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IN VITRO ALPHA2-ADRENERGIC AND DOPAMINERGIC RECEPTOR REGULATION OF GROWTH HORMONE-RELEASING HORMONE AND SOMATOSTATIN IN CATTLE

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By

Christine Ruth West

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ABSTRACT

IN VITRO α_2-ADRENERGIC AND DOPAMINERGIC RECEPTOR REGULATION OF GROWTH HORMONE-RELEASING HORMONE AND SOMATOSTATIN IN CATTLE

By

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An *in vitro* perifusion system was developed for bovine hypothalamic tissue to examine the role of hypothalamic α_2-adrenergic and dopaminergic receptors in the regulation of growth hormone-releasing hormone (GHRH) and somatostatin (SRIF) release and to determine if GHRH and SRIF regulate each other's release in cattle. Areas under GHRH and SRIF response curves (AUC), adjusted by covariance for pretreatment values, were calculated from samples of effluent collected every 20 min during the treatment/post-treatment period.

Activation of α_2-adrenergic receptors with clonidine and guanabenz increased AUC for GHRH relative to controls. In contrast, clonidine and guanabenz did not affect release of SRIF. An α_2-adrenergic receptor antagonist, idazoxan, blocked clonidine-induced release of GHRH without affecting release of SRIF.

Activation of D_1 receptors with SKF 38393 increased AUC for SRIF and decreased AUC for GHRH relative to controls. Blockade of D_1 receptors with SCH 23390 had no effect on basal release of either SRIF or GHRH, but prevented SKF 38393-induced release of SRIF and suppression of GHRH. In contrast, quinelorane, a D_2 receptor agonist, and haloperidol, which blocks D_2 receptors, did not affect release of SRIF or GHRH.

Perifusion of SRIF decreased AUC for GHRH relative to controls. GHRH increased release of SRIF when compared with controls. In addition, blockade of SRIF action with a SRIF antagonist, cyclo-[7-aminoheptanoyl-phe-D-trp-lys-thr(bzl)], increased release of GHRH and blocked SKF 38393-induced suppression of GHRH.

In conclusion, an *in vitro* perifusion system for bovine hypothalamic tissue was developed that can be utilized to study regulation of GHRH and SRIF release. Results support the hypothesis that an increase in release of GHRH, but not a decrease in release of SRIF, mediates α_2-adrenergic receptor stimulation of secretion of growth hormone in cattle. In addition, stimulation of D_1 receptors increases release of SRIF and decreases release of GHRH. Furthermore, SRIF inhibits release of GHRH, GHRH stimulates release of SRIF, and SRIF mediates the effects of activation of D_1 receptors to suppress release of GHRH from perifused, bovine hypothalamic slices. It is speculated that norepinephrine, via GHRH, stimulates release of growth hormone whereas dopamine, via SRIF, inhibits release of growth hormone in cattle.

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INTRODUCTION

A primary objective of animal agriculture is to increase efficiency of meat and milk production. Therefore, use of growth and lactation promotants that enhance efficiency of traditional animal production systems may lead to substantial benefits for livestock producers. Growth hormone is a primary regulator of postnatal growth and milk secretion in cattle (Bauman et al, 1982; Bauman, 1992). Administration of growth hormone stimulates protein accretion in muscle and reduces fat accretion in growing cattle and lactating cows (Eisemann et al., 1989; Binelli et al., 1995). In addition, administration of growth hormone markedly increases milk yield from 10 to 25% without affecting percentages of protein, fat, or lactose in milk (Bauman, 1992). Furthermore, exogenous growth hormone increases mammary development in heifers (Radcliff et al., 1996).

An alternative to administration of exogenous growth hormone to stimulate body growth and milk secretion is to enhance endogenous secretion of growth hormone. Such alternatives could be more cost-effective; however, relatively little is known about mechanisms involved in regulation of growth hormone in cattle. It is known that secretion of growth hormone from the anterior pituitary gland is under dual control of hypothalamic neuropeptides, growth hormone-releasing hormone (GHRH) and somatostatin (SRIF). Growth hormone-releasing hormone increases synthesis and release of growth hormone, whereas SRIF inhibits release of growth hormone (Frohman et al., 1992). Hypothalamic,

catecholaminergic neurotransmitters such as norepinephrine and dopamine are involved in regulation of endogenous secretion of GHRH and SRIF, and thus, secretion of growth hormone (Müller, 1987; Frohman et al., 1992; Gaynor et al., 1993). Therefore, an increase in understanding of basic neuroendocrine mechanisms regulating endogenous secretion of growth hormone could result in development of new methods to increase efficiency of meat and milk production.

The overall goal of this dissertation was to understand mechanisms whereby α_2-adrenergic and dopaminergic receptors within the hypothalamus mediate changes in release of GHRH and SRIF in cattle. Therefore, to achieve my overall goal, my initial objective was to develop and validate an in vitro perifusion system for culturing bovine hypothalamic tissue. Additional specific objectives of this dissertation were: 1) to determine the role of GHRH and SRIF in α_2-adrenergic receptor stimulation of growth hormone secretion in cattle; 2) to determine if pharmacological activation or inhibition of dopamine receptors regulates release of GHRH and SRIF from the bovine hypothalamus and; 3) to determine if GHRH and SRIF are involved in a reciprocal feedback relationship within the hypothalamus of cattle.

REVIEW OF LITERATURE

I. Characterization of Growth Hormone

Growth hormone is synthesized and stored in specific acidophilic cells in the anterior pituitary gland referred to as somatotropes, which comprise up to 50% of all anterior pituitary cells (Kuret and Murad, 1990). Growth hormone most commonly exists as a single linear chain of 191 amino acids with two disulfide bridges and a molecular weight of 21,500 daltons (Kuret and Murad, 1990). However, there is great variation in the growth hormone molecule between species and even within species. For example, variant forms of growth hormone have been reported in cattle as well as differences in galactopoietic activity of growth hormone variants in lactating cows (Hampson and Rottman, 1987; Krivi et al., 1989). Secretion of growth hormone is pulsatile, and primary control of its release is neuroendocrine in nature. Two hypothalamic neuropeptides, GHRH SRIF, primarily mediate the central nervous system's control of growth hormone secretion.

II. Hypothalamic Regulation of Growth Hormone Secretion

A. Discovery, Characterization, and Localization of GHRH

In 1964, Deuben and Meites first demonstrated growth hormone-releasing activity in extracts of rat hypothalamus. In 1982, Guillemin et al. and Rivier et al. independently

isolated human GHRH from pancreatic tumors of two patients with hypersecretion of growth hormone. Two of the three GHRH forms found in tumors were subsequently identified in human hypothalamus, GHRH(1-44)NH_2 and GHRH(1-40)OH, and differed only by the absence of the last four carboxyl-terminal amino acid residues (Ling et al., 1984). Following isolation of human GHRH, GHRH was identified in a number of mammalian species and later it became evident that GHRH(1-44)NH_2 was the predominant form among species (Frohman et al., 1992). The sequence of bovine, porcine, and ovine GHRH differs from human GHRH(1-44) by only three, five, and six amino acids, respectively (Esch et al., 1983; Bohlen et al., 1983; Brazeau et al., 1984). Despite these differences, there is little species specificity with regard to biological activity. This may be so because amino acid residues 1-29 are highly conserved among species and these residues elicit full biological activity of GHRH (Buonomo and Baile, 1990).

Neuronal perikarya containing GHRH, identified by immunohistochemical staining, are located primarily in the arcuate nucleus with a few cells present in the lateral basal hypothalamus, paraventricular, dorsomedial , and ventromedial nuclei in rats (Bloch et al., 1983; Merchenthaler et al., 1984). Later, mRNA for GHRH was identified in neuronal cell bodies in the arcuate nucleus confirming that these neurons synthesize GHRH (Mayo et al., 1986; Frohman et al., 1992). Most axonal projections from GHRH perikarya project to the outer layer of the median eminence (Frohman et al., 1992). Immunocytochemical studies in cattle revealed a similar regional distribution of GHRH as observed in rats. Indeed, a high proportion of GHRH-immunoreactive neurons are located in the arcuate nucleus along with a population of GHRH-immunoreactive neurons

being located lateral and dorsal to the arcuate nucleus within and around the ventromedial nuclei (Leshin et al., 1994). In addition, dense GHRH-immunoreactivity is localized in lateral regions of the external layer of the median eminence in cattle (Leshin et al., 1994).

Immunoreactive GHRH and GHRH mRNA have also been identified in limited amounts in extrahypothalamic tissues such as the gastrointestinal tract, testis, ovary, placenta, and lymphocytes (Bruhn et al., 1985; Berry and Pescovitz, 1988; Bagnato et al., 1991; Mizobuchi et al., 1995; Stephanou et al., 1991). Diverse biological activities for GHRH in these tissues have been suggested. For example, GHRH modulates gonadotropin-stimulated steroidogenesis in Leydig cell and granulosa cell cultures (Ciampani et al., 1992; Srivasta et al., 1993). In lymphocytes, GHRH appears to be involved in immune modulation (Weigent et al., 1991; Stephanou et al., 1991). However, in general, little is known about the role of GHRH in extrahypothalamic locations.

B. Effects of GHRH on Growth Hormone Synthesis and Secretion

Growth hormone is secreted in a pulsatile manner in all mammals studied, including cattle (Kraicer et al., 1988; Vasilatos and Wangsness, 1981). Growth hormone-releasing hormone, secreted from terminals of neurons in the median eminence, is transported via the hypophysial portal system to somatotropes in the anterior pituitary gland where GHRH stimulates secretion of growth hormone in several species (Frohman et al., 1992). Indeed, administration of GHRH and GHRH analogs elicits a rapid increase in serum concentrations of growth hormone in fetal calves, prepubertal bulls and heifers, steers, and lactating cows (Coxam et al., 1988; Enright et al., 1987; Scarborough et al., 1988; Moseley et al., 1984; Enright et al., 1988). In addition, GHRH increases secretion of growth hormone from bovine anterior pituitary cells in a dose-dependent manner

(Padmanabhan et al., 1987). In vivo, hypophysial stalk transection abolishes episodic secretion of growth hormone in calves; however, a GHRH challenge increases secretion of growth hormone in these calves (Plouzek et al., 1988). Furthermore, passive immunization against GHRH decreases serum concentrations of growth hormone in steers (Trout and Schanbacher, 1990). Thus, numerous *in vivo* reports in cattle indicate GHRH is a primary stimulator of secretion of bovine growth hormone.

In cultured rat anterior pituitary cells, GHRH increases growth hormone gene transcription, growth hormone mRNA levels, and somatotrope proliferation (Barinaga et al., 1983; Gick et al., 1984; Billestrup et al., 1986). In addition, GHRH stimulates accumulation of growth hormone mRNA in bovine anterior pituitary cells in culture (Silverman et al., 1988; Barinaga et al., 1983). In contrast, GHRH deficiency due to treatment with monosodium glutamate or GHRH antiserum decreases pituitary growth hormone content and mRNA levels, somatotrope cell number, and pituitary size (Maiter et al., 1991; Cella et al, 1990a; Frohman et al., 1992). In summary, the bulk of the evidence supports the hypothesis that GHRH not only increases secretion of growth hormone, but also increases synthesis of growth hormone and somatotrope proliferation.

C. Characterization and Signal Transduction Mechanism of GHRH Receptor

A critical step in mediating GHRH stimulation of synthesis and secretion of growth hormone is binding of GHRH to a specific pituitary cell surface receptor (Korytko et al., 1996). Specific, high affinity binding of GHRH to somatotrope cell membrane receptors has been demonstrated with intact cells or cell membrane preparations from rat and bovine pituitaries (Seifert et al., 1985; Velicelebe et al., 1986). Complementary DNAs of porcine, mouse, rat, and human GHRH receptor have been cloned and sequenced (Hsiung et al.,

1993; Lin et al., 1992; Mayo, 1992; Gaylinn et al., 1993). Comparison of predicted primary protein sequences between cloned GHRH receptor transcripts from different species demonstrates a 78% degree of homology, suggesting a highly conserved function for GHRH receptor during evolution (Tang et al., 1995).

Sequence analysis and deduced protein structure indicate GHRH receptors belong to the superfamily of guanine nucleotide-binding protein (G proteins)-coupled receptors, characterized by seven transmembrane helixes that are joined by three intracytoplasmic and three extracellular loops (Tang et al., 1995). Binding of GHRH activates the adenylate cyclase-cAMP-protein kinase A pathway via the stimulatory G protein, G_s (Mayo et al., 1995; Wong et al., 1995). Consistent with the notion that cAMP is an important second messenger for GHRH signaling, both GHRH and cAMP increase pituitary growth hormone secretion, elevate growth hormone gene expression, induce expression of the proto-oncogene *c-fos*, and stimulate proliferation of pituitary somatotrope cells (Barinaga et al., 1983; Gick et al., 1984; Billestrup et al., 1986, 1987).

The mechanism by which cAMP stimulates somatotrope proliferation and differentiation as well as synthesis of growth hormone is not completely elucidated. However, recent evidence suggests phosphorylation of CREB, a cAMP response element-binding protein that binds specific DNA sequences and stimulates gene transcription, may serve as an important biochemical intermediate in responses of somatotropes to GHRH (Bertherat et al., 1995b). Upon phosphorylation, CREB induces a number of target genes, such as c-*fos* and the pituitary-specific transcription factor, Pit-1, both of which are required for production of growth hormone and somatotrope proliferation (Mayo et al., 1995). Therefore, CREB binding to c-*fos* and Pit-1 provides a pathway for the signal of

GHRH binding to increase growth hormone synthesis and somatotrope proliferation in the anterior pituitary (Bertherat et al., 1995b). Although cAMP is accepted to be the primary mediator of growth hormone release, it is also proposed that GHRH depolarizes somatotropes in a Na^{+}-dependent manner and stimulates Ca^{+2} influx (Horváth et al., 1995).

D. Discovery, Characterization, and Localization of SRIF

Krulich et al. provided the first evidence for SRIF in 1968 when *in vitro* growth hormone-releasing and -inhibiting activity of anatomically fractionated regions of rat hypothalamus were determined. Later, SRIF was isolated from ovine hypothalami on the basis of its ability to inhibit growth hormone secretion from cultured rat pituitary cells (Brazeau et al., 1973). In mammals, two major biologically active forms of SRIF are synthesized: SRIF (14 amino acids) and a N-terminally extended form of the tetradecapeptide, SRIF-28 (Schally et al., 1980). Isolation and characterization of complementary DNA clones encoding SRIF indicate the primary translation product of SRIF mRNA is a 116-amino acid molecule, preprosomatostatin, which includes prosomatostatin (92 amino acids) and a 24-amino acid signal peptide (Reisine and Bell, 1995). Proteolytic processing of prosomatostatin generates SRIF and SRIF-28 (Reisine and Bell, 1995). Somatostatin contains two cysteine residues connected by a disulfide bond that is essential for biological activity, as are residues 6-9, which are contained within the ring structure of SRIF (Frohman et al., 1992).

In contrast to GHRH, SRIF is widely dispersed throughout the central and peripheral nervous systems, as well as in several types of endocrine cells (Chiodini et al., 1991; Meister and Hökfelt, 1992). Physiological studies and broad distribution of SRIF in

the central nervous system (CNS) suggest that SRIF may regulate many aspects of CNS function, including neuroendocrine control, autonomic function, nociception, and appetitive behavior (Breder et al., 1992; Giehl and Mestres, 1995). In addition, SRIF has been implicated in neuronal development and altered levels and distribution of SRIF immunoreactivity have been observed in neuronal degenerative states such as Alzheimer's disease (Leroux et al., 1992). Outside the CNS, SRIF has been isolated from pancreatic islets, epithelial cells of the stomach, small intestine, parafollicular cells of the thyroid, and kidney (Chiodini et al., 1991). In general, SRIF exerts inhibitory control on its target cell. For example, SRIF inhibits secretion of both insulin and glucagon as well as gastric, duodenal, and gallbladder motility (Reisine and Bell, 1995).

In the hypothalamus, SRIF-containing neuronal perikarya that establish connections with portal vessels of the median eminence/pituitary stalk are located primarily in the periventricular nucleus in rats, sheep, and cattle (Frohman et al., 1992; Willoughby et al., 1995; Leshin et al., 1994). In cattle, although most SRIF-immunoreactive cells in the periventricular nucleus are lateral to the ependymal cell layer, a number of SRIF-immunoreactive neurons appear to be bathed by the cerebrospinal fluid of the third ventricle (Leshin et al., 1994). In addition to the periventricular nucleus, SRIF-immunoreactive perikarya and axon terminals are also found in the hypothalamic arcuate nucleus in rats (Makara et al., 1983; Willoughby et al., 1989). However, in contrast to other arcuate neurons they lack a morphological connection to the median eminence (Makara et al., 1983). Leshin et al. (1994) also reported that immunoreactive SRIF fibers and varicosities were present in the arcuate nucleus in cattle. Therefore, SRIF has the potential to regulate GHRH that is primarily located in the arcuate nucleus.

E. Effects of SRIF on Growth Hormone Synthesis and Secretion

Both SRIF and SRIF-28 block the effect of GHRH on *in vitro* and *in vivo* release of growth hormone in many different species (Reisine and Bell, 1995). For example, in cultured bovine anterior pituitary cells, SRIF inhibits GHRH-stimulated release of growth hormone in a dose-dependent manner with higher concentrations of SRIF needed to suppress growth hormone responses to higher doses of GHRH (Padmanabhan et al., 1987). In addition, infusion of SRIF decreases GHRH-induced release of growth hormone in hypophysial stalk-transected calves (Plouzek et al., 1988). However, in the ruminant animal, the effect of SRIF on regulation of growth hormone secretion appears to be limited to modulation of the stimulatory actions of GHRH. Indeed, infusion of SRIF does not affect basal levels of growth hormone in sheep (Davis, 1975). In addition, neither active or passive immunization against SRIF in sheep, goats, or cattle affects basal release of growth hormone (Bass et al., 1987; VanKessel and Laarveld, 1992; Trout and Schanbacher, 1990). Furthermore, SRIF does not affect basal secretion of growth hormone in cultured bovine anterior pituitary cells (Padmanabhan et al., 1987).

Consistent with the lack of effects of SRIF alone on growth hormone release, SRIF effects on synthesis of growth hormone and somatotrope proliferation appear to be limited to modulating the actions of GHRH. For example, SRIF treatment alone does not affect basal levels of growth hormone synthesis, gene transcription, mRNA, or somatotrope proliferation in rat or bovine pituitary cell cultures (Billestrup et al., 1986; Fukata et al., 1985; Tanner et al., 1990; Frohman et al., 1992). In contrast, SRIF markedly reduces GHRH-stimulated increases in bovine growth hormone mRNA *in vitro* (Tanner et al., 1990). Somatostatin also attenuates GHRH-induced increases in

somatotrope proliferation and expression of the proto-oncogene c-*fos* in the rat (Billestrup et al., 1986, 1987).

F. Characterization and Signal Transduction Mechanisms of SRIF Receptors

Specific membrane-bound, high-affinity receptors on the surface of target cells mediate the physiological actions of SRIF (Schonnbrunn and Tashjian, 1978). Numerous receptor-binding autoradiography studies have demonstrated multiple receptor subtypes with differing affinities for SRIF and SRIF-28, as well as various chemically-synthesized agonists (Breder et al., 1992; Reisine and Bell, 1995). Studies utilizing biochemical and pharmacological techniques revealed two general types of SRIF receptors, SRIF-1 and SRIF-2 (Beaudet et al., 1995). Subsequent studies utilizing molecular biology tools, demonstrated the original two general types of SRIF receptors were comprised of at least five distinct SRIF receptor subtypes, designated SSTR1-SSTR5, all of which express mRNA in rodent hypothalami and anterior pituitary gland (Beaudet and Tannenbaum, 1995; Reisine et al., 1995). Based on structural and pharmacological profiles of the five receptors, they have been divided into two main classes of receptors: SSTR1/SSTR4 (pharmacologically analogous to SRIF-2 receptor type) and SSTR2/SSTR3/SSTR5 (pharmacologically analogous to SRIF-1 receptor type).

Cloned SSTR1-SSTR5 are approximately 50% identical in amino acid sequence and are capable of coupling to several effector systems via different types of G proteins, ones that are either pertussis toxin-sensitive or -insensitive (Reisine et al., 1995; Reisine and Bell, 1995; Hofland and Lamberts, 1996). Pertussis toxin catalyzes ADP ribosylation of the α-subunits of G_i and G_o thereby inactivating the α-subunits. Sensitivity to pertussis toxin has been used to reveal the importance of G_i and G_o in receptor signaling.

Somatostatin receptors mediate inhibition of adenylate cyclase activity, reduce conductance of voltage-dependent Ca^{+2} channels, and stimulate activity of inwardly and delayed rectifying K^+ channels via G_i and G_o proteins (Reisine et al., 1995; Hofland and Lamberts, 1996). Through pertussis toxin-insensitive pathways, SRIF receptors stimulate tyrosine phosphatase activity and a Na^+-H^+ exchanger (Delesque et al, 1995). Currently, it is unclear as to which molecular forms of SRIF receptors couple to which effector systems. However, depending on which G proteins are expressed in a given cell, an individual SRIF receptor could regulate several different effector systems (Reisine et al., 1995).

In somatotropes, SRIF inhibits activation of adenylate cyclase and(or) reduces intracellular Ca^{+2} levels to block the growth hormone-releasing effects of GHRH (Frohman et al., 1992). While all five SRIF receptor mRNAs are expressed in the anterior pituitary gland, SSTR2 is expressed in the highest amount (Reisine and Bell, 1995). In addition, the potencies of several SRIF analogs to bind cloned SSTR2 is positively correlated with their ability to inhibit secretion of growth hormone from cultured rat anterior pituitary cells (Reisine and Bell, 1995). In contrast, there is no relationship between the pharmacological characteristics of the four other SRIF receptor subtypes and inhibition of growth hormone secretion (Reisine and Bell, 1995). Furthermore, the SSTR2 subtype is expressed in a majority of human pituitary growth hormone adenomas (Greenman and Melmed, 1994; Hofland and Lamberts, 1996). Thus, Reisine and Bell (1995) suggest SSTR2 likely mediates SRIF's inhibition of growth hormone secretion from the anterior pituitary gland.

G. Interactions Between GHRH and SRIF in Regulation of Growth Hormone Release

The secretory pattern of growth hormone is dependent on an interaction between GHRH and SRIF at the level of the somatotrope (Frohman et al., 1992). Tannenbaum and Ling (1984) proposed a model in which pulsatile secretion of GHRH and SRIF are 180° out of phase with one another and determine the secretory profile of growth hormone. They proposed pulses of growth hormone result from pulses of GHRH release combined with troughs of SRIF release. In contrast, trough values of growth hormone occur when release of SRIF is elevated and release of GHRH is minimal. The major support for this model is based on the observation that growth hormone responses to GHRH are greater at times of spontaneous growth hormone pulses than during growth hormone troughs, presumably as a consequence of increased SRIF tone. The single report of portal GHRH and SRIF levels in rats is consistent with this model (Plotsky and Vale, 1985). However, GHRH and SRIF measurements were made in anesthetized, hypophysectomized rats, which were a different set of animals than those in which growth hormone was quantified. Therefore, this limits interpretation of the data, because even though growth hormone is released at regular 3 to 4 h intervals in rats, the pattern of secretion is not synchronized between different animals (Carlsson and Jansson, 1990).

The pattern of GHRH and SRIF secretion in sheep indicates regulation of growth hormone is more complex, because only 70% of growth hormone pulses occur coincident with or immediately following a GHRH pulse (Frohman et al., 1990; Thomas et al., 1991). Furthermore, SRIF pulses are not out of phase with those of GHRH and do not correlate with growth hormone troughs, and SRIF troughs do not correlate with growth hormone

pulses. Thus, it appears additional modulators contribute to pulsatile growth hormone secretion even though the Tannenbaum and Ling model is frequently referred to in the literature.

Morphological evidence suggests GHRH and SRIF may also regulate release of growth hormone through interactions with each other in the hypothalamus/pituitary stalk (Beaudet and Tannenbaum, 1995). For example, radioligand binding studies demonstrate high concentrations of high affinity SRIF binding sites throughout the mediobasal hypothalamus (Leroux et al., 1988). In addition, double labeling studies provide anatomical evidence for association of SRIF receptors with a subpopulation of neurons that both contain and synthesize GHRH within the arcuate nucleus of the hypothalamus in rats (Bertherat et al., 1992; McCarthy et al., 1992). Indeed, high concentrations of mRNA for two SRIF receptor subtypes, SSTR1 and SSTR2, are present in the arcuate nucleus of rats (Beaudet et al., 1995). Furthermore, synaptic contact between GHRH-containing nerve terminals and SRIF-containing dendrites has been reported in the periventricular nucleus where GHRH receptor mRNA is also present (Horvath et al., 1989; Takahashi et al., 1995). Leshin et al. (1994) demonstrated SRIF-immunoreactive axonal terminals surround GHRH-immunoreactive perikarya in the arcuate nucleus of cattle. Also, within the external layer of the median eminence, extensive intermingling of GHRH-immunoreactive and SRIF-immunoreactive fibers and terminals exists in cattle (Leshin et al., 1994).

Physiological studies also support the existence of a feedback relationship between GHRH and SRIF in regulation of growth hormone secretion. For example, intracerebroventricular injection of GHRH increases levels of SRIF in portal blood in rats

(Mitsugi et al., 1990). In addition, GHRH increases release of SRIF from rat hypothalami in culture as well as SRIF mRNA in the periventricular nucleus (Katakami et al., 1986; Richardson et al., 1988; Aguila, 1994). In contrast, central administration of SRIF antiserum increases concentrations of GHRH in hypophysial-portal plasma and SRIF inhibits release of GHRH in perifused rat hypothalami (Plotsky and Vale, 1985; Yamauchi et al., 1991).

III. α_2-Adrenergic Receptor Regulation of Growth Hormone Secretion

A. Characterization of Noradrenergic Innervation of the Hypothalamus

Dahlstrom and Fuxe (1964) initiated an alpha-numeric system for delineation of catecholamine groups A_1-A_{12}. Fuxe and Hokfelt (1969), Bjorklund et al. (1973), and Hokfelt et al. (1984) incorporated additional groups, A_{13}-A_{15}. Three noradrenergic pathways ascend from the brainstem to the diencephalon in rats: 1) the dorsal noradrenergic bundle (dorsal tegmental tract), originating in the locus coeruleus (A_6); 2) the ventral noradrenergic bundle (ventral tegmental tract) collecting fibers from the subcoeruleus (A_1, A_2, A_5, and A_7) and; 3) the dorsal periventricular tract also originating in the A_2 cell group and collecting fibers from noradrenergic cells of the locus coeruleus and subcoeruleus areas (Moore and Bloom, 1979; Palkovits et al., 1980).

In contrast to rats, noradrenergic perikarya in the locus coeruleus in cattle, sheep, and pigs are not clustered, but are scattered in a large area ventrolateral to the fourth ventricle (Tillet, 1995). In addition, in sheep, subcoeruleus (A_1 and A_2) noradrenergic neuronal axons project rostrally through the ventral tegmental and medial forebrain bundles to terminate in the mediobasal and lateral hypothalamus and pituitary stalk,

whereas noradrenergic neurons in the locus coeruleus (A_6) project axons via the dorsal tegmental bundle to provide innervation to the dorsal and periventricular regions of the hypothalamus (Tillet, 1995).

Leshin et al. (1995a) identified the anatomical location of catecholaminergic fibers in the hypothalamus of cattle with immunocytochemical staining for presence of the rate limiting enzyme for catecholamine synthesis, tyrosine hydroxylase, and the final enzyme of norepinephrine synthesis, dopamine β-hydroxylase. Cells that are immunoreactive for dopamine β-hydroxylase produce epinephrine as well as norepinephrine; thus, this immunocytochemical technique is used to identify adrenergic neurons. While epinephrine is present in hypothalamic tissue, norepinephrine fibers substantially outnumber epinephrine fibers in the hypothalamus (Moore and Bloom, 1979; Cooper et al., 1996).

Within the bovine mediobasal hypothalamus, dopamine β-hydroxylase-immunoreactive innervation is dense in the arcuate nucleus (Leshin et al., 1995a). In addition, dopamine β-hydroxylase-immunoreactive fibers innervate the periventricular nucleus in cattle (Leshin et al., 1995a). Compared to rats, dopamine β-hydroxylase-immunoreactive innervation is sparse and fibers do not appear to cluster about the capillaries in the bovine median eminence (Leshin et al., 1995a). Therefore, distribution of dopamine β-hydroxylase-immunoreactivity in cattle differs from that described in rats where dopamine β-hydroxylase-immunoreactive innervation of the median eminence is abundant (Swanson and Hartman, 1979; Leshin et al., 1995a).

B. Characterization and Signal Transduction Mechanisms of α_2-Adrenergic Receptors

Adrenergic receptors mediate physiological effects of epinephrine and norepinephrine. Pharmacological studies initially differentiated adrenergic receptors into α- and β-adrenergic responses and subsequently divided α-adrenergic responses into α_1 and α_2 subtypes (Berthelsen and Pettinger, 1977; Bylund et al., 1994; Milligan et al., 1994). Subsequently, more detailed pharmacological, biochemical, and molecular biological characterization indicated that each of the three broad classifications (α_1, α_2, and β) of adrenergic receptors contain multiple individual gene products (Bylund et al., 1994). With respect to regulation of growth hormone secretion, both α_2- and α_1-adrenergic receptors are involved, although effects of α_2-receptors appear to be dominant (Magnan et al., 1994).

α_2-Adrenergic receptors have been subdivided (α_{2A}, α_{2B}, α_{2C}, α_{2D}) based on differences in binding of ^{3}H-labeled antagonists, in amino acid sequence of the receptors, and chromosomal location of the genes encoding the receptor proteins (Bylund, 1988; MacKinnon et al., 1992). Both humans and rats possess α_{2A}, α_{2B}, and α_{2C} receptor subtypes, but only rats appear to possess the α_{2D} receptor (MacKinnon et al., 1992; Milligan et al., 1994). However, it is currently believed that α_{2A} in humans and α_{2D} in rats represent the same receptor; thus, there are species homologues (MacKinnon et al., 1992; Milligan et al., 1994).

Signaling mechanisms associated with members of the α_2-adrenergic receptor family are more complex than originally thought. The classically accepted mechanism of

α_2-adrenergic receptors is to inhibit adenylate cyclase via stimulation of the G protein, G_i (Cotecchia et al., 1990). However, while all α_2-adrenergic receptors appear to be capable of transducing this signal, it is not the only mechanism of signal transduction for α_2-adrenergic receptors (Bylund et al., 1994). For example, it has been reported that α_{2A}, α_{2B}, and α_{2C} receptors interact with both G_i and G_s depending on the concentration of agonist present (Eason et al., 1992). Furthermore, in studies where subtypes of α_2-adrenergic receptors have been transfected into fibroblast cell lines, agonist activation of a variety of phospholipases as well as regulation of $p21^{ras}$ and the mitogen-activated protein kinase cascade have also been reported (Cotecchia et al., 1990; MacNulty et al., 1992; Jones et al., 1991; Alblas et al., 1993). Therefore, the balance of α_2-adrenergic receptor regulation of adenylate cyclase and other signal transduction effector systems may depend on which receptor subtypes and effector proteins are expressed in the target cell, the level of expression, and the concentration range of norepinephrine or agonist to which the cell is exposed (Milligan et al., 1994).

A large number of α_2-adrenergic receptors have been described in the pituitary stalk/median eminence of cattle (Chen et al., 1981). Within the hypothalamus in rats, α_2-adrenergic receptors are localized in various nuclei, with the highest density being present in the medial basal hypothalmus (Cella et al., 1990). Based on in situ hybridization of receptor subtype-specific mRNAs for α_2-adrenergic receptors, α_{2A} is the predominant subtype in rat hypothalamus, but low levels of α_{2C} are also present, most notably in the ventromedial nucleus and the lateral hypothalamic area (Scheinin et al., 1994).

C. α_2-Adrenergic Receptor Regulation of Growth Hormone Release

Activation of α_2-adrenergic receptors increases and inhibition of α_2-adrenergic receptors decreases secretion of growth hormone in several species, including cattle (Gaynor et al., 1993). Indeed, clonidine, an α_2-adrenergic receptor agonist, has been used in clinical settings as a growth hormone secretagogue in humans (Bertherat et al., 1995). The growth hormone-releasing effect of clonidine is believed to be mediated primarily via release of GHRH from the hypothalmus. For example, clonidine has no effect on release of growth hormone from rat anterior pituitary cells in culture, or *in vivo* in rats with hypothalamic lesions (Cella et al., 1985; Becker and Conway, 1992; Katakami et al., 1984). In addition, clonidine stimulates secretion of growth hormone in both control- and SRIF-antiserum-treated rats and sheep, but fails to do so in animals pretreated with GHRH antiserum (Miki et al., 1984; Magnan et al., 1994). Furthermore, clonidine stimulates release of GHRH from perifused rat hypothalami (Kabayama et al., 1986). In sheep, administration of clonidine increases peripheral growth hormone and hypophysial portal GHRH without modulating release of SRIF (Magnan et al., 1994). It has been suggested also that decreases in levels of SRIF mediate effects of clonidine on release of growth hormone in rats and rabbits, but there are no direct data to support this idea (Minamitani et al., 1989; Lanzi et al., 1994; Strobl and Thomas, 1994; Arce et al., 1995).

IV. Dopamine Receptor Regulation of Growth Hormone Secretion

A. Characterization of Dopaminergic Innervation of the Hypothalamus

Unlike midbrain dopamine neurons that project widely to the forebrain areas, there are several locally-projected dopamine neurons in the hypothalamus (Wagner et al., 1995;

Pan, 1996). Hypothalamic dopamine neurons comprise four major groups: the A_{12} tuberinfundibular, A_{13} incertohypothalamic, A_{14} periventricular, and A_{15} ventral nuclear groups (Leshin et al., 1995b; Tillet et al., 1990; van den Pol et al., 1984). The A_{12} tuberinfundibular dopamine neurons, the most studied group, are located in the arcuate nucleus and have axons that project ventrally through the mediobasal hypothalamus and terminate in the median eminence region of the pituitary stalk. Dopamine released from these neurons is transported in hypophysial portal blood to the anterior pituitary gland where it inhibits secretion of prolactin (Ben-Jonathan, 1985). The remaining dopamine neuronal groups (A_{13}, A_{14}, and A_{15}) have short axons that project diffusely into adjacent hypothalamic regions, but little information is available regarding their function (Eaton et al., 1994; Wagner et al., 1995; Cooper et al., 1996).

Average concentrations of dopamine in the pituitary stalk/median eminence of bull calves is two to three times greater than concentrations in the mediobasal hypothalamus (Zinn et al., 1990). Indeed, Leshin et al. (1995a) reported the median eminence is extensively stained for tyrosine hydroxylase-immunoreactive fibers and varicosities in both internal and external layers in cattle. Furthermore, these neurons completely encompass the infundibular recess and are abundant in the ependymal layer of the median eminence. In contrast, dopamine β-hydroxylase-immunoreactivity is very low in the pituitary stalk/median eminence. Because immunostaining of tyrosine hydroxylase and absence of immunostaining of dopamine β-hydroxylase is commonly acknowledged to identify dopamine neurons, dopamine is probably the prevalent catecholamine in the pituitary stalk/median eminence in cattle (Hokfelt et al., 1984; Leshin et al., 1995a). In addition, tyrosine hydroxylase-immunoreactive innervation in the pituitary stalk/median eminence

most likely reflects innervation of axon terminals of A_{12} tuberinfundibular neurons (Leshin et al., 1995a). In the A_{14} periventricular nucleus, tyrosine hydroxylase-immunoreactive perikarya and varicosities are abundant adjacent to the ependymal cell layer of the third ventricle, and tyrosine hydroxylase-immunoreactive perikarya are present in the A_{15} ventral cell group in sheep and cattle (Leshin et al., 1995a; Tillet, 1995). Thus, dopamine innervation is located throughout bovine hypothalamic regions that contain the majority of GHRH and SRIF axons and cell bodies (Gaynor et al., 1995a).

B. Characterization and Signal Transduction Mechanisms of Dopamine Receptors

From studies using biochemical, pharmacological, and physiological techniques, Kebabian and Calne (1979) proposed that there were two subtypes of dopamine receptors, D_1 and D_2. Recent cloning of multiple subtypes of dopamine receptors reveal that there are at least five distinct receptor subtypes (D_1-D_5) that comprise the original D_1-like and D_2-like families (Bunzow et al., 1988; Dearry et al., 1990; Sokoloff et al., 1990; Van Tol et al., 1991; Sunahara et al., 1991).

The D_1 family of dopamine receptors (D_1-like) presently contains only two members, D_1 and D_5 subtypes, that exhibit similar pharmacological and functional characteristics (Civelli et al., 1993; Strange, 1996). Thus far, however, no selective pharmacological agents capable of differentiating the D_1 from the D_5 receptor have been described (Cooper et al., 1996). Both receptors exhibit saturable, high-affinity binding of SKF 38393, a D_1-like selective agonist, and SCH 23390, a D_1-like selective antagonist used in autoradiographic binding studies (Cooper et al., 1996). The most distinguishing characteristic of D_5 receptors is they bind dopamine with a higher affinity than do D_1 receptors (Sunahara et al., 1991; Civelli et al., 1993).

The D_2 family (D_2-like) consists of D_2, D_3, and D_4 subtypes, all of which have generally similar D_2-like pharmacology (Strange, 1996). In addition, alternate splicing of mRNA encoding the D_2 subtype generates two isoforms designated D_{2s} (short) and D_{2L} (long; Giros et al., 1989). Currently it is not possible to distinguish pharmacologically between the D_{2s} and D_{2L} splice variants (Sokoloff and Schwartz, 1995). However, with development of new antipsychotic drugs that are selective for D_2, D_3, and D_4 subtypes, differences in binding affinities for the various compounds allows for pharmacolgical identification of the different D_2-like receptor subtypes. For example, human D_4 receptor has greater affinity for an atypical neuroleptic, clozapine, than either D_2 or D_3 receptors (Civelli et al., 1993; Cooper et al., 1996).

Dopamine receptors with D_2-like pharmacology have been subdivided into pre- and postsynaptic receptors (Civelli et al., 1993). Postsynaptic receptors inhibit the cAMP second messenger system to convey dopamine messages in postsynaptic cells. Presynaptic receptors, or autoreceptors, are present on cells that release dopamine. Stimulation of autoreceptors inhibits neuronal activity, thereby regulating dopamine production via a feedback mechanism (Civelli et al., 1993). In contrast, D_1-like receptors are classified as only postsynaptic receptors (Cooper et al., 1996).

Similar to adrenergic receptors, dopamine receptors contain seven regions of hydrophobic amino acids that form membrane-spanning α-helices that are characteristic of G protein-coupled receptors (O'Dowd, 1995; Strange, 1996). In general, D_1-like and D_2-like receptors induce two types of signal transduction pathways, one obligatory and several cell-specific (Civelli et al., 1993). The obligatory pathway is detected in every cellular environment. The D_1-like receptors stimulate adenylate cyclase via G_s; conversely,

D_2-like receptors inhibit adenylate cyclase via G_i and G_o (Strange, 1996). When dopamine receptors are expressed in fibroblasts or secretory cells, dopamine induces additional and sometimes different signal transduction pathways (Sokoloff and Schwartz et al., 1995). For example, in GH4C1 somatomammotropes, D_1 receptors potentiate activation of L-type voltage-dependent calcium channels in a cAMP-dependent manner (Civelli et al., 1993). In addition, in chinese hamster ovary cells, D_2 receptors potentiate release of arachidonic acid via a protein kinase C mechanism that is independent of the concurrent adenylate cyclase inhibition (Kanterman et al., 1990). Even though observations of these differential effects are from heterologously expressed receptors, it appears various dopamine receptor suptypes may activate multiple signal transduction pathways.

Northern analysis and *in situ* hybridization have been used extensively to study distribution of dopamine receptor mRNA in brain. Distinct patterns of mRNA expression for the various dopamine receptor subtypes are observed throughout the CNS (Levant, 1996). In general, D_1 and D_2 receptor subtype mRNAs are present in all dopamine projection fields of the rat brain, with high levels present in the caudate-putamen, nucleus accumbens, and olfactory tubercule and lower levels in the septum, hypothalamus, and cortex (Meador-Woodruff et al., 1991; Civelli et al., 1993). In contrast, gene transcripts of D_3, D_4, and D_5 subtypes are much less abundant and display more discrete expression in brain (Sokoloff and Schwartz, 1995).

Based on autoradiography binding and *in situ* hybridization studies utilizing hypothalamic tissue, all dopamine receptor subtypes except for D_4 are expressed in the hypothalamus (Fremeau et al., 1991; Meador-Woodruff et al., 1992; Civelli et al., 1993;

Schambra et al., 1994). Cells in the hypothalamus express high levels of D_3 and D_5 mRNA as compared to other brain regions (Sibley and Monsma, 1992). However, this is not to say these are the predominant dopamine receptors in hypothalamic tissue, because expression of D_1 and D_2 mRNA is one to two orders of magnitude greater in abundance than D_3, D_4, and D_5 mRNA (Civelli et al., 1995). D_2 receptor mRNA is expressed at high levels in the pituitary where its physiological role in regulating prolactin secretion is well-known (Civelli, et al., 1993). No D_1, D_3, or D_5 receptor mRNA is detected in the pituitary, whereas D_4 mRNA exists at low levels (Civelli et al., 1993).

C. Dopamine Regulation of Growth Hormone Release

In contrast to the well-known inhibitory action of dopamine on prolactin secretion, the role of dopamine in control of growth hormone secretion is less well understood., Dopamine appears to inhibit secretion of growth hormone directly at the pituitary level. For example, dopamine and D_2-like receptor agonists inhibit growth hormone secretion in cultured or perifused pituitary cells of humans, rats, and sheep (Tallo and Malarkey, 1981; Cronin et al., 1984; Lindström and Ohlsson, 1987; Soyoola et al., 1994). At the hypothalamic level, dopamine stimulates SRIF release from dispersed rat hypothalamic cells, median eminence fragments, mediobasal hypothalamic fragments, and hypothalamic slices (Richardson et al., 1983; Negro-Vilar et al., 1978; Maeda and Frohman, 1980; Kitajima et al., 1989). This stimulatory effect of dopamine on SRIF secretion is consistent with *in vivo* data showing intracerebroventricular administration of dopamine increases release of SRIF into rat hypophysial portal blood (Chihara et al., 1979).

CHAPTER 1

Development of a Perifusion System for Bovine Hypothalamic Slices

INTRODUCTION

In cattle, much of the information regarding regulation of secretion of GHRH and SRIF has been determined indirectly through measurements of growth hormone concentrations in serum. However, changes in release of either GHRH of SRIF may alter secretion of growth hormone. In previous *in vitro* experiments in laboratory animals, incubation of hypothalamic fragments has been a useful approach to study release of hypothalamic hormones (Harter and Ramirez, 1980). Tissue can be maintained in either a static or perifusion system. An advantage of a perifusion system is medium is continuously supplied to the tissue and secretory products from the tissue are continuously removed from the system, thereby minimizing exposure of the tissue to potential regulatory factors. In addition, the dynamic nature of a perifusion system allows analysis of the time course and magnitude of a secretory response. Therefore, to determine directly the role of GHRH and SRIF mediation of α_2-adrenergic and dopaminergic receptor regulation of growth hormone secretion in cattle, my initial objective was to develop an *in vitro* perifusion system for culturing hypothalamic tissue.

Most perifusion systems developed for culturing hypothalamic tissue from laboratory species involve incubation of the entire hypothalamus. However, because brain tissue is critically dependent upon adequate levels of oxygen, the large size of bovine hypothalami, relative to those of laboratory species, precludes this practice as tissue would

be too thick to allow for ample diffusion of oxygen (Teyler, 1980). For electrophysiology recordings, it is recommended that hypothalamic slices be between 400-700 μm thick for the highest viability (Hatton et al., 1980). Cutting of fresh tissue into slices of uniform thickness varies in difficulty directly with the size of the tissue block to be cut (Hatton, 1983). To aid in cutting tissue uniformly and thinly, vibrating microtomes are used to slice brain tissue for electrophysiology recordings (Hatton, 1983). Thus, a goal in development of this *in vitro* system for culturing hypothalamic tissue was to prepare 400-700 μm thick pieces of tissue with a vibrating microtome to maximize the likelihood of oxygen penetration of tissue.

Prior to development of the perifusion system, radioimmunoassays for GHRH and SRIF were developed to quantify the neuropeptides in medium effluent.

MATERIALS AND METHODS

Radioimmunoassay for GHRH

Assay buffer (pH 7.0) was 0.05 M tris buffer containing 0.1% gelatin (Grayslake Gelatin CO., Grayslake, IL), 0.05% Tween 20 (Bio-Rad Laboratories, Richmond, CA), 0.01% sodium azide (Sigma Chemical Co., St. Louis, MO), and 2 mg/L aprotinin (#981 532; Boehringer Mannheim, Indianapolis, IN). Antiserum to bovine GHRH, kindly provided by Upjohn-Pharmacia Co. (Kalamazoo, MI) was raised in a rabbit. Characterization of the antiserum is described in Mehigh et al. (1993). Antiserum, used at

a final dilution of 1:90,000, was diluted in assay buffer. Radiolabeled antigen, 3-[^{125}I]iodotyrosyl^{10} GHRH 1-44 amide (human), was purchased from Amersham Life Science Inc. (Arlington Heights, IL). Lyophilized ^{125}I-GHRH was reconstituted with 100 µl 0.1% acetic acid. Aliquots were placed in polypropylene tubes and frozen at -20°C for use in separate assays. Standards were bovine GHRH 1-44 (Bachem California, Torrance, CA).

For the assay, samples (up to 200 µl), diluted with assay buffer to a total volume of 400 µl, were added to 12 x 75 mm borosilicate glass test tubes. Standards (10 pg/µl) plus a volume of MEM-α (hypothalamic perifusion medium described later) equal to the amount of unknown medium sample assayed were diluted to a final volume of 400 µl with assay buffer. Anti-GHRH (100 µl, diluted 1:15,000 with assay buffer) and ^{125}I-GHRH (100 µl, diluted to 0.006 µCi/100 µl with assay buffer) were added to tubes to measure total binding, standards, or samples. Tubes to measure non-specific binding received 300 µl assay buffer, 200 µl MEM-α, and 100 µl ^{125}I-GHRH. Tubes were incubated at 4°C for 24 h.

Goat anti-rabbit γ-globulin (250 µl; Antibodies Incorporated, Davis, CA) diluted 1:60 in 0.05 M tris buffer (pH 7.0) containing 0.025 M disodium EDTA, 0.01% sodium azide (Sigma Chemical Co.), 0.05% Tween 20 (Bio-Rad Laboratories), and 6% polyethylene glycol 8000 (Mallinckrodt Chemical Co., Paris, KY) was used to separate antibody-bound hormone from free hormone. One hundred microliters of assay buffer containing normal rabbit serum (1:400; Life Technologies, Grand Island, NY) were also added to each tube. After 2 h incubation at approximately 25°C, 2 ml assay buffer were

added to each tube. Tubes were then centrifuged at 1,600 x *g* (3,000 rpm) for 30 min at 4°C to precipitate antigen-antibody complexes. Supernatants were decanted and radioactivity in precipitates was counted in a gamma counter.

Radioimmunoassay for SRIF

Assay buffer (pH 7.0) was 0.1% bovine serum albumin (A3059; Sigma Chemical CO.) in 0.01 M sodium phosphate, 0.1 M disodium EDTA, 0.1% Tween 20, and 2 mg/L aprotinin. Antiserum to SRIF-14 was raised in a rabbit and was kindly provided by Dr. T.H. Elsasser (USDA-ARS, Beltsville, MD). Characterization of the antiserum is described in Elsasser et al. (1990). Antiserum, used at a final dilution of 1:10,000, was diluted in assay buffer. Radiolabeled antigen [^{125}I]Tyr^{1}-SRIF (NEX-129), was purchased from NEN Research Products (Wilmington, DE). Upon thawing of radiolabeled antigen, aliquots were placed in polypropylene tubes and frozen at -20°C for use in separate assays. Standards were SRIF-14 (Peninsula Labs, Belmont, CA).

For the assay, samples (up to 250 µl), diluted with assay buffer to a total volume of 500 µl, were added to 12 x 75 mm borosilicate glass test tubes. Standards (6.4 pg/µl) plus a volume of MEM-α equal to the amount of unknown medium sample assayed were diluted to a final volume of 500 µl with assay buffer. Anti-SRIF (200 µl, diluted 1:2,500 with assay buffer) and ^{125}I-SRIF (100 µl, diluted to 0.006 µCi/100 µl with assay buffer) were added to tubes to measure total binding, standards, or samples. Tubes to measure non-specific binding received 500 µl assay buffer, 250 µl MEM-α, and 100 µl ^{125}I-SRIF. Tubes were incubated at 4°C for 24 h.

Sheep anti-rabbit γ-globulin (250 µl; Antibodies Incorporated) diluted 1:100 in assay buffer containing 6% polyethylene glycol 8000 was used to separate antibody-bound hormone from free hormone. One hundred microliters of assay buffer containing normal rabbit serum (1:400) were also added to each tube. After 2 h incubation at approximately 25°C, tubes were centrifuged at 1,600 x *g* for 30 min (3,000 rpm) at 4°C to precipitate antigen-antibody complexes. Supernatants were decanted and radioactivity in precipitates was counted in a gamma counter.

Preparation of Medium and Balanced Salt Solution for Perifusion System

Perifusion medium, minimum essential medium-α (pH 7.4, MEM-α; Life Technologies Inc.), was supplemented with 25 mM N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid (HEPES; Sigma Chemical Co.), 0.1% bovine serum albumin (A3859; Sigma Chemical Co.), penicillin (10U/ml; Life Technologies Inc.), streptomycin (10 µg/ml; Life Technologies Inc.), ascorbic acid (1 g/L; Merck and Co., Inc., Rahway, NJ), bacitracin (25 mg/L; Sigma Chemical Co.), diprotin A (10 mg/L; Peninsula Laboratories), and soybean trypsin inhibitor (150 mg/L; Sigma Chemical Co.). Medium was prepared on the day of tissue collection and sterilized by filtration with a 0.22 µm Sterivex®-GS filter (Millipore®, Bedford, MA). Medium was saturated with 95% O_2/5% CO_2 and warmed to 37°C before use. Gas was filtered with 25 mm, 0.2 µm Acrodisc® CR PTFE filters (Gelman Sciences, Ann Arbor, MI). Bottles used for medium were pre-sterilized in a steam autoclave for 20 min.

Hank's balanced salt solution without calcium or magnesium (pH 7.4, CMF-HBSS; Life Technologies, Inc.) was supplemented with 25 mM HEPES, penicillin

(10U/ml), and streptomycin (10 μg/ml), sterilized by filtration, and saturated with 95% O_2/5% CO_2.

Preparation of Perifusion Chambers for Hypothalamic Slices

Five milliliter Luer Lok® syringe barrels (Becton-Dickinson and Co., Rutherford, NJ) were used as perifusion chambers (Figure 1). Steam-sterilized glass wool was aseptically placed in the bottom of each syringe up to the 0.5 ml mark and moistened with oxygenated MEM-α. Cytodex I (0.5 ml; Pharmacia Biotech, Uppsala, Sweden) was placed in syringes on top of the glass wool. Before addition to syringes, Cytodex I was swollen in 0.9% NaCl for 2 h, rinsed 2X with 0.9% NaCl, resuspended in 0.9% NaCl, and steam autoclaved for 20 min. Sterile Cytodex I beads were then rinsed with sterile MEM-α before use. Top ends of syringes were plugged with rubber stoppers fitted with 19 ga tubing adapters (Becton-Dickinson and Co.) and plugs. Bottom ends of syringes were plugged with serum stoppers (Fisher Scientific, Pittsburgh, PA).

Collection of Bovine Brain Tissue

Brains were obtained from cattle (> 12 mo of age) killed via stunning followed by exsanguination at a local abattoir. Within 10 min of death, brains with the pituitary stalk attached were removed from the cranial cavity (Figure 2). A scalpel was used to cut a block of tissue containing the thalamus, hypothalamus, and pituitary stalk, utilizing the optic chiasm (anterior), optic nerves (posterior), and hypothalamic sulci (lateral) as landmarks for dissection (Figure 2). Most coagulated blood was removed from the pituitary stalk and dorsal surface of the tissue block with scissors and forceps. The block of tissue was bisected with a scalpel along the sagittal midline through the thalamus, third ventricle, and pituitary stalk (Figure 3). The thalamus, optic chiasm, mammillary bodies,

Figure 1. Hypothalamic perifusion chamber containing glass wool (a), Cytodex I beads (b), and MEM-α (c). Top end of chamber is plugged with a rubber stopper (d) fitted with a 19 ga tubing adapter (e) and plugged with the male end of a 1-cc tuberculin syringe (f). Bottom end of chamber is plugged with a serum stopper (g).

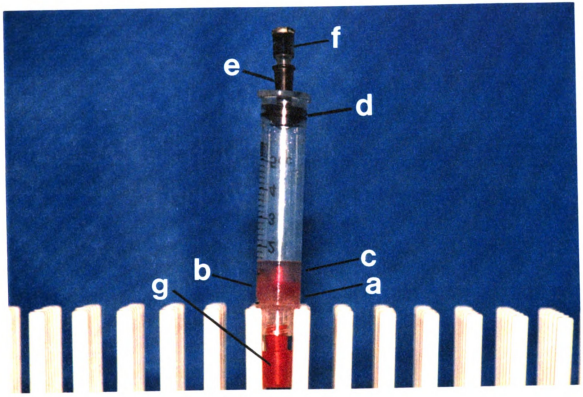
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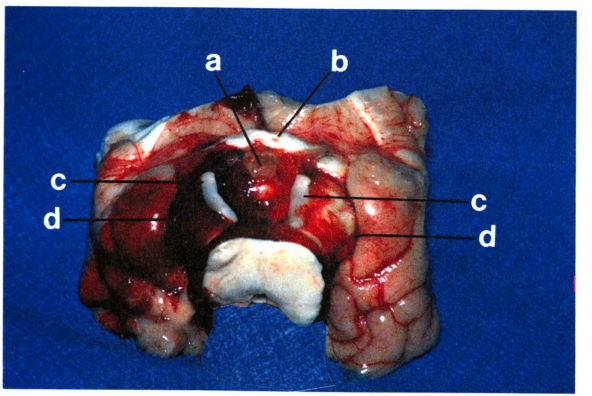
Figure 2. Top panel. Ventral piece of bovine brain with the pituitary stalk (a) attached. Structures utilized as landmarks during dissection include: optic chiasm (b), optic nerves (c), and hypothalamic sulci (d). **Bottom panel.** Block of tissue containing thalamus (not visible), hypothalamus (not visible), pituitary stalk (a), optic chiasm (b), and optic nerves (c).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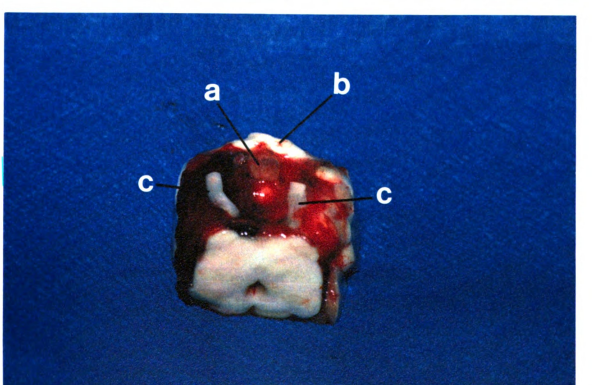
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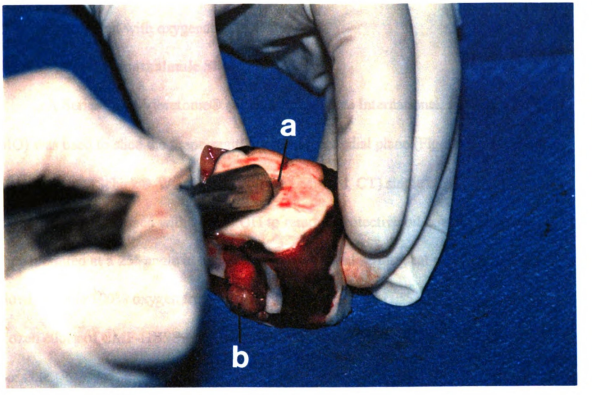
Figure 3. Top panel. Initial bisection of tissue block along the sagittal midline through the thalamus (not visible), third ventricle (a), and pituitary stalk (b). **Bottom panel.** Later stage of bisection of tissue block along the sagittal midline through the thalamus (c), third ventricle (a), pituitary stalk (not visible), optic chiasm (d), and mammillary body (e). Pineal gland is also visible (f).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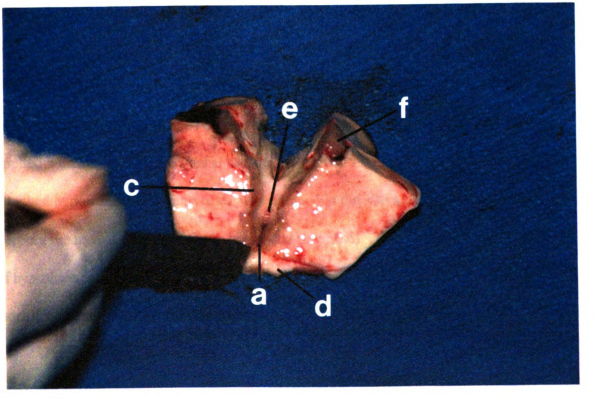
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and other extrahypothalamic tissue was removed (Figure 4). During dissection, tissue was frequently irrigated with oxygenated CMF-HBSS.

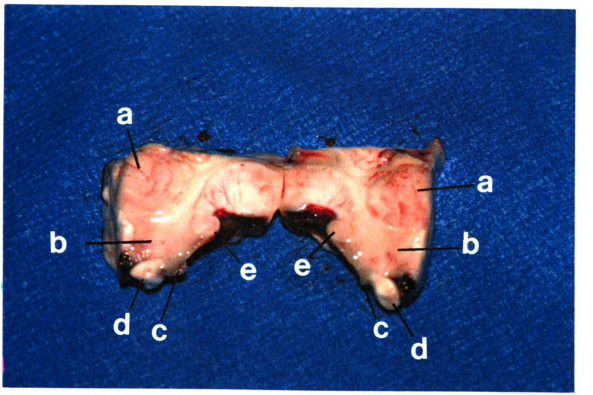
Preparation of Hypothalamic Slices

A Series 1000 Vibratome® (Technical Products International, Inc., St. Louis, MO) was used to slice the tissue sagittally from the medial plane (Figure 5). Schick® Platinum Plus (Schick®-Warner-Lambert Co., Milford, CT) single edge-injector type razor blades were wiped with 70% EtOH to remove protective oil coating. Blades were then mounted at a 25° angle in the Vibratome. A 10-ml serological pipette was used to slowly bubble 100% oxygen from a size E tank to the CMF-HBSS bath. In addition, frozen cubes of CMF-HBSS were added to maintain the specimen bath as close to 4°C as possible.

The most lateral side (furthest from the third ventricle) of each piece of hypothalamus/pituitary stalk was blotted with paper to remove excess fluids to allow for adhesion, and glued to a Vibratome mounting block with Loctite® 404 cyanoacrylate-based adhesive (Loctite Corp., Newington, CT). To aid in adhesion, slight pressure was applied with a spatula to the medial aspect (top) of the tissue. The mounting block with attached hypothalamus/pituitary stalk was clamped in the Vibratome specimen vise, keeping the medial surface of tissue horizontal (Figure 6). Two or three 600 μ thick slices of hypothalamus/pituitary stalk were cut until the fornix was visible. The first slice was most medial and the third slice was most lateral to the sagittal midline.

Hypothalamic slices were removed carefully from the specimen bath with paint brush and spatula, then each slice was placed in a perifusion chamber. Chambers were

Figure 4. Top panel. Bisected blocks of thalamus (a), hypothalamus (b), and pituitary stalk (c). Optic chiasm (d) and mammillary bodies (e) are visible also. **Bottom panel-left.** Hemihypothalami (b)/pituitary stalk (c) before removal of optic chiasm (d) and mammillary body (e). **Bottom panel-right.** Hemihypothalami (b)/pituitary stalk (c) after removal of optic chiasm and mammillary body in preparation for tissue slicing.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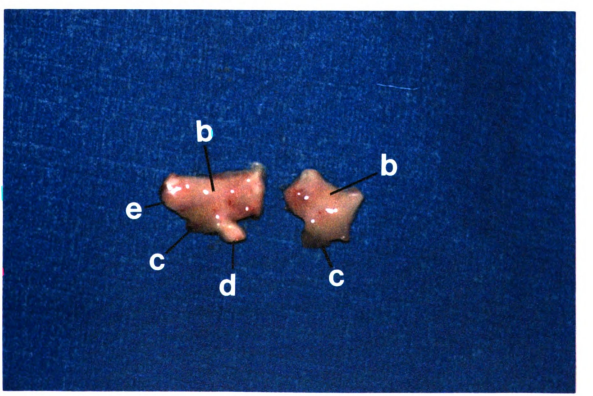
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Figure 5. A Series 1000 Vibratome® with a 10-ml serological pipette (a) placed in specimen bath (b) to deliver 100% oxygen from a size E tank (c) to the CMF-HBSS bath.

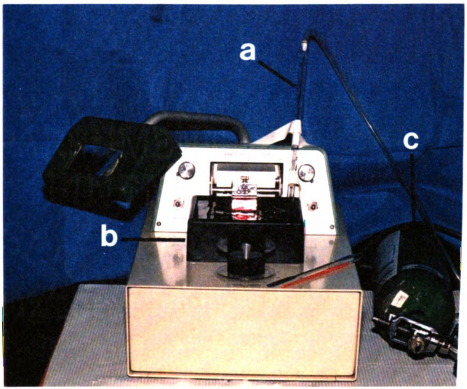
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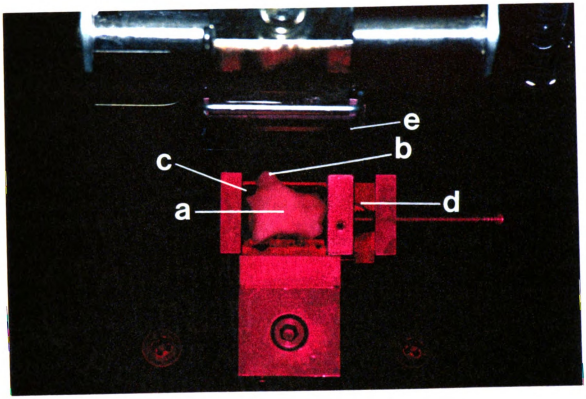
Figure 6. Hypothalamus (a)/pituitary stalk (b) attached to a mounting block (c) which is clamped in the Vibratome specimen vice (d) in the Vibratome specimen bath. The mounted razor blade (e) is also visible.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filled with ice-cold, oxygenated MEM-α and re-plugged with a rubber stopper fitted with a 19 ga tubing adapter and plug (Figure 7). These steps were completed within approximately 30 min of the animals' death. Chambers containing hypothalamic slices were placed in a Kool Mate™ Series Thermoelectric Cooler and Warmer (Igloo Products Corp., Houston, TX) that was maintained at approximately 4°C and transported to the laboratory.

Perifusion System for Hypothalamic Slices

Within 2 h of the animals' death, perifusion chambers containing slices of hypothalamus/pituitary stalk were placed in an incubator (Sherer-Dual Jet, Marshall, MI) maintained at 37°C. Chambers were unplugged and connected to a peristaltic pump (Masterflex L/S™ 07524; Cole-Parmer Instrument Co., Niles, IL) and perifused at 0.15 ml/min with MEM-α gassed continuously with 95% O_2/5% CO_2 (Figure 8). Gas was filtered with 25 mm, 0.2 μm Acrodisc® CR PTFE filters. The perifusion tubing was Masterflex® Pharmed® tubing (size 13; Cole-Parmer Instrument Co.) with an internal diameter of 0.8 mm. Length of tubing from the stock media bottle to the bottom of the perifusion chamber was 85 cm with a volume of 0.43 ml. Tubing was connected to the bottom of perifusion chambers with female polypropylene luer fittings (#06359-27, Cole-Parmer Instrument Co.); thus, medium flowed from the bottom of the chamber and out the top. Three-way stopcocks were placed between the bottle containing medium and perifusion chambers so tissues could be perifused with treatments during experiments.

Tubing carrying medium effluent was connected to the top of perifusion chambers with male polypropylene luer fittings (#06359-07, Cole-Parmer Instrument Co.). When

Figure 7. Hypothalamic slice (a) in a perifusion chamber containing glass wool (b), Cytodex I beads (c) and filled with ice-cold, oxygenated MEM-α (d). Top end of chamber is plugged with a rubber stopper (e) fitted with a 19 ga tubing adaptor (f) and plugged with the male end of a 1 cc tuberculin syringe (g). Bottom end of chamber is plugged with a serum stopper (h). Chambers containing hypothalamic slices, plugged at both ends, were transported at approximately 4° C to the laboratory.

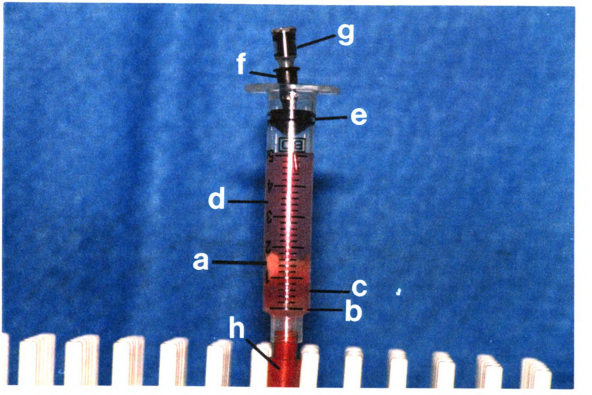
g
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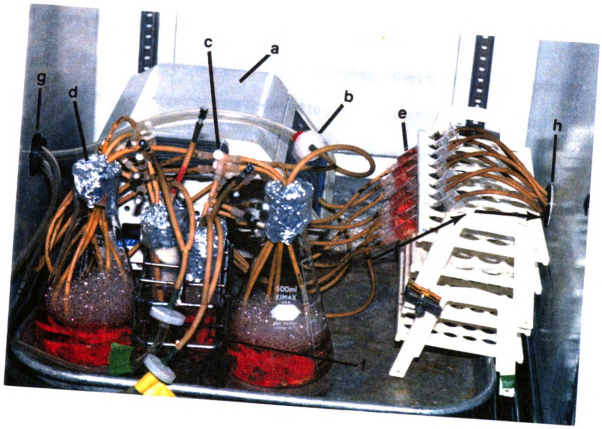
Figure 8. Perifusion chambers placed in incubator and connected to a peristaltic pump (a) and perifused with medium gassed continuously with 95% O_2/5% CO_2. Gas was filtered with 25 mm, 0.2 μm Acrodisc® CR PTFE filters (b). Medium flowed from the bottom of the chamber and out the top (direction of flow indicated by arrows). Three-way stopcocks (c) were placed between the bottle containing medium (d) and perifusion chambers (e) so treatments could be delivered from 50 ml conical tubes (f) at the appropriate times during experiments. Gas lines enter incubator on left side (g) and tubing carrying medium effluent exits incubator on right side (h).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medium effluent tubing was connected to perifusion chambers, the rubber stoppers were pushed down to the 2-ml mark. Length of media effluent tubing was 65 cm with a volume of 0.33 ml. Total volume of perifusion tubing and chambers was 2.76 ml. Perifusion tubing was steam sterilized before each use.

When tissue was to be perifused with treatments, valves on three-way stopcocks were positioned so treatments were delivered to perifusion chambers. Treatments, contained in 50-ml polypropylene conical tubes (Sarstedt Inc., Newton, NC), were continuously gassed with filtered 95% O_2/5% CO_2. Length of tubing delivering treatments to perifusion chambers was 73.5 cm with a volume of 0.37 ml.

During experiments, medium effluent was collected in 12 x 75 mm polypropylene test tubes (Sarstedt Inc.) that were adjacent to the incubator. Samples of medium effluent were stored at -20°C until assayed.

Recovery of GHRH and SRIF from Perifusion System

All components of the perifusion system were assembled except tissue was not placed in perifusion chambers. MEM-α was perifused at 0.15 ml/min and samples of medium effluent were collected at 10 min intervals for 150 min. Three chambers were perifused with medium containing GHRH (10 ng/ml) and three chambers were perifused with medium containing SRIF (6.4 ng/ml). Two pretreatment samples were collected during the first 20 min of the experiment. Treated chambers then received MEM-α containing either GHRH or SRIF for 10 min (1.5 ml). For the remainder of the experiment, all chambers were perifused with MEM-α. Samples of medium effluent were frozen at -20°C until assayed.

Effect of Animal and Location of Slice on Release of GHRH and SRIF

To determine if the animal from which hypothalami were obtained or the location from which the slice was cut (i.e. first, second, or third slice cut from the sagittal midline) effected basal release of GHRH or SRIF, values for basal release representing 140 slices and 36 animals from different experiments were compared. Mean basal release for each slice was calculated from two 20-min samples collected before treatments were imposed.

Effect of Potassium Chloride on Release of GHRH and SRIF

To establish that hypothalamic slices remained viable when cultured in the perifusion system, slices were perifused with either vehicle medium (MEM-α) for controls (n=6) or 60 mM KCl (n=6). Duration of the experiment was 180 min. Throughout the experiment, slices were perifused with MEM-α and medium effluent was collected at 20-min intervals. Two basal samples were collected during the first 40 min of the experiment. Treatments were imposed between 41 and 60 min. Slices then were perifused with MEM-α for the remaining 120 min after perifusion of treatments. Both treatment groups included slices from each animal.

Statistical Analyses

All data were subjected to analysis of variance using Statistical Analysis System (SAS Institute). Differences among means were determined with Bonferroni *t* tests (Gill, 1978). Probability values ≤ 0.05 were considered significant. Animal, location of slice, and experiment were included in the statistical model to test the effect of animal and location of slice on pretreatment release of GHRH and SRIF.

When analyzing the effect of potassium chloride on release of GHRH and SRIF, areas under GHRH and SRIF response curves (AUC; $ng \cdot ml^{-1}$ min) were calculated from concentrations representing the interval between 41 and 180 min using the trapezoidal rule and were utilized as indicators of GHRH and SRIF release. To account for differences in pretreatment release between slices, pretreatment GHRH and SRIF values at 20 min were used as covariates in the statistical analysis of their respective measurements because P was ≤ 0.25 for the covariate term in the statistical model. When appropriate, AUC were transformed to $\log_{10}$ before statistical analysis to achieve homogeneous variances among treatments (Gill, 1978). Perifusion chamber, animal, location of slice, pretreatment covariate, and treatment were included in statistical models when P was ≤ 0.25 for the statistical term (Gill, 1978). Interactions between animal and treatment, and slice location and treatment were also analyzed.

RESULTS

Standard Curves and Assay Parallelism for GHRH and SRIF Radioimmunoassays

Based on data from 20 radioimmunoassays, binding in tubes containing no GHRH was between 50 and 60%, with nonspecific binding between 7 and 11% of total counts added. Sensitivity of the assay was 10 pg/tube and 50% of maximal displacement on the standard curve was 306 pg/tube (Figure 9). Slope of the dilution curve of effluent medium collected from the hypothalamic perifusion system (3 replicates; $b = -38.8$; $r^2 = 0.95$) was parallel ($P > .10$) to that of the bovine GHRH standard between 40 and 1500 pg/tube (20 replicates; $b = -39.4$; $r^2 = 0.96$). In subsequent experiments, all samples from

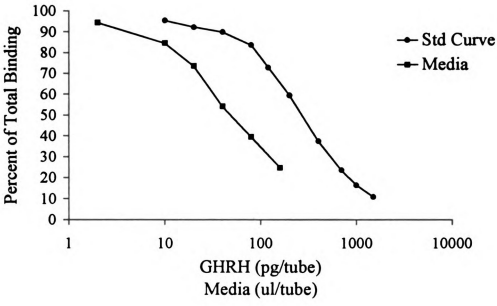

Figure 9. Standard curve for GHRH radioimmunoassay (20 replicates) and dilution curve of effluent medium from the hypothalamic perifusion system (3 replicates). Slope for standard curve of GHRH between 40 and 1500 pg/tube paralleled slope for dilution curve of medium effluent ($P > 0.10$).

a given experiment were analyzed in a single assay. Intra-assay variation averaged 9.1±0.4%.

Based on data from 20 radioimmunoassays, binding in tubes containing no SRIF was between 20 and 30%, with nonspecific binding between 1 and 2% of total counts added. Sensitivity of the assay was 5 pg/tube and 50% of maximal displacement on the standard curve was 169 pg/tube (Figure 10). Slope of the dilution curve of effluent medium collected from the hypothalamic perifusion system (3 replicates; b = -38.3; r^2 = 0.98) was ($P > 0.10$) parallel to that of the SRIF (1-14) standard between 5 and 1000 pg/tube (20 replicates; b = -41.1; $r^2 = 0.99$). In subsequent experiments, all samples from a given experiment were analyzed in a single assay. Intra-assay variation averaged 6.2±0.7%.

Recovery of GHRH and SRIF from Perifusion System

Recovery of GHRH and SRIF from the perifusion system was 78 and 86%, respectively (Table 1).

Effect of Animal and Location of Slice on Release of GHRH and SRIF

Location of slices relative to the sagittal midline had no effect on basal release of GHRH and SRIF (Figure 11). The minimum and maximum values for GHRH and SRIF were different from each other (Table 2).

Effect of Potassium Chloride on Release of GHRH and SRIF

Compared with perifusion of medium alone, medium containing 60 mM KCl increased AUC for GHRH and SRIF in slices maintained in culture for 14 h (Figures 12 and 13). There were no interactions between animal and treatment or slice location and treatment, therefore all slices from all animals and slice locations responded similarly.

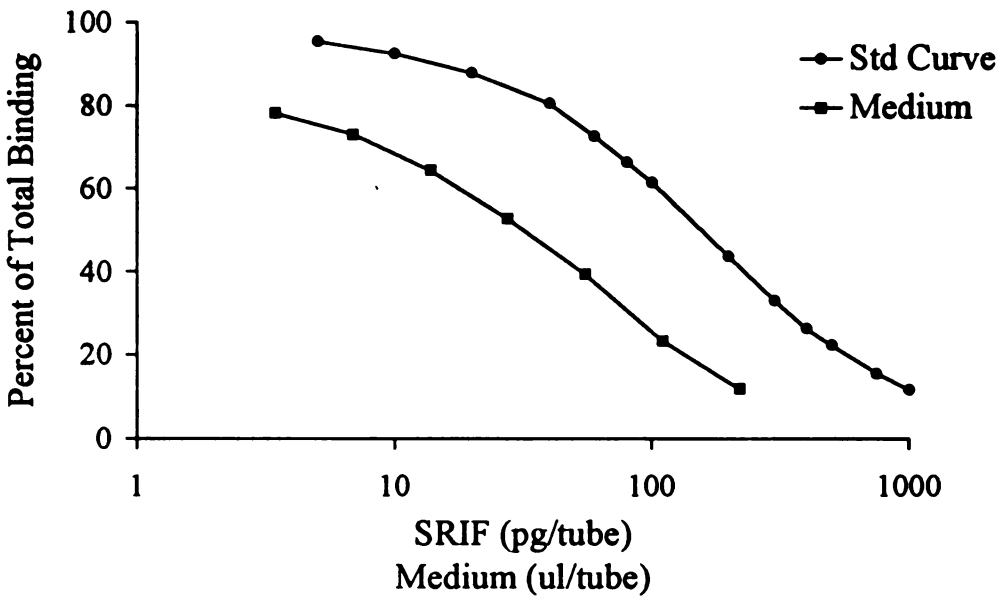

Figure 10. Standard curve for SRIF radioimmunoassay (20 replicates) and dilution curve of effluent medium from the hypothalamic perifusion system (3 replicates). Slope for standard curve of SRIF between 5 and 1000 pg/tube paralleled slope for dilution curve of medium effluent ($P > 0.10$).

Table 1. Recovery of GHRH and SRIF from perifusion system (3 replicates/peptide).

	GHRH	SRIF
Peptide perifused through system (ng)	15	9.6
Peptide recovered from system (ng)	11.7	8.3
% Recovery	78	86

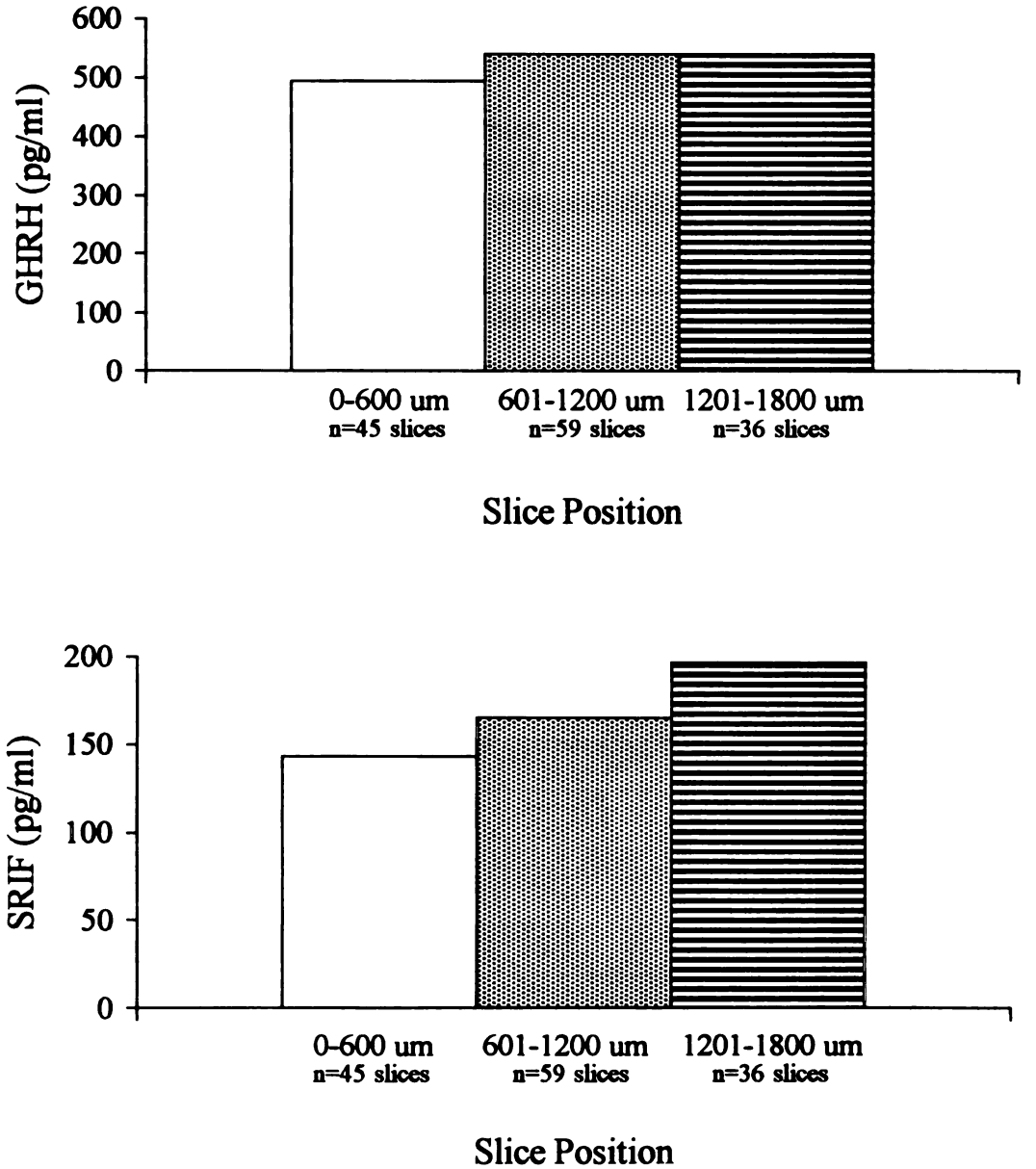

Figure 11. Mean basal release of GHRH (top panel) and SRIF (bottom panel) from bovine hypothalamic slices perifused at 0.15 ml/min. Mean basal release was calculated from two 20 min samples. Slices were cut 0-600 um (n=45 slices), 601-1200 um (n=59 slices), or 1201-1800 um (n=36 slices) from the sagittal midline. Means within peptide were not different from each other ($P > 0.20$; pooled SEM for GHRH = 31 pg/ml; pooled SEM for SRIF = 22 pg/ml).

Table 2. Mean, median, minimum, and maximum basal concentrations for GHRH and SRIF release from bovine hypothalmic slices perifused at 0.15 ml/min. Basal release was calculated from two 20-min samples. Values were pooled for 140 slices collected from 36 animals.

	GHRH (pg/ml)	SRIF (pg/ml)
Mean (±SEM)	525.6 ± 47.0	168.2 ± 18.7
Median	423.9	141.7
Minimum	230.5[a]	44.4[b]
Maximum	1297.0[a]	455.4[b]

[a,b]Differences among animals within a peptide = $P < 0.0001$

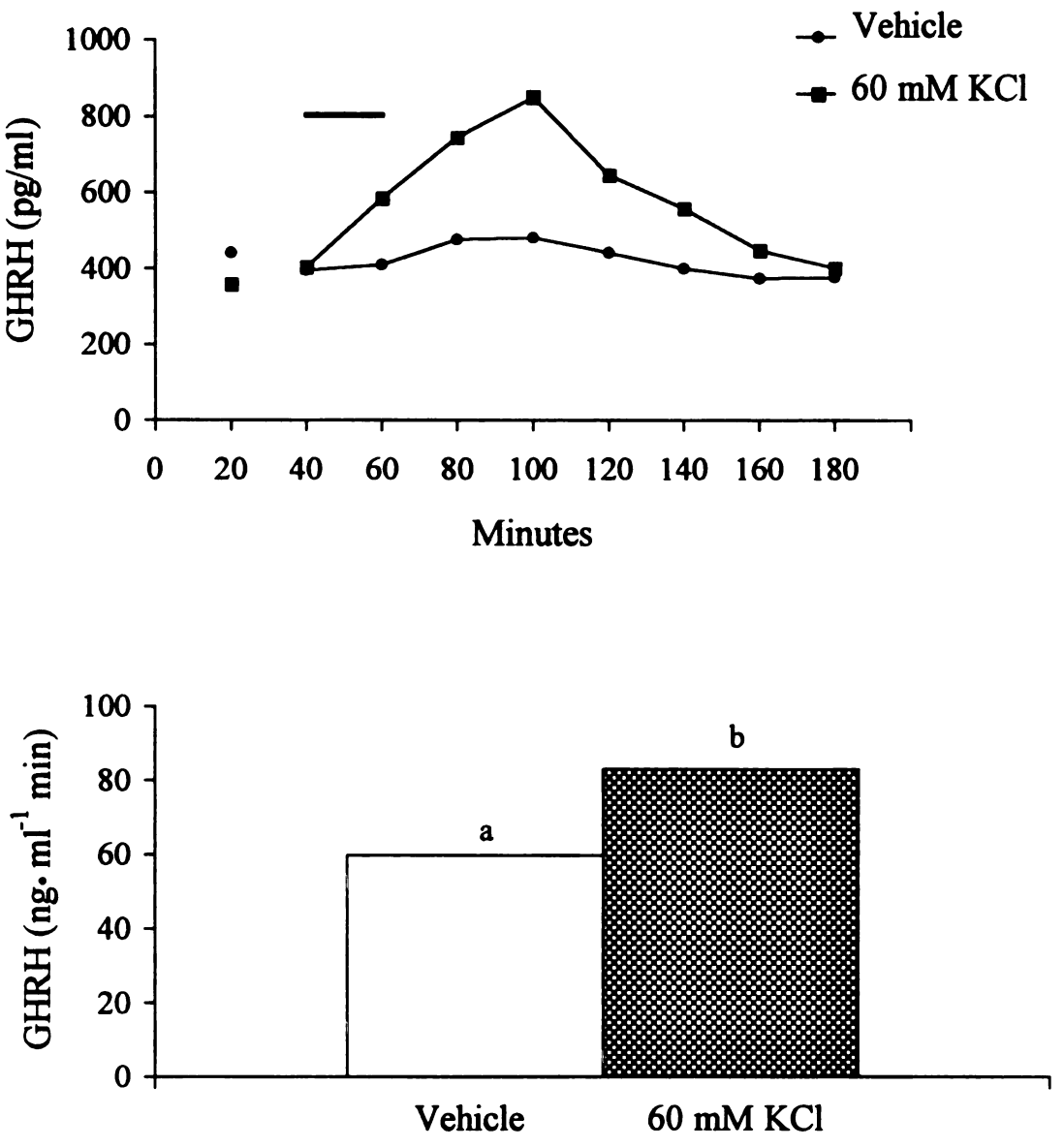

Figure 12. Concentrations of GHRH in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=6 slices) or 60 mM KCl (n=6) from 41 to 60 min (indicated by horizontal line). Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for GHRH calculated from concentrations representing the interval between 41 and 180 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.001$; pooled SEM = 2.2 ng · ml^{-1} min).

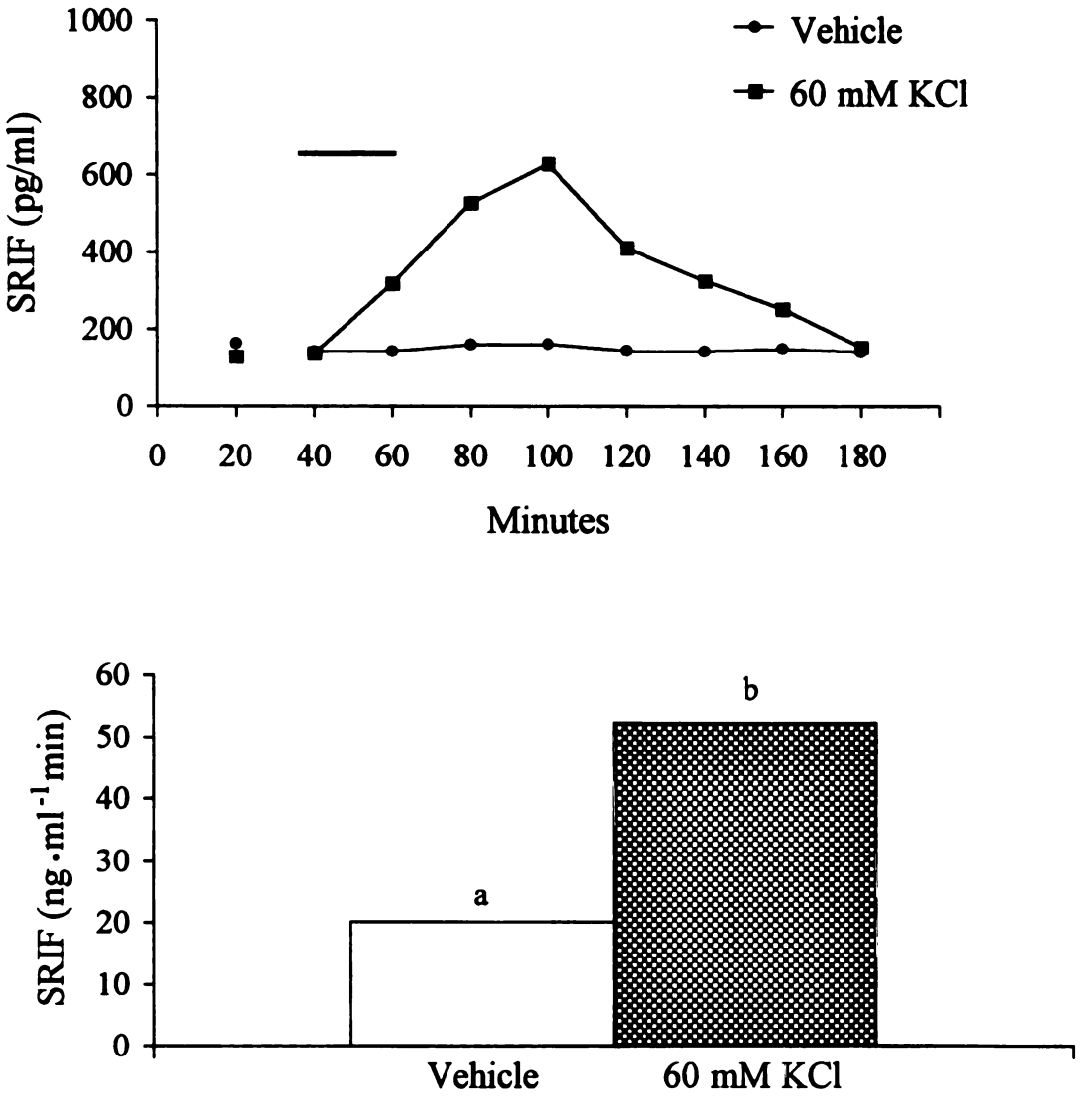

Figure 13. Concentrations of SRIF in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=6 slices) or 60 mM KCl (n=6) from 41 to 60 min (indicated by horizontal line). Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for GHRH calculated from concentrations representing the interval between 41 and 180 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.001$; pooled SEM = 1.1 ng $\cdot$ ml^{-1} min).

DISCUSSION

Freshly dissected hypothalamic preparations have been widely used in laboratory animals to examine regulatory mechanisms of hypothalamic hormone release (Maeda and Frohman, 1980). In the present chapter, I described radioimmunoassays that were developed for GHRH and SRIF and a perifusion system using bovine hypothalami, all which will be utilized to investigate the roles of α_2-adrenergic and dopaminergic receptors in regulation of GHRH and SRIF release. Assay sensitivies for both GHRH and SRIF allow for quantification of basal levels of GHRH and SRIF in samples of medium effluent. In addition, parallel displacement of ^{125}I-GHRH and ^{125}I-SRIF by medium effluent indicates that the apparent peptide content of a medium sample is independent of the dilution at which it is assayed.

Because secretory products from tissue are continuously removed from a perifusion system, regulatory factors should have minimal feedback effects on secretion of hormones. This is particularly important with respect to GHRH and SRIF, because previous studies suggested that GHRH and SRIF modulate their own release and exert feedback effects on one another (Mitsugi et al., 1990; Yamauchi et al., 1991; Aguila, 1994).

Membrane depolarization with potassium is a highly potent stimulus for hormone release because it triggers sodium and calcium influx, thereby leading to exocytosis of hormone (Harter and Ramirez, 1980). Furthermore, depolarization of neurosecretory terminals is a preliminary event in neuropeptide release (Shimatsu et al., 1982). Therefore, the ability of tissue to respond to depolarization with potassium is utilized as an indicator

of tissue viability in *in vitro* systems. Hypothalamic slices maintained in the perifusion system were viable for at least 14 h after tissue was collected because release of GHRH and SRIF increased when tissue slices were exposed to depolarizing concentrations of potassium. The large amount of animal variation in basal release of GHRH and SRIF may have been due to differences in sex, age, and breed of the animals used in the present experiments. Even though there was variation in basal release of GHRH and SRIF, slices from all animals and slice locations responded similarly to depolarization with KCl . Thus, I concluded that this system could be utilized to examine regulation of GHRH and SRIF secretion.

CHAPTER 2

α_2-Adrenergic Receptor Regulation of Growth Hormone-Releasing Hormone and Somatostatin from Perifused, Bovine Hypothalamic Slices

INTRODUCTION

Growth hormone is secreted from the anterior pituitary gland in a pulsatile manner under regulation of GHRH and SRIF (Frohman et al., 1992). Growth hormone-releasing hormone is synthesized in neurons located primarily in the arcuate nucleus of the hypothalamus, transported to nerve terminals in the median eminence, and released into hypophysial portal vessels to stimulate both synthesis and release of growth hormone (Leshin et al., 1994; Ibata et al., 1986). Somatostatin neuronal cell bodies are located primarily in the periventricular nucleus and project to the median eminence (Leshin et al., 1994). Portal vessels also transport SRIF from the median eminence to the anterior pituitary gland where it inhibits secretion of growth hormone (Frohman et al., 1992).

Neurons of the central noradrenergic system play a dominant stimulatory role in regulation of growth hormone secretion of several species (McCann and Krulich, 1989). For example, in vivo stimulation of α_2-adrenergic receptors increases secretion of growth hormone, whereas blockade of α_2-adrenergic receptors decreases secretion of growth hormone in cattle (Gaynor et al., 1993). A hypothalamic site of action is likely, because perifusion of rat anterior pituitary cells with an α_2-adrenergic receptor agonist does not affect release of growth hormone (Becker and Conway, 1992). However, analogous experiments have not been conducted in cattle. Thus, the first objective was to determine if an α_2-adrenergic receptor agonist directly affects secretion of growth hormone from

bovine somatotropes. The second objective was to determine if pharmacological activation of α_2-adrenergic receptors regulates release of GHRH and SRIF from the bovine hypothalamus.

MATERIALS AND METHODS

Perifusion of Bovine Anterior Pituitary Cells

Pituitary glands from cattle were collected at slaughter and enzymatically dispersed as described previously (Padmanabhan et al., 1987). Briefly, 1-mm^3 pieces of adenohypophyseal tissue were digested in a solution of CMF-HBSS and 0.3% collagenase (Type I, Worthington Biochemical, Freehold, NJ) for 45 min at 37°C. After enzymatic digestion, the tissue was triturated 20 times by passage through a 10-ml serological pipette. The resulting cell suspension was filtered through sterile, presoaked gauze and then centrifuged (400 x *g* for 5 min) to recover the cells. Cells were washed three times with CMF-HBSS and one time with Dulbecco's modified Eagle's medium (DMEM) with low glucose (<1,000 mg/L, Life Technologies) containing 1% newborn calf serum (Life Technologies), 25 mM HEPES, 10 U penicillin/ml, 10 μg streptomycin/ml, and 0.25 μg fungizone/L. Cells were then suspended in DMEM at a concentration of 10^6 cells/ml. After dispersion and resuspension, cell viability was greater than 90% as determined by trypan blue exclusion.

The perifusion system of Hassan et al. (1994) was modified for use in this experiment. Barrels of 3-ml syringes (Becton-Dickinson and Co.) served as perifusion chambers. Rubber serum stoppers were placed on the tips of syringes and a layer of sterile

glass wool was inserted to retain Cytodex I beads. Syringes were packed by gravity to a volume of 0.5 ml with sterile Cytodex I beads, which had been swollen in 0.9% NaCl. Aliquots (1 ml) of the cell suspension in media were pipetted into perifusion chambers. Syringes were then filled to the 3-ml mark with DMEM and placed in a humidified atmosphere of 95% O_2-5% CO_2 at 37°C for 24 h.

After the initial incubation, perifusion chambers were connected to peristaltic pumps and perifused with DMEM, gassed with 95% O_2-5% CO_2, at a flow rate of 0.15 ml/min for 360 min. After this flushing period, chambers were ready for experimental protocols. During experiments, medium effluent was collected in 12 x 75 mm polypropylene tubes that were adjacent to the incubator. Samples of medium effluent were stored at -20°C until assayed. Radioimmunoassay was used to quantify concentrations of growth hormone in medium effluent as described previously (Gaynor et al., 1995b).

Perifusion of Bovine Hypothalamic Slices

Brains were obtained from cattle (>12 mo of age) killed via stunning followed by exsanguination at a local abattoir. Tissue was processed and cultured in a perifusion system as described in Chapter 1. Radioimmunoassays were used to quantify concentrations of GHRH and SRIF in medium effluent as described in Chapter 1.

Experimental Protocols

Experiment 1 was conducted to test the effect of the α_2-adrenergic receptor agonist, clonidine on release of growth hormone from anterior pituitary cells. Duration of the experiment was 200 min and medium effluent (3 ml) was collected at 20-min intervals. Two pretreatment samples were collected during the first 40 min of the experiment and

treatments were imposed from 41 to 60 min. Control cells (n=6) were perifused with vehicle medium and treated cells received either 10^{-8} M GHRH 1-44 (Bachem California; n=6), 10^{-8} M (n=6), 10^{-6} M (n=6), or 10^{-4} M (n=6) clonidine (Hoffman and Lefkowitz, 1993; Sigma Chemical Co.). Cells were perifused with vehicle medium from 61 to 120 min. To test cell viability, cells were perifused with DMEM containing 60 mM KCl from 121 to 140 min. For the remaining 60 min after perifusion of KCl, cells were perifused with vehicle medium.

Experiments 2 and 3 were conducted to test the effect of activation of α_2-adrenergic receptors with either clonidine or guanabenz on release of GHRH and SRIF. Duration of experiments 2 and 3 was 180 min, and medium effluent (3 ml) was collected at 20-min intervals. In each experiment, two pretreatent samples were collected during the first 40 min of the experiment. Treatments were imposed from 41 to 60 min. In experiment 2, control slices (n=8) were perifused with vehicle medium and treated slices received either 10^{-8} M (n=8), 10^{-6} M (n=8), or 10^{-4} M (n=8) clonidine. In experiment 3, control slices (n=8) received vehicle medium and treated slices were perifused with either 10^{-8} M (n=8), 10^{-6} M (n=8), or 10^{-4} M (n=8) guanabenz (Hoffman and Lefkowitz, 1993; Sigma Chemical Co.). In both experiments, slices were perifused with vehicle medium for the remaining 120 min after perifusion of treatments.

A fourth experiment was conducted to determine if idazoxan, an α_2-adrenergic receptor antagonist, would block the effects of clonidine. Duration of the experiment was 200 min and medium effluent was collected at 20-min intervals with pretreatment samples collected during the first 40 min of the experiment. Treatments consisted of vehicle-perifused controls (n=4), 10^{-6} M clonidine (n=4), 10^{-6} M idazoxan (n=4; Moberg et al.,

1996; Sigma Chemical Co.), or a combination of 10^{-6} M idazoxan plus 10^{-6} M clonidine (n=4). Treatments were imposed from 41 to 100 min in controls and in the idazoxan-alone group. Treatment was perifused from 61 to 80 min in the clonidine-alone group. In the combination group, slices were perifused with idazoxan alone from 41 to 60 min, idazoxan plus clonidine from 61 to 80 min, and idazoxan alone from 81 to 100 min. After perifusion of treatments, all slices were perifused with vehicle medium for the remainder of the experiment.

Statistical Analyses

In experiment 1, areas under growth hormone response curves were calculated from concentrations representing the interval between 61 and 120 min using the trapezoidal rule and were subjected to analysis of variance using Statistical Analysis System (SAS Institute). To account for differences in pretreatment release between chambers of cells, pretreatment growth hormone values at 20 min were used as covariates in the statistical analysis. To achieve homogeneous variances among treatments, AUC were transformed to $\log_{10}$ before statistical analysis (Gill, 1978). Depolarization-stimulated (60 mM KCl) release of growth hormone was calculated as the average of concentrations in medium samples collected at 140 and 160 min. Comparisons between basal (samples collected at 20 and 40 min) and depolarization-stimulated secretion of growth hormone were determined using Student's paired *t* test (Gill, 1978).

All data from hypothalamic perifusions were subjected to analysis of variance using Statistical Analysis System. Differences among means were determined with Bonferroni *t* test or Dunnett's *t* test (Gill, 1978). Probability values ≤ 0.05 were considered to be significant. In experiments 2 and 3 AUC for GHRH and SRIF were calculated from

concentrations representing the interval between 41 and 180 min using the trapezoidal rule and were utilized as indicators of GHRH and SRIF release. In experiment 4, AUC for GHRH and SRIF were calculated from concentrations representing the interval between 61 and 200 min. To account for differences in pretreatment release between slices, pretreatment GHRH and SRIF values at 20 min were used as covariates in the statistical analysis of their respective measurements because P was ≤ 0.25 for the covariate term in the statistical model. When appropriate, AUC were transformed to $\log_{10}$ before statistical analysis to achieve homogeneous variances among treatments (Gill, 1978). Perifusion chamber, animal, location of slice, covariate, and treatment were included in statistical models when P was ≤ 0.25 for the statistical term (Gill, 1978). Interactions between animal and treatment, and slice location and treatment were also analyzed.

RESULTS

Effect of Clonidine on Release of Growth Hormone

Compared with perifusion of medium alone, medium containing clonidine had no effect on release of growth hormone from dispersed anterior pituitary cells (Figure 14). In contrast, perifusion of media containing 10^{-8} M GHRH increased release of growth hormone compared with perifusion of medium alone. For all treatments, perifusion of media containing 60 mM KCl increased concentrations of growth hormone in samples collected at 140 and 160 min compared with basal secretion (means of samples collected at 20 and 40 min).

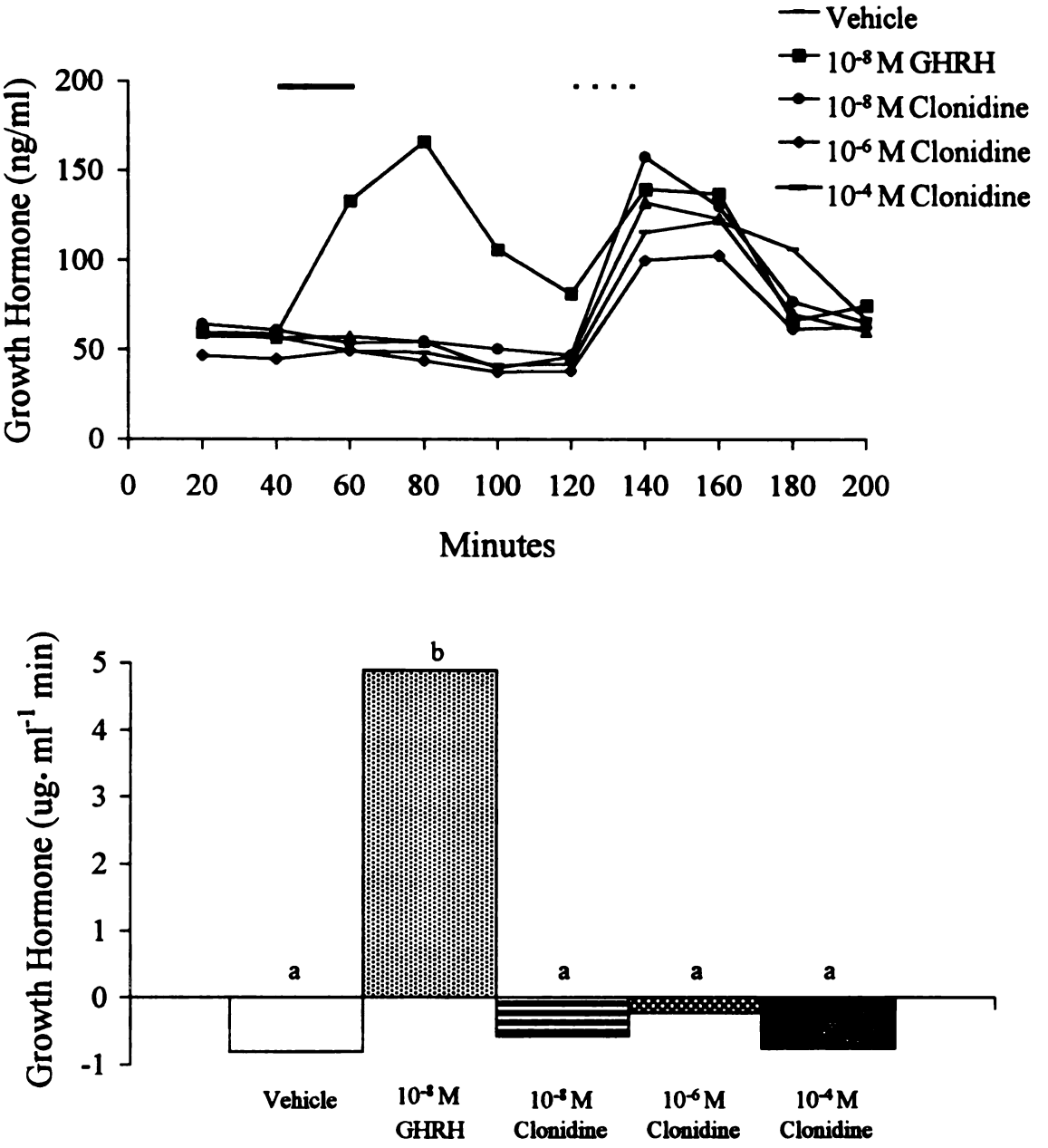

Figure 14. Concentrations of growth hormone in medium effluent (top panel) from bovine anterior pituitary cells perifused with vehicle medium (control, n=6), 10^{-8} M GHRH (n=6), 10^{-8} M (n=6), 10^{-6} M (n=6), or 10^{-4} M (n=6) clonidine from 41 to 60 min (indicated by solid horizontal line). Flow rate was 0.15 ml/min. All groups were perifused with 60 mM KCl from 121 to 140 min (indicated by dashed horizontal line). Statistical analysis was performed on AUC for growth hormone calculated from concentrations representing the interval between 61 and 120 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Negative values reflect declines in secretion of growth hormone. Means without a common letter differed ($P < 0.05$; pooled SEM = 0.5 ug· ml^{-1} min).

Effect of Clonidine on Release of GHRH and SRIF

Relative to vehicle-perifused controls, clonidine at 10^{-6} and 10^{-4} M increased AUC for GHRH (Figure 15). In addition, the increase in AUC for GHRH was 37% greater in the 10^{-4} M group when compared with the 10^{-6} M group. Clonidine at 10^{-8} M was ineffective. AUC for SRIF were not different among treatments (Figure 16). There were no interactions between animal and treatment or slice location and treatment, therefore all slices from all animals and slice locations responded similarly.

Effect of Guanabenz on Release of GHRH and SRIF

Compared with vehicle-perifused controls, guanabenz increased release of GHRH (Figure 17). Furthermore, it was a dose-responsive increase as AUC for GHRH in the 10^{-4} M group was greatest and the increase in the 10^{-8} M group was smallest. Medium containing guanabenz had no effect on release of SRIF (Figure 18). There were no interactions between animal and treatment or slice location and treatment, therefore all slices from all animals and slice locations responded similarly.

Effect of Idazoxan and Clonidine on Release of GHRH and SRIF

Relative to vehicle-perifused controls, clonidine at 10^{-6} M increased AUC for GHRH 27% (Figure 19). Idazoxan alone at 10^{-6} M did not affect release of GHRH. However, idazoxan blocked clonidine-induced release of GHRH. Release of SRIF was similar among groups (Figure 20).

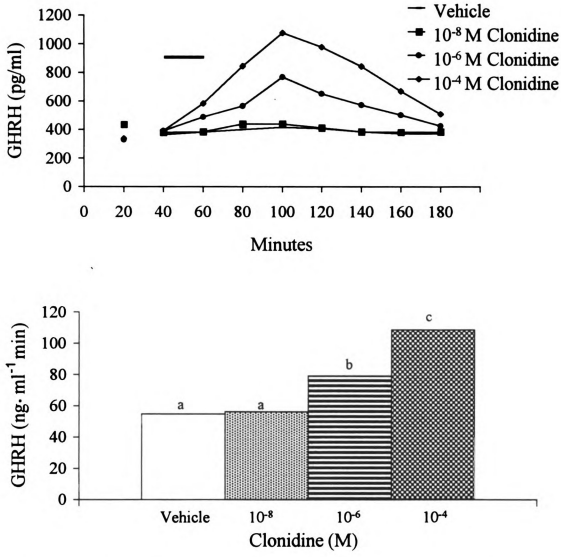

Figure 15. Concentrations of GHRH in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=8), 10^{-8} M (n=8), 10^{-6} M (n=8), or 10^{-4} M (n=8) clonidine from 41 to 60 min (indicated by horizontal line). Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for GHRH calculated from concentrations representing the interval between 41 and 180 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.001$; pooled SEM = 2.5 ng $\cdot ml^{-1}$ min).

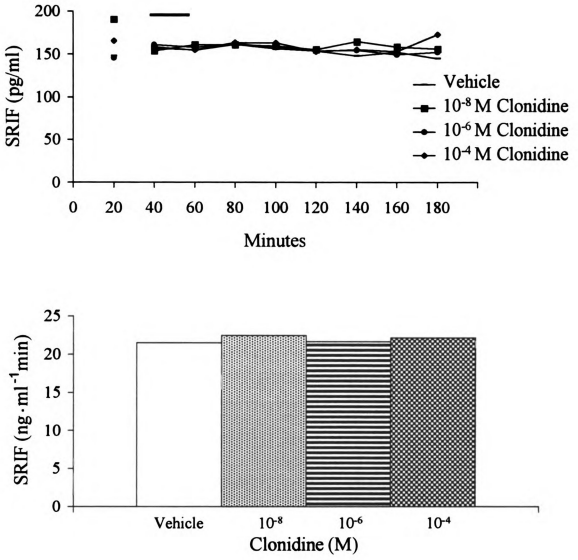

Figure 16. Concentrations of SRIF in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=8), 10^{-8} M (n=8), 10^{-6} M (n=8), or 10^{-4} M (n=8) clonidine from 41to 60 min (indicated by horizontal line). Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for GHRH calculated from concentrations representing the interval between 41and 180 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means were not different from each other (P = 0.7; pooled SEM = 0.6 ng $\cdot$ ml^{-1} min).

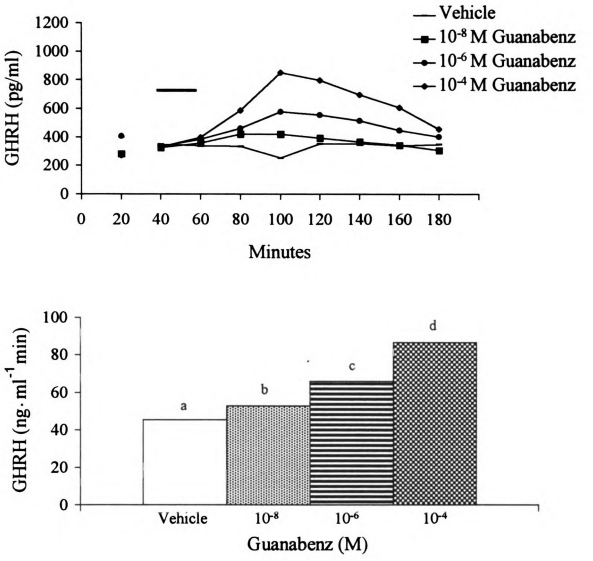

Figure 17. Concentrations of GHRH in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=8), 10^{-8} M (n=8), 10^{-6} M (n=8), or 10^{-4} M (n=8) guanabenz from 41to 60 min (indicated by horizontal line). Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for GHRH calculated from concentrations representing the interval between 41 and 180 min (bottom panel). All data are reported as least squares meáns and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.03$; pooled SEM = 1.6 ng $\cdot ml^{-1}$ min).

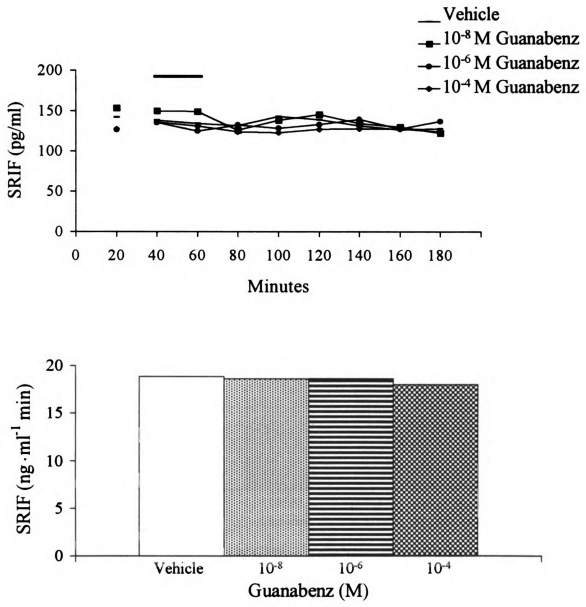

Figure 18. Concentrations of SRIF in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=8), 10^{-8} M (n=8), 10^{-6} M (n=8), or 10^{-4} M (n=8) guanabenz from 41 to 60 min (indicated by horizontal line). Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for SRIF calculated from concentrations representing the interval between 41 and 180 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means were not different from each other (P = 0.8; pooled SEM = 0.7 ng $\cdot$ ml^{-1} min).

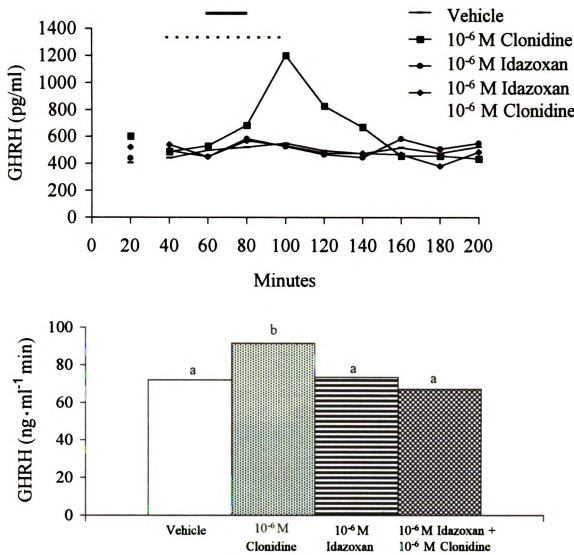

Figure 19. Concentrations of GHRH in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=4), 10^{-6} M clonidine (n=4), 10^{-6} M idazoxan (n=4), or 10^{-6} M idazoxan plus 10^{-6} M clonidine (n=4). Treatments were imposed from 41 to 100 min in controls and in the idazoxan alone group (indicated by dashed horizontal line). Treatment was perifused between 61 and 80 min in the clonidine alone group (indicated by solid horizontal line). In the combination group, slices were perifused with idazoxan between 41 and 60 min, idazoxan plus clonidine between 61 and 80 min, and idazoxan between 81 and 100 min. Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for GHRH calculated from concentrations representing the interval between 61 and 200 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.03$; pooled SEM = 4.1 ng · ml^{-1} min).

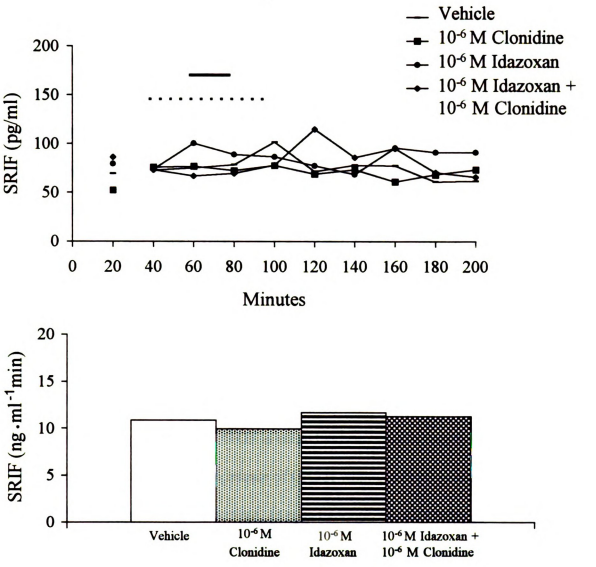

Figure 20. Concentrations of SRIF in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=4), 10^{-6} M clonidine (n=4), 10^{-6} M idazoxan (n=4), or 10^{-6} M idazoxan plus 10^{-6} M clonidine (n=4). Treatments were imposed from 41 to 100 min in controls and in the idazoxan alone group (indicated by dashed horizontal line). Treatment was perifused between 61 and 80 min in the clonidine alone group (indicated by solid horizontal line). In the combination group, slices were perifused with idazoxan between 41 and 60 min, idazoxan plus clonidine between 61 and 80 min, and idazoxan between 81 and 100 min. Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for SRIF calculated from concentrations representing the interval between 61 and 200 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means were not different from each other (P = 0.3; pooled SEM = 0.7 ng·ml^{-1} min).

DISCUSSION

Our laboratory showed previously that systemic administration of clonidine, a centrally acting α_2-adrenergic receptor agonist, increases secretion of growth hormone in cattle (Gaynor et al., 1993). This effect could have been the result of an increase in GHRH, a decrease in SRIF, or a direct effect on somatotropes in the anterior pituitary gland. Because clonidine did not stimulate release of growth hormone directly from bovine somatotropes, I hypothesized that the clonidine-induced increase in growth hormone observed in vivo was due to an increase in GHRH or a decrease in SRIF. Thus, my objective was to determine if clonidine regulates release of GHRH and SRIF.

The present studies are the first, to my knowledge, in which α_2-adrenergic receptor regulation of release of GHRH and SRIF in cattle has been directly examined. Both clonidine and guanabenz stimulated release of GHRH without affecting release of SRIF from the bovine hypothalamus. Idazoxan, an α_2-adrenergic receptor antagonist, was able to block clonidine-induced release of GHRH, suggesting that clonidine was acting via α_2-adrenergic receptors. These observations support the hypothesis that α_2-adrenergic receptor stimulation of growth hormone secretion in cattle is mediated via an increase in GHRH release and not a change in SRIF release.

Results from other studies on the action of α_2-adrenergic receptor agonists also suggest activation of α_2-adrenergic receptors stimulates release of GHRH rather than inhibits release of SRIF. For example, in rats passively immunized against GHRH, clonidine is unable to stimulate release of growth hormone, whereas immunoneutralization of endogenous SRIF does not alter α_2-adrenergic receptor-mediated stimulation of growth

hormone secretion (Miki, et al., 1984). In addition, clonidine stimulates GHRH release from perifused hypothalamic fragments in rats (Kabayama et al., 1986). Magnan et al. (1994) measured GHRH and SRIF in hypophysial portal blood after intravenous injection of clonidine in sheep. Clonidine increased peripheral growth hormone and hypophysial portal GHRH without modulating SRIF release. Furthermore, immunization of sheep against GHRH completely abolished clonidine-induced growth hormone secretion, whereas immunization against SRIF was without effect (Magnan et al., 1994). In other *in vivo* studies in rats and rabbits, however, it has been suggested that clonidine stimulates secretion of growth hormone via inhibition of hypothalamic SRIF release (Lanzi et al., 1994; Minamitani et al., 1989). However, GHRH and SRIF were not directly measured in either of these studies. Therefore, my data in the present studies are in agreement with a bulk of the evidence, which suggests that activation of α_2-adrenergic receptors stimulates release of GHRH but not SRIF.

Within the central nervous system, clonidine acts on postsynaptic receptors as well as presynaptic autoreceptors which inhibit norepinephrine synthesis and release through negative feedback mechanisms (Cooper, 1996). With respect to α_2-adrenergic receptor regulation of release of GHRH, it is unclear whether presynaptic or postsynaptic α_2-adrenergic receptors are involved. Therefore, in the present study, clonidine and guanabenz may have acted at the presynaptic level to inhibit the firing rate of terminals of noradrenergic neurons. In this case, inhibition of noradrenergic neurons may have relieved a tonic suppression of GHRH neurons thereby increasing the release of GHRH. Alternatively, clonidine may have acted at the postsynaptic level directly on hypothalamic neurons to stimulate release of GHRH. Because the in vitro preparation used in these

studies did not include intact norepinephrine neurons, noradrenergic tone may not have been present in the tissue. In addition, in rats and monkeys activation of postsynaptic α_2-adrenergic receptors most likely mediates the stimulatory action of clonidine on growth hormone release, because depletion of brain catecholamines does not prevent clonidine-induced growth hormone release (Müller, 1987). Thus, I speculate pharmacological agents used in these studies acted on postsynaptic α_2-adrenergic receptors.

A large number of α_2-adrenergic receptors have been described in the pituitary stalk/median eminence of cattle (Chen et al., 1981). In addition, binding sites for α_2-adrenergic receptor agonists are present in the arcuate nucleus of rats (Unnerstall et al., 1984; Cella et al., 1990b). Our laboratory has reported that activity of noradrenergic neurons terminating in the arcuate nucleus and pituitary stalk/median eminence is elevated when concentrations of growth hormone are elevated in steers possibly due to an increase in GHRH tone (Gaynor et al., 1995a). Therefore, the pharmacological agents used in the present experiments may have acted within the arcuate nucleus on GHRH neuronal cell bodies and(or) within the pituitary stalk/median eminence on terminals of GHRH neurons.

In conclusion, my findings support the hypothesis that noradrenergic neurons within the CNS increase release of GHRH, but not SRIF, to mediate α_2-adrenergic receptor stimulation of secretion of growth hormone in cattle.

CHAPTER 3

Dopaminergic Receptor Regulation of Growth Hormone-Releasing Hormone and Somatostatin from Perifused, Bovine Hypothalamic Slices

INTRODUCTION

Hypothalamic GHRH stimulates and SRIF inhibits secretion of growth hormone from the anterior pituitary gland (Frohman et al., 1992). Growth hormone-releasing hormone is synthesized in cell bodies located in the hypothalamic arcuate nucleus and released from axon terminals in the pituitary stalk/median eminence where it is transported in hypophysial portal blood to the anterior pituitary gland (Leshin et al., 1994; Ibata et al., 1986). Somatostatin is synthesized in cell bodies located in the periventricular nucleus of the hypothalamus and released from axon terminals in the pituitary stalk/median eminence (Leshin et al., 1994). Portal vessels also transport SRIF from the median eminence to the anterior pituitary gland (Frohman et al., 1992).

In rats, both *in vitro* and *in vivo* data suggest dopaminergic pathways are involved in regulation of hypothalamic release of SRIF (Kitajima et al., 1989; Chihara et al., 1979). There is little information regarding the role of dopamine neurons in the control of GHRH release. However, results from studies utilizing immunocytochemical staining for the presence of catecholamine-synthesizing enzymes suggest the periventricular and arcuate nuclei as well as the pituitary stalk/median eminence of cattle contain dopaminergic innervation (Leshin et al., 1995a). Therefore, dopamine may regulate release of SRIF and GHRH from the bovine hypothalamus.

Dopamine receptors have been divided on the basis of pharmacological, biochemical, and functional differences into two general subtypes referred to as D_1 and D_2 receptors (Cooper et al., 1996). Data from studies utilizing radioligand-binding autoradiography and in situ hybridization of mRNA encoding D_1 and D_2 receptors indicate the hypothalamus contains both D_1 and D_2 dopamine receptors (Cooper et al., 1996; Schambra et al., 1994). However, tuberoinfundibular dopamine neurons in the mediobasal hypothalamus lack D_2 receptors (Durham et al., 1996). Therefore in contrast to the well-established role of pituitary D_2 receptors in the control of prolactin secretion, much less is known about the potential involvement of D_1 and D_2 dopamine receptors in regulation of other neuroendocrine secretions. Thus, my objective was to determine if pharmacological activation or inhibition of either D_1 or D_2 dopamine receptors regulates release of SRIF and GHRH from the bovine hypothalamus.

MATERIALS AND METHODS

Perifusion of Bovine Hypothalamic Slices

Brains were obtained from cattle (>12 mo of age) killed via stunning followed by exsanguination at a local abattoir. Tissue was processed and cultured in a perifusion system as described in Chapter 1. Radioimmunoassays were used to quantify concentrations of SRIF and GHRH in medium effluent as described in Chapter 1.

Experimental Protocols

To test the effect of activation of D_1 dopamine receptors with SKF 38393 (Dubois et al., 1986), and blockade of D_1 dopamine receptors with SCH 23390 (Schambra et al.,

1994) on release of SRIF and GHRH, three experiments were conducted. Duration of experiments 1and 2 was 180 min, and medium effluent (3 ml) was collected at 20-min intervals. In each experiment, two pretreatment samples were collected during the first 40 min of the experiment. Treatments were imposed from 41 to 60 min. In experiment 1, control slices (n=8) were perifused with vehicle medium and treated slices received either 10^{-10} M (n=8), 10^{-8} M (n=8), or 10^{-6} M (n=8) SKF 38393 (Research Biochemicals International, Natick, MA). In experiment 2, control slices (n=8) received vehicle medium and treated slices were perifused with either 10^{-10} M (n=8), 10^{-8} M (n=8), or 10^{-6} M (n=8) SCH 23390 (Research Biochemicals International). In both experiments, slices were perifused with vehicle medium for the remaining 120 min after perifusion of treatments.

Experiment 3 was conducted to determine if a D_1 antagonist (SCH 23390) would block the effects of a D_1 agonist (SKF 38393) on release of SRIF and GHRH. Duration of this experiment was 200 min and medium effluent was collected at 20 min-intervals with pretreatment samples collected during the first 40 min of the experiment. Treatments consisted of vehicle-perifused controls (n=6) and slices perifused with 10^{-6} M SKF 38393 (n=6), 10^{-6} M SCH 23390 (n=6), or a combination of 10^{-6} M SCH 23390 plus 10^{-6} M SKF 38393 (n=6). Treatments were perifused from 41 to 100 min in controls and in the SCH 23390 alone group. Treatment was perifused from 61 to 80 min in the SKF 38393 alone group. In the combination group, slices were perifused with SCH 23390 alone from 41 to 60 min, SCH 23390 plus SKF 38393 from 61 to 80 min, and SCH 23390 alone from 81 to 100 min. After perifusion of treatments, all slices were perifused with vehicle medium until the end of the experiment at 200 min.

Experiments 4 and 5 were designed to test the effect of activation of D_2 dopamine receptors with quinelorane (Foreman et al., 1989) and blockade of D_2 dopaminergic receptors with haloperidol (Cooper et al., 1996) on release of SRIF and GHRH. Duration of experiments 4 and 5 was 180 min and medium effluent (3 ml) was collected at 20-min intervals. In each experiment, two pretreatment samples were collected during the first 40 min of the experiment. Treatments were imposed from 41 to 60 min. In experiment 4, control slices (n=8) were perifused with vehicle medium and treated slices received either 10^{-10} M (n=8), 10^{-8} M (n=8), or 10^{-6} M (n=8) quinelorane (Research Biochemicals International). In experiment 5, control slices (n=8) received vehicle medium and treated slices were perifused with either 10^{-10} M (n=8), 10^{-8} M (n=8), or 10^{-6} M (n=8) haloperidol (Sigma Chemical Co.). In both experiments, slices were perifused with vehicle medium for the remaining 120 min after perifusion of treatments.

Statistical Analyses

All data were subjected to analysis of variance using Statistical Analysis System (SAS Institute). Differences among means were determined with Bonferroni *t* tests and Dunnett's *t* test (Gill, 1978). Probability values ≤ 0.05 were considered to be significant. In experiments with duration of 180 min and that utilized SKF 38393, SCH 23390, quinelorane, or haloperidol alone, areas under SRIF and GHRH response curves (AUC, ng· ml^{-1} min) were calculated from concentrations representing the interval between 41 and 180 min using the trapezoidal rule and were utilized as indicators of SRIF and GHRH release. In the experiment utilizing the combination of SKF 38393 and SCH 23390, AUC for SRIF and GHRH were calculated from concentrations representing the interval between 61 and 200 min. To account for differences in basal release between slices,

pretreatment SRIF and GHRH values at 20 min were used as covariates in the statistical analysis of their respective measurements when P was ≤ 0.25 for the covariate term in the statistical model (all analyses except for SRIF in experiment 3). When appropriate, AUC were transformed to $\log_{10}$ before statistical analysis to achieve homogeneous variance among treatments (Gill, 1978). Perifusion chamber, animal, location of slice, pretreatment covariate, and treatment were included in statistical models when P was ≤ 0.25 for the statistical term (Gill, 1978). Interactions between animal and treatment, and slice location and treatment were also analyzed.

RESULTS

Effect of SKF 38393 and SCH 23390 on Release of SRIF and GHRH

Compared with perifusion of medium alone, medium containing 10^{-8} and 10^{-6} M SKF 38393 increased AUC for SRIF an average of 82-fold (Figure 21). At 10^{-10} M, SKF 38393 had no effect on SRIF. AUC for GHRH was decreased 50% in the 10^{-6} M SKF 38393 group relative to controls (Figure 22). Neither 10^{-10} or 10^{-8} M SKF 38393 had a significant effect on release of GHRH, but the numerical decrease in GHRH in the 10^{-8} M group tended to be significant (P = 0.18). There were no interactions between animal and treatment or slice location and treatment, therefore all slices from all animals and slice locations responded similarly.

The D_1 receptor antagonist, SCH 23390, had no effect on AUC for either SRIF or GHRH (Table 3).

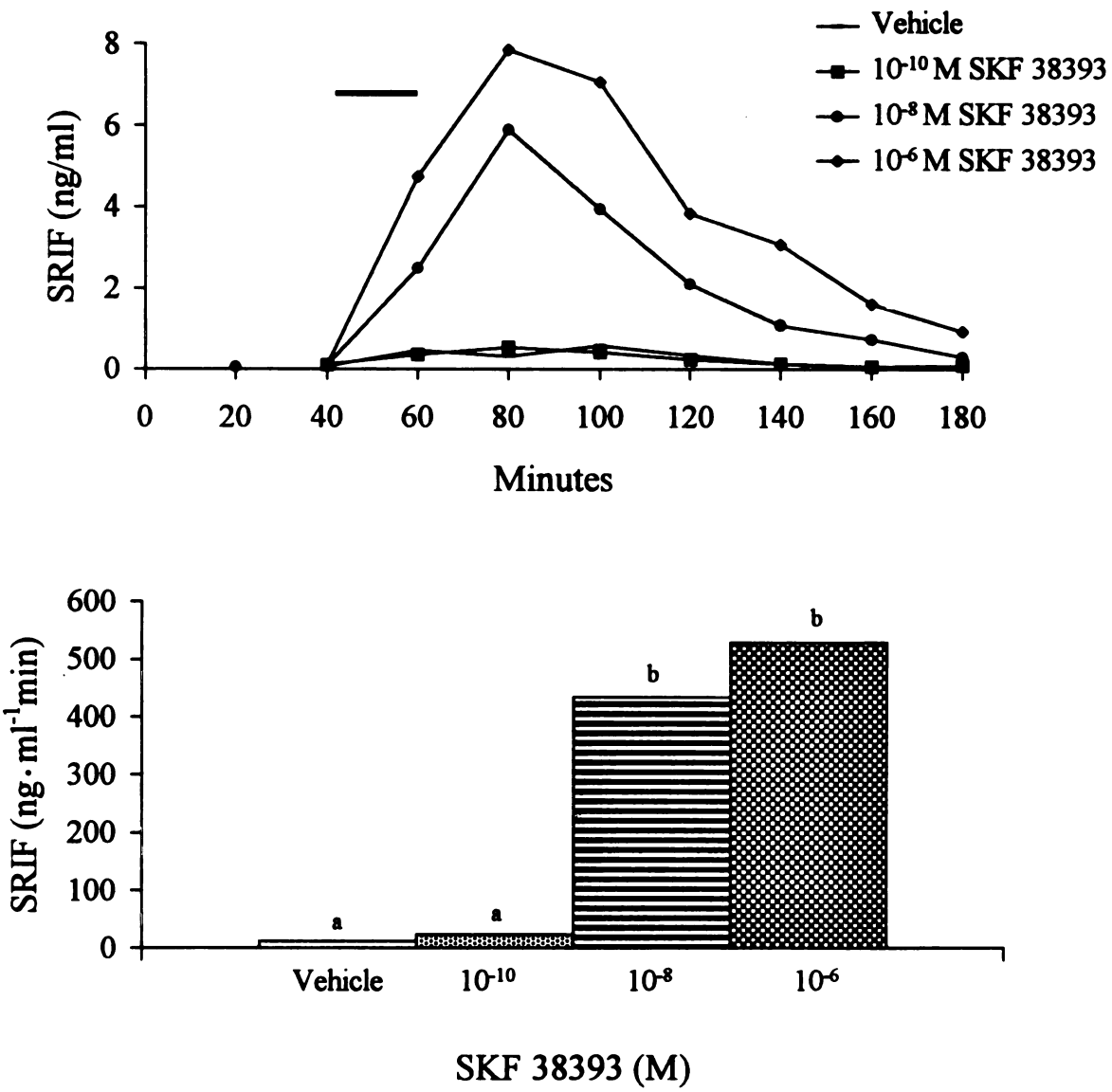

Figure 21. Concentrations of SRIF in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=8), 10^{-10} M (n=8), 10^{-8} M (n=8), or 10^{-6} M (n=8) SKF 38393 from 41 to 60 min (indicated by horizontal line). Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for SRIF calculated from concentrations representing the interval between 41and 180 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.001$; pooled SEM = 0.6 ng · ml^{-1} min).

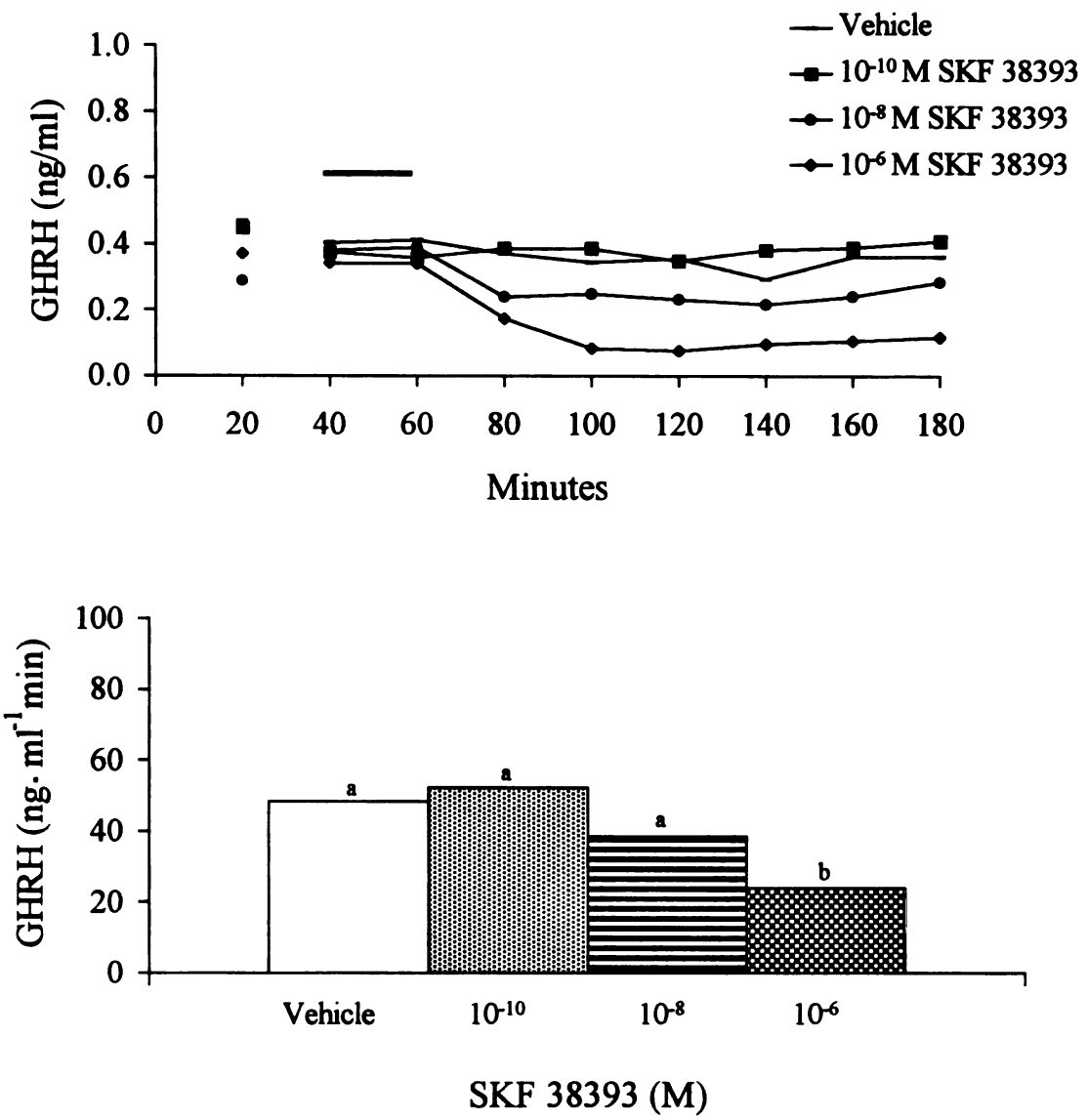

Figure 22. Concentrations of GHRH in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=8), 10^{-10} M (n=8), 10^{-8} M (n=8), or 10^{-6} M (n=8) SKF 38393 from 41 to 60 min (indicated by horizontal line). Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for GHRH calculated from concentrations representing the interval between 41and 180 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.002$; pooled SEM = 0.4 ng·ml^{-1} min).

Table 3. AUC for SRIF and GHRH (least squares means) calculated from concentrations representing the interval between 41and 180 min for slices perifused with vehicle medium (control) or SCH 23390 at 0.15 ml/min. Data are reported as least squares means and adjusted by covariance using pretreatment release of SRIF or GHRH at 20 min.

	SRIF ($ng^{-1}ml \cdot min$)	GHRH ($ng^{-1}ml \cdot min$)
Vehicle (n=8)	5.8	40.3
10^{-10} M SCH 23390 (n=8)	6.3	38.2
10^{-8} M SCH 23390 (n=8)	6.3	41.5
10^{-6} M SCH 23390 (n=8)	6.0	41.9
Pooled SEM	1.1	2.9
P	0.9	0.8

At 10^{-6} M, SKF 38393 increased AUC for SRIF from 5.1 ng·ml^{-1}min in controls to 470.3 ng·ml^{-1}min (Figure 23). SCH 23390 alone at 10^{-6} M did not affect release of SRIF, but SCH 23390 reduced the SKF 38393-induced release of SRIF to only 42.9 ng·ml^{-1}min. In other words, the increase in SRIF with SKF 38393 alone was 11 times greater than the increase with SCH 23390 plus SKF 38393. Release of GHRH was decreased from 80.3 ng·ml^{-1}min in controls to 45.0 ng·ml^{-1}min in the group perifused with SKF 38393 alone (Figure 24). SCH 23390 did not affect AUC for GHRH. However, the decrease in release of GHRH due to SKF 38393 was blocked with SCH 23390 treatment.

Effect of Haloperidol and Quinelorane on Release of SRIF and GHRH

Medium containing quinelorane had no effect on release of SRIF or GHRH (Table 4). In addition, haloperidol did not affect AUC for SRIF or GHRH (Table 5).

DISCUSSION

Cell bodies of hypothalamic SRIF and GHRH neurons are located primarily in the periventricular and arcuate nuclei respectively, and terminate in the pituitary stalk/median eminence of cattle (Leshin et al., 1994). In addition, all of these regions contain dopaminergic innervation (Leshin et al., 1995a; Zinn et al., 1990). The present studies are the first, to my knowledge, in which dopamine receptor regulation of release of SRIF and GHRH in cattle has been directly examined. Perifusion of SKF 38393, a D_1 receptor agonist, increased release of SRIF in a dose responsive manner. This result is consistent with *in vivo* and *in vitro* reports in rats that dopamine stimulates release of SRIF (Kitajima et al., 1989; Chihara et al., 1979; Bennett et al., 1979; Richardon et al., 1983). In

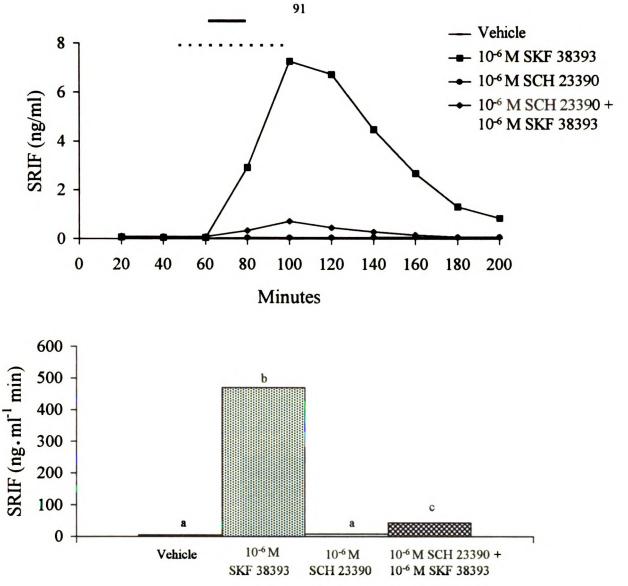

Figure 23. Concentrations of SRIF in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=6), 10^{-6} M SKF 38393 (n=6), 10^{-6} M SCH 23390 (n=6), or 10^{-6} M SCH 23390 plus 10^{-6} MSKF 39393 (n=6). Treatments were imposed from 41 to 100 min in controls and in the SCH 23390 alone group (indicated by dashed horizontal line). Treatment was perifused from 61 to 80 min in the SKF 38393 alone group (indicated by solid horizontal line). In the combination group, slices were perifused with SCH 23390 alone from 41 to 60 min, SCH 23390 plus SKF 38393 from 61 to 80 min, and SCH 23390 alone from 81 to 100 min. Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for SRIF calculated from concentrations representing the interval between 61and 200 min (bottom panel). All data are reported as least squares means. Means without a common letter differed ($P < 0.002$; pooled SEM = 0.6 ng·ml^{-1} min).

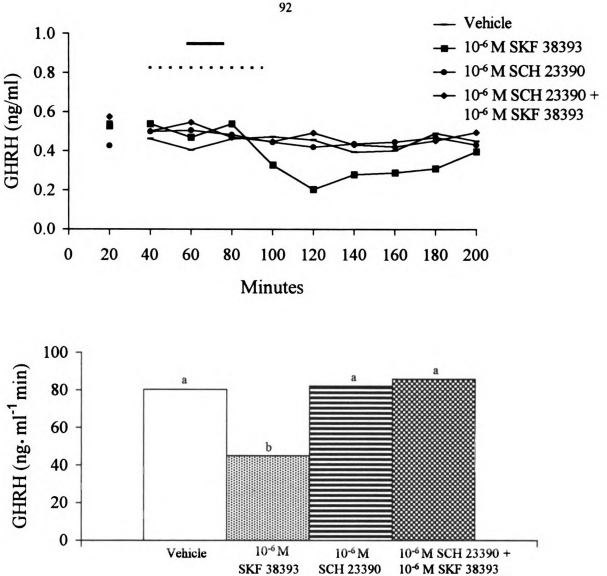

Figure 24. Concentrations of GHRH in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=6), 10^{-6} M SKF 38393 (n=6), 10^{-6} M SCH 23390 (n=6), or 10^{-6} M SCH 23390 plus 10^{-6} MSKF 39393 (n=6). Treatments were imposed from 41 to 100 min in controls and in the SCH 23390 alone group (indicated by dashed horizontal line). Treatment was perifused from 61 to 80 min in the SKF 38393 alone group (indicated by solid horizontal line). In the combination group, slices were perifused with SCH 23390 alone from 41 to 60 min, SCH 23390 plus SKF 38393 from 61 to 80 min, and SCH 23390 alone from 81 to 100 min. Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for SRIF calculated from concentrations representing the interval between 61and 200 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.001$; pooled SEM = 0.7 ng · ml^{-1} min).

Table 4. AUC for SRIF and GHRH (least squares means) calculated from concentrations representing the interval between 41and 180 min for slices perifused with vehicle medium (control) or quinelorane at 0.15 ml/min. Data are reported as least squares means and adjusted by covariance using pretreatment release of SRIF or GHRH at 20 min.

	SRIF ($ng^{-1}ml \cdot min$)	GHRH ($ng^{-1}ml \cdot min$)
Vehicle (n=8)	6.9	60.3
10^{-10} M quinelorane (n=8)	7.4	62.2
10^{-8} M quinelorane (n=8)	6.1	58.5
10^{-6} M quinelorane (n=8)	7.1	60.9
Pooled SEM	.9	5.1
P	0.5	0.6

Table 5. AUC for SRIF and GHRH (least squares means) calculated concentrations representing the interval between 41and 180 min for slices perifused with vehicle medium (control) or haloperidol at 0.15 ml/min. Data are reported as least squares means and adjusted by covariance using pretreatment release of SRIF or GHRH at 20 min.

	SRIF ($ng^{-1}ml \cdot min$)	GHRH ($ng^{-1}ml \cdot min$)
Vehicle (n=8)	9.6	50.6
10^{-10} M haloperidol (n=8)	7.4	65.5
10^{-8} M haloperidol (n=8)	10.7	58.6
10^{-6} M haloperidol (n=8)	8.5	63.4
Pooled SEM	1.2	5.1
P	0.4	0.3

addition, SKF 38393 decreased release of GHRH. This is the first report, to my knowledge, of dopaminergic suppression of GHRH in any species. The D_1 receptor antagonist, SCH 23390, did not affect basal release of SRIF or GHRH, indicating that D_1 receptors do not tonically suppress SRIF or GHRH. However, SCH 23390 blocked SKF 38393-induced release of SRIF and blocked SKF 39393-induced suppression of GHRH, suggesting that SKF 38393 was acting via D_1 dopamine receptors. In contrast, neither activation or blockade of D_2 receptors affected release of SRIF or GHRH from bovine hypothalamic slices, suggesting that D_2 dopamine receptors are not involved in regulation of SRIF or GHRH in cattle. Because haloperidol acts on both D_2 and D_1, an experiment utilizing a selective D_2 receptor antagonist may be necessary to verify my finding. Regardless, taken together, these data support the hypothesis that dopamine increases release of SRIF and decreases release of GHRH from the bovine hypothalamus via D_1 dopamine receptors.

Presynaptic dopamine receptors, or autoreceptors, are present on dopamine neurons and modulate release and synthesis of dopamine. Because dopamine autoreceptors are D_2 receptors and in the present experiments neither the D_2 agonist or antagonist affected GHRH or SRIF, data from the present experiments suggest pharmacological agents used in these studies did not act presynaptically (Cooper et al., 1996). If autoreceptors are involved in regulatation of dopamine neurons involved in regulation of SRIF and GHRH, then D_2 receptor agonists and antagonists should have affected endogenous dopamine which then could have acted on D_1 receptors. Furthermore, cells expressing D_1 receptor mRNA are detected throughout the hypothalamus in rats and are located on postsynaptic neurons (Fremeau et al., 1991,

Cooper, 1996). Therefore, postsynaptic D_1 dopamine receptors most likely mediated effects of the D_1 agonist and antagonist used in the present experiments.

Little information is available to elucidate the site of action for D_1 dopamine-receptor regulation of SRIF and GHRH. According to the alpha-numeric system of Dahlström and Fuxe (1964), dopamine neurons in the arcuate nucleus, which have axons that terminate in the median eminence/pituitary stalk, are referred to as the A_{12} cell group and those in the periventricular nucleus are termed the A_{14} cell group (Tillet, 1995). Our laboratory has reported that activity of the periventricular A_{14} cell group is elevated, whereas activity of the arcuate A_{12} cell group is unchanged when concentrations of growth hormone in serum are reduced in steers, possibly due to an increase in SRIF tone (Gaynor et al., 1995a). Therefore, A_{14} periventricular dopamine neurons acting through D_1 dopamine receptors located in the periventricular nucleus may have mediated the increase in SRIF observed in the present experiments.

An alternative site of action for dopamine regulation of SRIF and GHRH may occur in the pituitary stalk/median eminence via the A_{12} cell group. In cattle, Zinn et al. (1990) reported average concentrations of dopamine in the pituitary stalk of bull calves were two to three times greater than concentrations in the mediobasal hypothalamus. Therefore, it is possible that the A_{12} cell group has a role in regulation of SRIF and GHRH release. However, currently, it is unclear whether D_1 receptors are present in the pituitary stalk/median eminence. Regardless, the present experiments showed that D_1 receptor regulation of release of SRIF and GHRH occurs within the hypothalamus/pituitary stalk/median eminence, because tissue preparations in the present experiments did not contain extrahypothalamic tissue.

In conclusion, I demonstrated that SKF 38393, a D_1 agonist, increases release of SRIF and decreases release of GHRH from perifused, bovine hypothalamic slices. My findings support the hypothesis that dopamine increases release of SRIF and decreases release of GHRH from the bovine hypothalamus via D_1, not D_2, dopamine receptors.

CHAPTER 4

Reciprocal Feedback Between Growth Hormone-Releasing Hormone and Somatostatin within the Bovine Hypothalamus

INTRODUCTION

Growth hormone-releasing hormone and SRIF mediate the central nervous system's regulation of secretion of growth hormone from the anterior pituitary gland (Frohman et al., 1992). Growth hormone-releasing hormone neurons in the arcuate nucleus and SRIF neurons in the periventricular nucleus of the hypothalamus project to the median eminence and release the neuropeptides into hypophysial portal circulation (Frohman et al., 1992; Plotsky and Vale, 1985). Growth hormone-releasing hormone stimulates and SRIF inhibits secretion of growth hormone (Frohman et al., 1992).

Several studies have established a morphological basis for the existence of a reciprocal feedback relationship between GHRH and SRIF within the hypothalamus/ pituitary stalk. For example, in rats mRNA for SRIF receptors is heavily expressed in neurons containing GHRH in the arcuate nucleus (Beaudet et al., 1995). In addition, SRIF-immunoreactive nerve terminals synapse on GHRH cell bodies (Horváth et al., 1989). Furthermore, synaptic contact between GHRH-containing nerve terminals and SRIF-containing dendrites has been reported in the periventricular nucleus (Horváth et al., 1989). While ultrastructural studies identifying synaptic connections between SRIF and GHRH neurons have not been conducted in cattle, Leshin et al. (1994) reported that fibers and varicosities of SRIF-immunoreactive neurons lie in close apposition to GHRH-

immunoreactive perikarya in the arcuate nucleus and GHRH-immunoreactive fibers in the median eminence in cattle.

Physiological evidence in rats also supports the hypothesis that a GHRH-SRIF reciprocal-feedback relationship exists within the hypothalamus. For example, intracerebroventricular injection of GHRH increases levels of SRIF in portal blood in rats (Mitsugi et al., 1990). In addition, GHRH increases release of SRIF from rat hypothalamic tissue in culture (Richardson et al., 1988). Furthermore, SRIF inhibits *in vitro* release of GHRH (Yamauchi et al., 1991).

Hypothalamic interactions of GHRH and SRIF have not been addressed with physiological studies in cattle; however, I have indirect evidence that suggests this relationship may exist in the bovine hypothalamus. Specifically, as I described in Chapter 3, in perifused, bovine hypothalamic slices, release of GHRH decreases after stimulation of endogenous release of SRIF with a D_1 dopamine receptor agonist, SKF 38393. Therefore, I speculate SRIF may mediate the decrease in GHRH observed with SKF 38393 treatment, supporting the hypothesis that GHRH and SRIF are involved in a reciprocal-feedback relationship within the bovine hypothalamus. Thus, my objectives were to determine the effects of: 1) SRIF on release of GHRH, 2) GHRH on release of SRIF, and 3) a SRIF antagonist on SKF 38393-induced suppression of GHRH release from perifused, bovine hypothalamic slices.

MATERIALS AND METHODS

Perifusion of Bovine Hypothalamic Slices

Brains were obtained from cattle (>12 mo of age) killed via stunning followed by exsanguination at a local abattoir. Tissue was processed and cultured in a perifusion system as described in Chapter 1. Radioimmunoassays were used to quantify concentrations of SRIF and GHRH in medium effluent as described in Chapter 1.

Experimental Protocols

The first experiment was conducted to test the effect of SRIF on release of GHRH. Duration of this experiment was 180 min and medium effluent (3 ml) was collected at 20-min intervals. Two pretreatment samples were collected during the first 40 min of the experiment and treatments were imposed from 41 to 60 min. Control slices (n=8) were perifused with vehicle medium, and treated slices received either 10^{-8} M (n=8), 10^{-6} M (n=8), or 10^{-4} M (n=8) somatostatin 1-14 (Sigma Chemical Co.). Slices were perifused with vehicle medium for the remaining 120 min after perifusion of treatments.

To test the effect of GHRH on release of SRIF, a second experiment was conducted. Duration of the experiment was 180 min, and medium effluent (3 ml) was collected at 20-min intervals. Two pretreatment samples were collected during the first 40 min of the experiment. Treatments were imposed from 41 to 60 min. Control slices (n=8) were perifused with vehicle medium and treated slices received either 10^{-8} M (n=8), 10^{-6} M (n=8), or 10^{-4} M (n=8) growth hormone-releasing hormone 1-44 (kindly provided by Upjohn-Pharmacia, Kalamazoo, MI). Slices were perifused with vehicle medium for the remaining 120 min after perifusion of treatments.

The third experiment was conducted to determine if the SRIF antagonist, cyclo-[7-aminoheptanoyl-phe-D-trp-lys-thr(bzl)] (cyclo-SRIF; Fries et al., 1982; Bachem California, Torrance, CA) would block the effects of a D_1 agonist (SKF 38393; Dubois et al., 1986; Research Biochemicals International, Natick, MA) on release of GHRH. Duration of this experiment was 200 min and medium effluent was collected at 20 min-intervals with pretreatment samples collected during the first 40 min of the experiment. Treatments consisted of vehicle-perifused controls (n=6) and slices perifused with 10^{-6} M SKF 38393 (n=6), 10^{-6} M cyclo-SRIF (n=6), or a combination of 10^{-6} M cyclo-SRIF plus 10^{-6} M SKF 38393 (n=6). Vehicle or treatment was perifused from 41 to 100 min in controls and in the cyclo-SRIF alone group, respectively. Treatment was perifused from 61 to 80 min in the SKF 38393 alone group. In the combination group, slices were perifused with cyclo-SRIF alone from 41 to 60 min, cyclo-SRIF plus SKF 38393 from 61 to 80 min, and cyclo-SRIF alone from 81 to 100 min. After perifusion of treatments, all slices were perifused with vehicle medium until the end of the experiment at 200 min.

Statistical Analyses

All data were subjected to analysis of variance using Statistical Analysis System (SAS Institute). Differences among means were determined with Bonferroni *t* tests and Dunnett's *t* test (Gill, 1978). Probability values ≤ 0.05 were considered to be significant. In experiments with durations of 180 min and that utilized SRIF and GHRH alone, areas under GHRH and SRIF response curves (AUC, ng· ml^{-1} min) were calculated from concentrations representing the interval between 41 and 180 min using the trapezoidal rule and were utilized as indicators of GHRH and SRIF release. In the third experiment, which utilized the combination of SKF 38393 and cyclo-SRIF, AUC for GHRH and SRIF were

calculated from concentrations representing the interval between 61 and 200 min. To account for differences in basal release between slices, pretreatment SRIF and GHRH values at 20 min were used as covariates in the statistical analysis of their respective measurements when P was ≤ 0.25 for the covariate term in the statistical model (experiments 1 and 3). When appropriate, AUC were transformed to $\log_{10}$ before statistical analysis to achieve homogeneous variances among treatments (Gill, 1978). Perifusion chamber, animal, location of slice, pretreatment covariate, and treatment were included in statistical models when P was ≤ 0.25 for the statistical term (Gill, 1978). Interactions between animal and treatment, and slice location and treatment were also analyzed.

RESULTS

Effect of SRIF on Release of GHRH

Compared with perifusion of medium alone, medium containing 10^{-6} and 10^{-4} M SRIF decreased AUC for GHRH an average of 28% (Figure 25). At 10^{-8} M, SRIF had no effect on release of GHRH. There were no interactions between animal and treatment or slice location and treatment; therefore, all slices from all animals and slice locations responded similarly.

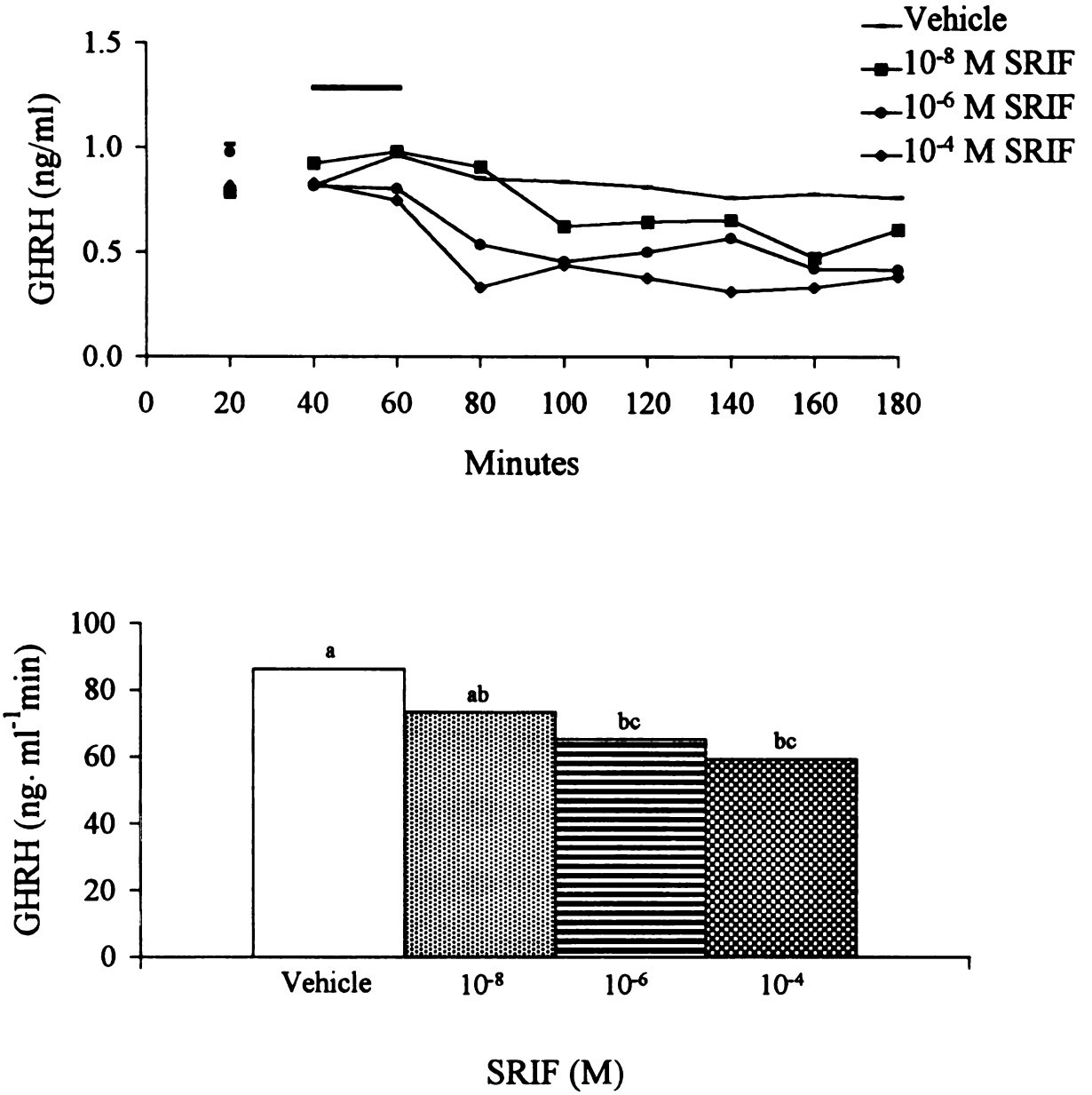

Figure 25. Concentrations of GHRH in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=8), 10^{-8} M (n=8), 10^{-6} M (n=8), or 10^{-4} M (n=8) SRIF from 41 to 60 min (indicated by horizontal line). Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for GHRH calculated from concentrations representing the interval between 41 and 180 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.05$; pooled SEM = 6.3 ng· ml^{-1} min).

Effect of GHRH on Release of SRIF

Relative to vehicle-perifused controls, GHRH increased release of SRIF (Figure 26). Furthermore, it was a dose-responsive increase as AUC for SRIF in the 10^{-4} M group was greatest and the increase in the 10^{-8} M group was smallest. There were no interactions between animal and treatment or slice location and treatment; therefore, all slices from all animals and slice locations responded similarly.

Effect of Cyclo-SRIF and SKF 38393 on Release of GHRH and SRIF

At 10^{-6} M, SKF 38393 increased AUC for SRIF from 12.5 $ng \cdot ml^{-1}min$ in controls to 484.9 $ng \cdot ml^{-1}min$ (Figure 27). Cyclo-SRIF alone at 10^{-6} M did not affect release of SRIF relative to controls. Furthermore, cyclo-SRIF had no effect on SKF-38393-induced release of SRIF because AUC for SRIF was not different between the SKF 38393 alone group and the cyclo-SRIF plus SKF 38393 group. Release of GHRH was decreased from 36.4 $ng \cdot ml^{-1}min$ in controls to 18.2 $ng \cdot ml^{-1}min$ in the group perifused with SKF 38393 alone (Figure 28). Relative to controls, cyclo-SRIF alone increased AUC for GHRH 90%. In addition, the decrease in release of GHRH due to SKF 38393 was blocked with cyclo-SRIF; in fact, the combination of cyclo-SRIF and SKF 38393 increased release of GHRH relative to vehicle-perifused controls. AUC for GHRH was similar between the cyclo-SRIF alone group and the cyclo-SRIF plus SKF 38393 group.

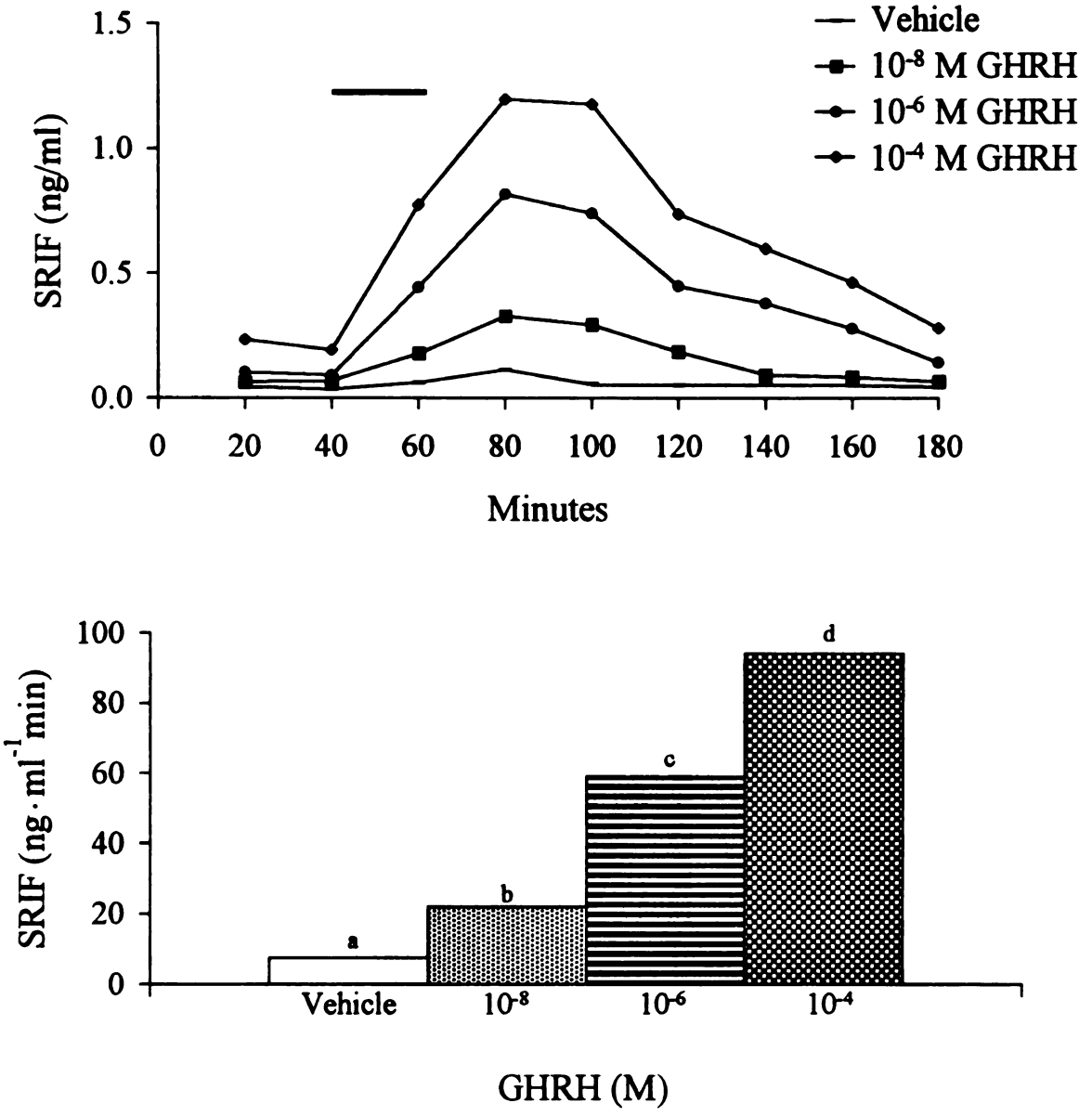

Figure 26. Concentrations of SRIF in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=8), 10^{-8} M (n=8), 10^{-6} M (n=8), or 10^{-4} M (n=8) GHRH from 41 to 60 min (indicated by horizontal line). Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for SRIF calculated from concentrations representing the interval between 41and 180 min (bottom panel). All data are reported as least squares means. Means without a common letter differed ($P < 0.05$; pooled SEM = 3.8 ng· ml^{-1} min).

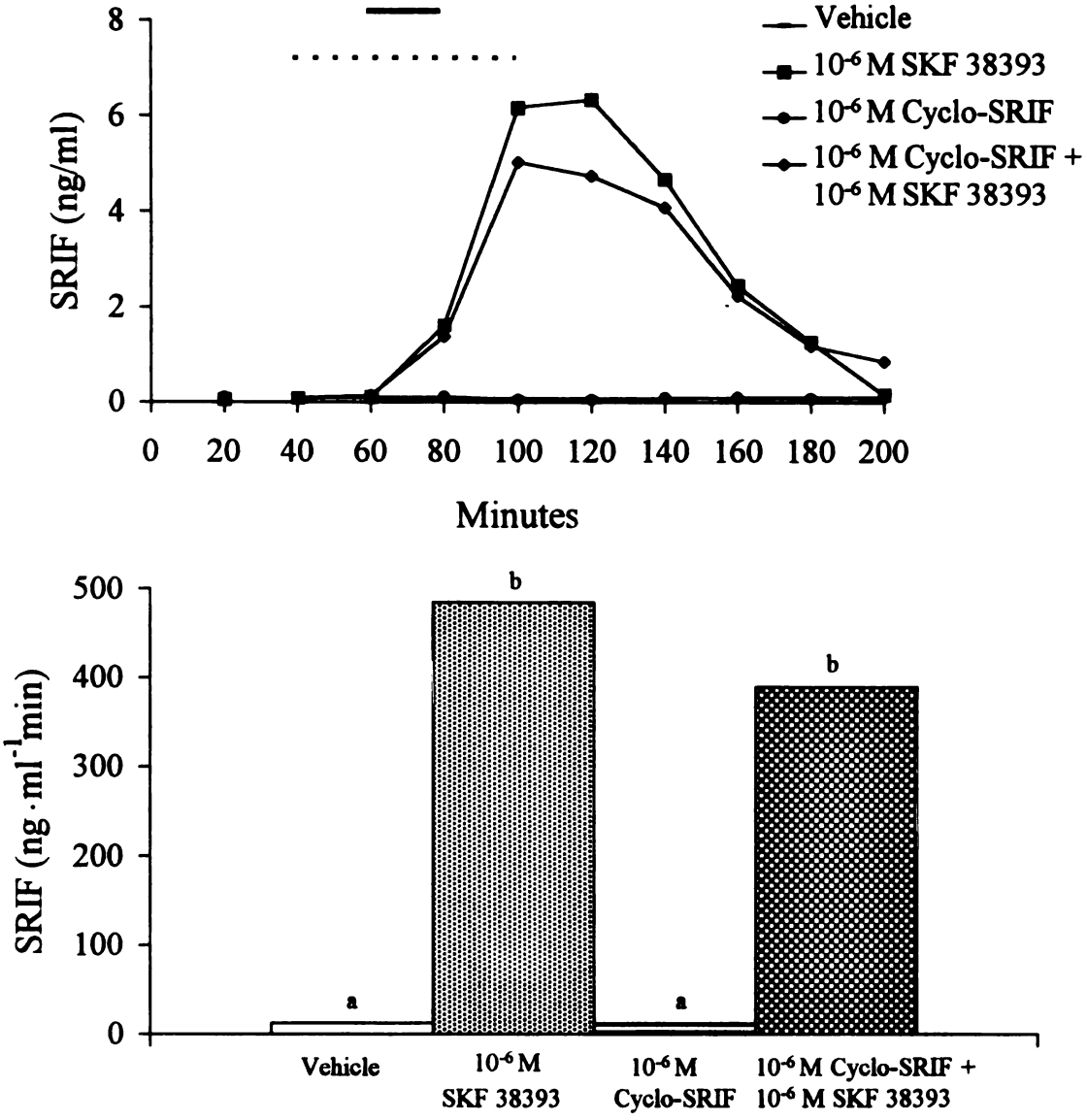

Figure 27. Concentrations of SRIF in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=6), 10^{-6} M SKF 38393 (n=6), 10^{-6} M cyclo-SRIF (n=6), or 10^{-6} M cyclo-SRIF plus 10^{-6} M SKF 39393 (n=6). Treatments were imposed from 41 to 100 min in controls and in the cyclo-SRIF alone group (indicated by dashed horizontal line). Treatment was perifused from 61 to 80 min in the SKF 38393 alone group (indicated by solid horizontal line). In the combination group, slices were perifused with cyclo-SRIF alone from 41 to 60 min, cyclo-SRIF plus SKF 38393 from 61 to 80 min, and cyclo-SRIF alone from 81 to 100 min. Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for SRIF calculated from concentrations representing the interval between 61 and 200 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.05$; pooled SEM = 3.8 ng·ml^{-1} min).

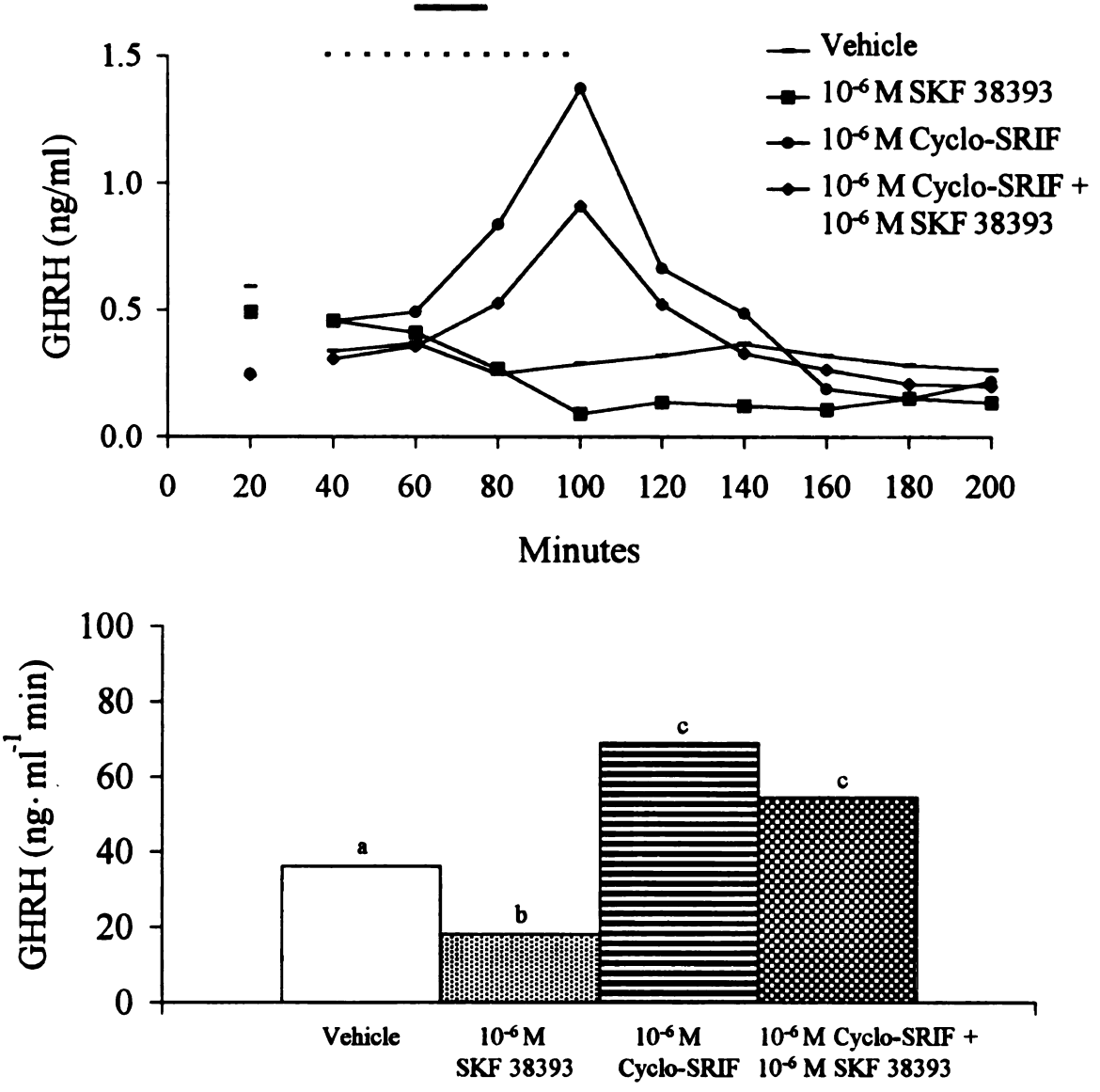

Figure 28. Concentrations of GHRH in medium effluent (top panel) from bovine hypothalamic slices perifused with vehicle medium (control, n=6), 10^{-6} M SKF 38393 (n=6), 10^{-6} M cyclo-SRIF (n=6), or 10^{-6} M cyclo-SRIF plus 10^{-6} M SKF 39393 (n=6). Treatments were imposed from 41 to 100 min in controls and in the cyclo-SRIF alone group (indicated by dashed horizontal line). Treatment was perifused from 61 to 80 min in the SKF 38393 alone group (indicated by solid horizontal line). In the combination group, slices were perifused with cyclo-SRIF alone from 41 to 60 min, cyclo-SRIF plus SKF 38393 from 61 to 80 min, and cyclo-SRIF alone from 81 to 100 min. Flow rate was 0.15 ml/min. Statistical analysis was performed on AUC for GHRH calculated from concentrations representing the interval between 61 and 200 min (bottom panel). All data are reported as least squares means and adjusted by covariance using pretreatment release at 20 min (depicted in top panel). Means without a common letter differed ($P < 0.05$; pooled SEM = 6.8 ng·ml^{-1} min).

DISCUSSION

In addition to the well-documented, stimulatory actions of GHRH and inhibitory actions of SRIF at the anterior pituitary gland, GHRH and SRIF may interact within the hypothalamus/pituitary stalk to modulate episodic secretion of growth hormone (Beaudet et al., 1995; Horváth et al., 1989; Mitsugi et al., 1990; Richardson et al., 1988; Yamauchi et al., 1991). Indeed, I have demonstrated in cattle, for the first time to my knowledge, that GHRH and SRIF interact within the hypothalamus/pituitary stalk to regulate each others release. Perifusion of GHRH increased release of SRIF, whereas, perifusion of SRIF decreased release of GHRH. These results are consistent with *in vivo* and *in vitro* reports in rats that indicate GHRH stimulates secretion of SRIF and SRIF inhibits secretion of GHRH. For example, central administration of GHRH increases levels of SRIF in hypophysial portal blood (Mitsugi et al., 1990). In addition, GHRH increases release of SRIF from rat hypothalami, as well as increases SRIF mRNA in the periventricular nucleus (Richardson et al., 1988; Aguilla et al., 1994). Moreover, perifusion of SRIF inhibits release of GHRH from rat hypothalami (Yamauchi et al., 1991).

In the present study, blockade of SRIF action with the SRIF antagonist increased release of GHRH, implying that endogenous SRIF tonically inhibits GHRH release. Because the SRIF antagonist utilized in these experiments previously has been shown to inhibit actions of SRIF in a variety of tissues including the anterior pituitary gland, cortex, hippocampus, and pancreas, presumably in the present experiments, the SRIF antagonist also blocked the actions of SRIF (Fries et al., 1982; Shibata et al., 1993; Li et al., 1995).

Thus, my findings are in agreement with previous *in vivo* and *in vitro* reports in rats indicating SRIF exerts tonic inhibition on release of GHRH. For example, central administration of SRIF antiserum increases concentrations of GHRH in portal blood in rats (Plotsky and Vale, 1985). In addition, anterolateral-hypothalamic deafferentation and cysteamine treatment, both of which deplete endogenous SRIF, increase release of GHRH from hypothalamic fragments in culture and increase levels of GHRH mRNA in the arcuate nucleus (Katakami et al., 1988; Bertherat et al., 1991). Several, neurotransmitter and neuropeptide inputs relaying metabolic and endocrine influences modulate neurosecretory SRIF neurons (Müller, 1987). Therefore, it is unclear as to which neural inputs drive tonic SRIF release in the bovine hypothalamus.

SRIF does not appear to inhibit its own release in cattle because the SRIF antagonist had no effect on release of SRIF in the present experiments. My results are in contrast to observations in rats where exogenous SRIF inhibits release of endogenous SRIF from dispersed hypothalamic cells (Richardson and Twente, 1986). Therefore, a species difference may exist between rats and cattle with respect to SRIF autoregulation.

My results support the hypothesis that SRIF mediates the suppression of GHRH observed after elevation of endogenous SRIF with D_1 agonist treatment. SKF 38393 increases release of SRIF and the SRIF antagonist blocked SKF 38393-induced suppression of GHRH, suggesting that SRIF mediates the effects of activation of D_1 receptors on release of GHRH. Furthermore, these data also support the hypothesis that GHRH and SRIF interact within the hypothalamus/pituitary stalk to modulate each others release.

Cell bodies of hypothalamic GHRH and SRIF neurons are located primarily in the arcuate and periventricular nuclei respectively, and terminate in the pituitary stalk/median eminence in cattle (Leshin et al., 1994). Studies addressing hypothalamic localization of mRNA encoding GHRH and SRIF receptors or presence of synaptic contacts between GHRH and SRIF neurons have not been conducted in cattle as have been done in rats. However, Leshin et al. (1994) reported SRIF-immunoreactive axonal and terminal varicosities closely surround GHRH-immunoreactive perikarya in the bovine arcuate nucleus. In addition, GHRH- and SRIF-immunoreactive fibers and terminals lie in close apposition to one another in the pituitary stalk/median eminence (Leshin et al., 1994). In contrast to findings in rats, Leshin et al. (1994) did not detect rostrally projecting GHRH-immunoreactive terminals in the periventricular nucleus in cattle. Therefore, in the present studies, it appears GHRH must have acted on SRIF nerve terminals in the pituitary stalk/median eminence to stimulate release of SRIF. However, based on Leshin et al.'s (1994) findings that SRIF innervation is present in both the arcuate nucleus and pituitary stalk/median eminence, SRIF and the SRIF antagonist may have acted either in the arcuate nucleus on GHRH perikarya or at the pituitary stalk/median eminence on GHRH nerve terminals to decrease and increase release of SRIF, respectively.

In conclusion, I have demonstrated that SRIF inhibits release of GHRH, GHRH stimulates release of SRIF, and SRIF mediates the effects of activation of D_1 receptors on release of GHRH from perifused, bovine hypothalamic slices. My findings support the hypothesis that GHRH and SRIF interact within the hypothalamus/pituitary stalk to modulate each others release in cattle.

SUMMARY AND CONCLUSIONS

My overall goal in this dissertation was to understand mechanisms whereby α_2-adrenergic and dopaminergic receptors within the hypothalamus mediate changes in release of GHRH and SRIF in cattle. Specifically, my objectives were: 1) to determine the role of GHRH and SRIF in α_2-adrenergic receptor stimulation of growth hormone secretion in cattle; 2) to determine if pharmacological activation or inhibition of dopamine receptors regulates release of GHRH and SRIF from the bovine hypothalamus and; 3) to determine if GHRH and SRIF are involved in a reciprocal feedback relationship within the hypothalamus of cattle. To address my objectives, first I developed a perifusion system for culturing bovine hypothalamic tissue that was utilized in the subsequent studies described in Chapters 2-4.

Results presented in Chapter 2 indicated that stimulation of α_2-adrenergic receptors had no effect on release of growth hormone from anterior pituitary cells in culture. However, α_2-adrenergic receptor agonists stimulated release of GHRH without affecting release of SRIF from perifused, bovine hypothalamic slices. These observations support the hypothesis that α_2-adrenergic receptor stimulation of growth hormone secretion in cattle is mediated via an increase in GHRH and not a change in SRIF release.

In Chapter 3, activation of D_1 receptors increased release of SRIF and decreased release of GHRH from the bovine hypothalamus. In addition, studies described in Chapter

4 indicated that GHRH increased release of SRIF, whereas SRIF decreased release of GHRH. Furthermore, I showed that a SRIF antagonist could block the effects of activation of D_1 receptors to suppress release of GHRH from perifused, bovine hypothalamic slices. Taken together, data from Chapters 3 and 4 support the hypothesis that dopamine increases release of SRIF, which in turn suppresses release of GHRH from the bovine hypothalamus. Moreover, it appears that GHRH and SRIF interact within the hypothalamus via a reciprocal feedback relationship to regulate each other's release in cattle.

In general, results from this dissertation provide direct evidence that hypothalamic receptors for the catecholaminergic neurotransmitters, norepinephrine and dopamine, regulate release of GHRH and SRIF. Presumably pharmacological agents utilized in the present experiments mimicked actions of norepinephrine and dopamine, thereby implying that norepinephrine and dopamine regulate release of GHRH and SRIF from the bovine hypothalamus. Indeed, Gaynor et al. (1995a) demonstrated that change in levels of growth hormone secretion in steers is associated with selective alterations in activity (nerve impulse travel) of norepinephrine and dopamine neurons in regions of the hypothalamus containing GHRH and SRIF neurons. For example, when concentrations of growth hormone are elevated, activities of norepinephrine neurons in the arcuate nucleus (which contains most GHRH neuronal cell bodies) and pituitary stalk (which contains GHRH nerve terminals) are elevated compared to neuronal activities when concentrations of growth hormone are low (Gaynor et al., 1995a). In addition, activity of dopamine neurons in the periventricular nucleus (which contains most SRIF neuronal cell bodies) is concurrently decreased when concentrations of growth hormone are elevated, relative to

activities when concentrations of growth hormone are low (Gaynor et al., 1995a). Collectively, observations from this dissertation and observations of Gaynor et al. (1995a) suggest that norepinephrine increases release of GHRH and that dopamine increases release of SRIF in cattle (Figure 29, pathways 1 and 2).

The conclusion drawn from experiments described in Chapter 4 that a reciprocal feedback relationship exists between GHRH and SRIF within the bovine hypothalamus, to my knowledge, has not been addressed with *in vivo* studies in cattle or any other livestock species (Figure 29, pathways 3 and 4). Because in rats this reciprocal feedback relationship between GHRH and SRIF appears to be an important aspect of neuroendocrine regulation of growth hormone secretion, confirmation of the presence of this relationship in *in vivo* studies in cattle is pertinent. In addition, it is unclear as to the location of SRIF and GHRH neuronal cell bodies which are involved in this reciprocal feedback relationship. It is possible SRIF neurons located in the arcuate nucleus and not the periventricular nucleus (as depicted in the proposed model) are responsible for regulating GHRH release.

Exogenous GHRH increased release of SRIF in Chapter 4. Therefore, on the surface, it would seem that in Chapter 2, the clonidine-induced increase of endogenous GHRH should have increased release of SRIF as well. But, clonidine did not affect SRIF. However, when considering the anatomical organization of GHRH and SRIF neurons within the bovine hypothalamus combined with the continuous removal of secretory products from perifusion chambers, these results actually may not be contradictory. This speculation is discussed below in greater detail.

Figure 29. Proposed model for neuroendocrine regulation of growth hormone secretion within the hypothalamus/pituitary stalk in cattle. Rectangles represent the periventricular (PeVN) and arcuate (ARC) nuclei of the hypothalamus. Circles with connected lines represent dopamine (DA), norepinephrine (NE), somatostatin (SRIF), and growth hormone-releasing hormone (GHRH) neurons. The cylinder represents a hypophysial portal vessel and the ellipse represents the anterior pituitary gland. The model depicts proposed neuronal pathways (numbered) involved in regulation of GHRH and SRIF neurons. Pathway 1 suggests NE increases release of GHRH. Pathway 2 suggests DA increases release of SRIF. Pathways 3 and 4 suggest GHRH and SRIF regulate each other's release via a reciprocal feedback relationship within the hypothalamus/pituitary stalk.

Proposed Neuroendocrine Regulation of GH Secretion in Cattle
NE
ARC
1
GHRH
3
4
DA
2
SRIF
PeVN
Hypothalamus
Pituitary Stalk
Portal Vessel
Anterior Pituitary
GH
Meat, Milk

In cattle, Leshin et al. (1994) reported GHRH- and SRIF-immunoreactive fibers and terminals lie in close apposition to one another in the pituitary stalk/median eminence. In addition, SRIF-immunoreactive axonal and terminal varicosities closely surround GHRH-immunoreactive perikarya in the arcuate nucleus (Leshin et al., 1994). However, GHRH-immunoreactive terminals do not appear to innervate areas around SRIF-immunoreactive perikarya in the periventricular nucleus (Leshin et al., 1994). Therefore, it appears SRIF can regulate GHRH at both the cell body and terminal levels, whereas GHRH can regulate SRIF neurons at the terminal level in the pituitary stalk/median eminence. Furthermore, it appears that GHRH released in response to clonidine would have to act on SRIF neuronal terminals at the pituitary stalk/median eminence to increase release of SRIF. Because in the present experiments medium was continuously pumped through perifusion chambers, clonidine-induced release of GHRH was continuously removed from perifusion chambers, thus precluding accumulation of GHRH in chambers and possibly preventing action of GHRH on SRIF neuronal terminals. However, when exogenous GHRH was perifused, much higher concentrations of GHRH (10^{-8} to 10^{-4} M) were achieved in perifusion chambers for a longer duration than in experiments utilizing clonidine, because the highest clonidine-induced concentration of GHRH was only 2×10^{-10} M. Granted, higher concentrations of GHRH than were measured in samples of medium effluent may have occurred transiently in perifusion chambers, but apparently they were not great enough and(or) sustained long enough to increase release of SRIF. This theory could be tested utilizing clonidine in a static culture system for hypothalamic tissue if tissue could be maintained under these conditions.

In contrast, in Chapter 3, dilution of endogenous SRIF in medium effluent did not appear to be a limitation in observing suppression of GHRH with SKF 38393-induced release of endogenous SRIF. As stated earlier, SRIF neurons terminate in both the arcuate nucleus and pituitary stalk/median eminence in cattle; thus, SRIF may act at one or both sites to suppress release of GHRH. Furthermore, SKF 38393 may have increased release of SRIF from neurons that terminate in the pituitary stalk/median eminence and(or) in the arcuate nucleus. As a result of SKF 38393-induced release of SRIF from neurons that terminate in the arcuate nucleus, local concentrations of SRIF in the arcuate nucleus would be elevated , thereby allowing for direct suppression of GHRH neuronal cell bodies in the arcuate nucleus and hence decreased release of GHRH. Because SKF 38393-induced release of SRIF from neurons that terminate in the pituitary stalk/median eminence would have been flushed from perifusion chambers in a similar manner as was clonidine-induced release of GHRH, I speculate that with the perifusion system utilized in the present studies SKF 38393-induced-SRIF release in the arcuate nucleus was responsible for decreased levels of GHRH observed in medium effluent. Immunohistochemical localization of SRIF receptors and GHRH receptors within the bovine hypothalamus as well as localization of SKF 38393-responsive SRIF neurons would shed light on this speculation.

In conclusion, I developed an *in vitro* perifusion system for bovine hypothalamic tissue that can be utilized to study regulation of GHRH and SRIF release. My findings support the hypothesis that an increase in release of GHRH, but not a decrease in release of SRIF, mediates α_2-adrenergic receptor stimulation of secretion of growth hormone in cattle. In addition, I demonstrated that stimulation of D_1 receptors increases release of

SRIF and decreases release of GHRH. Furthermore, I determined that SRIF inhibits release of GHRH, GHRH stimulates release of SRIF, and SRIF mediates the effects of activation of D_1 receptors on release of GHRH from perifused, bovine hypothalamic slices. Finally, I speculate that norepinephrine, via GHRH, stimulates release of growth hormone whereas dopamine, via SRIF, inhibits release of growth hormone in cattle.

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