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Development of a Hypothalamo-Pituitary Perifusion System to Study the Effect of Gonadal Steroids on Growth Hormone Regulation

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Hazem Abd Al-Rhman Hassan

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Robert A. Merkel
Major professor
Robert A. Merkel

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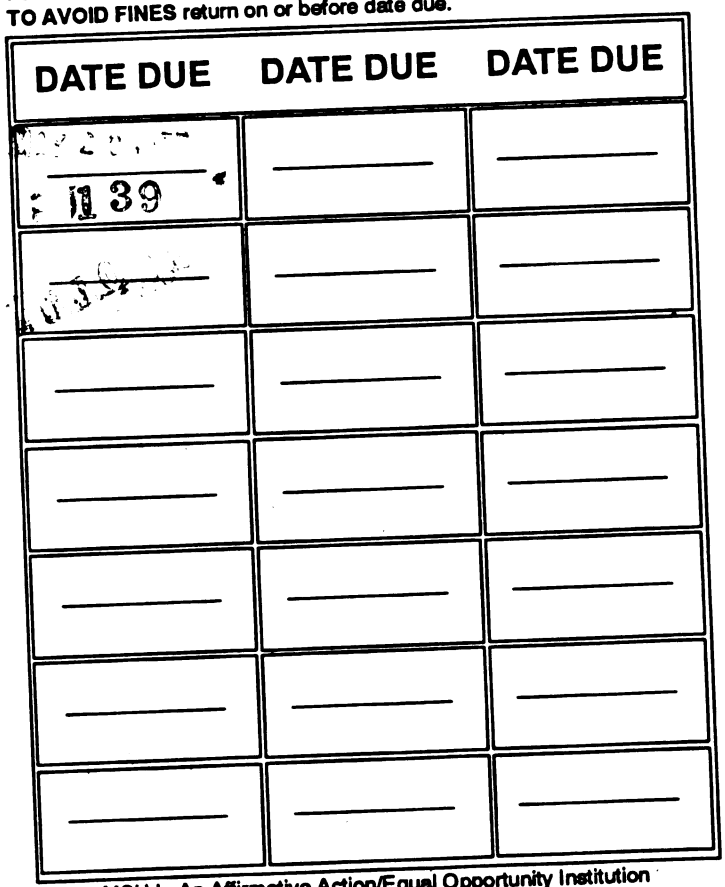

DEVELOPMENT OF A HYPOTHALAMO-PITUITARY PERIFUSION SYSTEM TO STUDY THE EFFECT OF GONADAL STEROIDS ON GROWTH HORMONE REGULATION

BY

Hazem Abd Al-Rhman Hassan

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ABSTRACT

An in vitro perifusion system was developed to examine effects of gonadal steroids on growth hormone (GH) release. Anterior pituitary (AP) cells in perifusion chambers (CH) had 4.8- and 3.3-fold greater basal and GH-releasing factor (GRF)-stimulated GH release than static cultures. When CH containing hypothalamic (HYP) slices were connected to AP-CH, release of GH mimicked the in vivo pulsatile pattern. All tissues responded to KCl challenge after 120 h in cultures thus indicating their viability.

Estradiol-17β (E_2, 10^{-11}M), testosterone (T, 10^{-9}M), dihydrotestosterone (DHT, 10^{-11}M) and 3α-androstandiol (3α-diol, 10^{-12}M) were applied to either AP-CH or HYP- plus AP-CH for 24 h. E_2 increased GH release when applied to the AP- plus HYP-CH, but not the AP-CH alone, via increased GRF and reduced somatostatin (SRIF) from the HYP. Both DHT and 3α-diol increased GH release from the AP cells and at the HYP; GRF was increased more than SRIF.

Perifusion of 10^{-9}M T to AP cells alone at constant or pulsatile rate had no effect on GH release. Constant rate T induced a similar increase in both GRF and SRIF from the HYP, thus no change in GH release from AP cells was observed. However, pulsatile T increased GRF more than SRIF, and consequently GH release was increased.

Inhibition of T aromatization to E_2 using the aromatase inhibitor, LY43578, had no effect on the AP cells but at the

HYP increased SRIF more than GRF. A complementary effect of DHT plus E_2 resulted in greater GH release from the AP cells, and increased GRF and decreased SRIF release from the HYP.

These studies indicated that the perifusion system was responsive to physiological stimuli and provides a useful tool to study the HYP-AP axis on the regulation of GH release. These data clarify the mechanism whereby gonadal steroids increase GH release from the anterior pituitary.

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REVIEW OF LITERATURE

Introduction. Growth is considered the most obvious long term process in animal production (Bauman et al., 1982). The ultimate goal of the beef industry is to achieve maximum growth in cattle and to supply high quality food for humans. Several metabolic and endocrine manipulations have been used to improve the production of meat animals with the objective of attaining more protein and less fat deposition in their carcasses (Gopinath and Kitts, 1984).

Growth. Growth is defined as the increase in the mass of an individual or the coordinated increase of all animal tissues over time (Beitz, 1985). Growth occurs as a result of organized processes of hyperplasia and hypertrophy when the balance between anabolic and catabolic processes in a tissue favors net anabolism (Weekes, 1986). The growth process involves interactions between nutrients, genotype, environment and the influence of a number of hormones on nutrient availability to cells and cell division (Spencer, 1986). Adequate nutrients are needed to increase body mass as well as to meet basal energy requirements (Gluckman, 1986).

The endocrine system is the mechanism whereby these interactions are modulated and cellular replication is regulated (Baile et al., 1986). Postnatal growth is directly

controlled by a multitude of hormones and growth factors such as insulin, glucagon, thyroid hormones, gonadal steroids, prolactin and insulin-like growth factors, but most notably growth hormone (GH; Oddy and Lindsay, 1986). Directly or indirectly, GH stimulates anabolic processes such as cell division, skeletal growth and protein synthesis (growth promoting activity), while increasing the oxidation of fat (lipolytic activity) and inhibiting the transport of glucose into body tissues (diabetogenic activity, Hart and Johnsson, 1986).

Consequently, a variety of growth stimulants, acting directly or by stimulating GH, have been widely used to improve growth efficiency. These include anabolic steroids (Tucker and Merkel, 1987), ß-adrenergic agonist (Bergen and Merkel, 1991), growth hormone (Moseley et al., 1992) and growth hormone modifiers (Frohman, 1991).

As alternatives to exogenous hormone administration, several other approaches have been undertaken to improve growth efficiency. These include, immunological approaches to enhance the rate of secretion of GH (Trout and Schanbacher, 1990), enhancement of the physiological action of GH with monoclonal antibodies with specific antigenic determinants or idiotypic antibodies which mimic the effect of GH (Gardner et al., 1990), enhance productive function by changing the genome of meat animals, including transgenic animals (Pinkert, 1991), and regulation of oncogenes and anti-oncogenes as regulators

of cell growth (Novakofski, 1991). Implants containing estrogenic or androgenic compounds improve growth efficiency and alter metabolism towards increased protein and decreased fat production (Tucker and Merkel, 1987). However, the mechanism/site of action has not been fully elucidated. It is possible that estrogenic and the androgenic compounds cause slight changes in total endogenous hormone pattern are sufficient to account for their growth promoting properties (Buttery and Sinnett-Smith, 1984).

Sexual Dimorphism of Growth and Growth Hormone. Males of most vertebrate species, including humans (Knigge et al., 1990), laboratory rodents (Wehrenberg et al., 1985) and ruminant animals (Irvine and Trenkle, 1971) are larger than their female counterparts. Gonadal steroids, which constitute a fundamental difference between sexes, mediate their effects on growth through regulating GH synthesis and release (Cronin and Rogol, 1984; Jansson et al., 1985; Wehrenberg et al., 1985). In cattle, intact males compared with females have consistently higher plasma GH concentrations at all ages (Irvin and Trenkle, 1971; Reynaert et al., 1976); however, the difference becomes most pronounced near puberty. Also, the difference in pituitary GH content (Birge et al., 1967) and release (McLeod et al., 1969; Yamamoto et al., 1970; Burek and Frohman, 1970) occurs around time of puberty, when concentrations of GH in males exceed those in females.

Gonadally intact males grow faster than females or castrated males (Dubreuil et al., 1989). The endocrine basis for this difference in growth rate has logically been attributed to testosterone (Gortsema et al., 1974). However, the mechanism by which testosterone increases growth rate has not been fully explained. One possible explanation for the growth promoting effect of testosterone may be through regulation of GH production (Davis et al., 1977). Increased testosterone concentrations are associated with increased circulating GH concentrations in bulls (Convey et al., 1971). In cattle, plasma GH secretory spikes of bulls are greater in magnitude than those in steers (Anfinson et al., 1975). Also, anterior pituitaries of males are capable of secreting more GH than those of females (Evans et al., 1985). These differences have been attributed to the secretory capacity of individual somatotropes rather than differences in their number (Hoefler and Frawley, 1986). Moreover, previous data showed that rate of GH synthesis by adult male pituitaries is greater than that of adult females (Burek and Frohman, 1970). Additionally, somatotropes from male pituitaries have greater responsiveness and(or) sensitivity to GH-releasing factor (GRF) stimulation than those of females (Evans et al., 1985; Wehrenberg et al., 1985; Krieg et al., 1986). Also, GRF induced greater cellular cAMP accumulation and GH release in males than in females (Cronin and Rogol, 1984; Simard et al., 1986). The response to GRF in males vs females in vivo is not clear; however, bulls

tend to respond more to GRF than heifers or steers (Plouzek and Trenkle, 1991).

Exogenous estrogenic compounds have been utilized for some time to improve growth rate and meat production in cattle and sheep (Gopinath and Kitts, 1984); however, the underlying mechanism has not been clarified. It has been suggested that estrogenic compounds may exert their effect on growth via regulation of GH secretion (Enright et al., 1990). Treatment with estrogenic compounds increases weight of the anterior pituitary gland (Elias and Weiner, 1987) and increases number of acidophilic cells within the pituitary (Clegg and Cole, 1954). Administration of estradiol-17β increases plasma GH concentrations (Breier et al., 1988).

Estrogen apparently influences GH secretion during adult life by increasing basal GH concentration and GH pulse frequency, and by decreasing pulse amplitudes (Borger et al., 1973; Trenkle, 1976; Ohlson et al., 1977; Donaldson et al., 1981; Eden et al., 1987). It has been suggested that estrogen exerts a stimulatory effect on GH production via direct action on the rat pituitary gland (Jansson et al., 1983; Simard et al., 1986). Trenkle (1981) suggested that the increase in GH may be due to an increase in secretion rate not to a decrease in clearance rate. The estrogen-induced increase in GH has been reported to be independent of circulating concentrations of the insulin-like growth factors (IGF's; Weidemann et al., 1976).

Sexual Dimorphism of GH Secretory Pattern. Sex specific patterns in circulating concentrations of GH have been clearly demonstrated in postpubertal rats (Eden et al., 1987) and mice (MacLeod et al., 1991). The most striking gender difference is the very regular pattern of GH pulses observed in males rats (Ohlsson et al., 1987). The adult male rat exhibits a regular pulsatile pattern of GH secretion with high amplitude pulses occurring at intervals of 3 to 3.5 h separated by low or undetectable baseline concentrations (Hertz et al., 1989). In contrast, mature female rats exhibit irregular, low amplitude GH pulses, and elevated baseline concentrations (Eden et al., 1987). This sexual dimorphism in rats becomes evident with the onset of puberty (Evans et al., 1985).

The pattern of GH in meat animals is not as well characterized as that in rats. However, the pattern of GH secretion in rams and bulls exhibits discrete episodes of release, while after castration the frequency of episodes increases and the amplitude decreases (Davis et al., 1977 and Anfinson et al., 1975). No difference was observed in the GH secretory pattern between prepubertal male and female calves (Anfinson et al., 1975). However, postpubertal heifers have fewer episodes of lower amplitude of GH release than prepubertal heifers (Zinn et al., 1986). Bulls have greater magnitude of GH release than steers or prepubertal males (Anfinson, 1975). However, Vasilatos and Wangsness (1981)

reported pulsatile secretion of GH in lactating cows with greater amplitude and higher baseline in early lactation than in late lactation when amplitude was decreased and frequency was increased. The baseline of GH secretion was greater in these cows than those in the bulls or steers reported by Anfinson et al. (1975).

Sexual Dimorphism of Carcass Composition. Gonadally intact males exhibit greater weight gain, feed efficiency, lean tissue growth and deposit less fat than females or castrates (Buttery and Dawson, 1990). In addition, intact Frisian bulls have much smaller adipocytes at any given age than steers (Vernon, 1986). Also, ovariectomized heifers or those immunized against estradiol-17β had reduced numbers of adipocytes compared with gonadally intact heifers (St.John et al., 1987).

Reducing fat in meat products has become a great challenge in the last decade for the U.S. beef industry (Bergen and Merkel, 1992). Estrogenic and androgenic compounds are used extensively in the U.S. to improve lean gain in ruminants (Buttery and Dawson, 1990). The mechanism of action of gonadal steroids on modifying carcass composition is not well established; however, evidence suggests that both estrogens and androgens act via modulating the pattern of GH secretion (Plouzek and Trenkle, 1991), which in turn greatly

affects growth and carcass composition (Gopinath and Kitts, 1984).

Growth Hormone Secretion and Regulation

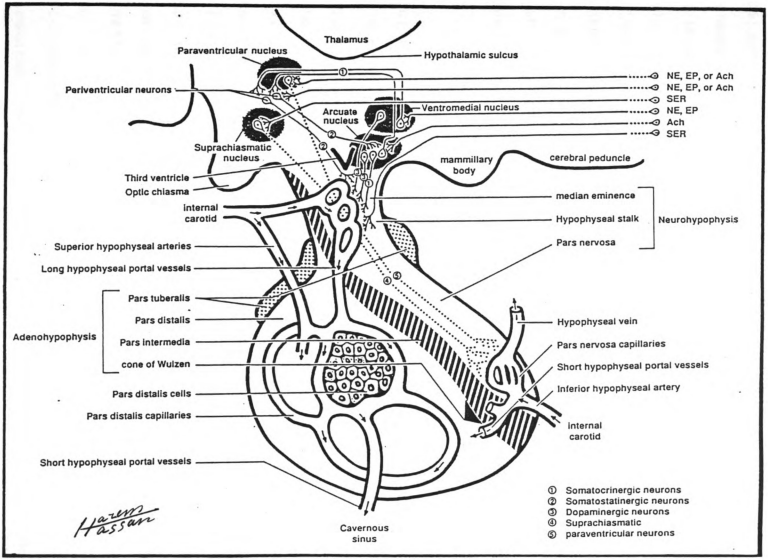
Growth Hormone Production and Clearance. Growth hormone (GH) is the most abundant hormone of all active principles of the human anterior pituitary (Murad and Haynes, 1985). It is secreted by specific anterior pituitary cells, the somatotropes (Daughaday et al., 1975). Somatotropic cells predominate in the lateral portion of the anterior pituitary (Dellmann, 1981). Within the somatotrope, synthesis of GH takes place in the endoplasmic reticulum and the nascent hormone is packed into secretory granules within the Golgi apparatus (Phillips, 1987). Somatotropic granules, which average 300 to 400 nm in diameter, have affinity for acidophilic stains and stain immunohistochemically for GH (Banks, 1981). There are several variant forms of GH arising from: different amino acid sequences, post-translational modifications or from fragmentation of the GH molecule (Lewis et al., 1980). The most abundant form of human GH has a molecular weight of 22 k Da and is composed of 191 amino acids (Niall et al., 1971). Bovine GH also has 191 amino acids. It is a globular protein with two disulfide bridges, one linking amino acids 53 and 165 which forms a large loop and the other near the carboxy terminus linking amino acids 182 and 189 to

form a small loop (Wallis, 1978). A smaller variant of GH (20 k Da) constitutes 10 to 15% of the total GH concentration in the anterior pituitary (Lewis et al., 1980). A third form of GH (45 k Da) is formed by dimerization of two 22 k Da-peptides which are linked by inter-chain disulfide bonds (Lewis et al., 1978). The later form is less abundant (1%) in the pituitary, yet comprises 30% of the hormone in the plasma (Lewis et al., 1987). The isoelectric point of GH is pH 4.9, and it is stable in pH 7.0 solution at 100°C (Li, 1987).

In nonlactating Holstein cows, the GH secretion rate is 19.1 mg/d, and the serum half-life ($t_{1/2}$) of bovine GH is 22.5 min (Yousef et al.,1969). Gopinath and Kitts (1984) estimated the metabolic clearance rate of GH to be 74.5 ml^{-1}. kg body $weight^{-1}$. h^{-1} and a secretion rate of .91 ug. kg body $weight^{-1}$. h^{-1} in steers. Trenkle (1976) reported a secretion rate of 2.6 ug. kg body $weight^{-1}$. h^{-1} in calves.

Regulation of Growth Hormone Secretion. Regulation of adenohypophyseal function is mediated mainly by hypothalamic regulatory peptides (Cryer and Daughaday, 1977). These peptides are transported from the hypothalamus to the anterior pituitary gland by the hypothalamo-hypophyseal portal system (Figure I), which originates from a capillary network in the median eminence (Murad and Haynes, 1985). Also, secretion of hypothalamic peptides is under the influence of biogenic amines, which are transmitted from extra-hypothalamic regions

Figure I. Schematic illustration of the bovine hypothalamo-pituitary axis.


Thalamus
Paraventricular nucleus
Hypothalamic sulcus
Periventricular neurons
NE, EP, or Ach
NE, EP, or Ach
SER
Ventromedial nucleus
Arcuate nucleus
NE, EP
Ach
SER
Suprachiasmatic nucleus
mammillary body
cerebral peduncle
Third ventricle
Optic chiasma
median eminence
Internal carotid
Hypophyseal stalk
Neurohypophysis
Pars nervosa
Superior hypophyseal arteries
Long hypophyseal portal vessels
Pars tuberalis
Pars distalis
Adenohypophysis
Pars intermedia
cone of Wulzen
Hypophyseal vein
Pars nervosa capillaries
Short hypophyseal portal vessels
Inferior hypophyseal artery
Pars distalis cells
Pars distalis capillaries
Internal carotid
Short hypophyseal portal vessels
Cavernous sinus
① Somatocrinergic neurons
② Somatostatinergic neurons
③ Dopaminergic neurons
④ Suprachiasmatic
⑤ paraventricular neurons
Hanem Hassan

to act at the hypothalamus (Baile and Della-Fera, 1983). Accumulated evidence indicates that GH secretion is under the stimulatory and inhibitory influences of hypothalamic (Guillemin et al., 1984) and extra-hypothalamic factors (Casanueva et al., 1986). In addition, GH is regulated by feedback autoregulation (i.e., GH itself, Kraicer et al., 1988 or by pituitary derived IGF's, Fagin et al., 1988) as well as other humoral factors including sex steroids (Hassan et al., 1992).

Hypophysiotropic Hormones. The secretion of GH is pulsatile in all species studied so far, and is believed to be controlled primarily by a complex interplay of the hypothalamic neuropeptides GRF and somatotropin release inhibiting factor or somatostatin (SRIF, Jansson et al., 1987). Concentrations of SRIF at or near its baseline sets the timing of the episodic bursts of GH secretion (Kraicer et al., 1986), while the magnitude of the bursts of GH is set by the amount of GRF impinging upon the somatotropes, before and during the return of SRIF to its baseline (Kraicer et al., 1988).

Growth Hormone-Releasing Factor. Human pancreatic GRF was isolated and characterized as a 44- and as a 40-amino acid peptide by Guillemin et al. (1982) and Rivier et al. (1982), respectively. Bovine GRF was isolated by Esch (1983) and

identified as a 44 amino acid peptide that differs from the human forms in five amino acid residues. Peptidergic neurons synthesize GRF in their cell bodies in the hypothalamus and it is transported along axons to nerve terminals in the median eminence where it is secreted into the hypophyseal portal circulation (Meister et al., 1987). The majority of the GRF-immunoreactive cell bodies are found in the arcuate nucleus and medial perifornical region of the lateral hypothalamus (Merchenthaler et al., 1984). The nerve fibers form a fan-like projection to the median eminence where a dense accumulation of GRF-containing terminals are found (Martin and Reichlin, 1987). A schematic representation in Figure I shows the distribution of GRF neurons in the hypothalamus.

Human pancreatic GRF is highly specific for release of GH from dispersed rat anterior pituitary cells (Brazeau et al., 1982). In addition, in vivo studies show that plasma concentrations of GH increased proportionally to the log dose of GRF and reached a peak in 5 to 10 min with iv injection and in 15 min with sc injection. The peak with sc injection was 37% of that for iv injection (Johke et al., 1984). Virtually full biological activity resides in the first 29 residues of GRF (Rivier et al., 1982) and the 1-29-amidated structure is the most extensively used form for structure-activity studies. Secondary structure studies indicate that GRF(1-29)-NH_2 is amphiphilic and likely has an α-helical conformation both in solution and in the receptor-bound state (Velicebili et al.,

1986). Several GRF-like peptides elicit identical dose-response curves for GH release (Guillemin et al., 1984).

It is generally agreed that GRF is the major physiological secretagogue of GH (Brazeau et al., 1982b) and that it stimulates GH secretion, synthesis, transcription of the GH gene and proliferation of somatotropes (Billestrup et al., 1987). Treatment with GRF increases intracellular concentrations of Ca^{2+} (Holl et al., 1988). In cattle most of this effect is on the amplitude of Ca^{2+} spikes with a lesser effect on spike frequency (Cuttler et al., 1992). Ohlsson and Lindstrom (1990) found that increased Ca^{2+} concentration is directly involved in GH exocytosis. In addition, Cuttler et al. (1986) reported increased cyclic adenosine monophosphate (cAMP) synthesis and(or) stability which was correlated with increased GH-mRNA. Tanner et al. (1988) demonstrated a stimulatory effect for the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA) which they suggested was the mechanism whereby GRF stimulated GH synthesis and secretion from bovine somatotropes. Moreover, activation of voltage sensitive Ca^{2+} channels by cAMP-dependent protein kinase (PKA) has been reported (Lussier et al., 1991a). These authors suggested a role for PKA in GRF-induced GH synthesis and release.

Somatotropin Release-Inhibiting Factor (SRIF). Brazeau et al. (1973) isolated and purified the 14-amino acid peptide

form of SRIF from ovine hypothalamic tissue. Several forms of SRIF had been detected in the brain and other tissues. The two major forms are the 14- and 28- amino acids containing peptides (Benoit et al., 1982). Patel (1987) reported that SRIF-14 is less potent than SRIF-28 in its effect on GH-regulation. In the pituitary, SRIF may be categorized as a neurohormone, whereas in the nervous system it may be described as a neurotransmitter or neuromodulator (Reichlin, 1983).

Lussier et al. (1991b) reported that SRIF inhibits some common cellular events, e.g., reduced intracellular Ca^{2+} , in the pituitary as well as other endocrine organs such as the pancreas. Decreased intracellular Ca^{2+} inhibits release of peptide hormones like GH from the pituitary and insulin from the pancreas (KoeKer et al., 1974). In the pituitary, SRIF inhibits basal and GRF-stimulated GH release (Wehrenberg et al., 1982). It also reduces the amplitude and frequency of calcium spikes (Holl et al., 1987) in somatotropes, which is associated with inhibition of GH secretion. In addition, SRIF decreases Ca^{2+} conductance (Lussier et al., 1991b) and reduces cellular cAMP (Borgeat et al., 1974). The latter effect is correlated with inhibition of release of stored and newly synthesized GH (Sheppard et al., 1979). Tanner et al. (1988) concluded that SRIF does not interfere with GH synthesis since GH-mRNA abundance did not change with SRIF concentration. In addition, SRIF enhances K^{+} conductance in rat pituitary cells

(Koch and Schonbrunn, 1988). This event accelerates the rate of repolarization and shortens the period of refractoriness after stimulation. In vitro studies have shown that SRIF or its analog exert an antiproliferative effect on some cultured cell lines, e.g., thymocytes (Mascardo et al., 1984), breast cancer cells (Setyono-Han et al., 1987), human hepatoma cells (Chou et al., 1987), spleen lymphocytes (Pawlikowski et al., 1985), and a rat pancreatic acinar tumor cell line (Viguerie et al., 1989). Also, SRIF reduces the proliferative action of epidermal growth factor (Mascardo and Sherline, 1982). Martin and Reichlin (1987) suggest that SRIF may be involved in short-loop feedback control of GH secretion, since incubation of hypothalamic slices with GH (Berelowitz et al., 1981a) or IGF-I (Berelowitz et al., 1981b) elevated SRIF secretion.

Sex Steroids and Hypothalamic Function. Experimental evidence has shown the hypothalamus to be an important site for gonadal steroid action in modulating GH regulatory peptides. Immunohistochemical studies have demonstrated that estradiol acts directly on hypothalamic GRF neurons (Shirasu et al., 1990); however, few SRIF cells in the periventricular nucleus were found to have nuclear concentrations of radioactive estradiol. In addition, immunoneutralization studies have shown the importance of GRF in maintaining elevated basal GH concentrations in females, and suggested that the pattern of SRIF secretion into the hypophyseal portal

circulation to be more or less continuous rather than cyclic (Painson and Tannenbaum, 1991). Also, these authors presented evidence for the importance of GRF in the peak amplitude, and of SRIF in maintaining the nadir concentrations in males.

Autoradiographic studies of the periventricular nucleus showed that testosterone stimulated SRIF gene expression, however SRIF-mRNA abundance was lower in female than in male rats (Chowen-breed et al., 1989). Additionally, Werner et al. (1988) reported a reduction in SRIF-mRNA abundance in both male and female rats as a result of gonadectomy. Painson et al. (1992) suggested that the mechanism of estradiol action at the hypothalamic level is via its control on the mode of hypothalamic SRIF signaling to the pituitary. Zeitler et al. (1990) reported stimulation of GRF-mRNA expression by testosterone, but estradiol had no effect on the message. Argenta et al. (1991) found that male rats had greater abundance of SRIF-mRNA than females at all ages studied. The authors reported that developmental changes in GRF-mRNA levels were similar for both sexes, and both had a slight increase in GRF over the course of maturation.

Methods Used to Study Regulation of GH Secretion. A variety of procedures has been employed to study regulation of GH secretion in vivo. It is well established that hormone secretion from the anterior pituitary is regulated by neural elements of the hypothalamus (Guillemin, 1978). The mechanism

by which the two hypothalamic peptides, GRF and SRIF, contribute to the pulsatile pattern of GH secretion has been studied extensively. The individual neuropeptides have been examined by intravenous injection (Brazeau et al., 1974; Lanes et al., 1989); and by elimination of their effects using passive immunization with specific antisera (Spencer and Gassen, 1983). Hypothalamic influence can be removed by isolating the pituitary gland from the hypothalamus (Jansson et al., 1983), by lesions of some areas in the hypothalamus either electrically (Martin, 1974), surgically (Krieg et al., 1988), or chemically via monosodium glutamate (Maiter et al., 1991). In all cases, a reduction in circulating GH concentrations was observed.

Direct evidence of GRF and SRIF pulsatile secretion has been reported for rats (Plotsky and Vale, 1985) and sheep (Frohman et al., 1990). The latter authors studied the GRF/SRIF/GH axis but they might overestimated GRF and SRIF concentrations since the retrograde flow from the pituitary to the hypothalamus carrying GRF and SRIF back, was included in the original concentration released from the hypothalamus. These observations illustrate the formidable technical difficulties associated with collection and measurement of GRF and SRIF from the hypothalamic-pituitary portal circulation (Frohman, 1991).

In vitro methods to study the regulation of GH secretion have utilized either primary explants or dispersed cell

suspensions (Freshney, 1987). Primary explant implies that the tissue architecture is retained, as slices or fragments are cultured on either a grid or gel to maintain their three-dimensional morphology (Horng and McLimans, 1975). Dispersed cells are cultured either as an adherent monolayer on a solid substrate or as a suspension in culture media (Folkman and Moscona, 1978).

Either primary explants or dispersed cells can be utilized in a static monolayer culture or in a perifusion system to study GH secretion. Cells in static cultures, adhere to the culture substratum and form stratified sheets (Watt et al., 1988). In static culture, cells are under restricted hormonal and nutritional milieu and this creates an environment which favors the spreading, migration and proliferation of unspecialized cells rather than expression of differentiated functions (Freshney, 1987). Cells in a perifusion system have greater capacity to release GH because they have a continuous supply of nutrients and hormones from the circulating media. Also, in the perifusion system, accumulation of metabolic and secretory products is greatly reduced which allows for an increase in cell number to occur and for enhancement of cellular machinery (Oosterom et al., 1983; Lapp et al., 1987a).

CHAPTER 1

DEVELOPMENT OF AN IN VITRO PERIFUSION SYSTEM THAT INCLUDES HYPOTHALAMIC SLICES AND ANTERIOR PITUITARY CELLS

Abstract

Dispersed bovine anterior pituitary (AP) cells were placed either in static or perifusion cultures for 48 h. Cells in both static culture and the perifusion system were incubated for an additional 24 h to assess basal, GRF (10^{-8}M) and SRIF (10^{-6}M) treatment on GH release. Basal GH concentrations after 12 h were 10-fold higher in perifused than in static cultures (2034 ± 160 vs 197 ± 17 ng.$12h^{-1}$). Challenge with GRF (10^{-8}M) for 1 h induced a 3.3- and 2.6-fold increase in GH release in perifused and static cultures, respectively, while SRIF (10^{-6}M) had no effect on GH release in either culture.

Seven hypothalamic (HYP) slices (1mm thick) removed laterally from the sagittal midline of each hemihypothalamus were extracted and assayed for GRF and SRIF. Concentrations of GRF and SRIF tended to be greater nearest the sagittal midline. These HYP slices were perifused in series with AP cells and media effluent was collected, frozen and assayed for GH concentrations. The GH release from HYP slices perifused in series with AP cells appear to mimic the characteristic pulsatile pattern of GH secretion in vivo.

Viability of AP cells and HYP slices after 120 h was determined by depolarization with KCl which increased GRF and SRIF release from the hypothalamus and GH from the pituitary. SRIF immunoreactivity decreased more rapidly than GRF following depolarization. The HYP slices and the AP cells resumed their normal secretory pattern following depolarization. These data indicate that the HYP slices and the AP cells were viable, for at least 120 h. Thus, the hypothalamo-pituitary dual chamber perifusion system can be used to study regulation of GH secretion.

Introduction

The physiological function of anterior pituitary cells has been investigated using hemipituitary explants placed in a controlled environment in static culture (Saffran and Schally, 1955; Guillemin et al., 1957). However, Guillemin et al. (1970) described this procedure as an unreliable method for studying growth hormone (GH) secretion since no more than two treatments could be compared within each experiment. Portanova et al. (1970) introduced the concept of cell dispersion, which was validated by Vale et al. (1972) for rats. The procedure has since been adapted for sheep (Miller et al., 1977) and cattle (Padmannabhan et al., 1979). In general, two types of pituitary cell preparations have been used: 1) the "classic" monolayer cell culture system in which cells are kept in static media for 3 or 4 d, or 2) a perifusion system which allows for continuous flow of media nutrients and removal of secretory and metabolic products from the cells. Use of these culture systems have explained the stimulatory effect of GH-releasing factor (GRF) and the inhibitory role of somatostatin (SRIF) on GH secretion (Reichlin, 1983). While, the interaction between GRF and SRIF has been studied (Tannenbaum and Ling, 1984; Stachura et al., 1988), the exact interrelation between endogenous GRF and SRIF in regulating the ultradian rhythm of GH secretion has not fully elucidated.

The hypothalamo-pituitary complex functions as a single unit in the control of GH secretion (Plotsky and Vale, 1985); the hypothalamus is the principal regulator and the pituitary is the accessory. Combining hypothalamic tissue and anterior pituitary cells in a single system may more closely resemble the in situ state for use to characterize the hypothalamo-pituitary axis on GH secretion.

Hypothalamic neurons release GRF and SRIF from nerve endings in the zona externa of the median eminence (Guillemin, 1978). The neuropeptides are then transported by the hypothalamo-hypophyseal portal circulation to the anterior pituitary cells (Figure I) where they affect GH release (Guillemin et al., 1984). In rats, SRIF immunoreactive cell bodies are found in the periventricular area with greater amounts located caudally than rostrally (Brownstein et al., 1975). While GRF immunoreactivity is found in the entire medial hypothalamic area, greatest concentrations are present in the infundibular nucleus within the medial hypothalamic area in humans (Leidy and Robbins, 1986). The objectives of the present study were: 1) to define the boundaries of the bovine hypothalamus and 2) to develop and validate a perifusion system that combines hypothalamic slices and anterior pituitary cells for use in the study of the regulation of GH secretion.

Materials and Methods

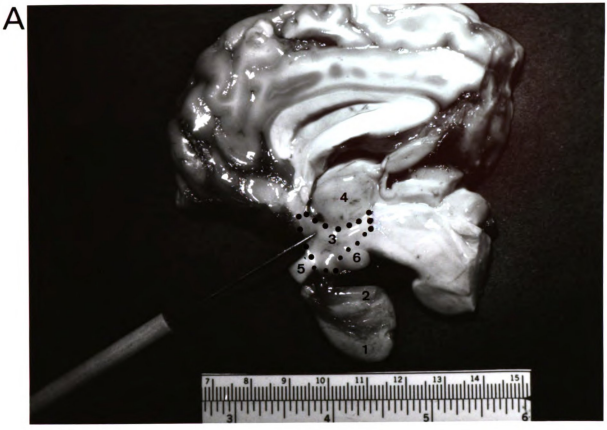
Hormones and Chemicals. Minimum essential medium-alpha (MEM-Alpha) with ribonucleosides and deoxyribonucleosides, Dulbecco's modified Eagle medium with low glucose (DMEM), Hank's balanced salt solution (HBSS), calcium-magnesium free HBSS (CMF-HBSS), MEM amino acid solution 10X, MEM non-essential amino acid solution 10X and newborn calf serum (lot# 4705) were purchased from GIBCO Laboratories, Grand Island, NY. Collagenase type 1A, pancreatin 4X N.F., antibiotic antimycotic solution, Nystatin, penicillin-streptomycin solution, trypan blue stain .4%, bovine serum albumin (BSA, A-7888), DEAE-Sephadex G-50, Hoechst 33258, calf thymus DNA and somatostatin-14 were purchased from Sigma Chemical Company, St. Louis, MO. The bovine growth hormone-releasing factor 1-44-NH_2 (GRF) was generously donated by Lilly Research Laboratories, Greenfield, IN.

Tissue Preparation. Calf or heifer pituitaries and hypothalami were obtained from the Michigan State University Meat Laboratory. At slaughter bovine brains and pituitaries were rapidly removed as aseptically as possible, placed in ice-cold oxygenated HBSS (see Appendix A), and transferred (within 20 min) to the tissue culture laboratory. All subsequent procedures were performed under sterile conditions.

Preparation of Hypothalamic Slices. Brains were sectioned mid-sagittally (Figure 1-1A) and hypothalami dissected out and sagittally sliced (≤1 mm thick). Schoenemann et al. (1985) described the hypothalamic block in cattle, as bordered rostrally by the optic chiasm, lamina terminalis and anterior commissure; caudally by the mammillary bodies (Figure 1-1A); dorsally by the thalamic border (9-11 mm from base of the brain); and laterally by the hypothalamic sulci. The lateral dimension of the ovine (Moss et al.,1980) and bovine (Schoenemann et al., 1985) hypothalamus is approximately 3-5 mm from the midline of the brain.

The hypothalamic slices were weighed, coated with DEAE-Sephadex (DEAE-S, see Appendix B), loosely folded and placed in 5cc (B-D) polypropylene syringes with a Luer-Lok tip which served as the perifusion chamber (Figure 1-2). A 3 cc syringe was used as an insertion device. The tip of the syringe was removed, and the body of the cylinder was cut into half longtudinally. The HYP slice was gently laid inside the half cylinder of the syringe and coated with Biogel. The plunger of the syringe was used to gently push the slice into the perifusion chamber. The HYP slice was then covered with biogel and the syringe (perifusion chamber) was stoppered tightly. Small glass wool plugs were placed at each end of the perifusion chamber to retain the slices and Biogel. Chambers were placed in a test tube rack inside a CO_2 incubator (Model 3028, Forma Scientific, Marietta, OH). Chambers were then

Figure 1-1. A. Median sagittal section of the bovine brain, 1. anterior pituitary, 2. posterior pituitary, 3. hypothalamus, 4. thalamus, 5. optic chiasm, 6. mammillary body.
B. Cross-section of the brain shows the third ventricle; V. 1. anterior pituitary, 2. posterior pituitary, 3. hypothalamus, 4. thalamus.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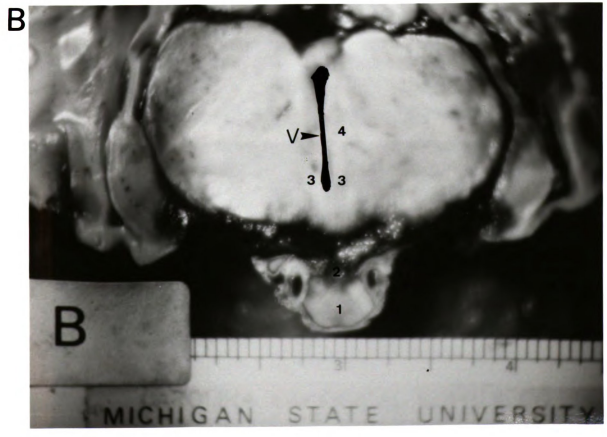
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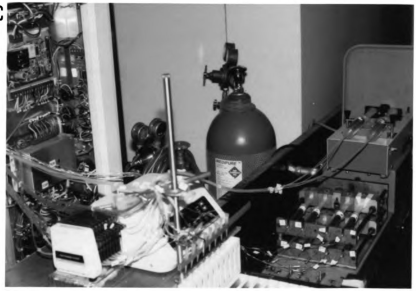
Figure 1-2. **A.** Shown are anterior pituitary (AP) cells in eight chambers of the perifusion system inside a CO_2-incubator.
B. Shown are chambers containing hypothalamic (HYP) slices (bottom) connected in series by tubing to chambers containing AP cells (top) inside the incubator.
C. Pumps are shown outside the incubator which are used to perifuse the media.

A

B

C



perifused (.2 $ml.min^{-1}$) with oxygenated MEM-alpha (see Appendix C) for 24 h. The perifusion flow rate is consistent with that reported by Stachura et al. (1986). The stock media bottle was placed inside the incubator (Figure 1-2A) and media were perifused using a Master Flex microprocessor pump drive (Cole-Parmer, Chicago, IL). Media were perifused from the bottom to ensure that the slices were covered with media. The perifusion apparatus (4 ml volume) consisted of the chambers that contained the hypothalamic slices, Tygon tubing (.08 mm ID; .24 mm OD; Fisher, Pittsburg, PA), straight polypropylene miniature barb fittings (Cole-Parmer, Chicago, IL) and Precision Glide 18G disposable needles (B-D) Baxter S/P (McGaw Park, IL). Ten minute fractions of media effluent from the hypothalamic chambers were collected (LKB 2070, Ultrorac II, Broma) for 12 h and frozen (-20°C). The fractions were thawed at room temperature and the six fractions of each hour were pooled and assayed for GRF and SRIF concentrations.

Preparation of Anterior Pituitary Cells. Bovine anterior pituitary cells were prepared following the procedure reported by Hassan et al. (1992). Connective tissue surrounding the pituitary gland and the posterior pituitaries was removed. Anterior pituitaries were sliced (1 mm thick) with a Stadie Riggs tissue slicer. The slices were incubated at 37°C in CMF-HBSS containing .3% collagenase and 3% BSA with gentle stirring. After 45 min the cells were washed with CMF-HBSS

containing 3% BSA. This was followed by incubation with .25% pancreatin in CMF-HBSS for 8 min. The resulting cell suspension was filtered through sterile gauze and cells were recovered by centrifugation at 400 x g for 5 min. Cells were washed four times with plating media (see Appendix D), and suspended in the media at a final concentration of 10^6 cells. ml^{-1}. Total yield was approximately 3 x 10^8 cells per pituitary gland. Cell viability determined by trypan blue exclusion was greater than 90%. Three cc (B-D) polypropylene syringes with a Luer-Lok tip served as the perifusion chambers for AP cells. They were plugged with glass wool and packed by gravity with DEAE-S to a volume of .5 ml. Aliquots (1 ml) of the cell suspension in media (10^6cells.ml^{-1}) were pipetted into the perifusion chambers and then covered with sterile gauze and left for 24 h before treatments were imposed.

Hypothalamo-Pituitary Perifusion in Series. After 24 h, chambers containing the AP cells were connected in series to the chambers containing the hypothalamic slices, and perifused for another 24 h (.03 $ml.min^{-1}$; Stachura et al., 1986) with DMEM, using a Dekastalitic pump (Buchler, Fort Lee, NJ), as well as with media effluent from the hypothalamic chamber for a combine flow rate of .23 $ml.min^{-1}$. The perifusion apparatus (6 ml volume) consisted of the chambers that contained AP cells, the chambers that contained the hypothalamic slice,

Tygon tubing (.08 mm ID; .24 mm OD; Fisher, Pittsburg, PA), straight and Tee polypropylene miniature barb fittings (Cole-Parmer, Chicago, IL) and Precision Glide 18G disposable needles (B-D) Baxter S/P (McGaw Park, IL). Media effluent from the AP chambers were collected (LKB 2070 Ultrorac II, Broma) every 20 min for 12 h, frozen (-20°C) and assayed for GH concentration.

Extraction of Bovine Hypothalami. The acid extraction procedure described by Vale et al. (1986) was modified as follow. Brains were removed within 5 min of exsanguination and hypothalami were dissected and sliced with a Stadie Riggs tissue slicer. Seven hypothalamic slices (1 mm thick) were removed laterally from each half of the hypothalamus starting at the third ventricle (Figure 1-2B). Each slice (approximately 100 mg) was immersed in liquid nitrogen immediately and kept frozen (-70°C) until extracted. Five slices (100 mg each) from the cerebral cortex also were frozen to be used for recovery studies. Frozen slices were lyophilized, washed in a 10-15 volume of acetone and maintained overnight (4°C). Purified bGRF (1 $ng.ml^{-1}$) and SRIF (1 $ng.ml^{-1}$) were added to the lyophilized cerebral cortex slices after acetone wash and run simultaneously with the unknown tubes to estimate recovery. The acetone precipitate was subsequently extracted with 1 ml hot (>90°C) mixture of 1 N acetic acid/ .1 N HCl/ .2% 2-mercaptoethanol/ 10 mM EDTA/ .4

$mg.ml^{-1}$ diprotein A/ .4 $mg.ml^{-1}$ leupeptin/ 4 $mg.ml^{-1}$ soybean trypsin inhibitor and heated for 5 min in boiling water bath. Then the tissue was homogenized (5 sec) with a Brinkmann Polytron (Westbury, NY). The samples were cooled on ice and centrifuged at 2000 x g for 30 min. The supernatants were lyophilized, resuspended in 1 ml RIA-buffer (Peninsula Laboratories, Belmont, CA) and frozen (-20°C) until assayed for GRF and SRIF. The calculated recovery for GRF was 78% and for SRIF was 91%.

Bovine GH Radioimmunoassay. Bovine GH (bGH) was measured in duplicate by double antibody radioimmunoassay (Purchas et al., 1970). All reagents used in this assay were kindly provided by Dr. H.A. Tucker (Department of Animal Science, Michigan State University). Purified bGH (The Upjohn Company) was labeled with ^{125}I using the chloramine T method (Greenwood et al., 1963) and the labeled hormone was separated from free ^{125}I on a Biogel P-60 column. The column yielded two radioactive peaks; the materials from the first peak (^{125}I-GH) was used in the assay. Rabbit anti-bovine gamma globulin (1st antibody, AB) was diluted 1:10 with rabbit control sera, which had been diluted 1:400 using phosphate buffer saline-EDTA (PBS-EDTA, .05 M and pH 7). Sheep anti-rabbit gamma globulin (2nd AB) was diluted 1:15 using PBS-EDTA (.05 M, pH 7). Standard tubes (one set /250 assay tubes) containing .1, .3, .4, .5, .6, .7, .8, 1, 1.5, 2, 2.5, 3, 4, and 5 ng standard GH

(The Upjohn Company) were assayed in each experiment. Samples were pre-screened for appropriate concentration and then all samples from one experiment were run in the same assay at relevant dilutions. Media were then incubated at 4°C with 1st-AB for 24 h, followed by another 24 h incubation with the labeled hormone, and finally incubation with the 2nd-AB for 72 h. Thereafter, all tubes were centrifuged at 1800 x g for 30 min and the supernatants were decanted. The tops of the tubes were washed, left to dry overnight and then counted in a gamma counter (Gamma Trac 1290, Tm Analytic, Elk Grove Village, IL). Radioimmunoassay standard curves were obtained from a multiple regression equation with linear, quadratic and cubic components.

Bovine GRF Radioimmunoassay. The concentrations of GRF were determined using a commercially available RIA kit (Peninsula Laboratories, Belmont, CA). The kit was developed for human pancreatic GRF (hpGRF). The antiserum used for this assay was raised against synthetic hpGRF, which differs from the bovine GRF in 5 amino acid residues. The validity for GRF measurements in the bovine has been conducted in Peninsula Laboratories. They reported a 100% cross reactivity with bovine and ovine GRF. This kit was validated for bovine GRF in culture media (Appendix E) in cooperation with Dr. Heiman's Laboratory (Lilly Research Laboratory, Greenfield, IN).

Somatostatin Radioimmunoassay. The concentration of SRIF was determined using SRIF RIA-kits (Peninsula Laboratories, Belmont, CA). The antiserum used for this assay was raised against synthetic somatostatin-14, and was found to have 100% cross reactivity with SRIF-14, SRIF-28, and SRIF-25. The kits were validated to measure SRIF in media (Appendix E) in corporation with Dr. Heiman's Laboratory (Lilly Research Laboratory, Greenfield, IN).

Determination of Cellular DNA Content. Cell number was monitored at dispersion and at the termination of the culture to assess the maintenance of cell viability. Since, the AP cells were mixed with Biogel beads, it was difficult to quantitatively recover them using hemocytometer methods. Thus, a crude homogenate was made for quantitation of DNA by a modification of the fluorescence method of Labarca and Paigen (1980). Immediately after dispersion, aliquots of AP cells (10^6 cells.ml^{-1}) were washed three times with CMF-HBSS, resuspended in 1 ml of the same buffer and stored (-70°C) until assayed for DNA. After 72 h, cells in static cultures were washed with ice-cold CMF-HBSS, recovered by scraping with a rubber policeman, and the cells of each well were resuspended in 1 ml of the same buffer, and frozen (-70°C) until assayed. Wells were further examined by microscopy to ensure complete recovery of cells. After 72 h, cells mixed

with biogel in the perifusion chambers were washed three times with CMF-HBSS, the contents of each chamber were resuspended in 1 ml of the same buffer, and frozen (-70°C) until assayed. At the time of assay, the frozen cells were thawed, washed with PBS (see Appendix F), and cell membranes were disrupted using a Branson Sonifier model 350 (Branson Sonic Power Company, Danburg, CT). Sonication was performed for 5 s at 50% duty cycle and an output of 7. Then the samples were diluted 1:10 in Hoechst buffer (see Appendix F). Fluorescence was read at 356 nm excitation and 458 emission using calf thymus DNA as standard (see Appendix F).

A similar procedure was undertaken for determination of DNA content in the hypothalamic slices. The slices were washed with CMF-HBSS, weighed, cut with scissors into approximately 20 small pieces, washed three times with CMF-HBSS and recovered by centrifugation (400 x g for 5 min). The pellet was then homogenized in PBS (100 mg tissue.ml^{-1}) using a polytron (Brinkmann Instruments, Westbury, NY), followed by sonication for 5 s at 50% duty cycle and an output of 7. The sample of each chamber was diluted 1:100 in PBS, then 1:10 in Hoechst buffer. Fluorescence was read as described previously.

Experimental Protocols. The exact protocol for each experiment is explained in the figure legend for each experiment.

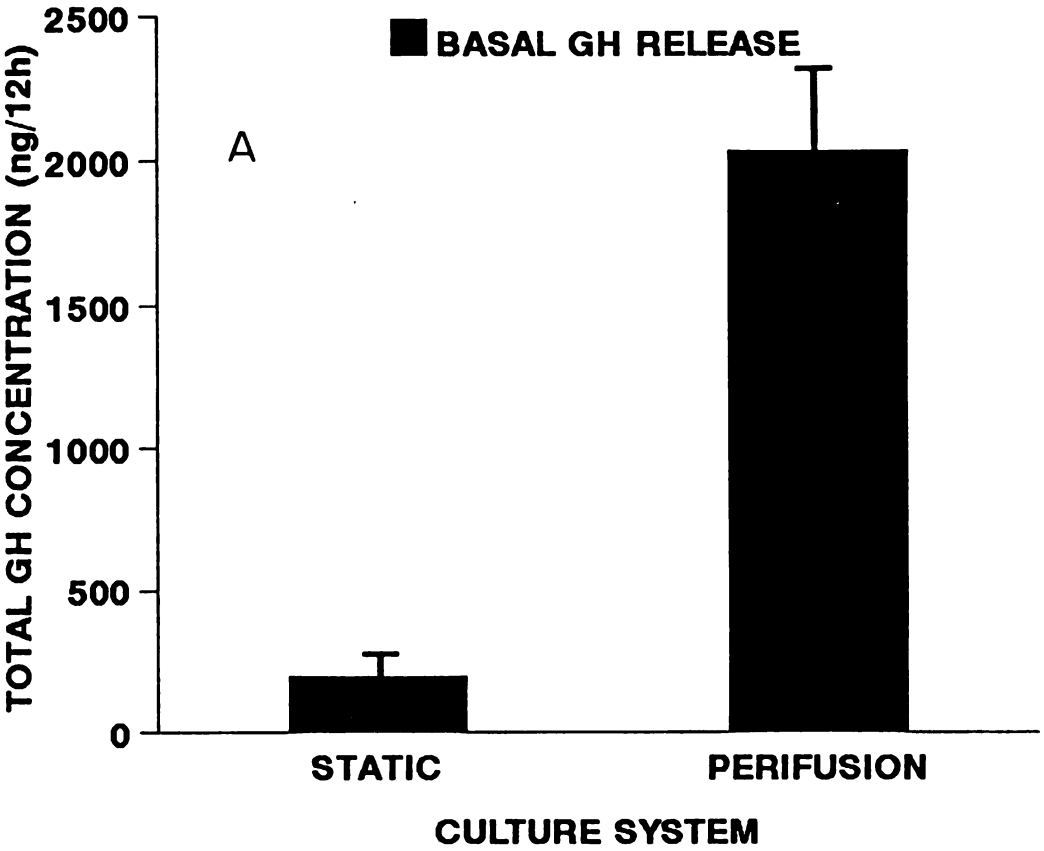
Statistical Analysis. Data were analyzed as for a randomized complete block design (Gill, 1978). Differences among groups were analyzed by two-way analysis of variance. Student's t test was used where appropriate to detect differences among individual means. The criterion for significance was either $P<.05$ or $P<.01$. The results are presented as mean $\pm$ SEM of five experiments, unless otherwise stated.

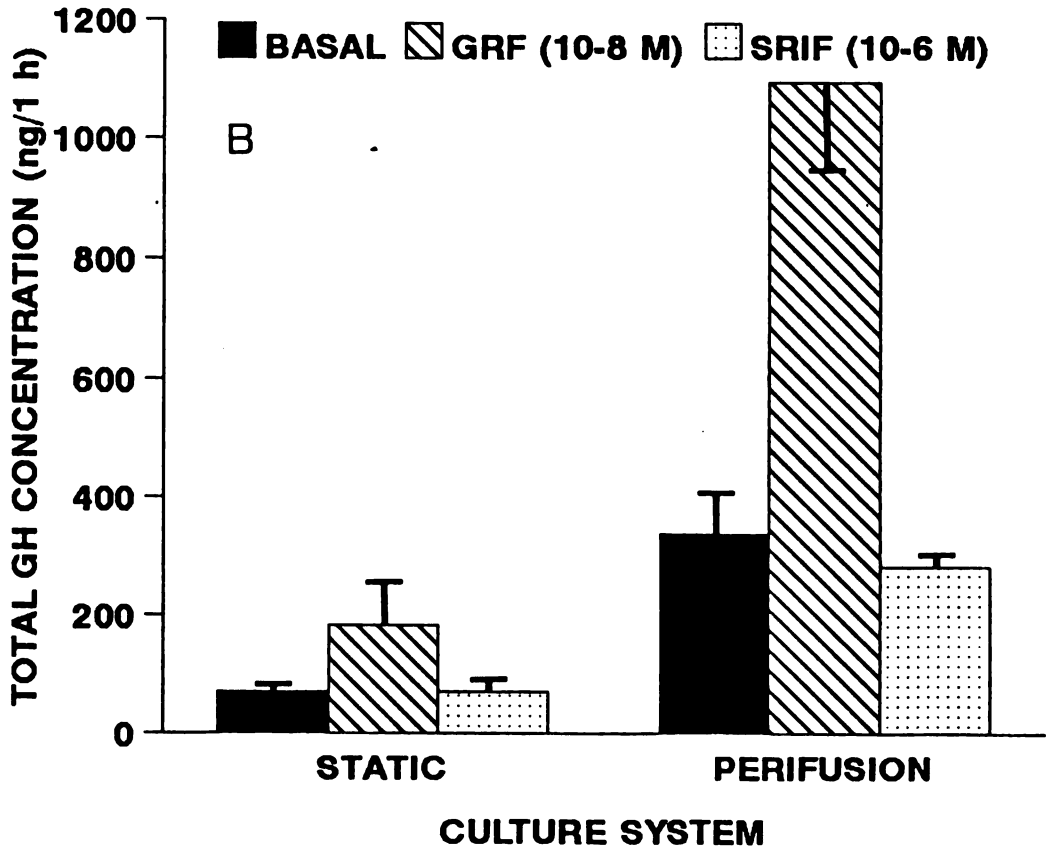
Results and Discussion

Basal GH release during 12 h was 10-fold higher in perifusion system as compared with static culture (Figure 1-3A). Basal and GRF-stimulated (10^{-8}M) GH release for 1 h was 4.8- and 5.9-fold greater, respectively, for AP cells in the perifusion system than those in static culture (Figure 1-3B). In contrast, SRIF (10^{-6}M) for 1 h did not affect ($P>.05$) GH concentration in either the perifusion or static culture. The greater capacity of somatotropes to release GH in the perifusion system may be attributed to any of several factors: 1) continuous nutrient supply with circulating media; 2) less accumulation of secretory and metabolic products; 3) increase in cell number and 4) enhancement of cellular machinery (Oosterom et al., 1983; Lapp et al., 1987a).

Lapp et al. (1987b) reported that factors present in serum-containing culture media (cortisol, epidermal growth factor, insulin, leucine and other nutrients) influence GH secretion. These factors may be limiting in static monolayer cultures, whereas they are more abundant in perifused cultures and may enhance GH release. Fagin et al. (1988) reported that accumulation of secretory products from AP cells may inhibit GH secretion in the limited volume of media in static cultures as a result of negative feedback on somatotropes. In the perifusion system there is continuous removal of inhibitory products (Lapp et al., 1987). While accumulation of GH in

Figure 1-3. Comparison of static vs perifusion culture of calf anterior pituitary (AP) cells. Static: AP cells (10^6cells.well^{-1}) were maintained at 37°C in a humidified atmosphere for 48 h, then media were replaced with fresh media and incubated for an additional 24 h. Perifusion: AP cells were plated on top of a DEAE-Sephadex column, maintained at 37°C in a humidified atmosphere for 48 h, then perifused (.2 ml.min^{-1}) with media for another 24 h. **A)** Basal media collected for 12 h and total GH released was calculated, **B)** Cells in static and perifusion were challenged with GRF (10^{-8}M) or SRIF (10^{-6}M) for 1 h, media were collected and total GH concentrations were calculated. Each bar represents the average of 12 replicates ± SEM.


BASAL GH RELEASE
A
TOTAL GH CONCENTRATION (ng/12h)
2500
2000
1500
1000
500
0
STATIC
PERIFUSION
CULTURE SYSTEM


BASAL
GRF (10-8 M)
SRIF (10-6 M)
B
TOTAL GH CONCENTRATION (ng/1 h)
1200
1000
800
600
400
200
0
STATIC
PERIFUSION
CULTURE SYSTEM

culture media inhibits its secretion (Wilfinger et al., 1979; Oosterom et al., 1983), GH does not directly affect pituitary somatotropes (Oosterom et al., 1983) but acts via stimulating IGF-I synthesis (Fagin et al., 1988). Increased IGF-I has been reported to inhibit GH secretion (Goodyer et al., 1984), pituitary GH-mRNA abundance (Yamashita and Melmed, 1986) and GH gene transcription (Yamashita et al., 1986). Thus, accumulation of IGF-I or possibly other products secreted from the anterior pituitary into the culture media may account for the decreased GH release in static cultures.

An increase in number of somatotropes may contribute to greater GH concentrations in the perifusion than in static cultures (Horng and McLimans, 1975). Cells in static cultures grow to confluency and are limited to the surface area, whereas, in the perifusion system cells have a much greater surface area which is provided by the biogel beads. In the present study, DNA analysis (Table 1-1) indicated that 96 h after plating total cell number, although nonsignificant, tended to be slightly greater ($P<.2$) in perifused than in static culture (2.4×10^8 vs 1.9×10^8 cells, respectively).

TABLE 1-1. TOTAL CELLS NUMBER AS DETERMINED BY HEMOCYTOMETER COUNT AT DISPERSSION AND BY CELL DNA CONTENT AFTER 96 H IN CULTURE

Time	Static Culture	Perifusion Culture
At disperssion	1×10^6	1×10^6
After 96 h in culture	1.9×10^8	2.4×10^8

However, DNA may have been underestimated in the perifusion system because of inherent difficulties in removing the cells from the beads. Ohlsson et al. (1988) found no change in cell number in a highly enriched population of somatotropes during 72 h in static culture.

There are six different types of endocrine cells in the anterior pituitary along with several other cell types (Dellmann, 1981) It is possible that the increase in cell number may include somatotropes as well as other cell types.

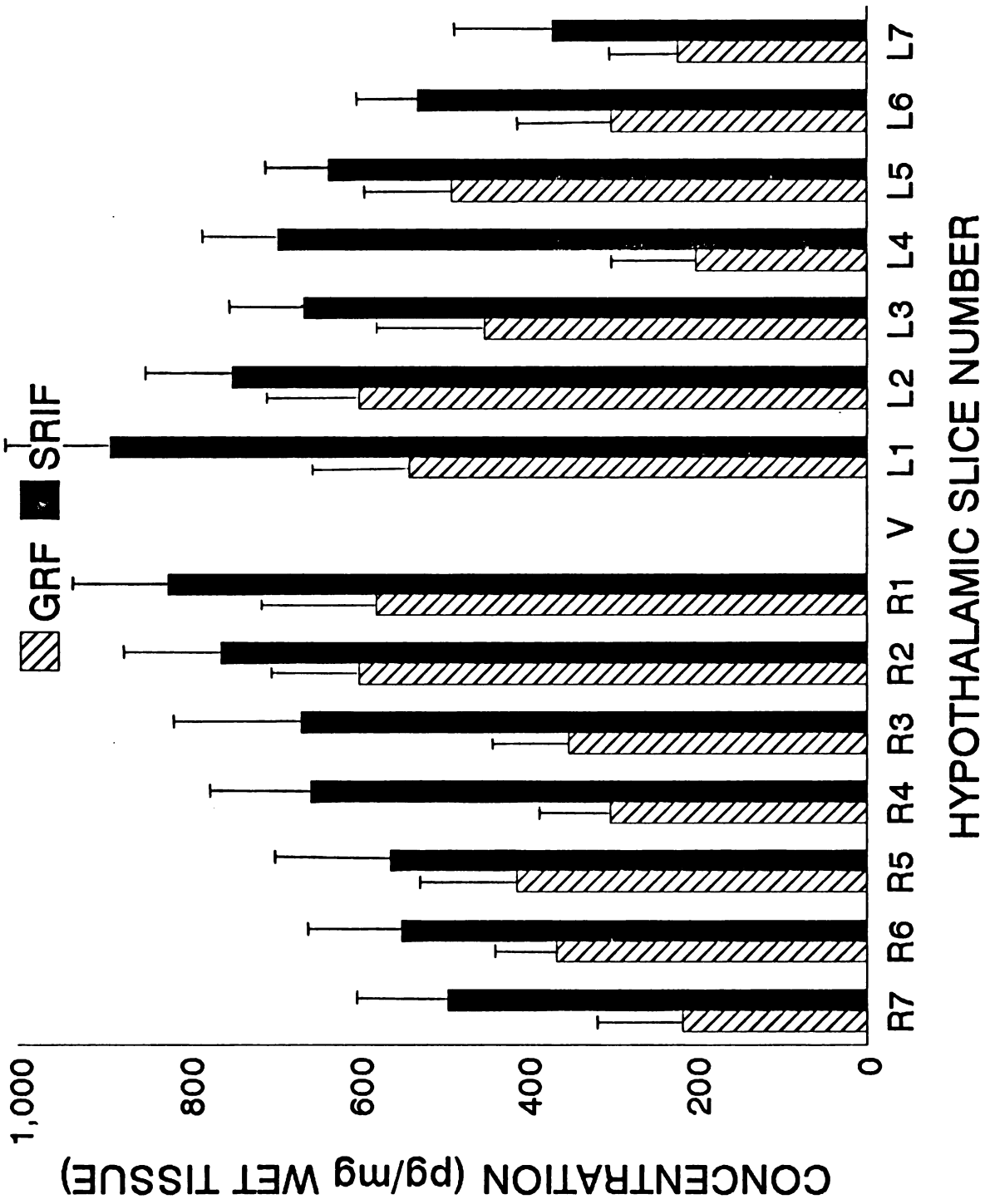
The role of cell density (Sugimoto et al., 1991) and cell-to-cell communication (Baes and Denef, 1987) in maintaining cell integrity and function is well documented. Sugimoto et al. (1991) emphasized the role of cell-to-cell interaction in the regulation of pituitary hormone secretion. In addition, dispersed AP cells tend to aggregate and form colonies in static culture in order to mimic the in vivo cellular arrangement (Hassan, 1990). Electron microscopy showed that cell volume tends to increase within cell colonies in static cultures, but granule profile area decreases (Ohlsson et al., 1988). Cells in static cultures attach to the plate surface (Horng and McLimans, 1975) and become flat or extremely extended in shape (Folkman and Moscona, 1978). While when cells grow on Biogel beads, their morphology is normal as they attach to the bead surface (Horng and McLimans, 1975). The anchorage sites and normal topology have been reported to reflect normal function (Banavar et al., 1991) for cells in a

perifusion system compared with those in static cultures. Electron microscopy of perifused cells mixed with beads showed the presence of active mitochondria, endoplasmic reticulum, tight cell junctions, and endoplasmic projections between cells and the Biogel beads (Horng and McLimans, 1975). Moreover, cell shape is tightly coupled to DNA synthesis and cellular growth (Folkman and Moscona, 1978; Watt et al., 1988).

The data in Figure 1-4 show the concentration of GRF and SRIF in each of the seven slices removed from the right and left hemihypothalamus. In order to determine the relative concentrations of these neuropeptides in each slice as well as to identify the lateral limits of their neurons, all seven slices (right and left) were assayed for GRF and SRIF immunoreactivity. The highest concentration of immunoreactive SRIF was found in slices in closest proximity to the third ventricle. Concentrations of SRIF decreased with each slice obtained laterally from the brain midline. This agrees with observations of Brownstein et al. (1975) who described the regional distribution of SRIF in the rat brain.

Immunoreactive GRF was less symmetrical in its lateral distribution, but it also had highest concentrations nearest the third ventricle. Leidy and Robbins (1986) reported similar results for GRF distribution in the human hypothalamus. While the lateral extent of the hypothalamus from the brain midline was reported to be 3 mm in sheep (Moss et al., 1980) and 5 mm

Figure 1-4. Hypothalamic content of GRF and SRIF. Hypothalamic (HYP) slices ($\leq$ 1 mm thick) were dissected laterally from the sagittal midline of the third ventricle (V). Seven slices from the right (R1 to R7) and seven slices from the left (L1 to L7) half of the HYP were removed, individually extracted and assayed for GRF and SRIF concentrations. Recovery of GRF ($1ng.ml^{-1}$) and SRIF ($1ng.ml^{-1}$) added to a 100 mg homogenate from the cerebral cortex was 78 and 91%, respectively. Each bar represents the average of five replicates $\pm$ SEM.


GRF
SRIF
1,000
800
600
400
200
0
CONCENTRATION (pg/mg WET TISSUE)
R7 R6 R5 R4 R3 R2 R1 V L1 L2 L3 L4 L5 L6 L7
HYPOTHALAMIC SLICE NUMBER

in cattle (Schoenemann et al., 1985), it was found to be at least 7 mm in the present study. Because concentrations of both neuropeptides were highest in the first two slices on either side of the sagittal midline of each hemihypothalamus, only these four slices were utilized in subsequent experiments.

The pattern of GH release from AP cells and AP cells cultured in series with HYP slices is presented in Figure 1-5. When AP cells were perifused without HYP slices, they had a low baseline (30 $ng.ml^{-1}$), frequent pulses (3.4 peaks. $12h^{-1}$), and low amplitude (50 $ng.ml^{-1}$). Lapp et al., (1987b) found that the somatotropes maintained their pulsatile behavior in perifusion culture when isolated from the hypothalamic influence. However, in the present study AP cells cultured in series with HYP slices had a higher baseline (55 $ng.ml^{-1}$), less frequent pulses (2.6 peaks. $12h^{-1}$), and greater pulse amplitude (150 $ng.ml^{-1}$) than AP cells without HYP slices. These data suggest that the hypothalamus regulates GH release, and that this perifusion system is capable of mimicking the in vivo hypothalamo-pituitary complex secretory pattern of GH.

Viability of the AP cells as well as the cells of the HYP slices was assessed by depolarization of the cells which was induced by adding a high concentration of K^+ (56 mM KCl) to the media. The effect of a high K^+ concentration (56 mM) on GRF and SRIF release from the hypothalamic slices is shown in Figure 1-6a. Stimulation with high K^+ markedly increased the

Figure 1-5. The pattern of GH released from AP cells (PIT) or AP cells cultured in series with HYP slices (HYP+PIT). PIT: AP cells were maintained in culture for 48 h and then perifused (.23 $ml.min^{-1}$) for another 48 h with media. HYP+PIT: HYP slices were placed in chambers, perifused (.2 $ml.min^{-1}$) for 48 h with media, and then the chambers containing HYP slices were connected in series to the chambers containing the AP cells and perifused (.03 $ml.min^{-1}$) with media as well as the media effluent (.2 $ml.min^{-1}$) from the chambers containing the HYP slices. The secretory pattern shown is one of five replications conducted.

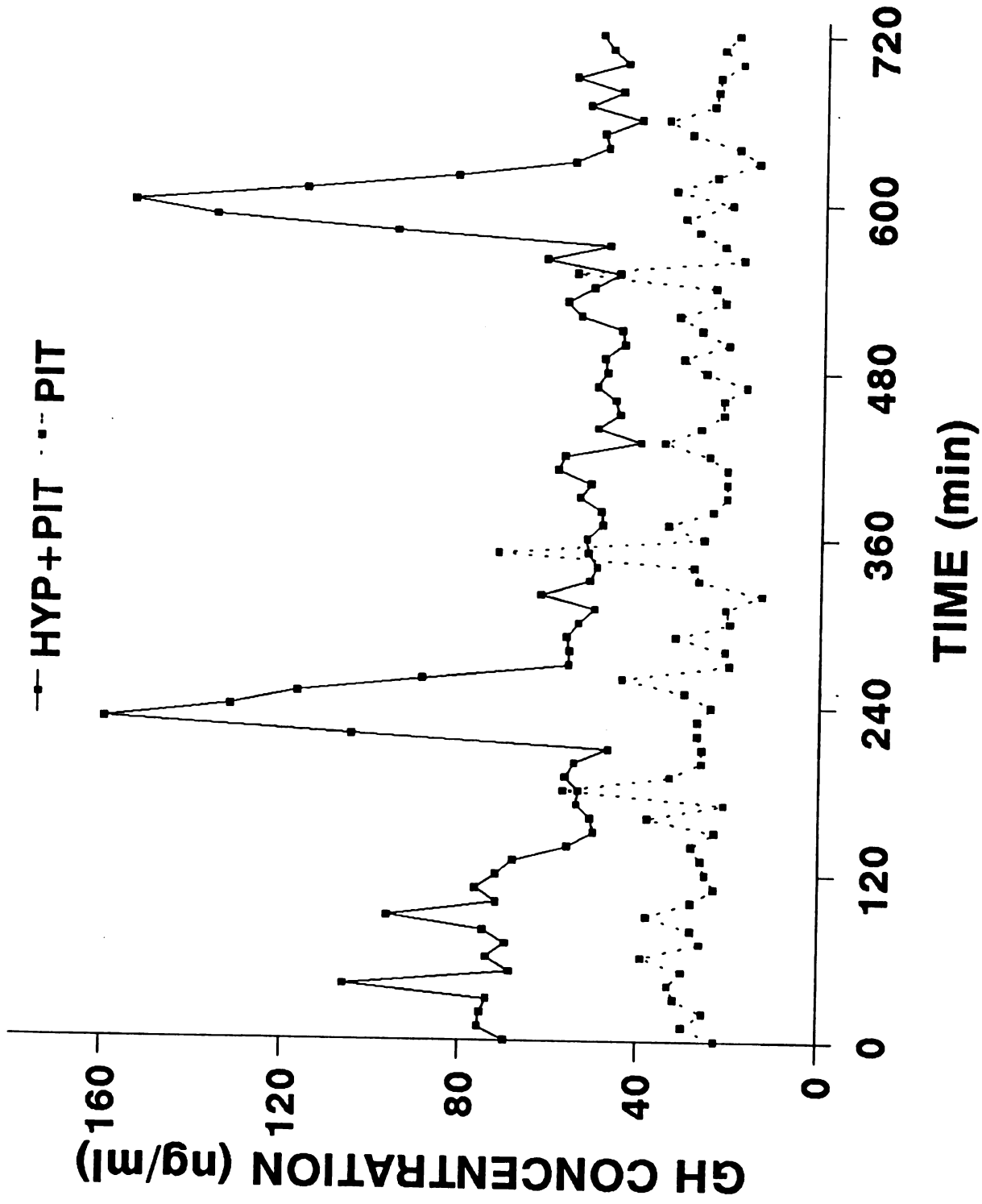
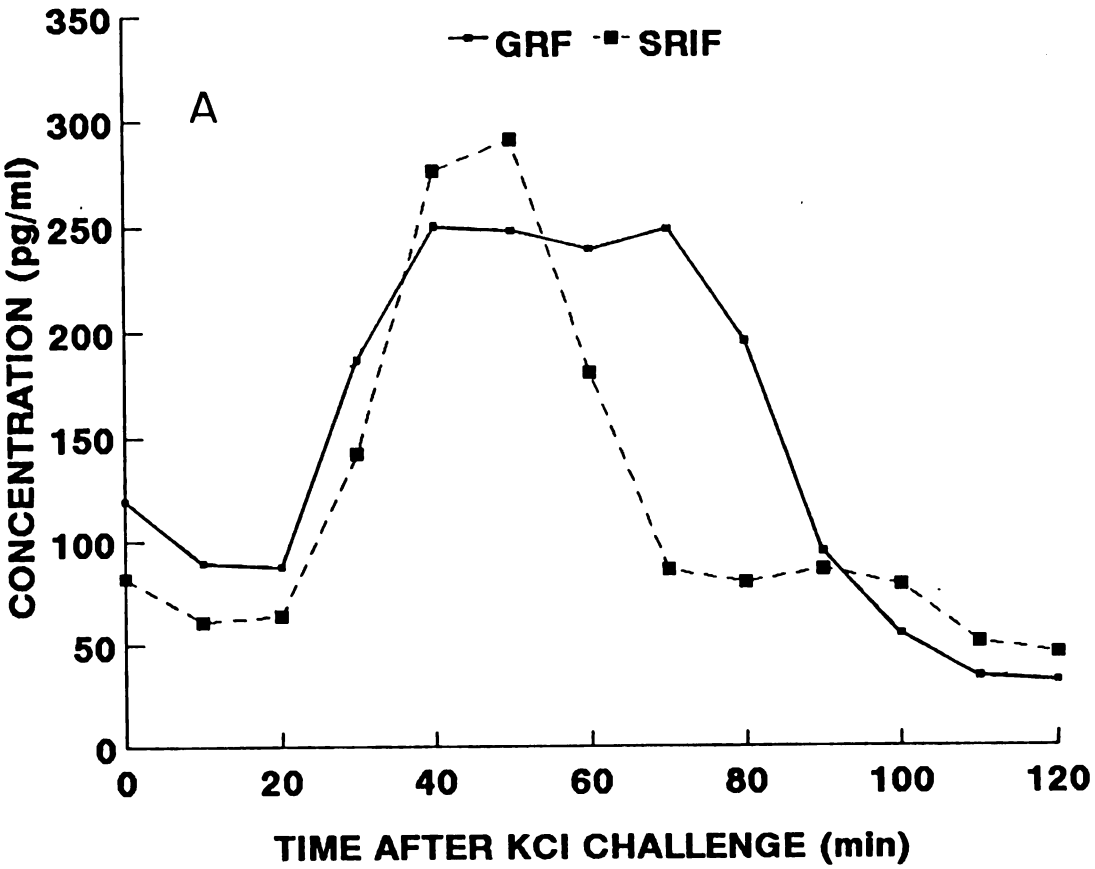
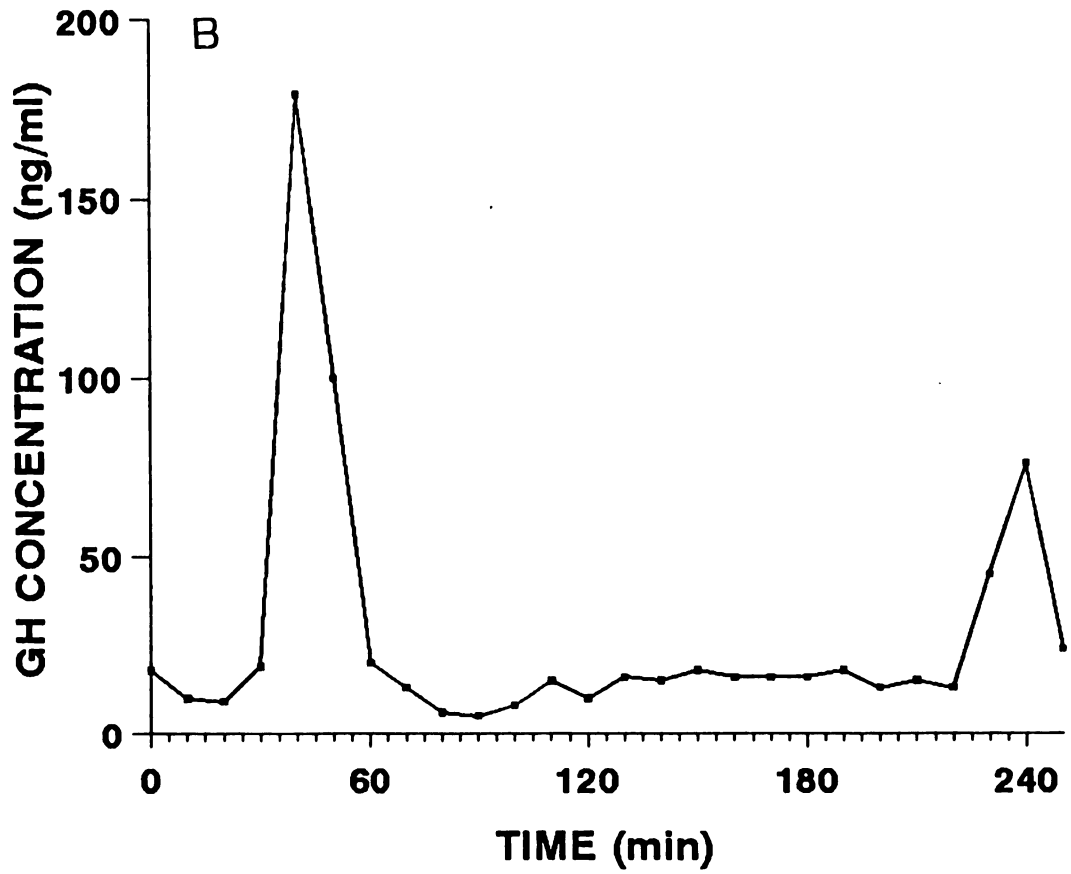

HYP+PIT
PIT
GH CONCENTRATION (ng/ml)
160
120
80
40
0
0
120
240
360
480
600
720
TIME (min)

Figure 1-6. The effect of KCl challenge on : **A)** HYP GRF and SRIF. HYP slices were maintained in perifusion system (.2 $ml.min^{-1}$) for 120 h then challenged with 56 mM KCl for 20 min, fractions were collected every 10 min for 120 min; **B)** Pituitary GH released in response to 56 mM KCl challenge. AP cells were maintained in media for 48 h, perifused (.2 $ml.min^{-1}$) with media for another 72 h then with media containing 56 mM KCl for 20 min, fractions were collected every 10 min for 250 min. These plots are one of five replications conducted.


A
GRF
SRIF
CONCENTRATION (pg/ml)
350
300
250
200
150
100
50
0
0
20
40
60
80
100
120
TIME AFTER KCl CHALLENGE (min)


B
GH CONCENTRATION (ng/ml)
200
150
100
50
0
0
60
120
180
240
TIME (min)

release of both peptides. Elevated K^+ facilitates the release of hormones through membrane depolarization coupled to an influx of Ca^{2+} ions (Henquin, 1978). It is worth noting that the increase in K^+ concentration increases media osmolarity. The effect of high osmotic pressure influences the neuropeptides, vasopressin and oxytocin (Hashimoto et al., 1985). Thus, an effect of osmolarity on GRF and SRIF release cannot be excluded.

In the present study, SRIF neurons released greater ($P<.05$) concentrations in response to high K^+ than did GRF neurons; however, SRIF concentrations decreased to basal values more rapidly (20 min) than GRF concentrations (40 min). This difference in neuron responsiveness to increased K^+ concentration has been reported previously (Stern et al., 1986). Figure 4b illustrates a rapid increase in GH release in response to 56 mM KCl, with a peak value of 180 $ng.ml^{-1}$. Thereafter, concentrations of GH rapidly declined to basal values. Potassium is thought to induced GH secretion through depolarization of somatotropes leading to opening of voltage-sensitive calcium channels (Ohlsson and Lindstrom, 1990). Rapid increases in intracellular Ca^{2+} induce GH release (Cuttler et al., 1992). Secretion of GH was monitored for 250 min; 180 min after the K^+-induced episodes, a spontaneous episode occurred. These observations indicate that the cells in the perifusion system are viable for at least 120 h.

This perifusion system responds to physiological

perturbations similar to those in vivo. It is concluded that this in vitro system was a valid tool to study the hypothalamo-pituitary axis in the regulation of growth hormone secretion.

SUMMARY

An in vitro perifusion system that includes hypothalamic slices and anterior pituitary cells placed in series was developed to understand the regulation of GH release from the somatotropes. Anterior pituitary cells in perifusion system had greater capacity to release GH than those in static cultures. When hypothalamic slices were placed in series with anterior pituitary cells, the pattern of GH release from anterior pituitary cells appeared to mimic the characteristic pulsatile pattern of GH secretion in vivo. Under the experimental conditions employed in this study, both anterior pituitary cells and hypothalamic slices are viable for at least 120 h. The hypothalamo-pituitary dual chamber perifusion system can be used to study regulation of GH release from the pituitary somatotropes.

CHAPTER 2

THE EFFECT OF SEX STEROIDS ON GH RELEASE FROM ANTERIOR PITUITARY CELLS PERIFUSED IN SERIES WITH HYPOTHALAMIC SLICES

ABSTRACT

The mechanism and site of action whereby sex steroid hormones alter GH secretion in cattle is not well understood. In the present study, hypothalamic (HYP) slices in perifusion chambers (HYP-CH) were connected in series via tubing with anterior pituitary (AP) cells in chambers (AP-CH). The latter were perifused with fresh media plus media effluent from the HYP-CH. Media effluent from the AP-CH was collected, assayed for GH and area under the curve (AUC-GH) was calculated. Estradiol-17ß (E_2, 10^{-11}M), testosterone (T, 10^{-9}M), dihydrotestosterone (DHT, 10^{-11}M) or 3α-androstane-3α,17ß-diol (3α-diol, 10^{-12}M) were applied to either the AP-CH or to the HYP-CH plus AP-CH. When applied to AP cells DHT and 3a-diol each increased ($P<.01$) AUC-GH compared with controls (26.3 vs 34.8 ug.min.ml^{-1} for DHT) and (28.9 vs 38.6 ug.min.ml^{-1} for 3α-diol). When E_2, DHT or 3α-diol were applied to the HYP-CH plus AP-CH, AUC-GH was increased ($P<.01$) compared with controls (24.7 vs 43.6 and 44.3 ug.min.ml^{-1} for E_2 and DHT, respectively) and (27.6 vs 43.1 ug.min.ml^{-1} for 3α-diol). In contrast, perifusion of T to AP cells or to the HYP-CH plus AP-CH had no effect ($P>.05$) on AUC-GH.

To mimic the endogenous T pulsatile pattern in post-pubertal males, T (10^{-9}M) was administered either at a constant rate or as a pulsatile pattern to AP cells or the HYP-CH plus AP-CH. Constant rate T had no effect (P>.05) on AUC-GH from AP-CH or AP-CH plus HYP-CH. Pulsatile T administration to AP-CH had no affect (P>.05); but to the AP-CH plus HYP-CH it increased (P<.01) AUC-GH 75.4% above controls.

The effect of T aromatization to E_2 was also studied. Pulsatile T was applied with or without an aromatase inhibitor (AI) to the combined HYP-CH plus AP-CH. Pulsatile T with AI decreased AUC-GH to control values. When T metabolites, E_2 plus DHT, were perifused together, they increased AUC-GH more than either alone. These data indicate that, estradiol-17β increases GH secretion by acting at the hypothalamus but not at the anterior pituitary. Androgenic metabolites act at both the AP and HYP to increase GH secretion. The pulsatile pattern of testosterone secretion in males appears to be an important factor in modulating GH secretion in vivo.

INTRODUCTION

Growth is an essential process in the livestock and meat industry. The endocrine control of growth involves a complex interaction of several hormones along with nutrient supply, genetic potential and environmental factors. Principal among the hormones is growth hormone (GH), which has a central role in the control of postnatal growth. The increase in gonadal steroid secretion coincides with the pubertal growth spurt (Bourguignon, 1988). The mechanism of gonadal steroid action has been proposed to be either a direct effect on target tissues (Itagane et al., 1991) or indirectly through modulation of GH secretion (Jansson et al., 1984).

Cattle (Anfinson et al., 1975), sheep (Davis et al., 1977), and pigs (Dubreuil et al., 1988) exhibit a sexual dimorphic pattern of GH secretion, which is related to the gonadal steroid status of the animal. In ewes, in which estrogen predominates, GH secretion exhibits higher baseline and more frequent episodes than in castrates (Davis and Borger, 1974). They also reported that treatment of ovariectomized ewes with E_2 enhanced GH secretory activity. Circulating androgens in bulls is responsible for the increase in plasma GH concentrations as well as magnitude of the GH secretory spikes (Anfinson et al., 1975). Similar results have been reported for rams (Davis et al., 1977); and they also found that testosterone propionate (TP) replacement in wethers enhanced plasma GH concentrations.

Intracellular metabolism of testosterone to 5α-reduced metabolites in the hypothalamus (Roselli and Resko, 1989) and the pituitary (Cohen et al., 1980) is an important factor in androgen regulation of GH secretion. Within the anterior pituitary as well as various areas in the hypothalamus, testosterone is reduced to 5α-dihydrotestosterone (DHT) which in turn is converted to 3α- and 3ß-androstandiol (3α- and 3ß-diol, Aakvaag and Haug, 1979). The 5α-reducing complex in the hypothalamus and the anterior pituitary is similar to that described for the peripheral androgen-dependent tissues (Martini, 1982). The only significant difference resides in the fact that 20-40% of the reduced metabolites formed in the hypothalamus and the anterior pituitary is 3α-diol (Celotti et al., 1979), while it represents only 10% in peripheral androgen-sensitive tissues (Martini, 1976). In the brain the predominant metabolite of 5α-reductase activity is DHT (Aakvaag and Haug, 1979). In the hypothalamus, but not the pituitary, the reductase pathway co-exists with the aromatase pathway which also can convert T to E_2 (Simerly and Young, 1991).

The endogenous secretion of T in bulls is pulsatile (McCarthy et al., 1978). The pulsatile pattern of T is necessary to maximize growth performance in sheep (Schanbacher et al., 1980). In addition, the mode of T replacement (continuous vs episodic) in castrated rats influences regulation of FSH and LH secretion (Tilbrook et al., 1991).

Implants that provide constant concentrations of T elicit a different response than that observed with pulsatile T in gonadally intact males (D'Occhio et al., 1983; Schanbacher, 1985). The mechanism of the T secretory pattern on GH regulation has not been established. Understanding the effect of estrogen and testosterone and its metabolites on GH production is necessary in order to take advantage of any potential growth-promoting effects these steroids might have on GH secretion and(or) synthesis.

The overall objectives of the present study were to: 1) assess the effect of E_2, T, and its metabolites, DHT and 3α-diol, on GH secretion from anterior pituitary cells and on those perifused in series with hypothalamic slices; 2) investigate the importance of pulsatile administration of T to E_2 on GH release; and 3) demonstrate the role of aromatization of T on GH release in a perifusion system.

MATERIALS AND METHODS

Hormones and Chemicals. Minimum essential medium (MEM)-Alpha with ribonucleosides and deoxyribonucleosides (MEM-alpha), Dulbecco's modified Eagle medium with low glucose (DMEM), Hank's balanced salt solution (HBSS), calcium-magnesium free HBSS (CMF-HBSS), MEM amino acid solution 10X, MEM non-essential amino acid solution 10X and newborn calf serum (lot# 4705) were purchased from GIBCO Laboratories, Grand Island, NY. Collagenase type 1A, pancreatin 4X N.F., antibiotic antimycotic solution, Nystatin, penicillin-streptomycin solution, trypan blue stain .4%, bovine serum albumin (BSA, A-7888), DEAE-Sephadex G-50, and somatostatin-14 were purchased from Sigma Chemical Company, St. Louis, MO. The bovine growth hormone-releasing factor 1-44-NH_2 (GRF) and the aromatase inhibitor (LY43578) was generously donated by Lilly Research Laboratories, Greenfield, IN.

Tissue Preparation. Heifer pituitaries and hypothalami were obtained from the Michigan State University Meat Laboratory. At slaughter brains and pituitaries were rapidly removed, as aseptically as possible and placed in ice-cold oxygenated HBSS (see Appendix A). The samples were then transferred (within 20 min) to the tissue culture laboratory. All subsequent procedures were performed under sterile conditions.

Preparation of Hypothalamic Slices. Brains were sectioned mid-sagittally and hypothalami dissected out as previously described (Chapter 1).

Preparation of Anterior Pituitary Cells. Heifer anterior pituitary cells were prepared as previously described (Chapter 1). The cells were then packed in perifusion chambers and incubated for 24 h before treatments were imposed.

Hypothalamo-Pituitary Perifusion In Series. The exact procedure for the dual chamber perifusion system is described in Chapter 1.

In preliminary studies, several concentrations of each steroid were used. The GH response for each steroid was similar among the different concentrations. Thus, a physiological concentration of each steroid hormone was used; i.e., 10^{-11}M for E_2, 10^{-9}M for T, 10^{-11}M for DHT and 10^{-12}M for 3α-diol.

Bovine GH RIA. The RIA protocol is described in Chapter 1. Concentrations of GH released every 20 min for 12 h were determined. Total concentration of GH released was determined by estimating area under the response curve (AUC) using the trapezoidal summation method. The pulsatile patterns of GH secretion were analyzed using the PULSAR program (Merriam and Wachter, 1982). Exclusion criteria for pulse identification

were 4.4, 2.9, 1.9, 1.3 and .9 within assay standard deviations for pulses with a duration of 1, 2, 3, 4 and 5 time points, respectively. The within-assay standard deviations was determined by assaying in quadruplets five samples with hormone concentrations distributed across the assay range. Assay standard deviation was equal to $(.09\ X^2 + 3.33\ X + 12.43)/\ 100$. The peak splitting cut-off value was assaigned as 3 standard deviations.

Experimental protocol. The protocol of each experiment is presented in the figure legend for each experiment.

Statistical Analysis. Data were analyzed by a randomized complete block design (Gill, 1978). Differences among groups were analyzed by two-way analysis of variance, followed by student's t test where appropriate to detect differences among individual means. The criterion for significance was either $P<.05$ or $P<.01$. The results are presented as the mean $\pm$ SEM of five experiments, unless otherwise stated.

RESULTS AND DISCUSSION

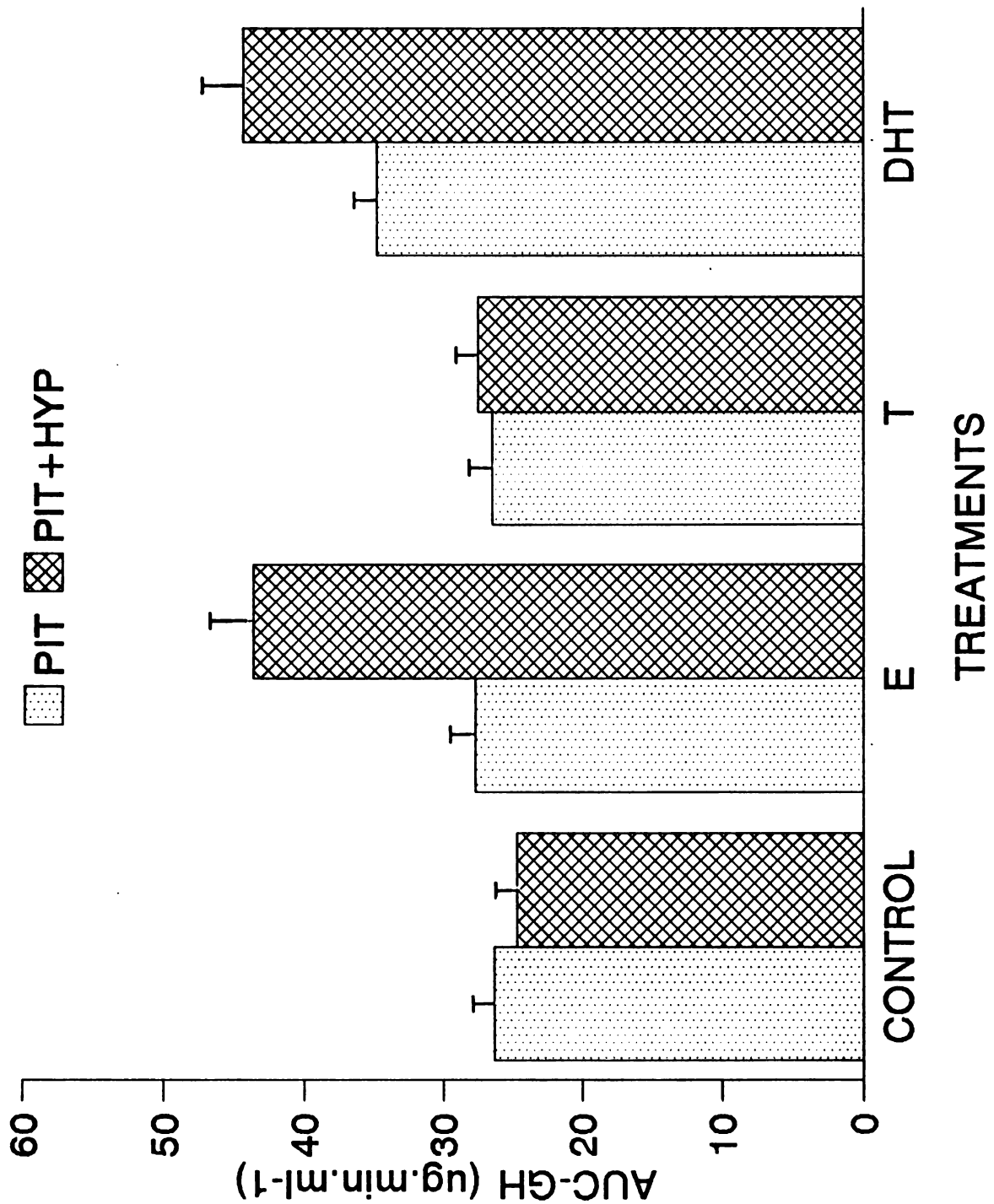
Physiological concentrations of either E_2 (10^{-11}M), T (10^{-9}M) or DHT (10^{-11}M) were added to the media and perifused to AP cells or to the AP-CH plus HYP-CH. Results of perifusion of these steroids for 24 h on AUC-GH are presented in Figure 2-1. When E_2 was applied to AP cells, AUC-GH was not different (P>.05) from the non-estradiol treated AP cells (26.3±2.3 vs 27.7±2.9 ug.min.ml^{-1}). However, when E_2 was perifused to the combined AP-CH plus HYP-CH, a 76% increase (P<.01) in AUC-GH was observed (24.7±2.1 vs 43.6±4.1 ug.min.ml^{-1}). The data in Table 2-1, show that E_2 increased

TABLE 2-1. EFFECT OF GONADAL STEROIDS ON GH RELEASE FROM AP CELLS CULTURED IN SERIES WITH HYP SLICES[1]

Variable[2,3]	Control	E_2	T	DHT
Baseline (ng.ml^{-1})	29.4±1.8	45.2±4**	7.1±3.1	43.1± 3**
Number of peaks (12h)	2.4±1.1	4.0±1.2*	2.2±.84	1.8±.45
Amplitude (ng.ml^{-1})	52.2±22	62.7±14	55.6±7	97.3± 28*
Peak length (min.)	61.7±11	71.1±12	69.8±24	94±24
Inter-peak interval (min)	281.7±76	196.0±65	255.0±87	364.0±86

[1]AP cells were cultured in series with HYP slices; perifused with E_2: estradiol-17β (10^{-11}M); T: testosterone (10^{-9}M); OR DHT: dihydrotestosterone (10^{-11}M) for 24 h. Media were collected every 20 min for 12 h, frozen and assayed for GH.
[2]Values represent the effect of steroids on the GH secretory pattern.
[3]Values are means ± SEM of five replicates.
* Significant (P<.05), ** Significant (P<.01) from controls.

Figure 2-1. The effect of testosterone (T, 10^{-9}M), dihydrotestosterone (DHT, 10^{-9}M), estradiol-17β (E, 10^{-11}M) and non-steroid treated controls (C) on area under the curve (AUC) of GH release from hypothalamo-pituitary cultures perifused in series. Tissues were obtained from heifers. Gonadal steroids were applied to anterior pituitary (AP) cells (PIT) or to AP cells plus hypothalamic slices (PIT+HYP) for 24 h. Then , 20 min fractions were collected for 12 h, frozen, assayed for GH, and AUC-GH was calculated. Each bar represents the average of five replicates (two animals per replicate) ± SEM.


PIT
PIT+HYP
AUC-GH (ug.min.ml-1)
0
10
20
30
40
50
60
CONTROL
E
T
DHT
TREATMENTS

(P<.01) the baseline of GH release (29.4±1.8 vs 45.2±4.3 $ng.ml^{-1}$), as well as increased (P<.05) peak frequency (2.4±1.1 vs 4.0±1.2 $peaks.12h^{-1}$). Peak amplitude of GH release tended to be greater (P<.10) in E_2-treated cells than controls. These data indicate that E_2 influences GH release via direct action on the hypothalamus, but not at the level of the pituitary. Shirasu et al., (1990) reported that certain populations of GRF neurons in the hypothalamus are targets for estrogen action. In addition, Painson and Tannenbaum (1991) indicated that estrogen alters the pattern of hypothalamic SRIF secretion and hence GRF/SRIF signaling to somatotropes in the pituitary. The observation that E_2 does not have a direct effect on pituitary GH-mRNA abundance (Silverman et al., 1988) or on GH secretion from either bovine (Hassan et al., 1992) or rats somatotropes in static culture (Wehrenberg et al., 1985; Fukata and Martin 1986; Hertz et al., 1989) agree with the present data. Implants containing E_2 increased GH baseline and pulse frequencies in ovariectomized ewes (Davis and Borger, 1974). Marynick et al. (1976) reported that E_2 crosses the blood brain barriers readily to reach the hypothalamus. Thus, it appears that the effect of E_2 on GH-release occurs via direct action of E_2 at the hypothalamus to modulate the release of GRF and SRIF.

Administration of physiological concentrations of T to AP cells (Figure 2-1) had no effect (P>.05) on AUC-GH (26.3±2.3 vs 26.5±3.1 $ug.min.ml^{-1}$) or other GH release criteria (Table

2-1). These data agree with several other reports for cattle (Silverman et al., 1988; Hassan et al., 1992) and rats (Wehrenberg et al., 1985; Fukata and Martin 1986; Hertz et al., 1989). In contrast, perifusion of AP cells with DHT, a metabolite of T, increased ($P<.01$) AUC-GH by 32.4% above controls (26.3 ± 2.3 vs 34.8 ± 3.1 ug.min.ml^{-1}, Figure 2-1). In static cultures, DHT had no effect on basal GH secretion of AP cells from cattle (Hassan et al., 1992) or rats (Wehrenberg et al., 1985). However, physiological concentrations of DHT (10^{-11}M) or supraphysiological concentrations of T (10^{-5}M) increased somatotropes responsiveness to GRF (Hassan et al., 1992). Under the experimental conditions employed in the present study, DHT also may have increased cell responsiveness to GRF released from the HYP slices; hence, the increase in AUC-GH from AP cells.

Cohen et al. (1980) have reported that pituitary gonadotropes are able to converte T to DHT and 5α-androstandiols (3α- and 3ß-diol) through the 5α-reductase pathway (Appendix G). It is generally accepted that these metabolites are the principal mediators of T action in androgen-sensitive tissues. Activity of this pathway in the anterior pituitary is reported to be next in order to the prostate and seminal vesicles which have the highest activity (Martini, 1982). The reduction of T at the 5-position by 5α-reductase is influenced by several hormones; i.e., glucocorticoids, thyroxine and GH in humans (Roelfsema et al.,

1983) and in rats (Mode et al.,1981). Excision of the anterior pituitary markedly decreases 5α-reductase activity in rats (Martini, 1982). In the latter study, the anterior pituitary was autotransplanted under the kidney capsule, and the 5α-reductase activity was low in all grafted animals for 14 days. In the present study, it appears that there was little or no 5α-reductase activity within the dispersed pituitary somatotropes because perifusion of T did not elicit an effect on GH release similar to that observed for DHT. In addition, the stimulatory action of DHT (10^{-11}M) on somatotropes suggests that T at physiological concentrations (10^{-9}M) is less potent than 100-fold lower concentrations of the 5α-reduced androgens in eliciting the androgenic response (Johnson et al., 1991; Hassan et al., 1992).

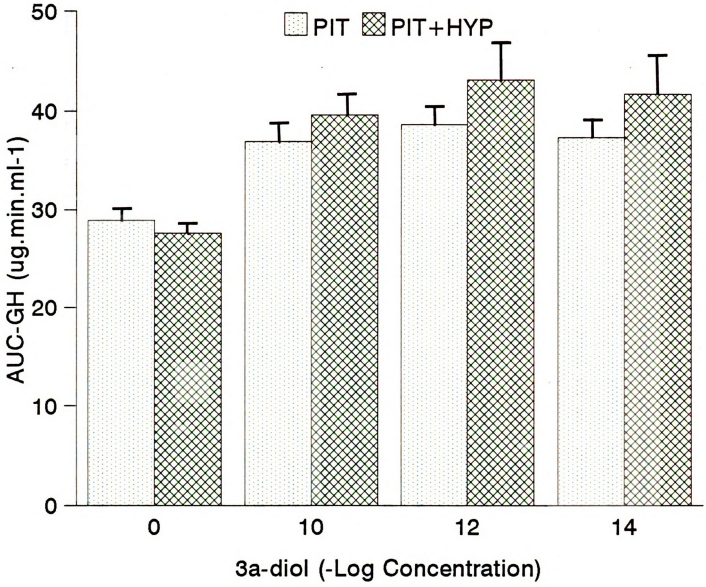
Perifusion of AP cells plus HYP slices with T, also had no effect (P>.05) on AUC-GH (24.7±2.1 vs 27.5±3.1 ug.min.ml^{-1}). Data in Table 2-1 show that T did not alter (P>.05) the GH secretory profile from that of controls. These observations are not consistent with published evidence indicating that T stimulates rat GRF-mRNA expression (Zeitler et al., 1990) and increases rat SRIF-mRNA abundance (Argente et al., 1991). Also, T is metabolized in the hypothalamus via 5α-reductase and(or) the aromatase pathway (Naftolin and Ryan, 1975). Since T is considered less potent in eliciting the androgenic response than its metabolites (Martini, 1982), the data in the

present study suggest that there is little or no conversion of T to its active metabolites.

In contrast to T, perifusion of the AP-CH plus HYP-CH with DHT (Figure 2-1) increased ($P<.01$) AUC-GH (24.7 ± 2.1 vs 44.3 ± 3.9 $ug.min.ml^{-1}$). Administration of DHT to AP cells increased AUC-GH by 32.3%, while perifusion of AP-CH plus HYP-CH with DHT increased AUC-GH by 79.4%. Table 2-1 also shows that DHT increased ($P<.01$) the baseline of GH secretion (29.4 ± 1.8 vs 43.1 ± 3.7 $ng.ml^{-1}$), as well as increased ($P<.05$) the amplitude of GH spikes (52.2 ± 22 vs 97.3 ± 28 $ng.ml^{-1}$). These data suggest that DHT acts on AP cells as well as on HYP slices to increase GH release.

The effect of 3α-diol at all concentrations studied on AUC-GH was similar to that observed with DHT (Figure 2-2). The AP cells perifused with 3α-diol, had increased ($P<.05$) AUC-GH which was 33.6% above controls (28.9 ± 2.4 vs 38.6 ± 3.6 $ug.min.ml^{-1}$). In addition, when 3α-diol was perifused on the AP cells plus HYP slices, it increased ($P<.05$) AUC-GH to approximately 56.2% above control (27.6 ± 2.3 vs 43.1 ± 4.2 $ug.min.ml^{-1}$). The response was similar at all three concentrations of 3α-diol. In some androgen-sensitive peripheral tissues, 3α-diol is an inactive metabolite (Wilson, 1972); however, 3α-diol has been shown to be a more potent suppressor of LH in the pituitary than other androgens (Zanisi et al., 1973). Moreover, 3α-diol is present in higher

Figure 2-2. The effect of 3α-diol (0, 10^{-10}, 10^{-12}, 10^{-14} M) on area under the curve (AUC) of GH release from hypothalamo-pituitary cultures perifused in series. Tissues were obtained from heifers. 3α-Diol were applied to anterior pituitary (AP) cells (PIT) or to AP cells plus hypothalamic slices (PIT+HYP) for 24 h. Then , 20 min fractions were collected for 12 h, frozen, assayed for GH, and AUC-GH was calculated. Each bar represents the average of three replicates (two animals per replicate) ± SEM.


PIT
PIT+HYP
AUC-GH (ug.min.ml-1)
50
40
30
20
10
0
0
10
12
14
3a-diol (-Log Concentration)

concentrations in the anterior pituitary and the hypothalamus than in peripheral tissues (Martini 1982).

The conversion of DHT to 3α-diol is irreversible in peripheral tissues (George and Peterson 1988), but reversible in the pituitary and hypothalamus (Noma et al, 1975). Thus it is possible that the effect of 3α-diol in the present study represents that of its reconversion to DHT. Also, it is possible that 3α-diol and DHT have different affinities for the androgen receptor (Barley et al., 1975; Hannouch et al., 1978) or they may act through other steroid receptors, since all sex steroids have wide stereospecificity for their receptors (Schmidt and Katzenellenbogen, 1979). Another possibility is that 3α-diol might act via a non-receptor mechanism, i.e., interaction with the cell membrane as proposed by Baulieu (1979). However, the biological significance of 3α-diol in the central nervous system remains to be determined.

The present study indicates that E_2 does not have a direct effect on the anterior pituitary but exerts its effects on the hypothalamus to regulate GH secretion. In contrast, the androgen metabolites, but not T, influenced GH secretion by direct action on the pituitary as well as on the hypothalamus.

In order to mimic the endogenous secretory pattern of T in bulls, the effect of pulsatile administration of T on GH secretion from AP cells and from AP cells plus HYP slices was studied. When the same total concentration of T was delivered

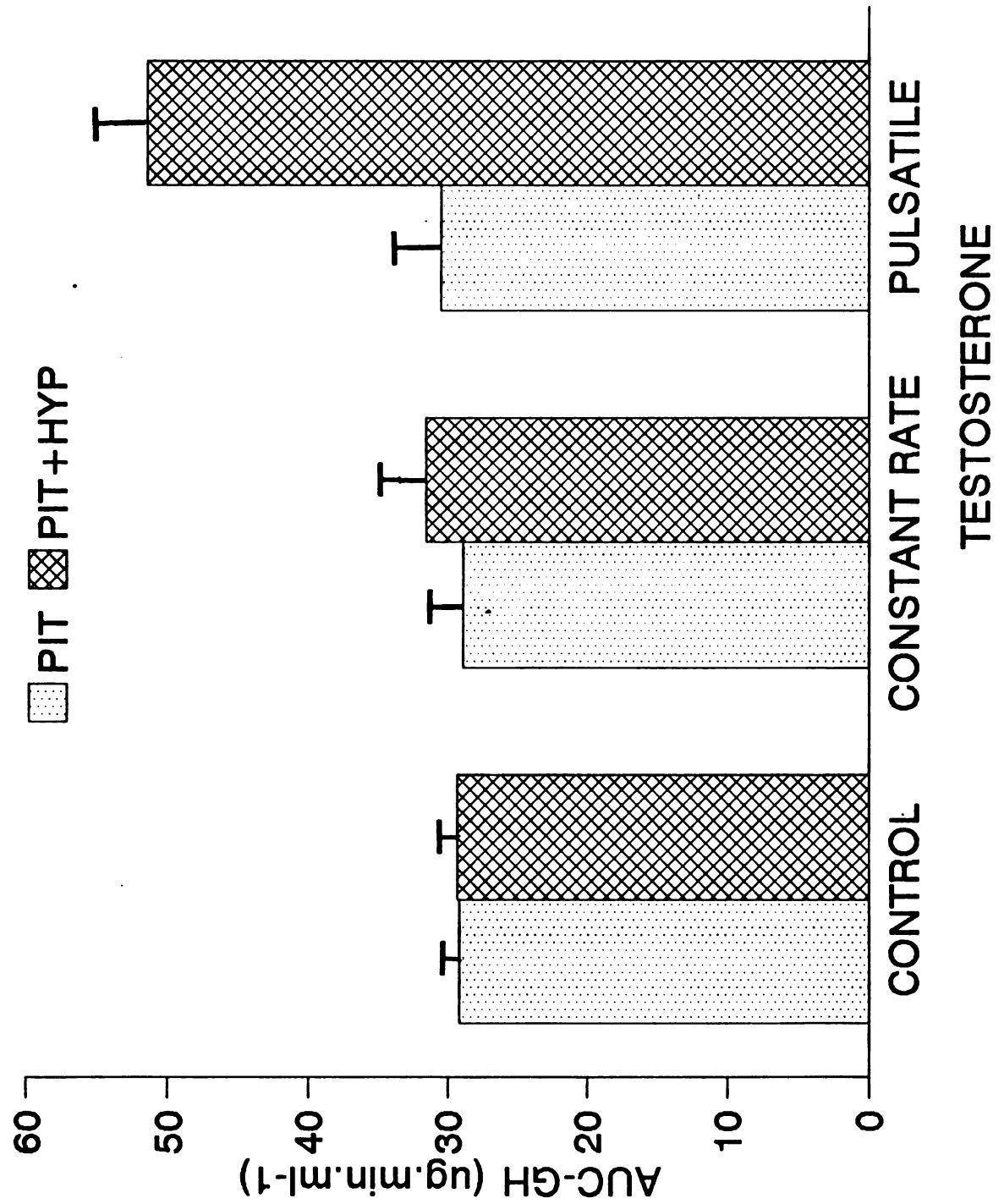
at a constant rate (2.9 $ng.ml^{-1}$) or in pulses (baseline .1 $ng.ml^{-1}$, peak amplitude 10 $ng.ml^{-1}$, and 3 $peaks.12h^{-1}$, Appendix H) to AP cells, neither had an effect (P>.05) on AUC-GH (29.2±2.5 for control vs 28.9±2.5 and 30.5±2.7 $ug.min.ml^{-1}$ for constant rate and pulsatile T, respectively, Figure 2-3). Moreover, when T was applied at a constant rate to AP cells plus the HYP slices, no effect (P>.05) on AUC-GH (29.3±2.3 vs 31.6±2.7 $ug.min.ml^{-1}$) was observed. Likewise, data in Table 2-2 show no effect (P>.05) of constant rate T on GH secretory

TABLE 2-2. EFFECT OF CONSTANT RATE AND PULSATILE TESTOSTERONE ADMINISTRATION ON GH RELEASE FROM AP CELLS CULTURED IN SERIES WITH HYP SLICES[1]

		Method of T Administration	
Variable[2,3]	Control	CONSTANT RATE	PULSATILE
Baseline ($ng.ml^{-1}$)	24.4±1.9	27.4±1.9	38.7±3.3 **
Number of Peaks (12h)	3.8±1.2	4.2±.45	2.8±.2 **
Amplitude ($ng.ml^{-1}$)	48.8±19.7	46.1±21.1	101.2±35.7
Peak length (min)	80.0±15.7	60.0±19.7	93.3±27.3
Inter-peak interval (min)	140±76.2	112±67.1	290±63.9 *

[1]AP cells were cultured in series with HYP slices; perifused with testosterone (10^{-9}M) at constant rate or pulsatile (baseline, .5 $ng.ml^{-1}$; peak amplitude, 10 $ng.ml^{-1}$, frequency, .0038 $peak.min^{-1}$) for 24 h. Media were collected every 20 min for 12 h, frozen and assayed for GH.
[2]Values represent the effect of steroids on GH secretory pattern.
[3]Values are means ± SEM of five replicates.
* Significant (P<.05), ** significant (P<.01) from control.

Figure 2-3. The effect of testosterone (T, 10^{-9}M) on area under the curve (AUC) of GH release from hypothalamo-pituitary cultures perifused in series. Tissues were obtained from heifers. T was administered at a constant rate (2.9 ng.ml^{-1}) or pulsatile (baseline .5 ng.ml^{-1}, peak amplitude 10 ng.ml^{-1}, with six peaks per 24 h). T was applied to anterior pituitary (AP) cells (PIT) or to AP cells plus hypothalamic slices (PIT+HYP) for 24 h. Then , 20 min fractions were collected for 12 h, frozen, assayed for GH, and AUC-GH was calculated. Control: non-steroid treated chambers. Each bar represents the average of five replicates (two animals per replicate) ± SEM.


PIT
PIT+HYP
AUC-GH (ug.min.ml-1)
0
10
20
30
40
50
60
CONTROL
CONSTANT RATE
PULSATILE
TESTOSTERONE

profile criteria. However, pulsatile T applied to AP cells plus HYP slices increased ($P<.01$) AUC-GH by 75.4% over controls (29.3 ± 2.3 vs 51.4 ± 3.7 ug.min.ml^{-1}) as shown in Figure 2-3. The data in Table 2-2 show that pulsatile T administration increased ($P<.01$) the baseline of GH release (24.4 ± 1.9 vs 38.7 ± 3.3 ng.ml^{-1}), peak GH amplitude (48.8 ± 19.7 vs 101.2 ± 35.7 ng.ml^{-1}), and inter-peak interval (140 ± 76.2 vs 290 ± 63.9 min).

These data indicate that the method of T administration, i.e., constant rate or pulsatile, influences GH secretion significantly. In general, the pattern of T secretion in gonadally intact post-pubertal males is episodic (McCarthy et al., 1978). Schanbacher et al. (1980) suggested that the endogenous pattern is needed to maximize growth performance in sheep. Also, intact bulls, that have episodic T secretion, have greater circulating GH concentrations than steers, which essentially have non-detectable T concentrations (Anfinson et al., 1975; Davis et al., 1977). Bulls also have greater GH concentrations than steers that have received T replacement. Daily injection of T or T implants in wether lambs maintain androgen concentrations above those of postpubertal rams; however, the latter still have a greater circulating GH concentrations (Davis et al., 1977).

Roselli et al. (1991) reported similar results in adult rhesus monkeys. Tilbrook et al. (1991) suggested that the mode

of T replacement (constant rate or episodic) is important for maximizing T action in the regulation of LH and FSH secretion. D'Occhio et al. (1983) and Schanbacher (1985) suggested that providing a constant concentration of T through the use of implants elicits a different response than similar concentrations derived from episodic pulses on hypothalamic LHRH in gonadally intact rams. Taken together these observations suggest that the pattern of T secretion is an important regulator of GH secretion. There is a clear need to determine the relation between hypothalamic neuropeptides, growth hormone and testosterone secretory pattern in gonadally intact males. To date no such studies have been reported.

Because pulsatile T administration increased GH release while constant rate administration did not, it was necessary to determine if the difference in results was due to the metabolites produced by pulsatile T. Low concentrations of T induce 5α-reductase activity (Pasmanik and Callard, 1988); whereas, high concentrations induce aromatase activity (Roselli et al., 1987). Thus, we speculate that pulsatile T administration may have induced these enzymes to produce the metabolites of T while constant rate T administration did not.

These latter possibilities could be studied by using selective inhibitors of 5α-reductase and aromatase, respectively. Unfortunately only the aromatase inhibitor was available for these studies. Thus, pulsatile T was administered to AP cells and to AP cells plus HYP slices in

the presence or absence of an inhibitor of aromatase (AI) and these were compared with control cultures (no T). Because metabolites of T include E_2 as well as DHT, equimolar concentrations (10^{-11}M) of E_2 and DHT were added to the same media and perifused on AP cells and on AP cells plus HYP slices to assess the combined effects of both metabolites on GH release.

The effect of pulsatile T (10^{-9}M) administration in the presence or absence of the aromatase inhibitor (AI, 10^{-7}M), as well as the combined effect of DHT (10^{-11}M) and E_2 (10^{-11}M) on AUC-GH is presented in Figure 2-4. When T with or without AI was applied to the AP cells, no effect ($P>.05$) on AUC-GH was observed (25.6 ± 2.3 for control vs 24.8 ± 2.3 and 27.2 ± 2.4 ug.min.ml^{-1}, for T with or without AI, respectively). These data support the previous finding in the present study, i.e., T administered to AP cells had no effect on GH release because it does not appear to be aromatized to E_2 or reduced to any of the androgenic metabolites. These data agree with previous reports that AP cells do not possess aromatase activity (Aakvaag and Haug, 1979; Cohen et al., 1980; Martini, 1982). The data also indicate that the AI used in this study did not affect GH release from the anterior pituitary cells.

As observed previously in this study, administration of pulsatile T to AP cells plus HYP slices increased ($P<.01$) AUC-GH (27.8 ± 2.1 vs 47.7 ± 3.1 ug.min.ml^{-1}, Figure 2-4). Table 2-3 shows that pulsatile T increased ($P<.01$) the baseline of GH

Figure 2-4. The effect of pulsatile testosterone (T, 10^{-9}M) in the presence or absence of aromatase inhibitor (AI) as well as the combined effect of dihydrotestosterone (DHT, 10^{-11}M) and estradiol-17β (E, 10^{-11}M) on area under the curve (AUC) of GH release from hypothalamo-pituitary cultures perifused in series. Tissues were obtained from heifers. Control chambers did not receive steroid treatment. Gonadal steroids were applied to anterior pituitary (AP) cells (PIT) or to AP cells plus hypothalamic slices (PIT+HYP) for 24 h and 20 min fractions were collected for 12 h, frozen, assayed for GH, and AUC-GH was calculated. Each bar represents the average of five replicates (two animals per replicate) ± SEM.

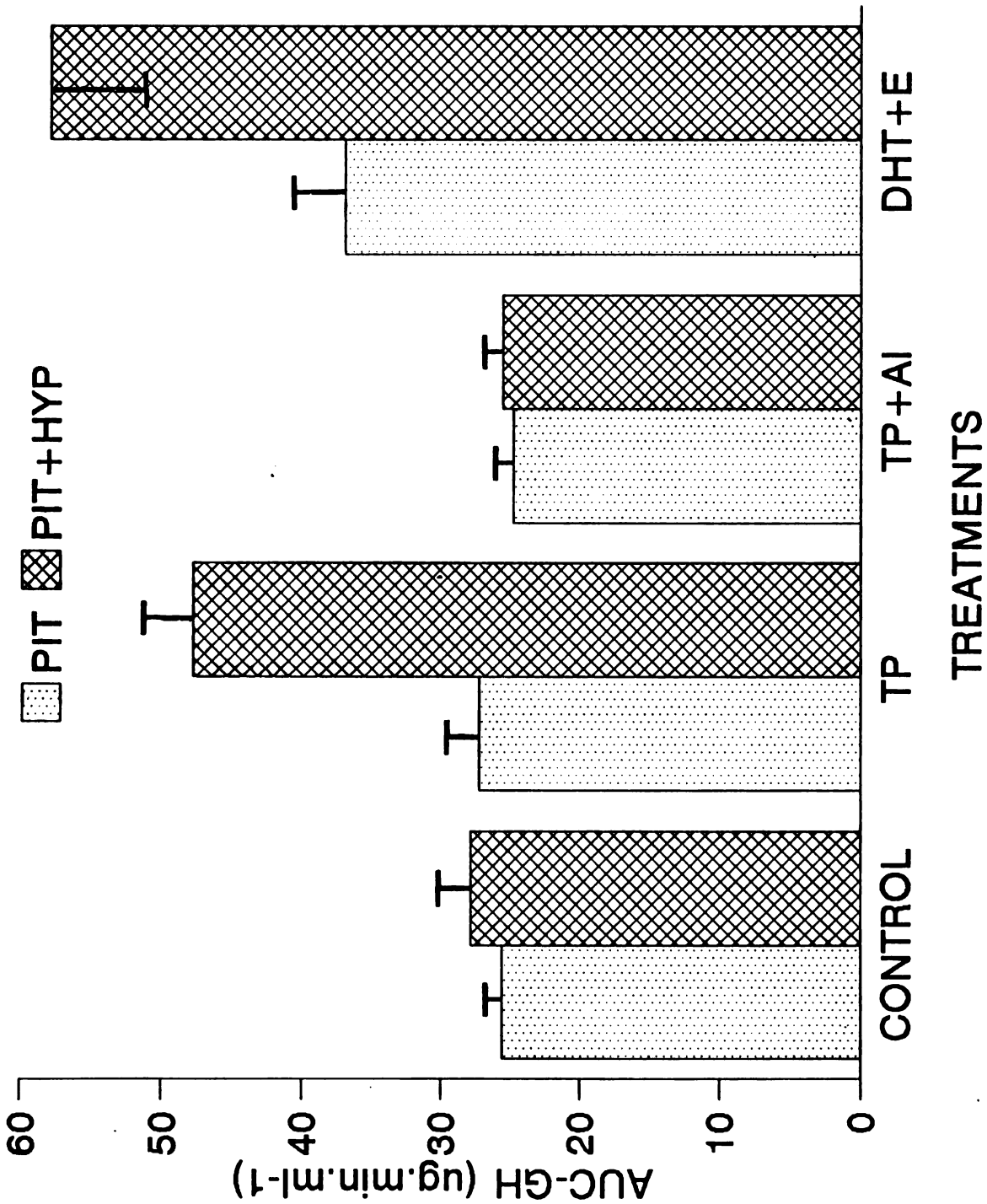

PIT
PIT+HYP
AUC-GH (ug.min.ml-1)
0
10
20
30
40
50
60
CONTROL
TP
TP+AI
DHT+E
TREATMENTS

TABLE 2-3. EFFECT OF PULSATILE T ADMINISTRATION IN THE PRESENCE OR ABSENCE OF AI AND THE COMBINED EFFECT OF E_2 AND DHT ON GH RELEASE FROM AP CELLS CULTURED IN SERIES WITH HYP SLICES[1]

Variable[2,3]	Control	TP	TP+AI	DHT+E_2
Baseline ($ng.ml^{-1}$)	20.8±1.8	* 33.7±3.1	18.1±4.3	* 34.6±3.7
Number of peaks (12h)	3.8±1.1	3.0±.84	4.0±1.2	4.2±.45
Amplitude ($ng.ml^{-1}$)	35.1±22	** 112.9±7	64.1±14	** 142.1±28
Peak length (min.)	46.7±11	75.8±24	70.1±12	* 90.1±24
Inter-peak interval (min)	187.7± 76.1	233.7± 87.6	219.3± 65.6	184.0± 86.1

[1]AP cells were cultured in series with HYP slices; perifused with TP: pulsatile T (baseline, .5 $ng.ml^{-1}$; peak amplitude, 10 $ng.ml^{-1}$, frequency, .0038 $peak.min^{-1}$); TP+AI: pulsatile testosterone in the presence of an aromatase inhibitor (100 $ng.ml^{-1}$); DHT+E_2: combined dihydrotestosterone (10^{-11}M) and estradiol-17β (10^{-11}M) for 24 h. Media were collected every 20 min for 12 h, frozen and assayed for GH.
[2]Values represent the effect of steroids on GH secretory pattern.
[3]Values are means ± SEM of five replicates.
* Significant (P<.05), ** significant (P<.01) from controls.

release and GH peak amplitude. This effect appears to be the net result of the combined action of T as well as its metabolites E_2, DHT and(or) 3α-diol. We speculate that blocking aromatization of T eliminated the effect of E_2. Thus, the effect of T in the presence of AI, was the net result of T and its reduced metabolites DHT and(or) 3α-diol. The data in Figure 2-4 show that the presence of the AI abolished the pulsatile T effect (P>.05) on AUC-GH (27.8±2.1 vs 25.5±2.1).

The GH release profile data in Table 2-3 support the data in Figure 2-4. The present experiments indicate that aromatase activity is present in the HYP slices and suggest that aromatization of T to E_2 may be at least in part the mechanism whereby T regulates GH secretion. The possibility that the AI used in this experiment may act through the steroid receptors can be excluded because it does not interact with any steroid receptors (Jones, 1992). These data suggest that the aromatization pathway may be responsible for the increase in GH secretion in response to pulsatile administration of T. The proportion of the T metabolites and the interactions between the metabolites in GH regulation remains to be established.

When DHT was combined with E and applied to the AP cells a 44.2% increase ($P<.05$) in AUC-GH was observed (25.6 ± 2.3 vs 36.9 ± 3.2 ug.min.ml^{-1}, Figure 2-4). Since, previous results in this study indicate that E_2 had no direct effect on GH secretion from AP cells, the data suggest that the increase in GH secretion is the result of DHT action on the AP cells.

When the combination of DHT and E_2 was applied to AP cells plus HYP slices, a marked increase ($P<.01$) in AUC-GH was observed (27.8 ± 2.1 vs 57.8 ± 3.9 ug.min.ml^{-1}, Figure 2-4). This increase is greater than that observed for either E_2 or DHT applied individually to the AP cells plus HYP slices (107.1% for DHT plus E_2 vs 76.5 and 79.4% as compared with controls, for E_2 and DHT, respectively). In the present study, it was

not possible to determine if the observed increase was due to an additive effect of these steroids on GH secretion. However, E_2 increases the sensitivity of specific brain regions to DHT by regulating androgen receptor binding in the hypothalamus (Handa et al., 1987). Johnson et al. (1991) suggested that DHT may potentiate the effect of E_2 by increasing its binding to the receptor. It is also possible that DHT may compete with estradiol for the cytosolic estrogen receptor and after translocation to the nucleus it acts as the E_2-receptor complex (Ruh et al., 1975). This androgen-mediated translocation of estrogen receptors may be followed by induction of estrogen-stimulated specific proteins (Garcia and Rochefort, 1977). Further studies are needed to determine the precise role of the interactions between steroids on the regulation of GH secretion.

SUMMARY

The influence of E_2 on GH secretion is by direct action at the hypothalamus, but it had no direct effect on the pituitary. The active metabolites of androgens affect GH release by direct effects on the pituitary as well as the hypothalamus. The pulsatile pattern of T perifusion markedly stimulated GH release but continuous administration had no effect. The stimulatory effects of pulsatile T appear to be due to the metabolites of T, i.e., E_2, DHT and(or) 3α-diol. However, the mechanism(s) of these observations has yet to be elucidated.

CHAPTER 3

ROLE OF SEX STEROIDS IN MODULATING GROWTH HORMONE-RELEASING HORMONE AND SOMATOSTATIN IN PERIFUSED BOVINE HYPOTHALAMIC SLICES

ABSTRACT

Bovine hypothalamic (HYP) slices were perifused (.02 $ml.min^{-1}$) with media containing either estradiol-17β (E_2, $10^{-11}M$), testosterone (T, $10^{-9}M$), dihydrotestosterone (DHT, $10^{-11}M$) or androstandiol (3α-diol, $10^{-12}M$) for 24 h. Ten minute fractions for each hour were pooled, and assayed for growth hormone (GH)-releasing hormone (GRF) and somatostatin (SRIF). In control chambers area under the curve (AUC) of GRF and SRIF release were (63.8 and 45.4 $ng.min.ml^{-1}$, respectively). Compared with controls, E_2 increased GRF 27% and reduced SRIF 33%, while constant rate perifusion of T and DHT, respectively, increased both GRF (66 and 104%) and SRIF (60 and 55%). Also, 3α-diol increased GRF and SRIF (84 and 45%).

With pulsatile T administration AUC of GRF and SRIF in control chambers was 61.2 and 43.9 $ng.min.ml^{-1}$. Both constant rate and pulsatile T perifusion increased GRF (71 and 125%) and SRIF (79 and 46%). The effect of pulsatile T in the presence or absence of an aromatase inhibitor (AI) had no effect on GRF release, but the AI increased SRIF (64% over pulsatile T without AI). When DHT and E_2 were perifused together they increased GRF and reduced SRIF. These data indicate that: 1) androgens stimulate GRF and SRIF while

estrogen stimulate GRF and inhibit SRIF, 2) pulsatile T appears to maintain elevated GRF compared with SRIF, 3) aromatization of T to E_2 maintains low concentrations of SRIF similar to E_2 perifusion.

INTRODUCTION

Growth hormone (GH)-releasing factor (GRF) and somatostatin (SRIF) are the principal hypothalamic peptides that regulate GH secretion from the anterior pituitary (Guillemin et al., 1984). While exhibiting independent rhythmicity, when GRF predominates it stimulates GH and when SRIF predominates it inhibits GH secretion (Frohman, 1990). Evidence to date suggests that the pulsatile secretion of GH is a consequence of a complex interdependent modulation by GRF and SRIF (Plotsky and Vale, 1985). The pulsatile pattern of GH secretion is sexually dimorphic in sheep and cattle (Davis and Borger 1974; Anfinson et al., 1975; Davis et al., 1977). In addition, modulation of GH secretion by gonadal steroids has been reported (Davis and Borger, 1974; Davis et al., 1977). Some evidence indicates the effect on pituitary regulation is indirect (Silverman et al., 1988; Hassan et al., 1992). Several studies have shown that the hypothalamus is an important site of gonadal steroid action in modulating GH secretion (Werner et al., 1987; Tsagarakis et al., 1989). In male rats, T stimulates hypothalamic GRF-mRNA (Zeitler et al., 1990) and SRIF-mRNA abundance (Baldino et al., 1988; Chowen-Breed et al., 1989). Immunohistological studies with female rats have demonstrated the presence of E_2 receptors on hypothalamic GRF neurons and suggested a direct action of E_2 on those neurons (Shirasu et al., 1990). Moreover, in situ hybridization studies revealed that E_2 treatment increases

SRIF gene transcription (Werner et al., 1988). Males generally have greater GRF-mRNA as well as SRIF-mRNA abundance than females (Argente et al., 1991). It is worth noting that measuring the transcription of any gene may not necessarily indicate hormone bioavailability. Transcription of a gene is only one step in a general scheme that starts with steroid hormone penetration of the plasma membrane (Li, 1987), and ends with processing the hormone (Silverman et al., 1988), which is secreted into the hypophyseal portal circulation (Maertens and Denef, 1987).

The precise interrelation between GRF, SRIF and GH secretion is not well understood. The technical difficulty in assaying GRF and SRIF from the hypothalamo-hypophyseal portal circulation has contributed to this paucity of information (Frohman, 1990). However, with hypothalamic explants in a perifusion system, it is possible to study the effects of gonadal steroids on release of GRF and SRIF. Also, hypothalamo-pituitary co-culture provides a system for studying the steroid-hypothalamo-pituitary interdependent modulation.

Thus, the objectives of the present study were to: 1) assess the effect of T, E_2, DHT and 3α-diol on GRF and SRIF release from hypothalamic slices; 2) clarify the relation between these steroids on the hypothalamus and the anterior pituitary in modulating the GH secretory pattern.

MATERIALS AND METHODS

Hormones and Chemicals. Minimum essential medium (MEM)-Alpha with ribonucleosides and deoxyribonucleosides (MEM-alpha), Dulbecco's modified Eagle medium with low glucose (DMEM), Hank's balanced salt solution (HBSS), calcium-magnesium free HBSS (CMF-HBSS), MEM amino acid solution 10X, MEM non-essential amino acid solution 10X and newborn calf serum (lot# 4705) were purchased from GIBCO Laboratories, Grand Island, NY. Collagenase type 1A, pancreatin 4X N.F., antibiotic antimycotic solution, Nystatin, penicillin-streptomycin solution, trypan blue stain .4%, bovine serum albumin (BSA, A-7888), DEAE-Sephadex G-50, and somatostatin-14 were purchased from Sigma Chemical Company, St. Louis, MO. The bovine growth hormone-releasing factor 1-44-NH_2 (GRF) and the aromatase inhibitor (LY43578) were generously donated by Lilly Research Laboratories, Greenfield, IN.

Tissue Preparation. Heifer hypothalami were obtained from the Michigan State University Meat Laboratory. At slaughter bovine brains were rapidly removed, as aseptically as possible, placed in ice-cold oxygenated HBSS (see Appendix A), and transferred (within 10 min) to the tissue culture laboratory. All subsequent procedures were performed under sterile conditions.

Preparation of Hypothalamic Slices. Brains were sectioned mid-sagittally and hypothalami dissected out and sagittally sliced (≤1 mm thick). Schoenemann et al. (1985) described the hypothalamic block in cattle, as bordered rostrally by the optic chiasm, lamina terminalis and anterior commissure; caudally by the mammillary bodies (Figure 1-2.A); dorsally by the thalamic border (9-11 mm from base of the brain); and laterally by the hypothalamic sulci. The lateral dimension of the ovine (Moss et al.,1980) and bovine (Schoenemann et al., 1985) hypothalamus has been reported to extend approximately 3-5 mm from the midline of the brain.

The hypothalamic slices were weighed, coated with DEAE-Sephadex (DEAE-S, see Appendix B), loosely folded and placed in 5cc (B-D) polypropylene syringes (with a Luer-Lok tip) which served as the perifusion chambers (Figure 1-3). The chambers were placed in a rack inside a CO_2 incubator (Model 3028, Forma Scientific, Marietta, OH). The chambers were then perifused (.2 $ml.min^{-1}$) with oxygenated MEM-alpha (see appendix C) for 24 h. The stock media bottle was placed inside the incubator and media were perifused using a Master Flex microprocessor pump drive (Cole-Parmer, Chicago, IL). The perifusion apparatus (4 ml volume) consisted of the chamber that contained the hypothalamic slices, Tygon tubing (.08 mm ID; .24 mm OD; Fisher, Pittsburg, PA), straight polypropylene miniature barb fittings (Cole-Parmer, Chicago, IL), Precision Glide 18G disposable needles (B-D) Baxter S/P (McGaw Park,

IL). Media effluent from the hypothalamic chamber was collected (LKB 2070, Ultrorac II, Broma) every 10 min for 12 h, frozen (-20°C) and assayed for GRF and SRIF concentrations.

Bovine GRF Radioimmunoassay. The concentrations of GRF were determined using a commercially available RIA kit (Peninsula Laboratories, Belmont, CA). The kit was developed for human pancreatic GRF (hpGRF). The antiserum used for this assay was raised against synthetic hGRF(1-44-NH_2), which differs from the bovine GRF by 5 amino acid residues. The validity for GRF measurements in the bovine has been conducted in Peninsula Laboratories. They reported a 100% cross-reactivity with bovine and ovine GRF. These kits were validated for bovine GRF in culture media (details are in Appendix D) in cooperation with Dr. Heiman's Laboratory (Lilly Research Laboratory, Greenfield, IN).

Somatostatin Radioimmunoassay. The concentration of SRIF was determined using SRIF RIA-kits (Peninsula Laboratories, Belmont, CA). Antiserum used for this assay was raised against synthetic somatostatin-14, and was found to have 100% cross-reactivity with SRIF-14, SRIF-28, and SRIF-25. The kits were validated for bovine SRIF in media (details are presented in Appendix E) in cooperation with Dr. Heiman's Laboratory (Lilly Research Laboratory, Greenfield, IN).

Experimental Protocols. The protocol for each experiment is explained in the figure legend for each experiment.

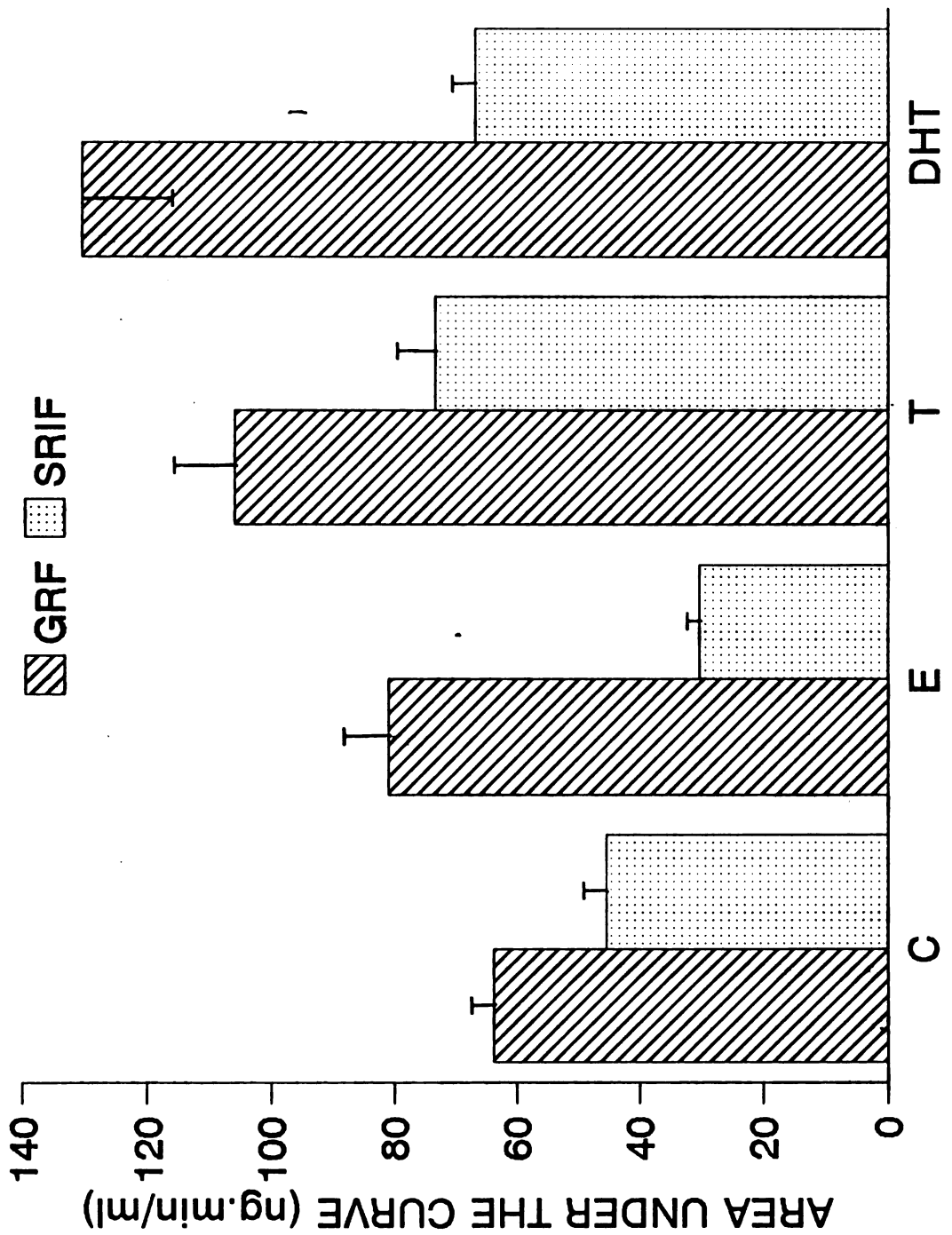
Statistical Analysis. Data were analyzed by a randomized complete block design (Gill, 1978). Differences among groups were analyzed by two-way analysis of variance. Student's t test was used where appropriate to detect differences among individual means. The criterion for significance was either $P<.05$ or $P<.01$. The results are presented as the mean $\pm$ SEM of 5 experiments, unless otherwise stated.

RESULTS AND DISCUSSION

The effect of T (10^{-9}M), DHT (10^{-11}M) and E_2 (10^{-11}M) perifusion on GRF and SRIF release from hypothalamic (HYP) slices is illustrated in Figure 3-1. Average area under the curve (AUC) of GRF and SRIF release in non-steroid treated control chambers was 63.8±4.1 and 45.4±2.7 ng.min.ml^{-1}, respectively. Perifusion with E_2 for 24 h increased ($P<.01$) GRF release and reduced ($P<.01$) SRIF release 27 and 33%, respectively, as compared with controls. These results are consistent with the increased GH release observed in Chapter 2 when AP cells plus HYP slices were perifused with E_2. The results of the present study support the observations of Painson and Tannenbaum, (1991) and the study of Shirasu et al., (1990) both of whom demonstrated E_2 receptors on hypothalamic SRIF and GRF neurons and suggested a regulatory role for E_2 in the secretion of both peptides.

The data reported to date on the effect of E_2 on SRIF synthesis and secretion from the hypothalamus in vivo are conflicting. Miki et al. (1988) and Werner et al. (1988) reported an increase in SRIF secretion and SRIF-mRNA abundance in response to E_2 replacement in ovariectomized rats. However, Tsagarakis et al. (1989) found no difference in hypothalamic content and potassium-stimulated secretion of SRIF between male and female rats. Chowen-Breed et al. (1989) found that female rats had lower SRIF-mRNA abundance than male rats which

Figure 3-1. The effect of control (C), testosterone (T, 10^{-9}M), dihydrotestosterone (DHT, 10^{-9}M) and estradiol-17β (E, 10^{-11}M) on area under the curve (AUC) of GRF and SRIF release from hypothalamic slices. Tissues were obtained from calves. HYP slices were perifused (.2 $ml.min^{-1}$) with media plus gonadal steroids for 24 h. Then, 10 min fractions were collected for 12 h. Fractions of each hour were pooled, frozen, assayed for GRF and SRIF, and AUC was calculated. Each bar represents the average of three replicates (one animal per replicate) ± SEM.


AREA UNDER THE CURVE (ng.min/ml)
140
120
100
80
60
40
20
0
GRF
SRIF
C
E
T
DHT
TREATMENT

they attributed to the influence of E_2 on SRIF gene expression.

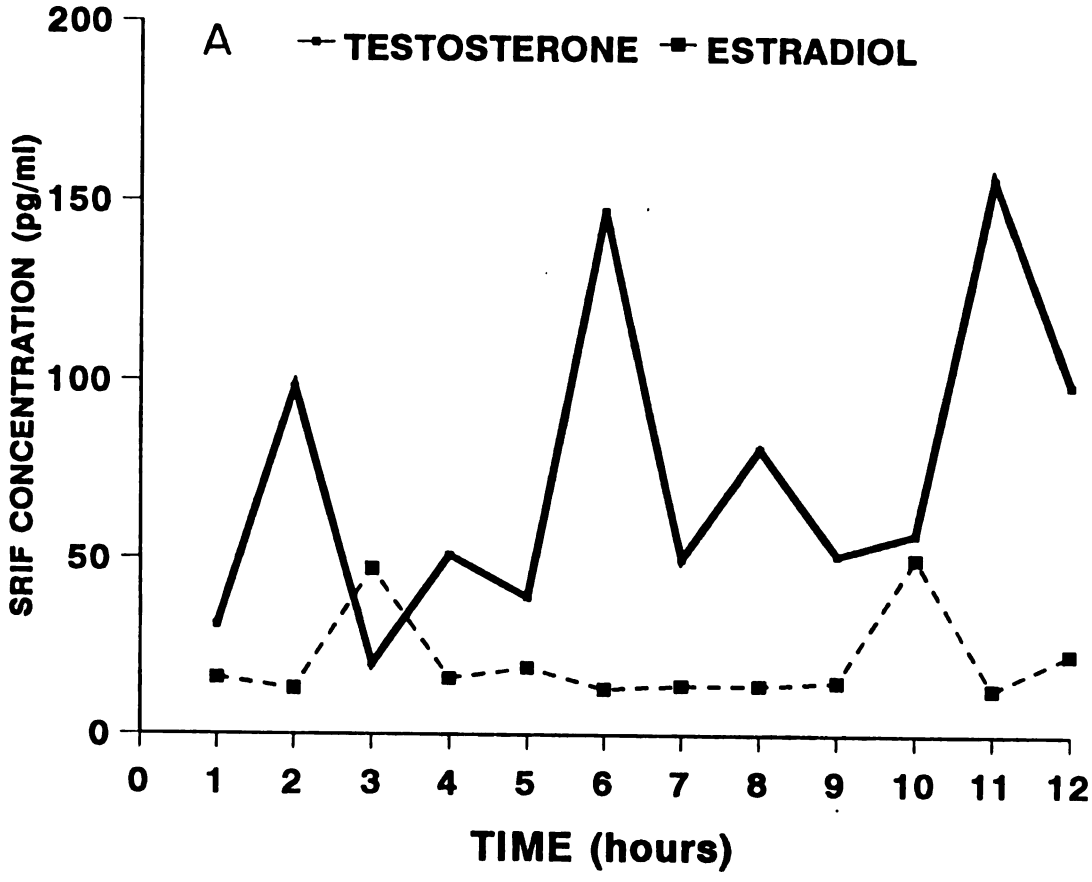
Several factors may account for the discrepancy of the E_2 effects on hypothalamic SRIF concentrations. In situ hybridization techniques are reported to be more sensitive than Northern blot hybridization analysis and thus allows the detection of low concentrations of mRNA in single cells (Shivers et al., 1986; Penshow et al., 1986). Also, availability of mRNA is an important factor in protein biosynthesis; however, it cannot be assumed that all SRIF-mRNA produced is translated into the mature peptide or that the peptide produced is actually released from the nerve terminals (Falvey and Schibler, 1991). Thus, it may be invalid to compare the results from studies that estimate the activity by mRNA abundance and those that determine the secretion of the peptide. A second factor is that the E_2 concentration of females changes over the course of an estrous cycle, although the GH secretory pattern of female rats does not appear to change during the cycle (Saunders et al., 1976). Gross (1980) reported changes in SRIF-mRNA abundance during the estrous cycle in rats which he suggested was the result of the E_2 concentrations. The data in the present study are probably of physiological relevance, since the increase in GRF and the decrease in SRIF were observed at picomolar concentrations of E_2 (10^{-11}M), which corresponds to plasma concentrations of E_2 (7 pg.ml^{-1}) in diestrus heifers (Wettemann et al., 1972).

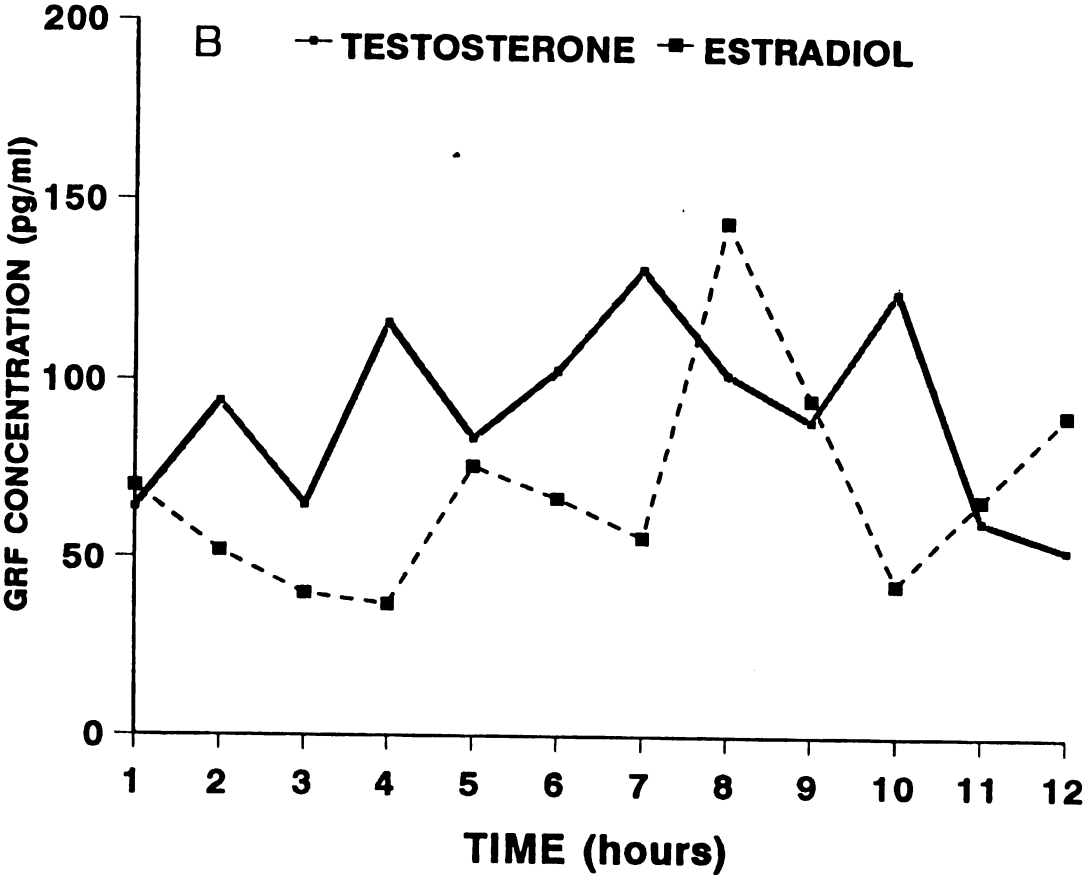
The observed increase in hypothalamic GRF release as a result of E_2 perifusion in the present study agrees with other data. The general consensus is that E_2 stimulates GRF synthesis (Werner et al., 1988) and secretion (Tsagarakis et al., 1989). This stimulatory effect of E_2 on GRF neurons has been reported to be direct (Shirasu et al., 1990) as well as indirect (Maiter et al., 1991). The presence of nuclear receptors for E_2 in the GRF-containing neurons in the hypothalamus is evidence of a direct effect on these neurons (Shirasu et al., 1990). The effect of E_2 on GRF neurons also may be mediated via biogenic amines, since E_2 receptors were found on dopamine (DA) neurons (Shirasu et al., 1990) and E_2 is known to alter DA secretion (Sar, 1984; Elis and Weiner,1987). It has been reported that DA influences GRF secretion (Kitajima et al., 1989). In addition, E_2 induces neutritic outgrowth which could conceivably lead to synaptic modulation and plasticity between GRF, SRIF and DA neurons (Toran-Allerand et al., 1983; Frankfurt et al., 1990). The synaptic communication between SRIF axons and GRF neurons in the arcuate nucleus of the rats (Liposits et al 1988) suggests a direct inhibitory effect of SRIF on GRF secretion (Katakami et al., 1988). Since E_2 reduced SRIF release in the present study, this presumably could increase GRF secretion as was observed (Figure 3-1). Also the synaptic interaction between DA and GRF neurons has been suggested to have a stimulatory

effect on GRF secretion via E_2 stimulation of DA secretion (Shirasu et al., 1990).

Painson and Tannenbaum (1991) suggested that SRIF is secreted into the hypophyseal portal blood at a relatively constant concentration in female rats rather than in a cyclical pattern as is characteristic of males. In the present study, the pattern of SRIF release from the hypothalamic slices (Figure 3-2A) with perifusion of E_2 tended to be more or less constant with a low baseline. In contrast, constant rate of perifusion of T caused SRIF release to be more pulsatile, compared with E_2, with greater peak heights (85.1 vs 25.3 $ng.ml^{-1}$), a higher baseline (50 vs 20 $ng.ml^{-1}$) and consequently greater concentrations of SRIF release (10.5 vs 3.1 $ng.12h^{-1}$). However, the 1 h interval of sampling used in this study may be inadequate to draw definite conclusions with respect to the secretory pattern. Nonetheless the present findings for E_2 effects on the hypothalamus, together with available evidence are consistent with the elevated baseline and frequent pulses observed in the GH secretory profile of ewes (Davis et al., 1977). The basline of GRF secretion tended to be higher with perifusion of T than with E_2 (Figure 3-2B). These data indicate that the effects of E_2 on GH release appear to be mediated by direct action on the HYP where it increases GRF release and decreases SRIF release to a low, relatively constant secretory profile. The secretory pattern of these two neuropeptides is the determinant of GH release as

Figure 3-2. The effect of testosterone (T, 10^{-9}M), and estradiol-17β (E, 10^{-11}M) on pattern of SRIF release from hypothalamic slices. Tissues were obtained from calves. HYP slices were perifused (.2 ml.min^{-1}) with media plus gonadal steroids for 24 h. Then, 10 min fractions were collected for 12 h, fractions of each hour were pooled, frozen, assayed for GRF and SRIF. The secretory pattern shown is one of three replications conducted for (A) SRIF and (B) GRF.


A
TESTOSTERONE
ESTRADIOL
SRIF CONCENTRATION (pg/ml)
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
TIME (hours)


B
TESTOSTERONE
ESTRADIOL
GRF CONCENTRATION (pg/ml)
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12
TIME (hours)

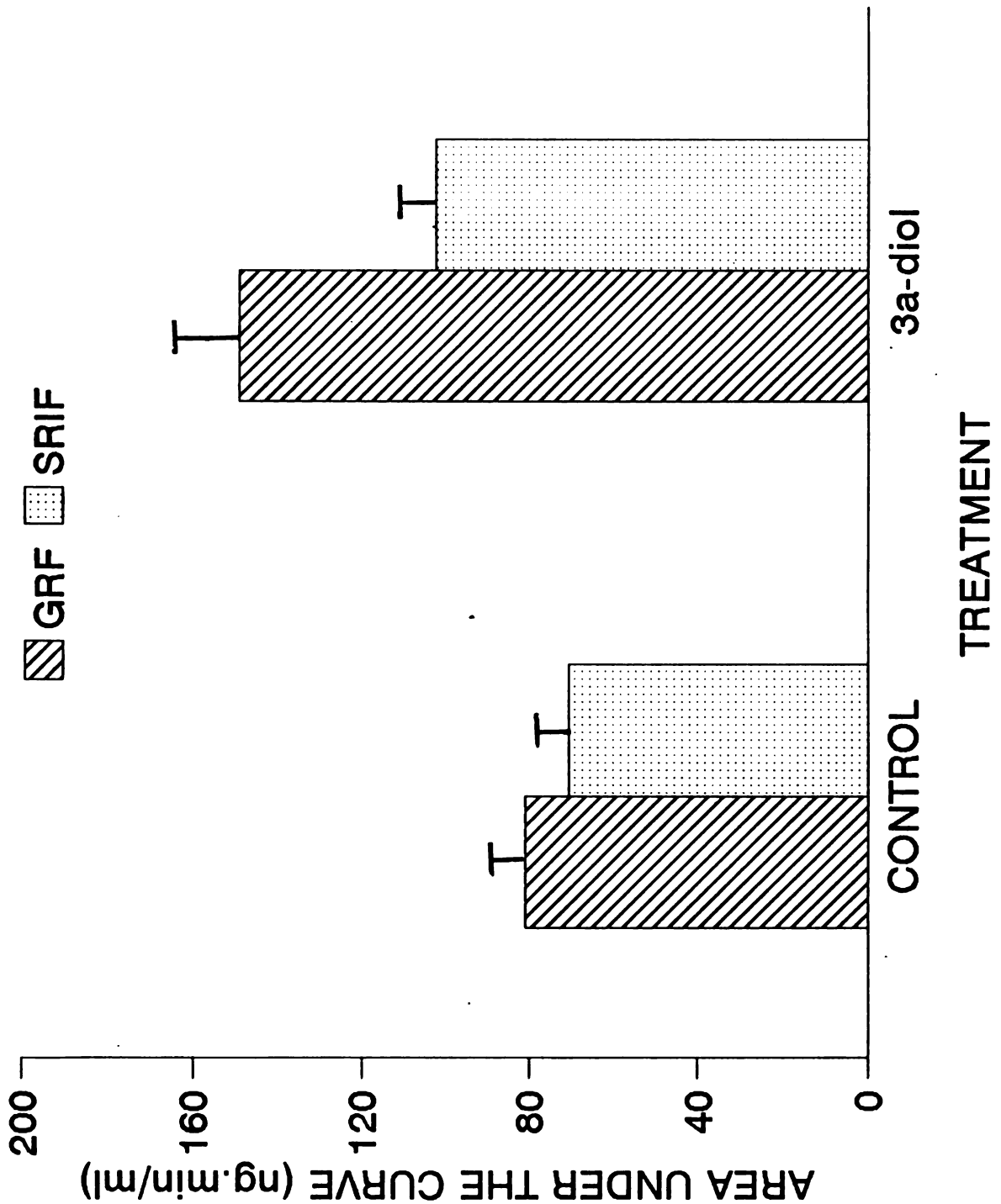
they signal the pituitary somatotropes (Guillemin et al., 1984).

The data in Figure 3-1 show that perifusion of T (10^{-9}M) at a constant rate increased (P<.01) both GRF and SRIF (66 and 60% above control, respectively). These results agree with the observations where T stimulated GRF-mRNA abundance in rats (Zeitler et al., 1990) as well as SRIF-gene expression (Baldino et al., 1988; Chowen-Breed et al., 1989). The data from the present study as well as previous findings support the contention that the hypothalamus may be a major site of androgen effects on GH secretion.

In the hypothalamus, T may exert its effects through its metabolites, i.e., via activation of the 5α-reductase pathway or the aromatase pathway or both (Martini, 1982). The reductase pathway yields DHT and 3α-diol, while the product of the aromatase pathway is E_2. In the hypothalamus 3α-diol is reconverted to DHT (Noma et al., 1975). Thus, the effect of the reduced metabolite, DHT which is a non-aromatizable androgen, on GRF and SRIF release was studied. Perifusion of DHT (10^{-11}M) for 24 h increased (P<.01) both GRF and SRIF release (104 and 55%, respectively, compared with controls, Figure 3-1).

The effects of 3α-diol (10^{-12}M) on GRF and SRIF release also was studied. Perifusion with 3α-diol increased (P<.01) AUC of GRF release (81 vs 149 ng.min.ml^{-1}) as well as SRIF release (70 vs 102 ng.min.ml^{-1}, Figure 3-3). These data

Figure 3-3. The effect of control (C), 3α-diol (10^{-12}M) on area under the curve (AUC) of GRF and SRIF release from hypothalamic slices. Tissues were obtained from calves. HYP slices were perifused (.2 ml.min^{-1}) with media plus gonadal steroids for 24 h. Then, 10 min fractions were collected for 12 h. Fractions of each hour were pooled, frozen, assayed for GRF and SRIF, and AUC was calculated. Each bar represents the average of three replicates (one animal per replicate) ± SEM.


AREA UNDER THE CURVE (ng.min/ml)
200
160
120
80
40
0
GRF
SRIF
CONTROL
3a-diol
TREATMENT

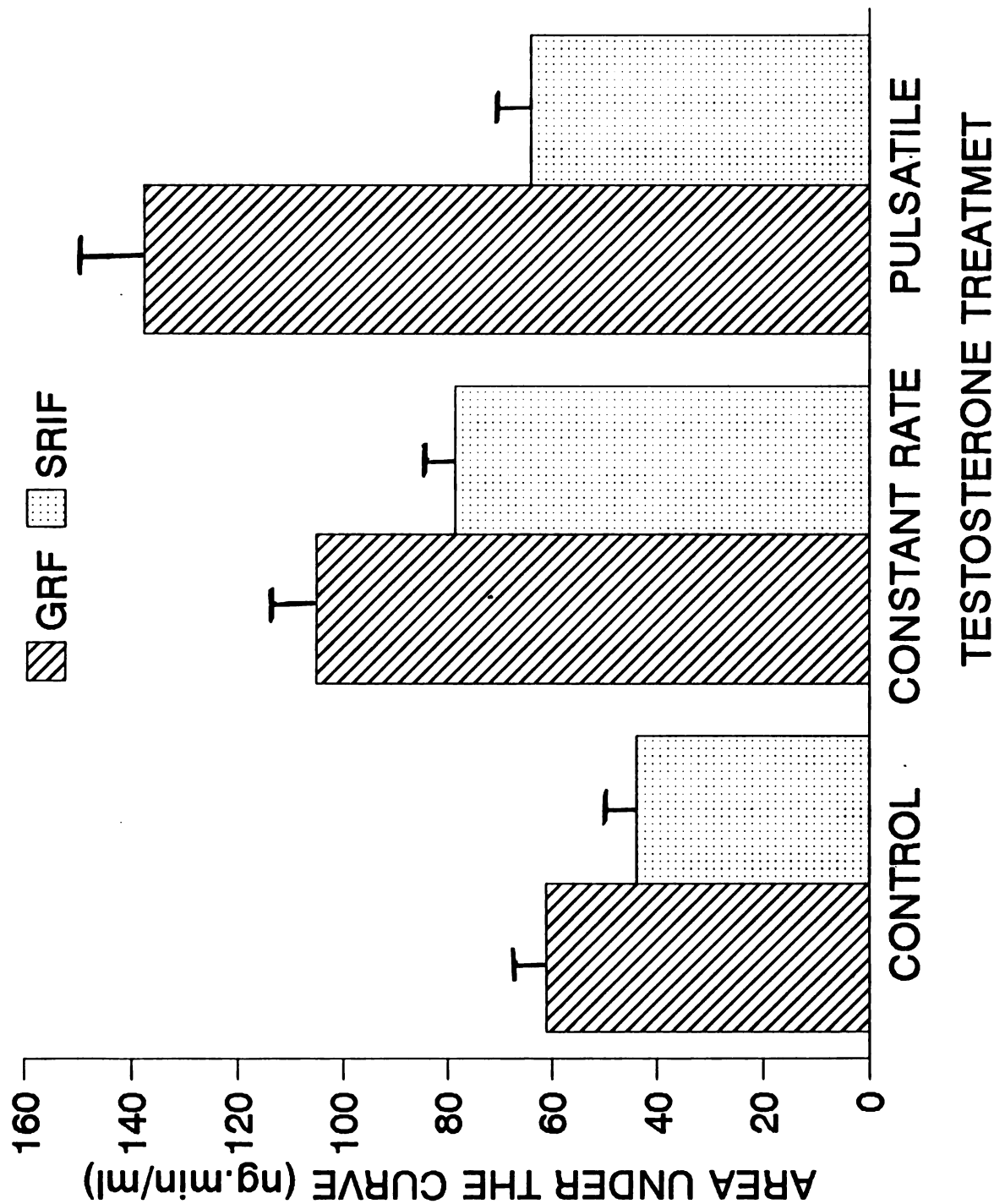
suggest that both T and its reduced metabolites DHT and 3α-diol, act through a similar mechanism to increase both GRF and SRIF. However, DHT and 3α-diol induced an even greater ($P<.05$) increase in GRF than T. This response is consistent with other data which show that DHT is the active metabolite of T action, and that DHT is more potent in stimulating androgen receptors than T (Grino et al., 1990). Since, 3α-diol may be reconverted to DHT in the hypothalamus (Noma et al., 1975), it was not possible to determine if the effects on GRF and SRIF are due to 3α-diol or DHT. The effects of 3α-diol on GRF and SRIF have not been reported previously. No differences ($P>.05$) in DHT and T effects on SRIF release were observed. Since DHT increased GRF more than T, and both DHT and T caused a similar increase in SRIF. We speculate that GRF and SRIF neurons have different sensitivities to these steroids. Coincident with these findings, Zeitler et al. (1990) suggested that different areas of the hypothalamus have different sensitivities to DHT.

Aromatization of T to E_2 is an important step in amplification of T action in the hypothalamus by enhancing DHT binding to its receptor (Noma et al., 1975; DeVries et al., 1986; Johnson et al., 1991). A possible explanation for the difference in response between T and DHT on GRF release may be due to a low conversion of T to DHT or to E_2. The 5α-reductase activity is optimal at subphysiological concentrations of T (Pasmanik and Callard, 1988) while aromatase activity is

optimal at supraphysiological concentration of T (Roselli et al., 1987). Since physiological concentrations of T were perifused at a constant rate in the present study, activation of these enzymes may have been minimal. Moreover, the possible indirect effect of DHT on GRF secretion through its effect on endogenous opioid peptides cannot be excluded (Adams et al., 1991), since ß-endorphin stimulates GRF secretion (Armstrong and Spears, 1988). These data suggest that DHT and(or) 3α-diol increase pituitary cell sensitivity to GRF as well as increase hypothalamic GRF and SRIF release. Continuous exposure of SRIF neurons to a constant rate of T may have resulted in low conversion to DHT and(or) E_2 . Thus, the persistently elevated SRIF with constant rate of T abolished the GRF-induced GH release and maintained the basal rate of GH release as was suggested by Stachura et al. (1988).

Since T is secreted episodically in gonadally intact post-pubertal males (McCarthy et al., 1978), the effects of a pulsatile pattern of T perifusion on GRF and SRIF release was studied. The effects of T (10^{-9}M) administered in a pulsatile manner or at a constant rate for 24 h on AUC of GRF and SRIF release are presented in Figure 3-4. Both perifusion rates of T increased (P<.01) AUC of GRF release (61.2±5.2 for control vs 105.1±8.9 and 137.5±11.7 ng.min.ml^{-1}, respectively). Average AUC of SRIF release in control chambers was 43.9 ng.min.ml^{-1}. When compared to control, constant rate and pulsatile perifusion of T increased (P<.01) SRIF 79 and 46%,

Figure 3-4. The effect of testosterone (T, 10^{-9}M) on area under the curve (AUC) of GRF and SRIF release from hypothalamic slices. Tissues were obtained from calves. T was administered at a constant rate (2.9 $ng.ml^{-1}$) or pulsatile (baseline .5 $ng.ml^{-1}$, peak amplitude 10 $ng.ml^{-1}$, with 6 peaks per 24 h). T was applied to anterior pituitary (AP) cells (PIT) or to AP cells plus hypothalamic slices (PIT+HYP) for 24 h. Then , 10 min fractions were collected for 12 h. Fractions of each hour were pooled, frozen, assayed for GRF and SRIF, and AUC was calculated. C: non-steroid treated chambers. Each bar represents the average of three replicates (one animal per replicate) $\pm$ SEM.


GRF
SRIF
AREA UNDER THE CURVE (ng.min/ml)
0
20
40
60
80
100
120
140
160
CONTROL
CONSTANT RATE
PULSATILE
TESTOSTERONE TREATMET

respectively. Comparing constant rate with pulsatile T, the constant rate of T administration caused a greater increase ($P<.05$) in SRIF and smaller increase ($P<.01$) in GRF secretion than pulsatile administration. Maximal 5α-reductase activity occurs in the hypothalamus when circulating concentrations of T are low (Pasmanik and Callard, 1988), and higher androgen concentrations, presumably during T peaks, stimulate aromatase activity (Roselli et al., 1987). It is tempting to speculate that the episodic profile of T secretion is necessary for optimal activity of the 5α-reductase and aromatase enzymes. It appears that androgen stimulation of SRIF is a possible mechanism by which T amplifies the GRF signal on somatotropes; since, pre-exposure of AP cells to SRIF for 30 to 60 min enhances subsequent GRF-induced GH secretion (Richardson and Twente, 1991). The pulsatile secretion of T in bulls (McCarthy et al., 1978) appears to coincide with the characteristic surge of GH secretion and is associated with the intermittent stimulation of GRF and SRIF to cause the surge in amplitude of GH secretion (Anfinson et al., 1975). The pulsatile pattern of T secretion appears to be important in generating maximal T action and probably the sexual dimorphic pattern of GH secretion.

Selective enhancement of androgen sensitivity in certain neurons in the hypothalamus has been reported (Schumacher and Balthazart, 1986). In the present study, both GRF and SRIF neurons were influenced by the pattern of T administration. A

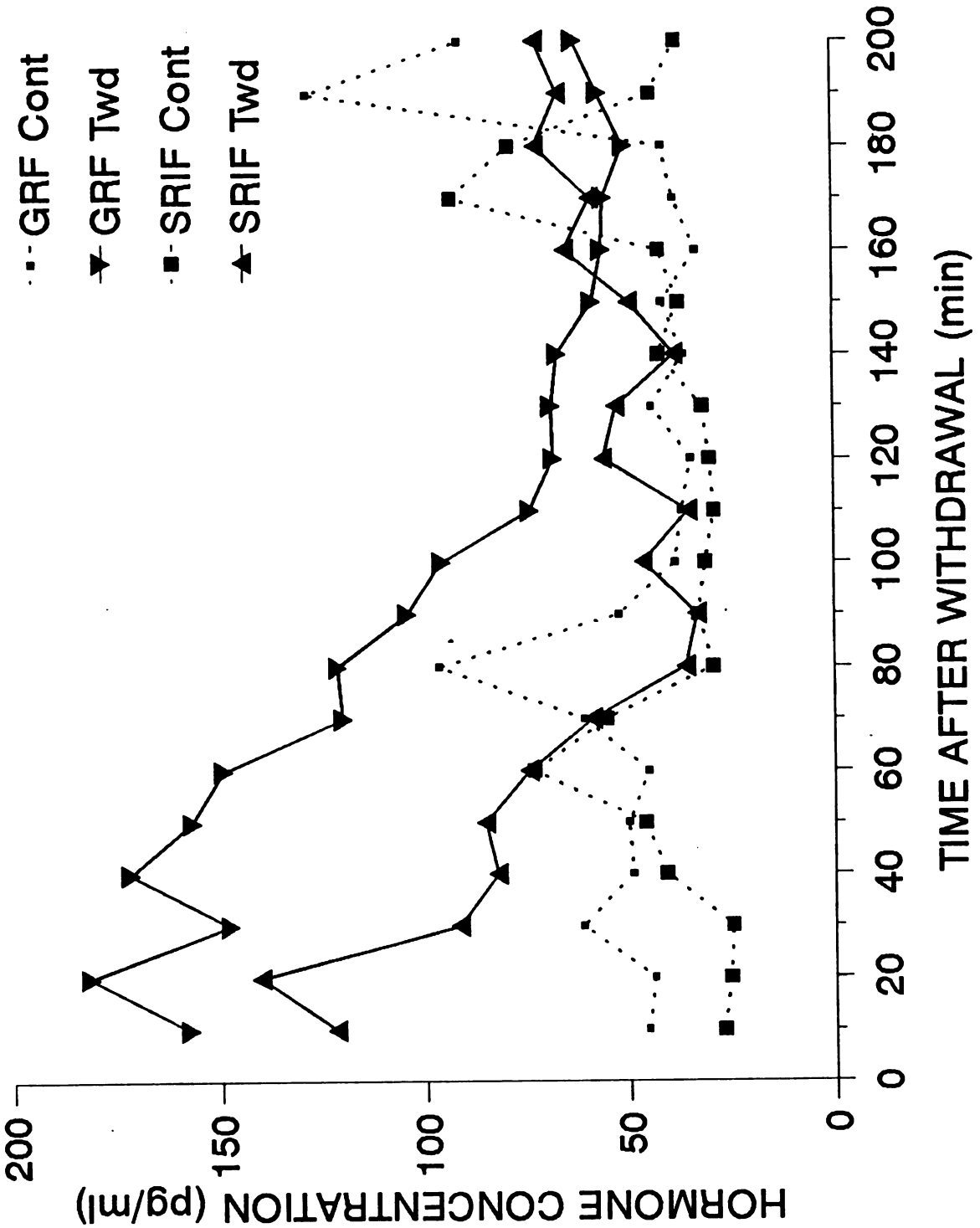
more dramatic effect on GRF was observed with the pulsatile pattern. These data are consistent with in vivo observations in that constant circulating concentrations of T as achieved with implants, provide different signals than do similar concentrations derived from episodic pulses in intact male animals (D'Occhio et al., 1983). Moreover, Roselli (1991) mentioned that peripheral administration of DHT and E_2 to castrates does not always achieve the same cellular concentrations as locally produced metabolites in intact male rats. In the present study, administration of T at a constant rate elevated GRF and SRIF in approximately equal proportions. This response did not change the ratio of GRF to SRIF, hence the signaling to the pituitary somatotropes was insufficient to change GH release. On the other hand, episodic T administration caused substantial increases in GRF and a smaller increase in SRIF. This response increased the ratio of GRF to SRIF and consequently increased somatotrope output of GH. Data in Chapter 2 demonstrated no effect of constant rate T on GH release; whereas, pulsatile T increased GH release.

The mechanism whereby episodic T influences the marked increase in GH release was investigated. Since the previous experiment showed that T, whether constant rate or pulsatile, increased both GRF and SRIF concentrations, it became necessary to determine the rate at which GRF and SRIF concentrations would decrease in the media following removal of T from the perifusate. Both GRF and SRIF concentrations

were reduced when T was removed from the culture media, (Figure 3-5). The data shows that SRIF concentrations decreased sharply to the baseline value within 60 min after the peak. Even though GRF concentration had a greater peak value, it decreased more gradually and reached the baseline value 140 min after the peak. These data suggest that the large magnitude of GH release observed with pulsatile T administration may be attributed to any of the following reasons: 1) the increase in SRIF concentration for a short period of time (30 to 60 min) has been reported to amplify the GRF action on pituitary somatotropes (Richardson and Twente, 1990), 2) the large increase in GRF in relation to SRIF (3.5-fold) results in a large increase in GH secretion, and 3) the concurrent increase in GRF and SRIF for 30 to 60 min followed by a rapid decrease in SRIF results in an overshoot of GH secretion (Kraicer et al., 1988). This overshoot of GH secretion is a result of accumulation of intracellular concentrations of GH at high concentrations of GRF and SRIF, which is followed by a low SRIF and high GRF concentrations. The present finding along with other data suggest that pulsatile T increases the magnitude of GH secretion by stimulating the secretion of both GRF and SRIF, which is followed by a more rapid decrease of SRIF concentration in relation to a more gradual decrease of GRF concentration.

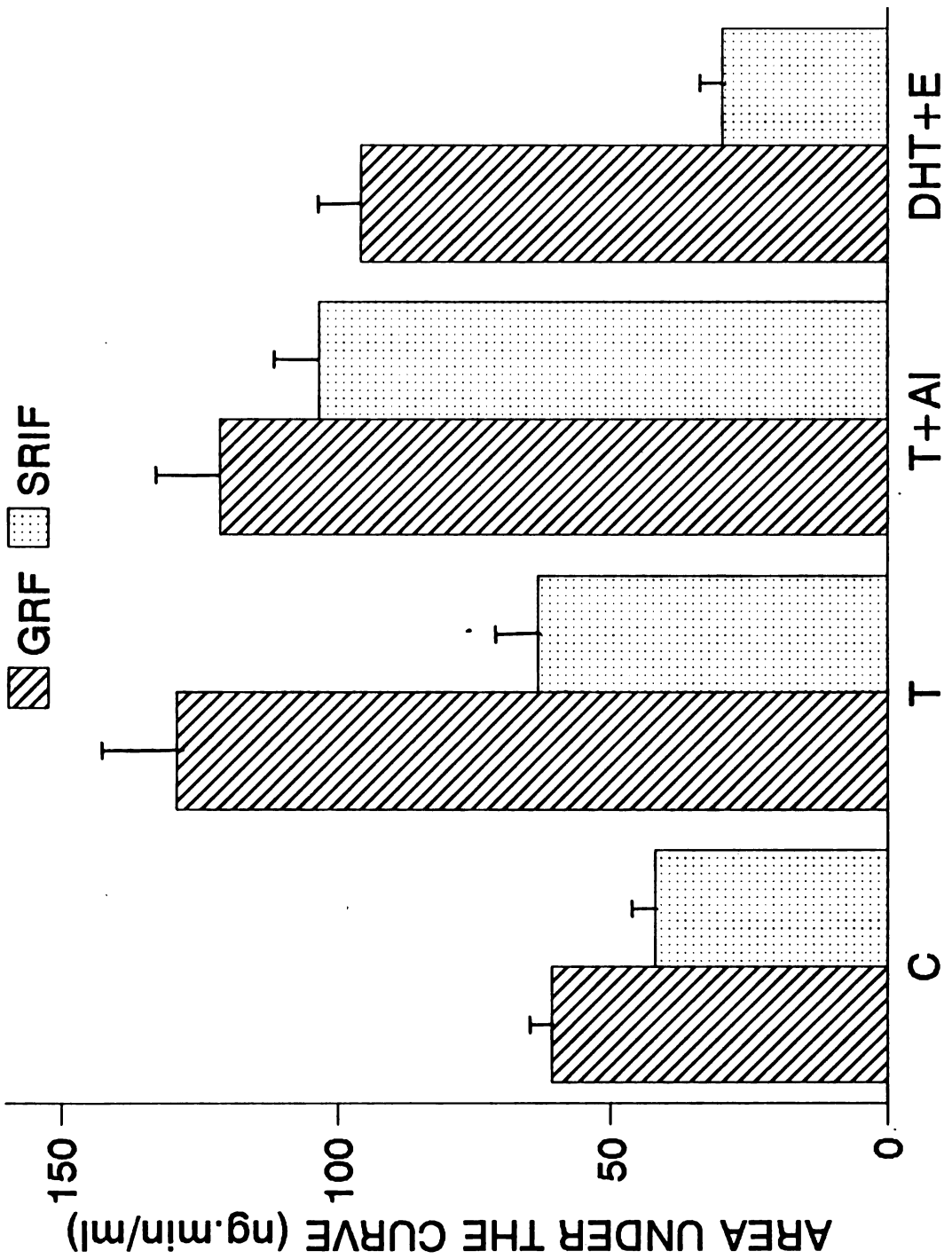
In order to investigate whether T activation of GRF and SRIF release from the hypothalamus is mediated through

Figure 3-5. The effect of testosterone (10^{-9}M) withdrawal (Twd) on GRF and SRIF concentrations. Pulsatile T was administered for 24 h, then T was withdrawn from the perifusate after the last peak. Concentrations of GRF and SRIF were monitored every 10 min for 200 min. Control (C, no T) GRF and SRIF concentrations are also shown. The secretory pattern shown is one of three replications conducted.


GRF Cont
GRF Twd
SRIF Cont
SRIF Twd
HORMONE CONCENTRATION (pg/ml)
200
150
100
50
0
0
20
40
60
80
100
120
140
160
180
200
TIME AFTER WITHDRAWAL (min)

androgenic receptors via 5α-reductase pathway or through estrogenic receptors via aromatase pathway, an experiment was conducted using an aromatase inhibitor (LY43578). Unfortunately, an inhibitor of 5α-reductase was not available. Figure 3-6 illustrates the effects of pulsatile T administration in the presence or absence of the aromatase inhibitor (AI), as well as the combined effects of DHT and E_2 on GRF and SRIF release. The AUC of GRF and SRIF release in control chambers was 60.8±5.1 and 41.8±3.7 $ng.min.ml^{-1}$, respectively. Pulsatile T administration increased ($P<.01$) both GRF and SRIF secretion (112 and 51%, respectively, over controls). These effects are similar to those for pulsatile T perifusion shown in Figure 3-4. No differences ($P>.05$) in GRF release were observed when T was applied with or without AI (168.7 and 179.4 $pg.ml^{-1}$, respectively). These results indicate that aromatization of T does not influence the T action on GRF release. However, the presence of AI increased ($P<.01$) AUC of SRIF release (63.3±5.7 for T without AI vs 103.5±9.4 $ng.min.ml^{-1}$ for T with AI). These data agree with data shown earlier (Figure 2-4) where T with AI reduced AUC-GH to control values. The observed increase in SRIF release abolished the GRF effects on GH release. Since the aromatase inhibitor used in this study does not interact with the steroid receptors (Jones, personal communication), it appears that aromatization of T occurred in the hypothalamus and the resultant E_2 is responsible for maintaining the low SRIF

Figure 3-6. The effect of pulsatile testosterone (T, 10^{-9}M) in the presence or absence of aromatase inhibitor (AI) as well as the combined effects of dihydrotestosterone (DHT, 10^{-9}M) and estradiol-17β (E, 10^{-11}M) on area under the curve (AUC) of GRF and SRIF release from hypothalamic slices. Tissues were obtained from calves. Controls (C) are non-steroid treated chambers. Gonadal steroids were applied to the hypothalamic slices for 24 h. Then , 10 min fractions were collected for 12 h. and frozen. Fractions of each hour were pooled and assayed for GRF and SRIF, and AUC was calculated. Each bar represents the average of three replicates (one animal per replicate) ± SEM.


AREA UNDER THE CURVE (ng.min/ml)
150
100
50
0
GRF
SRIF
C
T
T+AI
DHT+E
TREATMENT

release. The data herein suggest that T increases SRIF release via the androgenic pathway; however, the presence of E_2 (through the aromatase pathway) decreases SRIF. This agrees with the data in Figure 3-1 indicating that E_2 had an inhibitory effect on SRIF release. The complementary effects of E_2 and DHT have been reported previously (Zeitler et al., 1990). In the presence of E_2, DHT competes with estradiol for the cytosolic estrogen receptor and mimics E_2 action (Ruh et al., 1975). In addition, it has been reported that DHT potentiates the effect of E_2 by increasing E_2 binding to its receptors (Johnson et al., 1991). It is also possible that E_2 acts through alteration of biogenic amines to reduce SRIF secretion, since E_2 reduces DA (Elis and Weiner, 1987). Increased DA concentrations have been reported to increase SRIF release (Negro-Vilar et al., 1979). However, dual immunohistochemical staining for GRF and DA revealed that the cells that contain both GRF and DA do not have E_2 receptors (Shirasu et al., 1990). It is also worth noting that these same authors reported clear synaptic interactions between individual GRF, SRIF and DA neurons all of which contain E_2 receptors. These observations suggest a three-way synaptic interaction in modulating GH secretion. Liposits et al. (1988) reported a negative feedback interaction for SRIF neurons on GRF secretion. The slight decrease observed in GRF may be due to negative feedback interaction between SRIF nerve terminals and GRF neurons.

SUMMARY

At the hypothalamic level, estrogen increased GRF and reduced SRIF. Androgens stimulate the release of both GRF and SRIF. The pulsatile pattern of testosterone perifusion markedly maintained elevated GRF compared with SRIF. Aromatization of T to E_2 maintained low concentrations of SRIF similar to E_2 perifusion.

OVERALL SUMMARY

Secretion of GH from somatotropes of the anterior pituitary is under the influence of complex interaction of the hypothalamic peptides, GRF and SRIF. Using anterior pituitary cells in vitro to study GH regulation is not satisfactory, since the hypothalamo-pituitary complex functions as a single unit in the control of GH secretion. In order to understand the influence of gonadal steroids on GH secretion, it is necessary that the hypothalamic tissue and the anterior pituitary cells be combined in a single system. I developed such a system and the results of these studies are:

1- The hypothalamo-pituitary-in-series perifusion system responds to physiological perturbation and provides a useful tool to study the regulation of GH secretion.

2- Estrogen influences GH secretion by acting at the hypothalamus, but not at the anterior pituitary, to increase GRF and reduce SRIF and thereby increase GH release.

3- Continuous perifusion of testosterone increased both GRF and SRIF resulting in no change in GH release. However, pulsatile testosterone increased both GRF and SRIF but SRIF decreased more rapidly than GRF between testosterone episodes which increased the magnitude of GH spikes.

4- Testosterone metabolites, DHT and 3α-diol, increased GH release by acting at the anterior pituitary and at the hypothalamus. At the hypothalamus, these metabolites increase

both GRF and SRIF but GRF is increased more than SRIF, hence greater GH release.

5- Aromatization of testosterone to estrogen is responsible for maintaining low SRIF concentrations and the increased GH release.

6- These data are the first to show the effects of gonadal steroids as well as the testosterone metabolites on GRF and SRIF release from the hypothalamus.

7- These data more clearly explain the specific effects of gonadal steroids on GH release than those reported previously.

APPENDICES

APPENDIX A

Hank's Balanced Salt Solution

(HBSS)

Hank's Balanced Salt Solution	1L pkg
$NaHCO_3$	.35 g
HEPES (25mM)	5.96 g
Penicillin (10U/ml), Streptomycin (10μg/ml)	10 ml
Nystatin (10,000U/ml)	10 ml
pH	7.2

APPENDIX B

DEAE-Sephadex A-50

DEAE-Sephadex A-50 (Diethylaminoethyl Sephadex, Cat# A 2-50-120, Sigma Chemical Company, St Louis, MO.).

Sephadex beads are sequentially washed with .5 N NaOH, .5 N HCl, phosphate buffer (PBS, .1M, pH 7.4). Then beads are washed 3 times with PBS, sterilized (121°C, 20 min), and stored at 4°C in 1% BSA-PBS.

Just prior to use, the phosphate buffer component of the bead suspension is replaced with culture media and incubated in the CO_2 incubator (at least 2 h).

APPENDIX C

Minimum Essential Medium (MEM-alpha)

Minimum Essential Medium (MEM-alpha) with ribonucleosides and deoxyribonucleosides (GIBCO 410-1900)	1L pkg
$NaHCO_3$	2.2 g
HEPES (25mM)	5.96 g
MEM-Essential amino acids (1%)	10 ml
MEM-Non-Essential amino acids	10 ml
Penicillin (10U/ml)-Streptomycin (10μg/ml)	10 ml
Nystatin (10,000 U/ml)	10 ml
Bovine serum albumin (Sigma A7888, St Louis, MO)	1 g
Newborn calf serum (Lot # 4705)	50 ml
Ascorbic Acid (200μM)	.035 g
Aprotinin* (Sigma A3428, St Louis, MO)	100 KIU/ml
Diprotein A* (Peninsula 4132, Belmont, CA)	2 mg/100ml
Leupeptin* (Peninsula 4041, Belmont, CA)	2 mg/100ml
Soybean trypsin inhibitor* (Sigma T6522, St. Louis, MO)	30 mg/100ml

* Enzyme inhibitors added only when samples were assayed for GRF and SRIF and omitted with the co-culture system.

APPENDIX D

Dulbecco's Modified Eagle's Medium (DMEM)

Dulbecco's modified Eagle's medium	1L pkg
$NaHCO_3$	3.7 g
HEPES (25mM)	5.96 g
MEM-Essential amino acids (1%)	10 ml
MEM-Non-Essential amino acids (1%)	10 ml
Pencillin (10U/ml)-Streptomycin (10μg/ml)	10 ml
Nystatin (10,000 U/ml)	10 ml
Newborn calf serum (Lot# 4705)	100 ml
pH	7.4

APPENDIX E

VALIDATION OF GRF AND SRIF RIA

Validation precedures were done with pools of basal media collected from incubated hypothalamic slices. To assess dilution parallelism 50,100 and 200 µl of media were diluted to 1 ml final volume with assay buffer. Aliquots of 100 µl each were assayed as per kit instructions. Displacement of radioligand by these various dilutions is shown in Figures 1a and 1b, for GRF and SRIF, respectively.

To assess recovery of GRF and SRIF, 50, 100, 200 and 500 pg were added to 100 µl of MEM-alpha (see Appendix C). Results from recovery studies are in Figures 2a and 2b for GRF and SRIF, respectively.

Sensitivity and specificity are in Table 1a and 1b for GRF and SRIF, respectively.

(Table 1a) Sensitivity*: IC_{50} 15 pg/tube (10pM)

Specificity*:

PEPTIDE	% CROSS-REACTIVITY
hpGRF (1-44) NH_2	100
hpGRF (1-40) NH_2	100
hpGRF (1-40)	60
hpGRF (1-37)	24
hpGRF (1-29)	12
hpGRF (30-44)	<1
porcine GRF	67
rat GRF	<1
bovine GRF	100

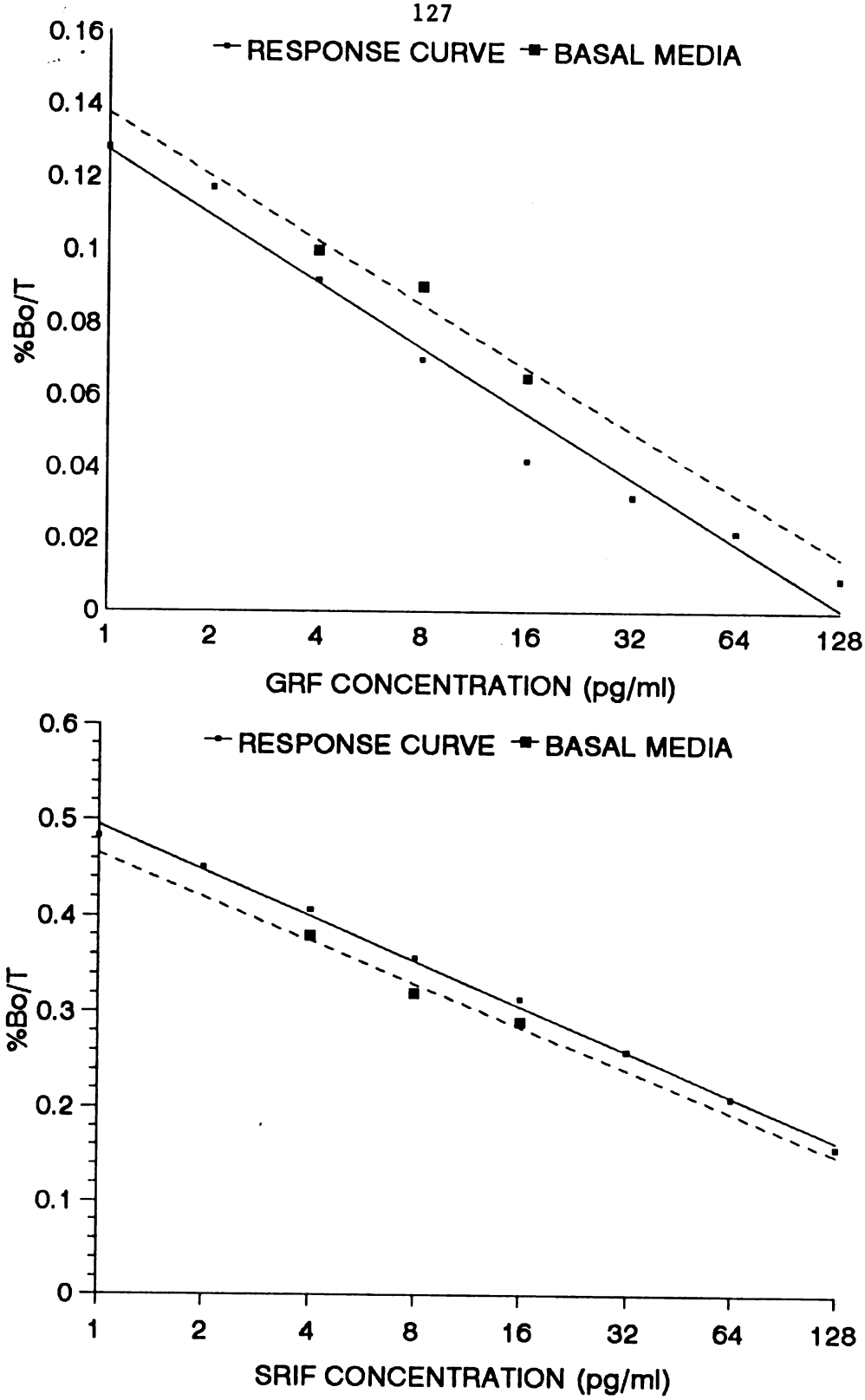
* assayed by Peninsula Laboratories.

(Table 1b) Sensitivity*: 41pg/tube (84 pM)

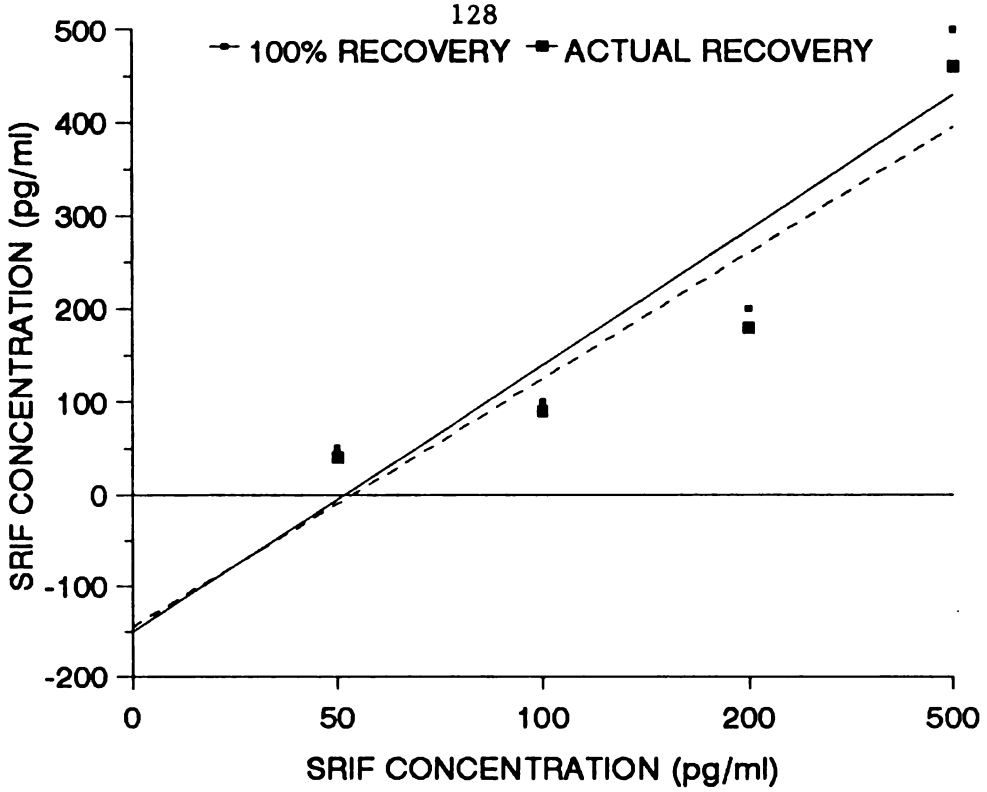
Specificity*:

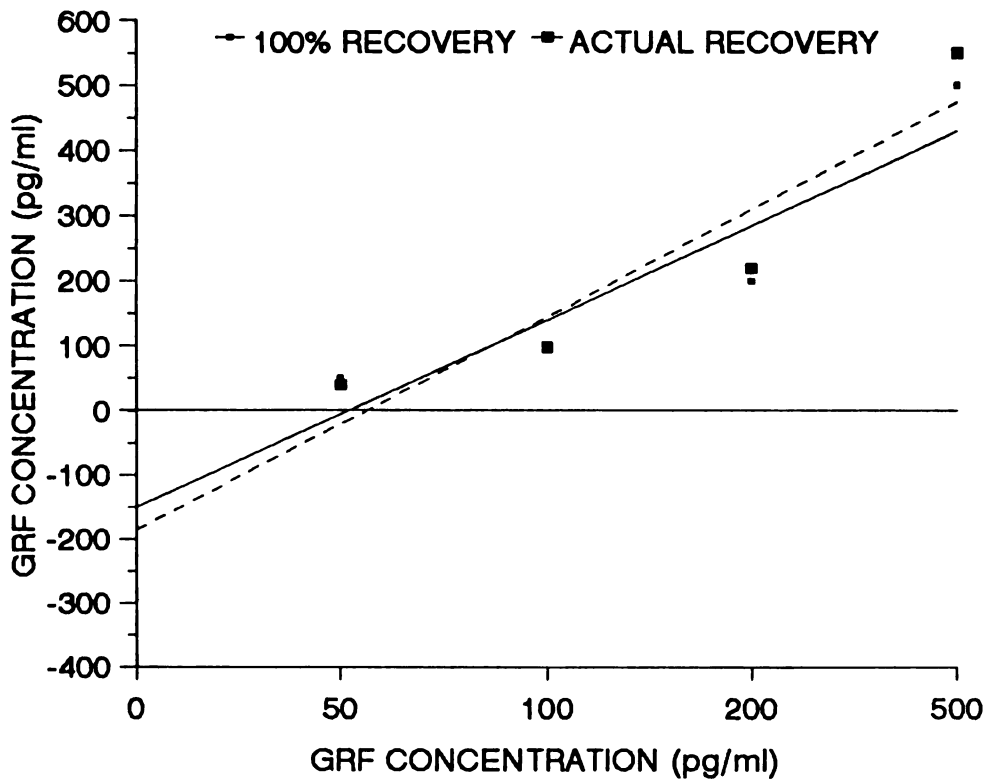
PEPTIDE	% CROSS-REACTIVITY
Somatostatin	100
Somatostatin-28	100
Somatostatin-25	100
Prosomatostatin 1-32 (Procins)	.002
Substance P	0
NPY (Porcine)	0
VIP	0

* assayed by Peninsula Laboratories.



(Figure 1) Displacement curves of various concentrations of media





(Figure 2) Recovery of various bovine GRF concentrations.

APPENDIX F

Determination of Cellular DNA Content

PBS: phosphate buffer saline (.05 M, pH 7.4).

- Stock solution A. 4.8 g NaH_2PO_4 (monobasic) in 200 ml deionized double distilled water (dd H_2O).

- Stock solution B. 28.4 g Na_2HPO_4 (dibasic) in 1000 ml dd H_2O

- PBS working solution (500 ml):

solution A	23.75 ml
solution B	101.25 ml
EDTA (2mM)	.37 g
NaCl (2M)	58.44 g

Hoechst H33258 reagent: Prepare final concentration of 200$\mu g.ml^{-1}$ in dd H_2O. As working solution make 3$\mu g.ml^{-1}$ in PBS.

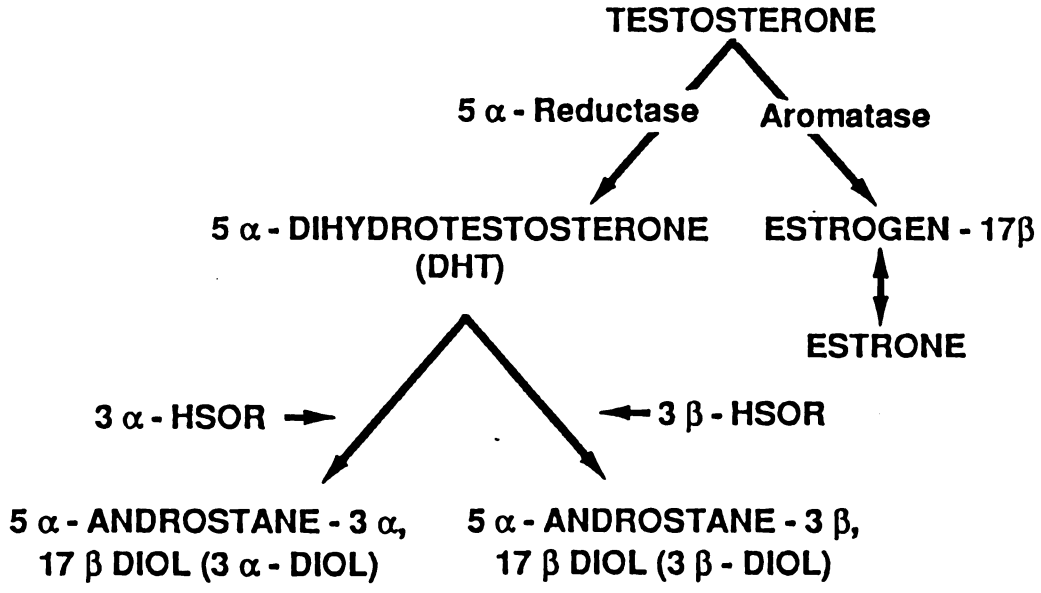
DNA Standard: Dilute to final concentration 500$\mu g.ml^{-1}$ in dd H_2O. Different concentrations of DNA are prepared as a DNA stock solution (i.e., 500, 50, 20 and 2 $\mu g.ml^{-1}$). Stock solution vials were then stored at -70°C.

DNA standard curve.

DNA stock solution	DNA stock (μl)	PBS Buffer (μl)	Final Conconcentration
500 $\mu g.ml^{-1}$	100	900	50 $\mu g.ml^{-1}$ stock
50 $\mu g.ml^{-1}$	80	1920	20 $\mu g.ml^{-1}$ stock
20 $\mu g.ml^{-1}$	400	3600	2 $\mu g.ml^{-1}$ stock
			Standard curve
20 $\mu g.ml^{-1}$	250	1750	Std 1, 5 μg
20 $\mu g.ml^{-1}$	200	1800	Std 2, 4 μg
20 $\mu g.ml^{-1}$	150	1850	Std 3, 3 μg
20 $\mu g.ml^{-1}$	100	1900	Std 4, 2 μg
2 $ug.ml^{-1}$	500	1500	Std 5, 1 μg
2 $ug.ml^{-1}$	400	1600	STd 6, 800 ng
2 $ug.ml^{-1}$	300	1700	Std 7, 600 ng
2 $ug.ml^{-1}$	200	1800	Std 8, 400 ng
2 $\mu g.ml^{-1}$	100	1900	Std 9, 200 ng
0	0	2000	Std 10, 0 ng

APPENDIX G

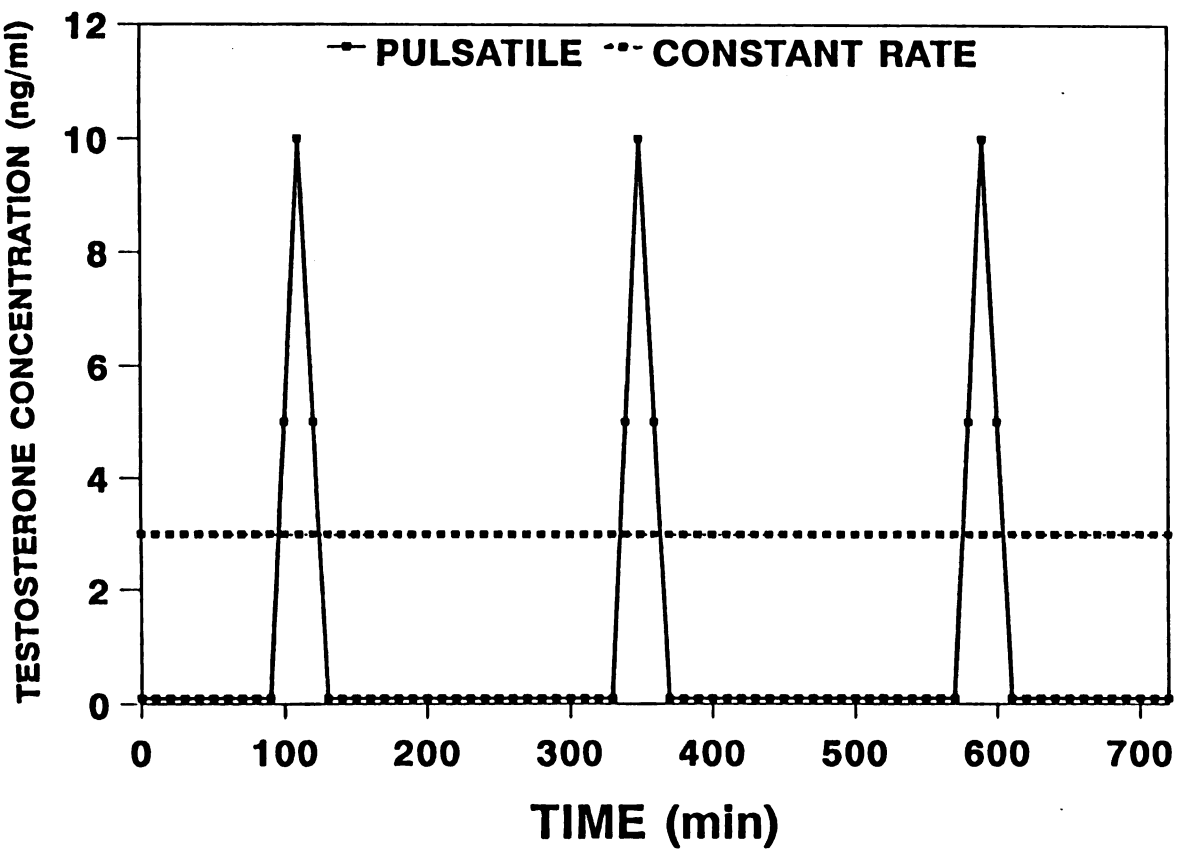
Testosterone Metabolism



APPENDIX H

Constant and Pulsatile Testosterone

The same concentration of T (10^{-9}M) was perifused either at constant or pulsatile rate. Constant rate T was 2.9 $ng.ml^{-1}$ continuously. Pulsatile T rate was .1 $ng.ml^{-1}$ baseline, 10 $ng.ml^{-1}$ peak amplitude and duration of each peak was 30 min. Peak frequency was programmed every 240 min.



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