AN INVESTIGATION INTO THE MECHANISM OF THE LIPOLYTIC ACTION OF GROWTH HORMONE IN ADIPOSE TISSUE OF SHEEP AND RAT

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by

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SUMMARY

The studies presented in this thesis were designed to evalulate the mechanism of action of the lipolytic effects of growth hormone in both sheep and rats. The initial study was carried out with virgin rats and involved manipulating levels of endogenous growth hormone using a specific antiserum to growth hormone (anti-rGH). The results showed that lowering levels of endogenous growth hormone resulted in an increase in the inhibition of isoprenaline-stimulated lipolysis, of isolated adipocytes, by submaximal concentrations of both PIA and PGE_1. There was also an increase in the amount of the inhibitory G-protein G_i2 and these effects were reversed upon replacement of growth hormone. Therefore growth hormone in vivo, specifically suppresses the expression of α-G_i2, in rat adipocytes leading to decreased sensitivity to anti-lipolytic agonists.

Further studies carried out with lactating and litter removed rats, again involving manipulation of endogenous growth hormone. Chronic treatment of lactating rats with growth hormone had no effect on maximum response or sensitivity to PIA, however, a diminished ability of antilipolytic agents after growth hormone treatment was seen in rats in which lactation was prematurely terminated but did not appear to involve a change in either adenosine receptor number or the amount of G_i1 plus G_i2. Therefore, it would appear that G_i in lactating rats in response to growth hormone is subject to covalent modification.

That the G_i-mediated antilipolytic system is a major target of growth hormone action was investigated further by treating sheep with recombinant bovine growth hormone (Monsanto) at 10 mg per day for 7 days *in vivo*. Treatment of sheep with the hormone decreased the maximum inhibition of isoprenaline-stimulated lipolysis. This effect of growth hormone was not accompanied by any discernable change in the number of adenosine receptors or amounts of the various isoforms of G_i. Therefore these studies also suggested that a decrease in G_i activity was occuring.

Further investigations were carried out using sheep adipose tissue *in vitro*. Sheep adipose tissue explants were preincubated in culture for 24 hours with no hormones and then growth hormone was added for the next 24 hours. As for the *in vivo* experiments, growth hormone treatment resulted in a decreased maximum response to PIA. Again there was no change in either adenosine receptor number or amounts of G_i. Further experiments were carried using this tissue culture system using a range of inhibitors to elucidate the mechanism of growth hormone action. As αG_i2 can be phosphorylated by at least two kinases the effect of protein kinase and phosphatase inhibitors were tested in the tissue culture system. Addition of H7, a protein serine kinase inhibitor, diminished the effects of growth hormone while the protein-serine phosphatase inhibitor, okadaic acid mimicked the effect of growth hormone, and therefore suggested that the effect of growth hormone involves protein serine phosphorylation. Further experiments with the phorbol ester PMA suggested involvement of protein kinase C isoforms that can be down-regulated by PMA in growth hormone actions, but use of D609, a phosphatidylcholine phospholipase C inhibitor suggested that diacylglycerol production via this pathway is not involved.

In time course experiments the maximum effect of growth hormone was reached after 24 hours demonstating that growth hormone is not simply activating a phosphorylation cascade. These results were supported by data from experiments with actinomycin D, an inhibitor of gene transcription, which was shown to knock out the effects of growth hormone.

More detailed studies were carried out to elucidate the nature of the altered activity of G_i. To do this ADP-ribosylation experiments were carried out with adipocyte membranes using Pertussis toxin and PIA to determine if growth hormone altered the ability of the adenosine receptor to cause dissociation of G_i. The results obtained show that growth hormone treatment does not affect receptor coupling to G_i. G_i activity was further assessed in adipocyte membranes using the diterpene forskolin or isoprenaline to activate adenylate cyclase, and p[NH]ppG or

PIA to inhibit this activation through a G_i-mediated response. The results from these experiments indicated that functional G_i was detected in membranes from control sheep adipose tissue but not in growth hormone treated tissue, indicating that the attenuation of the antilipolytic effect of adenosine may thus be due to changes in the interaction of G_i with adenylate cyclase.

Thus growth hormone decreases signalling through G_i by at least two mechanisms, altering both the amount of one isoform of this key protein and also the activity of the protein. Effects of growth hormone on the system vary with physiological state and are species specific.

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ABBREVIATIONS

AC	adenylate cyclase
ADP	adenosinediphosphate
ARF	ADP-ribosylation factor
ArGH	antiserum to rat growth hormone
ATP	adenosinetriphosphate
BSA	bovine serum albumin
cAMP	cyclic AMP
D609	tricyclodean-9-yl-xanthogenate
DNA	deoxyribonucleic acid
DTT	dithiothretol
EDTA	ethylene diaminetetra-acetic acid
EGF	epidermal growth factor
GH	growth hormone
GHR	growth hormone receptor
GLUT	glucose transporter
GMP	guanine monophosphate
G-3-PDH	glycerol-3-phosphate dehydrogenase
GTP	guanine triphosphate
H7	[1-{5-isoquinolinesulfonyl}-2-methyl piperazine, HCl]
IAP	islet activating protein
IGF-I	insulin-like growth factor
JAK	Janus kinase
MAP kinase	mitogen-activated protein kinase
MEK	MAP kinase kinase
MGF	mammary gland factor
MTT	[3-{4,5dimethylthiosal-2-yl}-2,5-diphenyl-tetrazoliumbromide]
PDGF	platelet-derived growth factor

PEG	polyethylene glycol
PGE_1	prostaglandin E_1
PIA	phenylisopropyladenosine
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonylfluoride
p[NH]ppG	guanosine 5-[βγ-imido]triphosphate
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide hydrogen phosphate
RIA	radioimmunoassay
SDS	sodiumdodecylsulphate
SIE	sis-inducible element
SRE	serum response element
STAT	signal transducers and activators of transcription
TEMED	tetramethylethylenediamine
TYK	tyrosine kinase

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CHAPTER ONE

INTRODUCTION

1.1 General Introduction

In recent years considerable interest has been expressed in reducing adiposity in domestic animals by the use of recombinant growth hormone (Boyd and Bauman, 1989; Bauman, 1992; Steele and Evock-Clover, 1993), and it has been shown that chronic administration of growth hormone decreases adiposity in farm animals (Boyd and Bauman, 1989; Hart and Johnsson, 1986). Other studies using transgenic animals expressing alien growth hormone genes have shown that pigs with rat growth hormone (Ebert *et al*, 1988) or bovine growth hormone (Wieghart *et al*, 1988), exhibited reduced adiposity.

Research into the control of milk production during recent times has focused principally upon the nutritional requirements and the central role that the endocrine system plays in stimulating milk yield. In the course of these studies it became apparent that, at least in rumimants, growth hormone is capable of inducing increases in milk yield of up to 50% (see Bauman and Vernon, 1993). Bovine growth hormone significantly enhances milk production of lactating cows at least in part by coordinating tissue nutrient utilisation to preferentially divert nutrients away from tissues such as adipose tissue to the mammary glands (Bauman and Vernon, 1993). Such chronic regulation of metabolism is known as homeorhesis, and homeorhetic controls shift nutrient partitioning by altering tissue response to acute, homeostatic signals such as insulin and the catecholamines epinephrine and norepinephrine and through tissue specific changes in gene expression (Bauman and Currie, 1980).

Resolution of the control processes of the signal transduction in adipocytes may provide novel means for manipulating both milk production and the degree of adiposity in animals and may also provide means for selective manipulation of the amounts of fat in different parts of the body.

Although adipose tissue is a major target of growth hormone, the mechanisms by which this hormone achieves its effects in adipose tissue are still not

fully understood. However, there is a growing body of evidence to suggest that the hormone acts both directly to alter the amount of key metabolic enzymes and in addition alters the ability of the adipocyte to respond to acutely acting metabolic hormones. These aspects, in particular effect on the lipolytic system, are considered in more detail below.

1.2 Adipose tissue

The major form of adipose tissue in adult animals is white adipose tissue. White adipose tissue is located at various sites throughout the body, for example: within the abdomen, omental, perirenal, under the skin (subcutaneous), within the musculature. In some animals adipose tissue can constitute as much as 50% of the body weight and in some grotesquely obese humans can be even higher (Flint and Vernon, 1993). In all mammals including marsupials the distribution of fat depots is essentially the same, and the average cell size, which varies from depot to depot, shows the same pattern of variation amongst different species (Pond and Mattacks,1985).

Adipose tissue comprises of adipocytes and a number of other cells. The adipocytes constitute approximately 25% of the cell population, but due to the enormous size of the adipocytes when bloated with fat actually contributes about 90% of the mass of adipose tissue in adult animals. The other cells are usually referred to as the stromovascular fraction and consist of adipocyte precursor cells at different stages of development, endothelial cells, mast cells and blood cells (Flint and Vernon, 1993).

While the major role of white adipose tissue is an energy store, the individual depots develop at different rates and different times (Flint and Vernon, 1993). This has led to numerous hypotheses as to its possible functions other than that of an enery store. The role of insulation has been considered as an important role,

especially for subcutaneous fat. While it is plausible that the function of subcutaneous adipose tissue is for insulation this is not always the case as in most land animals and ducks this fat is the first to be utilised in conditions of poor nutirition (see Vernon and Flint, 1993). Therefore the accumulation of large amounts of subcutaneous adipose tissue seen in artic animals may be for the physical reasons that really large amounts of fat can only be stored in the adominal cavity and under the skin. It has also been considered that the presence of adipose tissue around vital organs serves as protection. However, it has been suggested that cardiac and some intermuscular adipose tissue depots may play a critical, localized role in providing fatty acids as a fuel source for adjacent muscle fibres (Marchington *et al,* 1989). A role for adipose tissue for buoyancy in fish has been demonstrated in many species and studies described by Howell (1930) revealed a `lipid circulation system' with ducts and vessels that may allow rapid movement of lipids to aid in buoyancy.

Distinct adipose tissue depots play an important role in secondary sexual characteristics in some species. The mammary gland develops in a bed of adipose tissue (Sheffield, 1988) and produces such paracrine factors as prostaglandins and insulin-like growth factor that promote mammary growth (Levine and Stockdale, 1984). Adipose tissue is also required for the normal function of menstrual/estrous cycles and makes a major contribution to oestrogen production in postmenopausal women (Cleland *et al,* 1983).

Adipose tissue may also be important for the immune system as it has been shown to secrete adipsin, a serine protease (Cook *et al.*, 1987), which is a member of the alternative complement pathway, (Rosen *et al.*, 1989).

The second form of adipose tissue is brown adipose tissue; it is more prevalent in young animals and has an important role in heat production by nonshivering thermogenesis. It is typically found in a small number of defined depots (Himms-Hagen *et al,* 1972), has dense blood and nerve supplies (Fawcett,

1952; Ballard, 1974) and large numbers of mitochondria and the protein thermogin (Cannon *et al*, 1982). These and other differences found between white and brown adipose tissue indicate the major functional distinction between these tissues. However, all following information will focus on white adipose tissue.

1.2.1 Adipose tissue development

The growth of tissues involves an increase in cell number and cell size. In adipose tissue increased cell size is primarily due to lipid accumulation. Studies using 3T3-L1cells have led to a model of adipocyte development (Green, 1978; Green *et al*, 1985), which propose, the existence of pluripotent mesenchymal cells, which can differentiate into the precursors of adipocytes (adipoblasts) and also chondrocytes and myocytes (Sager and Kovac, 1982; Grigoradis *et al*, 1988). Adipoblasts are thought to be small, undifferentiated cells with little or no lipogenic activity and lacking lipid droplets, and are thought to proliferate and differentiate under multihormonal control into preadipocytes. Preadipocytes contain both lipogenic and lipolytic enzymes and accummulate fat as a number of small droplets that eventually fuse to form one single, large droplet, which occupies the centre of the cell surrounded by a thin film of cytoplasm.

It is now generally accepted that mature, lipid-filled adipocytes do not divide, and there is evidence for loss of actin filaments and other components required for mitosis during preadipocytes differentiation (Roncari, 1984), however, mitosis has been described *in vitro* in mature adipocytes, which lose their lipid and dedifferentiate (Sugihara *et al*, 1986, 1987). During later life increases in fat cell number are thought to be due to the proliferation and differentiation of precursor cells or to the accummulation of lipid in differentiated cells devoid of lipid.

Cell lines and primary cultures have been used for studying the development of the adipocyte and its endocrine control, however, these approaches are not

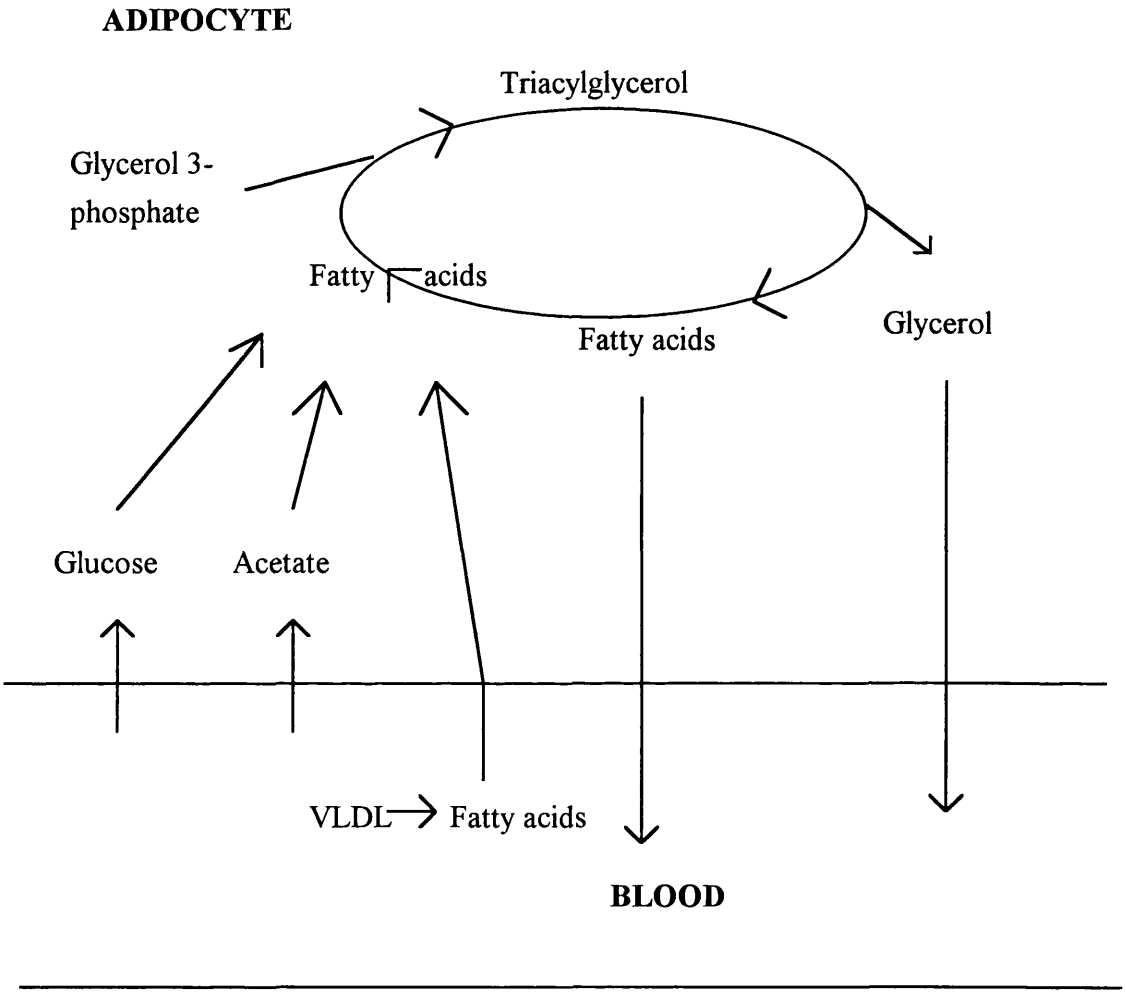
without problems. The requirement for serum in the medium bathing the cells in early studies made it difficult to investigate the importance of particular exogenous agents with confidence. There is still a requirement for serum in certain systems, but an increasing number of techniques involve serum-free cultures.

In vitro studies have shown differences in growth rate of stromovascular fractions derived from different adipose tissue depots. For example perirenal rat adipocytes have a higher mitotic rate than epididymal adipocytes (Djian *et al*, 1983; Kirkland *et al*, 1990), while in sheep, adipocytes from channel (lumbar) fat showed greater replication rates than several other types of adipocytes (Cryer and Cryer, 1989), whereas subcutaneous and popliteal preadipocytes showed a greater tendency to differentiate than omental preadipocytes (Adams *et al*, 1996). In broiler chickens precursor cell proliferation was much more rapid *in vitro* than that of precursors from (leaner) layer strain chickens, a difference that can be sustained through several passages (Donnelly et al, 1989). *In vivo* studies involving transplantation of adipose tissue between lean and obese mice suggest that lipid accummulating adipocytes is entirely dependent on cellular enviroment rather than any intrinsic factor (Ashwell *et al*, 1977; Ashwell and Meade, 1979). Therefore both intrinsic and enviromental factors influence the rate of adipose tissue formation, with their relative importance depending on situation. These site-specific differences then, offer considerable opportunities to manipulate the amounts of individual adipose tissue depots.

1.2.2 Adipose tissue metabolism

Food energy in animals is stored primarily as triacylglycerols in lipid storage droplets of adipocytes. Lipolysis is the process that liberates fatty acids and glycerol from stored lipid, while esterification, is the priniciple pathway involved in the synthesis of triacylglycerol, and the relative rates of the two processes determine if there is a net loss or accumulation of lipid (see figure 1.1).

Figure 1.1 Pathways for synthesis and hydrolysis of triacylglycerol



Adipose tissue synthesizes triacylglycerol using glycerol-3-phosphate which is derived from glucose and fatty acids which may be synthesised *de novo* in adipocytes or derived from plasma lipoproteins by the action of lipoprotein lipase. Adipose tissue has little or no glycerol kinase activity and so cannot re-use glycerol released by lipolysis as a precursor (Vernon, 1980). The enzyme lipoprotein lipase is secreted by adipocytes and serves to hydrolyse plasma triacylglycerols prior to uptake into the adipocyte. This enzyme is transported to the endothelial cells lining the capillaries and attaches to the outer surface of the plasma membrane facing the lumen of the capillary. The greater the amount of fat in the diet the greater is the importance of lipoprotein lipase, but even in ruminants which normally consume relatively low amounts of fat, lipoprotein lipase activity may supply in excess of fifty percent of fatty acids deposited (Vernon, 1980). Transport into the cell of the resulting fatty acids and monoglycerols is thought to be facilitated by a fatty acid translocase located in the plasma membrane (Scow and Blanchette-Mackie, 1985). Monoacylglycerols taken up by the cell are hydrolysed to fatty acid and glycerol.

1.2.2.1 Lipogenesis

Lipogenesis (fatty acid synthesis) requires a source of cytosolic acetyl CoA and NADPH. The most important precursors of acetyl CoA are acetate, glucose and possibly lactate but it can also be produced from a number of amino acids. The significance of these precursors varies from species to species and with age within a species (see Vernon. 1980, 1986). While glucose and lactate are most important in non-ruminants and may make a more substantial contribution in the foetal animal, for adult ruminants acetate is the most important precursor (Vernon, 1986).

The NADPH required for lipogenesis can be derived from three systems; glucose-6-phosphate and 6-phosphogluconate dehydrogenases, malic enzyme and NADP-isocitrate dehydrogenase. Their relative importance varies with species with isocitrate dehydrogenase being of special importance in ruminants (Vernon, 1980).

As cytosolic acetyl CoA and NADPH may be used for syntheses other than lipogenesis, the most important control point of lipogenesis is thought to be at acetyl CoA carboxylase which catalyses the conversion of acetyl CoA to malonyl CoA (Vernon, 1992). The enzyme exists in active and inactive states where phosphorylation of key serine residues results in inactivation (Hardie, 1989).

1.2.2.2 Lipolysis

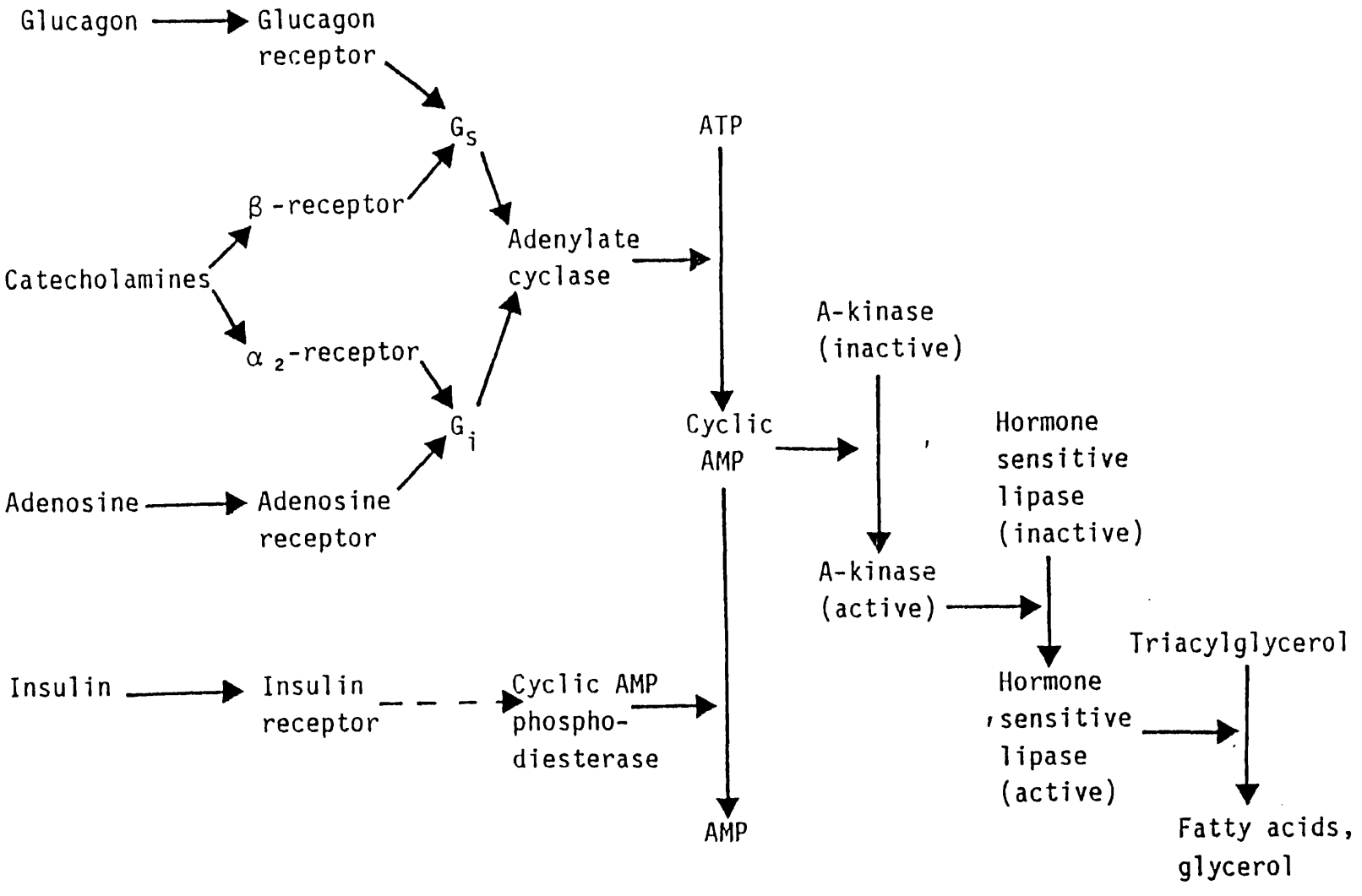
A central feature of the metabolism of white adipose tissue is lipolysis, brought about by the action of hormone-sensitive lipase which is under complex acute and chronic endocrine control (Vernon, 1992).

Triacylglycerols are triesters of glycerol with three long chain carboxylic acids, thus hydrolysis of fat yields glycerol and three fatty acids. Some of the fatty acids released from ester linkage are re-esterified and so retained in the cell. As adipocytes are deficient in the the glycerol kinase free glycerol produced by lipolysis cannot be phosphorylated and used for reesterification and so is released from the cell, thus providing a useful measure of lipolysis (Margolis and Vaughan, 1962).

1.2.2.3 The lipolytic pathway

Adipocytes possess both α_2 and β-adrenergic-receptors (Vernon and Sasaki, 1991). Catecholamines acting via β-adrenergic receptors are stimulators of lipolysis, details of which are outlined in figure 1.2. These, and glucagon, utilise the same pathway to cause triacylglycerol mobilisation. Binding of catecholamines to the β-adrenergic receptor in the plasma membrane causes it to interact with the heterotrimeric GTP-binding protein G_s which then binds GTP and dissociates to yield free, GTP-bound activated G_s-α subunit and a βγ subunit. The α-subunit interacts with, and subsequently activates adenylate cyclase (AC). All of these

Figure 1.2 Lipolytic Signal Transduction cascade



steps occur at the level of the plasma membrane. AC then catalyses the synthesis of cyclic AMP (cAMP) from ATP, which is released into the cytosol where it causes the dissociation and activation of cAMP-dependent Kinase (A-kinase). cAMP concentration is modulated by the activities of several cAMP phosphodiesterase enzymes which degrade cAMP to AMP (Beavo, 1995; Bolger, 1994). In addition, insulin, activates cyclic AMP-phosphodiesterase activity and decreases cAMP concentration (Conti *et al*, 1991; Morganiello, 1995). Therefore the cAMP concentration and hence the activation status of A-kinase will not only depend on the strengths of the various signalling through G_s, but also that through cAMP phosphodiesterase activity. A-kinase has a pivotal role, integrating a variety of signals into one, inhibiting lipogenesis and promoting lipolysis by phosphorylating specific serine residues of hormone-sensitive lipase causing its activation and thereby increases the rate of lipoysis (Vernon, 1992). Studies have suggested that phosphorylation of hormone-sensitive lipase results in a translocation of the lipase to its substrate at the surface of the lipid storage droplet (Egan *et al*, 1992). Perilipin is a protein located on the surface of the fat droplet and is phosphorylated by A-kinase, and studies suggest that perilipin is a `docking protein' for hormone sensitive lipase (Londos *et al*, 1990; Greenberg *et al*, 1991). Triacylglycerols are hydrolysed by the action of hormone sensitive lipase that cleaves two molecules of fatty acids. The resulting monoacylglycerol is hydrolysed to glycerol and fatty acid by monoacylglycerol lipase (Vernon, 1992). The cleavage of the first fatty acid moiety by hormone sensitive lipase is thought to be the rate-limiting step, while the monoacylglycerol lipase is very active and monoacylglycerol is normally present in very low amounts in the tissue (Vernon, 1992).

Essentially all the glycerol and some fatty acids produced are released from the cell, whereas some fatty acid is re-esterified. Esterification and lipolysis occur simultaneously and continuously giving a constant turnover of triacylglycerol, and the relative rates of the two processes determine if there is a net loss or accumulation of lipid (Vernon, 1992).

Other hormones and some locally produced factors bind to receptors linked to isoforms of another heterotrimeric GTP-binding protein termed G_i. An occupied receptor interacts with the G_i α-subunit, causing it to bind GTP. The G-protein then undergoes a conformational change, leading to the dissociation of the βγ complex and the release of the GTP-bound α-subunit which interacts with and inhibits adenylate cyclase activitity, thereby reducing cAMP formation. Adenosine (Vernon *et al*, 1991) and prostaglandin E (PGE) (Reichelson, 1992) which are produced in adipose tissue, act through their own receptors to inhibit lipolysis while catecholamines, by acting through the α2-receptor can also inhibit lipolysis by this mechanism. Hence lipolysis in the adipocyte is under complex, acute control, (Rodbell, 1980). That is the activity of hormone sensitive lipase at any moment is determined by the balance struck between stimulatory and inhibitory agonists.

The involvement of a G-protein in transmembrane signaling was first suggested by the requirement for GTP for hormonal activation of adenylyl cyclase (Rodbell *et al*, 1971a). At the same time it was found that GTP interfered with detection of the hormone glucagon binding to receptors responsible for regulation of adenylyl cyclase activity (Rodbell *et al*, 1971b). It was subsequently found that the effect of guanine nucleotides on receptor binding was specific for agonists and that their affinity for the receptor was reduced (Maguire *et al*, 1976). Further experiments with turkey erythrocyte membranes involving catecholamine-stimulated GTPase activity (Cassel and Sellinger, 1976, 1977), showed that G-protein-linked systems are activated on binding of GTP; hydrolysis of GTP initiates or is responsible for deactivation and dissociation of GDP is linked with the rate-limiting step and is controlled by the receptor. G-proteins and their receptors are considered in more detail in section 1.4.

1.2.3 Hormonal regulation of lipolysis

As described above, the hydrolysis of triacylglycerol by hormone sensitive lipase is under complex acute and chronic endocrine control. In mammals including ruminants (see Vernon, 1980) catecholamines are potent stimulators of lipolysis whereas glucagon has only a small affect; in poultry the converse is seen with glucagon being the major lipolytic hormone (Butterwith, 1988). Insulin is the major anabolic hormone of the body (Vernon and Flint, 1989) promoting lipid synthesis and inhibiting lipolysis in adipose tissue, and appears to decrease lipolysis by both cyclic AMP-dependent and independent mechanisms, the former probably involving activation of cyclic AMP phosphodiesterase and the later activation of a phosphatase and dephosphorylation of hormone sensitive lipase (Stralfors and Honnor, 1989). In contrast to insulin, IGF-I and IGF-II have been claimed to have acute lipolytic effects in sheep adipose tissue (Lewis *et al*, 1988), although these results have not been found to be reproducable (Houseknecht *et al*, 1996).

Studies with rats have shown that glucocorticoids increase β-receptor number but decrease maximum adenylate cyclase activity (Giudicelli *et al*, 1989; Ros *et al*, 1989). These results were supported by studies of that showed prolonged incubation of sheep adipose tissue with the glucocorticoid analogue, dexamethasone, increased response and sensitivity to isoprenaline and decreased maximum adenylate cyclase activity (Finley *et al*, 1990). Prolonged exposure to dexamethasone also increases response to α2-adrenergic agents and to adenosine in sheep adipose tissue (Vernon *et al*, 1991b). Conversely, studies with rat adipose tissue have shown that adrenalectomy increases response to adenosine (Saggerson, 1980; DeMazancourt *et al*, 1989). Hypothyroidism in rats has been shown to decrease the response to β-agonists and increase the response to adenosine and prostaglandin E_2 (Malbon *et al*, 1988; Vernon *et al*, 1991a). These effects were not due to receptor level changes, but the effects of adenosine and prostaglandin E_2 were found to involve an increase in G_i activity (Malbon *et al*, 1988, Milligan and

Saggerson, 1990). Conversely hyperthyroidism in rats increased the response to β-agonists and, due to a decrease in adenosine receptor number, decreased the response to adenosine (Malbon *et al*, 1988).

Insulin has a chronic as well as an acute effect on lipolysis (Vernon, 1992). Studies have shown that diabetes appears to decrease sensitivity to adenosine (Saggerson, 1989; Green and Johnson, 1991), possibly due to a change in G_i activity (Strassheim *et al*, 1990; Green and Johnson ,1991). Diabetes also appears to cause a decrease in maximum adenylate cyclase acivity in the rat (Lacasa *et al*, 1983; Strassheim *et al*, 1990).

1.2.4 Effects of lacatation on adipose tissue in sheep

A change in physiological state may alter a number of components of systems transmitting stimulatory and inhibitory signals. For lipolysis such changes could adjust the range of operational cyclic AMP concentrations within the cell and hence the strength of signal transmitted to A-kinase. Lactation provides an example of such a state.

The cycle of pregnancy and lactation causes profound changes in adipocyte metabolism. While lipid is usually accumulated during pregnancy, onset of lactation in ruminants results in a substantial transient loss of lipid from adipocytes as extra nutrients are needed to meet the requirements for milk production, which is replenished during the declining phase of lactation (Vernon and Flint, 1984; Vernon and Finley, 1988).

One complication is that the lipolytic response varies with fat-cell size (Vernon and Finley, 1985) and season, (Larsen *et al*,1985) and these have to be taken into account when comparing responsiveness in situations that lead to substantial changes in fat cell size, such as lactation, (Vernon and Flint, 1984).

Studies with the β-agonist, isoprenaline have shown evidence for an increase in maximum response and sensitivity to catecholamines during late pregnancy and

early lactation in sheep (Guesnet *et al*, 1987; Iliou and Demarne, 1987). In these studies mean cell volume did not change during lactation, indicating that changes in response and sensitivity were not due to a change in cell size. Studies with subcutaneous adipose tissue from lactating sheep showed both an increase in the response to noradrenaline, which has both β- and α2-adrenergic effects, and an increase in response to isoprenaline (Vernon and Finley, 1985; Vernon *et al*, 1995). These results showed that the enhanced response was due to an increased β-adrenergic response with no apparent in the α2-adrenergic response. It has also been shown that no change in the response to the α2-adrenergic agents with lactation occured in cattle (Smith and McNamara, 1989). While studies showed that lactation increased both the maximum response and the sensitivity to β-agonists in sheep omental and subcutaneous adipose tissue, paradoxically it has also been shown that there was a concomitant increase in response to adenosine (Vernon *et al*, 1995). Lactation thus results in both the adrenergic β-adrenergic and adenosine signal transduction systems of adipocytes becoming more responsive to agonists. Why lactation should enhance the response to antilipolytic factors is unclear. However, the paradox remains that lactation alters the responsiveness of both the stimulatory and inhibitory arms of the adrenergic-adenosine signal transduction system.

The studies, previously mentioned, (Vernon *et al*, 1995) also revealed that while an increase in β-receptor numbers in omental tissue was observed, this was not the case for subcutaneous tissue. In contrast, there was an increase in total adenylate cyclase activity in subcutaneous but not omental adipose tissue, while for both tissues the amounts of $G_s\alpha$-subunits were increased during lactation. The differences in signal thus seem to due to adaptations at the plasma membrane as activities of both PDE and A-kinase were unchanged by lactation. The increased response of omental and subcutaneous adipose tissue to adenosine may be due to an increase in the amounts of G_i α-subunits (Vernon *et al*, 1995).

1.2.5 Effect of lactation on adipose tissue in rats

There is some lipid accumulation in adipose tissue during pregnancy in rats, but much of this is lost around parturituion with some further loss during lactation (Vernon and Flint, 1984). Lipid lost during lactation is replaced on removal of young, so reproductively active female animals undergo cycles of lipid loss and accumulation (Johnson, 1973).

In contrast to sheep, no change in response to β-agonists was observed during lactation in the rat indicating a variation between species. However, the response of adipocytes to the anti-lipolytic effect of adenosine was again paradoxically, increased despite the increased mobilisation of fat reserves during lactation (Vernon *et al*, 1983). Further studies have shown an increase in β-receptor number and decreased cAMP phosphodiesterase activity during lactation but there is also a decrease in maximum adenylyl cyclase activity and activation of A-Kinase was unaltered (Vernon *et al*, 1993). Therefore lactation induced changes in some individual components of the β-adrenergic signal-transduction system which apparently cancelled each other out, so that there was no overall change in response or sensitivity to the signal.

Litter-removal from lactating rats decreased the response to β-agonists (Vernon *et al*, 1987; 1993; Ros *et al*, 1992). Ros *et al* (1992) suggested that this was due to a decrease in β-receptor number and amount of G_s. These results were refuted by Vernon *et al.* (1993), who showed that the fall in β-receptor number acted to restore the receptor numbers to levels found in virgin rats; more importantly, they showed that activation of protein kinase A was unimpaired and that the diminished response was due to a failure of hormone sensitive lipase to translocate to the fat droplet or in response to catecholamines.

1.3 Growth hormone

Growth hormone (GH) is secreted by acidophil cells of anterior pituitary gland and this secretion is mainly controlled by hypothalamic GH-releasing hormone which stimulates release, and somatostatin, which inhibits release (Page *et al*, 1989). GH is a single chain peptide hormone of 191 (Wallis, 1989) amino acids that is secreted in a pulsatile, circadian pattern that occurs about seven or eight times a day, usually associated with exercise, the onset of sleep or in response to falling plasma glucose levels about an hour after meals (Rawlings and Mason, 1989). GH promotes protein synthesis in muscle tissue and in conjunction with insulin stimulates amino acid uptake by muscle cells (Pell, 1989).

1.3.1 Growth hormone and lipid metabolism

The relationship between growth hormone and the metabolism of lipids was first investigated by Lee and Shaeffer in the 1930s when they showed that a decrease in total body fat occurred when rats were given prolonged treatment of pituitary extracts, while still observing an overall increase in body size (Lee and Shaeffer, 1934). Further experiments were carried out to isolate the factor responsible for the decrease in body fat and this resulted in the isolation and purification of growth hormone (Li *et al*, 1945). Other studies have also established that prolonged treatment of rats with preparations rich in growth hormone decreased the amount of body fat (Goodman and Schwartz, 1974; Rao and Ramachandran, 1977), while the availability of recombinant growth hormone has shown similar effects in ruminants (see section 1.1). For some tissues, for example the mammary gland, it would appear that the effects of growth hormone are mediated by IGF-I (Bauman and Vernon, 1993). However, the presence of growth hormone receptors and the absence of IGF-I receptors in rat and human adipocytes suggests that the mode of growth hormone on the adipocyte is direct. Further

evidence for this comes from studies such as those of Schoenle *et al* (1983) who found that effects of hypophysectomy in rats on glucose metabolism of adipose tissue could be prevented by infusion of growth hormone but not IGF-I for IGF-II. The effects of growth hormone on adipose tissue metabolism can also be observed with the addition of growth hormone to *in vitro* chronic cultures of adipose tissue (Vernon and Flint, 1989; Bauman and Vernon, 1993), providing further evidence for direct effects of growth hormone on adipocytes.

1.3.1.1 Effect of growth hormone on lipolysis

The role of growth hormone in the regulation of lipolysis is not fully understood but although it is still controversial, it appears that the hormone does not have direct lipolytic effects (Vernon and Flint, 1989). Several lines of evidence suggest that growth hormone probably exerts its effects indirectly by altering the ability of lipolytic and also antilipolytic factors to alter the rate of lipolysis. Chronic treatment of steers (Peters, 1986) or lactating cows (McCutcheon and Bauman, 1986; Sechen *et al*, 1990; Houseknecht *et al*, 1985) increased the lipolytic response to catecholamines *in vivo* (assessed from changes in plasma fatty acid and glycerol concentrations). Chronic treatment with growth hormone *in vivo* had no effect on sensitivity to catecholamines (Sechen *et al*, 1990). Studies *in vitro* involving maintenance of sheep adipose tissue in culture for 48 hours in the presence of growth hormone also increased the response and sensitivity to the β-agonist, isoprenaline, and increased ligand binding to the β-adrenergic receptor (Watt *et al*, 1991). Further evidence suggesting that growth hormone enhances signal transduction by increasing the number of β-adrenergic receptors was seen in studies by Vernon *et al*, (1993) and Watt *et al*, 1990, who showed that varying serum growth hormone in rats *in vivo* altered the ligand binding to the β-receptor of adipocytes. Additional evidence comes from studies with lactating rats (see section 1.3.2).

In contrast to the above, chronic treatment with growth hormone *in vivo* had no effect on lipolytic response to catecholamines *in vitro* in bovine adipose tissue (Peters, 1986; Lanna *et al*, 1992). This raised the possibility that some effects of growth hormone may be on the antilipolytic system. Treatment of lactating rats after litter removal with growth hormone reduced response to adenosine in adipocytes *in vitro* (Vernon *et al*, 1987) while tissue culture of sheep adipose tissue with growth hormone for 48 hours decreased the response to both adenosine (PIA) and α2-agonists (Vernon *et al*, 1991b). Thus growth hormone appears able to modulate signalling through both the lipoytic and antilipolytic systems.

1.3.1.2 Effect of growth hormone on lipogenesis

Growth hormone affects carbohydrate and fat metabolism in adipocytes: it acts chronically to antagonise the insulin-mediated cellular uptake of glucose and stimulates lipolysis (Vernon and Flint, 1989). The insulin-antagonistic effects of GH may also be important in the development of insulin resistance which is associated with obesity and diabetes mellitus (Fowelin *et al*, 1991). Growth hormone alters the activities of enzymes involved in lipid metabolism in adipocytes. Studies with intact rats showed that chronic elevation of growth hormone decreased rates of glucose oxidation and fatty acid synthesis in adipose tissue (Davidson, 1987; Goodman and Schwartz, 1974) while in intact growing pigs, growth hormone treatment reduced the rate of lipogenesis and the activity of several lipogenic enzymes (Magri *et al*, 1987). Decreases in acetyl CoA carboxylase and fatty acid synthase activities occur following chronic treatment of adipocytes or adipose tissue with growth hormone (Dietz and Schwartz, 1991; Bauman and Vernon, 1993; Vernon *et al*, 1993). Chronic growth hormone has also been found to decrease the expression of the GLUT1 glucose transporter in both rat adipocytes and 3T3-F442A adipocytes (Kilgour *et al*, 1993; KuTai *et al*, 1990). Growth hormone is a powerful insulin antagonist and inhibits the activation

of acetyl CoA carboxylase by insulin, possibly by inhibiting the synthesis of a protein required for mediation of the insulin-induced activation of the enzyme (Vernon *et al*, 1991a).

Time-course experiments of growth hormone effects on adipose tissue in rats has shown that abstinence from growth hormone for a few hours, *in vivo* or *in vitro*, or more results in a transient stimulation of lipogenesis and inhibition of lipolysis (Goodman, 1993). As these effects are only seen in cells previously deprived of growth hormone for several hours the physiological relevance of the findings is doubtful, except perhaps in young male rats in which growth hormone secretion is highly pulsatile (Vernon and Flint, 1989). This insulin-like effect is short lived, after which inhibition of glucose utilisation and enhanced lipolysis typical of the chronic effects of growth hormone are seen, however the development of these chronic effects are prevented by the addition of actinomycin D, an inhibitor of gene transcription (Goodman *et al*, 1987; Goodman, 1993). Studies with sheep adipose tissue showed that the full chronic effects of growth hormone on lipogenesis were seen after 12 hours following a 3-4 hour lag phase. This lag-phase was extended when insulin was present (Borland *et al*, 1994).

Polyamines have been implicated in the insulin-like antilipolytic effect of growth hormone on lipolysis in chicken adipose tissue (Campbell and Scanes, 1988). The effects of polyamines include a role in gene transcription (Scalabrino *et al*, 1991; Sjoholm, 1993), activation of a number of protein kinases and phosphatases (Morgan, 1990) and enhancing hormone binding to plasma membrane receptors (Pedersen *et al*, 1989). Exposure of sheep adipose tissue to growth hormone also increases the activity of S-adenosyl methionine decarboxylase, an enzyme of polyamine metabolism (Snoswell and Vernon, 1990). Studies carried out by Borland *et al*, (1994) showed that the inhibition of lipogenesis is sheep adipose tissue by growth hormone required polyamines. Further studies using actinomycin D, an inhibitor of gene transcription, implicated a protein with a half

life of less than three hours in the inhibition of lipogenesis by growth hormone (Borland *et al*, 1994). As the effect of actinomycin was not overcome by adding spermidine to cultures, it would appear that this putative protein is not ornithine decarboxylase (Borland *et al*, 1994). Very recent studies (Vernon, 1996) using the inhibitors H7, staurosporine and okadaic acid indicate a role for both protein serine phosphorylation and dephosphorylation in the inhibition of lipogenesis by growth hormone.

1.3.2 Growth hormone and lipid metabolism at lactation

Serum growth hormone is increased during lactation in ruminants but not in rats (Vernon, 1989). While it has not been proven directly, it would seem probable that this increase in serum growth hormone contributes to the enhanced response and sensitivity to catecholamines and the increased number of β-adrenergic receptors of adipocytes in lactating ruminants. The elevated levels of serum growth hormone are not sufficient to achieve a maximum effect, in lactating cows at least, as treatment with growth hormone enhances response, but not sensitivity to catecholamines (Sechen *et al*, 1990; Houseknecht *et al*, 1995). Elevated serum growth hormone is also thought to contribute to the decreased adipocyte lipogenesis in lactating ruminants (Vernon, 1988; Bauman and Vernon, 1993).

The role of growth hormone in lactating rats is less clear. The lack of increase in lipolytic response to catecholamines is consistent with lack of an increase in the serum growth hormone concentration. However, in rats, replenishment of lipid reserves following litter removal is associated with a decreased ability of catecholamines to stimulate lipolysis and an increase in lipid synthesis. The diminished response to catecholamines can be mimicked in lactating rats by treatment with a specific antiserum to rat growth hormone (Vernon *et al*, 1987). Similarly, the decrease in response at litter removal can be prevented with treatment of growth hormone. Serum growth hormone concentrations are very

variable due to pulsatile secretion, however, serum IGF-I which provides an indirect assessment of serum growth hormone rose on litter removal (Vernon *et al*, 1993), suggesting that the changes in the lipolytic system were not due to fall in serum growth hormone; it is possible that the sensitivity or response of the cells to growth hormone is decreased. This study also showed that growth hormone altered several components of the adrenergic signal transduction system in these animals, increasing the number of β-receptors and hormone-sensitive lipase activity and decreasing cAMP phosphodiesterase activity. The key effect of growth hormone was, however, on the association of hormone-sensitive lipase with the lipid droplet following catecholamine stimulation.

While in ruminants at least, changes in serum growth hormone may contribute to changes in lipogenesis and lipolytic response to catecholamines during lactation, the increased response to adenosine in adipocytes during lactation must be due to some other factor.

1.3.3 Growth hormone and differentiation

During differentiation, preadipocytes progressively accumulate lipid and acquire sensitivity to agents which regulate lipid metabolism. GH promotes the differentiation of adipocyte precursor cells (preadipocytes) into mature adipoctyes Evidence for this comes primarily from studies using preadipocyte cell lines such as 3T3-F442A and Ob1771 which both exihibit GH-dependent differentiation (Zezulak and Green, 1986; Grimaldi *et al*, 1984). It appears that GH can increase the transcription of IGF-1 (Doglio *et al*, 1987) and primes cells to respond to the mitogenic effects of IGF-1 and other agents which then stimulate a limited clonal expansion of the young adipocytes prior to terminal differentiation (Zezulak and Green, 1986).

GH has also been shown to influence the expression of other proteins involved in adipocyte differentiation. In 3T3-F422A cells GH alters the expression

of various cytoskeletal proteins (Guller *et al*, 1992) which could explain the morphological changes seen in the initial stages of differentiation. GH also increases transcription of early response genes *c-fos* and *c-jun* in preadipocytes (Doglio *et al*, 1987; Gurland *et al*, 1990), the increase in expression of these transcription factors may regulate the expression of further genes involoved in the differentiation process.

While many of the recent advances in understanding growth hormone signallling systems have been derived using growth hormone-sensitive preadipocyte cell lines, where growth hormone responses are studied in isolation after the removal of other serum components, cellular responses to growth hormone *in vivo* occur against a background of effects elicited by other hormones and growth factors. Therefore more challenging work on signalling on primary cultures of growth hormone-sensitive cells and *in vivo* is also necessary to confirm cell line-derived data.

1.3.4 The Growth hormone receptor

Growth hormone exerts direct effects on adipose tissue by interacting with receptors which have been demonstrated to be present on adipocytes of rats (Fagin *et al*, 1980) and man (DiGirolamo *et al*, 1986). The growth hormone receptor has been cloned from several species including man (Leung *et al*, 1987), rat (Mathews *et al*, 1989), mouse (Smith *et al*, 1989), cow (Hauser *et al*, 1990) and sheep (Adams *et al*, 1990). It is characterised as a single peptide of between 630 and 650 amino acids (Waters *et al*, 1990) comprising an N-terminal signal sequence followed by an extracellular hormone-binding domain, a single hydrophobic membrane spanning region and a large intracellular domain. The number of receptors varies with depot, sex and age, (Vernon and Flint, 1989). The growth hormone receptor possesses several structural features which classifies it as a

member of the cytokine or haematopoeitin receptor superfamily. This group also includes receptors for prolactin, erythropoeitin and several interleukins (2,3,4,6,7) (Bazan, 1989).

Due to alternative splicing there are short and long forms of the growth hormone receptor: the short form is a binding protein that ciculates in the blood and interacts with growth hormone (Kelly *et al*, 1994). A recent study indicated that rat adipocytes express both the full length receptor and a short form which remains tethered to the external membrane face (Goodman *et al*, 1994). The pool of growth hormone receptors on the adipocyte cell surface is made up of about 20% of the short form compared to 80% of the long form. They have different turnover rates and appear to be independently regulated. Immunoneutralisation of the short form decreased the magnitude of the effect of growth hormone on glucose metabolism, suggesting that the short form may mediate some of the responses to growth hormone (Goodman *et al*, 1994).

Studies by de Vos *et al* (1992) and Cunningham *et al* (1991) demonstrated that one molecule of human growth hormone (hGH) binds two molecules of the solubilised extracellular domain of the receptor. Thus there appears to be two distinct receptor binding sites on hGH. The formation of the growth hormone/receptor complex occurs sequentially, the receptor binding first to the higher affinity site 1 on growth hormone then to site 2. Studies suggest that dimerization of receptor is a necessary prerequisite for the formation of an active signal-transducing complex (Fuh *et al*, 1992). Since receptor oligomerisation has been shown to be important in signalling by other growth factors, eg. insulin, EGF and PDGF (Ullrich and Schlessinger, 1990), it seems likely that it will play some key role in growth hormone signallling.

1.3.5 JAK2 and growth hormone signalling

Several distinct signalling pathways which link the cell surface growth hormone receptors to effects on the nucleus have been tentatively identified (Anderson, 1994; Carter-Su *et al*, 1996). Although the growth hormone receptor possesses no intrinsic tyrosyl kinase activity itself agonist binding results indicated the rapid tyrosyl phosphorylation of a number of intracellular proteins (Anderson *et al*, 1990; Wang *et al*, 1993). Tyrosyl phosphorylation is initiated by the ability of the receptor to sequester the tyrosyl kinase JAK2 (Argetsinger *et al*, 1993). This kinase was initially shown in 3T3-F442A cells to be able to associate with the occupied growth hormone receptor resulting in activation of JAK2 kinase and tyrosyl phosphorylation of the growth hormone receptor (Argetsinger *et al*, 1993). JAK2 is a member of the Janus tyrosine kinase family that also includes JAK1, TyK2 and JAK3. All these proteins share the unusual feature of having two kinase-like domains (see Ihle *et al*, 1994). Studies using mutated growth hormone receptor (GHR) have been carried out to determine which region of the GHR associates with JAK2 and to examine whether activation of JAK2 is the only requirement for biological responses to growth hormone (Vanderkuur *et al*, 1994). These studies showed that the proline-rich Box 1 motif of the cytoplasmic domain, is primarily required for association of JAK2 with GHR and GH-dependent activation of JAK2. However, the N-terminal quarter of the cytoplasmic domain of the GHR appears to augment the interaction with JAK2. Although the sites of tyrosyl phosphorylation of the GHR have not been unequivocally identified, mutational studies suggest (Vanderkuur *et al*, 1994) that one or more of the tyrosyl residues present in the N-terminal half of the cytoplasmic domain of GHR are phosphorylated by JAK2.

It is now apparent that members of the Janus tyrosyl kinase family serve as receptor associated kinases for the multiple members of the cytokine/haemopoietin receptor family. In addition to the growth hormone receptor, JAK2 has been

shown to associate with the erythropoietin receptor (Witthuhn *et al*, 1993), IL-3 receptor (Silvennoinen *et al*, 1993) and prolactin receptor (Lebrun *et al*, 1994).

1.3.6 Growth hormone and signal transduction via STATS

Signal transducers and activators of transcription (STATs) participate in growth hormone signalling between the receptor and the nucleus (Darnell *et al*, 1994). STAT proteins contain both SH2 and SH3 (Src homology) domains (Fu, 1992; Schindler *et al*, 1992a; 1992b; Shuai *et al*, 1992) a specific region within target effector molecules that have considerable sequence similarity to the non-catalytic region of the *src* family of protein tyrosine kinases (Pawson and Gish, 1992). A large number of signalling molecules have been shown to contain SH2 domains which bind directly to tyrosine-phosphorylated receptors (see Malarkey *et al*, 1995).

Growth hormone has been shown to induce tyrosyl phosphorylation and nuclear accummulation of several STAT or STAT-related proteins: STAT1/p91; STAT3/APRF (acute-phase response factor; and/or STAT5/MGF (mammary gland factor) (see Carter-Su *et al*, 1996). STAT1 is a subunit of the ISGF3 complex which comprises of a 48-kDa DNA-binding component, and 84/91-kDa protein and a 113-kDa protein, termed p48, p84, p91 and p113 respectively, and these protein subunits of ISGF3 are localised in the cytoplasm of unstimulated cells. Formation of the complex and its migration to the nucleus requires tyrosine phosphorylation of p91/84 and p113 and it is hypothesised that this tyrosine phosphorylation is mediated by a JAK (Meyer *et al*, 1994). Following phosphorylation, STAT1 and STAT3 migrate to the nucleus and interact with the sis-inducible element (SIE). The *c-fos* promoter possesses a SIE, and these STATs probably contribute to GH-induced *c-fos* expression although growth hormone probably also regulates the SRE (serum response element) in the c-fos promoter via the MAP kinase pathway

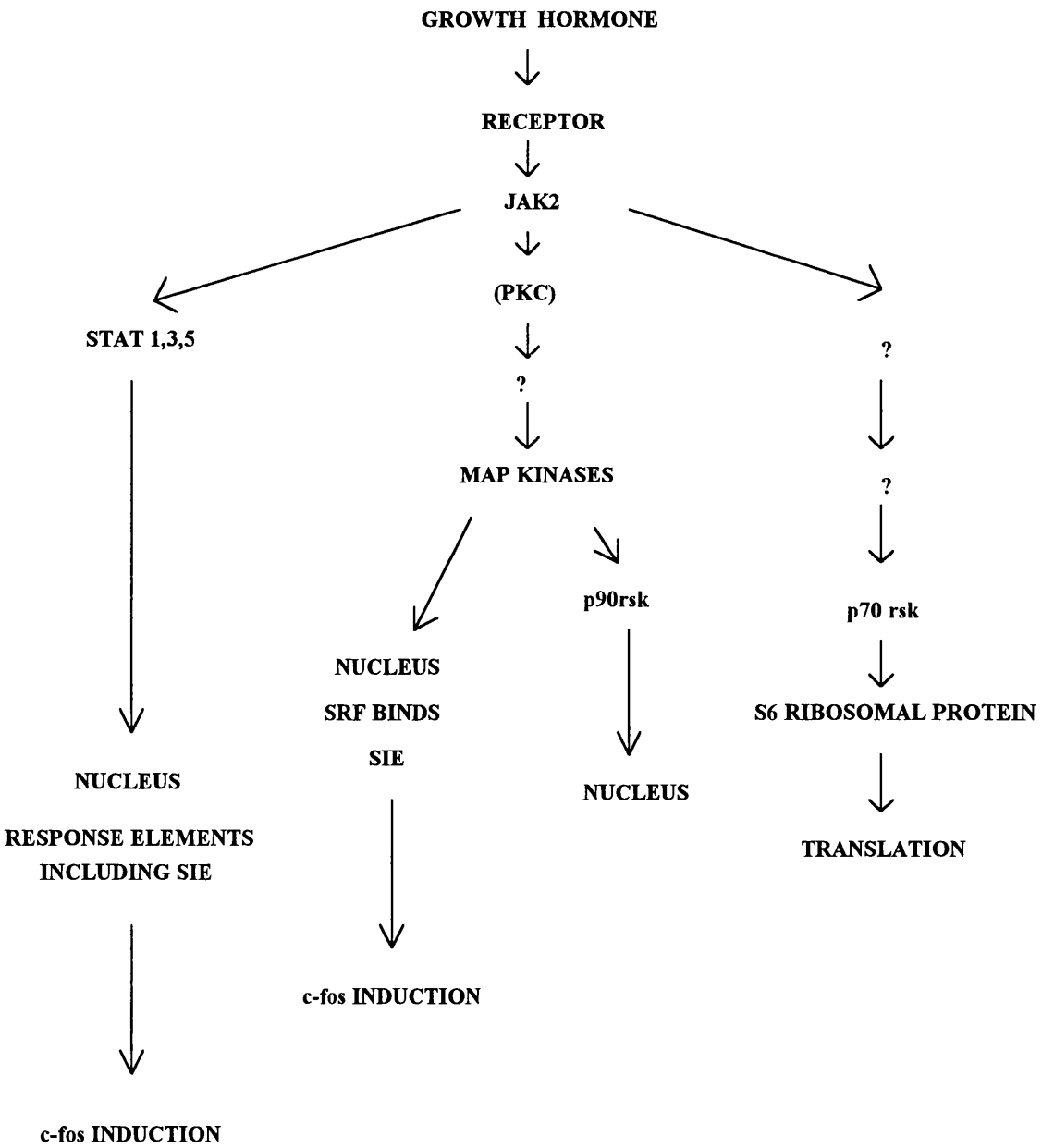
(see below). STAT5 binds to the growth hormone response element in the serum protease inhibitor gene (see Carter-Su *et al*, 1996).

1.3.7 Growth hormone signal transduction via MAP kinases

The second receptor-nucleus pathway involves the mitogen-activated protein (MAP) kinases. MAP kinase was first identified as a serine/threonine-directed kinase that utilised microtubule-associated protein 2 as a substrate following stimulation of adipocytes with insulin (Ray and Sturgill, 1987). MAP kinases are expressed in all cells and can be activated by hormones, growth factors, neurotransmitters and other cell stimuli (Anderson, 1992b). The activation of MAP kinase requires phosphorylation upon both tyrosine and threonine residues (Anderson *et al*, 1990; Gomez and Cohen, 1991) by dual specificity MAP kinase kinases or MEKs (Nakielny *et al*, 1992; Crews and Erikson, 1992).

Activation of MAP kinases can result in their translocation to the nucleus where they phosphorylate several transcription factors including the p62 ternary complex factor which participates in formation of the serum response element binding complex (Treisman, 1995). Growth hormone has been shown to activate two MAP kinases, namely the p42 and p44 isoforms (Anderson, 1992b; Winston and Bertics, 1992; Campbell *et al*, 1992) in 3T3-F442A preadipocytes. Administation of growth hormone to hypophysectomised rats induces the translocation of p42 and p44, and other tyrosine phosphorylated proteins, to the nucleus in hepatocytes (Gronowski and Rotwein, 1994). Growth hormone has also been shown to phosphorylate and activate the S6 kinase $p90^{rsk}$ which can also translocate to the nucleus (Chen *et al*, 1992) and has been shown to phosphorylate and activate several transcription factors (Blenis, 1993). This S6 kinase lies downstream of the p42 and p44 MAP kinases, and some studies suggest a role for protein kinase C (PKC) in the growth hormone-dependent activation of MAP kinases and p90rsk (Anderson, 1992a). Growth hormone also phosphorylates and

Figure 1.3 Summary of Growth hormone signalling pathways



activates the S6 ribosomal kinase $p70^{S6K}$ in 3T3-F442A cells (Anderson, 1993) which is involved in the regulation of translation via phosphorylation of the S6 ribosomal proteins; activation of $p70^{S6K}$ is via a MAP kinase independent pathway.

Therefore, the GH signalling pathway, a summary of which is outlined in Figure 1.3, apparently includes the involvement of activation of JAK2, a non receptor tyrosine kinase. The subsequent phosphorylated GHR and JAK2 serve as docking sites for SH2 domain-signalling molecules (Carter-Su *et al*, 1996) and includes the activation of MAP kinase, which are known to phosphorylate transcription factors, and STATs which, when combined with an active sis-inducible factor, are able to activate transcription by direct interaction with response elements on target DNA (Larner *et al*,1993, Sadowski *et al* 1993 and Shuai *et al*, 1993).

1.4 Guanine nucleotide binding Proteins

1.4.1 Identification of the stimulatory G-protein G_s

The identification of G_s was achieved by reconstitution with *cyc-* S49 cells (Bourne *et al,* 1975) which are deficient in this G-protein. This provided the first assay and purification of G_s, yielding a heterotrimer of α,β,γ subunits. Subsequent to this the isolation of certain exotoxins from cultures of both *vibrio cholerae* and *Bordetella pertussis* aided the identification of the stimulatory and inhibitory G-proteins, termed G_s and G_i respectively. Gill and Meren demonstrated that the cholera toxin activation of adenylyl cyclase in pigeon erythrocytes correlated with the radiolabelling of a 42kDa polypeptide, in the presence of [^{32}P]-NAD+ (Gill and Merren, 1978). The presumed reaction of cholera toxin was an ADP-ribosylation similar to that already detailed for the action of diphtheria toxin on a protein component required for protein synthesis (Collier, 1975). ADP-ribosylation by

cholera toxin, of a key arginine residue (R201/202 or R187/188, depending upon which of the four splice variants is referred to) in its α-subunit leads to the loss of GTPase activity and concomitant activation of adenylate cylase (Gilman, 1987; Birmbauer *et al*, 1990; Kaziro *et al*, 1991; Bray *et al*, 1986). Mutation of this arginine residue in $G_s\alpha$ (Freissmuth & Gilman, 1989), as seen with the *gsp* oncogene (Landis *et al*, 1989), also leads to the constitutive activation of adenylate cyclase. Cholera toxin requires the presence of a protein co-factor termed ADP-ribosylation factor (ARF) which is itself able to bind GTP (Kahn and Gilman, 1984) in order to catalyse the ADP-ribosylation of G_s. The effect of cholera toxin was similar to that obtained by non-hydrolysable analogues of GTP, thus allowing the identification of $G_s\alpha$ both a substrate for cholera toxin catalysed ADP-ribosylation and a protein capable of binding and hydrolysing guanine nucleotides.

1.4.2 Identification of the inhibitory G-protein G_i

Pertussis toxin, which is also termed islet activating protein (IAP), was shown to produce alterations in receptor mediated control of cyclic AMP production (Katada and Ui, 1981). Initial experiments in rat C6 glioma cells treated with pertussis toxin showed and increase in GTP activation of adenylyl cyclase, concomitant with the transfer of [^{32}P]-ADP-ribose from [^{32}P]NAD+ to a 41kDa membrane associated polypeptide (Katada and Ui, 1982). The release of tonic inhibition of adenylyl cyclase activity paralled by the modification of the 41kDa protein thus identified this protein as the previously unidentified inhibitory G-protein of the adenylyl cyclase cascade (G_i).

Pertussis toxin functions in a similar manner to cholera toxin in that it is an ADP-ribosyl transferase however, in this instance it catalyses the transfer of ADP-ribose from NAD+ onto a C-terminal cysteine on the G-protein α-subunit. The functional effects of pertussis toxin catalysed ADP-ribosylation of $G_i\alpha$ is to prevent productive coupling between the receptor and the G-protein, thus receptor

mediated inhibition of adenylyl cyclase is attenuated after pretreatment with pertussis toxin (Katada & Ui, 1979; Burns *et al*, 1983). This led to the over-simplistic hypothesis that any event which was attenuated by prior treatment with pertussis toxin was taken as indicative of a role for G_i in mediating the response.

1.4.3 Stimulatory G-proteins

At least four forms of $G_s\alpha$ exist, which are obtained from alternative splicing of a transcript from a single gene (Bray *et al*, 1986; Kozasa *et at*, 1988). These have been grouped into two subtypes which are termed long (46-kDa) and short (44-kDa) $G_s\alpha$. The cDNAs that encode two of the forms of $G_s\alpha$ have been isolated from a bovine adrenal cDNA library and are identical except for a sequence of 46 nucleotides in which the shorter form of $G_s\alpha$ contains alterations in 4 nucleotides and a deletion of 42 others (Robishaw *et al*, 1986). Whereas at least two forms of G_s are expressed in most tissues, their relative amounts usually vary (Mumby *et al*, 1986). Although G_s was originally identified as the G-protein required for receptor mediated stimulation of adenylyl cyclase, the protein has also been shown to couple to the activation of the dihydropyridine-sensitive Ca^{2+} channels (Yatani *et al*, 1987), the inhibition of the calcium pump in liver membranes (Jouneauz *et al*, 1993) and the inhibition of Na+ channels in cardiac myocytes (Schubert *et al*, 1989). $G_s\alpha$ has also been shown to mediate epidermal growth factor-elicited stimulation of rat cardiac adenylyl cyclase (Nair *et al*, 1990). A protein with 88% amino acid homology to $G_s\alpha$ has been cloned from olfactory epithelia and termed G_{olf}. Its sole location is in the olfactory neuroepithelia where it is responsible for olfactant regulation of adenylyl cyclase (Jones and Reed, 1987; Bruch, 1990).

1.4.4 Inhibitory G-proteins

$G_i\alpha$. Three genes encode the family of G-proteins, known as G_i1, G_i2 and G_i3 (Kaziro,1990; Jones and Reed, 1987). In addition to the three forms of $G_i\alpha$ mentioned, other G-protein α-subunits have been termed G_i-like due to their degree of amino acid homology with these proteins. However, it is not certain as to whether all or just certain of these species can inhibit adenylate cyclase under physiological conditions (Raymond *et al*, 1994; Houslay, 1991a). Recently cDNAs encoding persistently activated $G_i\alpha$ were expressed in several cell systems, where inhibition of some subtypes of adenylyl cyclase by all three $G_i\alpha$ subunits was shown to occur (Wong *et al*, 1991; Lowdnes *et al*, 1991; Hermout *et al* 1991; Wong *et al*, 1992; Taussig et al, 1993), indicating a possibe direct role for all three G_i forms in the inhibition of adenylyl cyclase. These conclusions were based upon inferences made by studying cAMP metabolism in intact transfected cells rather than any direct measure of adenylate cyclase activity. However, the overexpression of mutant G-proteins might attenuate adenylate cyclase functioning in a manner which does not necessarily reflect that seen *in vivo*. For example such a strategy showed that mutant $G_z\alpha$ inhibited forskolin-stimulated cAMP activity (Wong *et al*, 1992). This result was surprising as G-protein controlled inhibitory regulation of adenylate cyclase in native systems had uniformly been shown to be blocked by pertussis toxin, whereas G_z is insensitive to the action of this toxin (Gilman, 1987; Birnbauer *et al*, 1990; Kaziro *et al* ,1991; Bray *et al*, 1986; Freissmuth and Gilman, 1989). However, studies using a variety of approaches suggest that at least $G_i2\alpha$ can mediate such an inhibitory action in cells which have not been transfected with Giα-subunits (Bushfield *et al*,1990; Mckenzie and Milligan, 1990; Simonds *et al*, 1989; Watkins *et al*, 1992; Goetzel *et al*, 1994; Carter and Medrihadsky, 1993; Raymond *et al*, 1993; Milligan *et al*, 1991).

Activation of protein kinase C causes the phosphorylation of the α-subunit of G_i2 (Pyne *et al*, 1989; Rothenberg *et al*, 1988). Phosphorylation of $G_i2\alpha$ in

hepatocytes (Houslay, 1991b; Morris *et al*, 1994) and other cells (Katada *et al*, 1985; Watanabe *et al*, 1988; Daniel-Issakani *et al*, 1989; Strassheim and Malbon, 1994; Yatomi *et al*, 1992) appears to cause loss of GTP-dependent inhibition of adenylate cyclase. This effect, however, may be a cell-specific phenomenon, as it is not observed in all cell tyes. It has been suggested that hepatocyte G_i2 is controlled by a futile cycle of phosphorylation and dephosphorylation involving protein kinase C and protein phosphatase activity (Houslay, 1991b; Bushfield *et al*, 1991). Thus either stimulation of protein kinase C or inhibition of protein phosphatase activity may inhibit the functioning of G_i2. Phosphorylation of $G_i2\alpha$ also occurs at a second site, suggested to be ser^{207} (A-site), which results upon elevation of cAMP levels (Morris *et al*, 1994).

$G_t\alpha$. At least two forms of transducin occur, transducin 1 (G_t1) and transducin 2 (G_t2), which are encoded by seperate genes, and are found in rod and cone cells respectively (Lerea *et al*, 1986) and have an approximate 80% homology with the Gi-like proteins. They serve to couple rod and cone opsins to cGMP phospodiesterases. A novel transducin-like G-protein (G_g) has recently been identified from taste tissue (McLaughlin *et al*, 1992). Due to sequence homology in those regions of the G-protein α-subunit known to interact with receptors and effectors, it is known that a cGMP phosphodiesterase is involved in its means of signal transduction.

G_o. Studies using purified preparations of pertussis toxin substrates from bovine brain (Sternweis and Robishaw, 1984; Milligan and Klee, 1985; Neer *et al*, 1984) in conjunction with the purification of rat brian substrates (Katada *et al*, 1986), led to the identification of the 39 kDa G-protein α-subunit termed G_o for other (Nukada *et al*, 1986; Itoh *et al*, 1986). cDNA clones of $G_o\alpha$ have been isolated from bovine retina (Van Meurs *et al*, 1987), rat C6BU1 glioma cells (Itoh *et al*, 1986) and rat olfactory epithelium (Jones and Reed, 1987). Additional $G_o\alpha$

isoforms have also been identified (Goldsmith *et al*, 1988; Kobayashi *et al*, 1989) immunologically (Goldsmith *et al*, 1988) and biochemically (Inanobe *et al*, 1990; Hsu *et al*, 1990). While the function of G_o remains unresolved, early evidence exists to suggests that this G-protein may be involved in the regulation of receptor-mediated inhibition of voltage operated Ca^{2+} channels (Hescheler *et al*, 1987; Harris-Warrick *et al*, 1988; Ewald *et al*, 1988). More recent studies demonstrate a role for G_o in coupling to somatostatin receptors (Law *et al*, 1991, 1993) and the $\alpha 1\beta$-adrenergic receptor (Blizter *et al*, 1993) which serve to inhibit adenylyl cyclase and stimulate phospholipase C activity respectively.

$G_z\alpha$ Gxα cDNA was isolated from rat brain and was identical to one isolated from human retina (Fong *et al*, 1988). This protein is pertussis toxin insensitive and exhibits a remarkably slow rate of guanine nucleotide exchange and a very slow intrinsic GTPase activity when compared to $G_i\alpha$. The biochemical properties of G_za appear, therefore, to differ from thoses of $G_i\alpha$, although as yet the physiological significance of this remains undetermined.

1.4.5 G-protein linked receptors

Receptors span the cell membrane and have at their externally facing side a site specific for a particular hormone, growth factor, neurotransmitter, antigen or other ligand. Binding to the receptor generates a signal within the cell itself. Cell surface receptors can be divided into two classes, one of which possesses the machinery for the generation of an intracellular signal, while the second is reliant upon interaction with other plasma membrane proteins to elicit a signal.

For the second group of receptors mentioned, G-proteins function as intermediaries in transmembrane signalling (Houslay, 1992). The receptors that participate in such reactions are many and one of best charactized is the β-adrenergic receptor which has for example the agonist adrenaline and the more

selective agent isoproterenol (Gilman, 1987). Isolation, purification, molecular cloning and sequencing of the β-adrenoreceptor, (Dixon *et al.*, 1986) allowed models to be formulated regarding the disposition of the receptor in the plasma membrane. Subsequent cloning of other G-protein linked receptors identified a similar seven-membered hydrophobic transmembrane helices and because of this, such receptors were given the mnemonic 'R7G receptor' (Strosberg, 1991).

R7G receptors are formed from a single peptide chain of about 400 to 600 amino acids in length (Fraser, 1991; Raymond *et al*, 1990; Strosberg, 1991). Characteristic features are seven stretches of 22-28, essentially hydrophobic, amino acids that are thought to form distinct transmembrane domains as deduced from the electron microscopy imaging studies of bacteriorhodopsin (Henderson and Unwin, 1975). Bacteriorhodopsin, like the visual photoprotein rhodopsin, exerts its physiological effect by binding to the G-protein transducin causing it to bind GTP and dissociate (Hingorani and Ho, 1990). Once bound the GTP-Tα causes the activation of its specific effector/signal generator, a cyclic GMP phosphodiesterase. Therefore the R7G receptors are thought to consist of seven interacting transmembrane cylinders with loops of hydrophilic residues extending out into the extracellular space at the N-terminus and between cylinders 2/3, 4/5 and 6/7. The C-terminus of the protein and the cytosolic loops formed between the cylinders 1/2, 3/4 and 5/6 are at the cytosolic space. Greatest homology is seen within the seven individual transmembrane domains and this has been useful in cloning other members of this family.

1.4.6 The GTPase cycle

Using the available data on the efects of cholera toxin and non-hydrolysable GTP analogues on G_S, together with the ability to measure hormonal stimulation of

high affinity GTPase activity, Cassel and Selinger (1977) were able to propose a cyclical model to account for G-protein function which is still applicable.

Binding of a ligand to its receptor causes the α-subunit to lose its GDP and bind GTP in a Mg^{2+} dependent manner. Agonists act to reduce the concentration of Mg^{2+} required for activation, for example glucagon lowers the magnesium concentration required for GTPγS activation of Gsα from 25mM to 10uM. As the intracellular Mg^{2+} is estimated to be of the order of 2mM it can then be utilised (Iyengar and Birnbaumer, 1982). The binding of GTP reduces the affinity of the receptor for agonist, resulting in a dissociation of the receptor-G-protein complex and a magnesium dependent dissociation of the α-subunit from the βγ (Maguire *et al*, 1976). The Gα-GTP is now in an 'active' conformation suitable for specific interaction with an effector protein. On completion of effector activation, GTP is hydrolysed to GDP by the intrinsic GTPase of the α-subunit (Rodbell, 1980). The now inactive Gα-GDP can reassociate with its βγ subunits. This system is cyclical and dependent on ligand binding, GTP and Mg^{2+} (Ransas *et al*, 1992).

1.4.7 Covalent modification of G-Proteins

G-proteins can be covalently modified in several ways. These modifications are believed to be potentially important in regulating the function and cellular targetting of the G-protein.

1.4.7.1 Lipid modification

The association of G-proteins with cellular membranes is crucial in ensuring efficient signal transduction for essentially all these molecules (Helper & Gilman, 1992; Bourne *et al* 1991). Three general types of lipids can be found covalently linked to G-protein. The first is a saturated 16-carbon fatty acyl group, palmitoyl which was detected on products of the RAS proto-oncogenes (Chen *et al*, 1985).

Palmitoylation of proteins is a post-translational event, generally occurring through labile thioester bonds to cysteine residues (Schmidt, 1989). The lability of this bond is thought to be a critical facet of palmitoylation; it is the only one of the three major types of lipid modifications that is reversible. The second is a 14-carbon myristoyl group (Gordon *et al* 1991). Analysis of $G_i\alpha$ and $G_o\alpha$ subunit polypeptides by both chemical and metabolic labellling techniques initially revealed the presence of myristic acid on the proteins (Buss *et al*, 1987). Subsequent studies confirmed that the Giα1, $G_i\alpha$2, $G_i\alpha$3, $G_o\alpha$ and $G_z\alpha$ subtypes were all myristolated at conserved amino terminal residues, (Mumby *et al*, 1990; Jones *et al*, 1990). Indeed, mutagenesis of this glycine residue produced proteins which did not incorporate [^{3}H]myristate and did not become associated with cellular membranes. The third lipid type involves the 15- or 20-carbon unsaturated isoprenoid lipids to cysteine residues at or near the carboxyl terminus (Clarke, 1992). Ras proteins, which are monomeric `mini' G-proteins are prenylated and the modification is required for oncogenic forms of these proteins to transform cells (Hancock *et al*, 1989; Casey *et al*, 1989). The γ-subunits of all heterotrimeric G-proteins are also prenylated, this is believed to promote a means of anchoring the $\beta\gamma$ complexes to the plasma membranes (Clarke, 1992; Casey *et al*, 1989). These lipid modifications influence interactions between the proteins or between each protein and the plasma membrane.

1.4.7.2 ADP ribosylation

Pertussis toxin and cholera toxin catalysed mono ADP-ribosylation of G-protein α–subunits has been well characterised (sections 1.4.1, 1.4.2). However it is still uncertain if endogenous ADP-ribosylation occurs under normal cellular physiological conditions and thus promotes cellular control. Both arginine (Moss and Vaughn, 1988; Inageda and Tanuma, 1991) and cysteine specific (Tanuma *et al*, 1987; Tanuma *et al* 1988) mono-(ADP-ribosyl) transferases have been idenified

in several cell types. Enzymes that catalyse the removal of ADP-ribose from ADP-ribosylated arginine residues have also been identified (Moss *et al*, 1992), suggesting that ADP-ribosylation may be a reversible physiological event (Moss *et al*, 1985).

1.4.7.3 Phosphorylation

Phosphorylation of G-proteins is the least well defined of the covalent modifications. The G-protein α-subunits contain consensus sites for phosphorylation by a number of protein kinases and thus may be subject to regulation by hormones that do not interfere directly with a certain G-protein-linked pathway (Spiegel *et al*, 1992). It was shown by Jakobs and co-workers that a purified preparation of brain G_i, consisting of multiple forms, could be phosphorylated by PKC, suggesting that this may provide a basis for an alteration in G_i functioning (Katada *et al*, 1985). This suggests that phosphorylation might stabilise the inactive GDP-bound form of G_i and would be consistent with the hypothesis that phorbol esters serve to attenuate the rate of activation of G_i (Bell and Brunton, 1986). This is also consistent with the observation that the ability of pertussis toxin to mediate ADP-ribosylation of $G_i\alpha$, which can only occur on the intact GDP-bound holomeric form of this G-protein, was considerably greater in cells treated with phorbol ester for 15 minutes (Choi and Toscano, 1988). Furthermore in several cell systems (Daniel-Issakani and Spiegel, 1989; Gordelatze *et al*, 1989), and hepatocytes (Houslay, 1991) it has been demonstrated that PKC is able to phosphorylate $G_i2\alpha$, which leads to increased cAMP synthesis. The phosphorylation of the α-subunit of this G-protein is thought to occur on ser^{144} (C-site) (Morris *et al*, 1994). Studies with recombinant G-proteins have shown that the α-subunit of Gz is stoichiometrically phosphorylated by partially purified PKC on a serine residue near the N-terminal, whereas recombinant $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ were not labelled under the same conditions (Lounsbury *et al*, 1991).

Studies have also shown that G-proteins of the G_i family are phosphorylated by insulin and EGF-elicited tyrosine kinases (Cerione, 1991) when the G-protein is in its holomeric, GDP-bound state.

Evidence that the α-subunits of G_s can be phosphorylated on serine residues by protein kinase C (Pyne *et al*, 1992) and protein kinase A (Pyne *et al*, 1992b) has also been reported.

Although is has been demonstrated that the α-subunit of certain G-proteins can be phosphorylated *in vitro*, a link with physiological events has yet to be conclusively demonstrated and the biological implications of such G-protein phoshphorylation remain unclear. However phosphorylation of G-proteins may provide the basis for `cross-talk' between distinct signalling systems.

1.4.8 G-protein interaction with Adenylyl cyclase

Adenylyl cyclase catalyses the conversion of intracellular ATP to cAMP, increasing the intracellular levels of cAMP. It was only recently that detailed molecular information about the adenylyl cyclase enzyme has been acquired, following the cloning of several isoforms of the enzyme. Most adenylyl cyclases are membrane-associated although certain bacterial enzymes (and perhaps one form in mammalian sperm [Rojas *et al*, 1993]) are cytosolic.

The cloning of the first adenylyl cyclase revealed a large complex structure (Krupinski *et al*, 1989). This and all subsequently cloned adenylyl cyclases comprise 1,080 - 1,248 amino acid polypeptides. These membrane associated adenylyl cyclases have short cytoplasmic N- and C-terminal regions and two 40kDa cytoplasmic domains (C1 and C2) punctuated by two intensley hydrophobic stretches (M1 and M2), each of which are hypothesised to contain six transmembrane helices (figure 1.4). The two cytosolic regions include putative ATP-binding domains, which are homologous to each other (Krupinski *et al*, 1989)

Figure 1.4 Structure of adenylate cyclase isoforms

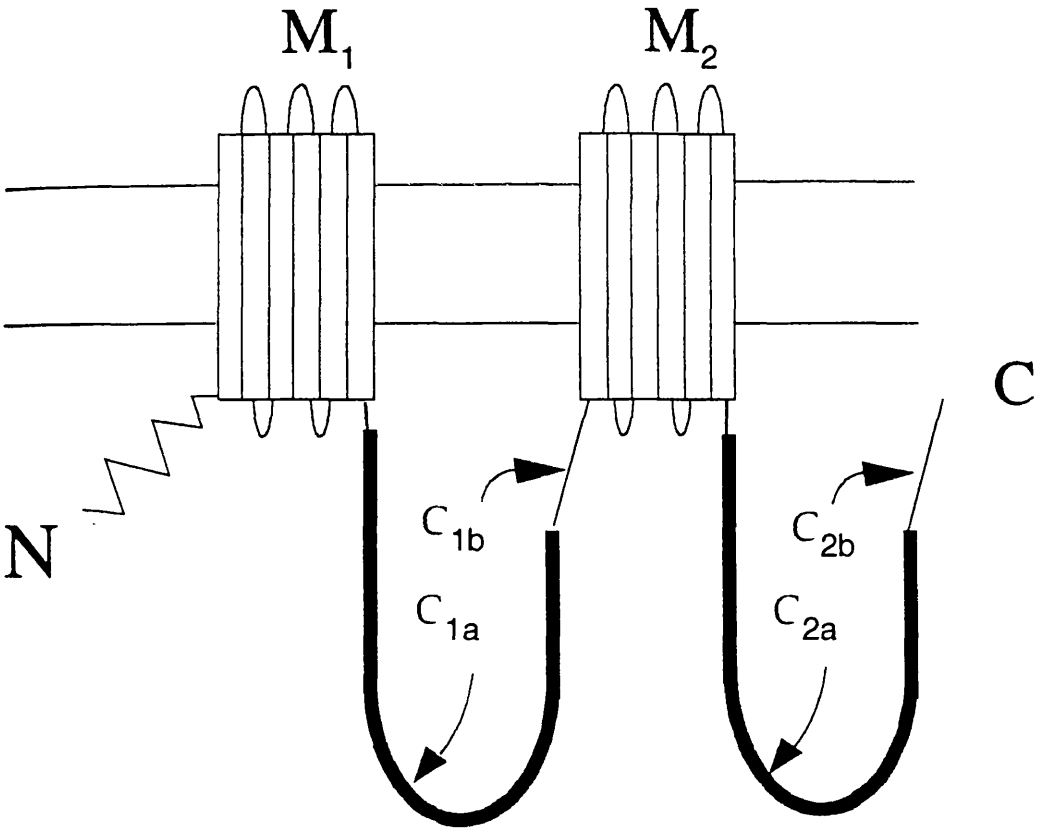


Figure 1.4 represents the structure of the eukaryotic adenylate cyclases (adapted from Tang and Gilman, 1992 [Cell 70, 869-872]).

and between different adenylyl cyclases (50-92%) (Tang and Gilman, 1992; Iyengar, 1993). Accumulating data indicate that non-overlapping regions exist on these enzymes, which allow separate interaction with $G_s\alpha$, $G_i\alpha$ and $\beta\gamma$ subunits of G-proteins, although the precise domains have not yet been defined, (Taussig *et al*, 1993; Taussig *et al*, 1994). cDNAs encoding numerous adenylyl cyclases of this type have now been cloned, 6 of which are from mammalian sources:

Type I from brain (Krupinski *et al*, 1989)

Type II from brain and lung (Feinstein *et al*, 1991)

Type III from olfactory specific neurons (Bakalyar and Reed, 1990)

Type IV from peripheral tissue and Brain (Gao and Gilman, 1991)

Type V from dog heart (Ishikawa *et al*, 1992) and rat liver and kidney (Premont *et al*, 1992)

Type VI from dog heart (Katsushika *et al*, 1992), rat heart, liver and kidney (Premont *et al*, 1992), Mouse S49 lymphoma cell (Premont *et al*, 1992b) and mouse hamster hybrid NCB-20 cells (Yoshimura and Cooper, 1992).

In addition, a cDNA from mouse S49 lymphoma cells that encodes a portion of an adenylyl cyclase related to the type II enzyme has been idendified and named the type VII enzyme (Kruprinski *et al*, 1992). Also a cDNA has been isolated from a human brain lilbrary that appears to partially encode a distinct species of adenylyl cyclase (Parma *et al*, 1991) and corresponding rat cDNA sequences have also been obtained for this form (Kruprinski *et al*, 1992) and have been termed type VIII.

All the mammalian adenylyl cyclase enzymes are activated by $G_s\alpha$. For a long time it was thought that inhibition of adenylyl cyclase could not be obtained with activated a-subunits of inhibitory G-proteins and a mechanism of hormonal inhibition was proposed, whereby $\beta\gamma$ dimers generated by the inhibitory receptor quenched the activity of $G_s\alpha$. Inhibition was thus proposed to come about by removal of the stimulatory signal. However, the finding that cyc-S49 lymphoma cells which lacked a functional $G_s\alpha$ and still exhibited normal inhibitory regulation of adenylyl cyclase argued against this as the sole mechanism of inhibition (Katada

et al, 1984). It was later shown that antibodies specific to $G_i2\alpha$ suppressed receptor mediated inhibition of adenylyl cyclase in NG108-15 cells (McKenzie *et al*, 1988).

The emerging picture of adenylyl cyclase regulation is extremely complex. In peripheral tissues such as fat, liver and heart, adenylyl cyclase subtypes are insensitive to $\beta\gamma$ subunits and α subunits probably mediate hormonal effects, whereas in the central nervous system as well as in some endocrine, exocrine and epithelial cells signalling by α and $\beta\gamma$ subunits may occur (Birnbauer, 1992).

1.5 Aims

Growth hormone has long been regarded as a lipolytic hormone however, the mechanism whereby it facilitates lipolysis is not fully explained. Treatment of cattle with growth hormone makes adipose tissue more susceptible to the lipolytic effects of catecholamines *in vivo* (Sechen *et al*, 1990) and in some circumstances, for example litter removal from lactating rats, growth hormone treatment renders adipocytes more responsive to catecholamines *in vitro* due to changes in the amount or activity of components of the β-adrenergic signal transduction system (Vernon *et al*, 1993). As well as acutely acting lipolytic agents, lipolysis is subject to acute control by antilipolytic agents, for example adenosine and prostaglandins.

Preliminary studies suggested that this antilipolytic system is chronically regulated by growth hormone, hence the objective of this study was to characterise the effects of growth hormone on this antilipolytic system, with emphasis on determining at a molecular level the mechanism whereby growth hormone exerts its effects.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

Bovine growth hormone was a generous gift given by Monsanto Europe, B-1150, Brussels, Belgium, and was used in some studies, while ovine growth hormone NIADDK-oGH-13 (National Institute of Arthritis, Diabetes, Digestive and Kidney diseases, Bethesda, MD, USA) was used in some studies. All radio chemicals were purchased from Amersham International, plc (Buckinghamshire, UK) except [^{32}P]NAD which was purchased from ICN Biochemicals Ltd. (Thame, Oxon, UK). All other reagents were obtained from Sigma Chemical Company (Poole, Dorset, UK), Gibco BRL (Renfrewshire, Scotland) or BDH (Poole, Dorset, UK) unless otherwise stated.

2.2 Animals

2.2.1 Sheep

Sheep were all Finn x Dorset Horn cross-breds. The animals were given hay and water *ad libitium* plus 400 g/day of a cereal mix for at least 4 weeks before slaughter. Both wether lambs (6-9 months old) and 3-4 year old ewes were used . Sheep were anaesthetised with an intrajugular injection of 20-30 ml of Sagatal (May & Baker, Dagenham, Essex, UK), given by Dr R.G. Vernon. The animals were exsanguinated when unconcious and samples of adipose tissue were removed asceptically and placed in isotonic saline at about 37^{o}C and taken to the laboratory as quickly as possible (less than 5 minutes).

2.2.2 Rats

Female Wistar rats from A.Tuck and Son (Rayleigh, Essex, U.K.) were given Labsure irradiated CRM diet (Labsure, Poole, Dorset, U.K.) and water *ad libitum*. Virgin rats used in studies described in chapter 3 weighed about 170 g; lactating rats used in studies described in chapter 4 weighed about 250 g while control, virgin rats used in chapter 4 were the same age as the lactating rats and weighed about 200 g. To minimise stress, animals were accustomed to handling before the commencement of experimentation. Injections were administered twice daily, at 09.00 and 17.00 h, for 2 days. On day 3 Rats were anaesthetised by an intraperitoneal injection of Sagatal (see Vernon *et al*, 1993) and adipose tissue taken and transported to the laboratory in isotonic saline at 37^{o}C. Following fat removal the rats were killed by exanguination.

2.3 Methods

2.3.1 Preparation of sheep adipocyte membranes

Subcutaneous or omental adipose tissue was removed and transported to the laboratory in 0.9% (w/v) saline at 37^{o}C as described in section 2.2.1. Adipocytes were prepared by collagenase digestion using the method of Rodbell (1964). Adipose tissue (approximately 20 g) was minced in a petri-dish containing medium 199 (containing Earle's salts, L-glutamine, 25 mM-Hepes [4-{2-hydroxyethyl}-1-piperazine-ethansulphonic acid] (pH7.3) supplemented with 2mM acetate (final concentration 2.6 mM) and antibiotics (Penicillin G 60 μg/ml; Streptomycin sulphate 100 μg/ml; neomycin sulphate 10 μg/ml); (this is referred to as Medium 199), and then transferred to flasks containing 15 ml of Krebs Ringer

bicarbonate buffer containing 1.2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 1.5 mM KH_2PO_4 and 25 mM $NaHCO_3$, supplemented with 25 mM Hepes (pH 7.4), 2 mM Na acetate, 0.3 mg/ml D-glucose, 1 mg/ml collagenase (type II, cat. no. 6885, Sigma) and 30 mg/ml BSA (dialysed and fatty acid free). The tissue was then digested for 45 minutes - 1 hour at 37°C in a shaking water bath. The digest was then passed through a plastic strainer, and the floating adipoctes washed three times with 30 mls of Krebs- Ringer bicarbonate containing 900 mg BSA.

Upon removal of Krebs buffer, adipocytes were lysed by addition of 15 ml of extraction buffer [10 mM Tris-HCL (pH 7.4), 20 mM EDTA, 0.2 M sucrose containing 1 μg/ml leupeptin, 0.3 mg/ml benzamidine-HCL and 100 μM phenyl-methylsulphonylfluoride (PMSF)] at 37°C. The suspension was vortexed for 2 minutes, transferred to warmed 50 ml plastic (polypropylene) centrifuge tubes and centrifuged at 2800 rpm for 5 minutes at 22°C to minimize trapping of membranes within the lipid layer. The tubes were then put on ice until the fat solidified, and the infranatant removed and diluted to 50 ml with ice-cold 10 mM Tris/HCl pH 7.4, containing 90 mM NaCl and centrifuged at 32,000 rpm at 4°C for 1 hour in a Sorvall RC5B (SS34 rotor). The resulting membrane pellet was resuspended in 50 mM Tris/HCl pH 7.4, containing 10 mM $MgCl_2$, 1 μg/ml leupeptin and 100 μM PMSF and then rapidly frozen to -80°C and stored in liquid nitrogen.

2.3.2 Preparation of rat adipocyte membranes

Rat adipose tissue was removed and transported to the laboratory in 0.9% saline at 37°C. Adipose tissue was minced in 30 mm petri-dishes and transferred to flasks containing Medium 199 supplemented with antibiotics (as described in section 2.3.1). After a 1 hour incubation at 37°C the resulting adipocytes were passed through a plastic strainer and washed once in medium 199 at 37°C, after

which they were washed a further three times in TES buffer (Tris 20 mM, EDTA 1 mM, sucrose 0.255 mM) at room temperature. Membranes were then prepared (Strassheim *et al*, 1990). The adipocytes were transferred to a 5 ml homogeniser where the cells were ruptured, placed in centrifuge tubes and spun at 2,000 rpm (1500 g) for ten minutes to separate the fat, the infranatant was then transferred to fresh tubes and spun for a further 20 minutes at 19,000 rpm (15,000 g). Once the supernatant had been discarded the pellet was resuspended in 3 ml of ice cold TES buffer and the tubes centrifuged at 19,000 rpm (15,000 g) for 20 minutes, and the resulting pellet was then resuspended in 1 mM Tris/ 1 mM EDTA pH 7.4 and frozen rapidly to -80^{o}C and stored in liquid nitrogen.

2.3.3 Tissue Culture

Pieces of sheep adipose tissue (approximately 20 mg) were cut with sterilised scissors and then used immediately for the isolation of adipocytes or maintained in tissue culture. Tissue pieces of about 20 mg (total weight about 100 mg) were maintained in culture at 37^{o}C under air/CO_2 (19:1, v/v) in 2 ml of Medium 199 containing Earle's salts, L-glutamine and 25mM Hepes (pH 7.3) and supplemented with 2 mM acetate (pH 7.3) and antibiotics (Penicillin G, 60 μg/ml; streptomycin sulphate, 100 μg/ml; neomycin sulphate 10 μg/ml) for 24 hours (Wastie *et al* 1995). The tissue was then transferred to fresh medium 199 for a further 24 hours containing hormones and other additions as described in the results chapters. Following this second period of culture the adipose tissue pieces were used for membrane preparations or in lipolysis studies.

2.3.4 Sheep Lipolysis Assay

Adipose tissue was prepared (as described above) and the rate of lipolysis was measured as glycerol release during a 3 hour incubation period at 37°C in Krebs -Ringer bicarbonate buffer (see section 2.3.1) supplemented with 25 mM Hepes (pH 7.3), 2 mM Na-acetate, 0.3 mg/ml D-glucose and 30 mg/ml BSA (dialysed and fatty acid free) as described by Vernon & Finley, (1985). The incubation medium was supplemented with hormones and other agents as indicated in the results chapters. Following incubation, 1 ml of the medium was taken and deproteinised by adding 300 µl of 45% (w/v) perchloric acid and following a 15 minute centrifugation (2500 rpm), 800 µl of the resulting supernatant was neutralised with 85 µl KOH (5M) and 170 µl saturated $KHCO_3$. Following a further 15 minute centrifugation (2500 rpm), 800 µl of each sample supernatant was transferred to fresh LP3 tubes and stored at -20°C prior to the determination of glycerol levels.

2.3.5 Rat Lipolysis assay

Immediately after killing, parametrial adipose tissue was removed and adipocytes were prepared (Aitchison *et al*, 1982). The parametrial pads were removed and placed in Medium 199 with 1 mg/ml collagenase and 30 mg/ml BSA and adipocytes prepared as described section 2.3.2.

The resulting adipocytes (final cell number 10^5 cells/ml) were incubated in 2.5 ml Medium 199 plus additions that will be detailed in the results chapters. Adipocytes were incubated for 60 min at 37°C. The reaction was terminated by addition of 0.3 ml of 45% (w/v) $HClO_4$ followed by deproteinisation and neutralisation as described in the sheep lipolysis section above.

2.3.6 Glycerol assay

Glycerol contcentration was measured as described previously (Aitchison *et al.*, 1982). Samples prepared in the lipolysis assay outlined above were used to determine glycerol levels. Samples were defrosted and centrifuged at 2500 rpm for 15 minutes, following which 30 µl of each sample was placed in duplicate into a 96 well plate with 200 µl of assay mix containing: 13.5 ml of 0.25 M triethanol ammonium chloride buffer pH 7.5, 2.5 ml of 2 mg/ml NAD, 2.7 ml of 5 mg/ml ATP, 250 µl of 1 M $MgCl_2$, 12 µl Triton X-100 (10%), 215 µl of 1 mg/ml diaphorase, 20 µl of 10 mg/ml Glycerol-3-phosphate dehydrogenase (G-3-PDH), 2.5 mg/ml [3-{4,5dimethylthiosal-2-yl}-2,5-diphenyl-tetrazoliumbromide] (MTT) and 810 µl distilled H_2O. The assay mix was prepared in a light-protected bottle containing a magnetic flea and was stirred at 4^oC. Glycerol levels were determined by adding 50 µl of glycerol kinase (4 units/ml) and the plates were incubated at room temperature in the dark for 45 minutes. Following incubation the plate was read at 600nm on a Titertek plate reader. Glycerol levels were calculated from a glycerol standard curve that was measured on each 96-well plate over a concentration range of 2-20nm.

2.3.7 Ligand binding assays

Binding of 1-[propyl-2,3-[^{3}H]dihydroalprenolol (specific radioactivity 61 Ci/mmol), [O-methyl-^{3}H]raulwolscine (specific radioactivity 66 Ci/mmol) and (-)N^6-R-[G-H^3]phenylisopropyladenosine (specific radioactivity 36 Ci/mmol) to sheep (Watt *et al*, 1991), and rat adipocyte membranes was performed in 50 mM-Tris/HCl (pH 7.4)/ 10 mM $MgCl_2$ at 37^oC in a final volume of 200 µl containing 20-200 µg of membrane protein and the appropriate radioligand prepared in 1 mM

ascorbic acid to a final concentration of 20 nM (sheep membranes only) or up to 100 mM (rat membranes), (Malbon *et al.*, 1978). 13 ml tubes were placed in a water bath and 40 µl of buffer was added to each tube, followed by 40 µl of labelled ligand. 40 µl of appropriate ligand (isoprenaline, yohimbine and PIA, in 1 mM ascorbic acid were used to assess β-adrenergic receptors, α2-receptors and adenosine receptors respectively) or 40 µl 1 mM ascorbic acid (control) was then added to the tubes and the reaction was initiated by addition of membrane suspensions. Incubations were stopped after 10 mins by addition of 5 ml of ice-cold incubation buffer. The contents of each assay tube were then rapidly filtered under vacuum through Whatman GF/C filter discs (2.5cm diameter). The filters were then washed with 2 x 5 ml of ice-cold incubation buffer and dried under vacuum. Filters were then placed in 10 ml of Opti-Fluor scintillant and counted for a radioactivity on an LKB 1215 Rackbeta liquid-scintillation counter. Assays were performed in duplicate. Non-specific binding was determined by incubating corresponding tubes with 100 µM -(-)isoprenaline, 100 µM-yohimbine or 100 µM-N6-phenylisopropyladenosine to measure β-, α2- and adenosine receptor ligand binding respectively. Specific binding of radioligands was defined as the total [^{3}H]ligand bound minus [^{3}H]ligand bound in the presence of 100 µM competing ligand.

For binding studies using [^{3}H]phenylisopropyladenosine, plasma membranes (20-100 µg) were first resuspended in incubation buffer (50 nM-Tris/HCl, pH 7.4 and 1 mM $MgCl_2$) and incubated with adenosine deaminase (1 µg/ml) for 10 min at 30^{o}C, final volume of asay mix was 70 µl (Trost and Schwabe, 1981). Adenosine deaminase treated plasma membranes were used immediately to assay adenosine-receptor binding. 20 nM ligand was sufficient to saturate the binding capacity of sheep fat-cell membranes whereas 100 nM ligand was required to saturate the binding capacity of rat-cell membranes.

2.3.8 Protein determination

Protein was measured by the method of Bradford (1976) using the Bio-rad protein assay (Cat. No. 500-0006). BSA (fraction V) was used as a protein standard (1-5 μg standard curve). Assays were performed in 96-well microtitre plates and the sample volume was 50 μl. The protein standard was prepared in distilled H_2O at a concentration of 0.1 mg/ml and was arranged in the first two lanes of each plate, with each point in triplicate. When both the standard curve and samples had been added, 240 μl of diluted Bio-rad protein reagent was added to each well. The concentrate was diluted in the ratio 1 part concentrate : 4 parts H_2O. The plate was then left for 5 minutes before being read using a Titertek Multiskan at 620nm.

2.3.9 Preparation of bovine serum albumin

All bovine serum albumin used was fraction V, essentially fatty acid free, from Sigma chemical Company, Poole, Dorset, and was dialysed prior to use as described (Hanson & Ballard, 1968). A 10% solution of the albumin was dialysed over 3 days against 0.9% NaCl at $4^{o}C$. The 0.9% NaCl was changed 5 times over the three days. The albumin was finally dialysed for at least 6 hours against distilled water to remove NaCl. The dialysed solution was lyophilised and the dry powder stored at $4^{o}C$ until required.

2.3.10 Determination of adipocyte volume and number of adipocytes per gram tissue

Cells were prepared and washed as described earlier (2.3.2) and samples (5 μl) of the cell suspension were transferred to a warm haemocytometer and the diameter of 100 cells was measured using a Projectina microscope at 145 x magnification. The mean cell volume was then calculated as described by Girolamo *et al* (1971). An estimate of the number of cells per gram tissue was obtained by dividing the total lipid content of the tissue by the mean cell volume x density of lipid (where density of lipid was taken to be 1.1). For rat adipose tissue, the total lipid content was taken to be equal to the dry weight of the tissue. The means of determining the lipid content for sheep adipose tissue is described below in section 2.3.11.

2.3.11 Determination of lipid content of sheep adipose tissue

Adipose tissue pieces (prepared as described in section 2.3.3) were weighed and added to 30 ml centrifuge tubes containing two drops of 1 M HCl in 8 ml $CHCl_3$/MeOH and shaken. The tubes were then left at room temperature for two hours. After this time 4 ml of $CHCl_3$ was added to each tubes, the tubes were shaken and then left overnight at room temperature. 3 ml of 0.88% KCl was then added to the tubes, the tubes were shaken and centrifuged at 2500 rpm for 15 minutes and the two phases allowed to seperate. The upper phase was then removed and the lower phase was taken to dryness using a rotary evaporator. The remaining lipid was then dissolved in 2-3 ml of chloroform, filtered into a weighed scintillation vial and the chloroform was removed with air whilst the vial was on a

heating block at 65^{o}C. The vials containing the lipid were then left to cool and weighed.

2.3.12 Insulin Radioimmunoassay

The insulin RIA assay was used to determine levels on endogenous insulin in rat experiments following manipulation of growth homone levels. The buffer used in the assay comprised 0.05 M Na_2PO_4 pH7.4, 0.15 M NaCl, 0.05% (w/v) sodium azide and 0.5% (w/v) BSA. The insulin standard, natural porcine insulin was dissolved in 10 mM HCl, diluted in RIA buffer and batched in 100 µl aliquots (10 µg/ml) in eppendorf tubes and stored at -20^{o}C. The first antibody, anti-bovine insulin antiserum, was raised in guinea pigs by D.J. Flint, Hannah Research Institue, and used at a final dilution in the assay at 1:20000. The second antibody was prepared in 140 mg ethylene diaminetetra-acetic acid (EDTA) (BDH Ltd., Glasgow, UK) in 15 ml RIA buffer (adjusted to pH 7.4 after adding EDTA), then 5 µl normal guinea pig serum and 125 µl anti-guinea pig precipitating serum (Scottish Antibody Production Unit, Glasgow, UK) and 15 ml of 16% (w/v) polyethylene glycol 6000 grade (PEG) (FSA Laboratory Supplies, Loughborough, Leics. UK). The insulin assay was based on a method described by Vernon, Clegg and Flint (1981). Insulin standards ranged from 0.16 to 5 ng/ml. A volume of 100 µl was taken, in duplicate, for standards and samples and this was added to 100 µl of anti-bovine insulin antiserum to give a final antiserum dilution of 1:20000. 100 µl of buffer and 100 µl antiserum was added to the zero tubes, whereas non-specific binding tubes received only 200 µl of buffer. After a 24 incubation at 4^{o}C, ^{125}I-insulin was added to all tubes (100 µl: 10000 cpm). The tubes were incubated for 24 hours at 4^{o}C, following which 300 µl of second antibody was added and the tubes were incubated for a further 4 hours at room temperature. After this time the

tubes were centrifuged at 3000 rpm for 30 minutes and the supernatant was decanted. The protein precipitate, containing antibody-bound ^{125}I-insulin, was counted on a gamma counter (Cobra Auto-gamma, Packard, Pangbourne, Berks. UK). The concentration of insulin in the samples was determined by interpolation from a standard curve.

2.3.13 IGF-I Radioimmunoassay

Determination of endogenous levels of rat IGF-I was achieved using IGF-I RIA. The RIA buffer used for the assay consisted: 0.05 M Na_2PO_4, O.15 M NaCl, 0.1 mg/ml thimersal and 0.5% (w/v) BSA. The IGF-I standard (Bachem) was dissovled in acid ethanol (250 mM HCl in ethanol) and then diluted in RIA buffer, batched in 100 µl aliquots (0.01 µg/µl) in ependorf tubes and stored at -20^{o}C. The first antibody anti-IGF-I (NIDDK and NHPP, University of Maryland School of Medicine, USA) was stored at a dilution of 1:10, which when diluted 1:200 gave a final dilution in the assay of 1:20000. The second antibody, anti-rabbit precipitating serum (SAPU, Carluke, UK) was prepared in 140 mg EDTA in 15 ml of RIA buffer (adjusted to pH 7.4 after adding EDTA), then 60 µl normal rabbit serum (SAPU) and 900 µl anti-rabbit precipitating serum and 15 ml of 16% (w/v) polyethylene glycol (PEG). IGF-I standards ranged from 0.3 to 10 ng/ml. A volume of 100 µl was taken, in duplicate, for standards and samples and 100 µl of anti-IGF-I was added to give a final antiserum dilution of 1:2000. 100 µl of buffer and 100 µl antiserum was added to zero tubes, whereas non-specific binding tubes received only 200 µl of buffer, the tubes were then vortexed and incubated at room temperature overnight. Following this, 100 µl ^{125}I-IGF-I was added to all tubes (100 µl : 20,000 cpm), the tubes were then vortexed and incubated at room temperature overnight. 300 µl of second antibody was then added, the tubes were

vortexed, incubated for 4 hours at room temperature, centrifuged for 30 minutes at 3,000 rpm and the supernatant removed. The protein precipitate, containing antibody bound ^{125}I-IGF-I, was counted on a gamma counter (Cobra Auto-gamma, Packard, Berks, UK). The concentration of IGF-I in the samples was determined by interpolation from a standard curve.

2.3.14 Standard size Polyacrylamide Gels

12% SDS/polyacrylamide gels were used to resolve proteins of both rat and sheep adipocyte membranes, prepared as described earlier. For 20 cm gels, 30 ml of a 12% separating gel containing: 10.08 ml distilled H_2O, 7.5 ml Tris-HCl (1.5M) pH 8.8, 10 ml acrylamide (30%), 0.3 ml SDS (sodiumdodecylsulphate) (10%), 0.1 ml ammonium persulphate (10%) and 20 µl tetramethylethylenediamine (TEMED) was cast and left to set at room temperature for about 1 hour. To prevent the gel drying out a thin layer of saturated butanol was added to the top of the gel. Once the gel was set the butanol was poured off and the gel washed 3 times with distilled water, a 5% stacking gel comprising: 5.7 ml deionised H_2O, 2.5 ml Tris/HCl (0.5M) pH 6.7, 1.6ml 30% acrylamide, 0.1 ml SDS (10%), 0.1 ml ammonium persulphate and 7.5 µl TEMED was poured, and the well-forming comb was carefully inserted avoiding air bubbles. Once the stacking gel had set after about 30 minutes, the comb was removed, the wells washed with distilled H_2O and the gel assembled ready for sample loading and running. The plates were then secured to the cooling system of the tank and tank buffer containing: 14.4 g/l glycine, 1.0 g/l SDS and 3.0 g/l Tris, poured into the top tank, and the main tank . The wells were loaded with sample using a blunt-ended Hamilton syringe, and the tank was placed on a stirrer the water cooling system was set in operation. As a means of protein size and identification a Bio-Rad (Bio-Rad Laboratories, Watford,

Herts, UK) standard (broad range; 7,200 to 208,000 daltons) was run on each gel. The gels were then run at 30-40 milliamps constant current per gel for 3-4 hours.

2.3.15 Mini-gels

7.5 cm-length mini-gels in a Bio-rad mini-gel tank were used for the detection of G_i. 3.5 ml of seperating gel, prepared as described above were used with 1.5 ml of stacking gel and gels were run in about 500 ml of tank buffer at about 25 milliamps for approximately 1 hour. Instead of using a water cooling system to keep the gels from heating the tank buffer was cooled to $4^{o}C$, this was sufficient due to short gel- running time.

2.3.16 Western Blotting

Western blotting was performed essentially as described previously (Mitchell *et al*, 1989; Strassheim *et al*, 1990, 1991). While gels were running (see above), transfer buffer was prepared with 86.4 g glycine, 18 g Tris in 4.8 l of deionised H_2O and placed in the cold room at $4^{o}C$ and just prior to use 1.2 l of methanol was added. Transferring of proteins from the gel to nitrocellulose paper was performed using a Transfor elecrophoretic transfer unit. The system was assembled by placing one half of a cassette in a tray containing cold transfer buffer, followed by a sponge, a piece of blotting paper and then the piece of nitrocellulose paper. The gel was then placed on top of the nitrocellulose paper and particular attention was paid to the removal of all air bubbles so as to ensure the transferring of all proteins from the gel. A further piece of blotting paper followed by another sponge was then overlaid and the second half of the cassette was then secured and

the entire unit was placed in the tank containg cold transfer buffer, and the transfer of the proteins was carried out overnight at a constant current of 130 mamps.

Following the transfer of the proteins, the nitrocellulose paper was placed in 100 ml of 2.5% Marvel blocking buffer: 2.5 g Marvel, 5 ml 1M Tris (pH 7.5), 0.9g NaCl, 0.1g azide, 100 µl Tween and 95 ml deionised H_2O. The nitrocellulose was then left in the blocking buffer overnight at room temperature.

The blocking buffer was removed the following day and the nitrocellulose was placed in a buffer containing the first antibody, which had been prepared in 1% marvel buffer: 1 g Marvel, 5 ml 1M Tris (pH 7.5), 0.9 g NaCl, 0.1 g azide, 100 µl Tween and 95 ml deionised H_2O, on a shaker at room temperature for 4 hours. After this 4 hour incubation the antibody was poured off and the nitrocellulose was vigorously washed 4 times in 1% marvel solution (as above), and each wash consisted of 5 minutes of shaking. Detection of the proteins for analysis was carried out using the ^{125}I - labelled protein A in 1% Marvel . The nitrocellulose was incubated for 1.5 hours on a shaking table at room temperature, following which the ^{125}I was removed and the blot washed 4 times in 1% and 10 times in Tris buffered saline (2.42 g/l Tris, 9.0 g/l NaCl, pH 7.4). The blot was then dried overnight and autoradiographed at -80^{o}C. Quantification of reactive proteins was achieved by γ-radiation counting of excised bands or by densitometric scanning using a Bio-Rad 620 densitometer.

2.3.17 ADP ribosylation

Pertussis toxin ADP ribosylation of the inhibitory G-proteins in sheep adipocyte membranes was carried out using 70 µl of pertussis toxin 0.1 µg/ul was activated by incubating with 7 µl of 5 mM dithiothreitol (DTT) and 7 µl of 1 mM ATP for 60 minutes at room temperature. After this time the activated toxin was

placed on ice until used. The reaction mix (per tube): 25 µl 1M $NaPO_4$ buffer pH 7.0, 10 µl 150 mM thymidine (made fresh in H_2O), 2 µl 50 mM ATP and 2 µl 50 mM GTP was prepared and stored on ice prior to use. 39 µl of reaction mix was then added to each labelled ependorf tube with 25 µl membranes prepared as detailed in section 2.3.1 with 31 µl of activated pertussis toxin mix. The reaction was started upon addition of 7 µl [^{32}P]NAD (5 µl unlabelled 100 µM NAD plus 2 µl 1.4 µCi/ul NAD) and incubated in a water bath for 2 hours at 37^{o}C. The reaction was halted by addition of 1 ml ice cold 50 mM Tris (containing the protease inhibitors PMSF 100 µM and leupeptin 1 µg/ml). The samples were then centrifuged at 14,000 rpm at 4^{o}C for five minutes, the supernatant discarded and 50 µl of 1x sample buffer (0.06 M Tris, 0.06 M β-mercaptoethanol, 2% (w/v) SDS, 10% glycerol and 0.02% (w/v) phenol red) was added, mixed and boiled for 5 minutes. The samples were then run on a 10% gel as described in section 2.3.11, the gel was then dried on a X gel drier following which autoradiography was performed overnight at -80^{o}C.

In experiments where PIA was employed, H_2O was omitted from the reaction mix and PIA was added to give final concentrations of 1 - 1000 nM.

2.3.18 Adenylate Cyclase Assay

To determine levels of adenylate cyclase in adipocyte membranes the method of Houslay *et al* (1976) was used with modifications (Strassheim *et al*, 1990). Sheep adipocyte membranes were prepared as described in section 2.3.1, with the exception that isolated adipocytes were suspended in 0.25 M sucrose, 10 mM Tris/HCl and 3mM ATP (Strassheim *et al*, 1990). A stock incubation medium: 25 mM Triethanolamine, 5 mM $MgSO_4$, 10 mM theophylline, 1 mM EDTA and 1 mM DTT was made as a 4x concentration solution and stored at

room temperature. This stock was then be diluted 1:2 with distilled H_2O for use in an assay where it occupied 50% of the volume, giving a correct final concentration. The ATP regeneration system for the assay was prepared in 2x concentration incubation medium plus 1.5 mM ATP, 7.4 mg/ml creatine phosphate (Boehringer), 0.2 mg/ml creatine kinase (Boehringer) and 0.8 mg/ml BSA. To 1.5 ml eppendorf tubes (on ice), 50 µl regeneration system, 10 µl 10^{-3}M GTP and 10 µl other agents (see chapter 6) or 10 µl incubation medium, was added. Finally, while the tubes were still on ice, 30 µl of membranes at 2 µg/µl protein concentration or 30 µl of H_2O (control) was added and the tubes were quickly vortexed and immediately transferred to a water bath at 30°C for 10 minutes. At the end of the incubation time the reaction was stopped by placing the tubes in a boiling water bath for 3-4 minutes. The tubes were then cooled and centrifuged at 14,000g for 5 minutes to precipitate the protein and the supernatant was removed and used for cAMP determination.

In experiments where PIA was employed, theophylline was omitted from the assays and the non-methylxanthine cAMP phosphodiesterase inhibitor Ro-07-2956 was added at 0.1 mM, together with 1 unit of adenosine deaminase/ml.

2.3.19 cAMP Binding Assay

The determination of cAMP binding was carried out as described by Whetton *et al* (1983). An assay buffer was prepared: 50 mM Tris/HCl and 4 mM EDTA, pH 7.4 and stored at 4°C. 10 mg of the binding protein, protein kinase cAMP was added to a solution of 25 ml assay buffer and 125 mg BSA, stirred and 1.1 ml aliquots were snap frozen and stored at -20°C. cAMP to be used for standards was stored at -20oC in 1.1 ml, aliquots at 3.2 µM. At the time of experimentation the aliquot was diluted 1:10 to give 320 nM cAMP which is

equivalent to 16 pmol/50 µl, and from this concentration a serial dilution was carried out. [3H]cAMP was diluted in assay buffer at 2 µl 0.5 µCi/ml in 4 ml assay buffer and was stored on ice prior to use. The charcoal suspension used to stop the reaction was prepared approximately 30 minutes before use and comprised 520 mg activated charcoal in 20 ml assay buffer containing 400 mg BSA. The suspension was kept stirring on ice prior to the assay.

For the assay of cAMP binding, eppendorf tubes were placed on ice. To the tubes, 50 µl of membrane/ 50 µl cAMP standard, 50 µl [3H]cAMP and 80 µl assay buffer, were added. The reaction was started upon addition of 20 µl of binding protein, the tubes were vortexed and incubated in the cold room at $4^{o}C$ on ice for 4 hours. The reaction was stopped upon the addition of 100 µl of charcoal suspension. The samples containing the charcoal suspension must sit on ice for at least 90 seconds. Following this the tubes were spun at 14,000 g for 5 minutes and then 400 µl of supernatant (without disturbing charcoal sediment in tube) was removed and placed in a scintillation vial with 4 ml of Opti-fluor scintillant and counted for radioactivity on an LKB 1215 Rackbeta liquid-scintillation counter.

Statistics

Statistical significance of results was assessed by analysis of variance or Student's t-test for either paired or unpaired observations as appropriate. Unless otherwise stated, the results are given as means either $\pm$ SED (standard error of difference) or $\pm$ SEM.

CHAPTER THREE

STUDIES ON THE LIPOLYTIC ACTION OF GROWTH HORMONE IN RAT ADIPOSE TISSUE

3.1 Introduction

In several species, including the rat, growth hormone has been shown to decrease adiposity through changes in the rate of both lipid synthesis and also lipolysis (Goodman *et al*, 1988; Vernon and Flint, 1989; Cuneo *et al*, 1992; Flint and Gardner, 1993). Recent studies suggest that growth hormone effects on lipolysis are indirect and are achieved by altering the maximal response or sensitivity of adipocytes to acutely acting lipolytic or antilipolytic agents.

Adipocyte lipolysis is under acute regulatory control from both stimulatory and inhibitory agonists. The rate limiting enzyme, hormone-sensitive lipase can be phosphorylated and activated by cyclic-AMP-dependent kinase. Therefore changes in adenylate cyclase (AC) activity, which modulate cAMP levels, are intimately connected with the control of lipolysis (Belfrage, 1985).

AC activity can be regulated by both stimulatory and inhibitory receptors which exert their actions through the heterotrimeric G-proteins, G_s and G_i respectively. Changes in the expression of adipocyte G_i and G_s have been shown to occur in a number of animal models of pathophysiological states, such as chemically induced hypothyroidism (Milligan and Saggerson, 1990), genetic obesity (Strassheim *et al*, 1991; Begin-Heick, 1992) and insulin resistance (Strassheim *et al*, 1990, 1991; Saggerson *et al*, 1991; Begin-Heick, 1991), and also in obese humans (Ohiosalo and Milligan, 1989; Milligan and Saggerson, 1990; Strassheim *et al*, 1991, Begin-Heick, 1992).

This prompted the following investigations into whether growth hormone can influence the regulation of lipolysis by altering the expression of G_i and G_s in rat adipocytes, and how this may correlate with the functioning of inhibitory receptors.

3.2 Experimental Procedure

Virgin female Wistar rats (200-250g) were handled and injections given as described previously in materials and methods (section 2.2). The rats received the following injections twice daily at 09:00 and 17:00 h: γ-globulin fraction of antiserum to rat growth hormone (anti-rGH; 150mg/injection, equivalent to 4.5 ml of serum), either alone or in combination with ovine growth hormone (NIADDK-oGH-13), 0.5 mg per injection. Control animals received carrier solutions (Madon *et al*, 1986). On day 3, between 10.00 and 11.00 h, rats were anaesthestized by injection of 0.5ml of Sagatal (May and Baker, Dagenham, Essex, UK), as previously described (Vernon *et al*, 1993).

Immediately after the induction of anaesthesia, samples of blood and parametrial adipose tissue were taken, after which rats were killed by exsanguination. There were ten rats in each treatment group; five were used for measurement of lipolysis (section 2.3.5) and five for preparation of membranes (section 2.3.2) to be used in receptor binding assays and western blotting for analysis of G-proteins.

Analysis of results

Statistical significance was assessed by analysis of variance. Unless otherwise stated, the results are given as means either ± S.E.D (standard error of difference) or ± SEM.

3.3 Results

3.3.1 Changes in rat growth rate

The effectiveness of treatments in changing serum GH concentration was assessed by measuring changes in growth rate. The rats were weighed at 10.00 hours before the injection regime commenced and then subsequently each day at this time over the following 48 hours. The body weight of the control group did not change over the 48h period of treatment (-1.22 $\pm$ 1.7g) whereas a significant decrease ($P < 0.05$) was observed with the group treated with anti-rGH (-4.5 $\pm$ 1.7g) (Fig. 3.1). In contrast, the group receiving ovine growth hormone along with the anti-rGH increased body weight by 7.3 $\pm$ 1.7g (Fig. 3.1). All the values are mean $\pm$ SED for 11 observations. While confirming the actions of the anti-rGH, these results suggest that the animals injected with oGH were receiving slightly higher than normal levels of GH.

3.3.2 Serum IGF-1

Effectiveness of the anti-rGH, and also of injections of oGH, were checked by assaying for serum IGF-1. Treatment of rats with anti-rGH alone significantly ($P< 0.001$) decreased the serum IGF-1 concentration, as compared with that in control animals, and concurrent injection of oGH prevented this decrease. Serum IGF-1 levels were 496 ng/ml for control animals, 233 ng/ml for anti-rGH-treated animals and 644ng/ml for anti-rGH + oGH-treated animals (SED = 51, n = 11 in each case).

3.3.3 Lipolytic response to PIA and PGE_1

In the lipolysis assays the maximal stimulation of lipolysis was achieved, as reported previously by Vernon *et al* (1987), by including 0.8μg/ml adenosine

deaminase and 100 nM isoprenaline in the incubations. Lipolysis was then inhibited in a dose-response manner using the adenosine analogue N^6-phenyl-isopropyladenosine (PIA) or prostaglandin E_1 (PGE_1).

Treatment of rats with anti-rGH, either with or without oGH, had no effect either on basal lipolysis or on the stimulation of lipolysis by isoprenaline (Fig. 3.2). Basal glycerol release, measured in the presence of adenosine deaminase, was 0.5 ± 0.1 μmol/min per 10^6 cells, and 100 nM isoprenaline increased ($P < 0.001$) the level of glycerol release to 5.4 ± 0.1 μmol/min per 10^6 cells (results are means $\pm$ S.E.M. of 15 observations ie. for all rats).

Lowering of serum GH levels by injecting rats with anti-rGH resulted in a significant increase ($P< 0.05$) in the inhibition of lipolysis induced by both 3nM and 10nM PIA (Fig. 3.3). This effect was prevented by concurrent injections with oGH. In contrast, anti-rGH had no significant effect on the inhibition of lipolysis by 100nM PIA (Fig. 3.3).

Treatment with anti-rGH also significantly increased ($P < 0.02$) the inhibition of lipolysis observed with 100nM PGE_1 (Fig. 3.4, Table 3.1). As described above, this effect was prevented by concurrent treatment with oGH. At levels of 1μM PGE_1 the apparent increase in the inhibition of lipolysis in animals treated with anti-rGH was not statistically significant.

These results show that GH supresses the anti-lipolytic effects of both PIA and PGE_1, by decreasing the sensitivity of adipocytes to sub-maximal concentrations of these agents. However, GH appears to have little effect on the response of lipolysis to maximally effective concentrations of either PIA or PGE_1.

3.3.4 Ligand binding to adenosine receptors

Measurement of ligand binding was performed on adipocyte membranes to determine if the differences in sensitivity to PIA was attributable to a change in receptor number. Initially receptor-binding studies were carried out using a single

maximum concentration of [^{3}H]-PIA at 20nM, based on the previous studies with sheep adipocyte membranes. The amount of [^{3}H]-PIA bound to the membranes was 83.0 fmol/mg protein in the control animals, 96.7 fmol/mg protein in the anti-rGH treated rats and 71.0 fmol/mg protein in the anti-rGH + oGH-treated rats. The results are means of 5 observations where the S.E.D. was 15.5 fmol/mg protein, therefore indicating no significant effect of treatments.

A subsequent experiment was carried out using a range of [^{3}H]-PIA (Fig. 3.5, Table 3.2) and showed that maximal binding was achieved at concentrations of about 100nM [^{3}H]-PIA. No significant effect of manipulation of serum GH levels was observed with any concentrations of [^{3}H]-PIA used. These results suggest that the changes in sensitivity are due to a post-receptor change; this might be expected as sensitivity to both adenosine (PIA) and prostaglandin is affected.

3.3.5 Immunoblotting of α-subunits of G_i1, G_i2, G_i3 and G_s

Having determined GH has no effect on receptor number, experiments were carried out to look at the levels of the G-proteins as the next protein in the lipolytic cascade. To ascertain changes in the levels of α-subunits of individual G-proteins in adipocyte membranes anti-peptide antisera were used.

Rat adipocytes express three forms of G_i (G_i1, G_i2 and G_i3), which are distinct gene products (Milligan, 1988; Mitchell et al, 1989). They also express two forms of G_s (42 kDa and 45 kDa), derived by alternative splicing (Robishaw *et al*, 1986; Strassheim *et al*, 1990, 1991). Injection of rats with anti-rGH had no effect on the expression of the α-subunit of G_i3 or on the 45 kDa and 42 kDa α-subunit forms of G_s, and this was also the case in those animals treated with anti-rGH + oGH (Fig. 3.6, Table 3.3). The similarity in size of the α-subunits of G_i1 (α-G_i1; 41 kDa) and G_i2 (α-G_i2; 40 kDa) did not permit their separation under the electrophoresis conditions used. The antiserum used , SG1, recognises

both α-G_i1 and α-G_i2 and therefore detected only one immunoreactive band which presumably represents both of these α-subunits. Injection of rats with anti-rGH resulted in a 3 + 0.27 increase ($P< 0.001$; mean $\pm$ S.E.M. for 5 observations) in the amount of α-G_i1 plus α-G_i2, as detected by antiserum SG1, in adipocyte membranes (Fig. 3.6, Table 1). This increase was prevented by concurrent administration of oGH. Immunoblotting with I1C, which recognises only α-G_i1, showed that anti-rGH had no effect on the amount of this α-subunit (Fig. 3.6, Table 1).

From these results it can be concluded that the increase in intensity of the immunoreactive band, revealed by antiserum SG1, after injection of rats with anti-rGH, can be attributed entirely to an increase in α-G_i2. This suggests that the increase in G_i2, elicited by depletion of endogenous rat GH, must be over 3-fold. These results then demonstrate that GH specifically and profoundly suppresses the amount of α-G_i2 in adipocyte membranes.

3.4 Discussion

Growth hormone has long been regarded as a lipolytic hormone, but the mechanism whereby it increases lipid mobilisation in fat cells has been a subject of considerable controversy. There is now growing support for the theory that GH alters the responsiveness of fat cells to acutely-acting lipolytic agents, and that this indirect effect may be the major mechanism whereby GH enhances lipolysis.

Measurement of serum GH itself is problematic, as the hormone is secreted in a highly pulsatile manner (Jannson *et al*, 1985) and the anti-rat GH serum interferes with the radioimmunoassay. In this study, the changes measured in growth rate and serum IGF1 indicate that significant changes in serum GH were being achieved, as found in previous studies using this antiserum and GH-therapy regime (Flint and Gardner, 1993; Vernon *et al*, 1993).

This study provides further evidence for GH modulating lipolysis by altering the ability of acutely acting factors to influence the process, and suggests that a major efect of GH is on the anti-lipolytic signalling system which operates via G_i. In this study using non-lactating virgin rats, GH treatment failed to alter the maximum response to β-agonists. This is in accordance with other studies that have shown that GH treatment failed to increase the response to β-agonists in lactating rats (Vernon *et al*, 1991) and cows (Lanna *et al*, 1992). These results may therefore imply that the only effect on the system as a whole is on the anti-lipolytic signalling system. However, GH has been shown to affect the response to β-agonists in other states: both litter-removal form lactating rats (Vernon *et al*, 1991, 1993) or cessation of lactation by removal of both serum prolactin and GH (Barber *et al*, 1992) leads to a substantial fall in the response to β-agonists which is prevented by concurrent treatment with GH (but not prolactin).

In the present study show that the predominant effect of GH is on the sensitivity to anti-lipolytic agents which act via G_i. However various observations suggest that GH can have a spectrum of effcts on the G_S-G_i-based signalling systems which regulate lipolysis. In lactating rats in which lactation was abruptly and completely curtailed either by litter removal (Vernon *et al*, 1987) or by endocrine manipulation (Barber *et al* 1992), GH markedly suppressed maximum response to PIA (sensitivity was not measured). In contrast, by giving GH injections or injections to anti-rGH, and thereby varying serum GH, showed that the effect on response to maximal concentrations of PIA in normally lactating rats (Vernon *et al*, 1987) and lactating cows (Lanna *et al*, 1992), was only small. However in lactating cows, as in this study, GH increased sensitivity to sub-maximal concentrations of PIA.

The possibility that varying GH serum may affect receptor number was investigated by looking at the effect of adenosine binding using [^{3}H]-PIA. These studies show that GH has no affect on adenosine binding, therefore indicating no change in the number or affinity of adenosine receptors.

The results from the lipolysis experiments using PIA and PGE_1 showed that manipulation of endogenous GH levels revealed a decrease in sensitivity to both agonists (Figs. 3.3 and 3.4). PIA and PGE_1 couple to distinct receptors, therefore the results suggest that GH modifies a post-receptor step, common to both agents. As both receptors presumably exert inhibitory action on adenylate cyclase by coupling to the inhibitory G-protein, G_i, it was thought that changes in this protein might account for the changes in anti-lipolytic effects.

An examination of the amounts of the various G-proteins of adipocyte membranes were determined by immunoblotting following separation by SDS gel electrophoresis, using antisera which are specific for the various isoforms of G_s and G_i, except for G_i2 where the antisera used recognised both G_i1 and G_i2. The immunological analysis of the adipocyte membranes revealed that treatment of animals with anti-rGH enhanced, by more than 3-fold, the amount of α-G_i2, but had little effect on the amounts of α-G_i1, α-G_i3 and α-G_s. Prevention of the levels of α-G_i2 were achieved upon administration of oGH, a treatment which in itself did not alter the levels of the other G-proteins. Therefore the observation that GH down-regulates the levels of α-G_i2 in adipocyte membranes provides an explanation for the decreased sensitivity to the anti-lipolytic agonists, PIA and PGE_1, seen in adipocyte membranes.

Therefore GH appears to affect a specific form of G_i, that being G_i2, of the G-protein family. Various investigations have suggested that G_i2 is responsible for the inhibition of adenylate cyclase in physiological systems. Studies carried out by McKenzie and Milligan (1990) showed that the δ-opoid receptor of mouse neuroblastoma x rat glioma hybrid cells (NG108-15) interacts directly and specifically with G_i2 to cause inhibition of adenylate cyclase. Other studies with platelet membranes identified G_i2 as the dominant mediator of cyclase inhibition (Simonds *et al*, 1989). Thus the data obtained in this study imply that both the adenosine and PGE_1 receptors exert inhibitory effects by coupling to G_i2, although it cannot be ruled out that they may also react with G_i1 and/or G_i3.

The similar maximal degree of inhibition of lipolysis by PIA and PGE_1, achieved in adipocytes from control and GH- deficient rats (Fig. 2 and Fig. 3) could be explained if the receptors for these agonists couple to a pool of G_i2 which, in itself, is of sufficient size, in both groups of animals to achieve maximal inhibition of lipolysis.

The use of anti-rGH to deplete GH levels would seem a more reliable indicator of events when looking at the GH-deficient state of the animal. Previous studies by Goodman *et al* (1988) looked at levels of G-proteins in hypophysectomized rats and found increased intensity of a 40 kDa band labelled by pertussis toxin-catalysed ADP-ribosylation. The 40 kDa band in this case could have represented the α-subunits of G_i1, G_i2, G_i3 and Go, all of which are expressed in rat adipocytes and exhibit similar molecular sizes of about 40 kDa (Milligan, 1988; Mitchell *et al*, 1989). While these results may indicate a supression of inhibitory G-protein levels by GH, the number of endocrine deficiences induced by hypophysectomy makes a precise interpretation of the results difficult. Further problems arise when trying to resolve ribosylated forms of G_i/G_o on gels and the fact that changes in dissociation of G-protein subunits can alter their ability to be ADP-ribosylated by pertussis toxin. Therefore the use of anti-rGH to deplete GH specifically and oGH to replace it is a simpler and more reliable way of looking at GH effects, and suggests that GH deficiency is a principal cause in the changes seen in G-protein expression.

In conclusion, these studies have demonstrated that GH, *in vivo*, specifically suppresses the levels of α-G_i2 in rat adipocytes and this leads to an attenuated response to the anti-lipolytic agents PIA and PGE_1. This will contribute to the ability of GH to decrease body fat mass. Other studies have shown that in several animal models of genetically acquired obesity (Strassheim *et al*, 1991; Begin-Heick, 1992) and in obese humans (Ohisalo and Milligan, 1989) the levels of G_i and G_s in adipocytes are lowered. The results from this study would then also indicate that G-protein expression in adipocytes are important in

determining fat mass, and that modulation of these proteins can lead to profound changes in body fat composition.

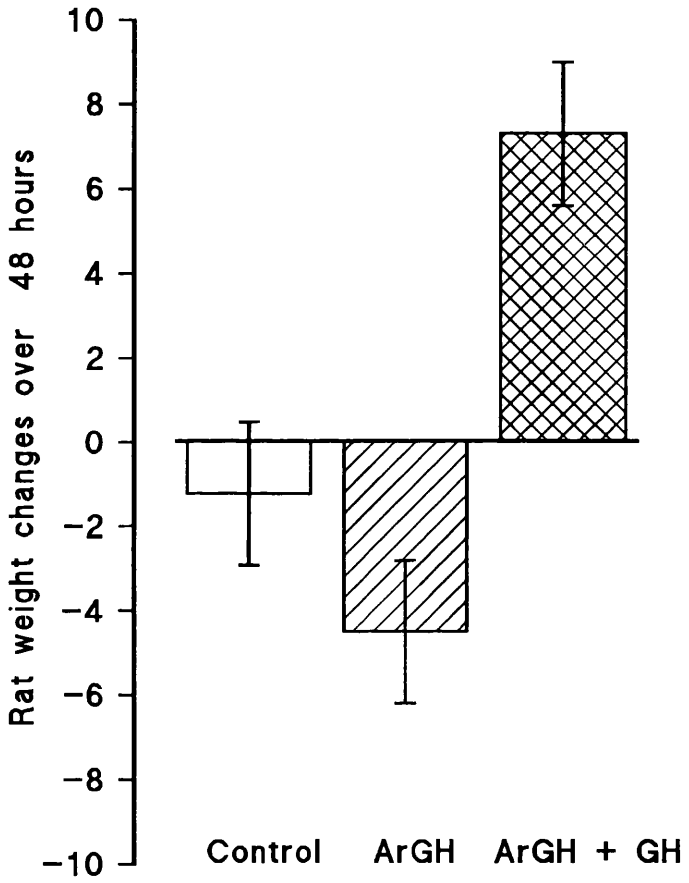


Figure 3.1 Rats were injected twice daily for 2 days with with either saline (controls), anti rat GH (ArGH) or anti rat GH + ovine GH (ArGH + GH). Changes in body weight over the 48h period of treatment were measured. Results are the means ± SED of 11 observations.

Basal and maximally–stimulated lipolysis of rat adipocytes

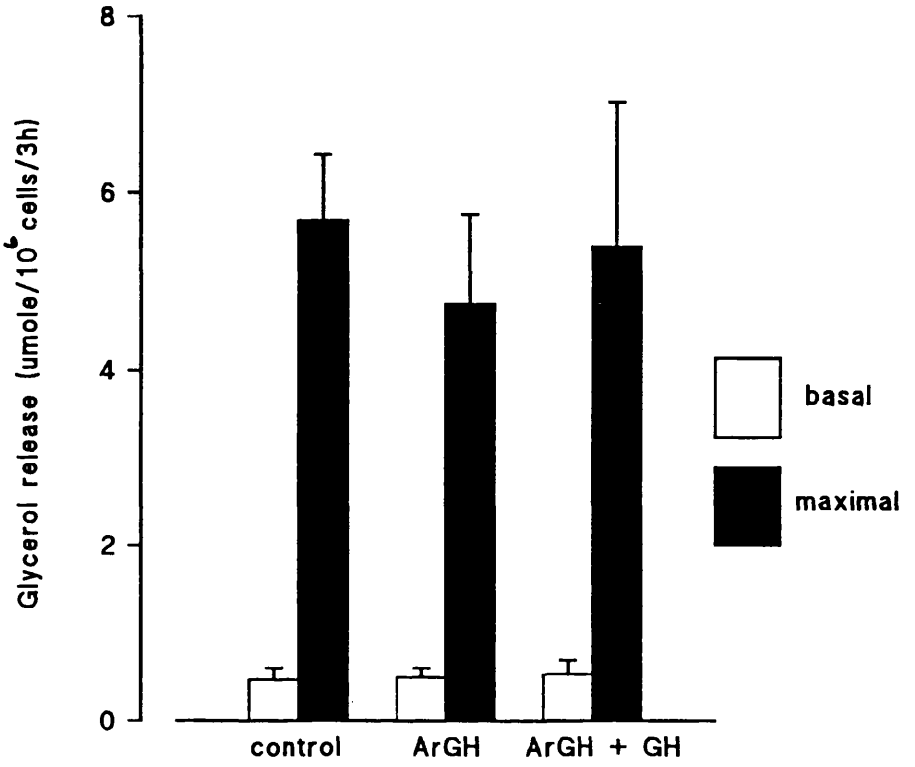


Figure 3.2 Adipocytes were prepared from rats treated as decsribed in legend to Fig 3.1. Glycerol release was measured, in the presence of 0.8 ug/ml adenosine deaminase and for maximal conditions, plus 100 nM isoprenaline. Results are the means ± SEM of 5 observations.

#Effect of PIA on Isoprenaline-stimulated lipolysis in virgin rats

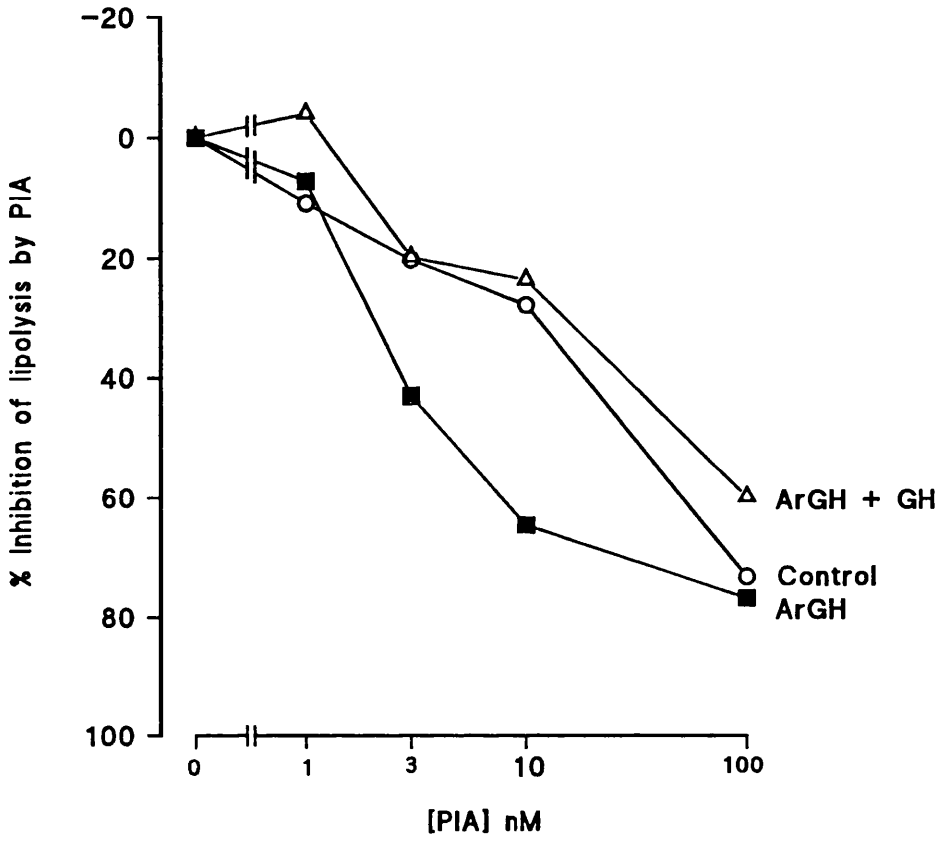


Figure 3.3 Adipocytes were prepared from rats treated as in Fig 3.1. Glycerol release was measured, in the presence of 0.8 ug/ml adenosine deaminase, 100 nM isoprenaline and the indicated concentration of PIA, as described in the experimental section. Results are the means of 5 observations, and the SED for comparing values at the same PIA concentration is 10.15.

Effect of PGE1 on Isoprenaline-stimulated lipolysis
following manipulation of GH levels in vivo in virgin rats

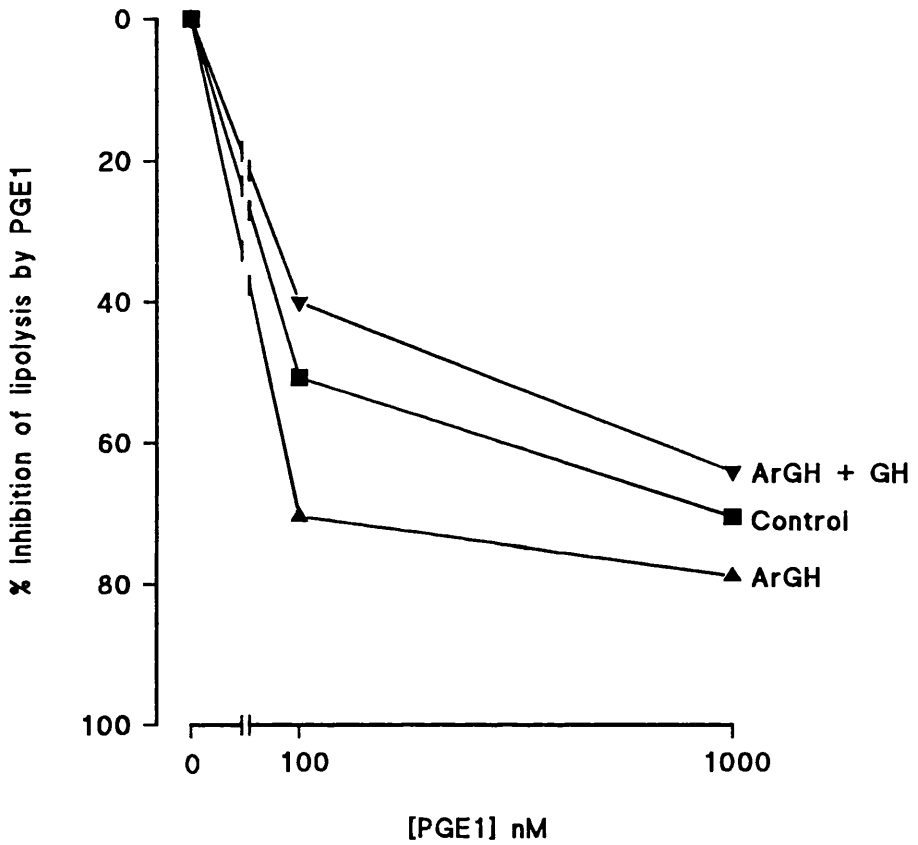


Figure 3.4 Adipocytes were prepared from rats treated as described in legend to Fig 3.1. Glycerol release was meaured, in the presence of 0.8 ug/ml adenosine deaminase,100 nM isoprenaline and the indicated concentration of PGE1, as described in the experimental section. Results are means of 5 observations and the SED for comparing values at the same PGE1 concentration is 7.3.

Table 3.1 Effect of varying serum GH *in vivo* on inhibition of isoprenaline-stimulated lipolysis by PGE_1 *in vitro*

Adipocytes were prepared from rats injected with anti-rGH, with or without oGH, or carrier solutions. Glycerol release was measured, in the presence of indicated concentrations of PGE_1 as described in the legend to Fig. 3.4. Results are the means of 5 observations and the SED for comparing values is 7.3; a and b indicate that the value is significantly different from the appropriate value for rats which received carrier solutions ($P < 0.02$) or anti-rGH ($P < 0.01$) respectively.

		Glycerol release (% inhibition)	
Injection	PGE_1 concentration	100 nM	1000 nM
Carrier solutions		50.6	70.4
Anti-rGH		70.4[a]	78.8
Anti-rGH, oGH		40.0[b]	64.0

Effect of varying GH concentration on the ability of adipocyte membranes to bind ^{3}H–PIA

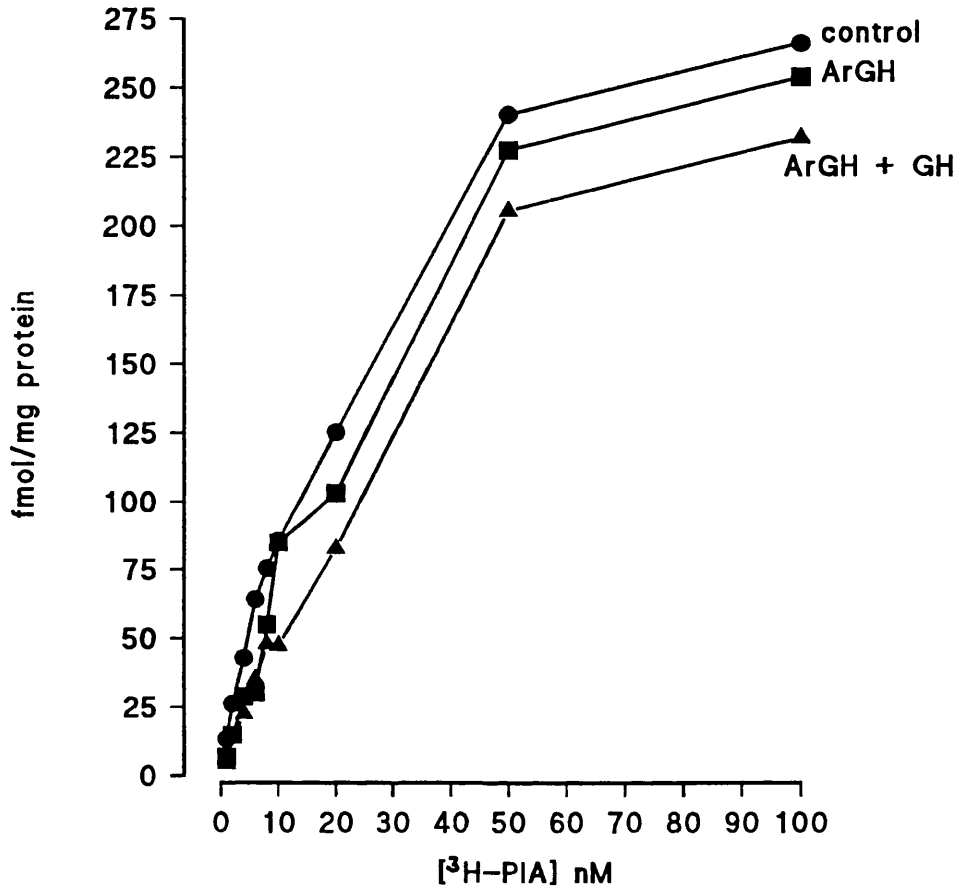


Figure 3.5 Rats were injected twice daily for 2 days as described in legend to Fig 3.1, after which they were killed, adipocyte membranes prepared and the ability of membranes to bind concentrations of [^{3}H]PIA was assessed. Each value is the mean of 3 seperate observations. SED range from 5.2 for 1 mM ^{3}H–PIA to 50 for 100 nM ^{3}H–PIA.

Table 3.2 Effect of varying serum GH concentration on the ability of adipocyte membranes to bind [^{3}H] PIA

Rats were injected twice daily for 2 days with either saline (0.15 M), an antiserum to rat GH (ArGH) or antiserum plus ovine GH after which they were killed, adipocyte membranes prepared and th addition of membranes bind concentrations of [^{3}H]PIA (1 to 100 nM) was assessed as described in the experimental section. Each value is the mean of 3 seperate observations (adipocytes from 2 rats were pooled for each preparation); pooled SEM values were derived from analysis of variance.

Treatment	[3H] PIA bound (fmol/mg protein)								
PIA (nM)...	1	2	4	6	8	10	20	50	100
Saline	13.33	26.19	42.86	64.17	75.48	85.71	125.24	240.48	266.43
ArGH	6.78	14.76	28.81	39.29	55.12	84.76	103.21	227.62	254.17
ArGH + GH	4.52	13.57	22.62	35.24	48.21	47.50	82.74	205.48	232.26
SEM (pooled)	3.69	7.74	12.50	16.07	20.60	23.21	29.17	33.10	35.36

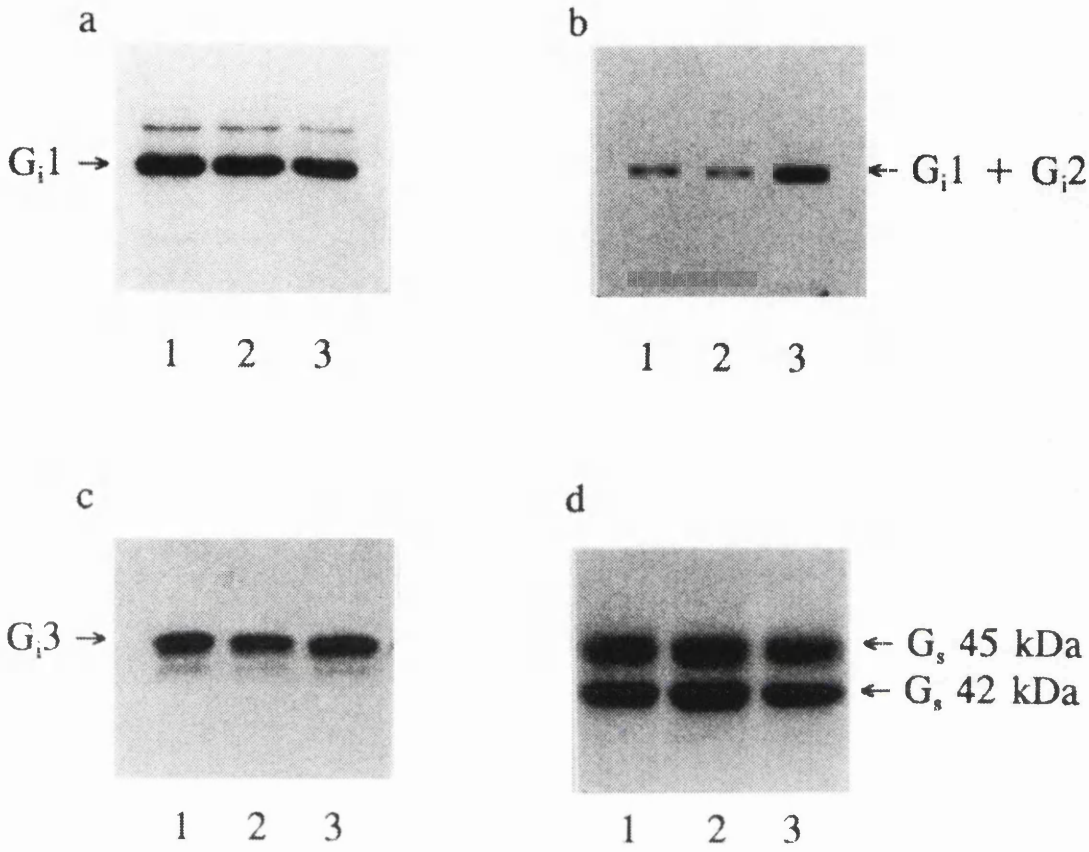


Figure 3.6 Immunoblotting of G-protein α-subunits in adipocyte membranes

(a) Detection of α-Gi1 with antiserum IIC (10 μg per track). (b) Detection of α-Gi1 plus α-Gi2 with antiserum SG1 (2.5 μg per track). (c) Detection of a α-Gi3 (10 μg per track). (d)Detection of the 42 kDa and 45 kDa forms of α-Gs with antiserum CS1 (30 μg per track). Adipocyte membranes were prepared from rats injected with (1) carrier solutions, (2) anti-rGH plus oGH or (3) anti-rGH alone. Immunoblotting was performed as described in the material and methods section. Data shown are typical of experiments done five times using membranes from different rats.

Table 3.3 Expression of G-protein α-subunits in adipocyte membranes

Adipocyte membranes were prepared from rats which had received the indicated injections. Levels of α-subunits, detected by immunoblotting, are given in arbitrary units (see experimental section). Results are means + SEM for 5 observations. a and b indicate that the value is significantly different ($P < 0.001$) from the value for animals treated with carrier solutions or anti-rGH respectively.

Injections G-protein.....	G_i1	$G_i1 + G_i2$	G_i3	G_s (45 kDa)	G_s (42 kDa)
Carrier solutions	189	333	0.89	3503	3314
Anti-rGH	233	911[a]	0.88	3488	3842
Anti-rGH, oGH	173	206[b]	0.76	3255	3527
SEM	48	78.1	0.2	288	430

CHAPTER FOUR

STUDIES ON THE LIPOLYTIC ACTION OF GROWTH HORMONE IN LACTATING RAT ADIPOSE TISSUE

4.1 Introduction

Lactation usually results in a partial depletion of the lipid reserves of white adipose tissue, which are susequently restored on removal of the litter or during natural weaning (Vernon and Flint, 1984). The restoration of lipid reserves in the rat after litter removal is of interest because it is associated with a transient fall in the lipolytic response to catecholamines (Vernon and Finley, 1986), which may be due to a defect in growth hormone action (Vernon *et al*, 1987). Curiously despite there being a loss of lipid from adipocytes during lactation in the rat, the response and sensitivity to the lipolytic effects of catecholamines is unchanged but response to adenosine is increased (Aitchison *et al*, 1982; Vernon and Finley, 1986; Vernon *et al*, 1995). Response to adenosine decreases slowly on litter removal (Vernon and Finley, 1986) and this decrease is markedly accelerated by treatment with growth hormone, but not prolactin (Vernon *et al*, 1987). Effects of litter removal on the lipolytic response to catecholamines can be mimicked by treating rats with antiserum to rat growth hormone plus bromocriptine (to lower serum prolactin) (Vernon *et al*, 1987; Barber *et al*, 1992).

In light of the results obtained in chapter 3 (Doris *et al*, 1994) showing the inhbitory G-protein G_i as a target of growth homone action it was decided to investigate and identify the components involved in response to growth hormone treatment in lactating rats and in rats in which lactation had been prematurely terminated. The study was carried out to determine if changes are due to alterations in the amount of G_i, or in the case of adenosine, receptor number and to see if the increased response to adenosine (PIA) seen during lactation is due to a change in the amount of a component of the antilipolytic signal transduction system.

4.2 Experimental procedure

Wistar rats (A. Tuck and Son, Rayleigh, Essex, UK) were fed on Labsure irradiated CRM diet (57% carbohydrate, 18% protein, 2.4% fat; Labsure, Poole, Dorset, UK) and water ad libitum. They were mated 2-3 months of age, and the number of pups per mother was adjusted to ten within 24 h after birth. Injections were begun on days 10-14 of lactation. Four groups of lactating rats were used: some lactating rats were injected with a γ-globulin fraction of antiserum to rat growth hormone (220 mg/injection, equivalent to 4.5ml antiserum) plus bromocriptine (500 μg/injection), some were injected as mentioned and were also given 1.0 mg of sheep growth hormone (a gift from NIADDK, Bethesda, MD, USA), the third group were injected with growth hormone only and the fourth group of controls were injected with a carrier solution (Madon *et al*, 1986). For litter-removal rats one group were injected with growth hormone as described earlier and the second group of controls were injected with carrier solutions, starting at the time of litter removal. Control age-matched, virgin rats were injected with carrier solutions only. All injections were subcutaneous and were administered twice daily at 09:00 and 17:00 h for two days: rats were not injected at 09:00 h on day 3 and were anaesthetized. Immediately after induction of anaesthesia, samples of blood and parametrial adipose tissue were taken, after which rats were killed by exsanguination. There were ten rats in each treatment group; five were used for measurement of lipolysis and five for preparation of membranes to be used in receptor binding studies and G-protein concentration determination (Chapter 2, sections 2.3.4, 2.3.7 and 2.3.16 respectively).

4.3 Results

4.3.1 Litter weights

To determine effectiveness of the treatments litter weights were measured at 10.00h each day at time of injections. The litter weight of the lactating rats increased by 22.7 $\pm$ 2.18 g over the 48 h, treatment of lactating rats with growth hormone showed a litter weight gain of 23.4 $\pm$ 1.29 g (Fig. 4.1). Treatment with anti-rGH plus bromocriptine resulted in a smaller ($P < 0.01$) increase in litter weight of 3.71 $\pm$ 1.32 g and replacement therapy with ovine growth hormone resulted in an intermediate increase in litter weight of 12.86 $\pm$ 0.58 g (Fig. 4.1). Therefore, treatment of lactating rats with growth hormone appeared to have no effect on pup litter weights. However, growth hormone replacement partially reversed the effects of bromocriptine and anti-rGH indicating a positive response to the hormone.

4.3.2 Serum IGF-I and Insulin levels

Lactation reduced serum IGF-I concentration and this was decreased further by treatment of rats with anti-rGH plus bromocriptine for 2 days (Table 4.1). In contrast, two days after litter removal, the serum IGF-I levels were increased although not back to levels found in vigin rats (Table 4.1). Treatment with growth hormone increased serum IGF-I concentrations in litter removed rats and in rats treated with anti-rGH plus bromocriptine (Table 4.1). Growth hormone treatment was less effective in lactating rats, and although a significant effect ($P < 0.05$) was apparent when log transformed values were analysed, the level achieved was significantly less than found in rats in which lactation had been terminated either by litter removal or endocrine manipulation.

Lactation had no apparent effect on serum insulin concentrations, but litter removal showed an increase in serum insulin levels, while treatment with anti-rGH

plus bromocriptine resulted in an intermediate value (Table 4.1). Treatment with growth hormone had no effect on serum insulin concentrations (Table 4.1).

4.3.3 Basal and Maximally stimulated lipoysis

Basal lipolysis was increased by lactation ($P < 0.01$) but was seen to return to the levels seen in virgin rats upon litter removal or treatment with anti-rat growth hormone plus bromocriptine. Treatment with growth hormone had no effect on the levels of basal lipolysis (Table 4.2).

Maximal stimulation of lipolysis was achieved, as reported previously (Vernon *et al*, 1987), by including 0.8 μg/ml adenosine deaminase and 100 nM isoprenaline in incubations. Lactation itself had no effect on maximal lipolysis (Table 4.2). At maximal lipolysis, litter removal and treatment with anti-rat growth hormone plus bromocriptine resulted in a significant decrease in lipolysis ($P < 0.05$). ANOVA showed that growth hormone increased the maximal rate of lipolysis ($P < 0.005$) (pooled data for all states) (Table 4.2). Comparison of individual treatment means showed a significant effect of growth hormone with lactating rats treated with anti-rGH plus bromocriptine. Analysis of log transformed data also showed a significant ($P < 0.05$) effect of growth hormone in litter removed rats.

4.3.4 Lipolytic response to PIA

Maximum stimulation of lipolysis was achieved by including 0.8 μg/ml adenosine deaminase and 100 nM isoprenaline. Lipolysis was then inhibited in a dose-response manner using PIA (1 to 100 nM). A concentration of 100 nM was found previously to induce essentially maximum inhibition in adipocytes from lactating rats (Vernon *et al*, 1993) and this is apparent in the present study if results are expressed on a linear scale. Lactation increased the maximum response to PIA

($P < 0.05$) (Fig. 4.2), and the response was further increased by either litter removal or treatment with anti-rGH plus bromocriptine ($P < 0.05$) (Fig. 4.2). Sensitivity did not change with state (ED_{50} mean pooled values for all states = 20.4 $\pm$ 3.3 nM); treatment of lactating rats with anti-rGH plus bromocriptine resulted in an apparent decrease in sensitivity but was not significant (ED_{50} = 24.8 nM). Treatment with growth hormone had no effect on the inhibition of isoprenaline-stimulated lipolysis by PIA in adipocytes from lactating rats (Fig. 4.2), but growth hormone markedly decreased PIA induced inhibition in adipocytes from either litter-removed or anti-rGH plus bromocriptine treated rats (Fig. 4.2). Treatment with growth hormone had no apparent effect on sensitivity to PIA.

4.3.5 Lipolytic response to Prostaglandin E_1

In contrast to the effect of PIA, lactation had no effect on the ability of prostaglandin E_1 (PGE_1) to inhibit isoprenaline-stimulated lipolysis (Fig. 4.3). However, the inhibition was enhanced ($P < 0.05$) by litter-removal or treatment of rats with bromocriptine and anti-rGH (Fig. 4.3). As found with PIA treatment, treatment of lactating rats with growth hormone had no effect on inhibition of lipolysis by PGE_1, but markedly suppressed the antilipolytic effect of PGE_1 in the litter removed and anti-rGH plus bromocriptine treated rats (Fig. 4.3); indeed after growth hormone treatment the degree of inhibition induced by PGE_1 in these groups of rats was less than that found in either growth hormone-treated or untreated lactating rats.

4.3.6 Ligand binding to adenosine receptors

Measurement of ligand binding was carried out using rat adipocyte membranes to ascertain if the changes in response to PIA was due to changes in levels of receptor numbers. Studies by Doris *et al*, (1994) showed that, essentially

maximum binding is achieved with [^{3}H]-PIA 100 nM, therefore in the present study the number of adenosine receptors was assessed by measuring the binding of [^{3}H]-PIA to adipocyte membranes using two concentrations of PIA, 20 nM and 100 nM. Lactation, treatment of lactating rats with bromocriptine and anti-rGH or litter-removal had no effect on PIA binding (Table 4.3), but ANOVA showed that growth hormone did decrease binding to membranes from litter removed rats (Table 4.3). The results are the means of 5 observations.

4.3.7 Immunoblotting of α-subunits of G_i and G_s

The amounts of GTP-binding protein α-subunits were determined by immunoblotting following seperation by SDS gel electrophoresis. Lactation, litter removal and treatment with anti-rGH plus bromocriptine had no effect on the amount of G_i1+2 or either isoform of G_s (Table 4.4). Similarly treatment of rats with growth hormone had no effect on the amounts of G_i1+2 or either isoform of G_s (Table 4.5). These results differ from those of Doris *et al* (1994) that showed that treatment of virgin rats with antiserum to growth hormone increased the levels of the inhibitory G-protein G_i2.

4.4 Discussion

The present study was carried out to investigate further the effects of growth hormone on the G_i-based antilipolytic system in the lactating rat. In addition the opportunity was also taken to explore the molecular basis for the enhanced response to antilipolytic factors during lactating (Vernon *et al*, 1983) and premature cessation of lactation in rats (Vernon *et al*, 1987).

This study shows that, in the rat, the increased response to antiloplytic factors during lactation does not appear to be due to either an increase in the number of receptors in the case of adenosine or a change in the amount of G_i1 plus

G_i2. As response to prostaglandin E_1 is also enhanced, this would suggest a change in some common component of both the adenosine and prostaglandin E systems, probably G_i. An increased response to adenosine is also found in lacatating sheep (Vernon and Finley, 1985; Vernon *et al*, 1995) with no change in the number of adenosine receptors observed, however, a small increase in the amount of G_i1 and G_i2 was found (Vernon *et al*, 1995). As G_i activity is known to be subject to phosphorylation by both protein kinase A and protein kinase C (Morris *et al*, 1994), a more subtle change such as this may account for the increased ability of adenosine and prostaglandin E_1 to inhibit lipolysis during lactation in the rat.

Effects of lactation on the amount of G_s isoforms has not been described previously for rats, but in studies with lactating sheep (Vernon *et al*, 1995), a significant increase in the amount of the 42 kDa form was found, in omental adipose tissue (also an abdominal depot). However in sheep, unlike rats (Vernon *et al*, 1993), lactation increases both the lipolytic response and sensitivity to β-adrenergic agonists (Vernon and Finley, 1985; Vernon *et al*, 1995).

The decrease in maximum lipolytic response to isoprenaline on either litter removal or treatment with anti-rGH plus bromocriptine agrees with previous studies (Vernon *et al*, 1987; Barber *et al*, 1992), but the lack of effect of litter removal on the amounts of G_i is in contrast with the studies of Ros *et al* (1992) who reported a decrease in the amount of both G_i (isoforms 1 plus 2) and G_s upon litter removal. However, the latter observation at least is perhaps surprising as the litter removal has no effect on the ability of isoprenaline to activate protein kinase A, so signalling through G_s would not appear to be altered in this state (Vernon *et al*, 1993). It would appear that the diminished lipolytic effects of β-agonist is due to a failure of hormone sensitive lipase translocation to the lipid droplet (Vernon *et al*, 1993).

The present study also shows that the ability of growth hormone to modulate signalling through the G_i-based antilipolytic system is species specific and depends on the physiological state of the animal.

Serum growth hormone levels were assessed indirectly by weighing litters and determining serum IGF-I and insulin levels. Litter weights were unaffected by treatment of lactating rats with growth hormone but when both growth hormone and prolactin are removed using antiserum to growth hormone and bromocriptine respectively, litter weight gain dropped dramatically, an effect that was partially relieved upon the addition of growth hormone and therefore indicated a response to growth hormone treatment. These results were also reflected in the serum concentrations of IGF-I and this was also evident in litter removed rats.

In contrast to a previous study in lactating cows (Lanna *et al*, 1995), chronic treatment of lactating rats with growth hormone had no effect on maximum response or sensitivity to PIA, thus showing clearly that there is a species difference in this effect of growth hormone. The reason for the difference is unclear but could reflect the much higher mean serum growth hormone concentration in rats than in cattle, even when the latter are lactating (Harvey and Daughaday, 1995).

In contrast to lactating rats, the ability of growth hormone to decrease the ability of both PIA and PGE_1 to inhibit lipolysis was markedly enhanced by premature termination of lactation, either by litter removal or by endocrine manipulation. In these rats, growth hormone treatment decreased inhibition by these antilipolytic agents to levels lower than that found in untreated lactating rats so that the levels of inhibition achieved were at least that seen in virgin rats. This would suggest that the ability of growth hormone to exert its effects in lactating rats is prevented by an unknown factor; the identity of this putative factor would not appear to be prolactin as neither treatment of lactating rats with bromocriptine alone nor treatment of litter removed rats with prolactin had any effect on the ability of PIA to inhibit lipolysis (Vernon *et al*, 1987).

The diminished ability of antilipolyltic agents to inhbit lipolysis after growth hormone treatment in rats in which lactation is prematurely terminated does not appear to involve a change in either the number of adenosine receptors or the amount of G_i1 plus G_i2. These results are consistent with studies in castrated male sheep (Chapter 5; Doris *et al*, 1995) suggesting that a change in activity of one of these components is involved. However, these findings contrast with those outlined in Chapter 3 of this thesis that showed that lowering growth hormone with a specific antiserum in virgin rats markedly increased the amount of G_i2. It is unclear why treatment of bromocriptine-treated rats with this same antiserum to growth hormone should have no effect on the amount of G_i1 plus G_i2, but presumably this reflects other endocrine differences in these states.

Interestingly, it would appear that in rats, at least the amount of G_i modulates sensitivity to PIA and prostaglandin E_1, whereas a maximum response to these agents is under different, more subtle control involving no change in protein concentration but probably involving covalent modification.

Effect of growth hormone treatment on rat litter weights

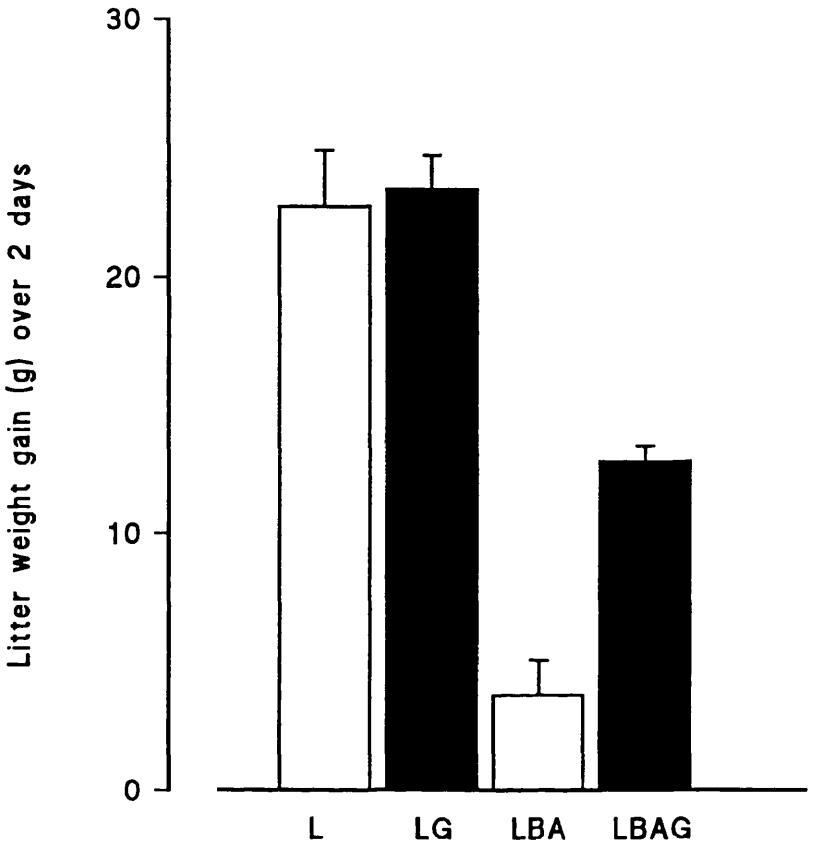


Figure 4.1 LItters from Lactating rats (L), lactating rats treated for 2 days with GH (LG), lactating rats which had been treated with ArGH plus bromocriptine (LBA),or treated with ArGH plus bromocriptine plus sheep GH (LBAG), were weighed before and after 48h treatments. Results are the means ± SEM of 10 observations.

Table 4.1 Effect of growth hormone treatment on serum IGF-I and Insulin levels

Blood samples was obtained from virgin rats, lactating rats, lactating rats which had been pretreated for 2 days with antiserum to rat growth hormone (ArGH) plus bromocriptine (Br), and lactating rats 2 days after litter removal, treated with or without sheep growth hormone or carrier solution. Serum IGF-I and insulin was measured (sections 2.3.13 and 2.3.12); results are the means of 10 observations ± SEM (from analysis of variance). Values within a column which do not have the same superscript (a, b, c, d) differ significantly ($P < 0.05$).

State	Treatment	IGF-I (ng/ml)	Insulin (ng/ml)
Virgin	carrier	555^{c} ± 31	0.27^{a} ± .061
Lactating	carrier	337^{ab} ± 46	0.28^{ab} ± .055
Lactating	GH	466^{a} ± 46	0.20^{a} ± .054
Lactating	ArGH + Br	283^{b} ± 48	0.41^{bc} ± .058
Lactating	ArGH + Br + GH	653^{c} ± 46	0.31^{ab} ± .054
Litter removed	carrier	448^{a} ± 46	0.54^{c} ± .054
Litter removed	GH	777^{c} ± 48	0.57^{c} ± .054

Table 4.2 Effects of lactation, litter removal and varying serum GH concentration on basal and maximal lipolysis

Adipose tissue was obtained from virgin rats, lacatating rats, lactating rats which had been pre-treated for 2 days with an antiserum to rat GH (ArGH) plus bromocriptine, and lactating rats 2 days after litter removal treated, with or without sheep GH. Glycerol release was measured in the presence of 0.8 μg/ml adenosine deaminase (basal) or adenosine deaminase plus 100 nM isoprenaline (maximal). The results are the means of five observations and the SEM is determined by analysis of variance of all values, except for virgin rat where n = 4. Values in the same column without the same superscript a and b are significantly different ($P < 0.05$).

Physiological state	Basal	SEM	Maximal	SEM
Lactating	0.25a	0.025	3.80a	0.41
Litter removed	0.11b	0.025	2.00b	0.41
Lactating + ArGH + Br	0.17ab	0.025	2.28b	0.41
Virgin	0.14ab	0.028	3.11ab	0.46

State	Basal		Maximal	
	-GH	GH	-GH	+GH
Lactating	0.25^{a}	0.24^{a}	3.80^{ab}	4.50^{a}
Litter removed	0.11^{b}	0.14^{b}	2.00^{b}	2.88^{bc}
Lactating + AGs + Br	0.17^{b}	0.17^{b}	2.28^{b}	3.51^{ac}
SEM	0.025	0.025	0.39	0.39

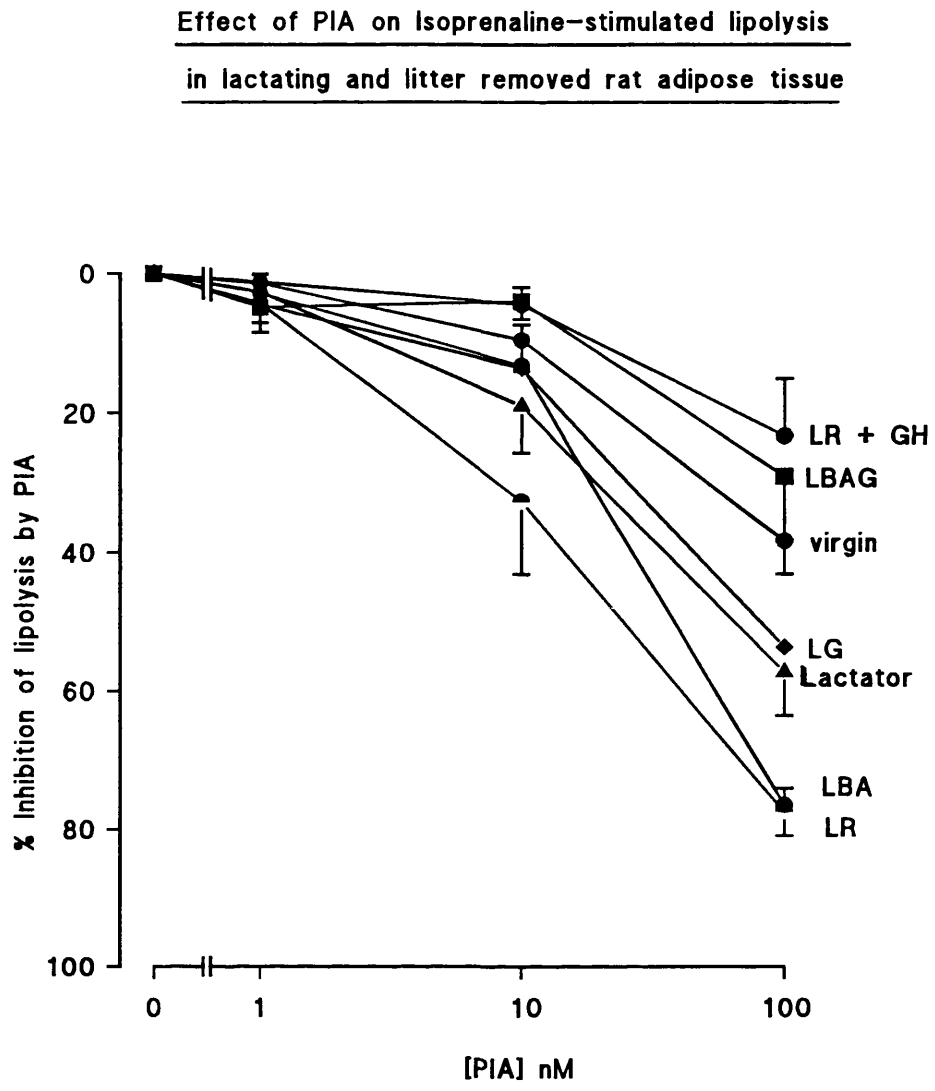


Figure 4.2 Adipose tissue was obtained from virgin rats, lactating rats, lactating rats treated with GH (LG), or with bromocriptine plus ArGH (LBA), or LBA rats treated with GH (LBAG), and litter-removed rats (LR) or LR rats treated with GH (LR + GH). Lipolysis was measured in the presence of 0.8 ug/ml adenosine deaminase and isoprenaline 100 nM and the concentration of PIA indicated above. Results are the means ± SEM of 5 observations.

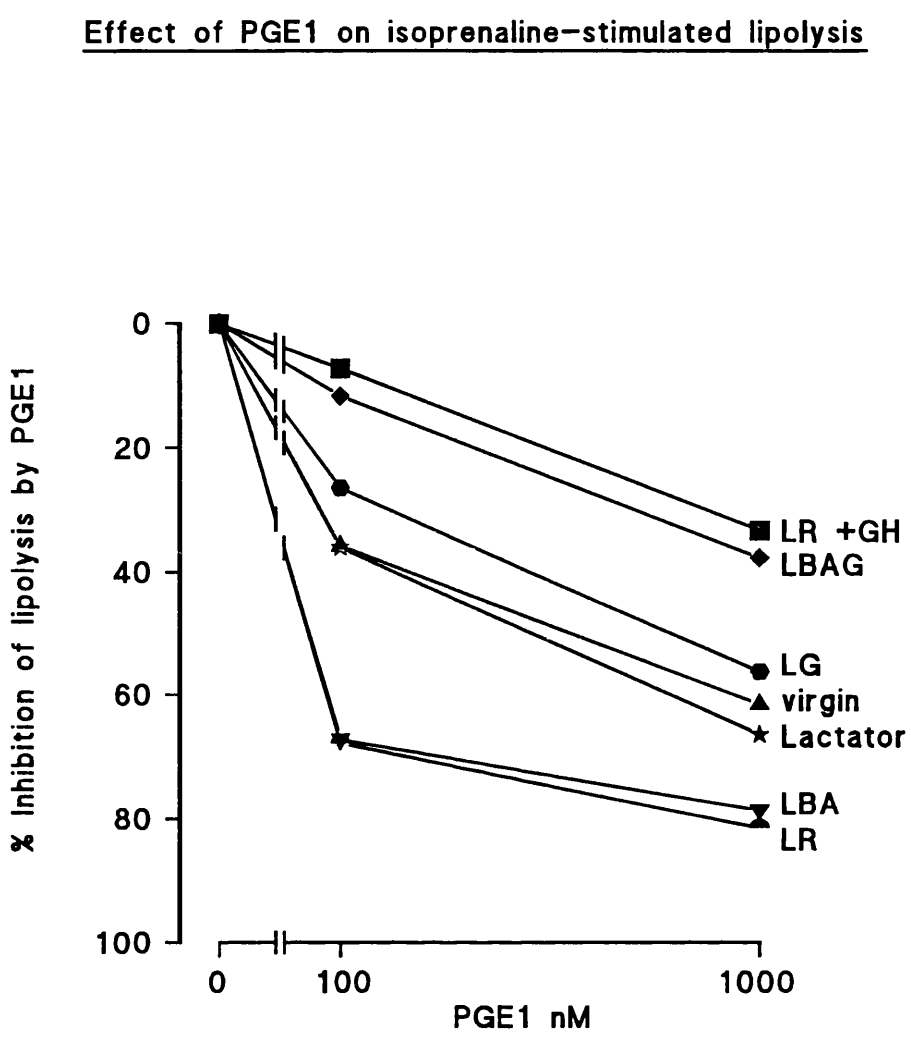


Figure 4.3 Adipose tissue was obtained from virgin rats, lactating rats, lactating rats treated awith GH (LG), or with bromocriptine plus anti-rGH (LBA), or LBA rats treated with GH (LBAG), and litter-removed rats (LR) or LR rats treated with GH (LR + GH). Lipolysis was measured in the presence of 0.8 ug/ml adenosine deaminase and isoprenaline 100 nM and the concentration of PGE1 indicated above. Results are the means of 5 observations and the SED for comparing values is 7.20.

Table 4.3 Effects of lactation, litter removal and varying serum GH concentration on adenosine receptor number in the rat

Adipose tissue was obtained from virgin rats, lactating rats, lactating rats which had been pre-treated for 2 days with an antiserum to rat GH (ArGH) plus bromocriptine, and lactating rats 2 days after litter removal treated, with or without sheep GH. Adipocyte membranes were prepared as described in the experimental section and levels of adenosine receptors were determined using [^{3}H]-PIA. The results are the means of 5 observations and the SEM was determined by analysis of variance of all values. Values without the same superscript a and b are significantly different ($P < 0.05$).

Physiological state	(fmol ligand bound/ mg of protein)	20 nM [^{3}H]PIA	100 nM [^{3}H]PIA	SEM
Lactating		36.5	62.3	10.75
Litter removed		28.4	47.6	10.75
Lactating + ArGH + Br		22.8	50.0	10.75
Virgin		28.9	50.7	10.75

State	(fmol ligand bound/ mg of protein)	-GH	+GH
Lactating		49.4^{a}	31.9^{ab}
Litter removed		38.0^{ab}	22.5^{b}
Lactating + AGs + Br		36.4^{ab}	51.3^{a}
SEM		5.85	5.85

Table 4.4 Expression of G-protein α-subunits in adipocyte membranes

Adipocyte membranes were prepared from lactating rats, lactating rats which had been pre-treated for 2 days with an antiserum to rat GH (ArGH) plus bromocriptine (Br), and lactating rats after litter removal. Levels of α-subunits of G-proteins, detected by immunoblotting, were determined by counting γ-radiation of excised bands. Results are the means ± SEM of 4 (virgin) or 5 (rest) observations.

	Arbitrary units / mg protein		
State — G-protein	G_i1+2	G_s (45kDa)	G_s (42kDa)
Lactating	655 ± 127[a]	2692 ± 424[a]	1510 ± 318[a]
Litter removed	588 ± 127[a]	1907 ± 424[a]	1494 ± 318[a]
Lactating + ArGH + Br	428 ± 127[a]	1586 ± 424[a]	1176 ± 318[a]
Virgin	399 ± 142[a]	1879 ± 474[a]	1497 ± 356[a]

Table 4.5 Effect of growth hormone on expression of G-protein α-subunits in rat adipocyte membranes

Adipocyte membranes were prepared from lactating rats, lactating rats which had been pre-treated for 2 days with an antiserum to rat GH (ArGH) plus bromocriptine, and lactating rats after litter removal treated, with or without sheep GH. Levels of α-subunits of G-proteins, detected by immunoblotting, were determined by counting γ-radiation of excised bands. Results are the means ± SEM for 4 or 5 observations.

		Arbitrary units / mg protein					
State	G-protein....	G_i1+2		G_s (45 kDa)		G_s (42 kDa)	
		-GH	+GH	-GH	+GH	-GH	+GH
Lactating		655 ± 190	1025 ± 190	2692 ± 378	2072 ± 378	1570 ± 274	1612 ± 274
Lactating + ArGH + Br		428 ± 190	694 ± 190	1586 ± 378	1758 ± 378	1176 ± 274	1461 ± 274
Litter removed		588 ± 190	587 ± 208	1907 ± 378	1775 ± 378	1494 ± 274	1496 ± 301

CHAPTER FIVE

STUDIES ON THE LIPOLYTIC ACTION OF GROWTH HORMONE IN SHEEP ADIPOSE TISSUE *IN VIVO*

5.1 Introduction

Growth hormone increases lipolysis in adipose tissue, but this appears to be achieved via indirect mechanisms. The chronic treatment with growth hormone *in vivo* have been shown to increase the lipolytic response to catecholamines *in vitro* in rats (Vernon *et al*, 1987) and *in vivo* in cows (McCutcheon and Bauman, 1986; Peters, 1986; Sechen *et al*, 1990). Maintaining sheep adipose tissue in culture with growth hormone again increased maximum response to β-agonists (Watt *et al*, 1991). In contrast, chronic treatment of cattle with bovine growth hormone *in vivo* had no effect on the response of subcutaneous adipose tissue to catecholamines *in vitro* (Peters, 1986; Lanna *et al*, 1992). Chronic treatment with bovine growth hormone *in vivo* decreases the response to the antilipolytic effects of the adenosine analog N^6-phenylisopropyladenosine (PIA) in rat (Vernon *et al*, 1987; Doris *et al*, 1994) and bovine (Lanna *et al*, 1992) adipose tissue *in vitro*.

The effects of growth hormone on the lipolytic signalling system were explored futher by chronically treating sheep for up to 7 days with bovine growth hormone and then assessing the lipolytic rate *in vitro* in subcutaneous adipose tissue. To probe the mechanism of the effect of chronic growth hormone treatment, the numbers of β- and α2-adrenergic receptors and adenosine receptors amd the amounts of the heterotrimeric GTP-binding proteins, which transmit signals from the above receptors to adenylate cyclase, were determined.

5.2 Experimental

Ten female Finn x Dorset Horn sheep, mean body weight 55.2 kg (range 44 to 64) were used. The sheep were individually housed in metabolism crates, in sight of other sheep, for 7 days. During this time they were injected with either bovine somatotropin (10 mg in 2 ml buffer i.m. daily) or vehichle (control). The animals were fed at 0800 and 1500 and the meals consisted of 250g of a cereal mix

plus hay to eat for 2 to 3 hours; drinking water was always available. One control and one growth hormone-treated (bovine growth hormone was a gift from Monsanto Europe, B-1150, Brussels, Belgium) sheep were used each week of the expiment. On day 7, pentobarbital (20-30 ml) was injected intravenously and sheep killed by exsanguination. Samples of subcutaneous tissue were removed and placed in 0.15M NaCl at 37^{o}C for transportation to the laboratory (less than five minutes), and used in lipolysis experiments and the preparation of membranes for receptor and G-protein analysis.

Analysis of results

Results were analysed by ANOVA with week and treatment , or treatment and PIA concentration and their interaction as factors when analysing data. A P value of less than 0.05 was considered significant.

5.3 Results

5.3.1 Basal and Maximal Lipolysis

Adenosine deaminase 0.8 ug/ml was added to remove any endogenously produced adenosine and this caused a small ($P < 0.01$) increase in the rate of basal lipolysis (Figure 5.1). Lipolysis was increased by the addition of the β-agonist isoprenaline ($P < 0.001$) (Figure 5.1). Chronic treatment of sheep with growth hormone increased basal, adenosine deaminase-stimulated, and isoprenaline-stimulated lipolysis (Figure 5.1). The increase in the rate of lipolysis induced by isoprenaline was determined as the rate of lipolysis in the presence of isoprenaline minus the rate in the absence of isoprenaline, and was increased ($P < 0.05$) by chronic growth hormone treatment (5.37 and 7.18 $\pm$ 0.43 μmol/ 3 h/10^6 cells, respectively, for control and growth hormone-treated sheep).

5.3.2 Lipolytic response to PIA and PGE_1

Chronic treatment with growth hormone *in vivo* also diminished the response ot acutely acting antilipolytic factors. Effects of adenosine and prostaglandin E_1 were assessed by stimulating tissue with isoprenaline plus adenosine deaminase and adding various concentrations of either the adenosine analogue PIA or PGE_1.

Pre-treatment *in vivo* with growth hormone markedly decreased ($P < 0.01$) the response to PIA (Figure 5.2) (results are the mean of 5 observations; SEM are 8.02 and 4.45 for comparing values between control and growth hormone-treated sheep respectively). With PGE_1, a concentration of 10 nM had no effect on the rate of isoprenaline-stimulated lipolysis in either control or growth hormone-treated sheep. However 100 nM PGE_1 reduced ($P < 0.01$) the rate of lipolysis in control sheep (5.79 and 4.32 $\pm$ 0.23 μmol/ 3 h/ 10^6 cells in the absence and presence of PGE_1 respectively) (Figure 5.3), but not in growth hormone-treated sheep (7.85 and 7.62 $\pm$ 0.40 μmol/ 3 h/ 10^6 cells in the absence and presence of PGE_1 respectively) (Figure 5.3).

5.3.3 Ligand binding to adenosine, β-adrenergic and α2-adrenergic receptors

Binding studies were carried out using a single maximum concentration of labelled ligand at 20 nM, as previous studies had shown that concentrations of ligand at this level was sufficient to achieve essentially maximum binding with each of the three receptors studied (Vernon *et al*, 1995). The amount of [^{3}H]dihydroalprenolol bound to the β-adrenergic receptors was 50.4 and 70.1 fmol/mg protein ($P < 0.05$) for control and growth hormone-treated sheep respectively (Figure 5.4), (results are means of 5 observations; SEM was 4.9 fmol/mg protein), indicating a small increase in the number of β-receptors. The

amount of [^{3}H]rauwolscine bound to α2-adrenergic receptors was 10.9 and 21.5 fmol/mg protein ($P > 0.10$) for control and growth hormone-treated sheep respectively (Figure 5.4), (results are means of 5 observations, SEM was 3.1 fmol/mg protein) and indicated no significant effect of treatment. Similarly, no effect of growth hormone-treatment was seen in [^{3}H]PIA binding to adenosine receptors and was 21.1 and 17.6 fmol/mg protein ($P > 0.75$) for control and growth hormone-treated sheep respectively (Figure 5.4), (results are means of 5 observations, SEM was 7.4 fmol/mg protein).

5.3.4 Immunoblotting of α-subunits of G_i1, G_i2, G_i3 and G_s

Anti-peptide antisera were used to ascertain changes in the levels of α-subunits of individual G-proteins in adipocyte membranes from control and growth hormone-treated sheep. The results are expressed as arbitrary units (Table 5.1) and it should be noted that, as the affinity of the different antisera are unknown, the absolute amounts of Gα subunits cannot be commented upon.

Immunoblotting revealed two isoforms of G_s with molecular weights of about 42- and 45-kDa as found previously in sheep (Vernon *et al*, 1995) and rat (Strassheim *et al*, 1990; Doris *et al*, 1994) adipocytes; sheep, however, differ from rats in that there is relatively more 42-kDa than 45-kDa G_s, whereas in rats both isoforms are present in roughly equal amounts. Treatment of growth hormone *in vivo* had no effect on the amount of either isoform of G_s (Table 5.1). Immunoblotting with the antiserum to G_i1 and G_i2 revealed a single band as found previously for sheep (Vernon *et al*, 1995) and rat (Mitchell *et al*, 1989; Doris *et al*, 1994) adipocytes. Pretreatment of sheep with growth hormone *in vivo* had no effect on the amount of G_i1 plus G_i2 (Table 5.1)

5.4 Discussion

Catecholamines and adenosine operate via a very similar mechanism of binding to a receptor in the plasma membrane that activates a G-protein, which then interacts with adenylate cyclase causing a change in cAMP concentration. This in turn alters the activity of protein kinase A, an enzyme that phosphorylates a number of key enzymes of which hormone-sensitive lipase in adipose tissue is an example (Vernon and Sasaki, 1991). Catecholamines acting via the β-adrenergic receptor cause activation of adenylate cyclase, but via another distinct receptor, the α2-adrenergic receptor, they can inhibit adenylate cyclase (Vernon and Sasaki, 1991). Depending on the tissue, adenosine can cause either activation or inhibtion of adenylate cyclase; in adipose tissue it causes inhibition, while in brain tissue it causes activation (Stiles, 1986).

Studies on the response to catecholamines in ruminants have focused on their well-defined effects on lipolysis in adipose tissue. Earlier studies *in vitro* suggested that the response to catecholamines in ruminant adipose tissue was less than that in the rat (see Vernon, 1980) but other studies have shown that catecholamines can elicit a considerable increase in the rate of lipolysis *in vivo* (Gooden et al, 1986; Thompson, 1986) and *in vitro* (Vernon and Finley, 1985; McNamara, 1991).

In the present studies, chronic treatment of sheep with growth hormone resulted in a small increase in the rate of basal and catecholamine-stimulated lipolysis *in vitro*. However, studies carried out in which cattle were chronically treated with bovine growth hormone, resulted in a large increase in response to catecholamines *in vivo* (McCutcheon and Bauman, 1986; Peters, 1986; Sechen *et al*, 1990) but no apparent change in response to catecholamines in subcutaneous adipose tissue *in vitro* (Peters, 1986; Lanna *et al*, 1992). The latter difference between sheep and cattle may reflect the fact that the increased response to

catecholamines *in vitro* is small and may not have been detected in earlier studies in cattle where SEM values were relatively high.

The apparent lack of increased response of subucutaneous tissue at least to catecholamines *in vitro* following growth hormone treatment has promoted a search for an alternative mechanism to account for the *in vivo* effect, and this has led to a focus on the adenosine antilipolytic system (Lanna *et al*, 1992). Earlier studies have showed that growth hormone treatment decreased the response to PIA in rats (Vernon *et al*, 1987) *in vitro* and sheep adipose tissue *in vitro* (Vernon *et al*, 1991). This study shows that growth hormone treatment of sheep *in vivo* markedly diminishes the response of subcutaneous adipose tissue to adenosine (PIA).

Prostaglandins of the E series are known to be antilipolytic, at least in rats and humans, and act via G_i (Richelsen, 1992). Growth hormone treatment in sheep resulted in a diminished response to prostaglandin E_1 but the concentrations needed to achieve this result were higher than those for PIA. These results then indicate that growth hormone modifies a post-receptor step common to both agents.

The increased rate of catecholamine-stimulated lipolysis could be due to an increased number of β-adrenergic receptors, and these results correspond to those of Watt *et al* (1991) that showed that incubation of sheep adipose tissue *in vitro* with growth hormone over 48 hours resulted in an increase in β-receptor numbers. Although it has been proposed that a change in receptor number is likely to change sensitivity rather than maximum response to the agonist (Kahn, 1978), this will depend on which component is limiting maximum response. In the various cell types examined, the number of molecules of G_s exceeds the number of molecules of β-adrenergic receptors by at least 100-fold (Ransas and Insel, 1988; Post *et al*, 1995). If G_s is in such excess in adipocytes, then the number of β-adrenergic receptors could be limiting and so the increase in β-receptor number could account for the enhanced rate of catecholamine-stimulated lipolysis in growth hormone

treated sheep. However, studies by Vernon *et al*, (1993) suggest that other mechanisms should not be excluded, and show that the association of hormone sensitive lipase with the lipid droplet may play a role in the altered response to catecholamines.

The lack of effect of growth hormone *in vivo* on the number of α2-adrenergic receptors agrees with the previous findings of Watt *et al* (1991) *in vitro* in which chronic exposure to growth hormone had no effect on either the number of α2-adrenergic receptors or response to the α2-adrenergic effect of noradrenaline. Although this study showed that growth hormone treatment *in vivo* markedly diminishes the response to PIA of subcutaneous tissue, this effect did not seem to be due to a change in either the number of adenosine receptors or the amount of the GTP-binding proteins, G_i1 and G_i2 that transmit inhibitory signals to adenylate cyclase. This may suggest a change in activity by one or more components of the system. Studies have shown that loss of G_i function occurs as a result of protein kinase C activation (Houslay, 1991), and this has been attributed to phosphorylation of G_i2 (Morris *et al*, 1994). In this study there was an apparent lack of effect of growth hormone on the amount of G_i. This contrasts with the previous findings, detailed in chapter 3, with rats that showed that by lowering serum growth hormone levels with a specific antiserum to growth hormone, the amount of the G-protein G_i2 was markedly increased (Doris *et al*, 1994) and was a phenomenon that could be reversed upon growth hormone replacement. This may reflect a species difference or may imply that growth hormone at low concentrations reduces G_i2 levels and at higher concentrations decreases its activity.

Therefore treatment of sheep *in vivo* alters the cyclic AMP-based signalling system of subcutaneous adipocytes by more than one mechanism. That is, an increased maximum rate of β-adrenergic-stimulated lipolysis is seen, possibly due to an increase in the number of β-adrenergic receptors. At the same time, a decrease in response to at least two antilipolytic agents, adenosine and

prostaglandin E_1 is seen. This study shows that the effect of growth hormone in sheep adipose tissue is likely to be by altering the responsiveness of adipocytes to acutely acting factors.

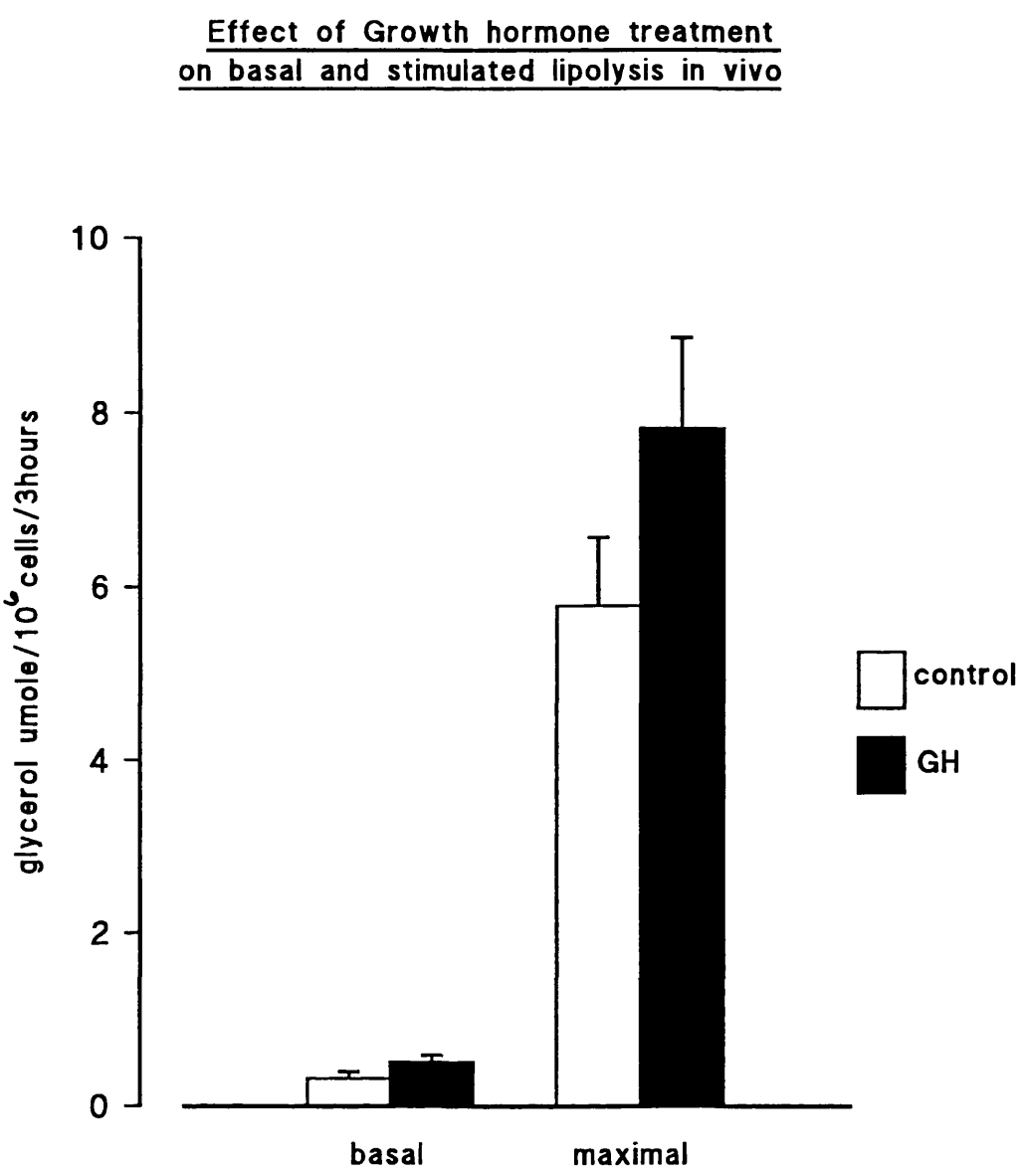


Figure 5.1 Adipose tissue was taken from GH-treated and control sheep. Glycerol release was measured, in the presence of 0.8 ug/ml adenosine deaminase (basal) or plus 100 nM isoprenaline (maximal). Results are the means ± SEM of 5 observations.

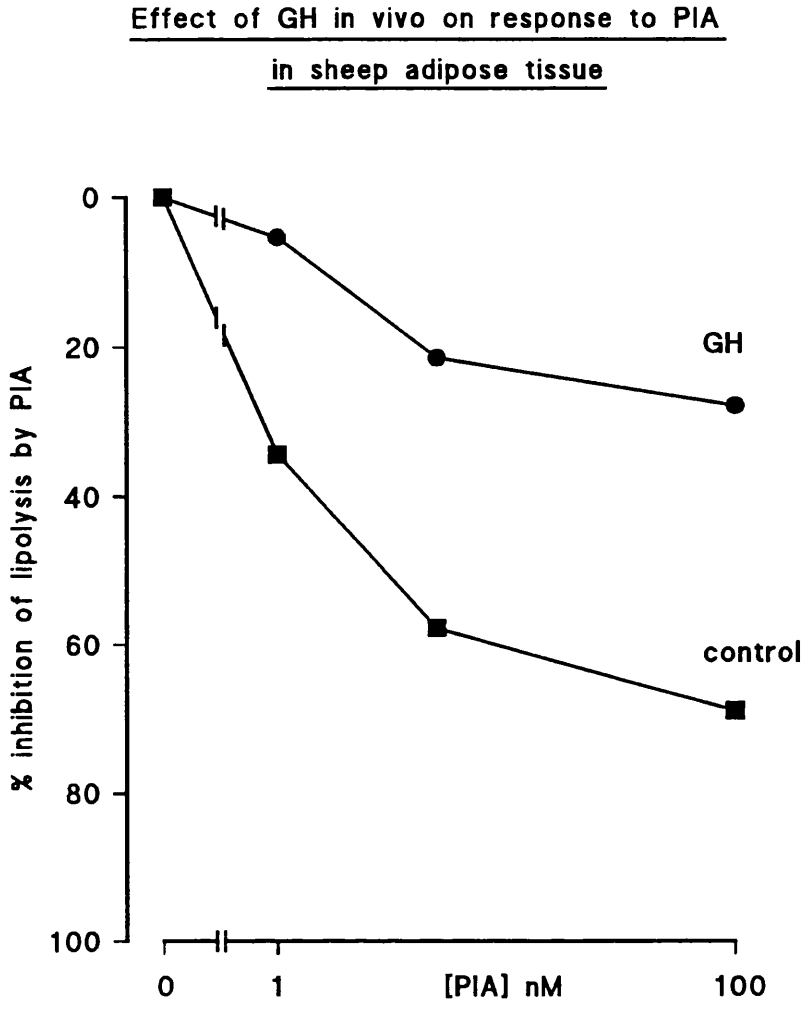


Figure 5.2 Adipose tissue was taken from GH-treated and control sheep. Glycerol release was measured,in the presence of 0.8 ug/ml adenosine deaminase, 100 nM isoprenaline and the indicated conc. of PIA. The results are the means of 5 observations. SEM are 8.02 and 4.45 for comparing values between control and GH-treated sheep respectively.

Effect of PGE1 on isoprenaline–stimulated lipolysis following manipulation of GH levels in sheep in vivo

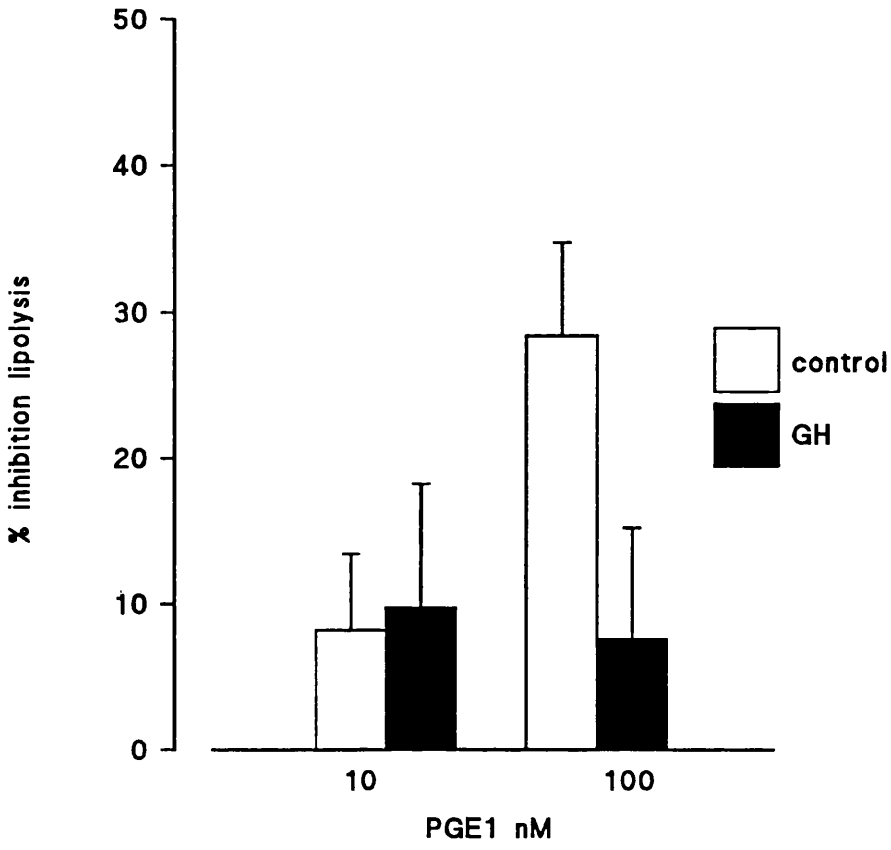


Figure 5.3 Glycerol release was measured in sheep adipose tissue in the presence of 0.8 ug/ml adenosine deaminase, 100 nM isoprenaline and the indicated concentration of PGE1. Results are the means ± SEM of 5 observations.

Effect of GH treatment on ligand binding to beta alpha-2 and adenosine receptors

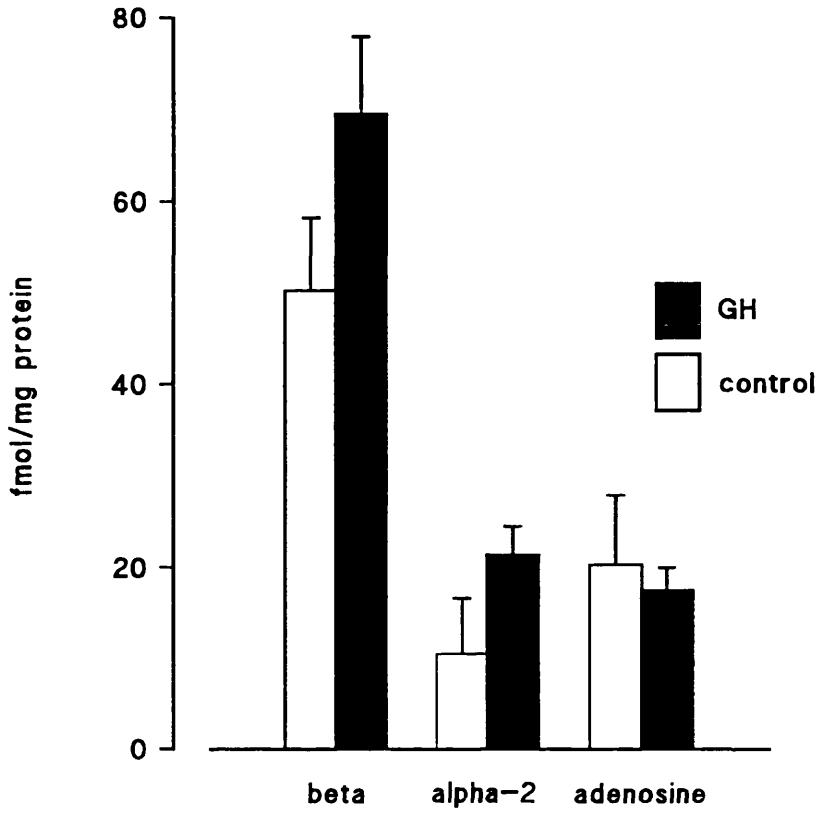


Figure 5.4 Adipose tissue was taken from control and GH-treated sheep, after which adipocyte membranes were prepared, and their ability to bind [^{3}H]dihydroalprenalol, [^{3}H]rauwolscine and [^{3}H]PIA was assessed. Results are the means $\pm$ SEM of 5 observations.

Table 5.1 Expression of G-protein α-subunits in sheep adipocyte membranes

Adipocyte membranes were prepared from control or growth hormone-treated sheep. Levels of α-subunits of G-proteins, detected by immunoblotting, are given in arbitrary units. Quantification of reactive proteins was achieved by densitometric scanning using a Bio-Rad 620 video densitometer. Results are the means of 5 observations.

Treatment	G-protein... $G_i1 + 2$	G_s (45-kDa)	G_s (42 k-Da)
Control	2.35	2.65	4.32
GH	2.47	2.93	4.46
SEM	0.10	0.21	0.31
P	0.404	0.795	0.757

CHAPTER SIX

STUDIES ON THE LIPOLYTIC ACTION OF GROWTH HORMONE IN SHEEP ADIPOSE TISSUE *IN VITRO*

6.1 Introduction

The mechanism whereby growth hormone facilitates lipolysis is not fully explained, however it is possible that growth hormone exerts its effects by modulating the acute regulation of this process by other hormones rather than having a direct effect itself. Initially studies focused on modulation of response of adipocytes to catecholamines, but more recent studies (Lanna *et al*, 1992; Doris *et al*, 1994) suggest that the G_i-mediated antilipolytic system is a major target of growth hormone action. The effect of growth hormone is of considerable economic importance, contributing to the galactopoetic effect of growth hormone (bovine somatotrophin (Bauman and Vernon, 1993)) and is being developed as a potential means of producing leaner meat animals (Boyd and Bauman, 1989; Etherton *et al*, 1993; Steele and Evock-Clover, 1993).

Growth hormone enhances the responsiveness of adipocytes to acutely acting lipolytic agents by several mechanisms. One of the more important mechanisms appears to be to decrease the ability of anti-lipolytic agents, for example adenosine and prostaglandin E, to inhibit lipolysis (Doris *et al*, 1994). Treatment of sheep *in vivo* with growth hormone leads to a diminished response to the adenosine analogue N^6-phenylisopropyladenosine (PIA) but with no apparent change in the number of adenosine receptors or amounts of G_i isoforms (Doris *et al*, 1994). This suggested a more subtle change, perhaps due to phosphorylation of G_i, altering its ability to interact with either its receptor or adenylate cyclase. Further investigations were, therefore, carried out using a tissue culture system to explore further the possible mechanism whereby growth hormone exerts its effects.

6.2 Experimental procedure

Sheep were 6-9 month old castrated males (wethers) and were fed on a diet of hay and cereals for at least one month before slaughter. On the day of killing,

sheep were anaesthetized by injection of 20-30 ml of Sagatal (May and Baker, Dagenham, Essex, UK) into the jugular vein, after which they were exsanguinated and samples of subcutaneous adipose tissue was removed asceptically from the flank fat-pads immediately anterior to the hind limbs. Adipose tissue was placed immediately in sterile 0.15 M NaCl at about 37^{o}C.

Pieces of adipose tissue (about 20 mg) were cut with scissors and then either used immediately for measurement of the rate of lipolysis or preparation of membranes, or maintained in tissue culture for up to 48 h at 37^{o}C under air/CO2 (19:1) in Medium 199 with Earle's salts, 25 mM Hepes (pH 7.3), 2 mM acetate and antibiotics (see section 2.3.3). The medium was supplemented with ovine growth hormone (a gift from NIAMDD, Bethesda, MD, USA). Adipose tissue explants were preincubate for 24 h with no hormones and then the tissue was transferred to fresh medium in the presence and absence of growth hormone (4.5 nM). Following the 48 h incubation adipose tissue was used in lipolysis experiments or in the preparation of plasma membranes. In the lipolysis experiments, PMA and okadaic acid were dissolved in dimethyl sulphoxide (DMSO) and subsequently diluted with Medium 199; maximum DMSO concentration in the culture medium was 1 μl/ml; this concentration had no effect on the variables measured in the study.

For the immunoblotting experiments, antipeptide antisera CS1 and I1C, which specifically recognise the α-subunits of the G-proteins G_s and G_i respectively, were kindly provided by Dr. G. Milligan, Dept. of Biochemistry, University of Glasgow. An antiserum which recognizes G_i1 plus G_i2 was generated against the C-terminal decapeptide of the α-subunit of rod transducin and characterized as described by Mitchell *et al* (1989).

Analysis of results

Statistical significance was assessed by analysis of variance or by Student's t-test for paired observations. The results are given as means either ± SED (standard error of difference) or ± SEM.

6.3 Results

6.3.1 Maximal Lipolysis

Sheep subcutaneous adipose tissue was cultured for 24 h in the absence of additons after which it was transferred to fresh medium in the presence and absence of growth hormone as described in section 2.3.3., after which lipolysis experiments were carried out as detailed in section 2.3.4. `Do' tissue was assayed at the time explants were initially prepared. The maximal response to isoprenaline was achieved with 100 nM agonist in the presence of 0.8 μg/ml adenosine deaminase, and increased over the 48 h incubation period from 2.09 ± 0.29 (Do) to 3.96 ± 1.17 μmol / 3h /10^6cells in control tissue and to 3.89 ± 0.80 in growth hormone-treated tissue (Fig. 6.1). Therefore, culture tended to increase maximum rate of lipolysis but the effect was not significant. The results are the means of 4 observations. In this initial experiment with 4 sheep, culture with growth hormone had no effect on maximum response to isoprenaline; however, analysis of pooled results from further experiments using tissue from 20 sheep (Table 6.1) revealed a small, but significant ($P < 0.01$) effect of growth hormone on this variable (mean rate of isoprenaline-stimulated lipolysis after culture with and without growth hormone was 4.96 and 4.22 μmol glycerol release /3h /10^6 cells respectively; SED = 0.22)

6.3.2 Lipolytic response to PIA

Maximum stimulation of lipolysis was achieved by including 0.8 μg/ml adenosine deaminase and 100 nM isoprenaline as described above. Lipolysis was then inhibited in a dose-response manner using PIA (1 nM - 100 nM).

Culture of adipose tissue in the absence of growth hormone had no effect on the response to PIA (Fig. 6.2). Treatment of sheep adipose tissue with growth hormone *in vitro* decreased ($P < 0.05$) the maximum response to adenosine from 72.8% to 40.2% inhibition of isoprenaline-stimulated lipolysis. The results are the

means of 4 observations, SED = 9.8 (Fig. 6.2). These results show that growth hormone supresses the anti-lipolytic effects of PIA by decreasing the response to maximal concentrations of this agent.

6.3.3 Ligand binding to adenosine-, α2-, and β-receptors

Measurement of ligand binding was performed with a single maximum concentration of ligand in each case at 20 nM, as reported by Watt *et al* (1991). The amount of [^{3}H]-PIA bound to adenosine receptors was 21.1 fmol/mg protein in control tissue and 22.1 fmol/mg protein in growth hormone-treated tissue respectively (Fig. 6.3). Tissue culture in the presence of growth hormone had no effect on [^{3}H]dihydroalprenalol binding to β-receptors, although a tendency toward an increase in β-receptor was apparent which may have been confirmed with further experiments. No effect of growth hormone was seen on [^{3}H]rauwolscine binding to α2-receptors (Fig. 6.3) These results suggest that the changes in response to PIA are due to post-receptor changes.

6.3.4 Immunoblotting of α-subunits of G_i andG_s

The amounts of GTP-binding protein α-subunits were determined by immunoblotting following separation by SDS gel electrophoresis. Sheep adipocytes express three forms of G_i (G_i1, G_i2 and G_i3) and two forms of G_s (42 kDa and 45 kDa) (Vernon *et al*, 1995). Incubation of sheep adipose tissue with growth hormone had no effect on the expression of the α-subunits of G_i1 plus G_i2 (Table 6.1). The similiarity in size of the α-subunits of G_i1 and G_i2 did not permit their seperation under the electrophoresis conditions used. Growth hormone treatment also had no effect on the expression of either form of G_s (Table 6.1). These results are consistent with those from the sheep *in vivo* study (Doris *et al*, 1994) and suggest a possible covalent modification of G_i.

6.3.5 Lipolytic response to H7

As there is evidence that $G_i2\alpha$ can be phosphorylated by protein kinase C and at least one other kinase (Bushfield *et al*, 1990), the effects of the protein serine kinase inhibitor [1-(5-isoquinolinesulfonyl)-2-methyl piperazine, HCl] (H7) (Scientific Marketing, Barnet, Herts, UK) was tested in the tissue culture system. Tissue was cultured for 24 h without additions after which growth hormone and H7 or growth hormone were added, singly or in combination, for the next 24 h. Lipolysis was then measured in the presence of isoprenaline at 100 nM and adenosine at 0.8 μg/ml and various concentrations of PIA. Addition of 100 μM H7 completely abolished the effect of growth hormone on response to PIA (Fig. 6.4) while having no apparent effect on control tissue. The results are the means of 4 observations and SED = 7.2.

6.3.6 Effect of Okadaic acid on lipolysis

To further investigate the role of protein phosphorylation in the effect of growth hormone on adipose tissue, the protein-serine phosphatase inhibitor, okadaic acid (Calbiochem-Novabiochem (UK) Ltd, Nottingham, Notts, UK) was used in the tissue culture system. Addition of this agent (10 nM) to the culture medium mimicked the effect of growth hormone and when added in combination with growth hormone the effects of the two agents were additive ($P < 0.05$) (Fig. 6.5). The results are the means of 4 observations and the SED = 7.3. These studies thus show that growth hormone can enhance lipolysis by decreasing the effects of agents acting via the G_i-based antilipolytic system, and provide further evidence for the effect of growth hormone to involve protein serine phosphorylation.

6.3.7 Effect of PMA on lipolysis

Phorbol 12-myristate 13-acetate (PMA) has been shown to activate PKC isoforms in hepatocytes, leading to the phosphorylation of $G_i2\alpha$ (Bushfield *et al*, 1991). In the short term PMA activates PKC, while at times after about 7 h PMA is thought to down-regulate some isoforms PKC (Hug and Sarre, 1993; Dekker and Parker 1994). Therefore, studies were carried out to look at the role of PMA in sheep adipocytes over a 48 h period.

PMA (10 μg/ml) was added to the culture system and lipolysis assays were carried out at 5, 24 and 48 h. Addition of PMA or growth hormone to the culture system for 5 hours had no effect on the ability of PIA to inhibit lipolyis (Fig. 6.6). Incubation of tissue in the presence of PMA for 24 h resulted in a decrease ($P < 0.05$) in inhibition of isoprenaline-stimulated lipolysis from 60.26% in control tissue to 44.5% (Fig. 6.6). Addition of both PMA and growth hormone combined for 24 h resulted in the same degree of inhibition of the PIA effect as growth hormone alone (Fig. 6.6). PMA was then added to the culture medium for the full 48 h of incubation and growth hormone was added for the final 24 h. Although over the 48 h period, addition of PMA resulted in an apparent decrease in the inhibition of lipolysis by PIA from 60.25 to 50.75%, the effect was not significant (Fig. 6.7), again, the degree of inhibition of the PIA effect by growth hormone was the same regardless of whether the tissue had been preincubated with PMA or not (Fig. 6.7). The results are the means of 3 observations and SED = 7.6.

6.3.8 Effect of D609 on lipolysis

It has been proposed that growth hormone increases diacylglycerol production and studies (Catalioto *et al*, 1990) have shown that growth hormone increases diacylglycerol concentration in ob1771 (preadipocytes) cells by increased phosphatidylcholine breakdown. Addition of tricyclodean-9-yl-xanthogenate

(D609) (Calbiochem-Novabiochem (UK) Ltd, Nottingham, Notts, UK), a phosphatidylcholine phospholipase C inhibitor (40 μg/ml) had no effect on the ability of growth hormone to inhibit the actions of PIA, however, addition of D609 to tissue culture itself tended to enhance the effects of PIA (Fig. 6.8). The results are the means of 4 observations and SEM = 5.95. These results suggests that phospholipase C activation does not play a role in the effects of growth hormone on lipolysis.

6.3.9 Lipolysis: time course

Adipose tissue was maintained in culture for 24 h, the tissue was then transferred to fresh medium in the presence or absence of growth hormone for up to 24 hours. Lipolysis was stimulated with isoprenaline and assayed every three hours up to 12 hours and then at 24 hours in the presence of 100 nM PIA. Maintenance of sheep adipose tissue explants in culture in the absence of exogenous hormones for up to 24h had no effect on the ability of PIA to inhibit lipolysis (Fig. 6.9). The presence of growth hormone decreased the maximum response to PIA ($P < 0.05$) after a 24 h incubation. However, at all other time points measured, growth hormone had no effect on the response to adenosine. These results suggest that for sheep adipose tissue at least, the chronic effects of growth hormone on lipolysis require about 24 hours to become fully manifest and probably involve signalling to the nucleus, rather than simply causing the activation of a protein which then goes on to exert its effects on the lipolytic cascade.

6.3.10 Effect of Actinomycin D

Actinomycin D is an inhibitor of gene transcription and was used in the tissue culture system to determine if it could prevent the decreased inhibition of lipolysis by adenosine in the presence of growth hormone. Analysis of variance

showed that addition of actinomycin D (100 ng/ml) to tissue after maintenance in culture in the absence of growth hormone decreased the ability of PIA to inhibit lipolysis Fig. 6.10). When actinomycin D was added in combination with growth hormone, the level of inhibition of lipolysis by PIA was the same as that seen in the presence of actinomycin D alone (Fig. 6.10), that is, the effect of growth hormone was no longer apparent. These results indicate a role for gene transcription for growth hormone effects on the lipolytic cascade.

6.3.11 ADP ribosylation of sheep adipocyte membranes

ADP ribosylation studies were carried out to look at the interaction between the adenosine receptor and G_i. Pertussis toxin stimulates ribosylation of the undissociated (heterotrimeric) form of G_i. Experiments were therefore carried out in the presence and absence of PIA to determine the ratio of dissociated and undissociated form of G_i.

Explants of sheep adipose tissue were maintained in culture for 24 h without hormones and then for a further 24 h plus or minus 100 ng/ml ovine growth hormone. Subsequently, adipocytes were isolated, disrupted and a membrane fraction prepared (see section 2.3.1). The ability of pertussis toxin to stimulate ADP-ribosylation of the membranes was assessed (see section 2.3.17). Incubation of membranes with 1 μM PIA decreased the amount of ADP-ribosylation due to receptor induced dissociation of G_i from 3154 to 1965 cpm/mg protein (Fig. 6.11). Tissue culture with growth hormone had no effect on the levels of ADP-ribosylation or on the ability of PIA to decrease ADP-ribosylation (Fig. 6.11). The results are the means of 5 observations and SED = 377. Further studies were carried using PIA at 1 nM and 100 nM and at both these concentrations, growth hormone again had no effect on the ability of PIA to reduce levels of ADP-ribosylation (Fig. 612). This suggests that growth hormone does not alter the ability of G_i to interact with the adenosine receptor.

6.3.12 Adenylate cyclase activity in sheep adipocyte membranes

That growth hormone does not affect G_i interaction with the adenosine receptor, suggests that the attenuation of the antilipolytic effect of adenosine by growth hormone may be due to changes in the interaction of G_i with adenylate cyclase. Therefore, G_i function was assesssed directly using low concentrations (10^{-12} - 10^{-7} M) of the non-hydrolysable GTP analogue 5-[βγ-imido]triphosphate (p[NH]ppG) to inhibit the forskolin-stimulated adenylyl cyclase activity.

A small increase in stimulation of adenylate cyclase by both isoprenaline and forskolin was seen in membraned prepared from tissue that had been cultured with growth hormone (Table 6.3) reflecting the results seen at maximal lipolysis (Table 6.1) in experiments using inhibitors. After amplifying basal adenylate cyclase activity using the diterpene forskolin (10^{-4}M), low concentrations of p[NH]ppG were used to cause inhibition of adenylate cyclase activity through a G_i-mediated response. Treatment of sheep adipocyte membranes with growth hormone completely prevented the inhibition of adenylate cyclase activity by p[NH]ppG ($P < 0.01$) (Fig. 6.13). The results are the means of 4 observations.

Further studies were carried out using 5 x 10^{-4}M isoprenaline to stimulate adenylate cyclase activity and the ability of PIA to inhibit this effect was assessed. The ability of PIA at 10^{-7}M to inhibit adenylate cyclase activity via G_i in tissue treated with growth hormone was reduced from control levels of 26.25% inhibition to 0% inhibition.($P < 0.005$) (Fig. 6.14). Whereas at 10^{-5}M the level of inhibition of adenylate cyclase acivity by PIA in growth hormone-treated tissue returned to that seen in the controls (Fig 6.14). The results are the means of 4 observations, SEM = 5.5.

6.4 Discussion

Studies carried out by Watt *et al* (1991) showed that chronic endocrine control of the adrenergic system of sheep adipose tissue can be achieved in tissue culture. Therefore, in the present study the same tissue culture system was used to investigate the effect of chronic exposure of sheep adipose tissue to growth hormone. Chronic exposure of sheep adipose tissue to growth hormone resulted in a small increase in maximal isoprenaline-stimulated lipolysis. These results agree with Watt *et al* (1991) who reported an increase in response and sensitivity of sheep adipose tissue to β-agonists, and also correspond with the *in vivo* studies of Doris *et al* (1996) (chapter 4 of this thesis) that also showed a small increase in response to isoprenaline in growth hormone-treated sheep.

The present study shows, that as with sheep adipose tissue *in vivo* (Doris *et al*, 1996), treatment of sheep adipose with growth hormone *in vitro* results in a decrease in inhibition of isoprenaline-stimulated lipolysis by the adenosine analogue PIA. Adenosine receptor number was not affected by hormone treatment and therefore, could not account for the decreased response to adenosine. Similarly growth hormone treatment had no effect on either G_s or G_i isoforms: thus suggesting a decrease in G_i activity; these findings and this conclusion are in agreement with results of Chapter 5 (Doris *et al*, 1996).

It has been shown that a purified preparation of brain G_i, which will consist of multiple G_i subtypes, could be phosphorylated by protein kinase C (Katada *et al*, 1985). The phosphorylation of G_i has since been shown to occur in a number of cell types, including intact hepatocytes (Pyne *et al*, 1989) and U973 cells (Daniel-Issakani *et al*, 1989), where immunoprecipitation studies are consistent with it being G_i2 that is modified on serine residues. As other studies have shown that G_i2 can be phosphorylated by PKC and at least one other kinase (Bushfield *et al*, 1990), the effect of protein kinase and phosphatase inhibitors were tested in the culture system. Once described as a specific PKC inhibitor (Hidaka *et al* 1984), H7 is now

known as a more general protein serine kinase inhibitor and in the present study, H7 was shown to completely abolish the effects of growth hormone. Therefore, these results would suggest that the effects of growth hormone on sheep adipocytes involves an H7-sensitive step(s), possibly an isoform of PKC.

The activity of G_i2 is thought to be controlled by a phosphorylation-dephosphorylation cycle (Houslay, 1994), where activation of PKC results in phosphorylation of G_i2 and activation of phosphatases ensures a dynamic turnover. Thus either protein kinase activation or phosphatase inhibition can be expected to drive an increase in G_i2 phosphorylation. Okadaic acid is a potent inhibitor of phosphatases 2A and 1 (MacKintosh and Mackintosh, 1994), and addition of this agent to the tissue culture system in the present study resulted in inhibition of the antilipolytic effects of adenosine, as seen with the addition of growth hormone; effects of the two agents were additive and therefore support the theory of a phosphorylation-dephosphorylation regulatory system for G_i. These results are consistent with those of Morris *et al* (1995), who showed that treatment of hepatocytes with okadaic acid increased the labelling of α-G_i2.

Phorbol esters initially activate certain isoforms of PKC, but prolonged exposure to these agents leads to down-regulation (loss) of these isoforms (Hug and Sarre, 1993; Dekker and Parker, 1994). The tumour-promoting phorbol esters bind directly to PKC, but with a far greater affinity than endogenously produced DAG (Nishizuka, 1988; Sando *et al*, 1992). However, while DAG is metabolised phorbol esters are only slowly removed and chronic treatment with phorbol esters leads to down regulation of PKC (Nishizuka, 1988; Sando *et al*, 1992; Stabel and Parker, 1992). In human platelets treated with the phorbol ester, PMA, the phosphorylation of G_z appeared to occur very rapidly, with maximal labelling being achieved within 1 minute (Carlson *et al*, 1989). Studies with hepatocytes also showed an increase in labelling of α-G_i2 after challenge of cells with PMA (Morris *et al*, 1994; Morris *et al*, 1995). The use of PMA, in the present study showed no effect in the culture system when added alone for 5 h at a time it would be expected

to activate PKC. Under similar conditions 5h exposure to PMA increased the rate of lipogenesis of sheep adipose tissue (Vernon, 1996). After 24h, PMA partly mimicked the effect of growth hormone and the effect of these two agents was not additive. Whether the effect of PMA is due to a loss of an isoform of PKC which enhances the PIA effect or whether PMA induced phosphorylation of G_i is long lasting and continues after subsequent down-regulation of PKC isoforms is not clear, but the latter possibility is consistent with known effects of PKC on G_i and the H7 study. This is also consistent with the finding that culture with PMA for 48h did not cause a significant decrease in PIA inhibition of lipolysis; over this longer period there could have been a gradual dephosphorylation of G_i along with a loss of PKC isoforms involved. That some effect of growth hormone was apparent after pretreatment with PMA for 24h suggests that part of the effect of growth hormone at least does not involve a PMA-sensitive isoform of PKC.

Effects of PMA and growth hormone on lipolysis in sheep adipose tissue differ from previous findings with rat adipose tissue (Gorin *et al*, 1990) in that they are slower to appear in sheep than in the rat study and they are additive in the rat but not in the sheep. The present results are consistent with a previous finding with sheep adipose tissue which showed that both PMA and growth hormone increased glycerol release into the culture medium over a 22h period, but again effects were not additive (Vernon, 1996).

PKC is activated by diacylglycerol (DAG) which is produced as a consequence of the receptor-mediated hydrolysis of phospholipids, such a phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine (Shears, 1990; Cooke and Wakelam, 1992). Such processes are initiated by the action of a diverse range of biologically active substances, including hormones and growth factors whose receptors then stimulate appropriate phospholipases (Sando *et al*, 1992; Shears, 1990; Cooke and Wakelam, 1992). The present study shows that addition of D609 a phosphatidylcholine phospholipase C inhibitor, failed to abolish the

ability of growth hormone to inhibit the actions of adenosine, thereby ruling out a role for phospholipase C activation in growth hormone effects on lipolysis.

In the time course experiments the maximum effect of growth hormone was reached after 24 hours demonstrating that growth hormone is not simply activating a phosphorylation cascade. These results were supported by data from experiments with actinomycin D, which was shown to knock-out the effects of growth hormone. These studies then show that growth hormone can enhance lipolysis by decreasing the effects of agents acting via the G_i-based antilipolytic system by a mechanism that requires gene transcription and probably results in an increase in the phosphorylation status of G_i and thereby decreasing its activity.

More detailed studies have been carried out to elucidate the nature of the altered activity of G_i. To do this ADP-ribosylation studies were carried out in the presence of PIA which causes a decrease in ADP-ribosylation and is hence a measure of adenosine receptor binding to G_i. The results show that the ability of pertussis toxin to stimulate ADP-ribosylation of the adipocytes was unchanged by growth hormone; as pertussis toxin stimulates the ribosylation of undissociated (heterotrimeric) form of G_i, this suggests that the ratio of dissociated to undissociated was unchanged by growth hormone. Incubation of membranes with PIA decreased the amount of ADP-ribosylation due to receptor-induced dissocation of G_i but this was not altered by culture with growth hormone. This suggests that growth hormone does not alter the ability of G_i to interact with the adenosine receptor, the attentuation of the antilipolytic effect of adenosine by growth hormone was therefore thought to be due to changes in the interaction of G_i with adenylate cyclase.

Analysis of G_i interaction with adenylate cyclase was done by assessing receptor-mediated G_i action or by activating G_i directly with low concentrations of the non-hydrolysable analogue p[NH]ppG as has been described previously by Strassheim *et al* (1990). Low concentrations of p[NH]ppG were employed to detect functional G_i activity by inhibiting basal adenylate cyclase activity that had

been amplified by forskolin. Using such an approach, functional G_i activity was detected in membranes from control adipose tissue but not in growth hormone-treated tissue. The experiments involving PIA showed that the magnitude of receptor-mediated G_i inhibition, elicited through adenosine receptors, was eliminated in membrane preparations from adipose tissue treated with growth hormone at 100 nM PIA, but was no different at 10 μM PIA. Similar results have been found in studies with adipocytes from obese (*fa/fa*) Zucker rats (Strassheim *et al*, 1991) where functional G_i was detected in membranes from lean, but not obese animals in experiments using p[NH]ppG in adenylate cyclase assays. However, in the same studies, receptor-mediated inhibition of adenylate cyclase achieved by PIA and PGE_1 was similar in membranes from both lean and obese animals (Strassheim *et al*, 1991). A further difference between the present study and that of Strassheim *et al* (1991) is seen in the stimulatory and inhibitory G-protein levels. In the present study, no apparent difference in levels of G_i or G_s was seen, however, in (*fa/fa*) Zucker rats, levels of both forms of G_s and those of G_i1 and G_i3 were lower in obese animals compared to lean animals (Strassheim *et al*, 1991).

These studies would then suggest that the point at which growth hormone exerts its effects on the antilipolytic pathway in sheep adipocytes is at the point of interaction between G_i and adenylate cyclase; the mechanism appears to involve the phosphorylation of G_i possibly by a PKC isoform and also gene transcription.

Effect of Growth hormone on maximally-stimulated lipolysis

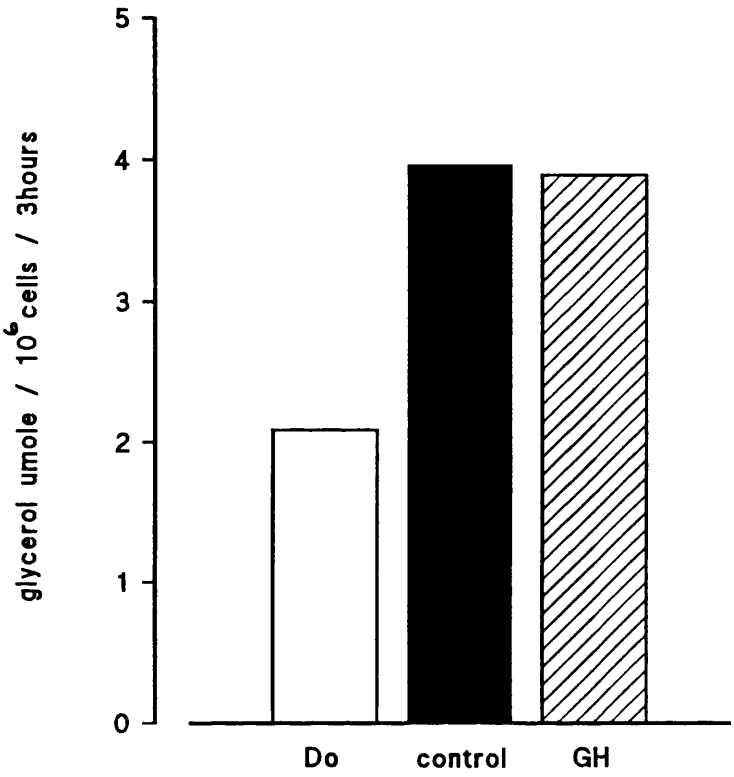


Figure 6.1 Sheep adipose tissue was cultured for 24h without additions, after which GH (4.5 nM) was added for the next 24h. Do tissue was assayed at time tissue was taken. Lipolysis was measured in the presence of 0.8 ug/ml adenosine deaminase and isoprenaline (100 nM). Results are the means of 4 observations and SEM = 0.73.

Table 6.1 Effect of Treatments on maximal lipolysis

Sheep subcutaneous adipose tissue was cultured for 24h without additions after which it was transferred to fresh medium in the presence and absence of growth hormone and other additions as described below, as described in section 2.3.3. Lipolysis was measured in the presence of 0.8 μg/ml adenosine deaminase and 100 nM isoprenaline. Results are the means ± SEM of 4 observations.

	Glycerol μM / 10^6 cells / 3 hours			
	control	inhibitor	GH	inhibitor + GH
H7	4.23 ± 0.77	4.43 ± 0.82	4.96 ± 0.80	5.82 ± 0.73
Okadaic acid	6.23 ± 0.89	6.62 ± 0.52	6.69 ± 0.58	6.30 ± 0.58
PMA	3.78 ± 0.30	3.67 ± 0.34	4.66 ± 0.41	4.54 ± 0.54
D609	3.61 ± 0.54	3.44 ± 0.45	4.63 ± 0.63	3.59 ± 0.39
Act. D	2.83 ± 0.67	2.97 ± 0.61	3.57 ± 0.60	2.82 ± 0.58

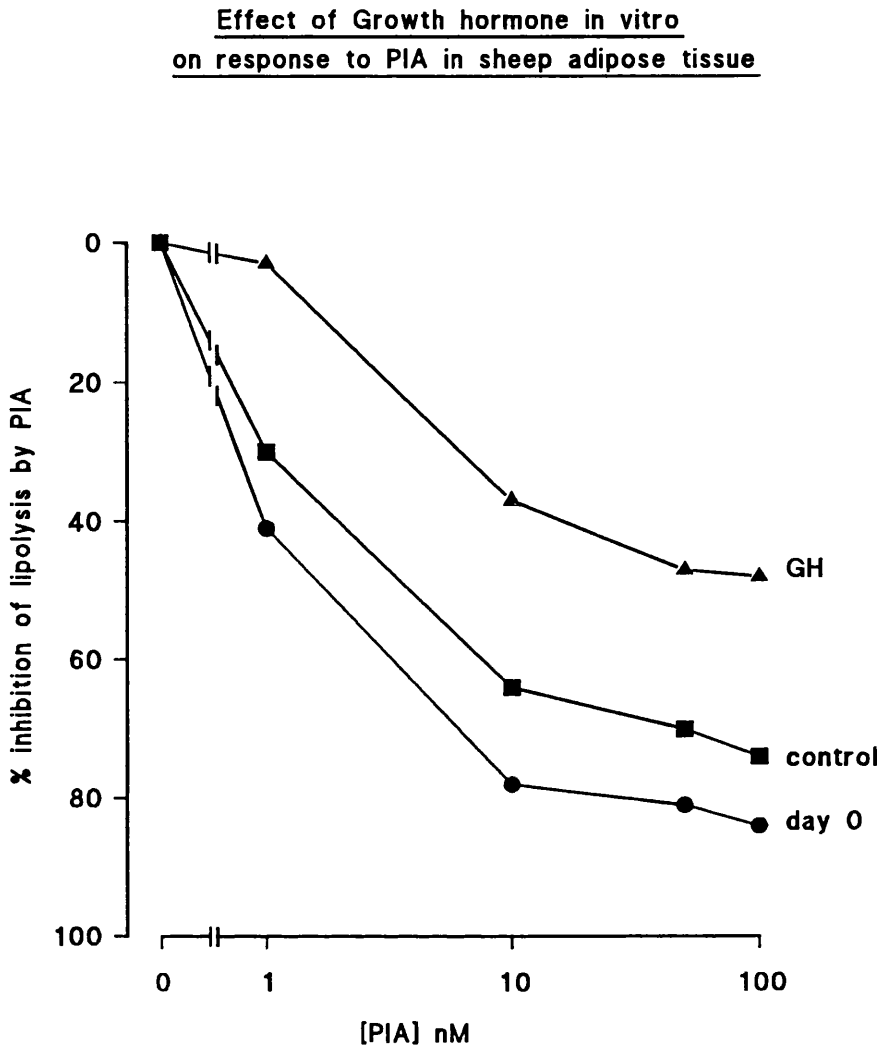


Figure 6.2 Tissue was cultured for 24h without addtions after which GH (4.5 nM) was added for the next 24h. Lipolysis was measured in the presence of 0.8 ug/ml adenosine deaminase, 100 nM isoprenaline and the concentration of PIA indicated. Day 0 tissue was assayed at time tissue was taken. Results are means of 4 observations,SED = 9.8.

Effect of GH treatment on ligand binding to beta alpha-2 and adenosine receptors

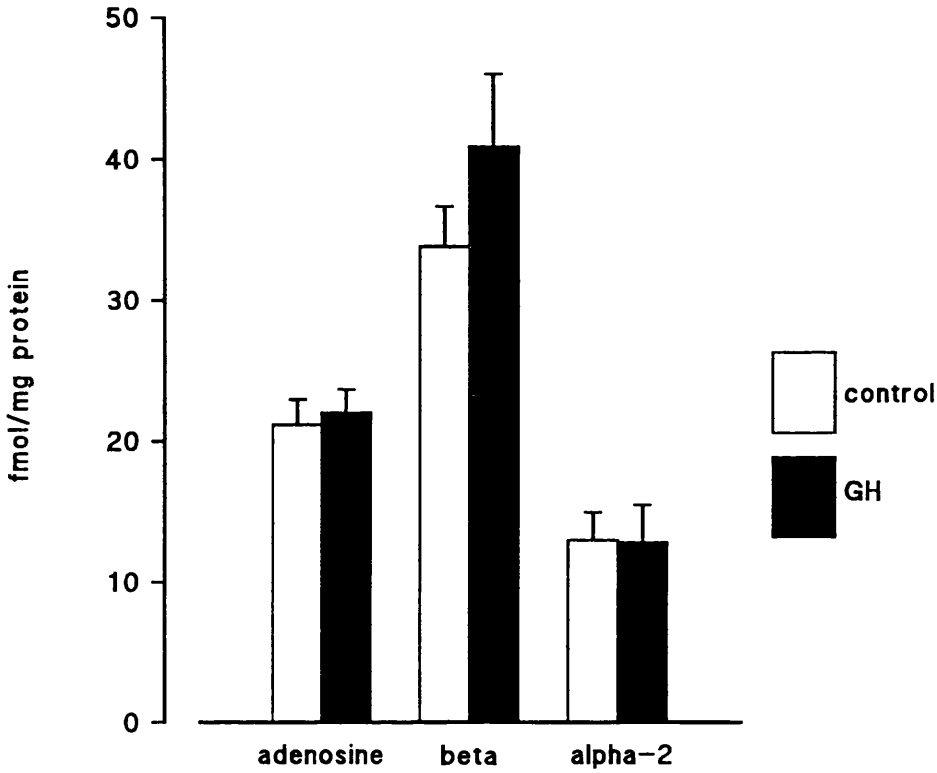


Figure 6.3 Sheep adipose tissue was cultured for 24h without additions after which GH (4.5 nM) was added for the next 24h. Membranes were prepared and the ability of the membranes to bind 20 nM-[^{3}H]dihydroalprenalol,20 nM-[^{3}H]rauwolscine and 20 nM-[^{3}H] PIA to beta, alpha-2 and adenosine receptors respectively was measured as described in methods section. Results are the means $\pm$ SEM of 3 observations.

Table 6.2 Expression of G-protein α-subunits in sheep adipose tissue

Tissue was cultured for 24 h without additions after which it was transferred to fresh medium in the presence and absence of growth hormone. Adipocyte membranes were prepared and immunoblotting carried out as described in the experimental section. Quantification of reactive proteins was achieved by γ radiation counting of excised bands. Results are means ± SEM of 3 observations.

		Arbitrary units / mg protein		
Treatment	G-protein	$G_i1 + 2$	G_s (45 kDa)	G_s(42 kDa)
Control		2587 ± 661	11496 ± 393	22873 ± 548
Growth hormone		2071 ± 405	12713 ± 1034	25389 ± 664

Effect of Growth hormone and H7 on lipolysis

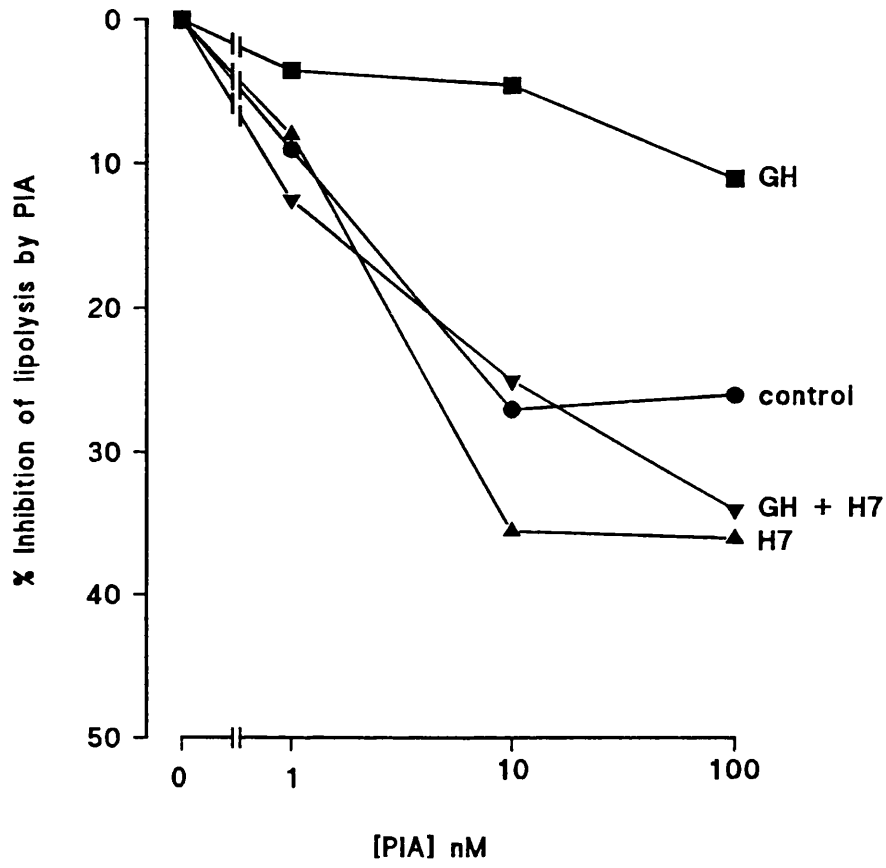


Figure 6.4 Sheep adipose tissue was cultured for 24h without additions after which GH (4.5 nM) and H7 (100 uM) were added, singly or in combination, for the next 24h. Lipolysis was measured in the presence of 0.8 ug/ml adenosine deaminase and 100 nM isoprenaline. Results are the means of 4 observations, SED = 7.2

Effect of okadaic acid and growth hormone on lipolysis

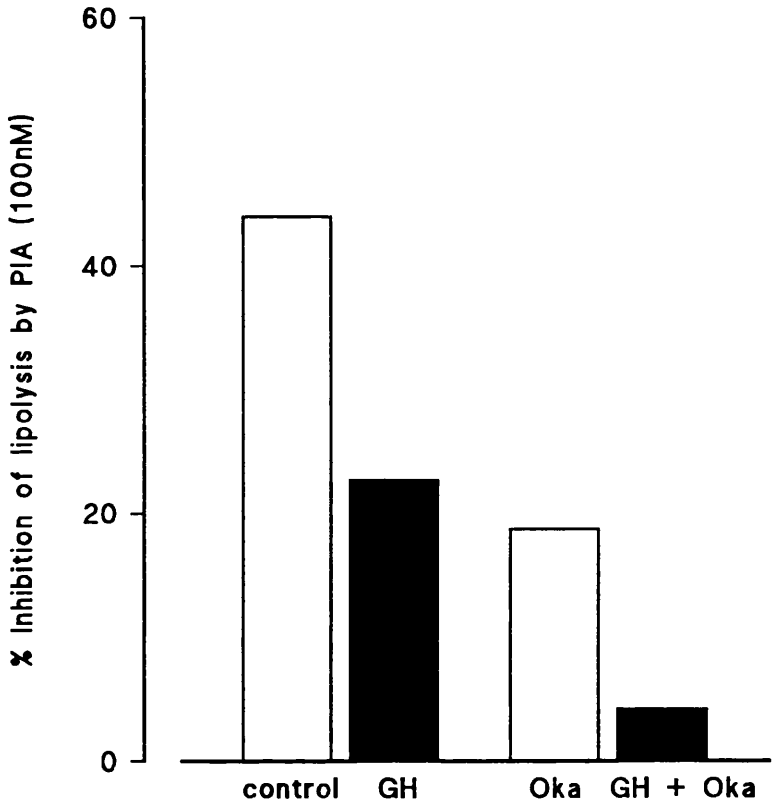


Figure 6.5 Sheep adipose tissue was cultured for 24h without additions after which GH (4.5 nM) and okadaic acid (10 nM) were added,in combination, for the next 24h. Lipolysis was measured in the presence of 0.8 ug/ml adenosine deaminase and 100 nM isoprenaline. Results are means of 4 observations, SED = 7.3.

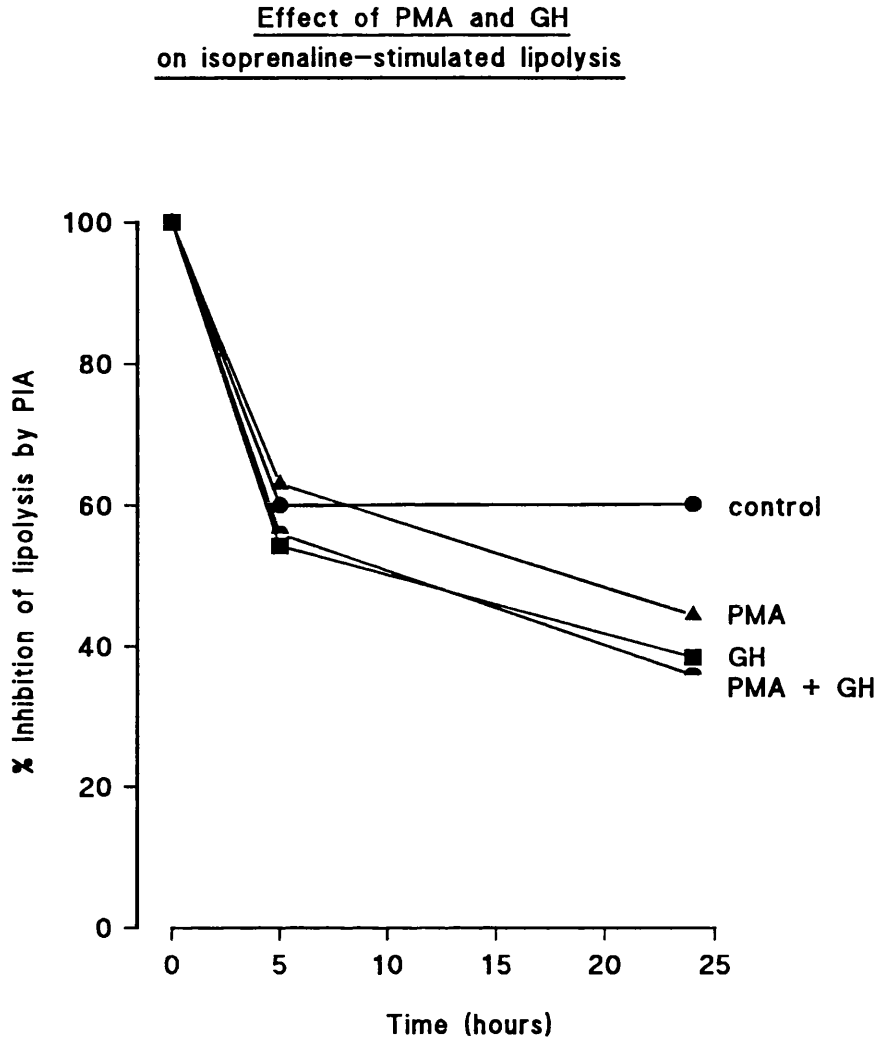


Figure 6.6 Sheep adipose tissue was incubated for 24h without addtions after which it was maintained in culture for 5 or 24h with GH (4.5 nM) or PMA (10 ug/ml) singly or in combination. Lipolysis was measured in the presence of 0.8 ug/ml adenosine adenosine deaminase and 100 nM isoprenaline. Lipolysis was inhibited using 100 nM PIA. Results are means of 3 observations, and SED = 7.6.

Effect of PMA and GH on isoprenaline–stimulated lipolysis over 48 hours

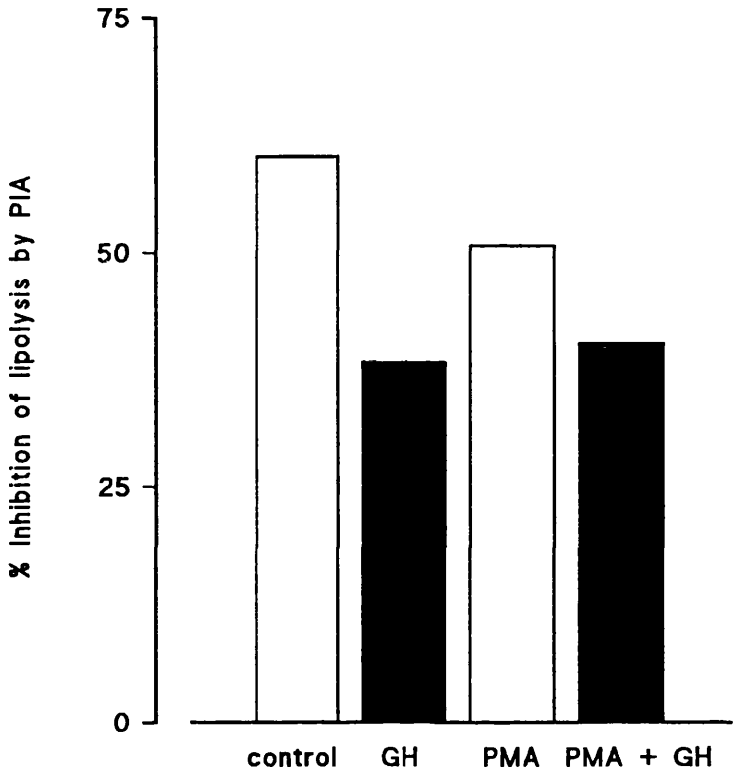


Figure 6.7 Tissue was incubated in the presence of PMA for 48h, and for the second 24h GH was added. Lipolysis was measured in the presence of 0.8 ug/ml adenosine deaminase and 100 nM isoprenaline. Lipolysis was inhibited using 100 nM PIA. Results are the means of 3 observations and SED = 7.6.

Effect of Growth hormone and D609 on lipolysis

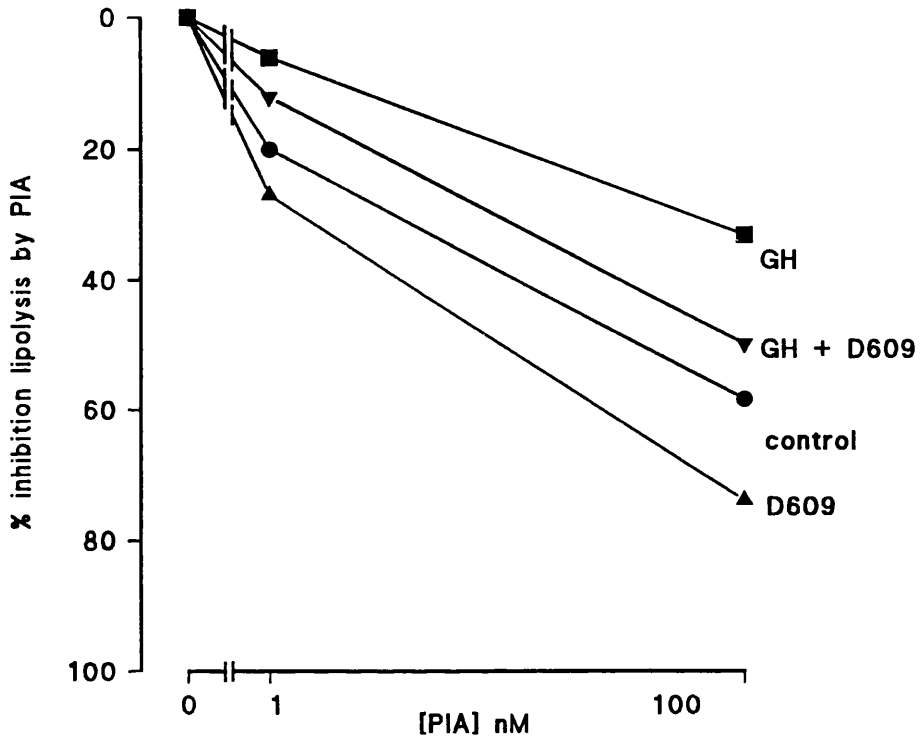


Figure 6.8 Sheep adipose tissue was cultured for 24h without additions after which GH (4.5 nM) and D609(40ug/ml) were added, singly or in combination, for the next 24h. Lipolysis was measured ub the presence of 0.8 ug/ml adenosine deaminase and 100 nM isoprenaline. Results are means of 4 observations and SEM = 5.95.

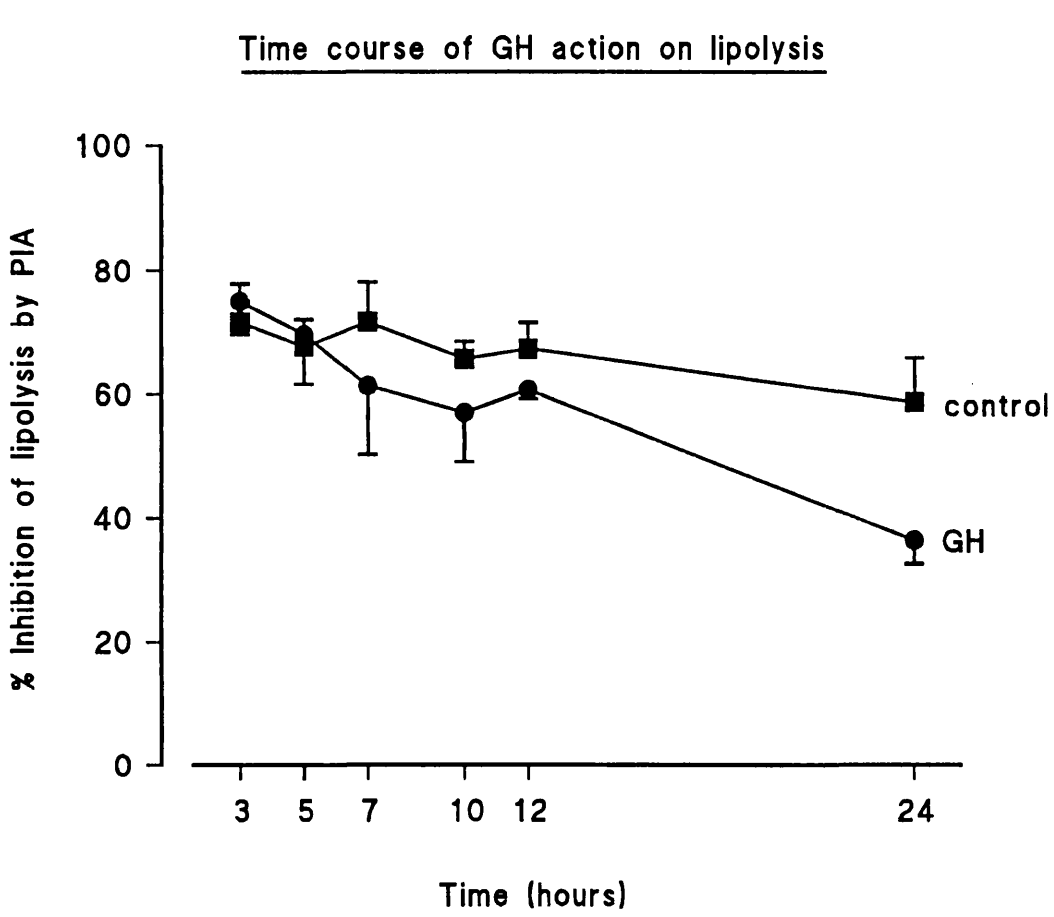


Figure 6.9 Sheep adipose tissue was precincubated with no hormones for 24h, after which it was maintained for a further 24h in the presence and absence of GH. Lipolysis was measured at the times indicated. Results are the means + SEM of 3 observations. Values in the presence of GH were significantly lower ($P < 0.05$) at 24h than those of controls.

Effect of Growth hormone and Actinomysin D on lipolysis

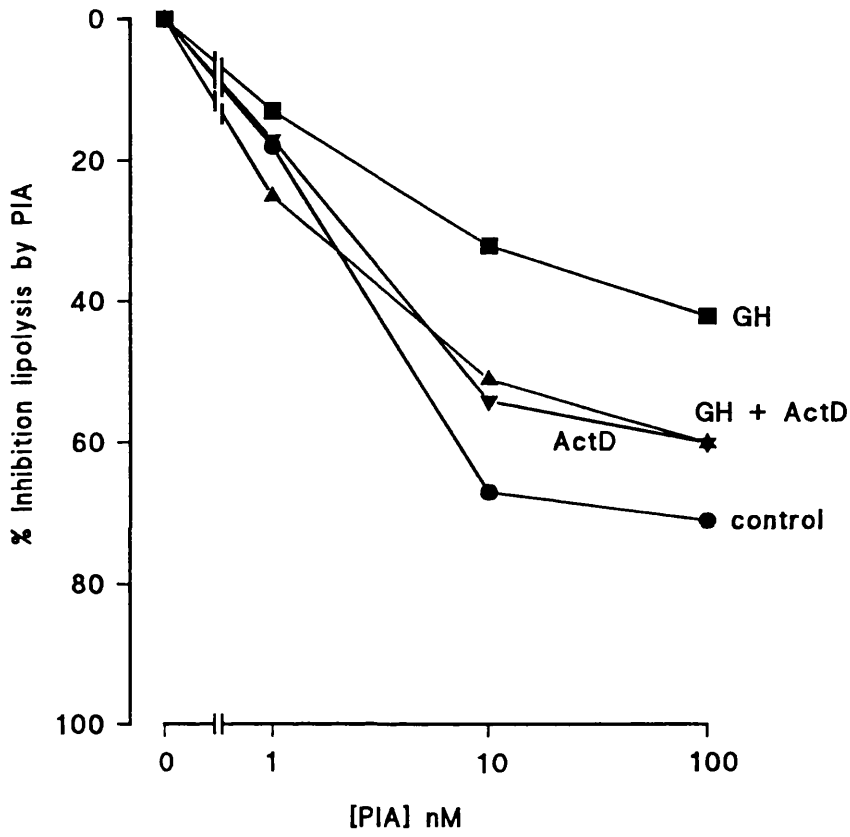


Figure 6.10 Sheep adipose tissue was cultured for 24h without additions after which GH (4.5 nM) and actinomycin D (100 ng/ml) were added, singly or in combination, for the next 24h. Lipolysis was measured in the presence of 0.8 ug/ml adenosine deaminase and 100 nM isoprenaline. Results are the means of 4 observations, SEM = 5.2.

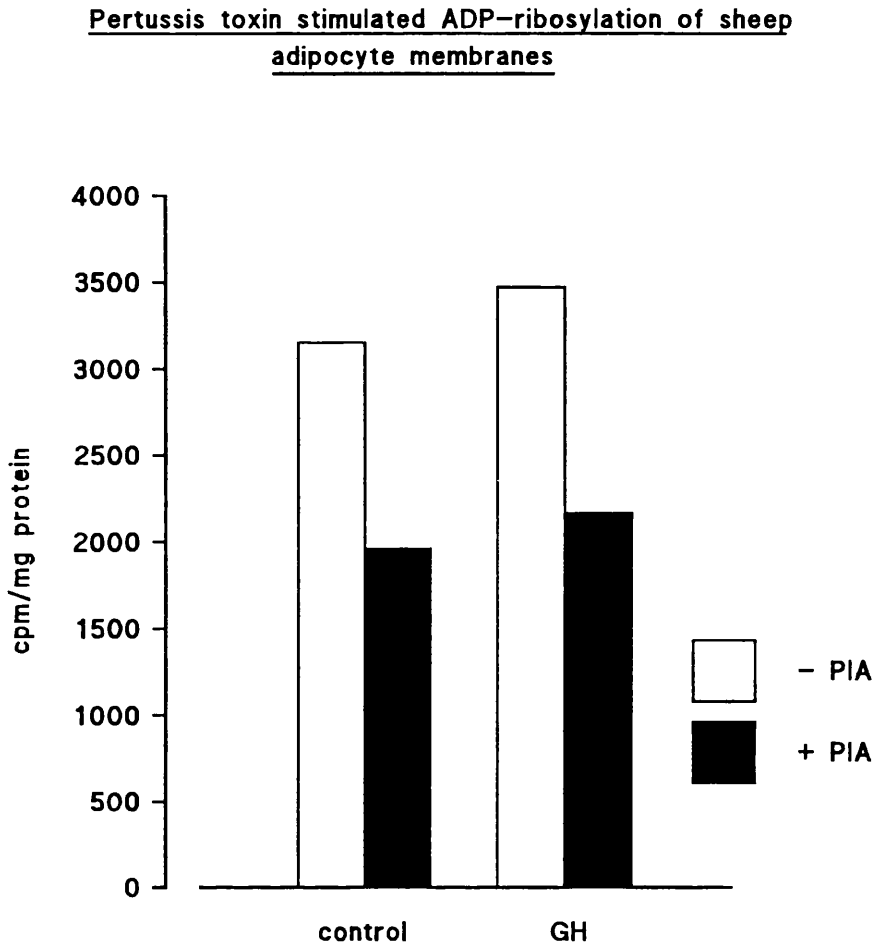


Figure 6.11 Sheep adipose tissue was cultured for 24h without addtions after which GH (4.5 nM) was added for the next 24h. Membranes were prepared and the ability of pertussis toxin to stimulate ADP–ribosylation of the membranes was assessed in the presence and absence of 1 uM PIA. The results are means of 5 observations and SED = 311.

Effect of PIA on ADP-ribosylation of Gi in sheep membranes

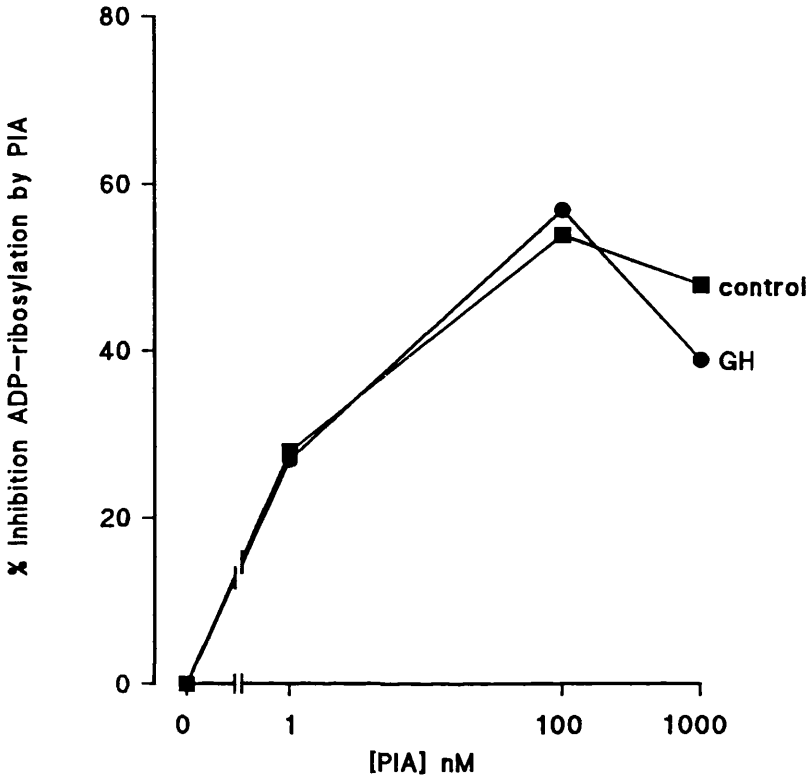


Figure 6.12 Sheep adipose tissue was cultured for 24h without additions after which GH (4.5 nM) was added for the next 24h. Membranes were prepared and levels of ADP-ribosytion were assessed in the presence and absence of PIA at concentrations indicated above. Results are means of 3 observations and SEM = 4.00.

Table 6.3 Effect of Growth hormone on Adenylate Cyclase activity

Adipocytes were isolated from subcutaneous sheep adipose tissue that was cultured for 24h without additions after which it was transferred to fresh medium in the presence and absence of growth hormone, and used for preparation of membranes as described in section 2.3.1. Membranes were assayed for adenylate cyclase activity in the absence of exogenous agents (basal) in the presence of 100 μM forskolin or 5 x 10^{-4}M isoprenaline (Isop) alone or with 10 μM GTP. Results are the means ± SEM of 4 observations.

	pmol / min / mg protein	
	-GH	+GH
Basal	61.75 ± 13.02	72.00 ± 10.48
+ Isop	216.25 ± 45.79	225.25 ± 75.87
+ Forskolin	805.00 ± 49.14	865.00 ± 85.68
+ GTP	87.85 ± 6.39	76.00 ± 2.97
+ Isop + GTP	358.75 ± 27.26	401.25 ± 100.96

Dose–effect experiments for p[NH]ppG on forskolin–stimulated adenylate cyclase activity in sheep adipocyte membranes

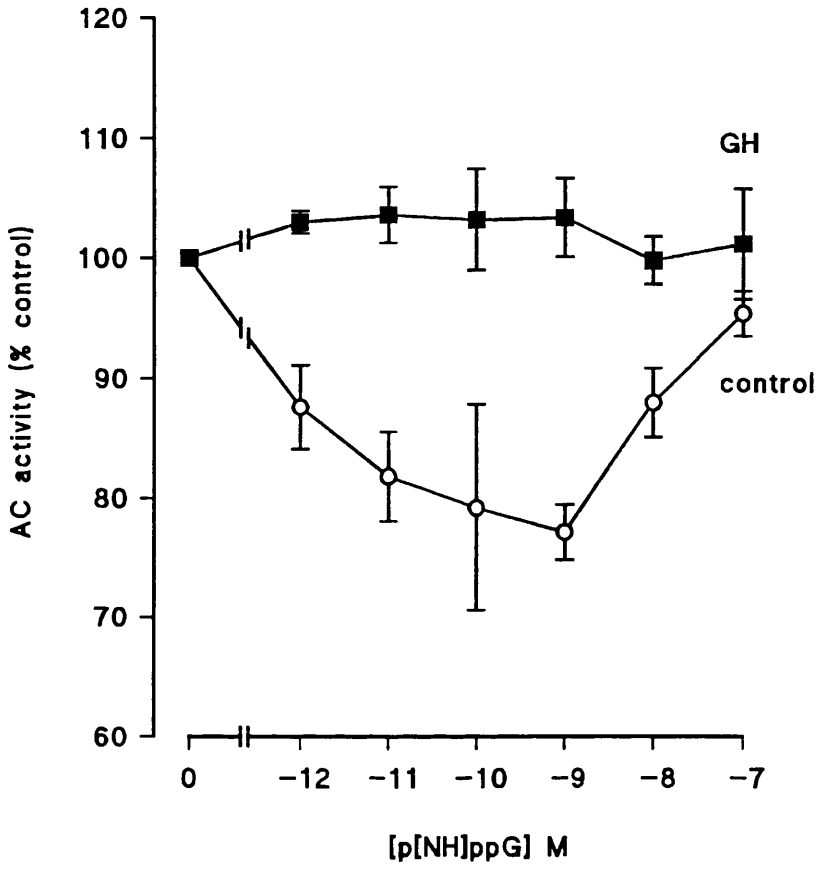


Figure 6.13 Sheep adipose tissue was cultured for 24h without additions after which GH (4.5 nM) was added for the next 24h. Membranes were prepared and increasing concentrations of p[NH]ppG were added to assays employing membranes from control and GH–treated tissue. Results are the means of ± SEM of 4 observations.

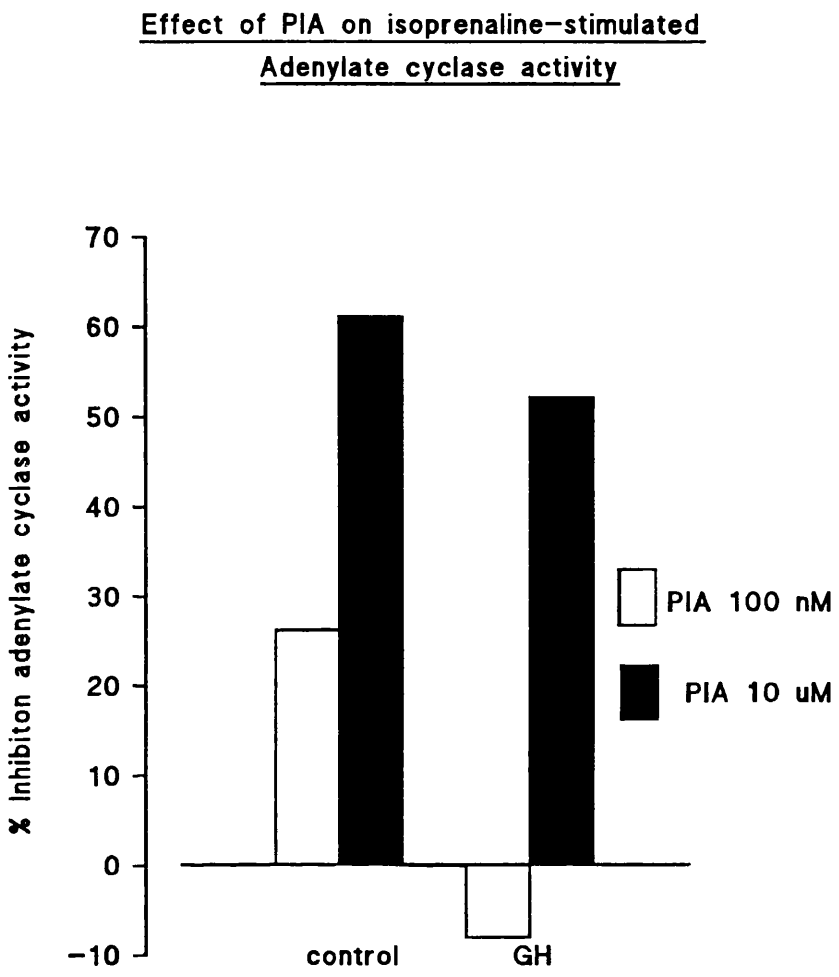


Figure 6.14 Sheep adipose tissue was maintained in tissue culture for 48h in the presence and absence of growth hormone, after which membranes were prepared. PIA was then added to assays employing membranes from control and GH-treated tissue. Results are the means of 4 observations, SEM = 5.5

CHAPTER SEVEN

GENERAL DISCUSSION

The aim of the studies presented in this thesis was to determine the mechanisms whereby growth hormone exerts its lipolytic effect in adipose tissue in both rat and sheep. The results presented in this thesis, show that the G_i-based antilipolytic signal transduction system is an important target of growth hormone action. The effects of growth hormone on this system are complex and vary with physiological state.

Both *in vivo* and *in vitro* approaches have been adopted to resolve the factors and mechanisms which regulate the adrenergic-adenosine signal transduction cascade in adipocytes. Studies with both sheep and rats have involved manipulation of growth hormone concentrations *in vivo* and further studies with sheep adipose tissue have involved exposure of adipose tissue to the hormone in a tissue culture system.

In accordance with other studies involving lactating rats (Vernon *et al*, 1991) and cows (Lanna *et al*, 1992), the experiments involving growth hormone treatment of virgin rats had no effect on the maximum response to β-agonists. However, growth hormone treatment of sheep *in vivo*, resulted in a small increase in response to β-agonists *in vitro* (Doris *et al*, 1996); also studies by Watt *et al* (1991) showed that incubation of sheep adipose tissue in the presence of growth hormone resulted in an increase in response to β-agonists. In contrast Growth hormone has a large effect on response to β-agonists in adipocytes from rats in which lactation is prematurely terminated either by litter removal or endocrine manipulation as found previously by Vernon *et al*, (1991; 1993) and Barber *et al* (1992) respectively. These studies imply that growth hormone can enhance signalling via the β-adrenergic pathway which stimulates lipolysis, but in most states, effects are small and may be species dependent. Studies by Lanna *et al* (1992) and the results in this thesis show that the major effect of growth hormone treatment is on the inhibitory system regulating lipolysis.

Manipulation of serum growth hormone levels in virgin rats, using an antiserum to rat growth hormone, resulted in an increase in the inhibition of isoprenaline-stimulated lipolysis by submaximal concentrations of both PIA and

PGE_1, but no effect on the maximum response to these factors was seen. The experiments with lactating rats in which lactation was abruptly and completely curtailed either by litter removal or by endocrine manipulation showed that treatment with growth hormone resulted in a decrease in the ability of maximal concentrations of PIA and PGE_1 to inhibit lipolysis. However, in the same experiments treatment of lactating rats with growth hormone showed no effect on the maximum response or sensitivity to PIA. While these results support the theory that growth hormone exerts its effects on the antilipolytic arm of the signal transduction system, it is apparent that the effect of growth hormone is dependent on the physiological state of the animal.

Investigations into the mechanism whereby growth hormone exerts its effects on these antilipolytic factors showed that changes in adenosine receptor number in both virgin and lactating rats was not responsible. However, treatment of virgin rats with anti-rGH resulted in an increase in levels of the inhibitory G-protein G_i2, and this effect was reversed upon replacement therapy with sheep growth hormone. Interestingly, this effect was not observed in the litter removed rats treated with anti-rGH plus bromocriptine, and indeed no change in levels of G-proteins was seen in either lactating or litter removed rats. In experiments carried out by Ros *et al* (1992), involving lactating rats, levels of both G_i1 plus G_i2 and G_s were shown to decrease upon litter removal. In these studies control animals (virgin rats) were not used and it is probable that the decrease in G-protein levels seen at litter removal restored the protein levels to that of the controls. Levels of both G_i and G_s however, have been shown to increase in lactating sheep, (Vernon *et al*, 1995), indicating that G-proteins are a target of regulation in this species.

Previous studies showed that sheep adipose tissue survives well in culture maintaining metabolic activities and response to hormones (Vernon and Sasaki, 1991), offering an alternative to *in vivo* manipulations to investigate the effects of growth hormone on the lipolytic system. As shown in the present studies (Chapter 5 and Chapter 6) culture with growth hormone for 24h does indeed mimic the effects of growth hormone treatment *in vivo*.

Growth hormone treatment of sheep adipose tissue, both *in vivo* and *in vitro*, resulted in a small increase of catecholamine-stimulated lipolysis which was also reflected in the increase seen in number of β-adrenergic receptors. These results are consistent with those of Watt *et al* (1991) who also showed an up-regulation of the stimulatory arm of the lipolytic pathway upon growth hormone treatment of sheep adipose tissue after a 48 hour incubation in tissue culture. Treatment of sheep *in vivo* with growth hormone decreased the maximum inhibition of isoprenaline-stimulated lipolysis by PIA, an effect that was not accompanied by any discernable change in the number of adenosine receptors or amounts of the various forms of G_i. Growth hormone also decreased the response to PGE_1, which also activates G_i but via a distinct receptor; the levels of which were not measured. Maintenance of sheep adipose tissue in culture with growth hormone again decreased the maximum response to adenosine with no change in either the number of adenosine receptors or amounts of G_i. This contrasts with the studies in the virgin rat which show that growth hormone *in vivo* decreases sensitivity to adenosine by decreasing the amount of G_i (Doris *et al*, 1994). The results are consistent with those found in the lactating and litter-removed rat study of this thesis, in which changes in response to PIA and PGE_1 occurred without a change in G_i levels. These studies showed that growth hormone can alter signalling through the G_i-based antilipolytic system by at least two mechanisms: a change in the amount of G_i2 and by a change in activity of some component. ADP-ribosylation studies involving sheep adipocyte membranes from cultured tissue showed that the interaction of the activated adenosine receptor with G_i in sheep adipose tissue is not affected by growth hormone treatment.

In the adenylate cyclase assays, using sheep adipocyte membranes, the results from experiments examining G_i interaction with adenylate cyclase were rather surprising in that at the higher concentration of 10 μM PIA the effect of growth hormone is diminished. Experiments to investigate the effects of growth hormone on lipolysis were all carried out at a maximal concentration of 100 nM PIA, and at this concentration in the adenylate cyclase assays, the results are consistent with those of

the lipolysis assays which show a decrease in efficiency of signal transduction via the antilipolytic pathway. Initial experiments to determine the maximum concentration of PIA in lipolysis assays included higher concentrations of PIA up to 10 μM and no reversal of growth hormone effect, as seen in the adenylate cyclase assays, was observed. The reason for the difference between isolated membranes and intact cells is not clear.

It would be useful to look at the interaction between G_i and adenylate cyclase in the lactating rat to see if receptor-mediated inhibition of adenylate cyclase is achieved when using PIA, as in studies carried out with lean and obese rats by Strassheim *et al* (1991). In this way it could be determined if the results obtained from experiments with sheep adipose tissue, where growth hormone treatment affects G_i inhibition of adenylate cyclase, elicited through adenosine receptors, is species specific.

Thus a second mechanism whereby growth hormone alters signalling through the G_i-based antilipolytic signalling system is through a change in G_i activity, in particular, in its ability to interact with and inhibit adenylate cyclase.

Paradoxically, despite an increase in serum growth hormone, response to PIA is enhanced during lactation in sheep (Vernon *et al*, 1995). Also response to PIA is increased during lactation in rats despite serum growth hormone being unchanged (Chapter 4). These observations show that some additional factor must be modulating the response to PIA during lactation and may be responsible for the failure of exogenous growth hormone to decrease response and/or sensitivity to PIA during lactation in rats. Exogenous growth hormone can decrease response and sensitivity to PIA in lactating cows (Lanna *et al*, 1995); why lactating rats and cows differ in this respect is not clear.

A possible reason for the change in G_i activity is phosphorylation, perhaps by protein Kinase C. This was investigated using inhibitors in the *in vitro* sheep adipose tissue culture system. Although the specificity of all signal transduction inhibitors have their limitations, they provide a valuable insight into possible mechanisms.

Protein kinase C activity is associated with proteins of a set of monomeric proteins of molecular mass around 80 kDa (Houslay, 1994). These structurally related isoforms are encoded by a large gene family (Stabel and Parker, 1991). Molecular cloning and enzymological analysis have identified at least twelve structurally related protein kinase C isozymes, (Stabel and Parker, 1991; Nishizuka, 1989; Nixon *et al*, 1992; Parker *et al*, 1992; Parker and Dekker, 1994) and appear to belong to three categories: conventional PKCs (cPKC α, βI, βII and γ) that are activated by phospholipid, diacylglycerol and calcium; those that are insensitive to Ca^{2+}, novel PKCs (nPKC δ, ε, θ and η), and atypical PKCs that require neither Ca^{2+} or DAG (aPKC ζ, λ, ι, and μ) for review see Dekker and Parker (1994). Most isoforms are soluble and found in the cytosol, however, depending on the isoform, treatment of cells with Ca^{2+}, DAG or phorbol esters (Sando *et al*, 1992) results in membrane-associated activity. It is possible that individual isoforms have distinct functional roles in the cell which may be characterised by differences in activation; for example phosphorylation which seems to be particularly important for PKC-α (Pears *et al*, 1992).

Protein kinase C modification of G_i2 can be observed in intact cells although it does not appear to be a universally apparent phenomenon (see Houslay, 1994), and may require a specific isoform. The ability of H7 to inhibit the action of growth hormone on G_i activity in sheep adipose tissue suggest that a PKC isoform is involved. The abililty of okadaic acid to mimic the effects of growth hormone provides further evidence for a phosphorylation-dephosphorylation cycle as a means of G_i2 regulation.

The role of PKC was further investigated using PMA. Observations have been made in cases where treatment with phorbol esters led to the sensitization of adenylate cyclase activity, suggesting that this activator of PKC might be able to phosphorylate and thus inactivate G_i (Katada *et al*, 1985). More recent experiments with human platelets (Carlson *et al*, 1989) and hepatocytes (Morris *et al*, 1994; 1995) have shown that treatment of cells with PMA increases labelling of G_i2.

Effects of PMA alone over 24h on PIA inhibition of lipolysis could be due to activation of PKC isoforms and phosphorylation of G_i although it is surprising that no effect was seen after 5h of exposure to PMA. However, as pretreatment with PMA for 24h, which should down-regulate at least some isoforms of PKC, did not prevent the effect of growth hormone on PIA inhibition of lipolysis, it would appear that if PKC is involved, then at least part of the effect of growth hormone involves PMA-insensitive isoforms, or at least isoforms which are only slowly down-regulated by PMA. More specific approaches, for example use of antisense technologies to selectively eliminate specific PKC isoforms, are needed to resolve the putative role of PKC. Experiments involving D609 also showed that phospholipase C is not involved in the effects of growth hormone on G_i activity. From the time course experiments and those with actinomycin D showing that the full effect is not seen for 24 hours, it can be speculated that at least one protein required for the phosphorylation and deactivation of G_i may not be constitutively present but requires gene transcription.

There are a number of similarities between the effects of growth hormone on lipolysis and growth hormone inhibition of lipogenesis in mature sheep adipocytes. In general, agents preventing the lipolytic effects of growth hormone have also been shown to have effects on lipogenesis. The effects of growth hormone are slow to develop in both lipogenesis (Borland *et al*, 1994) and lipolysis (Chapter 6) and the effect of growth hormone on both processes is blocked by actinomycin D (Borland *et al*, 1994; Chapter 6). Although D609 shows no effect on growth hormones action on lipolysis, addition of this phospholipase C inhibitor prevented the antilipogenic effects of growth hormone (Vernon, 1996). As with the studies detailed in this thesis, treatment of sheep adipose tissue in culture with H7 also decreased the antilipogenic effects of growth hormone (Vernon, 1996). However, while okadaic acid has been shown to mimic the effects of growth hormone on lipolysis, this protein serine phosphatase inhibitor diminished the antilipogenic effects of growth hormone (Vernon, 1996). Phosphatases 2A and 1 dephosphorylate a number of proteins (Mumby and Walter, 1993; Walter and Mumby, 1993) so okadaic acid may influence

growth hormone signalling at several points. Therefore, while growth hormone effects on both lipolysis and lipogenesis may involve one or more isoforms of PKC plus gene transcription, the effects of growth hormone on these systems do not occur via identical mechanisms.

Recent studies have provided evidence for proteins that regulate G-proteins known as RSG proteins (for Regulators of G-protein signalling) (see Roush, 1996). It is thought that RSG proteins work by binding to one of the three G-protein subunits and thereby prevent it from participating in signal transduction. Given that there is a high degree of sequence conservation between species including yeast, worms and mammals, it is reasonable to speculate that these proteins may be involved in regulating the G-proteins of the lipolytic pathway. At the moment very little is known about these proteins, including what regulates their own action, but perhaps they play a role in growth hormones actions in the modification of G_i.

In conclusion, these studies show that growth hormone can enhance lipolysis by decreasing the effects of agents acting via the G_i-based antilipolytic system and that the inhibitory G-protein G_i is a major target of growth hormone action. The experiments involving virgin and lactating rats also show that the effects of growth hormone are dependent on physiological state.

The studies involving sheep adipose tissue reveal a species specificity of growth hormone action, where G_i interaction with adenylate cyclase is a target for modification by growth hormone. It is possible that growth hormones mechanism of action in lactating rats and sheep adipose tissue is similar, in that G_i is a target of covalent modification rather than being subjected to down regulation as seen in virgin rats.

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