1991

The Phosphoinositide Second Messenger System And The Regulation Of The Release Of Growth Hormone From Purified Rat Somatotrophs

Michelle Brigid French

THE PHOSPHOINOSITIDE SECOND MESSENGER SYSTEM AND THE REGULATION OF THE RELEASE OF GROWTH HORMONE FROM PURIFIED RAT SOMATOTROPHS

by

Michelle Brigid French

Department of Physiology

Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
September, 1991

ISBN 0-315-66307-3

ABSTRACT

The release of growth hormone (GH) from the somatotrophs of the anterior pituitary is controlled by two hypothalamic hormones growth hormone-releasing factor (GRF) and somatostatin (SRIF). The former stimulates, while the later inhibits the release of GH. cAMP and Ca^{2+} are second messengers for GRF. Previous reports suggested that the phosphoinositide second messenger system was an additional pathway for GRF. Our purpose was to examine the role of this system in GRF action. We used perifused dispersed anterior pituitary cells and purified somatotrophs from male rats to ask: (1) Does activation of protein kinase C stimulate the release of GH and are cAMP and Ca^{2+} involved? (2) Does SRIF inhibit the effect of protein kinase C activation? (3) Is protein kinase C present in somatotrophs and does GRF activate the enzyme? (4) Does inactivation of protein kinase C alter the GH response to GRF? (5) Does GRF activate phospholipase C?

Activation of protein kinase C with dioctanoyl-rac-glycerol (diC_8) and phorbol 12-myristate 13-acetate (PMA) caused the immediate and transient release of GH from somatotrophs. The increase was accompanied by a small increase in cAMP accumulation. Removal of extracellular Ca^{2+} and the Ca^{2+} channel blocker nifedipine reduced protein kinase C activator-induced GH release but not cAMP accumulation. SRIF reduced the release of GH induced by diC_8 and PMA. These results suggest that cAMP and Ca^{2+} may mediate the action of the protein kinase C activators. Protein kinase C activity was present in somatotrophs. Protein kinase C activators caused a translocation of enzyme activity indicating that protein kinase C can be activated in these

cells. GRF, however, did not activate protein kinase C. Also, the GH response to GRF was not altered in cells that were unresponsive to diacylglycerol. GRF did not activate phospholipase C in somatotrophs as determined by measuring the levels of individual radiolabelled phosphoinositides and inositol phosphates following GRF treatment.

We conclude that while all of the components of the phosphoinositide second messenger system are present in somatotrophs, GRF does not act through this system to stimulate GH release.

To the memory of my mother

Brigid French

ACKNOWLEDGEMENTS

I thank my supervisor Dr. J. Kraicer for his instruction, guidance and patience. I appreciate the excellent scientific environment that he provided which enabled me to achieve my academic goals.

I also thank the members of my advisory committee, Dr. T.G. Kennedy, Dr. N. Narayanan, and Dr. F. Possmayer for their helpful suggestions and guidance.

I would like to thank the members of Dr. Kraicer's laboratory. In particular, I am indebted to Bruce Moor, head technician, for his technical advice and assistance. He helped me with all of the experiments described in this thesis and his contributions to this project were invaluable. I thank Benoit Lussier, former graduate student, for his assistance, advice and encouragement. The skilled technical assistance of Leigh Yardley, Kathi James and Cathy Cleave is gratefully acknowledged. Alicia Sue-Tang, as a fourth year research student in the laboratory, assisted me with the dual column perifusion experiments.

Dr. E. Ball of the Department of Biochemistry at the University of Western Ontario and Dr. R.J. Haslam of the Department of Pathology at McMaster University provided essential technical advice.

I thank my mother and father, Brigid and Maurice French, for their love, encouragement and support. I thank my brother, Sean French, for his love, advice and encouragement.

The Medical Research Council of Canada provided the financial support for this project.

I am grateful to the Ministry of Colleges and Universities of the Province of Ontario for Ontario Graduate Scholarships and the Faculty of Graduate Studies for a Special University Scholarship and Graduate Research Award.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BSA	bovine serum albumin
$[Ca^{2+}]_i$	free cytosolic Ca^{2+} concentration
cAMP	adenosine 3',5'-monophosphate
CDP	cytidine diphosphate
cGMP	cyclic guanosine 3',5'-monophosphate
CRF	corticotropin-releasing factor
CTP	cytidine trisphosphate
dbcAMP	dibutyryl cAMP
diC_8	1,2-dioctanoyl-rac-glycerol
DMSO	dimethylsulfoxide
E_m	membrane potential
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ER	extra release
G_i	pertussis toxin-sensitive GTP-binding protein which inhibits adenylate cyclase
G_k	pertussis toxin-sensitive GTP-binding protein which increases K^+ conductance
G_p	GTP-binding protein which increases phospholipase C activity
G_s	cholera toxin-sensitive GTP-binding protein which stimulates adenylate cyclase
G_t	GTP-binding protein which increases transducin activity
GDP	guanosine diphosphate
GH	growth hormone
GRF	growth hormone-releasing factor
GTP	guanosine triphosphate
GTPγS	guanosine-5'-O-(3'-thiotrisphosphate)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hGH	human growth hormone
hGRF	human growth hormone-releasing factor
hpGRF	human pancreatic growth hormone-releasing factor

IBMX	3-isobutyl-1-methylxanthine
IGF	insulin-like growth factors
IP	inositol-4-monophosphate
IP_2	inositol-1,4-bisphosphate
IP_3	inositol-1,4,5-trisphosphate
IP_4	inositol-1,3,4,5-tetrakisphosphate
IP_5	inositol-1,3,4,5,6-pentakisphosphate
IP_6	inositol-hexakisphosphate
K_d	dissociation constant
K_i	inhibition constant
kDa	kilodalton
M199	medium 199
M199A	medium 199 containing 0.1% BSA, 28 mM $NaHCO_3$, pH = 7.35 - 7.40 at 37 C gassed with 95% O_2 - 5% CO_2
M199AH/RT	medium 199 containing 0.1% BSA, 20 mM $NaHCO_3$, and 20 mM HEPES, pH = 7.35 - 7.40 at room temperature in air
M199AH/37	medium 199 containing 0.1% BSA, 20 mM $NaHCO_3$, and 20 mM HEPES, pH = 7.35 - 7.40 at 37 C in air
NIF	nifedipine
PA	phosphatidic acid
PDBu	phorbol dibutyrate
PGD	prostaglandin D
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2
PI	phosphatidylinositol
PIP	phosphatidylinositol-4-monophosphate
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKC-A	active protein kinase C
PKC-I	inactive protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PSR	peak secretion rate
rGRF	rat growth hormone-releasing factor
RIA	radioimmunoassay
SRIF	somatostatin (14 amino acid peptide)
SRIF-20	somatostatin (20 amino acid peptide)

SRIF-25	somatostatin (25 amino acid peptide)
SRIF-28	somatostatin (28 amino acid peptide)
TRH	thyrotropin-releasing hormone

CHAPTER 1: INTRODUCTION

Growth hormone (GH) is a protein hormone which is synthesized in and released from the somatotrophs of the anterior pituitary gland. GH increases lean body mass, linear bone growth, and organ size and decreases liposity. In addition to its growth promoting effects, GH also alters metabolism causing an increase in plasma concentrations of glucose and free fatty acids and a decrease in plasma concentrations of amino acids. GH stimulates the production (mainly from the liver) of a family of peptides called somatomedins or insulin-like growth factors. Some of the actions of GH are direct and some are mediated by insulin-like growth factors. The release of GH from somatotrophs is directly controlled by two hypothalamic hormones, growth hormone-releasing factor (GRF) and somatostatin (SRIF, somatotropin release-inhibiting factor). The former stimulates GH release, while the latter inhibits the release of GH. GRF and SRIF are peptide hormones which bind to their receptors on the plasma membrane of somatotrophs and exert their effects via changes in the levels of intracellular second messengers. The purpose of our work was to examine the intracellular second messengers that mediate the action of GRF and SRIF.

It is well established that cyclic adenosine 3',5'-monophosphate (cAMP) and calcium (Ca^{2+}) are second messengers for GRF. SRIF decreases the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) through a mechanism that has not been entirely elucidated but may involve hyperpolarization of the cell. At the start of this research project, there was some limited evidence that another second messenger system, the phosphoinositide second messenger system, might be involved in the regulation of GH release. The

subject of this thesis is the investigation of the role of the phosphoinositide second messenger system in the regulation of the release of GH from the somatotrophs of the anterior pituitary.

In this introductory chapter, the physiology of GH is reviewed with emphasis on the intracellular mechanisms involved in GRF and SRIF action. Also included is a general description of the second messenger systems that mediate the action of hormones and neurotransmitters and the process of exocytosis. Finally, the rationale for our studies and the questions that we addressed are outlined.

1.1 GH structure and function

The role of the pituitary in the promotion of growth was first suggested by the experiments of Aschner (1912) and Cushing (1912) who found that hypophysectomy of puppies caused growth retardation. Cushing also observed that some patients with tumours of the anterior pituitary had acromegaly, a condition characterized by excessive growth. In 1921, Evans and Long demonstrated the presence of a growth promoting principle in extracts of bovine anterior pituitary glands. Li and colleagues first isolated GH from ox anterior pituitaries in 1945 and provided evidence that it was a protein hormone since its growth promoting activity was lost following treatment with trypsin, heat and urea. Human GH (hGH), first sequenced in 1969 by Li and co-workers with subsequent modifications (Li, 1975), is a single polypeptide chain having a molecular weight of 22 kDa consisting of 191 amino acid residues with two disulfide bridges formed between residues 53-165 and 182-189 (Figure 1). There is considerable homology of the primary structure between GHs from different species but differences

exist which affect their tertiary structure, making them immunologically and often biologically distinct (Hayashida, 1972). While the 22 kDa form of hGH is the most prevalent and possesses full biological activity, a number of variants of hGH, originally thought to be contaminants of the extraction procedures, have since been described. These variants include a 20 kDa form, deaminated isomers of the 20 kDa and 22 kDa forms, an acylated form, and GH aggregates and GH fragments; all are found in the anterior pituitary and blood stream (Lewis et al., 1980; Brostedt et al., 1990; Baumann, 1990; Farrington and Hymer, 1990).

The growth promoting effects of GH, including increased linear growth, lean body mass and organ size, are mediated by insulin-like growth factors which are secreted by the liver and other tissues (Hall and Tally, 1989; Clemmons, 1989; Sara and Hall, 1990). In addition to its growth promoting ability, many metabolic effects, insulin-like and diabetogenic, of GH have been described (Knobil, 1966; Isaksson et al., 1985; Davidson, 1987; Gustafsson, 1989; Goodman et al., 1990; Sirek and Sirek, 1990). The insulin-like effects occur within the first few hours of GH administration and include decreased plasma concentrations of amino acids, free fatty acids and glucose; increased incorporation of amino acids into protein in muscle; and decreased lipolysis in adipose tissue. The diabetogenic effects are observed after several hours of GH treatment and include increased plasma concentrations of free fatty acids and glucose, and increased lipolysis in adipose tissue. There is also a long term effect on increased protein synthesis.

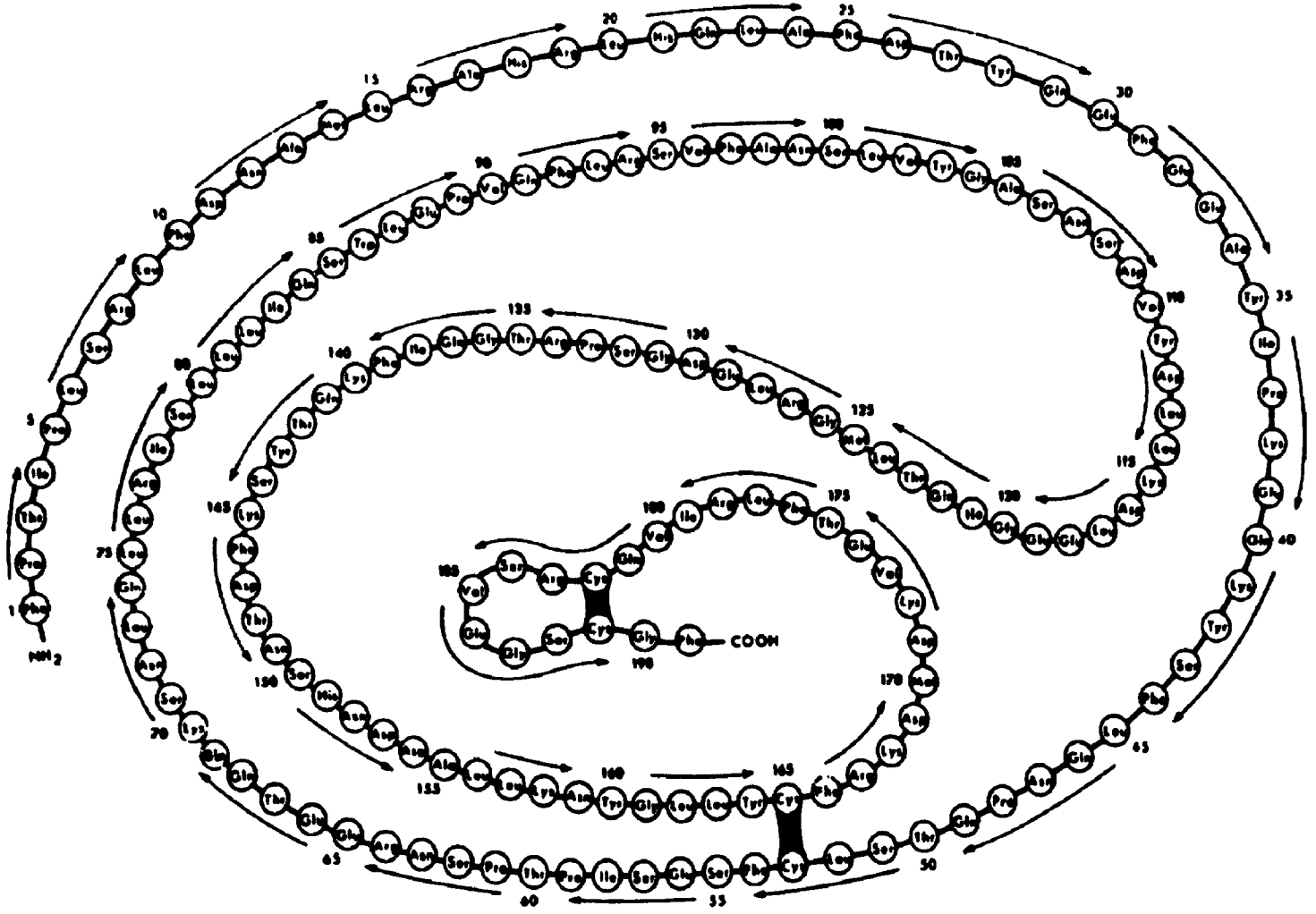


Figure 1: Structure of human GH. Taken from Li (1975). Reproduced with permission from Academic Press Inc. (©) 1975 Academic Press Inc.

1.2 Pattern of GH secretion

The development of radioimmunoassays for GH in the 1960s with improved efficiency and sensitivity compared to older bioassays allowed for measurement of GH in small volume samples and facilitated investigation of the control and pattern of GH secretion (Hunter and Greenwood, 1964; Glick et al., 1965). In one of the first studies employing radioimmunoassay, fasting and exercise were found to be strong stimuli for GH secretion but peaks of GH in the human subjects, not directly associated with the experimental perturbation, were also observed (Glick et al., 1965). Serial sampling of blood for prolonged periods of time, established that fluctuations of plasma GH can occur independently of external stimuli. For example, in humans, GH was released following the onset of sleep but smaller pulses were observed throughout the rest of the night and the following day that were independent of metabolic state (Takahashi et al., 1968; Honda et al., 1969; Alford et al., 1973). In 1976, Tannenbaum established that an endogenous ultradian rhythm of circulating GH with a period of 3.3 h is present in adult male rats. In recent years, the analysis of the data from serial blood sampling has become more rigorous. Various mathematical techniques for the analysis of the raw time series data have been employed, since several rhythms may be present which would not be discernible upon visual inspection of the data. Parker (1979) and Steiner (1978) have calculated the autocovariance function of their time series data from humans and male baboons and found that the GH rhythm has a frequency of 2-5 cycles/24 h and 1 cycle/4 h respectively. Power spectral analysis of serial plasma GH values from male dogs revealed a GH rhythm with a frequency of 1 cycle/4.5 h (French et al., 1987). Recently, sensitive immunoextracted radioimmunoassays for GH have been developed in which the

lower limits of detection are 0.2 μg/l compared to 1.0 μg/l of conventional radioimmunoassays. These assays have demonstrated the presence of GH pulses in adult humans in a previously undetectable range. Time series analysis of these plasma GH values has demonstrated the presence of a GH rhythm with a frequency of 13/day with a dominant but not strictly periodic 2 h rhythmicity (Drobny et al., 1983; Winer et al., 1990). The results from these studies have established that there is an endogenous ultradian rhythm of GH secretion.

There are age and sex differences in the pattern of GH release. In humans (13 males and 23 females ranging from 7 to 65 years of age), blood was withdrawn continuously for 24 h, collection tubes were replaced every 30 min and plasma GH was determined. These values were used to calculate 24 h integrated concentrations of GH release. The highest integrated concentration was observed between the ages of 15 and 20 years of age in both males and females with no difference in the amount of GH released when male and females were age matched (Zadik et al., 1985). The disadvantage of measurement of integrated GH concentrations compared to the measurement of serial GH samples is that the details of the pattern of GH release are missed. In another study (Winer et al., 1990), blood samples were withdrawn at 20 min intervals from 6 men and 6 women (20 to 47 years of age), and plasma GH was immunoextracted and measured by radioimmunoassay. Higher pulse amplitudes and higher baselines were observed in the women compared to the men. Although the sample size was limited, there was no difference in GH pattern in the follicular compared to the luteal phase of the menstrual cycle. A similar result has been found for other animals. In both male and female immature rats (22 days of age), plasma GH levels

were low and did not exceed 35 ng/ml. The pulsatile pattern seen in male rats, with low nadir (less than 1 ng/ml) levels and large peaks occurring at regular 3-4 hour intervals, developed by 30 days of age. On the other hand, peak GH levels were lower in 30 day old female rats compared to males of the same age. Vaginal opening occurred in these rats between 31 and 41 days of age. By 45 days, peak levels in the females were similar to males but the period between peaks was shorter and there is no distinct rhythm. At 90 days, female rats had higher nadir levels with lower peak levels and no rhythmicity of GH secretion. The effect of the oestrous cycle was not considered in these experiments and blood samples were taken at 30 min intervals so that some of the resolution of the GH pattern was probably lost (Edén, 1979). Tannenbaum et al. (1976) also found that adult female rats had higher basal levels and lower and more frequent peaks of GH compared to adult male rats. They also reported no differences in the secretory pattern of GH during different phases of the oestrous cycle but no mathematical analysis was performed on their data. The sex differences are thought to be due to the influence of sex steroids since neonatal gonadectomy decreased GH pulse height in male rats and testosterone replacement reversed these effects (Jansson et al., 1984).

1.3 Control of GH release

In 1947, Green and Harris suggested that the control of anterior pituitary hormone secretion was dependent on the close proximity of the pituitary to the hypothalamus and on the presence of the hypophysial portal system. The hypophysial portal system, first described by Popa and Fielding (1930), is a series of vessels which collect blood from the capillary plexus in the median eminence of the hypothalamus, travel down the

pituitary stalk and branch into the capillary plexus of the anterior pituitary. Green and Harris suggested that hypothalamic releasing and inhibiting factors are released into the capillaries of the median eminence, and travel via the hypophysial portal system to alter hormone release from the anterior piutitary. Transplantation of the anterior pituitary to the anterior chamber of the eye caused a decrease in growth of rats, even though the graft remained viable, suggesting that the hypothalamus and hypophysial circulation are essential for growth hormone release (Martini et al., 1959).

The possibility that the hypothalamus was a source for GRF prompted many groups to examine the effect of hypothalamic extracts on growth and GH release in an attempt to isolate and characterize this releasing factor. Franz et al. (1962) reported that an acid extract of porcine hypothalami had GH releasing activity in rats. Acid extracts from rat hypothalami added to rat pituitaries in vitro stimulated the secretion of bioassayable GH (Deuben and Meites, 1964). Pecile et al. (1965) reported that injections of ovine and rat hypothalamic extracts caused, within 15 min, a depletion of bioassayable GH in anterior pituitary extracts indicating that GH had been released from the pituitary. The GH releasing ability of hypothalamic extracts was also observed by Krulich and colleagues (1968) who reported that ovine and rat hypothalamic extracts increased the release of bioassayable GH from rat pituitaries in vitro. Studies of this type were continued when radioimmunoassays for the measurement of GH became available. Wilber et al. (1971) showed that extracts from rat and porcine hypothalami stimulated radioimmunoassayable GH release from rat pituitaries in vitro. Porcine hypothalamic extracts were found to increase plasma radioimmunoassayable GH when injected intravenously in rats (Malacara et al., 1972). Frohman et al. (1971) and Sandow et al.

(1972) demonstrated GH releasing ability in hypothalamic extracts when delivered close to the pituitary via intrapituitary injections or microcannulae inserted into hypophysial portal vessels. These experiments indicated that the hypothalamus contained GRF(s) but the isolation and characterization of GRF eluded researchers until the early 1980s (see section 1.3.1).

The hypothalamus as a site of GRF production was also implicated by experiments concerning the effect of hypothalamic lesions and electrical stimulation on GH release and growth. Reichlin (1960) demonstrated that large lesions of the anterior hypothalamus, including the ventromedial nucleus, decreased growth rate in rats that were supplemented with thyroxine, corticosterone, testosterone, vasopressin and deoxycorticosterone acetate. There was also a reduction in the content of bioassayable GH in the pituitaries of these animals, indicating increased release and/or decreased synthesis of GH (Reichlin, 1961). In subsequent experiments, lesions to the anterior hypothalamus of squirrel monkeys reduced insulin-induced GH release but failed to alter basal GH release (Abrams et al., 1966). Bernardis and Frohman (1968a, 1970) demonstrated that lesions limited to the ventromedial nucleus of the hypothalamus caused decreased growth, pituitary GH content and plasma GH concentration in weanling female rats. Pulsatile GH secretion was also abolished in adult male rats with lesions to the ventromedial nucleus of the hypothalamus (Martin et al., 1974). As expected, stimulation of the ventromedial or arcuate nuclei of the hypothalamus had the opposite effect, causing an increase in plasma GH levels (Frohman et al., 1968b; Bernardis and Frohman, 1971; Martin, 1972). Taken together, the results from these studies indicated

that the ventromedial and arcuate nuclei of the hypothalamus are involved in the control of GH release and are sites that most likely contain GRF neurons.

The first evidence that GH secretion might be under the control of an inhibitory factor came from Krulich and co-workers (1968) who, in attempts to isolate GRF, demonstrated the presence of a GH release-inhibiting factor in fractions of partially purified hypothalamic extracts. In 1973, the GH release-inhibiting factor, SRIF, was isolated and sequenced by Brazeau and colleagues (see section 1.3.3).

1.3.1 Structure of GRF

In November 1982, two groups identified and sequenced peptides with GH releasing ability from pancreatic islet cell tumours from patients with acromegaly (Guillemin et al., 1982; Rivier et al , 1982). Guillemin et al. (1982) described the structure of the major peptide as a 44 amino acid amide [hpGRF(1-44)-NH_2]. Two other minor components with GRF activity, hpGRF(1-40)-OH and hpGRF(1-37)-OH, consisting of 40 and 37 amino acids respectively were also isolated. Injection of the peptides into rats and dogs increased GH release. The major peptide sequenced by Rivier et al. (1982) was identical to hp(1-40)-OH described by Guilleman and co-workers and stimulated the release of GH from cultured rat pituitary cells.

Following the description of human pancreatic tumour GRF, human hypothalamic GRF (hGRF) was characterized and was reported to be identical to hpGRF(1-44)-NH_2 (Böhlen et al., 1983; Ling et al., 1984). Hypothalamic GRF has been isolated from several other species including pig, cow, sheep, goat and rat (Böhlen et al., 1983, 1984a; Esch et al., 1983; Spiess et al., 1983; Brazeau et al., 1984). Rat GRF (rGRF) is a 43

amino acid peptide and has 67% homology with hGRF (Figure 2) (Spiess et al., 1983; Böhlen et al., 1984b).

Since the initial reports describing the structure of GRF many laboratories have confirmed that GRF stimulates the release of GH both in vivo and in vitro. The effect is specific, rapid and dose-dependent (Wehrenberg et al., 1982b; Brazeau et al., 1982a; Gelato et al., 1983; Tannenbaum et al., 1983a; Arimura and Culler, 1985; Gelato and Merriam, 1986).

1.3.2 GRF localization

With the elucidation of the structure of GRF, antisera were produced and were used to study its localization. Block and co-workers (1983a,b), using antibodies to hpGRF(1-40), found immunoreactive cell bodies in the arcuate and ventromedial nuclei of monkeys having immunoreactive fibers terminating in the median eminence. Similar results were observed in brains from humans and rats (Jacobowitz et al., 1983; Okamura et al., 1985). Other areas of the brain that also contain GRF-immunoreactive cell bodies or fibers include the paraventricular and dorsomedial nuclei and areas of the lateral hypothalamus in the rat (Merchenthaler et al., 1984). Outside the hypothalamus, GRF fibers are found in the upper intestinal tract in humans and rats (Bosman et al., 1984; Christofides et al., 1984).

1.3.3 Structure of SRIF

SRIF, a tetradecapeptide, was isolated and characterized from ovine hypothalami by Brazeau and colleagues (1973) and named because of its ability to inhibit GH release from anterior pituitary cells in vitro. In the early 1980s, a SRIF immmunoreactive -

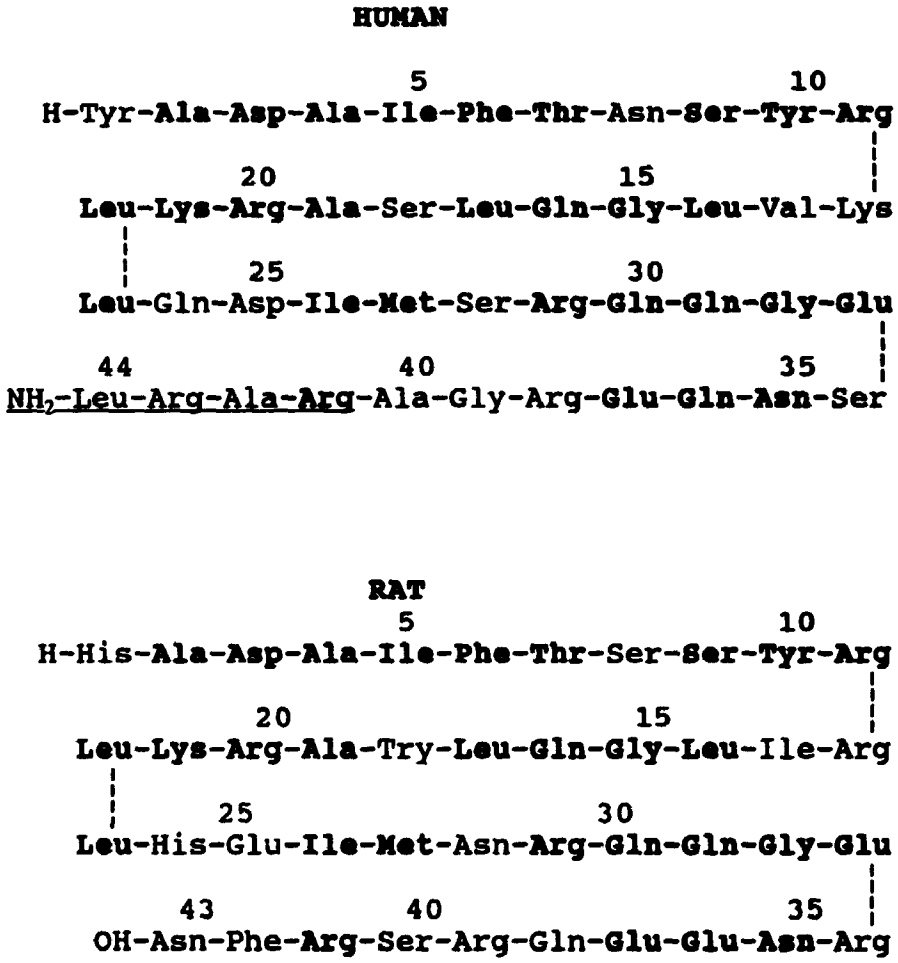


Figure 2: Structure of human and rat GRF. The first 40 amino acids of hGRF are identical for both forms. The difference between the two forms of hGRF is indicated by the underlined region. Bold amino acids are common to both rat and human GRF.

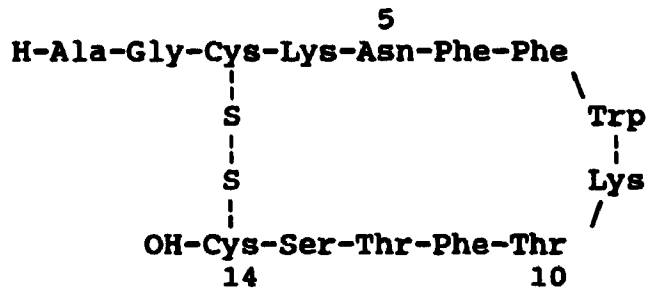
peptide was isolated from intestinal and hypothalamic tissues (SRIF-28). SRIF-28 consisted of 28 amino acids and contained a sequence identical to SRIF-14 at its COOH terminal (see Figure 3) (Pradayrol et al., 1980; Esch et al., 1980; Brazeau et al., 1981). SRIF-28 is reported to be more potent in vitro compared to the 14 amino acid form of SRIF (Brazeau et al., 1981; Srikant and Heisler, 1985). SRIF and SRIF-28 are formed from post-translational processing of prosomatostatin (Benoit, 1987). SRIF-14 is the predominant form in rats (Patel et al., 1985).

Since the initial description of SRIF, many studies have demonstrated that SRIF inhibits basal and stimulated GH release in vivo and in vitro (Siler et al., 1973; Martin et al., 1974; Lovinger et al., 1974; Martin, 1974; Martin et al., 1975; Stachura, 1976; Kraicer and Spence, 1981; Kraicer and Chow, 1982; Brown et al., 1983; Arimura and Culler, 1985; Sheppard et al., 1985; Lamberts, 1988; Frohman et al., 1990b; Thorner et al., 1990). Other physiological roles for SRIF were subsequently described and include inhibition of thyroid-stimulating hormone, insulin and glucagon release; neuromodulation in the central nervous system; and regulation of a variety of gastrointestinal functions (Arimura and Fishback, 1981; Brown and Fisher, 1985; Larsson, 1985; Makhlouf, 1987; Yamada, 1987; Owyang and Wiley, 1987; Dharmsathaphorn, 1987; Solomon, 1987).

1.3.4 SRIF localization

Unlike GRF, SRIF neurons and fibers are widely distributed. The SRIF neurons that regulate GH release are primarily located in the rostral part of the periventricular nucleus of the hypothalamus and project to the median eminence (Elde and Parsons,

SRIF-14



SRIF-28

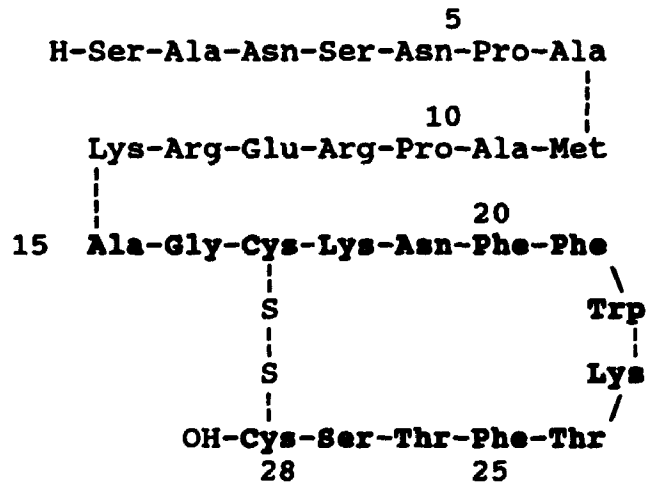


Figure 3: Stucture of SRIF-14 and SRIF-28. SRIF-28(15-28) is identical to SRIF-14.

1975; Kawano et al., 1982; Ishikawa et al., 1987). Other areas of the hypothalamus with SRIF neurons include the preoptic, suprachismatic, ventromedial and arcuate nuclei (Krisch, 1979; Johansson et al., 1984; Vincent et al., 1985). SRIF is found in many other areas of the brain and is located in the digestive tract and in the δ cells of the islets of Langerhans in the pancreas (Finley et al., 1981; Johansson et al., 1984; Vincent et al., 1985).

1.3.5 Control of GH release by neurotransmitters, neuropeptides, hormones and metabolites

A number of neurotransmitters, neuropeptides, hormones and metabolites have been identified as having effects on the release of GH. In addition, a number of neurotransmitters and neuropeptides are co-localized within GRF and SRIF neurons. These include dopamine, galanin, neuropeptide Y, acetylcholine, α-melanotropin and melanin (Meister et al., 1986; Risold et al., 1989; Niimi et al., 1990; Ciofi et al., 1990). The effects of these compounds for the most part, involve the stimulation and/or inhibition of GRF and/or SRIF release from the hypothalamus, although some direct effects on somatotroph function have been described (Martin, 1973; Arimura and Fishback, 1981; Brown et al., 1983; Arimura and Culler, 1985; De Gennaro Dieguez et al., 1988; Colonna et al., 1989).

It is difficult to reach conclusions about the effects of these factors for a number of reasons. For example, there may be more than one site of action and the factor may have different effects on GH release at different sites. The various methods employed to test the involvement of a particular factor, such as administration of the compound of interest at different sites, use of agonists and antagonists, and use of different species,

contribute to the difficulty. It is possible, however, to make some general statements about the effect of various neurotransmitters, neuropeptides, hormones and metabolites on GH release.

There is general agreement that stimulation of α_2-adrenergic pathways in vivo causes the release of GH and is important for the generation of episodic GH release. Other biogenic amines that also have been shown to increase GH release include dopamine and serotonin (Martin, 1973; Weiner and Ganong, 1978; Arimura and Culler, 1985; Dieguez et al., 1988). Dopamine and norepinephrine also stimulate SRIF release when administered into the third ventricle or to hypothalamic fragments in vitro (Arimura and Fishback, 1981). The predominant effect of these compounds, however, is stimulation of GH release and their action is at the level of the hypothalamus or above (Weiner and Ganong, 1978).

Acetylcholine also causes GH release. A direct effect of acetylcholine on anterior pituitary cells has been reported since acetylcholine stimulated the release of GH from bovine anterior pituitary cells (Young et al., 1979). But the effect of acetylcholine on GH release could not be demonstrated in rat anterior pituitary cells (Kraicer, 1979). The effect of acetylcholine in vivo appears to be via an inhibition of SRIF release rather than a stimulation of GRF release (Dieguez et al., 1988; De Gennaro Colonna et al., 1989).

Galanin, a neuropeptide that is found in GRF neurons stimulates the release of GH when administered in vivo. The effect of galanin also seems to be due to an inhibition of SRIF release from the hypothalamus (Ottlecz et al., 1986; Davis et al., 1987).

The hormones that alter GH release include thyroid hormones, glucocorticoids, estrogens and testosterone. Thyroid hormones are critical for GH release and act at the level of the hypothalamus and the pituitary to modulate GH release (Gelato and Merriam, 1986; Dieguez et al., 1988). Glucocorticoids have predominantly inhibitory effects on the release of GH when administered in vivo, although at the pituitary level in vitro they are potent stimulators of GH release (Martin, 1973; Gelato and Merriam, 1986; Dieguez et al., 1988). Testosterone stimulates while estrogens inhibit the release of GH (Dieguez et al., 1988).

There are reports that GH secretion is also under metabolic control. Plasma glucose levels influence GH release in a species-specific way. In primates, hypoglycaemia stimulates the release of GH, while GH release is reduced as a result of hypoglycaemia in rats (Gelato and Merriam, 1986; Dieguez et al., 1988). Increased plasma free fatty acid levels have been reported to inhibit GH release (Gelato and Merriam, 1986; Dieguez et al., 1988).

1.3.6 Negative feedback regulation

High concentrations of plasma GH inhibit endogenous GH release and also the GH response to GRF administration indicating that the release of GH is under negative feedback control. Negative feedback regulation of GH release may occur at the hypothalamic and somatotroph level and involve GH, insulin-like growth factors, GRF and SRIF (see Figure 4).

At the hypothalamic level, increased plasma concentrations of GH result in decreases in hypothalamic GRF and increases in hypothalamic SRIF content and release. There are reports that GH can enter the hypothalamus from the peripheral circulation

suggesting that GH can inhibit its own secretion by direct effects on GRF and SRIF neurons (Oliver et al., 1977). Maiter et al. (1990) observed that rats bearing growth hormone secreting MtTW15 tumours, which caused a ten fold increase in plasma GH levels, had decreased hypothalamic GRF content and increased SRIF hypothalamic content. The effect of GH on the hypothalamic content of SRIF was also observed in rats treated with rGH for 1 week (Berelowitz et al., 1981). Miki and co-workers (1989) showed that hypothalami removed from rats treated for 1 week with rGH had lower basal and K^+-stimulated release of GRF. One possible mechanism appears to be a direct effect of GH on GRF and SRIF producing neurons. Incubation of hypothalamic fragments with GH stimulates the content and the release of SRIF and administration of GH into the third ventricle increases the release of SRIF into hypophysial portal blood (Patel, 1979; Chihara et al., 1981; Frohman, 1988). Insulin-like growth factors have been detected in the rat hypothalamus and anterior pituitary (Goodyer et al., 1984b). Abe and colleagues (1983) reported that injection of insulin-like growth factors into the lateral ventricle inhibited episodic GH secretion in male rats for at least 6 h. The primary effect of insulin-like growth factors at the hypothalamic level is via stimulation of SRIF release.

Thus, elevated peripheral levels of GH result in changes in GRF and SRIF neuronal function. The inhibitory effect of GH may be direct or indirect via increases in insulin-like growth factors.

GH does not have a direct effect at the somatotroph since incubation or perifusion of anterior pituitary cells or somatotrophs in vitro in the presence of varying concentrations of GH does not alter basal or GRF-induced GH release (Goodyer et al., 1984a; Kraicer et al., 1988a). However, insulin-like growth factors appear to act at the

pituitary to inhibit the release of GH. Incubation of anterior pituitary cells with insulin-like factors for periods of 3 h or greater inhibits basal and GRF-induced GH release (Tannenbaum et al., 1983b; Goodyer et al., 1984a; Lamberts et al., 1989; Namba et al., 1989).

1.3.7 Generation of pulsatile GH release

Both GRF and SRIF are required for episodic GH release. This dual control of pulsatile GH has been demonstrated in experiments in which endogenous GRF or SRIF is removed by administration of antiserum to these hormones. GRF antiserum in rats abolishes pulsatile GH release (Wehrenberg et al., 1982a), while administration of SRIF antiserum in rats results in increased basal levels of GH and more frequent GH peaks (Ferland et al., 1976; Steiner et al., 1978). Similarly, deafferentation of the anterolateral hypothalamus, which removes SRIF input to the median eminence, also causes increased basal and more frequent bursts of GH (Minami et al., 1990). Thus, GRF and SRIF individually play crucial roles in the generation of episodic GH secretion.

It has been proposed that the release of GRF and SRIF is rhythmic and about 180° out of phase, so that when GRF levels are high, SRIF levels are low when GRF levels are low, SRIF levels are high (Tannenbaum et al., 1990). Indirect evidence for rhythmic SRIF release comes from experiments in which the effect of an injection of a GRF analogue on GH release in rats was tested during periods of low and periods of high endogenous GH release. The GRF analogue stimulated more GH release during periods of high endogenous GH release than during periods of low endogenous GH release suggesting that endogenous SRIF concentrations are higher during periods of low

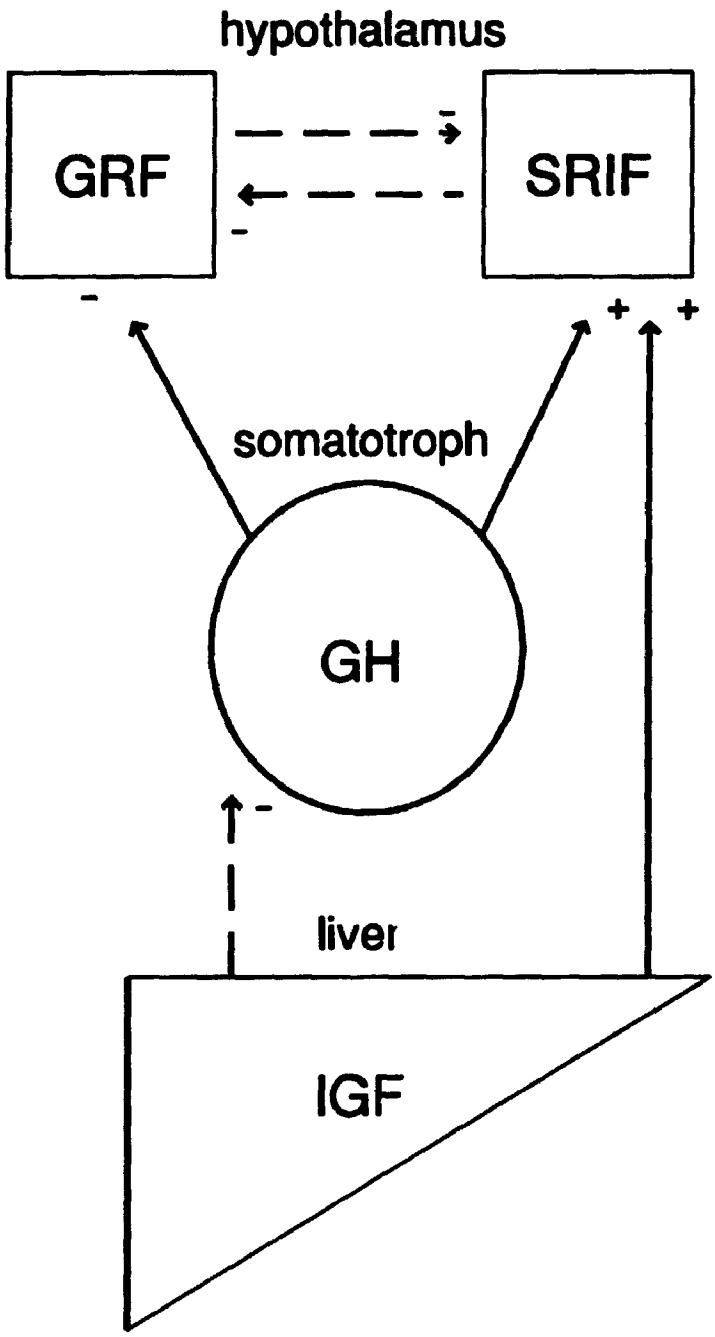


Figure 4: Negative feedback control of GH secretion. Solid lines indicate negative feedback loops for which there is good experimental evidence. Dashed lines indicate proposed negative feedback loops.

GH release. In fact, neutralization of endogenous SRIF eliminated this difference indicating that SRIF is increased during periods of low GH secretion (Tannenbaum and Ling, 1984).

Several attempts have been made to directly measure GRF and SRIF in hypophysial portal blood. Plotsky and Vale (1985) sampled hypophysial portal blood at 20 min intervals in anaesthetized male rats. Secretion of GRF was episodic with maximal concentrations present during expected GH secretory periods (GH was not actually measured in the same animals). The increase in GRF was accompanied by a moderate reduction in SRIF. While these results suggest that GRF and SRIF are released episodically, the anaesthesia may have altered the pattern of GRF and SRIF release and it is not possible to correlate changes in GRF and SRIF with changes in GH release since plasma GH release was not determined at the same time. Frohman and co-workers (1990a) have measured GRF and SRIF concentrations in hypophysial portal blood and GH in peripheral blood in unanesthetized ewes and found that GRF and SRIF secretion was pulsatile. There was an association between GRF and GH peaks but not between GRF and SRIF or SRIF and GH. The relationship between SRIF, GRF and GH may have been easier to demonstrate in rams which would have had more distinct GH peaks. Therefore, while the available evidence is limited, these results indicate that the rhythmic release of GRF and SRIF contributes to the episodic pattern of GH release.

The effects of GRF and SRIF at the pituitary level also appear to be important for the generation of episodic GH release. For example, the relative concentrations of GRF and SRIF impinging on the somatotrophs determine the amount of GH released. In perifusion, withdrawal of SRIF results in an immediate burst of GH release. The

subsequent overshoot in GH release is increased when the cells are perifused with GRF during the SRIF perifusion period (Kraicer et al., 1988b). This and other data has lead Cowan and colleagues (1984) to postulate that the timing of GH peaks is set by SRIF withdrawal, while the magnitude of the peaks in GH secretion is determined by GRF. It appears that SRIF may also sensitize the somatotrophs to GRF since, in perifusion, pre-exposure of cells to prolonged SRIF perifusion prevents the attenuated response that is observed with repeated GRF pulses (Sato et al., 1988).

To summarize, episodic release of GH appears to be dependent on the rhythmic release of GRF and SRIF from the hypothalamus and on their interactions at the level of the somatotroph.

1.4 Stimulus-secretion coupling

There are two major types of extracellular signalling molecules, lipid and water soluble. Lipid soluble extracellular signals, such as steroid and thyroid hormones, readily enter cells, bind to intracellular receptors and exert their effects by altering the rate of gene transcription (Alberts et al., 1989a). On the other hand, water soluble extracellular signals such as protein and peptide hormones and neurotransmitters cannot diffuse across the plasma membrane and, therefore, exert their effects by binding to their receptors on the plasma membrane of target cells. The extracellular signal is transduced by different components of the cell which may be arbitrarily divided into two categories. These are signal transduction enzymes and ion channels. Activation of the signal transduction enzymes, adenylate cyclase, guanylate cyclase, phospholipase A_2 or phospholipase C, leads to the production of intracellular second messengers which in turn

exert effects, often by activation of other enzymes. Another group of signal transduction enzymes are the tyrosine kinases which make up part of receptors for insulin, epidermal growth factor and platelet-derived growth factor and increase in activity with ligand binding (Sefton and Hunter, 1984). Extracellular signals may also alter ion channel opening and closing resulting in changes in membrane potential and/or the concentration of specific ions within the cell. Changes in membrane potential and intracellular concentrations of specific ions regulate a number of cellular processes. The effect of the hormone or neurotransmitter on ion channels may be direct or indirect via second messengers.

The purpose of this section is to review the mechanisms through which hormones and neurotransmitters act to alter cell function. The discussion is limited to the action of water soluble compounds since GRF and SRIF belong to this group. In addition, emphasis is placed on the regulation of GH release and on the systems involved, or proposed to be involved, in the mediation of GRF and SRIF action. Firstly, there is a description of G proteins and their role in signal transduction. This is followed by a review of the various types of intracellular effector systems. Finally, the process of exocytosis and the factors which regulate it are outlined.

1.4.1 G proteins

G proteins belong to a family of guanine nucleotide binding proteins that plays key roles in a variety of signalling pathways (Stryer and Bourne, 1986; Gilman, 1987; Birnbaumer, 1990). They link signals from activated membrane receptors to effector enzymes and channels. G proteins consist of 3 polypeptide subunits designated α, β and γ and cycle between an inactive GDP state and an active GTP state (see Figure 5).

When GDP is bound, α associates with β and γ to form a $G_{\alpha\beta\gamma}$ complex. When GTP replaces GDP, G_{α}-GTP is released from $G_{\beta\gamma}$ and alters the activity of a target enzyme or channel. The rate of the exchange of GTP for GDP is increased in the presence on an activated receptor. G_{α} has GTP-ase activity which converts GTP to GDP thereby returning the G_{α} subunit to its inactive bound state. There are a number of different G proteins and the heterogeneity comes primarily from variability of the α subunits.

The discovery of G proteins began with the observation by Rodbell et al. (1971) that GTP was necessary for glucagon-induced stimulation of adenylate cyclase activity. Other evidence for the involvement of GTP in hormone action came from experiments in which the GTP analogue, 5'-guanylylimidodiphosphate, was shown, in combination with glucagon, to be a potent activator of adenylate cyclase activity (Londos et al., 1974). In addition, Cassel and Selinger (1976) demonstrated that GTP hydrolysis was associated with catecholamine-stimulated adenylate cyclase activity. These results suggested that GTP was important for hormone-induced activation of adenylate cyclase.

Pfeuffer and Helmbreich (1975) demonstrated that GTP bound to a membrane protein that could be separated from adenylate cyclase. The first purification of a G protein came in 1979 with the description of G_t which was shown to play a role in signal transduction in the photoreceptor of the retina (Godchaux and Zimmerman, 1979). A number of other G proteins were subsequently identified including G_s and G_i, the G proteins that mediate hormonal stimulation and inhibition of adenylate cyclase activity (Stryer and Bourne, 1986; Gilman, 1987; Birnbaumer, 1990). G proteins are involved in a number of signal transduction systems which include: adenylate cyclase, retinal cyclic GMP, phospholipase A_2, phospholipase C and the regulation of ion channel

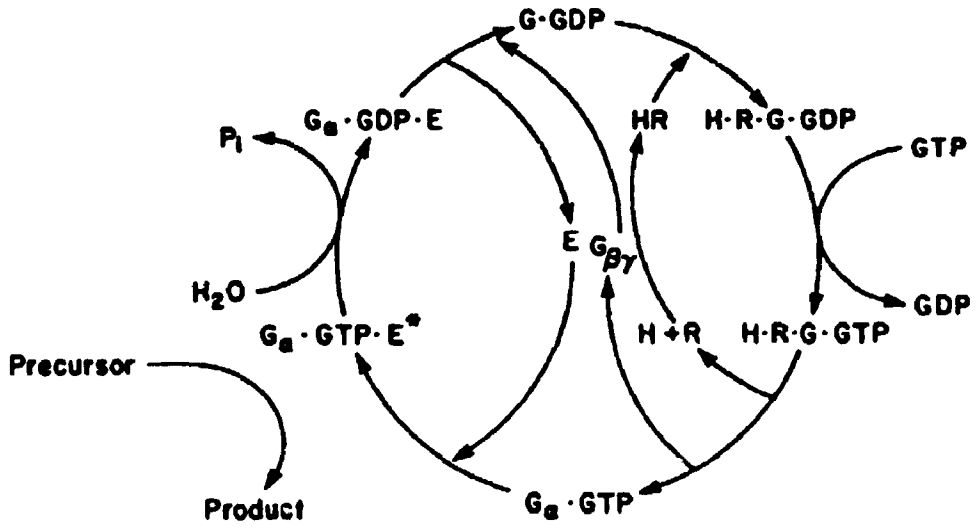


Figure 5: Diagram showing G protein activation. Abbreviations: G protein (G), hormone (H), receptor (R), effector (E), inorganic phosphate (P_i). Association of the H and R promotes the exchange of GTP for GDP. The α-subunit of G_s dissociates from the $\beta\gamma$-subunit. Gα-GTP then interacts with the effector. GTPase catalyses the breakdown of GTP and the subunits reassociate. Taken from Gilman (1987). Reproduced, with permission, from the Annual Review of Biochemistry, Vol. 56, © 1987 by Annual Reviews Inc.

activity (Stryer and Bourne, 1986; Gilman, 1987; Birnbaumer, 1990; Schultz et al., 1990).

Bacterial toxins which interfere with G protein function have been used as tools to examine the involvement of G proteins in signal transduction. For example, cholera toxin catalyzes the transfer of the ADP-ribosyl moiety of nicotinamide adenine dinucleotide to the α-subunit of the G_s protein causing persistent activation of the G_s protein through inhibition of its GTPase activity. On the other hand, the GDP bound $\alpha\beta\gamma$ trimer of G_i is ADP ribosylated by pertussis toxin, inactivating G_i (Ui and Katada, 1990).

1.4.2 Signal transduction enzymes

A group of enzymes have been identified as having key roles in signal transduction. In the sections that follow, the reactions that these enzymes catalyze are described. In addition, the effects of the reaction products or second messengers, are outlined.

1.4.2.1 Adenylate cyclase

Many hormones and neurotransmitters, among them adrenocorticotropic hormone, follicle stimulating hormone, luteinizing hormone, glucagon and the catecholamines (β_1 and β_2 receptors), exert their effects, at least in part, via activation of the plasma membrane-bound enzyme adenylate cyclase (see Figure 6) (Harper, 1988; Taylor et al., 1988). The stimulation of adenylate cyclase activity by receptor binding is mediated by the G protein G_s (Robison et al., 1971a,b; Harper, 1988). Adenylate cyclase converts ATP to cyclic 3',5'-adenosine monophosphate (cAMP). cAMP in turn activates protein kinase A (also called cAMP-dependent protein kinase) which phosphorylates target

proteins to exert biological effects. cAMP is converted to inactive 5'-AMP by the enzyme phosphodiesterase (Harper, 1988).

The importance of cAMP in signal transduction was first described by Sutherland and colleagues in studies concerning the effect of epinephrine and glucagon on liver phosphorylase activity (Rall et al., 1957; Robison, 1971a). Following their early reports, cAMP has been shown to be a second messenger for a number of hormones (Harper, 1988; Taylor et al., 1988).

Pharmacological tools are available to study the role of cAMP in the mediation of hormone action. For example, membrane-permeable analogues of cAMP such as dibutyryl cAMP (dbcAMP) may be added in vitro to determine whether they produce the same physiological effect as the hormone of interest. In addition, phosphodiesterase inhibitors such as theophylline and 3-isobutyl-1-methylxanthine may be added to increase the levels of endogenous cAMP by inhibiting its breakdown. If cAMP mediates the action of a particular hormone, then the phosphodiesterase inhibitor should enhance the biological response (Robison et al., 1971b,c; Sheppard et al., 1979).

These tools have been used to test the criteria, originally outlined by Sutherland, which should be met before cAMP can be accepted as a second messenger for a particular hormone. They are: (1) The hormone should stimulate adenylate cyclase activity in broken cell preparations; (2) The hormone should increase the intracellular level of cAMP and this increase should precede or be concurrent with the physiological response; (3) The action of the hormone should be potentiated by the addition of a phosphodiesterase inhibitor; and (4) The physiological effect of the hormone should be mimicked by the addition of cAMP analogues (Robison et al., 1971b).

The primary mechanism of cAMP action is through activation of the enzyme protein kinase A. Protein kinase A phosphorylates a number of target proteins to produce biological responses. Some of the substrates for protein kinase A are enzymes such as hormone-sensitive lipase and phosphorylase kinase which undergo conformational changes upon phosphorylation resulting in a change in their activity. Protein kinase A may also alter the rate of gene transcription by phosphorylating and changing the conformation of a protein bound to DNA called the cAMP response element binding protein (CREB). This results in an increase in gene transcription (Montminy et al., 1990). Other targets for protein kinase A are ion channels which undergo changes in activity upon phosphorylation (Curtis and Catterall, 1985).

There is another group of hormones and neurotransmitters that inhibit adenylate cyclase activity to cause a decrease in cAMP accumulation. The general effect of the decrease in cAMP accumulation is an inhibition of cellular processes such as hormone release. The G protein, G_i, links the activated receptor with adenylate cyclase reducing the activity of the enzyme. Often compounds that inhibit adenylate cyclase activity also have effects on other signal transduction systems (Limbird, 1988).

1.4.2.2 Guanylate cyclase

With the elucidation of cAMP as a second messenger for hormone and neurotransmitter action, other cyclic nucleotides were studied to determine whether they had a role in signal transduction. Cyclic guanosine 3',5'-monophosphate (cGMP) is also a second messenger. It regulates retinal function, blood vessel tone and mediates the action of atrial natriuretic peptide (ANP) (Tremblay et al., 1988; Corbin et al., 1990; Schmidt et al., 1990). cGMP is synthesized from GTP by two different forms of

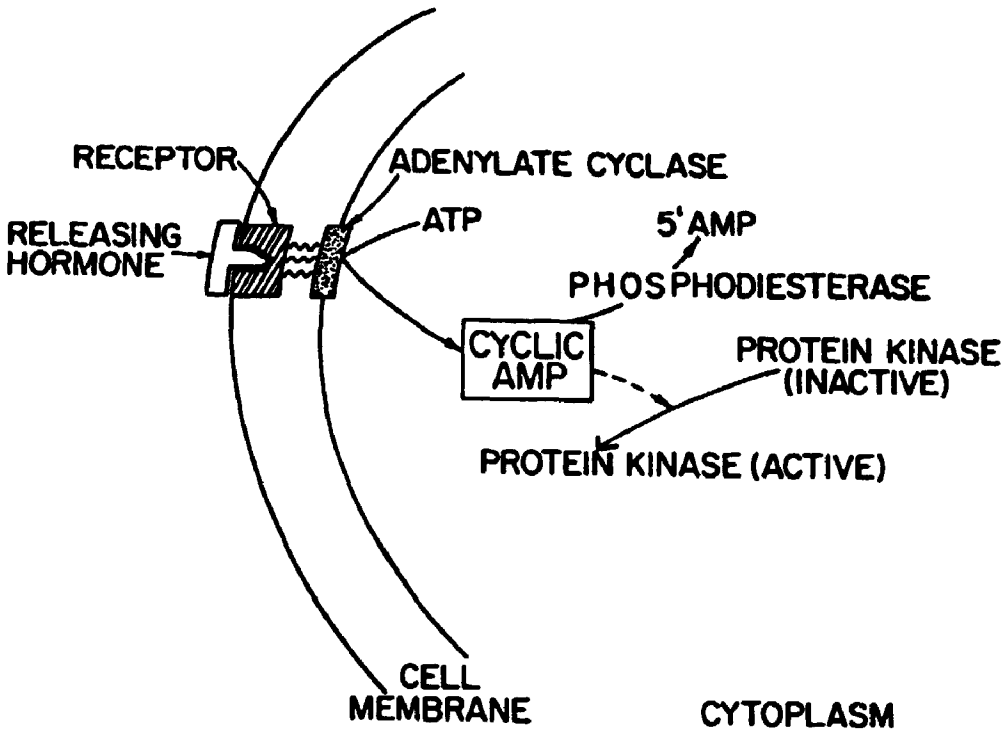


Figure 6: Simplified diagram of the cAMP second messenger system.

guanylate cyclase, a membrane-bound and a soluble heme-containing guanylate cyclase. Different extracellular signals increase the activity of each of these enzymes. For example, it is believed that ANP binds directly to guanylate cyclase on the cell surface to increase the activity of the enzyme. On the other hand, soluble guanylate cyclase activity may be increased by nitric oxide (previously known as endothelium-derived relaxing factor) which diffuses into the cell and binds directly to the enzyme. cGMP is hydrolysed to inactive 5'-GMP by phosphodiesterases (Goldberg and Haddox, 1977; Tremblay et al., 1988; Schmidt et al., 1990; Corbin et al., 1990).

Like cAMP, cGMP acts by binding to target proteins. Examples of these binding proteins are protein kinase G, cGMP binding phosphodiesterase and Na^+ channels in photoreceptors (Tremblay et al., 1988).

1.4.2.3 Phospholipase A_2

A varied group of arachidonic acid derivates, collectively called eicosanoids, are produced through the activation of phospholipase A_2 and have a wide range of biological effects (Shimizu and Wolfe, 1990; Foegh et al., 1991). The eicosanoids may act within the same cell or alternatively may leave the cell and exert effects on neighbouring cells by binding to cell surface receptors. The pathways for arachidonic acid metabolism leading to the production of the eicosanoids are complex (see Figure 7). Arachidonic acid is converted to prostaglandins, thromboxanes, leukotrienes, lipoxins, H(P)ETEs and epoxides. Of interest in stimulus-secretion coupling is the ability of the prostaglandins (PGE_2, PGD and PGI_2) to stimulate adenylate cyclase activity and of $PGF_{2\alpha}$, TXA_2, epoxides and leukotrienes to increase $[Ca^{2+}]_i$. The mechanism of activation of

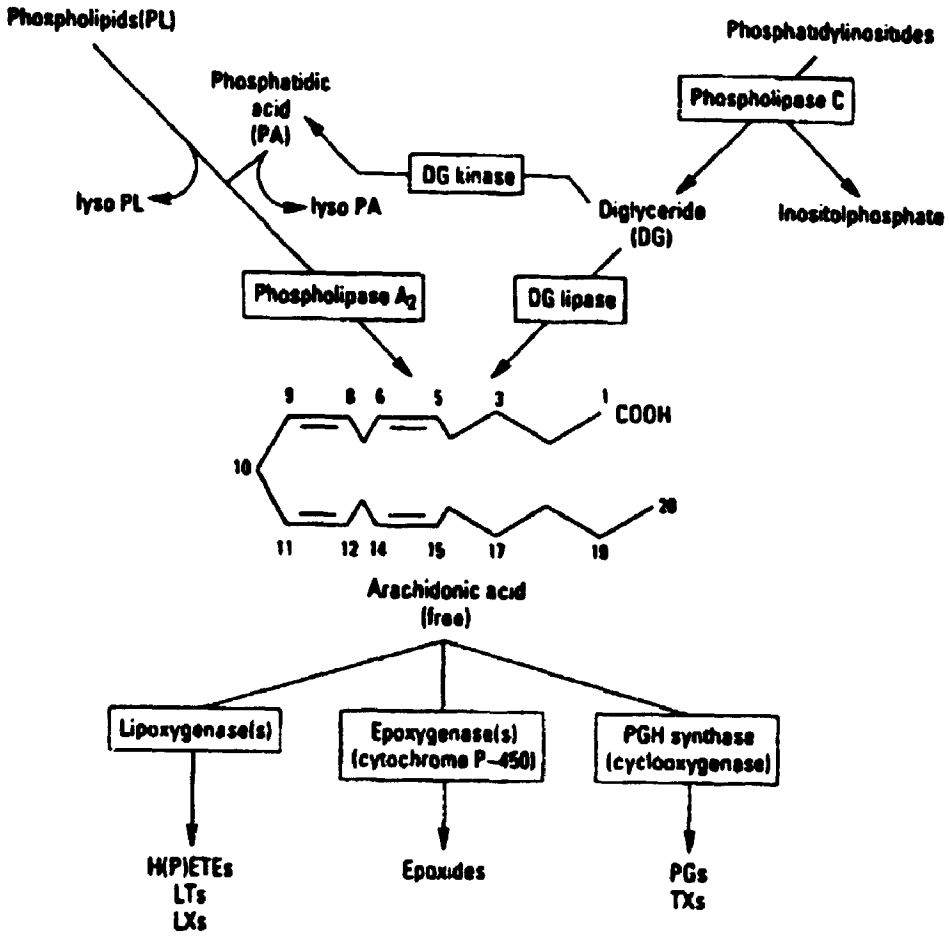


Figure 7: Pathways of arachidonic acid release and metabolism. The two possible sources of aracidonic acid are from the hydrolysis of phospholipids by phospholipase A_2 or from the sequential action of phospholipase C on phosphoinositides and DG lipase on diglycerides. Arachidonic acid can then be converted to H(P)ETEs, LTs and LXs by lipoxygenases, to epoxides by epoxygenases or to PGs and TXs by prostaglandin synthetase. Abbreviations: leukotrienes (LTs), lipoxins (LXs), prostaglandins (PGs), thromboxanes (TXs). Taken from Foegh et al. (1991). Reproduced with permission from Basic and Clinical Endocrinology, 3rd edition, pp. 53-65. (©) 1991 Appleton & Lange

phospholipase A_2 is poorly understood but may involve G proteins (Lapetina and Crouch, 1989; Laychock, 1989; Shimizu and Wolfe, 1990; Foegh et al., 1991).

1.4.2.4 Phospholipase C

The phosphoinositide second messenger system mediates the action of a number of hormones and neurotransmitters including: vasopressin (Creba et al., 1983), thyrotropin-releasing hormone (TRH) (Leung et al., 1982; Drummond et al., 1984; Macphee and Drummond, 1984), gonadotropin-releasing hormone (Raymond et al., 1984; Hirota et al., 1985; Naor et al., 1985a; Quilliam et al., 1985), angiotensin II (Farese et al., 1984; Wang and Chan, 1991), acetylcholine (muscarinic receptors) (Durell et al., 1969; Young et al., 1979; Buck and Fraser, 1990), norepinephrine (α_2 receptors) (Malhotra et al., 1990) and serotonin (Hokin, 1985; Van Rooijen et al., 1985). The binding of these ligands to specific cell membrane receptors activates the enzyme phospholipase C. Phospholipase C hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) producing diacylglycerol and inositol-1,4,5-trisphosphate (IP_3). Diacylglycerol activates the enzyme protein kinase C, and IP_3 mobilizes Ca^{2+} from intracellular stores to increase $[Ca^{2+}]_i$. The phosphorylated proteins, and the rise in $[Ca^{2+}]_i$ subserve a number of biological actions (Nishizuka, 1986; Berridge and Irvine, 1989). This section includes a history of the discovery of this signal transduction system. The components of the phosphoinositide second messenger system are described. In addition, the mechanisms of action of the second messengers, IP_3 and diacylglycerol, are discussed.

1.4.2.4.1 Historical perspective

The characterization of the phosphoinositide second messenger system began in the 1950s with reports from Hokin and Hokin (1953) that acetylcholine stimulated ^{32}P

incorporation into phospholipids in slices of pigeon pancreas. The phospholipid that was preferentially labelled was phosphatidylinositol (PI) along with phosphatidic acid (PA) and this effect of acetylcholine was also observed in brain cortical slices (Hokin and Hokin, 1955, 1956, 1958). The enhanced incorporation of ^{32}P was not accompanied by increased incorporation of labelled glycerol indicating that the diacylglycerol backbone was being reused. These experiments suggested that certain extracellular signals stimulated the turnover of phosphoinositides.

The enzymatic step in the phosphoinositide pathway that was regulated by receptor binding was investigated over a number of years. The first evidence that the site of regulation was phospholipase C came from experiments performed by Durell (1969) who showed that incubation of brain homogenates with acetylcholine for short periods of time led to a decrease in the labelling of PI and an increase in the labelling of PA. These results were confirmed in pancreatic tissue (Hokin-Neaverson, 1974) and subsequently observed for other agonists and in other cells (Michell, 1975; Bell and Majerus, 1980; Igarashi and Kondo, 1980; Hasegawa-Sasaki and Sasaki, 1982). Thus, the binding of extracellular signals was postulated to increase the activity of phospholipase C which would result in a decrease in the labelling of PI and an increase in the labelling of PA. At later times, the increase in flux through the phosphoinositide pathway would result in the increase in ^{32}P incorporation into PI that had been observed in earlier experiments. There were also reports that the polyphosphoinositides might serve as substrates for phospholipase C. For example, Abdel-Latif et al. (1977, 1980) observed that acetylcholine decreased labelling of PIP_2 and increased levels of labelled IP_3 and inositol-4-monophosphate (IP).

The development of techniques for the reliable and rapid separation of the individual inositol phosphates in the early 1980s allowed for the determination of the initial rate of breakdown of the individual phosphoinositides and accumulation of the individual inositol phosphates (Michell et al., 1981; Creba et al., 1983; Hokin, 1985; Danoff and Young, 1987; Downes et al., 1989). PIP_2 breakdown occurred the most rapidly, was detectable within 5 s of stimulation, and reached a new steady state between 50 and 80% of its control value within 1 min. Phosphatidylinositol-4-monophospate (PIP) levels usually were seen to decrease more slowly and PI levels did not normally decrease within the first few minutes. The breakdown of PIP_2 was accompanied by a rapid increase in IP_3 and inositol-1,4-bisphosphate (IP_2) formation. The formation of IP was slower and usually occurred after a lag of 1 min (Creba et al., 1983; Hokin, 1985; Downes et al., 1989). There are reports that PI and PIP may also serve as substrates for phospholipase C (Downes et al., 1989; Shears, 1989). If PI or PIP are hydrolysed then there would be an increase in diacylglycerol without the increase in IP_3 and only one arm of the phosphoinositide second messenger pathway would be activated.

While the effect of extracellular signals on phosphoinositide turnover had been described in the 1950s, the signal transduction function of this system was not determined until the late 1970s and early 1980s. Previously ascribed functions included roles related to membrane dynamics during exocytosis, long term adaptive changes within the cell, the transport of materials across the cell surface, or the function of acetylcholine receptors (Hokin and Hokin, 1958; Durell et al., 1969; Michell, 1975).

In 1975, Michell published a review in which he suggested that phosphoinositide turnover played a role in signal transduction. He believed that the breakdown of PI (at

that time accepted as the primary substrate of phospholipase C) led to an increase in $[Ca^{2+}]_i$ by stimulation of Ca^{2+} influx through the plasma membrane or perhaps from mobilization of intracellular stores. The general concept of the Michell hypothesis has since proven to be correct although some of the details have been modified and the concept has been expanded.

The expansion of this concept began in the late 1970s with the seemingly unrelated report of a novel cyclic nucleotide-independent protein kinase from bovine cerebellum (Takai et al., 1977). This protein kinase, called protein kinase M, was considered to be a proteolytic fragment of a proenzyme called protein kinase C (Inoue et al., 1977). Takai et al. (1979a) reported that protein kinase C could be fully activated with Ca^{2+} and phospholipid, particularly phosphatidylserine and PI. The importance of this enzyme in signal transduction was realized with the observation that diacylglycerol greatly enhanced the reaction velocity and decreased the concentration of Ca^{2+} required for maximal enzyme activation (Kishimoto et al., 1980). It was subsequently shown that protein kinase C occurs in a variety of tissue types throughout the animal kingdom (Kuo et al., 1980). In addition, protein kinase C phosphorylated a number of different cellular proteins suggesting that it might have a number of biological actions (Nishizuka et al., 1979). The characterization of protein kinase C provided evidence that diacylglycerol, formed upon PIP_2 hydrolysis, is a second messenger in signal transduction.

A function for IP_3, the other product of PIP_2 breakdown, in signal transduction was demonstrated by Streb and colleagues in 1983 . They reported that IP_3 released Ca^{2+} from non-mitochondrial intracellular stores in permeabilized rat pancreatic acinar cells. This result was confirmed in other tissues (see below) and provided the missing

link between the phosphoinositide turnover-induced increase in $[Ca^{2+}]_i$ proposed by Michell (1975).

The following general consensus emerged from these experiments. The binding of agonists to their cell surface receptors activates phospholipase C. Phospholipase C hydrolyses PIP_2 to produce diacylglycerol and IP_3. Diacylglycerol activates protein kinase C and IP_3 mobilizes Ca^{2+} from intracellular stores to increase $[Ca^{2+}]_i$. This scheme provided the foundation for further study of the phosphoinositide second messenger system and the sections that follow describe the current knowledge of the components of this signalling system.

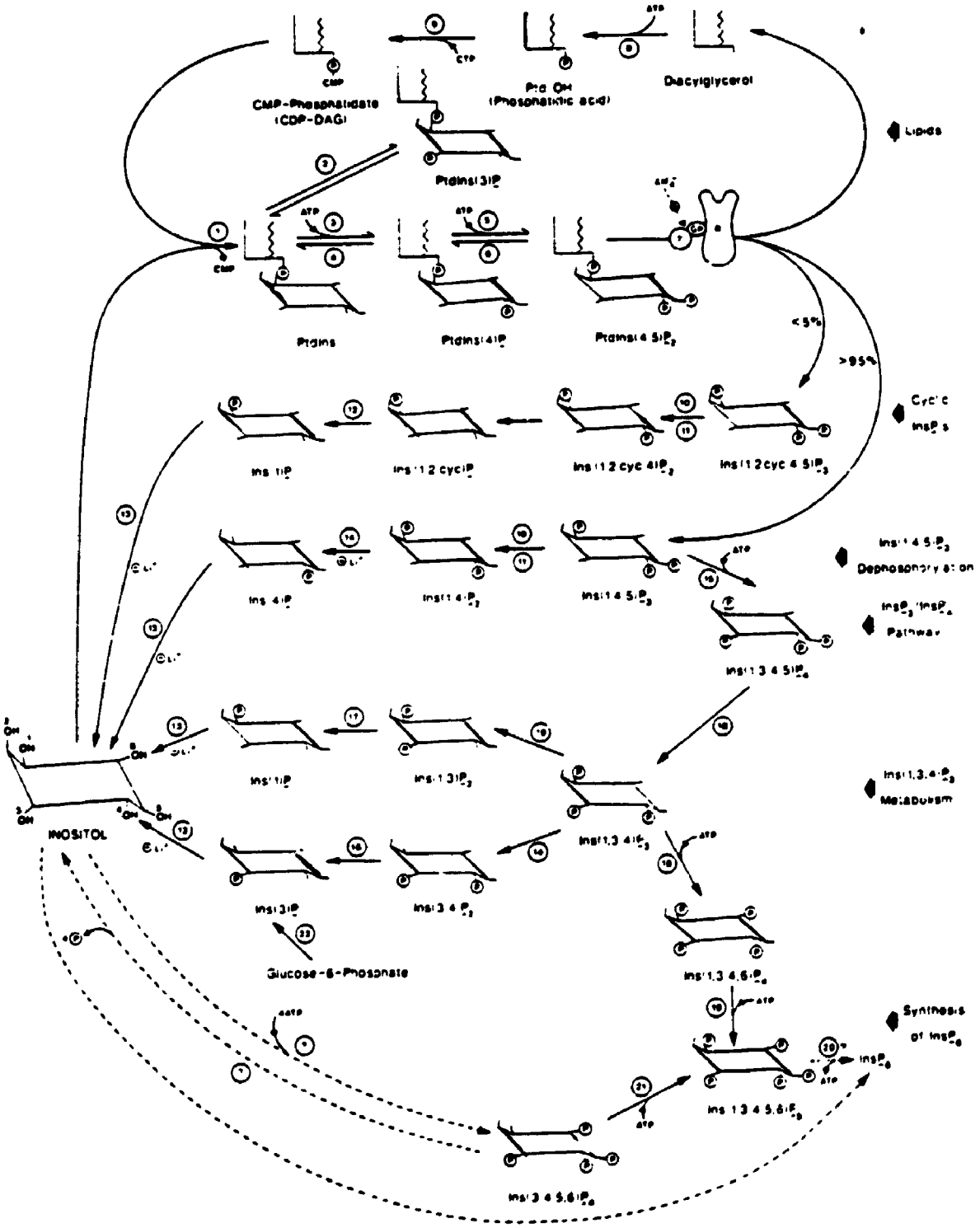
1.4.2.4.2 Metabolism of phosphoinositides and inositol phosphates

The phosphoinositides constitute 2-12% of the total phospholipids of the cell (Michell, 1975). PIP and PIP_2 are found in smaller amounts compared to phosphatidylinositol (PI) and are believed to be localized to the plasma membrane with a small amount of the total cellular PI (Michell, 1975).

PIP and PIP_2 are synthesized from PI by specific kinases that are primarily plasma membrane-bound enzymes. The reactions are bidirectional; the phosphoinositides may be dephosphorylated by specific phosphatases (Figure 8) (Michell, 1975; Majerus et al., 1986; Downes et al., 1989).

As mentioned above, hydrolysis of PIP_2 produces IP_3 and diacylglycerol. Diacylglycerol can then be a source for arachidonic acid or alternatively it, along with the free inositol, can be cycled back into PI. For this to occur, diacylglycerol is first phosphorylated to phosphatidic acid by diacylglycerol kinase. Phosphatidic acid is then primed by interaction with CTP to produce cytidine diphosphate-diacylglycerol (CDP-

Figure 8: Phosphoinositide second messenger system. Summary of known (solid arrows and suspected (dashed arrows) routes of metabolism of compounds containing inositol and phosphate. The enzymes are: (1) PtdIns synthetase; (2) PtdIns-3-kinase (type 1); (3) PtdIns-4-kinase (type II); (4) PtdIns(4)P phosphomonoesterase; (5) PtdIns(4)P-5-kinase; (6) PtdIns(4,5)P_2 phosphomonoesterase; (7) phospholipase C; (8) diacylglycerol kinase; (9) CMP-PA synthetase: (10) Ins(1,4,5)P_3/Ins(1,3,4,5)IP_4-5-phosphatase; (11) Ins(1,4,5)P_3-5-phosphatase; (12) Ins(1:2cyc)P phosphodiesterase; (13) InsP phosphatase; (14) inositol polyphosphate-1-phosphatase; (15) Ins(1,4,5)P_3 kinase; (16) inositolpolyphosphate-4-phosphatase; (17) Ins(1,3)P_2-3-phosphatase; (18) Ins(1,3,4)P_3-6-kinase; (19) Ins(1,3,4,6)P_4-5-kinase; (20) Ins(1,3,4,5,6)P_5-2-kinase; (21) Ins(3,4,5,6)P_4-1-kinase (22) Ins(3)-synthetase. Abbreviations: inositol (Ins) and phosphatidylinositol (Ptd Ins). Taken from Berridge and Irvine (1989). Reprinted by permisson from NATURE vol. 341, pp. 197-205. Copyright (C) 1989 Macmillan Magazines Ltd.


CMP-Phosphatidate
(CDP-DAG)
Pro OH
(Phosphatidic acid)
Diacylglycerol
Lipids
PtdIns(3)P
PtdIns
PtdIns(4)P
PtdIns(4,5)P2
<5%
>95%
Cyclic InsP s
Ins 1 P
Ins(1,2)cyclP
Ins(1,2)cyc 4,5)P2
Ins(1,2)cyc 4,5)P3
Ins 4 P
Ins(1,4)P2
Ins(1,4,5)P3
Ins(1,4,5)P3 Dephosphorylation
InsP3/InsP4 Pathway
Ins(1,3,4,5)P4
Ins 1 P
Ins(1,3)P2
Ins(1,3,4)P3
Ins(1,3,4)P3 Metabolism
INOSITOL
Ins 3 P
Ins(3,4)P2
Glucose-6-Phosphate
Ins(1,3,4,6)P4
Synthesis of InsP6
InsP6
Ins(1,3,4,5,6)P5
Ins(3,4,5,6)P4

diacylglycerol). PI is synthesized from CDP-diacylglycerol and inositol through the action of PI synthetase (Figure 8) (Berridge, 1984).

The I(1,4,5)P_3 isomer of IP_3 is produced by PIP_2 hydrolysis and is the most biologically active inositol phosphate. Another isomer of IP_3, I(1,3,4)P_3, is also found in cells and is formed via metabolism of I(1,4,5)P_3. I(1,4,5)P_3 is dephosphorylated to I(1,4)P_2 by a specific 5-phosphatase which is mostly confined to the plasma membrane and subsequently dephosphorylated to free inositol through a series of enzymatic steps (Majerus et al., 1986; Downes et al., 1989; Shears, 1989). Alternatively, IP_3 is phosphorylated to inositol-1,3,4,5-tetrakisphosphate [I(1,3,4,5)P_4] by I(1,4,5)P_3 kinase. IP_4 may be phosphorylated further to I(1,3,4,5,6)P_5 and IP_6. The inositol phosphates are sequentially dephosphorylated to free inositol through a series of enzymatic steps. A further complication of the pathway is that a family of inositol 1:2 cyclic phosphates, I(1:2 cyc)P, I(1:2 cyc,4)P_2 and I(1:2 cyc,4,5)P_3, with the 1-phosphate cyclized between the 1- and 2-hydroxyl groups also exists (Figure 8) (Majerus et al., 1986; Downes et al., 1989; Shears, 1989).

1.4.2.4.3 Characterization of phospholipase C

There are multiple forms of phosphoinositide-specific phospholipase C. To date, 4 isoenzymes of phospholipase C have been identified and sequenced. There is little homology in the primary structure of these isoenzymes and this makes them immunologically distinct (Rhee et al., 1989; Fain, 1990). Antibodies to the different forms of phospholipase C have been used to show that different forms of phospholipase C may exist together or in separate tissues (Majerus et al., 1986; Rhee et al., 1989; Fain, 1990). For example, in the rat brain, phospholipase C-γ immunoreactivity was

detected in all of the neurons of the brain, while phospholipase C-β immunoreactivity was localized in the striatum, hippocampus, dentate gyrus and thalamic nuclei and phospholipase C-α was confined to astroglia. The isoenzymes of phospholipase C are reported to display different specificities for PIP_2 and have different requirements for Ca^{2+} in vitro. However, it is difficult to relate the in vitro requirements to the intact cell.

A G protein called G_p is proposed to link receptor binding to the activation of phospholipase C (Cronin and Canonico, 1985; Verghese et al., 1985). Indirect evidence for the involvement of a G protein comes from a variety of experiments. Firstly, hormones and neurotransmitters, among them TRH, acetylcholine (muscarinic receptors) and bradykinin, that stimulate phosphoinositide hydrolysis, have been shown to increase GTPase activity (Harden, 1989). Secondly, GTP and analogues stimulate PI hydrolysis when added to permeabilized anterior pituitary cells or to membrane preparations of thymocytes, cerebral cortex or rat 7315C cells (Aub et al., 1987; Litosch, 1987; Limor et al., 1989; Sommermeyer et al., 1989). And finally, pertussis toxin is reported to alter phosphoinositide hydrolysis in some cell types although the effect of this toxin is not ubiquitous (Cockcroft and Stutchfield, 1989; Harden, 1989; Robberecht and Denef, 1990; Tohkin et al., 1990; Diaz-Laviada et al., 1991; Thomas et al., 1991). This suggests that the G_p proteins linked to phospholipase C have different structures in different cell types and that, in some cells, the proteins are similar in structure to the G_i protein that inhibits adenylate cyclase activity (Harden, 1989; Tohkin et al., 1990; Thomas et al., 1991). There are recent reports describing the purification of G_p, but these reports are contradictory and final confirmation will require reconstitution experiments with the

purified G_p, phospholipase C and receptor (Wang et al., 1989; Taylor et al., 1990). Taken together, these results indicate that G proteins are involved in the stimulation of phospholipase C activity (Rhee et al., 1989; Fain, 1990).

The activity of phospholipase C may be modulated by different protein kinases. For example, treatment of intact cells with activators of protein kinase C decreases agonist-induced PIP_2 hydrolysis. The decrease may be due to phosphorylation of phospholipase C or G_p proteins by protein kinase C (Smith et al., 1987; Sortino et al., 1987). In fact, phosphorylation of phospholipase C by protein kinase C has been reported (Rhee et al., 1989). Protein kinase A may also inhibit phospholipase C activity. For example, treatment of some cells with agents that elevate cAMP blocks phosphoinositide hydrolysis (Watson et al., 1984; Rhee et al., 1989). On the other hand, the activity of phospholipase C-γ is increased by tyrosine kinases (Rhee et al., 1989). The action of tyrosine kinases may be by direct phosphorylation of phospholipase C-γ (Wahl et al., 1989). The modulation of phospholipase C activity by these protein kinases suggests that there are complex interactions between different signalling pathways.

There are reports of a limited number of hormones and neurotransmitters that inhibit phospholipase C activity. Often, the mechanism of action of these compounds involves a reduction in $[Ca^{2+}]_i$ and is associated with a decrease in the breakdown of phosphoinositides. For example, dopamine has two effects on anterior pituitary cells. It inhibits the TRH-induced increase in inositol phosphates and reduces influx of Ca^{2+} via voltage-gated Ca^{2+} channels (Enjalbert et al., 1990). The mechanism of phospholipase C inhibition is not understood, but it may involve an inhibitory G protein that would be analogous to the G_i protein in the adenylate cyclase system, or

alternatively, may be secondary to the decrease in $[Ca^{2+}]_i$ (Linden and Delahunty, 1989; Brooks, 1988; Enjalbert et al., 1990).

1.4.2.4.4 Activation of protein kinase C

Diacylglycerol increases protein kinase C activity by increasing the affinity of the enzyme for Ca^{2+}, making it fully active at basal $[Ca^{2+}]_i$. Only diacylglycerols with a 1,2 *sn* configuration can activate protein kinase C and diacylglycerols with an unsaturated fatty acid in position 2 are most potent (Takai et al., 1979b; Kishimoto et al., 1980).

Generally, in the unstimulated state, the majority of protein kinase C activity is found in the cytosol or loosely associated with the plasma membrane so that cell homogenization in the presence of Ca^{2+} chelators leads to recovery of the enzyme in the soluble fraction (Jaken, 1989; Leach and Blumberg, 1989). Activation of protein kinase C in most cases involves a translocation of enzyme activity from the cytosol to the plasma membrane (Leach and Blumberg, 1989; Lussier et al., 1991a). The mechanism behind this translocation is not well understood, but diacylglycerol may stabilize the membrane association of the enzyme so that homogenization in the presence of Ca^{2+} chelators leads to recovery of the enzyme in the membrane fraction of cell homogenates (Leach and Blumberg, 1989). Whatever the mechanism, translocation of protein kinase C from the cytosol to the plasma membrane is an indication of increased protein kinase C activity.

Protein kinase C exists as a family of enzymes (Nishizuka, 1988). Initially, four cDNA clones coding for protein kinase C, α, β_1, β_2 and γ, were isolated. The recombinant proteins were similar to the proteins purified from brain by Huang et al. (1986). Another group of protein kinase C isoenzymes (δ, ϵ and ζ) from cDNA clones

isolated from a rat brain library were subsequently reported (Ono et al., 1987, 1988; Ohno et al., 1988). Diacylglycerol and phospholipid, but not Ca^{2+}, are required for full activation of the second group of protein kinase C isoenzymes. The different forms of protein kinase C have distinctive tissue and cellular localization and, may as a result have different functions (Nishizuka, 1986, 1988; Huang, 1989; Parker et al., 1989; Farago and Nishizuka, 1990; Hannun and Bell, 1990; Bell and Burns, 1991). The pituitary is reported to contain α and β_2 forms of protein kinase C although the second group of protein kinase C isoenzymes was not considered in this study (Naor, 1990).

Although the phosphoinositides were once believed to be the only source of diacylglycerol, it is now thought that there are other pathways for diacylglycerol generation. Diacylglycerol may be produced by a phosphatidylcholine-specific phospholipase C or by the sequential action of phospholipase D and a phosphatidase (Farago and Nishizuka, 1990). Activation of protein kinase C by fatty acids has been reported (McPhail et al., 1984; Seifert et al., 1988). But these experiments were performed with isolated enzyme preparations and it is unlikely that fatty acids are physiological activators for the majority of the protein kinase C isoenzymes (Chauhan et al., 1990).

Protein kinase C may be activated by the application of a diverse group of tumour promoters. These compounds, which include phorbol esters and diesters, teleocidins, bryrostatins, diterpenes and ingenol monoesters, are not carcinogenic but instead increase the incidence of tumour formation in the presence of carcinogens (Blumberg, 1980; Nakamura et al., 1989). Castagna et al. (1982) demonstrated that the phorbol ester, phorbol 12-myristate 13-acetate (PMA), could substitute for diacylglycerol and activate

protein kinase C in human platelets. Subsequently, Niedel and colleagues (1983) showed that the phorbol ester receptor co-purified with protein kinase C. Although these compounds are structurally dissimilar, they share common sites with diacylglycerol allowing them to interact with protein kinase C at the diacylglycerol binding site (Rando, 1988; Nakamura et al., 1989). In addition to the tumour promoters, membrane-permeable diacylglycerol analogues have been developed and these compounds have been used extensively to study the effects of protein kinase C activation on cellular processes (Conn et al., 1986).

While the tumour promoters are valuable tools to study protein kinase C mediated effects, one must be careful interpreting the results from the experiments in which they are used, since they do not have all of the same properties as diacylglycerol. For example, unlike diacylglycerol, the phorbol esters and other tumour promotors are not readily metabolized and therefore remain in the plasma membrane activating protein kinase C for longer periods of time (Blumberg, 1980; Leach and Blumberg, 1989). This prolonged activation of protein kinase C is probably not physiological. Also, prolonged treatment of cells with phorbol esters leads to desensitization to further stimulation by protein kinase C activators (Ballester and Rosen, 1985; Krug et al., 1987; Stabel et al., 1987). Phillips and Jaken (1983) have described this as a 2 stage process. They found that exposure of AtT-20/D_{16} cells to phorbol esters for less than 2 h resulted in a decrease in phorbol ester-induced adrenocorticotropic hormone release. The decrease in responsiveness was not accompanied by changes in the amount of protein kinase C in the plasma membrane as determined by [^{3}H]phorbol dibutyrate binding. On the other hand, treatment of cells with phorbol esters for periods greater that 2 h resulted in a decrease

in [^{3}H]phorbol dibutyrate binding indicating, down-regulation of protein kinase C at these later times. The mechanism of down-regulation is not fully understood, but may involve proteolysis of protein kinase C. It has been suggested that the Ca^{2+}-dependent enzyme, calpain, removes the regulatory domain of protein kinase C producing protein kinase M which, in turn, is rapidly removed by further proteolysis (Adachi et al., 1990; Kishimoto, 1990).

The functions of protein kinase C are diverse and include regulation of receptor binding, secretion, smooth muscle contraction, gene expression, cell proliferation and modulation of ionic conductances (Nishizuka, 1986; Reuter, 1987; Nishizuka, 1988; Maruyama, 1989; Parker et al., 1989; Huang et al., 1990; Weinstein, 1990; Houslay, 1991). This suggests that there are a variety of substrates for protein kinase C including receptor proteins, contractile and cytoskeletal proteins, membrane proteins and enzymes. Some of these proteins have been characterized (Padel and Soling, 1985; Woodgett et al., 1986; Represa et al., 1990; Baudier et al., 1991). The functions may prove to be under the control of different forms of protein kinase C although there is currently little evidence for substrate specificity (Nishizuka, 1986; Nishizuka, 1988; Shearman et al., 1989; Farago and Nishizuka, 1990).

1.4.2.4.5 Functions of inositol phosphates

The mobilization of Ca^{2+} from intracellular stores by IP_3 was first demonstrated in permeabilized pancreatic acinar cells (Streb et al., 1983). Similar observations were then reported for other permeabilized cells and microsomal preparations including microsomal preparations from anterior pituitary cells (Burgess et al., 1984a,b; Brass and Joseph, 1985; Berridge, 1987; Guillemette et al., 1987). Consistent with these

observations, high affinity binding sites for IP_3 have been described for a number of different tissues (Hirata et al., 1985; Baukal et al., 1985; Spat et al., 1986a,b; Nunn et al., 1990). In 1988, Supattapone and co-workers purified and characterized a 260 kDa IP_3 binding protein from rat cerebellum which was functionally reconstituted in lipid vesicles (Ferris et al., 1989). Proteins of similar size have also recently been isolated from rat liver and brain (Mignery et al., 1990; Nunn and Taylor, 1990). The IP_3 receptor has domains that are structurally similar to Ca^{2+} channels, suggesting that the IP_3 binding site and Ca^{2+} channel are part of the same protein (Mignery et al., 1990).

The nature and location of the IP_3-sensitive Ca^{2+} store has not been firmly established. It is unlikely, however, that the mitochondria are sensitive to IP_3 since inhibitors of mitochondrial function do not affect IP_3 function (Berridge, 1987). Consistent with a non-mitochondrial site, early reports suggested that the endoplasmic reticulum was the IP_3-sensitive Ca^{2+} store (O'Rourke et al., 1985; Berridge, 1987, Dunlop and Larkins, 1988). Recently, this has been challenged on the basis of results showing the lack of endoplasmic reticulum enzyme markers in IP_3-sensitive cell fractions (Volpé et al., 1988; Rossier et al., 1991). Volpé et al. (1988) are proponents of this view and have suggested that the IP_3-sensitive organelles stores be called calciosomes. The location, however, remains controversial (Irvine, 1989).

While the most widely accepted function of IP_3 is to cause mobilization of Ca^{2+} from intracellular stores, IP_3 and other inositol phosphates may also cause Ca^{2+} influx (Berridge, 1987; Irvine, 1989). The mechanisms for the increase in Ca^{2+} influx by the inositol phosphates have not been elucidated. In some cells, it may involve IP_4. Irvine and Moor (1986, 1987) observed that IP_4 injection into sea urchin eggs caused the

formation of a fertilization envelope. The effect was dependent on the presence of $I(2,4,5)P_3$ (an inactive inositol phosphate) and extracellular Ca^{2+}. They suggested that IP_4 controlled influx of Ca^{2+} across the plasma membrane. The stimulation of Ca^{2+} entry by IP_3 and IP_4 in combination was also observed for mouse lacrimal cells (Morris et al., 1987). A binding site has been reported for IP_4 in HL-60 cell membranes (Bradford and Irvine, 1987), so it is possible that this compound may have second messenger actions in certain cell types. On the other hand, Putney and colleagues (1989) have hypothesized that the emptying of the IP_3-sensitive store of intracellular Ca^{2+} stimulates Ca^{2+} entry. In their model, empty IP_3-sensitive Ca^{2+} stores are filled with Ca^{2+} that enters from the extracellular space through pores between the plasma membrane and the membrane of the IP_3-sensitive Ca^{2+} store. This Ca^{2+} is then released from the IP_3-sensitive Ca^{2+} stores resulting in an increase in $[Ca^{2+}]_i$.

1.4.3 Ion channel-linked receptors

Ions, particularly Na^+, K^+ and Ca^{2+}, play key roles in cell signalling pathways and in the regulation of cell function. In all cells, a resting membrane potential exists across the plasma membrane such that the inside of the cell is negative with respect to the outside of the cell. The magnitude of the resting membrane potential is dependent on the chemical and electrical gradients across the membrane and on the permeability of the plasma membrane to different ions. Na^+ and Ca^{2+} are found in greater concentrations in the extracellular fluid, while K^+ is found in higher concentrations inside the cell. The chemical gradients favour movement of Na^+ and Ca^{2+} into the cells and K^+ out of the cell, whereas the electrical gradient favours the movement of Na^+, Ca^{2+} and K^+ into the cell. At the resting membrane potential the cell is most permeable to

K^+. Ions move in and out of cytosol of cells via proteins called channels that are embedded in the plasma membrane and in some intracellular organelles. The opening and closing of these channels, which are usually specific for a particular type of ion, result in changes in the membrane potential and/or ion concentration within the cell.

The ion channels that participate in signal transduction may be divided into two major categories ligand-gated and voltage-gated channels. Ligand-gated channels may in turn be divided into primary and secondary ligand-gated channels. Primary ligand-gated channels change in conformation upon binding of the ligand to a receptor site directly on the channel. For example, the binding of acetylcholine to nicotinic receptors causes a conformational change and allows passage of cations through a channel that is part of the receptor protein (Alberts et al., 1989b). On the other hand, secondary ligand-gated channels are gated by an intracellular second messenger or G protein which becomes available following ligand receptor binding (Reuter, 1983; Reuter, 1987).

Voltage-gated ion channels open or close in response to changes in membrane potential. Voltage-gated Na^+ and K^+ channels are essential for the generation and propagation of action potentials. Voltage-gated Ca^{2+} channels are present on a number of cell types and have been shown to be involved in the regulation of secretion of a variety of hormones. Three types of Ca^{2+} channels, L-type, N-type and T-type, have been characterized based on their pharmacology, electrophysiology and distribution (Reuter, 1983; Fox et al., 1987a,b). L-type (long lasting) are distinguished by activation at more positive membrane potentials and longer periods of opening. These channels are blocked by some of the dihydropyridines (e.g. nifedipine), the phenylalkylamines (e.g. verapamil) and the benzodiazepines (e.g diltiazem) (Nicholls, 1986; Triggle, 1990). On

the other hand, the dihydropyridine, Bay K 8644, is an agonist for these channels (Stanfield, 1986). T-type channels (transient) are characterized by activation and inactivation at more negative membrane potentials and faster channel closing (Nowycky et al., 1985; Fox et al., 1987a,b). N-type (neuronal) channels, found in neurons, are activated over a range of potentials between those of L- and T-type channels and are inactivated over a broad range of membrane potentials (Nowycky et al., 1985; Fox et al., 1987a,b; Miller, 1987; Bean, 1990; Tsien and Tsien, 1990).

Like ligand-gated channels, voltage-gated channels may also participate in signal transduction. In the case of voltage-gated channels, receptor activation increases the availability of intracellular messengers which alters channel activity. This alteration in channel activity can occur in different ways. For example, intracellular messengers can modulate the kinetics, voltage dependence or conductance of the ion channels or alternatively can recruit another population of previously inactive channels (Kaczmarek, 1988; Brown and Birnbaumer, 1990).

1.4.3.1 Ca^{2+}

Since Ca^{2+} is a key ion in intracellular signalling and especially in the control of secretion, the regulation of $[Ca^{2+}]_i$ and the mechanisms through which an increase in $[Ca^{2+}]_i$ acts to alter cell function are reviewed in this section.

1.4.3.1.1 Regulation of $[Ca^{2+}]_i$

The concentration of free cytosolic Ca^{2+} in most cells ranges between 1.0 and 2.0 x 10^{-7} M in the basal state (Nicholls, 1986; Cobbold, 1989; Putney et al., 1989; Berridge, 1990; Sanchez-Bueno et al., 1990; Rink, 1990; Tsien and Tsien, 1990). In contrast, the extracellular concentration of Ca^{2+} is much greater (1.2 x 10^{-3} M). Thus,

in the permeability of the plasma membrane to Ca^{2+} result in rapid and relatively large increases in $[Ca^{2+}]_i$ (Reuter, 1991). There are also intracellular stores of Ca^{2+} in the endoplasmic reticulum, calciosomes and mitochondria which can serve as sources and sinks for Ca^{2+}. Some of the Ca^{2+} inside the cell is bound to membranes and to Ca^{2+} binding proteins. The low concentration of $[Ca^{2+}]_i$ is maintained by Ca^{2+}-ATPases, Na^+/Ca^{2+} exchangers and Ca^{2+} uniporters (Nicholls, 1986; Carafoli, 1987, 1989, 1991a,b; Alvarez et al., 1991).

Ca^{2+}-ATPases are present in the plasma membrane and in membranes of certain cellular organelles. They transport Ca^{2+} with high affinity but with low capacity against its electrochemical gradient. The energy for transport is derived from ATP hydrolysis (Reuter, 1991).

Na^+/Ca^{2+} exchangers have large capacity and low affinity and are located on the plasma and mitochondrial membranes. The exchange is bidirectional and electrogenic with a stoichiometry of 3 Na^+ per 1 Ca^{2+}. The exchange is driven by the Na^+ gradient that is established by Na^+/K^+ ATPase. The Na^+/Ca^{2+} exchanger will cause Ca^{2+} influx if the cell membrane is depolarized and Ca^{2+} efflux as the membrane repolarizes (Nicholls, 1986; Carafoli, 1987).

The Ca^{2+} uniporter transports Ca^{2+} into the mitochondria. Under physiological conditions, the uptake of Ca^{2+} into mitochondria has only a minor role in Ca^{2+} homeostasis. But when there is a massive increase in $[Ca^{2+}]_i$ due to cell damage the mitochondria has a high capacity for Ca^{2+} uptake (Nicholls, 1986; Carafoli, 1987).

1.4.3.1.2 Mechanisms of Ca^{2+} action

The intracellular Ca^{2+} signal that is generated upon stimulation is detected by a number of Ca^{2+} binding proteins within the cell. These binding proteins have specific, high affinity binding sites for Ca^{2+} and undergo conformational changes which alter their interaction with other proteins or their catalytic activity in the case of Ca^{2+} binding enzymes (Alberts et al., 1989).

Calmodulin is an example of a Ca^{2+} binding protein which mediates the action of Ca^{2+} in a number of Ca^{2+}-dependent processes (Alberts et al., 1989). For example, the Ca^{2+}-calmodulin complex regulates the activity of cyclic nucleotide phosphodiesterase, adenylate cyclase, calmodulin-dependent protein kinases, phosphorylase kinase, myosin light chain kinase and Ca^{2+}-ATPase (England, 1986; Harper, 1988). Calmodulin is but one of a number of Ca^{2+}-binding proteins and these Ca^{2+} response elements in turn regulate many different cell processes.

To summarize, Ca^{2+} is a second messenger in a number of cell types regulating a variety of cellular functions and its free intracellular concentration is tightly controlled.

1.4.4 Exocytosis

Exocytosis is generally divided into two types, constitutive and regulated (Knight et al., 1989; Maruyama, 1989; De Camilli and Jahn, 1990; Zimmermann, 1990; Miller and Moore, 1991). Constitutive exocytosis occurs continuously and is not initiated by an extracellular stimulus. On the other hand, with regulated exocytosis, secretory granules remain in the cytoplasm until their release has been stimulated by an extracellular signal. The mechanisms involved in exocytosis and its control are poorly understood. The proposed steps for regulated exocytosis are the following: (1) The

secretory vesicle, with fusion proteins in the vesicular membrane adheres to the plasma membrane; (2) The fusion proteins undergo conformational changes following stimulation; (3) A pore forms in the centre of the fusion protein; and (4) The contents of the secretory vesicle escape into the extracellular space (Almers, 1990). There are a variety of candidates for fusion proteins but none have been found in all cell types. It is possible, however, that different fusion proteins exist in different cell types (Burgoyne, 1990).

While the precise mechanisms involved in the regulation of exocytosis await elucidation, it is clear that intracellular second messengers are involved in this release process. The role of Ca^{2+} as a regulator of exocytosis was first described by Douglas (1968) who found that removal of extracellular Ca^{2+} inhibited acetylcholine-induced catecholamine release from chromaffin cells. He hypothesized that an increase in $[Ca^{2+}]_i$ was essential for stimulation of exocytosis in a wide variety of cell types and called the process stimulus-secretion coupling. In fact, a rise in $[Ca^{2+}]_i$ usually accompanies exocytosis and exocytosis is triggered by increases in $[Ca^{2+}]_i$ (Knight et al., 1989). The effect of Ca^{2+} may be mediated by calmodulin or other Ca^{2+} binding proteins (Harper, 1988). Alternatively, Ca^{2+} might bind directly to the fusion proteins thereby altering their conformation to result in hormone release (Almers, 1990).

There is evidence that protein kinase C plays a role in secretion. Activators of protein kinase C alter the Ca^{2+} sensitivity of the secretory process by increasing Ca^{2+} affinity or increasing the extent of release without changing Ca^{2+} affinity (Gomperts, 1986; Knight et al., 1989). cAMP is also involved in the regulation of exocytosis (Knight et al., 1989), either directly, or via the activation of protein kinase A.

G proteins may also be involved in the regulation of exocytosis. In mast cells, GTPγS stimulates exocytosis independently of Ca^{2+} suggesting that an as yet unidentified G protein is directly involved at the membrane fusion step (Maruyama, 1989). GTP analogues also enhance Ca^{2+}-dependent exocytosis (Knight et al., 1989). Gomperts (1990) has called this G protein G_E and postulates that it is involved in the regulation of secretion. In summary, the process of exocytosis is controlled and modulated by different intracellular second messengers, but the precise mechanisms involved in the regulation of exocytosis await elucidation.

1.5 Mechanisms of GRF and SRIF action

The binding of GRF and SRIF to their receptors on the plasma membrane of somatotrophs activates signal transduction systems to respectively stimulate and inhibit the release of GH. In this section, the intracellular mechanisms of GRF and SRIF action are reviewed.

1.5.1 GRF

It is well established that GRF increases cAMP and $[Ca^{2+}]_i$ to stimulate the release of GH. Other possible mechanisms of GRF action are depolarization, stimulation of the eicosanoid pathway and activation of the phosphoinositide second messenger system. The following sections present the evidence for these different systems in the mechanism of GRF action. The review will be confined to the effect of GRF on acute GH release. Other effects of GRF such as stimulation of GH synthesis will not be considered (Gelato and Merriam, 1986).

1.5.1.1 GRF receptors

A single class of high affinity binding sites for GRF on anterior pituitary membranes have been reported. Veliçelbi and co-workers (1985) observed that hpGRF(1-40-OH) binds specifically to bovine anterior pituitary homogenates with a K_D of 3 nM. Seifert et al. (1985) used the GRF analogue [His^1,Nle^{27}]hpGRF(1-32)-NH_2 to demonstrate the presence of specific binding sites on rat anterior pituitary cells (K_D = 41 x 10^{-12}M).

Chemical crosslinking of ^{125}I labelled GRF to rat pituitary cells and bovine pituitary membranes have implicated a 26 kDa protein (Zysk et al., 1986) and a 75 kDa protein (Veliçelebi et al., 1986) as possible receptors for GRF. To date, however, the GRF receptor has not been purified.

1.5.1.2 GRF and adenylate cyclase

cAMP is a second messenger for GRF since: (1) GRF stimulates the activity of adenylate cyclase in anterior pituitary cells (Labrie et al., 1983; Harwood et al., 1984; Schettini et al., 1984; Reyl-Desmars et al., 1985; Giannattasio et al., 1987) and somatotroph membranes (Narayanan et al., 1989); (2) GRF increases cAMP accumulation and the increase precedes the increase in GH release (Cronin et al., 1982; Bilezikjian and Vale, 1983; Michel et al., 1983; Harwood et al., 1984; Schettini et al., 1984; Sheppard et al., 1985); (3) The phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), stimulates GH release and potentiates the release of GH induced by submaximal concentrations of GRF (Spence et al., 1980; Kraicer and Chow, 1982; Harwood et al., 1984; Sheppard et al., 1985); and (4) dibutyryl cAMP stimulates the release of GH from purified somatotrophs (Sheppard et al., 1979; Kraicer and Chow,

1982). G proteins most likely mediate the action of GRF on adenylate cyclase activity since cholera toxin and forskolin both stimulate the release of GH from cultured anterior pituitary cells (Brazeau et al., 1982b). Also, the activation of adenylate cyclase is dependent on the presence of GTP (Spada et al., 1984; Narayanan et al., 1989). Taken together, these experiments demonstrate that cAMP is a second messenger for GRF.

1.5.1.3 GRF and $[Ca^{2+}]_i$

While cAMP mediates the action of GRF, there is considerable evidence to support a role for Ca^{2+} as another second messenger for GRF. Firstly, the Ca^{2+} ionophore A23187 and high concentrations of extracellular K^+, recently shown to increase $[Ca^{2+}]_i$ (Holl et al., 1989b; Lussier et al., 1991a), stimulate the release of GH even though cAMP levels remain unchanged (Kraicer and Spence, 1981; Kraicer and Chow, 1982). Secondly, GRF causes a concentration-related increase in $[Ca^{2+}]_i$, measured by Ca^{2+}-sensitive fluorescent dyes, in dispersed anterior pituitary cells (Schöfl et al., 1987), in somatotrophs identified by reverse haemolytic plaque assay (Holl et al., 1988a) and in purified preparations of somatotrophs (Snyder et al., 1989; Lussier et al., 1991b). And thirdly, prevention of an increase in $[Ca^{2+}]_i$, by incubation of somatotrophs in low-Ca^{2+} medium or the use of calcium channel antagonists (Lussier et al., 1991b), blocks GRF-induced GH release (Lussier et al., 1988). Thus, Ca^{2+} is a second messenger for GRF.

It is likely that GRF increases $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx since removal of extracellular Ca^{2+} inhibits the GRF-induced increase in $[Ca^{2+}]_i$ and GH release (Sheppard et al., 1987). In addition, the Ca^{2+} channel blockers nifedipine and diltiazem inhibit the increase in $[Ca^{2+}]_i$ and the release of GH induced by GRF (Lussier et al.,

1988, 1991b). And finally, GRF stimulates ^{45}Ca uptake by purified rat somatotrophs (Lussier et al., 1991b). These results indicate that the increase in $[Ca^{2+}]_i$ is primarily dependent on Ca^{2+} influx.

Ca^{2+} entry into somatotrophs is principally via voltage-gated Ca^{2+} channels since the increase in $[Ca^{2+}]_i$ and GH release is inhibited by nifedipine and diltiazem, agents known to block L-type Ca^{2+} channels (Sheppard et al., 1987; Lussier et al., 1988, 1991b). The voltage-gated Ca^{2+} channels are opened by decreases in membrane potential. Alternatively, second messengers may modify their characteristics resulting in opening at more negative membrane potentials (Reuter, 1983, 1987).

An effect of GRF on membrane potential has not been consistently observed. Chen et al. (1989), using enriched preparations of rat somatotrophs and electrophysiological techniques, found that hpGRF(1-44) induced a dose-dependent depolarization in 38% of cells recorded in the whole cell mode. hGRF also depolarized dispersed rat anterior pituitary cells as monitored by the voltage-sensitive fluorsecent dye *bis*-[1,3-diethylthiobarbiturate]trimethineoxonol. The depolarization was dependent on the presence of extracellular Na^+ and was observed even in the absence of extracellular Ca^{2+} (Kato and Suzuki, 1989). On the other hand, GRF did not affect ion currents in immunocytochemically identified bovine somatotrophs (Mason and Rawlings, 1988). The authors attributed the failure to observe a change in ionic currents as due to dialysis of cytoplasmic contents needed for GRF action.

1.5.1.4 GRF and phospholipase A_2

While the function of eicosanoids in the mediation of GRF action has not been fully investigated, experiments suggest that some of the eicosanoids have roles, although

they are probably small. Arachidonic acid, PGE_2 and epoxides stimulate the release of GH from anterior pituitary glands, dispersed anterior pituitary cells and purified somatotrophs (Schofield, 1970; Hertelendy, 1971; Sheppard et al., 1979; Dray et al., 1980). In addition, GRF stimulates the release of arachidonic acid and PGE_2 from dispersed anterior pituitary cells (Fafeur et al., 1985; Judd et al., 1985; Canonico et al., 1986). These results suggest that some of the metabolites of aracidonic acid, especially PGE_2, are second messengers for GRF. But they probably play a small role since indomethacin only partially inhibits GRF-induced GH release in some experiments and has no effect in others (Dray et al., 1980; Canonico et al., 1986; Snyder et al., 1989). Also, inhibition of phospholipase A_2 with quinacrine reduces, but does not abolish, GRF-induced GH release. PGE_2 acts, at least in part, via activation of adenylate cyclase (Sheppard et al., 1979; Michel et al., 1983).

1.5.1.5 GRF and phospholipase C

There is some evidence suggesting that GRF also acts by increasing phospholipase C activity. Protein kinase C activity is present in anterior pituitary cells (Turgeon et al., 1984) and activators of protein kinase C stimulate the release of GH from mixed populations of anterior pituitary cells and tumour cell lines (Smith and Vale, 1980; Osborne and Tashjian Jr., 1981; Ohmura et al., 1984; Aizawa and Hinkle, 1985; Barinaga et al., 1985; Ikeda et al., 1985; Summers et al., 1985; Ohmura and Friesen, 1985; Negro-Vilar and Lapetina, 1985; Judd et al., 1986; Ray et al., 1986; Sheppard et al., 1987; Ikuyama et al., 1987). There have been only limited studies concerning the effect of GRF on phospholipase C activity. Raymond et al. (1984) observed that GRF, at 10^{-7} M, did not alter ^{32}P labelling of PI in rat anterior pituitary cell cultures at 20 or

60 min of incubation. Escobar et al. (1986) reported no change in [^{3}H]myoinositol labelling of total inositol phosphates in cultures of rat anterior pituitary cells that were incubated with up to 10^{-5} M GRF for 15 min. In contrast, an increase in ^{32}P labelling of PI was observed in cultured rat anterior pituitary cells following incubation with GRF for 15, 30, and 60 min (Canonico et al., 1983). While these experiments were limited, incomplete and the results not consistent, they prompted us to further examine the role of this system in GRF action (see rationale for the research project, section 1.6).

1.5.2 SRIF

SRIF inhibits basal and GRF-stimulated GH release. SRIF acts by reducing $[Ca^{2+}]_i$ through mechanisms which may involve modulation of voltage-gated Ca^{2+} channels, hyperpolarization and/or a reduction in cAMP accumulation. Many studies concerning the mechanism of SRIF action employ mixed populations of anterior pituitary cells or pituitary tumour cell lines. However, results from these experiments must be interpreted with caution, since other anterior pituitary cell types respond to SRIF and pituitary tumour cells do not always share the same characteristics as normal cells.

1.5.2.1 SRIF receptors

Specific, high affinity binding sites for SRIF on anterior pituitary cell membranes have been demonstrated (Aguilera and Parker, 1982; Enjalbert et al., 1982; Reubi et al., 1982; Srikant and Patel, 1982). The reported binding constants ranged from 0.47 x 10^{-9} M to 8.6 x 10^{-9} M. The binding sites are thought to represent a single class of receptors, although Kimura et al. (1989) reported that there are two types of SRIF binding sites, one of which is expressed by treatment with 17-β-estradiol. The purification and characterization of the SRIF receptor in the anterior pituitary has been

described but the results are not consistent. Lewis and Williams (1987) report that the rat anterior pituitary receptor is a glycoprotein with a molecular weight of 88 kDa. On the other hand, Patel et al. (1990) have identified a 58 kDa protein as the SRIF receptor of the rat anterior pituitary. Therefore, complete characterization of the somatotroph receptor awaits further study.

1.5.2.2 SRIF and adenylate cyclase

SRIF only partially reduces GRF-stimulated adenylate cyclase activity and has no effect on basal adenylate cyclase activity in mixed populations of anterior pituitary cells (Harwood et al., 1984; Schettini et al., 1984; Spada et al., 1984; Reyl-Desmars et al., 1985). A decrease in GRF-induced cAMP accumulation is also observed in these cells (Harwood et al., 1984; Wreggett and Irvine, 1989). In contrast, SRIF, at a wide range of concentrations, did not inhibit basal or GRF-stimulated adenylate cyclase activity in membranes or homogenates prepared from purified somatotrophs (Narayanan et al., 1989). The reasons for the discrepancy are not clear but the effect seen with mixed cell preparations may reflect an effect of SRIF on another cell type. In purified intact somatotrophs, however, GRF-induced cAMP accumulation is modestly reduced by SRIF (Sheppard et al., 1985), suggesting that SRIF may have a small effect on adenylate cyclase which was not detected (Narayanan et al., 1989). SRIF completely blocks GRF-induced GH release even though it only partially reduces cAMP accumulation. In addition, SRIF blocks GH release induced by dibutyryl cAMP, phosphodiesterase inhibitors and Ca^{2+} ionophores (Kraicer and Spence, 1981; Kraicer and Chow, 1982). These observations point to an additional site of action for SRIF, independent of, or distal to cAMP.

1.5.2.3 SRIF and $[Ca^{2+}]_i$

SRIF lowers basal $[Ca^{2+}]_i$ in pituitary tumour cell lines (Schlegel et al., 1984; Koch et al., 1985, 1988; Lewis et al., 1986; Luini et al., 1986; Koch and Schonbrunn, 1988) and in rat somatotrophs (Holl et al., 1988a,b, 1989a; Lussier et al., 1991c). SRIF also inhibits basal and the GRF-induced increase in $[Ca^{2+}]_i$ in rat somatotrophs (Holl et al., 1988a; Lussier et al., 1991c). The decrease in $[Ca^{2+}]_i$ involves a reduction in Ca^{2+} currents through L- and T-type Ca^{2+} channels (Chen et al., 1990a) and a decrease in Ca^{2+} influx in rat somatotrophs measured by ^{45}Ca uptake (Lussier et al., 1991c).

The mechanisms through which SRIF acts to reduce $[Ca^{2+}]_i$ have not been completely elucidated. One possible mechanism comes from the observation that SRIF hyperpolarizes somatotrophs (Israel et al., 1983; Chen et al., 1989,1990b). The increase in resting membrane potential would reduce Ca^{2+} influx through voltage-gated Ca^{2+} channels resulting in a decrease in $[Ca^{2+}]_i$. The effect of SRIF on membrane potential has also been observed for pancreatic β cells (Pace and Tarvin, 1981) and pituitary tumour cell lines (Koch et al., 1988). In these experiments, the hyperpolarization was thought to be due to an increase in K^+ conductance induced by SRIF. Further evidence for a role of increased K^+ conductance in SRIF action comes from experiments in GH_4C_1 cells in which the effect of SRIF on $[Ca^{2+}]_i$ is reduced by increasing the concentrations of extracellular K^+ from 4.6 mM to 50 mM (Koch et al., 1988). Also, SRIF does not inhibit the high K^+-induced increase in $[Ca^{2+}]_i$ or ^{45}Ca influx while markedly reducing the high K^+-induced GH release (Kraicer and Chow, 1982; Lussier et al., 1991a). These results suggest that one of the mechanisms of SRIF action is an increase in K^+ conductance. Recently it has been reported that SRIF may increase K^+ conductance by

increasing the activity of phosphatases within the cells which dephosphorylate K^+ channels causing them to open (White et al., 1991).

1.5.2.4 SRIF and phospholipase C

The few reports concerning the effect of SRIF on the turnover of the phosphoinositides suggest that SRIF does not act by altering the activity of phospholipase C. Bicknell et al. (1979) showed that the acetylcholine-induced increase in the incorporation of ^{32}P into PI in bovine anterior pituitary cells at 60 min of incubation was not effected by SRIF. A similar result was obtained by Leung and colleagues (1982) who found that incubation of rat anterior pituitary cells with SRIF for periods up to 60 min did not alter the TRH-induced increase in the incorporation of ^{32}P into PA and PI. Also, Canonico et al. (1983) showed that SRIF had no effect on basal or hpGRF(1 40)OH-induced ^{32}P labelling in rat anterior pituitary cells measured at 30 min. In a more recent experiment, SRIF did not alter the basal or carbachol-induced increase in [^3H]-inositol labelling of IP, IP_2 and IP_3 that was detected in rat anterior pituitary cells at 30 min of incubation (Schrey and Read, 1988). A similar lack of effect of SRIF on phosphoinositide turnover has been observed for GH_3 cells, canine gastric parietal cells and rat hippocampal slices (Yajima et al., 1986; Park et al., 1987; Eva and Costa, 1987). Although these results suggest that SRIF does not alter phosphoinositide turnover in somatotrophs, further experiments examining the effect of SRIF at shorter incubation periods on individual phosphoinositides and inositol phosphates in purified somatotrophs are necessary.

1.5.2.5 SRIF and a post second messenger site of action

Although one of the mechanisms of SRIF action is a reduction of $[Ca^{2+}]_i$, SRIF may also inhibit GH secretion independently of changes in $[Ca^{2+}]_i$. For example, SRIF inhibits GH release induced by the Ca^{2+} ionophore A23187 (Kraicer and Spence, 1981). Also, SRIF reduces, but does not abolish, the release of GH induced by high extracellular K^+ concentrations (Kraicer and Spence, 1981). In both cases, entry of Ca^{2+} would remain elevated. There are two possible explanations for this effect. Firstly, SRIF may stimulate Ca^{2+} efflux at a rate greater than the rate of Ca^{2+} entry resulting in a net reduction of $[Ca^{2+}]_i$. It is unlikely, however, that this is so since, in the case of high K^+, an increase in $[Ca^{2+}]_i$ is still observed (Lussier et al., 1991c). A second explanation is that SRIF exerts direct effects on the exocytotic process. Luini and De Matteis (1990), using AtT20 cells, have shown that SRIF reduces the release of ACTH induced by Ca^{2+} ionophores and permeabilized cells exposed to buffers of increasing Ca^{2+} concentrations. The effect of SRIF was still observed in cells exposed to cAMP analogues and phorbol esters, but was inhibited by pertussis toxin. Thus, SRIF may also act at a site distal to a reduction in the levels of of second messengers and that this effect may be mediated by a G protein. Gomperts (1990) has suggested that the inhibitory G protein associated with ligand-mediated inhibition of exocytosis be called G_{Ei}.

1.6 Rationale for the research project

In September of 1986, at the start of this research project, there was limited evidence for a role for the phosphoinositide second messenger system in the regulation of the release of GH. Others had shown that protein kinase C activators do stimulate GH release from mixed populations of anterior pituitary cells and from pituitary tumour cell

lines (Smith and Vale, 1980; Osborne and Tashjian Jr., 1981; Ohmura et al., 1984; Barinaga et al., 1985; Ikeda et al., 1985; Negro-Vilar and Lapetina, 1985; Ohmura and Friesen, 1985; Summers et al., 1985; Judd et al., 1986; Ray et al., 1986; Sheppard et al., 1987). The heterogeneity of these cell preparations, however made interpretation of these results difficult since the effect of protein kinase C activators could not be pinpointed directly to the somatotroph. In addition, protein kinase C activators were reported to alter levels of cAMP in different cell types and this must be considered particularly when studying GH secretion (Phillips and Jaken, 1983; Cronin and Canonico, 1985). There were no studies demonstrating the presence of protein kinase C in somatotrophs or determining its distribution between the cytosol and membrane. Also, the effect of GRF on the distribution of protein kinase C activity in mixed populations of anterior pituitary cells or somatotrophs had not been investigated.

Previous studies by others concerning the effect of GRF and SRIF on the turnover of the phosphoinositides were limited, incomplete and the results were not consistent. There had not been a study of the effect of GRF on the individual phosphoinositides (PI, PIP and PIP_2) and inositol phosphates (IP, IP_2 and IP_3). The times of incubation with GRF were limited (greater than 15 min) and may have been inappropriate since activation of phospholipase C occurs very quickly (within one min) following stimulation. Also, the effect of GRF was only studied in mixed populations of anterior pituitary cells and not in purified somatotrophs.

The limited evidence prompted us to investigate the role of the phosphoinositide second messenger system in the regulation of GH release. We hypothesized that the phosphoinositide second messenger system is an additional pathway for GRF in the

somatotrophs of the anterior pituitary (Figure 9). Before the phosphoinositide second messenger system can be accepted as a second messenger system for GRF, the following criteria should be met:

1. GRF should stimulate the activity of phospholipase C.
2. GRF should increase the concentration of IP_3 and diacylglycerol and their increase should precede or be concurrent with an increase in GH release.
3. GRF should stimulate the activity of protein kinase C.
4. Diacylglycerol analogues and other protein kinase C activators should stimulate GH release.
5. IP_3 should stimulate the release of GH.
6. Activation of phospholipase C and/or phosphoinositide turnover stimulated by any means should stimulate GH release. Inhibition of phospholipase C and/or phosphoinositide turnover by any means should inhibit GH release.

For this research project, we examined criteria 2-4 directly, and criterion 1 indirectly. We did not measure phospholipase C activity directly in somatotrophs membrane preparations but instead studied the effect of GRF on the levels of individual radiolabelled phosphoinositides and inositol phosphates for an indirect indication of phospholipase C activity. Criterion 6 was not tested because of the lack of specific stimulators and inhibitors of phospholipase C.

1.7 Questions Addressed

To establish a role for the phosphoinositide second messenger system in the regulation of GH release we asked the following questions:

1) Does activation of protein kinase C stimulate the release of GH from somatotrophs?

   (a) Does protein kinase C activation alter cAMP levels in somatotrophs?

   (b) Does SRIF inhibit the effect of protein kinase C activation?

   (c) What are the dynamics of the GH response to protein kinase C activation?

   (d) Is extracellular Ca^{2+} required for protein kinase C activator action?

2) Does protein kinase C play a role in GRF action?

   (a) Do somatotrophs made unresponsive to diacylglycerol still respond to GRF?

   (b) Do protein kinase C inhibitors alter the GH response to GRF?

   (c) Does GRF activate protein kinase C in somatotrophs?

3) Does GRF stimulate phospholipase C activity in somatotrophs?

To answer these questions we used two in vitro techniques, static incubation and perifusion. In the static incubation system, somatotrophs in suspension were divided into beakers, test substances were added and aliquots of cell suspension were taken for the measurement of cummulative GH released into the medium and for the measurement of intracellular components such as cAMP, phosphoinositides and inositol phosphates, or protein kinase C activity. The somatotrophs were prepared from dispersed male rat anterior pituitary cells using a two stage, discontinuous gradient procedure. With this procedure, a cell preparation containing greater than 94% somatotrophs was obtained. The advantage of this system is that we can attribute changes in intracellular components directly to the somatotroph. In the perifusion system, dispersed anterior pituitary cells from male rats were applied to a column of Biogel. Medium containing test substances

was pumped through the column and fractions of effluent were collected for the measurement of GH. The advantage of this system is that we can study the dynamics and repeatability of the GH response.

The static incubation system was chosen when we wanted to measure the concentration of intracellular metabolites. The amount of GH measured in the static incubation experiments represents the amount of GH that has accumulated throughout the experiment and, as such, does not represent true basal GH release from somatotrophs. Therefore, GH responses above this background GH concentration can not be expressed in terms of this background level. On the other hand, the perifusion system gives a true measure of basal GH secretion and allowed us to determine the secretion rate of GH. Thus, GH responses to secretagogues can be expressed in terms of this basal GH release. The perifusion system was used when we wanted to study the dynamics and repeatability of the GH response.

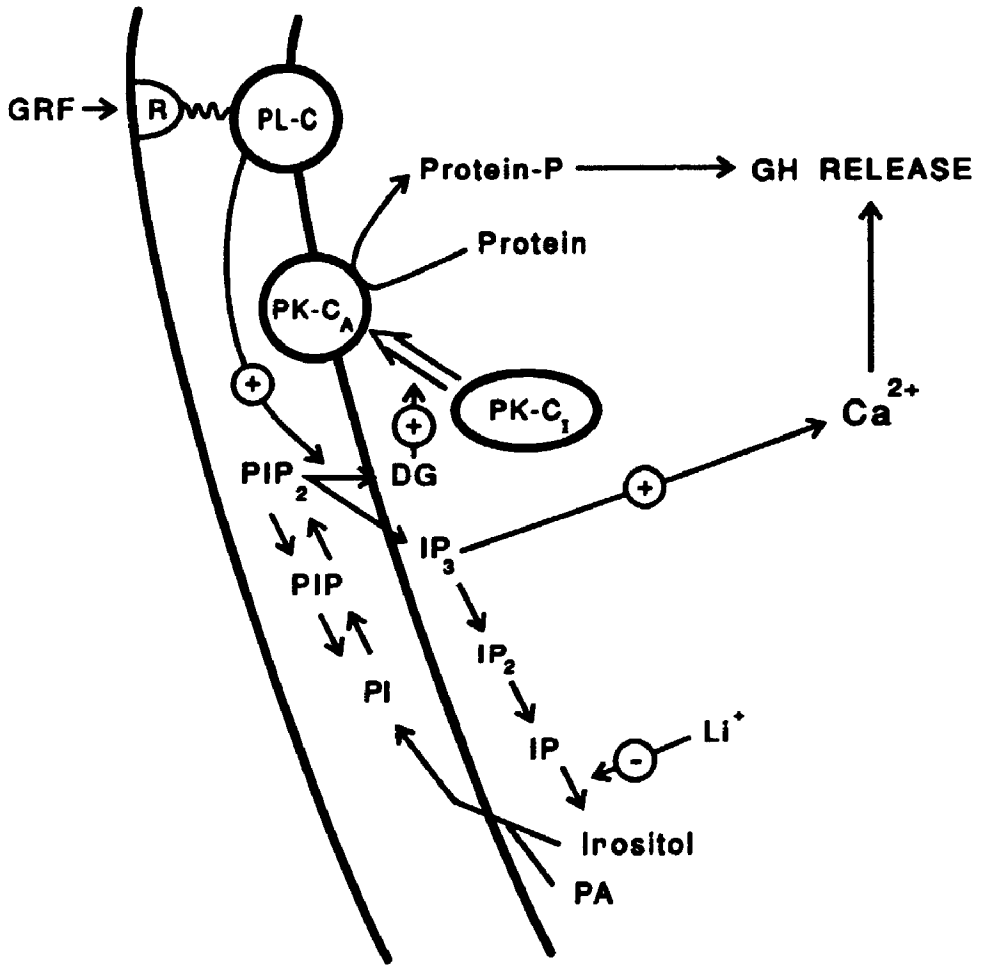


Figure 9: Proposed additional pathway for GRF. Abbreviations: receptor (R), lithium chloride (Li^+), inactive protein kinase C ($PK\text{-}C_I$) and active protein kinase C ($PK\text{-}C_A$).

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Young adult male rats (Charles River, St-Constant, Quebec, Crl:CD(SD)BR), weighing 175-200 g on arrival, were maintained and housed for at least 2 weeks in group cages in a temperature-controlled (25.0 ± 0.5 C) sound-proofed room on a 14 h/10 h light/dark cycle with feeding ad libitum.

2.2 Dispersion of anterior pituitary cells

On the afternoon preceding each experiment, animals were placed in single cages. Rats were killed by decapitation within 20 s of removal from the rat room between 0800-0930 h. The pars nervosa-intermedia was discarded, and the partes distales were minced into small (1-2 mm^3) fragments and dissociated at 37 C for 2.5 - 3.0 h in Minimum Essential Medium (Eagle's; 330-1650, Gibco, Grand Island, NY) containing 0.1% bovine serum albumin (BSA, Bovine Albumin Powder, Cohn fraction V, Armour Pharmaceutical Co. Chicago, IL; dialysed as previously described (Shortman, 1968) and lyophilized), 0.1% trypsin (0152-13-1; 1:250, Difco Laboratories, Detroit, MI) and 28.5 mM $NaHCO_3$ brought to pH 7.35 - 7.40 with humidified 95% O_2 - 5%CO_2.

2.3 Perifusion of dispersed anterior pituitary cells

The dispersed cells (2.5 x 10^6 - 4.0 x 10^6 / rat) were centrifuged at 500 *g* for 10 min and resuspended in 3.0 ml of Medium 199 (400-1200, Gibco) containing 0.1 % BSA and 22.3 mM $NaHCO_3$ (M199A, pH = 7.35 - 7.40 at 37 C) and incubated in a

siliconized 25 ml Erlenmeyer flask for 30 min at 37 C in a Dubnoff incubator-shaker gassed with humidified 95% O_2 - 5% CO_2. After centrifugation (500 *g* for 10 min), the supernatant solution was discarded, the cells were resuspended in M199A, and applied to the perifusion system (Kraicer and Chow, 1982). About 0.5 x 10^6 cells were applied to each column. The perifusion medium was M199A, pH 7.35 - 7.40, gassed with humidified 95% O_2 - 5% CO_2 in a 37 C water bath and the flow rate was 1 ml/min. The first 30 min of effluent was discarded. Fractions were then collected, usually at 1 min (single column) or 2 min (dual column) intervals, immediately frozen on dry ice, and stored at -20 C for subsequent GH assay.

2.3.1 Data analysis of the perifusion studies

To compare GH responses we calculated the extra release (ER) and peak secretion rate (PSR) of the GH responses. ER was the amount of GH above interpolated baselines during application of secretagogue and was calculated on a histogram basis. The interpolated baselines were the means of values over 10 min immediately before the application of secretagogue. PSR during ER were the maximum responses above the interpolated baselines. In the dual column perifusion studies, statistical significance was tested using analysis of variance and covariance with repeated measures and the experiments were performed 4 times. Usually each experiment was performed at least twice.

2.4 Purification of somatotrophs

Two different two stage, discontinuous density gradient centrifugation procedures were used to produce purified preparations of somatotrophs. In the initial experiments,

in which we examined the effect of activation of protein kinase C on GH release and cAMP accumulation, we used density gradients consisting of solutions of bovine serum albumin (BSA). In all subsequent experiments, we purified somatotrophs using gradients containing Percoll. The later method enabled us to increase our final somatotroph yield by 2-3 times without loss of purity.

2.4.1 Bovine serum albumin gradients

We followed the method, with some modifications, of Snyder and Hymer (1975). The dispersed anterior pituitary cells were centrifuged (at 500 *g* for 10 min at room temperature), resuspended in 1.5 ml of Medium 199 containing 1 % BSA and 28.5 mM $NaHCO_3$ (pH = 7.35 - 7.40 at 4 C) and applied to the first gradient. The gradients consisted of solutions of BSA in unbuffered balanced salt solution (UBSS; 137.0 mM NaCl, 4.5 mM KCl, 4.5 mM $CaCl_2$, 1.0 mM KH_2PO_4 and 1.0 mM $MgSO_4$ [Gradient 1: layer 1 = 25.5% BSA; layer 2 = 35% BSA; Gradient 2: layer 1 = 28% BSA; layer 2 = 35% BSA]. To prepare the gradients, 1.5 ml of 35% BSA was placed at bottom of 13 x 51 mm Ultra Clear centrifuge tubes (Beckman, Palo Alto, CA) and 1.5 ml of 25.5% BSA (Gradient 1) or 28% BSA (Gradient 2) was layered on top. Finally the cells were applied on top of the BSA layers. No more than 3×10^7 cells were applied to each gradient. The gradients were centrifuged at 2000 *g* for 40 min. The cells obtained from the first gradient were washed with 10 ml of phosphate buffered saline (PBS; 137 mM NaCl, 8 mM $NaHPO_4$, 1.5 mM KH_2PO_4, 2.5 mM $MgCl_2$ and 2.7 mM KCl, pH = 7.40 at 25 C) and resuspended in M199 containing 1% BSA (pH = 7.35 - 7.40 at 4 C) before being applied to the second gradient. In both cases, the cells at the interface of the two

BSA layers were collected. The final cell yield was 8 - 10% of initial cell yield. All of the purification procedures were performed at 4 C.

2.4.2 Percoll gradients

After the experiments described in section 2.5.1, we changed to Percoll gradients to purify somatotrophs. The dispersed cells were centrifuged, resuspended in 3 ml of M199 containing 20 mM HEPES, 0.1% BSA and 4.2 mM $NaHCO_3$ adjusted to a pH of 7.35 - 7.40 at room temperature with NaOH (M199AH/RT) and placed at the bottom of siliconized COREX test tubes (8441, Corning Glass Works, Corning, NY). The gradient layers were then applied beneath the cells using a syringe fitted with PE160 tubing. The gradients consisted of Percoll (17-0891-01, Pharmacia, Uppsala, Sweden):M199 10x (330-1181, Gibco) containing 200 mM HEPES and 42 mM $NaHCO_3$ (9:1, v/v) that was diluted to the appropriate density with M199 - 20 mM HEPES - 4.2 mM $NaHCO_3$ (pH 7.35 - 7.40 at room temperature) [Gradient 1: layer 1 = 1.069 g/cm^3 (53.7% v/v); layer 2 = 1.086 g/cm^3 (67.7% v/v); Gradient 2: layer 1 = 1.076 g/cm^3 (58.8% v/v); layer 2 = 1.086 g/cm^3]. The densities of the solutions were measured by weighing a 1 ml volume of solution dispensed with an Eppendorf repeater pipette using a previously calibrated Eppendorf Combitip. The densities were adjusted when necessary. No more than 3 x 10^7 cells were applied to each gradient. The gradients were centrifuged at 2,000 *g* for 30 min at room temperature. The cells obtained from the first gradient were washed with 10 ml of M199AH/RT and resuspended in 3 ml of M199AH before being applied to the second gradient. In both cases the cells at the interface of the two Percoll layers were collected. The final cell yield was 20 - 25% of the initial cell yield.

2.4.3 Determination of homogeneity of somatotroph preparation

The dispersed cells and purified somatotrophs were examined to determine the purity of the cell preparation. Slides of cells were prepared by centrifugation of cells in suspension at 1500 rpm for 5 min using a cytocentrifuge (Shannon Elliot Inc., Pittsburg PA). Control slides were prepared from cross sections of anterior pituitaries from lactating female rats. Lactating female rats were used to increase the number of mammotrophs to ensure that the stain differentiated mammotrophs and somatotrophs. The slides were fixed and stained with Herlant's Tetrachrome using techniques previously described with some modifications (Kraicer et al., 1967). A description of the modified procedure is found in Appendix 1.

2.5 Static incubation

2.5.1 Static incubation with protein kinase C activators

We first tested the effect of activation of protein kinase C on GH release and cAMP accumulation. We used the diacylglycerol analogue, dioctanyl-rac-glycerol (diC_8; D1270, Sigma, St. Louis, MO), and the phorbol ester, phorbol 12-myristate 13-acetate (PMA; P8139, Sigma), to activate protein kinase C. We also examined the effect of somatostatin (SRIF; 14 aa, S9129, Sigma) on the GH and cAMP responses to these protein kinase C activators. The somatotrophs (purified using BSA gradients) were resuspended in M199A and incubated in a 25 ml siliconized Erlenmeyer flask for 30 min at 37 C in a Dubnoff incubator-shaker and gassed with humidified 95% O_2 - 5% CO_2. Following centrifugation and resuspension in M199A, aliquots of cells in suspension, 2.5 x 10^5 - 5.0 x 10^5 cells/2.0 ml/beaker, were transferred to Teflon beakers and incubated

in a Dubnoff incubator-shaker at 37 C, gassed with humidified 95% O_2 - 5% CO_2. After 10 min, diC_8 (3 x 10^{-6} M to 10^{-4} M) or PMA (10^{-10} M to 10^{-7} M) was added. Aliquots of suspended cells (500 μl) were removed from the beakers at 4, 8, 16, and 32 min and transferred to siliconized Pyrex test tubes. An aliquot of the sample was immediately removed and centrifuged for 15 s at 9,250 *g*. A sample of the supernatant solution was removed to test tubes containing 1 or 2 ml of PBS - 1% BSA - 25 mM EDTA (pH = 7.6 at 25 C), and stored at -20 C for subsequent GH assay. The rest of the cell sample was frozen in a dry ice/alcohol bath and stored at -80 C for cAMP assay.

We also examined the effect of SRIF on protein kinase C activator-induced GH release and cAMP accumulation. "Start" samples were taken 5 min before the addition of SRIF (10^{-9} M and 10^{-7} M) and PMA (10^{-8} M) or diC_8 (10^{-5} M) was added 10 min after the addition of SRIF. Samples of cells in suspension were withdrawn at 4, 8 and 16 min for diC_8 experiments and at 8, 16 and 32 min for PMA experiments and processed as described above for the measurement of GH and cAMP.

2.5.2 Static incubation in low-Ca^{2+} medium

To examine the role of extracellular calcium in protein kinase C activator-induced GH release and cAMP accumulation, we compared the GH and cAMP responses to protein kinase C activation in the presence and absence of Ca^{2+} in the incubation medium. Somatotrophs were purified using Percoll gradients as described in section 2.4.2 except that the normal M199 was replaced by a specially prepared low-Ca^{2+} M199. The low-Ca^{2+} medium was prepared from a supply of M199 (Hank's salts free, 880122 AG, Gibco) to which we added Hank's salts without $CaCl_2$. The normal Ca^{2+} containing M199AH used in this experiment was prepared from the same stock M199 (Hank's salts

free) to which Hank's salts (including $CaCl_2$) were added. Following purification, the somatotrophs were divided into two equal volumes, centrifuged, resuspended in M199AH/37 (pH = 7.35 -7.40 at 37 C) or low-Ca^{2+} M199AH (pH = 7.35 -7.40 at 37 C). The cells were then incubated in two siliconized 25 ml Erlenmeyer flasks for 30 min at 37 C in a Dubnoff incubator-shaker. The cell suspensions were centrifuged (500 *g* for 10 min), resuspended in M199AH/37 or low-Ca^{2+} M199AH and then transferred to Teflon beakers (5.0 x 10^5 cells/2 ml/beaker). The cells were incubated at 37 C for 10 min before the addition of the protein kinase C activators, diC_8 (3 x 10^{-5} M) or PMA (10^{-8} M). Samples of cell suspension were taken and processed as described in section 2.5.1 for the measurement of GH and cAMP.

2.5.3 Static incubation with nifedipine

A second approach used to examine the role for extracellular Ca^{2+} in protein kinase C activator-induced GH release and cAMP accumulation was to study the effect of nifedipine (a Ca^{2+} channel antagonist) on the GH and cAMP responses to the protein kinase C activators. The somatotrophs were purified using Percoll gradients and incubated in two 25 ml siliconized Erlenmeyer flasks at 37 C for 30 min in a Dubnoff incubator-shaker. Following centrifugation (at 500 *g* for 10 min) and resuspension in M199AH/37, aliquots of somatotrophs in suspension were transferred to Teflon beakers (5.0 x 10^5 cells/2 ml/beaker) and incubated at 37 C for 10 min. Nifedpine (10^{-6} M, N7634, Sigma) was added 10 min before the addition of diC_8 (3 x 10^{-5} M) or PMA (10^{-8} M). Samples of cell suspension were taken from the beakers at 8, 16 and 32 min for the measurement of GH and cAMP as previously described (see section 2.5.1). These experiments were performed in subdued light since nifedipine is light-sensitive.

2.5.4 Static incubation with protein kinase C inhibitors

Protein kinase C inhibitors have been used to examine the role of protein kinase C in a number of different systems. A disadvantage of this approach is that the protein kinase C inhibitors also inhibit protein kinase A (Hidaka et al., 1984; Ruegg and Burgess, 1989). We decided to test two different protein kinase inhibitors, staurosporine and HA1004, to determine whether one would preferentially block protein kinase C while the other would preferentially block protein kinase A as assessed by examining their effects on diC_8- and dbcAMP-induced GH release. If these compounds preferentially blocked diC_8- and dbcAMP-induced GH release, we would then study their effect on GRF-induced GH release to determine the relative roles of protein kinase C and protein kinase A in GRF-induced GH release. (Staurosporine: K_i for protein kinase C = 0.00076 μM, K_i for protein kinase A = 0.00700 μM; HA1004: K_i for protein kinase C = 40.0 μM, K_i for protein kinase A = 2.3 μM; (Hidaka et al., 1984)). We first tested the specificity and efficacy of the protein kinase inhibitor, HA1004 (12804, Seikugaku America Ltd., St. Petersburg FL). Somatotrophs were purified using Percoll gradients and incubated in two 25 ml siliconized Erlenmeyer flasks in a Dubnoff incubator-shaker for 30 min at 37 C. Following centrifugation and resuspension in M199AH/37, aliquots of cells in suspension were transferred to 2 ml polystyrene beakers (2.0×10^5 cells/0.9 ml/beaker) and incubated for 10 min in a Dubnoff incubator-shaker at 37 C. HA1004 (10^{-7} M to 10^{-4} M) was then added and dibutyryl cyclic AMP (10^{-3} M; dbcAMP; N^6,2'-O-dibutyryladenosine 3':5' cyclic monophosphate; D0627, Sigma) or diC_8 (5×10^{-5} M) was then added 15 min later. Aliquots of cells in suspension (350 μl)

were withdrawn from the beakers at 20 and 40 min and processed as described in section 2.5.1 for the measurement of GH.

Another approach to test HA1004 was to examine its effect on GH release induced by endogenously generated cAMP. We used 3-isobutyl-1-methylxanthine (IBMX; Aldrich Chemical Company Inc., Milwaukee, WI) which inhibits the breakdown of cAMP by inhibiting phosphodiesterase and causes an increase in cAMP levels and the release of GH. HA1004 (10^{-7} M to 10^{-4} M) was added 30 min before IBMX (0.1 and 0.5 mM) and samples of cells in suspension were taken 30 and 60 min later for the measurement of GH as described in section 2.5.1.

2.5.5 Data analysis of the static incubation studies

Each secretagogue was tested in 4-5 beakers. Differences among groups in the static incubation studies were tested using analysis of variance and Duncan's multiple range test. Experiments were repeated at least twice.

2.6 Phospholipase C

The purpose of this series of experiments was to examine the effect of GRF on the activity of phospholipase C in somatotrophs. Phospholipase C activity was assessed by examining the changes in levels of radiolabelled phosphoinositides (PIP_2, PIP and PI) and inositol phosphates (IP_3, IP_2 and IP) following GRF. In preliminary experiments, we incubated somatotrophs with [^{3}H]-inositol to label the phosphoinositides and inositol phosphates. We were unable to detect labelling of PIP_2, IP_3 or IP_2 with [^{3}H]-inositol so we then switched to ^{32}P-orthophosphate for labelling (see Appendix 2 for details regarding the [^{3}H]-inositol experiments). Incubation of somatotrophs with ^{32}P-

orthophosphate allowed for detectable labelling of all of the phosphoinositides and inositol phosphates. The method that we used in the subsequent GRF experiments follows.

2.6.1 Labelling of phosphoinositides and inositol phosphates with ^{32}P-orthophosphate

The somatotrophs (purified using Percoll gradients) were centrifuged (500 *g* for 10 min), resuspended in M199AH-phosphate free (88-0122AG, Gibco) (pH 7.35 - 7.40 at 37 C) containing 0.1 mCi/ml of ^{32}P-orthophosphate (PBS-13A, Amersham Corporation, Arlington Heights, IL; 10 mCi/ml), and incubated in a 25 ml siliconized Erlenmeyer flask for 4 h at 37 C in a Dubnoff incubator-shaker. The somatotrophs were then washed twice with 10 ml of M199AH-phosphate free and resuspended at a concentration of 1.0 - 1.5 x 10^6 cells/ml in M199AH-phosphate free containing 5 mM LiCl. LiCl was included to inhibit the conversion of IP to inositol (Berridge and Irvine, 1989). Cells were distributed in 1 ml aliquots to Teflon beakers and incubated for 15 min at 37 C in a Dubnoff incubator-shaker before the addition of GRF. Rat GRF (rGRF; synthetic, 8068, Peninsula Laboratories, Belmont, CA) (10^{-7} M) or an equal volume of vehicle (1.0 mM ascorbic acid -0.1% BSA - 10 mM acetic acid) was added for 0.33, 1, 3, 10, 30, or 90 min before the addition of 4 ml of chloroform:methanol (1:2, v/v). The number of somatotrophs per cell preparation was limited, so only three incubation times were used in a given experiment. The cell lysate was mixed, sonicated, and centrifuged (at 1000 *g* for 10 min). Chloroform (1 ml) and 0.1N HCl (1 ml) were added to the supernatant solution to extract the phosphoinositides and inositol phosphates as previously described with some modifications (Martin, 1986; Sortino et al., 1988; Naor et al., 1986; Bonvini et al., 1987). The aqueous layer was removed and the

chloroform layer was washed with an equal volume of methanol:0.1N HCl (1:1, v/v). The aqueous and chloroform layers were dried under vacuum (Savant Speed Vac Concentrator, Farmingdale, NY) and stored at -20 C for further processing. In some experiments, a 10 μl sample of cell suspension was withdrawn from the Teflon beakers 10 s before the addition of chloroform:methanol (1:2, v/v) for the determination of GH in the medium. The sample was placed in microfuge tubes containing 1 ml of M199AH-phosphate-free, mixed, and centrifuged at 9,250 *g* for 15 s. The supernatant was stored at -20 C for GH assay.

2.6.2 Separation of phosphoinositides

The chloroform layers were reconstituted in chloroform:methanol:water (20:9:1, v/v/v) containing 100 μg of a mixture of phosphoinositides (P 6023, Sigma) and applied to silica gel G-plates (LK5DF, Whatman, Clifton, NJ) that had been dipped in 2.5% potassium oxalate and heat-activated for 1 h at 110 C. The mixture of phosphoinositides was added to act as a carrier and to aid in the identification of the phosphoinositides. In addition, L-α-phosphatidylinositol-4,5-bisphosphate (P9763, Sigma), L-α-phosphatidylinositol-4-monophosphate (P9638, Sigma) and L-α-phosphatidylinositol (P2517, Sigma) were run concurrently on separate lanes to identify the phosphoinositides and to ensure clean separation of the individual phosphoinositides. The plates were developed in chloroform:methanol:acetone:acetic acid:water (40:13:15:12:8, v/v) and visualized under ultraviolet light after spraying the plates with 1 mM 2-p-toluidinyl-naphthalene-6-sulfonic acid (T8753, Sigma) in 50 mM Tris base (T1503, Sigma) (Martin, 1986; Sortino et al., 1988; Raymond et al., 1984; Bonvini et al., 1987) (see Appendix 3 for details regarding the characterization of the thin layer chromatography). The

appropriate areas were scraped into scintillation vials containing 1 ml of water and 10 ml of ACS II (196295, Amersham) and radioactivity was determined by liquid scintillation spectroscopy.

2.6.3 Separation of inositol phosphates

The aqueous layers were reconstituted in 1 ml of 0.5 M perchloric acid containing a tritiated mixture of IP, IP_2, and IP_3 (0.081 μCi/ml, 1 Ci/mmol, TRK.882, Amersham). The samples were brought to a pH of 6.0 - 7.0 with 2 M K_2CO_3, mixed, and centrifuged (9,250 *g* for 2 min). To remove nucleotides, 20 mg of charcoal in 50 μl of water (Darco G-60, D127-500, Fisher Scientific, Fair Lawn, NJ) was added to the supernatant, samples were mixed, refrigerated for 5 min, and then the charcoal was removed by centrifugation at 9,250 *g* (Beckman, Microfuge B) for 2 min (Meek, 1986; Daniel et al., 1987). The selectivity of this method was tested by measuring the absorbance (λ = 259 nm) of solutions containing 0.2 M ATP (A5394, Sigma), ADP (A5410, Sigma), AMP (A1877, Sigma) and cAMP (A9501, Sigma). The charcoal treatment removed 100% of the ADP, AMP and cAMP and 95% of the ATP, while 95% of the tritiated inositol phosphate mixture remained in solution. Details regarding the charcoal treatment method are given in Appendix 4. The charcoal-treated samples were applied to 1 ml Dowex columns (AG 1-X8, formate form, mesh 200-400, BioRad, Richmond, CA), washed with water and 5 mM disodium tetraborate/60 mM sodium formate, and IP, IP_2 and IP_3 were eluted with 0.15 M, 0.6 M and 1.0 M ammonium formate in 0.1 M formic acid (Martin, 1986; Downes and Michell, 1981; Bonvini et al., 1987). Appendix 5 describes the characterization of the column chromatography. The effluent from the first 5 ml of 0.15 M ammonium formate in 0.1 M formic acid was collected in 0.5 ml fractions while

the rest of the effluent was collected in 1 ml fractions. The radioactivity in each fraction was determined by liquid scintillation spectroscopy.

2.6.4 Data analysis of the phospholipase C studies

The radioactivity in PIP_2, PIP and PI was normalized for the amount of radioactivity that was applied to each lane on the plates. The ^{32}P radioactivity in IP, IP_2 and IP_3 was calculated by summing the dpm in the fractions containing the tritiated IP, IP_2 and IP_3 standard and the values were normalized for the amount applied to each column. The GRF values were expressed as a percentage of control. The mean and SEM of these percentages from all of the experiments were calculated for each time point. Differences between control and GRF values were calculated using a Student's t-test. Differences were considered significant for $P < 0.05$.

2.7 Protein kinase C

The purpose of this series of experiments was to examine the role of protein kinase C in GRF action by directly measuring protein kinase C activity in somatotrophs following GRF treatment.

2.7.1 Static incubation with rGRF and protein kinase C activators

The somatotrophs were resuspended in M199AH/37 and incubated in two 25 ml siliconized Erlenmeyer flasks for 30 min at 37 C in a Dubnoff incubator-shaker. After centrifugation and resuspension in M199AH, aliquots of cells in suspension (3 - 6 x 10^6/aliquot) were transferred to microfuge tubes and incubated at 37 C in a Dubnoff incubator-shaker 10 min prior to the addition of rGRF. rGRF (10^{-7} M) or vehicle was added for 0.33, 1, 3, 10, 30 or 90 min. The microfuge tubes were then centrifuged for

15 s at 9,250 *g* (Beckman Microfuge B) and the supernatant was removed and stored at -20 C for GH assay. In addition to the GRF experiments, we also examined the effect of 3 min of incubation with PMA (10^{-8} M) or diC_8 (5×10^{-5} M) on protein kinase C activity to ensure that protein kinase C could be activated in these experiments. Protein kinase C in soluble and particulate fractions was partially purified using procedures previously described with some modifications (McArdle and Conn, 1986; Zatz et al., 1987; Drouva et al., 1990). The cell pellet was resuspended in 3 ml of Buffer A (20 mM Tris-HCl, 0.32 M sucrose, 2 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 0.01% leupeptin, pH = 7.5 at 4 C). The cells were homogenized by 35 strokes of a Potter-Elvehjem homogenizer set at 1000 rpm. The cell lysate was centrifuged at 100,000 *g* for 30 min at 4 C (TL1000, Beckman). The supernatant solution (soluble fraction) was removed and retained. The pellet was washed with 3 ml of Buffer A, resuspended in 3 ml of Buffer A containing 1% Nonidet (N3516, Sigma), and dispersed with 3 strokes of a Potter-Elvehjem homogenizer set at 1000 rpm. The suspension was mixed at 4 C for 45 min and then centrifuged at 100,000 *g* for 10 min. The supernatant solution (solubilized particulate fraction) was retained.

2.7.2 Partial purification of protein kinase C

The soluble and particulate fractions were purified using 1 ml DEAE-cellulose columns (DE 52, Whatman, Maidstone, UK) that had been equilibrated with Buffer B (20 mM Tris-HCl, 0.32 M sucrose, 50 mM 2-mercaptoethanol, pH = 7.5 at 4 C). The gel was prepared as follows. 10 g of gel was suspended in 110 ml of 0.1 M Tris HCl and mixed for 20 s at 3 min intervals for 15 min. The gel mixture was centrifuged at

500 *g* for 3 min and redispersed in 50 ml of Buffer B. The gel was recentrifuged and resuspended in 50 ml of Buffer B. This wash was repeated twice. Finally, the gel was resuspended in 44 ml of Buffer B. The soluble and particulate fractions were applied to the columns and washed with 10 ml of Buffer B and 3 ml of Buffer B containing 0.02 M NaCl. Protein kinase C was eluted with 2 ml of Buffer B containing 0.1 M NaCl. The purification procedure was performed at 4 C.

2.7.3 Protein kinase C assay

Protein kinase C was assayed by the determination of the rate of transfer of ^{32}P from [$\gamma^{32}P$] ATP to a peptide substrate using a protein kinase C assay kit (RPN 77, Amersham, Oakville, Ontario). Protein kinase C activity was taken as the difference between the amount of phosphorylation in the presence or absence of both Ca^{2+} and lipid. The assay was performed according to the instructions provided. The kit contained the following solutions:

Reagent 1: 12 mM Ca^{2+} acetate in 50 mM Tris HCl with 0.05% w/v sodium azide.

Reagent 2: 8 mole % L-α-phosphatidyl-L-serine and 24 μg/ml phorbol 12-myristate 13-acetate in 50 mM Tris HCl with 0.05% w/v sodium azide.

Reagent 3: 900 μM peptide in 50 mM Tris HCl with 0.05% w/v sodium azide. The sequence of the substrate, Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu, is part of the sequence of a peptide called EGF-R_3 (Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu; residues 650-658 of the EGF receptor). EGF-R_3 is a substrate for protein kinase C and is selectively phosphorylated by the enzyme (Woodgett et al., 1986).

Reagent 4: 30 mM dithiothreitol in 50 mM Tris HCl with 0.05% w/v sodium azide.

Reagent 5: 150 μM ATP and 45 mM magnesium acetate in 50 mM Tris HCl with 0.05% w/v sodium azide.

Reagent 6: stop reagent described by the company as a weak acid.

Two components, one containing Ca^{2+} and lipid and the other without Ca^{2+} and lipid, were prepared from the first 4 reagents and were part of the reaction mixtures. Component X consisted of reagent 1: reagent 2: reagent 3: reagent 4 (1:1:1:1, v/v). For component Y, reagent 1 and reagent 2 were replaced with 6 mM EGTA in 50 mM Tris HCl. The reaction mixture contained 25 μl of component X or 25 μl of component Y and 25 μl of column effluent [0.05 - 0.15 μg protein /25 μl (soluble fraction) and 0.02 - 0.06 μg protein / 25 μl (particulate fraction)]. The reaction was started by the addition of 25 μl of reagent 5 which contained [γ^{32}P]-ATP (Dupont-NEN, NEG-002H, Boston, MA, 3.0 x 10^6 dpm / 25 μl, specific activity = 800 dpm/pmol). The reaction was carried out at 25 C and stopped 15 min later by the addition of 100 μl of reagent 6. 150 μl of the mixture was then applied to phosphocellulose paper measuring 3 cm by 3 cm and washed 3 times for 10 min with 1 % phosphoric acid. The papers were transferred to vials, 12 ml of scintillation cocktail (BCS, Amersham) was added and radioactivity was measured by liquid scintillation spectroscopy. Protein concentration was determined using a protein assay kit (500-0006, BioRad, Richmond, CA). All of the samples from one experiment were measured within one assay with an intra-assay coefficient of variation of 10%. The lowest detectable concentration of protein (BSA standards) was 0.5 μg/assay tube and the mid range concentration of the assay was 6.0 μg/assay tube.

2.7.4 Data analysis of the protein kinase C studies

Data were expressed as mean ± SEM of 3 separate observations. Differences between control and treatment groups for each time point were calculated using a Student t-test. Differences were considered significant for $P < 0.05$.

2.8 Preparation of chemicals

rGRF was initially dissolved and stored in 1.0 mM ascorbic acid-0.1% BSA-10 mM acetic acid. SRIF was dissolved in M199A just before use. PMA was initially dissolved and stored in dimethylsulfoxide (DMSO) and the final concentration of DMSO in the incubation system was less than 0.1%. diC_8 was initially dissolved in absolute ethanol and the final concentration of ethanol in the incubation system was less than 0.1%. Nifedipine was initially dissolved in DMSO and the final concentration of DMSO in the incubation system was 0.0059%. HA1004 was dissolved in distilled water and as a consequence the osmolarity of the medium was reduced by 1%. dbcAMP was dissolved in M199AH/37 just before use. IBMX was initially dissolved in 1 N NaOH, made up to the appropriate volume with M199AH/37 and adjusted to a pH for 7.50 with 1 N HCl. Experiments were performed in subdued light when PMA or nifedipine was used.

2.9 Radioimmunoassays

2.9.1 GH Assay

GH was measured in triplicate using the rat RIA kit supplied by NIDDK National Hormone and Pituitary Program, University of Maryland School of Medicine. Results are expressed as ng/1000 cells (static incubation studies) or ng/ml (perifusion studies) of

the rat GH RP-2 standard. Samples from each experiment were measured within one assay, with a intra-assay coefficient of variation of less than 5%. A single label RIA program was used to calculate the GH content of each assay tube using a linear regression line of the logit B/B_0 versus log concentration of rat GH standard. The lowest detectable GH concentration was 0.1 ng/assay tube and the mid range concentration of the assay was 0.5 ng/assay tube.

2.9.2 cAMP Assay

cAMP was extracted and assayed as previously described with some modifications (Sheppard et al., 1979). Anti-cAMP antibody was kindly supplied by Dr. D.T. Armstrong, Department of Physiology, University of Western Ontario (Reddoch et al., 1986). Results are expressed as fmol/1000 cells. Samples from each experiment were measured within one assay, with an intra-assay coefficient of variation of less than 10%. The lowest detectable concentration and the mid range concentration of the assay was respectively 10 and 60 fmol/assay tube. The extraction and assay protocols are found in Appendix 6.

CHAPTER 3: RESULTS

Histological examination of the purified somatotroph preparations (Appendix 1) revealed a routine purity of over 94%. We assumed that the somatotrophs were homogenous in that the same population of cells responded to all of the secretagogues studied.

3.1 Effect of protein kinase C activation

The purpose of this series of experiments was to determine whether activation of the protein kinase C arm of the phosphoinositide pathway would cause GH release in purified somatotrophs. We also examined the effect of the protein kinase C activators on cAMP accumulation. In addition, we studied the dynamics and repeatability of the GH response to protein kinase C activation using our perifusion system. The effect of SRIF on protein kinase C activator action was also determined. Finally, we studied the role of extracellular Ca^{2+} in protein kinase C activator action.

3.1.1 Effect of protein kinase C activation on GH and cAMP responses from purified somatotrophs

We first examined the effect of protein kinase C activation on the GH response from, and cAMP accumulation in, purified somatotrophs. To activate protein kinase C we used the diacylglycerol analogue, diC_8, and the phorbol ester, PMA. Figure 10 shows the time course of cAMP accumulation (upper panel) and cumulative GH released (lower panel) in response to diC_8 added at zero time. In this and in subsequent figures, unless otherwise stated in the figure legend, the points represent the means of four

separate determinations and the error bars are the SEM. Where bars do not appear, the SEM was too small to plot. diC_8, at 3 x 10^{-6} M or less did not alter GH release. There was a small but significant concentration-related increase in GH release at 10^{-5} and 3 x 10^{-5} M, with a massive response at 10^{-4} M. The GH response to 10^{-4} M was significant by 4 min, while the responses to 10^{-5} and 3 x 10^{-5} M were small but significant by 8 min. cAMP accumulation was increased by 4 min, and this increase was concentration-related at 10^{-5} M or greater. At 32 min, the response to 3 x 10^{-6} M, though small, was also significant. Figure 11 shows the GH and cAMP responses to PMA. PMA at 10^{-10} M did not alter GH release, but the three higher concentrations of PMA elicited small but significant increases in GH release by 4 min, which were greater than control values at 8, 16 and 32 min. The response to PMA was concentration-related between 10^{-10} M and 10^{-9} M, with no further increase in GH release at higher concentrations of PMA. cAMP accumulation was significantly increased by 4 min, and this increase was concentration-related at concentrations greater than 10^{-10} M.

3.1.2 Dynamics and repeatability of the GH response to protein kinase C activation

Since GH release was stimulated by protein kinase C activation, we then examined the dynamics and repeatability of the GH response to diC_8 and PMA. To compare GH responses we calculated the extra release (ER) and peak secretion rate (PSR) of the GH responses. ER was the amount of GH above interpolated baselines during application of secretagogue and was calculated on a histogram basis. The interpolated baselines were the means of values over 10 min immediately before the application of secretagogue. PSR during ER were the maximum responses above the interpolated baselines. diC_8 or PMA was administered for two 30 min periods, separated

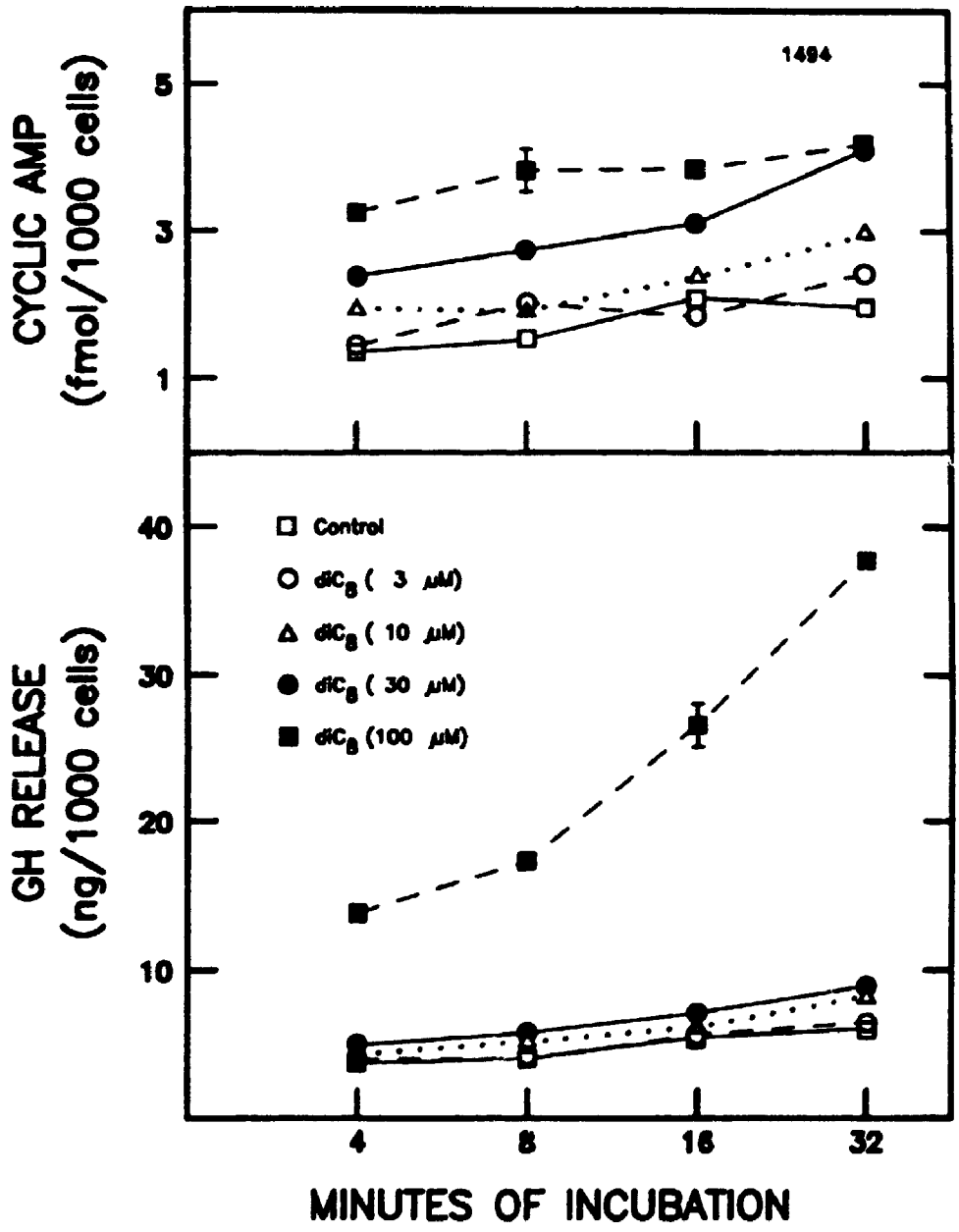


Figure 10: Effect of graded concentrations of diC_8 on cAMP accumulation (upper panel) and GH release (lower panel) from purified somatotrophs at 4, 8, 16 and 32 min. Representative of two experiments.

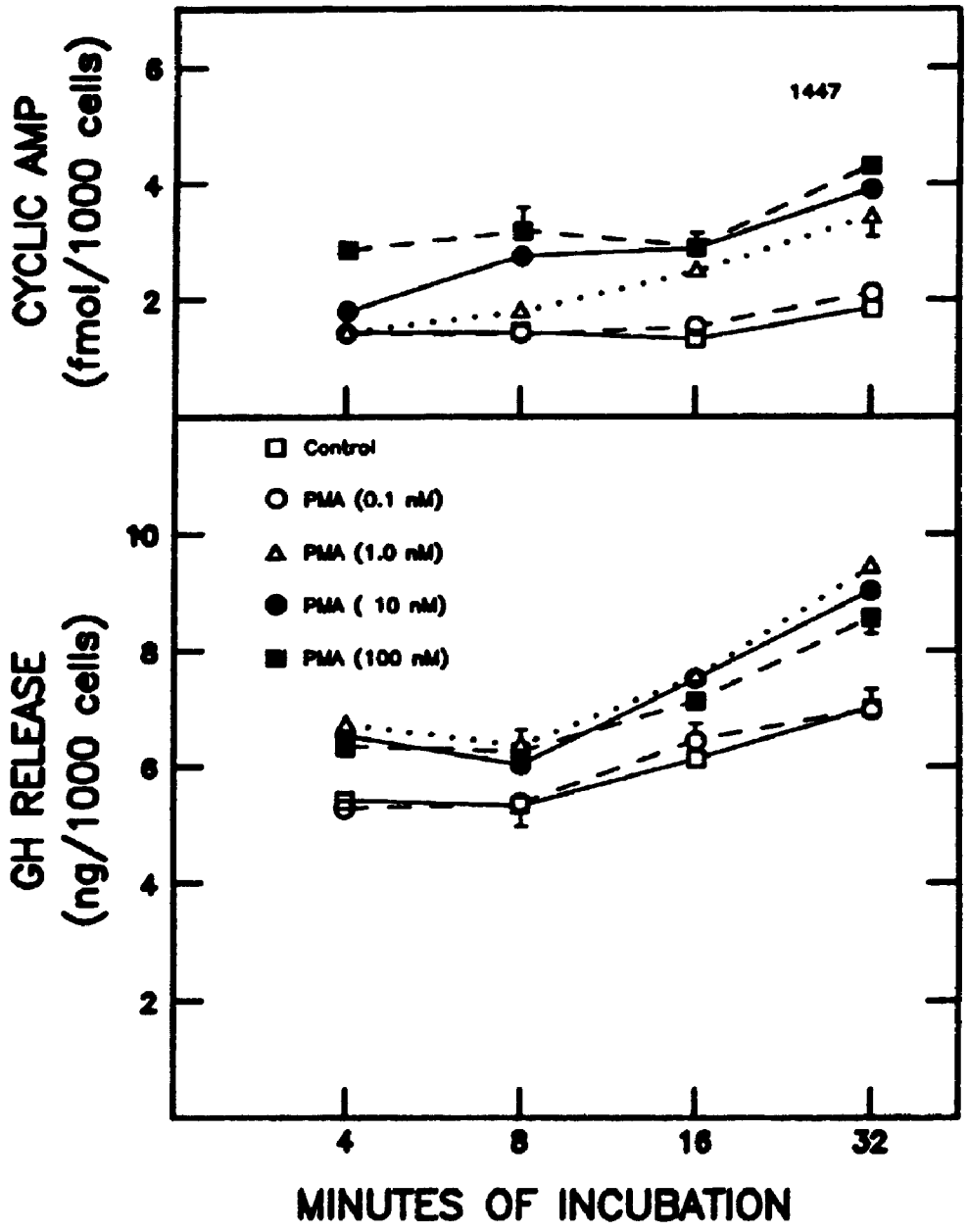


Figure 11: Effect of graded concentrations of PMA on cAMP accumulation (upper panel) and GH release (lower panel) from purified somatotrophs at 4, 8, 16 and 32 min. Representative of two experiments.

by 1 h of perifusion with M199A. diC_8 at 2 x 10^{-6} M (Figure 12), a submaximal concentration, elicited an immediate (within 1 min) burst of GH release (ER=294 ng, PSR=32 ng/min) which then fell progressively while diC_8 was maintained, remaining above the initial baseline until the end of the period of perifusion with diC_8. The second response was similar in magnitude and form to the first (ER=266 ng, PSR=23 ng/min). In 3 similar experiments, the initial ER / PSR were 144 ng / 14 ng/min, 422 ng / 41 ng/min and 838 ng / 97 ng/min, and the second ER and PSR were respectively 75 $\pm$ 13% and 73 $\pm$ 17% of the first. PMA at 10^{-9} M (Figure 13), again a submaximal concentration, elicited an immediate (within 1 min) burst of GH release (ER=784 ng, PSR=66 ng/min), which fell rapidly over 6 min, and then more slowly over the remainder of the first 30 min period of perifusion. After the cessation of the first PMA pulse, the baseline remained slightly, but consistently, higher than the original baseline. The second 30 min application of PMA (10^{-9} M), 60 min after the cessation of the first, elicited a much reduced and gradually increasing GH response, when compared to the initial response, which was maintained throughout the 30 min period (ER=180 ng, PSR=8 ng/min). Following the second application of PMA, GH release decreased slowly. Since reduced responsiveness to PMA may have been due to a depletion of releasable GH, we studied the effect of two 30 pulses of PMA at a reduced concentration of 5 x 10^{-10} M. Figure 14 shows that the reduced concentration of PMA elicited a similar but smaller initial response compared to the response to PMA at 10^{-9} M (ER= 539 ng, PSR=29 ng/min). This initial response was comparable to that seen with diC_8 (Figure 12). The GH response to the second application was again much reduced, (ER=104 ng, PSR=5 ng/min), when compared to the initial response.

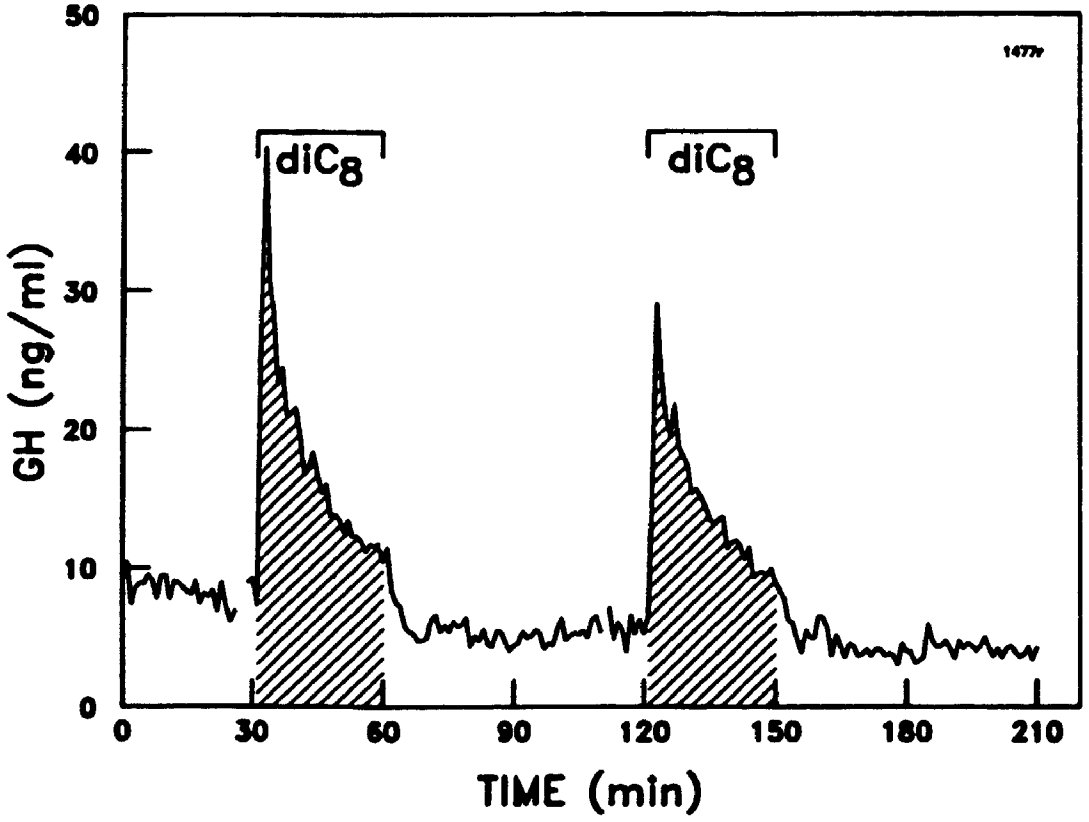


Figure 12: GH response from dispersed anterior pituitary cells to two 30 min periods of perifusion with diC_8 (2×10^{-6} M). Representative of five experiments.

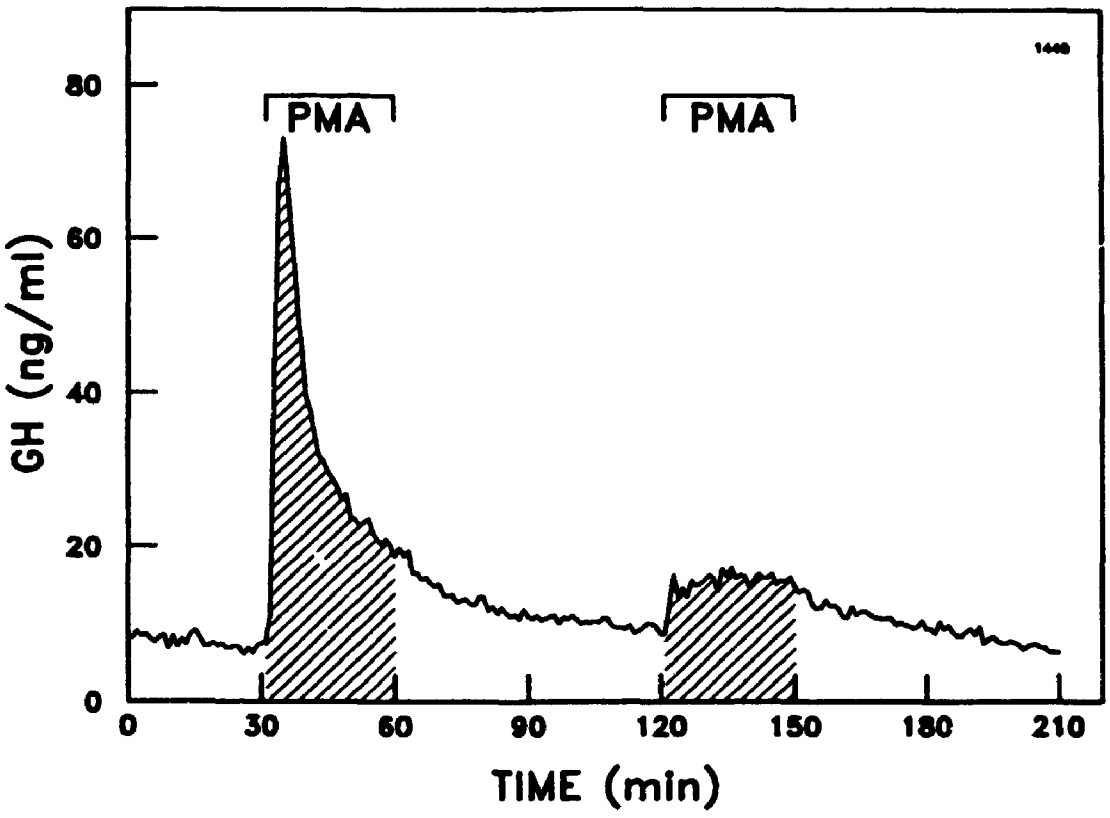


Figure 13: GH response from dispersed anterior pituitary cells to two 30 min periods of perifusion with PMA (10^{-9} M). Experiment was performed once.

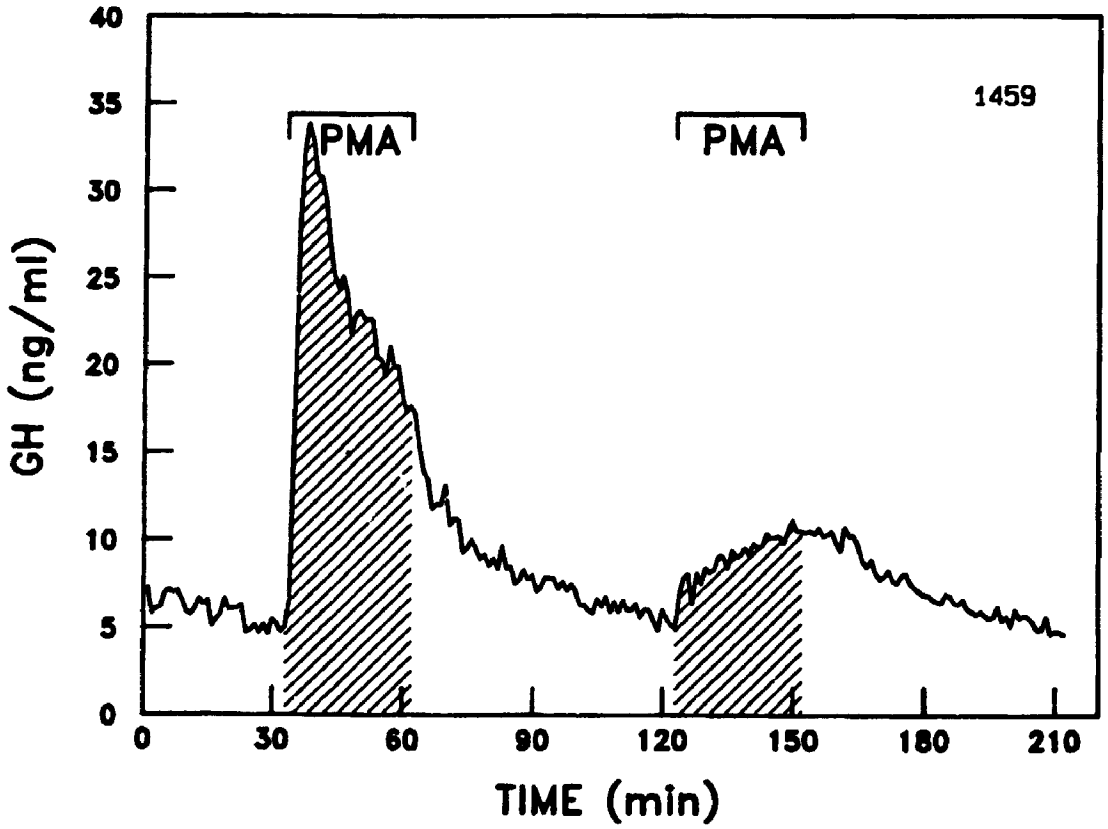


Figure 14: GH response from dispersed anterior pituitary cells to two 30 min periods of perifusion with PMA (5 x 10^{-10} M). Experiment was performed once.

3.1.3 Effect of SRIF on the GH and cAMP responses to protein kinase C activation

We then examined whether SRIF would suppress the increase in GH release and cAMP accumulation induced by diC_8 (Figure 15) and PMA (Figure 16). "Start" samples were taken 5 min before addition of SRIF, and PMA or diC_8 was added 10 min after the addition of SRIF. There were no significant differences in GH or cAMP content within the "starts". GH release and cAMP accumulation with SRIF (10^{-7} M) alone was not different from control (Figure 15 and Figure 16). diC_8 ($5x10^{-5}$ M) elicited a significant increase in GH release by 4 min (Figure 15). SRIF significantly reduced the diC_8-induced increase in GH release in a concentration-related manner, though the reduction was not complete (15% inhibition at 16 min with SRIF at 10^{-9} M). Even at a concentration of SRIF of 10^{-7} M, the GH response to diC_8 was still significant (30% inhibition at 16 min). diC_8 ($5x10^{-5}$ M) elicited a significant increase in cAMP accumulation by 4 min. Neither concentration of SRIF altered the diC_8-induced increase in cAMP accumulation, except for a small but significant reduction with SRIF (10^{-7} M) at 4 min.

PMA (10^{-8} M) produced a significant increase in GH release by 8 min (Figure 16). SRIF (10^{-9} M and 10^{-7} M) blocked the augmented GH release at 8 and 16 min, while the reduction with SRIF (10^{-9} M and 10^{-7} M), although significant, was incomplete at 32 min. PMA (10^{-8} M) elicited a significant increase in cAMP accumulation by 8 min. Neither concentration of SRIF reduced the PMA-induced increase cAMP accumulation at 4 min. SRIF (10^{-7} M) caused a small but significant reduction in cAMP accumulation at 16 min. At 32, both concentrations of SRIF caused a small, but significant reduction in cAMP accumulation induced by PMA.

We next examined, using perifusion, the time base for the SRIF-induced block on GH release induced by diC_8 (Figure 17). diC_8 ($2x10^{-6}$ M), applied for 30 min, elicited the expected burst of GH release (ER=189 ng, PSR=16 ng/min). Fifty min after the switch to M199A, SRIF (10^{-9} M) was applied, followed in 10 min by a concurrent application of a second 30 min of diC_8 ($2x10^{-6}$ M). SRIF induced an immediate small decrease in basal GH release and then, in contrast to the static incubation results, completely blocked the response to the second application of diC_8. After the cessation of SRIF and diC_8, there was an immediate and large burst of GH release with a return to baseline within 10 min.

3.1.4 Role of Ca^{2+} in the GH and cAMP responses to protein kinase C activation

Since Ca^{2+} plays an essential role in the mediation of GRF-induced GH release, we next asked whether an influx of Ca^{2+} is essential for the increase in GH release induced by protein kinase C activators. We therefore studied the effect of removal of extracellular Ca^{2+} and also the effect of nifedipine, a Ca^{2+} channel antagonist, on diC_8- and PMA-induced GH release and cAMP accumulation.

3.1.4.1 Effect of removal of extracellular Ca^{2+} on the GH and cAMP responses to protein kinase C activation

To study the effect of removal of extracellular Ca^{2+}, diC_8 (3×10^{-5} M) or PMA (10^{-8} M) was added to somatotrophs in normal M199AH and in M199AH containing no added Ca^{2+}. Samples of cell suspension were taken at 8, 16 and 32 min for the measurement of GH released into the medium and cAMP accumulation (Figure 18). Basal GH release was constantly but not significantly lower in low-Ca^{2+} media. diC_8 in normal M199AH elicited a significant increase in GH release by 8 min. The GH

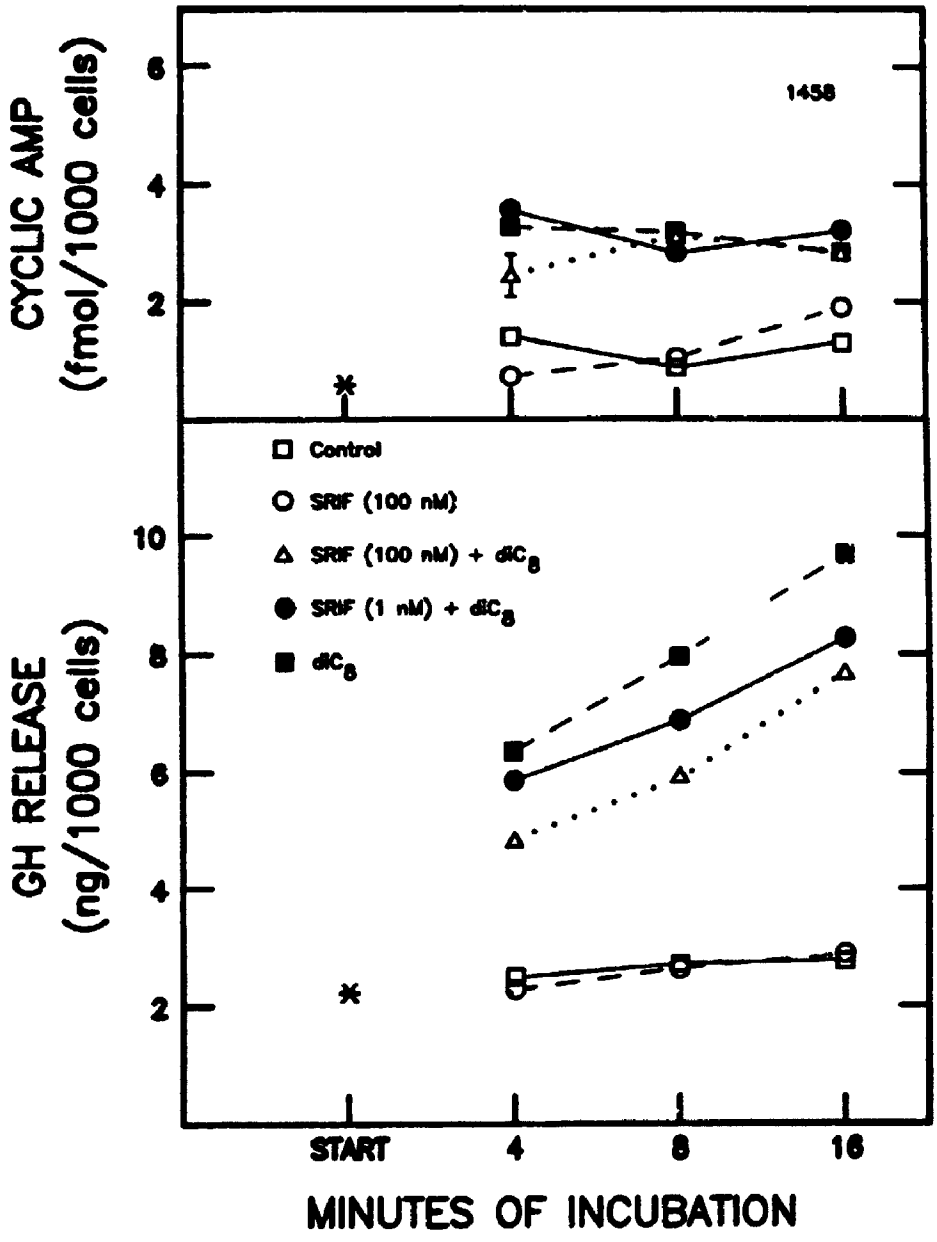


Figure 15: Effect of SRIF (10^{-9} M, 10^{-7} M) on cAMP accumulation (upper panel) and GH release (lower panel) from purified somatotrophs induced by diC_8 (5 x 10^{-5} M). "Starts" (*) were withdrawn 5 min before the addition of SRIF. SRIF was added 10 min before the addition of diC_8 and samples were withdrawn at 4, 8, and 16 min. Representative of two experiments.

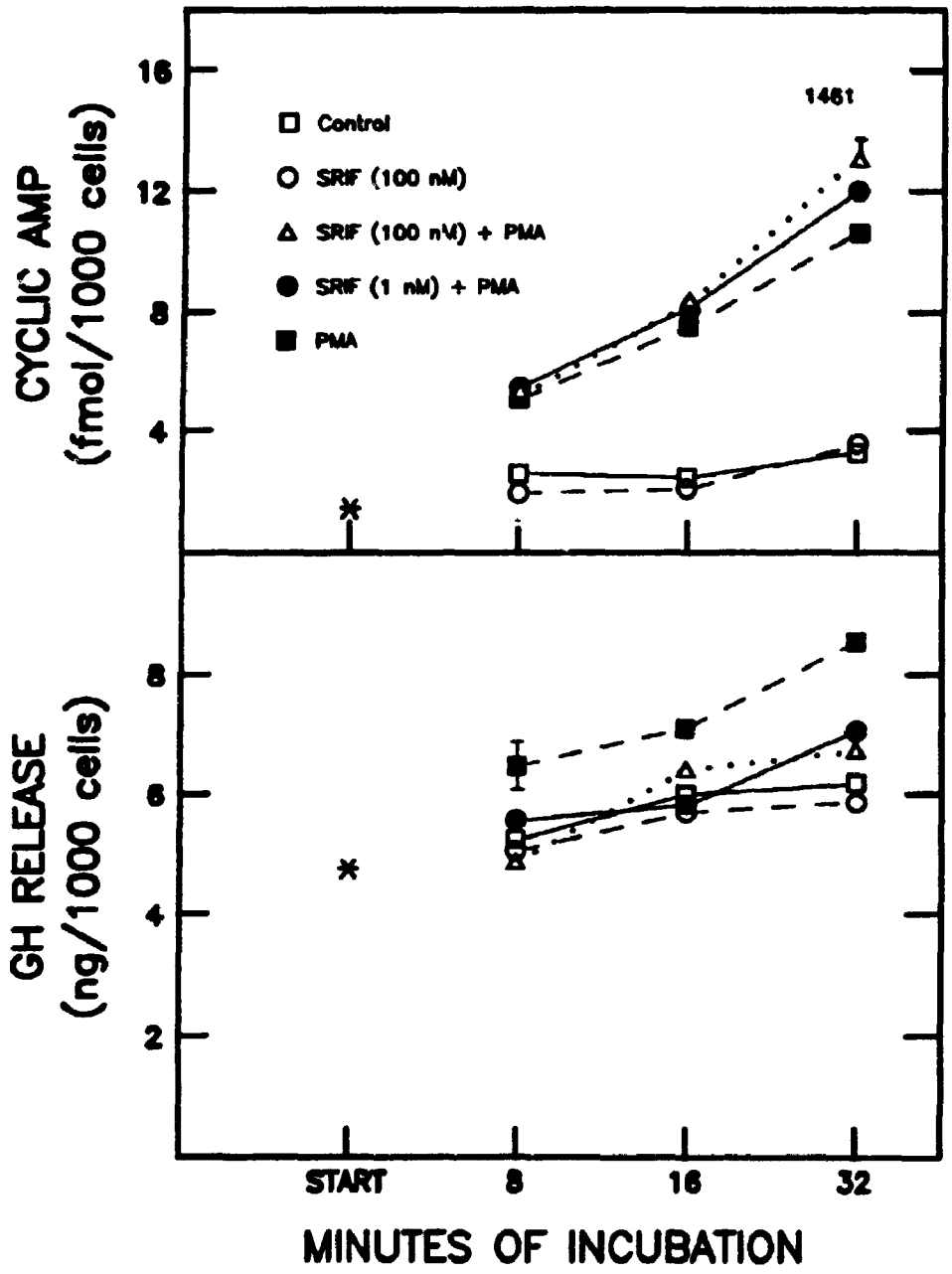


Figure 16: Effect of SRIF (10^{-9} M, 10^{-7} M) on cAMP accumulation (upper panel) and GH release (lower panel) from purified somatotrophs induced by PMA (10^{-8} M). "Starts" (*) were withdrawn 5 min before the addition of SRIF. SRIF was added 10 min before the addition of PMA and samples were withdrawn at 8, 16 and 32 min. Representative of two experiments.

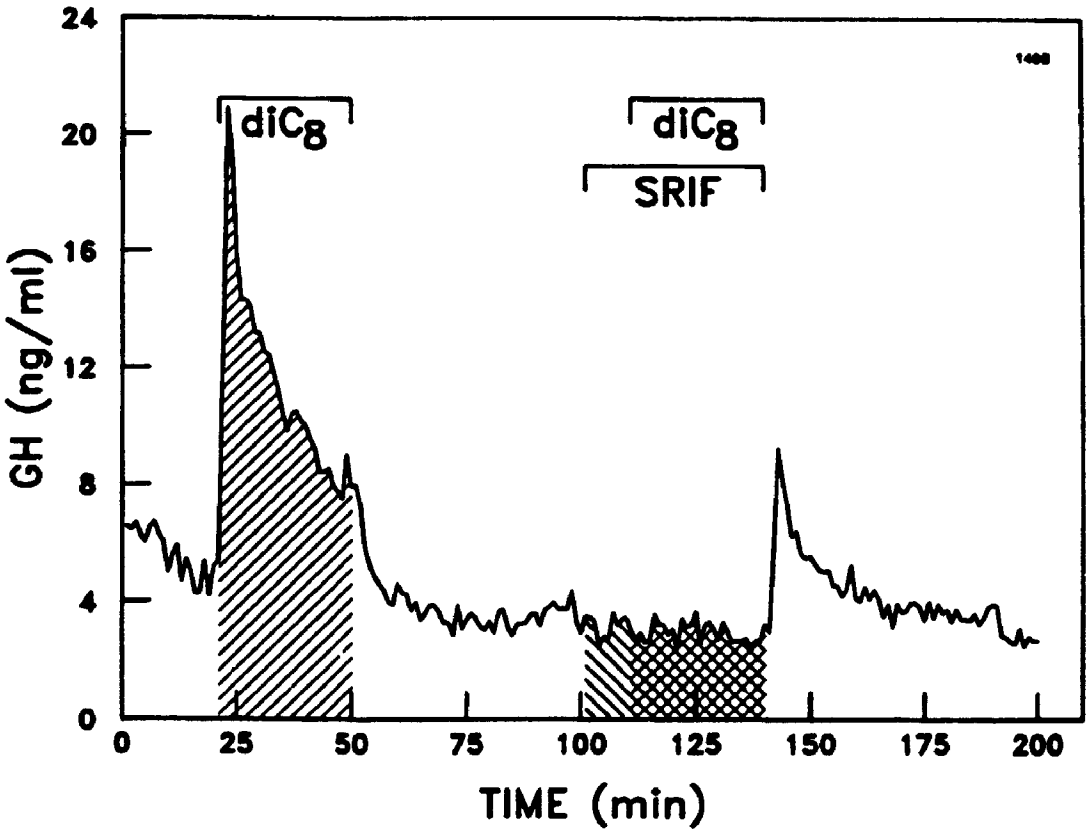


Figure 17: GH response from dispersed anterior pituitary cells to 30 min of perifusion with diC_8 (2×10^{-6} M), and a later concurrent perifusion with SRIF (10^{-9} M) and diC_8 (2×10^{-6} M). SRIF perifusion began 10 min before diC_8. Representative of three experiments.

response to diC_8 in low-Ca^{2+} M199AH was significantly reduced, but remained significantly greater than control. PMA caused a significant increase in GH release by 8 min which was abolished, at all times, in low-Ca^{2+} M199AH. The GH response to diC_8 was significantly greater than the response to PMA at 8 and 16 min, but not at 32min. Basal cAMP levels were constantly higher in low-Ca^{2+} M199AH compared to normal M199AH. The difference was significant at 8 and 16 min but not at 32 min. diC_8 in M199AH caused a significant increase in cAMP when compared to M199AH alone at 16 and 32 min. Surprisingly, cAMP levels in low-Ca^{2+} M199AH with diC_8 were significantly lower than cAMP levels in low-Ca^{2+} M199AH without diC_8. The cAMP response to diC_8 at 8 min, but not at 16 and 32 min, was significantly greater in low- Ca^{2+} M199AH compared to the response to diC_8 in normal M199AH. As expected, PMA in M199AH significantly increased cAMP compared to M199AH alone. cAMP accumulation induced by PMA in low-Ca^{2+} M199AH was significantly greater compared to low-Ca^{2+} M199AH alone and also significantly greater than cAMP accumulation elicited by PMA in normal M199AH at all times.

3.1.4.2 Effect of nifedipine on the GH and cAMP responses to protein kinase C activation

We also examined the effect of a Ca^{2+} channel antagonist, nifedipine, on the GH and cAMP responses to diC_8 and PMA, to further investigate the role for extracellular Ca^{2+} in protein kinase C activator action. Nifedipine (10^{-6} M) was added to purified somatotrophs 10 min before diC_8 (10^{-5} M) or PMA (10^{-8} M) and cell samples were withdrawn at 8, 16 and 32 min for the measurement of GH and cAMP (Figure 19). Nifedipine did not alter basal GH release. Both diC_8 and PMA elicited a significant

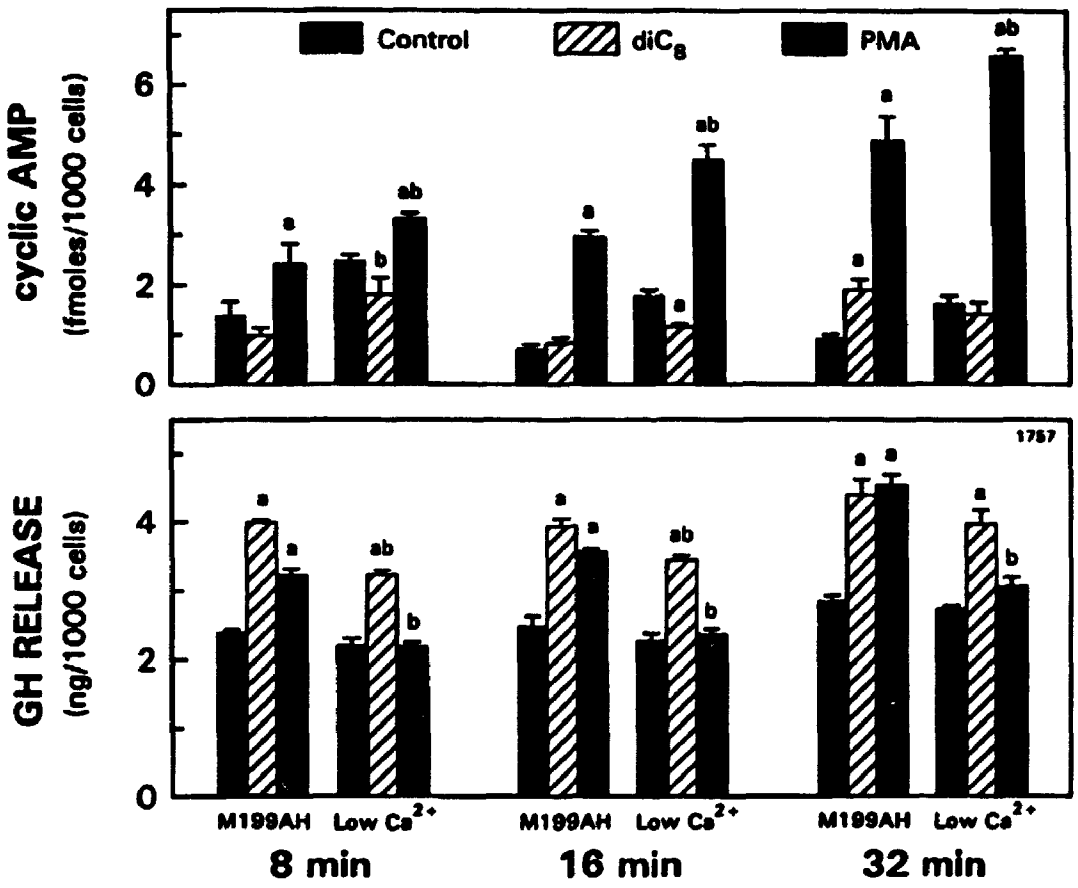


Figure 18: The effect of low-Ca^{2+} M199AH on cAMP accumulation (upper panel) and GH release (lower panel) from purified somatotrophs induced by diC_8 (3×10^{-5} M) and PMA (10^{-8} M) at 8, 16 and 32 min. Values significantly different from appropriate controls are indicated by "a", while values for responses to diC_8 or PMA in low-Ca^{2+} that are significantly different from responses in M199AH are indicated by "b" ($P < 0.05$). Representative of three experiments.

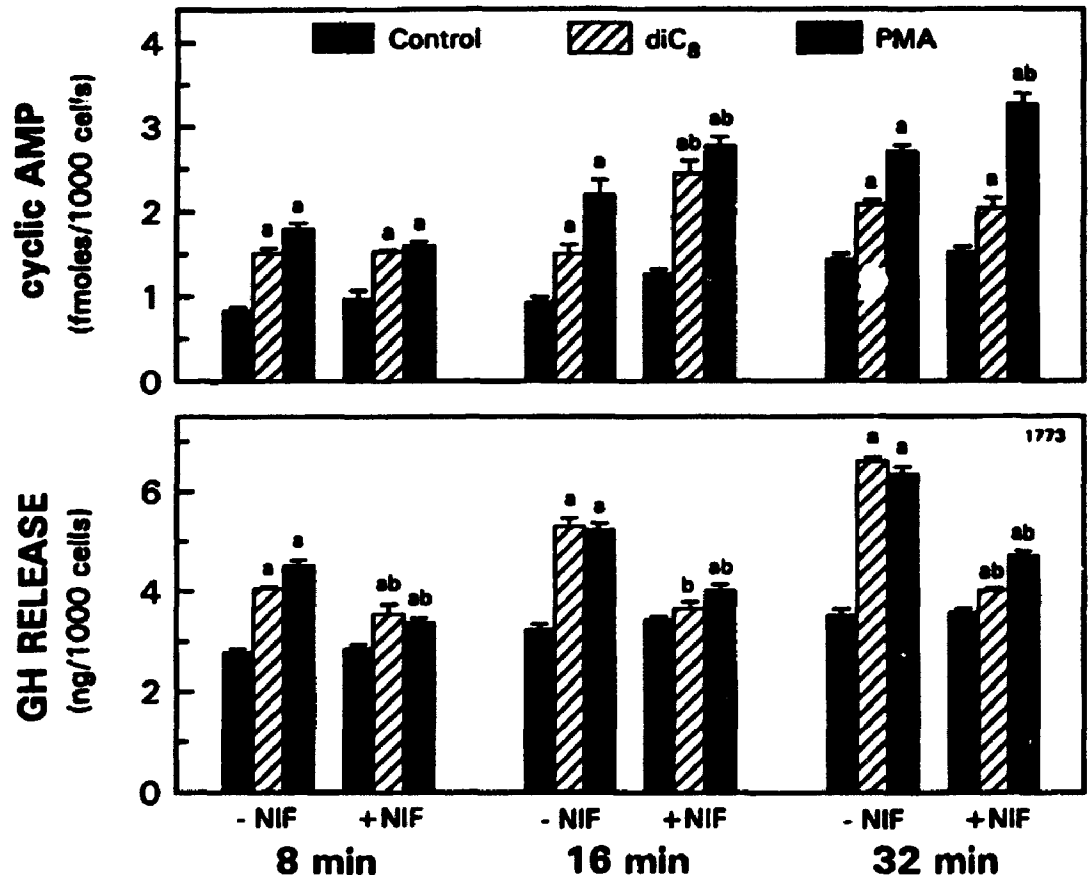


Figure 19: The effect of nifedipine (10^{-6} M) on cAMP accumulation (upper panel) and GH release (lower panel) from purified somatotrophs induced by diC_8 (10^{-5} M) and PMA (10^{-8} M) at 8, 16 and 32 min. Nifedipine was added 10 min before the addition of diC_8 or PMA. Values significantly different from appropriate controls are indicated by "a", while values for responses to diC8 or PMA with nifedipine that are significantly different from responses without nifedipine are indicated by "b" ($P<0.05$). Representative of three experiments.

increase in GH release at all three times that was similar in magnitude. Nifedipine significantly reduced, but did not abolish, the GH response to both diC_8 and PMA, except at 16 min, when the GH response to diC_8 was abolished. Nifedipine alone did not alter cAMP levels at 8 and 32 min, although it did significantly increase cAMP at 16 min. Both diC_8 and PMA significantly increased cAMP at all three times. Nifedipine did not significantly alter the cAMP response to diC_8 or PMA at 8 min, but significantly augmented the response at 16 min. At 32 min, the cAMP response to diC_8 was not altered with nifedipine, while the cAMP response to PMA was significantly increased by nifedipine.

3.2 Role of protein kinase C in GRF-induced GH release

The purpose of this series was to examine the role of protein kinase C in GRF-induced GH release. We used three approaches. Firstly, the effect of inactivation of protein kinase C on the subsequent GH response to GRF was studied. Secondly, we attempted studies using protein kinase C inhibitors. And finally, we studied the effect of GRF on protein kinase C activity in somatotrophs (see section 3.4).

3.2.1 Role of protein kinase C in the mediation of the GH response to GRF

The reduced responsiveness of cells in perifusion to a second 30 min application of PMA (Figure 13 and Figure 14) suggested that protein kinase C activity could not be stimulated further. Others have shown that treatment of cells with phorbol esters for this period of time causes them to be refractory to a second stimulation by protein kinase C activators (Phillips and Jaken, 1983). We thought that the lack of responsiveness to protein kinase C activators might provide a useful strategy to examine the role of protein

kinase C in GRF-induced GH release. To ensure that protein kinase C activity could not be further stimulated, we increased the concentration and duration of the initial period of PMA perifusion to 10^{-9} M for 1 h and then examined the GH response to a second 1 h application of PMA (10^{-9} M) (Figure 20). The first 60 min application of PMA produced a GH response (ER=1544 ng, PSR=98 ng/min) similar to that already described. Basal release remained elevated at about 2 times the initial basal level during the 30 min before the second application of PMA. The GH response to the second 1 h pulse of PMA was virtually abolished (ER=149 ng, PSR=7 ng/min). We then asked whether 1 h of perifusion with PMA (10^{-9} M) would also block the subsequent response to diC_8. One h of perifusion with PMA was followed by 30 min of basal release in M199A, which was followed by 30 min of perifusion with diC_8 (10^{-5} M) (Figure 21). The expected initial response to PMA was seen (ER=1330 ng, PSR=83 ng/min). The introduction of diC_8 elicited a small, immediate and transient increase in GH release and then GH release dipped and remained below baseline. The cessation of diC_8 was followed by an immediate 2-fold burst of GH release, which gradually returned to baseline. Thus, pretreatment of cells with PMA for 1 h renders them unresponsive to protein kinase C activation and would be a useful tool to examine the role of protein kinase C in GRF-induced GH secretion.

We then asked whether GRF requires protein kinase C to cause the release of GH. The cells were initially perifused with PMA (10^{-9} M) for 1 h and then, after a 30 min period of perifusion with M199A, GRF (10^{-10} M) was applied for 30 min. PMA elicited the expected increase in GH release, (ER=1280 ng, PSR=83 ng/min), which fell during the PMA perifusion period and remained at 2 times the basal levels with the

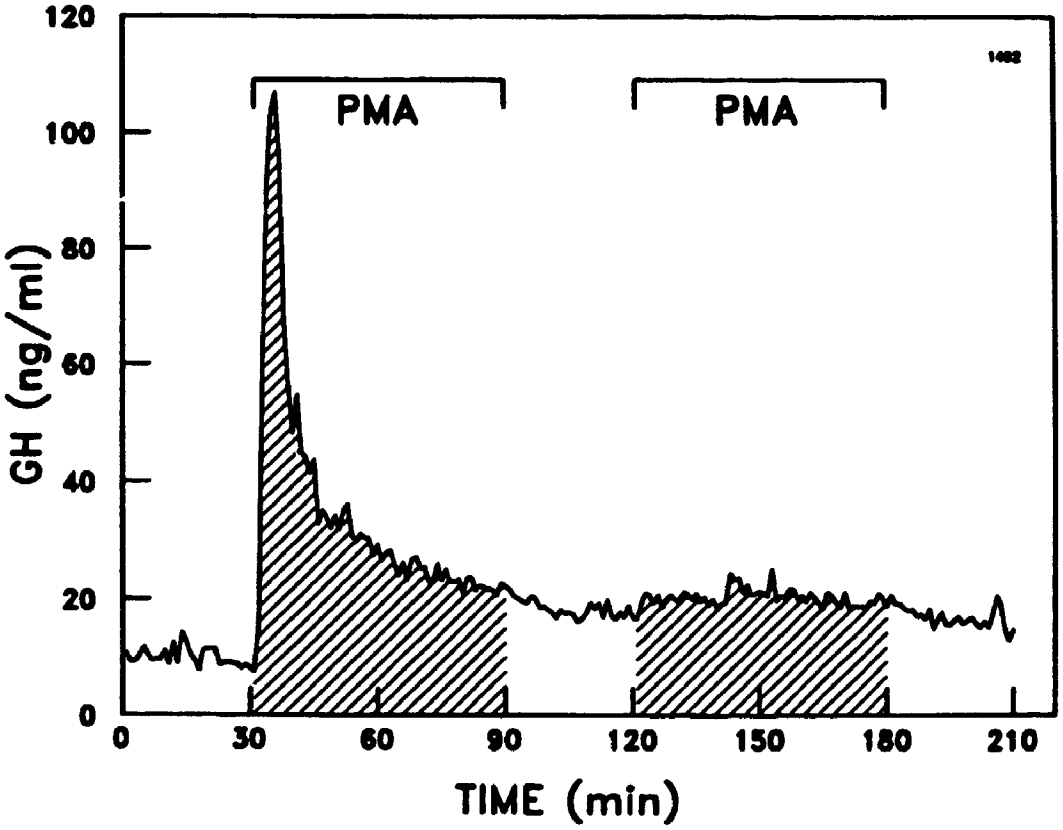


Figure 20: GH response from dispersed anterior pituitary cells to two 1 h periods of perifusion with PMA (10^{-9} M), separated by 30 min of perifusion with M199A. Representative of two experiments.

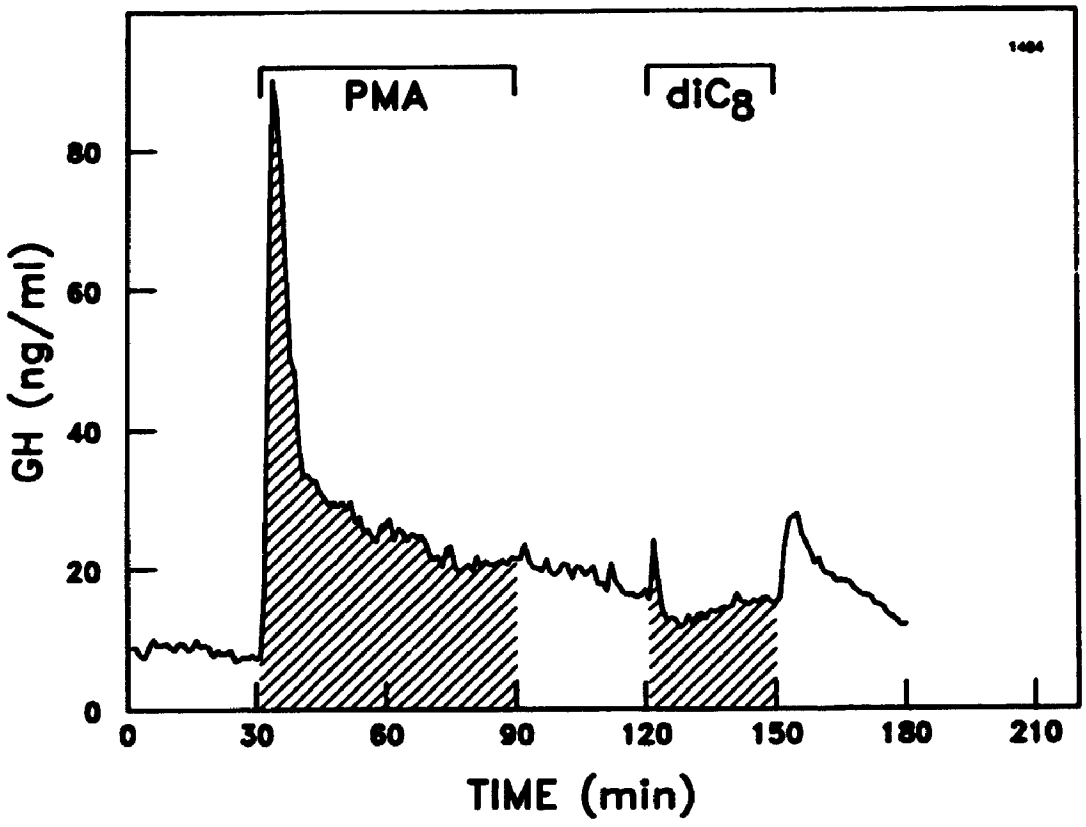


Figure 21: GH response from dispersed anterior pituitary cells to to an initial 1 h period of perifusion with PMA (10^{-9} M), and a subsequent 30 min period of perifusion with diC_8 (10^{-5} M), separated by 30 min of perifusion with M199A. Representative of two experiments.

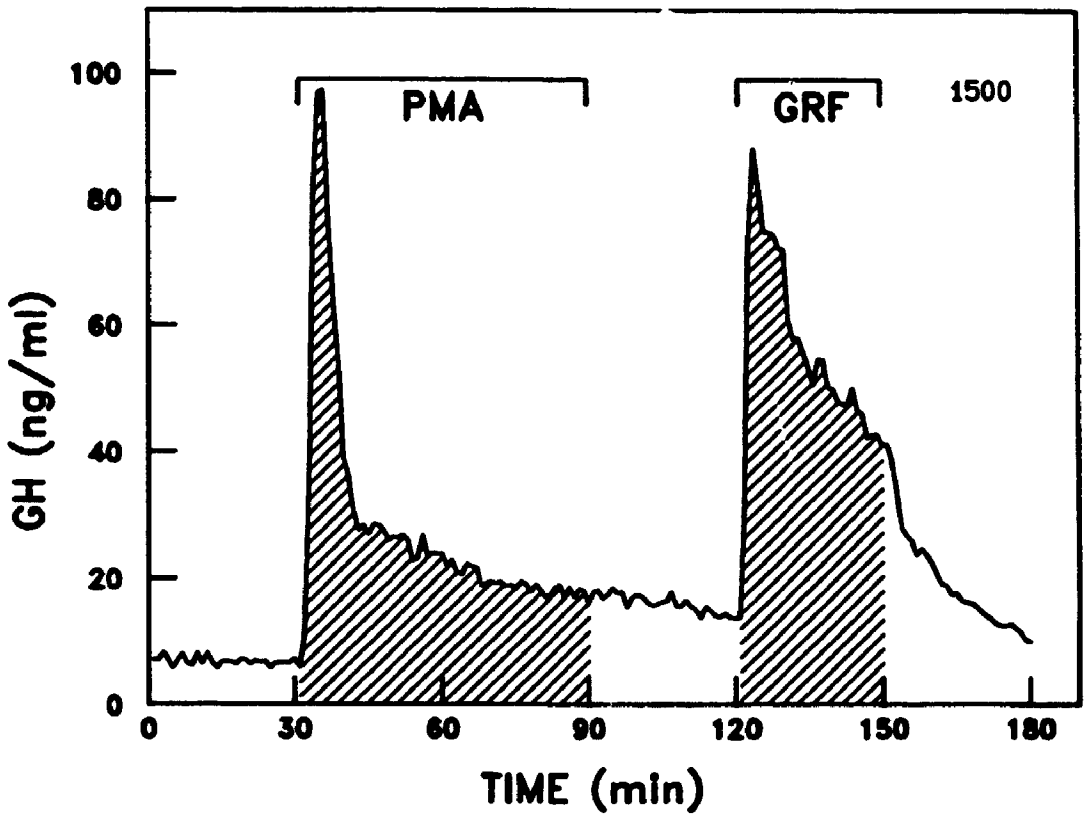


Figure 22: GH response from dispersed anterior pituitary cells to an initial 1 h period of perifusion with PMA (10^{-9} M) and a subsequent 30 min period of perifusion with GRF (10^{-10} M), separated by 30 min of perifusion with M199A. Representative of two experiments.

cessation of PMA (Figure 22). GRF elicited an immediate 6-fold increase in GH release, (ER=1268 ng, PSR=74 ng/min), which fell during the GRF perifusion period and then returned slowly to basal levels following the cessation of GRF perifusion. Therefore, GRF was effective in stimulating the release of GH even though protein kinase C activity could no longer be further stimulated.

We then examined whether pretreatment of cells with PMA modifies the GH response to GRF. This required simultaneous perifusion of 2 columns of cells. We first ensured that the GH responses would be identical from two simultaneously perifused columns. Figure 23 shows the GH responses to three 20 min periods of perifusion with 3 different concentrations of GRF (2.5×10^{-12} M, 5.0×10^{-12} M and 1.0×10^{-11} M) from two columns of cells simultaneously perifused. Increasing concentrations of GRF elicited increasing GH responses. The GH responses (ER and PSR) to a given concentration of GRF from the two columns were not significantly different when compared using a Student t-test. We also calculated the means of the differences between the GH values at each minute from the two columns during basal and peak periods and found that they were not significantly different when compared using a Student t-test.

Having confirmed that both columns of cells responded identically to the same stimulus, we asked whether GRF stimulates GH release, in part, through the activation of protein kinase C. The control column received the vehicle for PMA while the other column was perifused with medium containing PMA (10^{-9} M) throughout the experiment. After 46 min of perifusion with PMA, increasing concentrations of GRF (10^{-12} M, 10^{-11} M, 10^{-10} M and 10^{-9} M) were administered to both columns for 6 min intervals separated by 26 min periods of perifusion with the vehicle or PMA. Finally, diC_8

(10^{-5} M) was administered for the last 10 min of the experiment. This protocol was repeated in 4 separate experiments. Figure 24 shows the GH response to GRF and diC_8 from one such experiment, starting 10 min before the first application of GRF. In the control column (Figure 24A), GRF produced a concentration-related increase in GH secretion. The release of GH was immediate, declined over the 6 min perifusion period and returned to basal values 4 min after cessation of the GRF pulse. diC_8-induced an immediate increase in the release of GH, that reached a peak within 1 min and then declined over the period of perifusion. In the PMA column (Figure 24B), GRF elicited a concentration-related increase in GH release at concentrations between 10^{-12} M and 10^{-10} M. diC_8 (10^{-5} M) induced only a small increase in GH release (ER=37 ng, PSR=9 ng/min) when compared to the control column (ER=280 ng, PSR=75 ng/min). Table I summarizes the ER and PSR values from the four experiments. Analysis of variance and covariance with repeated measures showed no statistically significant differences between the GH responses in the control and PMA-treated cells (p=0.134 for ER and 0.069 for PSR), while the effect of GRF concentration was highly significant (p=0.018 for ER and 0.016 for PSR). Although not significant, the GH responses to the 2 highest concentrations of GRF were consistently reduced in the PMA-treated groups.

3.2.2 Effect of protein kinase C inhibitors

Another approach to examine the role of protein kinase C in GRF-induced GH release was to study the effect of protein kinase inhibitors on GRF-induced GH release. We first tested the effect of the protein kinase inhibitor HA1004 on diC_8- and dbcAMP-induced GH release from purified somatotrophs to determine whether it would preferentially block dbcAMP-induced GH release.

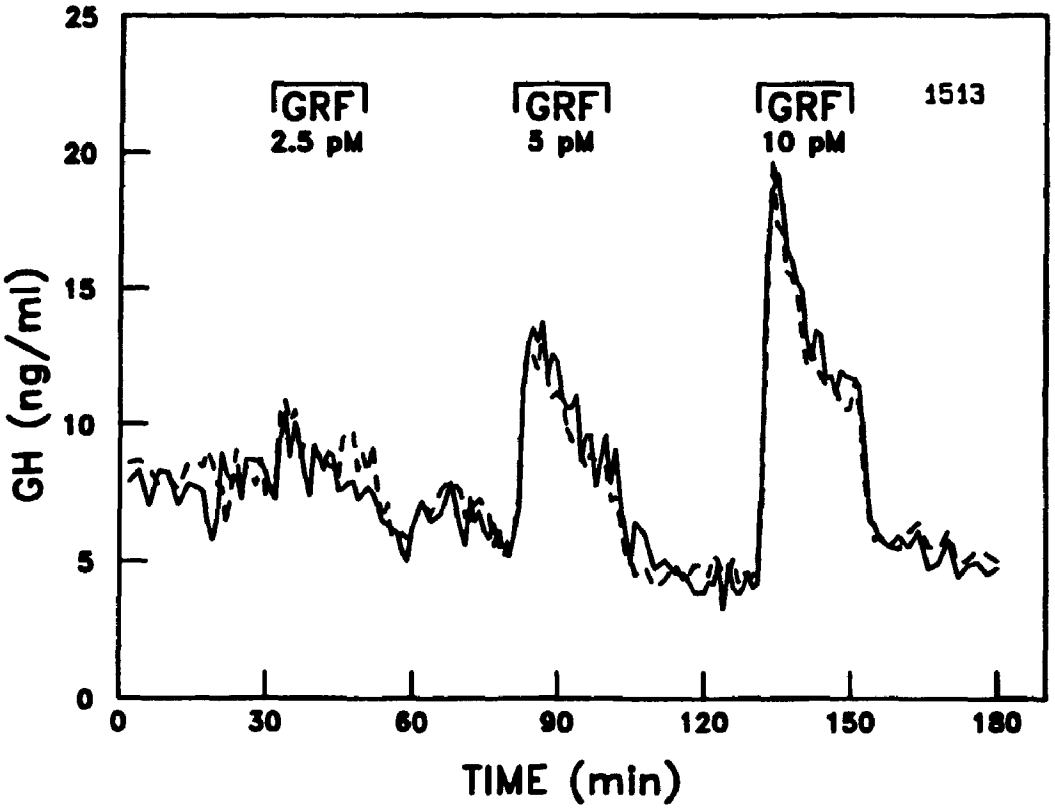


Figure 23: GH responses from two columns of dispersed anterior pituitary cells simultaneously perifused for three 20 min periods with increasing concentrations of GRF (2.5×10^{-12} M, 5.0×10^{-12} M and 1.0×10^{-11} M), separated by 30 min periods of perifusion with M199A. Solid line indicates GH values from one column, while the dashed line indicates GH values from the other column. Representative of two experiments.

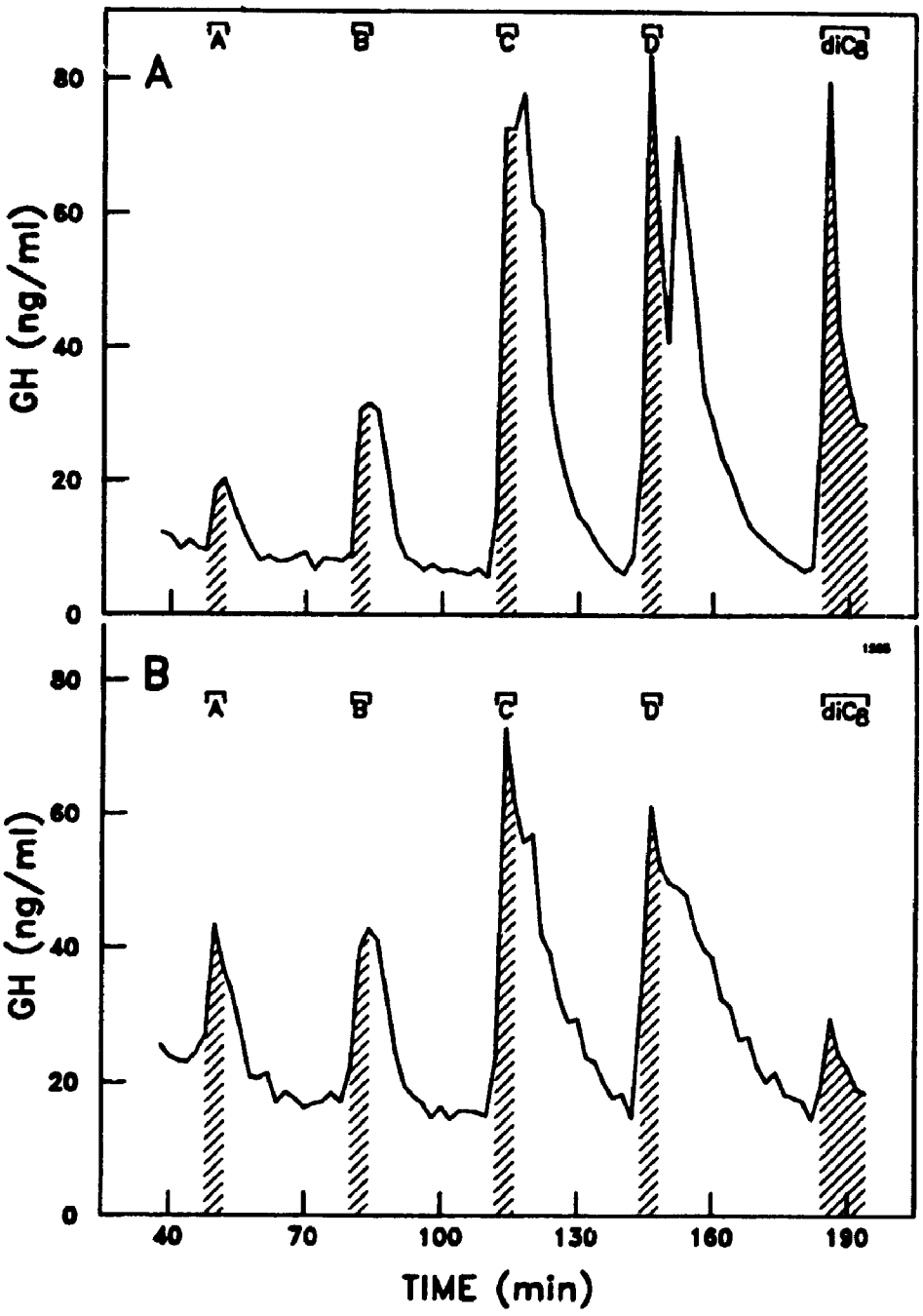


Figure 24: GH response from dispersed anterior pituitary cells to GRF (10^{-12} M, A; 10^{-11} M, B; 10^{-10} M, C; 10^{-9} M, D) and to diC_8 (10^{-5} M), without (panel A) and with (panel B) concurrent and continuous perifusion with PMA (10^{-9} M). Cells in panel B were perifused with PMA (10^{-9}M) for 46 min prior to the first application of GRF. Representative of four experiments.

Table I: GH responses to graded concentrations of GRF and to diC_8 from control and PMA treated dispersed anterior pituitary cells.

	GH release							
	Exp 1		Exp 2		Exp 3		Exp 4	
	Control	PMA	Control	PMA	Control	PMA	Control	PMA
GRF								
10^{-12}M	83/31	78/28	9/4	23/8	24/14	65/29	22/9	45/19
10^{-11}M	82/27	66/26	24/10	25/9	48/18	75/26	69/23	77/26
10^{-10}M	112/42	92/25	70/25	50/20	163/56	147/52	213/72	152/57
10^{-9}M	158/66	137/51	88/31	77/21	229/89	209/76	174/75	151/62
diC_8	179/87	25/13	156/75	16/9	187/89	46/22	130/73	22/13

The GH responses are expressed as ER (ng) / PSR (ng/min). One column of cells was perifused with PMA (10^{-9} M) for 46 min before and during the application of GRF and diC_8, while the other column of cells received the vehicle for PMA. GRF (10^{-12} M, 10^{-11} M, 10^{-10} M and 10^{-9} M) was administered to both columns for 6 min intervals separated by 26 min periods of perifusion with the vehicle or PMA. Finally, diC_8 (10^{-5} M) was administered to both columns for the last 10 min of the experiment.

Figure 25 (upper panel) shows the effect of increasing concentrations of HA1004 on the release of GH induced by diC_8 (5×10^{-5} M). diC_8 produced a significant 2.56 ± 0.14 fold increase in GH release at 20 min and a 3.04 ± 0.04 fold increase at 40 min. At 20 min, a small reduction in diC_8-induced GH release was seen only at the highest concentration of HA1004. HA1004 did not inhibit diC_8-induced GH release at 40 min. dbcAMP (10^{-3} M) (Figure 25B, lower panel) stimulated GH release at 20 min although the response was not significant. HA1004 at concentrations greater than 10^{-6} M appeared to increase the response to dbcAMP, and the increase was significant at 10^{-5} M. At 40 min, dbcAMP caused a significant 1.84 ± 0.12 fold increase GH release which was not significantly altered with HA1004.

It was possible that the lack of inhibition by HA1004 of dbcAMP-induced GH release was due to a massive accumulation of dbcAMP within the cells which saturated the effect of the inhibitor. Therefore, we increased endogenous cAMP with IBMX and examined the effect of HA1004 on IBMX-induced GH release. IBMX, which inhibits phosphodiesterase, was used to inhibit the breakdown of cAMP, and thereby increase cAMP levels. HA1004 (10^{-7} to 10^{-4} M) was added 30 min before IBMX (0.1 and 0.5 mM) and samples of cell suspension were taken 30 min and 60 min later for the measurement of GH released into the medium. IBMX produced a significant concentration-related increase in GH release (Figure 26). HA1004 did not alter the response except for a significant increase in GH release with HA1004 (10^{-4} M) and IBMX (0.1 mM) at 30 min and with HA1004 (10^{-4} M) and IBMX (0.5 mM) at 60 min.

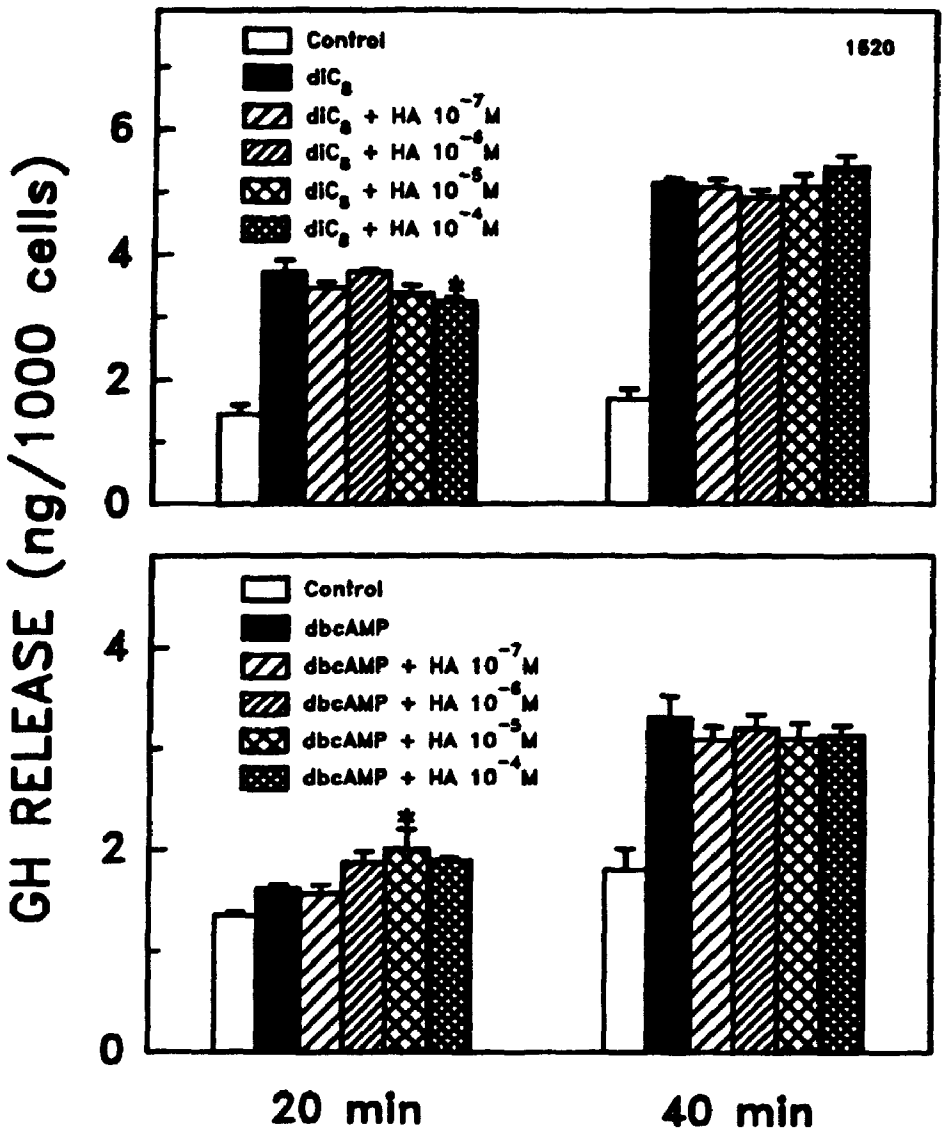


Figure 25: The effect of HA1004 (10^{-7} M to 10^{-4} M) on GH release from purified somatotrophs induced by diC_8 (5 x 10^{-5} M, upper panel) and dbCAMP (10^{-3} M, lower panel) at 20 min and at 40 min. HA1004 was added 15 min before the addition of diC_8 of dbcAMP. The error bars represent the SEM. Where no error bars appear the SEM was too small to plot. Representative of two experiments.

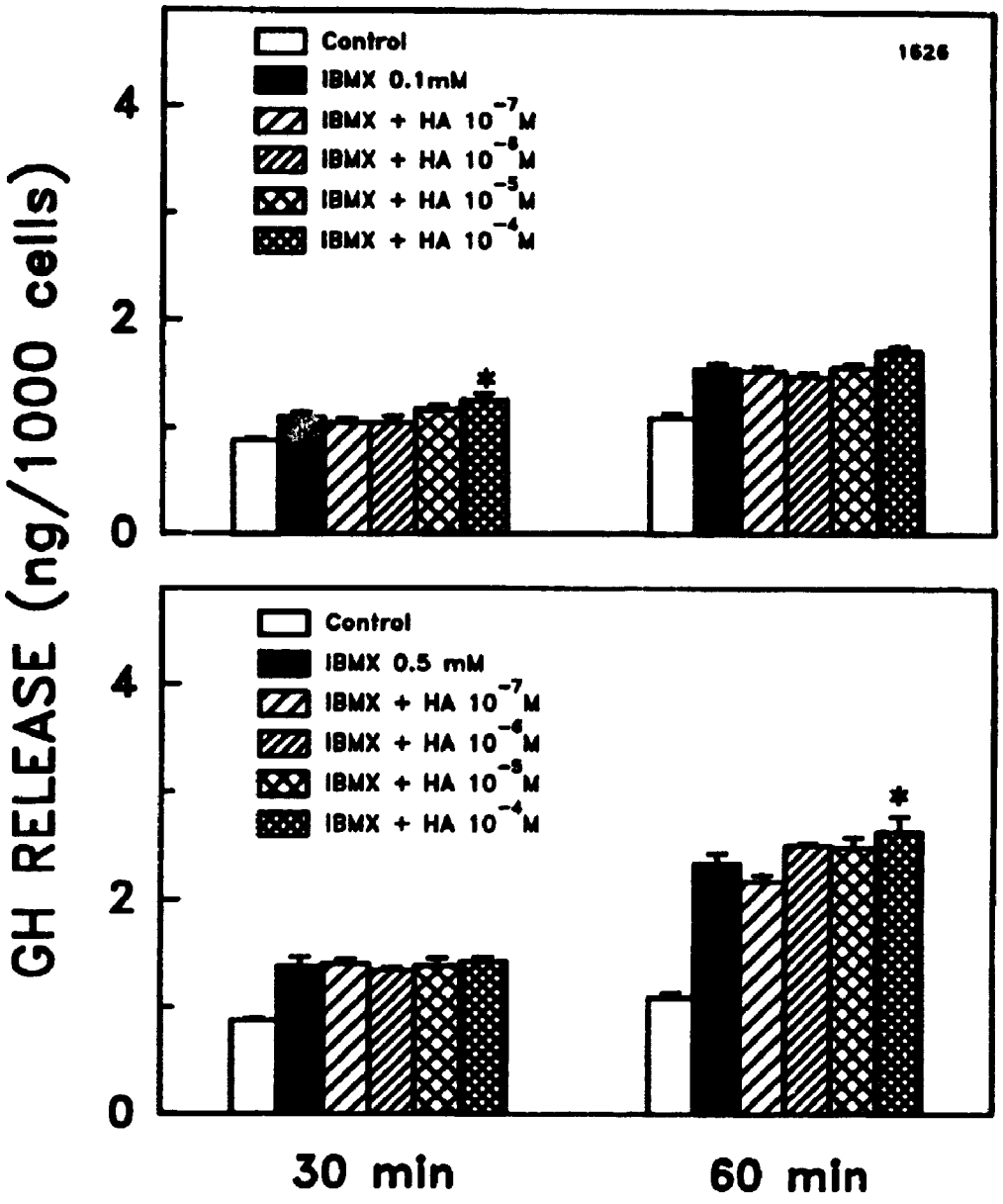


Figure 26: The effect of HA1004 (10^{-7} M to 10^{-4} M) on GH release from purified somatotrophs induced by IBMX (10^{-4} M, upper panel and 5 x 10^{-4} M, lower panel) at 30 min and 90 min. HA1004 was added 30 min before the addition of IBMX. Experiment performed once.

3.3 Phospholipase C

The purpose of these experiments was to examine the effect of GRF on phospholipase C activity by examining changes in the levels of radiolabelled phosphoinositides and inositol phosphates. As a first measure of the effect of GRF (10^{-7} M) on the phosphoinositides and inositol phosphates, the amount of radioactivity present in the organic and aqueous layers (following charcoal treatment) was determined for each GRF incubation time. GRF did not significantly alter the level of ^{32}P incorporation into the organic and aqueous layers (Table II) even though the amount of GH in the medium increased 57% by 3 min [3.6 (GRF) vs. 2.3 ng/1000 cells (control), n=2] and was 500% of control by 90 min [16.2 (GRF) vs. 2.7 ng/1000 cells control), n=2].

Figure 27 shows the effect of GRF on the incorporation of ^{32}P into PIP_2, PIP and PI. GRF did not significantly alter the incorporation of ^{32}P into any of the phosphoinositides except for a small decrease in ^{32}P labelling of PIP at 90 min.

The effect of GRF on the ^{32}P labelling of IP_3, IP_2 and IP is shown in Figure 28. In general, instead of increasing the incorporation of ^{32}P into the inositol phosphates, GRF caused small decreases in ^{32}P incorporation. The decrease in labelling of IP_3 was significantly different from control at 90 min of incubation with GRF. There were also small but significant reductions in ^{32}P incorporation into IP_2 at 20 s, 3 min and 30 min. GRF did not significantly alter labelling of IP at any time.

Table II: Effect of GRF on the incorporation of ^{32}P into the organic and aqueous phases of somatotroph extracts

TIME (min)	ORGANIC PHASE (% of control)	AQUEOUS PHASE (% of control)
0.33 (3)	104.6 ± 5.5	104.0 ± 3.8
1 (4)	101.7 ± 1.3	97.2 ± 2.6
3 (3)	101.2 ± 0.8	102.2 ± 4.3
10 (4)	102.0 ± 0.9	96.5 ± 2.8
30 (4)	101.0 ± 1.5	98.7 ± 2.6
90 (3)	102.4 ± 1.4	102.5 ± 1.9

Time indicates the period of incubation with GRF (10^{-7} M). The radioactivity in the aqueous phases was determined following charcoal treatment. The GRF values are expressed as a percentage of the control at each time. Data are the mean and SEM of these percentages. The number in parentheses denotes the number of experiments at that time point.

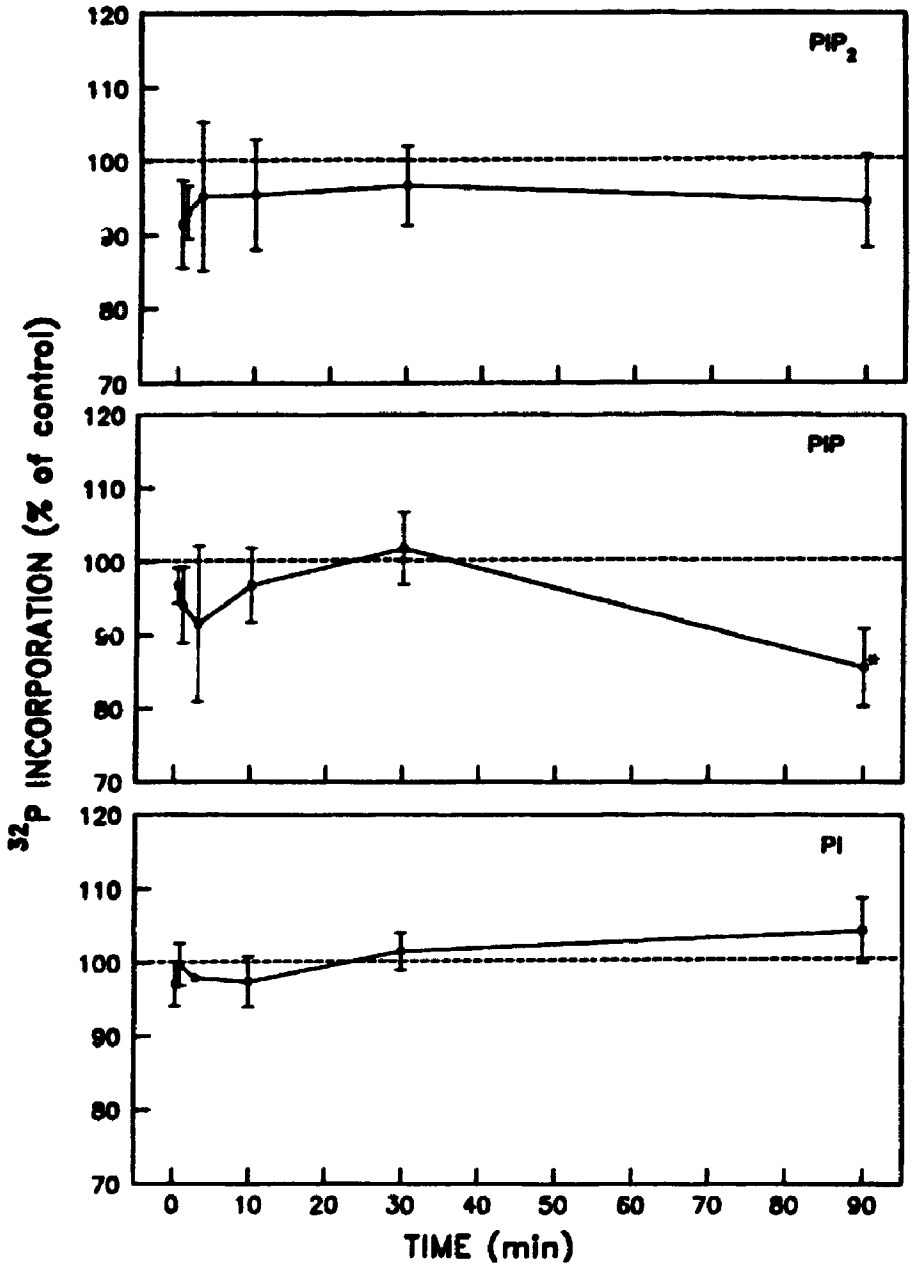


Figure 27: Effect of GRF on the incorporation of ^{32}P into PIP_2, PIP and PI. GRF (10^{-7} M) was added to somatotrophs at zero min. The mean ± SEM of the dpm values for the controls were 4456 ± 318 (PIP_2), 12300 ± 769 (PIP) and 196391 ± 3870 (PI). GRF values are expressed as a percentage of control values which were determined at each time point. Data are the means of the percentages from 3-4 experiments. Error bars represent the SEM. Asterisks denote values significantly different from control ($P < 0.05$).

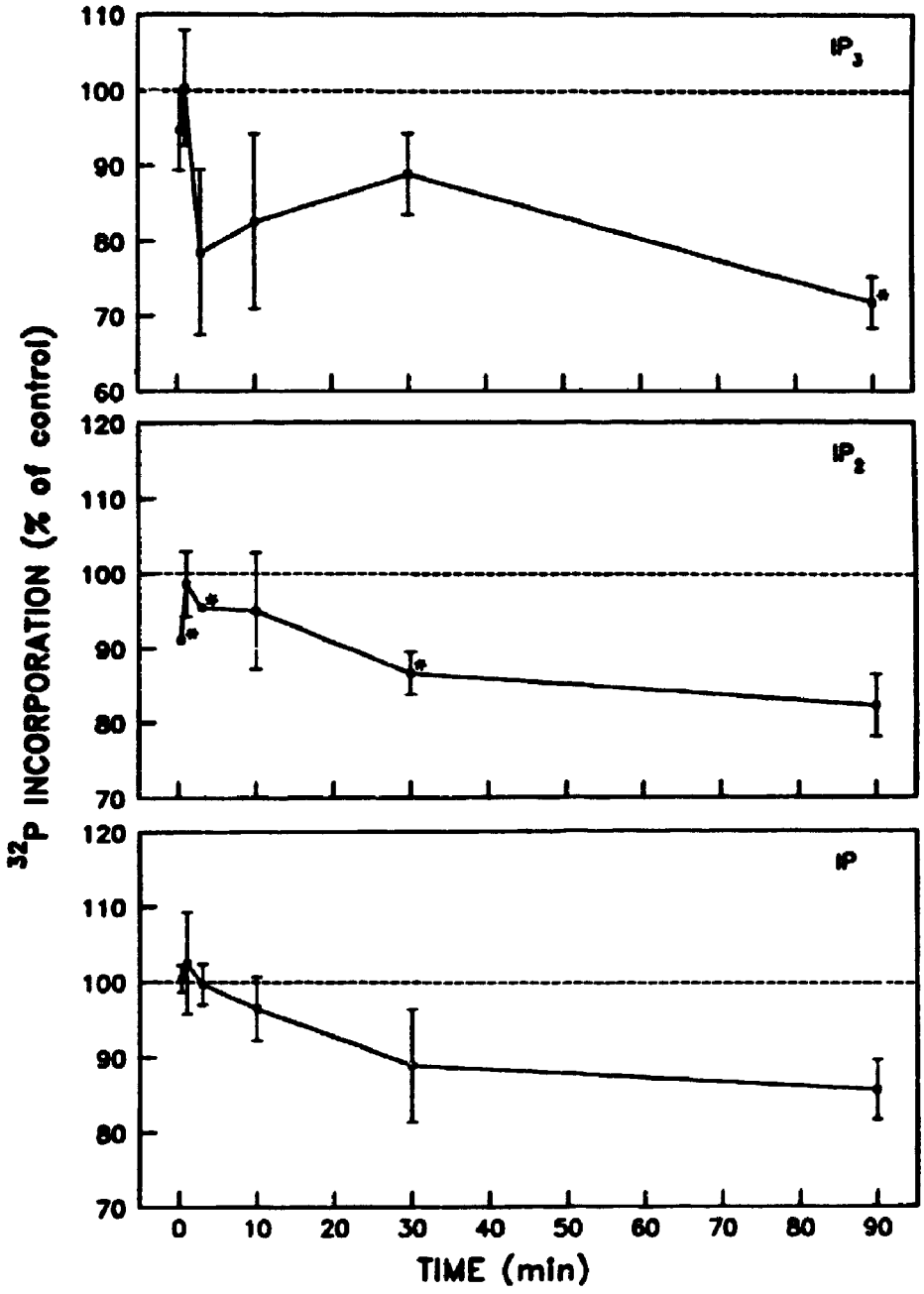


Figure 28: Effect of GRF on the incorporation of ^{32}P into IP_3, IP_2 and IP. GRF (10^{-7} M) was added to somatotrophs at zero min. The mean ± SEM of the dpm values for the controls were 10774 ± 389 (IP_3), 25711 ± 684 (IP_2) and 171126 ± 6063 (IP). GRF values are expressed as a percentage of control values which were determined at each time point. Data are the means of the percentages at each time point from 3-4 experiments. Error bars represent the SEM. Asterisks denote values significantly different from control ($P < 0.05$).

3.4 Protein kinase C

The purpose of this series was to study the role of protein kinase C in GRF action by first determining whether protein kinase C is present in somatotrophs and then examining the effect of GRF on protein kinase C activity. Protein kinase C activity was found in soluble and particulate fractions of somatotrophs. Measurement of full enzyme activity required the presence of Ca^{2+} and lipid in the assay incubation mixture. The relationship between the amount of enzyme activity to the amount of protein in the assay was linear over the range of protein concentrations used in the assay.

Protein kinase C activity was expressed as specific activity and total activity recovered in the soluble and particulate fractions. Specific activity was the amount of ^{32}P transferred divided by the amount of protein in the sample of column effluent in the reaction mixture divided by the number of minutes of assay incubation time. The specific activity in the soluble and particulate fractions following treatment was also expressed as the percentage of the specific activity detected in the soluble and particulate fractions from the control cells. The total activity recovered in the soluble and particulate fractions was the amount of ^{32}P transferred per million cells in the initial cell sample. The total activity recovered in the soluble and particulate fractions was also expressed as the percentage of the total detectable activity (the sum of the activity measured in the soluble and particulate fractions).

In control groups, specific activity of soluble protein kinase C was 2512.2 $\pm$ 232.9 pmol ^{32}P / mg protein / min (mean $\pm$ SEM from 24 observations) and specific activity of particulate protein kinase C was 1259.6 $\pm$ 87.3 pmol ^{32}P / mg protein / min (mean $\pm$ SEM from 24 observations). Most of the detectable protein kinase C activity

was present in the soluble fraction (91.0 ± 3.0 % of the total measurable activity; mean ± SEM from 24 observations).

3.4.1 Effect of protein kinase C activators

PMA caused a significant 1.82 ± 0.02 fold increase in the release of GH at 3 min. diC_8 produced a significant 1.62 ± 0.07 fold increase in the release of GH at 3 min. Table III shows the effect of PMA and diC_8 on protein kinase C activity in somatotrophs. Incubation of somatotrophs with PMA (10^{-8} M) for 3 min caused a significant reduction in the total activity recovered in soluble fractions and a significant increase in the total activity recovered in particulate fractions. In other words, PMA, as expected, caused a translocation of enzyme activity from the soluble to particulate fractions. PMA also significantly decreased the specific activity of protein kinase C in the soluble fractions and increased the specific activity of protein kinase C in particulate fractions. diC_8 (5×10^{-5} M) also caused a significant translocation of protein kinase C at 3 min. The specific activity of protein kinase C, with diC_8, in particulate fractions was significantly increased, while the reduction in specific activity of protein kinase C in soluble fractions was not significant. The concentration of protein in the soluble and particulate fractions was not significantly altered with PMA and diC_8 treatment. Protein concentrations (mean ± SEM, n=3) in the PMA experiment were: soluble fractions: control -- 0.139 ± 0.003 μg / 25 μl, PMA -- 0.137 ± 0.002 μg / 25 μl; particulate fractions: control -- 0.027 ± 0.003 μg / 25 μl, PMA -- 0.027 ± 0.001 μg / 25 μl. Protein concentrations (mean ± SEM, n=3) in diC_8 experiment were: soluble fractions: control -- 0.104 ± 0.003 μg / 25 μl, diC_8 -- 0.112 ± 0.005 μg / 25 μl; particulate fractions: control -- 0.023 ± 0.002 μg / 25 μl, diC_8 -- 0.025 ± 0.003 μg / 25 μl.

Table III: Effect of PMA and diC_8 on specific activity of protein kinase C and total protein kinase C activity recovered in soluble and particulate fractions of purified somatotrophs.

TREATMENT	FRACTION	SPECIFIC ACTIVITY (pmol ^{32}P/mg protein/min)		TOTAL ACTIVITY PER FRACTION PER MILLION CELLS (pmol ^{32}P/fraction/million cells)		PERCENTAGE OF TOTAL ACTIVITY RECOVERED	
		control	PMA or diC_8	control	PMA or diC_8	control	PMA or diC_8
PMA	soluble	3038.7 ± 111.6 (100)	623.0 ± 19.0* (20.5 ± 0.6)*	8.22 ± 0.17	1.64 ± 0.03*	93.3 ± 0.1	17.8 ± 0.3*
	particulate	1114.1 ± 74.4 (100.0)	14216.6 ± 491.7* (1276.0 ± 44.1)*	0.59 ± 0.02	7.58 ± 0.28*	6.7 ± 0.1	82.2 ± 0.3*
diC_8	soluble	2267.7 ± 74.1 (100)	2184.9 ± 61.0 (96.4 ± 2.3)	5.63 ± 0.18	5.85 ± 0.27	92.1 ± 0.7	82.9 ± 0.6*
	particulate	885.3 ± 123.1 (100)	2108.8 ± 264.5* (238.2 ± 29.9)*	0.48 ± 0.04	1.21 ± 0.10*	7.9 ± 0.7	17.1 ± 0.6*

PMA (10^{-6} M) or diC_8 (5×10^{-5} M) was added to somatotrophs for 3 min. The results shown are the mean ± SEM of triplicate determinations from 3 samples at each time point. The results in parentheses are the mean ± SEM of the specific activity with GRF treatment expressed as a percentage of control values at each time point. Asterisks denote values significantly different from control ($P < 0.05$).

GRF (10^{-7} M) was added to somatotrophs at zero min. The results shown are the mean $\pm$ SEM of triplicate determinations from 3 samples at each time point. The results in parentheses are the mean $\pm$ SEM of the specific activity with GRF treatment expressed as a percentage of control values at each time point. Asterisks denote values significantly different from control ($P < 0.05$).

Table IV: Effect of GRF on specific activity of protein kinase C and total protein kinase C activity recovered in soluble and particulate fractions of purified somatotrophs.

TIME (min)	FRACTION	SPECIFIC ACTIVITY (pmol ^{32}P/mg protein/min)		TOTAL ACTIVITY PER FRACTION PER MILLION CELLS (pmol ^{32}P/fraction/million cells)		PERCENTAGE OF TOTAL ACTIVITY RECOVERED	
		control	GRF	control	GRF	control	GRF
0.33	soluble	1567.4 ± 115.1 (100.0)	1630.9 ± 37.2 (104.1 ± 2.4)	4.43 ± 0.24	4.50 ± 0.12	89.6 ± 2.7	89.4 ± 2.3
	particulate	814.7 ± 212.7 (100.0)	709.7 ± 154.5 (87.1 ± 18.9)	0.51 ± 0.13	0.53 ± 0.12	10.4 ± 2.7	10.6 ± 2.3
1	soluble	1406.9 ± 30.7 (100)	1037.2 ± 167.1 (112.6 ± 7.2)	5.43 ± 0.46	5.64 ± 0.41	91.7 ± 1.4	92.4 ± 1.2
	particulate	1584.0 ± 101.7 (100)	1042.4 ± 53.6 (100.5 ± 5.2)	0.50 ± 0.09	0.46 ± 0.05	8.3 ± 1.4	7.6 ± 1.2
3	soluble	4950.0 ± 258.9 (100)	4861.3 ± 260.2 (98.2 ± 5.3)	13.82 ± 0.71	14.01 ± 0.48	92.8 ± 1.2	94.6 ± 0.7
	particulate	1348.4 ± 266.9 (100)	1206.1 ± 231.1 (89.4 ± 17.1)	1.09 ± 0.25	0.80 ± 0.09	7.2 ± 1.2	5.4 ± 0.7
10	soluble	1453.6 ± 90.3 (100)	1665.4 ± 99.3 (114.4 ± 7.6)	4.14 ± 0.36	4.74 ± 0.31	88.2 ± 1.2	90.3 ± 1.7
	particulate	553.2 ± 75.2 (100)	567.9 ± 89.4 (102.0 ± 16.1)	0.55 ± 0.03	0.52 ± 0.12	11.9 ± 1.2	9.7 ± 1.7
30	soluble	2795.4 ± 143.3 (100)	2674.2 ± 115.4 (95.6 ± 4.1)	5.03 ± 0.29	4.92 ± 0.16	88.8 ± 2.5	89.1 ± 0.4
	particulate	1577.6 ± 324.7 (100)	1564.0 ± 129.1 (99.1 ± 8.2)	0.63 ± 0.13	0.60 ± 0.03	11.2 ± 2.5	10.9 ± 0.4
90	soluble	2776.5 ± 29.3 (100)	3042.9 ± 64.0* (110.0 ± 2.3)*	8.49 ± 0.10	9.07 ± 0.04	91.3 ± 1.3	89.8 ± 0.5
	particulate	1087.2 ± 233.1 (100)	1381.6 ± 126.1 (127.1 ± 11.3)	0.81 ± 0.13	1.03 ± 0.06	8.7 ± 1.3	10.2 ± 0.5

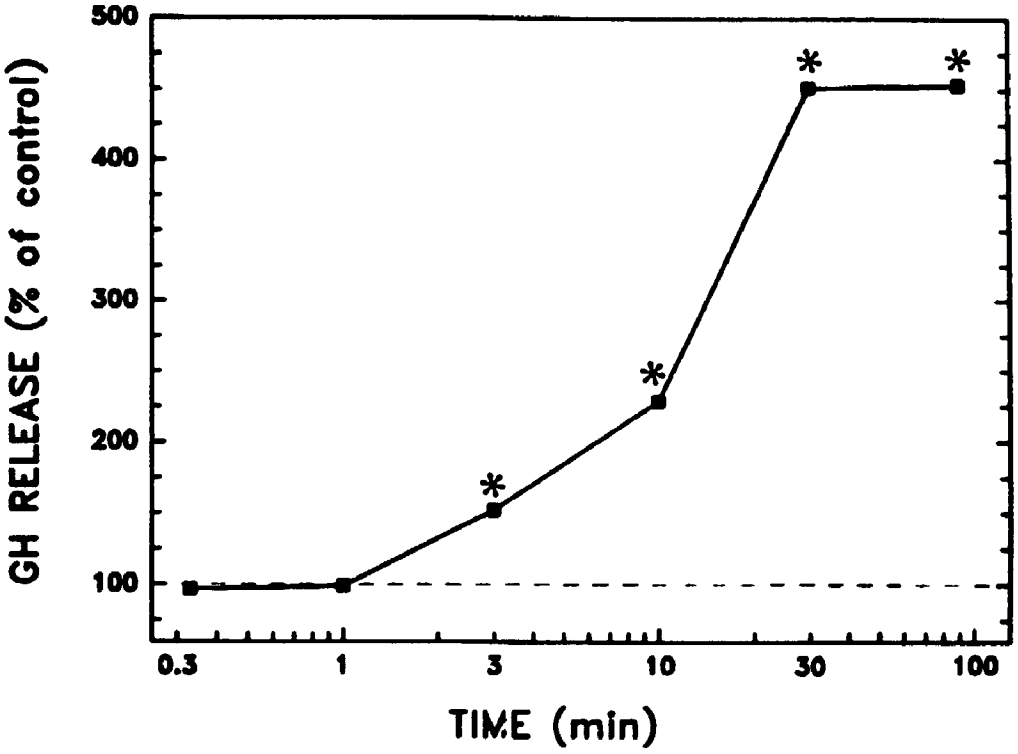


Figure 29: Effect of GRF on GH release from purified somatotrophs in protein kinase C experiments. GRF (10^{-7} M) was added to somatotrophs at zero min. GH release induced by GRF was expressed as a percentage of control values. Data are the mean ± SEM. Error bars were too small to plot. Asterisks denote values significantly different from control ($P < 0.05$).

3.4.2 Effect of GRF

GRF (10^{-7} M) induced a significant increase in GH release by 3 min (Figure 29). Table IV shows the effect of GRF (10^{-7} M) on protein kinase C activity in soluble and particulate fractions at 0.33, 1, 3, 10, 30 and 90 min of incubation. GRF did not significantly alter the distribution of protein kinase C activity in somatotrophs at any time point. In addition, GRF did not significantly alter the specific activity of protein kinase C in soluble and particulate fractions, except for a small (10%) but significant increase in specific activity of protein kinase C in soluble fractions at 90 min. The concentration of protein in the soluble and particulate fractions was not significantly altered with GRF treatment. Protein concentrations (mean $\pm$ SEM, n=18) in controls were: soluble fractions -- 0.146 $\pm$ 0.051 μg / 25 μl; particulate fractions -- 0.0365 $\pm$ 0.0046 μg / 25 μl and in GRF samples were: soluble fractions -- 0.144 $\pm$ 0.051 μg / 25 μl; particulate fractions 0.0349 $\pm$ 0.0043 μg / 25 μl.

3.4.3 Effect of prolonged PMA treatment on protein kinase C activity in somatotrophs

We also examined the effect of prolonged PMA treatment on protein kinase C activity in somatotrophs. We were interested in the activity of protein kinase C following PMA treatment since we had used this approach to study the role of protein kinase C in GRF-induced GH release in earlier experiments (see section 3.2). Table V shows the effect of PMA (10^{-9} M) on protein kinase C activity in soluble and particulate fractions at 1 and 4 h of incubation. PMA treatment significantly altered the distribution of protein kinase C activity. At both times, there was also a consistent decrease, although not statistically significant, in specific activity and total activity in the cytosolic

fractions and a statistically significant increase in specific and total activity in the membrane fractions. The total activity (the sum of the cytosolic and membrane activities expressed as pmol ^{32}P/fraction/million cells) was not significantly different at 1 and 4 h compared to controls. Protein concentrations in control and treatment groups were not significantly different. Protein concentrations (mean ± SEM, n=3) in the 1 h experiment were: soluble fractions: control -- 0.171 ± 0.002 μg / 25 μl, PMA -- 0.161 ± 0.007 μg / 25 μl; particulate fractions: control -- 0.043 ± 0.003 μg / 25 μl, PMA -- 0.037 ± 0.003 μg / 25 μl. Protein concentrations (mean ± SEM, n=3) in 4h experiment were: soluble fractions: control -- 0.254 ± 0.001 μg / 25 μl, PMA -- 0.253 ± 0.004 μg / 25 μl; particulate fractions: control -- 0.054 ± 0.002 μg / 25 μl, PMA -- 0.061 ± 0.003 μg / 25 μl.

Table V: Effect of long periods of PMA incubation on specific activity of protein kinase C and total protein kinase C activity recovered in soluble and particulate fractions of purified somatotrophs.

TIME (hour)	FRACTION	SPECIFIC ACTIVITY (pmol ^{32}P/mg protein/min)		TOTAL ACTIVITY PER FRACTION PER MILLION CELLS (pmol ^{32}P/fraction/million cells)		PERCENTAGE OF TOTAL ACTIVITY RECOVERED	
		control	PMA	control	PMA	control	PMA
1	soluble	3790.4 ± 236.0 (100.0)	2962.1 ± 225.8 (78.2 ± 6.0)	10.12 ± 0.60	1.64 ± 0.03*	95.7 ± 0.3	87.5 ± 0.6*
	particulate	683.8 ± 36.4 (100.0)	1833.5 ± 61.6* (268.2 ± 9.0)*	0.46 ± 0.06	7.58 ± 0.28*	4.3 ± 0.3	12.51 ± 0.6*
4	soluble	3764.0 ± 336.2 (100)	3231.0 ± 78.7 (85.8 ± 2.1)	14.52 ± 0.63	5.85 ± 0.27	97.1 ± 0.1	93.1 ± 0.4*
	particulate	518.3 ± 30.8 (100)	1006.6 ± 69.7* (194.2 ± 13.5)*	0.43 ± 0.03	1.21 ± 0.10*	2.9 ± 0.1	6.9 ± 0.4*

PMA (10^{-8} M) was added to somatotrophs for 1 or 4h. The results shown are the mean ± SEM of triplicate determinations from 3 samples at each time point. The results in parentheses are the mean ± SEM of the specific activity with GRF treatment expressed as a percentage of control values at each time point. Asterisks denote values significantly different from control ($P < 0.05$).

CHAPTER 4: DISCUSSION

The purpose of this project was to investigate the role of the phosphoinositide second messenger system in the regulation of GH release. Our hypothesis was that the phosphoinositide second messenger system is an alternative pathway for GRF action in the somatotrophs of the anterior pituitary. If this signalling system is a second messenger system for GRF, then the following criteria should be met (see Figure 9):

1) GRF should stimulate the activity of phospholipase C.

2) GRF should stimulate phosphoinositide turnover increasing the concentrations of IP_3 and diacylglycerol. Their increase should precede or be concurrent with an increase in GH release.

3) GRF should stimulate the activity of protein kinase C.

4) Diacylglycerol analogues and other protein kinase C activators should stimulate GH release.

5) IP_3 should stimulate GH release.

6) Activation of phospholipase C and/or stimulation of phosphoinositide turnover by any means should stimulate GH release. Inhibition of phospholipase C and/or phosphoinositide turnover by any means should inhibit GH release.

Criteria 1-4 were addressed in this project. We did not measure phospholipase C activity directly in somatotroph plasma membrane preparations, but, instead, determined the levels of the individual radiolabelled phosphoinositides and inositol phosphates following GRF treatment. The effect of IP_3 on GH release was not examined

since criteria 1-3 were not fulfilled. Criterion 6 was not tested because ot a lack of specific stimulators or inhibitors of phospholipase C.

4.1 Activation of protein kinase C does stimulate GH release

To study the role of the phosphoinositide second messenger system in the regulation of GH release, we first assessed whether activation of protein kinase C would stimulate the release of GH from purified somatotrophs (criterion 4). In previous studies, activation of protein kinase C stimulated the release of GH from mixed populations of anterior pituitary cells and pituitary tumour cell lines (Smith and Vale, 1980; Ohmura et al., 1984; Aizawa and Hinkle, 1985; Ikeda et al., 1985; Barinaga et al., 1985; Ohmura and Friesen, 1985; Negro-Vilar and Lapetina, 1985; Judd et al., 1986; Ray et al., 1986; Ikuyama et al., 1987; Sheppard et al., 1987; Richardson and Twente, 1988). However, the effect of protein kinase C activators in these experiments may have been through a paracrine effect of another cell type, in the case of the anterior pituitary cell preparations, and may not have been physiological in the case of the pituitary tumour cell lines. Therefore, we extended these experiments by examining the direct effect of the protein kinase C activators on GH release from purified somatotrophs. In addition, we looked for possible concurrent changes in cAMP accumulation in the somatotrophs.

Protein kinase C may be activated by the application of diacylglycerol analogues and a number of different types of tumour promoters. We chose two different compounds, the diacylglycerol analogue, diC_8, and the phorbol ester, PMA, to activate protein kinase C. diC_8 is a potent diacylglycerol analogue (Conn et al., 1986), while

PMA, a tumour promoter, is a potent phorbol ester (Leach and Blumberg, 1989). [In subsequent experiments, we confirmed that diC_8 (5×10^{-5} M) and PMA (10^{-8} M) do, in fact, activate protein kinase C in purified somatotrophs (Table III)].

We first demonstrated, in static incubation, that activation of protein kinase C with diC_8 and PMA does stimulate the acute concentration-related increase in GH release from purified somatotrophs (Figure 10 and 11). The massive GH response to diC_8 at 10^{-4} M compared to 3×10^{-5} M (Figure 10) may represent a steep dose response to diC_8 or alternatively may have been due, at least in part, to an alteration of the plasma membrane through a nonspecific lipid effect. This might be confirmed by measurement of cytoplasmic markers such as lactate dehydrogenase outside the cells. The GH response to PMA reached a maximum at 10^{-9} M (Figure 11). The apparent smaller GH response to PMA at higher concentrations and longer incubation times may reflect the inactivation of protein kinase C activity by PMA (see section 4.2.1). In general, the GH responses to diC_8 and PMA in static incubation were relatively small compared to the GH response to GRF (Sheppard et al., 1985). This is consistent with the transient nature of the GH response to diC_8 and PMA observed in perifusion (see Figure 12 and 13). We conclude that activation of protein kinase C stimulates the release of GH from somatotrophs.

Our results are consistent with previous studies. Ohmura et al. (1984) first studied the effects of PMA on GH release from cultured rat anterior pituitary cells and found that PMA (10^{-7} M) caused a maximal 3-4 fold stimulation of GH release at 6 h of incubation. The effect of PMA was apparent by 1 h of incubation and was observed at concentrations as low as 10^{-9} M. PMA-induced GH release from mixed populations of

anterior pituitary cells was subsequently observed in a number of studies. Effective concentrations of PMA ranged from 10^{-9} M to 10^{-7} M with incubation times as short as 15 min and as long as 4 h (Barinaga et al., 1985; Ohmura and Friesen, 1985; Summers et al., 1985; Judd et al., 1986; Ray et al., 1986; Sheppard et al., 1987). An effect of PMA on GH release was also observed in human pituitary adenoma cells (Ikuyama et al., 1987) and GH_4C_1 cells (Aizawa and Hinkle, 1985). Other protein kinase C activators also stimulate GH release. Negro-Vilar and Lapetina (1985) used the phorbol ester, phorbol 12,13-dibutyrate (PDBu), and the diacylglycerol analogue, didecanoylglycerol (diC_{10}), to activate protein kinase C in anterior pituitary cells. They found that PDBu at 10^{-9} M and diC_{10} at 10^{-6} M were minimally effective concentrations. Maximal release of GH was attained at 15 min with diC_{10} and 60 min with PDBu. Ohmura and co-workers (1987) observed that the tumour promoters mezerein and teleocidin, which activate protein kinase C, stimulated the release of GH from cultured rat anterior pituitary cells. Our present studies extend these results, since they demonstrate that the protein kinase C activators have a direct effect on somatotrophs.

The perifusion studies were carried out to define the time base for the stimulatory effects of diC_8 and PMA on GH release and to determine repeatability and reproducibility. Protein kinase C activation by diC_8 and PMA caused an immediate burst of GH release that declined quickly during the period of perifusion. The shape of the GH response to PMA was similar to that seen with diC_8 except that the GH response was not quite as rapid and GH release remained above basal values after discontinuation with PMA. Since diC_8 and PMA are relatively stable compounds in solution (Leach and Blumberg, 1989; Blumberg, 1980) the decline in GH release, while the secretagogue was

maintained, was not simply due to their rapid breakdown. The decline does not reflect depletion of GH since the total GH released during these studies was less than 5% of the cell content of GH. As well, GH responses of much greater magnitude that were maintained over prolonged periods of time have been previously observed for other GH secretagogues (Kraicer and Spence, 1981; Kraicer and Chow, 1982; Cowan et al., 1983). Possible explanations for the rapid decline in GH release during perifusion are that the protein kinase activators stimulate only a specific pool of releasable GH or that their effect is short lived (see also 4.1.1).

The apparent increased responsiveness of the dispersed anterior pituitary cells to diC_8 and PMA in perifusion compared to the responses in static incubation requires further comment. As discussed in the introduction, the background level of GH in the medium in static incubation does not reflect true basal GH release and, therefore, GH responses to diC_8 and PMA can not be related to this background level. On the other hand, the GH release in perifusion is a measure of basal release so that GH responses to diC_8 and PMA may can be related to the basal release. Thus, it is unlikely that the cells in perifusion are more responsive to diC_8 and PMA.

We applied diC_8 and PMA in repeated pulses to examine the reversibility and repeatability of the GH response to these secretagogues. The GH response to diC_8 was reversible and repeatable (Figure 12). On the other hand, the GH response to a second application of PMA was much reduced compared to the first response (Figure 13). The lack of repeatability was observed when we reduced the concentration of PMA to 5×10^{-10} M to produce an initial GH response of similar magnitude to that seen with diC_8 (Figure 14). Therefore, the refractoriness of the cells to PMA was not due to a depletion

of protein kinase C-releasable GH. Instead, we assumed that the lack of repeatability of the GH response to PMA was due to a desensitization or inactivation of protein kinase C by phorbol ester pretreatment since others had shown that incubation of cells with phorbol esters desensitizes cells to a subsequent stimulation with protein kinase C activators. We exploited this effect of PMA in later experiments (see section 4.2.1).

The dynamics of the GH response to PMA was first described by Smith and Vale (1980). They observed that PMA (10^{-7} M) caused an immediate burst of GH release from superfused rat anterior pituitary cells attached to cytodex beads which declined over the period of perifusion with PMA and remained elevated following the application of PMA. The refractoriness of the GH response to PMA was also observed by Richardson and Twente (1988). Perifusion of dispersed anterior pituitary cells with PMA (10^{-8} M) for 6 min caused an immediate burst of GH which declined slowly following the application of PMA. The cells did not respond to subsequent 6 min pulses of PMA, 20 and 40 min later. The dynamics and repeatability of GH response to diC_8 in perifusion, on the other hand, has not been previously described. A possible explanation for the difference in the repeatability of the GH response to diC_8 compared to PMA is that diC_8 is eventually metabolized while PMA is not (Jaken, 1989; Leach and Blumberg, 1989).

These results taken together with the results from the static incubation studies demonstrate that activation of protein kinase C stimulates the immediate and transient release of GH from somatotrophs.

4.1.1 Mechanism of protein kinase C activator-induced GH release

We also examined the possible mechanism(s) through which activated protein kinase C stimulates GH release. Since increases in intracellular cAMP and Ca^{2+}

concentrations stimulate the release of GH, we asked whether these second messengers mediate the action of protein kinase C. The effect of diC_8 and PMA or cAMP accumulation in purified somatotrophs was studied in static incubation. To examine the role of Ca^{2+}, two approaches were used. First, the effect of removal of extracellular Ca^{2+} on the protein kinase C activator-induced GH release and cAMP accumulation was studied by incubating cells in medium containing no added Ca^{2+}. Second, Ca^{2+} entry into somatotrophs was blocked with nifedipine and the effect of subsequent addition of diC_8 and PMA on GH release and cAMP accumulation was determined.

Activation of protein kinase C caused a significant and concentration-related, increase in cAMP accumulation in somatotrophs (Figure 10 and 11). The increase in cAMP occurred concurrently with the increase in GH release. These results indicate that cAMP may mediate the effects of diC_8 and PMA in stimulating GH release and is an example of "cross-talk" that may occur between second messenger systems.

Activation of protein kinase C is reported to have different effects on cAMP accumulation in different cell types which may be related to the different forms of protein kinase C present (Nishizuka, 1986). For example, Gasovsky and Gutkind (1991) have recently reported that protein kinase C-α inhibits, while protein kinase C-γ facilitates forskolin-mediated accumulation of cAMP in NIH 3T3 cells. The results from previous studies concerning the effect of protein kinase C activators on cAMP accumulation in anterior pituitary cells and pituitary tumour cell lines have not been consistent. Ohmura and Friesen (1985) reported that PMA (1.6×10^{-9} M) did not stimulate cAMP accumulation in cultures of rat anterior pituitary cells during 15 to 120 min of incubation. A similar result was observed by Baringa et al. (1985) who incubated cultures of rat

anterior pituitary cells for 1 h with PMA (10^{-7} M). Other researchers have shown that while PMA has no effect on basal cAMP accumulation, it potentiates the effect of corticotropin releasing hormone (Reisine and Guild, 1987; Reisine, 1989). In contrast, Ray and colleagues (1986) found that PMA (10^{-7} M) increased cAMP accumulation in cultured ovine pituitary cells at 2 min of incubation. Also, PMA increased basal cAMP levels in GH_3 and GH_4C_1 pituitary cell lines (Brostrom et al., 1983; Summers and Cronin, 1986). In our experiments, the effect of the protein kinase C activators on cAMP accumulation was small. The discrepancy in the results may have been due, in part, to differences in the sensitivity of the cAMP assays, to different incubation times, or may be masked by background levels in the six or more cells types in the anterior pituitary.

The advantage of the homogenous cell preparation used in our experiments is that the increase in cAMP accumulation induced by the protein kinase C activators may be attributed directly to the somatotroph. Thus, the release of GH induced by activation of protein kinase C may not be independent of the cAMP pathway.

The mechanism through which protein kinase C causes an increase in cAMP accumulation is not clear. Summers and Cronin (1986) reported that PMA stimulates adenylate cyclase activity in membrane preparations from the 235-1 pituitary cell line. PMA treatment of intact human platelets, through an action on protein kinase C activity, caused a loss of adenylate cyclase inhibition by GTP and inhibitory agonists (Jakobs et al., 1985). Consistent with this finding, purified protein kinase C phosphorylates the α subunit of G_i, thereby suppressing its function in hormonal inhibition of adenylate cyclase

(Katada et al., 1985). Thus, the increase in cAMP accumulation may be due to a direct effect of protein kinase C on a component of the adenylate cyclase system.

We also examined the involvement of Ca^{2+} in protein kinase C activator-induced GH release and cAMP accumulation, since all known GH secretagogues cause an increase in $[Ca^{2+}]_i$ (Lussier et al., 1991a,b). The increase in $[Ca^{2+}]_i$ is primarily due to influx of Ca^{2+} through voltage-gated Ca^{2+} channels, since removal of extracellular Ca^{2+} or blockage of voltage-gated Ca^{2+} channels inhibits the secretagogue-induced increases in $[Ca^{2+}]_i$ (Lussier et al., 1991a,b). Therefore, to assess the role for Ca^{2+} in protein kinase C activator-induced GH release, we studied the effect of removal of extracellular Ca^{2+} and the addition of nifedipine (a voltage-gated Ca^{2+} blocker) on the subsequent GH response to protein kinase C activation. Given the previous finding that these two manoeuvres block the increase in $[Ca^{2+}]_i$ induced by diC_8 and PMA (Lussier et al., 1991a), we expected that the increase in GH release would also be blocked.

Removal of Ca^{2+} blocked the GH response to PMA (10^{-8} M), suggesting that protein kinase C acts by increasing Ca^{2+} influx in somatotrophs. The GH response to diC_8 (5×10^{-5} M) was significantly reduced, but not abolished, at 8 and 16 min, while the response at 32 min was not significantly different from the response to diC_8 in normal medium (Figure 18). A caveat of the use of low-Ca^{2+} medium to prevent Ca^{2+} influx is that Ca^{2+} gradients are altered. Therefore, there may be leaching out of Ca^{2+} from an essential intracellular pool and/or mobilization of Ca^{2+} from an intracellular store.

Blockage of voltage-gated Ca^{2+} channels with nifedipine significantly reduced, but did not completely block diC_8- and PMA-induced GH release (Figure 19). The

concentration of nifedipine chosen had been shown to inhibit the GRF-induced increase in GH release and $[Ca^{2+}]_i$ in somatotrophs (Lussier et al., 1988, 1991b).

A reduction in protein kinase C activator-induced GH release by removal of extracellular Ca^{2+} or by Ca^{2+} channel blockers has been previously described for mixed populations of anterior pituitary cells. Ohmura et al. (1984) found that 2 mM Co^{2+}, a competitive antagonist of Ca^{2+}, completely inhibited and verapamil, a Ca^{2+} channel blocker, significantly reduced GH release induced by PMA from cultured rat anterior pituitary cells. Sheppard and co-workers (1987) reported that removal of extracellular Ca^{2+} or the Ca^{2+} channel blockers, nifedipine and diltiazem, reduced, but did not abolish GH release induced by PMA from dispersed rat anterior pituitary cells.

It is clear that the reduction in protein kinase C activator-induced GH release in low-Ca^{2+} medium or with nifedipine is not the result of a reduction in cAMP levels, since cAMP accumulation tended to increase under these conditions. The increase in cAMP accumulation in low-Ca^{2+} medium may reflect an increase in adenylate cyclase activity, since Narayanan et al. (1989) observed that Ca^{2+} has an inhibitory effect on basal or GRF-stimulated adenylate cyclase activity in purified somatotroph membrane preparations. The effect on cAMP accumulation is similar to results from previous experiments (Sheppard et al., 1987; Lussier et al., 1988) in which low-Ca^{2+} medium and Ca^{2+} channel blockers blocked GRF-induced GH release, and augmented cAMP accumulation.

The failure of removal of extracellular Ca^{2+} and Ca^{2+} channel blockers to completely inhibit diC_8-induced GH release or Ca^{2+} channel blockers to completely inhibit PMA-induced GH release may be due to the presence of residual Ca^{2+} in the

medium or to incomplete blockade of voltage-gated Ca^{2+} channels. Alternatively, protein kinase C might stimulate GH release by increasing Ca^{2+} influx through dihydropyridine-insensitive channels, by increasing $[Ca^{2+}]_i$ through a mobilization of Ca^{2+} from intracelluar stores or by acting independently of an increase in $[Ca^{2+}]_i$. Nevertheless, our results suggest that activation of protein kinase C stimulates the influx of Ca^{2+} through voltage-gated Ca^{2+} channels. This is consistent with the increase in $[Ca^{2+}]_i$ seen with activation of protein kinase C which is blocked in low-Ca^{2+} medium and with nifedipine (Lussier et al., 1991a).

Measurements of $[Ca^{2+}]_i$ following activation of protein kinase C have been made in a number of different cell types including anterior pituitary cells and pituitary tumour cell lines. The protein kinase C activators are reported to both increase and/or decrease $[Ca^{2+}]_i$. In some cases, an increase in $[Ca^{2+}]_i$ is followed by a drop in $[Ca^{2+}]_i$ to below basal levels. Holl and colleagues (1989a) reported that activation of protein kinase C results in a transient increase in $[Ca^{2+}]_i$ in somatotrophs identified by reverse haemolytic plaque assay. Lussier et al. (1991a) observed that $[Ca^{2+}]_i$ increased transiently following diC_8 and PMA addition, declined and then dropped below basal levels. This effect was also observed in GH_4C_1 cells (Albert and Tashjian Jr., 1985; Albert et al., 1987). The shape of the $[Ca^{2+}]_i$ response to diC_8 and PMA is similar to the GH response to these compounds seen in perifusion except that the GH response does not drop to below basal values unlike the Ca^{2+} response (Figure 12 and 13).

The transient increase in $[Ca^{2+}]_i$ induced by protein kinase C activation involves influx of Ca^{2+} via voltage-gated Ca^{2+} channels since the protein kinase C activator-induced increase in $[Ca^{2+}]_i$, like the increase in GH release, is reduced in low-Ca^{2+}

medium and in the presence of Ca^{2+} channel blockers (Holl et al., 1989a; Lussier et al., 1991a). The effect of protein kinase C may be by direct phosphorylation of voltage-gated Ca^{2+} channels. Alternatively, protein kinase C might reduce K^+ conductance through phosphorylation of K^+ channels to cause depolarization which would in turn open voltage-gated Ca^{2+} channels. Such a mechanism has been proposed for AtT-20 cells since K^+ antagonists inhibited PMA-stimulated Ca^{2+} influx (Reisine, 1989). The subsequent drop in $[Ca^{2+}]_i$ may be due to a decrease in Ca^{2+} influx and/or to an increase in Ca^{2+} efflux. This effect seems to require elevated $[Ca^{2+}]_i$ along with activated protein kinase C. The decrease in Ca^{2+} influx may be due to direct inactivation of voltage-gated Ca^{2+} channels. Lewis and Weigent (1988) report that activation of protein kinase C inhibits voltage-dependent Ca^{2+} currents in AtT-20 cells. There is also evidence to support a role for increased Ca^{2+} efflux, since Furukawa et al. (1989) observed that $[Ca^{2+}]_i$ was reduced and Ca^{2+} efflux was increased with PMA in cultured vascular smooth muscle cells previously stimulated with ionomycin. This effect may have been the result of enhanced activity of Ca^{2+}-ATPase (Smallwood et al., 1988).

We conclude that activation of protein kinase C causes a transient increase in $[Ca^{2+}]_i$ in part through Ca^{2+} influx via voltage-gated Ca^{2+} channels. This increase in $[Ca^{2+}]_i$ triggers GH release, since GH release is reduced by removal of extracellular Ca^{2+} and Ca^{2+} channels blockers. The transient nature of the GH response reflects the transient nature of the $[Ca^{2+}]_i$ response.

4.1.2 SRIF inhibits the release of GH induced by activation of protein kinase C

We then examined the effect of SRIF on protein kinase C-induced GH release and cAMP accumulation. SRIF was added 10 min before diC_8 and PMA in static incubation

so that we could detect the effect of SRIF. If diC_8 and PMA had been added before SRIF then the GH release induced by the protein kinase C activators would have accumulated in the medium and it would not have been possible to detect the reduction in GH released into the medium. SRIF, in static incubation, reduced both the diC_8- and PMA-induced GH release from purified somatotrophs (Figure 15 and 16). This result is in agreement with studies using mixed populations of anterior pituitary cells (Ohmura et al., 1984; Summers et al., 1985; Ray et al., 1986; Sheppard et al., 1987) and human pituitary adenoma cells (Ikuyama et al., 1987). The inability of SRIF to completely inhibit the GH response to the protein kinase C activators was probably due to the relative concentrations of SRIF and the protein kinase C activators and/or the experimental design, since SRIF (10^{-9} M) completely blocked the GH response to a second application of diC_8 (2×10^{-6} M) in perifusion (Figure 17). The effect of SRIF was not via a reduction in cAMP accumulation, since SRIF did not alter the increase in cAMP accumulation induced by both secretagogues. This observation is consistent with results from previous experiments in which SRIF completely inhibited the release of GH induced by GRF but only partially inhibited cAMP accumulation and suggests that SRIF acts at a post-cAMP site (Sheppard et al., 1985). The cAMP response to PMA in this experiment was higher than that seen previously (Figure 11). This probably was due to variability in the cell preparations.

While the mechanism of SRIF action has not been completely elucidated, SRIF acts, in part, by reducing basal and secretagogue-stimulated $[Ca^{2+}]_i$. In fact, it has been reported that SRIF blocks the increase in $[Ca^{2+}]_i$ induced by protein kinase C activators in somatotrophs identified by reverse haemolytic plaque assay as well as in purified

preparations of somatotrophs (Holl et al., 1989a; Lussier et al., 1991c). Thus, the ability of SRIF to inhibit GH release stimulated by protein kinase C activation is probably the result of its ability to inhibit the protein kinase C-induced increase in $[Ca^{2+}]_i$. It may also be due to a direct effect of SRIF on the process of exocytosis.

4.2 Protein kinase C does not mediate the action of GRF

Activation of protein kinase C does stimulate the release of GH from somatotrophs suggesting that protein kinase C may be involved in the regulation of GH release. Since GH release is stimulated by protein kinase C activation, we hypothesized that activation of protein kinase C may be an alternative pathway for GRF (criterion 3). To test this hypothesis we used 3 different approaches. We first inactivated protein kinase C by phorbol ester treatment and examined the subsequent GH response to GRF. Secondly, we explored the use of protein kinase inactivators. Finally, we measured the direct effect of GRF on the activity of protein kinase C in purified somatotrophs.

4.2.1 The GH response to GRF is not altered in cells made unresponsive to diacylglycerol

We first examined the GH response to GRF from cells made unresponsive to diacylglycerol. We assumed that the reduced responsiveness of the cells in perifusion to a subsequent application of PMA (Figure 13 and 14) was due to a reduced responsiveness of the somatotrophs to stimulation by diacylglycerol. To ensure that the cells were completely unresponsive to diacylglycerol, the concentration and duration of the initial PMA pulse was increased to 10^{-9} M for 1 h and the subsequent GH response to PMA and diC_8 was examined. This treatment was sufficient to abolish the GH

response to a second application of PMA and to markedly alter the GH response to diC_8 (Figure 20 and 21). These results suggested that protein kinase C had been made unresponsive by the phorbol ester treatment. However, this interpretation warrants further comment. Firstly, GH secretion remained elevated even after PMA was withdrawn, suggesting that protein kinase C was still activated. In addition, when we later measured protein kinase C activity directly in somatotrophs that were incubated with PMA (10^{-9} M) for 1 or 4 h, we found that protein kinase C activity was still detectable (Table VI). In fact, 87-93% of the total activity remained and the amount of protein kinase C activity in the plasma membrane was only slightly elevated. Therefore, PMA (10^{-9} M) for 1 or 4 h did not deplete or down-regulate protein kinase C activity. Indeed, depletion of protein kinase C activity with phorbol esters usually requires greater concentrations of PMA for longer incubation times (Phillips and Jaken, 1983; Krug and Tashjian Jr., 1987; McArdle et al., 1987; Leach and Blumberg, 1989; Jaken, 1989). The lack of repeatability of the GH response to protein kinase C activation, therefore, probably reflects a so-called desensitization of protein kinase C. Phillips and Jaken (1983) have described this phenomenon. It occurs rapidly following PMA treatment and continues for 2 h. During this period the cells are unresponsive to further activation of protein kinase C. After this initial period, protein kinase C activity begins to disappear from the cell and is said to be down-regulated or depleted. The mechanism by which protein kinase C desensitization occurs is not understood.

Although the stimulatory effects of diC_8 were abolished by pretreatment of cells with PMA (Figure 21), application of diC_8, to our surprise, caused an initial inhibition of GH release which was followed by an immediate burst of GH after perifusion with

diC_8 was ended. This GH response is similar to that seen with SRIF in perifusion (Kraicer and Chow, 1982). One of the actions of SRIF is to reduce $[Ca^{2+}]_i$. As discussed in section 4.1.1, diC_8 and PMA have both stimulatory and inhibitory effects on $[Ca^{2+}]_i$. PMA pretreatment may have uncoupled the two effects of the protein kinase C activators on $[Ca^{2+}]_i$. This suggests that the inhibitory action of diC_8 and PMA on $[Ca^{2+}]_i$ may be due to a different isoenzyme of protein kinase C that is not desensitized by PMA treatment or, alternatively, that this effect is not mediated by protein kinase C.

We then examined the effect of 1 h of perifusion with PMA (10^{-9} M) on the GH response to GRF (10^{-10} M) 30 min later. GRF stimulated the release of GH from dispersed anterior pituitary cells that were preperifused with PMA (Figure 22). This indicates that further activation of protein kinase C is not essential for GRF action.

The GH response, however, may have been altered by pretreatment with PMA, an effect that we would not have detected with our single column perifusion system. Therefore, to determine whether PMA pretreatment alters the GH response to GRF, we developed a two column perifusion system. The two columns of cells responded identically to graded concentrations of GRF (Figure 23) demonstrating that this system could be used to compare the GH responses to GRF from control and PMA treated cells.

We then compared the GH responses to graded concentrations of GRF and to diC_8 from control cells and those perifused with PMA (10^{-9} M) (Figure 24 and Table I). The GH response to diC_8 from the PMA treated cells at the end of each experiment was virtually abolished, indicating that the cells were unresponsive to further stimulation by diacylglycerol. The GH responses to graded concentrations of GRF were not significantly altered by the concurrent administration of PMA. Thus, somatotrophs retain

their responsiveness to GRF even though they are unresponsive to protein kinase C activators, indicating that further activation of protein kinase C is not essential for GRF-induced GH release. There was a small, consistent, though not significant, reduction in responsiveness to higher concentrations of GRF which may reflect a small reduction in the releasable pool of GH contained in the somatotrophs after PMA-induced GH release or, as in the case of epidermal growth factor receptors, a PMA-induced reduction in GRF binding (Lee and Weinstein, 1978; Brown et al., 1979).

Ohmura et al. (1988) also examined the effect of PMA pretreatment on the subsequent GH response to GRF using a very different protocol. In their experiments, cultured anterior pituitary cells were incubated with PMA (8.0×10^{-8} M) for 3 h, the cells were washed, incubated for 20 h, and then challenged with PMA (3.0×10^{-9} M) or GRF (5.0×10^{-9} M) for 3 h. The GH response to GRF was inhibited, however, the GH response to PMA was reduced but not abolished by this treatment. Therefore, the reduced responsiveness to GRF in these cells was not due to depletion of protein kinase C activity. These results indicate that activation of protein kinase C, 20 h before the application of GRF, causes other changes within the cells such that the GH response to GRF is altered.

4.2.2 Protein kinase inhibitors

Another approach to examine the role of protein kinase C in GRF action was to study the effect of protein kinase C inactivators on GRF-induced GH release. The disadvantage of this approach is the relative lack of specificity of these compounds for a specific type of protein kinase. Since protein kinase A is believed to mediate the action of cAMP in somatotrophs, the use of relatively nonspecific protein kinase C inhibitors

in these cells would yield inconclusive results. Therefore, we chose two protein kinase inhibitors, staurosporine and HA1004, and planned to test their relative efficacy in inhibiting diC_8 (i.e. protein kinase C)- and dbcAMP (i.e. protein kinase A)-induced GH release. Staurosporine is relatively more potent in blocking protein kinase C (Hidaka et al., 1984), while HA1004 is relatively more potent in blocking protein kinase A (Hidaka et al., 1984). Our strategy was to find a concentration of HA1004 that would block protein kinase A, but not protein kinase C, and then a concentration of staurosporine that would block protein kinase C, but not protein kinase A. If these compounds selectively inhibited the action of diC_8 or dbcAMP at lower concentrations, then the effect of these compounds on GRF-induced GH release would have been compared. The protein kinase inhibitor HA1004 was tested first. HA1004 (10^{-7} M to 10^{-4} M) did not inhibit diC_8- or dbcAMP-induced GH release, except for a small reduction in diC_8-induced GH release at 20 min with HA1004 (10^{-4} M). In addition, this compound at 10^{-5} M caused a small significant increase in GH release induced by dbcAMP at 20 min (Figure 25). The lack of effect of HA1004 may have been the result of a failure of this compound to enter the cells. However, HA1004 is reported to be effective when added to intact cells for incubation times as short as 5 min (Asano and Hidaka, 1984; Hidaka et al., 1984; Goodman et al., 1990). Alternatively, the concentrations of diC_8 and dbcAMP used may have "saturated" the effect of HA1004. To test this, we examined the effect of HA1004 on endogenously generated cAMP. Endogenous cAMP levels were modestly elevated by IBMX, a phosphodiesterase inhibitor. Again, HA1004 failed to inhibit GH release (Figure 26). Therefore, the lack of effect of HA1004 on GH release was not due to saturation with dbcAMP. Another interpretation of these results is that protein kinase C

and protein kinase A do not mediate the action of diC_8 and dbcAMP in somatotrophs. Taken together, however, our results showed that HA1004 was ineffective in somatotrophs. Therefore, we could not pursue this stategy to study the roles of protein kinase C and protein kinase A in GRF-induced GH release.

4.2.3 GRF does not activate protein kinase C

Although the results from the inactivation experiments (section 5.2.1) indicated that protein kinase C is not essential for GRF-induced GH release, the presence of, distribution of, and effect of GRF on protein kinase C activity in somatotrophs had not been examined directly. The direct measurement of protein kinase C activity in somatotrophs was necessary since this enzyme might mediate an effect of GRF other than stimulation of GH release which was the only action of GRF tested with the previous approach. Also, it was essential to measure protein kinase C activity directly following GRF in light of our later experiments which showed that protein kinase C activity was not depleted by treatment of cells with PMA at 10^{-9} M for 1 or 4 h.

We used a purified preparation of somatotrophs which allowed us to measure the basal levels of protein kinase C activity and to determine the effect of protein kinase C activators on the distribution of protein kinase C activity exclusively in the somatotrophs. In addition, changes in protein kinase C activity induced by GRF, if present, would not be obscured by basal levels of protein kinase C activity in non-responsive cell types.

Our results show (Table III and IV), for the first time, that protein kinase C activity is present in somatotrophs. Under basal conditions, as expected, the majority of the enzyme activity is located in the cytosol. This distribution is similar to that seen in mixed populations of anterior pituitary cells (Naor et al., 1985b; Hirota et al., 1986;

McArdle and Conn, 1986; Drouva et al., 1990). The values for basal protein kinase C activity were somewhat variable between experiments. This variability may reflect true variations in the level of protein kinase C in different somatotroph preparations or differences in the recovery of protein kinase C activity between experiments. The variability, however, within experiments was low and comparisons between control and treatment groups were made within the same experiment. Different isoenzymes of protein kinase C have been described for the anterior pituitary (Huang et al., 1986; Nishizuka, 1988). We did not attempt to separate the different isoenzymes of protein kinase C in this study.

In most cell types, under basal conditions, protein kinase C is present in the cytosol or loosely associated with the plasma membrane so that homogenization of cells in the presence of calcium chelators leads to recovery of the enzyme in soluble fractions (Jaken, 1989; Leach and Blumberg, 1989). Activation of protein kinase C results in the redistribution of protein kinase C activity from the cytosol to the plasma membrane and occurs following receptor-mediated generation of diacylglycerol (Jaken, 1989). Diacylglycerol may stabilize membrane association so that more protein kinase C activity is located in particulate fractions following homogenization (Leach and Blumberg, 1989). Whatever the mechanism, translocation of protein kinase C is an indicator of activation of the enzyme in many cell types (Jaken, 1989).

To ensure that activation of protein kinase C could be detected in somatotrophs, we first examined the effect of the protein kinase C activators diC_8 and PMA (Table III). As expected, these compounds did cause a significant translocation of protein kinase C activity from the soluble to the particulate fractions. PMA induced a marked reduction

in soluble protein kinase C activity and a concomitant increase in particulate protein kinase C activity. The effect of diC_8, although significant, was less dramatic. The difference between the effect of PMA and diC_8 probably reflect differences in the metabolism and solubility of the two compounds. diC_8 is rapidly metabolized and is not as soluble as PMA in aqueous solutions (Leach and Blumberg, 1989). The difference may also reflect the incubation time that was arbitrarily chosen for these experiments. For example, the effect of diC_8 may have been greater at shorter periods of incubation. These results indicate that activation of protein kinase C does cause detectable translocation of protein kinase C activity in somatotrophs. As expected, both diC_8 and PMA significantly increased GH release at 3 min of incubation.

A high concentration of GRF and a wide range of incubation times, from 20 s to 90 min, was chosen so an effect of GRF on the distribution of protein kinase C activity, if present, would not be missed. GRF, however, did not cause translocation of protein kinase C at any time point (Table IV), even though a significant increase in GH release was seen by 3 min (Figure 29). In addition, GRF did not significantly alter the specific activity of protein kinase C in soluble or particulate fractions except for a small increase in soluble specific activity at 90 min. It is unlikely that this increase is important for GH release per se since GRF-induced GH release is evident within 3 min. Since GRF did not cause a translocation of protein kinase C activity, we conclude that GRF does not stimulate protein kinase C activity in somatotrophs.

These results are consistent with reports concerning the lack of effect of GRF on the phosphoinositide second messenger system (Raymond et al., 1984; Escobar et al., 1986). Receptor-mediated hydrolysis of PIP_2 is a potential source for diacylglycerol.

However, GRF does not stimulate phosphoinositide turnover in somatotrophs, thereby ruling out PIP_2 as a source of diacylglycerol (see section 4.3).

However, there are other sources of diacylglycerol in cells. For example, diacylglycerol may be formed from phosphatidylcholine by the action of a phosphatidylcholine-specific phospholipase C (Jaken, 1989). Therefore, a lack of effect of GRF on phosphoinositide turnover did not preclude a lack of effect of GRF on the activation of protein kinase C activity. The results from these experiments, however, establish that GRF does not activate protein kinase C.

These results confirm and extend the conclusions of the previous experiments in which we demonstrated that activation of protein kinase C is not essential for GRF-induced GH release (section 4.2.1). Protein kinase C activity is present in somatotrophs and activation of protein kinase C does stimulate GH release. GRF however, does not cause translocation of protein kinase C in somatotrophs and a role for this enzyme in somatotrophs awaits elucidation.

4.3 GRF does not activate phospholipase C

The purpose of this series of experiments was to determine whether GRF stimulates phospholipase C activity in purified somatotrophs (criteria 1 and 2). To accomplish this, we measured changes in the ^{32}P labelling of the phosphoinositides and inositol phosphates following incubation with GRF. The use of a purified preparation of somatotrophs allowed us to attribute changes in phosphoinositides and inositol phosphates directly to the somatotrophs. This is particularly important since the phosphoinositide second messenger system mediates the release of other anterior pituitary hormones (Martin, 1983; Naor et al., 1986; Andrews and Conn, 1986).

We used a high concentration of GRF (10^{-7} M), a wide range of GRF incubation times, and measured the individual phosphoinositides and inositol phosphates so that an effect of GRF on phospholipase C activity, if present, should have been detected. If GRF does stimulate the activity of phospholipase C, then we would expect to see a decrease in the ^{32}P labelling of the phosphoinositides and an increase in the ^{32}P labelling of the inositol phosphates. In fact, GRF did not significantly alter labelling of the phosphoinositides (except for a small, but significant, reduction in PIP labelling at 90 min) (Figure 27). Also, in general, ^{32}P incorporation into the inositol phosphates, and in particular IP_2, was reduced, not stimulated, by GRF (Figure 28). Consistent with these observations, GRF did not alter the total radioactivity in the organic or aqueous layers of the somatotroph extracts at any incubation time (Table II). It is surprising that GRF should tend to produce a decrease in the level of both the phosphoinositides and inositol phosphates since one would expect a reciprocal relationship. These results, however, indicate that GRF does not stimulate, and may in fact inhibit, phospholipase C activity in somatotrophs.

The lack of an effect of GRF on the phosphoinositide system was not due to a lack of responsiveness of the somatotrophs since GH release was stimulated by GRF in these experiments.

These results resolve the discrepancies in reports from others who have carried out limited studies using mixed pituitary cell preparations on the effect of GRF on the phosphoinositide second messenger system. None of these studies attempted a separation of the individual phosphoinositides or inositol phosphates. Also, GRF incubation periods of less than 15 min were not included in these experiments. Raymond et al. (1984)

observed that GRF, at 10^{-7} M, did not alter ^{32}P labelling of PI in rat anterior pituitary cell cultures at 20 or 60 min of incubation. Escobar et al. (1986) reported no change in [^{3}H]myoinositol labelling of total inositol phosphates in cultures of rat anterior pituitary cells that were incubated with up to 10^{-5} M GRF for 15 min. In contrast, an increase in ^{32}P labelling of PI was observed in cultured rat anterior pituitary cells following incubation with GRF for 15, 30, and 60 min (1983).

The presence of the components of the phosphoinositide second messenger system in somatotrophs raises the possibility that agonists other than GRF act through this system to alter the release of GH and other somatotroph functions. Among the candidates for potential agonists (see section 4.6) are acetylcholine and ATP. It has been reported that acetylcholine and ATP stimulate the production of the inositol phosphates in mixed populations of anterior pituitary cells. There are conflicting reports, however, concerning the effect of acetylcholine on the release of GH (Kraicer, 1979; Young et al., 1979; Scarey and Read, 1988). Also, even though ATP does increase inositol phosphate production, it does not stimulate GH release (Davidson et al., 1990). Thus, a role for the phosphoinositide system in the somatotroph awaits identification.

4.4 Summary and conclusions

To summarize, activation of protein kinase C, by diC_8 and PMA, causes the immediate and transient release of GH from purified somatotrophs. The GH response to diC_8 in perifusion is repeatable while the GH response to PMA is not. The increase in GH release is accompanied by a small increase in cAMP accumulation. The stimulation of GH release by the protein kinase C activators is reduced by removal of

extracellular Ca^{2+} and the Ca^{2+} channel blocker nifedipine. Therefore, cAMP and Ca^{2+} may mediate the action of protein kinase C in somatotrophs and there is "cross-talk" between these second messenger systems.

Protein kinase C activity is present in somatotrophs, and under basal conditions the majority of enzyme activity is located in the cytosol. Protein kinase C activators cause a translocation of enzyme activity from the cytosol to the plasma membrane indicating that protein kinase C can be activated in these cells. However, GRF does not activate protein kinase C in somatotrophs. In addition, inactivation of protein kinase C by PMA treatment does not alter the GH response to GRF. GRF does not activate phospholipase C in somatotrophs as determined by measuring the levels of individual radiolabelled phosphoinositides and inositol phosphates following addition of GRF. The protein kinase inhibitor, HA1004, was ineffective in inhibiting dbcAMP-, diC_8- and IBMX-induced GH release.

We conclude that the phosphoinositide second messenger system does not mediate the action of GRF in somatotrophs, even though all of the components of this system are present in these cells.

The presence of the phosphoinositide second messengers in somatotrophs presents the intriguing possibility of the existence of agonists, other than GRF, which would act through this system to alter the release of GH and other somatotroph functions. In addition, the ability of protein kinase C to alter the levels of cAMP and Ca^{2+} suggests that there are interactions between the different signalling systems and adds another layer of complexity to the intracellular regulation of GH release from the somatotrophs of the anterior pituitary.

4.5 Current models of the intracellular mechanisms that regulate the release of GH

The following models of the intracellular mechanisms that regulate the release of GH are proposed and are based on the results from this research project and the current knowledge that was reviewed in the introduction.

4.5.1 Basal GH release

In the absence of GRF and SRIF the somatotroph is still active since somatotrophs in vitro continue to release GH. With electrophysiological techniques it is possible to characterize the basal ion channel activity of single somatotrophs. Sims et al. (1991) reported that rhythmic oscillations of membrane potential which gave rise to action potentials were present in 36 of 52 somatotrophs studied. The membrane potential oscillated between -70 mV and -25 mV with an average frequency of 0.9 ± 0.9 s^{-1}. There was evidence that the outward current was due to K^+ channels. Na^+ and Ca^{2+} currents were also observed when depolarizing commands were given. These oscillations may contribute to basal GH release since Ca^{2+} influx would presumably be increased during the depolarization phase of the oscillations. Thus, under basal conditions the somatotrophs are active and the effect of GH secretagogues and inhibitors is superimposed over the basal activity.

4.5.2 Stimulation of GH release

Figure 30 shows the proposed mechanisms involved in the stimulation of GH release. The binding of GRF to its receptors (Veliçelebi et al., 1985; Kato et al., 1988; Zysk et al., 1986; Veliçelebi et al., 1986) on the somatotroph plasma membrane would activate adenylate cyclase via the G_s protein (Labrie et al., 1983; Schettini et al., 1984; Harwood et al., 1984; Reyl-Desmars et al., 1985; Giannattasio et al., 1987; Narayanan

et al., 1989). This would increase cAMP which would in turn activate protein kinase A. Protein kinase A may phosphorylate Na^+ channels, causing Na^+ influx resulting in a depolarization of the cell (Costa et al., 1982; Costa and Catterall, 1984; Swandulla and Lux, 1984; Curtis and Catterall, 1985). The depolarization would open voltage-gated Ca^{2+} channels. cAMP may also bind directly to Na^+ channels to cause their opening (Nakamura and Gold, 1987). In addition, protein kinase A would phosphorylate voltage-gated Ca^{2+} channels to alter their characteristics causing them to open (Curtis and Catterall, 1985). GRF, binding to its receptor, may also open voltage-gated Ca^{2+} channels via a membrane-limited mechanism by interacting with G proteins in the plasma membrane (Brown and Birnbaumer, 1990). The increase in $[Ca^{2+}]_i$ and protein kinase A activity would stimulate exocytosis of GH secretory granules (Knight et al., 1989).

The results from the current study establish that GRF does not act through the phosphoinositide second messenger system. Yet this system is present in somatotrophs and we have demonstrated that protein kinase C may be stimulated in these cells. Also, activation of protein kinase C will result in the acute release of GH. Therefore, it is possible that, an as yet unidentified agonist binds to its receptor to activate phospholipase C. If this were the case, phospholipase C would hydrolyze PIP_2 to produce IP_3 and diacylglycerol. IP_3 would increase $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores. Diacylglycerol would activate protein kinase C (Nishizuka, 1984; Berridge and Irvine, 1989; Farago and Nishizuka, 1990). Protein kinase C would phosphorylate voltage-gated Ca^{2+} channels to increase Ca^{2+} influx. In addition, protein kinase C might reduce K^+ conductance (Shearman et al., 1989) to cause depolarization which would in turn open voltage-gated Ca^{2+} channels (Nishizuka, 1986, 1988; Shearman et al., 1989; Farago and

Nishizuka, 1990). Protein kinase C may also have a direct effect on the process of exocytosis that is independent of its effect on $[Ca^{2+}]_i$ (Gomperts, 1986; Knight et al., 1989). Protein kinase C would also increase cAMP concentration presumably through an effect on adenylate cyclase. Thus, activation of the phosphoinositide second messenger system would enhance basal or GRF-induced GH release.

After stimulation, other mechanisms would be used to return the cell to prestimulation conditions (Figure 31). The increase in $[Ca^{2+}]_i$ would exert negative feedback effects on the intracellular signalling systems. For example, Ca^{2+} would inactivate adenylate cyclase (Narayanan et al., 1989). This would decrease cAMP production resulting in a decrease in active protein kinase A. Ca^{2+} would also activate Ca^{2+}-sensitive K^+ channels to cause an increase in membrane potential which would close voltage-gated Ca^{2+} channels (Rogawski, 1989). Ca^{2+} efflux would be increased by a Ca^{2+}-dependent increase in Ca^{2+}-ATPase activity (Reuter, 1991). Protein kinase C may also participate in this phase, since activation of protein kinase C in the presence of increased $[Ca^{2+}]_i$ causes a decrease in $[Ca^{2+}]_i$ (Stojilkovic et al., 1991). This effect may occur by inactivation of voltage-gated Ca^{2+} channels (Shearman et al. 1989) or an increase in Ca^{2+}-ATPase activity (Smallwood et al., 1988). Thus, it is possible that activation of protein kinase C, and perhaps phospholipase C, following stimulation would exert negative feedback effects on $[Ca^{2+}]_i$.

4.5.3 Inhibition of GH release

Figure 32 outlines the current model for the mechanisms involved in the inhibition of GH release. SRIF would bind to its receptor on the somatotroph plasma membrane (Enjalbert et al., 1982; Leung et al., 1982; Reubi et al., 1982; Srikant and Patel, 1982)

and via a G_k protein would open K^+ channels to increase K^+ conductance (Pace and Tarvin, 1981; Israel et al., 1983; Koch et al., 1988; Sims et al., 1991). SRIF may also increase K^+ conductance by increasing the activity of phosphatases which specifically dephosphorylate K^+ channels causing them to open (White et al., 1991). This would hyperpolarize the cell and close voltage-gated Ca^{2+} channels to cause a decrease $[Ca^{2+}]_i$. Since one type of receptor may couple to more than one type of G protein (Brown et al., 1990), SRIF may also have a direct effect on voltage-gated Ca^{2+} channels, via G_i and a membrane-limited mechanism, causing their closure (Lewis et al., 1986; Bean, 1989). SRIF may also inhibit GH release by blocking the process of exocytosis at steps subsequent to its effect on $[Ca^{2+}]_i$ perhaps via the G protein G_{Ei} (Gomperts, 1990; Luini and De Matteis, 1990; Lussier et al., 1991c). A minor effect of SRIF would be to decrease cAMP levels which would have small inhibitory effects on the processes regulated by protein kinase A (Harwood et al., 1984; Wreggett and Irvine, 1989). Therefore, the effects of cAMP, including the effect of cAMP on Na^+ channels, would be reduced. There are no data to suggest that the phosphoinositide second messenger system mediates the action of SRIF, but the involvement of this system in SRIF action has not been thoroughly examined in somatotrophs.

4.6 New questions and future experiments

The results from this project and the preceding descriptions of the current models of the intracellular mechanisms involved in the stimulation and inhibition of GH release raise a number of additional questions.

At present, the physiological role of the phosphoinositide second messenger system in somatotrophs has not been elucidated. There are a number of candidate agonists which may exert their effects through this system. For example, neurotransmitters and neuropeptides present in GRF neurons would probably be released at the same time as GRF. In addition, there are a number of compounds in hypothalamic neurons that project to the median eminence which are released into the hypophysial portal circulation and a number of compounds located within the anterior pituitary that may exert paracrine effects. A partial list includes acetylcholine, ATP, pituitary adenylate cyclase activation polypeptide, endothelin, TRH and galanin. Information regarding the type of receptors on somatotrophs would be beneficial in identifying potential agonists for the phosphoinositide second messenger system.

Although it is unlikely that SRIF acts through the phosphoinositide second messenger system, a detailed study has not been carried out for somatotrophs and is warranted. Many of the techniques described in this thesis could be used for this purpose.

Once the physiological role for the phosphoinositide second messenger system is established, it would be of interest to determine whether there are changes in this system throughout development and whether there are differences between males and females. These differences might help to explain the various patterns of GH release seen during development and between the sexes (Edén, 1979).

The effect of IP_3 on GH release should also be determined before the phosphoinositide second messenger system is accepted as a second messenger system for somatotrophs. A photolabile derivate of IP_3 (*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-

nitrophenyl) ethyl ester (Calbiochem Corp., San Diego, CA), called caged IP_3, has been developed and might be used for this purpose (Gilroy et al., 1990). This compound is not metabolized by endogenous phosphatases. This would require a permeabilized cell preparation to allow entry of IP_3 into the cell. Once inside the cell, IP_3 would be released from the cage with a flash of laser light (360 nm) and subsequent GH release could be measured. Also, the presence of IP_3 receptors in somatotrophs should be examined.

The models outlined above predict that protein kinase C has two different roles in the regulation of GH release through stimulatory and inhibitory actions on $[Ca^{2+}]_i$. Addition of GRF at the same time as the addition of protein kinase C activators should enhance the release of GH. The effect might be additive or synergistic. A synergistic effect would mean that there is interaction between the different second messenger systems. The model also predicts that activation of protein kinase C during or after stimulation of cells with GRF would reduce the GH response to GRF or would reduce the GH response to a subsequent application of GRF. The perifusion system is ideally suited to study these effects.

Activation of protein kinase C does stimulate the release of GH from somatotrophs. We found that cAMP and Ca^{2+} may mediate the action of protein kinase C. It would be of interest to identify the substrates for protein kinase C in somatotrophs. This could be accomplished by incubating cells, previously loaded with ^{32}P-orthophosphate, with protein kinase C activators, homogenizing the cells, separating the phosphorylated proteins, and comparing the pattern to that from control cells.

To determine whether the protein kinase C activator-induced increase in cAMP accumulation is due to activation of adenylate cyclase activity, adenylate cyclase activity could be measured in membrane preparations of somatotrophs that were treated with protein kinase C activators. Also, electrophysiological techniques could be used to assess the effects of protein kinase C activation on voltage-gated Ca^{2+} channels and other ion channels in purified somatotrophs. It would also be of interest to determine whether protein kinase C activators stimulate Ca^{2+}-ATPase activity.

The different isoenzymes of protein kinase C were not separated in our experiments. There is increasing evidence that the isoenzymes of protein kinase C have different effects (Nishizuka, 1988; Huang et al., 1989, 1990). These isoenzymes could be identified with the use of specific antibodies that have only recently been developed (Huang et al., 1989).

The mechanisms involved in the translocation, desensitization and down-regulation of protein kinase C activity have not been determined.

There are other aspects concerning the regulation of GH release that are not directly related to the phosphoinositide second messenger system. For example, although the ionic characteristics of the somatotroph are beginning to be described, a more complete description is required. The types of G proteins in somatotrophs should be determined and would provide additional information about the intracellular mechanisms involved in the regulation of GH release. The substrates for protein kinase A should also be identified. Finally, the mechanisms of exocytosis are poorly understood. More information about this process including its regulation by Ca^{2+}, protein kinase A, protein kinase C and other second messengers is required.

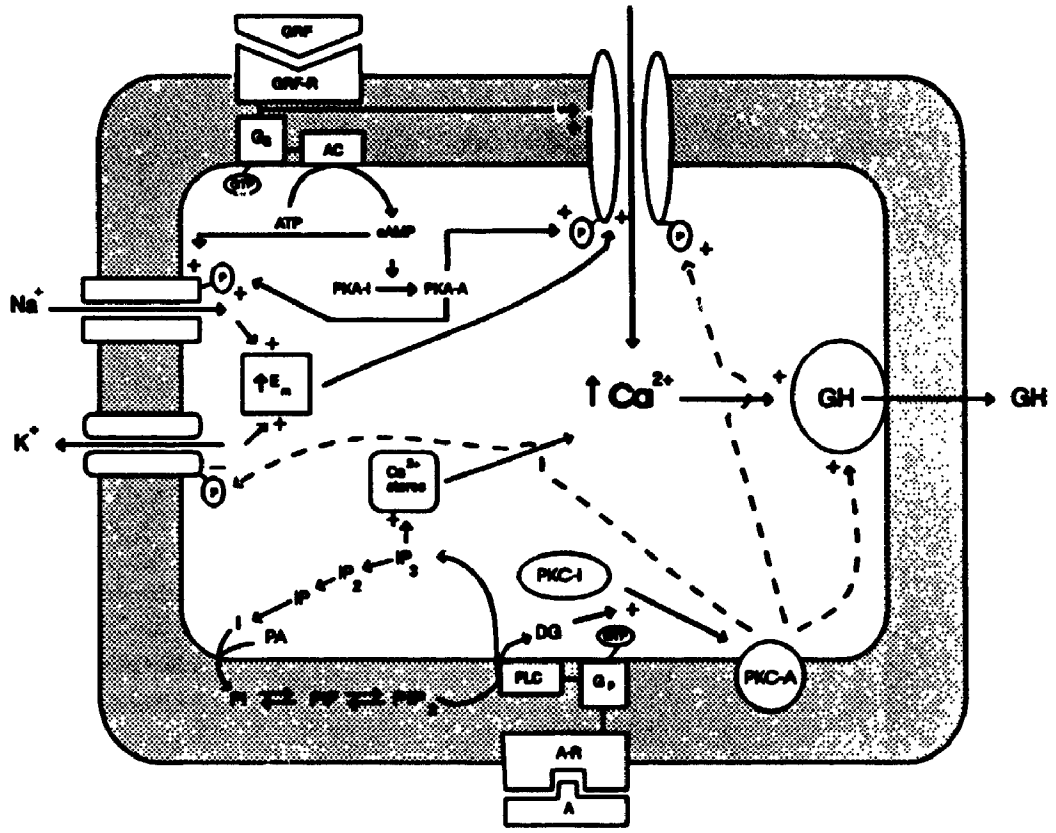


Figure 30: Model of the intracellular mechanisms involved or proposed to be involved in the stimulation of GH release from somatotrophs. Abbreviations: agonist (A), adenylate cyclase (AC), agonist receptor (A-R), depolarization (E_m with an upward pointing arrow), GRF receptor (GRF-R), active protein kinase A (PKA-A), inactive protein kinase A (PKA-I), stimulation (+), inhibition (-), phosphorylation (encircled P).

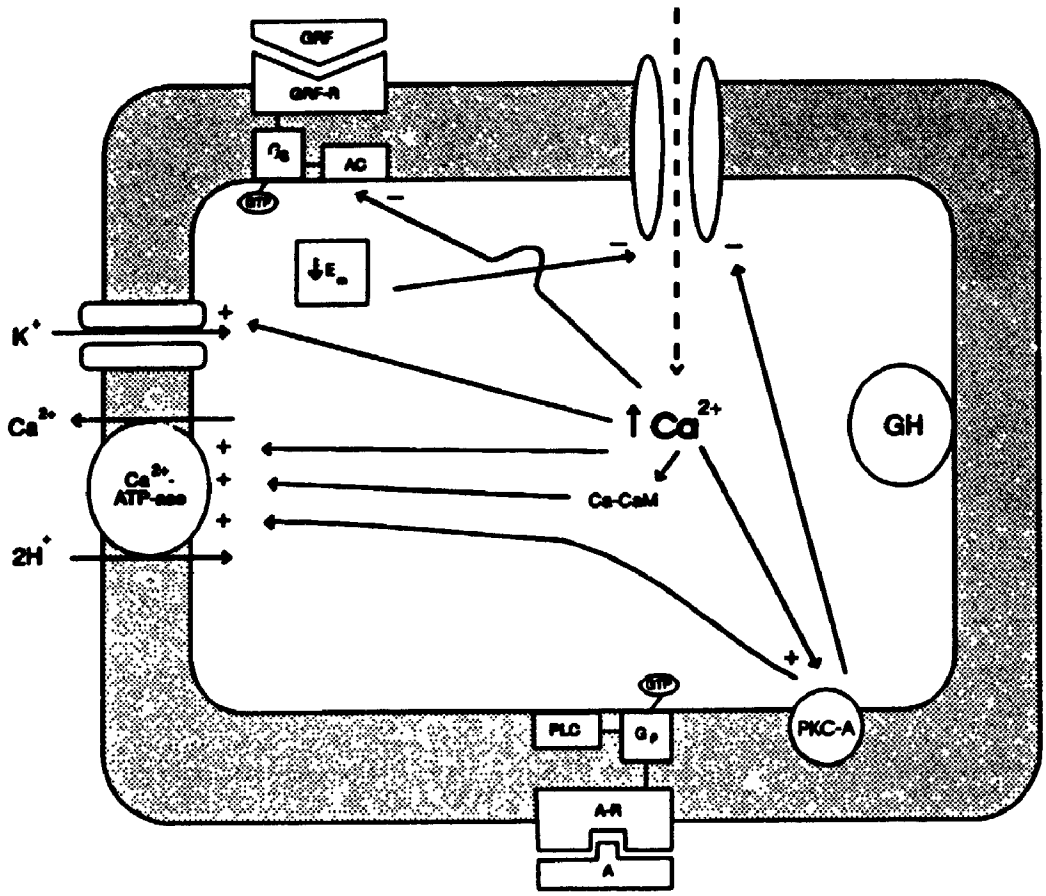


Figure 31: Model of the intracellular mechanisms involved or proposed to be involved in the return of somatotrophs to basal state following stimulation. Abbreviations: agonist (A), adenylate cyclase (AC), agonist receptor (A-R), hyperpolarization (E_m with a downward pointing arrow), GRF receptor (GRF-R), stimulation (+), inhibition (-), phosphorylation (encircled P). Dashed lines indicate a reduction in the process depicted by the line.

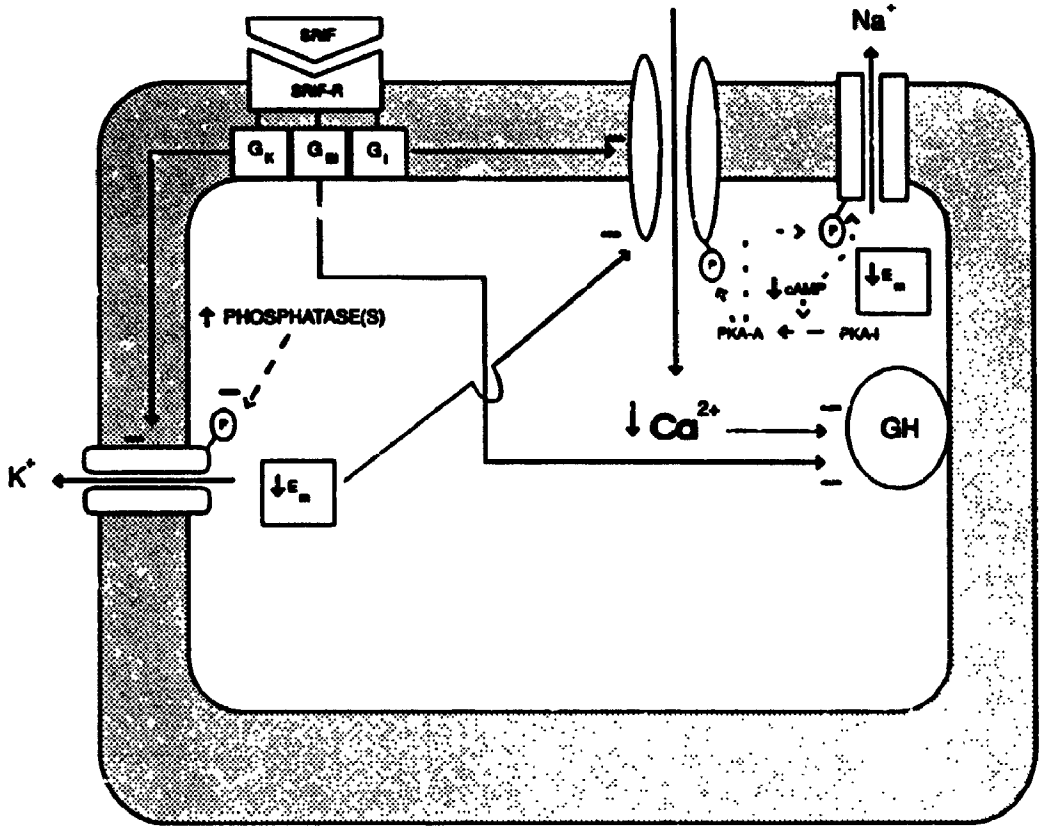


Figure 32: Model of the intracellular mechanisms involved or proposed to be involved in the inhibition of GH release from somatotrophs. Abbreviations: agonist (A), adenylate cyclase (AC), agonist receptor (A-R), hyperpolarization (E_m with a downward pointing arrow), GTP-binding protein associated with an inhibition of release (G_{E_i}), GRF receptor (GRF-R), active protein kinase A (PKA-A), inactive protein kinase A (PKA-I), stimulation (+), inhibition (-), phosphorylation (encircled P). Dashed lines indicate a reduction in the process depicted by the line.

APPENDIX 1: HISTOLOGICAL PROCEDURES

The following protocol, modified from Kraicer et al. (1967), was used to fix and stain slides for determination of the purity of the somatotroph preparations. With this procedure the different types of anterior pituitary cells are differentiated. The somatotrophs are yellow with fine orange-yellow granules.

1) Slides fixed in Bouin-Hollande Sublimate for at least 24 h.

2) Slides of fixed cells were quickly rinsed 3 times and then washed 3 times for 10 min in aqueous lithium carbonate.

3) Slides of fixed cells washed in 70% alcohol for 6 times 3 min.

4) Slides of control sections from lactating female rats washed with xylene (2 times 3 min), absolute alcohol (2 times 3 min), 95% alcohol for 3 min, and 70% alcohol (3 min).

5) Washed with 1% iodine in 70% alcohol for 5 min.

6) Bleached with sodium thiosulphate and washed in running tap water for 15 min.

7) Stained in erythrosin solution for 30 min: erythrosin B, (773, Anachemia Ltd., Montréal, Canada) 1.0 g; M/5 acetate buffer, pH = 6.21 to 100 ml.

8) Rinsed in tap water.

9) Stained 10 min in Mallory Blue II: Aniline Blue, water soluble, Michrome (E. Gurr Ltd., London, United Kingdom) 0.5 g; Orange G

(27, Anachemia Ltd.) 2.0 g; distilled water to 100 ml; when dissolved add 8.0 ml glacial acetic acid. Used 1:1 dilution with distilled water.

10) Rinsed in tap water.

11) Stained 5 min with Acid Alizarine Blue solution: Acid alizarine Blue, BB, Michrome 9 (E Gurr Ltd.) 0.5 g; aluminium sulfate or chloride 10.0 g; distilled water to 100 ml. Brought to boiling, boiled until blue-purple, cooled, brought up to 100 ml with distilled water.

12) Rinsed in tap water.

13) Slides into 5% aqueous phosphomolybdic acid for 15 min.

14) Transferred to 1% phosphomolybdic acid in 70% alcohol for 10 s.

15) Transferred to 1% phosphomolybdic acid in 90% alcohol for 8 s.

16) Transferred to absolute alcohol and cleared for mounting.

APPENDIX 2: [^{3}H]-INOSITOL LABELLING OF PHOSPHOINOSITIDES AND INOSITOL PHOSPHATES

We initially chose to label the phosphoinositides and inositol phosphates with [^{3}H]-inositol since these compounds are the primary components of the cell that are labelled with [^{3}H]-inositol. However, the disadvantage of labelling with [^{3}H]-inositol for us is that relatively long incubation times or large amounts of tissue are required to get detectable labelling (Martin, 1986). These are disadvantages because we use purified somatotrophs prepared on the same day as the experiment and have not set up or characterized a culture system in the laboratory. In addition, we obtain a relatively small amount of tissue with our purification procedures. Nevertheless, we first studied the effect of incubating cells with [^{3}H]-inositol for periods up to 8 h, to determine whether we could get detectable labelling of the phosphoinositides and inositol phosphates.

A.1 Effect of loading time on [^{3}H]-inositol incorporation

Somatotrophs were prepared with Percoll gradients using the procedure previously described (section 2.4.2) except that M199 was replaced with inositol-free M199 (87-014AG, Gibco). The somatotrophs were suspended in M199AH/37-inositol free containing 5 mM LiCl and [^{3}H]-inositol (10 μCi / ml, 20 Ci / mmol, NET 114, New England Nuclear, Boston, MA) and incubated for 2, 4, 6, or 8 h in a Dubnoff incubator-shaker at 37 C. The cells were then centrifuged for 10 min at 500 *g*, washed with 10 ml M199AH/37-inositol free containing 5 mM LiCl, and made up to 2.2 ml with the same medium. One ml aliquots (1.0×10^6 - 2.0×10^6 cells / ml/ beaker) were transferred to Teflon beakers. The cells were lysed by the addition of 4 ml of chloroform:methanol

(1:2, v/v) and the phosphoinositides and inositol phosphates were extracted and separated as described in sections 2.6.2 and 2.6.3, except that the aqueous layers were not treated with charcoal.

The uptake of [^{3}H]-inositol into the somatotrophs was linear and ranged from 0.77 - 3.4% per million cells. There was good incorporation of [^{3}H]-inositol into PI even at 2 h. The radioactivity in PI as expressed as a percentage of the total radioactivity from the each plate lane ranged from 93-97%. PIP was labelled to a much lesser extent and counts in PIP_2 were not above background. The amount of labelling of PI and PIP increased with time but there was no detectable labelling of PIP_2 even at 8 h. Table VI gives the results of a representative separation of the phosphoinositides following loading of cells for 4 h with [^{3}H]-inositol.

Chromatography of the aqueous layers with Dowex anion exchange columns yielded 5 detectable peaks. Figure 33 shows a representative elution pattern of the aqueous layer from cells incubated with [^{3}H]-inositol for 4 h. Peak 1 was inositol, peak 2 was probably glycerophosphatidylinositol and peak 3 was IP. The identity of peaks 4 and 5 is not known but could be, at least in part, glycerophosphatidylinositol-phosphate and glycerophosphatidylinositol-bisphosphate. IP was detectable by 2 h of incubation, while IP_2 or IP_3 were not detected at any time point.

Therefore, even at the longest incubation time, we were unable to get [^{3}H]-inositol labelling of PIP_2, IP_2, or IP_3.

A.2 Effect of GRF on [^{3}H]-inositol labelling of inositol phospholipids

Although we did not observe labelling of PIP_2, IP_2 or IP_3, we reasoned that we might be able to see an effect of GRF on PI, PIP or IP labelling and that this would give

Table VI: Radioactivity in phosphoinositides from somatotrophs incubated with [^{3}H]-inositol for 4 h.

Segment	Radioactivity (dpm)	% of Total Radioactivity Recovered
	93.9	0.07
	82.8	0.06
PIP_2	141.6	0.11
	423.0	0.33
PIP	2188.2	1.69
	750.0	0.58
PI	122,562.0	94.54
	1,986.7	1.53
	1,405.8	1.08

The phosphoinositides were separated by thin layer chromatography. The radioactivity in the entire lane of the plate is shown with segments representing the individual phosphoinositides labelled PIP_2, PIP, and PI. The radioactivity was also expressed as a percentage of the total radioactivity recovered from the entire lane of the plate.

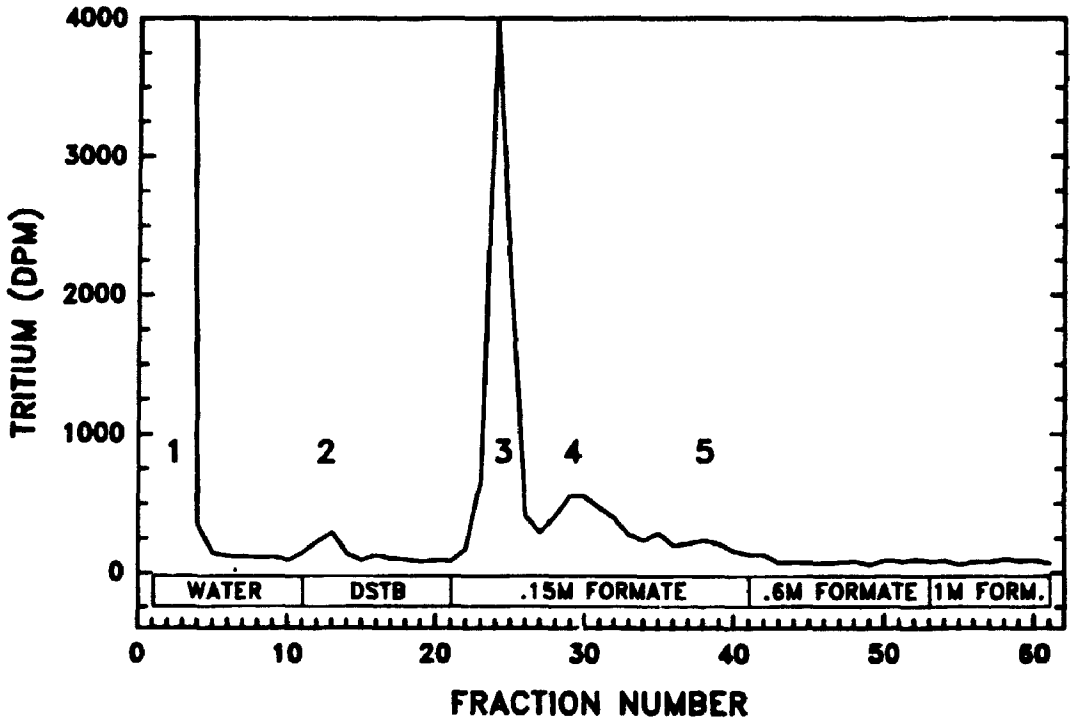


Figure 33: Representative elution pattern from Dowex anion exchange columns of an aqueous layer extracted from somtatrophs incubated with [^{3}H]-inositol for 4 h. Abbreviations: 5 mM disodium tetraborate/60 mM sodium formate (DSTB), 0.15 M ammonium formate in 0.1 M formic acid (.15M FORMATE), 0.6 M ammonium formate in 0.1 M formic acid (.6M FORMATE) and 1.0 M ammonium formate in 0.1 M formic acid (1 M FORM).

us an indication of the effect of GRF on phospholipase C activity. We chose to incubate the cells with [^{3}H]-inositol for 4 h in these experiments, because there was no appreciable difference in the labelling between 4 and 8 h and we were concerned about the health of the cells with incubation times as great as 8 h. We used two different protocols.

Protocol 1

The effect of incubation of cells with GRF (10^{-7}M) for 20 s or 1 min was first examined. The somatotrophs were incubated for 4 h with [^{3}H]-inositol using the protocol described in A.1. The somatotrophs were washed with M199AH/37-inositol free containing 5 mM LiCl and divided into Teflon beakers (1.0 - 2.0 x 10^6 cells/ ml /beaker). After 10 min of incubation in a Dubnoff incubator-shaker at 37 C, GRF (10^{-7} M) was added for 20 s or 1 min. The cells were lysed with the addition of 4 ml of chloroform:methanol (1:2, v/v) and then the phosphoinositides and inositol phosphates were separated as described in section A.1.

There were no detectable differences in the radioactivity present in the organic or aqueous layers with GRF (Table VII). GRF did not alter the labelling of PI, PIP or IP at either time point (Table VIII). Again, we did not detect labelled PIP_2, IP_2 or IP_3.

Protocol 2

We reasoned that the lack of observed response using protocol 1 may have been due to (a) the inclusion of LiCl during the period of incubation of the cells with [^{3}H]-inositol or (b) insufficient time of incubation of cells with GRF. Therefore, we omitted LiCl in the loading medium and added it only 20 min before the addition of GRF and examined the effect of GRF (10^{-7} M) at 15 and 30 min of incubation. We used a similar procedure to the one described in protocol 1, except that LiCl was omitted from the

incubation medium and, only after the loaded cells were washed, were they suspended in M199AH/37-inositol free containing 5 mM LiCl. The cells in suspension were aliquoted and then incubated for 20 min before the addition of GRF. GRF was added for 15 and 30 min and the cell lysate was processed as described in section A.1.

Omission of LiCl until the final 20 min before the addition of GRF did not markedly alter the basal labelling of PI, PIP or PIP_2. The labelling of IP, however, was reduced to compared protocol 1 but was still readily detectable. PIP_2, IP_2, and IP_3 were not labelled above background. GRF did not alter the amount of radioactivity in the organic and aqueous layers of the cell extracts (Table VII) or the labelling of PIP, PI and IP (Table VIII). These results suggest that GRF does not stimulate phospholipase C activity, but the lack of labelling of PIP_2, IP_2, and IP_3 make these results inconclusive. It was essential to get good labelling of all of the inositol phospholipids before a complete study of the effect of GRF on phospholipase C activity could be initiated. There were two ways to improve the labelling of the minor inositol phospholipids. One was to incubate the somatotrophs for 48-76 h with [^{3}H]-inositol but would involve setting up a primary culture system in the laboratory. The other way was to label with [^{32}P]-orthophosphate since this compound becomes incorporated into the inositol lipids in a shorter period of time (Martin, 1986). We wished to compare and correlate the phosphoinositide studies with previous studies from the laboratory. Therefore, we decided to switch to ^{32}P-orthophosphate labelling since this would not require cultured somatotrophs which might be altered by prolonged periods of incubation and with exposure to serum during culture.

Table VII: Effect of GRF on the amount of radioactivity in the chloroform and aqueous layers of the somatotroph extracts.

TIME	RADIOACTIVITY IN LAYER (% of CONTROL)	
(min)	CHLOROFORM LAYER	AQUEOUS LAYER
0.33	101.8 (2)	102.5 (2)
1	108.6 (2)	103.0 (2)
15	101.9 (1)	99.2 (1)
30	102.2 ± 2.8 (3)	99.8 ± 1.4 (3)

The values represent the mean ± SEM of the GRF dpm expressed as a percentage of the control dpm at each time point. LiCl (5 mM) was present in the loading medium in the 0.33 and 1 min experiments. LiCl (5 mM) was present in the medium 20 min prior to the addition of GRF in the 15 and 30 min experiments. GRF (10^{-7}M) was added at time zero. The numbers in parentheses indicate the number of observations at that time point.

Table VIII: Effect of GRF on the incorporation of [^{3}H]-inositol into the individual phosphoinositides and inositol phosphates

TIME	INCORPORATION OF [3H]-INOSITOL (% of CONTROL)		
(MiN)	PIP	PI	IP
0.33	89.3 ± 9.4 (4)	97.9 ± 5.8 (4)	97.0 (2)
1	111.3 ± 13.4 (4)	92.9 ± 7.5 (4)	105.8 (2)
15	103.4 (2)	97.9 ± 9.0 (2)	110.4 (1)
30	94.9 ± 5.1 (6)	100.2 ± 3.1 (6)	109.1 ± 2.5 (3)

The values represent the mean ± SEM of the GRF dpm expressed as a percentage of the control dpm at each time point. LiCl was present in the loading medium in the 0.33 and 1.0 min experiments. LiCl was present in the medium 20 min prior to the addition of GRF in the 15 and 30 min experiments. GRF (10^{-7}M) was added at time zero. The numbers in parentheses indicate the number of observations at that time point.

APPENDIX 3: CHARACTERIZATION OF THIN LAYER CHROMATOGRAPHY TECHNINIQUES FOR THE SEPARATION OF PIP_2, PIP AND PI

We tested two previously described thin layer chromatography protocols to separate the phosphoinositides, PIP_2, PIP, and PI. The two methods involved the use of different developing solutions while the same procedures were used for the preparation of the plates. The developing solution for the first method (system 1) consisted of chloroform:methanol:acetone:acetic acid:water (40:13:15:12:8, v/v) and is reported to resolve PIP_2 (R_f = 0.19), PIP (R_f = 0.26) and PI (R_f = 0.43), while phosphatidylserine and phosphatidylcholine (R_f = 0.54), and phosphatidylglycerol and phosphatidyletanolamine (R_f = 0.74) are not resolved (Martin, 1986). The developing solution for the second method (system 2) consisted of chloroform:methanol: 4N NH_4OH (45:35:10, v/v) which is reported to give good separation of PIP_2 (R_f = 0.18), PIP (R_f = 0.32), and PI (R_f = 0.48) but phosphatidylserine migrates close to PI (Martin, 1986).

The two systems were tested using the following protocol. Prelayered silica gel G-plates (LK5DF, Whatman, Clifton, NJ) were run in methanol: 2% potassium oxalate in water: water (40:24:36) overnight. The plates were allowed to dry in the air and then were heat activated for 1 h at 110 C. The following standards were then run on different lanes on the plates: 50 μg of L-α-phosphatidylinositol-4,5-bisphosphate (P9763, Sigma), L-α-phosphatidylinositol-4-monophosphate (P9638, Sigma) and L-α-phosphatidylinositol (P2517, Sigma) and 100 μg of a brain extract that contained all of the phosphoinositides (P6023, Sigma). One of the plates was developed in system 1 while the other was developed in system 2. Both of the chambers were pre-equilibrated for 1 h. The plates

were removed from the developing chambers, air dried, the phosphoinositides were visualized with phosphomolybdic spray (Martin, 1986) and the R_f values were calculated. Table IX gives the observed R_f values for the two developing systems along with their published values (Martin, 1986).

There was good separation of the PIP_2, PIP and PI with system 1 even though the R_f values for the different phosphoinositides were consistently higher than the published values. With system 2, we did not get migration of PIP_2 and migration of PIP was less than the published value. From these results we concluded that we would use system 1 in our studies since it gave the best resolution of PIP_2, PIP and PI.

We also determined the migration pattern of the chloroform layers of the somatotroph extracts using system 1. The chloroform layers were reconstituted in chloroform:methanol:water (20:9:1) and applied to the plates. There were 4 spots that migrated close to PI (R_f = 0.69 in this experiment). One spot was below PI (R_f = 0.64) and the other spots were above PI (R_f = 0.79, 0.86, and 0.95). These spots most likely represent other phospholipids such as phosphatidylserine, phosphatidylcholine, phosphatidylglycerol and phosphatidylethanolamine that would be present in the chloroform layer. PIP_2 and PIP were not detectable probably because they are present is very small amounts in the cell. In the subsequent experiments, in which we studied the effect of GRF on phospholipase C activity, we ran brain extract with the chloroform layers so that we could identify PIP_2 and PIP on the lane. In addition, we ran the PIP_2, PIP and PI standards on separate lanes to ensure that there was complete separation of these phosphoinositides.

Table IX: Calculated and published R_f values of the different phosphoinositides using two different developing systems.

Compound Applied	System 1 R_f value		System 2 R_f value	
	Measured	Published	Measured	Published
PIP_2	0.23	0.19	----	0.18
PIP	0.34	0.26	0.20	0.32
PI	0.54	0.43	0.48	0.48
Brain Extract	0.22, 0.31 0.55, 0.60		0.21, 0.49	

Published values taken from Martin (1986).

APPENDIX 4: CHARACTERIZATION OF TECHNIQUES FOR THE REMOVAL OF NUCLEOTIDES FROM AQUEOUS LAYERS

We tested two methods to separate the nucleotides from the inositol phosphates (Meek, 1986; Mayr, 1988).

Method 1

We followed the method outlined by Mayr (1988). Charcoal (Norit A , alkaline, B26997, BDH Canada Ltd., Toronto, Canada) was first pre-treated. 2 g of charcoal was boiled in 250 ml of 3 N HCl for 2 h, washed with 4 L of distilled water and dried at 120 C. The charcoal was then resuspended in 50 mM sodium acetate / 0.1 M NaCl (pH = 4.0 at room temperature). We tested the ability of the charcoal to adsorb a tritiated mixture of inositol phosphates (IP_3, IP_2 and IP, TRK.882, Amersham, 1 Ci / mmol, 12,000 dpm / 50 μl) and [γ^{32}P]-ATP (NEG-002X, NEN, 32 Ci / mmol, 12,000 dpm / 50 μl) in 10^{-7} M ATP. 50 μl of the tracer was added to 0.925 ml of 0.5 M perchloric acid and the pH was adjusted to 5.0 with 6 N KOH. 10 μl to 25 μl of charcoal solution (20%) was added to the neutralized samples. The samples were mixed, left at room temperature for 15 min and then centrifuged at 9,250 *g* for 3 min. The supernatant was removed to scintillation vials and radioactivity was determined by liquid scintillation spectroscopy.

With this technique, 67-75% of the IPs were left in solution, while 10% of the labelled ATP was left in solution.

Method 2

We also tested another previously described charcoal method (Meek, 1986; Daniel et al., 1987). The charcoal (1.0 g, Darco G-60, D127-500, Fisher Scientific Co. ltd., Fairlawn, NJ) was washed 4 times with 50 ml of distilled water and resupended in distilled water (1.0 g / 2.5 ml). The IP mixture and ATP was prepared as described in method 1. The samples were brought to a pH of 6 to 7 with 2 M K_2CO_3. 50 μl of charcoal suspension containing 20 mg of charcoal was added, the samples were mixed, refrigerated for 5 min, and centrifuged at 9,250 *g* for 2 min. The supernatant was removed to scintillation vials and counted. With this procedure, 95% of the [^{3}H]-inositol phosphate mixture remained in solution, while 95% of the [^{32}P]-ATP was adsorbed on the charcoal.

Therefore, method 2 was the most effective. We studied the effectiveness of this method further by measuring the absorbance (λ = 259) of solutions containing 0.2 M cAMP (A9501, Sigma), AMP (A1877, Sigma), ADP (A5410, Sigma) and ATP (A5394, Sigma) before and after charcoal treatment. Charcoal treatment removed 100 % of the cAMP, AMP and ADP and 95 % of the ATP. Charcoal treatment did not alter the elution pattern of the standard mixture of inositol phosphates.

We then studied the effect of charcoal treatment on the elution pattern of the aqueous layers from cells labelled with ^{32}P-orthophosphate for 4 h. The cells were labelled with ^{32}P-orthophosphate using the method described in section 2.6.1. Charcoal treatment only removed up to 16% of the total radioactivity in the aqueous layer. We would expect that free ^{32}P-orthophosphate would make up the largest proportion of the radioactivity in these samples and that this would not be removed by charcoal treatment.

Figure 34 shows the elution pattern of the charcoal-treated aqueous layer of cells incubated for 4 h with ^{32}P-orthophosphate. A large peak that did not correspond to any of the inositol phosphates was not removed with charcoal treatment. There were still two peaks that eluted with 0.6M ammonium formate in 0.1N formic acid. We expected that the large peak was ^{32}P-orthophosphate. To test this, we applied ^{32}P-orthophosphate to a separate column. We found that the ^{32}P-orthophosphate ran in the same place as the large peak confirming that this large peak was free ^{32}P-orthophosphate (Figure 35). To separate IP from phosphate in our subsequent studies we collected 0.5 ml fractions for the first 5 ml of the elution with 0.15 M ammonium formate in 0.1 M formic acid. In addition, we added the tritiated standard mixture of IP, IP_2, and IP_3 to each charcoal-treated aqueous layer to identify the fractions that contained these inositol phosphates.

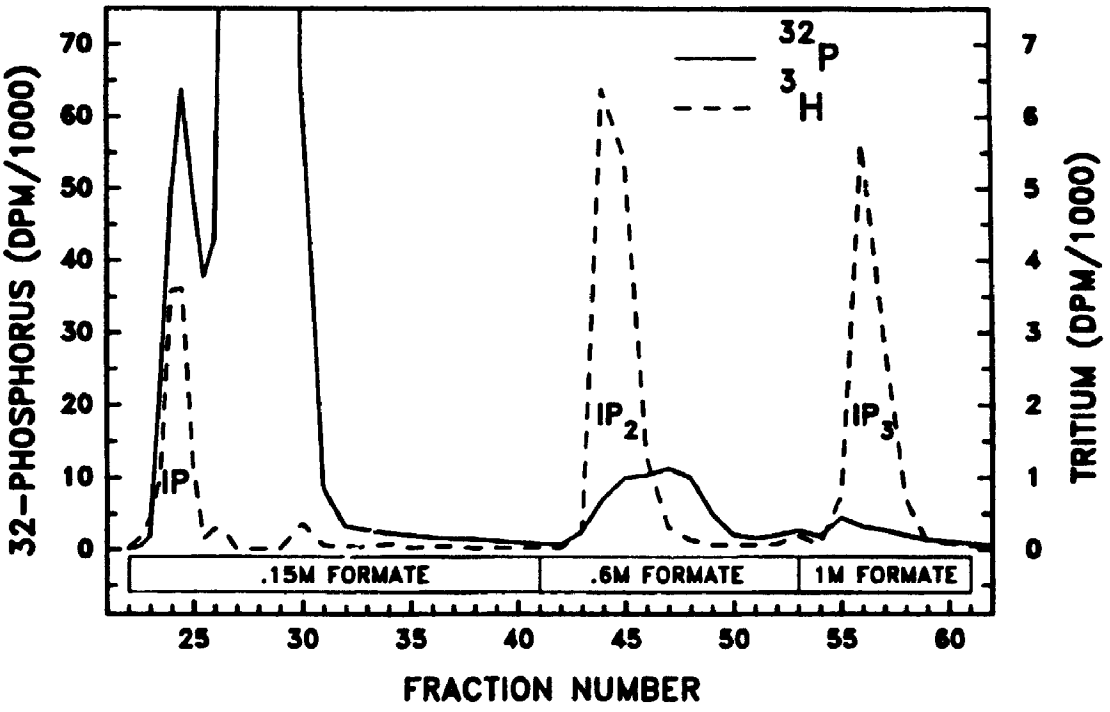


Figure 34: Elution pattern (solid lines) of charcoal-treated (method 2) aqueous layer from somatotrophs labelled with ^{32}P-orthophosphate for 4 h. A standard mixture of tritiated inositol phosphates (IP, IP_2, and IP^3) were added to the aqueous layers before chromatography and their elution pattern is also shown (dashed lines).

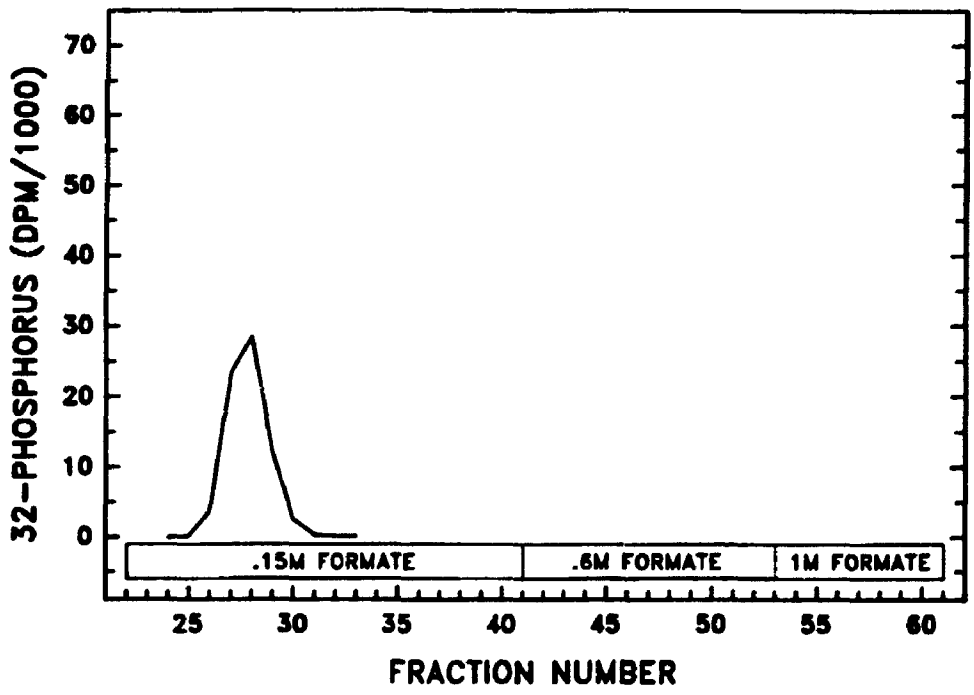


Figure 35: Elution pattern of ^{32}P-orthophosphate on Dowex columns.

APPENDIX 5: CHARACTERIZATION OF TECHNIQUES FOR THE SEPARATION OF IP_3, IP_2 AND IP

We used Dowex anion exhange chromatography to separate the inositol phosphates, IP, IP_2, and IP_3. The different isomers of the inositol phosphates are not separated by this method and their separation requires high performance liquid chromatography techniques. Dowex anion exchange chromatography, however, was sufficient for our purposes since we were simply asking whether GRF increases the production IP_3, IP_2 and IP_3. We tested two elution protocols (Martin, 1986). The standards that we used were radiolabelled inositol (NET 114, NEN, Boston, MA); IP and IP_2 (supplied by Dr. R.J. Haslam, Department of Pathology, McMaster University); and a mixture of IP, IP_2, IP_3 (TRK.882, Amersham).

Preparation of Columns

Dowex (AG1-X8, formate form, mesh 200-400, BioRad) was pretreated with ammonium formate using the following procedure. 200 g of Dowex resin was suspended in 300 ml of distilled water. The resin was poured into a 350 ml glass column. The water was drained and the resin was rinsed with 4 N ammonium formate in 6 N formic acid (2.5 l). The resin was then rinsed with 20 l of distilled water, resuspended in distilled water (resin:water, 2:1, v/v), and stored at 4 C. On the day of the chromatography, columns were prepared with 10 ml disposable columns (BioRad). A 1 ml resin bed was washed with 10 ml of distilled water before the application of the samples.

Elution Protocols

The radiollabeled standards were made up in 1 ml of water and applied to separate columns. The samples were washed with 5 ml of water (elutes inositol), 5 ml of 5 mM disodium tetraborate/60 mM sodium formate (elutes glycerophosphatidyl-inositol); 10 ml 0.15 M ammonium formate in 0.1 M formic acid (elutes IP); 15 ml 0.6 M ammonium formate in 0.1 M formic acid (protocol 1) or 0.4 M ammonium formate in 0.1 M formic acid (protocol 2) (elutes IP_2); and 6 ml 1.0 M ammonium formate in 0.1 M formic acid (elutes IP_3). 1 ml fractions of effluent were collected and transferred to scintillation vials. Radioactivity in each fraction was determined by liquid scintillation spectroscopy.

Inositol, IP and IP_2 were all eluted with the predicted solutions using either protocol. The IP_2 peak, however, was retarded and broader when 0.4 M ammonium formate in 0.1 M formic acid was used. Figure 36 shows the elution pattern of the radiolabelled inositol phosphate mixture using protocol 1 and 2. Again, the IP_2 peak was delayed and broader when 0.4M ammonium formate in 0.1M formic acid was used. We chose protocol 1 for subsequent experiments since it gave sharper IP_2 peaks.

We then tested the effect of addition of an aqueous layer of somatotroph extract to the tritiated inositol phosphate mixture to ensure that the compounds present would not interfere with the elution of the inositol phosphates. Figure 37 shows that the elution pattern of the inositol phosphates was not altered by the addition of the aqueous layer.

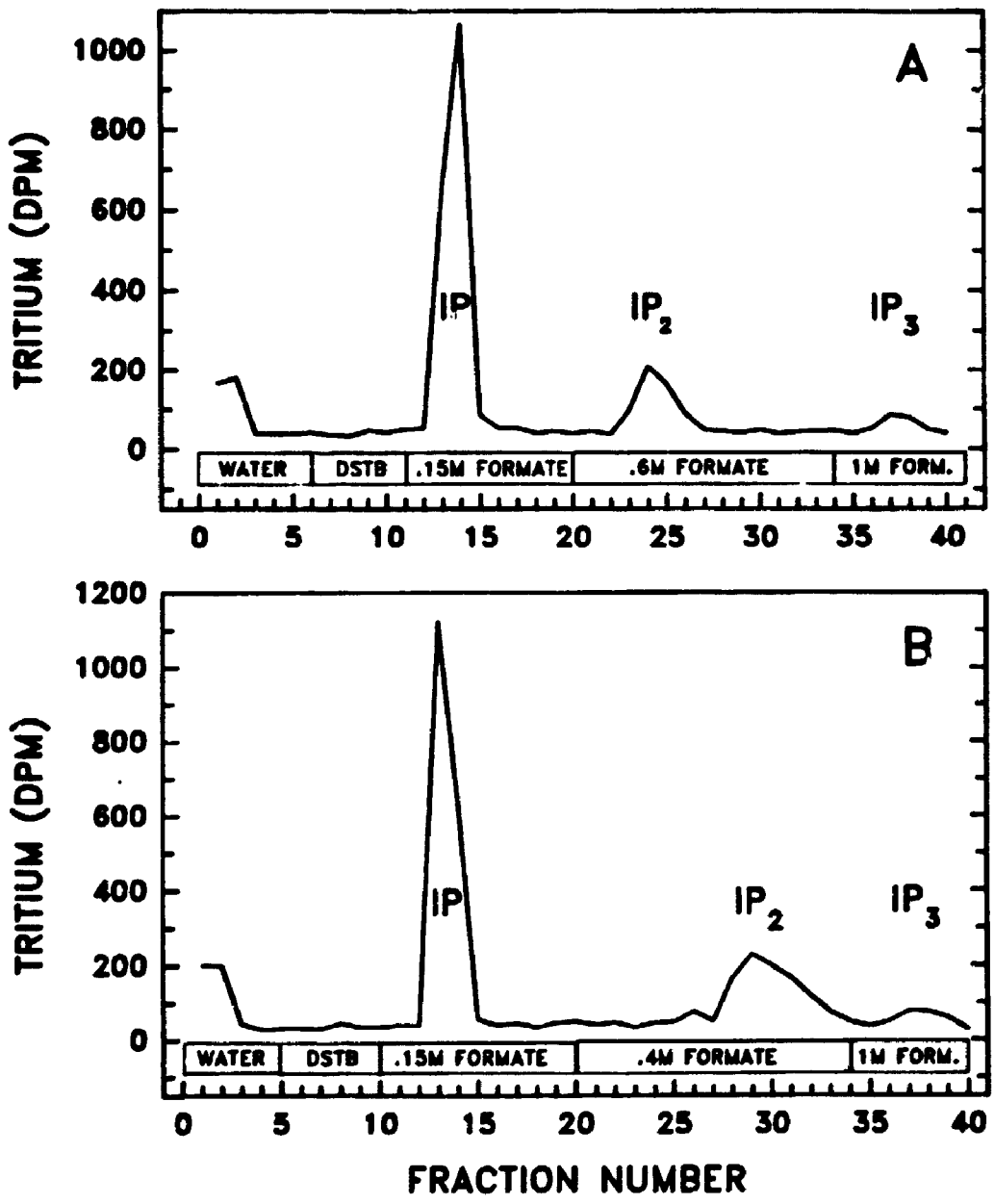


Figure 36: Elution pattern of tritiated inositol phosphate mixture using elution protocol 1 (panel A) and protocol 2 (panel B).

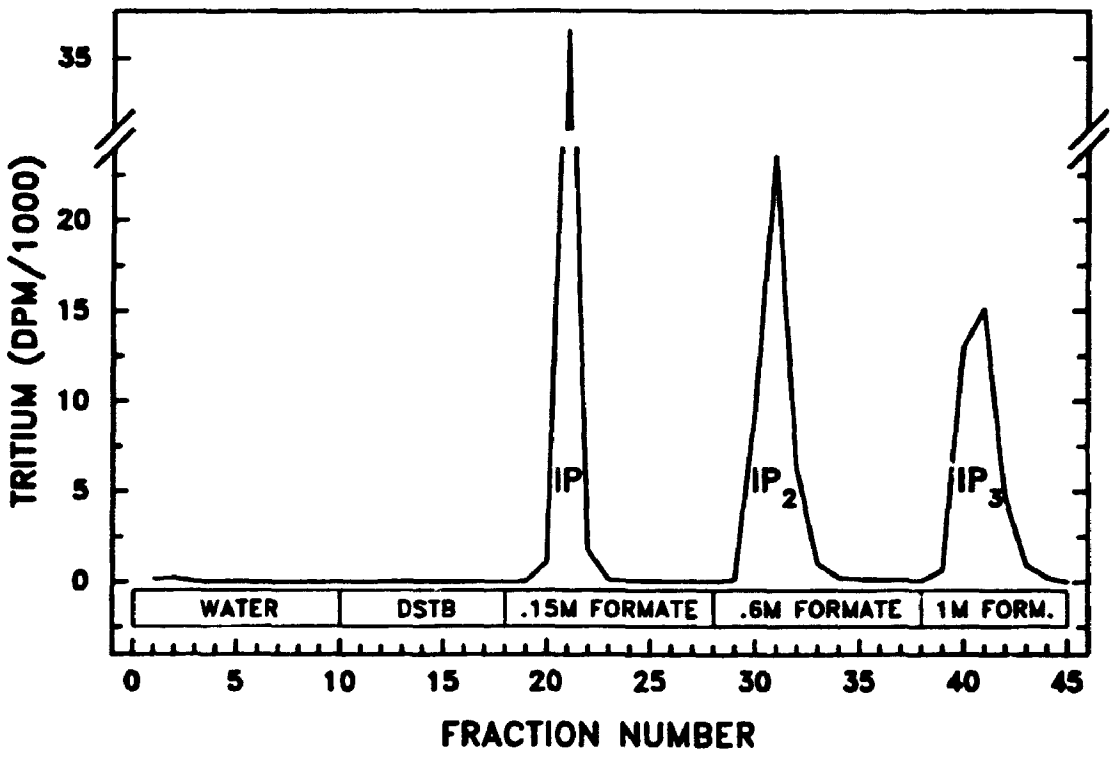


Figure 37: Elution pattern of tritiated inositol phosphate mixture containing aqueous layer from extraction of somatotrophs using protocol 1.

APPENDIX 6: EXTRACTION AND RADIOIMMUNOASSAY OF cAMP

A6.1 Extraction of cAMP

An equal volume of 10% perchloric acid was added to the frozen samples of cell suspension. To monitor recovery of cAMP, tritiated cAMP was added to each sample (2000 cpm/sample, NET-275, Dupont). The samples were sonicated in a bath type sonicator (Bransonic 220, Branson, Shelton, CT) for 10 min, transferred to microfuge tubes, and centrifuged at 10,000 rpm (Centra 4, with 749 rotor) for 15 min. The supernatant solution was transferred to microfuge tubes and neutralized with 6 N KOH and, if necessary, 0.6 N KOH and 2% perchloric acid were used to adjust the pH. The microfuge tubes were then centrifuged at 10,000 rpm for 15 min. The supernatant was applied to 1 ml Dowex columns (AG-1X8, formate form, 200-400 mesh, BioRad Laboratories, Richmond, CA) that had been washed with 10 ml of water. The resin was prepared as described in Appendix 5. After application of the sample, the columns were washed with 10 ml of water and 1 ml of 3 N formic acid. Finally cAMP was eluted with 3 ml of 3 N formic acid. 100 μl of 1% mannitol was added to each sample and then the samples were evaporated to dryness in a Savant Speed Vac Concentrator.

A6.2 cAMP assay

The dried extracts were reconstituted in 500 μl of acetate buffer (ABA; 0.05 M sodium acetate containing 0.015 M sodium azide brought to a pH of 6.20 at room temperature with glacial acetic acid), left at room temperature for 15 min, mixed and then centrifuged. 100 μl was transferred to 12 x 75 glass test tubes and 5 μl of acetylation reagent (2 ml triethylamine and 1 ml acetic anhydride, used within 10 min)

was added using a Hamilton syringe. 25 μl of acetyled sample was added to assay tubes containing 150 μl of ABA. 25 μl of tracer [adenosine 3',5'-Sc-phosphoric acid, 2'-o-S-TME-[^{125}I] (NEX-130, Du Pont NEN Products, Boston, MA) in 1% NRS (normal rabbit serum; 200-6120, Gibco) + ABA; 10,000 - 12,000 cpm /25 μl] was added along with 100 μl of antibody (0.6 mg of cAMP antibody kindly supplied by Dr. D.T. Armstrong, Department of Physiology, University of Western Ontario in 40 ml of 0.2% BSA-0.1 M phosphate buffer). The tubes were mixed and left overnight (16-18 h) at 4 C. The following day, 50 μl of second antibody (rabbit anti-goat γ-globbulin; 539845, Calbiochem, La Jolla, CA; 1:16 dilution in 0.2% BSA-ABA) and the tubes were left overnight at 4 C. The next day, 1 ml of 0.2% BSA-ABA was added to the tubes. The tubes were centrifuged at 2,700 rpm for 60 min (MSE Coolspin 2) at 4 C. The supernatant was decanted, the tubes were drained for 45 min, and radioactivity was determined.

REFERENCES

Abdel-Latif, A.A., Akhtar, R.A. and Hawthorne, J.N. (1977) Acetylcholine increases the breakdown of triphosphoinositide of rabbit iris muscle prelabelled with [^{32}P]phosphate. Biochem. J. 162:61-73.

Abe, H., Molitch, M.E., Van Wyk, J.J. and Underwood, L.E. (1983) Human growth hormone and somatomedian C supress the spontaneous release of growth hormone in unanesthetized rats. Endocrinology 113:1319-1324.

Abrams, R.L., Parker, M.L., Blanco, S., Reichlin, S. and Daughaday, W.H. (1966) Hypothalamic regulation of growth hormone secretion. Endocrinology 78:605-613.

Adachi, Y., Maki, M., Ishii, K., Hatanaka, M. and Murachi, T. (1990) Possible involvement of calpain in down-regulation of protein kinase C. Adv. Second Messenger Phosphoprotein Res. 24:478-484.

Aguilera, G. and Parker, D.S. (1982) Pituitary somatostatin receptors. Characterization by binding with a nondegradable peptide analogue. J. Biol. Chem. 257:1134-1137.

Aizawa, T. and Hinkle, P.M. (1985) Differential effects of thyrotropin-releasing hormone, vasoactive intestinal peptide, phorbol ester, and depolarization in GH_4C_1 rat pituitary cells. Endocrinology 116:909-919.

Akhtar, R.A. and Abdel-Latif, A.A. (1980) Requirement for calcium ions in acetylcholine-stimulated phosphodiesteratic cleavage of phosphatidyl-*myo*-inositol 4,5-bisphosphate in rabbit iris smooth muscle. Biochem. J. 192:783-791.

Albert, P.R. and Tashjian, A.H., Jr. (1985) Dual actions of phorbol esters on cytosolic free Ca^{2+} concentrations and reconstitution with ionomycin of acute thyrotropin-releasing hormone responses. J. Biol. Chem. 260:8765-8759.

Albert, P.R., Wolfson, G. and Tashjian Jr., A.H. (1987) Diacylglycerol increases cytosolic free Ca^{2+} concentration in rat pituitary cells. Relationship to thyrotropin-releasing hormone action. J. Biochem. 262:6577-6581.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1989a) Cell Signalling. In: Molecular Biology of the Cell, 2nd Ed. M. Robertson, ed., Garland Publishing Inc., New York, p. 681-726.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1989b) The Nervous System. In: Molecular Biology of the Cell, 2nd Ed. M. Robertson, ed., Garland Publishing Inc., New York, p. 1059-1136.

Alford, F.P., Baker, H.W.G., Patel, Y.C., Rennie, G.C., Youatt, G., Burger, H.G. and Hudson, B. (1973) Temporal patterns of circulating hormones as assessed by continuous blood sampling. J. Clin. Endocrinol. Metab. 36:108-116.

Almers, W. (1990) Exocytosis. Annu. Rev. Physiol. 52:607-624.

Alvarez, C.V., Mallo, F., Burguera, B., Cacicedo, L., Dieguez, C. and Casanueva, F.F. (1991) Evidence for a direct pituitary inhibition by free fatty acids of in vivo growth hormone responses to growth hormone-releasing hormone in the rat. Neuroendocrinology 53:185-189.

Andrews, W.V. and Conn, P.M. (1986) Gonadotropin-releasing hormone stimulates mass changes in phosphoinositides and diacylglycerol accumulation in purified gonadotrope cell cultures. Endocrinology 118:1148-1158.

Arimura, A. and Culler, M.D. (1985) Regulation of growth hormone secretion. In: The Pituitary Gland, H. Imura, ed., Raven Press, New York, p. 221-257.

Arimura, A. and Fishback, J.B. (1981) Somatostatin: regulation of secretion. Neuroendocrinology 33:246-256.

Asano, T. and Hidaka, H. (1984) Vasodilatory action of HA1004 [N-(2-guanidinoethyl)-5-isoquinolinesulfonamide], a novel calcium antagonist with no effect on cardiac function. J. Pharmacol. Exp. Ther. 231:141-145.

Aschner, B. (1912) über die funktion der hypophyse. Pflüg. Arch. Physiol. 146:1-146.

Aub, D.L., Gosse, M.E. and Cote, T.E. (1987) Regulation of thyrotropin-releasing hormone receptor binding and phospholipase C activation by a single GTP-binding protein. J. Biol. Chem. 262:9521-9528.

Ballester, R. and Rosen, O.M. (1985) Fate of immunoprecipitable protein kinase C in GH_3 cells treated with phorbol 12-myristate 13-acetate. J. Biol. Chem. 260:15194-15199.

Barinaga, M., Bilezikjian, L.M., Vale, W.W., Rosenfeld, M.G. and Evans, R.M. (1985) Independent effects of growth hormone releasing factor on growth hormone release and gene transcription. Nature 314:279-281.

Baudier, J., Deloulme, J.C., Van Dorsselaer, A., Black, D. and Matthes, H.W.D. (1991) Purification and characterization of a brain-specific protein kinase C substrate, neurogranin (p17). Identification of a consensus amino acid sequence between neurogranin and neuromodulin (GAP43) that corresponds to the protein kinase C phosphorylation site and the calmodulin-binding domain. J. Biol. Chem. 266:229-237.

Baukal, A.J., Guillemette, G., Rubin, R., Spät, A. and Catt, K.J. (1985) Binding sites for inositol trisphosphate in the bovine adrenal cortex. Biochem. Biophys. Res. Commun. 133:532-538.

Baumann, G. (1990) Growth hormone binding proteins and various forms of growth hormone: implications for measurements. Acta Paediatr. Scand. [Suppl.] 370:72-80.

Bean, B. (1990) Calcium channels: Gating for the physiologist. Nature 348:192-193.

Bean, B.P (1989) Neurotransmitter inhibiton of neuronal calcium currents by changes in channel voltage dependence. Nature 340:153-156.

Bell, R.L. and Majerus, P.W. (1980) Thrombin-induced hydrolysis of phosphatidylinositol in human platelets. J. Biol. Chem. 255:1790-1792.

Bell, R.M. and Burns, D.J. (1991) Lipid activation of protein kinase C. J. Biol. Chem. 266:4661-4664.

Benoit, R. (1987) Peptides derived from mammalian prosomatostatin. In: Somatostatin: Basic and Clinical Status, S. Reichlin, ed., Plenum Press, New York, p. 33-50.

Berelowitz, M., Firestone, S.L. and Frohman, L.A. (1981) Effects of growth hormone excess and deficiency on hypothalamic somatostatin content and release and on tissue somatostatin distribution. Endocrinology 109:714-719.

Bernardis, L.L and Frohman, L.A. (1970) Effect of lesion size in the ventromedial hypothalamus on growth hormone and insulin levels in weanling rats. Neuroendocrinology 6:319-328.

Bernardis, L.L. and Frohman, L.A. (1971) Plasma growth hormone responses to electrical stimulation of the hypothalamus in the rat. Neuroendocrinology 7:193-201.

Berridge, M.J. (1984) Inositol trisphosphate and diacylglycerol as second messengers. Biochem. J. 220:345-360.

Berridge, M.J. (1987) Inositol trisphosphate and diacylglycerol: two interacting second messengers. Annu. Rev. Biochem. 56:159-193.

Berridge, M.J. (1990) Calcium oscillations. J. Biol. Chem. 265:9583-9586.

Berridge, M.J. and Irvine, R.F. (1989) Inositol phosphates and cell signalling. Nature 341:197-205.

Bicknell, R.J., Young, P.W. and Schofield, J.G. (1979) Inhibition of the acetylcholine-induced secretion of bovine growth hormone by somatostatin. Mol. Cell. Endocrinol. 13:167-180.

Bilezikjian, L.M. and Vale, W.W. (1983) Stimulation of adenosine 3',5'-monophosphate production by growth hormone-releasing factor and its inhibition by somatostatin in anterior pituitary cells *in vitro*. Endocrinology 113:1726-1731.

Birnbaumer, L. (1990) Transduction of receptor signal into modulation of effector activity by G proteins: the first 20 years or so... FASEB J. 4:3068-3078.

Bloch, B., Brazeau, P., Bloom, F. and Ling, N. (1983a) Topographical study of the neurons containing hpGRF immunoreactivity in monkey hypothalamus. Neurosci. Lett. 37:23-28.

Bloch, B., Brazeau, P., Ling, N., Bohlen, P., Esch, F., Wehrenberg, W.B., Benoit, R., Bloom, F. and Guillemin, R. (1983b) Immunohistochemical detection of growth hormone-releasing factor in brain. Nature 301:607-608.

Blumberg, P.M. (1980) *In vitro* studies on the mode of action of the phorbol esters, potent tumor promoters: part 1. CRC Crit. Rev. Toxicol. 8:153-197.

Bonvini, E., Ruscetti, F.W., Ponzoni, M., Hoffman, T. and Farrar, W.L. (1987) Interleukin 2 rapidly stimulates synthesis and breakdown of polyphosphoinositides in interleukin 2-dependent, murine T-cell lines. J. Biol. Chem. 262:4160-4164.

Bosman, F.T., Van Assche, C., Nieuwenhuyzen Kruseman, A.C., Jackson, S. and Lowry, P.J. (1984) Growth hormone releasing factor (GRF) immunoreactivity in human and rat gastrointestinal tract and pancreas. J. Histochem. Cytochem. 32:1139-1144.

Böhlen, P., Brazeau, P., Bloch, B., Ling, N., Gaillard, R. and Guillemin, R. (1983a) Human hypothalamic growth hormone releasing factor (GRF): evidence for two forms identical to tumor derived GRF-44-NH_2 and GRF-40. Biochem. Biophys. Res. Commun. 114:930-936.

Böhlen, P., Esch, F., Brazeau, P., Ling, N. and Guillemin, R. (1983b) Isolation and characterization of the porcine hypothalamic growth hormone releasing factor. Biochem. Biophys. Res. Commun. 116:726-734.

Böhlen, P., Wehrenberg, W.B., Esch, F., Ling, N., Brazeau, P. and Guillemin, R. (1984) Rat hypothalamic growth hormone-releasing factor: isolation, sequence analysis and total synthesis. Biochem. Biophys. Res. Commun. 125:1005-1012.

Bradford, P.G. and Irvine, R.F. (1987) Specific binding sites for [^{3}H]inositol(1,3,4,5)tetrakisphosphate on membranes of HL-60 cells. Biochem. Biophys. Res. Commun. 149:680-685.

Brass, L.F. and Joseph, S.K. (1985) A role for inositol triphosphate in intracellular Ca^{2+} mobilization and granule secretion in platelets. J. Biol. Chem. 260:15172-15179.

Brazeau, P., Böhlen, P., Esch, F., Ling, N., Wehrenberg, W.B. and Guillemin, R. (1984) Growth hormone-releasing factor from ovine and caprine hypothalamus: isolation, sequence analysis and total synthesis. Biochem. Biophys. Res. Commun. 125:606-614.

Brazeau, P., Ling, N., Esch, F., Böhlen, P., Benoit, R. and Guillemin, R. (1981) High biological activity of the synthetic replicates of somatostatin-28 and somatostatin-25. Regul. Pept. 1:255-264.

Brazeau, P., Ling, N., Böhlen, P., Esch, F., Ying, S.-Y. and Guillemin, R. (1982a) Growth hormone releasing factor, somatocrinin, releases pituitary growth hormone *in vitro*. Proc. Natl. Acad. Sci. U.S.A. 79:7909-7913.

Brazeau, P., Ling, N., Esch, F., Böhlen, P., Mougin, C. and Guillemin, R. (1982b) Somatocrinin (growth hormone releasing factor) in vitro bioactivity; Ca^{++} involvement, cAMP mediated action and additivity of effect with PGE_2. Biochem. Biophys. Res. Commun. 109:588-594.

Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J. and Guillemin, R. (1973) Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science 179:77-79.

Brooks, C.M. (1988) The history of thought concerning the hypothalamus and its functions. Brain. Res. Bu'l. 20:657-667.

Brostedt, P., Luthman, M., Wide, L., Werner, S. and Roos, P. (1990) Characterization of dimeric forms of human pituitary growth hormone by bioassay, radioreceptor assay, and radioimmunoassay. Acta Endocrinol. (Copenh.) 122:241-248.

Brostrom, M.A., Brostrom, C.O., Brotman, L.A. and Green, S.S. (1983) Regulation of Ca^{2+}-dependant cyclic AMP accumulation and Ca^{2+} metabolism in intact pituitary cells by modulators of prolactin production. Mol. Pharmacol. 23:399-408.

Brown, A.M. and Birnbaumer, L. (1990) Ionic channels and their regulation by G protein subunits. Annu. Rev. Physiol. 52:197-213.

Brown, G.M, Cleghorn, J.M. and Boyne, T.S. (1983) Psychoneuroendocrinology of growth hormone: an update. In: The Anterior Pituitary Gland, A.S. Bhatnagar, ed., Raven Press, New York, p. 393-403.

Brown, K.D., Dicker, P. and Rozengurt, E. (1979) Inhibition of epidermal growth factor binding to surface receptors by tumor promotors. Biochem. Biophys. Res. Commun. 86:1037-1043.

Brown, M.R. and Fisher, L.A. (1985) Central nervous system actions of somatostatin-related peptides. In: Somatostatin. Advances in Experimental Medicine and Biology. Volume 188, Y.C. Patel and G.S. Tannenbaum, eds., Plenum Press, New York, p. 217-228.

Buck, M.A. and Fraser, C.M. (1990) Muscarinic acetylcholine receptor subtypes which selectively couple to phospholipase C: pharmacological and biochemical properties. Biochem. Biophys. Res. Commun. 173:666-672.

Burgess, G.M., Godfrey, P.P., McKinney, J.S., Berridge, M.J., Irvine, R.F. and Putney, J.W., Jr. (1984a) The second messenger linking receptor activation to internal Ca release in liver. Nature. 309:63-66.

Burgess, G.M., Irvine, R.F., Berridge, M.J., McKinney, J.S. and Putney, J.W., Jr. (1984b) Actions of inositol phosphates on Ca^{2+} pools in guinea-pig hepatocytes. Biochem. J. 224:741-746.

Burgoyne, R.D. (1990) Secretory vesicle-associated proteins and their role in exocytosis. Annu. Rev. Physiol. 52:647-659.

Canonico, P.L., Cronin, M.J., Thorner, M.O. and MacLeod, R.M. (1983) Human pancreatic GRF stimulates phosphatidylinositol labeling in cultured anterior pituitary cells. Am. J. Physiol. (Endocrinol. Metab. 8) 245:E587-E590.

Canonico, P.L., Speciale, C., Sortino, M.A., Cronin, M.J., MacLeod, R.M. and Scapagnini, U. (1986) Growth hormone releasing factor (GRF) increases free arachidonate levels in the pituitary: a role for lipoxygenase products. Life Sci. 38:267-272.

Carafoli, E. (1987) Intracellular calcium homeostasis. Annu. Rev. Biochem. 56:395-433.

Carafoli, E. (1989) The plasma membrane calcium pump: Structure, function and regulation. Biochem. Soc. Trans. 17:808-810.

Carafoli, E. (1991a) Calcium pump of the plasma membrane. Physiol. Rev. 71:129-153.

Carafoli, E. (1991b) The calcium pumping ATPase of the plasma membrane. Annu. Rev. Physiol. 53:531-547.

Cassel, D. and Selinger, Z. (1976) Catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. Biochim. Biophys. Acta 452:538-551.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257:7847-7851.

Chauhan, V.P.S., Chauhan, A., Deshmukh, D.S. and Brockerhoff, H. (1990) Lipid activators of protein kinase C. Life Sci. 47:981-986.

Chen, C., Israel, J.-M. and Vincent, J.-D. (1989) Electrophysiological responses of rat pituitary cells in somatotroph-enriched primary culture to human growth-hormone releasing factor. Neuroendocrinology 50:679-687.

Chen, C., Zhang, J., Vincent, J.-D. and Israel, J.-M. (1990a) Two types of voltage-dependent calcium current in rat somatotrophs are reduced by somatostatin. J. Physiol. (Lond.) 425:29-42.

Chen, C., Zhang, J., Vincent, J.-D. and Israel, J.-M. (1990b) Somatostatin increases voltage-dependent potassium currents in rat somatotrophs. Am. J. Physiol. 259:C854-C861.

Chihara, K., Minamitani, N., Kaji, H., Arimura, A. and Fujita, T. (1981) Intraventricularly injected growth hormone stimulates somatostatin release into rat hypophysial portal blood. Endocrinology 109:2279-2281.

Christofides, N.D., Stephanou, A., Suzuki, H., Yiangou, Y. and Bloom, S.R. (1984) Distribution of immunoreactive growth hormone-releasing hormone in the human brain and intestine and its production by tumors. J. Clin. Endocrinol. Metab. 59:747-751.

Ciofi, P., Tramu, G. and Bloch, B. (1990) Comparative immunohistochemical study of the distribution of neuropeptide Y, growth hormone-releasing factor and the carboxyterminus of precursor protein GHRF in the human hypothalamic infundibular area. Neuroendocrinology 51:429-436.

Clemmons, D.R. (1989) Structural and functional analysis of insulin-like growth factors. Br. Med. Bull. 45:465-480.

Cobbold, P.H. (1989) Oscillatory calcium signals in hormone-stimulated cells. NIPS 4:211-215.

Cockcroft, S. and Stutchfield, J. (1989) ATP stimulates secretion in human neutrophils and HL60 cells via a pertussis toxin-sensitive guanine nucleotide-binding protein coupled to phospholipase C. FEBS Lett. 245:25-29.

Conn, P.M., Ganong, B.R., Ebeling, J., Staley, D., Niedel, J.E. and Bell, R.M. (1986) Synthesis and use of diacylglycerols as activators of protein kinase C in neuroendocrine tissue. Methods Enzymol. 124:57-63.

Corbin, J.D., Thomas, M.K., Wolfe, L., Shabb, J.B., Woodford, T.A. and Francis, S.H. (1990) New insights into cGMP action. Adv. Second Messenger Phosphoprotein Res. 24:411-418.

Costa, M.R.C., Casnellie, J.E. and Catterall, W.A. (1982) Selective phosphorylation of the α subunit of the sodium channel by cAMP-dependent protein kinase. J. Biol. Chem. 257:7918-7921.

Costa, M.R.C. and Catterall, W.A. (1984) Cyclic AMP-dependent phosphorylation of the α subunit of the sodium channel in synaptic nerve ending particles. J. Biol. Chem. 259:8210-8218.

Cowan, J.S., Gaul, P., Moor, B.C. and Kraicer, J. (1984) Secretory bursts of growth hormone secretion in the dog may be initiated by somatostatin withdrawal. Can. J. Physiol. Pharmacol. 62:199-207.

Cowan, J.S., Moor, B.C., Chow, A. and Kraicer, J. (1983) Characteristics of the post-somatostatin rebound in growth hormone secretion from perifused somatotrophs. Endocrinology 113:1056-1061.

Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H. and Kirk, C.J. (1983) Rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in rat hepatocytes stimulated by vasopressin and other Ca^{2+}-mobilizing hormones. Biochem. J. 212:733-747.

Cronin, M.J. and Canonico, P.L. (1985) Tumor promoters enhance basal and growth hormone releasing factor stimulated cyclic AMP levels in anterior pituitary cells. Biochem. Biophys. Res. Commun. 129:404-410.

Cronin, M.J., Rogol, A.D., Dabney, L.G. and Thorner, M.O. (1982) Selective growth hormone and cyclic AMP stimulating activity is present in a human pancreatic islet cell tumor. J. Clin. Endocrinol. Metab. 55:381-383.

Curtis, B.M. and Catterall, W.A. (1985) Phosphorylation of the calcium antagonist receptor of the voltage-sensitive calcium channel by cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. U. S. A. 82:2528-2532.

Cushing, H. (1912) The Pituitary Body and its Disorders. J.B. Lippincott Co., Philadelphia,

Daniel, J.L., Dangelmaier, C.A. and Smith, J.B. (1987) Formation and metabolism of inositol 1,4,5-trisphosphate in human platelets. Biochem. J. 246:109-114.

Danoff, S.K. and Young, J.M. (1987) Is histamine potentiation of adenosine-stimulated cyclic AMP accumulation in guinea-pig cerebral cortical slices mediated by products of inositol phospholipid breakdown? Biochem. Pharmacol. 36:1177-1179.

Davidson, J.S., Wakefield, I.K., Sohnius, U., van der Merwe, P.A. and Millar, R.P. (1990) A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. Endocrinology 126:80-87.

Davidson, M.B. (1987) Effect of growth hormone on carbohydrate and lipid metabolism. Endocr. Rev. 8:115-131.

Davis, T.M.E., Burrin, J.M. and Bloom, S.R. (1987) Growth hormone (GH) release in response to GH-releasing hormone in man is 3-fold enhanced by galanin. J. Clin. Endocrinol. Metab. 65:1248-1252.

De Camilii, P. and Jahn, R. (1990) Pathways to regulated exocytosis in neurons. Annu. Rev. Physiol. 52:625-645.

De Gennaro Colonna, V., Cella, S.G., Locatelli, V., Loche, S., Ghigo, E., Cocchi, D. and Müller, E.E. (1989) Neuroendocrine control of growth hormone secretion. Acta Paediatr. Scand. [Suppl.] 349:87-92.

Deuben, R.R. and Meites, J. (1964) Stimulation of pituitary growth hormone release by a hypothalamic extract *in vitro*. Endocrinology 74:408-414.

Dharmsathaphorn, K. (1987) Somatostatin in gastrointestinal function: intestinal absorption and secretion. In: Somatostatin: Basic and Clinical Status, S. Reichlin, ed., Plenum Press, New York, p. 267-273.

Diaz-Laviada, I., Larrodera, P., Nieto, J.L., Cornet, M.E., Diaz-Meco, M.T., Sanchez, M.J., Guddal, P.H., Johansen, T., Haro, A. and Moscat, J. (1991) Mechanism of inhibition of adenylate cyclase by phospholipase C-catalyzed hydrolysis of phosphatidylcholine. Involvement of a pertussis toxin-sensitive G protein and protein kinase C. J. Biol. Chem. 266:1170-1176.

Dieguez, C., Page, M.D. and Scanlon, M.F. (1988) Growth hormone neuroregulation and its alterations in disease states. Clin. Endocrinol. 28:109-143.

Douglas, W.W. (1968) The First Gaddum Memorial Lecture. Cambridge: September 1967. Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. Br. J. Pharmac. 34:451-474.

Downes, C.P., Hawkins, P.T. and Stephens, L. (1989) Identification of the stimulated reaction in intact cells, its substrate supply and the metabolism of inositol phosphates.

In: Inositol Lipids in Cell Signalling, R.H. Michell, A.H. Drummond and C.P. Downes, eds., Academic Press, London, p. 3-38.

Downes, C.P. and Michell, R.H. (1981) The polyphosphoinositide phosphodiesterase of erythrocyte membranes. Biochem. J. 198:133-140.

Dray, F., Kouznetzova, B., Harris, D. and Brazeau, P. (1980) Role of prostaglandins on growth hormone secretion: PGE_2 a physiological stimulator. Adv. Prostaglandin Thromboxane Res. 8:1321-1328.

Drobny, E.C., Amburn, K. and Baumann, G. (1983) Circadian variation of basal plasma growth hormone in man. J. Clin. Endocrinol. Metab. 57:524-528.

Drouva, S.V., Gorenne, I., Laplante, E., Rérat, E., Enjalbert, A. and Kordon, C. (1990) Estradiol modulates protein kinase C activity in the rat pituitary *in vivo* and *in vitro*. Endocrinology 126:536-544.

Drummond, A.H., Bushfield, M. and Macphee, C.H. (1984) Thyrotropin-releasing hormone-stimulated [^{3}H]inositol metabolism in GH_3 pituitary tumor cells. Mol. Pharmacol. 25:201-208.

Dunlop, M.E. and Larkins, R.G. (1988) GTP- and inositol 1,4,5-trisphosphate-induced release of $^{45}Ca^{2+}$ from a membrane store co-localized with pancreatic-islet-cell plasma membrane. Biochem. J. 253:67-72.

Durell, J., Garland, J.T. and Friedel, R.O. (1969) Acetylcholine action: biochemical aspects. Science 165:862-866.

Edén, S. (1979) Age- and sex-related differences in episodic growth hormone secretion in the rat. Endocrinology 105:555-560.

Elde, R.P. and Parsons, J.A. (1975) Immunocytochemical localization of somatostatin in cell bodies of the rat hypothalamus. Am. J. Anat. 144:541-548.

England, P.J. (1986) Intracellular calcium receptor mechanisms. Br. Med. Bull. 42:375-383.

Enjalbert, A., Guillon, G., Mouillac, B., Audinot, V., Rasolonjanahary, R., Kordon, C. and Bockaert, J. (1990) Dual mechanisms of inhibition by dopamine of basal and thyrotropin-releasing hormone-stimulated inositol phosphate production in anterior pituitary cells. Evidence for an inhibition not mediated by voltage-dependent Ca^{2+} channels. J. Biol. Chem. 265:18816-18822.

Enjalbert, A., Tapia-Arancibia, L., Rieutort, M., Brazeau, P., Kordon, C. and

Epelbaum, J. (1982) Somatostatin receptors on rat anterior pituitary membranes. Endocrinology 110:1634-1640.

Esch, F., Böhlen, P., Ling, N., Benoit, R., Brazeau, P. and Guillemin, R. (1980) Primary structure of ovine hypothalamic somatostatin-28 and somatostatin-25. Proc. Natl. Acad. Sci. U.S.A. 77:6827-6831.

Esch, F., Böhlen, P., Ling, N., Brazeau, P. and Guillemin, R. (1983) Isolation and characterization of the bovine hypothalamic growth hormone releasing factor. Biochem. Biophys. Res. Commun. 117:772-779.

Escobar, D.C., Vicentini, L.M., Ghigo, E., Ciccarelli, E., Usellini, L., Capella, C. and Cocchi, D. (1986) Growth hormone-releasing factor does not stimulate phosphoinositides breakdown in primary cultures of rat and human pituitary cells. Acta Endocrinol. (Copenh.) 112:345-350.

Eva, C. and Costa, E. (1987) In rat hippocampus, somatostatin 14 and muscarinic receptor ligands modulate an adenylate cyclase belonging to a common domain of the receptor. J. Pharmacol. Exp. Ther. 242:888-894.

Evans, H.M. and Long, J.A. (1921) The effect of the anterior lobe administered intraperitoneally upon growth, maturity, and oestrous cycles of the rat. Anat. Rec. 21:62-63.

Fafeur, V., Gouin, E. and Dray, F. (1985) Growth hormone-releasing factor (GRF) stimulates PGE_2 production in rat anterior pituitary. Evidence for a PGE_2 involvement in GRF-induced GH release. Biochem. Biophys. Res. Commun. 126:725-733.

Fain, J.N. (1990) Regulation of phosphoinositide-specific phospholipase C. Biochim. Biophys. Acta 1053:81-88.

Farago, A. and Nishizuka, Y. (1990) Protein kinase C in transmembrane signalling. FEBS Lett. 268:350-354.

Farese, R.V., Larson, R.E. and Davis, J.S. (1984) Rapid effects of angiotensin II on polyphosphoinositide metabolism in the rat adrenal glomerulosa. Endocrinology 114:302-304.

Farrington, M. and Hymer, W.C. (1990) Growth hormone aggregates in the rat adenohypophysis. Endocrinology 126:1630-1638.

Ferland, L., Labrie, F., Jobin, M., Arimura, A. and Schally, A.V. (1976) Physiological role of somatostatin in the control of growth hormone and thyrotropin secretion. Biochem. Biophys. Res. Commun. 68:149-156.

Ferris, C.D., Huganir, R.L., Supattapone, S. and Snyder, S.H. (1989) Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles. Nature 342:87-89.

Finley, J.C.W., Maderdurt, J.L., Roger, L.J. and Petrusz, P. (1981) The immunocytochemical localization of somatostatin-containing neurons in the rat central nervous system. Neuroscience 6:2173-2192.

Foegh, M.L., Hecker, M. and Ramwell, P.W. (1991) The eicosanoids: prostaglandins, thromboxanes, leukotrienes, and related compounds. In: Basic and Clinical Endocrinology, F.S. Greenspan, ed., Appleton and Lange, Norwalk, p. 53-65.

Fox, A.P., Nowycky, M.C. and Tsien, R.W. (1987a) Single-channel recordings of three types of calcium channels in chick sensory neurones. J. Physiol. (Lond.) 394:173-200.

Fox, A.P., Nowycky, M.C. and Tsien, R.W. (1987b) Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. J. Physiol. (Lond.) 394:149-172.

Franz, J., Haselbach, C.H. and Libert, O. (1962) Studies of the effect of hypothalamic extracts on somatotrophic pituitary function. Acta Endocrinol. 41:336-350.

French, M.B., Vaitkus, P., Cukerman, E., Sirek, A. and Sirek, O.V. (1987) Secretory pattern of canine growth hormone. Am. J. Physiol. (Endocrinol. Metab. 15) 252:E268-E272.

Frohman, L.A. (1988) The role of hypothalamic hormones in the control of growth hormone secretion and of growth. Acta Paediatr. Scand. [Suppl.] 343:3-11.

Frohman, L.A. and Bernardis, L.L. (1968a) Growth hormone and insulin levels in weanling rats with ventromedial hypothalamic lesions. Endocrinology 82:1125-1132.

Frohman, L.A., Bernardis, L.L. and Kant, K.J. (1968b) Hypothalamic stimulation of growth hormone secretion. Science 162:580-582.

Frohman, L.A., Downs, T.R., Clarke, I.J. and Thomas, G.B. (1990a) Measurement of growth hormone-releasing hormone and somatostatin in hypothalamic-portal plasma of unanesthetized sheep. Spontaneous secretion and response to insulin-induced hypoglycemia. J. Clin. Invest. 86:17-24.

Frohman, L.A., Downs, T.R., Kelijman, M., Clarke, I.J. and Thomas, G. (1990b) Somatostatin secretion and action in the regulation of growth hormone secretion. Metabolism 39 [Suppl. 2]:43-45.

Frohman, L.A., Maran, J.W. and Dhariwal, A.P.S. (1971) Plasma growth hormone responses to intrapituitary injections of growth hormone releasing factor (GRF) in the rat. Endocrinology 88:1483-1488.

Furukawa, K.-I., Tawada, Y. and Shigekawa, M. (1989) Protein kinase C activation stimulates plasma membrane Ca^{2+} pump in cultured vascular smooth muscle cells. J. Biol. Chem. 264:4844-4849.

Gelato, M.C. and Merriam, G.R. (1986) Growth hormone releasing hormone. Annu. Rev. Physiol. 48:569-591.

Gelato, M.C., Pescovitz, O., Cassorla, F., Loriaux, D.L. and Merriam, G.R. (1983) Effects of a growth hormone releasing factor in man. J. Clin. Endocrinol. Metab. 57:674-676.

Giannattasio, G., Bianchi, R., Spada, A. and Vallar, L. (1987) Effect of calcium on adenylate cyclase of rat anterior pituitary gland. Endocrinology 120:2611-2619.

Gilman, A.G. (1987) G proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. 56:615-649.

Gilroy, S., Read, N.D. and Trewavas, A.J. (1990) Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphate initiates stomatal closure. Nature 346:769-771.

Glick, S.M., Roth, J., Yalow, R.S. and Berson, S.A. (1965) The regulation of growth hormone secretion. Rec. Prog. Horm. Res. 21:241-270.

Godchaux, W., III, and Zimmerman, W.F. (1979) Membrane-dependent guanine nucleotide binding and GTPase activities of soluble protein from bovine rod cell outer segments. J. Biol. Chem. 254:7874-7884.

Goldberg, N.D. and Haddox, M.K. (1977) Cyclic GMP metabolism and involvement in biological regulation. Annu. Rev. Biochem. 46:823-896.

Gomperts, B.D. (1986) Calcium shares the limelight in stimulus-secretion coupling. Trends Biochem. Sci. 11:290-292.

Gomperts, B.D. (1990) G_E: A GTP-binding protein mediating exocytosis. Annu. Rev. Physiol. 52:591-606.

Goodman, H.M., Schwartz, Y., Tai, L.R. and Gorin, E. (1990) Actions of growth hormone on adipose tissue: possible involvement of autocrine or paracrine factors. Acta Paediatr. Scand. [Suppl.] 367:132-136.

Goodman, H.M., Tai, L.R. and Chipkin, S.R. (1990) The isoquinoline sulfonamide inhibitors of protein phosphorylation, H-7, H-8, and HA-1004, also inhibit RNA synthesis: studies on responses of adipose tissue to growth hormone. Endocrinology 126:441-450.

Goodyer, C.G., De Stephano, L., Guyda, H.J. and Posner, B.I. (1984a) Effects of insulin-like growth factors on adult male rat pituitary function in tissue culture. Endocrinology 115:1568-1576.

Goodyer, C.G., De Stéphano, L., Lai, W.H., Guyda, H.J. and Posner, B.I. (1984b) Characterization of insulin-like growth factor receptors in rat anterior pituitary, hypothalamus, and brain. Endocrinology 114:1187-1195.

Green, J.D. and Harris, G.W. (1947) The neurovascular link between the neurohypophysis and adenohypophysis. J. Endocrinol. 5:136-146.

Guillemette, G., Balla, T., Baukal, A.J. and Catt, K.J. (1987) Inositol 1,4,5-trisphosphate binds to a specific receptor and releases microsomal calcium in the anterior pituitary gland. Proc. Natl. Acad. Sci. U. S. A. 84:8195-8199.

Guillemin, R., Brazeau, P., Böhlen, P., Esch, F., Ling, N. and Wehrenberg, W.B. (1982) Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. Science 218:585-587.

Gusovsky, F. and Gutkind, J.S. (1991) Selective effects of activation of protein kinase C isozymes on cyclic AMP accumulation. Mol. Pharmacol. 39:124-129.

Gustafsson, J. (1989) Possible metabolic side effects of high dose growth hormone treatment. Acta Paediatr. Scand. [Suppl.] 362:50-55.

Hall, K. and Tally, M. (1989) The somatomedin-insulin-like growth factors. J. Intern. Med. 225:47-54.

Hannun, Y.A. and Bell, R.M. (1990) Rat brain protein kinase C. Kinetic analysis of substrate dependence, allosteric regulation, and autophosphorylation. J. Biol. Chem. 265:2962-2972.

Harden, T.K. (1989) The role of guanine nucleotide regulatory proteins in receptor-selective direction of inositol lipid signalling. In: Inositol Lipids in Cell Signalling, R.H. Michell, A.H. Drummond and C.P. Downes, eds., Academic Press, New York, p. 113-133.

Harper, J.F. (1988) Stimulus-secretion coupling: second messenger-regulated exocytosis. Adv. Second Messenger Phosphoprotein Res. 22:193-318.

Harwood, J.P., Grewe, C. and Aguilera, G. (1984) Actions of growth hormone-releasing factor and somatostatin on adenylate cyclase and growth hormone release in rat anterior pituitary. Mol. Cell. Endocrinol. 37:277-284.

Hasegawa-Sasaki, H. and Sasaki, T. (1982) Rapid breakdown of phosphatidylinositol accompanied by accumulation of phosphatidic acid and diacylglycerol in rat lymphocytes stimulated by concanavalin A. J. Biochem. 91:463-468.

Hayashida, T. (1972) Comparative immunochemical studies of pituitary growth hormone. In: Growth and Growth Hormone, A. Pecile and E.E. Müller, eds., Excerpta Medica, Amsterdam, p. 25-37.

Hertelendy, F. (1971) Studies on growth hormone secretion. II. Stimulation by prostaglandins in vitro. Acta Endocrinol. 68:355-362.

Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23:5036-5041.

Hirata, M., Sasaguri, T., Hamachi, T., Hashimoto, T., Kukita, M. and Koga, T. (1985) Irreversible inhibition of Ca^{2+} release in saponin-treated macrophages by the photoaffinity derivative of inositol-1,4,5-trisphosphate. Nature 317:723-725.

Hirota, K., Hirota, T., Aguilera, G. and Catt, K.J. (1985) Hormone-induced redistribution of calcium-activated phospholipid-dependent protein kinase in pituitray gonadotrophs. J. Biol. Chem. 260:3243-3246.

Hirota, K., Hirota, T., Aguilera, G. and Catt, K.J. (1986) Gonadotropin release and redistribution of calcium-activated, phospholipid-dependent protein kinase in phorbol-stimulated rat pituitary cells. Arch. Biochem. Biophys. 249:557-562.

Hokin, L.E. (1985) Receptors and phosphoinositide-generated second messengers. Annu. Rev. Biochem. 54:205-235.

Hokin, L.E. and Hokin, M.R. (1955) Effects of acetylcholine on the turnover of phosphoryl units in individual phospholipids of pancreas slices and brain cortex slices. Biochim. Biophys. Acta 18:102-110.

Hokin, L.E. and Hokin, M.R. (1956) Metabolism of phospholipids in vitro. Can. J. Biochem. Physiol. 34:349-360.

Hokin, L.E. and Hokin, M.R. (1958) Phosphoinositides and protein secretion in pancreas slices. J. Biol. Chem. 233:805-810.

Hokin, M.R. and Hokin, L.E. (1953) Enzyme secretion and the incorporation of P^{32} into phospholipides of pancreas slices. J. Biol. Chem. 203:967-977.

Hokin-Neaverson, M. (1974) Acetylcholine causes a net decrease in phosphatidylinositol and a net increase in phosphatidic acid in mouse pancreas. Biochem. Biophys. Res. Commun. 58:763-768.

Holl, R.W., Thorner, M.O. and Leong, D.A. (1988a) Intracellular calcium concentration and growth hormone secretion in individual somatotropes: effects of growth hormone-releasing factor and somatostatin. Endocrinology 122:2927-2932.

Holl, R.W., Thorner, M.O., Mandell, G.L., Sullivan, J.A., Sinha, Y.N. and Leong, D.A. (1988b) Spontaneous oscillations of intracellular calcium and growth hormone secretion. J. Biol. Chem. 263:9682-9685.

Holl, R.W., Thorner, M.O. and Leong, D.A. (1989a) Cytosolic free calcium in normal somatotropes: effects of forskolin and phorbol ester. Am. J. Physiol. (Endocrinol. Metab. 19) 256:E375-E379.

Holl, R.W., Thorner, M.O., Zysk, J.R. and Leong, D.A. (1989b) Ionophore bromo-A23187 reveals cellular calcium stores in single pituitary somatotropes. Mol. Cell. Endocrinol. 64:105-110.

Honda, Y., Takahashi, K., Takahashi, S., Azumi, K., Irie, M., Sakuma, M., Tsushima, T. and Shizume, K. (1969) Growth hormone secretion during nocturnal sleep in normal subjects. J. Clin. Endocr. 29:20-29.

Houslay, M.D. (1991) 'Crosstalk': a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. Eur. J. Biochem. 195:9-27.

Huang, F.L., Yoshida, Y., Cunha-Melo, J.R., Beaven, M.A. and Huang, K.-P. (1989) Differential down-regulation of protein kinase C isozymes. J. Biol. Chem. 264:4238-4243.

Huang, K.-P. (1989) The mechanism of protein kinase C activation. Trends Neurosci. 12:425-432.

Huang, K.-P., Huang, F.L., Nakabayashi, H. and Yoshida, Y. (1989) Expression and function of protein kinase C isozymes. Acta Endocrinol. (Copenh.) 121:307-316.

Huang, K.-P., Huang, F.L., Nakabayashi, H. and Yoshida, Y. (1990) Roles of protein kinase C isozymes in cellular regulation. Adv. Exp. Med. Biol. 255:21-28.

Huang, K.-P., Nakabayashi, H. and Huang, F.L. (1986) Isozymic forms of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase. Proc. Natl. Acad. Sci. U. S. A. 83:8535-8539.

Hunter, W.M. and Greenwood, F.C. (1964) A radio-immunoelectrophoretic assay for human growth hormone. Biochem. J. 91:43-56.

Igarashi, Y. and Kondo, Y. (1980) Acute effect of thyrotropin on phosphatidylinositol degradation and transient accumulation of diacylglycerol in isolated thyroid follicles. Biochem. Biophys. Res. Commun. 97:759-765.

Ikeda, H., Mitsuhashi, T., Kubota, K., Kuzuya, N. and Uchimura, H. (1985) Effects of phorbol ester on GH, TSH and PRL release by superfused rat adenohypophysis. Endocrinol. Japon. 32:759-765.

Ikuyama, S., Nawata, H., Kato, K.-I., Natori, S. and Ibayashi, H. (1987) Phorbol ester and phospholipase C-induced growth hormone secretion from pituitary somatotroph adenoma cells in culture: effects of somatostatin, bromocriptine, and pertussis toxin. J. Clin. Endocrinol. Metab. 64:572-577.

Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. J. Biol. Chem. 252:7610-7616.

Irvine, R.F. (1989) Functions of inositol phosphates. In: Inositol lipids of cell signalling, R.H. Michell, A.H. Drummond and C.P. Downes, eds., Academic Press, London, p. 135-161.

Irvine, R.F. and Moor, R.M. (1986) Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. Biochem. J. 240:917-920.

Irvine, R.F. and Moor, R.M. (1987) Inositol(1,3,4,5)tetrakisphosphate-induced activation of sea urchin eggs requires the presence of inositol trisphosphate. Biochem. Biophys. Res. Commun. 146:284-290.

Isaksson, O.G.P., Edén, S. and Jansson, J.-O. (1985) Mode of action of pituitary growth hormone on target cells. Annu. Rev. Physiol. 47:483-499.

Ishikawa, K., Taniguchi, Y., Kurosumi, K., Suzuki, M. and Shinoda, M. (1987) Immunohistochemical identification of somatostatin-containing neurons projecting to the median eminence of the rat. Endocrinology 121:94-97.

Israel, J.-M., Denef, C. and Vincent, J.-D. (1983) Electrophysiological properties of normal somatotrophs in culture. An intracellular study. Neuroendocrinology 37:193-199.

Jacobowitz, D.M., Schulte, H., Chrousos, G.P. and Loriaux, L. (1983) Localization of GRF-like immunoreactive neurons in the rat brain. Peptides 4:521-524.

Jaken, S. (1989) Diacylglycerol: The role of stimulated production in activation of protein kinase C. In: Inositol Lipids in Cell Signalling, R.H. Michell, A.H. Drummond and C.P. Downes, eds., Academic Press, San Diego, p. 163-178.

Jakobs, K.H., Bauer, S. and Watanabe, Y. (1985) Modulation of adenylate cyclase of human platelets by phorbol ester. Impairment of the hormone-senstive inhibitory pathway. Eur. J. Biochem. 151:425-430.

Jansson, J.-O., Ekberg, S., Isaksson, O.G.P. and Edén, S. (1984) Influence of gonadal steroids on age- and sex-related secretory patterns of growth hormone in the rat. Endocrinology 114:1287-1294.

Johansson, O., Hökfelt, T. and Elde, R.P. (1984) Immunohistochemical distribution of somatostatin-like immunoreactivity in the central nervous system of the adult rat. Neuroscience 13:265-339.

Judd, A.M., Koike, K. and MacLeod, R.M (1985) GRF increases release of growth hormone and arachidonate from anterior pituitary cells. Am. J. Physiol. (Endocrinol. Metab. 11) 248:E438-E442.

Judd, A.M., Koike, K., Yasumoto, T. and MacLeod, R.M. (1986) Protein kinase C activators and calcium-mobilizing agents synergistically increase GH, LH, and TSH secretion from anterior pituitary cells. Neuroendocrinology 42:197-202.

Kaczmarek, L.K. (1988) The regulation of neuronal calcium and potassium channels by protein phosphorylation. Adv. Second Messenger Phosphoprotein Res. 22:113-138.

Katada, T., Gilman, A.G., Watanabe, Y., Bauer, S. and Jakobs, K.H. (1985) Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. Eur. J. Biochem. 151:431-437.

Kato, M., Hattori, M.-A. and Suzuki, M. (1988) Inhibition by extracellular Na^+ replacement of GRF-induced GH secretion from rat pituitary cells. Am. J. Physiol. (Endocrinol. Metab. 17) 254:E476-E481.

Kato, M. and Suzuki, M. (1989) Growth hormone releasing factor depolarizes rat pituitary cells in Na^+-dependent mechanism. Brain Res. 476:145-148.

Kawano, H., Daikoku, S. and Saito, S. (1982) Immunohistochemical studies of intrahypothalamic somatostatin-containing neurons in rat. Brain Res. 242:227-232.

Kimura, N., Hayafuji, C. and Kimura, N. (1989) Characterization of 17-β-estradiol-dependent and -independent somatostatin receptor subtypes in rat anterior pituitary. J. Biol. Chem. 264:7033-7040.

Kishimoto, A. (1990) Limited proteolysis of protein kinase C by calpain, its possible implication. Adv. Second Messenger Phosphoprotein Res. 24:472-477.

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) Activation of calcium and phospholipid-dependent protein kianse by diacylglycerol, its possible relation to phosphatidylinositol turnover. J. Biol. Chem. 255:2273-2276.

Knight, D.E., von Grafenstein, H. and Athayde, C.M., (1989) Calcium-dependent and calcium-independent exocytosis. Trends Neurosci. 12:451-458.

Knobil, E. (1966) Tenth Bowditch Lecture. The pituitary growth hormone: an adventure in physiology. The Physiologist 9:25-44.

Koch, B.D., Blalock, J.B. and Schonbrunn, A. (1988) Characterization of the cyclic AMP-independent actions of somatostatin in GH cells. I. An increase in potassium conductance is responsible for both the hyperpolarization and the decrease in intracellular free calcium produced by somatostatin. J. Biol. Chem. 263:216-225.

Koch, B.D., Dorflinger, L.J. and Schonbrunn, A. (1985) Pertussis toxin blocks both cyclic AMP-mediated and cyclic AMP-independent actions of somatostatin. Evidence for coupling of N_i to decreases in intracellular free calcium. J. Biol. Chem. 260:13138-13145.

Koch, B.D. and Schonbrunn, A. (1988) Characterization of the cyclic AMP-independent actions of somatostatin in GH cells. II. An increase in potassium conductance intiates somatostatin-induced inhibition of prolactin secretion. J. Biol. Chem. 263:226-234.

Kraicer, J. (1979) Acetylcholine does not stimulate the relase of growth hormone from perifused rat adenohypophyses. Can. J. Physiol. Pharmacol. 57:748-750.

Kraicer, J. and Chow, A.E.H. (1982) Release of growth hormone from purified somatotrophs: use of perifusion system to elucidate interrelations among Ca^{++}, adenosine 3',5'-monophosphate, and somatostatin. Endocrinology 111:1173-1180.

Kraicer, J., Herlant, M. and Duclos, P. (1967) Changes in adenohypophyseal cytology and nucleic acid content in the rat 32 days after bilateral adrenalectomy and the chronic injection of cortisol. Can. J. Physiol. Pharmacol. 45:947-956.

Kraicer, J., Lussier, B., Moor, B.C. and Cowan, J.S. (1988a) Failure of growth hormone (GH) to feed back at the level of the pituitary to alter the response of the somatotrophs to GH-releasing factor. Endocrinology 122:1511-1514.

Kraicer, J., Sheppard, M.S., Luke, J., Lussier, B., Moor, B.C. and Cowan, J.S. (1988b) Effect of withdrawal of somatostatin and growth hormone (GH)-releasing factor on GH release *in vitro*. Endocrinology 122:1810-1815.

Kraicer, J. and Spence, J.W. (1981) Release of growth hormone from purified somatotrophs: use of high K^+ and the ionophore A23187 to elucidate interrelations among Ca^{++}, adenosine 3',5'-monophosphate, and somatostatin. Endocrinology 108:651-657.

Krisch, B. (1979) Immunohistochemical results on the distribution of somatostatin in the hypothalamus and in limbic structures of the rat. J. Histochem. Cytochem. 27:1389-1390.

Krug, E., Biemann, H.P. and Tashjian, A.H., Jr. (1987) Evidence for increased synthesis as well as increased degradation of protein kinase C after treatment of human osteosarcoma cells with phorbol ester. J. Biol. Chem. 262:11852-11856.

Krug, E. and Tashjian, A.H., Jr. (1987) Time-dependent changes in protein kinase C distribution and disappearance in phorbol ester-treated human osteosarcoma cells. Cancer Res. 47:2243-2246.

Krulich, L., Dhariwal, A.P.S. and McCann, S.M. (1968) Stimulatory and inhibitory effects of purified hypothalamic extracts on growth hormone release from rat pituitary in vitro. Endocrinology 83:783-790.

Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackettt, N.L., Katoh, N., Shoji, M. and Wrenn, R.W. (1980) Calcium-dependent protein kinase: widespread occurrence in various tissues and phyla of the animal kingdom and comparison of effects of phospholipid, calmodulin, and trifluoperazine. Proc. Natl. Acad. Sci. U. S. A. 77:7039-7043.

Labrie, F., Gagné, B. and Lefèvre, G. (1983) Growth hormone-releasing factor stimulates adenylate cyclase activity in the anterior pituitary gland. Life Sci. 33:2229-2233.

Lamberts, S.W.J. (1988) The role of somatostatin in the regulation of anterior pituitary hormone secretion and the use of its analogs in the treatment of human pituitary tumors. Endocr. Rev. 9:417-436.

Lamberts, S.W.J., den Holder, F. and Hofland, L.J. (1989) The interrelationship between the effects of insulin-like growth factor I and somatostatin on growth hormone

secretion by normal rat pituitary cells: the role of glucocorticoids. Endocrinology 124:905-911.

Lapetina, E.G. and Crouch, M.F. (1989) Relationship of inositol phospholipid metabolism to phospholipase A_2. Adv. Prostaglandin Thromboxane Leukotriene. Res. 19:568-573.

Larsson, L.-I. (1985) Distribution and morphology of somatostatin cells. In: Somatostatin. Advances in Experimental Medicine and Biology, Volume 188, Y.C. Patel and G.S. Tannenbaum, eds., Plenum Press, New York, p. 383-402.

Laychock, S.G. (1989) Coordinate interactions of cyclic nucleotide and phospholipid metabolizing pathways in calcium-dependent cellular processes. Curr. Top. Cell. Reg. 30:203-242.

Leach, K.L. and Blumberg, P.M. (1989) Tumor prototers, their receptors and their actions. In: Inositol Lipids in Cell Signalling, R.H. Michell, A.H. Drummond and C.P. Downes, eds., Academic Press, San Diego, p. 179-205.

Lee, L.-S. and Weinstein, I.B. (1978) Tumor-protmoting phorbol esters inhibit binding of epidermal growth factor to cellular receptors. Science 202:313-315.

Leung, P.C.K., Raymond, V. and Labrie, F. (1982) Mechanism of action of TRH: involvement of the phosphatidylinositol (PI) response in the action of TRH in rat anterior pituitary cells. Life Sci. 31:3037-3041.

Lewis, D.L., Weight, F.F. and Luini, A. (1986) A guanine nucleotide-binding protein mediates the inhibition of voltage-dependent calcium current by somatostatin in a pituitary celll line. Proc. Natl. Acad. Sci. U. S. A. 83:9035-9039.

Lewis, D.L. and Weight, F.F. (1988) The protein kinase C activator 1-oleoyl-2-acetylglycerol inhibits voltage-dependent Ca^{2+} current in the pituitary cell line AtT-20. Neuroendocrinology 47:169-175.

Lewis, L.D. and Williams, J.A. (1987) Structural characterization of the somatostatin receptor in rat anterior pituitary membranes. Endocrinology 121:486-492.

Lewis, U.J., Singh, R.N.P., Tutwiler, G.F., Sigel, M.B., VanderLaan, E.F. and VanderLaan, W.P. (1980) Human growth hormone: a complex of proteins. Recent Prog. Horm. Res. 36:477-508.

Li, C.H. (1975) The chemistry of human pituitary growth hormone: 1967-1973. In: Hormonal Proteins and Peptides, C.H. Li, ed., Academic Press, New York, p. 1-40.

Li, C.H. and Dixon, J.S. (1971) Human pituitary growth hormone. XXXII. The primary structure of the hormone: revision. Arch. Biochem. Biophys. 146:233-236.

Li, C.H., Evans, H.M. and Simpson, M.E. (1945) Isolation and properties of the anterior hypophyseal growth hormone. J. Biol. Chem. 159:353-366.

Limbird, L.E. (1988) Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. FASEB J. 2:2686-2695.

Limor, R., Schvartz, I., Hazum, E., Ayalon, D. and Naor, Z. (1989) Effect of guanine nucleotides on phospholipase C activity in permeabilized pituitary cells: Possible involvement of an inhibitory GTP-binding protein. Biochem. Biophys. Res. Commun. 159:209-215.

Linden, J. and Delahunty, T.M. (1989) Receptors that inhibit phosphoinositide breakdown. Trends Pharmacol. Sci. 10:114-120.

Ling, N., Esch, F., Böhlen, P., Brazeau, P., Wehrenberg, W.B. and Guillemin, R. (1984) Isolation, primary structure, and synthesis of human hypothalamic somatocrinin: growth hormone-releasing factor. Proc. Natl. Acad. Sci. U.S.A. 81:4302-4306.

Litosch, I. (1987) Guanine nucleotide and NaF stimulation of phospholipase C activity in rat cerebral-cortical membranes. Studies on substrate specificity. Biochem. J. 244:35-40.

Londos, C., Salomon, Y., Lin, M.C., Harwood, J.P., Schramm, M., Wolff, J. and Rodbell, M. (1974) 5'-guanylylimidodiphosphate, a potent activator of adenylate cyclase systems in eukaryotic cells. Proc. Natl. Acad. Sci. U. S. A. 71:3087-3090.

Lovinger, R., Boryczka, A.T., Shackelford, R., Kaplan, S.L., Ganong, W.F. and Grumbach, M.M (1974) Effect of synthetic somatotropin release inhibiting factor on the increase in plasma growth hormone elicited by L-dopa in the dog. Endocrinology 95:943-946.

Luini, A. and De Matteis, M.A. (1990) Evidence that receptor-linked G protein inhibits exocytosis by a post-second-messenger mechanism in AtT-20 cells. J. Neurochem. 54:30-38.

Luini, A., Lewis, D., Guild, S., Schofield, G. and Weight, F. (1986) Somatostatin, an inhibitor of ACTH secretion, decreases cytosolic free calcium and voltage-dependent calcium current in a pituitary cell line. J. Neurosci. 6:3128-3132.

Lussier, B.T., French, M.B., Moor, B.C. and Kraicer, J. (1991a) Free intracellular Ca^{2+} concentration and growth hormone (GH) release from purified rat somatotrophs. III.

Mechanism of action of GH-releasing factor and somatostatin. Endocrinology 128:592-603.

Lussier, B.T., French, M.B., Moor, B.C. and Kraicer, J. (1991b) Free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and growth hormone release from purified rat somatotrophs. I. GH-releasing factor-induced Ca^{2+} influx raises $[Ca^{2+}]_i$. Endocrinology 128:570-582.

Lussier, B.T., Moor, B.C., French, M.B. and Kraicer, J. (1988) Release of growth hormone from purified somatotrophs: effects of the calcium channel antagonists diltiazem and nifedipine on release induced by growth hormone-releasing factor. Can. J. Physiol. Pharmacol. 66:1373-1380.

Lussier, B.T., Wood, D.A., French, M.B., Moor, B.C. and Kraicer, J. (1991c) Free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and growth hormone release from purified rat somatotrophs. II. Somatostatin lowers $[Ca^{2+}]_i$ by inhibiting Ca^{2+} influx. Endocrinology 128:583-591.

Macphee, C.H. and Drummond, A.H. (1984) Thyrotropin-releasing hormone stimulates rapid breakdown of phosphatidylinositol 4,5-bisphaophate and phosphatidylinositol 4-phosphate in GH_3 pituitary tumor cells. Mol. Pharmacol. 25:193-200.

Maiter, D.M., Gabriel, S.M., Koenig, J.I., Russell, W.E. and Martin, J.B. (1990) Sexual differentiation of growth hormone feedback effects on hypothalamic growth hormone-releasing hormone and somatostatin. Neuroendocrinology 51:174-180.

Majerus, P.W., Connolly, T.M., Deckmyn, H., Ross, T.S., Bross, T.E., Ishii, H., Bansal, V.S. and Wilson, D.B. (1986) The metabolism of phosphoinositide-derived messenger molecules. Science 234:1519-1526.

Makhlouf, G.M. (1987) Antral somatostatin: a paracrine regulator of gastrin secretion. In: Somatostatin: Basic and Clinical Status, S. Reichlin, ed., Plenum Press, New York, p. 239-251.

Malacara, J.M., Valverde-R., C., Reichlin, S. and Bollinger, J. (1972) Elevation of plasma radioimmunoassayable growth hormone in the rat induced by porcine hypothalamic extract. Endocrinology 91:1189-1198.

Malhotra, R.K., Bhave, S.V., Wakade, T.D., Bhave, A.S. and Wakade, A.R. (1990) Effects of neurotransmitters and peptides on phospholipid hydrolysis in sympathetic and sensory neurons. FASEB J. 4:2492-2498.

Martin, J.B. (1972) Plasma growth hormone (GH) response to hypothalamic or extrahypothalamic electrical stimulation. Endocrinology 91:107-115.

Martin, J.B. (1973) Neural regulation of growth hormone secretion. N. Engl. J. Med. 288:1384-1393.

Martin, J.B. (1974) Inhibitory effect of somatostatin (SRIF) on the release of growth hormone (GH) induced in the rat by electrical stimulation. Endocrinology 94:497-502.

Martin, J.B., Audet, J. and Saunders, A. (1975) Effects of somatostatin and hypothalamic ventromedial lesions on GH release induced by morphine. Endocrinology 96:839-847.

Martin, J.B., Renaud, L.P. and Brazeau, P., Jr. (1974) Pulsatile growth hormone secretion: suppression by hypothalamic ventromedial lesions and by long-acting somatostatin. Science 186:538-540.

Martin, T.F.J. (1983) Thyrotropin-releasing hormone rapidly activates the phosphodiester hydrolysis of polyphosphoinositides in GH_3 pituitary cells. Evidence for the role of a polyphosphoinositide-specific phospholipase C in hormone action. J. Biol. Chem. 258:14816-14822.

Martin, T.F.J. (1986) Measurement of phospholipid turnover in cultured hormone responsive pituitary cells. Methods Enzymol. 124:424-442.

Martini, L., De Poli, A., Pecile, A., Saito, S. and Tani, F. (1959) Functional and morphological observations on the rat pituitary grafted into the anterior chamber of the eye. J. Endocrinol. 19:164-173.

Maruyama, Y. (1989) Control of exocytosis in single cells. NIPS 4:53-56.

Mason, W.T. and Rawlings, S.R. (1988) Whole-cell recordings of ionic currents in bovine somatotrophs and their involvement in growth hormone secretion. J. Physiol. (Lond.) 405:577-593.

Mayr, G.W. (1988) A novel metal-dye detection system permits picomolar-range h.p.l.c. analysis of inositol polyphosphates from non-radioactively labelled cell or tissue specimens. Biochem. J. 254:585-591.

McArdle, C.A. and Conn, P.M. (1986) Hormone-stimulated redistribution of gonadotrope protein kinase C *in vivo*: dependence on Ca^{2+} influx. Mol. Pharmacol. 29:570-576.

McArdle, C.A., Huckle, W.R. and Conn, P.M. (1987) Phorbol esters reduce gonadotrope responsiveness to protein kinase C activators but not to Ca^{2+}-mobilizing secretagogues. Does protein kinase C mediate gonadotropin-releasing hormone action? J. Biol. Chem. 262:5028-5035.

McPhail, L.C., Clayton, C.C. and Synderman, R. (1984) A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase. Science 224:622-625.

Meek, J.L. (1986) Inositol bis-, tris-, and tetrakis(phosphate)s: analysis in tissues by HPLC. Proc. Natl. Acad. Sci. U. S. A. 83:4162-4166.

Meister, B., Hökfelt, T., Vale, W.W., Sawchenko, P.E., Swanson, L. and Goldstein, M. (1986) Coexistence of tyrosine hydroxylase and growth hormone-releasing factor in a subpopulation of tubero-infundibular neurons of the rat. Neuroendocrinology 42:237-247.

Merchenthaler, I., Thomas, C.R. and Arimura, A. (1984) Immunocytochemical localization of growth hormone releasing factor (GHRF)-containing structures in the rat brain using anti-rat GHRF serum. Peptides. 5:1071-1075.

Michel, D., Lefèvre, G. and Labrie, F. (1983) Interactions between growth hormone-releasing factor, prostaglandin E_2 and somatostatin on cyclic AMP accumulation in rat adenohypophysial cells in culture. Mol. Cell. Endocrinol. 33:255-264.

Michell, R.H. (1975) Inositol phospholipids and cell surface receptor function. Biochim. Biophys. Acta 415:81-147.

Michell, R.H., Kirk, C.J., Jones, L.M., Downes, C.P. and Creba, J.A. (1981) The stimulation of inositol lipid metabolism that accompanies calcium mobilization in stimulated cells: defined characteristics and unanswered questions. Philos. Trans. R. Soc. Lond. 296:123-138.

Mignery, G.A., Newton, C.L., Archer, B.T., III and Südhof, T.C. (1990) Structure and expression of the rat inositol 1,4,5-trisphosphate receptor. J. Biol. Chem. 265:12679-12685.

Miki, N., Ono, M., Miyoshi, H., Tsushima, T. and Shizume, K. (1989) Hypothalamic growth hormone-releasing factor (GRF) participates in the negative feedback regulation of growth hormone secretion. Life Sci. 44:469-476.

Miller, R.J. (1987) Multiple calcium channels and neuronal function. Science 235:46-52.

Miller, S.G. and Moore, H.-P.H. (1991) Reconstitution of constitutive secretion using semi-intact cells: regulation by GTP but not calcium. J. Cell Biol. 112:39-54.

Minami, S., Wakabayashi, I., Sugihara, H., Kamegai, J., Hasegawa, O., Kitamura, T. and Yamada, J. (1990) Effect of intermittent infusions of somatostatin on growth hormone secretion in unrestrained male rats with hypothalamic deafferentation. Brain Res. 509:293-298.

Montminy, M.R., Gonzalez, G.A. and Yamamoto, K.K. (1990) Characteristics of the cAMP response unit. Metabolism 39 [Suppl. 2]:6-12.

Morris, A.P, Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1987) Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channels. Nature 330:653-655.

Nakamura, H., Kishi, Y., Pajares, M.A. and Rando, R.R. (1989) Structural basis of protein kinase C activation by tumor promoters. Proc. Natl. Acad. Sci. U. S. A. 86:9672-9676.

Nakamura, T. and Gold, G.H. (1987) A cyclic nuleotide-gated conductance in olfactory receptor cilia. Nature 325:442-444.

Namba, H., Morita, S. and Melmed, S. (1989) Insulin-like growth factor-I action on growth hormone secretion and messenger ribonucleic acid levels: interaction with somatostatin. Endocrinology 124:1794-1799.

Naor, Z. (1990) Further characterization of protein kinase-C subspecies in the hypothalamo-pituitary axis: differential activation by phorbol esters. Endocrinology 126:1521-1526.

Naor, Z., Azrad, A., Limor, R., Zakut, H. and Lotan, M. (1986) Gonadotropin-releasing hormone activates a rapid Ca^{2+}-independent phosphodiester hydrolysis of polyphosphoinositides in pituitary gonadotrophs. J. Biol. Chem. 261:12506-12512.

Naor, Z., Molcho, J., Zakut, H. and Yavin, E. (1985) Calcium-independent phosphatidylinositol response in gonadotropin-releasing-hormone-stimulated pituitary cells. Biochem. J. 231:19-23.

Naor, Z., Zer, J., Zakut, H. and Hermon, J. (1985b) Characterization of pituitary calcium-activated, phospholipid-dependent protein kinase: redistribution by gonadotropin-releasing hormone. Proc. Natl. Acad. Sci. U. S. A. 82:8203-8207.

Narayanan, N., Lussier, B., French, M., Moor, B. and Kraicer, J. (1989) Growth hormone-releasing factor-sensitive adenylate cyclase system of purified somatotrophs: effects of guanine nucleotides, somatostatin, calcium, and magnesium. Endocrinology 124:484-495.

Negro-Vilar, A. and Lapetina, E.G. (1985) 1,2-didecanoylglycerol and phorbol 12,13-dibutyrate enhance anterior pituitary hormone secretion *in vitro*. Endocrinology 117:1559-1564.

Nicholls, D.G. (1986) Intracellular calcium homeostatsis. Br. Med. Bull. 42:353-358.

Niedel, J.E., Kuhn, L.J. and Vandenbark, G.R. (1983) Phorbol diester receptor copurifies with protein kinase C. Proc. Natl. Acad. Sci. U. S. A. 80:36-40.

Niimi, M., Takahara, J., Sato, M. and Kawanishi, K. (1990) Immunohistochemical identification of galanin and growth hormone-releasing factor-containing neurons projecting to the median eminence of the rat. Neuroendocrinology 51:572-575.

Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308:693-698.

Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. Science 233:305-312.

Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature. 334:661-665.

Nishizuka, Y., Takai, Y., Hashimoto, E., Kishimoto, A., Kuroda, Y., Sakai, K. and

Yamamura, H. (1979) Regulatory and functional compartment of three multifunctional protein kinase systems. Mol. Cell. Biochem. 23:153-165.

Nowycky, M.C., Fox, A.P. and Tsien, R.W. (1985) Three types of neuronal calcium channel with different calcium agonist sensitivity. Nature. 316:440-443.

Nunn, D.L., Potter, B.V.L. and Taylor, C.W. (1990) Molecular target sizes of inositol 1,4,5-trisphosphate receptors in liver and cerebellum. Biochem. J. 265:393-398.

Nunn, D.L. and Taylor, C.W. (1990) Liver inositol 1,4,5-trisphosphate-binding sites are the Ca^{2+}-mobilizing receptors. Biochem. J. 270:227-232.

O'Rourke, F.A., Halenda, S.P., Zavoico, G.B. and Feinstein, M.B. (1985) Inositol 1,4,5-trisphosphate releases Ca^{2+} from a Ca^{2+}-transporting membrane vesicle fraction derived from human platelets. J. Biochem. 260:956-962.

Ohmura, E. and Friesen, H.G. (1985) 12-*O*-tetradecanoyl phorbol-13-acetate stimulates rat growth hormone (GH) release through different pathways from that of human pancreatic GH-releasing factor. Endocrinology 116:728-733.

Ohmura, E., Okada, M., Ohba, Y., Onoda, N., Sano, T., Tsushima, T. and Shizume, K. (1988) Phorbol ester pretreatment attenuates the growth hormone (GH) response to GH-releasing factor in cultured rat pituitary cells. J. Endocrinol. 118:423-428.

Ohmura, E., Tsushima, T., Murakami, H., Wakai, K. and Shizume, K. (1984) Effect of phorbol esters on the release of growth hormone and prolactin from rat pituitary cells cultured in monolayer. Acta Endocrinol. (Copenh.) 107:185-191.

Ohmura, E., Tsushima, T., Emoto, N., Obuchi, E. and Shizume, K. (1987) Effects of tumor promoters (mezerein, teleocidin and palytoxin) on growth hormone secretion from rat anterior pituitary cells cultured in monolayer. Life Sci. 41:691-696.

Ohno, S., Akita, Y., Konno, Y., Imajoh, S. and Suzuki, K. (1988) A novel phorbol ester receptor/protein kinase, nPKC, distantly related to the protein kinase C family. Cell 53:731-741.

Okamura, H., Murakami, S., Chihara, K., Nagatsu, I. and Ibata, Y. (1985) Coexistence of growth hormone releasing factor-like and tyrosine hydroxylase-like immunoreactivities in neurons of the rat arcuate nucleus. Neuroendocrinology 41:177-179.

Oliver, C., Mical, R.S. and Porter, J.C. (1977) Hypothalamic-pituitary vasculature: evidence for retrograde blood flow in the pituitary stalk. Endocrinology 101:598-604.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1987) Identification of three additional members of rat protein kianse C family: δ-, ∈- and ζ-subspecies. FEBS Lett. 226:125-128.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1988) The structure, expression, and properties of additional members of the protein kinase C family. J. Biol. Chem. 263:6927-6932.

Osborne, R. and Tashjian Jr., A.H. (1981) Tumor-promoting phorbol esters affect production of prolactin and growth hormone by rat pituitary cells. Endocrinology 108:1164-1170.

Ottlecz, A., Samson, W.K. and McCann, S.M. (1986) Galanin: evidence for a hypothalamic site of action to release growth hormone. Peptides 7:51-53.

Owyang, C. and Wiley, J. (1987) Somatostatin inhibits intestinal motility via modulation of cyclic AMP-dependent cholinergic transmission. In: Somatostatin: Basic and Clinical Status, S. Reichlin, ed., Plenum Press, New York, p. 253-258.

Pace, C.S. and Tarvin, J.T. (1981) Somatostatin: mechanism of action in pancreatic islet β-cells. Diabetes 30:836-842.

Padel, U. and Soling, H.-D. (1985) Phosphorylation of the ribosomal protein S6 during agonist-induced exocytosis in exocrine glands is catalyzed by calcium-phospholipid-dependent protein kinase (protein kinase C). Experiments with guinea pig parotid glands. Eur. J. Biochem. 151:1-10.

Park, J., Chiba, T. and Yamada, T. (1987) Mechanisms for direct inhibition of canine gastric parietal cells by somatostatin. J. Biol. Chem. 262:14190-14196.

Parker, D.C., Rossman, L.G., Kripke, D.F., Gibson, W. and Wilson, K. (1979) Rhythmicities in human growth hormone concentrations in plasma. In: Endocrine Rhythms, D.T. Krieger, ed., Raven Press, New York, p. 143-173.

Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, C., Schaap, D., Stabel, S. and Webster, C. (1989) Protein kinase C--a family affair. Mol. Cell. Endocrinol. 65:1-11.

Patel, Y.C. (1979) Growth hormone stimulates hypothalamic somatostatin. Life Sci. 24:1589-1594.

Patel, Y.C., Murthy, K.K., Escher, E.E., Banville, D., Spiess, J. and Srikant, C.B. (1990) Mechanism of action of somatostatin: An overview of receptor function and studies of the molecular characterization and purification of somatostatin receptor proteins. Metabolism 39 [Suppl. 2]:63-69.

Patel, Y.C., Zingg, H.H. and Srikant, C.B. (1985) Somatostatin-14 like immunoreactive forms in the rat: characterization, distribution and biosynthesis. In: Somatostatin. Advances in Experimental Medicine and Biology, Volume 188, Y.C. Patel and G.S. Tannenbaum, eds., Plenum Press, New York, p. 71-87.

Pecile, A., Müller, E., Falconi, G. and Martini, L. (1965) Growth hormone-releasing activity of hypothalamic extracts at different ages. Endocrinology 77:241-246.

Pfeuffer, T. and Helmreich, E.J.M. (1975) Activation of pigeon erythrocyte membrane adenylate cyclate by guanylnucleotide analogues and separation of a nucleotide binding protein. J. Biol. Chem. 250:867-876.

Phillips, M.A. and Jaken, S. (1983) Specific desensitization to tumor-promoting phorbol esters in mouse pituitary cells. Evidence that desensitization is a two-step process. J. Biol. Chem. 258:2875-2881.

Plotsky, P.M. and Vale, W. (1985) Patterns of growth hormone-releasing factor and somatostatin secretion into the hypophysial-portal circulation of the rat. Science 230:461-463.

Popa, G. and Fielding, U. (1930) The portal circulation from the pituitary to the hypothalamic region. J. Anat. (Lond.) 65:88-91.

Pradayrol, L., Jörnvall, H., Mutt, V. and Ribet, A. (1980) N-terminally extended somatostatin: the primary structure of somatostatin-28. FEBS Lett. 109:55-58.

Putney, J.W., Jr., Takemura, H., Hughes, A.R., Horstman, D.A. and Thastrup, O. (1989) How do inositol phosphates regulate calcium signaling. FASEB J. 3:1899-1905.

Quilliam, L.A., Dobson, P.R.M. and Brown, B.L. (1985) Modulation of cyclic AMP accumulation in GH_3 cells by a phorbol ester and thyroliberin. Biochem. Biophys. Res. Commun. 129:898-903.

Rall, T W., Sutherland, E.W. and Berthet, J. (1957) The relationship of epinephrine and glucagon to liver phosphorylase. IV. Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates. J. Biol. Chem. 224:463-475.

Rando, R.R. (1988) Regulation of protein kinase C activity by lipids. FASEB J. 2:2348-2355.

Ray, K.P., Hart, G.R. and Wallis, M. (1986) Effects of dopamine and somatostatin on phorbol ester-stimulated prolactin and growth hormone secretion. Mol. Cell. Endocrinol. 48:205-212.

Raymond, V., Leung, P.C.K., Veilleux, R., Lèfevre, G. and Labrie, F. (1984) LHRH rapidly stimulates phosphatidylinositol metabolism in enriched gonadotrophs. Mol. Cell. Endocrinol. 36:157-164.

Reddoch, R.B., Pelletier, R.-M., Barbe, G.J. and Armstrong, D.T. (1986) Lack of ovarian responsiveness to gonadotropic hormones in infantile rats sterilized with busulfan. Endocrinology 119:879-886.

Reichlin, S. (1960) Growth and the hypothalamus. Endocrinology 67:760-773.

Reichlin, S. (1961) Growth hormone content of the pituitaries from rats with hypothalamic lesions. Endocrinology 69:225-230.

Reisine, T. (1989) Phorbol esters and corticotropin releasing factor stimulate calcium influx in the anterior pituitary tumor cell line, AtT-20, through different intracellular sites of action. J. Pharmacol. Exp. Ther. 248:984-990.

Reisine, T. and Guild, S. (1987) Activators of protein kinase C and cyclic AMP-dependent protein kinase regulate intracellular calcium levels through distinct mechanisms in mouse anterior pituitary tumor cells. Mol. Pharmacol. 32:488-496.

Represa, A., Deloulme, J.C., Sensenbrenner, M., Ben-Ari, Y. and Baudier, J. (1990) Neurogranin: immunocytochemical localization of a brain-specific protein kinase C substrate. J. Neurosci. 10:3782-3792.

Reubi, J.-C., Perrin, M., Rivier, J. and Vale, W. (1982) High affinity binding sites for somatostatin to rat pituitary. Biochem. Biophys. Res. Commun. 105:1538-1545.

Reuter, H. (1983) Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature. 301:569-574.

Reuter, H. (1987) Modulation of ion channels by phosphorylation and second messengers. NIPS 2:168-171.

Reuter, H. (1991) Sodium-calcium exchange: Ins and outs of Ca^{2+} transport. Nature 349:567-568.

Reyl-Desmars, F., Baird, A. and Zeytin, F.N. (1985) GRF is a highly potent activator of adenylate cyclase in the normal human, bovine and rat pituitary: interaction with somatostatin. Biochem. Biophys. Res. Commun. 127:977-985.

Rhee, S.G., Suh, P.-G., Ryu, S.-H. and Lee, S.Y. (1989) Studies of inositol phospholipid-specific phospholipase C. Science 244:546-550.

Richardson, S.B. and Twente, S. (1988) Evidence that diminished pituitary responsivity to GHRF is secondary to intracellular GH pool depletion. Am. J. Physiol. (Endocrinol. Metab. 17) 254:E358-E364.

Rink, T.J. (1990) Receptor-mediated calcium entry. FEBS Lett. 268:381-385.

Risold, P.Y., Fellmann, D., Lenys, D. and Bugnon, C. (1989) Coexistence of acetylcholinesterase-, human growth hormone-releasing factor(1-37)-, α-melanotropin- and melanin-concentrating hormone-like immunoreactivities in neurons of the rat hypothalamus: a light and electron microscope study. Neurosci. Lett. 100:23-28.

Rivier, J., Spiess, J., Thorner, M. and Vale, W. (1982) Characterization of a growth hormone-releasing factor from a human pancreatic islet tumour. Nature 300:276-278.

Robberecht, W. and Denef, C. (1990) Evidence for a pertussis toxin-sensitive signalling pathway in the dual action of angiotensin II on growth hormone release in pituitary cell aggregates. Cell Biol. Int. Rep. 14:1001-1012.

Robison, G.A., Butcher, R.W. and Sutherland, E.W. (1971a) An introduction. In: Cyclic AMP, Academic Press, New York, p. 1-16. Robison, G.A.,

Robison, G.A., Butcher, R.W. and Sutherland, E.W. (1971b) Cyclic AMP and hormone action. In: Cyclic AMP, Academic Press, New York, p. 17-47.

Robison, G.A., Butcher, R.W. and Sutherland, E.W. (1971c) Chemistry of cyclic nucleoside phosphates and synthesis of analogs. In: Cyclic AMP, Academic Press, New York, p. 48-71.

Rodbell, M., Birnbaumer, L., Pohl, S.L. and Krans, H.M.J. (1971) The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanyl nucleotides in glucagon action. J. Biol. Chem. 246:1877-1882.

Rogawski, M.A. (1989) Aminopyridines enhance opening of calcium-activated potassium channels in GH_3 anterior pituitary cells. Mol. Pharmacol. 35:455-465.

Rossier, M.F., Bird, G.St.J. and Putney, J.W., Jr. (1991) Subcellular distribution of the calcium-storing inositol 1,4,5-trisphosphate-sensitive organelle in rat liver. Possible linkage to the plasma membrane through the actin microfilaments. Biochem. J. 274:643-650.

Rüegg, U.T. and Burgess, G.M. (1989) Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol. Sci. 10:218-220.

Sanchez-Bueno, A., Dixon, C.J., Woods, N.M., Cuthbertson, K.S.R. and Cobbold, P.H. (1990) The hepatocyte calcium oscillator. Adv. Second Messenger Phosphoprotein Res. 24:115-121.

Sandow, J., Arimura, A. and Schally, A.V. (1972) Stimulation of growth hormone release by anterior pituitary perfusion in the rat. Endocrinology 90:1315-1319.

Sara, V.R. and Hall, K. (1990) Insulin-like growth factors and their binding proteins. Physiol. Rev. 70:591-614.

Sato, M., Takahara, J., Fujioka, Y., Niimi, M. and Irino, S. (1988) Physiological role of growth hormone (GH)-releasing factor and somatostatin in the dynamics of GH secretion in adult male rat. Endocrinology 123:1928-1933.

Saunders, A., Terry, L.C., Audet, J., Brazeau, P. and Martin, J.B. (1976) Dynamic studies of growth hormone and prolactin secretion in the female rat. Neuroendocrinology 21:193-203.

Schettini, G., Cronin, M.J., Hewlett, E.L, Thorner, M.O. and MacLeod, R.M. (1984) Human pancreatic tumor growth hormone-releasing factor stimulates anterior pituitary adenylate cyclase activity, adenosine 3',5'-monophosphate accumulation, and growth hormone release in a calmodulin-dependent manner. Endocrinology 115:1308-1314.

Schlegel, W., Wuarin, F., Wollheim, C.B. and Zahnd, G.R. (1984) Somatostatin lowers the cytosolic free Ca^{2+} concentration in clonal rat pituitary cells (GH_3 cells). Cell Calcium 5:223-236.

Schmidt, K., Graier, W.F. and Kukovetz, W.R. (1990) EDRF-formation increases cGMP-levels in cultured endothelial cells. Adv. Second Messenger Phosphoprotein Res. 24:455-460.

Schofield, J.G. (1970) Prostaglandin E_1 and the release of growth hormone. Nature 228:179-180.

Schöfl, C., Sandow. J. and Knepel, W. (1987) GRF elevates cytosolic free calcium concentration in rat anterior pituitary cells. Am. J. Physiol. (Endocrinol. Metab. 16) 253:E591-E594.

Schrey, M.P. and Read, A.M. (1988) Cholinergic stimulation of phosphoinositide hydrolysis in rat anterior pituitary. Mol. Cell. Endocrinol. 57:107-113.

Schultz, G., Rosenthal, W., Hescheler, J. and Trautwein, W. (1990) Role of G proteins in calcium channel modulation. Annu. Rev. Physiol. 52:275-292.

Sefton, B.M. and Hunter, T. (1984) Tyrosine protein kinases. Adv. Second Messenger Phosphoprotein Res. 18:195-226.

Seifert, H., Perrin, M., Rivier, J. and Vale, W.W. (1985) Binding sites for growth hormone releasing factor on rat anterior pituitary cells. Nature 313:487-489.

Seifert, R., Schächtele, C., Rosenthal, W. and Schultz, G. (1988) Activation of protein kinase C by *cis*- and *trans*-fatty acids and its potentiation by diacylglycerol. Biochem. Biophys. Res. Commun. 154:20-26.

Shearman, M.S., Sekiguchi, K. and Nishizuka, Y. (1989) Modulation of ion channel activity: A key function of the protein kinase C enzyme family. Pharmacol. Rev. 41:211-237.

Shears, S.B. (1989) Metabolism of the inositol phosphates produced upon receptor activation. Biochem. J. 260:313-324.

Sheppard, M.S., Eatock, B.A. and Bala, R.M. (1987) Characteristics of phorbol ester stimulated growth hormone release: inhibition by insulin-like growth factor I, somatostatin, and low calcium medium and comparison with growth hormone releasing factor. Can. J. Physiol. Pharmacol. 65:2302-2307.

Sheppard, M.S., Moor, B.C. and Kraicer, J. (1985) Release of growth hormone (GH) from purified somatotrophs: interaction of GH-releasing factor and somatostatin and role of adenosine 3',5'-monophosphate. Endocrinology 117:2364-2370.

Sheppard, M.S., Spence, J.W. and Kraicer, J. (1979) Release of growth hormone from purified somatotrophs: role of adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate. Endocrinology 105:261-268.

Shimizu, T. and Wolfe, L.S. (1990) Arachidonic acid cascade and signal transduction. J. Neurochem. 55:1-15.

Shortman, K. (1968) The separation of different cell classes from lymphoid organs. II. The purification and analysis of lymphocyte population by equilibration density gradient centrifugation. Aust. J. Exp. Biol. Med. 46:375-396.

Siler, T.M., VandenBerg, G. and Yen, S.S.C. (1973) Inhibition of growth hormone release in humans by somatostatin. J. Clin. Endocrinol. Metab. 37:632-634.

Sims, S.M., Lussier, B.T. and Kraicer, J. (1991) Somatostatin activates an inwardly rectifying K^+ conductance in freshly dispersed rat somatotrophs. J. Physiol. (Lond.) in press.

Sirek, A. and Sirek, O.V. (1990) The metabolic effects of plasma growth hormone. Diab. Nutr. Metab. 3:157-169.

Smallwood, J.I., Gügi, B. and Rasmussen, H. (1988) Regulation of erythrocyte Ca^{2+} activity by protein kinase C. J. Biol. Chem. 263:2195-2202.

Smith, C.D., Uhing, R.J. and Snyderman, R. (1987) Nucleotide regulatory protein-mediated activation of phospholipase C in human polymorphonuclear leukocytes is disrupted by phorbol esters. J. Biol. Chem. 262:6121-6127.

Smith, M.A. and Vale, W.W. (1980) Superfusion of rat anterior pituitary cells attached to cytodex beads: validation of a technique. Endocrinology 107:1425-1431.

Snyder, G. and Hymer, W.C. (1975) A short method for the isolation of somatotrophs from the rat pituitary gland. Endocrinology 96:792-796.

Snyder, G.D., Yadagiri, P. and Falck, J.R. (1989) Effect of epoxyeicosatrienoic acids on growth hormone release from somatotrophs. Am. J. Physiol. (Endocrinol. Metab. 19) 256:E221-E226.

Solomon, T.E. (1987) Effect of somatostatin on exocrine pancreas. In: Somatostatin: Basic and Clinical Status, S. Reichlin, ed., Plenum Press, New York, p. 275-283.

Sommermeyer, H., Behl, B., Oberdisse, E. and Resch, K. (1989) Effects of nucleotides on the activity of phospholipase C in rabbit thymus lymphocytes. Differences in assays using endogenous [^{3}H]inositol-prelabeled membranes or exogenous [^{3}H]phosphatidylinositol 4,5-bisphosphate as substrate. J. Biol. Chem. 264:906-909.

Sortino, M., Canonico, P.L., Summers, S.T. and Cronin, M.J. (1987) Protein kinase C inhibits TRH-stimulated phosphoinositide hydrolysis in GH_3 cells. Eur. J. Pharmacol. 135:77-83.

Sortino, M.A., Evans, W.S., Speciale, C., Thorner, M.O., Scapagnini, U., MacLeod, R.M. and Canonico, P.L. (1988) Gonadotropin-releasing hormone-stimulated

phosphoinositide hydrolysis in the anterior pituitary. Modulation by protein kinase C but not by cyclic nucleotides. Neuroendocrinology 48:544-550.

Spada, A., Vallar, L. and Giannattasio, G. (1984) Presence of an adenylate cyclase dually regulated by somatostatin and human pancreatic growth hormone (GH)-releasing factor in GH-secreting cells. Endocrinology 115:1203-1209.

Spät, A., Bradford, P.G., McKinney, J.S., Rubin, R.P. and Putney, J.W.,Jr. (1986a) A saturable receptor for ^{32}P-inositol-1,4,5-trisphosphate in hepatocytes and neutrophils. Nature. 319:514-516.

Spät, A., Fabiato, A. and Rubin, R.P. (1986b) Binding of inositol trisphosphate by a liver microsomal fraction. Biochem. J. 233:929-932.

Spence, J.W., Sheppard, M.S. and Kraicer, J. (1980) Release of growth hormone from purified somatotrophs: interrelation between Ca^{++} and adenosine 3',5'-monophosphate. Endocrinology 106:764-769.

Spiess, J., Rivier, J. and Vale, W. (1983) Characterization of rat hypothalamic growth hormone-releasing factor. Nature 303:532-535.

Srikant, C.B. and Heisler, S. (1985) Relationship between receptor binding and biopotency of somatostatin-14 and somatostatin-28 in mouse pituitary tumor cells. Endocrinology 117:271-278.

Srikant, C.B. and Patel, Y.C. (1982) Characterization of pituitary membrane receptors for somatostatin in the rat. Endocrinology 110:2138-2144.

Stabel, S., Rodriguez-Pena, A., Young, S., Rozengurt, E. and Parker, P.J. (1987) Quantitation of protein kinase C by immunoblot--expression in different cell lines and response to phorbol esters. J. Cell Physiol. 130:111-117.

Stachura, M.E. (1976) Influence of synthetic somatostatin upon growth hormone release from perifused rat pituitaries. Endocrinology 99:678-683.

Stanfield, P.R. (1986) Voltage-dependent calcium channels of excitable membranes. Br. Med. Bull. 42:359-367.

Steiner, R.A., Stewart, J.K., Barber, J., Koerker, D., Goodner, C.J., Brown, A., Illner, P. and Gale, C.C. (1978) Somatostatin: A physiological role in the regulation of growth hormone secretion in the adolescent male baboon. Endocrinology 102:1587-1594.

Stojilkovic. S.S., Chang, J.P., Izumi, S., Tasaka, K. and Catt, K.J. (1991) Mechanisms of secretory responses to gonadotropin-releasing hormone and phorbol esters in cultured

pituitary cells. Participation of protein kinase C and extracellular calcium mobilization. J. Biol. Chem. 263:17301-17306.

Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306:67-69.

Stryer, L. and Bourne, H.R. (1986) G proteins: a family of signal transducers. Annu. Rev. Cell Biol. 2:391-419.

Summers, S.T., Canonico, P.L., MacLeod, R.M., Rogol, A.D. and Cronin, M.J. (1985) Phorbol esters affect pituitary growth hormone (GH) and prolactin release: the interaction with GH releasing factor, somatostatin and bromocriptine. Eur. J. Pharmacol. 111:371-376.

Summers, S.T. and Cronin, M.J. (1986) Phorbol esters enhance basal and stimulated adenylate cyclase activity in a pituitary cell line. Biochem. Biophys. Res. Commun. 135:276-281.

Supattapone, S., Worley, P.F., Baraban, J.M. and Snyder, S.H. (1988) Solubilization, purification, and characterization of an inositol trisphosphate receptor. J. Biol. Chem. 263:1530-1534.

Swandulla, D. and Lux, H.D. (1984) Changes in ionic conductances induced by cAMP in *Helix* neurons. Brain Res. 305:115-122.

Takahashi, Y., Kipnis, D.M. and Daughaday, W.H. (1968) Growth hormone secretion during sleep. J. Clin. Invest. 47:2079-2090.

Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characteriation of an active enzyme from bovine cerebellum. J. Biol. Chem. 252:7603-7609.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979a) Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. J. Biol. Chem. 254:3692-3695.

Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979b) Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. Biochem. Biophys. Res. Commun. 91:1218-1224.

Tannenbaum, G.S., Eikelboom, R. and Ling, N. (1983a) Human pancreas GH-releasing factor analog restores high-amplitude GH pulses in CNS lesion-induced GH deficiency. Endocrinology 113:1173-1175.

Tannenbaum, G.S., Guyda, H.J. and Posner, B.I. (1983b) Insulin-like growth factors: a role in growth hormone negative feedback and body weight regulation via brain. Science 220:77-79.

Tannenbaum, G.S. and Ling, N. (1984) The interrelationship of growth hormone (GH)-releasing factor and somatostatin in generation of the ultradian rhythm of GH secretion. Endocrinology 115:1952-1957.

Tannenbaum, G.S. and Martin, J.B. (1976) Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. Endocrinology 98:562-570.

Tannenbaum, G.S., Painson, J.-C., Lapointe, M., Gurd, W. and McCarthy, G.F. (1990) Interplay of somatostatin and growth hormone-releasing hormone in genesis of episodic growth hormone secretion. Metabolism 39 [Suppl. 2]:35-39.

Taylor, S.J., Smith, J.A. and Exton, J.H. (1990) Purification from bovine liver membranes of a guanine nucleotide-dependent activator of phosphoinositide-specific phospholipase C. Immunologic identification as a novel G-protein α subunit. J. Biol. Chem. 265:17150-17156.

Taylor, S.S., Bubis, J., Toner-Webb, J., Saraswat, L.D., First, E.A., Buechler, J.A., Knighton, D.R. and Sowadski, J. (1988) cAMP-dependent protein kinase: prototype for a family of enzymes. FASEB J. 2:2677-2685.

Thomas, C.P., Kester, M. and Dunn, M.J. (1991) A pertussis toxin-sensitive GTP-binding protein couples endothelin to phospholipase C in rat mesangial cells. Am. J. Physiol. (Renal Fluid Electrolyte Physiol. 29) 260:F347-F352.

Thorner, M.O., Vance, M.L., Hartman, M.L., Holl, R.W., Evans, W.S., Veldhuis, J.D., Van Cauter, E., Copinschi, G. and Bowers, C.Y. (1990) Physiological role of somatostatin on growth hormone regulation in humans. Metabolism 39 [Suppl. 2]:40-42.

Tohkin, M., Yagami, T., Katada, T. and Matsubara, T. (1990) Possible interaction of α_1-adrenergic receptor with pertussis-toxin-sensitive guanine-nucleotide-binding regulatory proteins (G proteins) responsible for phospholipase C activation in rat liver plasma membranes. Eur. J. Biochem. 194:81-87.

Tremblay, J., Gerzer, R. and Hamet, P. (1988) Cyclic GMP and cell function. Adv. Second Messenger Phosphoprotein Res. 22:319-383.

Triggle, D.J. (1990) Calcium, calcium channels, and calcium channel antagonists. Can. J. Physiol. Pharmacol. 68:1474-1481.

Tsien, R.W. and Tsien, R.Y. (1990) Calcium channels, stores, and oscillations. Annu. Rev. Cell Biol. 6:715-760.

Turgeon, J.L., Ashcroft, S.J.H., Waring, D.W., Milewski, M.A. and Walsh, D.A. (1984) Characteristics of the adenohypophyseal Ca^{2+}-phospholipid-dependent protein kinase. Mol. Cell. Endocrinol. 34:107-112.

Ui, M. and Katada, T. (1990) Bacterial toxins as probe for receptor-G_i coupling. Adv. Second Messenger Phosphoprotein Res. 24:63-69.

Van Rooijen, L.A.A., Fisher, S.K. and Agranoff, B.W. (1985) Biochemical aspects of stimulated turnover of inositol lipids in the nervous system. In: Phospholipids in the Nervous System, Vol. 2: Physiological Roles, L.A. Horrocks, J.N. Kanfer and G. Porcellati eds., Raven Press, New York, p. 31-38.

Veliçelebi, G., Patthi, S., Provow, S. and Akong, M. (1986) Covalent cross-linking of growth-hormone releasing factor to pituitary receptors. Endocrinology 118:1278-1283.

Veliçelebi, G., Santacroce, T.M. and Harpold, M.M. (1985) Specific binding of synthetic human pancreatic growth hormone releasing factor (1-40-OH) to bovine anterior pituitaries. Biochem. Biophys. Res. Commun. 126:33-39.

Verghese, M.W., Smith, C.D. and Snyderman, R. (1985) Potential role for a guanine nucleotide regulatory protein in chemoattractant receptor mediated polyphosphoinositide metabolism, Ca^{++} mobilization and cellular responses by leukocytes. Biochem. Biophys. Res. Commun. 127:450-457.

Vincent, S.R., McIntosh, C.H.S., Buchan, A.M.J. and Brown, J.C. (1985) Central somatostatin systems revealed with monoclonal antibodies. J. Comp. Neur. 238:169-186.

Volpe, P., Krause, K.-H., Hashimoto, S., Zorato, F., Pozzan, T., Meldolesi, J. and Lew, D.P. (1988) "Calciosome," a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store of nonmuscle cells? Proc. Natl. Acad. Sci. U. S. A. 85:1091-1095.

Wahl, M.I., Nishibe, S., Suh, P.-G., Rhee, S.G. and Carpenter, G. (1989) Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-II independently of receptor internalization and extracellular calcium. Proc. Natl. Acad. Sci. U. S. A. 86:1568-1572.

Wang, P., Nishihata, J., Takabori, E., Yamamoto, K., Toyoshima, S. and Osawa, T. (1989) Purification and partial amino acid sequences of a phospholipase C-associated GTP-binding protein from calf thymocytes. J. Biochem. 105:461-466.

Wang, T. and Chan, Y.L. (1991) The role of phosphoinositide turnover in mediating the biphasic effect of angiotensin II on renal tubular transport. J. Pharmacol. Exp. Ther. 256:309-317.

Watson, S.P., McConnell, R.T. and Lapetina, E.G. (1984) The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin. J. Biol. Chem. 259:13199-13203.

Wehrenberg, W.B., Brazeau, P., Luben, R., Böhlen, P. and Guillemin, R. (1982a) Inhibition of the pulsatile secretion of growth hormone by monoclonal antibodies to the hypothalamic growth hormone releasing factor (GRF). Endocrinology 111:2147-2148.

Wehrenberg, W.B., Ling, N., Brazeau, P., Esch, F., Böhlen, P., Baird, A., Ying, S. and Guillemin, R. (1982b) Somatocrinin, growth hormone releasing factor, stimulates secretion of growth hormone in anesthetized rats. Biochem. Biophys. Res. Commun. 109:382-387.

Weiner, R.I. and Ganong, W.F. (1978) Role of brain monoamines and histamine in regulation of anterior pituitary secretion. Physiol. Rev. 58:905-976.

Weinstein, I.B. (1990) The role of protein kinase C in growth control and the concept of carcinogenesis as a progressive disorder in signal transduction. Adv. Second Messenger Phosphoprotein Res. 24:307-316.

White, R.E., Schonbrunn, A. and Armstrong, D.A (1991) Somatostatin stimulates Ca^{2+}-activated K^+ channels through protein dephosphorylation. Nature 351:570-573.

Wilber, J.F., Nagel, T. and White, W.F. (1971) Hypothalamic growth hormone-releasing activity (GRA): characterization by the *in vitro* rat pituitary and radioimmunoassay. Endocrinology 89:1419-1424.

Winer, L.M., Shaw, M.A. and Baumann, G. (1990) Basal plasma growth hormone levels in man: new evidence for rhythmicity of growth hormone secretion. J. Clin. Endocrinol. Metab. 70:1678-1686.

Woodgett, J.R., Gould, K.L. and Hunter, T. (1986) Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. Eur. J. Biochem. 161:177-184.

Wreggett, K.A. and Irvine, R.F. (1989) Automated isocratic high-performance liquid chromatography of inositol phosphate isomers. Biochem. J. 262:997-1000.

Yajima, Y., Akita, Y. and Saito, T. (1986) Pertussis toxin blocks the inhibitory effects of somatostatin on cAMP-dependent vasoactive intestinal peptide and cAMP-independent thyrotropin releasing hormone-stimulated prolactin secretion of GH_3 cells. J. Biol. Chem. 261:2684-2689.

Yamada, T. (1987) Gut somatostatin. In: Somatostatin: Basic and Clinical Status, S. Reichlin, ed., Plenum Press, New York, p. 221-228.

Young, P.W., Bicknell, R.J. and Schofield, J.G. (1979) Acetylcholine stimulates growth hormone secretion, phosphatidyl inositol labelling, ^{45}Ca efflux and cyclic GMP accumulation in bovine anterior pituitary glands. J. Endocrinol. 80:203-213.

Zadik, Z., Chalew, S.A., McCarter, R.J., Jr., Meistas, M. and Kowarski, A.A. (1985) The influence of age on the 24-hour integrated concentration of growth hormone in normal individuals. J. Clin. Endocrinol. Metab 60:513-516.

Zatz, M., Mahan, L.C. and Reisine, T. (1987) Translocation of protein kinase C in anterior pituitary tumor cells. J. Neurochem. 48:106-110.

Zimmermann, H. (1990) Neurotransmitter release. FEBS Lett. 268:394-399.

Zysk, J.R., Cronin, M.J., Anderson, J.M. and Thorner, M.O. (1986) Cross-linking of a growth hormone releasing factor-binding protein in anterior pituitary cells. J. Biol. Chem. 261:16781-16784.