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Signal Transduction Mechanisms for the Stimulation of Lipolysis by Growth Hormone

A Thesis Presented

by

Rupert Guk-Chor Yip

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences,
Worcester
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DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

August 1994

Signal Transduction Mechanisms for the Stimulation of Lipolysis by Growth Hormone

A Thesis

By

Rupert Guk-Chor Yip

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September, 1994

Dedication

This work is dedicated to my mother and father who are two of the most loving and patient people I am blessed to know. Their warm support and encouragement has allowed me to finally attain this achievement. I am grateful to them for teaching me the value of education and will continue to grow and learn because of them.

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First and foremost, an immense debt of gratitude is owed to Dr. Maurice "Moe" Goodman for providing a nurturing environment with which to pursue science. His enormous optimism and support can only be surpassed by his patience with my training. As a role model, Moe has provided a level of excellence for which I shall forever strive to achieve.

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(particularly Tufts School of Dental Medicine) for providing a much needed "support group" for some of the darkest moments in graduate training.

ABSTRACT

The purpose of this study was to investigate the mechanism of action of lipolysis by growth hormone in rat adipocytes. GH-induced lipolysis, in contrast to that of isoproterenol (ISO), is slow in onset (lag time >1h), small in magnitude (~2X basal) and requires corticosteroid. Evidence for direct coupling between GH receptors and adenylyl cyclase or G-proteins is lacking, and although we could detect no measurable change in cAMP content after treatment with GH + dexamethasone (Dex), it is likely that cAMP activation of protein kinase A is a central event in GH-induced lipolysis. Rp-cAMPS, a competitive antagonist of cAMP was equally effective in decreasing lipolysis in tissues treated with GH/Dex or a comparably lipolytic dose of ISO. Incorporation of ^{32}P from γ-^{32}P-ATP into kemptide, a synthetic oligopeptide substrate for protein kinase A, was increased in homogenates of GH/Dex-treated tissue. This increase was correlated with increased lipolysis. Earlier estimates based upon ^{32}P-ribosylation of G_i catalysed by pertussis toxin (PTx) suggested that the abundance of G_i in adipocyte membranes was decreased 4h after treatment of hypophysectomized rats with GH. We therefore examined the possibility that changes in amount or distribution of G-proteins in adipocyte membranes might account for the lipolytic action of GH.

Homogenates of GH/Dex-treated and control adipocytes were subjected to differential centrifugation and the abundance of G-proteins in low speed, 16k x g (16k), pellets and high speed, 100k x g (100k), pellets were determined by quantitative Western analysis with densitometry. A 35% loss of $G_i\alpha_2$ from the 16k pellet compared from tissues treated with GH/Dex was associated with a 70% increase of $G_i\alpha_2$ in the 100k pellet. No change in $G_s\alpha$ was observed in the 16k pellet but a 35% loss of $G_s\alpha$ was seen in the 100k pellet. The G proteins in the 16k pellet were fractionated on a continuous sucrose gradient followed by quantitation with Western analysis or autoradiography after ^{32}P-NAD ribosylation. $G_i\alpha_2$ was consistently shifted from heavier to lighter fractions of the 16k pellet after treatment with GH/Dex. Similar shifts of $G_s\alpha$ were not seen. The distribution of ^{32}P-labelled proteins was comparably altered after incubation of homogenates of control and GH/Dex treated adipocytes with PTx and ^{32}P-NAD. These shifts were blocked by treatment of adipocytes with 100μM colchicine which also blocked the lipolytic action of GH/Dex. We propose that an action of GH/Dex on the cytoskeleton of fat cells may change the cellular distribution of G-proteins in a manner that produces a relative decrease in the tonic inhibitory influence of G_i on adenylyl cyclase.

Table of Contents

List of Figures

List of Tables

Introduction and Background

Growth hormone (GH) was discovered and named for its growth promoting effects (Evans and Long, 1921; Knobil and Greep, 1959). If promoting growth were its only action, one would expect GH concentrations in blood to be greatest during childhood and adolescence and low or negligible in the non-growing adult. However, levels of GH remain high (~60% of that in adolescence) up until the 3rd decade of life when they drop to about 25% of the levels observed in adolescence (Zadik *et al.*,1985). In the non-growing individual, it is likely that GH's primary role may be as a regulator of metabolism. A role for GH in fat metabolism was first recognized in 1934 when Lee and Schaffer (1934) found that chronic treatment of rats with pituitary extracts rich in growth promoting activity increased lean body mass at the expense of body fat. Subsequent confirmation of decreased body fat after treatment with purified GH and findings of decreased respiratory quotient, increased ketogenesis, and accumulation of hepatic fat supported the notion that GH increases fat mobilization and oxidation (Goodman and Schwartz, 1974). Experiments with rats on limited food intake suggested that continued growth in response to GH was possible only as long as there was sufficient mobilizable fat to fuel the growth process (Greenbaum, 1953) and linked fat mobilization to growth stimulation. After

it was discovered that free fatty acids (FFA) in blood are the form in which fat is mobilized from adipose storage depots (Dole, 1956; Gordon and Cherkes, 1956), Raben and Hollenberg (1959) and Goodman & Knobil (1959) reported increases in FFA concentration in blood following injection of GH to non-growing humans or monkeys. Development of radioimmunoassay for GH led to the observation that GH levels fluctuate according to metabolic demands such as increased GH secretion during fasting (Glick *et al.*,1965). This evidence supports the notion that GH may act as a regulator of metabolism as well as of growth.

The metabolic effects of GH compliment its growth promoting effects in that GH tends to influence metabolism in ways that directly or indirectly enhance or defend the lean body mass. Thus GH directs the flow of metabolic substrates towards consumption of fat and away from the breakdown of proteins.

Some of the growth promoting effects of GH (for example, bone) are achieved through the insulin-like growth factor I (IGF-I), which is produced by liver and peripheral cells in response to GH. Insulin-like growth factor stimulates mitosis and/or differentiation of fibroblasts, prechondrocytes, and other progenitor cells (Isaksson *et al.*, 1987). Upon stimulation by GH, prechondrocytes release IGF-I which operates in an autocrine fashion (Isaksson *et al.*, 1987) to stimulate clonal expansion and differentiation. In

contrast, the metabolic effects of GH are thought to be achieved through a direct action, without an intermediary such as IGF-I, on cells in adipose tissue, liver, muscle, and pancreas. The metabolic effects of GH may be divided into two types: acute and chronic effects. The acute effects are rapid in onset (within minutes) and short lived. Acute effects are seen in cells that have been deprived of GH for at least 3 hours and are sometimes referred to as insulin-like effects because they resemble many of the actions of insulin (Goodman, 1965). For example, increased glucose uptake and oxidation to CO_2. Chronic effects are seen in the time frame of hours or days and are contrary to the effects of insulin; that is, glucose metabolism by muscle and fat are reduced and fat metabolism is accelerated (Goodman, 1968).

When physiological amounts of GH are administered to GH-deficient or normal experimental animals or human patients, characteristic changes are observed depending on duration of treatment and the prevailing physiological state. In non-growing subjects, GH maintains body carbohydrate reserves during starvation and thereby, perhaps prolongs survival. In the growing subject, nitrogen is retained in structural proteins and in enzymes involved in increasing lean body mass (Kostyo and Nutting, 1974). As muscle and liver mass increase, so too do nucleic acids and carbohydrate reserves (Altszuler, 1974). Although body fat increases somewhat during GH-stimulation of growth in well nourished young

subjects, the general metabolic trend is towards increasing the ratio of lean body mass to body fat. Therefore, in GH treated subjects, there is an increase in the number of fat cells but paradoxically, the total mass of stored triglyceride decreases relative to other body constituents (Morikawa *et al.*, 1982; Nixon and Green, 1984). In non-growing human adults, GH decreases body fat through the combined actions of decreasing fat synthesis and increasing fat mobilization (Saloman *et al.*,1989; Rudman *et al.*,1990).

Repeated injections of supra-physiological doses of GH have been reported to increase circulating glucose and insulin concentrations in humans (Davidson, 1987), dogs (Altszuler, 1974), and pigs (Gopinath and Etherton, 1989), as well as blood levels of FFA and glycerol. Increased insulin levels may contribute to decreased glucose tolerance seen in these subjects since high levels of insulin decrease the abundance of insulin receptors in target cells (down-regulation) (Davidson, 1987). In dogs, the pancreatic beta cells may be irreversibly damaged by chronic treatment with high concentrations of GH, perhaps as a result of prolonged hypersecretion of insulin due to increased circulating levels of glucose. (Pierluissi and Campbell, 1980).

Control of lipolysis

A wide variety of hormones has been shown to stimulate lipolysis in rat adipose tissue *in vitro*. The earliest investigations demonstrated sensitivity to catecholamine (Gordon and Cherkes, 1956; White and Engel, 1958a), glucagon (Steinberg *et al.*, 1959), ACTH, and GH (White and Engel, 1958b; Raben and Hollenberg, 1959a), and various pituitary hormones (Steinberg and Vaughan, 1965; Robison *et al.*, 1971). These results were obtained originally with adipose tissue segments, but similar effects are also evident in isolated fat cells.

Although many hormones appear to be lipolytic *in vitro*, few can be regarded as physiologically important regulators of lipolysis. The fact that catecholamines elicit lipolysis in many mammalian species suggests that they may be the principle lipolytic agents in mammals (Scow and Chernick, 1970). However, the concentrations effective *in vitro* (1-100 μg/L or 5.5-550 nM) are very high (perhaps to compensate for the rapid oxidation of catecholamine *in vitro*) relative to those in plasma (0.02-5.6 μg/L or 0.11-30.6 nM) (Dearborn and Skillman, 1976). It is possible that such high concentrations may be attained when noradrenaline is released locally by the sympathetic nervous system and thus may be more important in regulating lipolysis than circulating catecholamines of adrenal origin. Stimulation of nerves in

preparations, *in vitro*, of epididymal fat pads from rat (Correll, 1963) was shown to cause release of FFAs. Abolishing sympathetic activity by denervation or with ganglionic blockers was shown to attenuate exercise-induced lipid mobilization (Havel, 1968). On the other hand, Wirsen (1965) and Ballantyne & Raftery (1969) did not find histological evidence for adrenergic innervation of fat cells in several species, and proposed that nervous stimulation of lipolysis occurs by release of noradrenaline into the circulation and alteration of blood flow. Studies for the role of the sympathetic nervous system in the regulation of lipolysis have been reviewed by Havel (1968), Brodie *et al.*, (1965), Scow and Chernick (1970) and Fredholm (1970).

A lipolytic effect of GH was first demonstrated at only very high concentrations (>1 μg/ml) (Jungas and Ball, 1962) but Fain *et al.*, (1965) showed that much lower concentrations, as low as 10 ng/ml, were effective when glucocorticoids were also present. Under these conditions, 1-2 hrs elapsed before a measurable change in glycerol production was seen. The stimulation of lipolysis was slow to develop and was prevented by inhibitors of RNA and protein synthesis. In contrast, the almost immediate lipolytic effects of ACTH and catecholamine are insensitive to these inhibitors.

In addition to the rapid effects of many lipolytic hormones, there appear to be long-term endocrine controls of lipolysis. Adipose tissue from

adrenalectomized rats has a diminished capacity to respond to lipolytic hormones both *in vitro* and *in vivo* (Reshef and Shapiro, 1960; Allen and Beck, 1972), a defect that can be repaired by treatment with glucocorticoids. Hyperthyroid and hypothyroid animals show increased and decreased sensitivity to catecholamine both *in vitro* and *in vivo* (Debons and Schwartz, 1961; Armstrong *et al.*, 1974; Correze *et al.*, 1974). Thyroid hormone has no direct effect on lipolysis when added *in vitro* but correction of thyroid status in the hypothyroid animal also normalizes the sensitivity of adipose tissue to lipolytic agents (Elks and Manganiello, 1983).

Biochemistry of lipolysis in adipocytes

The cycle of fatty acid esterification to form triglycerides and of triglyceride breakdown (lipolysis) to release fatty acids and glycerol is an ongoing process within the adipocyte. Re-esterification of glycerol and free fatty acids requires the glycerol to be in the form of α-glycerol phosphate. Free glycerol produced by lipolysis cannot be phosphorylated because adipocytes are deficient in the enzyme α-glycerokinase (Margolis and Vaughan, 1962). The only source of α-glycerol phosphate is the triose phosphate pool which is derived from glucose metabolism. The rate of fatty acid release thus is sensitive to any factor that affects the rate of glucose

metabolism in adipocytes as well as any factor that accelerates lipolysis. In addition to promoting lipolysis, GH can inhibit re-esterification by limiting the availability of α-glycerol phosphate by decreasing glucose metabolism which could theoretically double or triple free fatty acid mobilization even if the rate of lipolysis is remaining constant (Goodman, 1968).

Before exploring the lipolytic actions of GH, it may be useful to review briefly current understanding of the lipolytic process (for detailed review, see Steinberg and Huttumen, 1972; Steinberg, 1976). Lipids are stored in adipose tissue in the form of triglycerides which are composed of 3 mol of long-chain fatty acids in ester linkage with 1 mol of glycerol. Lipolysis is the step-wise enzymatic cleavage of the ester bonds to release fatty acids and glycerol. The rate-limiting step seems to be cleavage of the ester bond at the α-carbon of glycerol and is catalysed by the hormone-sensitive lipase (see below). Successive cleavage of the remaining ester bonds by the hormone-sensitive lipase or other tissue esterases progresses rapidly with little or no accumulation of mono- and diglycerides. Fatty acids that are not esterified are released into the circulation as FFA. *In vitro*, only a small fraction of the fatty acids released from ester linkages escapes from the adipocyte (Goodman, 1968; Sinnett-Smith and Woolliams, 1989). Also *in vitro*, most fatty acids within adipocytes, usually more than two-thirds, are re-esterified and hence

trapped within the fat cell as triglyceride. The net rate of FFA mobilization is thus determined by the relative rates of the opposing processes of lipolysis and re-esterification. Although the FFAs released by lipolysis can be recycled to triglyceride, the glycerol cannot be recycled, since triacylglyceride synthesis requires α-glycerol phosphate rather than free glycerol, which escapes into the extracellular space. Therefore the rate of glycerol production, rather than of FFA production, is a good index of lipolysis (Figure 1).

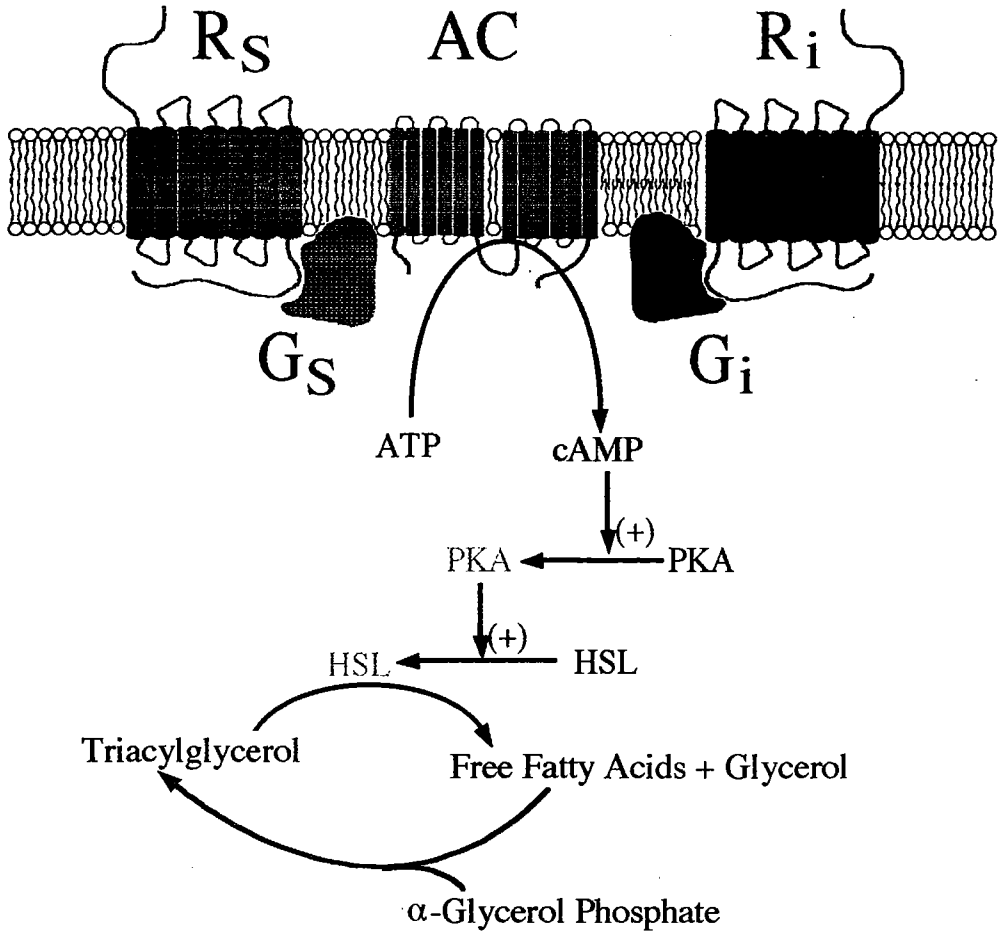


Figure 1

The cyclic AMP-mediated lipolytic pathway. Stimulatory (Rs) or inhibitory (Ri) receptors, through the intermediary actions of stimulatory (Gs) or inhibitory (Gi) G proteins stimulate or inhibit adenylyl cyclase (AC). Activation of adenylyl cyclase catalyses the conversion of ATP to cyclic AMP. Cyclic AMP acts as second messenger to activate protein kinase A, which phosphorylates and activates hormone-sensitive lipase. Hormone-sensitive lipase catalyses the breakdown of triacylglycerol to free fatty acids and glycerol. Free fatty acids may recycle to reform triacylglycerol but glycerol cannot recycle because the formation of triacylglycerol requires α -glycerol phosphate rather than free glycerol. Adipocytes cannot form α-glycerol phosphate from glycerol.

The adipocyte can respond to a wide variety of endocrine and paracrine signals that can either stimulate or inhibit lipolysis. Stimulatory agents such as adrenaline binds to stimulatory receptors which transduce the signal and activate adenylyl cyclase via stimulatory guanine nucleotide binding proteins or G_s proteins. Likewise, inhibitory agents such as adenosine bind to inhibitory receptors which transduce the signal to adenylyl cyclase via inhibitory G_i proteins (Figure 1).

Under resting conditions, adenylyl cyclase in the adipocyte is suppressed by inhibitory agonists such as adenosine and prostaglandin E_2 through an autocrine fashion (Kather *et al.*, 1985). These agents presumably produce a basal level of inhibition on adenylyl cycase which upon removal of these agents (for example with adenosine deaminase or indomethacin) relieves inhibition and hence an apparent stimulation of cyclase. Although it has long been recognized that the α subunit of G_s has a direct stimulatory effect on adenylyl cyclase, the ability of the α subunit of G_i to inhibit adenylyl cyclase has only been established recently (Taussig *et al.*, 1993; Wong *et al.*, 1991).

Lipid mobilizing effects of growth hormone

Growth hormone *in vitro* has been shown to decrease glucose metabolism in fat tissues of rodents (Goodman, 1968; Maloff *et al.*,1980), humans (Nyberg *et al.*,1980; Nyberg and Smith, 1977), and other species including sheep (Sunnett-Smith and Woolliams, 1989) and pigs (Magri *et al.*, 1990). This effect was slow in onset, required many hours and was seen both in the absence (Goodman, 1968; Maloff *et al.*, 1980; Nyberg *et al.*, 1980; Nyberg and Smith, 1977; Sunnett-Smith and Woolliams, 1989; Magri *et al.*, 1990) and in the presence of insulin (Walton *et al.*, 1986; Etherton *et al.*, 1987; Cameron *et al.*, 1987). Reduced glucose metabolism appears to be a direct action of GH as well as an indirect consequence of diminishing insulin sensitivity. Reduced glucose metabolism results in decreased *de novo* synthesis of fatty acids in the fat cell and decreases the availability of α-glycerol phosphate required for re-esterification. Therefore a higher proportion of newly liberated fatty acids escape into the circulation as FFA. Growth hormone also increases lipolysis through a direct action on the lipolytic apparatus and perhaps also by antagonizing the anti-lipolytic effects of insulin (Rosenbaum *et al.*, 1989). All these actions result in decreased body fat and increased FFA and glycerol in blood.

Increases in glycerol and FFA production of up to 10 times or greater, are seen within minutes of incubation of rat fat with catecholamine, ACTH, or TSH (Vaughan and Steinberg, 1963). Because GH failed to stimulate lipolysis within this time frame except when high concentrations of highly purified, but nevertheless impure hormone preparations were used (Goodman and Schwartz, 1974), the likelihood of a direct lipolytic action was questioned. The lipolytic effect of GH seldom exceeds twice the basal rate of lipolysis and has not been observed consistently.

Uncertainty of GH's ability to increase lipolysis arose in the era in which GH preparations were obtained from pituitary extracts which were often contaminated with other pituitary peptides known to be potent lipolytic agents (ex: ACTH, TSH). Although recombinant GH, derived from bacterial sources, is free of contamination by mammalian lipolytic hormones, uncertainty of GH's ability to increase lipolysis persists. Recombinant human or bovine GH were found to be lipolytic in rat (Goodman, 1984; Elsair *et al.*, 1985) and mouse (Fielder and Talamantes, 1987) adipose tissue, in cultured mouse 3T3-F422A adipocytes (Dietz and Schwartz, 1991), and in chicken adipose explants (Campbell *et al.*, 1990; Campbell and Scanes, 1985). Other investigators, however, found either inconsistent (Frigeri *et al.*, 1982) or no (Hart *et al.*, 1984) lipolytic action of recombinant bovine GH in rat adipose tissue.

Different findings may result from different experimental conditions. Hart *et al.*, (1984) obtained negative results in tissues from rats that had been fasted 48 hours prior to excision of adipose tissue, while the positive effects reported by Grichting and Goodman (1983) were seen in fed rats. Frigeri *et al.*, (1982) found small lipolytic effects in tissues of fed, but not starved rats. In addition, consistent findings of increasing lipolysis (Goodman, 1984; Grichting and Goodman 1983) reflect the behavior of adipose tissues only in the 4th hour after addition of GH, when the effect is fully developed. In contrast, other investigators averaged the rate of glycerol production over an entire 4 hour incubation period which minimizes any GH effect by including rates of glycerol production in the 2 hours preceding the appearance of the lipolytic effect. Furthermore, this approach compares glycerol production in response to GH with an inappropriately high control value since basal glycerol production often declines precipitously after the 1st hour *in vitro* (Goodman, 1981).

GH has been reported to have no lipolytic effects in rabbit (Bowden *et al.*, 1985; Barenton *et al.*, 1984), swine (Walton and Etherton, 1986), cattle (Peters, 1986; McDowell *et al.*, 1987), chicken (Duquette *et al.*, 1984), reindeer (Larsen and Nilssen, 1985), and human adipose tissue explants (Nyberg and Smith, 1977). Species differences may account for some of these findings; rabbit fat, unlike that of other species, is insensitive to isoproterenol (Fain *et*

al., 1984) as well as GH, but it is 10 times more sensitive to β-lipotropin than rat fat (Lis *et al.*, 1972). Other findings are inconclusive and most of the negative findings require critical re-evaluation. Although no effects of GH on lipolysis were observed in cultured swine fat (Walton and Etherton, 1986), no glucocorticoids were present and the culture conditions were such that the basal rate of lipolysis was 4 times higher than the maximum seen in rat fat. No data were included to demonstrate that these explants were sensitive to any lipolytic agent. In explants of bovine fat (Peters, 1986), mean rates of glycerol production were higher in some groups of tissues treated with GH, but with only 3 or 4 observations per group, statistical significance was not achieved. Lipolytic effects of GH on human adipose tissue were sought only in fragments of subcutaneous fat that had been cultured for 7 days with or without GH (Nyberg and Smith, 1977). These conditions may not have been optimal for demonstrating an increase in lipolysis in response to any agent since basal lipolysis was 4-25 times higher than in freshly isolated human subcutaneous adipocytes (Ostman *et al.*, 1979). Responsiveness to other lipolytic agents was not examined in these studies.

Except for rabbits, which have anomalous responses to lipolytic agents in general, available evidence against a direct lipolytic effect of GH in humans and farm animals is not convincing. A survey of the literature revealed only one study of the effects of GH in human adipose tissue (Nyberg

and Smith, 1977) and its conclusions are at odds with a wealth of data that support a lipolytic action for GH *in vivo* (Davidson 1987; Press, 1988). Because the lipolytic effect of GH is small in contrast to that of catecholamine, its successful demonstration *in vitro* requires careful optimization of experimental conditions with respect to the incubation medium, maintenance of low basal rates of lipolysis, and careful timing of the measurement of hormone action.

Role of glucocorticoids

The mechanism of action of glucocorticoids has interested investigators for over 50 years. Shortly after its role in carbohydrate metabolism was established, Ingle (1952) recognized that the glucocorticoids must be involved in a wide variety of seemingly unrelated processes and postulated that they act as general hormones permitting key metabolic processes to achieve maximal rates.

Glucocorticoids are required for maximal metabolic rates of gluconeogenesis (Exton *et al.*, 1972), glycogenolysis (Stalman and Laloux, 1979), glycogenesis (Plas and Nunez, 1976), and lipolysis (Fain, 1979). Since glucocorticoids are not direct agonists for these processes, the idea of a permissive role of glucocorticoid hormones evolved. The concept of

permissive action accounts for the observations that physiologic concentrations of glucocorticoids hormone that have no stimulatory or inhibitory effects on a specific process *per se* but nevertheless "permit" a maximal response to another hormone or stimulus.

The mechanism of action of glucocorticoids has been studied extensively (for review, see Baxter and Rousseau, 1979; Yamamoto, 1985) and appears to involve the binding of glucocorticoid to a glucocorticoid receptor located in the cytosol. The ligand-receptor complex translocates into the nucleus which then binds to DNA on specific DNA sequences called glucocorticoid response elements. Binding to DNA initiates transcription of mRNAs and eventually to translation and protein synthesis.

In addition to affecting processes in the nucleus, glucocorticoids have also been demonstrated to amplify the effects of non-steroid hormones. Glucocorticoids have been shown to amplify the rates of glucagon-, adrenaline-, ACTH, and GH-induced lipolysis. Yet the fact that glucocorticoids can amplify the effects of these hormones which presumably act through a cyclic AMP-mediated process suggest that the signal transduction pathway for glucocorticoids and peptide hormones may converge at some point.

The mechanism of the steroid-cyclic AMP interaction is unknown and although one or more steps in the action of these compounds could be

involved, most studies have focused on the pathway through which cyclic AMP acts. Possibilities which could be involved include (1) regulation of the intracellular level of cyclic AMP, (2) regulation of the action of cyclic AMP, and (3) interaction through a steroid dependent product.

Regulation of intracellular cyclic AMP levels

Glucocorticoids could enhance the action of cyclic AMP by allowing an increase in the intracellular concentration of this nucleotide if levels of cyclic AMP were limiting. However, data in the literature (Honnor *et al.*, 1985) and to be presented in this thesis indicates that , at least for lipolysis, the rate limiting component is the hormone sensitive lipase. If cyclic AMP were not rate-limiting, a cyclic AMP-mediated pathway may still be amplified by increasing the pre-existing levels of cyclic AMP. However, Granner *et al.*, (1968) have shown that glucocorticoids alone did not increase cyclic AMP production in liver cells after 4 hours of treatment. It is possible that glucocorticoids do not increase cellular levels of cyclic AMP *per se* but rather create conditions which promote the accumulation of cyclic AMP produced by other hormones. Such a condition may arise if glucocorticoids inhibit phosphodiesterase activity. Although glucocorticoids have been demonstrated to inhibit phosphodiesterase activity in liver cells, the inhibition was not sufficient to allow for cyclic AMP accumulation (Granner

et al., 1975). Thus cyclic AMP generation or degradation does not seem to be involved in the permissive effect of glucocorticoids.

The glucocorticoid and cyclic AMP pathway is thought to converge at some point although the exact point is unknown. There is evidence , however, that the site of interaction is distal to cyclic AMP. Friedman *et al.*, (1969) showed that the stimulatory effect of glucagon on gluconeogenesis noted in intact animals was not obtained in livers of adrenalectomized rats, despite that fact that identical increases in cyclic AMP were achieved in both. Schaeffer *et al.*, (1969) postulated that the metabolic lesion in adrenalectomized rats lies beyond adenylyl cyclase, since cyclic AMP was unable to activate phosphorylase in the liver of such animals. This result was confirmed in a perfused liver system by Exton *et al.*, (1972) who showed that, although the levels of cyclic AMP achieved by perfusing glucagon or adrenaline through livers from normal or adrenalectomized rats was normal, gluconeogenesis and glycogenolysis were impaired. These authors also showed that lipolysis was markedly blunted in adipose tissue from adrenalectomized rats (Exton *et al.*, 1972). Also, induction of tyrosine aminotransferase required pretreatment with dexamethasone although dexamethasone had no effect on basal levels of intracellular cyclic AMP (Granner *et al.*, 1975) further supporting the notion that glucocorticoids act distal to cyclic AMP accumulation.

Regulation of cyclic AMP action

There are several ways in which changes in the activity of the cyclic AMP-mediated pathway could occur in the absence of a change in cyclic AMP levels: (1) The amount of the regulatory subunit of protein kinase A or its affinity to cyclic AMP may change . (2) The amount of the catalytic subunit or its K_m of activation by $Mg^{++}ATP$ could change. (3) Protein kinase could be activated *in vivo* by changes in distribution of cyclic AMP, and/or kinase translocation. Finally, (4) the activity and/or amount of protein kinase inhibitor may be changed.

If the affinity to cyclic AMP were to increase in the presence of glucocorticoids, then one would expect pretreatment with glucocorticoids to increase dibutyryl cyclic AMP actions. This was not the case as reported by Fain (1968). Pretreatment of fat cells for 2 hours with dexamethasone failed to potentiate dibutyryl cyclic AMP-induced lipolysis which would argue against the possibility that dexamethasone increases sensitivity of protein kinase A to cyclic AMP. Although no one has looked at whether glucocorticoids could change the number of cyclic AMP binding sites to the number of protein kinase A catalytic subunits, there is evidence to indicate that the total kinase activity within liver cells (Rousseau and Wérenne, 1976) and fat cells (data to be presented) is unchanged. Also, if glucocorticoids were

able to alter K_m, then a given amount of cyclic AMP should be more effective in the presence of glucocorticoids but again this was not the case (Granner *et al.*, 1977; Rousseau and Wérenne, 1976). Thus the first 2 possible actions of glucocorticoid on cyclic AMP-dependent protein kinase seems unlikely.

Does dexamethasone promote protein kinase translocation? Translocation of protein kinase activity has been suggested to occur in heart (Corbin *et al.*, 1977) and liver (Rousseau *et al.*, 1976) and is a potential mechanism of control. Such a mechanism could be accomplished without significant changes in the intracellular concentration of cyclic AMP. Corbin *et al.*, (1977) demonstrated that the rabbit heart protein kinase associated with the particulate fraction. Addition of cyclic AMP causes cytosol catalytic activity to increase, with a corresponding decrease in activity in the particulate fraction. The authors use this data to postulate a model for the hormonal control of the compartmentalization, and activation, of protein kinase A (Corbin *et al.*, 1977). However, Rousseau *et al.*, (1976) had studied the intracellular distribution of protein kinase A in liver and found it to be the same in intact and adrenalectomized rats. Re-administration of glucocorticoids did not change the distribution of protein kinase A and thus does not appear likely that protein kinase A translocation is involved in the interaction of glucocorticoids and cyclic AMP.

Does dexamethasone modulate protein kinase A activity through an inhibitor? In addition to regulation by cyclic AMP, protein kinase A can be modulated by a heat-stable protein kinase inhibitor (Walsh *et al.*, 1971). This protein kinase A inhibitor appears to inhibit catalytic activity by binding to free catalytic subunits and inhibiting its enzymatic activity. Glucocorticoids could indirectly influence kinase catalytic activity by altering the amount of this inhibitory protein or the kinetics of binding of cyclic AMP to protein kinase A. However, the observation that kinase activation by cyclic AMP is identical in control and dexamethasone-treated cells argue against a regulatory effect of glucocorticoids via an inhibitor. As well, levels of the heat-stable protein kinase inhibitor appear to be unchanged with dexamethasone stimulation (Ashby *et al.*, 1977).

Interaction through a steroid dependent product

Other actions of glucocorticoid require a lag time presumably to allow for RNA and protein synthesis. Adrenalectomized animals has been shown to have a blunted hyperglycemic response to adrenaline. Administration of cortisol to these animals for 3 days restored the hyperglycemic response to adrenaline where an injection of cortisol only 15-20 min before adrenaline failed to restore the hyperglycemic response (Schaeffer *et al.*, 1969). This lag suggests that glucocorticoids induces the

synthesis of proteins for the hyperglycemic response. This finding supported the earlier finding by Fain (1967) in which he demonstrated that inhibitors of RNA and protein synthesis prevented the lipolytic actions of GH/Dexamethasone. Thus it seems the augmentation of the cyclic AMP-mediated pathway requires RNA and protein synthesis.

Finally, an additional mechanism by which glucocorticoids may potentiate the cyclic AMP pathway without activating it is by removal of tonic inhibition. Kather *et al.*, (1985) had shown that, *in vivo*, fat cells are under predominantly inhibitory control and prostaglandins was shown to be one of the inhibitory agents but did not address the question as to the source of the prostaglandin. Parker *et al.*, (1989) had found that isolated adipocytes were unable to synthesize prostaglandin E_2 but a more recent finding by Richelsen *et al.*, (1992) have shown that adipocytes do indeed synthesize prostaglandins.

Lewis and Piper (1975) had shown that glucocorticoids could inhibit the release of prostaglandins. By preventing the release of prostaglandins, glucocorticoids could potentiate the lipolytic effect of GH. In addition to preventing the release of prostaglandins, glucocorticoids have also been shown to have a potent blocking action on prostaglandin biosynthesis (Flower *et al*, 1972). It was eventually demonstrated that the blocking action of glucocorticoids was prevented by inhibitors of protein synthesis (Flower

and Blackwell, 1979). Eventually the protein which inhibited prostaglandin synthesis was identified (Blackwell *et al.*, 1982) and named lipocortin (Di Rosa *et al.*, 1984). Lipocortin inhibits prostaglandin biosynthesis by inhibiting the enzyme phospholipase A_2 which catalyses the formation of arachidonate, a precursor to prostaglandins. Thus glucocorticoids have a double action in suppressing prostaglandin action: inhibition of its synthesis and release, both of which can potentially augment the cyclic AMP-mediate pathway in a hormone response without actually triggering the cyclic AMP cascade.

G proteins

G protein coupled receptors

Before exploring the different mechanisms by which GH may transduce its signal for lipolysis, it may be useful to review what is known about the receptors and signalling mechanisms for the more extensively studied lipolytic agents such as catecholamine.

Catecholamine receptors, which belong to a superfamily of glycoprotein receptors that consist of approximately 400-600 amino acids, include receptors for adrenergic, dopaminergic, muscarinic, and serotoninergic ligands. Peptide hormone receptors in the same superfamily include tachykinins, glucagon, and somatostatin to name a few (for review

see Dohlman *et al.*, 1991). The most notable feature of this superfamily is the presence of 7 stretches of 24-28 predominantly hydrophobic amino acids arranged in α-helical structures that are thought to represent transmembrane domains by analogy with the known secondary and tertiary structure of bacteriorhodopsin (Henderson and Unwin, 1975). Thus it appears that these molecules thread back and forth through the cell membrane such that there are 4 extracellular regions and 4 intracellular regions. The amino acid homologies among the members of this gene family are greatest within the transmembrane domains (Dohlman *et al.*, 1991). The putative transmembrane domains are connected by alternating extracellular and intracellular domains (Wang *et al.*, 1989). The amino termini of G protein-coupled receptors are extracellular and contain sites for N-linked glycosylation while the cytoplasmic domains contain potential sites for phosphorylation. The C-terminus and the cytoplasmic segment between helices V and VI form domains that interact with G proteins. It now appears likely that any receptor that interacts with G proteins contains these key structural features (Figure 2).

Since most lipolytic agents utilize the cAMP-dependent pathway, which is regulated by G proteins, and because G proteins are potential targets for GH action it may be useful to review the current understanding of these

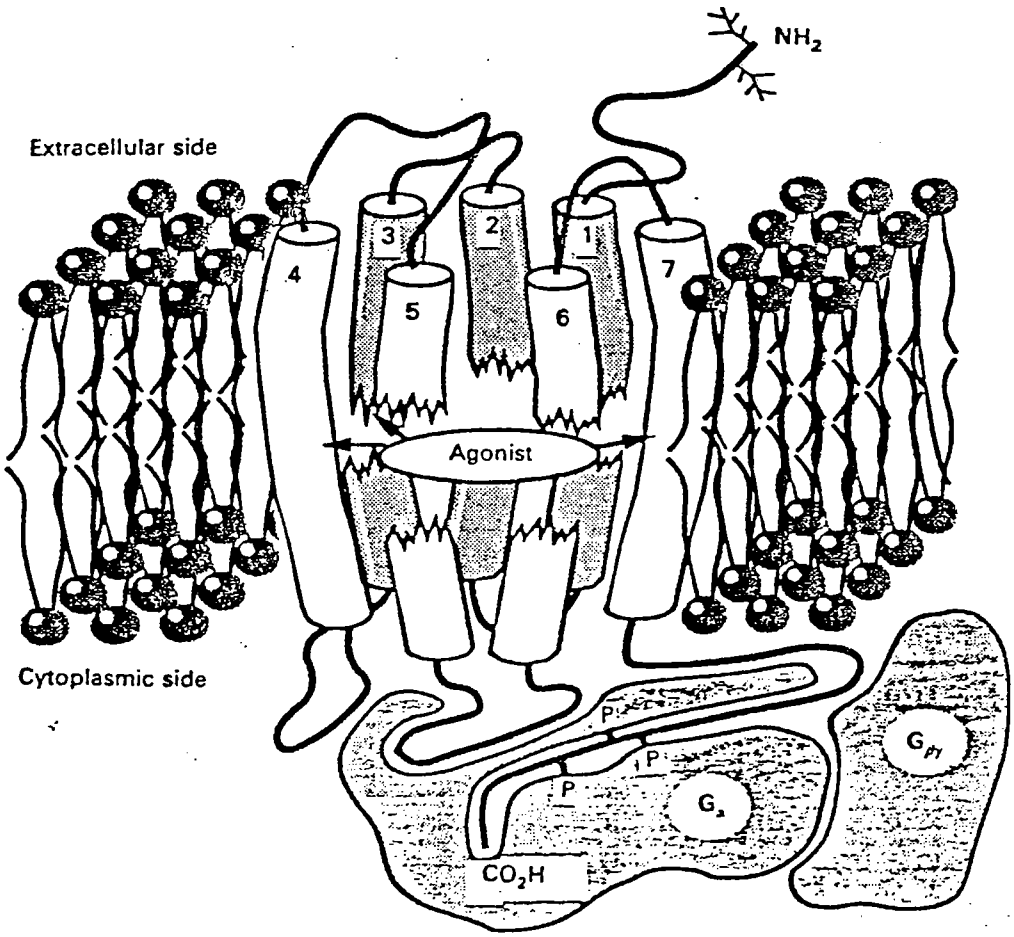


Figure 2

Shared features of receptors coupled to G proteins. The model illustrates some of the features predicted to be shared by all receptors that interact with G proteins. About half the residues are proposed to form seven transmembrane helices which appear to form the agonist binding pocket. Variable numbers of the extracellular asp residues in the N-terminal tail are glycosylated and in the intracellular C-terminal tail, several serine and threonine residues may be phosphorylated. Some of the regions proposed to interact with G proteins are also shown. (Taken from Taylor, 1990)

ubiquitous regulators. In 1971, Rodbell and co-workers demonstrated that the hormone glucagon (which exerts its effects through adenylyl cyclase) and its receptor are not sufficient to activate adenylyl cyclase. The nucleotide guanosine triphosphate (GTP) had to be present too (Rodbell *et al.*, 1971). This finding ultimately led to the discovery of the family of GTP-binding proteins, now known as G proteins, which play a pivotal role in transducing a wide variety of extracellular stimuli into intracellular responses. G proteins may transmit signals that stimulate or inhibit adenylyl cyclase and are accordingly designated G_s or G_i. It was subsequently found that G proteins also regulate other effectors besides adenylyl cyclase including phospholipase C and ion channel proteins (Cockcroft and Gomperts, 1985; Breitwiesser and Szabo, 1985). These G proteins are heterotrimers that consist of α, β, and γ subunits, of molecular mass 36-52, 35-36, and 6-8 kD, respectively and are believed to be bound to the cytoplasmic face of the membrane bilayer (Gilman, 1987). Regulation of adenylyl cyclase is mediated primarily by the α subunits which are therefore designated $G_i\alpha$ or $G_s\alpha$. β and γ subunits are shared by $G_s\alpha$ and $G_i\alpha$. To date, molecular cloning techniques have identified in mammalian tissues more than 17 different α subunits, 4 β subunits, and 4 γ subunits (Simon *et al.*, 1991). Rat adipocytes express three

forms of inhibitory G proteins (G_i1, G_i2, G_i3) which are distinct gene products (Mitchell *et al.*, 1989; Milligan, 1988). Some investigators have suggested $G_i\alpha_2$ as the $G_i\alpha$ species responsible for the inhibition of adenylyl cyclase (McKenzie and Milligan, 1990; Simonds *et al.*, 1989). Rat adipocytes also express two isoforms of G_s (42 and 45 kD), which are derived from a singlegene product by alternative splicing of mRNA (Strassheim, 1991, 1990; Robishaw *et al.*, 1986).

The β and γ subunits form a $\beta\ \gamma$ complex, which can only be dossociated under denaturing conditions. The major function of the $\beta\ \gamma$ complex appears to be the formation of the G protein trimeric complex, which constitutes the inactivated state of the protein. There is also some evidence that $\beta\gamma$ complexes may play a role in anchoring α subunits to the membrane (Sternweis, 1986). More recently, the $\beta\ \gamma$ complex has also been implicated as a mediator of intracellular signals such as activation of phospholipase A_2 (Kim *et al.*, 1989; Axelrod *et al.*, 1988; Jelsema *et al.*, 1987), adenylyl cyclase (Federman *et al.*, 1992; Tang and Gilman, 1991), and the mating response in yeast (Whiteway *et al.*, 1989). Although the different G proteins have traditionally been defined by their α subunits, the recent discoveries of greater numbers of β and γ subunits complicate this picture.

Signal transduction processes known to be mediated by G proteins include the hormone- and neurotransmitter-mediated regulation of adenylyl cyclase, phosphoinositide-derived second messengers and ion channels and activation of sensory systems including vision, olfaction, and taste. The molecular mechanism of G protein-mediated signal transduction has been elucidated in two systems: the hormone-stimulated increase in adenylyl cyclase activity and the light-stimulated phototransduction cascade in retinal rod cells (Neer and Claphman, 1988; Gilman, 1987).

The disaggregation theory, first proposed by Rodbell (1980) describes the mechanism by which G proteins may transduce hormonal signals. This theory suggests that a wide variety of receptors are coupled with a family of G proteins. In the resting state, G proteins, which consist of $\alpha\beta\gamma$ subunits, are associated with GDP. When a hormone binds to its G protein coupled receptor, G protein exchanges its bound GDP for GTP, and becomes activated. The G protein heterotrimer then dissociates into the free α monomer and the $\beta\gamma$ dimer. The GTP-bound α subunit then diffuses to and binds to an effector (eg: adenylyl cyclase or ion channel) and activates it. After a few seconds, the α subunit, which has intrinsic GTPase activity, converts GTP to GDP thereby inactivating itself. The inactivated α subunit then re-associates with the $\beta\gamma$ complex and resumes the resting state.

In spite of the extensive knowledge of G protein structure and actions, understanding of the topological relationship between receptors, G proteins and effectors, and their relative stoichiometry is limited. It is not even known whether the quantitative relationship between individual components of the G protein signalling pathway changes. There is evidence that G proteins are not confined to the inner surface of the membrane (Garty *et al.*, 1988). Those attached to the membrane are activated by hormones. Tolkovsky and Levitzki (1978) proposed a "collision-coupling" model in which the rate of adenylyl cyclase activation is proportional to the frequency and efficiency of collisions between agonist-bound receptor and G protein. In this way, a receptor may activate a number of G proteins due to the free mobility of each component. Although receptors may interact or "collide" with more than one G protein, each type of receptor interacts with a specific subtype of G protein. For example, stimulatory receptors interact exclusively with stimulatory G proteins.

An important characteristic of G protein-mediated signal transduction is the ability to amplify the primary signal (Gilman, 1987). For most systems, amplification is achieved in two stages: (1) the activation of 10 to several hundred G proteins by a single ligand bound receptor, and (2) the turnover of thousands of second-messenger molecules by each activated

effector enzyme (Gilman, 1987). In this way, the signal carried by a single ligand molecule may by amplified by more than 10,000-fold.

The function of many proteins is determined by the covalent addition of a variety of molecular entities to their polypeptide chains. Post-translational modifications may serve to target a protein to its cellular location or it may regulate its activity. Such modifications may be as simple as addition or removal of a phosphate group or addition of constituents as large and complex as glycophospholipid moieties. A large number of modifications which include prenylation, acylation, phosphorylation, and ADP-ribosylation may affect G proteins. Prenylation and acylation involves the attachment of a hydrophobic side-chain to the G protein allowing it to be anchored or attached to the plasma membrane. Prenylation involves the transfer of an isoprenoid moiety to a cysteine residue near the carboxyl terminus of the γ-subunit (Yamane and Fung, 1993) followed, in some cases, by proteolysis of three terminal residues to yield a carboxyl-terminal prenyl cysteine. Acylation is a more general process and typically includes the attachment of a myristate or palmitate group to α and γ subunits (Yamane and Fung, 1993). The two prominent acylation reactions are myristoylation and palmitoylation. In myristoylation, a myristate group is attached to an amino terminal glycine, a process that appears to be cotranslational and

irreversible. Myristoylation of α subunits appears to increase the apparent affinity of α subunits for the βγ complex or for effector molecules, and to facilitate association with cellular membranes (Mumby *et al.*, 1990; Linder *eet al.*, 1991; Taussig *et al.*, 1993; Jones *et al.*, 1990). In contrast, palmitoylation occurs on cysteine residues of α subunits, is posttranslational and reversible (Roach, 1991; Edelman *et al.*, 1987) and may function to regulate activity and membrane association of α subunits (Linder *et al.*, 1993)

The functional activity of G proteins may also be regulated by phosphorylation. Phosphorylation of G_i probably catalysed by protein kinase C (Martin *et al.*, 1992; Sagi-Eisenberg, 1989; Katada *et al.*, 1985) diminishes its ability to inhibit adenylyl cyclase (Pyne *et al.*, 1989; Katada *et al.*, 1985). The enhanced ability of glucagon to increase cyclic AMP in livers of diabetic rats is associated with increased phosphorylation of $G_i\alpha_2$ (Bushfield *et al.*, 1990) but the amounts of the G proteins are unchanged (Allard *et al.*, 1991; Bushfield *et al.*, 1990).

ADP-ribosylation

ADP ribosylation differs from other modifications in that G proteins do not appear to be ADP-ribosylated under normal physiological conditions. ADP-ribosylation does occur *in vivo* when cholera toxin and pertussis toxin,

from the bacteria *Vibrio cholerae* and *Bordetella pertussis*, catalyse ADP-ribosylation of G_s & G_i α subunits. ADP-ribosylation involves the transfer of an ADP-ribose moiety from NAD^+ to an acceptor residue, such as arginine, cysteine, or asparagine. The resulting ADP-ribosylated protein loses its ability to cycle between active and inactive forms.

ADP ribosylation catalysed by either cholera toxin or pertussis toxin is a useful tool for identifying which G proteins might participate in a process and for manipulating a process experimentally. Cassel and Selinger (1977) were the first to identify the component that regulates adenylyl cyclase, later known as G_s as the substrate for ADP-ribosylation by cholera toxin and deduced that ribosylation of G_s blocked the intrinsic GTPase activity of $G_s\alpha$ (Cassel and Selinger, 1978) rendering it constitutively active.

Pertussis toxin-mediated ADP-ribosylation affects a distinct subset of G protein α subunits, $G_i\alpha$, $G_o\alpha$ (olfactory) and $G_t\alpha$ (transducin) (Katada *et al.*, 1986; Van Dop *et al.*, 1984; Manning *et al.*, 1984; Watkins *et al.*, 1984; Sternweis and Robishaw, 1984). The ADP-ribosylation of G proteins by pertussis toxin is most efficient when the α subunit is in the $\alpha\beta\gamma$ heterotrimeric form (Moss and Vaughan, 1988). ADP-ribosylation blocks the interaction of the α subunits of the inhibitory G proteins with their receptors, thereby blocking the receptor mediated exchange of GDP for GTP (Cote *et al.*, 1984; Murayama

and Ui, 1983). As a result, inhibition of adenylyl cyclase is relieved, leading to elevation in cellular concentrations of cyclic AMP.

Agonist-regulated levels and distribution of G proteins

The relative abundance of G proteins, and their subtypes is a source of debate, but there is now evidence that levels of G proteins within cells are not fixed and may change depending upon prevailing physiological conditions (Milligan and Green, 1991; Milligan, 1993). If G proteins are or can be made to be the limiting component for the action of a transmembrane signalling cascade, then they provide an additional locus for regulation of desensitization (Milligan and Green, 1993). In the few cases reported, simple measurements of the levels of G proteins suggest the G proteins are present in substantially greater abundance than adenylyl cyclase. For example, it has been calculated that there is 30-80 times more $G_s\alpha$ than adenylyl cyclase in S49 lymphoma cells (Levis and Bourne, 1992; Alousi *et al.*, 1991). It thus might seem that even substantial down-regulation of G proteins would be unlikely to limit maximal responsiveness. However, agonist induced down-regulation of G proteins often correlates with development of a so-called heterologous desensitization (Milligan and Green, 1993) in which a decrease in $G_i\alpha$ is associated with a concomitant increase in $G_s\alpha$, or vice-versa

depending on the agonist. For example, in rat white fat cells, chronic treatment with phenylisopropyladenosine (PIA) or prostaglandin E_1 produced a time- and dose-dependent down-regulation of the relative amounts of membrane-associated α subunits of G_i1, G_i2, and G_i3 while increasing levels of $G_s\alpha$ (Green *et al.*, 1990, 1992). These changes occurred without any changes in the mRNAs for these proteins suggesting regulation at the translation or degradation levels. Loss of $G_i\alpha$ was detectable within 24 hours (Hadcock *et al.*, 1991; Green *at al*, 1990) of incubation of adipocytes with the adenosine receptor agonist. Conversely, inactivating G_i by treating rats with pertussis toxin for 3 days doubled $G_i\alpha_1$ and $G_i\alpha_2$ without changing $G_s\alpha$ (Ramkumar and Stiles, 1990). Hypothyroidism also decreased $G_i\alpha$ in adipocytes (Ros *et al.*, 1988; Milligan *et al.*, 1987), while adrenalectomy decreased $G_s\alpha$ and the β subunits common to both G_s and G_i (Ros *et al.*, 1989a,b).

An alternate means of regulating the functional activity of G proteins may be to control the relative amounts of G_i and G_s in the vicinity of adenylyl cyclase. G protein subunits have been shown to partition from plasma membranes to cytosol in response to physiological changes in some experimental models. Stimulation of human platelets with adrenaline

resulted in a shift of $G_i\alpha_2$ from a detergent-soluble fraction, presumably membranes, to a detergent-insoluble cytoskeletal fraction (Crouch *et al.*, 1989). Human neutrophils subjected to a mild degranulating stimulus responded with a shift of G_i from the granule fraction to the plasma membrane fraction (Rotrosen *et al.*, 1988). Stimulation of rat adipocytes with a high concentration of isoproterenol produced a shift in the distribution of $G_i\alpha_2$, $G_s\alpha$, and adenylyl cyclase from heavier plasma membranes to less dense membranes thought to represent pinocytic vesicles (Haraguchi and Rodbell, 1990).

Treatment of membranes with analogs of GTP (eg: GTPγS) with or without a relevant agonist, can cause a time- and dose-dependent "release" of G_s proteins from the membrane fraction to the soluble fraction (Milligan and Unson, 1989). Such agonist-induced G protein partitioning from membrane to the soluble fraction would appear to require that the affinity of interaction of the G protein α subunit with the membrane be reduced (Levis and Bourne, 1992). A possible mechanism for such a regulatory process may be a change in the fatty acylation status of the α subunit. For example, since palmitoylation is reversible and occurs with both G_s and G_i, agonist-induced membrane release of G proteins may involve depalmitoylation.

Palmitoylation is thought to anchor G protein α subunits to the plasma membrane (Mumby *et al.*, 1994). Such a mechanism could conceivably result in reduced abundance or accessibility of $G_i\alpha_2$ or $G_s\alpha$ in the vicinity of adenylyl cyclase and thus affect cyclic AMP production in response to agonist.

Possible growth hormone signalling mechanisms

Great strides have recently been made in our understanding of GH action. The human GH receptor is a 620 amino acid single chain protein containing a glycosylated 246 amino acid extracellular hormone-binding domain, a single 24 amino acid transmembrane domain, and a 350 amino acid cytoplasmic domain. The GH receptor is a member of the GH/prolactin/cytokine/hematopoietin receptor superfamily (Cosman *et al.*, 1990; Bazan, 1990) which also includes receptors for interleukins (IL)-2, IL-3, IL-4, IL-6, IL-7, granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin, ciliary neurotrophic factor, leukemia inhibitory factor, and interferons α, β, and γ. Although there is relatively little overall homology between these receptors, members of this receptor family possess in the extracellular region, 4 conserved cysteine residues which form 2 disulfide loops, a 7 amino acid proline-rich sequence and a peptide consensus

sequence, the so-called WSXWS motif, which is less conserved in the GH receptor, (Cunninghan *et al.*, 1991; Colosi *et al.*, 1993).

Growth hormone promotes the phosphorylation of its receptor on tyrosyl residues (Foster *et al.*, 1988) but unlike the insulin receptor (Becker and Roth, 1990; Wente and Rosen, 1990), the GH receptor has no intrinsic tyrosine kinase activity (Colosi *et al.*, 1993; Carter-Su *et al.*, 1989). The recent identification of JAK2 as a GH receptor associated tyrosine kinase (Argetsinger *et al.*, 1993) may resolve some of the issues of phosphorylation without tyrosine kinase activity. JAK2 is a member of the Janus family of tyrosine kinases which have both a catalytic tyrosine kinase domain and a tyrosine kinase-like region (Harpur *et al.*, 1992). Of particular importance are the observations that JAK2 also associates with other members of the cytokine/hematopoietin receptor superfamily including the receptors for erythropoietin, interleukin-3, GM-CSF, and prolactin and may transduce their signals (Argetsinger *et al.*, 1993). Thus it appears that the members of this receptor superfamily exhibit at least one common signalling pathway.

Signalling cascades involving protein kinase C have also been implicated in some actions of GH although the connection between the GH receptor and phospholipase C is unknown. Acridine orange, an inhibitor of protein kinase C, blocked the stimulation of lipogenesis by GH in rat adipocytes (Smal and De Meyts, 1989). Sphingosine, another protein kinase C

inhibitor, suppressed the insulin-like actions (increased glucose uptake, lipogenesis) of GH in rat adipocytes (Smal and De Meyts, 1989). Studies by Gorin *et al.*, (1990) showed that sphingosine and staurosporine, yet another protein kinase C inhibitor, blocked the lipolytic effects of GH in tissues of both normal and hypophysectomized rats. Gorin *et al.*, (1990) also showed that phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, was lipolytic, but its lipolytic effect was additive with the maximal lipolytic response of GH suggesting that activation of protein kinase C does not account for the lipolytic response to GH.

In unrelated studies induction of c-fos by GH in a mouse 3T3 adipocyte cell line appeared to be mediated in part by activation of protein kinase C (Doglio *et al.*, 1989; Slootweg *et al.*, 1990). One of the earliest events in the actions of many hormones and growth factors is the induction of early response proto-oncogenes. Growth hormone stimulates the induction of the c-myc proto-oncogene in the liver and kidney of hypophysectomized rats (Murphy *et al.*, 1987) as well as c-fos in OB1771 mice cells (Doglio *et al.*, 1989). Furthermore, continuous GH infusion has been shown to modulate the expression of c-myc and c-fos during early stages of rat liver carcinogenesis (Hallstrom *et al.*, 1989). Growth hormone has also been shown to rapidly and transiently induce the expression of c-fos and c-jun mRNA in preadipocytes (Gurland *et al.*, 1990). This induction was inhibited by prolonged incubation

with phorbol ester to deplete protein kinase C suggesting that GH induction of c-fos and c-jun may involve diacylglycerol and protein kinase C.

Despite recent progress in the understanding of early reactions in the GH signalling pathway, the cellular mechanisms that account for the lipolytic actions of GH are unknown. There is no evidence for a direct interaction between the GH receptor and G proteins, but nevertheless it is likely that GH acts by way of the cyclic AMP-dependent pathway (Goodman *et al.*, 1988). After treatment of adipocytes with GH, small increases in cyclic AMP were observed by some investigators (Fain and Saperstein, 1970, Moskowitz and Fain, 1970) but not others (Sengupta *et al.*, 1981; Harris and Bennun, 1976). Glycogen phosphorylase, whose activity depends upon cyclic AMP in a manner similar to hormone sensitive lipase was also activated by GH/Dex treatment after a delay of about 2 hours (Eisen and Goodman, 1969; Moskowitz and Fain, 1969). Inhibition of cyclic AMP breakdown potentiates the lipolytic action of GH (Goodman, 1968a, 1968b; Fain, 1968,1967), consistent with a role for cyclic AMP. If lipolysis induced by GH/Dex does indeed utilize the cyclic AMP dependent pathway, the actions of GH/Dex must precede protein kinase A activation since responsiveness to dibutyryl cyclic AMP and 8-bromo-cyclic AMP are unchanged in tissues treated with GH/Dex (Fain, 1968; Goodman, unpublished data)

Although GH may not interact with G proteins directly, it is possible that G proteins are targets of GH induced proteins. As already discussed, the amounts or activities of G proteins are not constant and appear to be regulated physiologically. Studies by Goodman *et al.*, (1988) have demonstrated that in the absence of GH (eg: hypophysectomy), G_i levels, as determined in studies of pertussis toxin catalysed ADP-ribosylation, increase and upon administration of GH, G_i levels decrease. More recently, Doris *et al.*, (1994) have shown GH to decrease the response to anti-lipolytic agents (PIA and prostaglandin E_1) and decrease the levels of G_i2 in rat adipocytes. As well, Roupas *et al.*, (1991) found that GH treatment of adipose tissue inhibited ribosylation by pertussis toxin (~60% inhibition) suggesting that the effect involves a large portion of pertussis toxin-sensitive G proteins.

Objectives

The objective of the research in this dissertation is to gain insight into signalling mechanisms by which GH increases lipolysis. To this end we tested the hypothesis that the increase in lipolysis induced by GH occurs through a cyclic AMP mediated pathway. We propose that GH acts to increase intracellular cyclic AMP levels thereby activating protein kinase A with the consequent activation of the hormone-sensitive lipase and lipolysis.

As our studies progressed, other hypotheses were formulated and tested as discussed in subsequent sections.

We approached this study by examining cellular and biochemical events that occur in fat tissue and adipocytes in response to GH. Growth hormone has been shown to activate several signal transduction pathways in adipocytes including tyrosine kinase (Argetsinger *et al.*, 1993), protein kinase C (Gorin *et al.*, 1990) and the cyclic AMP-dependent pathway (Goodman *et al.*, 1988) all of which may or may not be interrelated since, for example, activation of JAK2 is very rapid (within minutes) while activation of lipolysis may occur only after a delay of several hours. Although the cyclic AMP-mediated events may be the immediate events prior to lipolysis, the events between it and the receptor are not known and are explored in this thesis.

We elected to study GH effects on the cyclic AMP-dependent pathway because it was most likely to be involved in lipolysis. Parallel studies into the lipolytic effect of GH and isoproterenol, a catecholamine analog, was performed to compare the lipolytic effects of GH with what is known about lipolysis by catecholamine.

Materials and Methods

Animals- Normal male rats of the Charles River CD® strain (Charles River Breeding Laboratories, Kingston, NY) were used in all experiments in accordance with protocols approved by the UMMC Animal Care and Use Committee. Rats were fed Purina 5008 (Ralston-Purina, St. Louis, MO) from the time they were received until they were studied 1-2 weeks later and had attained a body weight of 180-250 g. The rats were maintained at constant temperature (23°C) and lighting with lights on from 0600-1800 h. Rats were killed by cervical dislocation and epididymal and perirenal fat pads were removed.

Incubations- Thin distal portions of epididymal fat pads (~100 mg), or isolated adipocytes (Rodbell, 1964) from epididymal and perirenal depots were incubated (1:10 dilution) in Krebs Ringer bicarbonate-buffer (pH 7.4) that contained 5.5 mM glucose (KRBG) and 1% w/v bovine serum albumin (BSA) (Introgen Co. NY). Incubations were carried out in a shaking water bath (37°C) under an atmosphere of 95% O_2/ 5% CO_2 for 4 hours When indicated, the following were added to incubation media: 30 ng/ml human GH (hGH; Genentech Corp., San Francisco, CA), 1 μg/ml dexamethasone (9α-fluoro-11β,17α-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione; Lypho-

Med, Inc., Chicago, IL), 0.1 ng/ml or 1 μg/ml (l)-isoproterenol (both with added 100 μg/ml ascorbate), 100 μM colchicine, 30 μM Rp-CAMPS (a generous gift from Dr. Bell of Sandoz Inc., NJ), or 2 μg/ml pertussis toxin (List Biological; Campbell, CA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Tissue or Cell Homogenization and Membrane Purification- Unless otherwise indicated, all tissues and cells were homogenized in 2 volumes of homogenization buffer (10 mM Tris-HCl, pH 7.4; 1 mM ethylenediaminetetracetic acid (EDTA); 100 mM phenylmethylsulfonyl fluoride (PMSF); 10 μg/ml leupeptin) at 4°C in ground glass homogenizers with 10 up/down strokes. The homogenates were allowed to settle for 10 min in an ice bath until a fat cake formed. After removal of the fat cake the infranatants were used to prepare purified membranes or were assayed for cyclic AMP content or protein kinase A.

Plasma membranes were purified from homogenates of isolated adipocytes according to procedures developed by McKeel and Jarret (1970). Briefly, homogenates were centrifuged in an SS-34 rotor in a Sorval® RC2-B centrifuge (Du Pont, Wilmington, DE) at 12,000 rpm (~16k x *g*) for 20 min. The resulting pellet is referred to as the "16k pellet". The remaining supernatant was further centrifuged in a Ti 60 rotor in a Beckman® L8-M Ultracentrifuge (Palo Alto, CA) at 50,000 rpm (~100k x *g*) for at least 1 hour to

obtain the "100k pellet". The 16k and 100k pellets were assayed for adenylyl cyclase activity (Salomon *et al.*, 1974), or studied by Western immunoblot analysis (Towbin *et al.*, 1979). In some experiments, the 16k pellets were resuspended in 1 ml and layered over linear sucrose density gradients (5-25%) and centrifuged in SW41 rotor at 37,000 rpm (~100k x *g*) for 90 min. Fractions were collected by pipetting successive 0.8 ml aliquots from the top. Hence, lower fraction numbers represent lighter fractions while higher fraction numbers represent denser fractions. The fractions were subsequently analyzed for their content of G proteins or 5'-nucleotidase (see below).

Western Blot Analysis- Both the 16k and 100k pellets as well as membrane fractions separated on sucrose gradients were solubilized (mixed and shaken with Laemmli buffer (1970) for 1 hour at room temperature to dissociate G proteins from membranes then boiled for 5 min before loading on 10% sodium dodecyl sulfate polyacrylamide gels for separation by electrophoresis (PAGE). After determining protein content (Lowry *et al.*, 1951) all lanes containing 16k or 100k samples were loaded with identical amounts of protein. Samples were resolved by electrophoresis with a Hoefer® vertical electrophoresis apparatus (SE600; Hoefer, San Francisco, CA) and transferred onto nitrocellulose (MSI, Westboro MA) by the method of Towbin (1979) with a Hoefer® transfer apparatus (TE42; Hoefer, San Francisco, CA). Non-specific antibody binding sites on the membrane were blocked

with 10% milk proteins in phosphate buffered saline (PBS) for 2 hours at room temperature followed by washing 3 times for 2 min, in PBS + 1% milk proteins. Membranes were then incubated with rabbit anti-$G_i\alpha_2$ diluted 1:1000 in PBS +1% milk proteins at 4°C for 2 hours and washed 3 times for 2 min and once for 20 min, in PBS + 1% milk proteins + 0.3% Tween 20. Polyclonal antibodies directed at the C-terminal decepeptide of $G_i\alpha_2$ [Lys-Glu-Asn-Leu-Lys-Asp-Cys-Gly-Leu-Phe] or $G_s\alpha$ [Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-Leu-Leu] and characterized by Rapiejko *et al.*, (1989) were a generous gift from Dr. C. Malbon. Membranes were then re-incubated with horseradish-peroxidase conjugated goat-anti-rabbit antibody diluted 1:10,000 in PBS + 1% milk proteins at 4°C for 2 hours followed by washing 3 times for 2 min, and once for 20 min, in PBS + 0.3% Tween 20. After soaking for 1 min in ECL® chemiluminescent reagent (Amersham; Arlington Heights, MO) to allow for chemiluminescent detection of antibodies, membranes were exposed to X-ray film for between 5 seconds to 1 hour, depending upon the intensity of luminescence. After development of the film, membranes were cleared of antibodies by soaking in stripping solution (60 mM Tris-HCl, pH 6.8; 2% w/v SDS; 0.1 M β-mercaptoethanol) for 30 min at 37°C with occasional agitation and were re-probed with anti-$G_s\alpha$ under identical

conditions as described above. Bands representing G proteins were quantitated by densitometry.

ADP-Ribosylation-Pertussis catalysed ADP-ribosylation was performed according to methods described by Roupas *et al.*, (1991). Briefly, the reaction was carried out in 100 mM Tris-HCl (pH 8.0) containing 2 µg/ml of activated pertussis toxin, 2 mM ATP, 25 mM dithiothreitol, 30-50 µg proteins and 5 µCi of [α–^{32}P]NAD [specific activity 5 µCi/µmole; Amersham, Arlington Heights, IL] in a total volume of 100 µl. The ribosylation reaction was carried out at 37°C for 30 min and terminated by addition of 50 µl 2X concentrated Laemmli buffer. Samples were solubilized, boiled and resolved on SDS-PAGE followed by autoradiography and quantitation by densitometry.

ASSAYS

Glycerol- Except where indicated, the rate of lipolysis was measured as the rate of glycerol production during the 4th hour of incubation. One ml aliquots of cell suspension or incubation media were removed at 180 min and 240 min of incubation, filtered through glass wool to remove cells. Samples of filtrate were assayed for glycerol content by the method of Wieland (Wieland, 1957). The difference in glycerol content between 240

min and 180 min represents the amount of glycerol produced in the fourth hour.

Cyclic AMP- Cyclic AMP was determined in duplicate by radioimmunoassay according to the manufacturer's specifications using a kit obtained from Amersham (Arglington Heights, IL). Samples were serially diluted 100- to 1000-fold to obtain concentrations of cyclic AMP that fell within the optimal detectable range of the radioimmunoassay.

Protein Kinase A- Protein kinase A was assayed by an adaptation of the procedures of Roskowski (1983) and Corbin (1983). Replicate tissues (~100 mg) were homogenized in an equal volume of PKA homogenizing buffer (10 mM potassium phosphate, pH 6.8; 10 mM EDTA; 0.5 mM 1-methyl-3-isobutylxanthine; 0.5 M NaCl) and centrifuged at 12,000 x*g* for 5 min at 4°C. 25 µl of infranatant (the aqueous phase) was added to the reaction mixture (50 mM morpholinopropanesulfonic acid (MOPS) pH 7.0, 10 mM $MgCl_2$, 0.25 mg/ml bovine serum albumin, 100 µM kemptide (Sigma, St. Louis, MO), 100 µM γ^{32}P-ATP (specific activity 5µCi/µmol; Amersham, Arlington Heights, IL), ±100 µM cyclic AMP, distilled water) to give a final volume of 100 µl and incubated for 10 min at 37°C. 20 µl aliquots of reaction mixture were spotted within 2 cm X 2 cm squares, drawn out in pencil, on a sheet of Whatman P81 chromatography filter paper and the reaction stopped by washing 3X in 75 mM phosphoric acid for 5 min and then with 95% ethanol. After drying at room temperature, the individual squares were cut out and counted in

scintillation cocktail (Optifuor®, Beckman Instruments). Protein content of the infranatants was determined by method of Lowry *et al.*, (1951). Data were expressed as cpm/10 µg protein/10 min or the ratio of specific activity of the enzyme in the absence of 100 µM cyclic AMP divided by maximally stimulated activity in the presence of cyclic AMP. The ratio thus represents the degree of protein kinase A activation by hormone, henceforth referred to as the activity ratio.

Adenylyl Cyclase- 50 µl of 16k and 100k pellets resuspended in homogenizing buffer were mixed with 300 µl of adenylyl cyclase assay mixture (25 mM Tris-HCl, pH 7.4; 12.5 mM $MgCl_2$; 20 mM creatine phosphate; 250 U/ml creatine phosphokinase; 1 mM ATP; 100 µM GTP) with and without 1 mM forskolin (Okuda *et al.*, 1992) and incubated at 30°C for 10 min. The reaction was terminated by addition of 50 µl of 25% ice-cold trichloroacetic acid (TCA) followed by centrifugation in an Eppendorf tabletop centrifuge (~16k x g) for 15 min. TCA was removed by extraction 3 times with 5 volumes of diethyl ethyl ether and the remaining aqueous layer was assayed for cyclic AMP as described above. Data were expressed as pmol/µg protein/10 min or the ratio of enzyme activity in the absence and presence of 1 mM forskolin. The ratio thus represents the degree of adenylyl cyclase activation by forskolin henceforth referred to as the activity ratio.

5'-Nucleotidase Assay- 5'-nucleotidase was used as an enzyme marker for the plasma membrane and was assayed with and without α,β–methyleneadenosine diphosphate (AOPCP), an agent that specifically inhibits plasma membrane associated 5'-nucleotidase (Burger and Lowenstein, 1970). Briefly, the reaction was carried out for 15 min at 37°C in 1 ml of 60 mM Tris-HCl (pH 7.4) containing 100 μM ATP, 0.015 μCi [U-^{14}C]5'-AMP (specific activity 0.2 μCi/pmol; Du Pont NEN, Boston MA), 1-15 μg protein, with or without AOPCP. The reaction was terminated by addition of 200 μl of 5% w/v $ZnSO_4$, mixing, and addition of 200 μl of 0.3 N $Ba(OH)_2$. Tubes were mixed and centrifuged for 10 min in an Eppendorf tabletop centrifuge and 0.9 ml of clear supernatant was removed and counted with a Packard Tri-Carb® Model 4530 scintillation counter in 5 ml Optifluor® scintillation cocktail. 5'-nucleotidase activity was measured as the rate of formation of ^{14}C-adenosine in the supernatant.

Statistics- For statistical evaluation, each experiment consisting of a tissue segment from a single rat or a separate population of adipocytes pooled from 3-8 rats was considered to be an independent observation. Means and standard errors from 4-16 replicate experiments were analysed by ANOVA for repeated measures (Winer, 1962; Bliss, 1967) followed by pair-wise t-test (Snedecor and Cochran, 1989) to determine statistical significance using the computer program StatsView® (Abacus Concepts, Inc. Berkeley, CA).

Results

The role of cyclic AMP in growth hormone-induced lipolysis

a. Effects GH/Dex on glycerol production

Typical lipolytic effects of GH and dexamethasone are shown in Figure 3. To obtain these data, duplicate segments of fat tissues from 8 rats were incubated in the absence or presence of GH (30ng/ml), dexamethasone (1 μg/ml), or both (GH/Dex) for 3 hours. One tissue segment of each pair was then removed for cyclic AMP assay while the other was transferred to fresh medium for a final hour of incubation. At the end of the 4th hour the tissues were prepared for cyclic AMP assay and the medium was assayed for glycerol. Lipolysis was significantly increased in tissues treated with GH alone (143%; $p<0.05$) or GH/Dex (307%; $p<0.01$). The small effect of dexamethasone was not significant. Note that the combination of GH/Dex produced a considerably greater increase in lipolysis than the sum of the effects of GH alone and dexamethasone alone. Growth hormone alone increased glycerol production from 1.85 to 2.8 μmoles/g/hr while the combination of GH/Dex increased glycerol production from 1.85 to 5.56 μmoles/g/hr, an effect 4 times greater than that of GH alone.

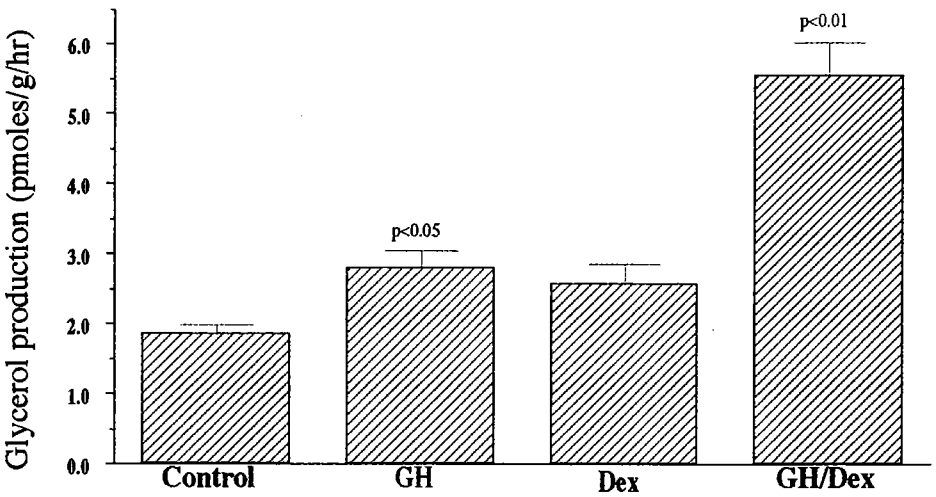


Figure 3

Effects of GH and dexamethasone on lipolysis in fat segments. Tissues were incubated with and without hormones for 3 hours then transferred to fresh medium of identical composition for 1 final hour. Medium from the final hour was assayed for glycerol content. Significant increases in lipolysis were seen in tissues treated with GH or GH/Dex. Each bar represents the mean and s.e.m. of 16 replicates.

b. Effects on cyclic AMP concentrations

Cyclic AMP was measured in tissues from the same rats as illustrated in Figure 3. Tissues were homogenized at the end of 3 or 4 hours of incubation with GH/Dex to provide an estimate of the cyclic AMP concentration at the beginning and end of the interval in which lipolysis was assessed and when the lipolytic action of GH/Dex is fully developed (Fain *et al.*, 1965). Despite increased rates of lipolysis seen in tissues incubated with GH alone, dexamethasone alone or the two hormones together, no measurable differences in cyclic AMP content were detected (Figure 4).

Effect of Rp-CAMPS on lipolysis

Failure to detect an increase in cyclic AMP in tissues treated with GH/Dex suggests two possibilities: (a) cyclic AMP may not play a role in GH induced lipolysis or (b) changes in cyclic AMP concentrations that are too small to detect are sufficient to stimulate lipolysis. Therefore as an alternative approach to determining if cyclic AMP has a role in GH induced lipolysis, we examined the effects of a competitive inhibitor of cyclic AMP, Rp-CAMPS (Rothermel *et al.*, 1983), on the ability of GH/Dex to increase lipolysis (Figure 3). Ten segments of epididymal fat from each of 8 normal rats were incubated for 3 hours in the absence of hormones, or with GH alone, dexamethasone alone or the combination of GH/Dex. At the end of

this preincubation period the tissues were transferred to fresh medium containing the same hormones and incubated for a final hour. In addition, some tissues that pre-incubated without hormones for the first three hours were transferred to medium that contained 0.3 ng/ml isoproterenol and 0.1 mg/ml ascorbate. This concentration of isoproterenol was chosen because it produces a similar increase in lipolysis as GH/Dex. Half the tissues were exposed to Rp-CAMPS during the last hour of incubation. Rp-CAMPS had no effect on basal lipolysis, but attenuated GH/Dex- or isoproterenol-induced lipolysis to a similar extent. Rp-CAMPS decreased the GH/Dex-dependent production of glycerol by ~50% and the isoproterenol-dependent production of glycerol by 40% (Figure 5). We did not attempt to block lipolysis more completely with higher concentrations of Rp-CAMPS because its inhibitory action is self-limiting. Rp-CAMPS also inhibits cyclic AMP phosphodiesterase activity and thus allows cyclic AMP to accumulate and compete for binding sites on protein kinase A. Thus higher concentrations of Rp-CAMPS are no more effective than the 30 μM used (Pereira *et al.*, 1987). The results of this experiment are consistent with the notion that GH increases lipolysis by a pathway that may be cyclic AMP dependent.

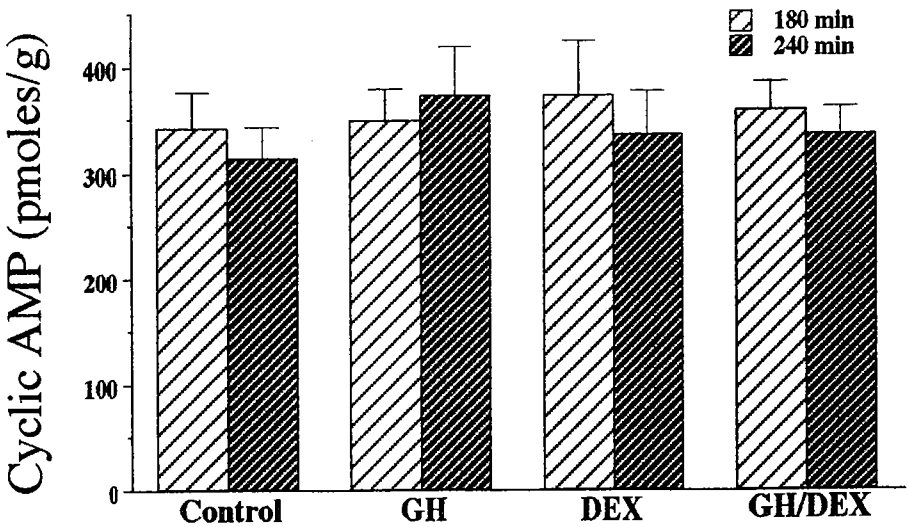


Figure 4

Effects of GH and dexamethasone on cyclic AMP accumulation in fat tissues. Tissue segments used in figure 3 were homogenized and assayed for cyclic AMP. No observable differences were seen between groups. Each bar represents the mean and s.e.m. of 16 replicates.

Activation of protein kinase A

The apparent discrepancy between the lack of an increase in cyclic AMP in GH/Dex treated tissues and the finding that Rp-CAMPS inhibited lipolysis caused by GH/Dex might be resolved with a more sensitive method for determining changes in cyclic AMP levels. Although cyclic AMP concentrations were unchanged when measured in tissue homogenates (i.e.: in total cell water), it is possible that local concentrations of cyclic AMP may have increased in the immediate vicinity of protein kinase A, the target of cyclic AMP action. Therefore measurement of protein kinase A activity might serve a more sensitive index of cyclic AMP concentration at physiologically relevant cellular loci.

Fat segments (~100 mg) were incubated in 1 ml KRB for 4 hours at 37°C. In GH/Dex-treated tissue segments, both GH (30 ng/ml) and dexamethasone (1 μg/ml) were present throughout the 4 hours of incubation. For isoproterenol treatment of tissue segments, two concentrations of isoproterenol were chosen: a lower concentration (0.1 ng/ml) to produce a similar increase in lipolysis as GH/Dex and a higher concentration (1 μg/ml) to produce maximal lipolysis. Isoproterenol was added only for the last hour of incubation. After incubation, tissue segments were assayed for protein kinase A activity and the media were assayed for glycerol.

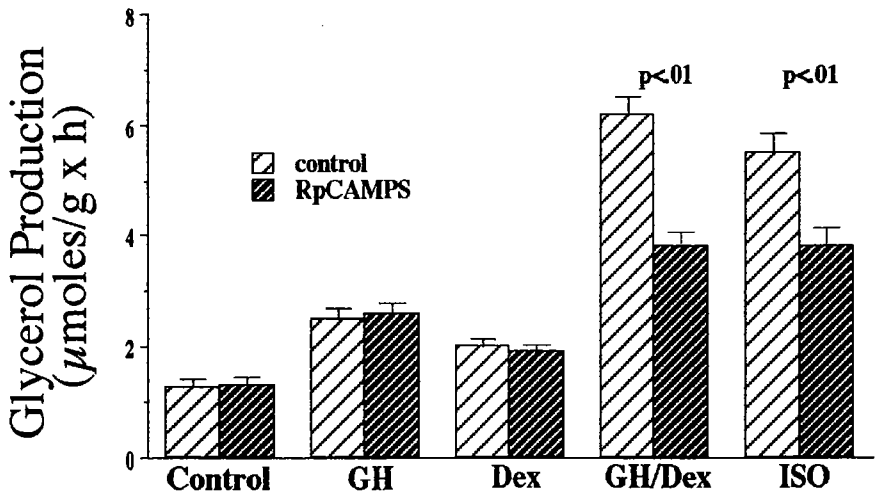


Figure 5

Effect of Rp-CAMPS on lipolysis. Rp-CAMPS (30 μM) was present in the last hour of incubation. Rp-CAMPS significantly reduced GH/Dex and 0.3 ng/ml isoproterenol-induced lipolysis in fat tissues. Growth hormone almost doubled lipolysis ($p<0.05$) while GH/Dex and isoproterenol increased lipolysis by almost 4-fold ($p<0.01$). Each bar represents the mean and s.e.m. of 16 replicates. p-values are for comparisons between control and Rp-CAMPS treatment for each conditions.

All hormone treatments significantly increased protein kinase A activity as determined in the absence of exogenous cyclic AMP (Table 1). In the presence of a saturating concentration of cyclic AMP, protein kinase A activity was increased to more than 5 times its basal activity, but was not further changed by any of the hormone treatments suggesting that GH/Dex did not affect either the synthesis or degradation of the enzyme. When expressed as a fraction of the maximum protein kinase A activity, GH/Dex and the higher concentration of isoproterenol significantly increased the activity ratios.

Table 1

Protein kinase A activity (cpm/10 μg protein/10 min)

	Control	ISO (0.1 ng/ml)	ISO (1 μg/ml)	GH/Dex
(-) cAMP	2775 ± 800	3097 ± 816 ($p<0.05$)	5080 ± 825 ($p<0.001$)	3158 ± 793 ($p<0.05$)
(+) cAMP	16392 ± 2548	15595 ± 1749 n.s.	15978 ± 1503 n.s.	15690 ± 1595 n.s.
Activity Ratio	14.9% ± 1.39	18.1% ± 2.11 n.s.	30.89% ± 3.19 ($p<0.0001$)	18.60% ± 1.92 ($p<0.05$)

Values represent the mean ± s.e.m. of 16 replicates. 100 mg tissue segments were incubated *in vitro*. GH/Dex was present throughout the 4 hours of incubation whereas isoproterenol was present only during the last hour. p-values were assessed by ANOVA and are for comparisons with control tissues.

Glycerol production by these same tissues paralleled the activity of protein kinase A. When glycerol production was plotted as a function of protein kinase A activity ratios, values for all three hormone treatments fell on the same straight line (Figure 6). Linear regression analysis of data for control, GH/Dex and two concentrations of isoproterenol revealed a linear relation between protein kinase A activity ratio and lipolysis with a correlation coefficient of 99.5%. The data are consistent with the premise that GH/Dex and isoproterenol stimulate lipolysis through activation of protein kinase A and suggest that, like isoproterenol, GH/Dex must act at a site in the lipolytic pathway that lies proximal to protein kinase A to regulate lipolysis.

Effect of GH/Dex on adenylyl cyclase activity

The findings that Rp-CAMPS successfully attenuated GH/Dex induced lipolysis and that treatment with GH/Dex increased protein kinase A activity in tissues strongly suggest a role for cyclic AMP in the lipolytic action of GH. However, the findings do not address the question of whether GH/Dex may (1) stimulate cyclic AMP production, (2) inhibit cyclic AMP breakdown or (3) increase protein kinase A sensitivity to cyclic AMP. In considering the first possibility, one would expect GH/Dex either to increase

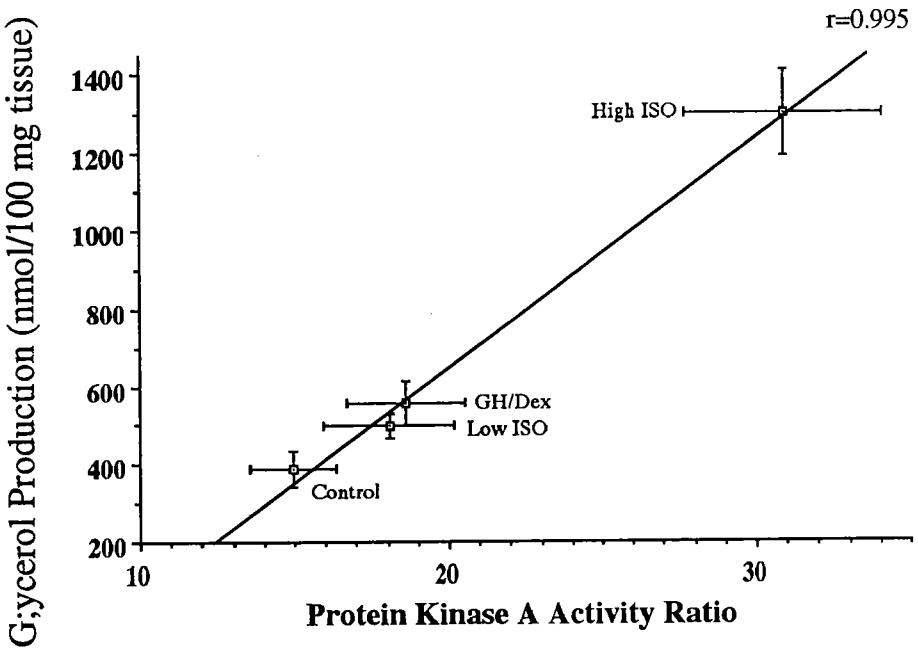


Figure 6

Glycerol production as a function of PKA activtiy. Each point represents the mean value obtained in the same 16 experiments as described in Table 1. Glycerol production was significantly ($p<0.05$) increased in all three hormone-treated groups.

the rate of cyclic AMP synthesis or to decrease the rate of cyclic AMP breakdown.

To determine whether treatment with GH/Dex has an effect on cyclic AMP synthesis, adenylyl cyclase activity was measured in cells that were incubated for 4 hours in the presence of GH/Dex. Immediately after incubation, the cells were homogenized and the aqueous phase of the homogenate was centrifuged to obtain the 16k and 100k pellets as described in Methods. Adenylyl cyclase was assayed with or without addition of 1 mM forskolin to the reaction mixture. Forskolin is a diterpene that directly stimulates adenylyl cyclase (Seamon *et al.*, 1981) and was used to obtain a maximal catalytic activity.

Growth hormone and dexamethasone increased adenylyl cyclase activity from 1.71 pmol/μg protein/10 min in 16k pellets of control tissues to 3.13 pmol/μg protein/10 min ($p<0.01$). This change corresponds to a doubling in activity ratio from 11.11% to 24 % ($p<0.01$). This increase in adenylyl cyclase activity in cells treated with GH/Dex cannot be attributed to an increase in the total amount of adenylyl cyclase since the maximal activity of the enzyme as determined in the presence of forskolin did not increase with GH/Dex treatment. More than 85% of the activatable adenylyl cyclase was recovered in the 16k pellet and this amount was unchanged by GH/Dex. The approximately 15% of adenylyl cyclase found in the 100k pellet was not

activated by GH/Dex. The finding that treatment of adipocytes with GH/Dex increases adenylyl cyclase activity is consistent with the hypothesis that lipolytic action of GH/Dex may be attributed to an increase in cyclic AMP production followed by activation of protein kinase A, of hormone-sensitive lipase, and ultimately lipolysis. This finding, however, raises the question of how GH/Dex-treatment increases adenylyl cyclase activity in fat cells.

TABLE 2

Adenylyl cyclase activity (pmol/μg protein/10 min)

	Control (n=6)	GH/Dex (n=6)	p-values*
16k pellet	1.71 ± 0.10	3.13 ± 0.34	0.0047
16k pellet +forskolin	15.64 ± 1.07	13.27 ± 1.03	n.s.
Activity Ratio	11.11% ± 1.08	23.98% ± 3.12	0.004
100k pellet	0.13 ± 0.03	0.32 ± 0.11	n.s.
100k pellet +forskolin	1.10 ± 0.26	2.95 ± 0.89	n.s.
Activity Ratio	11.64% ± 1.07	10.84% ± 1.27	n.s.

*p-values are for comparisons with control.

Effect of pertussis toxin

Adenylyl cyclase activity is under both stimulatory control mediated by G_s and inhibitory control, mediated by G_i. The work by Kather *et al.*, (1985) provides strong evidence that adenylyl cyclase in human adipocytes is

maintained at basal levels by tonic inhibition. It is thus apparent that adenylyl cyclase may be activated either through increased stimulatory input or through decreased inhibitory input. Growth hormone therefore might affect adenylyl cyclase activity by increasing or decreasing the relative amounts or effectiveness of G_s or G_i. Studies by Goodman *et al.,* (1988) indicated that GH preferentially decreased G_i in adipocyte ghosts. Such a decrease might somewhat relieve adenylyl cyclase of inhibition. In evaluating this possibility it was necessary first to determine if adenylyl cyclase in rat adipocytes is also under tonic G_i-mediated inhibition under basal conditions. We therefore incubated adipocytes with 1 μg/ml pertussis toxin to inactivate G_i and transferred the cells to fresh medium every hour (Figure 7). Glycerol assays were performed on samples of incubation medium from each hour. After a delay of ~2 hours pertussis toxin dramatically increased glycerol production to an apparently maximal rate as compared to the effects of high isoproterenol in Figure 4. These data support the idea that lipolysis is under tonic G_i-mediated inhibition in the basal state in rat adipocytes as well as in human fat (Kather *et al.,* 1985). This observation and the findings of Goodman *et al.,* (1988) support the possibility that GH might promote lipolysis in adipocytes by decreasing relative input from G_i.

Partitioning of G proteins

If stimulation of lipolysis by GH is achieved through decreasing G_i and results in increased adenylyl cyclase activity, then three possible mechanisms may account for such an action: (1) G_i synthesis may be decreased, (2) G_i degradation may be increased, or (3) G_i may be removed from the vicinity of adenylyl cyclase and translocated to another part of the cell. The slow turnover of G proteins in fat cells ($t_{1/2}$ 24-48 hours) as shown by Hadcock *et al.*, (1991), makes the first possibility unlikely and the second possibility untestable under circumstances that are relevant to our experimental model. We therefore examined the other possibility that G proteins may be selectively translocated from the plasma membrane to lighter membranous compartments (Haraguchi and Rodbell, 1990). We hypothesized that GH may decrease the accessibility of adenylyl cyclase to G_i and thereby increase adenylyl cyclase activity.

To explore this possibility four replicate experiments were performed. In each experiment, fat cells pooled from 10 rats were incubated for 4 hours; half with and the other half without GH/Dex. Immediately following incubation, cells from both groups were homogenized and the aqueous layer of the homogenate was separated into 16k and 100k pellets by centrifugation as described in Methods. Both pellets were resuspended in 1 ml of homogenization buffer. 100 µl aliquots of each were solubilized in

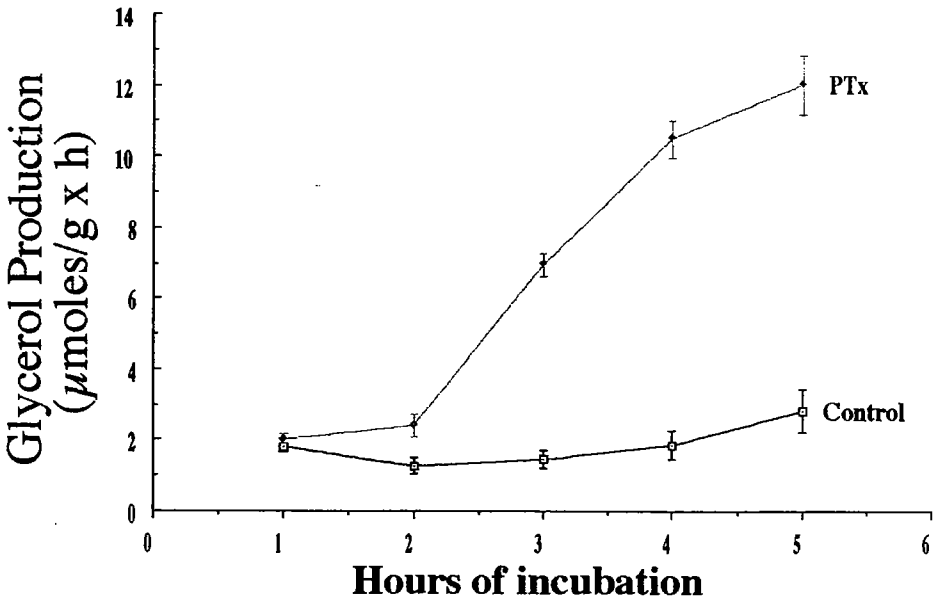


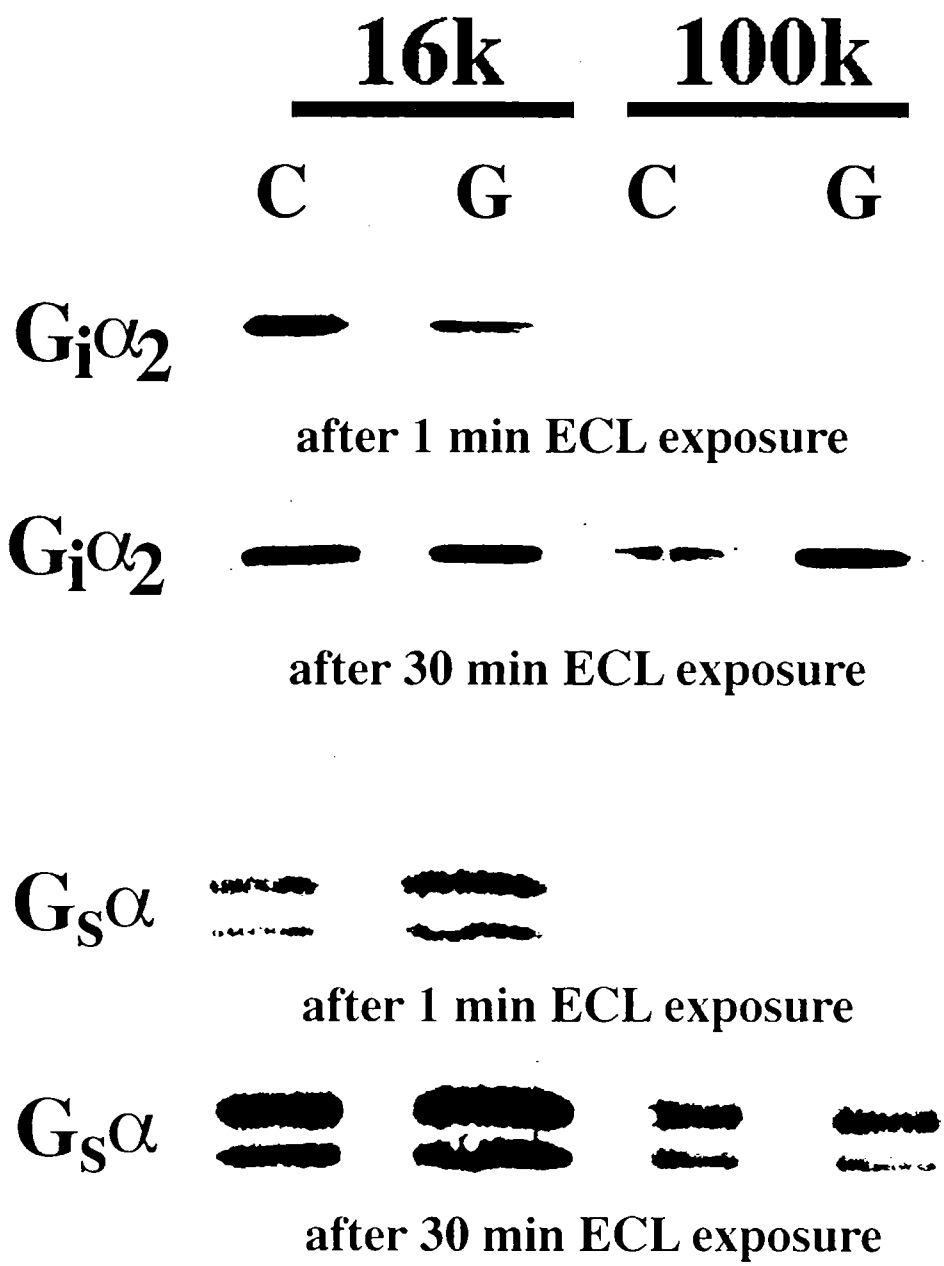
Figure 7

Effect of pertussis toxin on lipolysis in segments of adipose tissue from normal rats. Pertussis toxin (1 μg/ml) was present in incubation medium only during the 1st hour of incubation. Tissues were transferred to fresh medium every hour and samples of medium from each hour were assayed for glycerol released. Each point represents the mean and s.e.m. of 8 replicates.

Laemmli buffer and the proteins separated by electrophoresis in 10% polyacrylamide gels. Resolved proteins were transferred onto nitrocellulose membranes and probed by Western blotting with anti-$G_i\alpha_2$ and anti-$G_s\alpha$ as described in Methods and visualized by chemiluminescence. A typical experiment shows that the G proteins in the 16k pellets are sufficiently abundant to be observable after 1 min of exposure of the X-ray film, while the proteins in the 100k pellets were visible only after 30 min exposure (Figure 8). Since each band represents 10% of the G proteins present in either the 16k or 100k pellet, we can estimate their relative abundance from the intensity of the enhanced chemiluminescent reaction. To calibrate this reaction, we spotted aliquots of serially diluted horseradish peroxidase-conjugated antibody on nitrocellulose. After soaking for 1 min in ECL® reagent (Amersham, Arlington Heights, IL), the membranes were exposed to X-ray film for 1 min or 30 min. Comparable intensities were obtained after 1 or 30 min of exposure when the horseradish peroxidase-conjugated antibody differed by a factor of 4. That is, a 1:1000 dilution of horseradish peroxidase-conjugated antibody gave as dense a band after 1 min as a 1:4000 gave in 30 min. Therefore we estimate that the 16k pellet contains roughly 4 times more $G_i\alpha_2$ than the 100k band.

Figure 8

A representative experiment showing the changes in distribution of Gi and Gs in homogenates of control and GH/Dex treated cells. Cells were homogenized and after removal of the fat cake from the homogenates the infranatants were separated to 16k and 100k pellets. The pellets were dissolved in Laemmli buffer and proteins resolved on 10% SDS-PAGE, transferred onto nitrocellulose and probed with anti-$G_i\alpha_2$ and anti-$G_s\alpha$. Adipocytes treated with GH/Dex (G) had more $G_i\alpha_2$ in the 16k pellet than control (C) while the 100k pellet contained more $G_i\alpha_2$ in the control condition than in the GH/Dex condition. Similar data were obtained in 3 other experiments. The upper panel show the results after 1 minute of the chemiluminescence reaction and the lower panel shows exposure of the same nitrocelullose membrane for 30 min to permit visualization of G α in the 100k pellet.


16k
100k
C
G
C
G
$G_i\alpha_2$
after 1 min ECL exposure
$G_i\alpha_2$
after 30 min ECL exposure
$G_s\alpha$
after 1 min ECL exposure
$G_s\alpha$
after 30 min ECL exposure

Due to the variability in exposure times needed for the 16k and 100k pellets, as well as variability in exposure times from experiment to experiment, intensities of the $G_i\alpha_2$ and $G_s\alpha$ bands in aliquots of GH/Dex treated tissues were normalized to the intensities obtained simultaneously in controls. That is, for each experiment, the densities of bands representing treatment with GH/Dex in 16k and 100k pellets were expressed as a percentage of the bands representing untreated 16k and 100k pellets respectively. Accordingly, treatment with GH/Dex decreased $G_i\alpha_2$ in the 16k pellet by 35% when compared to control. This decrease was associated with a 70% increase in $G_i\alpha_2$ in the 100k pellet (Figure 9).

In an attempt to further refine these observations, particulate matter in the 16k pellet of control and GH/Dex treated fat cells were separated on linear sucrose gradients with densities ranging from 5-25%. These gradients were divided into 1 ml fractions (from lightest to heaviest) and each fraction was analyzed by quantitative Western blotting. Treatment of adipocytes with GH/Dex produced a shift of $G_i\alpha_2$ from heavier fractions of plasma membrane to lighter membrane fractions (Figure 10 and 11) but had no effect on the distribution of $G_s\alpha$. GH/Dex also had no effect on the partitioning of the membrane marker enzyme 5'-nucleotidase (Figure 12). In contrast,

isoproterenol (1μg/ml) produced a shift of 5'-nucleotidase towards lighter fractions, in agreement with the findings of Haraguchi and Rodbell (1990).

To determine whether GH/Dex-induced changes in the distribution of $G_i\alpha_2$ along the density gradient was restricted to the α-subunit or involved the entire trimeric complex, fractions of plasma membrane were subjected to ribosylation by pertussis toxin. The 16k pellets were prepared for sucrose gradients as described above. Each fraction was then incubated for 30 min with [^{32}P]-NAD and 2 μg/ml pertussis toxin. After ADP-ribosylation, fractions were separated on 10% SDS-PAGE followed by quantitative autoradiography as measured by densitometry. The ribosylation data confirmed the Western data and showed a GH/Dex-induced shift of G_i from heavier to lighter membrane fractions. The data further suggest GH/Dex causes the entire G protein complex to redistribute to a lower density fraction. However, the design of the experiment does not address the question of whether the observed shift of G_i distribution to lighter fractions was due to an overall change in the membrane preparation due to GH-treatment (for example, GH may change the distribution of actin adjacent to plasma membranes such that membranes from GH-treated cells are lighter) or whether there is a partitioning of G_i to lighter membrane vesicles. However, if GH were to cause plasma membrane to become lighter, we should expect to

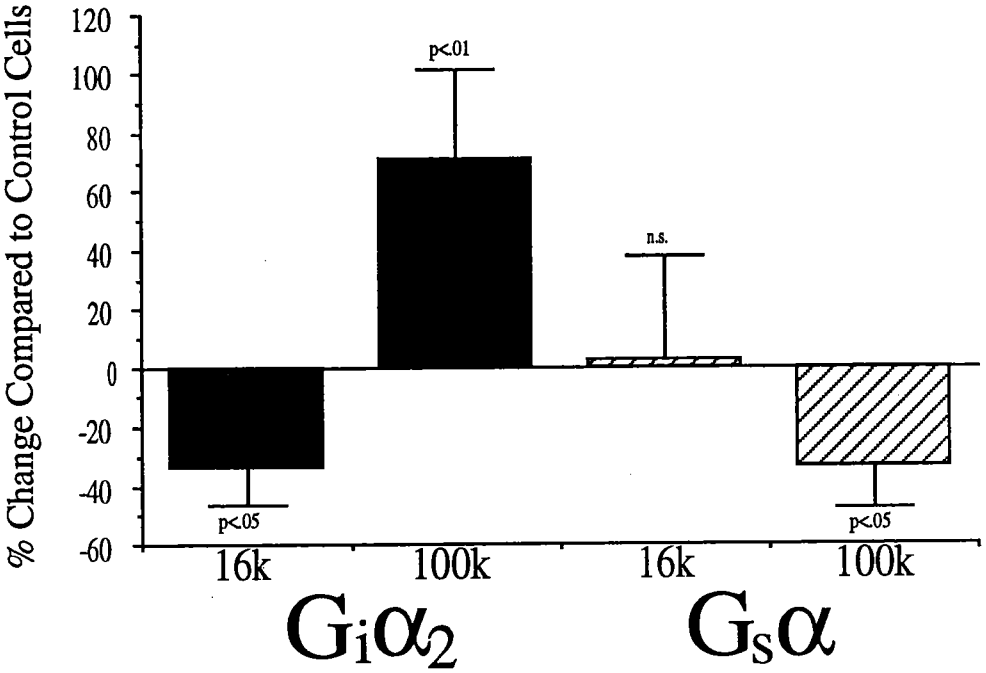


Figure 9

Changes in distribution of Gi and Gs in homogenates of GH/Dex treated cells. Preparation of samples for Western analysis was performed as described in Figure 8 and in Methods. Intensity of band staining, by immunoblotting with anti-$G_i\alpha_2$ and anti-$G_s\alpha$, was estimated by densitometry. Each bar represents 4 experiments. Due to the variability of exposure from experiment to experiment, 16k and 100k bands from GH/Dex treated samples were normalised with their control band. Values of cells treated with GH/Dex were expressed as a percentage of values from control cells.

Figure 10

Effect of GH/Dex on the distribution of $G_i\alpha_2$ in the 16k pellet. 16k pellets were fractioned on a linear sucrose gradient followed by Western analysis of the resultant fractions. Nitrocellulose membranes containing G proteins were probed with anti-$G_i\alpha_2$ and anti-$G_s\alpha$ as described in Methods. GH/Dex induced s selective re-distribution of $G_i\alpha_2$, but not $G_s\alpha$, from higher density fractions to lower density fractions. Shown are the results from a typical experiment of which a total of four replicated experiments were performed.

Distribution of G-proteins in purified plasma membranes separated on sucrose gradient column

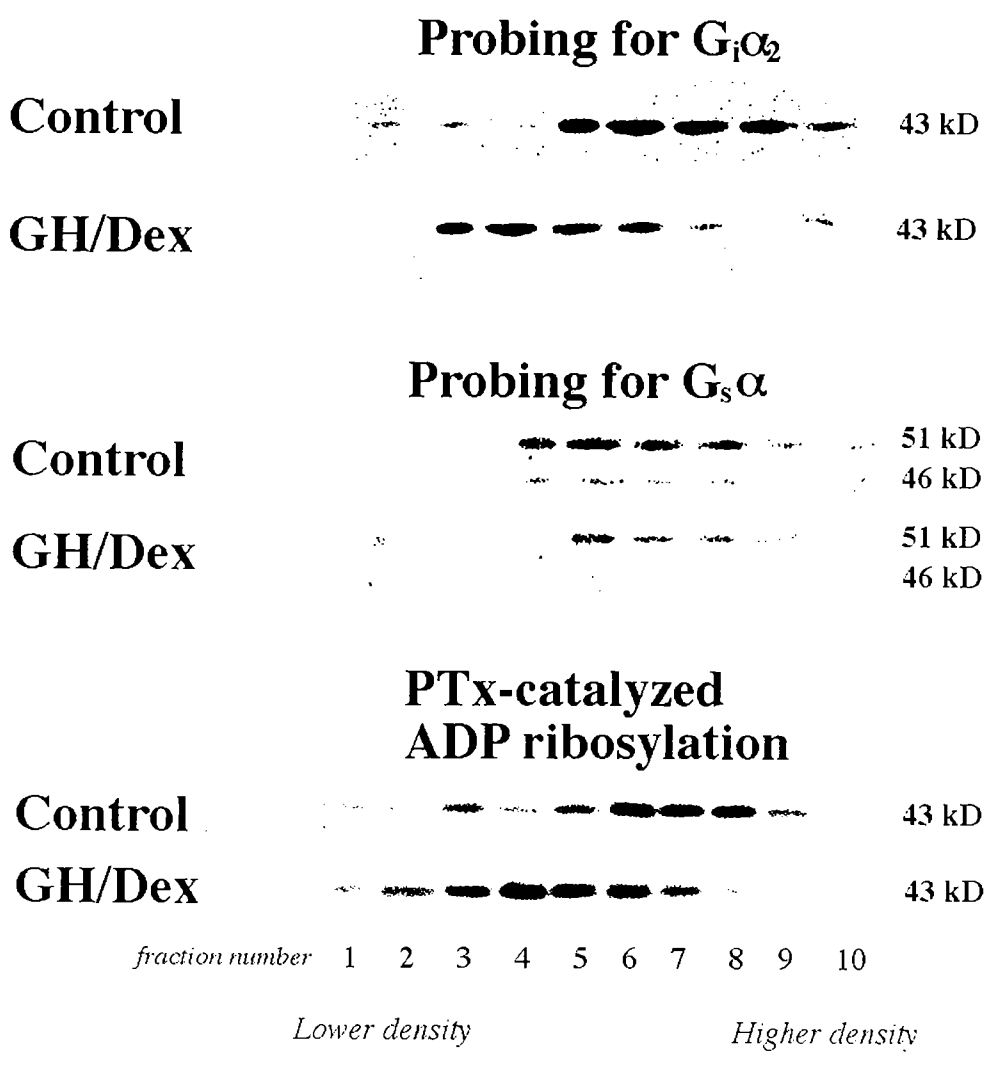
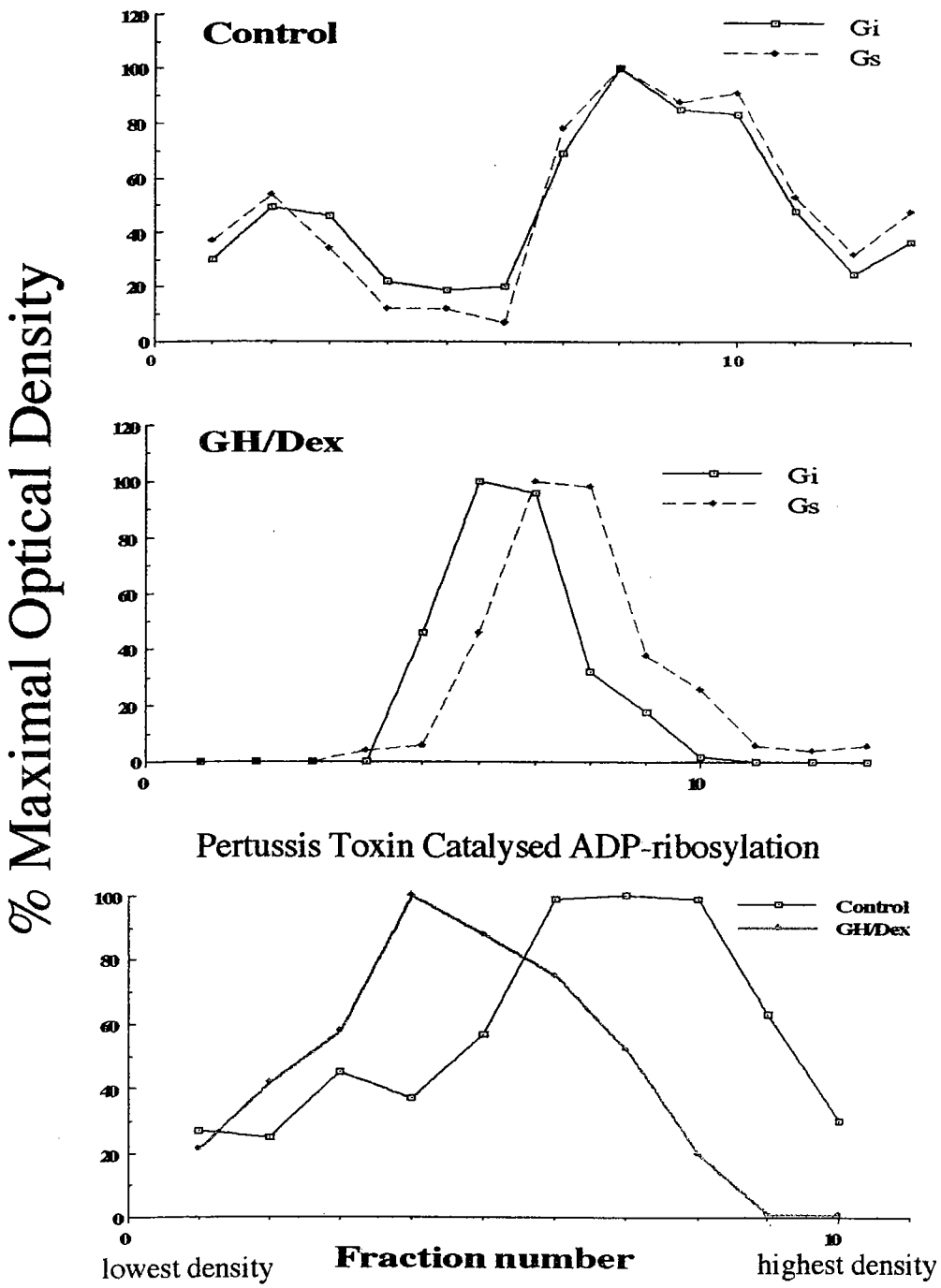


Figure 11

Graphical representation of the effect of GH/Dex on the distribution on the distribution of $G_i\alpha_2$ in the 16k pellet. 16k pellets were prepared as described in Figure 10. Due to variability of exposure from experiment to experiment, all bands on the same nitrocellulose membrane were normalised to the darkest band. GH/Dex induced a shift of $G_i\alpha_2$ to lower density fractions. This shift was also seen for G_i proteins labelled with ^{32}P-NAD after ribosylation with pertussis toxin. Shown are the densitometry results from all four experiments.

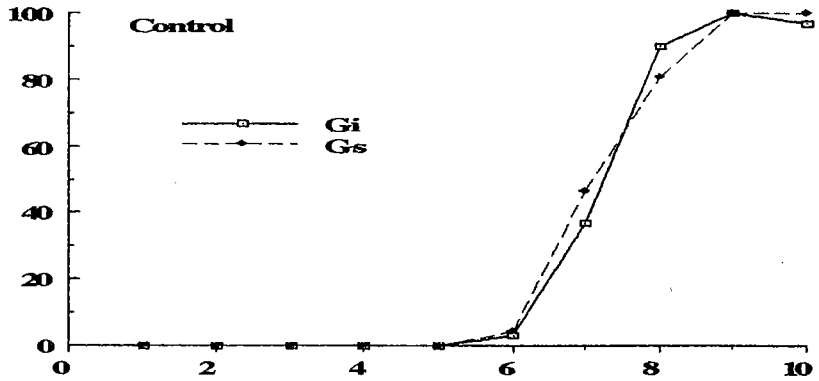
Western Analysis of G Protein Distribution

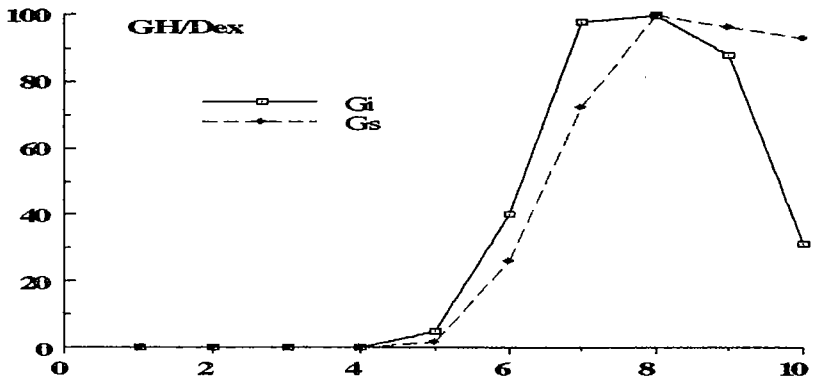
Trial 1



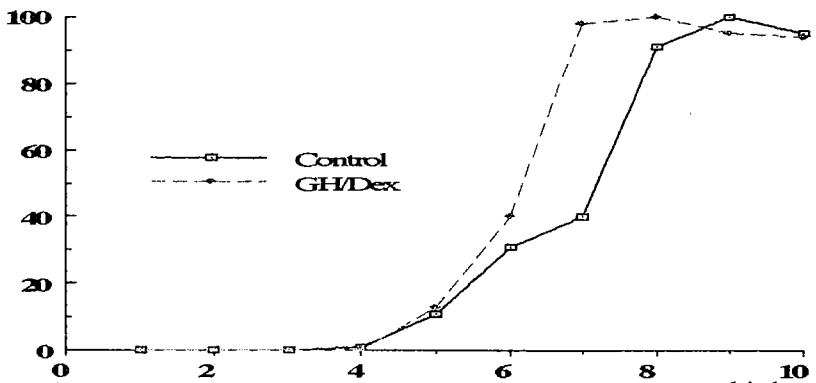
Western Analysis of G Protein Distribution

Trial 2





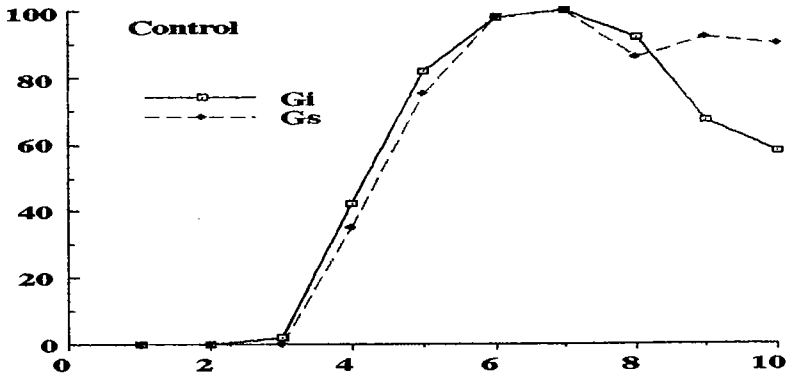
Pertussis Toxin Catalysed ADP-ribosylation

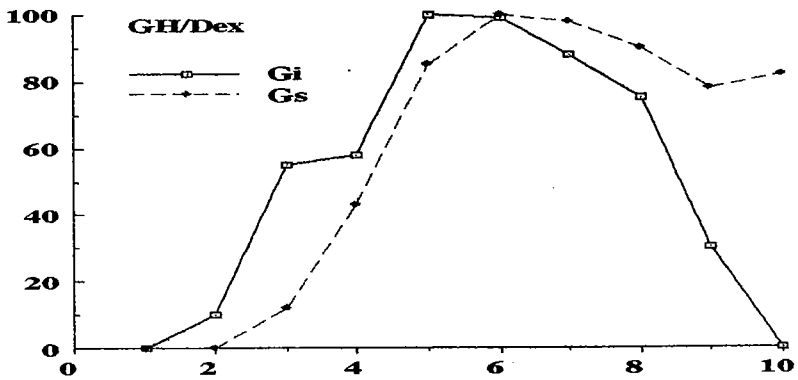


lowest density **FractionNumber** highest density

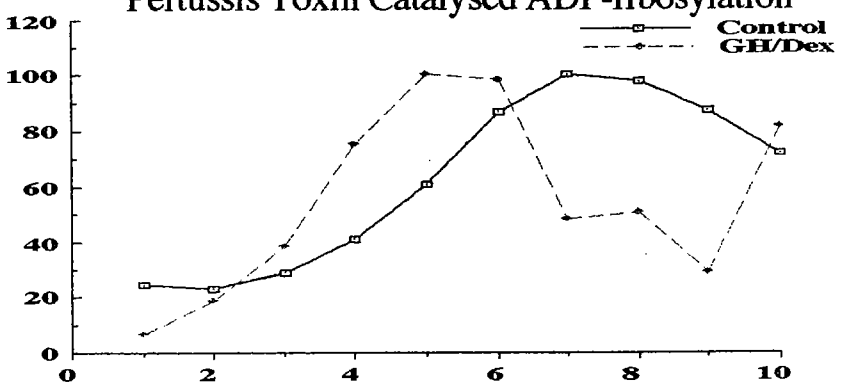
Western Analysis of G Protein Distribution

Trial 3





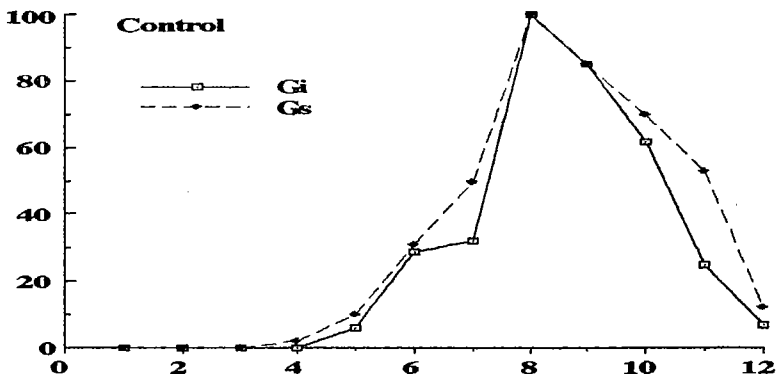
Pertussis Toxin Catalysed ADP-ribosylation

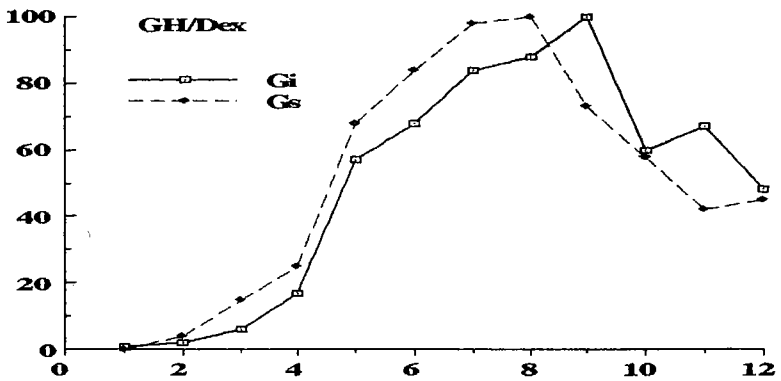


lowest density **Fractionnumber** highest density

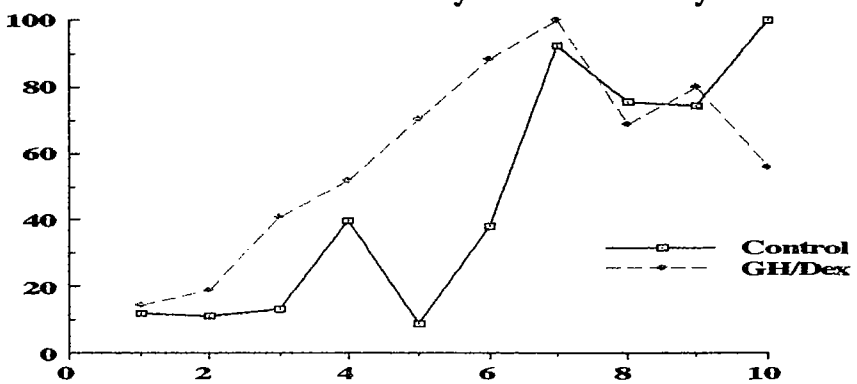
Western Analysis of G Protein Distribution

Trial 4





Pertussis Toxin Catalysed ADP-ribosylation



lowest density — Fraction Number — highest density

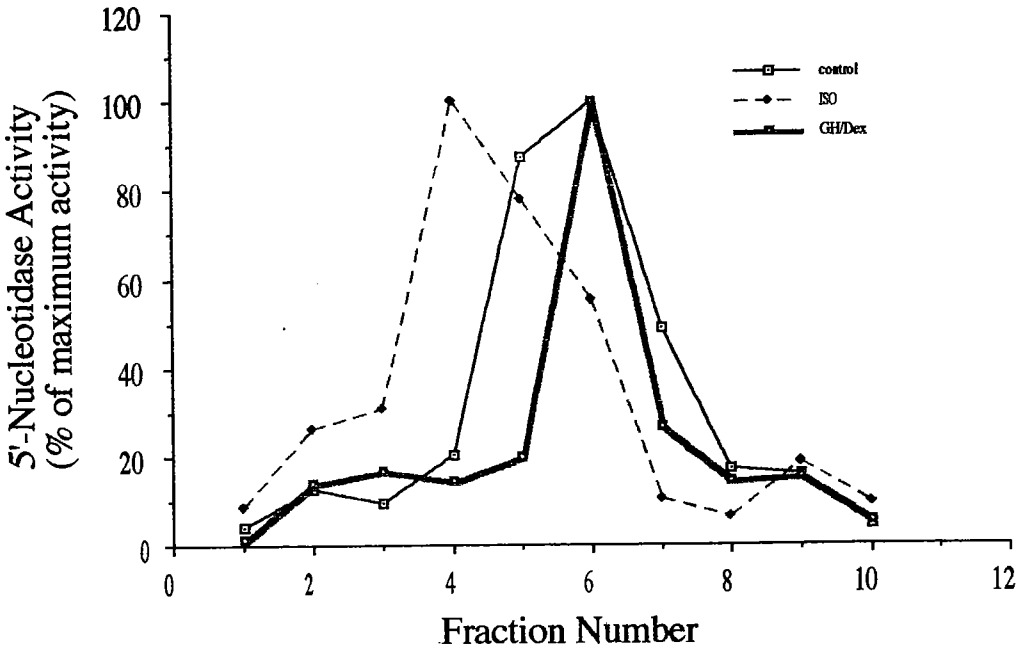


Figure 12

A representative experiment showing the effects of GH/Dex and isoproterenol on the distribution of 5'-nucleotidase in 16k pellet after separation on a sucrose gradient. Similar data were obtained in 3 other replicate experiments. Since maximal 5'-nuleotidase activity varied between conditions, fractions were normalised to the maximal activity encountered in each condition.

see all components of the membrane to shift evenly however only a selective change in the distribution of $G_i\alpha_2$ was observed; the distributions of $G_s\alpha$, and 5'-nucleotidase as well as adenylyl cyclase (Table 2) were unaffected.

Effect of colchicine on partitioning of G proteins and lipolysis

To determine how $G_i\alpha_2$ might be partitioned we next explored the possibility that the cytoskeleton might be involved. We therefore examined the effect of disruption of the cytoskeleton with colchicine on the distribution of $G_i\alpha_2$ and $G_s\alpha$. After the 4th hour of incubation with GH/Dex, the cells were homogenized. The homogenate was separated into 16k and 100k pellets and the pellets were analyzed on Western blots by using anti-$G_i\alpha_2$ and anti-$G_s\alpha$ as described above. Again treatment with GH/Dex decreased $G_i\alpha_2$ in the 16k pellet and increased $G_i\alpha_2$ in the 100k pellet. Colchicine completely abolished the changes by GH/Dex, that is, there was no reduction in $G_i\alpha_2$ in the 16k pellet nor accumulation in the 100k pellet (Figure 13).

If redistribution of $G_i\alpha_2$ is the causal event in GH stimulated lipolysis, then colchicine should also prevent GH/Dex induced lipolysis. To test this idea, we incubated adipocytes with and without GH/Dex for 4 hours in the absence or presence of 100 μM colchicine. Samples of medium were

removed at the beginning and at the end of the 4th hour of incubation for glycerol assay to determine the rate of glycerol release. Again, fat cells that were incubated with GH/Dex showed more than a 2-fold increase in lipolysis ($p<0.01$). Colchicine completely abolished the increase in glycerol production (Figure 14; $p<0.01$) while having no effect on the basal rate of lipolysis. To test whether the decreased glycerol release by colchicine was due to non specific inhibition, replicate aliquots of colchicine treated or control cells were treated with 1 μg/ml isoproterenol in the 4th hour of incubation. A typical lipolytic response to isoproterenol, 7-8 fold increase in lipolysis ($p<0.001$), was obtained even in the presence of colchicine suggesting that cells were still viable and that lipolysis induced by isoproterenol was insensitive to colchicine. These findings are consistent with the notion that GH/Dex regulates cyclic AMP and adenylyl cyclase by a different mechanism from that of the catecholamine.

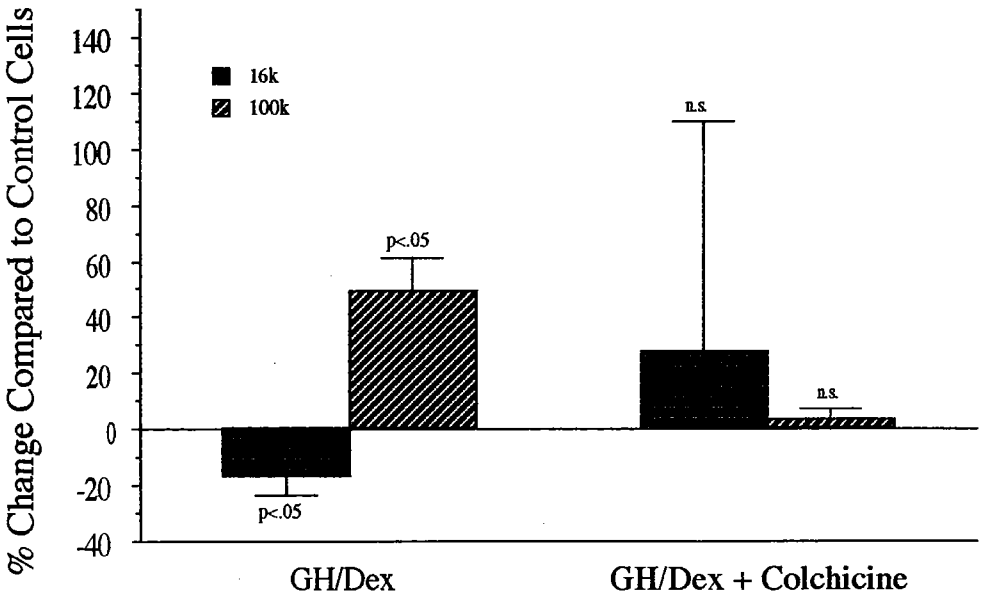


Figure 13

Changes in distribution of $G_i\alpha_2$ in homogenates of GH/Dex treated cells in the absence and presence of 100 μM colchicine. After 4 hours incubation in the presence of colchicine, samples were prepared as described in Figure 8 and Methods. Each bar represents the mean and s.e.m. of the ratio of $G\alpha_2$ content in GH/Dex treated and control cells for 4 experiments as determined by densitometry from Western analysis. p-values are for the differences between experimental values and 100%.

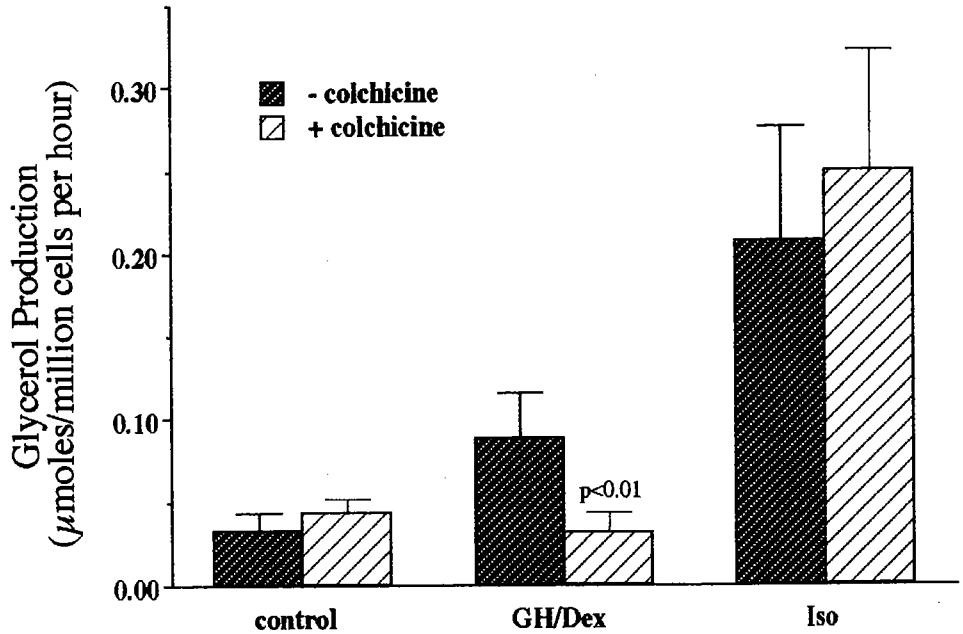


Figure 14

The effects of colchicine on lipolysis produced by GH/Dex or isoproterenol. Adipocytes were incubated for 4 hours in the absence or presence of 100 μM colchicine. Glycerol production was calculated as the difference in glycerol content in incubation medium between the beginning and the end of the 4th hour of incubation. As before GH/Dex increased glycerol production by 2.5 times greater than control while a high dose of isoproterenol (1 μg/ml) increased glycerol production by 6-fold. Each bar represents the mean and s.e.m. of 4 replicates. The p-value shown is for the comparison between (-)colchicine and (+)colchicine in GH/Dex treated cells.

Discussion

Various lines of evidence have been important in implicating cyclic AMP as the intracellular mediator of the lipolytic hormones. Fast-acting lipolytic hormones such as adrenaline increase cyclic AMP concentrations in adipocytes and stimulate adenylyl cyclase in broken-cell preparations. The presence of an adrenaline-sensitive adenylyl cyclase system in adipose tissue homogenates was reported by Sutherland and Rall (1960) and Klainer *et al.*, (1962). Also, Vaughan (1960) showed that phosphorylase activation, which might be taken as an indirect measurement of tissue levels of cyclic AMP, and lipolysis were both increased in fat pads incubated with catecholamine, ACTH, TSH, or glucagon. Vaughan and Steinberg (1963) provided additional and strong evidence when they reported that the methylxanthine, caffeine, an inhibitor of cyclic AMP phosphodiesterase, acted synergistically with adrenaline on lipolysis. Finally, Rizack (1964) reported the activation of an adrenaline sensitive lipase activity by cyclic AMP in cell-free preparations of fat pads incubated with ATP, Mg^{+2}, and caffeine. In 1965, Sutherland *et al.* show that the analog dibutyryl cyclic AMP activates lipolysis in fat tissues, however, this compound may act as a more permeant and stable form of cyclic AMP and may also inhibit cyclic AMP breakdown. The early studies on

the role of cyclic AMP in lipolysis have been comprehensively reviewed by Robinson *et al.*, (1971).

Previous studies of changes in cyclic AMP in adipocytes in response to GH produced conflicting or contradictory results. Sengupta *et al.*, (1981) found that GH (20 μg/ml) increased cyclic AMP levels in perifused fat cells but had no effect in flask-incubated fat cells. In these experiments, however, measurements of cyclic AMP were made only in the first hour of incubation with GH, bringing into question the significance of their findings since lipolysis is normally not increased by GH until considerably later (2-3 hours). Also, if the growth hormone preparation were contaminated with 0.1% ACTH or TSH, these agents would be enough to account for this lipolysis. Harris and Bennun (1976) also showed that GH was not capable of activating adenylyl cyclase in normal rat adipocytes but they too studied cells only after 15 min of incubation with GH and theophylline, well before any lipolytic effect might be expected (Fain *et al.*, 1965). Moskowitz and Fain (1970) were able to show an increase in cyclic AMP concentrations in fat cells after 4 hours of incubation with GH and dexamethasone but only when theophylline was also present. Theophylline, an inhibitor of cyclic AMP phosphodiesterase, is now known to also be an adenosine A_1 receptor antagonist (Ukena *et al.*, 1993; Daly, 1982). The adenosine A_1 receptor is coupled to G_i. By blocking the A_1 receptor it is possible that theophylline might also block or decrease the

inhibitory input to adenylyl cyclase. Thus, theophylline may elevate levels of cyclic AMP by relieving inhibition on adenylyl cyclase as well as by protecting cyclic AMP from enzymatic degradation. Therefore, experiments performed with GH in the presence of theophylline raise the question as to which agent, GH or theophylline or both, might produce substances that promote adenylyl cyclase activation thus increasing cyclic AMP.

The present experiments differed from earlier studies in that we restricted our measurements of response to GH/Dex to effects seen after 3 hours of incubation. We used no additional agents such as theophylline to preserve or amplify putative effects on cyclic AMP accumulation because those agents were not needed to preserve or amplify effects on lipolysis. We found no differences in cyclic AMP concentrations between control and tissues treated with GH/Dex, and can conclude one of two possibilities: (1) cyclic AMP is not involved in GH-induced lipolysis or (2) cyclic AMP is involved but does not require a measurable change in its bulk intracellular levels.

In considering the first possibility, a recent survey of the literature has shown no other lipolytic pathways than the classical cyclic AMP-protein kinase A-mediated pathway. However, recent work by Gorin *et al.*, (1990) have demonstrated a role of protein kinase C in isoproterenol- and GH-induced lipolysis but the authors concluded that the effects of protein kinase

C are likely proximal to cyclic AMP and are of a permissive nature rather than a direct stimulatory effect on lipolysis.

There are, however, some precedents for increased lipolysis without increased cyclic AMP. Despite the vast literature on cyclic AMP and lipolysis, there appears to be no firm experimental basis for evaluating the quantitative relationship between these two parameters. For example, Manganiello *et al.*, (1971), Schimmel (1974), and Fain *et al.*, (1979) have shown that exposure of adipocytes to lipolytic hormones results in a linear increase in the rate of lipolysis that, paradoxically, coincide with a transient rise and fall in cellular cyclic AMP concentrations. Manganiello *et al.*, (1971) found that the peak level of cyclic AMP was attained between 4 and 7 min after addition of adrenaline or ACTH while the rate of lipolysis remained unchanged even as the cyclic AMP levels rise and fall. They also found that the steady state level of cyclic AMP needed to produce a maximum lipolytic rate was quite low compared to the transient peak level but did find a close correlation between adenylyl cyclase activity and lipolysis. They suggested that cellular cyclic AMP may have a very short half-life (high turnover rate) (Manganiello *et al.*, 1971). Schimmel also observed a rise and fall in levels of cyclic AMP in adipose tissue within 15 min of addition of adrenaline while the rate of lipolysis continued to increase even 1 hour after addition of adrenaline. Similarly, Fain *et al.*, (1979) observed a sustained maximal rate of lipolysis 60 min after

addition of noradrenaline even though cyclic AMP levels had already dropped to near basal levels. Schimmel (1974) and Fain *et al.*, (1979) suggested that the observed discrepancy between lipolytic rate and cyclic AMP levels may be due to different pools of cyclic AMP and suggested that the pool of cyclic AMP measured in their studies was not closely coupled to the lipolytic machinery and therefore the lipolytic rate may be modified with no dramatic change in the measured cyclic AMP levels.

Our data reflect some very interesting discrepancies which cannot be explained by assuming that the cytosol is a uniform "soup", a condition reproduced by experiments using cell extracts in cell free solutions. We assume that rat adipocytes have type II protein kinase A (Beebe and Corbon, 1984), with affinity for cyclic AMP at K_a=78 nM (Ekanger *et al.*, 1985). Our assay shows both resting and hormone activated cyclic AMP levels to be roughly 0.3-0.4 μM and if so, we would expect all cyclic AMP binding sites on protein kinase A to be occupied and the kinase completely activated. However, because phosphodiesterase has a greater affinity ($K_m \leq 10$ nM; Barber and Sutherland, 1992) for cyclic AMP than Type II protein kinase A (K_a=78 nM; Ekanger *et al.*, 1985), and the rate of cyclic AMP synthesis (1.56 nmol/min/μg protein), as measured in the presence of forskolin, is much lower than the rate of degradation by Type IV phosphodiesterase (8.5 μmol/min/μg protein; Manganiello *et al.*, 1992), one would expect very little

cyclic AMP accumulation, if any, in the cytosol. This problem may be resolved if there exists a cellular architecture which places protein kinase A and adenylyl cyclase in close proximity to each other thus allowing the newly synthesized cyclic AMP to activate protein kinase A before degradation by phosphodiesterase.

Both Manganiello *et al.*, (1971) and Schimmel (1984, 1974) suggested that the discrepancy between cyclic AMP levels and lipolytic rates may be due to multiple pools of cyclic AMP in different functional compartments of the cytosol. Other evidence in support of compartmentalization of cyclic AMP signalling components within the adipocyte comes from the work of Honeyman *et al.*, (1979) who showed that adrenaline and serotonin both increase cellular levels of cyclic AMP and accelerated glycogenolysis but only adrenaline increased lipolysis. The interpretation of these findings by Honeyman *et al.*, (1979) was that adipocyte cyclic AMP must be sequestered in different "pools". Catecholamine may increase cyclic AMP accumulation in one pool, that is co-localized with protein kinase A, phosphorylase and hormone-sensitive lipase, while serotonin may promote cyclic AMP accumulation in another pool that is co-localized with protein kinase A and phosphorylase only. As well, Hollenga *et al.*, (1991) demonstrated that BRL 37344, a β_3-adrenergic receptor agonist (Hollenga and Zaagsma, 1989) and isoproterenol produced the same degree of maximal lipolysis in adipocytes.

However, the cyclic AMP levels required to produce maximal lipolysis by BRL 37344 was only 20% of the levels needed with isoproterenol. BRL 37344 increased lipolysis without an apparent increase in cyclic AMP or with only a very small increase in cyclic AMP may be explained by intracellular compartmentalization in which significant increases in cyclic AMP might be confined to small areas in the vicinity of protein kinase A as suggested by Hollenga *et al.*, (1991) and Pohl (1981). More recently, Ashida and Sakuma (1992) provided evidence for compartments of cyclic AMP in rat platelets by using specific phosphodiesterase inhibitors. In their studies, one pool of cyclic AMP was responsible for platelet aggregation and sensitive to degradation by a cyclic AMP-specific phosphodiesterase, while another pool of cyclic AMP was responsible for the platelet release reaction and sensitive to degradation by a cyclic GMP-specific phosphodiesterase (Ashida and Sakuma, 1992). These studies (Manganiello *et al.*, 1971; Schimmel, 1974, 1984; Honeyman *et al.*, 1979; Hollenga *et al.*, 1991; Ashida and Sakuma, 1992) and our findings further support the notion of functionally separate pools of cyclic AMP in intact cells.

Because it is possible for a small change in cyclic AMP to go undetected and yet to be sufficient to promote lipolysis, it was necessary to establish whether cyclic AMP plays a role in GH/Dex induced lipolysis by an approach other than direct measurement. To this end, the cyclic AMP antagonist Rp-CAMPS (Rothermel *et al.*, 1983), was used. Rp-CAMPS, the Rp

isomer of adenosine 3'5'-phosphorothioate, competes with cyclic AMP for binding sites on the regulatory subunits of protein kinase A, but unlike cyclic AMP, Rp-CAMPS cannot induce the conformational change in the regulatory subunits necessary for the release of the catalytic subunits (de Wit *et al.*, 1988; Parker-Botelho *et al.*, 1988), Although Rp-CAMPS decreased GH/Dex induced lipolysis, it did not completely abolish the lipolytic effect of GH/Dex. Others have also reported the partial effectiveness of Rp-CAMPS at blocking cyclic AMP-dependent processes. Adashi *et al.*, (1990) reported that Rp-CAMPS, at a concentration of 1 mM, only partially blocked FSH-stimulated progesterone accumulation in granulosa cells. This may be attributed to the ability of Rp-CAMPS to compete with cyclic AMP for binding to phosphodiesterase and thus inhibit cyclic AMP degradation leading eventually to a buildup of endogenous cyclic AMP which then overcomes the inhibition produced by Rp-CAMPS. Pereira *et al.*, (1987) had noted that at concentrations greater than 30 μM, Rp-CAMPS was not an effective cyclic AMP inhibitor. Therefore we chose a concentration (30 μM) of Rp-CAMPS that would effectively inhibit cyclic AMP while minimally inhibiting phosphodiesterase. Inhibition of GH/Dex induced lipolysis by Rp-CAMPS is strong and suggestive, but not conclusive evidence that cyclic AMP mediates the acceleration of lipolysis induced by GH.

In an attempt to further explore the role of cyclic AMP in GH induced lipolysis a more direct and sensitive technique was employed: the protein kinase A assay (Corbin, 1983). Whereas our assay of cyclic AMP measured the total cyclic AMP levels within the tissue segment, assay of protein kinase A activity would reflect the functional levels of cyclic AMP; i.e. those participating in protein kinase A activation. The protein kinase A holoenzyme consist of two catalytic and two regulatory subunits (Taylor *et al.*, 1990). Each regulatory subunit has two cyclic AMP binding sites. Occupation of the first cyclic AMP binding site on each regulatory subunit increases the affinity of the other. Because of this strong positive cooperativity of cyclic AMP binding, small changes in cyclic AMP can produce large changes in bound cyclic AMP and protein kinase A activation (Rannels and Corbin, 1981). Thus changes in cyclic AMP levels which might be too small to detect in radioimmunoassays, either in the whole cell or within the proximity of protein kinase A, might nevertheless be large enough to activate protein kinase A.

Following the work of Honnor *et al.*, (1985) we measured endogenously activated protein kinase A as a function of maximal available protein kinase A as determined in the presence of saturating amounts of cyclic AMP. Consistent with the notion that GH/Dex induces lipolysis through a cyclic AMP-dependent pathway, a greater fraction of protein kinase

A was activated in fat tissues in which lipolysis was accelerated by GH/Dex. Activation of protein kinase A might occur in several ways: (1) it may result from elevation of cyclic AMP levels within the adipocyte, (2) the affinity of protein kinase A for cyclic AMP may be increased so that there may be greater fractional activation in the presence of constant levels of cyclic AMP, or (3) sites of cyclic AMP production may be brought into closer proximity to protein kinase A so that activation of protein kinase A occurs more efficiently with only small changes in local levels of cyclic AMP.

Activation of protein kinase A by increased concentrations of cyclic AMP is well established in the literature (Taylor *et al.*, 1990). However, there is also evidence to support the hypothesis that sensitivity of protein kinase A to cyclic AMP may not be constant. Protein kinase A exists as 2 isoforms: type I and type II (for review see Taylor *et al.*, 1990) which have identical catalytic subunits but different regulatory subunits (Hanks *et al.*, 1988; Taylor *et al.*, 1990). The two isoforms of protein kinase A are functionally distinguishable on the basis of their interaction with MgATP (Taylor *et al.*, 1990). Only the type I holoenzyme binds MgATP with a high affinity and exists in the cell as a ternary complex containing a regulatory subunit, a catalytic subunit, and MgATP. In addition to increasing the amount of cyclic cAMP necessary to activate the holoenzyme, MgATP also promotes the reassociation of the regulatory and catalytic subunits of the type I holoenzyme thus hastening its

inactivation. The type II holoenzyme may exist in a phosphorylated and a dephosphorylated state. When phosphorylated the type II isozyme has a lower affinity for the catalytic subunit and thus dissociates at a lower concentration of cyclic AMP than when dephosphorylated (Rosen and Erlichman, 1975; Hoffmann *et al.*, 1975; Rangel-Aldao and Rosen, 1977). In epididymal adipocytes, the predominant isoform of protein kinase A is type II which comprises 90% of the total protein kinase A (Corbin, 1983). Therefore it is possible that the lipolytic actions of GH may be achieved through phosphorylation of protein kinase A resulting in a greater sensitivity for cyclic AMP for activation.

Although the idea that GH might sensitize protein kinase A to cyclic AMP through phosphorylation is attractive, this does not appear to be the case. The type II isoform of protein kinase A is present primarily in its phosphorylated form (Taylor *et al.*, 1990) and thus already sensitized to cyclic AMP in the absence of GH. Also, it has been shown that the lipolytic response to dibutyryl cyclic AMP, a membrane permeable analog of cyclic AMP, is not enhanced by GH pretreatment suggesting the sensitivity of protein kinase A to dibutyryl cyclic AMP was not enhanced with GH pretreatment (Fain, 1968; Goodman, unpublished data).

If GH directs cyclic AMP to a functional compartment in which protein kinase A is abundant and has access to hormone-sensitive lipase, one

might expect that GH would direct dibutyryl cyclic AMP or 8-bromo-cyclic AMP towards a compartment with protein kinase A and thus enhance their lipolytic actions. However, the work by Fain (1968) and Goodman (unpublished data) indicates that GH does not increase lipolysis induced by the cyclic AMP analogs. These studies are inconclusive, however, since exogenous dibutyryl cyclic AMP and 8-bromo-cyclic AMP may not distribute in the same way as endogenously produced cyclic AMP.

Honnor *et al.*, (1985) also examined a variety of lipolytic hormones including isoproterenol, glucagon, and ACTH, known to stimulate lipolysis through the cyclic AMP-dependent pathway (Birnbaumer *et al.*, 1985) to see what effect these agents had on protein kinase A activity. They found that regardless of the lipolytic agent tested, maximal lipolysis was achieved when 40% protein kinase A was activated. There was a direct correlation between the rate of glycerol production and the protein kinase A activity ratio up until maximal lipolysis was reached. Upon stimulation with even higher concentrations of agonist, the activity ratio of protein kinase A continued to increase suggesting that hormone-sensitive lipase, rather than protein kinase A or adenylyl cyclase had become rate-limiting. (Honnor *et al.*, 1985). This finding further indicated that the relationship between protein kinase A and hormone-sensitive lipase is independent of the agent used. Although we only examined the lipolytic effects of GH/Dex and of two concentrations of

isoproterenol we found that the same relationship between protein kinase A activity and lipolysis held for GH/Dex as for isoproterenol suggesting that GH/Dex must act prior to activation of protein kinase A. If GH does indeed activate protein kinase A by elevating cyclic AMP levels within the adipocyte it must also increase adenylyl cyclase activity.

Growth hormone could increase adenylyl cyclase activity by several possible mechanisms: (1) stimulation through the action of G_s on cyclase or (2) removal of inhibition mediated by G_i (Gilman, 1987) or (3) stimulation by a G protein independent mechanism. Interaction with G_s activates adenylyl cyclase and thus increases cyclic AMP formation. Alternatively, by decreasing the inhibitory influence of G_i on adenylyl cyclase, either through inactivation or decreased abundance, a state of partial stimulation may be achieved. Our finding that pertussis toxin increased lipolysis, after 2 hours, provides evidence that the activity of adenylyl cyclase may be increased by inactivation of G_i, or loss of receptor coupling and is in agreement with the findings in other investigators (Moreno *et al.*, 1983; Olansky *et al.*, 1983, Kather *et al.*, 1985) who also reported lipolytic effects of crude preparations of pertussis toxin. Finally, to our knowledge, there is no other mechanism other than G protein interaction which may regulate adenylyl cyclase.

Goodman *et al.*, (1988) reported that hypophysectomy increased G_i in the plasma membranes of adipocyte ghosts and that treatment of rats with GH

4 hours before sacrifice decreased the apparent amount of G_i in adipocyte membranes. They proposed that a diminished level of G_i in the plasma membrane of adipocytes may result in partial stimulation of adenylyl cyclase and hence increased lipolysis. Their report of diminished G_i in the plasma membrane by GH is supported by our findings and the recent report of Doris *et al.*, (1994) who found that $G_i\alpha_2$ increased whenever GH was removed from circulation for 2 days. Doris *et al.*, (1994) also showed that this action of GH was specific for $G_i\alpha_2$, the form of inhibitory G protein which various investigations have suggested is responsible for inhibition of adenylyl cyclase (Simonds *et al.*, 1989; McKenzie and Milligan, 1990) while leaving $G_s\alpha$ levels unchanged. Moxham *et al.*, (1993) also showed that $G_i\alpha_2$ mediates the inhibition of adenylyl cyclase when they showed that blocking expression of $G_i\alpha_2$ with antisense RNA blunted the inhibitory response to PIA and resulted in a 3.1-fold increase in basal cyclic AMP accumulation. The data of Moxham *et al.*, (1993) suggest that as $G_i\alpha_2$ decreases, cyclic AMP levels increase, presumably due to attenuated inhibition of adenylyl cyclase.

Several mechanisms might account for the decrease in $G_i\alpha_2$ in the plasma membrane: (1) GH might decrease the rate of synthesis of $G_i\alpha_2$, (2) GH might accelerate the rate of $G_i\alpha_2$ degradation, or (3) GH might cause a

dissociation of $G_i\alpha_2$ from adenylyl cyclase. In order for the first possibility to account for the lipolytic action of GH, a decrease in the synthesis of $G_i\alpha_2$ would have to produce a significant decrease in $G_i\alpha_2$ within 2-3 hours. The half-life of G proteins has been reported to range from 72 hours in cardiocytes (Silbert *et al.*, 1990) to 24 hours in hamster smooth muscle (Hadcock *et al.*, 1991) suggesting that even if synthesis was completely shut down, more than 95% of $G_i\alpha_2$ would still be present in the membrane 3 hours after addition of GH/Dex.

With regard to the alternative mechanism, enhanced $G_i\alpha_2$ degradation, Mitchell *et al.*, (1993) reported that the muscarinic M1 receptor agonist, carbachol, accelerated the degradation rate of $G_q\alpha/G_{11}\alpha$ in Chinese hamster ovary cells from a $t_{1/2}$ of 18 hours to 2.9 hours. They also reported that this enhanced rate of degradation was specific for $G_q\alpha/G_{11}\alpha$ since $G_s\alpha$ and $G_i\alpha_2$ were unaffected. Accelerated degradation occurred only during the first 8 hours even when exposure to carbachol was maintained for 60 hours (Mitchell *et al.*, 1993). Although it is possible that the decrease in $G_i\alpha_2$ in the plasma membrane of adipocytes caused by GH/Dex treatment may be due to enhanced $G_i\alpha_2$ degradation, the slow turnover of G_i precludes metabolic labelling in our experimental model and hence it was not possible to study

effects of GH on the rate of loss of label from immunoprecipitation of $G_i\alpha_2$. Other results make this unlikely, since the decrease of $G_i\alpha_2$ in the 16k pellet was accompanied by an increase in the 100k pellet suggesting translocation.

Stimulation of rat adipocytes with isoproterenol has been reported to induce translocation of $G_s\alpha$ from the dense plasma membrane fraction to lighter membrane fractions (Haraguchi and Rodbell, 1990). Our findings with GH/Dex are consistent with the notion that $G_i\alpha_2$ may be translocated from plasma membrane to lighter membrane fractions, but, our findings differ from those of Haraguchi and Rodbell in that translocation of $G_i\alpha_2$ from plasma membranes to lighter membrane fractions was selective. Haraguchi and Rodbell (1990) reported that stimulation of adipocytes with isoproterenol resulted in the translocation of $G_i\alpha$, $G_s\alpha$, adenylyl cyclase, and 5'-nucleotidase. In our hands, stimulation with GH/Dex resulted in partitioning of only $G_i\alpha_2$ leaving the distribution of $G_s\alpha$, adenylyl cyclase, and 5'-nucleotidase unchanged. Haraguchi and Rodbell (1990) attributed the redistribution of membrane proteins to pinocytosis. The selective redistribution of $G_i\alpha_2$ seen in our studies may also be explained by a pinocytic process in which vesicles selectively enriched in heterotrimeric $G_i\alpha_2$ are removed from the plasma membrane in much the same manner as vesicles containing the insulin

sensitive glucose transporters are removed from the plasma membrane after insulin dissociates from its receptor (Karnieli *et al.*, 1981). More work will be needed to address the question of whether selective redistribution of $G_i\alpha_2$ involves pinocytic vesicles. The data further suggest GH/Dex causes the entire G protein complex and not just the α-subunit to redistribute to a lower density fraction. However, the design of the experiment doesn't allow us to determine if the change in distribution of G proteins was due to: (1) an overall decrease in adipocyte membrane densities or (2) a partitioning of G proteins to lighter membranes. To address the first possibility, it is possible that GH/Dex treatment could result in plasma membranes having densities lighter than that of untreated cells resulting an apparent redistribution of G proteins to lighter fractions of a density gradient. However, if this were the case, then all components of the membrane should be shifted in the density gradient to an equal extent. This is not the case. We demonstrate that after treatment with GH/Dex, only the $G_i\alpha_2$ is shifted to a lighter fraction of the density gradient whereas $G_s\alpha$ was unaffected (Figure 9, 10 and 11). Even adenylyl cyclase, identified by its activity in the presence of forskolin, did not partition from 16k to 100k pellet as did $G_i\alpha_2$ (Table 2). This selective redistribution of $G_i\alpha_2$ argues against the possibility that hormone treatment

results in a non-specific partitioning of membrane components. Since this partitioning occurs in plasma membranes, the result is a change in the membrane composition thus $G_i\alpha_2$ and $G_s\alpha$ must be physically separated.

The next question is if $G_i\alpha_2$ is redistributed to lighter membrane components by treatment with GH/Dex, does this redistribution represent movement of individual molecules (i.e.: in soluble form) or molecules associated with membranes (for example pinocytosis)? If it is, indeed, due to dissociation of G_i proteins from the plasma membrane to intracellular vesicles, then this would mean that G_i is soluble for a period of time before anchoring itself to another lipid bilayer. Unless the time interval in which G_i is soluble is very short, we should lose some of the G_i in the supernatant above the 100k pellet. Upon examination of the data in Figure 9, we see that a 35% decrease in the amount of $G_i\alpha_2$ in the 16k pellet corresponds with a 70% increase in the 100k pellet. Since the 16k pellet represents roughly 4 times more $G_i\alpha_2$ than the 100k pellet, the amount of $G_i\alpha_2$ recovered in the 100k pellet after GH/Dex-treatment represents only half of the material lost from the 16k pellet. This very crude estimation is consistent with the notion that a portion of the $G_i\alpha_2$ is lost to the soluble portion of the cell extract and may support the contention that redistribution of $G_i\alpha_2$ to lighter membrane

components may be due to $G_i\alpha_2$ partitioning from plasma membrane to "microsomal" membranes. One may speculate upon a mechanism whereby the G proteins lose their fatty acid anchor, become unattached from the plasma membrane, and now that it is soluble within the cytoplasm, re-distributes to intracellular membrane vesicles and anchors themselves through re-acylation. However, from our knowledge of G protein structure and its post-translational modifications, it seems inconceivable for such a mechanism to be occurring yet partitioning may account for the apparent discrepancy between the amount of G protein lost in the 16k pellet and the amount gained in the 100k pellet. However, since we did not measure the levels of G proteins in the supernatant of the 100k pellet, we cannot conclude that G proteins actually become soluble and then re-anchor themselves to other membranous particles.

The alternate possibility is that $G_i\alpha_2$ is selectively removed from the vicinity of G_s and adenylyl cyclase. Such a separation could upset the balance of stimulation and inhibition to favor stimulation of adenylyl cyclase. A mechanism for such removal of $G_i\alpha_2$ from plasma membrane may be a special type of pinocytosis such that predominantly $G_i\alpha_2$ is removed from the plasma membrane rather than G_s or adenylyl cyclase. Precedence for selective translocation comes from the example of the glucose transporter (Karnielli *et*

al., 1981). Upon insulin stimulation, glucose transporters are translocated from cytosol to plasma membrane presumably through the fusion of intracellular vesicles with plasma membrane. When insulin is removed, the glucose transporters are removed from the plasma membrane by some sort of pinocytosis which selectively removes the glucose transporters away from the plasma membrane. The exact mechanism for this selective removal is unclear.

Recently, work by Anderson (1993a, 1993b) described a process in which cell membranes form flask-shaped invaginations called caveolae. Caveolae have the ability to concentrate small molecules and ions. The ability of caveolae to concentrate molecules before internalization may provide a mechanism for selective partitioning of $G_i\alpha_2$, although such a mechanism is purely speculative. We may speculate that GH/Dex treatment induces the formation of caveolae which allows for the selective concentration of $G_i\alpha_2$ within these caveolae. Since these flask-like structures can pinch off the plasma membrane to become intracellular vesicles, they may fractionate with the 100k pellet and result in the separation of $G_i\alpha_2$ from the plasma membrane. Alternatively, caveolae containing $G_i\alpha_2$ may not pinch off the plasma membrane but upon homogenization of the cells, the actual process of homogenizing cells may disrupte the plasma membrane in

such a way allowing the formation of small vesicles from caveolae which may partition with 100k pellet resulting in an apparent partitioning of $G_i\alpha_2$ to lighter membrane vesicles.

Interestingly, the molecular composition of caveolae indicates that they have the capacity to store and process messengers such as cyclic AMP, calcium, or adenosine and to use non-receptor tyrosine kinases to initiate crucial phosphorylation cascades (Anderson, 1993a). Therefore, in certain cells, caveolae may be important in signal transduction.

In 1956, Sir Rudolph Peters hypothesized that hormones act through the reorientation of the cytoskeletal system. The cytoskeleton, which consists of microtubules, microfilaments, and intermediate filaments, is known to have an important role in regulating cell growth and differentiated functions. In spite of considerable progress that has been made in understanding hormone-mediated signal transduction pathways, relatively little is known regarding the interaction of cytoskeletal components with these pathways. Among the cytoskeletal components, microtubules are of particular interest because they have been implicated in regulation of hormonal responses (for review see Zor, 1983; Yan and Rasenick, 1990). For example, it has been shown that disruption of microtubules with colchicine or vinblastine may increase cyclic AMP formation in several different cell systems (Zor, 1983). In

S49 lymphoma cells, colchicine and vinblastine enhanced β-adrenergic-stimulated cyclic AMP accumulation (Insel and Kennedy, 1978; Kennedy and Insel, 1979). This enhancement seems to be regulated at points proximal to cyclic AMP production, possibly at the level of G proteins (Yan and Rasenick, 1990). Recent reports have shown specific associations between tubulin and G proteins (Rasenick *et al.*, 1990;, Wang and Rasenick, 1991). Our data support the notion that an intact cytoskeleton is necessary for both the redistribution of $G_i\alpha_2$ and lipolytic effect of GH since colchicine blocked both the redistribution of $G_i\alpha_2$ and lipolysis induced by GH. As suggested by Haraguchi and Rodbell, (1990), G protein translocation may occur by pinocytosis and the pinocytic process may require an intact cytoskeleton. It is possible that GH causes the selective removal of G_i from plasma membranes by pinocytosis and this removal also requires an intact cytoskeleton. This could conceivably follow from GH-induced structural changes in the cytoskeleton resulting in invaginations of regions of plasma membranes rich in G_i leading to vesicles which may translocate to the cytosol.

Another way in which $G_i\alpha_2$ may be translocated away from the plasma membrane is through the "treadmilling" action of microtubules. Microtubules are simple, linear cylindrical assemblies of tubulin protein subunits with polarity, a plus end and a minus end. Subunits of tubulin are

added at the plus end and lost from the minus end yet the microtubule remains the same length (Hotani and Miyamoto, 1990). The plus end of the microtubule is located close to the plasma membrane while the minus end is situated near the nucleus (Hotani and Miyamoto, 1990). The treadmilling action results in movement of tubulin molecules from the periphery of the cell towards the nucleus (Hotani and Miyamoto, 1990). Since G proteins have been found to be associated with microtubules (Rasenick *et al.*, 1990), it is possible that a treadmilling action of microtubules is responsible for redistribution of G proteins and could result in transporting them from plasma membrane to the interior of the cell. Our findings that GH-treatment results in redistribution of $G_i\alpha_2$ from 16k pellet to 100k pellet, as well as redistribution to lighter fractions of the 16k pellet, suggest that $G_i\alpha_2$ does not dissociate from membranes to become a soluble protein. However, we did not assay the supernatant above the 100k pellet, for G proteins. The amount of $G_i\alpha_2$ gained in the 100k pellet may not completely account for the $G_i\alpha_2$ lost from the 16k pellet. Our estimates are crude and it appears that a significant part of $G_i\alpha_2$ lost from dense membranes appears in lighter fractions. Clearly, further studies will be needed to uncover the mechanism for redistribution of $G_i\alpha_2$.

Throughout the studies presented in this dissertation, dexamethasone has been added along with GH. Some comment about its role in the lipolytic action of GH is warranted. Although glucocorticoids were added with GH to amplify the lipolytic effect, there is evidence to indicate that they do not directly stimulate lipolysis. Fain *et al.*, (1965) demonstrated that glucocorticoids did not stimulate lipolysis but did enhance lipolysis induced by GH. Goodman (1968) showed that in the presence of theophylline, GH but not dexamethasone increased lipolysis. In addition to enhancing the lipolytic action of GH, glucocorticoids have also been shown to enhance the lipolytic action of catecholamine (Allen and Beck, 1972; Goodman, 1970). Goodman (1970) found that glucocorticoids potentiated the acceleration of lipolysis by catecholamine but this potentiation was delayed and was abolished by inhibitors of RNA synthesis.

Since lipolysis is thought to be cyclic AMP-dependent, it is possible that glucocorticoids act at some point along the cyclic AMP-dependent pathway. Evidence, as reviewed by Granner (1979) and Fain (1979), suggests that potentiation of lipolysis by glucocorticoids is not mediated through changes in cyclic AMP accumulation. No accumulation of cyclic AMP was found in rat adipocytes after several hours of incubation with glucocorticoids, however, we now know that very small changes in cyclic AMP may be sufficient to accelerate lipolysis. (Moskowitz and Fain, 1970; Fain *et al.*, 1971;

Fain and Saperstein, 1970). Under conditions in which glucocorticoids were absent (eg. after adrenalectomy), fat cells or fat pads displayed normal increases in cyclic AMP in response to hormones although the increase in lipolysis in response to catecholamine was blunted (Exton *et al.*, (1972; Allen and Beck, 1972; Werner and Low, 1974) suggesting that glucocorticoids had no direct effect on cyclic AMP production or degradation. Glucocorticoids did potentiate cyclic AMP accumulation in adipocytes treated with GH (Fain and Saperstein, 1970) further supporting the notion that it is GH and not glucocorticoids that is responsible for the increase in cyclic AMP levels.

A possible mechanism for enhancement of lipolysis by glucocorticoids could involve an increase in protein kinase A activity. Lamberts *et al.*, (1975) described such an effect in fat cells where prior incubation for 4 hours with dexamethasone resulted in a 28% increase in phosphorylation of histone by protein kinase A assayed in the presence of cyclic AMP. Dexamethasone did not increase basal protein kinase A activity (Lamberts *et al.*, 1975). These investigators also reported that 2 weeks of elevated cortisol levels, *in vivo*, resulted in enhanced lipolysis in response to dibutyryl cyclic AMP and suggested an increase in protein kinase A as the reason for the enhancement (Lamberts *et al.*, 1975). However, these results indicate chronic effects of glucocorticoids and were in contrast to those of Fain

(1968) and Goodman *et al.*, (1988) who reported no potentiation of lipolysis by dibutyryl cyclic AMP in cells pretreated with dexamethasone for 4 hours.

More recent evidence suggests that glucocorticoids can increase adenylyl cyclase activity (Chang and Bourne, 1987; de Mazancourt *et al.*, 1989; McLelland *et al.*, 1983), decrease phosphodiesterase activity (Schonhofer *et al.*, 1972; Elks *et al.*, 1983) or increase G_s (Chang and Bourne, 1987) but, again, these appear to be chronic effects of glucocorticoids which require several days of treatment and are unlikely to contribute directly to lipolysis in our experiments.

Glucocorticoids have also been shown to inhibit the release of prostaglandins, which have an inhibitory effect on adenylyl cyclase (Lewis and Piper, 1975). It is possible that the slight increase in lipolysis as well as the potentiating effects of glucocorticoids on lipolysis may be mediated by blocking the tonic release of prostaglandin.

Thus evidence in the literature does not support an immediate action (at least within 4 hours) of glucocorticoids on adenylyl cyclase or on any other component of the cyclic AMP-mediated pathway and is reasonable to suspect that the lipolytic actions of GH/Dex is mediated by the direct effects of GH rather than dexamethasone.

In summary, the findings presented in this study support the hypothesis that stimulation of lipolysis by GH occurs through a cyclic AMP-

mediated pathway. We also present evidence that cyclic AMP levels are elevated through activation or "de-inhibition" of adenylyl cyclase. Unlike catecholamine which activate adenylyl cyclase through the agency of stimulatory G proteins, our findings suggest GH/Dex activates adenylyl cyclase through relief of inhibition by selectively removing inhibitory G proteins from the vicinity of adenylyl cyclase. We further present evidence that the selective removal of $G_i\alpha_2$ is achieved through a mechanism that requires an intact cytoskeleton. Stimulation of lipolysis by GH/Dex but not by isoproterenol was inhibited by colchicine also suggesting an intact cytoskeleton is required for GH/Dex to stimulate lipolysis.

Future Directions

In addition to providing some insight into the mechanism of action of GH to stimulate lipolysis, these findings also prompt new questions which will be pursued in future studies. For example, if GH/Dex does indeed bring about a change in the structure of the cytoskeleton, how may it do so? As well, we have not yet demonstrated with what time course this particular action of GH/Dex takes place. Does redistribution occur immediately upon GH/Dex exposure to adipocytes or do cytoskeletal changes occur after 2-3 hours, the time increased lipolysis is seen? Are specific proteins induced by GH required for the redistribution phenomenon? Recently Meyer *et al.*, (1994) reported a signalling pathway between the GH receptor and the nucleus, consistent with the findings of Fain (1967) in which stimulation of lipolysis by GH required RNA and protein synthesis. It is possible that the newly synthesized proteins may be involved in redistribution.

Also there is the question of the role of glucocorticoids in stimulation of lipolysis by GH. We did not test the possibility that redistribution might be the result of GH alone or dexamethasone alone. We therefore will need to look for redistribution in adipocytes incubated with GH or dexamethasone alone. If glucocorticoids are responsible for the selective

redistribution of G_i then redistribution induced by glucocorticoids may explain some of its permissive effects. Fain (1967) showed that regardless of the time in which glucorticoids are added with GH, there is always a 1.5-2 hour delay before the enhancement of lipolysis induced by GH begins. It is possible that the glucocorticoid receptor, which can bind to DNA, may somehow facilitate the signalling between GH receptor and nuclear DNA to promote lipolysis.

References

Adashi EY, Resnick CE, and Jastorff B (1990) Blockade of granulosa cell differentiation by an antagonistic analog of adenosine 3',5'-cyclic monophosphate (cAMP): central but non-exclusive intermediary role of cAMP in follicle-stimulating hormone action. *Molecular & Cellular Endocrinology* 72(1):1-11

Allard WJ, Vicario PP, Saperstein R, Slater EE, and Stout HV (1991) The function but not the expression of rat liver inhibitory guanine nucleotide binding protein is altered in Streptozotocin-induced diabetes. *Endocrinology* 129(1):169-75

Allen DO, and Beck R (1972) Alterations in lipolysis, adenylate cyclase and adenosine 3'5'-monphosphate levels in isolated fat cells following adrenalectomy. *Endocrinology* 91:504-510

Alousi AA, Jasper JR, Insel PA, and Motulsky HJ (1991) Stoichiometry of receptor-Gs-adenylate cyclase interactions. *FASEB Journal* 5(9):2300-3

Altszuler N (1974) *In* Handbook of Physiology (E. Knobil and WH Sawyer, eds.) Sect. 7, Vol. 4, pp 233-252. American Physiological Society, Washington D.C.

Anderson RG. (1993a) Caveolae: where incoming and outgoing messengers meet. *Proceedings of the National Academy of Sciences of the United States of America.* 90(23):10909-13 Dec 1.

Anderson RGW (1993b) Potocytosis of small molecules and ions by caveolae. *Trends in Cell Biology* 3:69-72

Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN, and Carter-Su C (1993) Identification of JAK-2 as a growth hormone receptor-associated tyrosine kinase. *Cell* 74:237-244

Armstrong KJ, Stouffer JE, Van Inwegen RG, Thompson WJ, and Robison GA (1974) Effects of thyroid hormone deficiency on cyclic adenosine 3',5'-monophosphate and control of lipolysis in fat cells. *Journal of Biological Chemistry* 249(13):4226-31

Ashby CD, and Walsh DA (1973) Characterization of the interaction of a protein inhibitor with adenosine 3′:5′-monophosphate dependent protein kinase. *Journal of Biological Chemistry* 248:1255-61

Ashida S. and Sakuma K. (1992) Demonstration of functional compartments of cyclic AMP in rat platelets by the use of phosphodiesterase inhibitors. *Advances in Second Messenger & Phosphoprotein Research.* 25:229-39

Axelrod J, Burch RM, and Jelsema CL (1988) Receptor-mediated activation of phospholipase A_2 via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *Trends in Neurosciences* 11(3):117-23

Ballantyne B, and Raftery AT (1969) The neurochemistry of adipose tissue. *Journal of Physiology* - London 205(2):31P-33P

Barber R. Goka TJ. and Butcher RW. (1992) Cyclic AMP turnover in intact tissue. Role of cyclic nucleotide phosphodiesterases. *Advances in Second Messenger & Phosphoprotein Research.* 25:1-11

Barenton B, Batifol V, Combarnous Y, Dulor JP, Durand P, and Vezinhet (1984) A Reevaluation of lipolytic activity of growth hormone in rabbit adipocytes. *Biochemical & Biophysical Research Communications* 122(1):197-203

Baxter JD, and Rousseau GG (1979) Glucocorticoid Hormone Action. *Monographs on Endocrinology* vol. 12, Springer-Verlag, Berlin

Bazan JF (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proceedings of the National Academy of Sciences of the United States of America* 87(18):6934-8

Becker AB. and Roth RA. (1990) Insulin receptor structure and function in normal and pathological conditions. *Annual Review of Medicine.* 41:99-115

Beebe SJ, and Corbin JD (1984) Rat adipose tissue cAMP-dependent protein kinase: a unique form of type II. *Molecular and Cellular Endocrinology* 36:67-78

Birnbaumer L, Codina J, Mattera R, Cerione RA, Hildebrandt JD, Sunyer T, Rojas FJ, Caron MG, Lefkowitz RJ, and Iyengar R (1985) Regulation of hormone receptors and adenylyl cyclases by guanine nucleotide binding N proteins. *Recent Progress in Hormone Research* 41:41-99

Blackwell GJ, Carnuccio R, Di Rosa M, Flower RJ, Langham CSJ, Parente L, Persico P, Russell-Smith NC, and Stone D (1982) Glucocorticoids induce the formation and release of anit-inflammatory and anti-phospholipase proteins into the peritoneal cavity of the rat. *British Journal of Pharmacology* 76:185-94

Bliss CI (1967) *Statistics in Biology,* McGraw-Hill Book Co., New York

Bowden CR, White KD, Lewis UJ, and Tutwiler GF (1985) Highly purified human growth hormone fails to stimulate lipolysis in rabbit adipocytes *in vitro* or in rabbits *in vivo. Metabolism: Clinical & Experimental* 34(3):237-43

Breitwiesser GE, and Szabo G (1985) Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature* 317:538-40

Brodie BB, Maickel RP, and Stern DN (1965) *In* Handbook of Physiology, Section 5, pp.583-600

Burger RM, and Lowenstein JM (1970) Preparation and properties of 5'-nucleotidase from smooth muscle of small intestine. *Journal of Biological Chemistry* 245(23):6274-80

Bushfield M, Griffiths SL, Murphy GJ, Pyne NJ, Knowler JT, Milligan G, Parker PJ, Mollner S, and Houslay MD (1990) Diabetes-induced alterations in the expression, functioning and phosphorylation state of the inhibitory guanine nucleotide regulatory protein Gi-2 in hepatocytes. *Biochemical Journal* 271(2):365-72

Bylund DB (1992) Subtypes of α_1 and α_2-adrenergic receptors. *FASEB Journal* 6(3):832-9

Cameron CM, Kostyo JL, Adamafio NA, and Dunbar JC (1987) Metabolic basis for the diabetogenic action of growth hormone in the obese (ob/ob) mouse. *Endocrinology.* 120(4):1568-75

Campbell RM, and Scanes CG (1985) Lipolytic activity of purified pituitary and bacterially derived growth hormone on chicken adipose tissue *in vitro*. *Proceedings of the Society for Experimental Biology & Medicine* 180(3):513-7

Campbell RM, Kostyo JL, and Scanes CG (1990) Lipolytic and antilipolytic effects of human growth hormone, its 20-kilodalton variant, a reduced and carboxymethylated derivative, and human placental lactogen on chicken adipose tissue *in vitro*. *Proceedings of the Society for Experimental Biology & Medicine* 193(4):269-73

Carter-Su C, Stubbart JR, Wang XY, Stred SE, Argetsinger LS, and Shafer JA (1989) Phosphorylation of highly purified growth hormone receptors by a growth hormone receptor-associated tyrosine kinase. *Journal of Biological Chemistry* 264(31):18654-61

Cassel D, and Selinger Z (1977) Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. *Proceedings of the National Academy of Sciences of the United States of America* 74(8):3307-11

Cassel D, and Pfeuffer T (1978) Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proceedings of the National Academy of Sciences of the United States of America* 75(6):2669-73

Chang FH, and Bourne HR (1987) Dexamethasone increases adenylyl cyclase activity and expression of the alpha-subunit of Gs in GH3 cells. *Endocrinology* 121(5):1711-5

Cockcroft S, and Gomperts BD (1985) Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature* 314:534-6

Colosi P, Wong K, Leong SR, and Wood WI (1993) Mutational analysis of the intracellular domain of the human growth hormone receptor. *Journal of Biological Chemistry* 268:12617-12623

Corbin JD, Sugden PH, Lincoln TM, and Keely SL (1977) Compartmentalization of adenosine 3':5'-monophosphate and adenosine 3':5'-monophosphate dependent protein kinase in heart tissue. *Journal of Biological Chemistry* 252:3853-61

Corbin JD (1983) Determination of the cAMP-dependent protein kinase activity ratio in intact tissues. *Methods in Enzymology* 99, 277-232

Correll (1963) Adipose tissue: Ability to responsd to nerve stimulation *in vitro Science* 140:387-8

Correze C, Laudat MH, Laudat P, and Nunez J (1974) Hormone-dependent lipolysis in fat-cells from thyroidectomized rats. *Molecular & Cellular Endocrinology* 1(5):309-27

Cosman D, Lyman SD, Idzerda RL, Beckmann MP, Park LS, Goodwin RG, and March CJ (1990) A new cytokine receptor superfamily. *Trends in Biochemical Sciences* 15(7):265-70

Cote TE, Frey EA, and Sekura RD (1984) Altered activity of the inhibitory guanyl nucleotide-binding component (Ni) induced by pertussis toxin. Uncoupling of Ni from receptor with continued coupling of Ni to the catalytic unit. *Journal of Biological Chemistry*. 259(14):8693-8

Crouch MF, Winegar DA, Lapetina EG (1989) Epinephrine induces changes in the subcellular distribution of the inhibitory GTP-binding protein G_i α-2 and a 38-kDa phosphorylated protein in the human platelet. *Proceedings of the National Academy of Sciences of the United States of America* 86(6):1776-80

Cunningham BC, Ultsch M, De Vos AM, Mulkerrin MG, Clauser KR, and Wells JA (1991) Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. *Science* 254(5033):821-5

Daly JW (1982) Adenosine receptors: Targets for future drugs. *Journal of Medicinal Chemistry* 25(3):197-207

Davidson MB (1987) Effect of growth hormone on carbohydrate and lipid metabolism. *Endocrine Reviews* 8(2):115-31

Dearborn EC, and Skillman JJ (1976) *In* Hormones in Human Blood, HN Antoniades ed. pp 711-719, Harvard University Press, Cambridge MA

Debons AF, and Schwartz IL (1961) Dependence of the lipolytic action of epinephrine *in vitro* upon thyroid hormone. *Journal of Lipid Research* 2:86-9

de Mazancourt P, Lacasa D, Giot J, and Giudicelli Y (1989) Role of adenosine 3',5'-monophosphate and the Ri-receptor Gi-coupled adenylate cyclase inhibitory pathway in the mechanism whereby adrenalectomy increases the adenosine antilipolytic effect in rat fat cells. *Endocrinology* 124(3):1131-9

de Wit RJ, Hekstra D, Jasteroff B, Stec WJ, Baraniak J, Van Driel R, and Van Haastert PJ (1984) Inhibitory action of certain cyclophosphate derivatives of cAMP on cAMP-dependent protein kinases. *European Journal of Biochemistry* 142(2):255-60

Dietz J, and Schwartz J (1991) Growth hormone alters lipolysis and hormone-sensitive lipase activity in 3T3-F442A adipocytes. *Metabolism: Clinical & Experimental* 40(8):800-6

Di Rosa M, Flower RJ, Hirata F, Parente L, and Russo-Marie F (1984) Nomenclature announcement. Anti-phospholipase proteins. *Prostaglandins* 28:441-2

Doglio A, Dani C, Grimaldi P, and Ailhaud G (1989) Growth hormone stimulates c-fos gene expression by means of a protein kinase C without increasing inositol lipid turnover. *Proceedings of the National Academy of Sciences of the United States of America* 86:1148-1152

Dohlman HG, Thorner J, Caron MG, and Lefkowitz RJ (1991) Model systems for the study of seven-transmembrane-segment receptors. *Annual Review of Biochemistry* 60:653-688

Dole VP (1956) A relation between non-esterified fatty acids in plasma and metabolism of glucose. *Journal of Clinical Investigations* 35:150-156

Doris R, Vernon RG, Houslay MD, and Kilgour E (1994) Growth hormone decreases the response to anti-lipolytic agonist and decreases the levels of G_i2 in rat adipocytes. *Biochemical Journal* 297:41-5

Duquette PF, Scanes CG, and Muir LA (1984) Effects of ovine growth hormone and other anterior pituitary hormones on lipolysis of rat and ovine adipose tissue *in vitro*. *Journal of Animal Science* 58(5):1191-7

Edelman AM. Blumenthal DK. and Krebs EG. (1987) Protein serine/threonine kinases. *Annual Review of Biochemistry.* 56:567-613

Eisen HJ, and Goodman HM (1969) Growth hormone and phosphorylase activity in adipose tissue. *Endocrinology* 84:414-416

Ekanger R. Sand TE. Ogreid D. Christoffersen T. and Doskeland SO. (1985) The separate estimation of cAMP intracellularly bound to the regulatory subunits of protein kinase I and II in glucagon-stimulated rat hepatocytes. *Journal of Biological Chemistry.* 260(6):3393-401

Elks ML, Manganiello VC, and Vaughan M (1983) Hormone-sensitive particulate cAMP phosphodiesterase activity in 3T3-L1 adipocytes. Regulation of responsiveness by dexamethasone. *Journal of Biological Chemistry* 258(14):8582-7

Elsair J, Khelfat K, Aoul MT, and Ghouini A (1985) Biphasic action of human growth hormones, of hypophyseal origin or bacterial production, on the lipolysis of rat adipocytes *in vitro. Annales D'Endocrinologie* 46(6):389-97

Erneaux C, Van Sande J, Jasteroff B, and Dumont JE (1986) Modulation of cyclic AMP action in the dog thyroid by its agonist and antagonist Sp- and Rp-adenosine 3',5'-monophosphorothioate. *Biochemical Journal* 234:193-7

Etherton TD, Evock CM, and Kensinger RS (1987) Native and recombinant bovine growth hormone antagonize insulin action in cultured bovine adipose tissue. *Endocrinology* 121(2):699-703

Evans HM, and Long JA (1921) The effect of the anterior lobe of the hypophysis administered intraperitoneally upon growth and the maturity and oestus cycles of the rat. *Anatomical Records* 21:61-3

Exton JH, Friedmann N, Wong EH, Brineaux JP, Corbin JD, and Park CR (1972) Interaction of glucocorticoids with glucagon and epinephrine in the control of gluconeogensis and glycogenolysis in liver and lipolysis in adipose tissue. *Journal of Biological Chemistry* 247:3579-3588

Fain JN, Scow RO, and Chernick SS (1963) Effects of glucocorticoids on metabolism of adipose tissue *in vitro. Journal of Biological Chemistry* 238:54-58

Fain JN (1967) Studies on the role of RNA and protein synthesis in the lipolytic action of growth hormone in isolated fat cells. *Advances in Enzyme Regulation* 5:39-51

Fain JL (1968) Effect of dibutyry-3',5'-AMP, theophylline and norepinephrine on lipolytic action of growth hormone and glucocorticoid in white fat cells. *Endocrinology* 82:825-830

Fain JN (1979) Inhibition of glucose transport in fat cells and activation of lipolysis by glucocorticoids. In Monographs in *Endocrinology* 12:547069

Fain JN, Kovacev VP, and Scow RO (1965) Effect of growth hormone and dexamethasone on lipolysis and metabolism in isolated fat cells of the rat. *Journal of Biological Chemistry* 240(9):3522-3529

Fain JN, and Saperstein R (1970) Involvement of RNA synthesis and cyclic AMP in the activation of fat *Cell* lipolysis by growth hormone and glucocorticoids. *In: Adipose Tissue, Regulation and Metabolic Functions.* Jeanrenaud B, and Hepp D. (eds) Academic Press, New York, pp.20-27

Fain JN, Dodd A, and Novak L (1971) Enzyme regulation in gluconeogenesis and lipogenesis. Relationship of protein synthesis and cyclic AMP to lipolytic action of growth hormone and glucocorticoids. *Metabolism: Clinical & Experimental* 20(2):109-18

Fain JN. Li SY. and Moreno FJ. (1979) Regulation of cyclic AMP metabolism and lipolysis in isolated rat fat cells by insulin, N6-(phenylisopropyl) adenosine and 2',5'-dideoxyadenosine. *Journal of Cyclic Nucleotide Research.* 5(3):189-96

Fain JN, Garcia-Sainz JA, Mills I, and O'Donnell CJ (1984) Activation of lipolysis and cyclic AMP accumulation in rabbit adipocytes by isoproterenol in the presence of forskolin or pertussis toxin. *Biochimica et Biophysica Acta* 798(3):382-9

Federman AD, Conklin BR, Schrader KA, Reed RR, and Bourne HR. (1992) Hormonal stimulation of adenylyl cyclase through Gi-protein βγ subunits. *Nature* 356(6365):159-61

Fielder PJ, and Talamantes F (1987) The lipolytic effects of mouse placental lactogen II, mouse prolactin, and mouse growth hormone on adipose tissue from virgin and pregnant mice. *Endocrinology* 121(2):493-7

Flower RJ, Gryglewski R, Herbacyznska-Cedro K, and Vane JR (1972) The effects of anit-inflammatory drugs on prostaglandin biosynthesis. *Nature* 238:104-6

Flower RJ, and Blackwell GJ (1979) Anti-inflammatory steroids induce biosynthesis of a phsopholipase A_2 inhibitor which prevents prostaglandin generation. *Nature* 278:456-9

Foster CM, Shafer JA, Rozsa FW, Wang X, Lewis SD, Renken DA, Natale JE, Schwartz J, and Carter-Su C. (1988) Growth hormone promoted tyrosyl phosphorylation of growth hormone receptors in murine 3T3-F442A fibroblast and adipocytes. *Biochemistry* 27:326-34

Fredholm BB (1970) Studies on the sympathetic regulation of circulation and metabolism in isolated canine subcutaneous adipose tissue. *Acta Physiologica Scandinavica* Supplement. 354:1-47

Friedman N, Exton JH, and Park CR (1967) Interaction of adrenal steroids and glucagon on gluconeogenesis in perfused rat liver. *Biochmical and Biophysical Research Communications* 29:113-119

Frigeri LG, Robel G, and Stebbing N (1982) Bacteria-derived human growth hormone lacks lipolytic activity in rat adipose tissue. *Biochemical & Biophysical Research Communications* 104(3):1041-6

Garty NB, Galiani D, Aharonheim A, Ho YK, Phillips DM, Dekel N, and Salomon Y. (1988) G-proteins in mammalian gametes: an immunocytochemical study. *Journal of Cell Science* 91 (Pt 1):21-31

Gilman AG (1987) G proteins: transducers of receptor-generated signals. *Annual Review of Biochemistry* 56:615-49

Glick SM, Roth J, Yalow RS, and Berson SA (1965) Regulation of growth hormone secretion. *Recent Progress in Hormone Research* 21:241-283

Goodman HM (1965) Early and late effects of growth hormone on the metabolism of glucose in adopise tissue. *Endocrinology* 76:1134-40

Goodman HM (1968) Growth hormone and the metabolism of carbohydrate and lipid in adipose tissue. *Annals of the New York Academy of Sciences* 148(2):419-40

Goodman HM (1968a) Multiple effects of growth hormone on lipolysis. *Endocrinology* 83:300-308

Goodman HM (1968b) Effects of growth hormone on the lipolytic response of adipose tissue to theophylline. *Endocrinology* 81:1027-1034

Goodman HM (1970) Permissive effects of hormones on lypolysis. *Endocrinology* 86(5):1064-74

Goodman HM (1981) Separation of early and late responses of adipose tissue to growth hormone. *Endocrinology* 109(1):120-9

Goodman HM (1984) Biological activity of bacterial derived human growth hormone in adipose tissue of hypophysectomized rats. *Endocrinology* 114(1):131-5

Goodman HM, and Schwartz J (1974) *In* Handbook of Physiology- *Endocrinology* IV, part 2. pp.211-231

Goodman HM, and Knobil E (1959) Effects of fasting and of growth hormone on plasma fatty acids concentration in normal and hypophysectomized rhesus monkeys. *Endocrinology* 65:451-458

Goodman HM, Gorin E, and Honeyman TW (1988) Biochemical basis for the lipolytic activity of growth hormone. *In: Human Growth Hormone, Progress and Challenges* Underwood LE (ed) Marcel Dekker Inc., New York and Basel, pp.75-111

Gopinath R, and Etherton TD (1989) Effects of porcine growth hormone on glucose metabolism of pigs: II. Glucose tolerance, peripheral tissue insulin sensitivity and glucose kinetics. *Journal of Animal Science* 67(3):689-97

Gordon RS, and Cherkes A (1956) Unesterified fatty acids in human blood plasma. *Journal of Clinical Investigatons* 35:206-212

Gorin E, Tai LR, Honeyman TW, and Goodman HM (1990) Evidence for a role of protein kinase C in the stimulation of lipolysis by growth hormone and isoproterenol. *Endocrinology* 126(6):2973-2982

Granner D, Chase LR, Aurbach GD, and Tomkins GM (1968) Tyrosine aminotransferase: Enzyme induction independent of adenosine 3',5'-monophosphate. *Science* 162:1018-1020

Granner DK, Sellers L, Lee A, Butters C, and Kutine L (1975) A comparison of the uptake, metabolism, and action of cyclic adenine nucleotides in cultured hepatoma cells. *Archives Biochimica and Biophysica* 169:601-15

Granner DK, Lee A, and Thompson EB (1977) The interaction of glucocorticoid hormones and cyclic nucleotides in the induction of tyrosine aminotransferase in cultured hepatoma cells. *Journal of Biological Chemistry* 252:3891-7

Granner DK (1979) The role of glucorticoid hormones as biological amplifiers. In Monographs in *Endocrinology* 12:593-611

Green A, Johnson JL, and Milligan G (1990) Down-regulation of Gi sub-types by prolonged incubation of adipocytes with an A_1 adenosine receptor agonist. *Journal of Biological Chemistry* 265(9):5206-10

Green A, Milligan G, and Dobias SB. (1992) Gi down-regulation as a mechanism for heterologous desensitization in adipocytes. *Journal of Biological Chemistry* 267(5):3223-9

Greenbaum AL (1953) Changes in body composition and respiratory quotient of normal female rats treated with purified growth hormone. *Biochemical Journal* 54:400-407

Grichting G, and Goodman HM (1983) Growth hormone and lipolysis: a reevaluation. *Endocrinology* 113:1697-1702

Gurland G, Ashcom G, Cochran BH, and Schwartz J (1990) Rapid events in growth hormone action. Induction of c-myc and c-fos transciption in 3T3-F442A preadipocytes. *Endocrinology* 127:3187-95

Hadcock JR, Port JD, and Malbon CC (1991) Cross-regulation between G-protein-mediated pathways. *Journal of Biological Chemistry* 266(18):11915-11922

Hallstrom IP, Gustafsson JA, and Blanck A (1989) Effects of growth hormone on the expression of c-myc and c-fos during early stages of sex-differentiated rat liver carcinogenesis in the resistant hepatocyte model. *Carcinogenesis* 10:2339-2343

Hanks SK, Quinn AM, and Hunter T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* 241(4861):42-52

Haraguchi K, and Rodbell M (1990) Isoproterenol stimulated shift of G proteins from plasma membranes to pinocytotic vesicles in rat adipocytes: A possible means of signal dissemination. *Proceedings of the National Academy of Sciences of the United States of America* 87:1208-1212

Harpur AG, Andres AC, Ziemiecki A, Aston RR, and Wilks AF (1992) JAK2, a third member of the JAK family of protein tyrosine kinases. *Oncogene* 7:1347-1353

Harris R, and Bennun A (1976) Hormonal control of fat cells adenylate cyclase. *Molecular Cellular Biochemistry* 13(3):141-146

Hart IC, Chadwick PM, Boone TC, Langley KE, Rudman C, and Souza LM (1984) A comparison of the growth-promoting, lipolytic, diabetogenic and immunological properties of pituitary and recombinant-DNA-derived bovine growth hormone (somatotropin). *Biochemical Journal* 224(1):93-100

Havel RJ (1968) The autonomic nervous system and intermediary carbohydrate and fat metabolism. *Anesthesiology* 29(4):702-13

Henderson R, and Unwin PN (1975) Three-dimensional model of purple membrane obtained by electron microscopy. *Nature* 257(5521):28-32

Hoffmann F, Beavo JA, Bechtel PJ, and Krebs EG (1975) Comparison of adenosine 3':5'-monophosphate-dependent protein kinases from rabbit skeletal and bovine heart muscle. *Journal of Biological Chemistry* 250(19):7795-801

Hollenga CH, and Zaagsma J (1989) Direct evidence for the atypical nature of β-adrenoceptorsin rat adipocytes. *British Journal of Pharmacology* 98:1420-4

Hollenga C, Brouwer F, and Zaagsma J (1991) Differences in functional cyclic AMP compartments mediating lipolysis by isoprenaline and BRL 37344 and fopur adipocyte types. *European Journal of Pharmacology* 200:325-330

Honeyman TW, Levy LK, and Goodman HM (1979) Independent regulation of phosphorylase and lipolysis in adipose tissue *American Journal of Physiology* 237(1):E11-E17

Honnor RC, Dhillon GS, and Londos C (1985) cAMP-dependent proetin kinase and lipolysis in rat adipocytes. *Journal of Biological Chemistry* 260(28):15139-15145

Hotani H, and Miyamoto H (1990) Dynamic features of microtubules as visualized by dark-field microscopy. *Advances in Biophysics* 26:135-56

Ingle DJ (1952) The role of the adrenal cortex in homeostasis. *Journal of Endocrinology* 8:xxiii-xxxviii

Insel PA, and Kennedy MS (1978) Colchicine potentiates beta-adrenoreceptor-stimulated cyclic AMP in lymphoma cells by an action distal to the receptor. *Nature* 273(5662):471-3

Isaksson OGP, Lindhal A, Nilsson A, and Isgaard J (1987) Mechanism of the stumulatory effect of growth hormone on longitudinal bone growth. *Endocrine Reviews* 8:426-38

Jeanrenaud B (1967) Effect of glucocorticoid hormones on fatty acid mobilization and re-esterification in rat adipose tissue. *Biochemical Journal* 103(3):627-33

Jelsema CL, and Axelrod J (1987) Stimulation of phospholipase A2 activity in bovine rod outer segments by the beta gamma subunits of transducin and its inhibition by the alpha subunit. *Proceedings of the National Academy of Sciences of the United States of America.* 84(11):3623-7

Jones TLZ, Simonds WF, Merendino JJ Jr, Brann MR, and Spiegel AM (1990) Myristoylation of an inhibitory GTP-binding protein α subunit is essential for its membrane attachment. *Proceedings of the National Academy of Sciences of the United States of America* 87:568-72

Jungas RL, and Ball EG (1962) Studies on the metabolism of adipose tissue: The stimulation of oxygen consumption by TSH preparations in relation to growth hormone and other pituitary fractions. *Endocrinology* 71:68-76

Karnieli E, Zarnowski MJ, Hissin PJ, Simpson IA, Salans LB, Cushman SW (1981) Insulin-stimulated translocation of glucose transport systems in the isolated rat adipose cell. Time course, reversal, insulin concentration dependency, and relationship to glucose transport activity. *Journal of Biological Chemistry* 256(10):4772-7

Katada T, Gilman AG, Watanabe Y, Bauer S, and Jakobs KH (1985) Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *European Journal of Biochemistry* 151(2):431-7

Katada T, Oinuma M, and Ui M (1986) Two guanine nucleotide-binding proteins in rat brain serving as the specific substrate of islet-activating protein, pertussis toxin, Interaction of the α subunt with βγ-subunits in development of their biological activities. *Journal of Biological Chemistry* 261:8182091

Kather H, Bieger W, Michel G, Aktories K, and Jakobs KH (1985) Human fat cells is primarily regulated by inhibitory modulators acting through distinct mechanisms. *Journal of Clinical Investigations* 76:1559-1565

Kennedy MS, and Insel PA (1979) Inhibitors of microtubule assembly enhance beta-adrenergic and prostaglandin E1-stimulated cyclic AMP accumulation in S49 lymphoma cells. *Molecular Pharmacology* 16(1):215-23

Kim D, Lewis DL, Graziadei L, Neer EJ, Bar-Sagi D, and Clapham DE (1989) G-protein βγ-subunits activate the cardiac muscarinic K+-channel via phospholipase A_2. *Nature* 337(6207):557-60

Klainer LM, Chi Ym, Freidberg SL, Rall TW, and Sutherland EW (1962) Adenyl Cyclase: The effects of neurohormones on the formation of adenosine 3',5'-phosphate by preparations from brain and other tissues. *Journal of Biological Chemistry* 237:1239-43

Knobil E, and Greep RO (1959) The physiology of growth hormone with particular reference to its action in the rhesus monkey and the "species specificity" problem. *Recent Progress in Hormone Research* 15:1-69

Kostyo JL and Nutting DF (1974) *In* Handbook of Physiology (E. Knobil and WH Sawyer, eds.) Sect. 7, Vol. 4, pp 187-210. American Physiological Society, Washington D.C.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685

Lamberts SWJ, Timmermans HAT, Kramer-Blankenstijn M, and Birkenhager JC (1975) The mechanism of the potentiating effect of glucocorticoids on catecholamine-induced lipolysis. *Metabolism* 24:681-689

Larsen TS, and Nilssen KJ (1985) On the hormonal regulation of lipolysis in isolated reindeer adipocytes. *Acta Physiologica Scandinavica* 125(3):547-52

Lee MO, and Schaffer NK (1934) Anterior pituitary growth hormone and the composition of growth. *Journal of Nutrition* 7:337-363

Levis MJ, and Bourne HR (1992) Activation of the α subunit of Gs in intact cells alters its abundance, rate of degradation, and membrane avidity. *Journal of Cell Biology* 119:1297-1307

Lewis GP. and Piper PJ. (1975) Inhibition of release of prostaglandins as an explanation of some of the actions of anti-inflammatory corticosteroids. *Nature.* 254(5498):308-11

Lewis KJ, Molan PC, Bass JJ, and Gluckman PD (1988) The lipolytic activity of low concentrations of insulin-like growth factors in ovine adipose tissue. *Endocrinology* 122(6):2554-7

Linder ME, Pang IH, Duronio RJ, Gordon JI, Sternweis PC, and Gilman AG (1991) Lipid modification of G protein subunits. Myristoylation of Goα increases its affinity for βγ. *Journal of Biological Chemistry* 266:4654-9

Linder ME, Middleton P, Hepler JR, Taussig R, Gilman AG, and Mumby SM (1993) Lipid modification of G proteins: α subunits are palmitoylated. *Proceedings of the National Academy of Sciences of the United States of America* 90:3675-9

Lis M, Gilardeau C, and Chretien M (1972) Fat *Cell* adenylate cyclase activation by sheep-lipotropic hormone. *Proceedings of the Society for Experimental Biology & Medicine* 139(2):680-3

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the folin phenol reagent *Journal of Biological Chemistry* 239:265-275

Magri KA, Adamo M, Leroith D, and Etherton TD (1990) The inhibition of insulin action and glucose metabolism by porcine growth hormone in porcine adipocytes is not the result of any decrease in insulin binding or insulin receptor kinase activity. *Biochemical Journal* 266(1):107-13

Maloff BL, Levine JH, and Lockwood DH (1980) Direct effects of growth hormone on insulin action in rat adipose tissue maintained *in vitro*. *Endocrinology* 107(2):538-44

Mandelkow EM, and Mandelkow E (1985) Unstained microtubules studied by cryo-electron microscopy. Substructure, supertwist and disassembly. *Journal of Molecular Biology* 181(1):123-35

Manganiello VC. Murad F. and Vaughan M. (1971) Effects of lipolytic and antilipolytic agents on cyclic 3',5'-adenosine monophosphate in fat cells. *Journal of Biological Chemistry.* 246(7):2195-202

Manganiello VC. Degerman E. Smith CJ. Vasta V. Tornqvist H. and Belfrage P. (1992) Mechanisms for activation of the rat adipocyte particulate cyclic-GMP-inhibited cyclic AMP phosphodiesterase and its importance in the antilipolytic action of insulin. *Advances in Second Messenger & Phosphoprotein Research.* 25:147-64

Manning DR, Fraser BA, Khan RA, and Gilman AG (1984) ADP-ribosylation of transducin by islet-activating protein. Identification of asparagine as the site of ADP-ribosylation. *Journal of Biological Chemistry* 259:749-56

Margolis S, and Vaughan M (1962) α-Glycerol phosphate synthesis and breakdown in homogenates of adipose tissue. *Journal of Biological Chemistry* 237:44-48

Martin GA, Yatani A, Clark R, Conroy L, Polakis P, Brown AM, and McCormick F (1992) GAP domains responsible for ras p21-dependent inhibition of muscarinic atrial K+ channel currents. *Science* 255(5041):192-4

McDowell GH, Hart IC, Bines JA, Lindsay DB, and Kirby AC (1987) Effects of pituitary-derived bovine growth hormone on production parameters and biokinetics of key metabolites in lactating dairy cows at peak and mid-lactation. *Australian Journal of Biological Sciences* 40(2):191-202

McKeel DW, and Jarett L (1970) Preparation and characterization of plasma membrane fraction from isolated fat cells. *Journal of Cell Biology* 44:417-432

McKenzie FR, and Milligan G. (1990) Delta-opioid-receptor-mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding protein Gi2. *Biochemical Journal* 267(2):391-8

Meyer DJ, Campbell GS, Cochran BH, Argetsinger LS, Larner AC, Finbloom DS, Carter-Su C, and Schwartz J. (1994) Growth hormone induces a DNA binding factor related to the interferon-stimulated 91-kDa transcription factor. *Journal of Biological Chemistry* 269(7):4701-4

Milligan G (1988) Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. *Biochemical Journal* 255(1):1-13

Milligan G (1993) Agonist regulation of cellular G protein levels and distribution: Mechanisms and functional implications. *Trends in Pharmacological Sciences* 14:413-18

Milligan G, Spiegel AM, Unson CG, and Saggerson ED (1987) Chemically induced hypothyroidism produces elevated amounts of the alpha subunit of the inhibitory guanine nucleotide binding protein (Gi) and the beta subunit common to all G-proteins. *Biochemical Journal* 247(1):223-7

Milligan G, and Unson CG (1989) Persistent activation of the α subunit of Gs promotes its removal from the plasma membrane. *Biochemical Journal* 261:837-41

Milligan G, and Green A (1991) Agonist control of G protein levels. *Trends in Pharmacological Sciences* 12:207-9

Milligan G, and Green A (1993) *In* Regulation of cellular transduction pathways by desensitization and amplification. *Molecular Pharmacology of Cell Regulation.*
vol. 3 (Sibley DR and Houslay MD, eds.), pp234-7, John Wiley & Sons.

Mitchell FM, Griffiths SL, Saggerson ED, Houslay MD, Knowler JT, and Milligan G (1989) Guanine-nucleotide-binding proteins expressed in rat white adipose tissue. Identification of both mRNAs and proteins corresponding to G_i1, G_i2, and G_i3. *Biochemical Journal* 262(2):403-8

Mitchell FM, Buckley NJ, and Milligan G (1993) Enhanced degradation of the phosphoinositidase C-linked guanine-nucleotide-binding protein Gqα/G11α following activation of the human Mi muscarinic acetylcholine receptor expressed in CHO cells. *Biochemical Journal* 293:495-9

Moreno FJ. Mills I. Garcia-Sainz JA. and Fain JN. (1983) Effects of pertussis toxin treatment on the metabolism of rat adipocytes. *Journal of Biological Chemistry.* 258(18):10938-43

Morikawa M, Nixon T, and Green H (1982) Growth hormone and the adipose conversion of 3T3 cells. *Cell* 29(3):783-9

Moskowitz J, and Fain JL (1969) Hormonal regulation of lipolysis and phosphorylase activity in human fat cells. *Journal of Clinical Investigations* 48:1802-1808

Moskowitz J, and Fain JL (1970) Stimulation by growth hormone and dexamethasone of labeled cyclic adenosine 3',5'-monophosphate accumulation by white fat cells. *Journal of Biological Chemistry* 245(5):1101-1107

Moss J, and Vaughan M (1988) ADP-ribosylation of guanyl nucleotide-binding regulatory proteins by bacterial toxins. *Advances in Enzymology & Related Areas of Molecular Biology* 61:303-79

Moxham CM, Hod Y, and Malbon CC (1993) Gi alpha 2 mediates the inhibitory regulation of adenylylcyclase *in vivo*: analysis in transgenic mice with Gi alpha 2 suppressed by inducible antisense RNA. *Developmental Genetics* 14(4):266-73

Mumby SM, Heuckeroth RO, Gordon JI, and Gilman AG (1990) G protein α subunit expression, myristoylation, and membrane association in COS cells. *Proceedings of the National Academy of Sciences of the United States of America* 87:728-32

Mumby SM, Kleuss C, and Gilman AG (1994) Receptor regulation of G protein palmitoylation. *Proceedings of the National Academy of Sciences of the United States of America* 91:2800-4

Murayama T, and Ui M (1983) Loss of the inhibitory function of the guanine nucleotide regulatory component of adenylate cyclase due to its ADP ribosylation by islet-activating protein, pertussis toxin, in adipocyte membranes. *Journal of Biological Chemistry* 258(5):3319-26

Murphy LJ, Bell GI, and Freisen HG (1987) Growth hormone stimulates sequential induction of c-myc and insulin-like growth factor I expression *in vivo*. *Endocrinology* 120:180601812

Neer EJ, and Clapham DE (1988) Roles of G protein subunits in transmembrane signalling. *Nature* 333(6169):129-34

Nixon T, and Green H (1984) Contribution of growth hormone to the adipogenic activity of serum. *Endocrinology*. 114(2):527-32

Nyberg G, and Smith U (1977) Human adipose tissue in culture. VII. The long-term effect on growth hormone. *Hormone & Metabolic Research* 9(1):22-7

Nyberg G, Bostrom S, Johansson R, and Smith U (1980) Reduced glucose incorporation to triglycerides following chronic exposure of human fat cells to growth hormone. *Acta Endocrinologica* 95(1):129-33

Okuda H, Morimoto C, and Tsujita T (1992) Relationship between cyclic AMP production and lipolysis induced by forskolin in rat fat cells. *Journal of Lipid Research* 33:225-231

Olansky L, Meyers GA, Pohl SL, and Hewlett EL (1983) Promotion of lipolysis in rat adipocytes by pertussis toxin: reversal of endogenous inhibition. *Proceedings of the National Academy of Sciences of the United States of America* 80:6547-51

Ostman J, Arner P, Engfeldt P, and Kager L (1979) Regional differences in the control of lipolysis in human adipose tissue. *Metabolism: Clinical & Experimental* 28(12):1198-205

Parker-Botelho LH, Webster LC, Rothermel JD, Baraniak J, and Stec WJ (1988) Inhibition of cAMP-dependent protein kinase by adenosine cyclic 3′-5′-phosphorothioate, a second cAMP antagonist. *Journal of Biological Chemistry* 263:5301-5

Parker J, Lane J, and Axelrod L (1989) Cooperation of adipocytes and endothelial cells required for catecholamine stimulation of PGI_2 production by rat adipose tissue. *Diabetes* 38:1123-32

Pereira ME, Segaloff DL, Ascoli M, and Eckstein F (1987) Inhibition of choriogonadotropin-activated steroidogenesis in cultured Leydig tumor cells by the Rp diastereoisomer of adenosine 3',5'-cyclic phosphorothioate. *Journal of Biological Chemistry* 262(13):6093-100

Peters JP (1986) Consequences of accelerated gain and growth hormone administration for lipid metabolism in growing beef steers. *Journal of Nutrition* 116(12):2490-503

Peters RA (1956) Hormones and the cytoskeleton. *Nature* 177:426-8

Pierluissi J, and Campbell J (1980) Metasomatotrophic diabetes and its induction: basal insulin secretion and insulin release responses to glucose, glucagon, arginine and meals. *Diabetologia* 18(3):223-8

Plas C, and Nunez J (1976) Role of cortisol on the glycogenolytic effect of glucagon and on the glycogenic response to insulin in fetal hepatocyte culture. *Journal of Biological Chemistry* 251:1431-37

Pohl SL (1981) Cyclic Nucleotides and lipolysis. *Internation Journal of Obesity* 5:627-633

Press M (1988) Growth hormone and metabolism. *Diabetes-Metabolism Reviews* 4(4):391-414

Pyne NJ, Murphy GJ, Milligan G, and Houslay MD (1989) Treatment of intact hepatocytes with either the phorbol ester TPA or glucagon elicits the phosphorylation and functional inactivation of the inhibitory guanine nucleotide regulatory protein Gi. *FEBS Letters* 243(1):77-82

Raben MS, and Hollenberg CH (1959) Effects of growth hormone on plasma fatty acids. *Journal of Clinical Investigations* 38:484-488

Raben MA, and Hollenberg CH (1959a) Uptake and release of fatty acids by adipose tissue. *Journal of Clinical Investigations* 38:1032

Ramkumar V, and Stiles GL (1990) *In vivo* pertussis toxin administration: effects on the function and levels of Gi alpha proteins and their messenger ribonucleic acids. *Endocrinology* 126(2):1295-304

Rangel-Aldao R, and Rosen OM (1977) Effect of cAMP and ATP on the reassociation of phosphorylated and nonphosphorylated subunits of the cAMP-dependent protein kinase from bovine cardiac muscle. *Journal of Biological Chemistry* 252(20):7140-5

Rannels SR, and Corbin JD (1981) Studies on the function of the two intrachain cAMP binding sites of protein kinase. *Journal of Biological Chemistry* 256(15):7871-7876

Rapiejko PJ, Watkins DC, Ros M, and Malbon CC (1989) Thyroid hormones regulate G-protein β-subunit mRNA expression *in vivo*. *Journal of Biological Chemistry* 264(27):16183-9

Rasenick MM, Wang N, and Yan K (1990) Specific associations between tubulin and G proteins: Participation of cytoskeletal elements in cellular signal transduction. *In: The Biology and Medicine of Signal Transduction* Nishizuka Y (ed) Raven Press, New York, pp. 381-386

Reshef L, and Shapiro B (1960) Effect of epinephrine, cortisone and growth hormone on release of unesterified fatty acids by adipose tissue *in vitro*. *Metabolism: Clinical and Experimental* 9:551-5

Richelsen B, Borglum JD, and Sorensen SS (1992) Biosynthetic capacity and regulatory aspects of prostaglandin E_2 formation in adipocytes. *Molecular and Cellular Endocrinology* 85:73-81

Rizack MA (1964) Activation of an epinephrine-sensitive lipolytic activity from adipose tissue by adenosine 3',5'-phosphate *Journal of Biological Chemistry* 239:392-5

Roach PJ. (1991) Multisite and hierarchal protein phosphorylation. *Journal of Biological Chemistry*. 266(22):14139-42

Robinson GA, Butcher rW, and Sutherland EW (1971) Lipolysis in Adipose Tissue. *In Cyclic AMP* Academic Press, pp.285-316

Robishaw JD, Smigel MD, and Gilman AG (1986) Molecular basis for two forms of the G protein that stimulates adenylate cyclase. *Journal of Biological Chemistry*. 261(21):9587-90

Robison GA, Butcher RW, and Sutherland EW (1971) Lipoysis in adipose tissue. *In Cyclic AMP*. Academic Press, New York, pp.283-316

Rodbell M (1964) Metabolism of isolated fat cells. *Journal of Biological Chemistry* 239(2):375-380

Rodbell M, Birnbaumer L, Pohl SL, and Krans HMJ (1971) The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanyl nucleotides in glucagon action. *Journal of Biological Chemistry* 246:1877-82

Rodbell M (1980) The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* 284(5751):17-22

Ros M, Northup JK, and Malbon CC (1988) Steady-state levels of G-proteins and beta-adrenergic receptors in rat fat cells. Permissive effects of thyroid hormones. *Journal of Biological Chemistry* 263(9):4362-8

Ros M, Watkins DC, Rapiejko PJ, and Malbon CC (1989a) Glucocorticoids modulate mRNA levels for G-protein beta-subunits. *Biochemical Journal* 260(1):271-5

Ros M, Northup JK, and Malbon CC (1989b) Adipocyte G-protein and adenylate cyclase: Affects of adrenalectomy. *Biochemical Journal* 257:737-744

Rosen OM, and Erlichman J (1975) Reversible autophosphorylation of a cyclic 3',5'-AMP-dependent protein kinase from bovine cardiac muscle. *Journal of Biological Chemistry*. 250(19):7788-94

Rosenbaum M, Gertner JM, and Leibel RL (1989) Effects of systemic growth hormone (GH) administration on regional adipose tissue distribution and metabolism in GH-deficient children. *Journal of Clinical Endocrinology & Metabolism* 69(6):1274-81

Roskowski Jr R (1983) Assays of protein kinase. *Methods in Enzymology* 99:3-6

Rothermel JD, Stec WJ, Baraniak J, Jasteroff B, and Parker Botelho LH (1983) Inhibition of glycogenolysis in isolated rat hepatocytes by the Rp diastereomer of adenosine cyclic 3',5'-phosphorothioate. *Journal of Biological Chemistry* 258, 12125-12128

Rotrosen D, Gallin JI, Spiegel AM, and Malech HL (1988) Subcellular localization of Gi alpha in human neutrophils. *Journal of Biological Chemistry* 263(22):10958-64

Roupas P, Chou SY, Towns RJ, and Kostyo JL (1991) Growth hormone inhibits activation of phosphoinositol phospholipase C in adipose plasma membranes: Evidence for a growth hormone-induced change in G protein function. *Proceedings of the National Academy of Sciences of the United States of America* 88:1691-1695

Rousseau GG, and Wérenne J (1976) Possible mechanisms for the permissive action of glucocorticoid hormones. Studies on cyclic AMP-dependent protein kinase activity of rat liver and mouse L1210 cells. *Journal of Steriod Biochemistry* 7:1131-4

Rudman D, Feller AG, Nagraj HS, Gergans GA, Lalitha PY, Goldberg AF, Schlenker RA, Cohn L, Rudman IW, and Mattson DE (1990) Effects of human growth hormone in men over 60 years old. *New England Journal of Medicine* 323(1):1-6

Sagi-Eisenberg R (1989) GTP-binding proteins as possible targets for protein kinase C action. *Trends in Biochemical Sciences* 14(9):355-7

Salomon Y, Londos C, and Rodbell M (1974) A highly sensitive adenylate cyclase assay. *Analytical Biochemistry* 58:541-48

Schaeffer LD, Chenoweth M and Dunn A (1969) Adrenal corticosteroid involvement in the control of liver glycogen phosphorylase activity. *Biochimica et Biophysica Acta* 192:292-303

Schimmel RJ (1974) Responses of adipose tissue to sequential lipolytic stimuli. *Endocrinology* 94:1372-1380

Schimmel RJ (1984) Stimulation of cAMP accumulation and lipolysis in hamster adipocytes with forskolin. *American Journal of Physiology* 246:C63-C68

Schonhofer PS, Skidmore IF, Paul MI, Ditzion BR, Pauk GL, and Krishna G (1972) Effects of glucocorticoids on adenyl cyclase and phosphodiesterase activity in fat cell homogenates and the accumulation of cyclic AMP in intact fat cells. *Naunyn-Schmiedebergs Archiv fur Pharmakologie* 273(3):267-82

Scow RO, and Chernick SS (1970) Mobilization, transport, and utilization of free fatty acids. *In Comprehensive Biochemistry*, Vol. 18, Lipid Metabolism, M. Florkin and EH Stoltz eds., Elsevier, Amsterdam

Seamon KB, Padgett W, and Daly JW (1981) Forskolin: unique activator of adenylate cyclase in membrane and in intact cells. *Proceedings of the National Academy of Sciences of the United States of America* 78(6):3363-7

Sengupta K, Long KL, and Allen DO (1981) Growth hormone stimulation of lipolysis and cyclic AMP levels in perifused fat cells. *Journal of Pharmacology and Experimental Therapeutics* 216:15-19

Silbert S, Michel T, Lee R, and Neer EJ (1990) Differential degradation rates of the G protein alpha o in cultured cardiac and pituitary cells. *Journal of Biological Chemistry* 265(6):3102-5

Simon MI, Strathmann MP, and Gautam N (1991) Diversity of G proteins in signal transduction. *Science* 252(5007):802-8

Simonds WF, Goldsmith PK, Codina J, Unson CG, and Spiegel AM (1989) Gi2 mediates α_2-adrenergic inhibition of adenylyl cyclase in platelet membranes: in situ identification with Gα C-terminal antibodies. *Proceedings of the National Academy of Sciences of the United States of America* 86(20):7809-13

Sinnett-Smith PA, and Woolliams JA (1989) Antilipogenic but not lipolytic effects of recombinant DNA-derived bovine somatotropin treatment on ovine adipose tissue; variation with genetic type. *International Journal of Biochemistry* 21(5):535-40

Slootweg MC, Van Genesen ST, Otte A, Duursma SA, and Kruijer W (1990) Activation of mouse osteoblast growth hormone receptor: c-fos oncogene expression independent of phosphoinositide breakdown and cAMP. *Journal of Molecular Endocrinology* 4:265-274

Smal J, and De Meyts P (1989) Sphingosine, an inhibitor of protein kinase C, supresses the insulin-like effects of growth hormone in rat adipocytes. *Proceedings of the National Academy of Sciences of the United States of America* 86:4705-4709

Snedecor GW, and Cochran WG (1989) *Statistical Methods*, Iowa State University Press, Ames

Stalmans W, and Laloux M (1979) Glucocorticoids and hepatic glycogen metabolism. *In* Glucocorticoid hormone action. *Monographs in Endocrinology* 12:517-534

Steinberg D (1976) Interconvertible enzymes in adipose tissue regulated by cyclic AMP-dependent protein kinase. *Advances in Cyclic Nucleotide Research* 7:157-198

Steinberg D, Shafrir E, and Vaughan M (1959) Direct effect of glucagon on realse of unesterified fatty acids (UFA) from adipose tissue. *Clinical Research* 7:220

Steinberg D, and Vaughan M (1965) Release of free fatty acids from adipose tissue *in vitro* in relation to rates of triglyceride synthesis and degradation. *In Handbook of Physiology, Adipose Tissue,* (Renold Ae and Cahill FG Jr., eds.) Washington DC, pp.335-37

Steinberg D, and Huttumen JK (1972) The role of cyclic AMP in activation of hormone-sensitive lipase of adipose. *Advances in Cyclic Nucleotide Research 1:47-62*

Sternweis PC. (1986) The purified alpha subunits of Go and Gi from bovine brain require beta gamma for association with phospholipid vesicles. *Journal of Biological Chemistry.* 261(2):631-7

Sternweis PC, and Robishaw JD (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *Journal of Biological Chemistry* 259(2):13806-13

Strassheim D, Milligan G, and Houslay MD (1990) Diabetes abolishes the GTP-dependent, but not the receptor-dependent inhibitory function of the inhibitory guanine-nucleotide-binding regulatory protein (Gi) on adipocyte adenylate cyclase activity. *Biochemical Journal* 266(2):521-6

Strassheim D, Palmer T, Milligan G, and Houslay MD (1991) Alterations in G-protein expression and the hormonal regulation of adenylate cyclase in the adipocytes of obese (fa/fa) Zucker rats. *Biochemical Journal* 276 (Pt 1):197-202

Sutherland EW, and Rall TW (1960) The relation of adenosine 3',5'-phosphate and phosphorylase to the actions of catecholamines and other hormones. *Pharmacological Reviews* 12:265-99

Sutherland EW, Øye I, and Butcher RW (1965) The action of epinephrine and the role of the adenyl cyclase system in hormone action. *Recent Progress in Hormone Research* 21:623-46

Tang WJ, and Gilman AG (1991) Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. *Science* 254(5037):1500-3

Taussig R, Iñiguez-Lluhi J, and Gilman AG (1993) Inhibition of adenylyl cyclase by Giα. *Science* 216:218-21

Taylor SS, Buechler JA, Yonemoto W (1990) cAMP-Dependent protein kinase: Framework for a diverse family of regulatory enzymes. *Annual Review of Biochemistry* 59:971-1005

Tolkovsky AM, and Levitzki A (1978) Mode of coupling between the β-adrenergic receptor and adenylate cyclase in turkey erythrocytes. *Biochemistry* 17:3795-810

Towbin H, Stachelin T, and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* 76:4350-4354

Ukena D, Schudt C, and Sybrecht GW (1993) Adenosine receptor-blocking xanthines as inhibitors of phosphodiesterase isozymes. *Biochemical Pharmacology* 45:847-51

Van Dop C, Yamanaka G, Steinberg F, Sekura RD, Manclark CR, Stryer L, and bourne HR (1984) ADP-ribosylation of transducin by pertussis toxin blocks the light-stimulated hydrolysis of GTP and cGMP in retinal photoreceptors. *Journal of Biological Chemistry* 259:23-26

Vaughan M (1960) Effect of hormones on phosphorylase activity in adipose tissue. *Journal of Biological Chemistry* 235:3049-53

Vaughan M and Steinberg D. (1963) Effect of hormones on lipolysis and esterification of free fatty acids during incubtion of adipose tissue *in vitro*. *Journal of Lipid Research* 4(2):193-199

Vernon RG, Finley E, and Flint DJ (1987) Role of growth hormone in the adaptations of lipolysis in rat adipocytes during recovery from lactation. *Biochemical Journal* 242(3):931-4

Vernon RG and Finley E (1988) Roles of insulin and growth hormone in the adaptations of fatty acid synthesis in white adipose tissue during the lactation cycle in sheep. *Biochemical Journal* 256(3):873-8

Vikman K, Isgaard J, and Eden S (1991) Growth hormone regulation of insulin-like growth factor-I mRNA in rat adipose tissue and isolated rat adipocytes. *Journal of Endocrinology* 131(1):139-45

Walsh DA, Ashby CD, Gonzalez C, Calkins D, Fischer EH, and Krebs EG (1971) Purification and characterization of a protein kinase inhibitor of adenosine 3′:5′-monophosphate-dependent protein kinases. *Journal of Biological Chemistry* 246:1977-85

Walton PE, and Etherton TD (1986) Stimulation of lipogenesis by insulin in swine adipose tissue: antagonism by porcine growth hormone. *Journal of Animal Science* 62(6):1584-95

Walton PE, Etherton TD, and Evock CM (1986) Antagonism of insulin action in cultured pig adipose tissue by pituitary and recombinant porcine growth hormone: potentiation by hydrocortisone. *Endocrinology* 118(6):2577-81

Wang H, Lipfert L, Malbon CC, and Bahouth S (1989) Site-directed anti-peptide antibodies define the topography of the beta-adrenergic receptor. *Journal of Biological Chemistry* 264(24):14424-31

Wang N, and Rasenick MM (1991) Tubulin-G protein interactions involve microtubule polymerization domains. *Biochemistry* 30:10957-10965

Watkins PA, Moss J, Burns DL, Hewlett EL, and Vaughan M (1984) Inhibition of the bovine rod outer segment GTPase by *Bordatella pertussis* toxin. *Journal of Biological Chemistry* 259:1378-81

Watt PW, Finley E, Cork S, Clegg RA, and Vernon RG (1991) Chronic control of the beta- and alpha 2-adrenergic systems of sheep adipose tissue by growth hormone and insulin. *Biochemical Journal* 273(Pt 1):39-42

Wente SR. and Rosen OM. (1990) Insulin-receptor approaches to studying protein kinase domain. *Diabetes Care.* 13(3):280-7

Werner S. and Low H. (1974) Adenylate cyclase activity in rat adipocyte plasma membranes after adrenalectomy and administration of cortisone acetate. Effects of calcium ions and hormonal stimulations. *Hormone & Metabolic Research.* 6(5):365-70

White JE, and Engel FL (1958a) A lipolytic action of epinephrine and norepinephrine on rat adipose tissue *in vitro*. *Proceedings of the Society for Experimental Biology and Medicine* 99:375-378

White JE, and Engel FL (1958b) Lipolytic action of corticotropin on rat adipose tissue *in vitro*. *Journal of Clinical Investigations* 37:1556-1563

Whiteway M, Hougan L, Dignard D, Thomas DY, Bell L, Saari GC, Grant, FJ. O'Hara P, and MacKay VL (1989) The STE4 and STE18 genes of yeast encode potential beta and gamma subunits of the mating factor receptor-coupled G protein. *Cell* 56(3):467-77

Wieland O (1957) Eine enzymatisch methods zur die bestimming von glycerin. *Biochem. Z.* 329:313-319

Winer BJ (1962) *Statistical Principles in Experimental Design*, McGraw-Hill Book Co., New York

Wirsen C (1965) *In* Handbook of Physiology, Section 5, pp197-9

Wong YH, Federman A, Pace AM, Zachary I, Evans T, Pouyssegur J, and Bourne HR (1991) Mutant alpha subunits of Gi2 inhibit cyclic AMP accumulation. *Nature* 351(6321):63-5

Yamamoto KR (1985) Steroid receptor regulated transcription of specific genes and gene networks. *Annual Review of Genetics* 19:209-52

Yamane HK, and Fung BK (1993) Covalent modifications of G-proteins. *Annual Review of Pharmacology & Toxicology* 33:201-41

Yan K and Rasenick MM (1990) *In Biology of Cellular Transducing Signals* (Vandernoek JY, ed) Plenum Publishing Corp., New York, pp.163-72

Zadik Z, Chalew SA, McCarter RJ Jr, Meistas M, and Kowarski AA (1985) The influence of age on the 24-hour integrated concentration of growth hormone in normal individuals. *Journal of Clinical Endocrinology & Metabolism 60(3):513-6*

Zor U (1983) Role of cytoskeleton organization in the regulation of adenylate cyclase-cyclic adenosime monophosphate by hormones. *Endocrine Reviews* 4:1-21