g\ P.C.A.\ precipitate = \left(\left(\frac{R_A \times S}{R_S}\right) \times a\right) \div wt_A \qquad (2.3)$$

where R_A is the value for the acinar sample (mV), R_S is the value for the standard (mV) and wt_A is the weight of the acinar PCA pellet, a is the sample dilution constant (3600), and S is the standard dilution constant (19.7).

2.6. Measurement of cellular calcium using fura-2 fluorescent dye

Intracellular calcium was measured in mammary acini using the fura-2 fluorescent dye technique, adapted from Sudlow and Burgoyne (1997), and Duncan and Burgoyne (1996).

2.6.1 Preparation of acini

Acini were prepared as described in section 2.2.2. After the final wash the acini were re-suspended in a buffer containing: (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. 50µl of 1mM Fura-2 AM (Fura-2 acetoxymethyl ester in DMSO and 0.02% Pluronic w:v) was added and the acini were thereafter incubated in the dark and at room temperature for a minimum of 40 minutes. 2ml of the acini suspension was used to determine background fluorescence. Following the loading period, the acini were washed three times, using buffer (similar in composition to that described above), by centrifugation (1800 x g for 15 seconds – MSE Centaur 1) and re-suspension (10ml buffer). After the third wash the acini were re-suspended in buffer and were incubated for a further 40 minutes, to allow for leaching of fura-2 from damaged cells. The acini were then washed (x3) again. The fura-2 loaded acini were re-suspended in a buffer as described in the figure legends. The background acini were re-suspended in 2ml of buffer as described above.

2.6.2. Measurement of $[Ca^{2+}]_i$ in mammary acini

Acini were incubated in a fluorimeter (Perkin-Elmer LS-5 Luminescence Spectrometer) maintained at 37°C by a heated water circuit. Fluorescence was stimulated by excitation wavelengths of 340 and 380nm, and was recorded at an emission wavelength of 509nm. The background sample was read first. The fluorescence was measured for 1 minute at which point 1.5ml of buffer (as described in section 2.6.1) was added and the readings continued for a further minute. When calculating the change in fluorescence of the loaded samples the background before or after the dilution was subtracted, depending on the volume of the acini

suspension.

The fura-2 loaded acini were analysed for changes in $[Ca^{2+}]_i$ at different time points (see figure legends for details). The fluorescence was measured for a minimum of 4 minutes after the addition of the test solution, to ensure that the full profile of $[Ca^{2+}]_i$ change was recorded. Maximum and minimum fluorescences were calculated using a standard consisting of 5μM fura-2 free acid in the buffer (as described in section 2.6.1). The maximum fluorescence was measured in the presence of 10mM $CaCl_2$ and the minimum fluorescence in the absence of free Ca^{2+} (by the addition of 10mM Tris-EGTA, pH 7.4). The concentration of calcium was calculated using equation 2.4:

$$[Ca^{2+}]_i = Kd \times B \times [(R - R_{min})/(R_{max} - R)] \qquad (2.4)$$

where R is the ratio of light emitted following stimulation at 340/380nm. R_{max} and R_{min} represent the maximum and minimum ratios of fluorescence obtained using the standards. B is the ratio of the 380 signal in the absence of Ca^{2+} to the 380 signal in the presence of 10mM Ca^{2+}. Kd was taken to be 224nM.

2.7. LIPOGENESIS IN RAT MAMMARY EXPLANTS

Lipogenesis was measured in rat mammary tissue explants by [^{14}C]-acetic acid incorporation into lipid. The method for extraction and isolation of lipid was modified from that used by Travers and Barber (1999).

2.7.1. Preparation of tissue and sample collection

Explants were prepared as described in section 2.2.1. Explants were incubated in a water bath maintained at 37°C, the volume and composition of medium depended on the experiment (for details see figure legends). There was a pre-incubation period of 15 minutes during which the explants were gassed with 100%O_2. The [^{14}C]-acetic acid was added at a ratio of 1µl/ml of incubation medium, giving 1µCi/ml. Following addition of isotope, the explants were incubated for 1hr after which the tissue was removed, washed with ice cold buffer (containing (mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH7.4), blotted (Whatman No. 1 filter paper) and placed in a pre-weighed 1.5ml Eppendorf tube. The sample was weighed and then 400µl of GIT-RPA (5M guanidinium isothiocyanate and 0.1M EDTA, pH 8) was added. Samples were left for a minimum of 16 hours to solubilize the tissue.

2.7.2. Chloroform extraction of lipid from samples

400µl of dH_2O-saturated chloroform was added to each GIT-RPA sample and the mixture was vortexed for a minimum of 1 minute. The samples were centrifuged for 5 minutes at 10,000 rpm (MSE MicroCentaur), causing the suspension to form two distinct layers. 200µl of the chloroform (lower) layer was removed and transferred to a glass scintillation vial. The vials were then placed on a heating plate set at 60°C in a fume hood to dry the samples. After all of the chloroform had evaporated, 1ml of 'Toluene Scintillator' (Packard) was added, the samples were capped and shaken, and then left for an hour to re-solvate the lipid. Finally, 4ml of 'Ultima Gold' scintillation cocktail was added to each of the samples.

To correct for [^{14}C]-acetic acid bound to lipid, rather than incorporated, a background sample was taken 2 minutes after addition of tracer. It was then put through the same extraction procedure as the other lipid samples. When calculating the results, the background was removed from each of the other samples.

The specific activity of the tracer in each incubation medium was calculated by taking a 10µl sample. To this 1ml of 'Toluene Scintillator' and 4ml of 'Ultima Gold' scintillation agent were added and the tubes were vigorously shaken. This was done in duplicate for each incubation medium. A second sample was also taken from each medium for the calculation of the dry/wet weight ratios (as described in section 2.3.1). Results were calculated as a function of the mammary tissue dry weight and were expressed as % of the control values which in each case was the isosmotic medium.

2.8. Statistics

Differences were assessed by Student's paired or unpaired *t*-test as appropriate and were considered different when $P < 0.05$.

CHAPTER THREE

PROTEIN SYNTHESIS IN RAT MAMMARY TISSUE

3.1. INTRODUCTION

It is well established that a change in cell volume, also termed the cellular hydration state, can have a profound effect on the rate of various metabolic paths (Lang *et al.*, 1998a). Studies in a variety of tissue types have led to the conclusion that an increase in cell volume acts as a general anabolic signal whereas cell shrinking activates catabolic processes. It is predicted that during lactation, the large flux of nutrients across the mammary epithelium will result in frequent changes to the hydration state of the epithelial cells (Shennan and Peaker, 2000). In this connection, a preliminary study has shown that rat mammary protein synthesis is sensitive to a change in the cellular hydration state (Millar *et al.*, 1997). Thus, an increase in cell volume, caused by a hyposmotic challenge, resulted in a marked increase in total protein synthesis and cell shrinking, caused by hyperosmotic shock, inhibited protein synthesis. However, the mechanisms underlying these fundamental observations remain unknown. Therefore, the purpose of the present study was to confirm and extend the preliminary findings that cell volume regulates mammary protein synthesis. Therefore, the effect of cell volume on protein synthesis in rat mammary explants and acini was investigated.

3.2. METHODS

3.2.1. Protein synthesis in rat mammary explants

Protein synthesis in rat mammary explants was quantified by measuring the incorporation of a radiolabelled amino acid into T.C.A. precipitable material. The two tracers chosen for the experiments were [^{3}H]-leucine and [^{35}S]-methionine. Explants were incubated in solutions containing a full complement of amino acids similar to that found in rat plasma (see table 2.1). At predetermined time points, explants were removed and placed in a pre-weighed vial containing 1ml of 10%T.C.A. Following that, each vial was re-weighed and then left for a minimum of 60 min. The vials were then centrifuged at 13,000 x g and the resultant pellet washed thrice by centrifugation and re-suspension with fresh 10% T.C.A. After the last wash the samples were dissolved in 90% formic acid, mixed with distilled water and scintillation cocktail. The specific activity of tracer in each incubation vial was measured by counting the radioactivity in 100µl samples. The total tissue water was determined by drying explants at 110°C to constant weight.

3.2.2. Protein synthesis in rat mammary acini

The rate of protein synthesis was measured in rat mammary acini by incorporation of [^{3}H]-leucine into T.C.A-precipitable material. Acini were incubated in buffers (see figure legends for details) at 37°C, under constant gassing with 100% O_2. Prior to the addition of the tracer there was a 10 minute pre-incubation period. At predetermined times a sample of the acinar suspension was removed and centrifuged (13,000 rpm on pulse). The

supernatant was removed and the pellet was resuspended in 10% T.C.A. The sample was left for a minimum of 30 minutes and then washed twice more by centrifugation and resuspension. After the final wash the pellet was blotted and weighed before being dissolved in formic acid (90%).

3.2.3. Determination of ATP in rat mammary acini

The concentration of ATP in mammary acini was determined by a bioluminescent firefly luciferin luciferase assay (adapted from the technique of Clegg and Calvert, 1988). The enzyme, firefly luciferase, catalyses the activation of D-luciferin by ATP and its subsequent oxidation to electronically excited oxyluciferin. The transfer of the excited molecule to its ground state results in the emission of light (figure 3.1 shows the reaction). The reagents are designed such that the intensity of light emitted is directly proportional to the quantity of ATP available for the reaction. Acini were incubated in their respective buffers for a total of 30 minutes, maintained at 37°C and constantly gassed with 100% O_2. After the 30 minutes, the acini were treated with 12% PCA (v:v) to stop the reaction. The ATP was extracted from the acid by precipitation with an organic amine solution (see section 2.5 for details). The samples were measured on an LKB L1250 Luminometer and results were displayed as mV. Each data-point was given a molar value by comparison with a 10μM ATP standard.

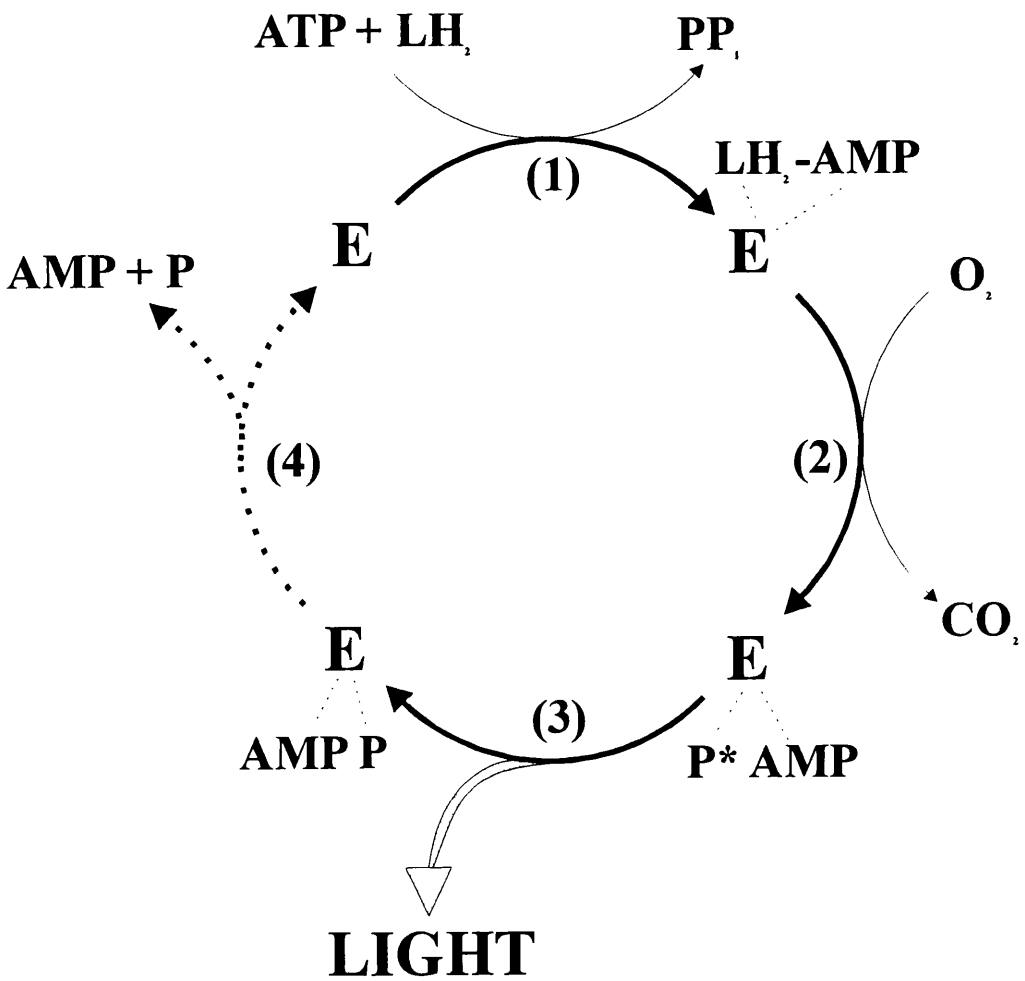

Figure 3.1: Schematic of the firefly luciferase reaction. (1) Firefly luciferase enzyme catalyses the activation of D-luciferin (LH_2) by ATP. (2) This is then oxidised to the electronically excited form, oxyluciferin (P*). (3) The transition of oxyluciferin to its ground state results in light emission. (4) Luciferase is regenerated from the enzyme-product complex (adapted from LKB-Wallac Luminometer 1250 application notes).

3.3. RESULTS - MAMMARY PROTEIN SYNTHESIS IS REGULATED BY CELL VOLUME

3.3.1. Effect of an osmotic challenge on protein synthesis in rat mammary tissue

The first step of the investigation was to confirm that altering cell volume changes the rate of protein synthesis in rat mammary explants. Thus, the incorporation of [^{3}H]-leucine into T.C.A.-precipitable material was measured over a 30 minute period in isosmotic, hyposmotic and hyperosmotic media. The results of these experiments are shown in figure 3.2. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4. Commonly, in studies of this nature, buffers are made hyposmotic by reducing the NaCl concentration. However, the trans-membrane Na^+ gradient is the driving force for the uptake of a number of amino acids by the mammary gland (Shennan and Peaker, 2000). Therefore, the extracellular concentration of NaCl was, under all conditions, maintained at 55mM to minimise the effect of changing the Na^+-gradient and thus the driving force for amino acid uptake by mammary cells. The hyposmotic buffer was similar in composition to the isosmotic buffer except that it contained no choline-Cl. The hyperosmotic buffer was also similar to the isosmotic buffer except that it contained an additional 200mM sucrose. In each of the buffers there was a full complement of amino acids at concentrations similar to that found in rat plasma (detailed in table 2.1). Radiolabelled leucine incorporation into T.C.A precipitable material was found to be linear under all conditions. Cell-swelling, induced by decreasing the osmolality of the incubation medium from 290 ± 4 mosmol/kg to 153 ± 1 mosmol/kg) resulted in a marked increase in the rate of protein synthesis ($P < 0.01$),

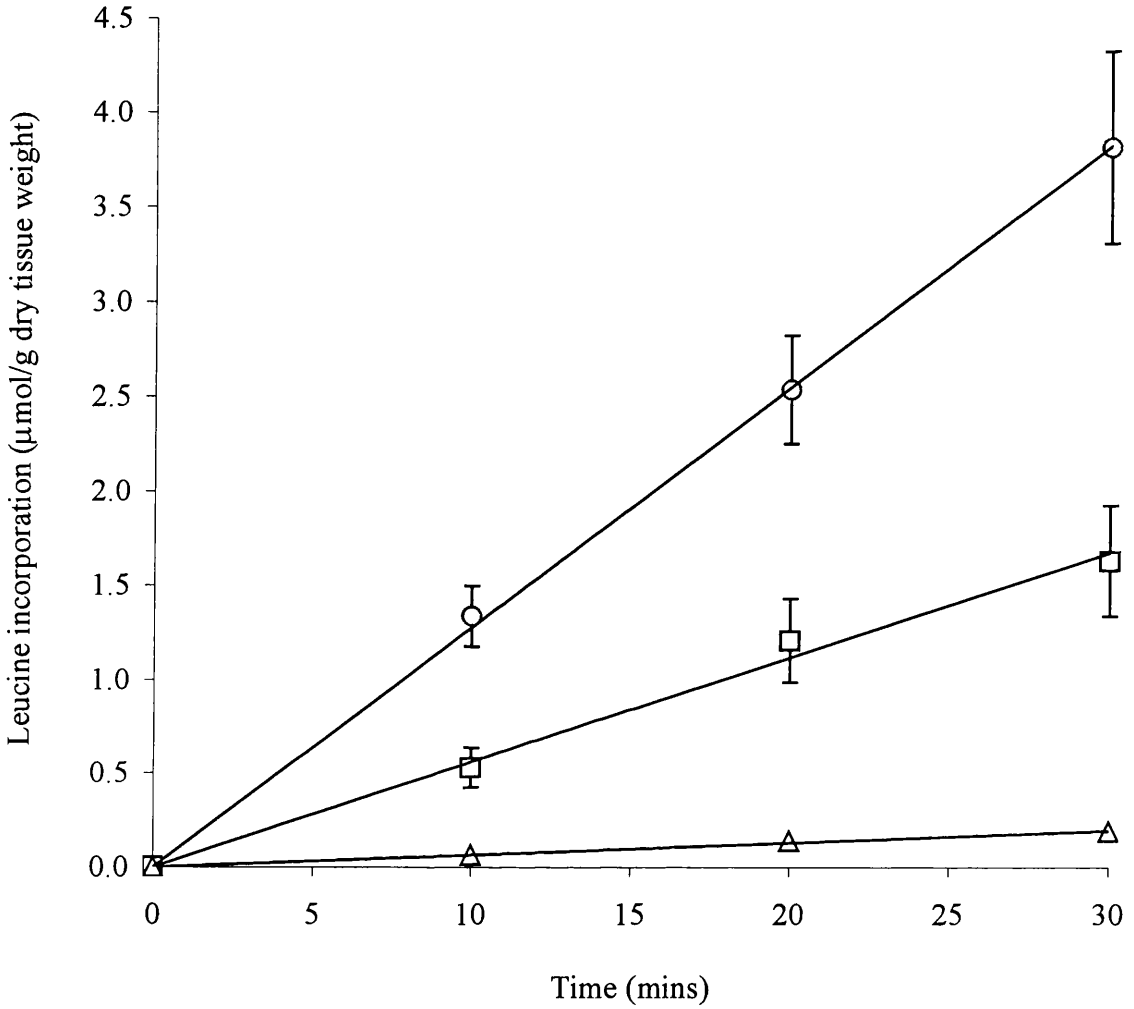

Figure 3.2: Time course of [^{3}H]-leucine incorporation into rat mammary explant protein under isosmotic (□), hyposmotic (○) and hyperosmotic (△) conditions. Isosmotic (290 ± 4 mosmol/kg water), hyposmotic (153 ± 1 mosmol/kg water) and hyperosmotic (492 ± 14 mosmol/kg water) conditions were used. All buffers contained amino acids at concentrations shown in table 2.1. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 4 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 (+1µCi/ml [^{3}H]-leucine). The hyposmotic buffer was similar in composition except that it did not contain any choline-Cl. The hyperosmotic buffer was similar in composition except that it also contained 200 mM sucrose. Each point is the mean ± SEM of six experiments using tissue from separate animals.

from 3.16 ± 0.64 to 7.83 ± 1.16 μmol/h/g dry tissue weight (±SEM, n = 6). Conversely, increasing the osmolality to 492 ± 14 mosmol/kg, hence decreasing the volume of the cells, resulted in a marked decrease in the rate of protein synthesis ($P < 0.01$), from 3.16 ± 0.64 to 0.43 ± 0.09 μmol/h/g (±SEM, n = 6). Therefore, cell swelling and shrinking respectively increased and decreased mammary protein synthesis by 147% and 86%.

[^{3}H]-leucine incorporation into protein was also measured in rat mammary acini in isosmotic (314 ± 1 mosmol/kg water), hyposmotic (173 ± 0 mosmol/kg water) and hyperosmotic (536 ± 1 mosmol/kg water) conditions. The data are shown in figure 3.3. The buffers for the experiments using acini were the same as those for the explant studies, except that the Tris-MOPS concentration was increased from 10 to 20mM. Protein synthesis was measured over a period of 20 min. Under isosmotic conditions the rate of incorporation of leucine into T.C.A.-precipitable material was 1.77 ± 0.24 μmol/h/g T.C.A. precipitate. Reducing the osmolality by 45% resulted in an increase in the rate of protein synthesis to 4.14 ± 0.69 μmol/h/g T.C.A. precipitate ($P < 0.05$). Conversely, hyperosmotic cell shrinking resulted in a marked decreased the rate of leucine incorporation from 1.77 ± 0.24 to 0.183 ± 0.02 μmol/h/g T.C.A. precipitate ($P < 0.01$, n = 5, ±SEM).

The effect of cell volume on the incorporation of [^{35}S]-methionine into rat mammary explant protein was also examined. Again, hyposmotic (154 ± 2 mosmol/kg), isosmotic (292 ± 3 mosmol/kg) and hyperosmotic (504 ± 4 mosmol/kg) buffers were used. The composition of the buffers was the same as those used for the leucine incorporation

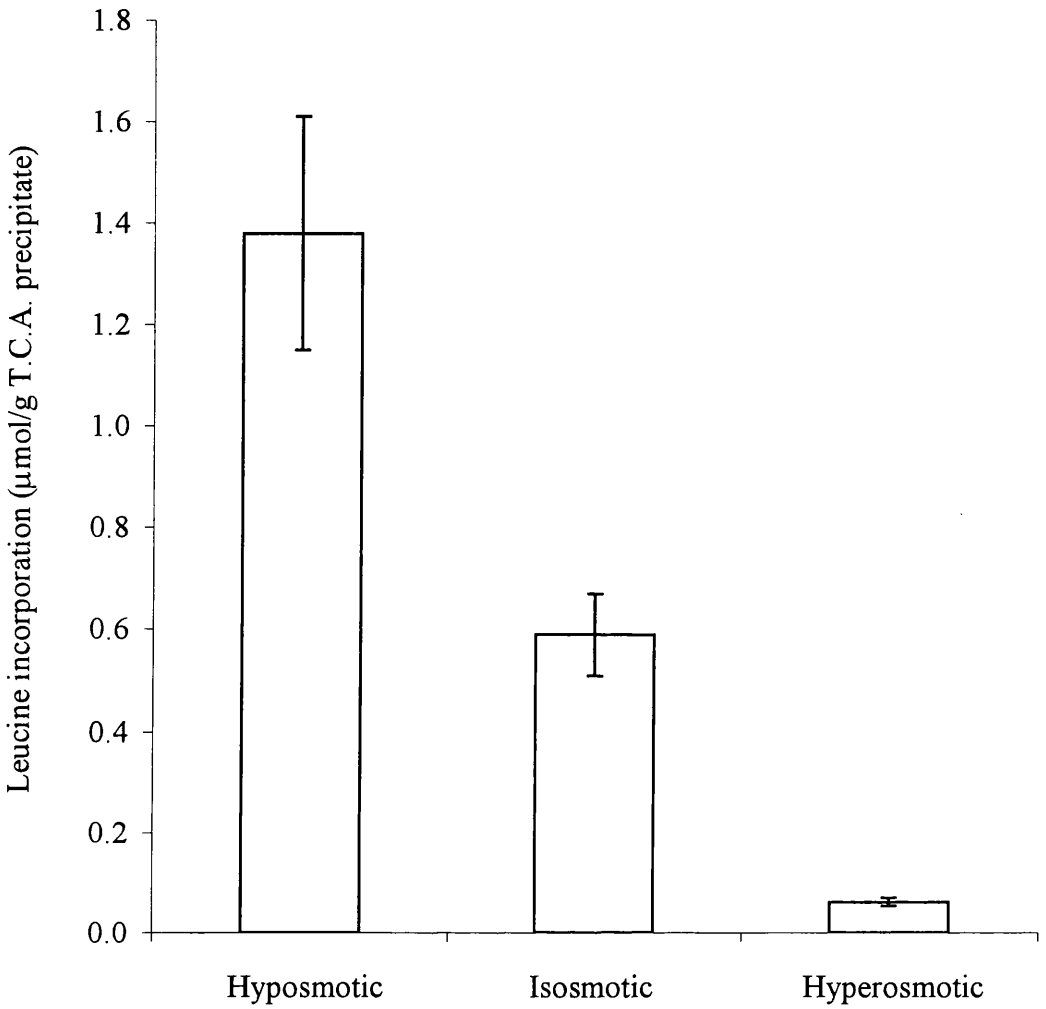

Figure 3.3: [^{3}H]-leucine incorporation into T.C.A.-precipitable material in mammary acini. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 4 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 314 ± 1 mosmol/kg water). The hyposmotic buffer was similar in composition except that it did not contain any choline-Cl (173 ± 0 mosmol/kg water). The hyperosmotic buffer was similar in composition except that it also contained 200mM sucrose (536 ± 1 mosmol/kg water). Incorporation was measured over 20 minutes. Each point is the mean ±SEM of five experiments using tissue from separate animals

experiments described above with the exception of the tracer. It can be seen from the results (figure 3.4) that a hyposmotic challenge increased ($P < 0.01$) the rate of incorporation of [^{35}S]-methionine into mammary protein. Thus, the rate of methionine incorporation under isosmotic and hyposmotic conditions was respectively 0.42 ± 0.02 and 0.95 ± 0.08 μmoles/h/g dry tissue weight (± SEM, n = 4). Conversely, increasing the osmolality of the incubation medium reduced ($P < 0.01$) the rate of methionine incorporation from 0.42 ± 0.02 to 0.03 ± 0.01 μmol/h/g dry tissue weight (±SEM, n = 4). Thus, protein synthesis was increased by 126% as a consequence of cell swelling and decreased by 93% when cell volume was reduced. Note from figure 3.4 that [^{35}S]-methionine incorporation into T.C.A.-precipitable material was linear under all the conditions tested. The ratio of leucine to methionine incorporation into rat mammary protein under isosmotic and hyposmotic conditions was 7.52 and 8.24 respectively.

Cell shrinking is known to increase proteolysis in hepatocytes (Lang *et al.*, 1998a). It could be that the observed decrease in protein synthesis shown in figures 3.2-3.4 as a result of a hyperosmotic challenge was due to an increase in the rate of proteolysis causing a dilution of the intracellular specific-activity of tracer. Therefore, the rate of incorporation of [^{3}H]-leucine into rat mammary protein in explants was measured under isosmotic, hyposmotic and hyperosmotic conditions in the presence of 4mM (as opposed to 225μM) unlabelled leucine. The rationale behind this approach was that the effect of unlabelled leucine derived from proteolysis on the intracellular specific activity of radiolabelled leucine would be reduced (all other amino acid concentrations were maintained at the levels described in table 2.1). Aside from the leucine, the

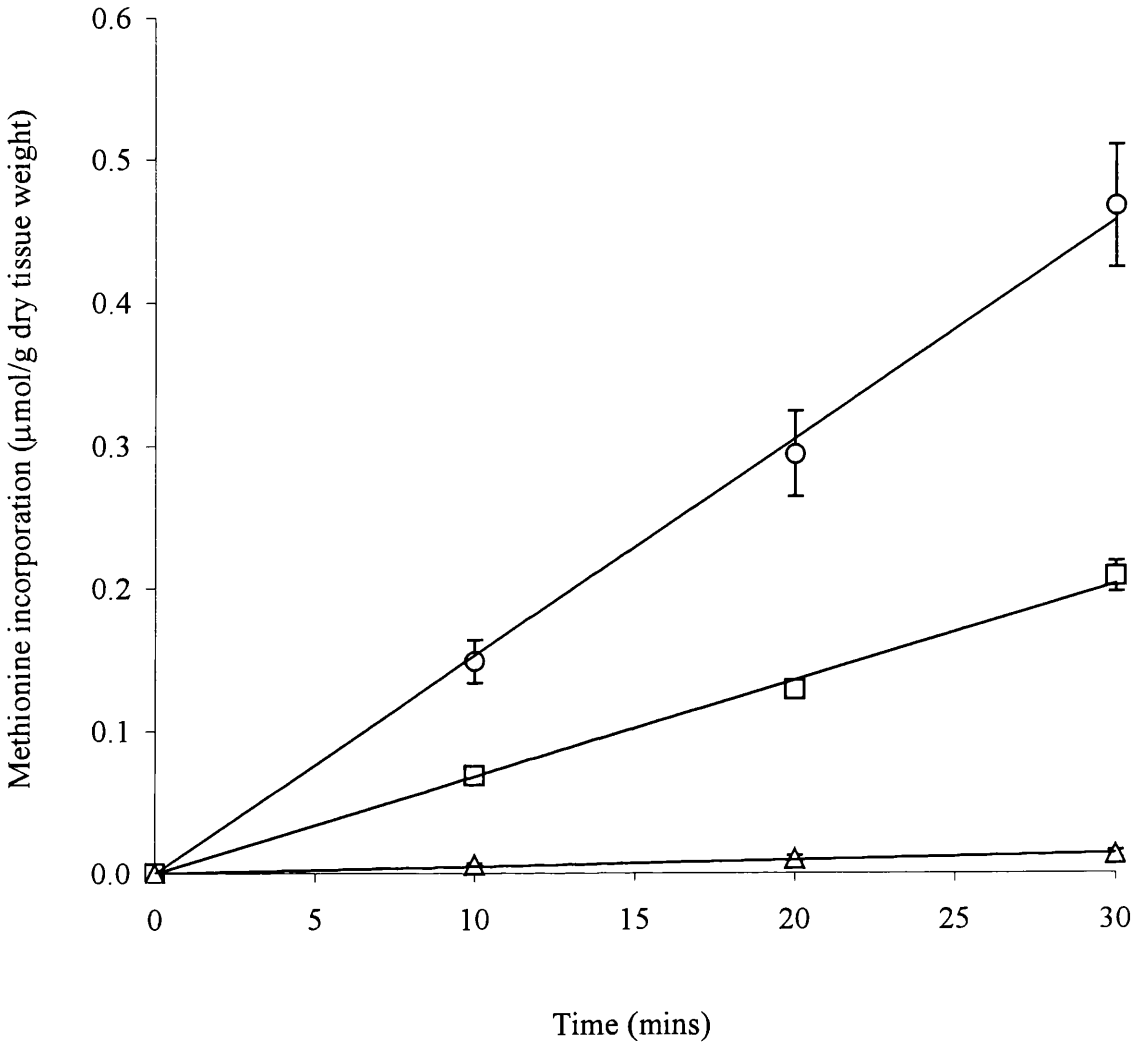

Figure 3.4: Time course of [^{35}S]-methionine incorporation into rat mammary explant protein under isosmotic (□), hyposmotic (○) and hyperosmotic (△) conditions. Isosmotic (292 ± 3 mosmol/kg water), hyposmotic (154 ± 2 mosmol/kg water) and hyperosmotic (504 ± 4 mosmol/kg water) conditions were used. All buffers contained amino acids at concentrations shown in table 2.1. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 4 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 (+2.5µCi/ml [^{35}S]-methionine). The hyposmotic buffer was similar in composition except that it did not contain any choline-Cl. The hyperosmotic buffer was similar in composition except that it also contained 200 mM sucrose. Each point is the mean ± SEM of four experiments using tissue from separate animals.

composition of the buffers was unchanged from the experiments shown in figure 3.2. The osmolalities were 294 ± 13, 159 ± 9 and 500 ± 10 mosmol/kg for isosmotic, hyposmotic and hyperosmotic buffers, respectively. Figure 3.5 shows that incorporation of label over the thirty minute incubation was linear under all three conditions. Reducing the osmolality of the incubation medium from isosmotic to hyposmotic caused an increase in the rate of incorporation of [^{3}H]-leucine into T.C.A.-precipitable material from 6.64 ± 1.07 to 11.43 ± 1.48 μmol/h/g dry tissue weight (± SEM, n = 4, $P < 0.002$). Conversely, exposure to a hyperosmotic medium decreased the rate of incorporation to 1.17 ± 0.03 μmol/h/g dry tissue weight (±SEM, n = 4, $P < 0.02$). Hence cell swelling increased protein synthesis by 72% and cell shrinking decreased synthesis by 82% under these experimental conditions.

In the experiments just described (figures 3.2-3.5), the incubation buffers were made hyposmotic by reducing the choline-Cl concentration. Therefore, there was a possibility that the difference found in protein synthesis between isosmotic and hyposmotic conditions was due to the presence of choline. Therefore, it was decided to use $NMDG^+$ instead of choline. The results are shown in figure 3.6. In this set of experiments the isosmotic buffer contained (mM) 55 NaCl, 80 NMDG, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 (plus an amino acid complement as described in table 2.1). The hyposmotic buffer was similar in composition to the isosmotic buffer except that is contained no $NMDG^+$. Under isosmotic conditions the rate of protein synthesis was 1.77 ± 0.55 μmol/h/g dry tissue weight(±SEM, n = 3). Mammary protein synthesis increased by 176% to 4.89 ± 0.89 μmol/h/g dry tissue weight when the

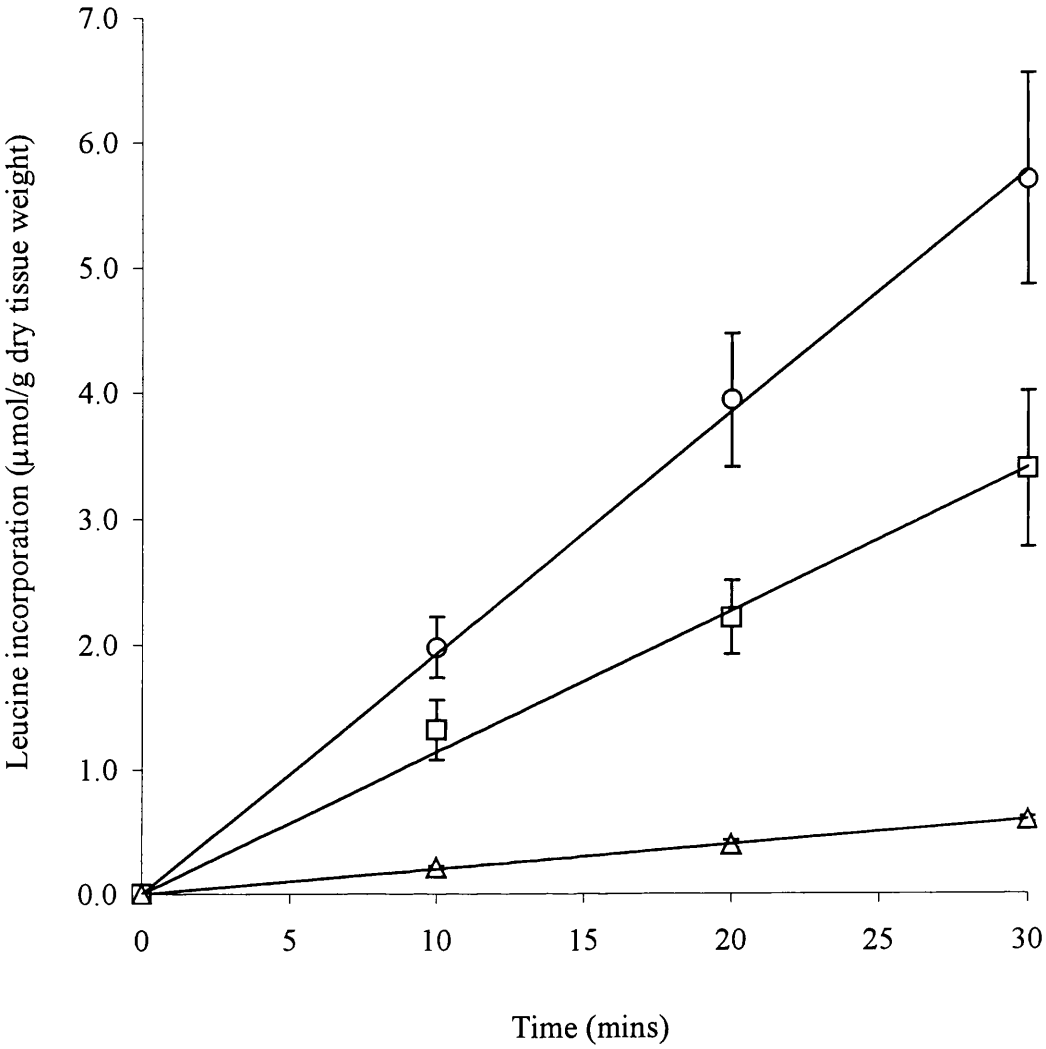

Figure 3.5: Time course of [^{3}H]-leucine incorporation into rat mammary explant protein in the presence of 4mM leucine under isosmotic (□), hyposmotic (○) and hyperosmotic (△) conditions. Explants were incubated in isosmotic (294 ± 13 mosmol/kg water), hyposmotic (159 ± 9 mosmol/kg water) and hyperosmotic conditions (500 ± 10 mosmol/kg water). The composition of the buffers was the same as described in figure 3.2 except that the concentration of unlabelled leucine was 4 mM. Each point is the mean ± SEM of four experiments using tissue from separate animals.

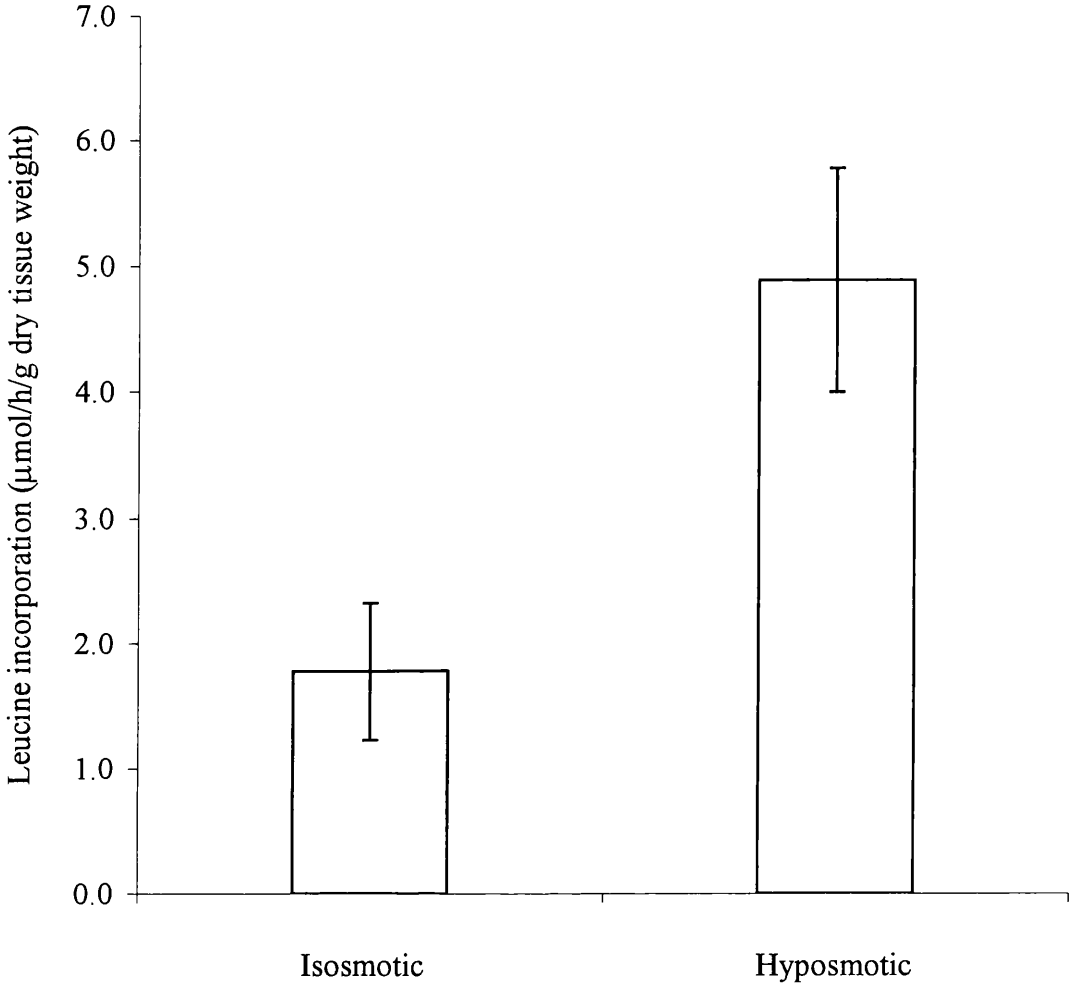

Figure 3.6: Effect of a hyposmotic challenge on [^{3}H]-leucine incorporation into mammary protein, compared to an isosmotic buffer containing NMDG. The isosmotic buffer contained (mM) 55 NaCl, 80 NMDG, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 287 ± 2 mosmol/kg water). The hyposmotic buffer was similar in composition except that it had no NMDG (osmolality = 155 ± 2 mosmol/kg water). Data are means ±SEM of three experiments using separate animals.

tissue was incubated in a hyposmotic buffer (±SEM, n = 3, $P < 0.02$).

3.3.2. Reversibility of volume sensitive protein synthesis

The decrease in protein synthesis observed under hyperosmotic conditions could be due to cell damage. Therefore, experiments were designed to test whether or not the effect of a hyperosmotic shock on rat mammary protein synthesis was reversible. Figure 3.7 shows the effect of incubating mammary explants first in a hyperosmotic buffer and then in an isosmotic buffer on the incorporation of radiolabelled leucine into protein. The hyperosmotic buffer consisted of (mM) 55 NaCl, 160 sucrose, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 (with a full complement of amino acids, as described in table 1). At t = 22 min an equal volume of a buffer containing (mM) 55 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 (plus a full complement of amino acids - see table 2.1) was added to the hyperosmotic solution. This addition reduced the osmolality from 461 ± 7 mosmoles/kg of water to 307 ± 5 mosmoles/kg of water. Note that the added buffer also contained radiolabelled leucine (1 μCi/ml), and therefore, the specific activity and concentration of leucine was not changed as a consequence of reducing the osmolality. For the first twenty minutes of incubation [^{3}H]-leucine incorporation into mammary protein was linear; the rate of synthesis under hyperosmotic conditions was 0.73 ± 0.13 μmol/h/g dry tissue weight (±SEM, n = 4). When the incubation buffer was made isosmotic the rate of leucine incorporation into mammary protein increased to 3.16 ± 0.29 μmol/h/g dry tissue weight (± SEM, n = 4, $P < 0.01$). Thus, protein synthesis was increased by 332%. It appears, therefore, that the effect of a hyperosmotic challenge on mammary protein synthesis in

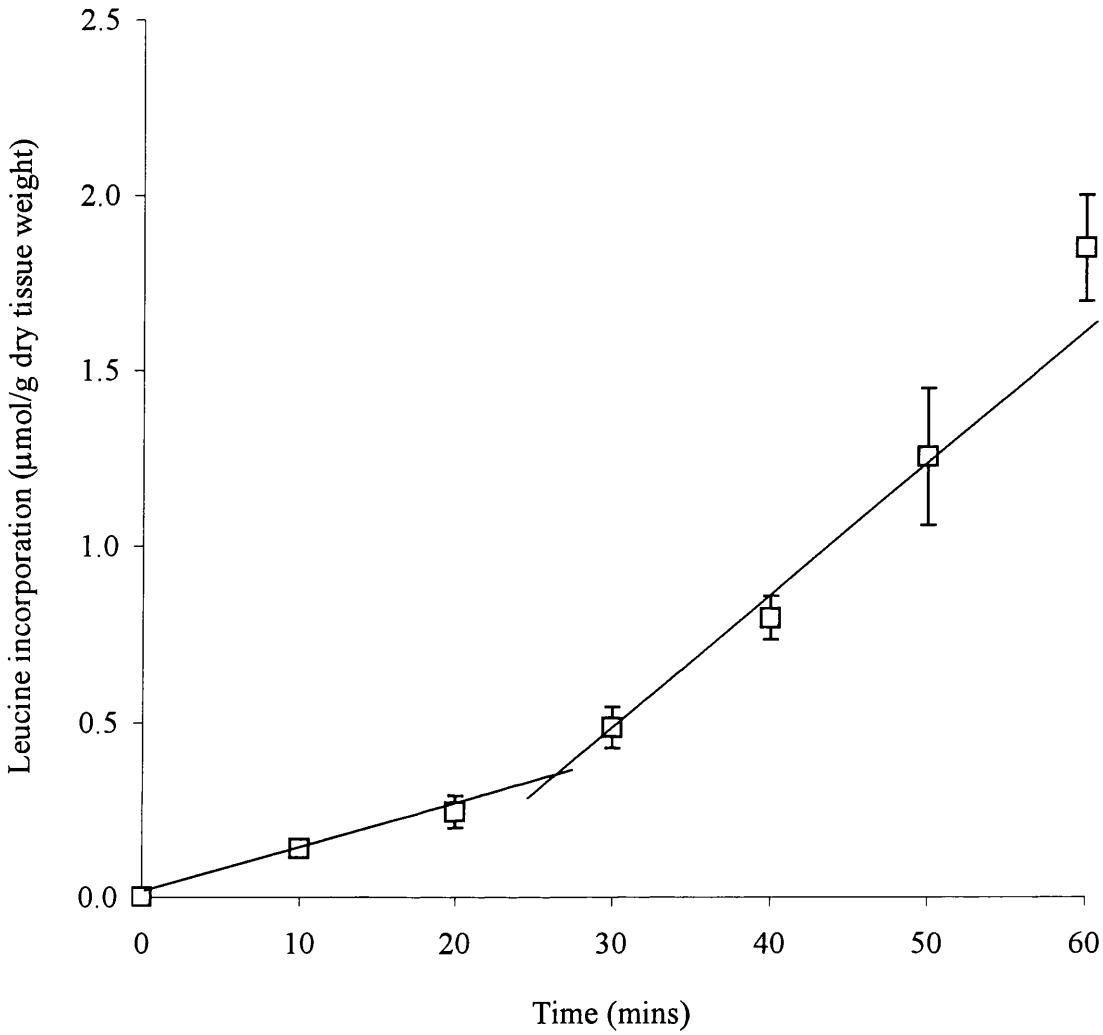

Figure 3.7: Reversibility of hyperosmotic inhibition of [^{3}H]-leucine incorporation into mammary protein (Explants). Explants were initially incubated in a medium containing (mM) 160 sucrose, 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 461 ± 7 mosmol/kg water). At t = 22 min the medium was made isosmotic by adding an equal volume of hyposmotic buffer, similar in composition to the hyperosmotic except that it had no sucrose or choline-Cl (final osmolality = 307 ± 5 mosmol/kg water). Each point is the mean ± SEM of four experiments using tissue from separate animals.

explants is fully reversed within 20 minutes of restoring isosmotic conditions.

The ability of protein synthesis in mammary acini to recover from a hyperosmotic challenge was also examined. As with the investigation using explants, acini were incubated for one hour: acini were incubated first in a hyperosmotic solution (473 ± 3 mosmol/kg) and then at t = 22 minutes the solution was diluted to an isosmotic level (316 ± 3 mosmol/kg). The buffers for the acinar experiments were the same as those used for the explant studies except that the Tris-MOPS concentration was increased from 10 to 20mM. The results can be see in figure 3.8. Under hyperosmotic conditions, the rate of incorporation of isotope was linear at 0.31 ± 0.06 μmol/h/g T.C.A. precipitate. Once the solution had been made isosmotic the rate of protein synthesis steadily increased to 1.10 ± 0.35 μmol/h/g T.C.A. precipitate, between 50 and 60 minutes (n = 6, ±SEM).

The reversibility of the hyposmotic stimulation of protein synthesis (shown in figures 3.2-5) was examined. The results are shown in figure 3.9. Radiolabelled leucine incorporation was measured under hyposmotic conditions (152 ± 1 mosmol/kg) for 22 minutes after which the solution was made isosmotic (283 ± 3 mosmol/kg) by the addition of a more concentrated solution. The hyposmotic buffer contained (mM) 55 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 (plus a full complement of amino acids as described in table 2.1). The buffer used to make the solution isosmotic was similar in composition except that it also contained 160 choline-Cl. Again, the specific activity of radiolabelled leucine was not changed by the addition. Consistent with the results shown in figure 3.2 mammary protein synthesis was linear under hyposmotic

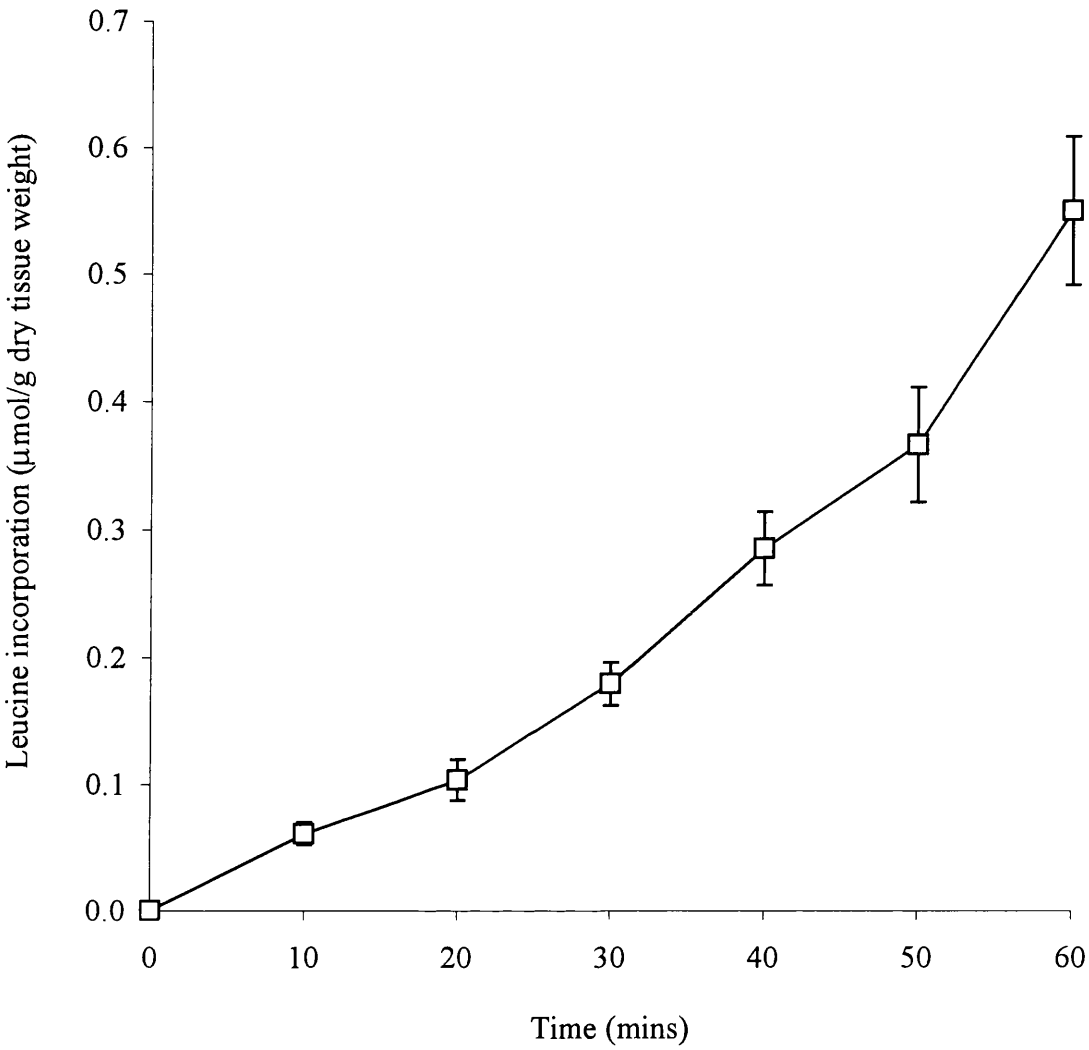

Figure 3.8: Reversibility of hyperosmotic inhibition of [^{3}H]-leucine incorporation into mammary protein (Acini). Acini were initially incubated in a medium containing (mM) 160 sucrose, 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine. (+ amino acids as described in table 2.1) (osmolality = 473 ± 7 mosmol/kg water). At t = 22 min the medium was made isosmotic by adding an equal volume of hyposmotic buffer, similar in composition to the hyperosmotic except that it had no sucrose or choline-Cl (final osmolality = 316 ± 8 mosmol/kg water). Each point is the mean ± SEM of six experiments using tissue from separate animals.

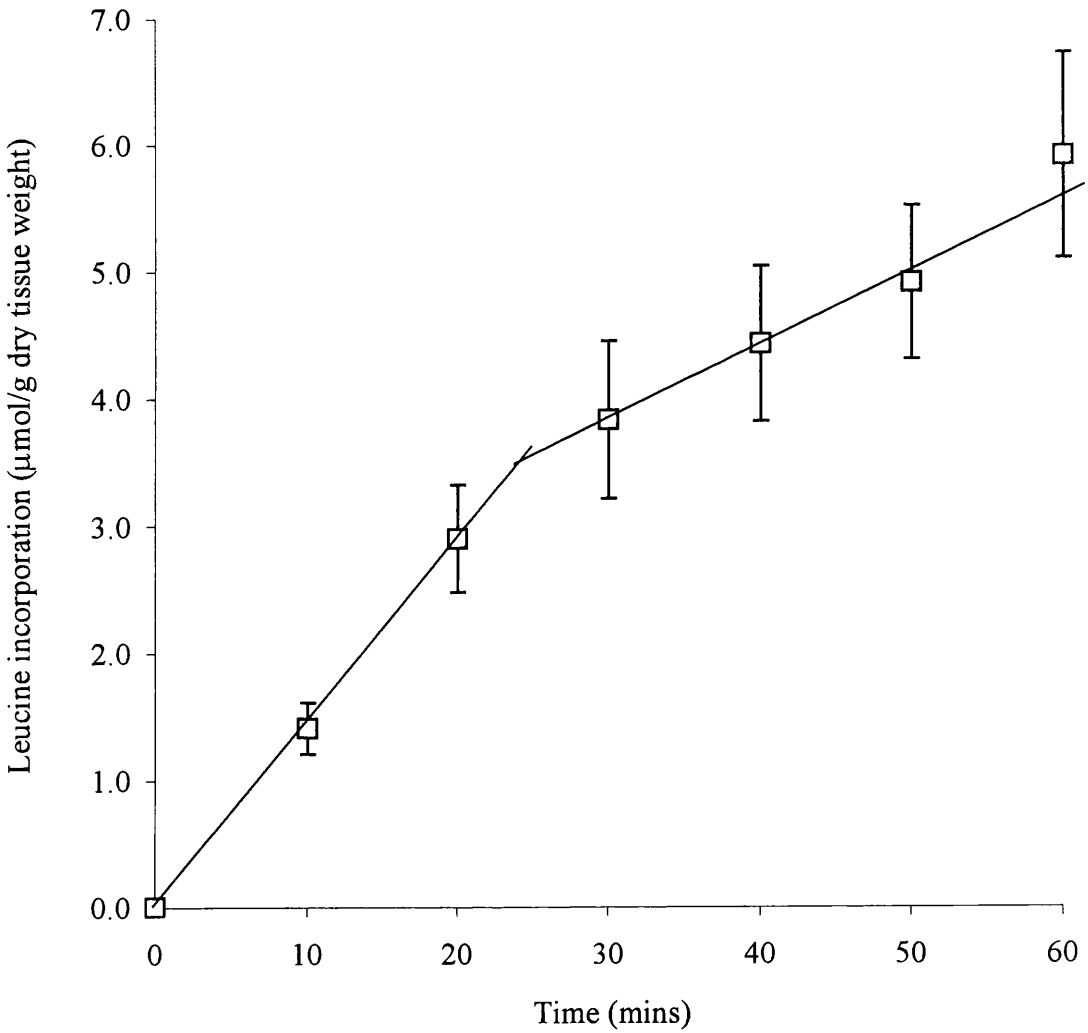

Figure 3.9: Reversibility of hyposmotic stimulation of [^{3}H]-leucine incorporation into mammary explant protein. Explants were initially incubated in a medium containing (mM) 55 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine. (+ amino acids as described in table 2.1) (osmolality = 152 ± 1 mosmol/kg). At t = 22 min the medium was made isosmotic by adding an equal volume of a buffer similar in composition to the hyposmotic buffer, except that it had additional 160mM choline-Cl (final osmolality = 283 ± 3 mosmol/kg). Each point is the mean ± SEM of four experiments using tissue from separate animals.

conditions; the rate of leucine incorporation was 8.70 ± 1.27 μmoles/h/g dry tissue weight (±SEM, n = 4). The rate of leucine incorporation into mammary protein was also linear under isosmotic conditions but at a lower rate of 4.44 μmoles/h/g dry tissue weight (n = 4, $P < 0.02$). There was a lag period of at least 8 min before protein synthesis again became linear after the buffer was made isosmotic. Evidently, the effect of a hyposmotic shock, and hence cell swelling, on mammary protein synthesis is reversible.

3.3.3. Latency of hyperosmotic-induced decrease in mammary protein synthesis

It is clear that cell shrinking, induced by a hyperosmotic shock, inhibits mammary protein synthesis. The question that arises is: how long does it take for this effect to manifest? To answer this question protein synthesis in mammary explants was measured first under isosmotic conditions (for 22 min) and then under hyperosmotic conditions (for the remainder of the time course). The incorporation of radiolabelled leucine into TCA-precipitable protein was taken as a measure of protein synthesis. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 (plus a full complement of amino acids as described in table 2.1). The buffer added to make the media hyperosmotic was similar in composition to the isosmotic buffer except that it contained an additional 300mM sucrose. Again the specific activity of radiolabelled leucine was not changed when the osmolality was increased. Incorporation of labelled amino acid was linear under both isosmotic and hyperosmotic conditions (figure 3.10). The rate of incorporation under isosmotic conditions was 5.54 ± 1.173 (± SEM, n = 4). Protein synthesis under hyperosmotic conditions was effectively reduced to zero. Figure 3.10 shows that the time taken for cell shrinking to cause an

inhibition of protein synthesis was approximately 10 minutes.

3.3.4. Sensitivity of protein synthesis to changes in the extracellular osmolality

The sensitivity of protein synthesis in rat mammary tissue explants to changes in extracellular osmolality was examined. The effect of decreasing the osmolality of the incubation medium from 295 to 156 mosmol/kg of water on leucine incorporation into mammary protein is shown in figure 3.11. The isosmotic buffer (295 mosmol/kg of water) contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 (and a full complement of amino acids as described in table 2.1). The osmolality was changed by reducing the concentration of choline-Cl in the incubation buffer. Incorporation of [^{3}H]-leucine was measured over a 20 minute period. Figure 3.11 shows that as the osmolality of the incubation medium decreases the rate of protein synthesis increases. Incorporation of tracer increased in a linear fashion between 295 to 226 mosmol/kg, and was maximal at 188 mosmol/kg.

The sensitivity of protein synthesis to increasing the osmolality of the incubation medium was also examined. The osmolality was increased from 298 to 500 mosmol/kg of water. The isosmotic buffer (298 mosmol/kg) was similar in composition to that described for the investigation into hyposmotic sensitivity. The osmolality of the incubation media was increased by the addition of sucrose. It is evident from the data in figure 3.12 that the reduction in protein synthesis was dependent upon the extent of the hyperosmotic challenge. The relationship between protein synthesis and the osmolality of the incubation

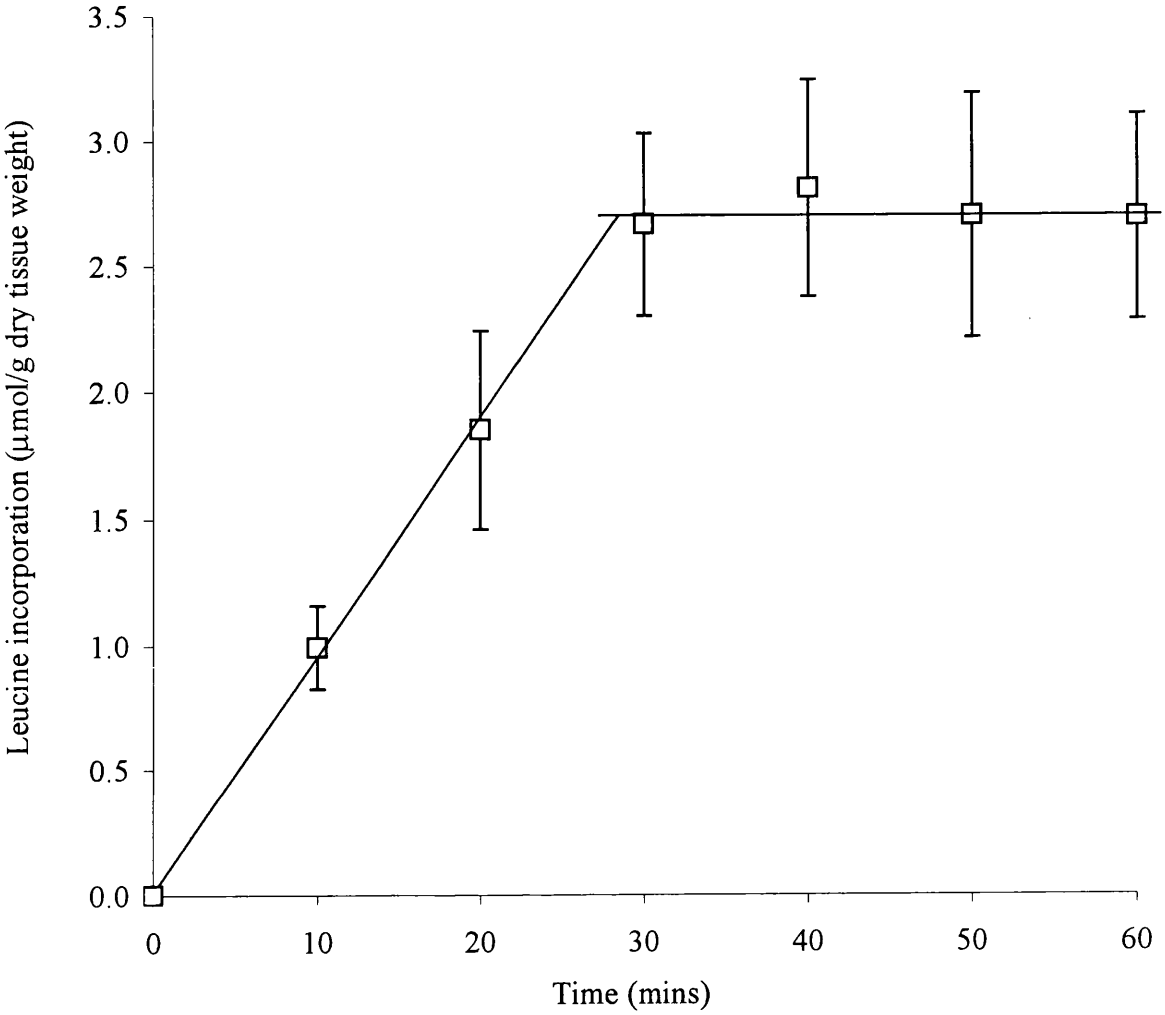

Figure 3.10: Time course of inhibition of [^{3}H]-leucine incorporation into mammary explant protein by application of a hyperosmotic challenge. Mammay tissue explants were initially incubated in an isosmotic medium containing (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 CaCl2, 1 MgSO4, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 295 ± 2 mosmol/kg water). At t = 22 min the medium was made hyperosmotic by the addition of a buffer similar to the isosmotic medium but with 300mM Sucrose added (final osmolality = 455 ± 1 mosmol/kg water). Data shown are means ± SEM of four experiments using tissue from separate animals.

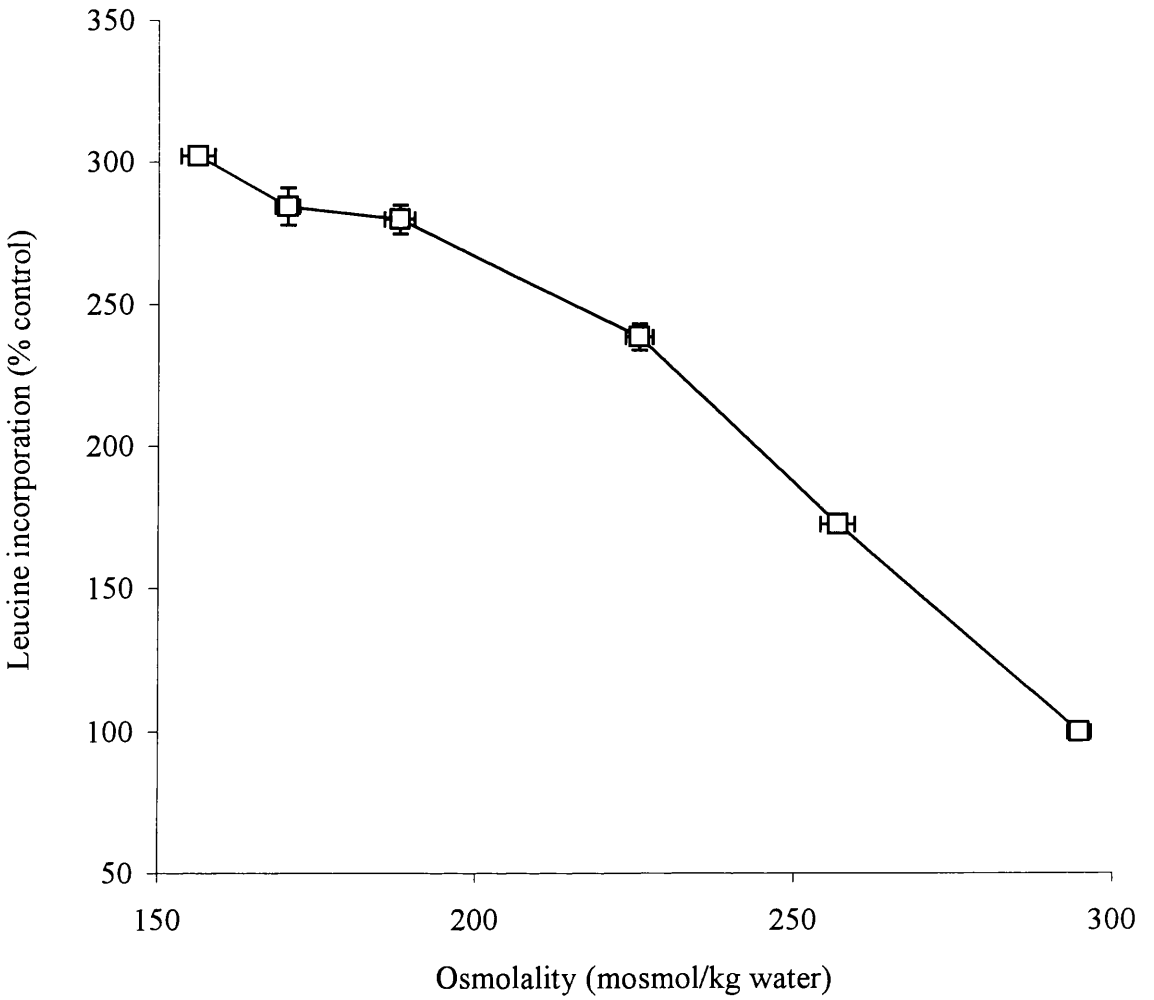

Figure 3.11: Sensitivity of mammary explant protein synthesis to decreasing osmolality. Mammary tissue explants were incubated in media of different osmolalities below and including an isosmotic control. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 CaCl2, 1 MgSO4, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1μCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 294.8 ± 1.8 mosmol/kg water). The concentration of choline-Cl was altered to give different osmolalities; 60, 40, 20 10 and 0mM (osmolalities = 256.8 ± 2.7, 225.7 ± 2.1, 187.8 ± 2.4, 170.2 ± 1.8, 156.2 ± 2.7 mosmol/kg water, respectively). [^{3}H]-leucine incorporation was measured over a 20 minute period. Data shown are means ± SEM of six experiments using tissue from separate animals.

buffer shown in figure 3.12 can be described by a mono-exponential equation (3.1):

$$P_r = ae^{-kr} \qquad (3.1)$$

where P_r is protein synthesis (%) at a given osmolality r (mosmol/kg), a is a constant (4924.5) and k is the decay constant (0.013 (mosmol/kg)$^{-1}$).

3.3.5. Isosmotic swelling increases protein synthesis

The effect of a hyposmotic solution on protein synthesis could be due to either cell swelling or the change in osmolality of the incubation buffer per se. To distinguish between these two possibilities, the effect of isosmotic cell swelling on protein synthesis in rat mammary explants was examined. To elicit an increase in cell volume under isosmotic conditions an isosmotic solution containing a relatively high concentration of urea was used. The rationale behind this method is that cells will swell, despite the isosmotic conditions, on account of the high permeability of urea. Protein synthesis was measured using radiolabelled leucine. The buffers contained (mM) 55 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 10 Tris-MOPS, pH 7.4 and either 80 choline-Cl or 160 urea (plus a full complement of amino acids as described in table 2.1). Figure 3.13 shows that isosmotic swelling increased the rate of protein synthesis by 106% ($P < 0.001$): [^{3}H]-leucine incorporation increased from 2.11 ± 0.30 to 4.35 ± 0.55 μmol/h/g dry tissue weight (± SEM, n = 6).

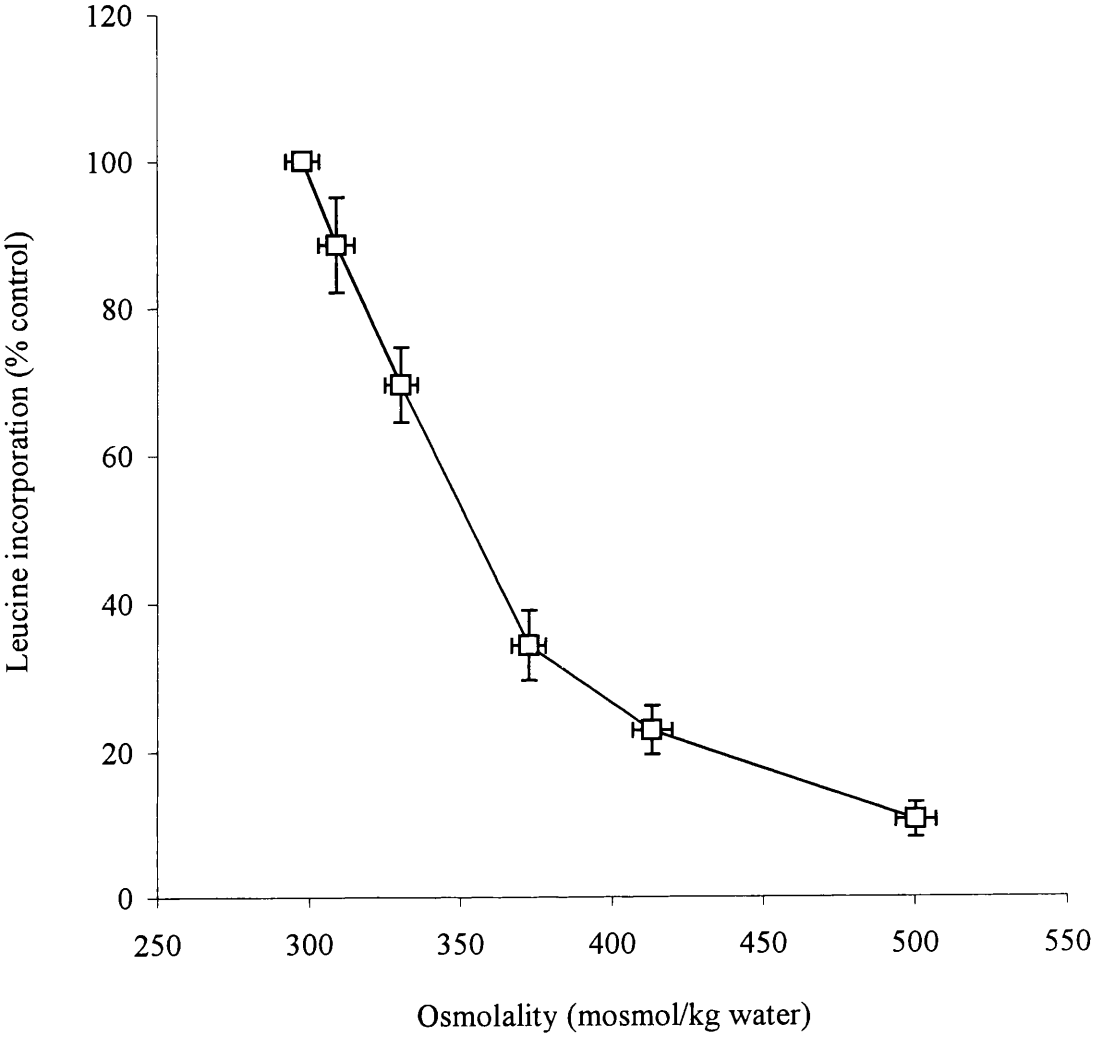

Figure 3.12: Sensitivity of mammary explant protein synthesis to increasing osmolality. Mammary tissue explants were incubated in media of different osmolalities above and including an isosmotic level. The isosmotic buffer contained (mM) 10 sucrose, 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1μCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 297.7 ± 5.4 mosmol/kg water). The concentration of sucrose was altered to give different osmolalities; 20, 40, 80, 120 and 200mM (osmolalities = 308.8 ± 6.0, 330.3 ± 5.5, 372.5 ± 5.7, 413.5 ± 6.4, 500.5 ± 6.6 mosmol/kg water, respectively). [^{3}H]-leucine incorporation was measured over a 20 minute period. Data shown are means ± SEM of six experiments using tissue from separate animals.

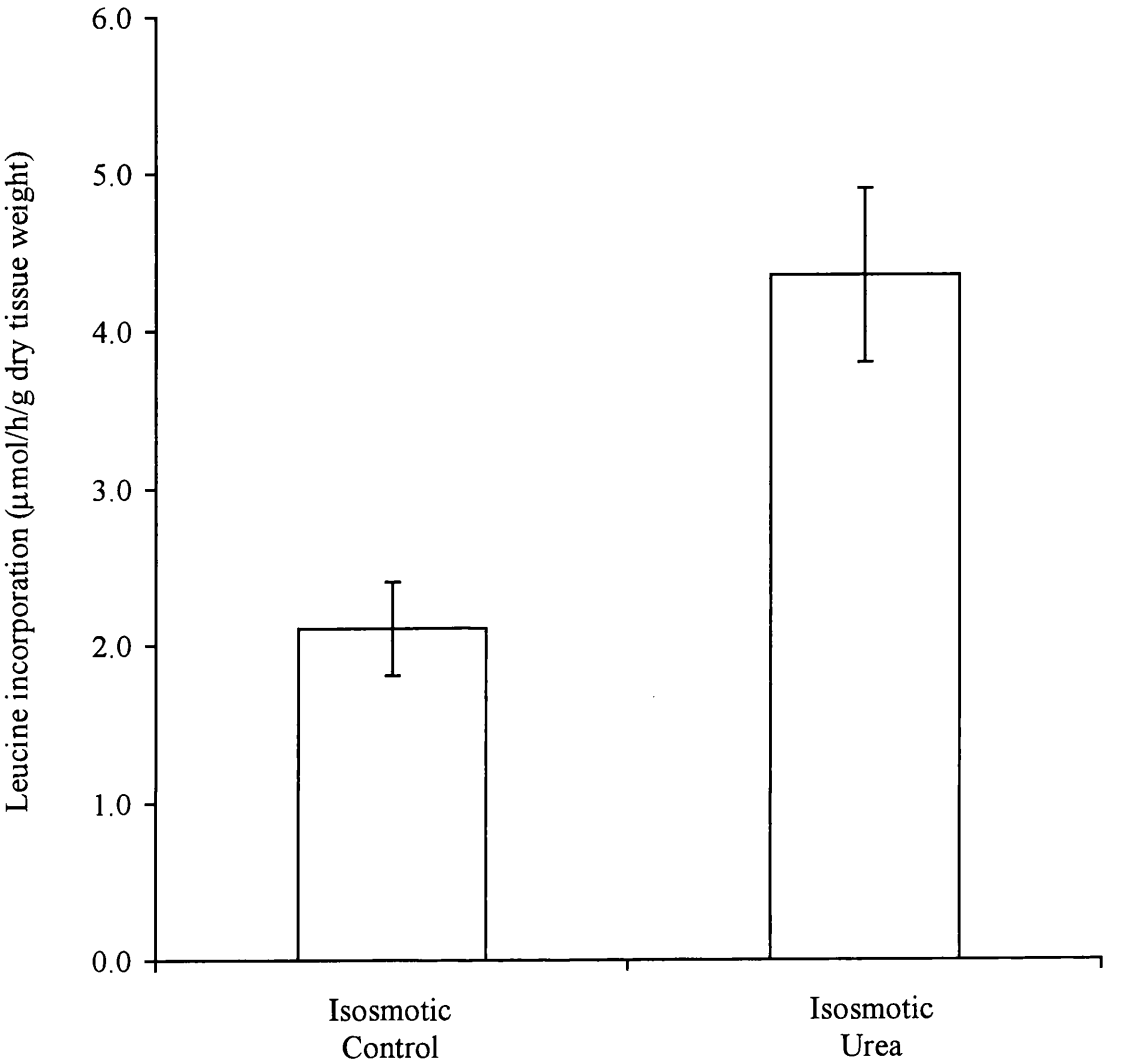

Figure 3.13: The effect of isosmotic swelling on [^{3}H]-leucine incorporation into mammary explant protein. The control isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in Table 2.1) (osmolality = 294 ± 1 mosmol/kg water). The buffer containing urea was similar in composition except that it contained 160mM urea and no choline-Cl (osmolality 306 ± 3 mosmol/kg water). Data shown are the means ± SEM of six experiments using tissue from separate animals.

In one experiment, the incorporation of [^{3}H]-leucine into rat mammary protein was measured under similar experimental conditions to that just described in the absence and presence of phloretin. Phloretin is an inhibitor of urea transport (Shayakul and Hediger, 1996) so it was predicted that phloretin should block the increase in protein synthesis due to urea-induced swelling. The results are shown in figure 3.14. The rates of protein synthesis under control isosmotic conditions (i.e. in the absence of urea) were 1.17 and 0.33 μmol/h/g dry tissue weight in the absence and presence of inhibitor, respectively. With urea present in the incubation buffer, the rates of leucine incorporation were 2.98 and 0.46 μmol/h/g dry tissue weight in the absence and presence of phloretin respectively. It is apparent that phloretin inhibits volume-sensitive protein synthesis. However, it appears that phloretin also inhibits protein synthesis under control isosmotic conditions (i.e. in the absence of urea).

3.4. Results - Role of Ca^{2+} in volume-sensitive protein synthesis

Several studies have shown that mammary protein synthesis under isosmotic conditions is dependent upon calcium (Wilde *et al.*, 1981; Smith *et al.*, 1982; Duncan and Burgoyne, 1996). In view of this the dependence of swelling-activated protein synthesis on calcium was examined.

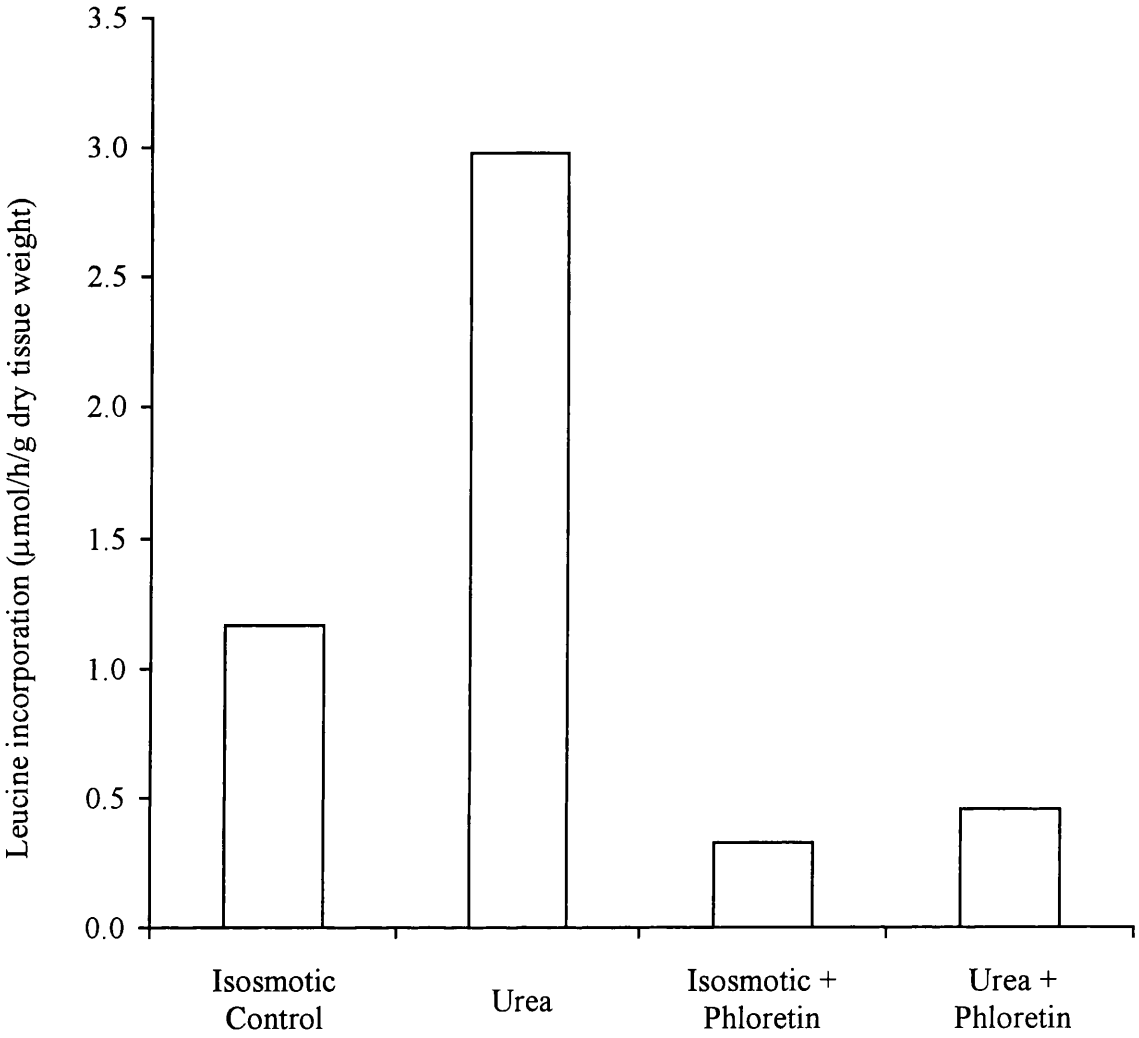

Figure 3.14: Effect of Phloretin on urea induced [^{3}H]-leucine incorporation into mammary explant protein. The isosomotic buffers contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in Table 2.1) (osmolality = 307 mosmol/kg water). The urea buffers were similar in composition to the isosmotic buffer except that it had 160mM urea instead of the choline-Cl (osmolality = 327 mosmol/kg water). When required, phloretin was used at 50µM. DMSO was present at 0.1% (v/v) in all buffers. Data shown are means from two experiments using tissue from a single animal.

3.4.1 Effect of removing extracellular Ca^{2+} on volume-activated protein synthesis

Figure 3.15 shows that removing Ca^{2+} (+EGTA) from the external solution caused a reduction in protein synthesis in rat mammary explants under isosmotic and hyposmotic conditions. Thus, removing Ca^{2+} (+EGTA) from the isosmotic medium decreased the incorporation of leucine into mammary tissue protein from 2.81 ± 0.27 to 1.29 ± 0.09 μmol/h/g dry tissue weight ($P < 0.02$). Similarly, removing Ca^{2+} (+EGTA) from the hyposmotic buffer reduced protein synthesis from 5.62 ± 0.41 to 2.20 ± 0.19 μmol/h/g dry tissue weight ($P < 0.001$). Therefore, removing calcium from the incubation medium reduced the volume-sensitive increase in protein synthesis (-68%) from 2.81 ± 0.40 to 0.91 ± 0.14 μmol/h/g ($n = 4$, ±SEM, $P < 0.02$).

The effects of removing extracellular calcium on protein synthesis were also examined using mammary acini. The buffers used in this investigation were the same as those used in the experiments using explants except that they contained 20 rather than 10mM Tris-MOPS. Figure 3.16 shows that under isosmotic conditions the rate of incorporation of leucine into mammary protein was reduced from 1.01 ± 0.11 to 0.75 ± 0.09 μmol/h/g T.C.A. precipitate ($P < 0.05$), when Ca^{2+} was absent from the incubation medium. Similarly, removing extracellular calcium (+EGTA) under hyposmotic conditions reduced protein synthesis. Thus, protein synthesis in the presence and absence of extracellular calcium was, respectively, 2.93 ± 0.49 and 0.95 ± 0.07 μmol/h/g T.C.A. precipitate (±SEM, $n = 4$, $P < 0.05$). Therefore, removing extracellular calcium decreased volume-activated protein synthesis in rat mammary acini by 90%.

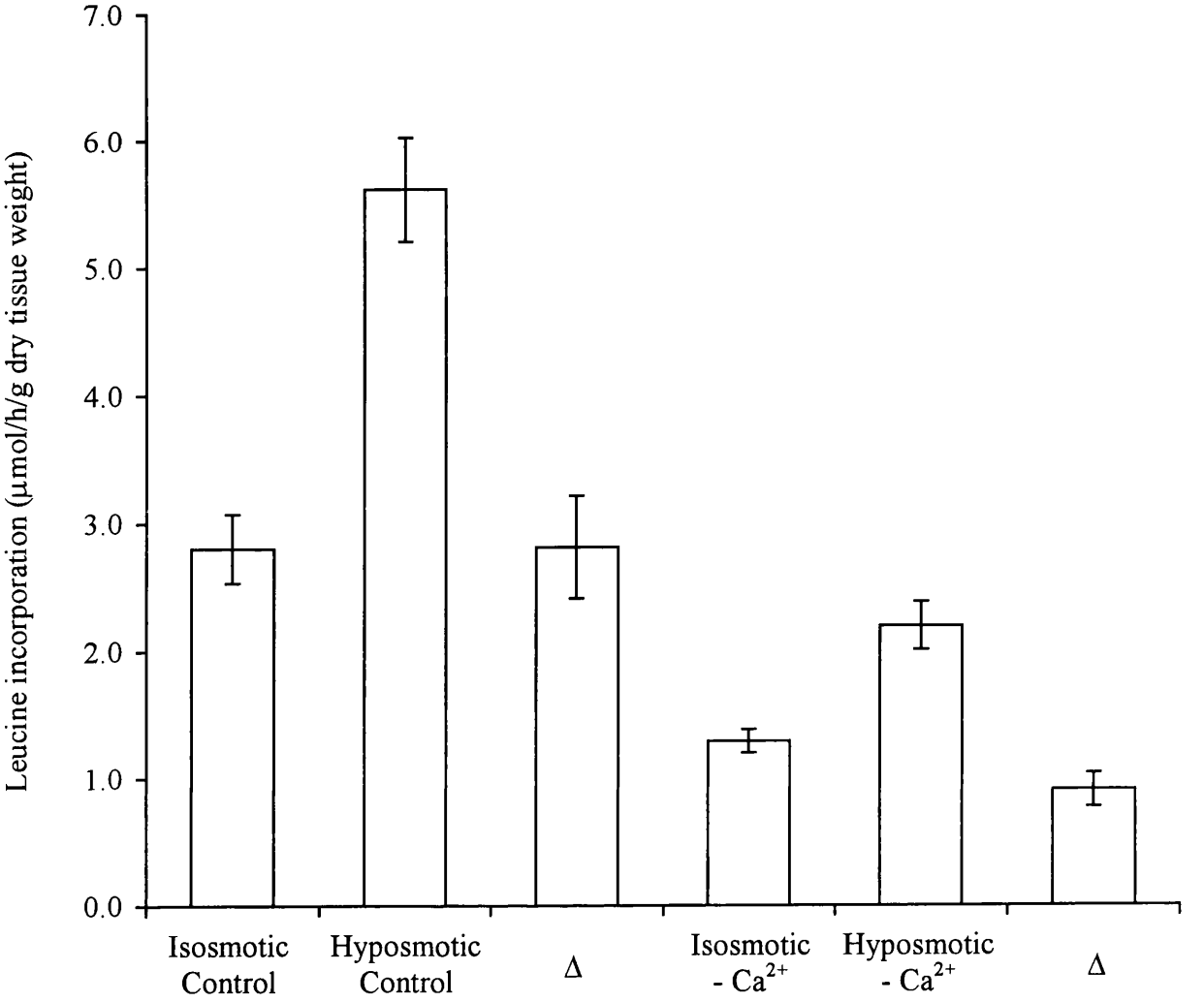

Figure 3.15: The effect of Ca^{2+} on swelling-induced [^{3}H]-leucine incorporation into mammary protein (explants). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, ± 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 303 ± 9 mosmol/kg water). The hyposmotic buffer was similar in composition except that it did not contain choline-Cl (osmolality = 159 ± 5 mosmol/kg water). Tris-EGTA (0.5mM) was also added to the Ca^{2+}-free buffers. Data shown are the means ±SEM of four experiments using tissue from separate animals.

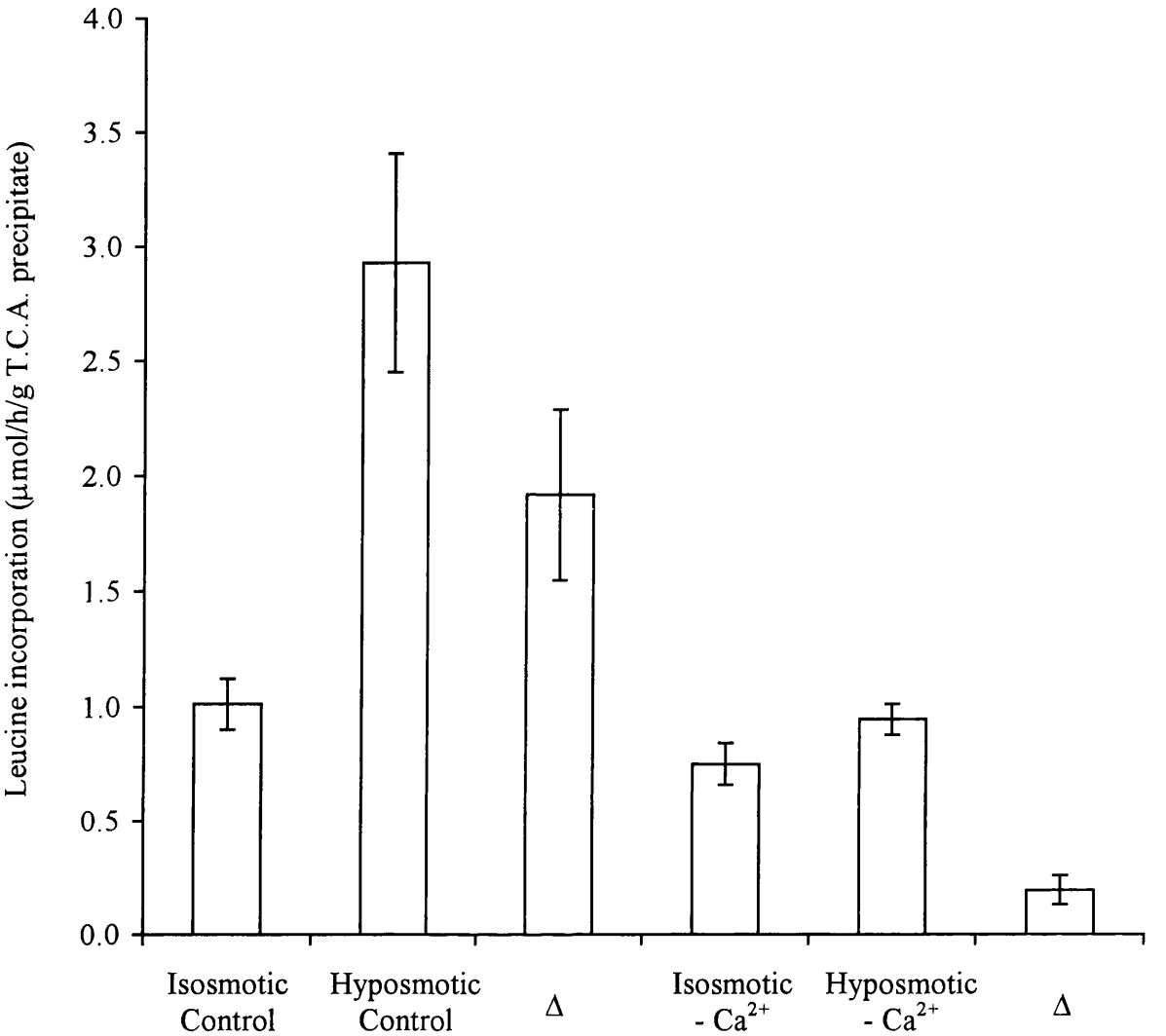

Figure 3.16: The effect of Ca^{2+} on swelling-induced [^{3}H]-leucine incorporation into mammary protein (acini). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, ± 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1). The hyposmotic buffer was similar in composition except that it did not contain choline-Cl. Tris-EGTA (0.5mM) was also added to the Ca^{2+}-free buffers. Data shown are the means ±SEM of four experiments using tissue from separate animals.

3.4.2 Control of protein synthesis by intracellular (lumenal) stores of Ca^{2+}

The results described in section 3.4.1 show that removing extracellular calcium reduces the incorporation of radiolabelled leucine into mammary protein under both isosmotic and hyposmotic conditions. This could be due to two factors. First, removing extracellular calcium could reduce the concentration of free ionised calcium in the cytosolic compartment of mammary cells and thus lead to a decrease in protein synthesis. Second, removing extracellular calcium may also deplete calcium stores within intracellular organelles particularly the endoplasmic reticulum. In light of this, experiments were designed to test the effect of depleting the intracellular calcium stores on the incorporation of radiolabelled leucine into rat mammary protein under isosmotic and hyposmotic conditions. The effect of depleting intracellular Ca^{2+} stores on rat mammary protein synthesis was studied utilising two inhibitors; thapsigargin (see appendix 1) and 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBHQ), both of which are pharmacological inhibitors of the endoplasmic reticulum (ER) Ca^{2+}-ATPase (Moore *et al.*, 1987; Thastrup *et al.*, 1990; Robinson *et al.*, 1992; Duncan and Burgoyne, 1996). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 (plus amino acids at concentrations described in table 2.1). The hyposmotic buffer was similar in composition except that it had no choline-Cl. When required, thapsigargin and tBHQ were was used at a concentration of 1 μM and 31 μM respectively. The incorporation of tracer into mammary protein was measured over 20 minutes.

Figure 3.17 shows that the addition of thapsigargin to the incubation medium decreased protein synthesis in mammary explants measured under isosmotic conditions from 2.11

± 0.32 to 1.36 ± 0.23 μmol/h/g dry tissue (n = 6, ±SEM, $P < 0.01$). Under hyposmotic conditions, thapsigargin reduced the incorporation of leucine into mammary protein from 4.99 ± 0.80 to 3.12 ± 0.50 μmol/h/g dry tissue (n = 6, ±SEM, $P < 0.01$). Thus, thapsigargin inhibited the volume-activated moiety of protein synthesis by 39 % (*n* = 6, ± SEM, $P < 0.01$).

tBHQ also inhibited mammary explant protein synthesis under isosmotic and hyposmotic conditions (figure 3.18). In the absence of tBHQ, the incorporation of leucine under isosmotic and hyposmotic conditions was respectively 1.31 ± 0.23 and 3.15 ± 0.44 μmol/h/g dry tissue weight ($P < 0.01$). In the presence of tBHQ, the rate of mammary tissue protein synthesis under isosmotic and hyposmotic conditions was respectively 0.80 ± 0.08 and 2.01 ± 0.20 μmol/h/g dry tissue weight ($P < 0.01$). Therefore, it is apparent that tBHQ inhibited mammary protein synthesis under both conditions. The volume-sensitive component of mammary protein synthesis was inhibited by 35% (n = 6, ±SEM, $P < 0.05$).

The effect of thapsigargin on protein synthesis in rat mammary acini under isosmotic and hyposmotic conditions was investigated (figure 3.19). In the absence of the inhibitor the rate of protein synthesis under isomotic and hyposmotic conditions was respectively 1.80 ± 0.26 and 3.53 ± 0.53 μmol/h/g TCA precipitate. Inclusion of thapsigargin in the isosmotic medium resulted in a 41% decrease in protein synthesis to 1.07 ± 0.17 μmol/h/g T.C.A. precipitate ($P < 0.02$). Similarly, thapsigargin reduced protein synthesis under hyposmotic conditions. Thus, the rate of incorporation was reduced by 49% to 1.81 ±

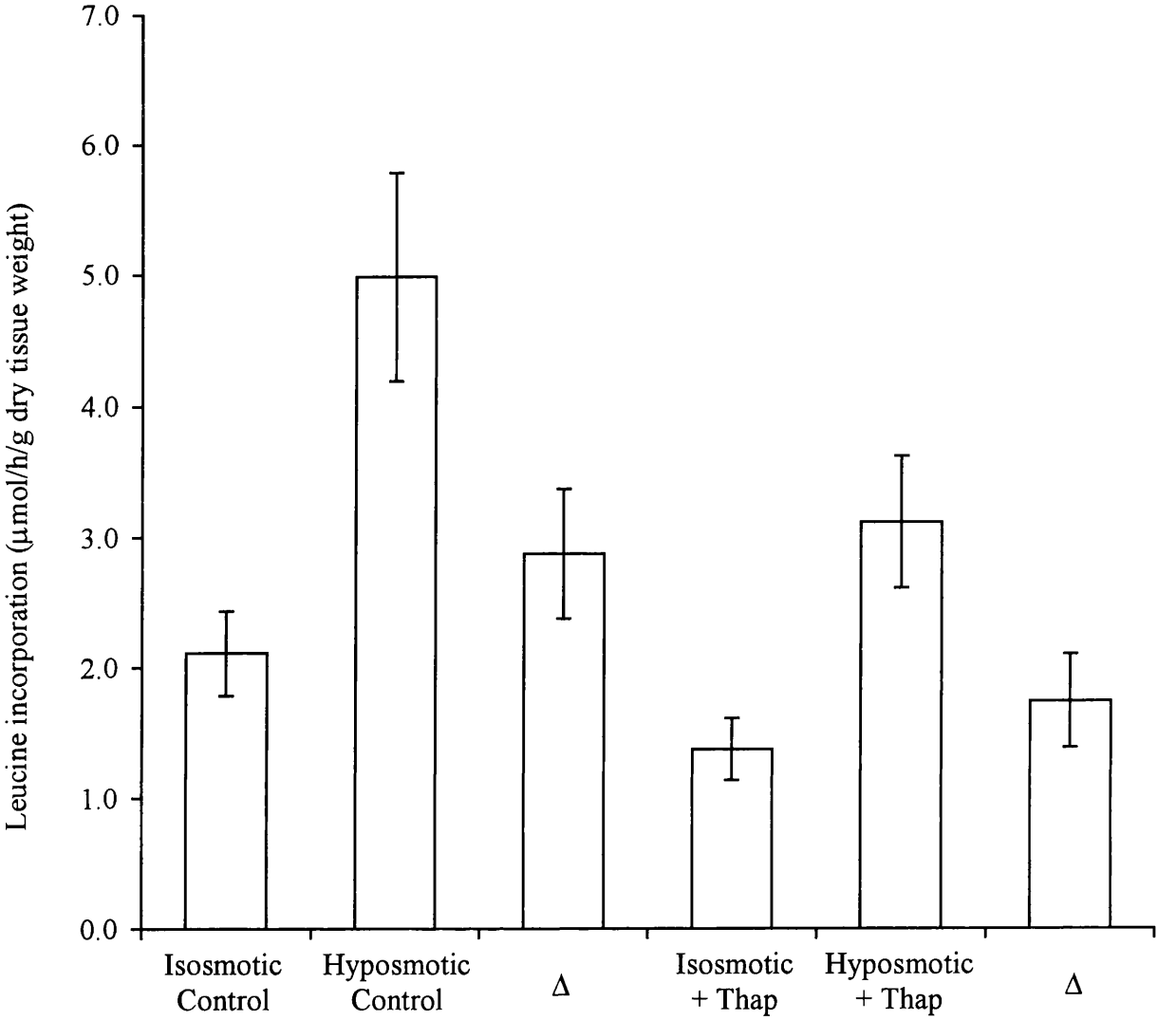

Figure 3.17: The effect of thapsigargin on swelling-induced [^{3}H]-leucine incorporation into mammary protein (explants). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 305 ± 1 mosmol/kg water). The hyposmotic buffer was similar in composition except that it did not contain choline-Cl (osmolality = 165 ± 1 mosmol/kg water). When required, thapsigargin was used at a final concentration of 1µM. DMSO was present at 0.1% (v/v) in all buffers. Data shown are the means ± SEM of six experiments using tissue from separate animals.

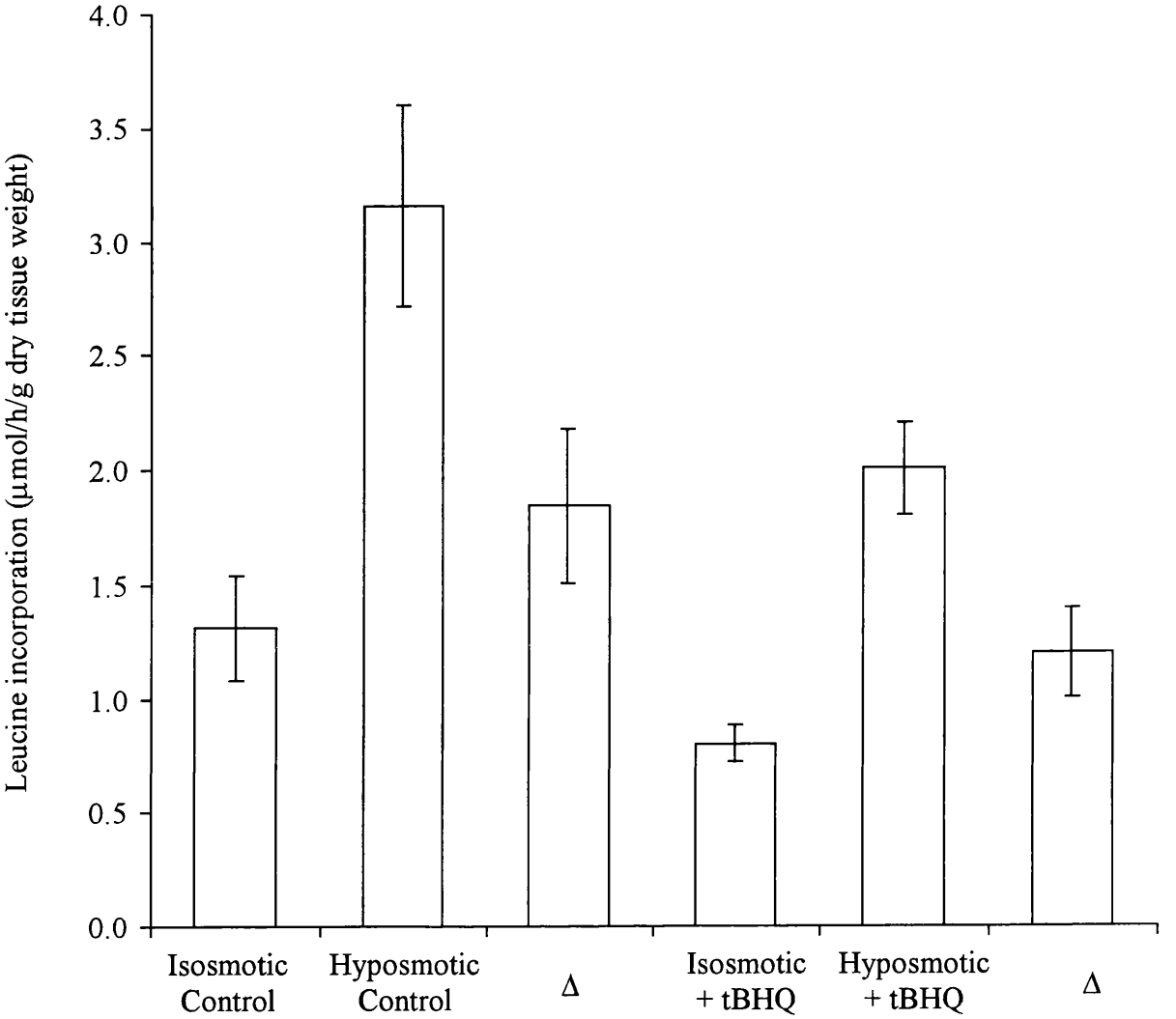

Figure 3.18: The effect of tBHQ on swelling-induced [^{3}H]-leucine incorporation into mammary protein (explants). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 311 ± 2 mosmol/kg water). The hyposmotic buffer was similar in composition except that it did not contain choline-Cl (osmolality = 171 ± 2 mosmol/kg water). When required, tBHQ was used at a final concentration of 31µM. DMSO was present at 0.1% (v/v) in all buffers. Data shown are the means ± SEM of six experiments using tissue from separate animals.

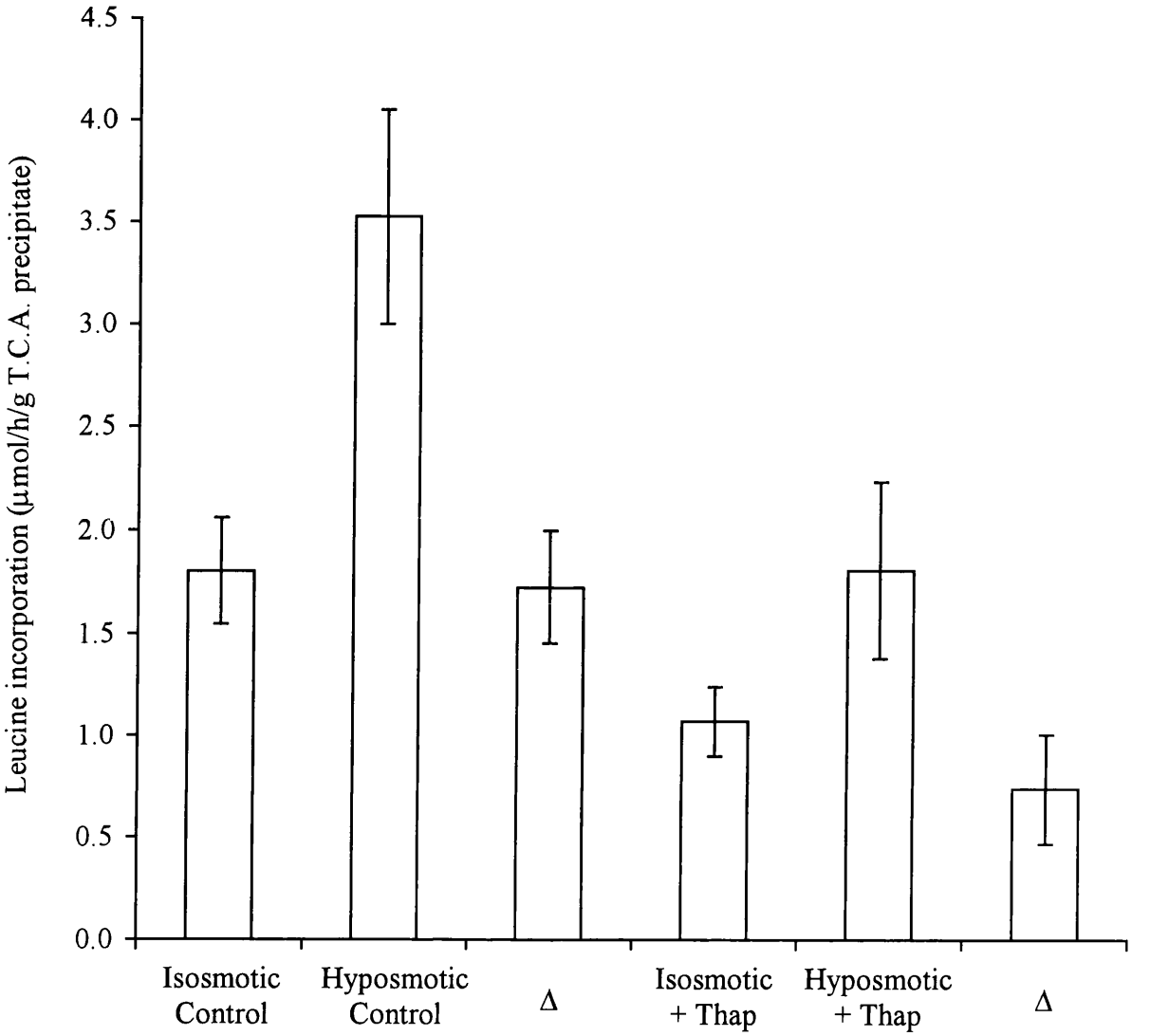

Figure 3.19: The effect of thapsigargin on swelling-induced [^{3}H]-leucine incorporation into mammary protein (acini). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1). The hyposmotic buffer was similar in composition except that it did not contain choline-Cl. When required, thapsigargin was used at a final concentration of 1µM. DMSO was present at 0.1% (v:v) in all buffers. Data shown are the means ±SEM of six experiments using tissue from separate animals.

0.43 μmol/h/g T.C.A. precipitate ($P < 0.001$). Therefore, volume-activated protein synthesis in rat mammary acini was reduced from 1.72 ± 0.27 to 0.74 ± 0.27 μmol/h/g TCA precipitate (n = 4, ±SEM, $P < 0.001$) by thapsigargin.

3.4.3. The effect of calcium channel blockers on mammary protein synthesis

Shennan and Gow (2000) have recently shown that a hyposmotic shock increases the cytosolic free calcium concentration in rat mammary acinar cells. It appears that the increase in calcium involves calcium uptake from the medium rather than release of calcium from intracellular stores. Therefore, it is possible that volume-sensitive mammary protein synthesis may be susceptible to compounds which are known to block calcium uptake into mammalian cells. The effect of two calcium channel inibitors, namely verapamil (see appendix 1) and gadolinium (Gd^{3+}) were tested on mammary protein synthesis. Verapamil is an inhibitor of L-type Ca^{2+} channels (Striessnig *et al.*, 1998) and Gd^{3+} has been shown to block passage of Ca^{2+} through stretch-activated channels situated in the plasma membrane (Caldwell *et al.*, 1998; Urbach *et al.*, 1999). Incorporation of [^{3}H]-leucine into mammary explant protein was measured over a 20 minute period in the absence and presence of the calcium channel blockers. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, $2CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 (plus amino acids as described in table 2.1). The hyposmotic buffer was similar in composition except that it contained no choline-Cl. Figures 3.20 and 3.21 show the results of the experiments. Verapamil (100 μM) had no effect on mammary protein synthesis under isosmotic or hyposmotic conditions. Under isosmotic conditions mammary protein synthesis was respectively 1.59 ± 0.15 and 1.43 ± 0.09 μmol/h/g dry

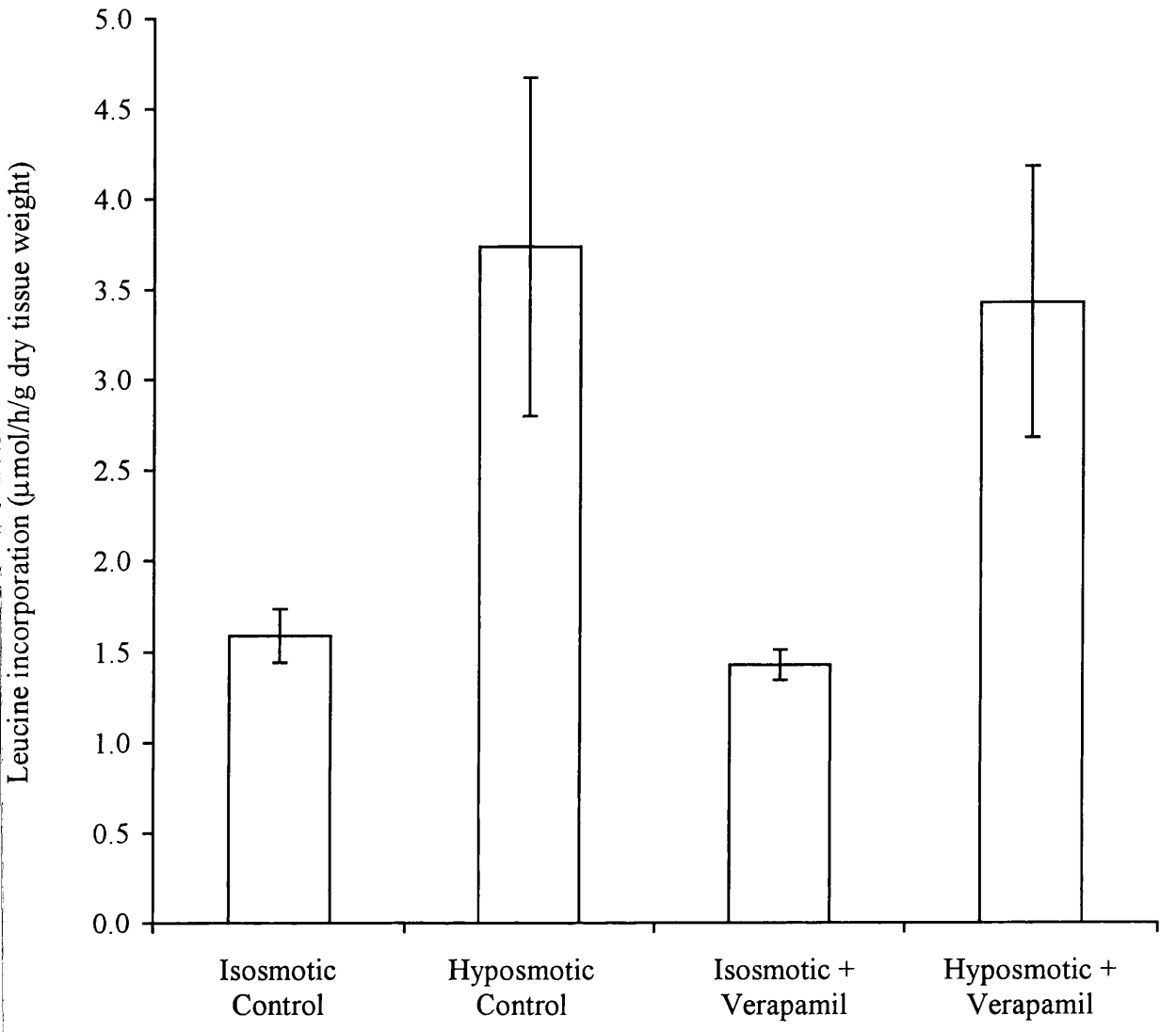

Figure 3.20: Effect of Verapamil on swelling-induced [^{3}H]-leucine incorporation into mammary explant protein. The isosomotic buffers contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 300 ± 5 mosmol/kg water). The hyposmotic buffers were similar in composition except that they had no choline-Cl (osmolality = 164 ± 6 mosmol/kg water). When required, verapamil was used at 0.1mM. Data shown are means ±SD from two experiments using tissue from separate animals.

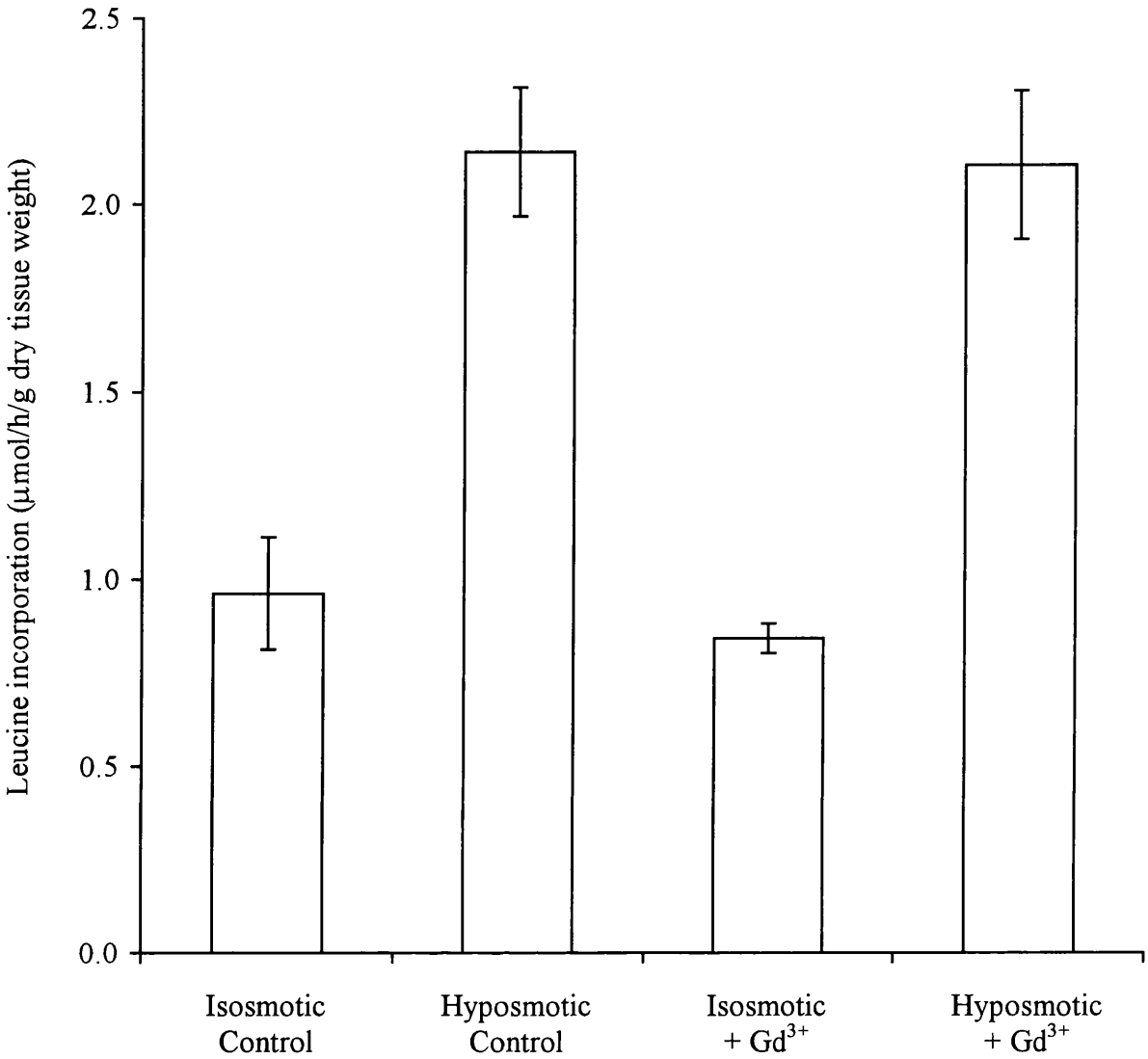

Figure 3.21: Effect of Gadolinium on swelling-induced [^{3}H]-leucine incorporation into mammary explant protein. The isosomotic buffers contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1µCi/ml [^{3}H]-leucine (+ amino acids as described in table 2.1) (osmolality = 303 ± 2 mosmol/kg water). The hyposmotic buffers were similar in composition except that they had no choline-Cl (osmolality = 166 ± 2 mosmol/kg water). When required, gadolinium was used at 0.1mM. Data shown are means ±SEM from three experiments using tissue from separate animals.

tissue weight (±SD, n = 2) in the absence and presence of verapamil. Under hyposmotic conditions, incorporation of leucine in the absence and presence of verapamil was respectively 3.74 ± 0.94 and 3.43 ± 0.75 μmol/h/g dry tissue (n = 2, ±SD).

Similarly, when 10 μM Gd^{3+} was added to the incubation medium there was no significant decrease in the rate of incorporation of tracer under both isosmotic and hyposmotic conditions (figure 3.21). In the absence of Gd^{3+} the rate of protein synthesis under isosmotic and hyposmotic conditions was respectively 2.89 ± 0.45 and 6.42 ± 0.52 μmol/h/g dry tissue weight. When Gd^{3+} was present the rate of mammary protein synthesis in isosmotic and hyposmotic media was respectively 2.53 ± 0.12 and 6.32 ± 0.60 μmol/h/g dry tissue weight (n = 3, ±SEM).

3.5 Results - Effect of cell volume change on cellular ATP in rat mammary tissue

The effect of changing cell volume on cellular ATP was studied to see if a change in the level of ATP could account for the inhibition of protein synthesis under hyperosmotic conditions. Rat mammary acini were incubated in isosmotic, hyposmotic and hyperosmotic buffers. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS. The hyposmotic buffer was similar in composition except that it had no choline-Cl. The hyperosmotic buffer was also similar in composition to the isosmotic buffer except that it had an additional 200mM sucrose. The effects of cell volume change on cellular ATP are illustrated in figure 3.22.

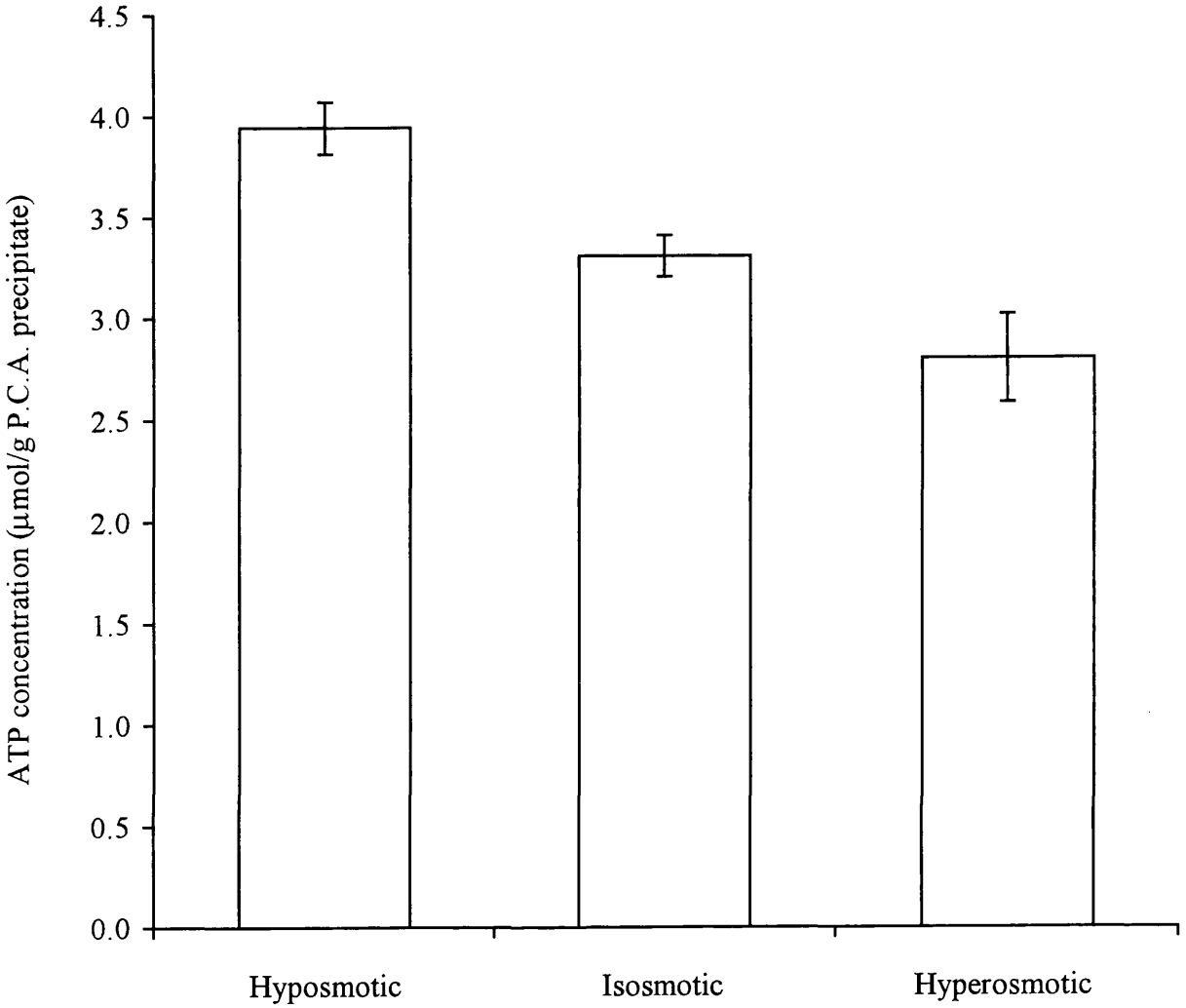

Figure 3.22: Modulation of intracellular ATP concentration by changes in mammary cell volume. Acini were incubated in isosmotic, hyposmotic and hyperosmotic conditions for 30 mins. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 4 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. The hyposmotic buffer was similar in composition except that it did not contain any choline-Cl. The hyperosmotic buffer was similar in composition except that it also contained 200 mM sucrose. Each point is the mean ±SEM of four experiments using tissue from separate animals.

When incubated in the hyposmotic buffer the ATP content of the acinar cells increased by 19%, from 3.31 ± 0.10 to 3.94 ± 0.13 μmol/g P.C.A. precipitate (±SEM, n = 4, $P < 0.001$). Conversely when the tissue was incubated in a hyperosmotic medium the quantity of ATP decreased by 15% to 2.80 ± 0.22 μmol/g P.C.A. precipitate (±SEM, n = 4, $P < 0.05$).

3.6. Discussion

The rate of protein synthesis in rat mammary tissue was quantified by measuring radiolabelled amino acid incorporation into T.C.A.-precipitable material. The amino acids used were [^{3}H]-leucine and [^{35}S]-methionine. The reason for using these amino acids was twofold. First, leucine and methionine transport into mammary cells is rapid which means that transport will not be a rate-limiting step. Both leucine and methionine use system L to cross the basolateral pole of the mammary epithelium (Shennan and Peaker, 2000): system-L is a Na^{+}-independent carrier that is sensitive to the amino acid analogue BCH. Importantly, leucine uptake has been shown to be unaffected by changes to the osmolality of the incubation medium (Millar *et al.*, 1997). Second, the intracellular concentrations of leucine and methionine are similar to those found in plasma. They are not markedly concentrated within mammary cells unlike glutamate, glycine and alanine (Shennan *et al.*, 1994, 1997). Therefore, it is reasonable to assume that the intracellular specific activities of radiolabelled leucine and methionine will be similar to their extracellular specific activities.

The rate of [^{3}H]-leucine and [^{35}S]-methionine incorporation was linear when mammary

explants were incubated in isosmotic, hyposmotic and hyperosmotic media, suggesting that the secretion of newly synthesised protein was negligible. In this connection, previous studies have shown that newly synthesised (labelled) mammary proteins were only detected in the medium after a period of 45-60 min (Turner *et al.*, 1992; Rennison *et al.*, 1992). The ratio of leucine to methionine incorporation, under isosmotic conditions, was 7.52 which is similar to the ratio of the two amino acids in casein (5.51), the primary component of newly synthesised mammary protein (Davis *et al.*, 1993). This suggests that the technique used for measuring mammary protein synthesis in explants was suitable.

3.6.1. Mammary protein synthesis is affected by cell volume

The results of the present study show that mammary protein synthesis is respectively increased and decreased by a hyposmotic and hyperosmotic shock. Therefore, it is reasonable to suggest that mammary protein synthesis is increased by cell swelling and decreased by cell shrinking. It appears that cell swelling acts as an anabolic signal whereas cell shrinking favours catabolism. This is in accordance with the results of Millar et al. (1997) who found that mammary protein synthesis was markedly affected by changes to cell volume. However, the overall rates of protein synthesis reported in this study are larger than that found by Millar et al. (1997). This may reflect that there was a difference between the way the TCA-precipitates were treated. In the study of Millar et al. (1997) TCA-precipitates were stored at -20°C until they were processed.

The influence of cell volume perturbations on mammary tissue protein synthesis was also

investigated using acini which had been prepared by collagenase digestion. As with explants, protein synthesis in mammary acini was stimulated by a hyposmotic challenge and inhibited by a hyperosmotic shock. It is generally accepted that mammary acini contain fewer stromal cells than explants. Therefore, it can be concluded that volume-sensitive protein synthesis occurs within the epithelial cells. In support of this notion is the finding that cell volume regulates casein synthesis by rat mammary tissue (Millar *et al.*, 1997).

In addition to having an effect on the rate of protein synthesis, changes in cell volume have also been shown to alter the rate of proteolysis (Haussinger *et al.*, 1993a; vom Dahl *et al.*, 1995, 1996; Lang *et al.*, 1998a). Indeed it has been suggested that dehydration and therefore reduction in muscle/liver cell volume is the cause of negative nitrogen balance in diseased states (Haussinger *et al.*, 1993a; Haussinger *et al.*, 1993b). Thus, there was a possibility that the decrease in mammary protein synthesis induced by a hyperosmotic challenge could be due to a decrease in the intracellular specific activity of radiolabelled leucine following proteolysis. However, it appears that this is not the case since a hyperosmotic shock markedly inhibited leucine incorporation into T.C.A.-precipitable material under conditions where the concentration of unlabelled leucine was increased to 4 mM (from 225 μM). Under these conditions it was predicted that unlabelled leucine, derived from hydrolysed protein, should have less effect on label dilution.

In the present investigation, the extracellular concentration of NaCl was maintained at 55 mM in order to minimise changes to Na^+-dependent substrate uptake. Therefore the osmolality of the test buffers was altered (between 150 and 300 mosmol/kg water) by the

addition of choline-Cl. It was possible, therefore, that the difference in protein synthesis between hyposmotic and isosmotic conditions reflected the presence of choline in the isosmotic buffer. However, similar results were found with another cation, namely $NMDG^+$.

The effect of cell swelling and shrinking on mammary protein synthesis was found to be reversible. This suggests that the decrease in mammary protein synthesis induced by a hyperosmotic shock cannot simply be attributed to cell damage. In mammary explants, the time taken to reverse the effect of cell shrinking on mammary protein synthesis was approximately 10-20 min. The effect of a hyposmotic challenge on rat mammary protein synthesis was also found to be reversible: it took approximately 10 min for protein synthesis to be reduced after the tissue was transferred from a hyposmotic to an isosmotic buffer. Furthermore, it took approximately 10 min for protein synthesis to be inhibited as a consequence of changing the osmolality of the incubation medium from 300 to 450 mosmol/kg water. These results suggest that mammary protein synthesis is rapidly regulated by cell volume changes.

3.6.2. Isosmotic swelling increases protein synthesis

Isosmotic cell swelling, using urea, also increased mammary protein synthesis. Urea is known to rapidly move across cell membranes including those of the mammary gland (Allen, 1988). Therefore, it is predicted that isosmotic buffers containing urea will swell mammary cells. This is based on the assumption that urea will move down its concentration gradient; osmotically obliged water will accompany the urea entering the

cells. In support of the notion that urea can swell mammary cells is the finding that urea can activate taurine efflux from lactating rat mammary tissue explants which has the same characteristics as taurine efflux activated by a hyposmotic challenge (Shennan *et al.*, 1994). In addition, hyposmotically-induced K^+(Rb^+) efflux from mammary tissue can be activated under isosmotic conditions by urea (Shennan and Gow, 2000). The finding that mammary protein synthesis can be stimulated under isomotic conditions suggest that mammary cells are responding to a change in their volume rather than sensing the osmolality of the incubation medium. Perhaps mammary cells are responding to a change in the intracellular osmotic pressure.

It is interesting to note that the effect of urea on mammary protein synthesis was inhibited by phloretin, a compound known to block urea transport. This is in accordance with the idea that urea has to cross the plasma membrane to exert its affect on mammary protein synthesis. However, we also found that phloretin inhibited mammary protein synthesis under control isosmotic conditions (i.e. in the absence of urea). Therefore, it is possible that phloretin inhibited mammary protein synthesis as a consequence of reducing glucose uptake by mammary cells; it is established that phloretin is an effective inhibitor of glucose transport via GLUT1, a protein known to mediate glucose transport in mammary tissue (Obermeier, 2000). Clearly, more experiments are needed to characterise the nature of inhibition of mammary protein synthesis by phloretin.

3.6.3. Sensitivity of protein synthesis to changes in osmolality

The rate of protein synthesis was dependent on the extent of the osmotic challenge.

Indeed, protein synthesis was markedly affected by relatively small changes in the osmolality of the incubation medium. This is an important finding as it suggests that mammary protein synthesis is a process which could be altered by small changes to cell volume. However, it must be borne in mind that cell volume changes per se may not be the factor that is being sensed by mammary cells. It is envisaged that mammary cells are able to regulate their volume following an osmotic perturbation. Indeed, mammary cells express several transport mechanisms which could help reduce cell volume following swelling. For example, mammary tissue possesses a volume-sensitive amino acid pathway and a volume-activated K^+ transport mechanism which could contribute to a regulatory volume decrease (Calvert and Shennan, 1998; Shennan and Gow, 2000). Therefore, it is possible that mammary protein synthesis is still affected even after mammary cells have regulated their volume. This means that cells are sensing factors other than or in addition to cell volume following an osmotic challenge. One such factor could be macromolecular crowding (Minton *et al.*, 1992; Summers *et al.*, 1997).

3.6.4. Role of Ca^{2+} in volume-sensitive protein synthesis in mammary tissue

It is established that reducing the calcium concentration outside the cell (Duncan and Burgoyne, 1996), in the cytosol or intracellular stores (Duncan and Burgoyne, 1996; Burgoyne *et al.*, 1998), can have a marked effect on mammary protein synthesis. The finding that rat mammary protein synthesis was reduced in a calcium-free (+EGTA) buffer is in agreement with the earlier studies. It is now shown that hyposmotically-activated protein synthesis is also reduced when the extracellular calcium concentration is diminished.

The effect of reducing the extracellular calcium concentration on mammary protein synthesis could be due to a reduction of a) cytosolic calcium or b) calcium in cell organelles. Indeed, incubating mammary acinar cells in a Ca^{2+}-free buffer reduces both cytosolic and eventually intracellular stores of Ca^{2+} (Sudlow and Burgoyne, 1997). The finding that thapsigargin and tBHQ inhibited mammary protein synthesis under isosmotic and hyposmotic conditions suggests that intracellular calcium stores (i.e. that within the ER) play an important role. Thapsigargin and tBHQ are agents that inhibit Ca^{2+} uptake from the cytosolic space into the ER (Thastrup *et al.*, 1989, 1990), by the sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Lytton *et al.*, 1991). However, both cytosolic and sequestered concentrations of calcium have been implicated as controlling factors in protein synthesis of other eukaryotic tissues (Palfrey and Nairn, 1995). Thus, a role for cytoplasmic Ca^{2+} in the regulation of mammary protein synthesis cannot be ruled out at this stage.

3.6.5. Effects of cell volume change on mammary ATP content

It is apparent that changing the volume of mammary cells has a significant effect on the rate of protein synthesis. Increasing cell volume markedly increased protein synthesis whereas decreasing mammary cell volume inhibited protein. Changing the hydration state of a cell effectively alters the environment surrounding each intracellular organelle and possibly their function. It is believed that a change in the osmotic environment surrounding mitochondria can have significant effects on the hydration state of the matrix (Lang *et al.*, 1998a). In this connection, several volume regulatory processes have been identified in mitochondria (Halestrap, 1989; Halestrap, 1994). In turn it has been shown

that a change in mitochondrial volume affects a change in the rate of energy production (Halestrap *et al.*, 1990; Halestrap, 1994). To determine if a change in the supply of ATP was a contributing factor in the alteration in rate of protein synthesis, ATP was quantified under isosmotic, hyposmotic and isosmotic conditions. Firstly, it was shown that increasing the hydration state of mammary acinar cells by hyposmotic challenge results in a 19% increase in ATP. Secondly it was also shown that there is a 15% reduction in ATP content following a hyperosmotic challenge. Therefore, it is apparent that mammary ATP is not particularly sensitive to cell volume pertubations. This suggests that the inhibition of protein synthesis following cell shrinking cannot be attributed to a lack of ATP.

CHAPTER FOUR

CONTROL OF INTRACELLULAR FREE CALCIUM CONCENTRATION ($[Ca^{2+}]_i$) IN MAMMARY TISSUE BY CELL VOLUME

4.1. INTRODUCTION

The results in Chapter 3 show that protein synthesis in rat mammary tissue is dependent upon extracellular calcium under both isosmotic and hyposmotic conditions. In this connection it has been shown that cell swelling, induced by a hyposmotc challenge increases the free cytosolic calcium concentration, measured using the fura-2 dye technique, in rat and mouse mammary epithelial cells (Sudlow and Burgoyne, 1997; Shennan and Gow, 2000). In both studies it was found that the increase in the $[Ca^{2+}]_i$ required the presence of extracellular calcium suggesting that a hyposmotic challenge stimulates Ca^{2+} uptake across the plasma membrane rather than release of Ca^{2+} from intracellular stores (Sudlow and Burgoyne, 1997; Shennan and Gow, 2000).

The purpose of the experiments described in this chapter was to examine further the effect of cell swelling on the intracellular $[Ca^{2+}]$ in rat mammary acinar cells given the relationship between protein synthesis and calcium. In particular two important points have been addressed. First, the swelling-induced increase in the cytosolic $[Ca^{2+}]$ of mammary cells found by Sudlow and Burgoyne (1997) and Shennan and Gow (2000) could have been due to a change in the electrochemical ion gradients (particulary that of Na^+) which accompanied the hyposmotic perturbation. Second, although a hyposmotic challenge is a useful tool to manipulate cell volume, the effect of isosmotic swelling on

mammary $[Ca^{2+}]_i$ remains to be examined. Therefore, experiments were conducted to a) confirm that cell swelling is the factor responsible for the hyposmotic-induced increase in cytosolic calcium and b) to test the effect of isosmotic cell swelling, using urea, on rat mammary $[Ca^{2+}]_i$.

4.2 METHODS

Changes in the concentration of cytosolic free calcium in rat mammary acinar cells were measured using the fura-2 dye technique (protocol described in detail in section 2.6). Fura2 is an UV-excitable, ratiometric Ca^{2+} indicator and therefore provides quantitative data independent of uneven dye loading, cell thickness, photo bleaching and dye leakage (Grynkiewicz *et al.*, 1984). The fluorescence excitation spectra for fura-2, measured at both zero and saturating concentrations of Ca^{2+} are shown in figure 4.1. This figure illustrates the difference in emission measured at 510nm when the dye is exposed to these two concentrations of calcium. The dye exhibits two distinct profiles with peaks at 335 and 362nm excitation, corresponding to calcium at saturating and zero concentrations respectively. In the present investigation, excitation wavelengths of 340 and 380nm were used and emission was measured at 509nm, in accordance with previous studies (Shennan and Gow, 1998; Sudlow and Burgoyne, 1997).

The cells were loaded with the dye through the use of the non-invasive fura-2 AM (acetoxymethyl ester) analogue. This form of the dye is membrane permeable and so can move freely into the cells. The rat mammary acini were incubated in a medium containing 50 μM fura-2 AM for 40 minutes, after which they were washed thrice by centrifugation

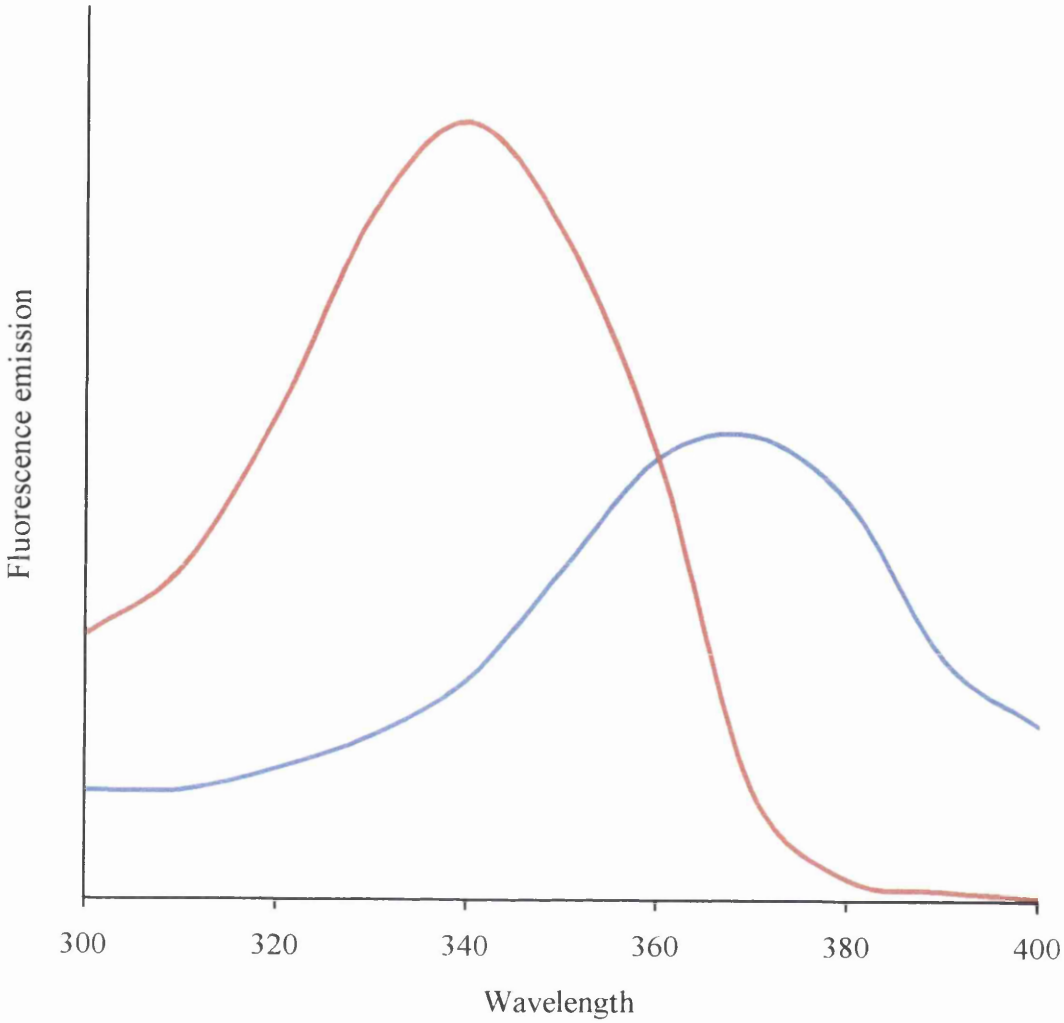

Figure 4.1: Schematic diagram of the excitation spectrum for the calcium sensitive dye Fura-2. Fura-2 was incubated either in the absence (—) of or at a saturating concentration (—) of calcium. Excitation wavelengths between 300 and 400nm were used and the emission of light was recorded at 509nm. The isosbestic point is the wavelength at which a change in the concentration of calcium has no effect on the fluorescence of the dye (approx 360nm)(adapted from Grynkeiwicz et al., 1984)

and resuspension (described in detail in chapter 2). Once inside the cell the AM-ester is hydrolysed leaving it in the impermeable acid form (see figure 4.2), trapping the dye in the cytosolic compartment. The acini were then left for a further 40 minutes, for two reasons: firstly, to maximise the hydrolysis of the ester form and secondly, to allow for leakage of dye from damaged cells. The rat mammary acini were then washed thrice more (as above).

Standards were calculated using the free acid form of fura2, solubilised in the standard isosmotic buffer, which contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. The maximum fluorescence was measured by saturating the dye with calcium (adding 10mM $CaCl_2$); the minimum was determined by addition of an excess of Tris-EGTA, pH 7.4 (10mM). Background fluorescence was calculated by the incubation of dye-free cells with and without additions.

It was important to ensure that the cells in each acinar preparation were intact and retaining the dye within the cytosolic compartment. Therefore the acini were treated with Triton X-100 to lyse the cells and release the dye. The rationale behind this control was that if there was no observed increase in the calcium concentration then the cells had not retained the dye. Conversely, a large increase in the concentration would mean that the cells had retained the dye. Figure 4.3 shows a typical trace. In this example, the acini were initially incubated in an isosmotic buffer containing (mM) 135 NaCl, 5 KCl, 2$CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 s, Triton X-100 was added to the acinar suspension to give a final concentration of 0.4% (w : v). It is evident that this manouvre increased the signal suggesting that the dye was within the acinar cells.

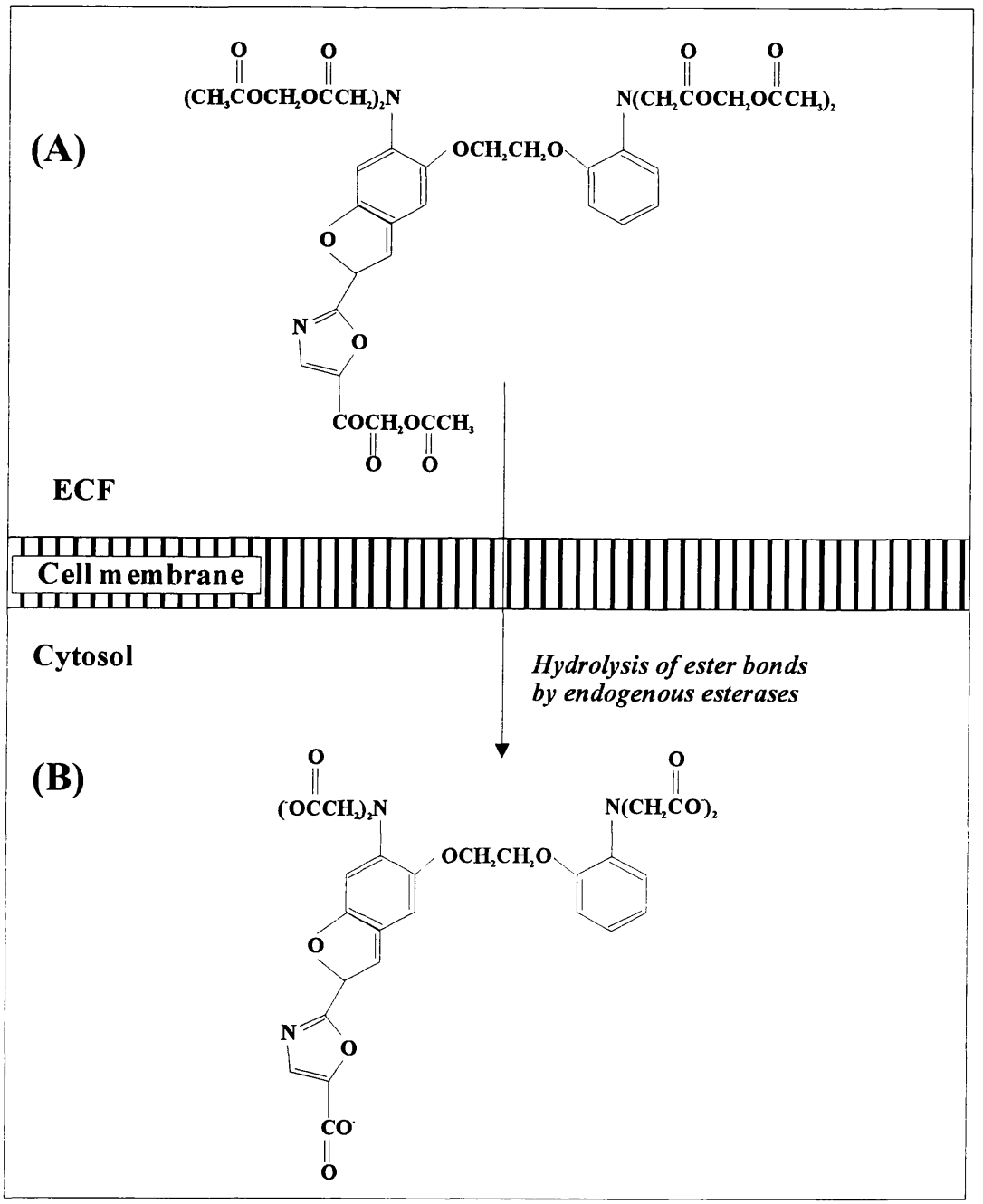

Figure 4.2: Non-invasive accumulation of fura-2 in cells. Fura-2 AM (A) freely diffuses across the plasma membrane into the cytosol. Ester bonds are hydrolysed by esterases within the cell. The anionic form of Fura-2 (B) is then trapped within the cell (adapted from Sigma manual).

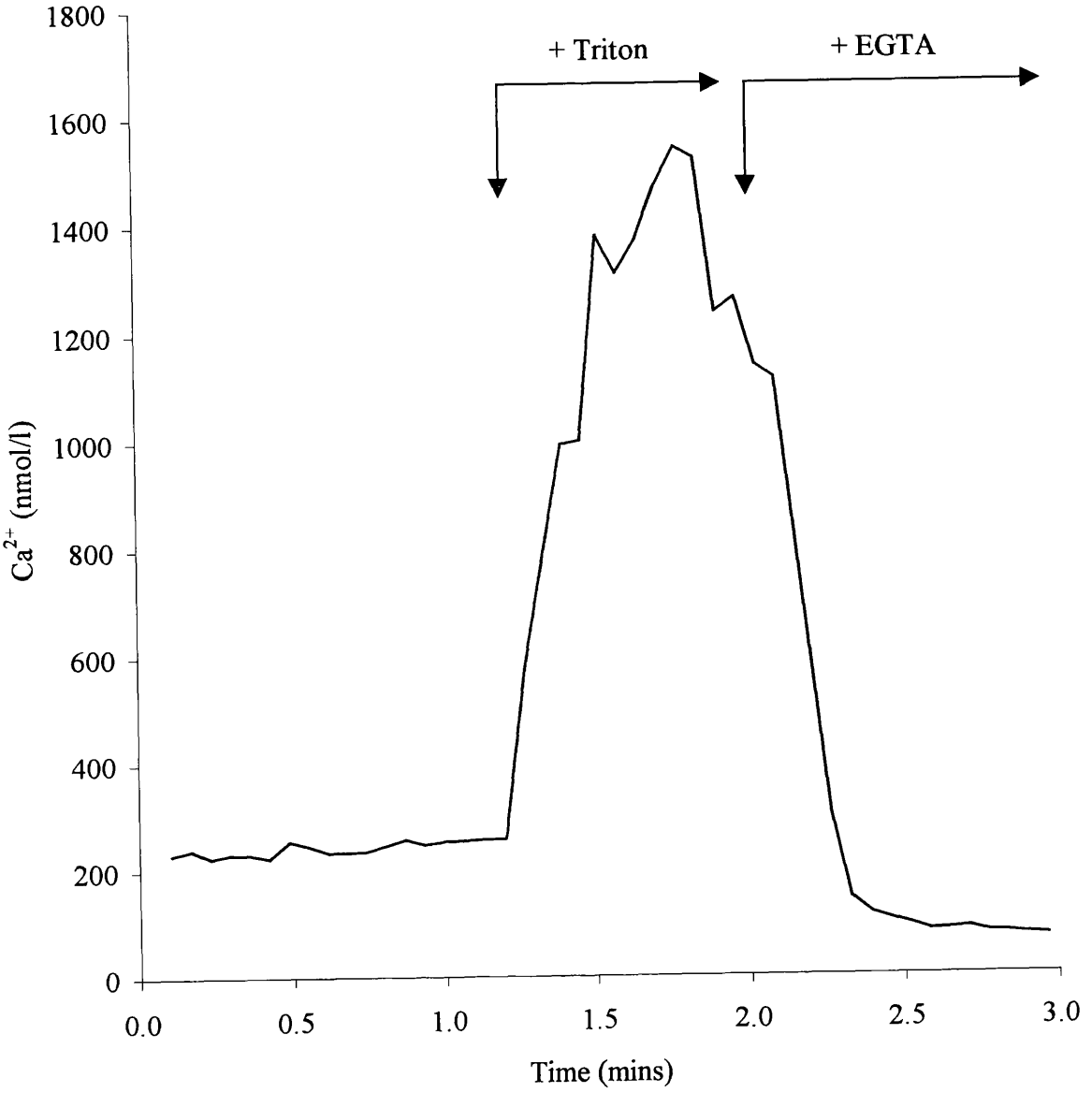

Figure 4.3: Release of fura-2 from Triton-x 100 lysed acini. Fura-2-loaded acini are initially incubated in a solution containing (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4 (osmolality = 314 mosmol/kg water). At t = 60 secs, Triton-x 100 is added to a final concentration of 0.4% (w : v). At t = 120 secs, Tris-EGTA is added to a final concentration of 15mM. Excitation wavelangths = 340 and 380nm, emission is recorded at 509nm. Results are from one experiment.

Statistical analysis of the data was carried out using paired Student's *t*-test. Data was analysed at single time points before and after a given challenge, or between two different experimental conditions where a control incubation was incorporated.

4.3. Results - The effect of cell volume change on intracellular free calcium concentration

4.3.1. Effect of a hyposmotic challenge on $[Ca^{2+}]_i$ in mammary acinar cells

It has previously been shown that lowering the osmolality of the incubation medium increases the intracellular calcium concentration in mouse and rat mammary acinar cells (Sudlow and Burgoyne, 1997, Shennan and Gow, 2000). The first step in the investigation was to confirm this observation using the same incubation conditions used by Sudlow and Burgoyne (1997) and Shennan and Gow (2000). Figure 4.4 shows the effect of cell swelling, induced by a hyposmotic challenge on $[Ca^{2+}]_i$ in rat mammary acinar cells. For comparison, figure 4.4 also shows the $[Ca^{2+}]_i$ in rat mammary acinar cells which were incubated in an isosmotic buffer throughout the entire time course. The isosmotic buffer contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 20 Tris-MOPS, pH 7.4 (osmolality = 314 mosmoles/kg of water). Under isosmotic conditions (at t = 52 s) the free cytosolic calcium concentration was 121 ± 5 nM. After 1 min the acinar suspension was diluted with an equal volume of distilled H_2O containing 2mM Ca^{2+}; the osmolality of the suspension was reduced to 160 mosmoles/kg of water. It is apparent that this manoeuvre increased ($P < 0.001$) the intracellular calcium

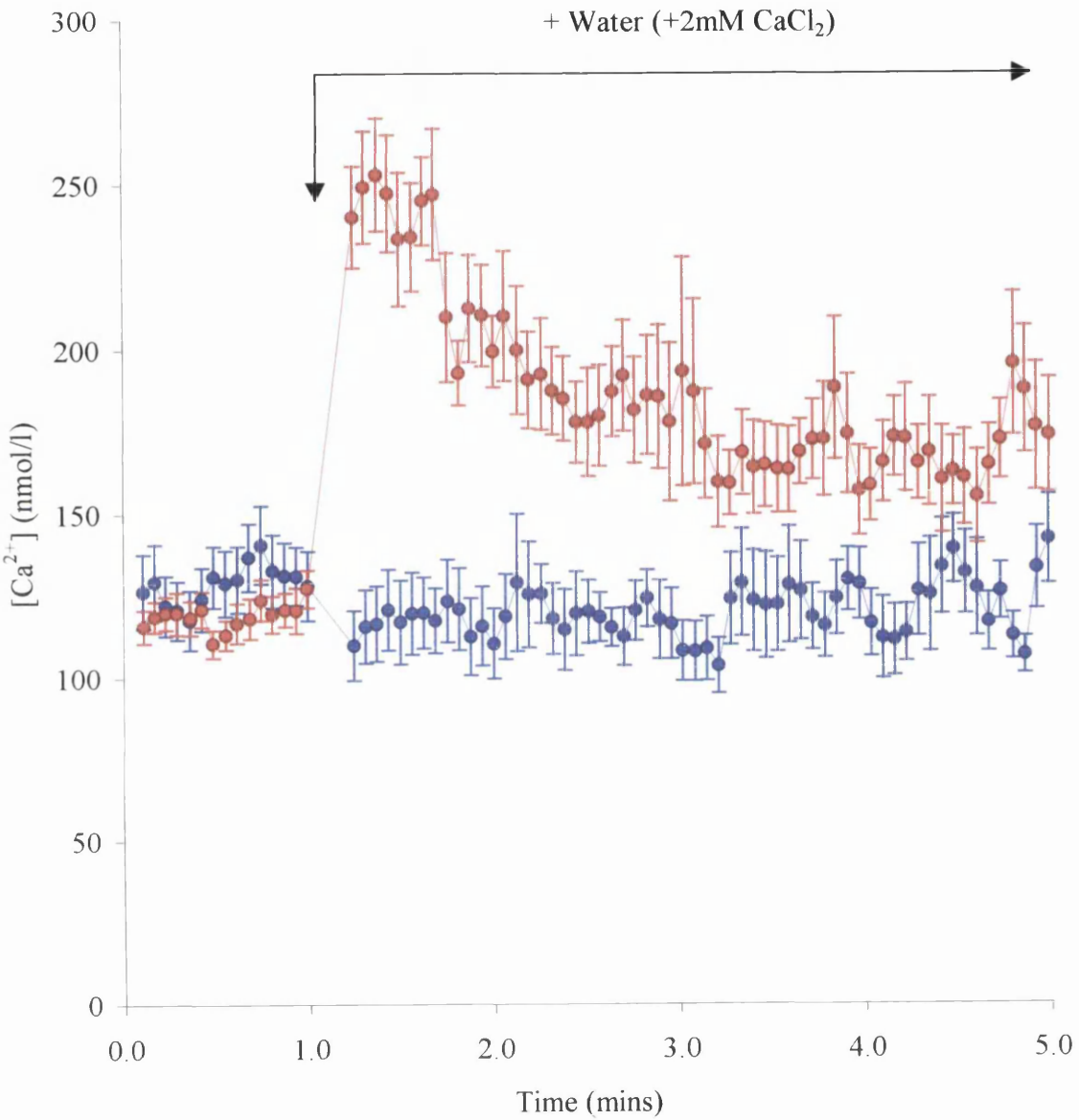

Figure 4.4: Effect of hyposmotic challenge on $[Ca^{2+}]_i$. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 secs, the solution was made hyposmotic (—) by the addition of an equal volume of H_2O + 2mM $CaCl_2$ (final osmolality = 160 mosmol/kg water). Fluorescence was recorded for a further 4 minutes. The control suspension (—) was maintained in isosmotic conditions throughout. The suspensions were constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 14 experiments with tissue obtained from different animals.

concentration to 253 ± 17 nM 14 s after the hyposmotic challenge was given. However, it is apparent from figure 4.4 that the effect of a hyposmotic shock on $[Ca^{2+}]_i$ was only transient. Thus $[Ca^{2+}]_i$ decreased to a level similar to that found under isosmotic conditions within 240 s.

The result shown in figure 4.4 confirms the finding that the $[Ca^{2+}]_i$ in rat mammary acinar cells is sensitive to a change in the osmolality of the incubation medium. However, it must be borne in mind that not only the osmolality of the incubation medium was altered. Thus, the transmembrane Na^+, K^+, Cl^- gradients would also have been changed (and hence the trans-membrane electrical potential difference). In addition the glucose concentration and the buffering capacity of the incubation medium would have been reduced under the prevailing experimental conditions. In the next set of experiments the incubation buffer was made hyposmotic by reducing the NaCl concentration whilst keeping the concentration of all other constituents constant. Figure 4.5 shows the effect of a hyposmotic challenge on the intracellular free Ca^{2+} concentration under these conditions. For the first minute the acini were incubated in an isosmotic solution containing (mM) 135 NaCl, 5 KCl, $2CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. After 60 seconds the acinar suspension was diluted with a buffer similar in composition to the isosmotic buffer except that it had no NaCl. This meant the final concentration of NaCl was 67.5mM. This manoeuvre decreased the osmolality of the incubation buffer by 39% from 314 to 190 mosmol/kg water. Prior to the osmotic challenge (t = 60 s), $[Ca^{2+}]_i$ was 126 ± 10 nM. Reducing the osmolality of the incubation medium markedly increased the concentration of free Ca^{2+} within the cell: $[Ca^{2+}]_i$ increased to 220 ± 17 nM (± SEM, n = 16, $P < 0.001$) 15 s following the osmotic shock. However, the increase in $[Ca^{2+}]_i$ was

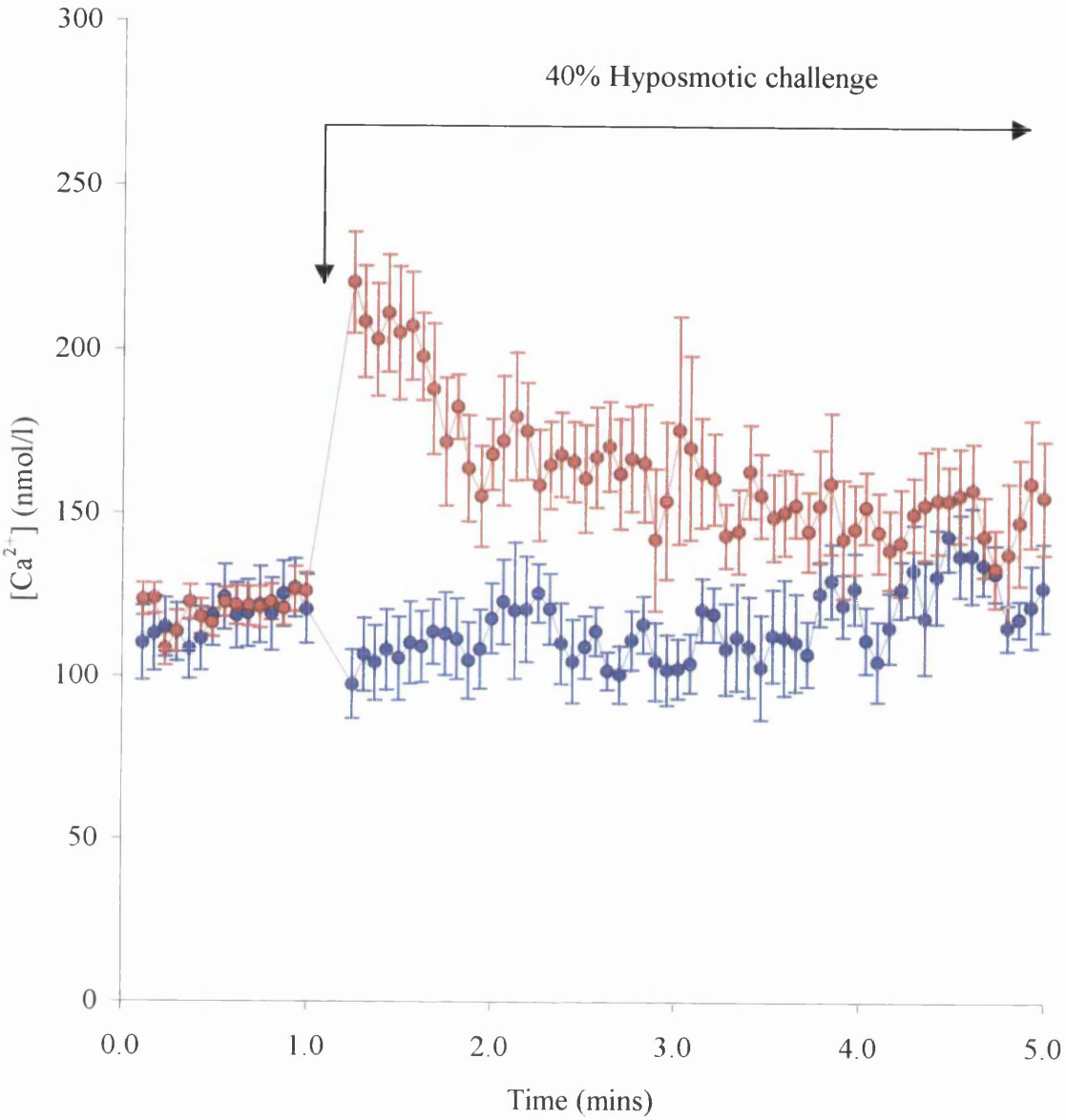

Figure 4.5: Effect of 40% hyposmotic challenge on $[Ca^{2+}]_i$. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 secs, the solution was made hyposmotic (—) by the addition of a buffer similar to the isosmotic one, except that it had no NaCl (final osmolality = 190 mosmol/kg water). Fluorescence was recorded for a further 4 minutes. The control suspension (—) was maintained in isosmotic conditions throughout. The suspensions were constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 16 experiments with tissue obtained from different animals.

transient: the cytosolic free calcium concentration in rat mammary acinar cells returned to a value similar to that found under isosmotic conditions within 180 s.

Decreasing the osmolality of the incubation buffer by 20% from 314 to 252 mosmol/kg water also increased $[Ca^{2+}]_i$ in rat mammary acinar cells (figure 4.6). The isosmotic buffer was the same as that used in the experiments illustrated in figure 4.5. Again, the osmolality was lowered by the addition of an equal volume of buffer similar in composition to the isosmotic buffer, except that the NaCl concentration was reduced to 67.5 mM, thus making the final concentration 101.3mM. Figure 4.6 shows that was an increase in $[Ca^{2+}]_i$, from 125 ± 9 nM to 182 ± 9 nM (± SEM, n = 8, $P < 0.001$). However the increase was only transient returning to a basal level within 34 s.

The effect of reducing the osmolality of the incubation buffer by 10% on $[Ca^{2+}]_i$ in rat mammary acinar cells was also tested. The acini were incubated initially in an isosmotic buffer containing (mM) 135 NaCl, 5 KCl, $2CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 s the osmolality of the incubation medium was reduced by lowering the NaCl concentration to 116.9mM. It is apparent from figure 4.7 that this manouvre had no significant effect on $[Ca^{2+}]_i$ in rat mammary acinar cells.

For comparison, the effect of the calcium ionophore ionomycin on $[Ca^{2+}]_i$ in rat mammary acinar cells was examined. The results of these experiments are shown in figure 4.8. The acini were incubated initially in an isosmotic buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. Ionomycin was added to the incubation medium at t = 60 s to give a final concentration of 10 μM. It is evident from

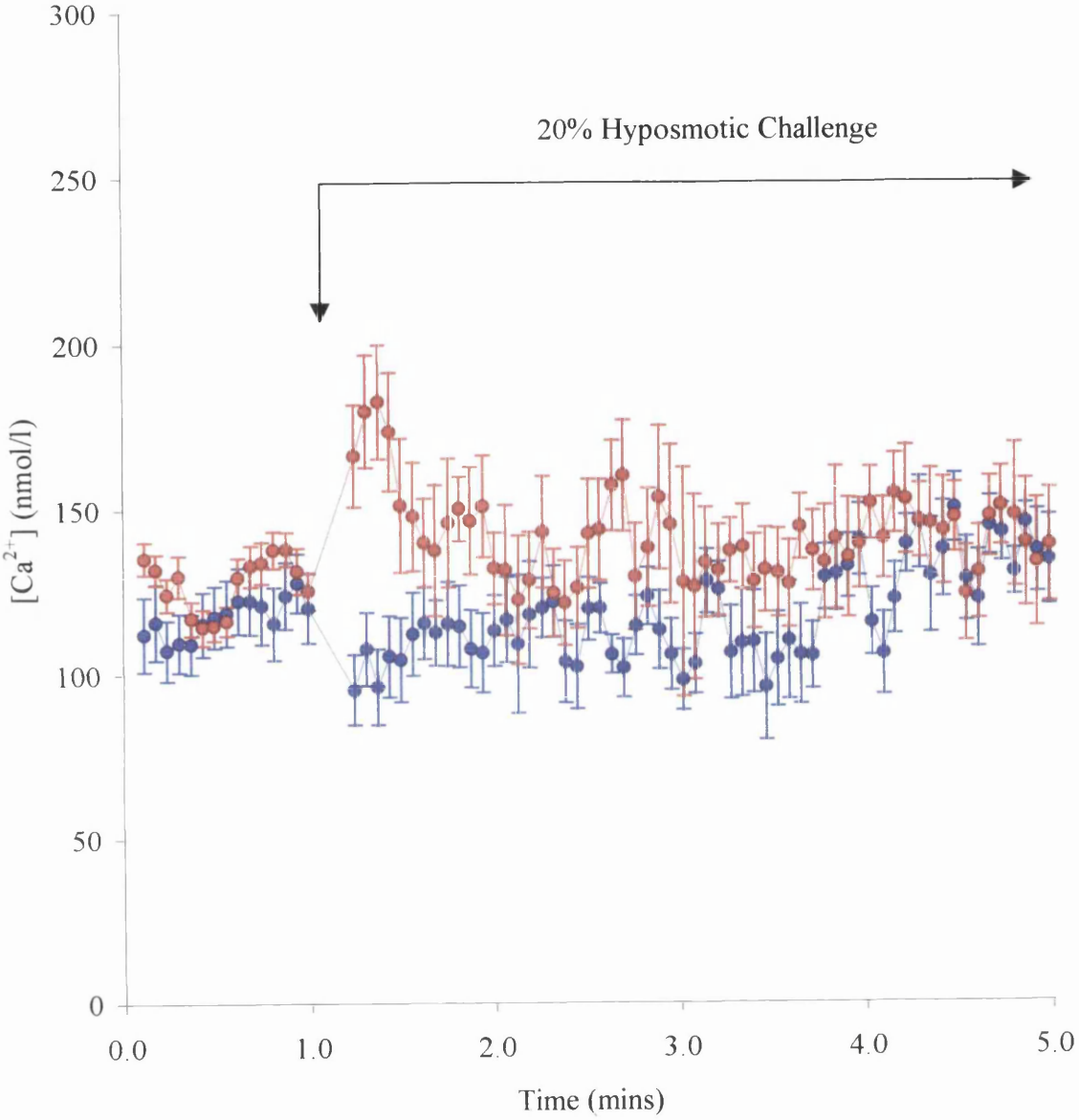

Figure 4.6: Effect of a 20% hyposmotic challenge on $[Ca^{2+}]_i$. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer was the same as in figure 4.5. At t = 60 secs, the solution was made hyposmotic (—) by the addition of a buffer similar to the isosmotic one, except that the NaCl concentration was reduced to 67.5mM (final osmolality = 252 mosmol/kg water). Fluorescence was recorded for a further 4 minutes. The control suspension (—) was maintained in isosmotic conditions throughout. The suspension was constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 8 experiments with tissue obtained from different animals.

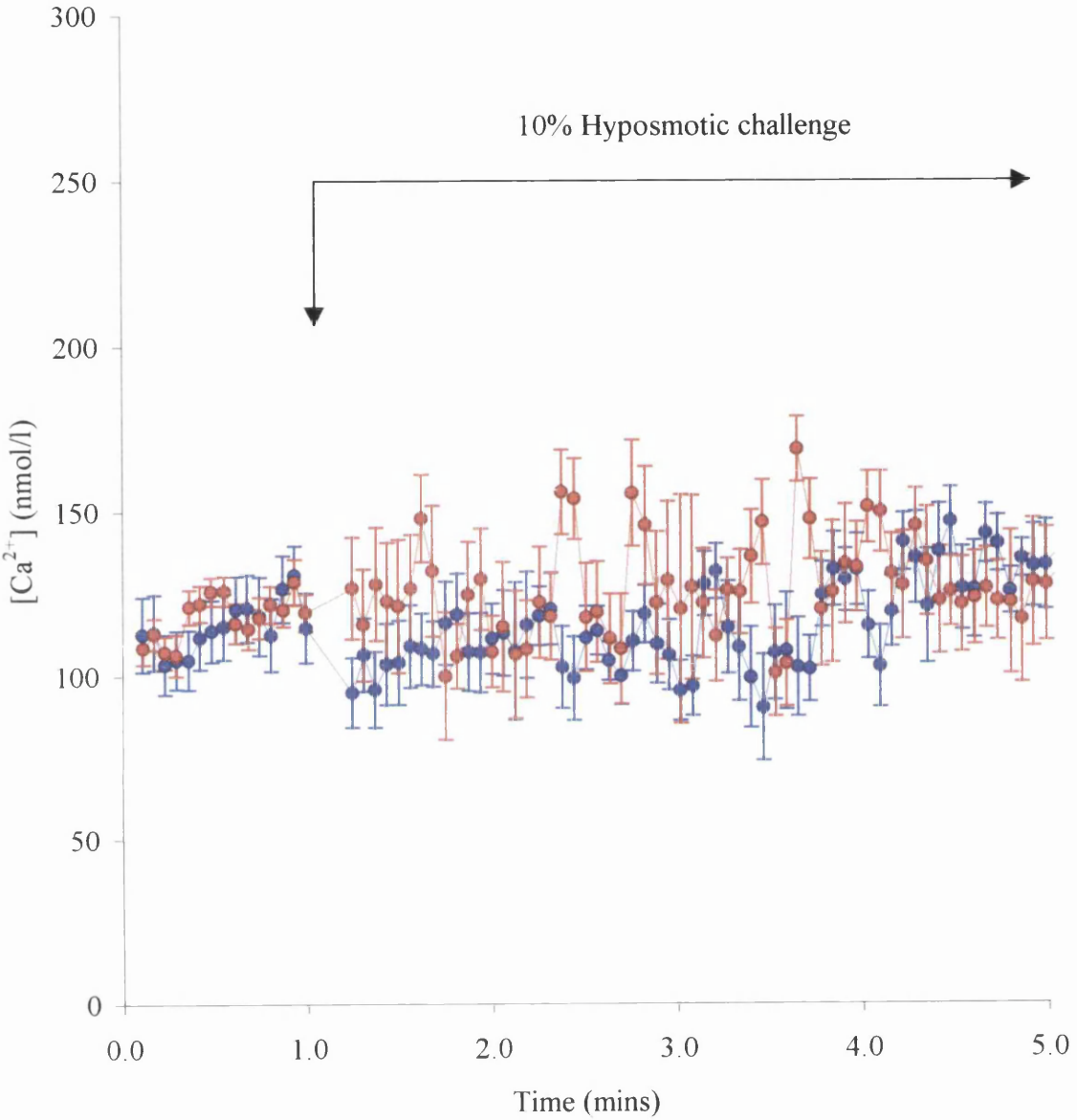

Figure 4.7: Effect of 10% hyposmotic challenge on $[Ca^{2+}]_i$. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer was the same as in figure 4.5. At t = 60 secs, the solution was made hyposmotic (—) by the addition of a buffer similar to the isosmotic one, except that the NaCl concentration was reduced to 101.5mM. Fluorescence was recorded for a further 4 minutes. The control suspension (—) was maintained in isosmotic conditions throughout. The suspension was constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 9 experiments with tissue obtained from different animals.

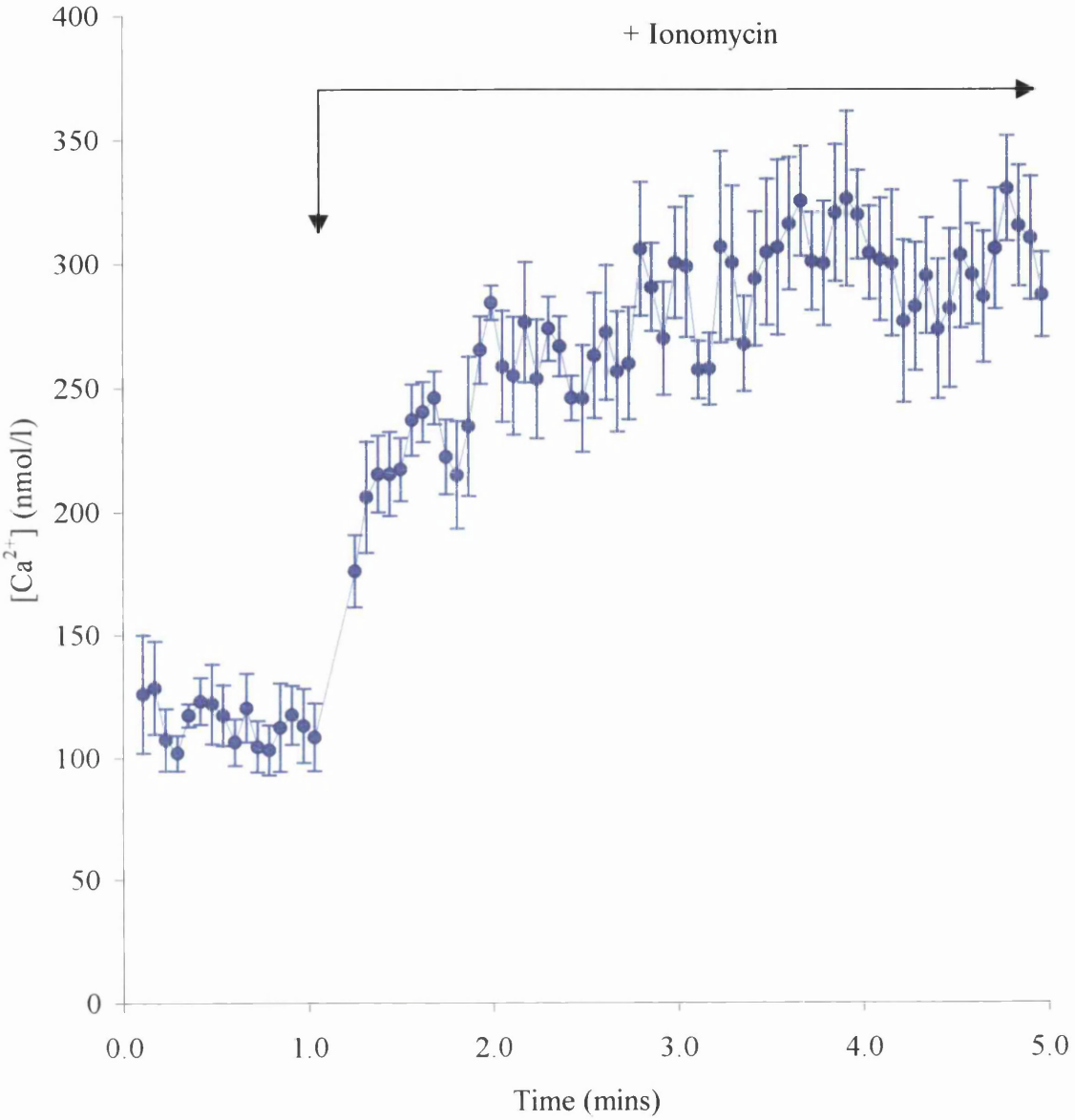

Figure 4.8: Effects of Ionomycin on cytosolic $[Ca^{2+}]_i$. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 secs, ionomycin was added to a final concentration of 10μM. Fluorescence was recorded for a further 4 minutes. The suspension was constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 6 experiments with tissue obtained from different animals.

figure 4.8 that there was a rapid, sustained increase in the concentration of free calcium following addition of the ionophore (±SEM, n = 6, $P < 0.001$).

4.3.2 Effect of reducing the Na^+ gradient on intracellular $[Ca^{2+}]$

It appears from the results described above that a hyposmotic challenge increases the $[Ca^{2+}]_i$ in rat mammary acinar cells. However, the increase in $[Ca^{2+}]_i$ shown in figures 4.4 to 4.6 could be due to a reduction in the extracellular Na^+ concentration *per se*. One mechanism of controlling $[Ca^{2+}]_i$ is through Na^+/Ca^{2+} exchange (Matsuda *et al.*, 1997): reducing the extracellular concentration of Na^+ could reduce the efflux of Ca^{2+} and thus lead to an increase in $[Ca^{2+}]_i$. In this connection, experiments were conducted to investigate the effects of reducing extracellular Na^+ without altering the osmolality of the incubation medium on $[Ca^{2+}]_i$ in rat mammary acini (figure 4.9). Acini were incubated initially in a buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. The buffer added to reduce Na^+, while maintaining an isosmotic environment, was similar in composition except that the NaCl was replaced with choline-Cl. Thus the final concentration of NaCl was 67.5 mM. Figure 4.9 shows that reducing the extracellular Na^+ concentration in this manner had no significant effect on the intracellular concentration of Ca^{2+} in rat mammary acinar cells.

4.3.3 Effect of reducing the ionic strength of the incubation medium on $[Ca^{2+}]_i$

The increase in the intracellular free $[Ca^{2+}]$ shown in figures 4.4 to 4.6 could also be due to a decrease in the ionic strength of the incubation medium. Therefore, the effect of

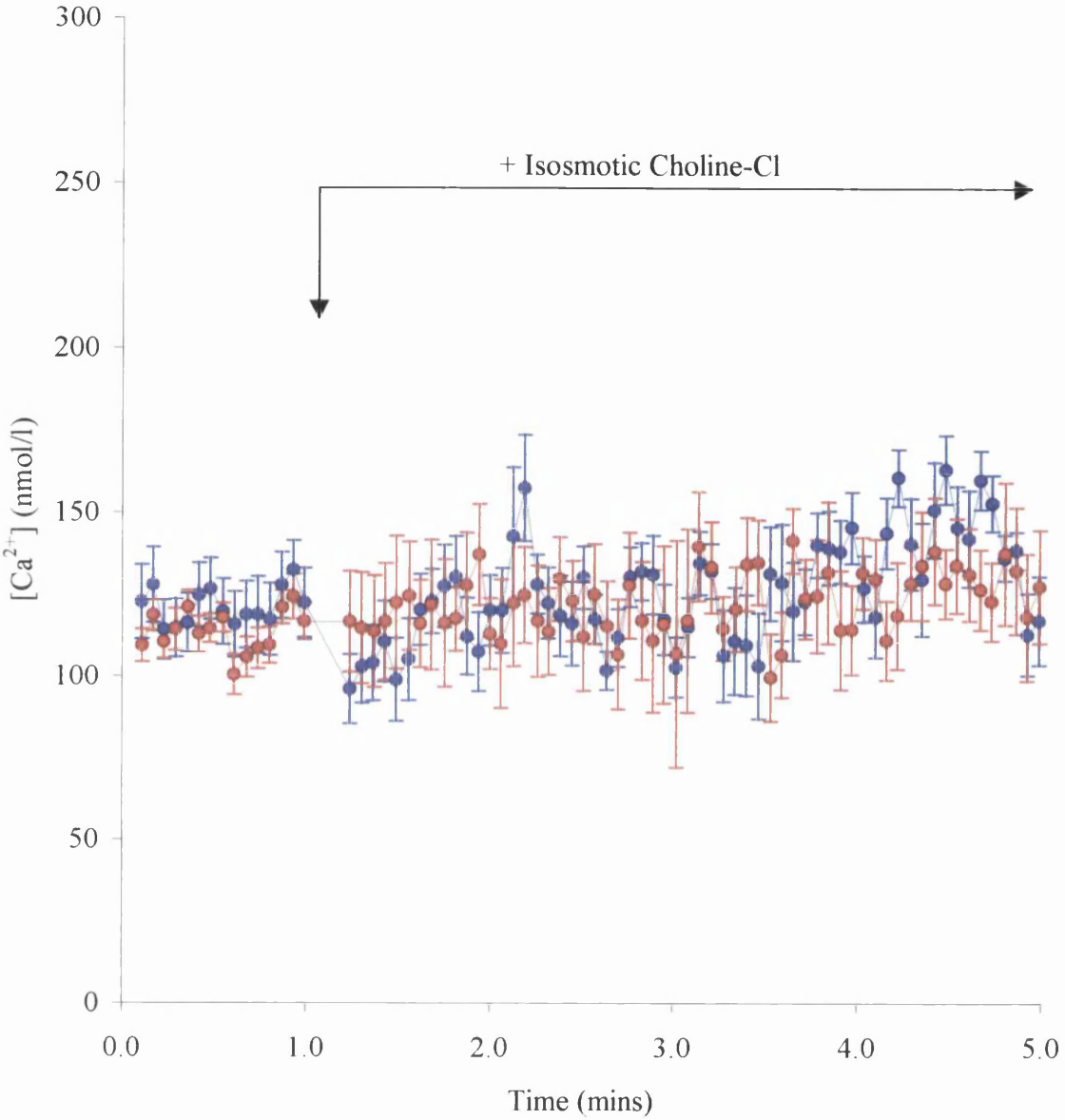

Figure 4.9: Effect of reducing the Na^+ gradient on $[Ca^{2+}]_i$. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 secs, the solution was mixed with a second isosmotic buffer similar to the first one except that it contained 135mM choline-Cl (—) and no NaCl. Fluorescence was recorded for a further 4 minutes. The control suspension (—) was maintained in isosmotic conditions throughout. The suspension was constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 5 experiments with tissue obtained from different animals.

changing the ionic strength of the medium under isosmotic conditions was examined. In this set of experiments the NaCl concentration of the incubation medium was reduced whilst sucrose was added to maintain osmolality. Initially, the acini were incubated in a buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS. At t = 60 s, the acinar suspension was mixed with an equal volume of a buffer similar in composition except that it contained 270mM sucrose and no NaCl. Therefore, the final concentration of NaCl was 67.5mM. It is apparent from the data shown in figure 4.10 that a reduction in ionic strength of the incubation buffer had no significant effect on the $[Ca^{2+}]_i$ of rat mammary acinar cells.

4.3.4 Effect of removing extracellular Ca^{2+} on $[Ca^{2+}]_i$

The results of Shennan and Gow (2000) suggest that the increase in $[Ca^{2+}]_i$ in rat mammary acinar cells following a hyposmotic shock is due to an increase in calcium influx across the plasma membranes. Experiments were designed to confirm this finding, thus, the effect of a hyposmotic challenge in the absence of extracellular Ca^{2+} on $[Ca^{2+}]_i$ in rat mammary acinar cells was examined. The calcium chelator EGTA was used to reduce the extracellular $[Ca^{2+}]$. Acini were incubated in an isosmotic medium containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4) for 1 minute at which point Tris-EGTA, pH7.4, was added to give a final concentration of 1mM. Then, at t = 120 s, the acinar suspension was made hyposmotic by the addition of a solution similar in composition to the isosmotic buffer except that it had no NaCl (+1mM Tris-EGTA, pH 7.4). Figure 4.11 shows that there was no increase in the cytosolic calcium concentration in response to the hyposmotic challenge in the absence of extracellular free

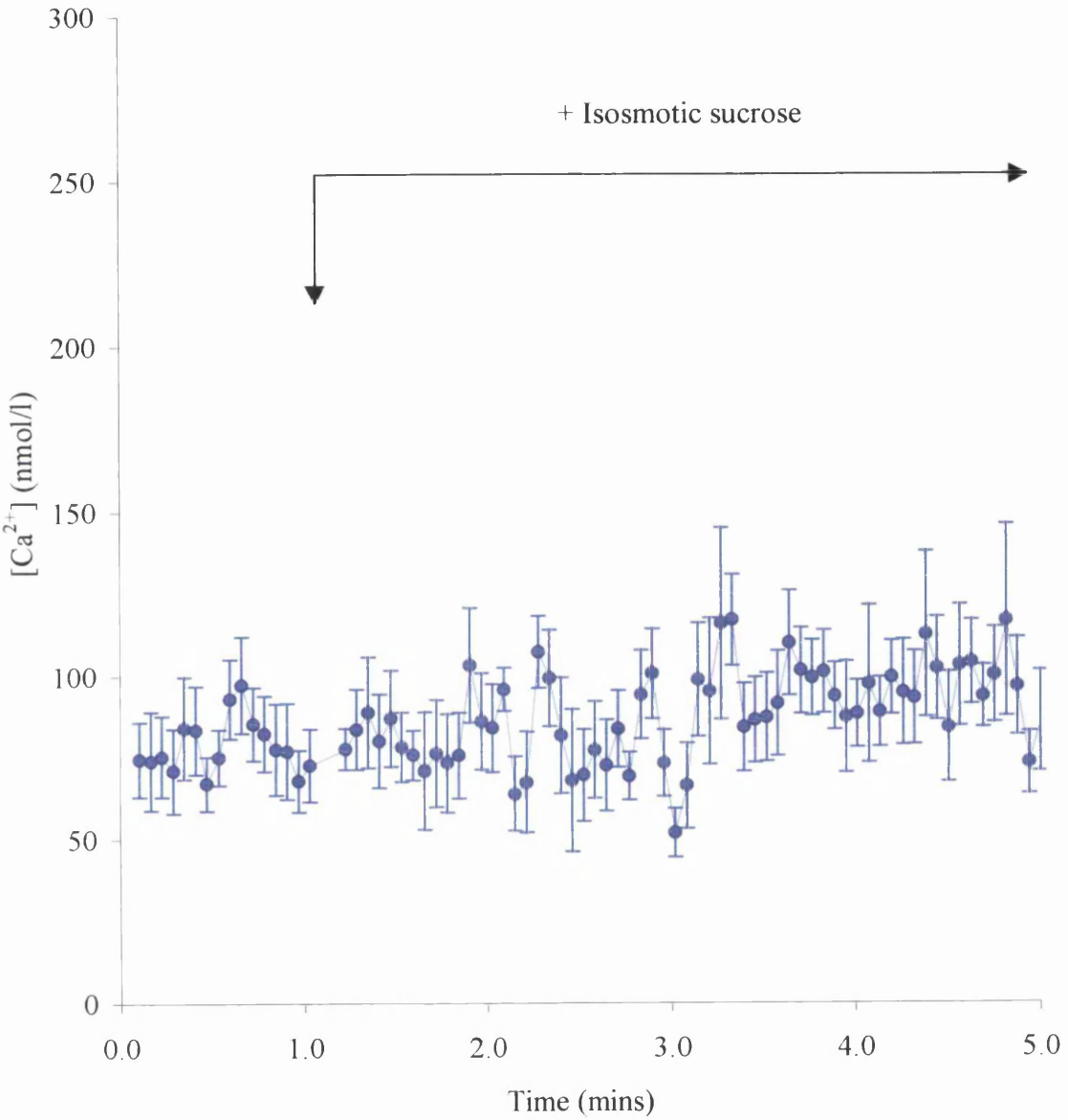

Figure 4.10: The effect of reducing ionic strength on $[Ca^{2+}]_i$. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 secs, the solution was mixed with a second isosmotic buffer similar to the first one except that it contained 270mM sucrose and no NaCl. Fluorescence was recorded for a further 4 minutes. The suspension was constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 6 experiments with tissue obtained from different animals.

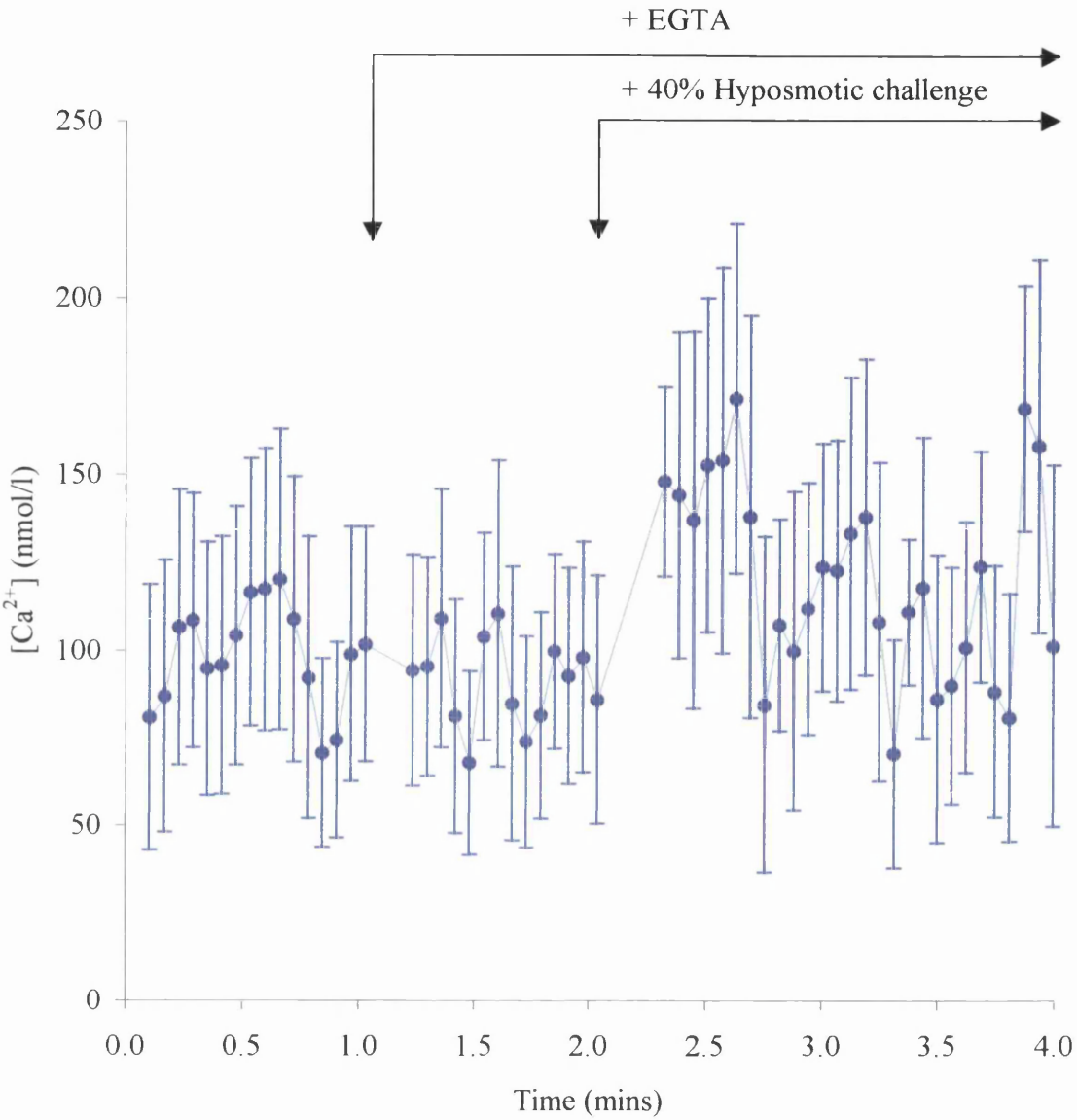

Figure 4.11: Effect of a hyposmotic challenge on $[Ca^{2+}]_i$ in the presence of 1mM EGTA. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 secs, Tris-EGTA was added to a final concentration of 1mM. At t = 120 secs the solution was rendered hyposmotic by the addition of a buffer similar to the isosmotic one, except that there was no NaCl (+15mM Tris-EGTA; final osmolality = 190 mosmol/kg water). Fluorescence was recorded for a further 4 minutes. The suspension was constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 3 experiments with tissue obtained from different animals.

Ca^{2+}.

In a further set of experiments the effect of a hyposmotic challenge on $[Ca^{2+}]_i$ in rat mammary acinar cells in the presence of a higher concentration of EGTA was studied. Figure 4.12 illustrates the effect of a hyposmotic shock, in the absence and presence of extracellular Ca^{2+}, on $[Ca^{2+}]_i$ in rat mammary acinar cells. It is apparent that adding EGTA (15mM) to the isosmotic buffer markedly reduced $[Ca^{2+}]_i$ ($P < 0.001$). Reducing the osmolality of the incubation medium from 314 to 190 mosmoles/kg of water in the presence of EGTA had no effect on the $[Ca^{2+}]_i$ in mammary acinar cells. In contrast, a hyposmotic shock markedly increased the $[Ca^{2+}]_i$ in the absence of EGTA (see figure 4.5).

4.3.5 Release of Ca^{2+} from intracellular stores

The increase in $[Ca^{2+}]_i$ following a hyposmotic challenge could be limited by Ca^{2+} pumps removing free Ca^{2+} from the cytosolic compartment. A number of different calcium pumps localised to the plasma and ER membranes are present in the rat mammary gland during lactation. Two (sarco-) endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) and three plasma membrane Ca^{2+}-ATPases (PMCAs) have already been identified (Reinhardt and Horst, 1999; Reinhardt *et al.*, 2000). Therefore the effect of the tumour promoter, and SERCA-specific inhibitor, thapsigargin was examined on $[Ca^{2+}]_i$. The results of the experiments are shown in figure 4.13. Acini were suspended initially in an isosmotic buffer (314 mosmol/kg water) which contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. Thapsigargin (1μM) was added to the suspension at t = 60 s; this addition caused a slight increase in $[Ca^{2+}]_i$. Subsequently

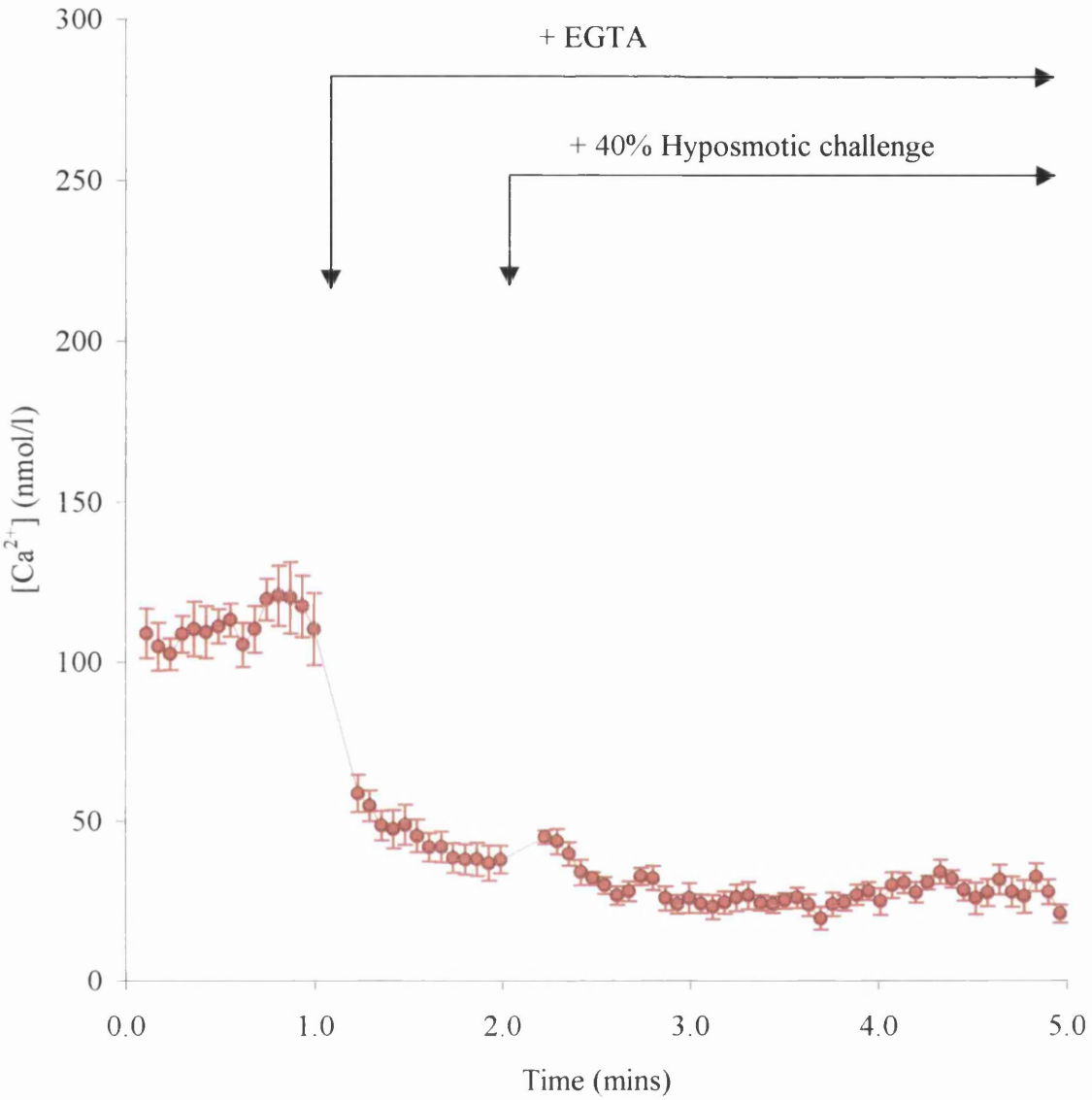

Figure 4.12: Effect of a hyposmotic challenge on $[Ca^{2+}]_i$ in the presence of 15mM EGTA. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer was the same as in figure 4.11. At t = 60 secs, Tris-EGTA was added to a final concentration of 15mM. At t = 120 secs the solution was rendered hyposmotic by the addition of a buffer similar to the isosmotic one, except that there was no NaCl (+ 15mM Tris-EGTA; final osmolality = 190 mosmol/kg water). Fluorescence was recorded for a further 4 minutes. The suspension was constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 9 experiments with tissue obtained from different animals.

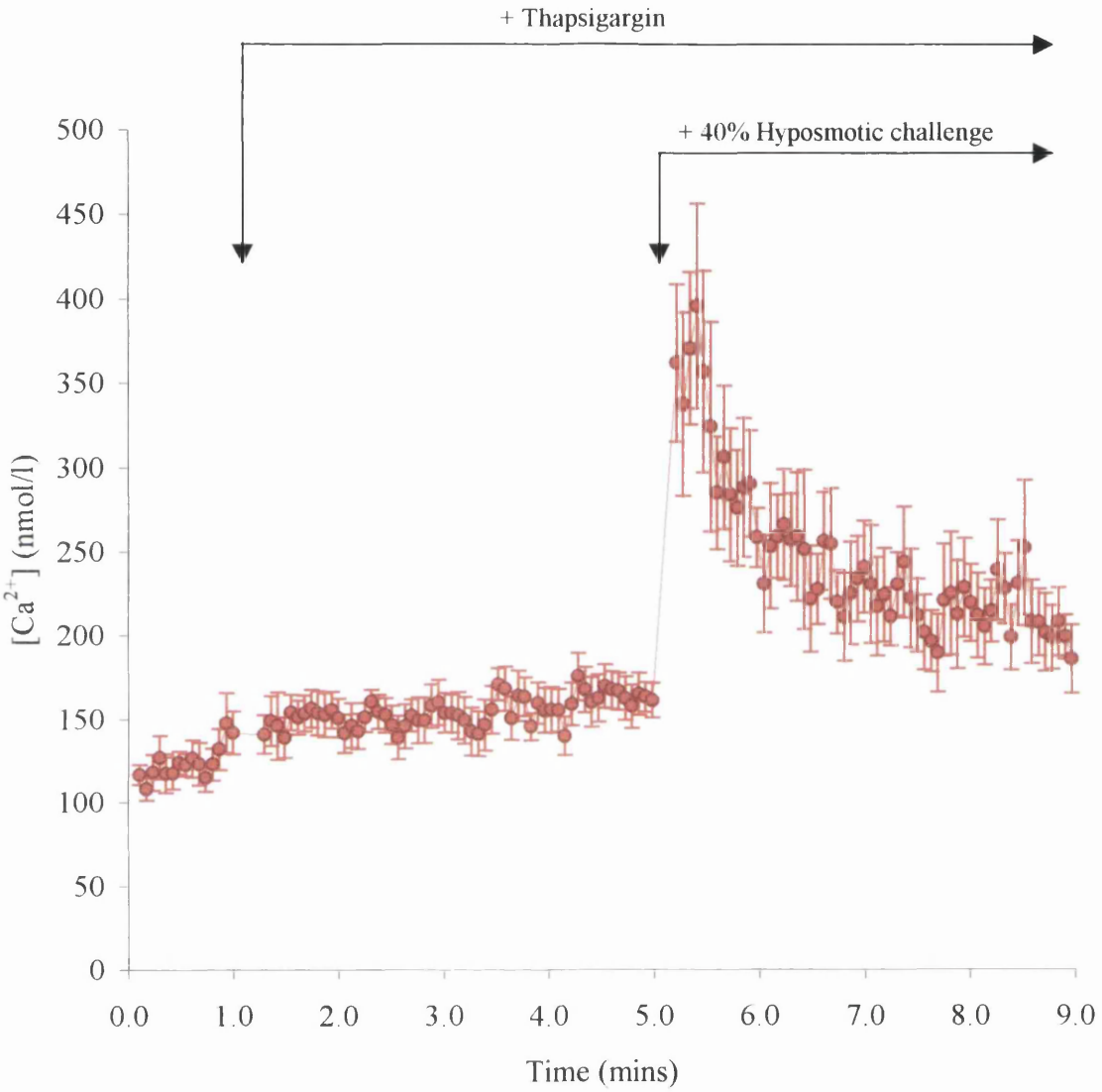

Figure 4.13: Effect of a hyposmotic challenge on $[Ca^{2+}]_i$ in the presence of thapsigargin. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer was the same as in figure 4.11. At t = 60 secs, thapsigargin was added to a final concentration of 1μM (DMSO = 0.1%, v:v). At t = 5 mins the solution was rendered hyposmotic by the addition of a buffer similar to the isosmotic one, except that there was no NaCl (final osmolality = 190 mosmol/kg water). Fluorescence was recorded for a further 5 minutes. The suspensions were constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 8 experiments with tissue obtained from different animals.

reducing the osmolality of the incubation medium to 190 mosmol/kg water (by addition of a buffer similar in composition to the isosmotic buffer but with no NaCl) increased the $[Ca^{2+}]_i$ (basal – peak) from 161 ± 10 nM to 396 ± 60 nM (± SEM, n = 9, $P < 0.01$). Therefore it is apparent that the hyposmotically-sensitive increase in $[Ca^{2+}]_i$ was larger in the presence of thapsigargin ($P < 0.05$).

4.3.6 Effect of Isosmotic cell swelling on $[Ca^{2+}]_i$

The effect of isosmotic cell swelling on the intracellular free Ca^{2+} concentration was also examined. The acini were initially incubated for one minute in a buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 s, isosmotic swelling was induced by the addition of a buffer similar in composition to the isosmotic buffer except that the NaCl was replaced with 270 mM urea. The rationale behind this procedure was that the high permeability of urea causes the acinar cells to swell despite the isosmotic nature of the incubation buffer. This procedure appears to effectively swell mammary cell volume since it activates volume-sensitive taurine and $K^+(Rb^+)$ efflux from lactating mammary tissue (Shennan *et al.*, 1994; Shennan and Thomson, 2000; Shennan and Gow, 2000). The effect of isosmotic swelling on $[Ca^{2+}]_i$ is shown in figure 4.14. It is evident that isosmotic cell swelling, had no significant effect on the intracellular concentration of free Ca^{2+}.

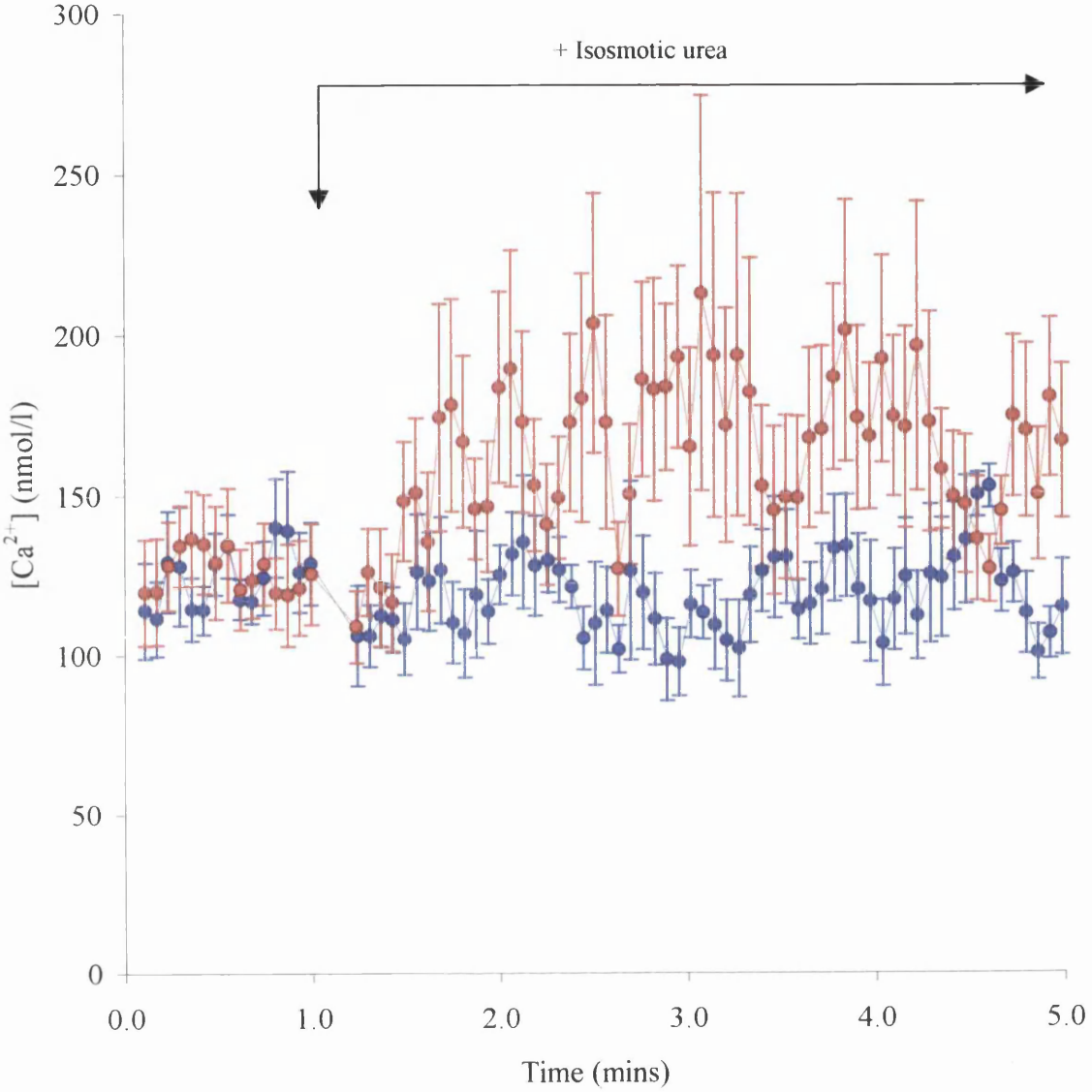

Figure 4.14: Effect of isosmotic swelling on $[Ca^{2+}]_i$. Fura-2-loaded acini were incubated at 37°C for 60 secs in an isosmotic buffer (314 mosmol/kg water). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 secs, a solution was added that was similar in composition to the isosmotic buffer, except that it contained 270mM urea (—) instead of NaCl. Fluorescence was recorded for a further 4 minutes. The control suspension (– –) was maintained in isosmotic conditions throughout. The suspension were constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 8 experiments with tissue obtained from different animals.

4.3.7 Effect of Gadolinium on hyposmotic-induced [Ca^{2+}] increase

Previous experiments have established that extracellular calcium is the source of the increase in cytoslic free Ca^{2+} in response to a hyposmotic challenge. However, the route of calcium entry into the cell under hyposmotic conditions is not known. Therefore the effect of the calcium channel blocker gadolinium (Gd^{3+}) was examined.

Figure 4.15 shows the effects of Gd^{3+} on $[Ca^{2+}]_i$ in rat mammary acinar cells under isosmotic and hyposmotic conditions. The acini were initially incubated in an isosmotic buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. After 60 s, Gd^{3+} was added to a final concentration of 20 μM. Gd^{3+} had no effect on $[Ca^{2+}]_i$ under isosmotic conditions. After a further minute the acinar suspension was rendered hyposmotic by the addition of a buffer, similar to that described above except that it had no NaCl (+20 μM Gd^{3+}). There was a marked increase in the $[Ca^{2+}]_i$ from 128 ± 8 nM to 231 ± 19 nM (±SEM, n = 13, $P < 0.001$). It is apparent that the addition of Gd^{3+} had no significant effect on the hyposmotically-induced change in the concentration of cytosolic calcium.

4.3.8 Effect of a hyperosmotic challenge on $[Ca^{2+}]_i$

The effect of cell shrinking, induced by hyperosmotic shock, on the intracellular free Ca^{2+} concentration was examined. The results from this set of experiments are shown in figure 4.16. Rat mammary acini were incubated in an isosmotic buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 s, the

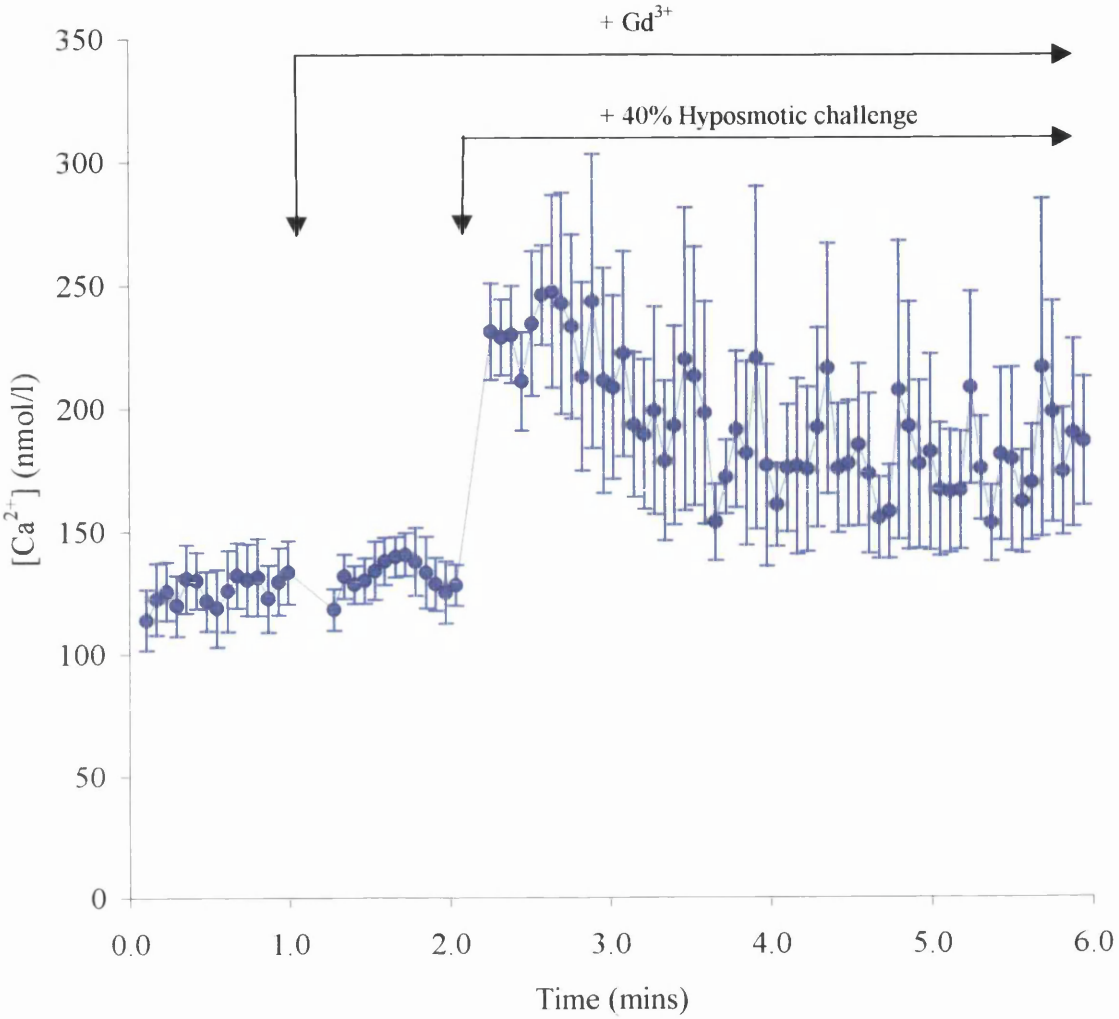

Figure 4.15: Effect of Gd^{3+} on hyposmotic-induced $[Ca^{2+}]_i$ increase. Fura-2-loaded acini were incubated at 37°C for 60 secs in an isosmotic buffer (314 mosmol/kg water). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 secs, Gd^{3+} was added to a final concentration of 10μM. Then, at t = 120 secs, the solution was made hyposmotic by the addition of a solution similar in composition to the isosmotic one except that it contained no NaCl (+10μM Gd^{3+}; final osmolality = 190 mosmol/kg water). The suspension were constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 13 experiments with tissue obtained from different animals.

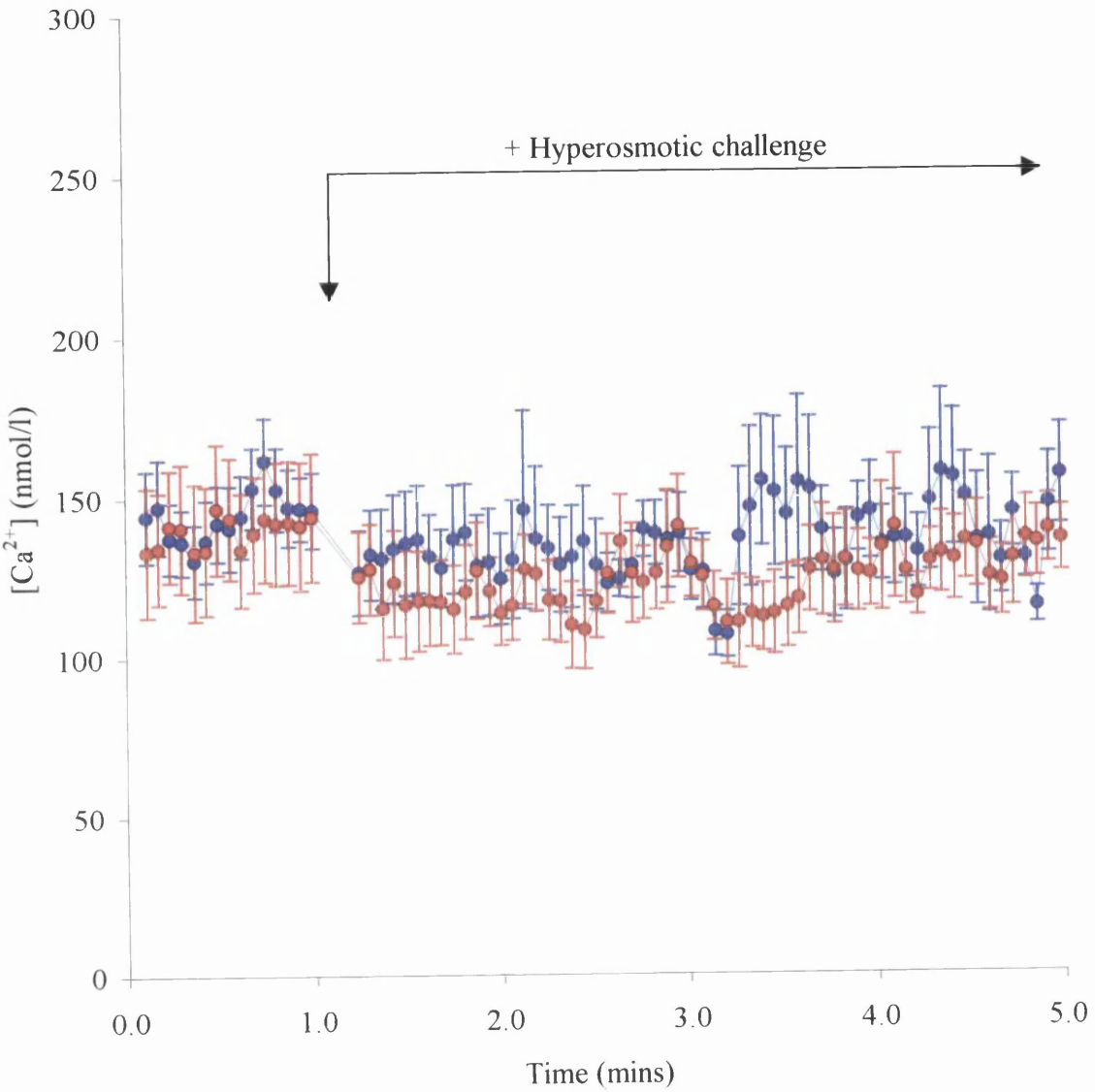

Figure 4.16: Effect of hyperosmotic challenge on $[Ca^{2+}]_i$. Fura-2-loaded acini were incubated at 37°C for 60 secs in isosmotic buffer (314 mosmol/kg water). The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. At t = 60 secs, the solution was rendered hyperosmotic (—) by the addition of an equal volume of a buffer similar in composition to the isosmotic buffer except that it contained an additional mM sucrose (final osmolality = 518 mosmol/kg water). Fluorescence was recorded for a further 4 minutes. The control suspension (—) was maintained in isosmotic conditions throughout. The suspension was constantly mixed with a micro-flea magnetic stirrer. The excitation wavelengths were 340 and 380nm, emission was measured at 509nm. Results are the means ±SEM of 9 experiments with tissue obtained from different animals.

acinar suspension was diluted with an equal volume of buffer similar in composition to the isosmotic buffer except that it also contained 400mM sucrose. This increased the osmolality of the suspension from 314 to 518 mosmol/kg of water. It is evident from the results shown in figure 4.16 that increasing the osmolality of the incubation medium had no significant effect on the cytosolic free Ca^{2+} concentration (±SEM, n = 9).

4.4. Discussion

4.4.1. Reducing extracellular osmolality increases cytosolic free Ca^{2+}

The results show that a reduction in osmolality, which in turn leads to an increase in cell volume, increases the intracellular free calcium concentration. This is in agreement with the observations of Sudlow and Burgoyne (1997) and Shennan and Gow, (2000) who respectively studied the effect of a hyposmotic shock on $[Ca^{2+}]_i$ in mouse and rat mammary acinar cells. Following the initial increase in $[Ca^{2+}]_i$, the concentration decreased in an exponential manner and returned to a level not significantly different from that measured under isosmotic conditions. Thus, the effect of a hyposmotic shock on $[Ca^{2+}]_i$ was transient. This is in agreement with the preliminary results of Shennan and Gow (2000). However, Sudlow and Burgoyne (1997) found that the increase in $[Ca^{2+}]_i$ in response to a hyposmotic challenge consisted of at least two phases: a transient large increase in $[Ca^{2+}]_i$ followed by a sustained plateau phase. The reason for the difference between the present results and those of Sudlow and Burgoyne (1997) is not immediately apparent but may simply reflect a species difference.

4.4.2. Sensitivity of $[Ca^{2+}]_i$ response to change in osmolality

Data from the present study also show that the hyposmotically-induced increase in cytosolic free calcium is dependent on the extent of the osmotic challenge. Therefore a 40% reduction in osmolality resulted in a 75% increase in $[Ca^{2+}]_i$ whereas a 20% hyposmotic challenge caused the $[Ca^{2+}]_i$ to rise by 46%. A 10% hyposmotic challenge had no discernible effect on intracellular calcium. When the mammary acini were subjected to the larger osmotic perturbation the intracellular calcium took longer to return to normal (180 and 34 seconds for 40 and 20% challenges respectively). The difference could be due to the possibility that the mammary cells were regulating their volume.

4.4.3. Changes to Na^+ and ionic gradients do not effect $[Ca^{2+}]_i$

Although the concentration of NaCl in the incubation medium was the only variable in most of the experiments, it should be borne in mind that administering a hyposmotic challenge in this way also changes the *trans*-membrane Na^+ gradient and the ionic strength of the medium. Thus, there was a possibility that the $[Ca^{2+}]_i$ was being altered as a consequence of changing the flux of ionised calcium via a Na^+/Ca^{2+} exchanger. Although preliminary evidence suggests that (mouse) mammary tissue does not express a Na^+/Ca^{2+} exchanger (Neville and Watters, 1983), it was necessary to design experiments to see if Na^+/Ca^{2+} exchange played a major role in controlling $[Ca^{2+}]_i$ in rat mammary tissue. To address this issue the effect of reversing the trans-membrane Na^+-gradient on $[Ca^{2+}]_i$ in rat mammary acinar cells was examined. The results show that $[Ca^{2+}]_i$ was not affected by changing the Na^+ electrochemical gradient. Therefore it can be concluded that the

hyposmotically-induced increase in $[Ca^{2+}]_i$ is not due to inhibition of a Na^+/Ca^{2+} exchanger. In addition, it appears that the effect of a hyposmotic shock on $[Ca^{2+}]_i$ cannot be attributed to a change in the ionic strength of the incubation medium.

4.4.4. Importance of extracellular calcium in the response to a hyposmotic challenge

It has previously been shown that removing extracellular calcium (using EGTA) attenuates the hypotonically-induced increase in $[Ca^{2+}]_i$ in mouse and rat mammary acinar cells (Sudlow and Burgoyne, 1997, Shennan and Gow, 2000). The results of the present study confirm the earlier findings. However, in the studies of Sudlow and Burgoyne (1997) and Shennan and Gow (2000) it is apparent that the addition of EGTA to the incubation medium decreased the $[Ca^{2+}]_i$ under isosmotic conditions. Therefore, there is the possibility that the intracellular stores of Ca^{2+} could have been lowered under the experimental conditions. This, in turn, could have masked any contribution of internally derived Ca^{2+} to the hyposmotically-induced increase in $[Ca^{2+}]_i$. Indeed, from the three experiments carried out it appears that at 1mM EGTA there is a small increase in intracellular calcium in response to a hyposmotic challenge, suggesting that stores of calcium within the cell are also involved in the response. However, this conclusion is drawn from a small set of data (compared to previous measurements of $[Ca^{2+}]_i$ - see figure 4.4 and 4.5, where n = 14 and 16 respectively) and analysis by *t*-test shows that the increase is not significant. Unfortunately, no more experiments of this nature were possible due to the fact that subsequent acini preparations were not viable (adjudged by trypan blue exclusion): this was due to using a new batch of collagenase in the tissue digestion. Thus, although the current statistical analysis suggests that intracellular stores

of calcium do not contribute to the increase in $[Ca^{2+}]$ in response to a hyposmotic challenge, it would be prudent to extend the number of observations to confirm this result.

It is worth noting that analysis of this data using an alternative statistical method, such as Anovar may have proved useful in determining the significance of the result. In this technique variability in the entire data-set is taken into account when determining the significance of an apparent change. However, given the experimental design, Student's *t*-tests were deemed appropriate. Had an alternative multifactorial approach to the investigation been used, a more complex analysis would have been suitable.

4.4.5. Transient nature of increase in $[Ca^{2+}]_i$ following a hyposmotic shock

The effect of a hyposmotic challenge on $[Ca^{2+}]_i$ in rat mammary acinar cells was transient: after a 40% hyposmotic challenge the cytosolic calcium concentration returned to a basal level within 3 minutes. This is in marked contrast to the effect of ionomycin on $[Ca^{2+}]_i$ in rat mammary acinar cells. To survive, cells need to maintain a low concentration of cytosolic Ca^{2+}. In this connection, there are a number of different systems which could be responsible for reducing $[Ca^{2+}]_i$ in rat mammary cells following cell swelling. Such mechanisms include the (sarcoplasmic) endoplasmic reticulum Ca^{2+}-ATPases (SERCAs), plasma membrane Ca^{2+}-ATPases (PMCAs) (Reinhardt and Horst, 1999; Reinhardt *et al.*, 2000), mitochondrial uniport uptake (Gunter and Pfeiffer, 1990; Gunter *et al.*, 1994; Babcock *et al.*, 1997) and possibly P-type Ca^{2+}-ATPases in the Golgi apparatus membrane (West, 1981; Neville and Watters, 1983; Virk *et al.*, 1985). Recently, Reinhardt and Horst (1999; *et al.*, 2000) have identified two different isoforms from the SERCA family

of enzymes in lactating rat mammary tissue: SERCA2 and SERCA3. They also found three isoforms of the PMCA enzymes: PMCA1b, PMCA2b and PMCA4b. The finding in the present study that the hyposmotically-induced rise in $[Ca^{2+}]_i$ was increased by thapsigargin suggests that at least one of the SERCA isoforms acts to limit the increase in $[Ca^{2+}]_i$ induced by cell swelling. However, it is apparent that $[Ca^{2+}]_i$ still decreased towards a basal level in the presence of thapsigargin suggesting that mechanisms in addition to the SERCAs are acting to pump calcium out of the cytosolic compartment. It would have been interesting to test the effect of inhibiting plasma membrane calcium ATPases either alone or in combination with thapsigargin on $[Ca^{2+}]_i$. However, there are no specific inhibitors currently available.

4.4.6. Isosmotic swelling does not markedly affect $[Ca^{2+}]_i$

It should be noted that a hyposmotic challenge was used simply as an experimental tool to swell the mammary acinar cells. However, under normal physiological conditions mammary tissue will never be subjected to such osmotic challenges. In spite of this fact it is believed that mammary cell volume could be markedly altered through substrate accumulation or through changes to the rate of oxidative metabolism (Shennan and Peaker, 2000). Substrate uptake via Na^+-dependent mechanisms such as Na^+-K^+-Cl^- co-transport and Na^+-dependent amino acid transport systems probably acts to swell mammary acinar cells (see chapters 1 and 6). However, the present results show that isosmotic swelling, using urea, did not markedly affect $[Ca^{2+}]_i$. It is beyond doubt that urea acts to swell mammary cells; previous studies have shown that volume regulatory mechanisms are activated in response to buffers containing urea (Shennan *et al.*, 1994,

Shennan and Gow, 2000). Thus, urea has been shown to increase both taurine and $K^+(Rb^+)$ efflux from lactating rat mammary tissue explants (Shennan *et al.*, 1994, Shennan and Gow, 2000). Moreover, swelling mammary cells using urea increases protein synthesis by rat mammary tissue (chapter 3). The reason for the different response of $[Ca^{2+}]_i$ to a hyposmotic shock and isosmotic swelling is not immediately apparent. However, it is possible that a hyposmotic shock activates a signaling pathway(s) which is not activated by isosmotic swelling which in turn regulates calcium entry into mammary acinar cells. In this connection Millar and Robson (2001) have recently shown that the activation of a Cl^- conductance in frog renal proximal tubule is dependent upon the way cells are swollen. Thus, hyposmotically induced swelling of frog renal cells activated a DIDS-sensitive Cl^- conductance via a conventional protein kinase C. In contrast, renal cells swollen under isosmotic conditions activated a chloride conductance via a non-conventional protein kinase C.

4.4.7. Cell shrinking does not affect $[Ca^{2+}]_i$

In agreement with the results of Sudlow and Burgoyne (1997), it was found that decreasing the volume of mammary acinar cells, by administering a hyperosmotic challenge, had no effect on the concentration of intracellular $[Ca^{2+}]$. This is an interesting result as it is predicted that decreasing cell volume would have an immediate concentrating effect on cytosolic calcium. The lack of an increase suggests that calcium is rapidly removed from the cytosolic compartment. Ca^{2+} pumps situated in the ER, Golgi and plasma membranes could be responsible for maintaining $[Ca^{2+}]_i$ following a hyperosmotic challenge.

4.4.8. Mechanism of Ca^{2+} influx in response to cell swelling

Volume-sensitive Ca^{2+} entry into some cell types is inhibited by calcium channel blockers such as nifedipine and gadolinium (Sudlow and Burgoyne, 1997). Gd^{3+} is a trivalent lanthanide that inhibits stretch-activated channel (SAC) activity (Caldwell *et al.*, 1998). Indeed, Sudlow and Burgoyne showed that Gd^{3+} partially inhibited the influx of calcium following a hyposmotic challenge into mouse mammary acinar cells. This suggests that there is more than one mode of calcium entry into the cell following exposure to a reduction in osmolality. The present data show that the hyposmotically-induced increase in $[Ca^{2+}]$ in rat mammary acinar cells was unaffected by Gd^{3+}. Therefore, the pathway(s) responsible for Ca^{2+} entry induced by a hyposmotic shock remains to be identified. The reason for the difference between the results of the present study and those of Sudlow and Burgoyne (1997) with respect to the effect of Gd^{3+} is not immediately apparent. However, it may be related to a species difference.

CHAPTER FIVE

LIPOGENESIS IN RAT MAMMARY TISSUE

5.1. INTRODUCTION

Cell swelling is generally held to be an anabolic signal whereas cell shrinking appears to favour catabolism (Lang *et al.*, 1998a). For example, cell swelling increases protein synthesis, lipogenesis, and glycogen synthesis (Baquet *et al.*, 1991a; reviewed in Lang *et al.*, 1998a). On the other hand, cell shrinking inhibits protein synthesis (Stoll *et al.*, 1992). Accordingly, the results in chapter 3 show that cell swelling and shrinking respectively increases and decreases rat mammary protein synthesis. Thus, a hyposmotic shock increased the rate of incorporation of radiolabelled amino acids into T.C.A.-precipitable material whereas a hyperosmotic challenge reduced mammary protein synthesis.

It is not clear if cell volume acts as a general regulator of mammary metabolism or whether it is only protein synthesis that is affected. To answer this, the effect of changing cell volume on mammary lipogenesis was examined since lipids, like protein, are one of the major components of milk. The effect of cell volume changes induced by osmotic perturbations on the rate of acetate incorporation into mammary lipid was studied.

5.2. METHOD

Lipogenesis in rat mammary tissue explants and acini, prepared as described in chapter 2, was measured by following the incorporation of [^{14}C]-acetate into chloroform-extractable

material (as described in section 2.7).

5.3. Results - Cell volume affects lipogenesis in mammary explants

5.3.1. Effects of an anisosmotic challenge on mammary lipogenesis

The effect of increasing or decreasing cell volume on the formation of lipid in mammary tissue was quantified by measuring the incorporation of [^{14}C]-acetate into chloroform extractable material. Incorporation of acetate was measured under isosmotic (311 ± 1 mosmol/kg water), hyposmotic (167 ± 1 mosmol/kg water) and hyperosmotic (526 ± 1 mosmol/kg water) conditions. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 20 Tris-MOPS, pH 7.4 and 2 acetate. The hyposmotic solution was similar to the isosmotic buffer except that it had no choline-Cl. The hyperosmotic buffer was also similar to the isosmotic medium except that it contained an additional 200mM sucrose. The results are shown in figure 5.1. Lipogenesis, under isosmotic conditions, was 4.91 ± 1.26 μmol/h/g dry tissue weight (±SEM, n = 6). Decreasing the osmolality of the incubation buffer increased the rate of lipogenesis ($P < 0.01$) to 8.29 ± 1.84 μmol/h/ g dry tissue weight (±SEM, n = 5). Thus, a hyposmotic shock increased the rate of lipogenesis by 68.8%. Conversely, reducing the osmolality of the incubation medium lowered the rate of lipogenesis ($P < 0.02$) to 0.33 ± 0.12 μmol/h/g dry tissue weight (±SEM, n = 5). Thus, cell shrinking inhibited mammary lipogenesis by 93.2%.

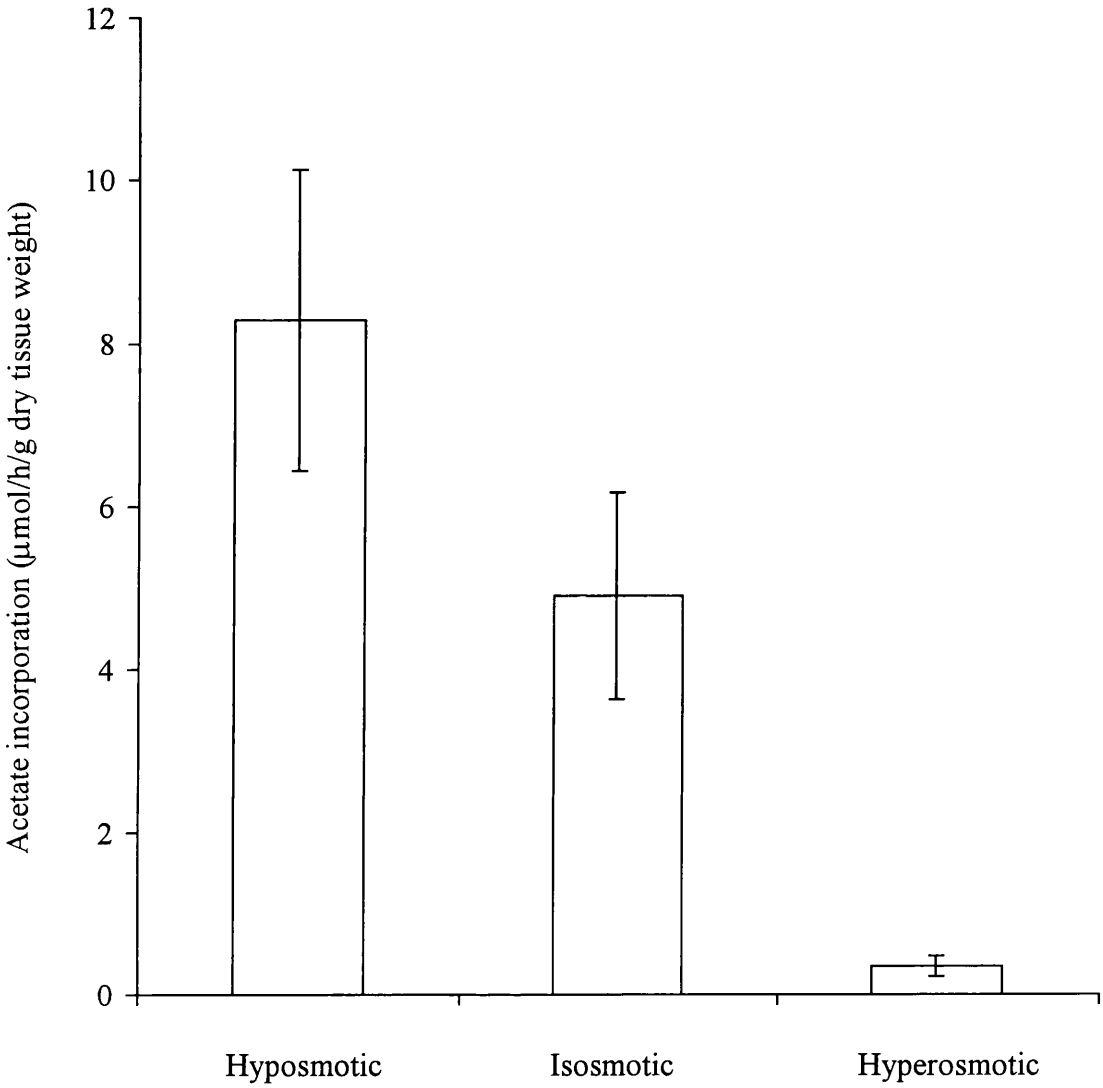

Figure 5.1: Effect of anisosmotic exposure on mammary explant lipogenesis. Explants were incubated in isosmotic (311 ± 1 mosmol/kg water), hyposmotic (167 ± 1 mosmol/kg water) and hyperosmotic (526 ± 1 mosmol/kg water) media for 60 mins (+ 15 mins pre-incubation), at 37°C with constant 100% O_2 gassing. The isosmotic medium contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 2 acetate and 20 Tris-MOPS, pH 7.4 (+1µCi/ml [^{14}C]-acetic acid). The hyposmotic solution was similar in composition except that it contained no choline-Cl. The hyperosmotic solution was also similar to the isosmotic medium except that it contained an additional 200mM sucrose. Data are means ± SEM of five experiments using tissue isolated from different animals.

5.3.2 Sensitivity of lipogenesis to changes in the extracellular osmolality

The sensitivity of lipogenesis to changes in extracellular osmolality was examined. The change in the rate of [^{14}C]-acetate incorporation into lipid, caused by varying the osmolality between 163 and 305 mosmol/kg water, is shown in figure 5.2. The isosmotic buffer (305 ± 2 mosmol/kg water) contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 20 Tris-MOPS, pH 7.4 and 2 acetate. The osmolality was decreased by lowering the concentration of choline-Cl in the incubation buffer. It is apparent that the rate of lipogenesis was dependent on the extent of the osmotic perturbation. In this set of experiments, the incorporation of [^{14}C]acetate increased from 6.51 ± 1.11 to 14.18 ± 2.71 μmol/h/g dry tissue weight (±SEM, n = 6, $P < 0.01$) in a linear manner ($P < 0.01$).

The sensitivity of mammary lipogenesis to an increase in the osmolality of the incubation medium was also determined. The isosmotic buffer (318 ± 5 mosmol/kg water) contained (mM) 10mM sucrose, 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 20 Tris-MOPS, pH 7.4 and 2 acetate. The osmolality of the incubation media was increased by the addition of sucrose. Figure 5.3 shows that the decrease in rate of mammary lipogenesis was dependent on the extent of the hyperosmotic challenge (±SEM, n = 4). Thus, increasing the osmolality of the incubation buffer decreased [^{14}C]-acetate incorporation into lipid from 4.32 ± 0.97 to 0.36 ± 0.07 μmol/h/g dry tissue weight (±SEM, n = 4, $P < 0.05$).

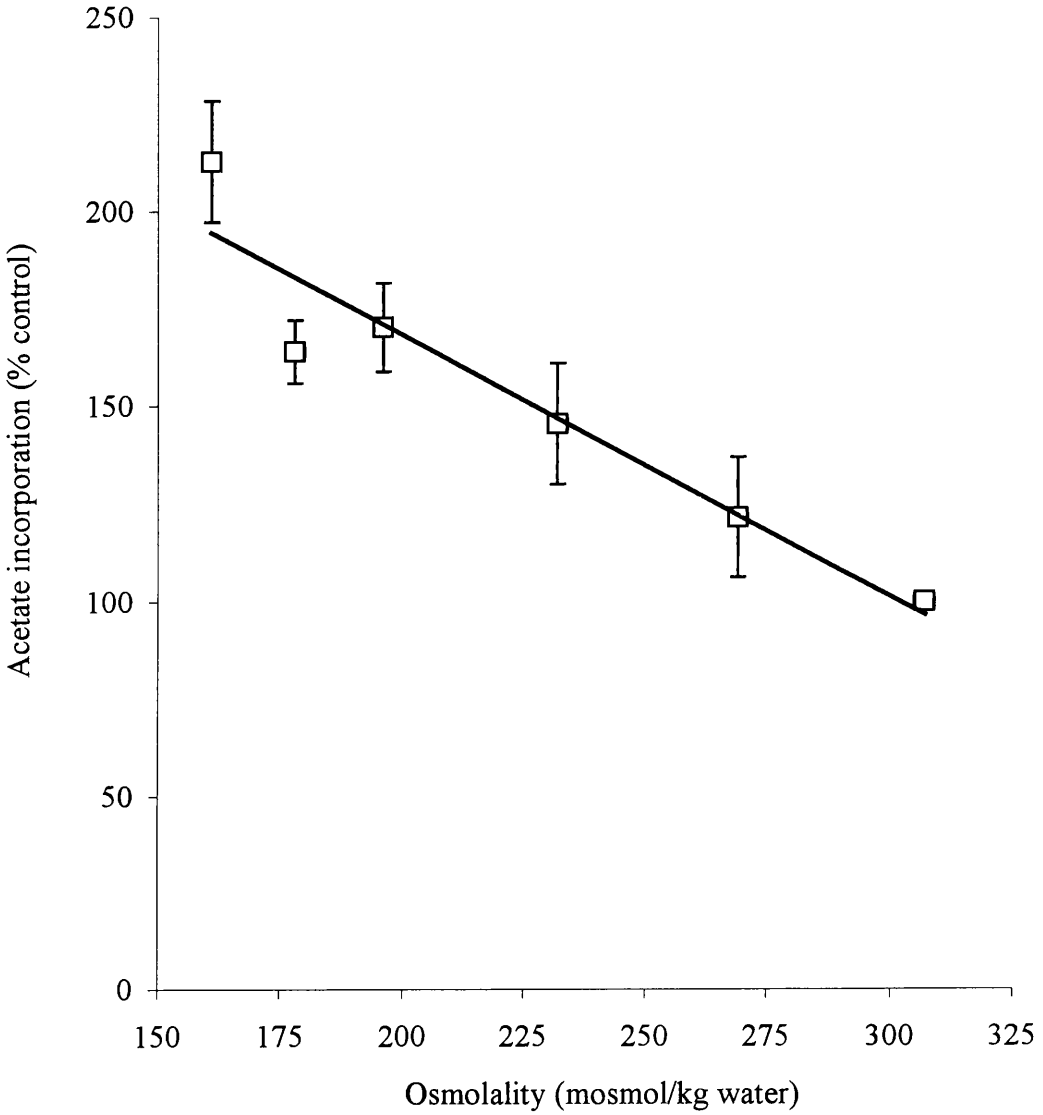

Figure 5.2: Sensitivity of mammary lipogenesis to decreasing osmolality. Mammary tissue explants were incubated in media of different osmolalities for 60 mins (+ 15 mins pre-incubation), at 37°C with constant 100% O_2 gassing. The isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 CaCl2, 1 MgSO4, 10 glucose, 2 acetate and 20 Tris-MOPS, pH 7.4 (+1μCi/ml [^{14}C]-acetic acid) (osmolality = 307 ± 2 mosmol/kg water). The concentration of choline-Cl was altered to give different osmolalities; 60, 40, 20, 10 and 0mM (osmolalities = 269 ± 2, 232 ± 2, 196 ± 1, 178 ± 2, 161 ± 1 mosmol/kg water, respectively). Data shown are means ± SEM of six experiments using tissue from separate animals. r = 0.934.

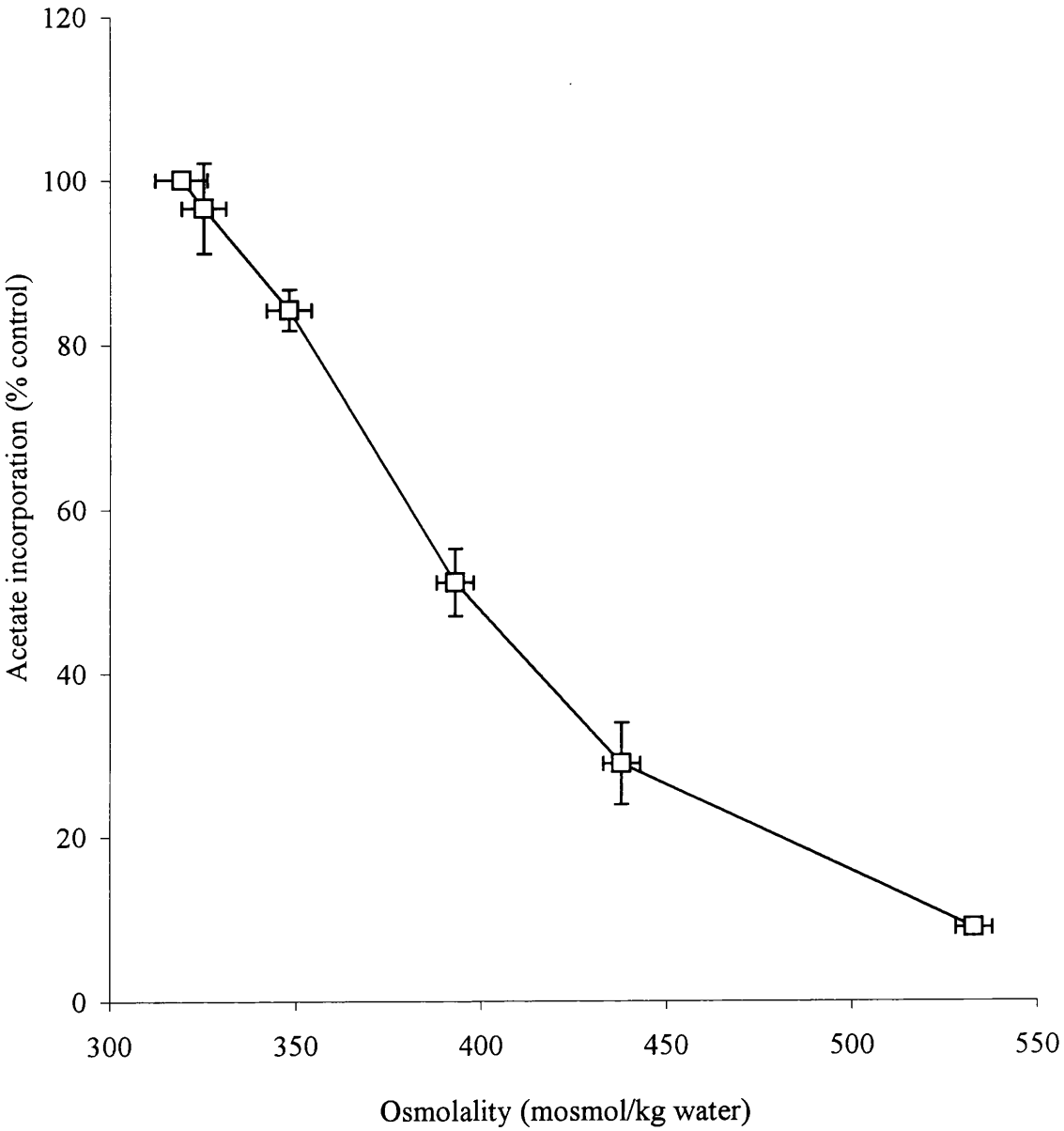

Figure 5.3: Sensitivity of mammary lipogenesis to increasing osmolality. Mammary tissue explants were incubated in media of different osmolalities for 60 mins (+ 15 mins pre-incubation), at 37°C with constant 100% O_2 gassing. The isosmotic buffer contained (mM) 10 sucrose, 55 NaCl, 80 choline-Cl, 5 KCl, 2 CaCl2, 1 MgSO4, 10 glucose, 2 acetate and 20 Tris-MOPS, pH 7.4 (+1μCi/ml [^{14}C]-acetic acid) (osmolality = 319 ± 7 mosmol/kg water). The concentration of sucrose was altered to give different osmolalities; 20, 40, 80, 120 and 200mM (osmolalities = 325 ± 6, 348 ± 6, 393 ± 5, 438 ± 5, 533 ± 5 mosmol/kg water, respectively). Data shown are means ± SEM of four experiments using tissue from separate animals.

The effect of increasing the osmolality of the incubation medium using NaCl instead of sucrose on mammary lipogenesis was examined. The results are illustrated in figure 5.4. In these experiments the isosmotic buffer (osmolality = 320 ± 3 mosmol/kg of water) contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4 plus 2 acetate. The hyperosmotic buffer (osmolality = 494 ± 11 mosmol/kg of water) was similar in composition except that the NaCl concentration was increased to 235 mM. The incorporation of [^{14}C]-acetate into mammary lipid under isosmotic and hyperosmotic conditions was respectively 12.42 ± 1.93 and 1.14 ± 0.34 µmol/h/g dry tissue weight (±SEM, n = 4). Thus, cell shrinking, induced by adding NaCl to the medium inhibited lipogenesis by 91% ($P < 0.02$).

5.3.3. Isosmotic swelling increases mammary lipogenesis

The effect of isosmotic swelling on the rate of lipogenesis in rat mammary explants was studied. Cell swelling under isosmotic conditions was achieved by the addition of a relatively high concentration of urea to the isosmotic buffer whilst keeping the osmolality constant (through the reduction of the choline-Cl concentration as described in section 3.2.4). Incorporation of [^{14}C]-acetate into lipid was measured over 60 minutes. The control isosmotic buffer (osmolality = 310 ± 3 mosmol/kg water) contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 2 acetate and 20 Tris-MOPS, pH 7.4. The isosmotic buffer used to swell the cells (osmolality = 326 ± 3 mosmol/kg water) was similar in composition except that it had 160mM urea in place of the choline-Cl. The data, shown in figure 5.5, shows that isosmotic cell swelling increased the rate of lipogenesis from 6.97 ± 0.84 to 10.30 ± 1.68 µmol/h/g dry tissue weight (±SEM, n =

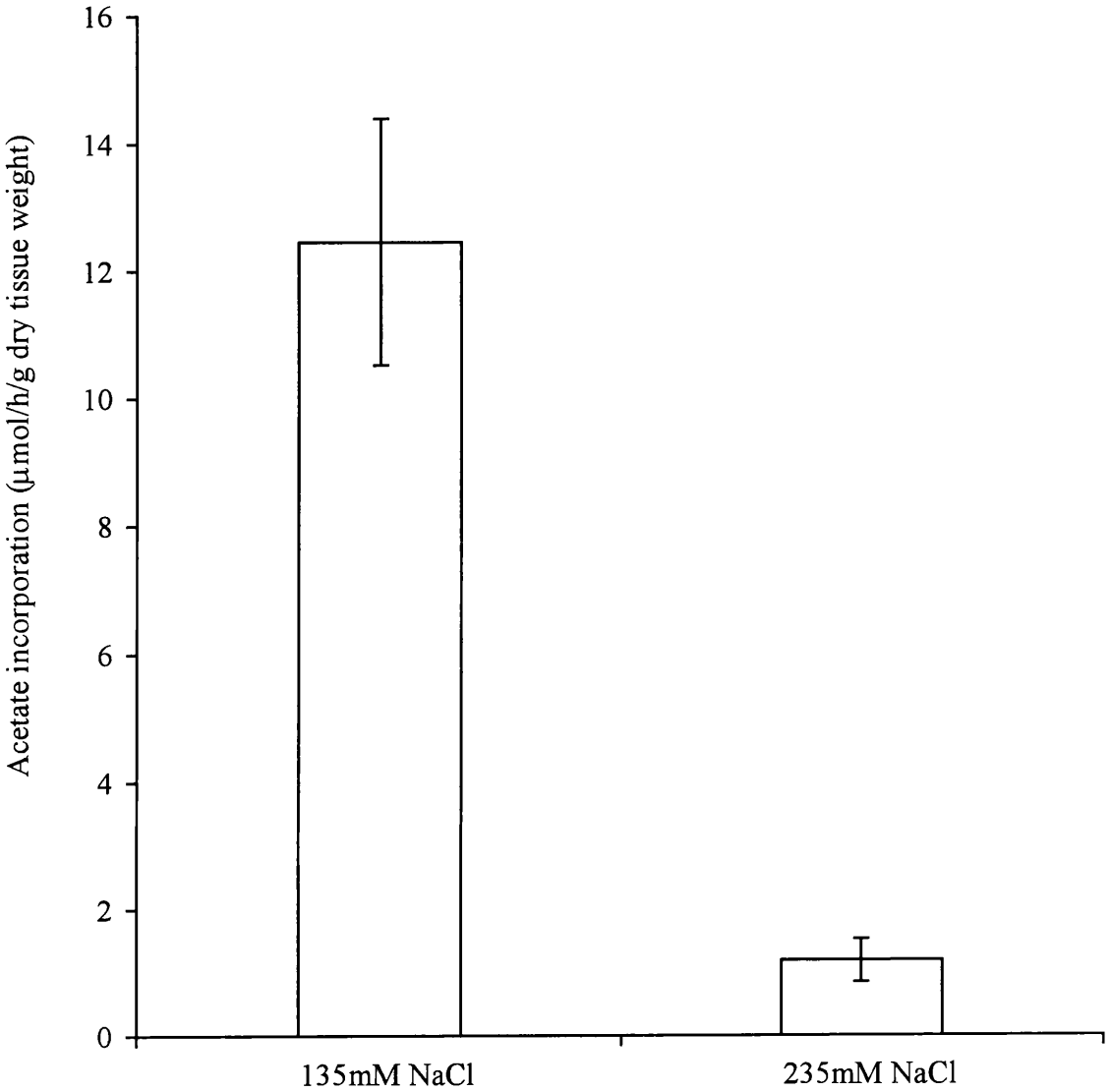

Figure 5.4: Effect of increasing the Na^+ concentration on lipogenesis. Mammary tissue explants were incubated in media for 60 mins (+ 15 mins pre-incubation), at 37°C with constant 100% O_2 gassing. The isosmotic buffer contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 2 acetate and 20 Tris-MOPS, pH 7.4 (+1µCi/ml [^{14}C]-acetic acid) (osmolality = 320.5 ± 3.3 mosmol/kg water). The increased Na^+ buffer was similar in composition to the isosmotic buffer except that it contained an additional 100mM NaCl (total [NaCl] = 235; osmolality = 494.8 ± 11.4 mosmol/kg water). Data shown are means ± SEM of four experiments using tissue from separate animals.

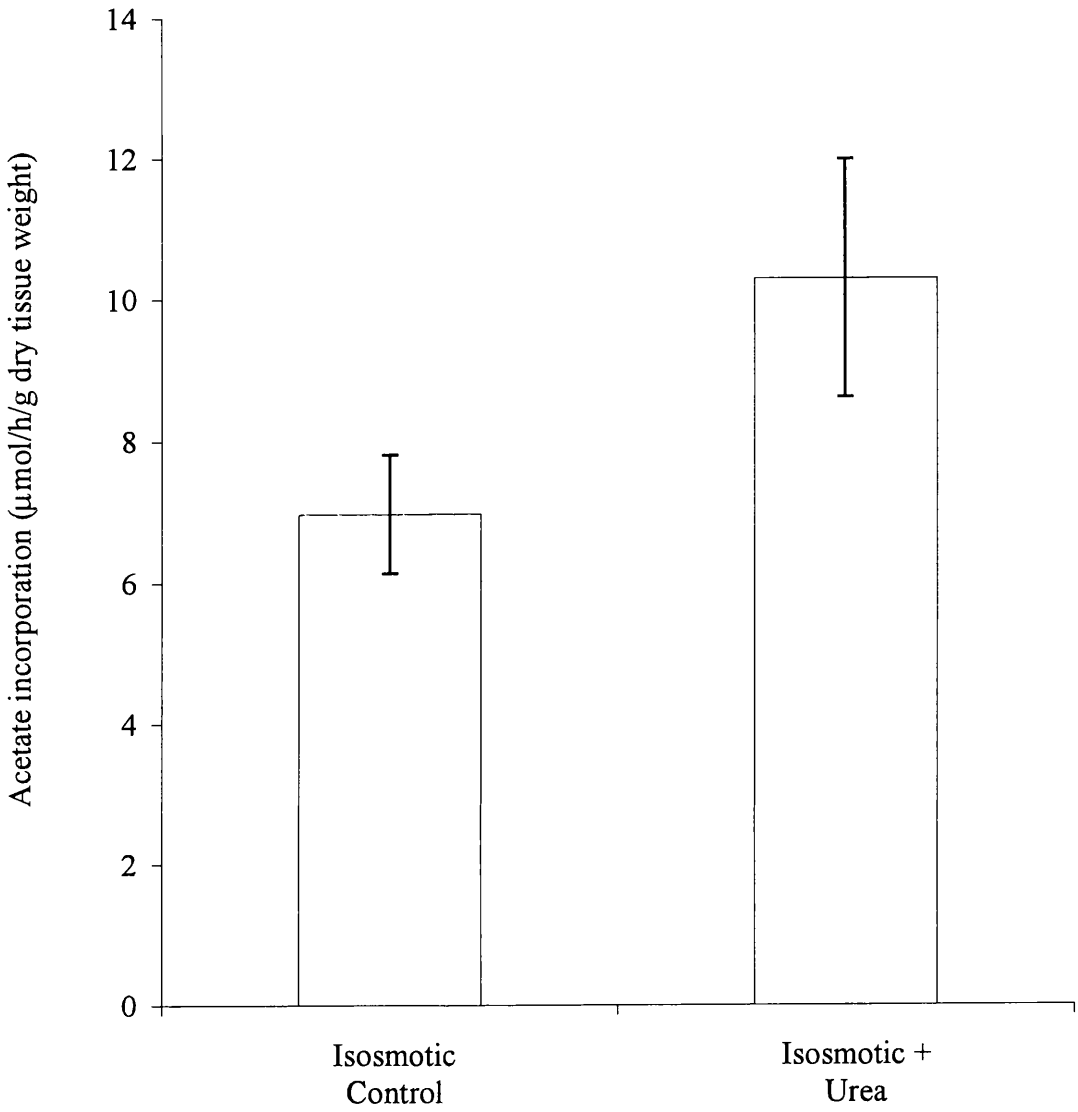

Figure 5.5: Effect of isosmotic cell swelling on mammary lipogenesis. Mammary tissue explants were incubated in media for 60 mins (+ 15 mins pre-incubation), at 37°C with constant 100% O_2 gassing. The control isosmotic buffer contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 2 acetate and 20 Tris-MOPS, pH 7.4 (+1µCi/ml [^{14}C]-acetic acid) (osmolality = 310 ± 3 mosmol/kg water). The isosmotic buffer used to swell cells was similar in composition to the control buffer except that it contained 160mM urea instead of choline-Cl (osmolality = 326 ± 3 mosmol/kg water). Data shown are means ± SEM of six experiments using tissue from separate animals.

6, $P < 0.05$). Thus isosmotic cell swelling increased the rate of lipogenesis by 47.7%.

5.3.4 Effect of removing extracellular Ca^{2+} on lipogenesis

In chapter 3 it was established that protein synthesis under both isosmotic and hyposmotic conditions is inhibited by the removal of extracellular calcium. In light of this, the dependence of lipogenesis on calcium was examined. Thus, mammary lipogenesis was measured in rat mammary explants incubated in isotonic and hypotonic solutions in the presence and absence of extracellular Ca^{2+}. The isosmotic buffers contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 1 $MgSO_4$, 10 glucose, 2 acetate and 20 Tris-MOPS, pH 7.4 with or without 2 $CaCl_2$. The hyposmotic solutions were similar to the isosmotic buffers except that they contained no choline-Cl. Each of the calcium-free buffers was supplemented with 0.5mM Tris-EGTA, pH 7.4. Lipogenesis was measure over a period of 60 min. The results are illustrated in figure 5.6. Removing extracellular calcium from the isosmotic buffer increased [^{14}C]-acetate incorporation from 6.97 ± 0.83 to 9.29 ± 1.47 µmol/h/g dry tissue weight (±SEM, $n = 6$, $P < 0.05$), an increase of 33.2%. Similarly, the rate of lipogenesis under hyposmotic conditions was increased by the removal of extracellular calcium ($P < 0.01$). Thus, the rate of [^{14}C]-acetate incorporation under hyposmotic conditions in the presence and absence of extracellular calcium was respectively 11.39 ± 1.75 and 13.84 ±1.69 µmol/h/g dry tissue weight. Under control and calcium-free conditions the volume-sensitive increase was respectively 4.42 ± 1.05 and 4.50 ± 1.02 µmol/h/g dry tissue weight (±SEM, $n = 6$) suggesting that the volume-sensitive component of lipogenesis was unaffected by the removal of extracellular calcium.

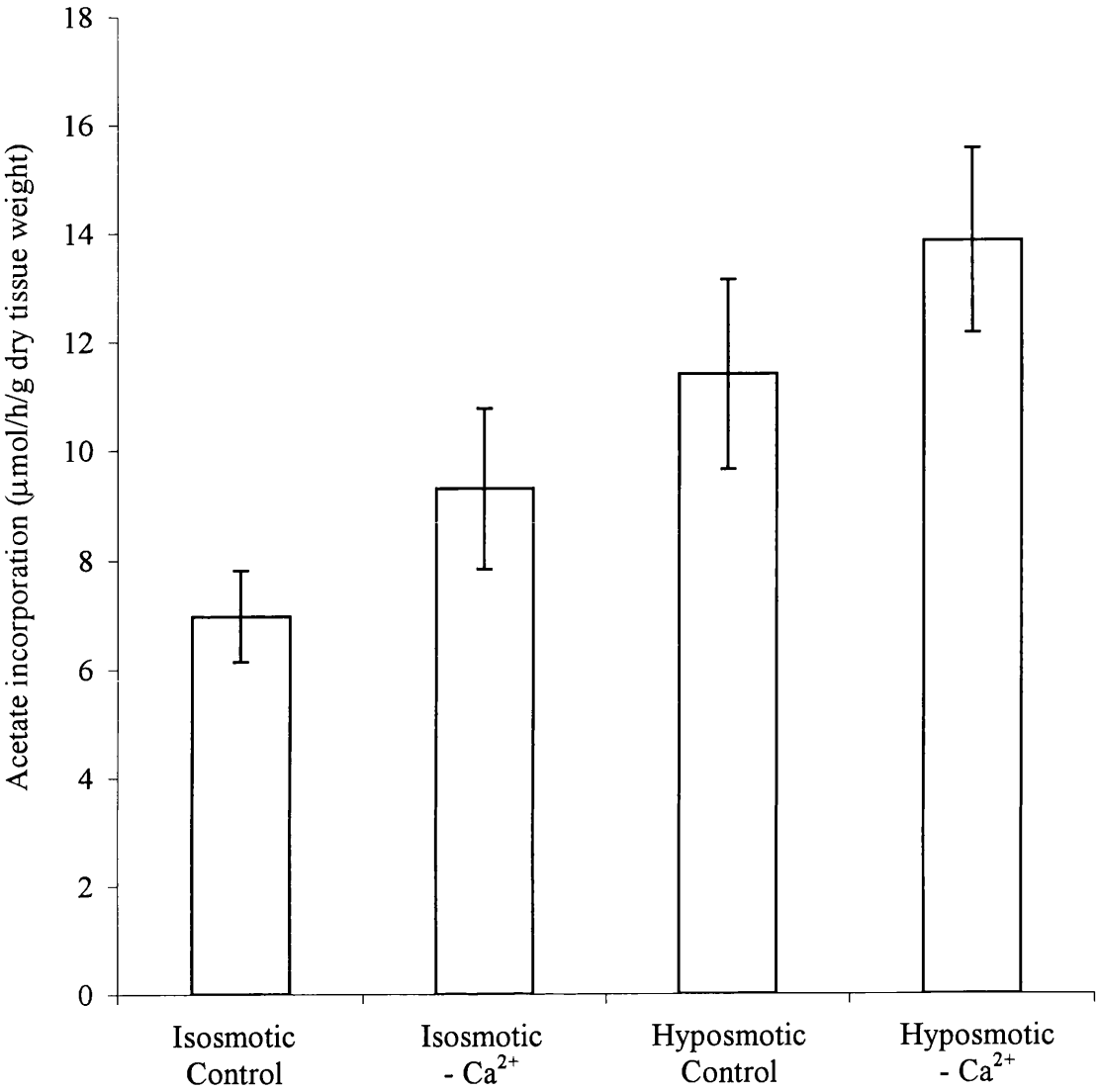

Figure 5.6: Effect of removing extracellular Ca^{2+} on mammary lipogenesis. Mammary tissue explants were incubated in media for 60 mins (+ 15 mins pre-incubation), at 37°C with constant 100% O_2 gassing. The isosmotic buffers contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 1 $MgSO_4$, 10 glucose, 2 acetate and 20 Tris-MOPS, pH 7.4 (+1µCi/ml [^{14}C]-acetic acid) ± 2 $CaCl_2$ (osmolality = mosmol/kg water). The hyposmotic buffers were similar in composition to the isosmotic buffers except that they contained no choline-Cl (osmolality = mosmol/kg water). The Ca^{2+}-free buffers also contained 0.5mM Tris-EGTA. Data shown are means ± SEM of six experiments using tissue from separate animals.

5.3.5. The effect of cell volume perturbations on lipogenesis in rat mammary acini

The effect of changing cell volume on the rate of lipogenesis in rat mammary acini was studied. The incorporation of [^{14}C]-acetate into mammary lipid under isosmotic, hyposmotic and hyperosmotic conditions was examined. The isosmotic buffer contained (mM) 80 choline-Cl, 55 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 2 acetate and 20 Tris-MOPS, pH 7.4. The hyposmotic buffer was similar in composition except that it contained no choline-Cl. The hyperosmotic buffer was similar in composition to the isosmotic solution except that it was supplemented with 200 mM sucrose. It is apparent from the data shown in figure 5.7 that cell swelling and shrinking respectively increased and decreased [^{14}C]-acetate incorporation into lipid in rat mammary acini. The rate of lipogenesis under isosmotic, hyposmotic and hyperosmotic conditions was respectively 473.4 ± 211.4, 788.5 ± 364.7 and 77.7 ± 43.2 μmol/h/mg DNA (±SD, n = 2).

5.4. Discussion

Lipogenesis in rat mammary explants was quantified by measuring the incorporation of [^{14}C]-acetate into chloroform extractable material. This method of measuring the synthesis of lipid has been used extensively in previous studies (e.g. see Cameron and Rillema, 1983; Bijleveld and Geelen, 1987; Waters and Rillema, 1988; Baquet *et al.*, 1993; Travers and Barber, 1999). The main source of carbon for fatty acid synthesis in the mammary gland is acetyl-coenzyme A (acetyl-CoA), which is converted into malonyl-CoA by acetyl-CoA carboxylase (ACC). This is then used by fatty acid synthetase (FAS) to form fatty acids. In ruminants the primary source of acetyl-CoA is acetate. However in

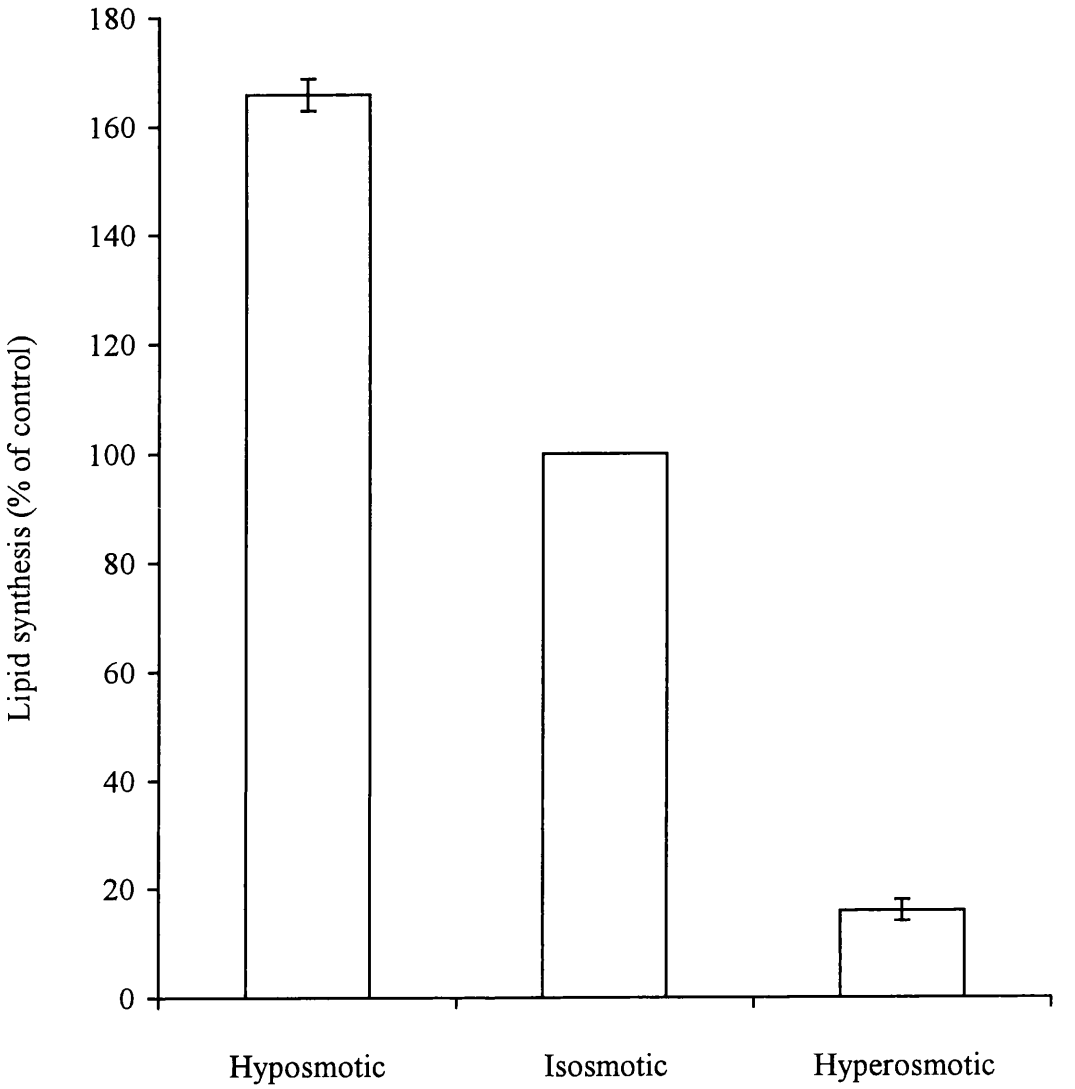

Figure 5.7: Effect of anisosmotic conditions on mammary acinar lipogenesis acini. Acini were incubated in isosmotic (311 ± 1 mosmol/kg water), hyposmotic (167 ± 1 mosmol/kg water) and hyperosmotic (526 ± 1 mosmol/kg water) media for 60 mins (+ 15 mins pre-incubation), at 37°C with constant 100% O_2 gassing. The isosmotic medium contained (mM) 55 NaCl, 80 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 2 acetate and 20 Tris-MOPS, pH 7.4 (+1μCi/ml [^{14}C]-acetic acid). The hyposmotic solution was similar in composition except that it contained no choline-Cl. The hyperosmotic solution was also similar to the isosmotic medium except that it contained an additional 200mM sucrose. Data are means ± SD of two experiments using tissue isolated from different animals.

non-ruminants, glucose also contributes to the acetyl-CoA pool, through metabolism in the mitochondria by pyruvate dehydrogenase (PDH) (Mepham, 1987). This means that the rate of lipogenesis measured by acetate incorporation may not represent the absolute rate of lipid synthesis. However, the concentration of acetate used (2 mM) should have been sufficiently high to ensure that the intracellular specific activity of acetyl-CoA would not have been markedly changed by the production of acetyl-CoA from glucose.

Incorporation of [^{14}C]-acetate into lipid was quantified after the rat mammary explants had been incubated for an hour. In caprine mammary tissue, labeled fatty acids become associated with fat droplets in secretory cells within minutes of intravenous injection, however, they do not appear to be secreted in to milk in less than 5 hours (Mepham, 1987). However, the possibility of interspecies differences should not be ignored here. It is also important to remember that newly formed fatty acids can diffuse across plasma membranes. In rats, triacylglycerols account for approximately 83-87% of lipid in milk, compared to free fatty acids that account for only 3-6% (Jenness, 1974; Mepham, 1987). Combined with the fact that newly formed fatty acids rapidly associate with fat droplets, it seems unlikely that diffusion of free fatty acids across the apical membrane could have a significant effect on results. In spite of this, the possibility of newly formed lipid being secreted from rat mammary tissue cannot be completely ruled.

5.4.1. Lipogenesis is altered by cell volume change

The results show that cell swelling increased the rate of lipogenesis in both rat mammary explants and isolated acini. This result corresponds with previous observations made in

hepatocytes (Baquet *et al.*, 1991a; Baquet *et al.*, 1991b; Baquet *et al.*, 1993; Hue, 1994) that showed a volume-sensitive component of lipogenesis. In these studies it was shown that an increase in cell volume resulted in an up-regulation of ACC activity and lipogenesis. ACC is deactivated and activated by phosphorylation and dephosphorylation respectively. In the hepatocyte studies, it appears that cell swelling leads to dephosphorylation of ACC by one of the enzymes in the protein phosphatase family (Baquet *et al.*, 1991b; Baquet *et al.*, 1993). The present study also shows that cell shrinking, induced by a hyperosmotic shock, markedly inhibited lipogenesis in rat mammary tissue explants and acini. Assuming that regulation of lipogenesis is occurring at the level of ACC, this could represent an increase in the activity of enzymes involved in the phosphorylation of ACC, or an inhibition of protein phosphatase activity. In subcellular liver fractions, the protein phosphatases implicated in the activation of ACC have been identified as PP-1, PP-2A and PP-2C (Baquet *et al.*, 1993).

Furthermore, lipogenesis in mammary explants was also shown to be dependent on the extent of the osmotic challenge. Here the osmolality of the incubation medium was varied between 150 and 550 mosmol/kg water. The data showed that only a small change in osmolality and therefore the hydration state of the cell was required to elicit a change in the rate of lipogenesis. The relationship between lipogenesis and the osmolality of the incubation medium in rat mammary tissue explants appeared to be relatively linear. This result differs from protein synthesis in mammary tissue (as described in chapter 3), which showed a sigmoidal relationship over a similar range of osmolalities. Thus, it appears that protein synthesis is more responsive to a change in cell volume than is lipogenesis. However it should be noted that protein synthesis was measured over a 20 min incubation,

whereas explants used for determination of lipogenesis were incubated for 1 hour. It is predicted that under conditions where volume is challenged, mammary cells will try to compensate through mechanisms analogous to regulatory volume decrease (RVD) (Calvert and Shennan, 1998; Shennan and Gow, 2000). In this connection, it is likely that after longer incubation times, volume perturbations will be partially corrected by RVD returning the cell towards a normal volume. This could account for the relatively small change in lipogenesis observed when the osmotic challenge was smallest: measurement of lipogenesis over a shorter time scale may have uncovered a larger response to the smaller changes in volume. Further studies looking at the relative rates of secretion of lipid and protein during cell volume alterations must be carried out to determine whether the effects of changing the rate of synthesis within the secretory epithelium will actually impact on milk composition.

As the cells swell, volume activated opening of Cl^- channels will result in a net decrease in the intracellular concentration of Cl^-. Investigations into the effects of volume on hepatic lipogenesis have implicated this decrease in the activation of ACC (Baquet *et al.*, 1993). In the present study Cl^- levels in the incubation medium were reduced in order to change the osmolality of the medium and hence elicit cell swelling. It is predicted that a reduction in the extracellular [Cl^-] will result in a decrease in the intracellular [Cl^-]. Therefore there is a possibility that lipogenesis in mammary tissue is also affected by the intracellular [Cl^-].

5.4.2. Isosmotic swelling increases mammary lipogenesis

Lipogenesis was also measured following isosmotic cell swelling with urea. In this investigation some of the salt in the isosmotic solution was replaced with urea. This made it possible to alter the volume of the mammary cells without changing the extracellular osmolality (as described in section 3.5.2). It was important to carry out this study to ensure that the change in rate of lipogenesis was in fact caused by a change to the hydration state of the cell and not due to a difference in the osmolality of the incubation medium per se. The rate of lipogenesis during isosmotic swelling was shown to increase by nearly 50%, which is comparable to the increase in lipid synthesis observed under hyposmotic cell swelling. Thus, it appears that it is indeed the change in cellular hydration that is affecting the increase in lipogenesis and not the reduction in extracellular osmolality. This data agrees with previous investigations (Baquet *et al.*, 1991; Baquet *et al.*, 1993; Hue, 1994; Krause *et al.*, 1996) which showed that cell swelling by either hyposmotic challenge or accumulation of amino acids resulted in an increase in lipid synthesis in hepatocytes.

5.4.3. The effect of Ca^{2+} on mammary lipogenesis

The rate of lipid synthesis under normal and anisosmotic conditions was also investigated in the absence of extracellular Ca^{2+} (+EGTA). It is fairly well established that for normal protein synthesis to occur, extracellular calcium must be present (Wilde *et al.*, 1981; Smith *et al.*, 1992; Cameron and Rillema, 1983; Brostrom *et al.*, 1983; Chin *et al.*, 1987; Palfrey and Nairn, 1995). Furthermore, experiments in rat mammary tissue (chapter 3)

showed that volume-sensitive protein synthesis was also dependent on the presence of extracellular calcium. Therefore the effects of removing extracellular calcium on normal and volume-activated lipogenesis were examined. When mammary explants were incubated in either isosmotic or hyposmotic conditions, in the absence of extracellular calcium, there was a significant increase in the rate of lipid synthesis. This is in stark contrast to the results from the protein synthesis experiments that showed such a marked decrease in the absence of extracellular Ca^{2+}. This result is interesting as it may identify a regulatory factor that could be used to manipulate the composition of milk. Down-regulation of phosphorylating enzymes may be the cause of the increase in ACC activity in the absence of calcium. There are at least 8 serine residues that act as phosphorylation sites on ACC, with two possible results: phosphorylation of three of them (ser-77/79, -1200 and -1215) results in an inhibition of ACC activity, the rest have no observed affect (Davies *et al.*, 1990; Ha *et al.*, 1994). The two enzymes that have the former effect have been identified as AMP-dependent protein kinase and cAMP-dependent protein kinase (Davies *et al.*, 1990; Hardie, 1992; Ha *et al.*, 1994; Hardie *et al.*, 1997). An alternative point of regulation of ACC as a consequence of cell swelling could be the up-regulation of protein phosphatase 2A. This enzymes has been identified as key in the dephosphorylation and hence activation of ACC (Ingebritsen *et al.*, 1983; Munday *et al.*, 1986; Gaussin *et al.*, 1996). Thus, the present data suggest that there is an increased activation of the lipogenic mechanism either through inhibition of enzymes designed to deactivate ACC, or an activation of protein phosphatase 2A, with the result of increasing ACC activity. It is interesting to note that the increase in lipogenesis caused by exposure to a hyposmotic solution remained constant between Ca^{2+}/Ca^{2+}-free media. This suggests that the activation of lipogenesis in Ca^{2+}-free media may be caused by an additional

mechanism to the volume-activated system. It is possible therefore that both of the mechanisms mentioned above are activated during cell swelling in the absence of calcium. However, the regulation of ACC activity through phosphorylation and dephosphorylation by different enzymes is extremely complex and until a more in-depth study is carried out, theories relating to the precise mechanism remain speculative.

CHAPTER SIX

GENERAL DISCUSSION

6.1. MAMMARY PROTEIN SYNTHESIS IS SENSITIVE TO CELL VOLUME CHANGE

It is evident from the present investigation that cell volume regulates mammary protein synthesis. Thus, protein synthesis in rat mammary explants and acini was increased under hyposmotic conditions. Conversely, cell shrinking induced by a hyperosmotic shock markedly inhibited mammary protein synthesis. Therefore, the results of this investigation are in accordance with those of Millar et al. (1997). The present study shows for the first time that volume-sensitive mammary protein synthesis was a) dependent upon the extent of the osmotic perturbation b) reversible and c) dependent upon the presence of extracellular calcium. In addition, the results show that protein synthesis could be stimulated by isosmotic cell swelling.

Volume-sensitive protein synthesis has been identified in other cell types. For example, protein synthesis in hepatocytes is affected by changing the osmolality of the incubation medium (Stoll *et al.*, 1992; Lang *et al.*, 1998a). However, there is one major difference between the effect of cell volume changes in mammary tissue and hepatocytes. The results of the present study show that a hyposmotic challenge increased mammary protein synthesis above and beyond that found under isosmotic conditions. In contrast, Stoll et al. (1992) found that hepatocyte protein synthesis, in the presence of extracellular amino acids, was unaffected by a hyposmotic shock. This could be due to the fact that the incubation buffers used by Stoll et al. (1992) contained amino acids at concentrations

twice that found under physiological conditions. Therefore, there is a possibility that the rate of protein synthesis was already maximal under isosmotic conditions. In the present study amino acid concentrations were similar to that found in rat plasma. Meijer et al. (1993) also found that a hyposmotic challenge, in the presence of amino acids at twice their physiological concentrations, did not increase protein synthesis in hepatocytes. However, it is notable that hepatocyte protein synthesis was increased by a hyposmotic shock when the incubation medium did not contain any amino acids (Meijer *et al.*, 1993). Another reason why Stoll et al. (1992) and Meijer et al. (1993) failed to find an effect of a hyposmotic challenge on hepatocyte protein synthesis could be due to the way the hyposmotic shock was administered. In their studies, the buffers were made hyposmotic by reducing the NaCl concentration. This could have reduced amino acid uptake via those systems that are Na^+-dependent and this, in turn, could have placed limitations on the rate of protein synthesis. In the present study a hyposmotic shock was administered without changing the external Na^+ concentration.

The inhibition of rat mammary protein synthesis by a hyperosmotic challenge agrees with results from similar experiments using other cell types (Brostrom *et al.*, 1983; Stoll *et al.*, 1992). A hyperosmotic challenge, hence cell shrinking, inhibits protein synthesis in hepatocytes (Stoll *et al.*, 1992). In an earlier study, Brostrom et al. (1983) found that a hyperosmotic shock inhibited the incorporation of radiolabelled methionine into protein in C-6 glioma cells, however, they failed to draw attention to the relationship between cell volume and the regulation of protein synthesis. It was established that the inhibition of mammary protein synthesis by a hyperosmotic shock was not due to increased proteolysis and hence dilution of the specific activity of the radiolabelled amino acids. This is an

important finding given that proteolysis in hepatocytes is stimulated by cell shrinking. Thus, proteolysis in rat liver tissue increases under the influence of hyperosmotic conditions and decreases when exposed to hyposmotic conditions (vom Dahl *et al.*, 1991a, 1995, 1996). Indeed, dehydration has been implicated as a key factor in liver and skeletal muscle protein catabolism observed in various chronic diseased states (Haussinger *et al.*, 1993a; Finn *et al.*, 1996; Waldegger *et al.*, 1997, 1998; Berneis *et al.*, 1999).

Mammary protein synthesis under isosmotic and hyposmotic conditions required extracellular calcium. A similar dependence on calcium has been documented in a variety of other eukaryotic cell types (Brostrom *et al.*, 1983; Palfrey and Nairn, 1995). In each case there was a significant reduction in the incorporation of radiolabelled amino acid into synthesised protein in the absence of calcium. There are two main theories as to the cause of the reduction in protein synthesis when calcium is absent. Firstly, the inhibition of 43 S pre-initiation complex formation by phosphorylation of eIF-2α (Chin *et al.*, 1987; Brostrom *et al.*, 1989; Palfrey and Nairn, 1995; Duncan and Burgoyne 1996) has been proposed as a mechanism. It is tempting to suggest that, in the absence of calcium, inhibition of translational initiation prevents protein synthesis in rat mammary tissue from proceeding. The effects of thapsigargin (and tBHQ) may also be attributable to a reduction in translational initiation. Addition of these inhibitors to GH3 pituitary cells prevented the accumulation of polysomes (Wong *et al.*, 1993). Subsequent studies identified the blockade as phosphorylation of eIF-2α by PKR (Srivastava *et al.*, 1995; Meldolesi and Pozzan, 1998; Kimball *et al.*, 2001). Secondly, a reduction in protein synthesis, in the absence of calcium could be due to the degradation of misfolded proteins in the ER. In HepG2 hepatoma cells it was shown that depletion of ER lumenal calcium

affects the folding and processing of proteins (Wong *et al.*, 1993; Cooper *et al.*, 1997). An earlier investigation into protein synthesis in a Chinese hamster ovary (CHO) cell line established that depletion of calcium stores resulted in an accelerated rate of protein degradation in the ER (Wileman *et al.*, 1991). Proteolysis in the ER takes place when incorrectly folded proteins accumulate. There are a number of ER resident chaperone proteins that are designed to minimise the likelihood of this happening (Pelham, 1989). So far, four such proteins have been identified in mammary tissue; BiP, GRP, protein disulphide isomerase (PDI) and calreticulin (Ghosal *et al.*, 1994). Recent studies have proposed that the concentration of Ca^{2+} in the ER is sensed by calreticulin (Corbett *et al.*, 1999, 2000). Changes in the lumenal concentration of Ca^{2+} were shown to modulate calreticulin binding to ERp-57 and PDI and in this manner the activity of these two chaperones (illustrated in figure 6.1). Thus, it is conceivable that proteolysis in rat mammary tissue increases as a consequence of calcium signaling in the ER. However, further studies are required to confirm the precise mechanisms controlling mammary protein synthesis, under isosmotic and hyposmotic conditions, in the absence of calcium.

6.2. MAMMARY LIPOGENESIS IS REGULATED BY CELL VOLUME

Lipogenesis in mammary tissue was also responsive to changes in the hydration state of the tissue. In both explants and acini, cell swelling and shrinking respectively increased and decreased the rate of lipogenesis. The extent of the change in lipogenesis was, as with the rate of protein synthesis, dependent on the degree of the osmotic perturbation.

A stimulation of lipogenesis by increased cell volume has also been observed in rat liver

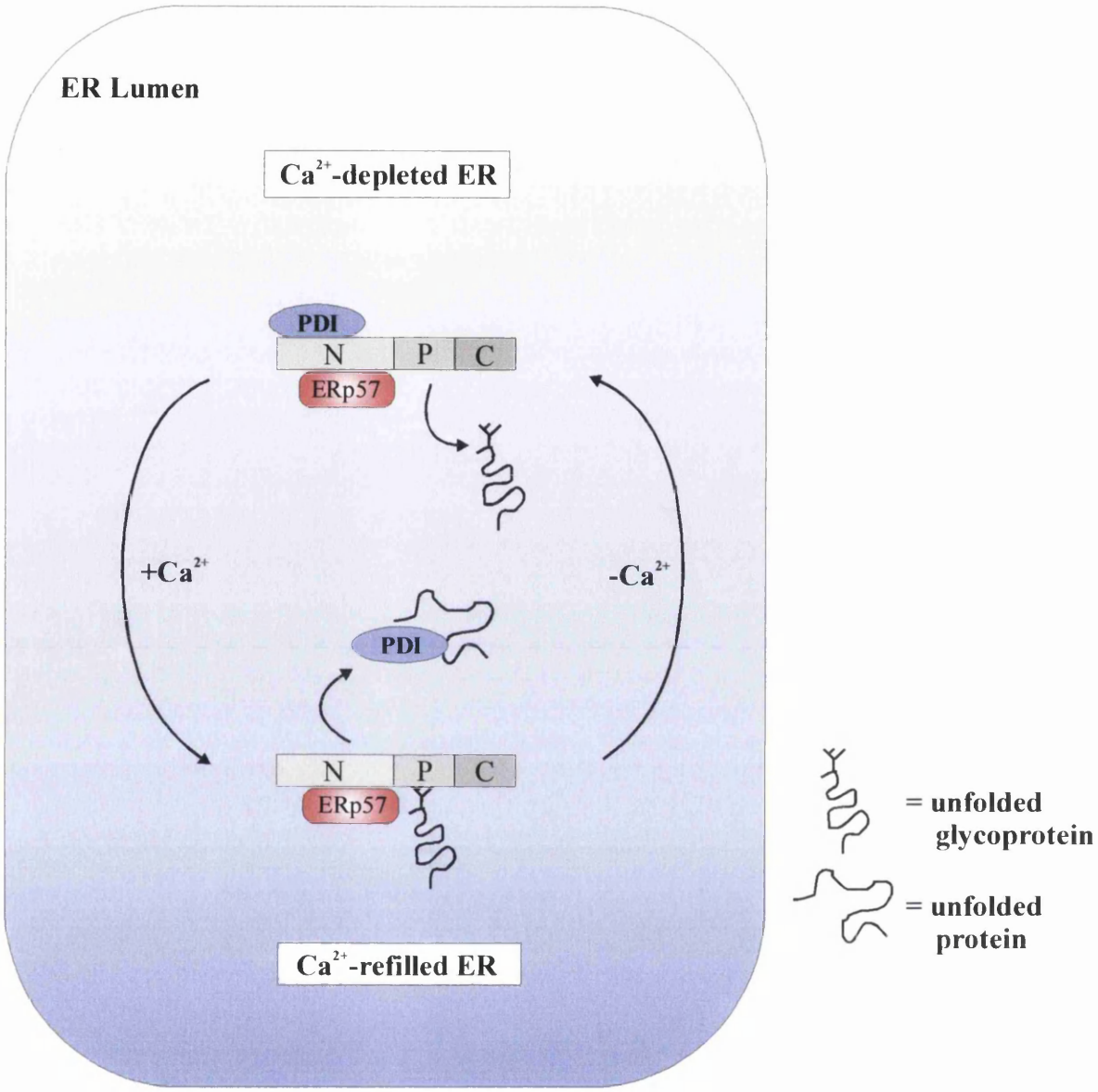

Fig. 6.1: Schematic diagram of the effect of luminal Ca^{2+} on chaperone function in the ER. As calcium is depleted from the ER lumen, calreticulin (NPC) binds protein disulphide isomerase (PDI), inhibiting its catalytic action. The affinity for unfolded glycoproteins is also reduced and they dissociate. As the calcium stores are refilled PDI dissociates from calreticulin and the affinities for unfolded proteins and glycoproteins are increased.

tissue (Baquet *et al.*, 1991a). Initially it was shown that supplementing incubation media with glutamine resulted in stimulation of both lipogenesis and glycogen synthesis (Lavoinne *et al.*, 1987). Subsequently, these stimulatory effects were attributed to the change in cell volume caused by Na^+-dependent amino acid accumulation (Baquet *et al.*, 1990, 1991a). As the osmolality of the incubation medium was reduced (by Na^+-depletion), the rate of lipogenesis increased (Baquet *et al.*, 1991a). The extent of Na^+-depletion in the incubation medium determined how much lipogenesis increased by in hepatocytes: when the Na^+ concentration was reduced to 105mM and 95mM lipogenesis was respectively stimulated by 55 and 90%.

Baquet and others (1991b) also showed that the anabolic effect of cell swelling on lipid metabolism was controlled by ACC activity. Dephosphorylation of the enzyme resulted in a marked increase in ACC activity in both hyposmotic and glutamine supplemented media. The enzyme implicated in controlling the activation of ACC was protein phosphatase-2A (PP2A; Baquet *et al.*, 1993). This was confirmed by Gaussin et al. (1996). At the same time, inhibitor studies led to the discovery that phosphatidylinositol 3-kinase was involved in ACC activation (Krause *et al.*, 1996), shedding some light on the mechanism that transmits the volume change signal. When wortmannin and LY294002 were added to the incubation medium the effects of cell volume on ACC activation were attenuated.

The effect of cell shrinking on lipogenesis has not been fully investigated in hepatocytes and thus, the present study is the first to describe a decrease in the rate of lipogenesis in response to cell shrinking. It has been shown that the addition of raffinose to a glutamine

supplemented medium prevented the activation of ACC (Baquet *et al.*, 1991b). However, ACC activity was not significantly inhibited by a hyperosmotic challenge, suggesting that there may be an alternative explanation for the decrease in lipogenesis observed in mammary tissue during cell shrinking.

The present study shows that volume-sensitive protein synthesis is dependent on calcium (chapter 3). However, the same was not true for lipogenesis in mammary tissue. Volume-sensitive lipogenesis was unaffected by removing extracellular calcium. Thus, it appears that under certain experimental conditions the rate of synthesis of these two milk components are differentially controlled.

6.3. THE SIGNIFICANCE OF VOLUME-SENSITIVE $[Ca^{2+}]_i$ IN MAMMARY ACINAR CELLS

The finding that extracellular calcium is required for mammary protein synthesis under isosmotic and hyposmotic conditions prompted an investigation into the effects of cell volume on $[Ca^{2+}]_i$ in mammary acinar cells. It was found that a reduction in osmolality of the incubation medium led to a transient increase in the cytosolic calcium concentration. In contrast, there was no significant change in $[Ca^{2+}]_i$ following cell shrinking. Removing calcium from the medium, by the addition of the EGTA, abolished the calcium response to an increase in cell volume. Subsequent experiments in which the mammary acini were treated with thapsigargin suggest that the intracellular stores of calcium were not involved in the calcium signal, but that SERCAs are involved in reducing cytosolic calcium following the initial rise.

A similar response to a hyposmotic stimulation has been recorded in numerous other tissue types, including cultured epithelial cells (Hazama and Okada, 1990), trout gill cells (Leguen and Prunet, 2001), cerebellar granule neurons (Morales-Mulia *et al.*, 1998), renal A6 cells (Urbach *et al.*, 1999) and vascular smooth muscle cells (VSMC; Mohanty *et al.*, 2001), to name but a few. However, this is by no means a ubiquitous response. The $[Ca^{2+}]_i$ in lymphocytes was found to be unaffected by cell swelling (Rink *et al.*, 1983) and in rat endothelial cells, shrinkage rather than swelling evoked a rise in $[Ca^{2+}]_i$ (Marchenko and Sage, 2000). The effect of cell swelling on the $[Ca^{2+}]_i$ profile also varies between cell types (see figure 6.2). In rat mammary tissue the response was rapid and transient, with the $[Ca^{2+}]_i$ returning to the basal level within 3 mins after the hyposmotic shock was administered (Shennan and Gow, 2000; chapter 4). In mouse mammary tissue the effect of cell swelling on $[Ca^{2+}]_i$ was biphasic: there was a rapid initial peak in the $[Ca^{2+}]_i$ followed by a sustained plateau phase (Sudlow and Burgoyne, 1997). In VSMCs the response of the $[Ca^{2+}]_i$ to cell swelling was different from that seen in mouse and rat mammary tissue. When subjected to a maintained hyposmotic challenge the $[Ca^{2+}]_i$ in VSMCs increased to reach a level which was sustained (Mohanty *et al.*, 2001).

The source of the volume-sensitive increase in the $[Ca^{2+}]_i$ also appears to depend on the cell type. In the present study, when calcium was depleted from the incubation medium using EGTA, the calcium response to a hyposmotic challenge was abolished. This suggests that the calcium signal is mediated by the opening of channels in the plasma membrane. The increase in $[Ca^{2+}]_i$ in VSMCs in response to a hyposmotic challenge appeared to be due to the release of calcium from an internal stores (Mohanty *et al.*, 2001). The study of Mohanty et al. (2001) eliminated IP_3-sensitive, ryanodine-sensitive

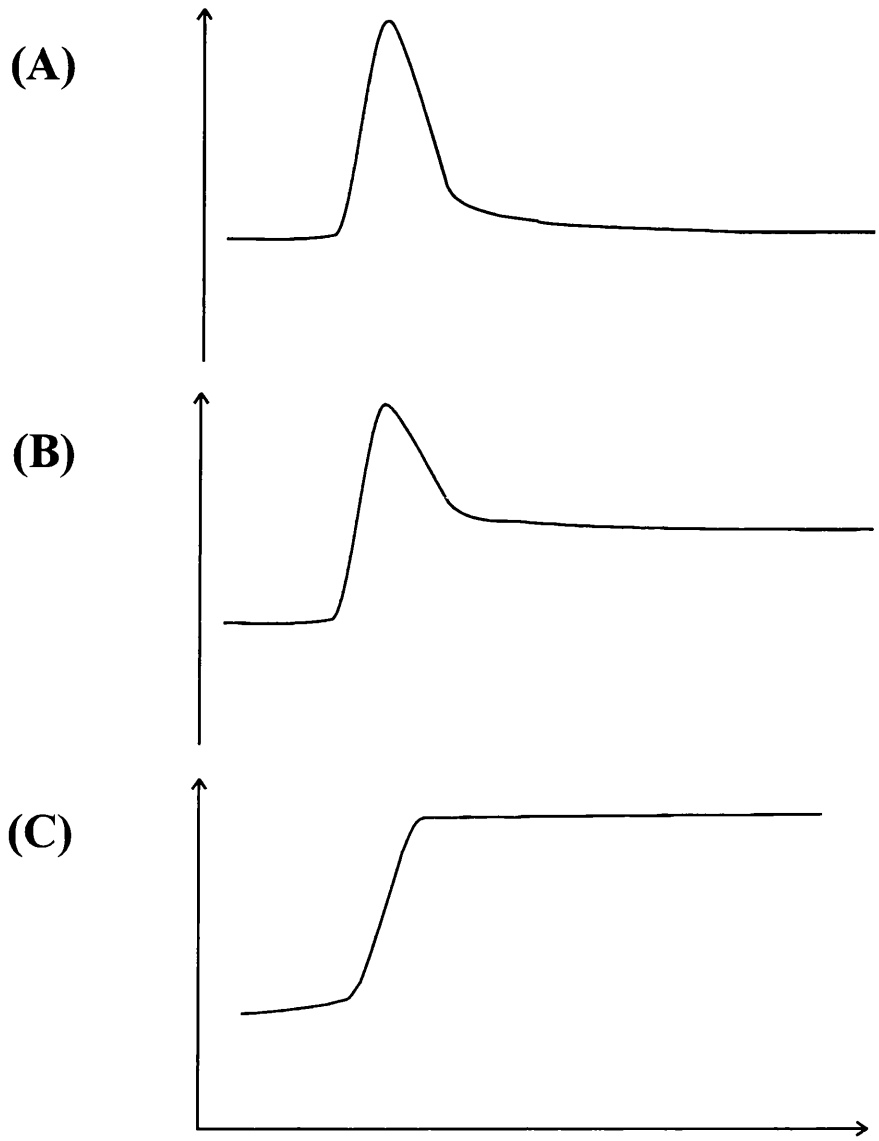

Fig. 6.2: Schematic diagrams of volume-sensitive changes in $[Ca^{2+}]_i$. (A) Transient response, (B) transient peak followed by plateau phase and (C) a sustained increase.

and NAADP-sensitive stores, suggesting the presence of a novel, unidentified calcium store. In intestinal epithelial cells (Hazama and Okada, 1990) each increase in $[Ca^{2+}]_i$ of the biphasic response was transient and it appeared that the peaks came from two separate sources. The initial increase was due to influx of calcium from the extracellular fluid and the second increase appeared to be due to release from an internal store, possibly evoked by the initial rise in $[Ca^{2+}]_i$.

The mechanism of calcium entry into mammary cells in response to a hyposmotic challenge remains to be identified. The present investigation showed that gadolinium had no effect on calcium entry, suggesting that stretch activated calcium channels are not involved in the response to anisosmolality.

One question which arises: what is the relationship between volume-activated Ca^{2+} uptake and volume-sensitive protein synthesis in mammary tissue? It is tempting to suggest that extra calcium needs to be taken up from the incubation medium to sustain hyposmotically-activated protein synthesis. However, there are three main findings that suggest there is no connection between the increase in cytosolic calcium concentration and protein synthesis. Firstly, isosmotic swelling using urea caused an increase in protein synthesis similar to that found during anisosmotic cell swelling. No such increase in calcium concentration was observed when acini were treated with a solution containing urea. Secondly, the sensitivity of volume-activated protein synthesis and swelling-induced calcium transport to cell swelling is markedly different. It is apparent that protein synthesis is stimulated by relatively small osmotic perturbations that have no effect on $[Ca^{2+}]_i$. Lastly, the timeframe over which the change in $[Ca^{2+}]_i$ occurs (lasting 3 minutes

- figure 4.5) appears to be too short compared to that of volume-activated protein synthesis, which is sustained for 30 minutes. Against this last point is the possibility that volume-activated Ca^{2+} uptake could be the first step in an enzyme signal cascade. In this connection, the calcium/calmodulin-dependent protein kinase II (CaM Kinase II), known to be involved in a number of enzyme cascades, is activated initially by the presence of both elevated calcium and calmodulin. Following this the enzyme undergoes autophosphorylation, ensuring that it remains in the active state even in the absence of a continued calcium signal.

Does volume-activated Ca^{2+} uptake play a role in the trans-epithelial transport of calcium? Milk is a calcium rich fluid. Accordingly, the mammary gland, during lactation, extracts large quantities of calcium from the systemic circulation. The transport mechanisms responsible for calcium uptake across the blood facing aspect remain to be fully characterised. If the volume-sensitive calcium transport pathway is situated in the basolateral membranes of mammary secretory cells then it could be in a position to contribute to transepithelial calcium transport. However, the transient nature of the hyposmotically induced increase in calcium appears to suggest that the capacity of this pathway is relatively limited.

It is evident that more experiments are required to elucidate the physiological significance of volume-sensitive changes to the $[Ca^{2+}]_i$ in mammary epithelial cells.

6.4. PHYSIOLOGICAL SIGNIFICANCE OF VOLUME-SENSITIVE MAMMARY METABOLISM

The results clearly show that mammary metabolism is markedly affected by changes to the osmolality of the incubation medium. It can be inferred that mammary protein synthesis and lipogenesis are regulated by cell volume changes as a result of the anisosmotic conditions. However, it must be borne in mind that mammary tissue, in vivo, will never be subjected to such osmotic perturbations under normal physiological conditions. The results do show, however, that mammary metabolic processes can be affected by isosmotic swelling. In this connection it is predicted that mammary cells will swell *in vivo* as a consequence of solute accumulation and/or changes to the rate of oxidative metabolism. In particular, solute uptake via sodium-dependent mechanisms, which are able to concentrate solutes within the cytosol with respect to the extracellular compartment will affect cell volume. Interestingly, the mammary gland expresses a variety of Na^+-dependent systems whose activity could influence cell volume (see table 6.1). For example, mechanisms such as (Na^+-K^+-Cl^-) cotransport could perhaps influence the hydration state of mammary cells. This transport mechanism has been implicated in regulating cell volume in a number of epithelia. Functional evidence suggests that the transporter is situated in the basolateral aspect of the mammary epithelium (Shennan, 1989, Shennan and Gow, 2000). Moreover, immunohistochemical studies have shown that the (Na^+-K^+-Cl^-) cotransporter in mouse mammary epithelial cells is situated in the blood-facing aspect (Shillingford, unpublished observations). The triple cotransporter is able to accumulate Cl^- within cells to a level above that predicted by the Nernst equation which in turn has a bearing on cell volume. Mammary tissue also expresses a variety of Na^+-dependent amino acids carriers (table 6.1). Amino acids, particularly the non-

Table 6.1: Na^+-dependent uptake systems in mammary tissue.

Na^+-dependent mechanism		Details	References
P_i uptake		Predominant Pi uptake system in rat mammary tissue	Shillingford *et al.*, 1996
Iodide uptake		Identified in pregnant tissue from mice, stimulated by prolactin	Rillema and Yu, 1996
Choline uptake		High affinity carrier in rat mammary tissue	Chao *et al.*, 1988
L-carnitine uptake		Identified in rat mammary tissue	Shennan *et al.*, 1998b; Zammit *et al.*, 1998.
SGLT1 (glucose uptake)		Glucose transport in rat, ovine, bovine and human mammary tissue	Shennan and Beechey, 1995; Shillingford *et al.*, 1997; Zhao *et al.*, 1999; Obermeier *et al.*, 2000
Amino acid uptake:	System X^-_{AG}	Anionic AA transport, possibly accounted for by GLAST and GLT-1 activity	Millar *et al.*, 1996, 1997; *Kansal et al.*, 2000; Martinez-Lopez *et al.*, 1998.
	System β	Taurine uptake in rat and pig mammary tissue	Shennan and McNeillie, 1994b; Hurley *et al.*, 2001
	System A	Neutral AA uptake identified in rat, mouse and bovine mammary tissue	Shennan and McNeillie, 1994a; Tovar *et al.*, 2000; Neville *et al.*, 1980; Sharma and Kansal, 1999; Baumrucker, 1985
	System ASC	Neutral AA transport in guinea pig and bovine mammary tissue	Mepham *et al.*, 1985; Baumrucker, 1985

essential ones, are concentrated in mammary tissue through the action of the sodium-dependent carriers. The concentration of free amino acids within mammary cells is large enough to make a significant contribution to the intracellular osmotic pressure. The anionic amino acids, L-glutamate and L-aspartate, are perhaps the amino acids which are concentrated most with respect to their extracellular concentrations (Shennan *et al.*, 1997). It is possible that the activity of system X^-_{AG}, the mechanism responsible for the

transport of anionic amino acids, contributes to the cellular hydration state. Another amino acid carrier which could also make a contribution to mammary cell volume is system β. This carrier is specific for the β-amino acids including taurine; it has been shown in other cell types that the activity of system β can have a marked effect on the cellular hydration state (Kwon and Handler, 1995). There is a possibility that cell volume could increase following a post-prandial rise in plasma nutrients such as amino acids. This would be a way of matching nutrient supply to demand. Thus, the uptake of amino acids into mammary cells could increase the hydration state and consequently protein synthesis.

There is also the possibility that swelling-activated protein synthesis could contribute to mammary cell volume regulation. Mammary tissue contains free amino acids at relatively high concentrations; indeed free amino acids make a significant contribution to the intracellular osmotic pressure. Therefore, the synthesis of proteins from the intracellular free amino acid pool will help to reduce the amount of osmotically active solutes within the cytosol which in turn could contribute to a regulatory volume decrease, or at least limit the extent of cell swelling. On the other hand a reduction in protein synthesis following cell shrinking could help facilitate a regulatory volume increase by maintaining the amount of osmotically active free amino acids within the cytosolic compartment. At the very least, the inhibition of protein synthesis by cell shrinking could limit the extent of shrinking.

Assuming that volume regulation does occur in mammary tissue, the question arises, how long do the effects of an increase in cell volume continue for if the volume of the cell is partially restored? The current investigation only shows that volume-activated mammary

protein synthesis is linear over a 30 minute period, due to the fact that longer incubations could have been affected by protein secretion. However, the significance of longer term effects of cell volume on protein synthesis and lipogenesis can only be determined through the use of extended incubations. If synthesis of protein and lipid remains linear for longer periods of time it is predicted that there will be a concomitant increase in the rate of secretion, preventing the build up of these products within the cell.

A recent review by Knight et al (2000) suggests that manipulation of milk composition *in vivo* may be possible. In this connection, the present study shows that a reduction in osmolality of 10% leads to increases in protein synthesis and lipogenesis of approximately 50% and 20%, respectively (figures 3.1 and 5.2). Thus, the ratio of protein to lipid in rat milk could be changed significantly. If similar effects of cell volume were observed in bovine mammary tissue, altering mammary metabolism through cell volume change would be of great commercial value. Cheese yield is known to be highly dependent on the content of protein and hence modulating bovine mammary cell volume *in vivo* could be beneficial to the dairy industry.

At this early stage of the investigation conclusions such as this remain speculative, as many other factors must be taken into account and duly investigated. Not least of all are the possible interspecies differences between rat and bovine tissue. Many different factors contribute to milk yield in dairy cattle: stage of lactation, seasonality, frequency of milking and diet are all controlling factors (Knight *et al*, 2000). The volume of milk secreted would also have a marked effect on the concentration of protein and lipid in milk. Even if the quantity of protein synthesised does increase by 50%, movement of water into the

alveolar lumen at a relative rate could dilute the protein concentration to the extent that the change may be undetectable. In connection with this, the rate of secretion of newly synthesised protein and lipid must also be taken into account. Thus, it is clear that further investigation is required before speculation as to the commercial value of altering mammary cell volume can be confirmed.

6.5. FUTURE STUDIES

The present results clearly show that the syntheses of proteins and lipids is affected by cell volume. However, it remains to be shown that protein and lipid secretion by mammary epithelial cells is also affected by cell volume perturbations. In this connection it has been demonstrated that protein secretion in other cell types is affected by cell volume. In particular, it has been shown that insulin secretion is very sensitive to changes in the hydration state of pancreatic β-cells (Blackard *et al.*, 1975; Marcstrom *et al.*, 1990; Miley *et al.*, 1997; Grant *et al.*, 2000). Indeed, it has been postulated that D-glucose uptake and metabolism by β-cells increases cell volume and hence insulin secretion (Miley *et al.*, 1997; Grant *et al.*, 2000).

Although it is predicted that mammary cell volume will be affected by the rate of solute uptake it remains to be demonstrated that the activity of mammary transport mechanisms has a direct effect on the hydration state of mammary epithelial cells. Mammary explants and acini will not be good models to do this because of the relatively large extracellular space. It is envisaged that the perfused lactating rat mammary gland could be used to test the effect of solute transport on mammary cell volume. This could be achieved by simply

measuring the weight of the perfused gland in the absence and presence of known substrates of mammary transport mechanisms. Such an approach has been used to successfully investigate the effect of amino acid transport on hepatocyte cell volume (Haussinger *et al.*, 1990). Although this method of measuring volume change was deemed suitable in liver studies it is likely to be of limited use in the mammary gland. Due to the macroscopic nature of the method there is no distinction between water retained within the cells and that in the ECF or luminal compartments of the tissue. Also, the exocrine function of the mammary gland means that water may be lost through the secretory pathway and therefore containment of such a system is more complex than with an endocrine or non-secreting tissue. Thus any measurements would have to be confirmed using a secondary method of determining cell volume.

An alternative method for measuring cell volume change is the use of a fluorescent dye. If the dye is internalised within the cell, the concentration, and thus fluorescence would change with an alteration in the hydration state. In this connection, it was speculated that the dye Fura-2 could have been used for such measurements. If the isosbestic excitation wavelength (see figure 4.1) had been used then a change in the $[Ca^{2+}]_i$ would have had no effect and thus, a change in fluorescence would have been due to alterations in the concentration of the dye. However, the size of the cellular aggregates in the acinar preparation is highly variable and thus inappropriate for such measurements. Use of single cells is preferable with such a technique, but the isolation procedure causes epithelial cells to both their shape and polarity. In addition to this an isolated cell may display different characteristics to one which has normal cell-to-cell contacts.

As mentioned in the introduction (chapter 1) it is becoming increasingly apparent that certain hormones are able to change cell volume and hence the metabolic activity of the target cell. For example, insulin can increase the volume of hepatocytes and thus protein synthesis (Lang et al., 1998a). In this connection, it is interesting to note that many mammary transport systems are regulated by prolactin, a hormone implicated in osmoregulation (Shennan, 1994). Interestingly, the mammary (Na^+-K^+-Cl^-) cotransporter requires prolactin for maximal activity (Shennan and Madon, 1991). As discussed above, the triple cotrransporter may help regulate the hydration state of mammary cells. Similarly, systems X_{AG}^- and A in rat mammary tissue are regulated by prolactin (Shennan and McNeillie, 1994c; Millar and Shennan, 1999). Therefore, it is conceivable that prolactin exerts its actions on mammary metabolism by controlling the cellular hydration state. Therefore, it would be interesting to test the effect of prolactin on the hydration state of mammary epithelial cells.

The study has shown that the synthesis of two major components of milk, namely protein and lipids, are affected by changing mammary cell volume. The effect of cell volume perturbations on the synthesis of lactose by mammary epithelial cells has yet to be examined. Such a study would reveal whether or not the synthesis of all the major milk components is affected by cell volume.

There is also a need to investigate the signalling pathways which are involved in the control of mammary protein synthesis and lipogenesis. Such a knowledge could lead to strategies being developed which could allow the quantitiy and possibly the quality of milk being manipulated.

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