IN VITRO AND *IN VIVO* ACTIVATION OF THE PITUITARY-ADRENAL AXIS BY CORTICOTROPIN-RELEASING FACTOR, VASOPRESSIN (VP), AND TWO VP ANALOGS

A Thesis

by

JEFFERY ALLEN CARROLL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 1993

Major Subject: Physiology of Reproduction

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ABSTRACT

In Vitro and *In Vivo* Activation of the Pituitary-Adrenal Axis by Corticotropin-Releasing Factor, Vasopressin (VP), and Two VP Analogs. (August 1993)

Jeffery A. Carroll, B.S., Texas A&M University

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This project's objectives were to: 1) compare the relative potency of a VP analog with that of CRF and VP in terms of ACTH secretion by cultured bovine corticotrophs; 2) evaluate a VP analog as an antagonist of VP-induced ACTH secretion by cultured bovine corticotrophs; 3) evaluate the ability of CRF and VP to induce ACTH secretion *in vivo* in cattle; and, 4) evaluate the ability of the antiglucocorticoid, RU486, to block the inhibitory effects of dexamethasone (DEX) on CRF- and VP-induced ACTH secretion *in vitro*.

Bovine and porcine adenohypophyses were enzymatically dispersed to yield cells for primary culture. On Day 5 of primary culture, bovine and porcine corticotrophs were challenged for 3 h and 4 h, respectively, with medium alone (Control) or various combinations and concentrations of bCRF, VP, the two VP analogs, { VPB: [Deamino'(D-3-(Pyridyl) Ala^2, Arg^8)-VP]; and VPP: [$d(CH_2)_5$,D-$Try(Et)^2$,Val^4,Cit^8-VP]}, DEX and RU486. Medium concentration of ACTH was determined by RIA. Bovine CRF, VP, and the VP analogs each increased ($P < .05$) ACTH secretion by bovine corticotrophs.

Maximal increases in ACTH secretion occurred in response to .1μM bCRF (5.1-fold) and 1μM VP (3.7-fold), relative to Control. VP and VPB were more potent than VPP in terms of stimulating secretion of ACTH. RU486 and DEX, added either alone or together to bovine or porcine corticotrophs did not affect ($P > .10$) basal concentration of ACTH. DEX reduced ($P < .01$) CRF-stimulated secretion of ACTH by 68%. Concurrent addition of RU486 negated 62% of the inhibitory effect of DEX on CRF-stimulated secretion of ACTH.

Cows were randomly assigned to one of four groups (n = 8 cows/group): 1) Control (saline); 2) bCRF (.3 μg/kg BW); 3) VP (1 μg/kg BW); and 4) bCRF (.3 μg/kg BW) + VP (1 μg/kg BW). Jugular blood samples were collected at 15-min intervals for 4 h pre- and for 6 h post-treatment; samples also were taken at 1, 5 and 10 min post-treatment. Plasma concentration of ACTH and cortisol did not differ among the groups for the 4-h pre- injection period. At 1 min post-injection, bCRF + VP, VP, and bCRF increased ACTH secretion 22.4-, 9.6-, and 2.2- fold respectively, relative to Control (.03 ng/ml). Maximal plasma concentration of ACTH occurred at 5, 10, and 15 min for VP (1.02 ng/ml), bCRF + VP (1.4 ng/ml), and bCRF (.32 ng/ml), respectively.

These data demonstrate that the two VP analogs are agonistic ACTH secretagogues *in vitro*. RU486 partially blocks DEX's inhibition of ACTH secretion of ACTH *in vitro*. VP acutely activates the bovine pituitary-adrenal axis whereas the ACTH response mediated by CRF is slower in onset longer duration.

DEDICATION

First and foremost, this thesis is dedicated to my loving and supportive wife, Jo Ann. Without her unconditional love, support and understanding this endeavor may never have been undertaken. For the willingness to postpone many events in her life, in order that I might pursue this dream, I am eternally grateful. The patience and understanding you exhibited during the long hours I spent in the laboratory is commendable. I am extremely fortunate to have been blessed with such a strong and courageous woman as my life long friend and wife.

This thesis is also dedicated to my wonderful children, Aces Brice and Kressa Lea. Their understanding and patience during the latter stages of this degree is greatly appreciated. Their unconditional love and confidence in me will always be a precious memory.

ACKNOWLEDGMENTS

I will always remember the friendship, guidance, understanding and patience expressed by my mentor, Dr. Thomas H. Welsh, Jr. during this period of my life. For the interest he expressed in both my personal and professional life, I will always be grateful. I will always be indebted to him for his guidance and continued effort to help me succeed with my professional career. I will always value our friendship and reflect on the enjoyable times spent out at the horse ranch.

I would also like to express my sincere appreciation to Dr. Fuller W. Bazer, Dr. Newell H. McArthur and Dr. Deborah T. Kochevar for their friendship and for serving on my advisory committee during this period of my life. I am very fortunate to have had the involvement of each of these revered professionals during this thesis work.

I was fortunate enough to be involved in several collaborative research projects and I would like to express my appreciation to Scott T. Willard and Dr. Paul G. Harms for their support and involvement. I will always be grateful to Scott, a fellow graduate student and special family friend, for his drive and determination which helped motivate and drive me during the so often demanding times. I am also very thankful for the times he was there to assist and support my family during Jo Ann's pregnancy.

Two other fellow graduate students that provided support and

friendship are Joe G. Gillespie and Caroline N. Kemper. I am grateful for their involvement in my research project and their friendship. I would also like to express my appreciation to Greg Graf and Devin Kieschnick for their support by maintaining a functional laboratory and for their understanding during the demanding times experienced throughout my research.

The technical support and guidance provided by Margaret Sutton and Pat Chen during my research is also greatly appreciated. I am also grateful for the cooperation and assistance of Dr. Chris G. Woelfel and the Dairy Center staff, especially Curtis and Mike, during the *in vivo* portion of my research.

The financial support provided by the Department of Animal Science through a partial assistantship is also greatly appreciated. I am fortunate to have worked for such a devoted and supportive staff as that of the Department of Animal Science here at Texas A&M University.

To my mother, Claudette, my two brothers, Chris and Ralph, and my sister, Lisa, I will always be grateful for all of the love and support they provided during this period of my life. Finally, I would like to acknowledge my father, Willie, who passed away in August 1987. His hard work and desire allowed me to take the first step in fulfilling this dream.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

INTRODUCTION

When an animal is exposed to a stressor, neurotransmitters are activated which mediate the secretion of corticotropin-releasing factor (CRF) and vasopressin (VP) by specific hypothalamic neurons. These two neurohormones (CRF and VP) stimulate the corticotrophs in the adenohypophysis to release adrenocorticotropic hormone (ACTH) which in turn stimulates the adrenal cortex to release glucocorticosteroids (cortisol and corticosterone; Figure 1). The secretion and plasma concentration of these stress-related hormones (e.g., ACTH and cortisol) have been used as classical diagnostic tests or biological endpoints to assess the functional integrity of the adrenal axis and/or characterize responsiveness to a stressor or stressful enviroment.

Elevated cortisol (CS) in response to a stressor has long been associated with detrimental effects on reproduction and animal productivity (e.g., average daily gain, milk production) and more recently with a compromised immune system. It has been estimated that shipping stress, a condition associated with increased secretion of ACTH and CS during transportation and marketing of beef cattle can be linked to approximately 80% of all feedlot deaths (McKercher, 1978; Hutcheson and Cole, 1986) resulting in a loss of $700 million per year for the beef industry.

Citations on the following pages follow the style of Journal of Animal Science.

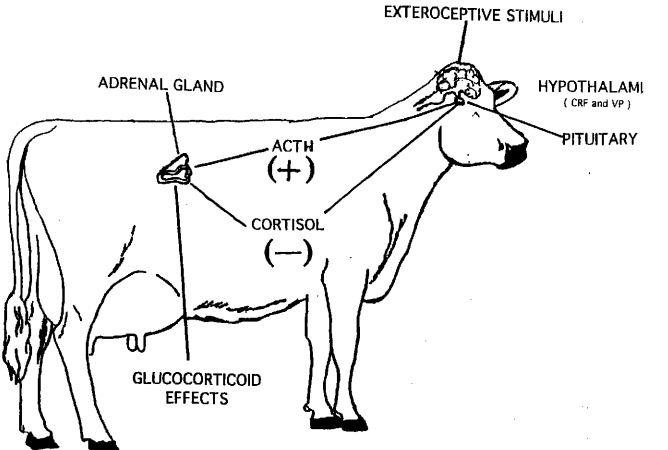

Figure 1. Diagramatic representation of the hypothalamic-pituitary-adrenal axis in the bovine. When an animal perceives an exteroceptive stimulus, the two hypothalamic neurohormones (e.g., CRF and VP) are released from the hypothalamus. CRF and VP stimulate the release of ACTH from the anterior pituitary which in turn stimulates the release of cortisol from the adrenal cortex.

In order to combat the detrimental biological and economic effects of stress, we must first identify and understand the factors that compromise the pathway of the hypothalamic-pituitary-adrenal axis (HPA). CRF and VP both stimulate the release of ACTH, however, the relative potency of these neurohormones is species specific. In cultured *bovine* corticotrophs, CRF is more potent, whereas in cultured *ovine* corticotrophs, VP is more potent. Recently, an analog of VP has been shown to be 1/36 as potent as VP in stimulating the release of ACTH by cultured ovine corticotrophs (Schwartz et al., 1991). The regulatory action of CRF and VP on the synthesis and/or secretion of CRF and/or VP is still under investigation. However, Aguilera et al. (1993) provided some insight when they reported that water deprivation-induced VP decreased CRF mRNA and increased the accumulation of CRF in the paraventricular nucleus of the rat. Apple et al. (1993) have also recently evaluated additional factors such as serum calcium and electrolytes in restraint-stressed sheep. Although there are several VP analogs available, (some reported to have antagonist activity peripherally) there has not been an analog reported which has antagonistic activity at the pituitary. Dexamethasone, however, has been utilized successfully to inhibit both CRF- and VP-induced release of ACTH (Wagner, 1987).

Therfore, the objectives of this study were to:

1) compare the relative potency of a VP analog with that of CRF and VP in terms of ACTH secretion by cultured bovine corticotrophs;

2) evaluate a VP analog as an antagonist of VP-induced ACTH secretion by cultured bovine corticotrophs;
3) evaluate the ability of CRF and VP to induce ACTH secretion *in vivo* in cattle;
4) evaluate the ability of the antiglucocorticoid, RU486, to block the inhibitory effects of dexamethasone on CRF- and VP-induced ACTH secretion *in vitro*.

LITERATURE REVIEW

Background. The physiological consequences of stress on the body have been of scientific interest for many years. The first scientist to introduce the term "stress" into the medical community was Hans Selye during the 1930s. Selye proposed that regardless of the stimuli, the body would respond in the same physiological manner. Selye's work attracted the interest of several other scientists into this field of study who strived to link the endocrine system to the stress response of the body (Sapolsky, 1988).

Early investigators concluded that the regulation of the secretion of glucocorticoids by the adrenal cortex depended on a linkage of the hypothalamus and pituitary gland. For example, Harris (1948), after studying factors which regulated the pituitary gland, suggested that neurons of the hypothalamus regulate the secretion of hormones from the adenohypophysis. Harris' findings led to further investigation into the hypothalamic-pituitary axis. In the 1950s, several others had provided convincing evidence that one of the factors produced in hypothalamic neurons regulated the secretion of adrenocorticotropic hormone (ACTH) from the adenohypophysis (Saffran et al., 1955; Guillemin and Rosenberg, 1955; Porter and Jones, 1956). According to conventional nomenclature, Saffran et al. (1955) named this adenohypophyseal regulator corticotropin-releasing factor.

During this same period, other scientists were conducting *in vivo* stress studies with VP in the rat (Martini and Morpurgo, 1955;

McCann, 1957). Initially, VP was thought to be the putative CRF. This led to a controversy in the scientific community as to which substance, CRF or VP, was the primary factor responsible for the regulation of ACTH secretion. Throughout the 1960s and 1970s, the controversy continued, although there was increasing evidence that VP was indeed not the primary stimulator of ACTH secretion (Arimura et al., 1967; Portanova and Sayers, 1973).

In addition to CRF and VP, there are at least two other regulatory factors that have been reported to induce ACTH secretion from the adenohypophysis; epinephrine (E; Giguere and Labrie, 1983) and oxytocin (OT; Link et al., 1993). The presence of high affinity receptors for both E (Petrociv et al., 1983) and OT (Antoni, 1986) have been identified in the rat pituitary. However, the focus of this report will be on the CRF and VP-induced ACTH secretion.

Localization of CRF and VP. Vale et al. (1981) characterized the chemical structure and sequenced ovine CRF as a 41-amino acid hypothalamic peptide with intrinsic ACTH-releasing activity. Land et al. (1982) isolated and sequenced the DNA complementary to the specific mRNA for vasopressin. Gillies et al. (1982) demonstrated that VP enhanced the ACTH-releasing potential of CRF. In women, Liu et al. (1983) demonstrated that VP and CRF acted synergistically in regard to stimulation of ACTH secretion. Eventually, with the use of immunohistochemical staining techniques, CRF was identified in various regions of the hypothalamus with the primary source localized in the parvocellular neurons of the paraventricular nucleus

(PVN; Bugnon et al., 1982; Merchenthaler et al., 1982; Olschowka et al., 1982; Figure 2).

These CRF neurons can be subdivided into two types: 1) a VP-containing neuron and 2) a VP-deficient neuron. The axons which transport CRF from these neurosecretory cells originate in the medial parvocellular subdivision of the PVN and project to the external zone of the infundibulum in the tuberohypophyseal tract where CRF is released into extracellular space around the fenestrated capillaries of the hypothalamo-hypophyseal portal system (Guillaume et al., 1992). Vasopressin has been localized in two regions of the hypothalamus, the magnocelllular neurons of both the PVN and the supraoptic nucleus (SON; Zimmerman et al., 1977; Figure 2). The neurons that synthesize VP in the PVN and SON project their axons in the paraventriculohypophyseal and supraopticohypophyseal tracts, respectively, and terminate in the neural lobe of the neurohypophysis (Figure 3).

The pathway by which magnocellular neuron synthesized VP reaches the corticotrophs in the adenohypophysis of the pituitary is still unresolved. Two of the most widely accepted viewpoints are provided by Holmes et al. (1986) and Oliver et al. (1977). Holmes et al. (1986) contend that VP is released from preterminal axons in the internal zone of the infundibulum. The other possible pathway proposed by Oliver et al. (1977) suggests that VP is transported by retrograde blood flow from the neurohypophysis to the portal system which facilitates delivery of VP to the adenohypophysis.

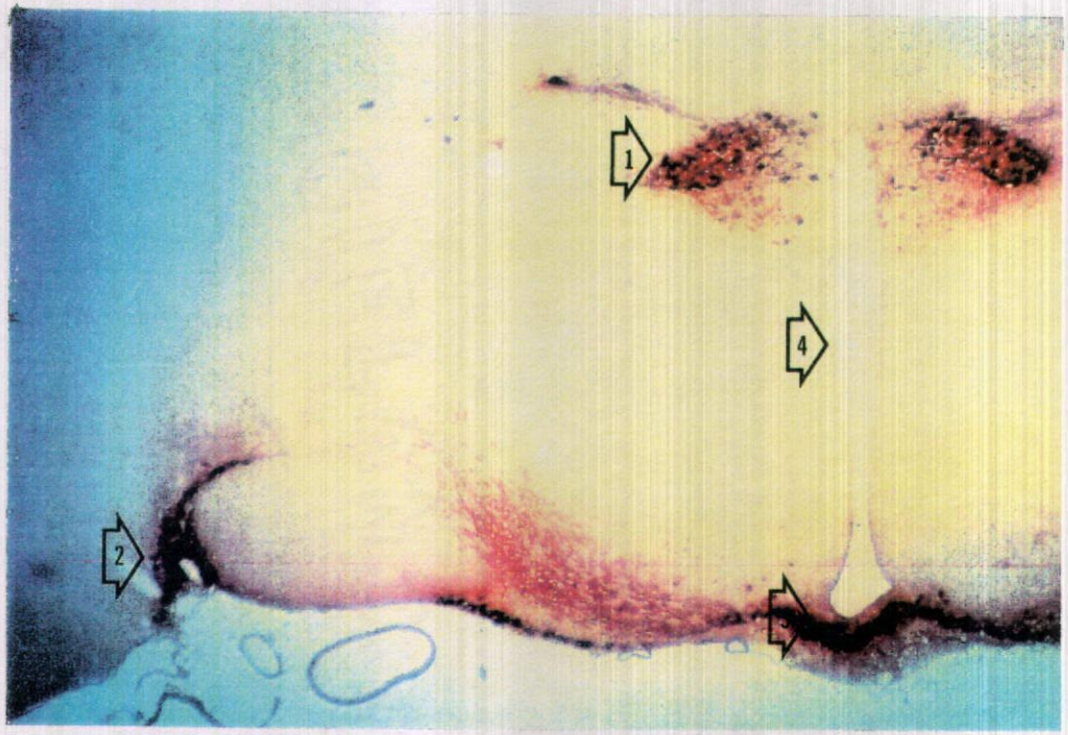

Figure 2. Cross section through the diencephalon region of the brain illustrating the location of the neurons that synthesize vasopressin and various anatomical regions: 1) Paraventricular nucleus, 2) Supraoptic nucleus, 3) Infundibulum and 4) 3rd Ventricle.

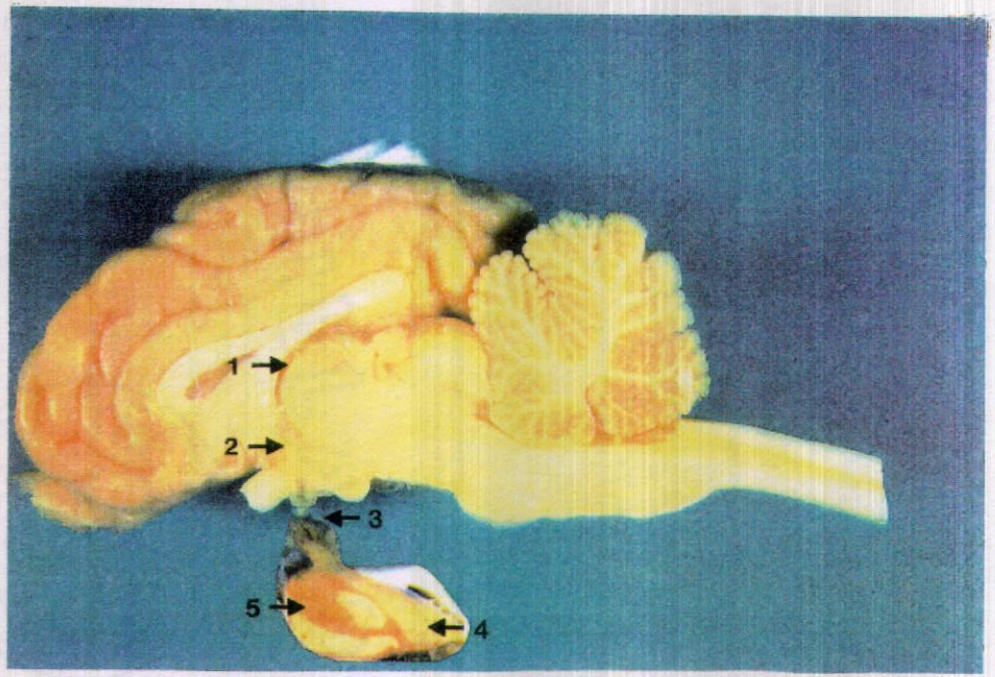

Figure 3. Sagittal view of the brain and the pituitary illustrating various anatomical regions:
1) Thalamus, 2) Hypothalamus, 3) Infundibulum, 4) Neurohypophysis and 5) Adenohypophysis.

CRF and VP Receptors. As of 1984, there were two types of peripheral vasopressin receptors identified (i.e., V1 and V2 receptors). The V1 receptor is located on smooth muscle and liver cells. The V2 receptor, however, has been shown to be responsible for the antidiuretic activities induced by VP in the kidneys. Although the V2 receptor transduction signal is via the adenylate cyclase system, the V1 receptor does not seem to be mediated through this particular second messenger pathway (Knepel et al., 1984).

Many scientists agreed with the theory of two separate VP receptors. The concept they did not agree upon, however, was which receptor was responsible for the CRF-like induced ACTH secretion. While some reported considerable evidence that the CRF-like activity of VP paralleled pressor potency (Mormede, 1983; Knepel et al., 1982), others had previously reported results which were in conflict with these findings (Doepfner et al., 1963; Arimura et al., 1969). Jard et al. (1988) provided more insight into this controversy when they suggested the presence of a pituitary vasopressin receptor with its own distinct form. They contended that this pituitary vasopressin receptor retained some of the V1 receptor properties and thus named it the V1b receptor (Jard et al., 1988).

As molecular biologists continue their work to isolate and identify this and other possible VP receptors with CRF-like activity, endocrinologists and physiologists continue the task of learning how stress affects the body.

The CRF receptor was identified in the brain (Wynn et al., 1984) and the anterior pituitary (Leroux and Pelletier, 1984) of the rat.

However, further investigation into the localization of CRF receptors has demonstrated the existence of the receptor in extra-hypothalamic tissues of the body (Herkenham, 1987).

Mechanism of Action for CRF and VP. CRF and VP are both polypeptide hormones that bind to their respective membrane bound receptors in the pituitary gland (DeSouza et al., 1985; Lutz-Bucher and Koch, 1983). Both receptors exhibit similiar properties in that they are desensitized and down-regulated in response to chronic stimulation by their specific ligand (Reisine and Hoffman, 1983; Koch and Lutz-Bucher, 1985).

When CRF binds to its specific receptor, it activates protein kinase A which is coupled to adenylate cyclase to produce 3',5' cyclic AMP (cAMP; Labrie et al., 1982). VP on the other hand has been shown to activate protein kinase C and does not directly stimulate the production of cAMP. However, Abou-Samra et al. (1987) did report that VP would augment the production of cAMP in CRF-stimulated corticotrophs.

It has been proposed, however, that increases in cAMP activate calcium channels in a variety of cells (Luini et al., 1985; Armstrong and Eckert, 1987). In 1988, Luini and DeMatteis reported a study in a permeabilized corticotroph mouse pituitary tumor cell line (AtT-20), a homogenous ACTH-secreting cell line, that showed exocytotic release of ACTH is calcium dependent and that cAMP had no direct effect on ACTH release from corticotrophs. Also, it has been shown that stimulation of corticotrophs with both CRF and VP results in increased intracellular calcium. Stimulation with CRF results in a

calcium influx from cytosolic origin whereas VP mobilizes calcium from intracellular stores. Therefore, Link et al. (1993) postulated that it may be the release of intracellular pool of calcium which is the main regulatory signal that activates ACTH release irrespective of the secretagoges.

ACTH and Corticotrophs. ACTH in the porcine, bovine, ovine, and human is a single-chain polypeptide that consists of 39 amino acids (Evans et al., 1966). The N-terminal amino acids numbered 1 through 24 are identical in each of the aforementioned species. This particular portion of the amino acid sequence is responsible for the full biological activity of the complete molecule. The major difference among species is in the C-terminus amino acids 25-33. ACTH is synthesized in corticotrophs of the pars distalis of the adenohypophysis (Baker, 1974).

Corticotrophs are classified as basophils due to their intense blue staining with aldehyde thionin and are diffusely distributed throughout the pars distalis. The percentage of all cells in the pars distalis that are corticotrophs is varible. However, most reports concur with Baker et al. (1969) that corticotrophs represent 4% of the cell population in the pars distalis. The corticotroph has been described as having: a cytoplasm of low electron opacity; sparse electron-opaque secretory granules approximately 200 mμ in diameter in a single row beneath the cell membrane; immature granules in the Golgi apparatus separated from their enveloping membranes by a clear halo; slender mitochondria with cristae that are often parallel to the long axis of the cell; Golgi complexes

scattered throughout the cytoplasm; and a modest amount of rough endoplasmic reticulum (Baker et al., 1969).

The identification of corticotrophs, however, is not a simple task. Other investigators have reported cell characteristics for the corticotroph that are inconsistent with the description by Baker et al. (1969). Kurosumi et al. (1962) described the corticotroph in the rat as having a dense core of secretory granules surrounded by a less dense zone and an outer limiting membrane. Also, according to Rennels and Shiino , the secretory granule in the corticotroph ranges from 126 to 154 mμ in diameter which is inconsistent with the report by Baker et al. (1974).

Mechanism of Action for Dexamethasone. The exact mechanism by which cortisol and dexamethasone (DEX), a synthetic glucocorticoid, inhibit CRF- and VP-induced ACTH secretion remains elusive. It has been demonstrated by use of a clonal cell line that intracellular receptors mediate the actions of glucocorticoids by modulating nuclear events in corticotrophs (Svec, 1984). Two classes of glucocorticoid receptors (i.e., Type I and Type II) have been reported (Canny et al., 1990) in the central nervous system. The Type I receptors have a high affinity for aldosterone, corticosterone and cortisol, but not for DEX. The Type I receptors are concentrated in the hippocampus and dentate gyrus (Reul et al., 1985).

Type II glucocorticoid receptors are quite different as compared to the Type I receptors. Type II receptors are the classical DEX-binding receptors and are abundant in the hypothalamus and the

anterior pituitary where they are clearly implicated in the control of ACTH secretion (Dallman et al., 1987).

There have been several hypotheses as to what cellular event(s) is(are) mediated by DEX to directly inhibit secretion of ACTH by the corticotroph cells of the anterior pituitary. Reports on the effects of glucocorticoids on cAMP production are controversial (Link et al., 1993). Also, the aforementioned studies reported in the section on mechanism of action for CRF and VP which refer to intracellular calcium involvement have recently been refuted (Clark and Kemppainen,1993). Clark and Kemppainen (1993) have suggested that glucocorticoid negative feedback occurs at a step (s) prior to the influx of extracellular calcium.

Glucocorticoid Receptors and RU486. Glucocorticoid receptors (GR) have been localized at multiple sites in the body including the hippocampus region of the brain, CRF producing neurons of the PVN and the anterior pituitary corticotrophs (Horiba et al., 1993). These locations provide the pathway by which cortisol feeds back to the hypothalamus and the anterior pituitary to inhibit further CRF and VP-induced ACTH secretion. Recent results (Pacak et al., 1993; Figure 1) suggested that glucocorticoids do indeed feedback to the PVN of the hypothalamus to inhibit the release of CRF and decrease hypothalamic turnover of catecholamines which are involved in regulation of CRF release.

The search for an effective antisteroid which would block the negative feedback of glucocorticoids with high affinity for the GR led scientists from Roussel Uclaf to the discovery of RU38486

(RU486) or mifepristone. RU486 exhibits both antiprogestin and antiglucocorticoid properties, however, a higher dosage is required to achieve the antiglucocorticoid effect (Baulieu, 1986). Both, *in vitro* and *in vivo* studies demonstrate that, RU486 possesses GR blocking activity without exhibiting agonistic corticoid effects (Gagne et al., 1985). The uniqueness of this particular antisteroid was that its affinity for the GR was three times greater than that of the synthetic glucocorticoid, dexamethasone. Experiments in humans and some animal models demonstrated that RU486 has the ability to block the inhibitory effect of CS on ACTH secretion in a dose-related manner (Spitz and Bardin, 1993). Sufficient evidence that may still be lacking however, is whether RU486 has the ability to block the negative feedback of CS on both CRF and VP-induced ACTH secretion especially in domestic farm animals. Therefore, development of an antistress agent would benefit the livestock industry.

Consequences of Stress. The concept that stress imposes some detrimental effects on the body is not novel. One of the first adverse conditions associated with stress was the development of ulcers in experimentally stressed monkeys in the 1950s (as reviewed by Sapolsky, 1988). Even as early as the late 1960s, researchers were linking elevated levels of glucocorticoids to damage of the hippocampal cells of the brain (Sapolsky, 1988). Other adverse conditions that have been associated with stress include reduced rates of reproduction, lower production (e.g., average daily gain, milk output) and a compromised immune system.

The detrimental effects associated with reduced reproduction seem to be connected to the inhibition of the release of luteinizing hormone-releasing hormone (LHRH) from the infundibulum (Ono et al., 1984). In males, the inhibitory effect of cortisol on the secretion of luteinizing hormone (LH) and testosterone appears to be species dependent. Increased CS in the male rat (Vreeburg et al., 1984), the bull (Welsh et al., 1979), and man (Doerr and Pirke, 1976) has been reported to decrease both LH and/or testosterone. However, in the male Rhesus monkey (Hayashi and Moberg, 1987) and the boar (Liptrap and Raeside, 1975; Juniewicz and Johnson, 1981), increases in ACTH and/or CS are associated with an increase in plasma concentration of LH and testosterone. Although an increased plasma concentration of cortisol has been reported to have inhibitory effects in both males and females, it appears that females are more susceptible to stress. Some studies describe a greater stress-induced secretion of ACTH in females than in males. Moberg suggested that female reproduction is more susceptible due to the dependency of successful reproduction on carefully timed hormonal secretions (Moberg, 1991). If the female is subjected to stressors near the time of ovulation, the elevation of plasma cortisol can diminish the release of LHRH, this may inhibit the release/surge of LH from the adenohypophysis that is needed for ovulation.

Reduced animal productivity and a compromised immune system are considered together for the purpose of this review. When an animal's immune system is compromised, it will usually become sick, and therefore, there is reduced production for that

animal [i.e., lower average daily gain, lower milk yield, etc. (Friend, 1991)]. In 1976, Selye described how stress affected not only the nervous and endocrine systems, but also the immune system (Selye, 1976). Stress-induced effects on the immune system stem from the hormonal secretions of the hypothalamic-pituitary-adrenal axis (Livnat et al., 1985). Several *in vitro* studies have demonstrated this connection by showing that CRF directly suppresses human peripheral blood natural killer cell activity (Pawlikowski et al., 1988) and monocyte chemotaxis (Stepien et al., 1987). Also, when the immune system is suppressed due to stress, animals are more susceptible to conditions such as shipping fever/bovine respiratory disease (McKercher, 1978) and viruses that cause infectious bovine rhinotracheitis (Narita et al., 1981).

Summary. Previous studies provide ample scientific evidence to warrant further research regarding the endocrinology and physiology of "stress" in domestic animals. In order to efficiently combat the detrimental effects associated with stress, we must first have a thorough and complete understanding of how stress interacts with the body's nervous, endocrine, and immune systems. The intent of the proposed research was not to address all of these areas. The main focus of this thesis project was on the endocrine system. Therefore, the outlined objectives were proposed to gain further insight into the interactions of CRF and VP as activators of the stress response in cattle and how these factors may be negated.

MATERIALS AND METHODS

Experiment 1: In Vitro Regulation of CRF- and VP-induced ACTH Secretion

Tissue. The pituitary glands were recovered from 30 slaughtered steers, 2 heifers and 3 six-month old boars at the Texas A&M University Rosenthal Meat Sciences and Technology Center within 10-20 min following stunning of the animals. The tissues were transferred immediately to a tissue culture room in the adjacent Kleberg Center. After exsanguination, the head was removed at the occipitoatlantal joint and transferred to a processing room where a band saw was used to expose the brain. The brain was removed from the neurocranium to expose the pituitary gland. The pituitary gland was removed with a sterile scalpel and hemostats, placed in a sterile plastic bag, and then transferred immediately to the tissue culture room in the adjacent Kleberg Center. Upon arrival at the culture room, the pituitary gland was split mid-sagitally and separated under sterile conditions into the pars distalis of the adenohypophysis (PDA) and neurohypophysis (with pars intermedia). The pars distalis (see Appendix A for weights) was weighed and prepared for cell culture (Appendix B); the weight of the neurohypophysis was recorded prior to disposal.

Cell Culture. The pars distalis was sliced into 1 mm thick sections with a Stadie-Riggs tissue slicer and placed into a 1X solution of Dulbecco's Modified Eagle Medium (DMEM) tissue culture medium until further processing. The slices were then minced into approximately 1 mm cubic portions with the blade of the tissue slicer and the pieces washed two times in 1X DMEM. Tissue from all steers, heifers and boars on a given culture day were pooled by species/sex class and enzymatically dispersed in 1X DMEM solution which contained 0.3% collagenase (350 units/mg of tissue; Sigma Chemical Co., St. Louis, MO) for 1 hour (37 °C water bath with the solution being mixed with a magnetic stir bar). After dispersal, the solution was filtered through a single layer of sterile gauze and the filtrate was then centrifuged for 15 min at 200 xg to precipitate the cells. The liquid was aspirated and the pellet of cells was resuspended in 1X DMEM and then centrifuged. This washing portion of the procedure was repeated three times.

After the third wash, the cells were resuspended and the concentration of cells determined using a hemocytometer. Viability of the cells was assessed by trypan blue stain exclusion (viability averaged 79%). The cells were then diluted to a concentration of 400,000 viable cells/ml (200,000 each for heifers and boars) in 1X DMEM containing 1.0 M L-glutamine (Gibco Laboratories, Chagrin Falls, OH) and 10% fetal calf serum (Whittaker M. A. Bioproducts, Inc., Walkerville, MD). The cell suspension was then dispensed in 1-ml fractions into 35 mm x 10 mm polystyrene 6-well tissue culture dishes (Corning Glass Works, Corning, NY.) and placed into a 37 °C

incubator (95% humidified air and 5% carbon dioxide). The day of plating the cells was designated as Day 0 of the culture.

On Day 2 (48 hours after plating cells), the medium was aspirated from the culture wells with sterile glass pipettes and a new 1 ml fraction of the supplemented 1X DMEM added to the well (medium was added gently to prevent dislodging of the cells from the culture wells). The medium was changed again on Days 3 and 4 as described above for Day 2. On Day 5, the cells were washed twice with serum-free medium prior to adding treatments. After the second wash, the medium was aspirated and a 1-ml fraction of serum-free 1X DMEM containing the treatment was added to the appropriate well and allowed to incubate for 3 h for primary cultures from the steers and the heifers, and 4 h for primary cultures from boars. After the 3-h and 4-h incubation periods, the medium was collected with plastic pipette tips, dispensed into polypropylene test tubes and stored at -20 °C until medium concentration of ACTH was determined by radioimmunoassay (RIA) (Wagner, 1987). Primary cell cultures orginating from boar pituitaries had a new 1 ml fraction of 1X DMEM plus serum added and continued in culture as described above until Day 14 at which time the cells were again treated as on Day 5 of the culture. After a 4-h incubation period, the medium was collected and analyzed as described above.

Cell Culture Treatments. Dosages used for bCRF (Penninsula Laboratories, Inc., Belmont, CA., Cat. #8568), VP (Calbiochem, La Jolla, CA., Cat. #676435) and DEX (Steraloids Inc., Wilton, NH., Cat.

#P500) were based on previous results from our laboratory (Wagner, 1987; Welsh et al., 1989) and ranged from 10^{-11} M to 10^{-6} M for CRF, 10^{-9} M to 10^{-6} M for VP, and 10^{-10} M to 10^{-7} M for the VP analog. In order to evaluate the efficiency of the two VP analogs (Figure 4) as potent agonists or antagonists at the level of the pituitary, dose concentrations were chosen to parallel those of the VPP analog (Manning et al., 1992). The RU486 (17B-hydroxy-11B-[4-dimethylaminophenyl-1]-17α[prop-1-ynyl]-estra-4,9-diene-2-one) dose concentration chosen by our laboratory to evaluate its antiglucocorticoid effect was 10^{-6} M (donated by Roussel-Uclaf).

Cell Culture Medium. The cell culture medium was prepared by supplementing Dulbecco's Modified Eagle Medium with the following constituents: 1) 50 ml of 50X MEM; 2) 50 ml of 100X MEM non-essential amino acids; 3) 5 ml Fungizone (Amphotericin-B 250 mg/ml); 4) 5 ml penicillin-streptomycin (10,000 U/ml penicillin, 10,000 mg/ml streptomycin); 5) 18.5 g HEPES (Gibco Laboratories, Chagrin Falls, OH); and 6) 18.5 g sodium bicarbonate (Fisher Scientific, Fair Lawn, NJ). and 7) 5 ml of glutamine as an energy source. In order to sterilize the medium, it was passed through a filtration system fitted with a .2 micron filter.

bovine CRF-Peninsula	Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Asn-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2		
	(M.W. 4694.48)		
(Arg^8)-VP	H-Cys-Tyr-	**Phe-Gln-Asn-Cys-Pro-Arg-Gly**	-NH_2
	(M.W. 1084.2)		
VP analog-Bachem (VPB)	Mpr-D3-Pal-	**Phe-Gln-Asn-Cys-Pro-Arg-Gly**	-NH_2
	(M.W. 1054.2)		
VP analog-Peninsula	Pmp-D-Tyr (Et)-	**Phe-Val-Asn-Cys-Pro-Cit-Gly**	-NH_2
	(M.W. 1135.04)		

Figure 4. Amino acid sequence and molecular weights for bovine CRF, arginine vasopressin, and two vasopressin analogs.

Experiment 2: In Vivo Activation of the Bovine HPA axis by bCRF and VP

Animals and Plasma Samples. The ability of CRF and VP to enhance plasma concentration of ACTH was studied in eight docile, proestrous Jersey cows (500 kg BW and 6 yr old) at the Texas A&M University Dairy Center. These particular cows had been exposed previously to extended periods of halter restraint and handling for teaching purposes. Blood samples were collected on four days during the experiment. Approximately 10 mls of blood were collected at each sampling time followed by an infusion of heparin (.07 g/liter; Sigma, St. Louis, MO., Cat.#H9133) and physiological saline (0.9%) to replace fluid volume.

Days 1 and 2 of the experiment were consecutive days after which the cows had a two-wk recovery period prior to the consecutive sampling on Days 3 and 4 of the experiment. Indwelling jugular cannulae were inserted in the cows 24 h prior to the onset of sample collection and remained intact for samples collected on the consecutive day. Blood samples were collected via jugular vein at 15-min intervals for 4 h prior to treatment (PRE) and for 6 h after treatment (POST). Blood samples were also collected at 1, 5, and 10 min POST.

At time of treatment (Time O), each cow received a 5-ml injection which consisted of one of the following four treatments in a saline vehicle: 1) Control (5 ml of saline alone; Control); 2) bCRF (.3 μg/kg BW); 3) VP (1 μg/kg BW); and 4) bCRF (.3 μg/kg BW) + VP (1 μg/kg BW). Cows were randomly assigned to treatment groups with 2

cows/treatment for each of the four treatment days. Days 1 and 2 (Experiment 2a) were separated from d 3 and d 4 (Experiment 2b) in order to maintain the cows in the proestrous phase of the estrous cycle during all four days of the experiment. Blood samples were centrifuged (2820 xg) for 30 min at 4 °C at the Dairy Center to recover plasma which was then transferred to laboratory facilities in the Kleberg Center for analysis of ACTH and CS by RIA.

In Vivo Treatments. To determine the ability of CRF and VP to induce secretion of ACTH *in vivo*, the cows were challenged with one of the following four treatments: 1) Control (saline alone); 2) bovine CRF (bCRF; 0.3 μg/kg BW); 3) arginine vasopressin (AVP; 1μg/kg BW); or 4) bCRF + AVP (0.3 μg/kg BW and 1 μg/kg BW, respectively). Each cow was evaluated with each of the four treatments on separate sampling days to allow for individual response variations. The experiment utilized cows in the proestrous phase of the estrous cycle; therefore, Experiments 2a and 2b were separated by at least 30 days.

Radioimmunoassays. Cell culture medium and plasma from blood samples were used to determine the concentration of ACTH by using a modified version (Wagner, 1987) of a double antibody radioimmunoassay (Appendix C) developed by Nicholson et al. (1984): 1) the first antibody was IgG-ACTH-1 rabbit anti-(1-24)ACTH (IgG Corporation, Nashville, TN) supplied at a dilution of 1:40 and diluted to 1:2000 for use; 2) the second antibody was goat anti-rabbit gamma-globulin (Calbiochem, La Jolla, CA., Cat. #539845) diluted to

1:20; 3) ^{125}I h(1-24)-ACTH (INC Biomedical, Carson, CA., Cat. #07106126) was reconstituted in double-distilled water to yield 10,000 cpm/100 µl and is used as the radioactive antigen, and 4) the standards were serially diluted (50 pg/100 µl to .0781 pg/100 µl) from h(1-24)-ACTH (Peninsula Laboratories, Inc., Belmont, CA., Cat. #8741). The primary antiserum does not crossreact with other peptides with a structure similar to that of ACTH (i.e., lipotropin, melanotropin, or endorphin). In addition, hormones used in these experiments (i.e., vasopressin, CRF, dexamethasone) do not interfere with the ability of the antiserum to bind radiolabeled ACTH. The sensitivity of the ACTH RIA was 2 pg/tube and the intra- and inter-assay coefficients of variation were 6 and 10%, respectively. The total volume of unknown assayed was 200 µl; the plasma or medium samples containing a higher quantity of ACTH needed to be diluted with assay buffer.

Plasma samples were also assayed for CS using a primary antibody assay procedure (Appendix D) with the following components (Anderson et al., 1986): 1) primary antibody was rabbit anti-cortisol antiserum received at a dilution of 1:400 and diluted further to 1:2500 (Pantex, Santa Montica, CA., Cat. #P44); 2) the standards (4-pregnen-11B,17,21-triol-3,20-dione; Steroids Inc., Wilton, NH.) were made by serial dilutions (4000 pg/500 µl to 3.9 pg/500 µl); 3) the tracer used was ^{3}H-Hydrocortisone (1,2-^{3}H; NEN, Boston, MA., Cat. #NET-185) and 4) the liquid scintillation fluid was Ecolume (ICN, Irvine, CA., Cat. #882470). The assay sensitivity was 62 pg/assay tube and the antibody crossreacted 60, 48, 0.01 and

0.01% with corticosterone, deoxycorticosterone, progesterone and estradiol, respectively. Intra- and inter-assay coefficients of variation were 8 and 12%, respectively.

Statistical Analysis. The concentration of ACTH and cortisol in unknowns was determined using the computer program Assay Zap (Biosoft, Cambridge, UK.) which utilizes a four parameter model [$y= a-d/1+(x/c)^b + d$]. All *in vitro* and *in vivo* data were arrayed and analyzed by Excel 4.0 (Microsoft Corporation) and Statview 1.04 (Abacus Concepts, Inc., Berkley, CA.). The Excel program was used to calculate means and the area under the hormone profile curves. Statview tests included in the analyses were: 1) unpaired T-test; 2) analysis of variance; 3) Fisher's r to z correlations; 4) Spearman's test for correlations; and 5) stepwise regression for prediction equations. The ANOVA models included time, culture replicate and interactions as sources of variation. Treatment differences with a P value less than or equal to .05 were considered statistically significant. Correlation analyses were used to describe temporal relationships between ACTH and cortisol.

RESULTS AND DISCUSSION

Experiment 1: In Vitro Regulation of CRF and VP-induced ACTH Secretion

CRF-induced ACTH Secretion. Five individual primary pituitary cell cultures were established from the 30 steers, as described in the Materials and Methods section, to investigate the ACTH releasing activity of *bovine* CRF. The following data are based on the treatment mean plus the standard error of the mean (S.E.M.) for 6-8 wells/culture. Figure 5 represents the stimulatory action of various concentrations of CRF (10^{-11} M to 10^{-7} M). Secretion of ACTH by cells treated with 10^{-9}M CRF did not differ ($P > .10$) from that of Control cells (284.9 ± 14.9 pg/ml).

Secretion of ACTH was increased by treating cells with 10^{-8} M CRF (3.1-fold increase; $P < .0001$ relative to Control). Medium concentration of ACTH was further enhanced ($P < .0001$) by treatment with 10^{-7} M CRF (1577.5 ± 98.6 pg/ml; a 5.5-fold increase relative to Control). These results differ from those reported by Wagner (1987) in which *ovine* CRF-induced secretion of ACTH was increased by 2.2-, 3.3-, and 3.7-fold, at 10^{-9} M, 10^{-8} M, and 10^{-7} M, respectively. A possible explanation for the diffences reported between the stimulatory effects of *bovine* and *ovine* CRF in cultured bovine corticotrophs may reside in the recognition of the ligand by the CRF-

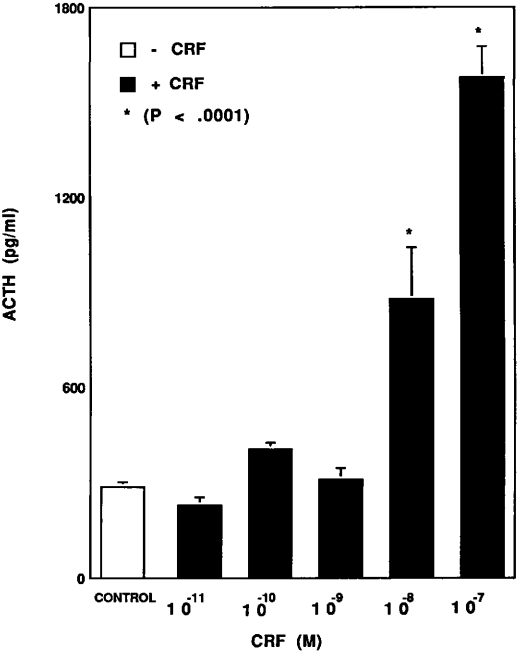

Figure 5. Ability of CRF to stimulate ACTH secretion by steer anterior pituitary cells (400,000 viable cells/well). Cells challenged on Day 5 of culture for 3 h (n = 6 wells/treatment/culture).

binding protein (i.e., CRF BP). In a recent study, Woods et al. (1993) demonstrated that *ovine* CRF does not bind appreciably with *human* CRF BP and therefore does not dimerize to form the CRF-CRF BP complex which may be associated with the clearance rate of the ligand. The cells which synthesize the CRF BP have not been identified at this time. Therefore, the possible presence of these CRF binding proteins in our culture system could explain the different degrees of ACTH secretion in response to *ovine* versus *bovine* CRF. If the *ovine* CRF is not being bound by CRF BP with an affinity equivalent to that for *bovine* CRF, it would be reasonable to assume that there is more ligand that can interact with its receptor.

Gibbs and Vale (1983) reported CRF concentration in the hypothalamic-hypophyseal portal blood of the rat to be approximately 1 ng/ml. In the ovine, Engler et al. (1989) reported that basal CRF concentration in the hyppophysial-portal circulation ranged from 18 $\pm$ 8 pmol/l to 127 $\pm$ 14 pmol/l. Given that *in vitro* studies for both the bovine and rat corticotroph demonstrate that CRF is a more potent stimulator of ACTH secretion than VP, it is possible that the concentration of CRF in the hypothalamic-hypophyseal portal blood of the bovine would be within the 1 ng/ml range. These levels are consistent with the reported Kd of CRF. Therefore, the stimulatory action of CRF 10^{-8} M may indeed be physiologically relevant in the bovine.

VP-induced ACTH Secretion. The stimulatory action of VP was also examined at various concentrations (10^{-10} M to 10^{-6} M) as represented in Figure 6. Neither 10^{-10} M, 10^{-9} M nor 10^{-8} M increased

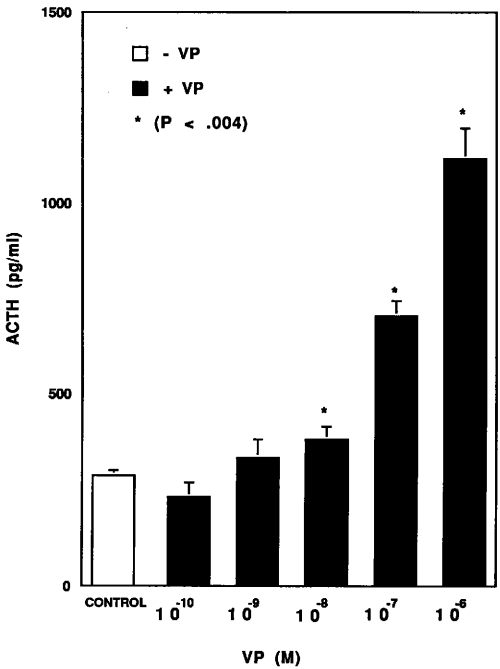

Figure 6. VP-induced ACTH secretion by cultured steer anterior pituitary cells (400,000 viable cells/well). Cells challenged on Day 5 of culture for 3 h (n = 6 wells/treatment/culture).

terms of stimulating secretion of ACTH. Medium concentration of ACTH was further enhanced ($P < .001$) with 10^{-6} M VP (1115.45 $\pm$ 79.4 pg/ml).

The coupling of these data (e.g., comparison of CRF- and VP-induced secretion of ACTH) with the data of Wagner (1987), indisputably demonstrates that CRF is indeed the more potent stimulator of ACTH secretion in cultured bovine corticotrophs. The minimum stimulatory concentration of VP (10^{-7} M) is similar to the concentration reported in the hypothalamic-hypophyseal portal blood of the sheep (50-2000 pmol/liter; Engler et al., 1989),the monkey (11-16 ng/ml; Zimmerman et al., 1973), the rat (1 ng/ml; Gibbs and Vale, 1983) and in the horse (25 pmol/liter; Alexander et al., 1991).

ACTH Secretion Induced by VP Analogs. Both VPB AND VPP have been reported to possess antagonistic activity peripherally. At the onset of the present study it was known that the VPB analog also demonstrated agonistic activity at the pituitary in terms of stimulating ACTH secretion. We evaluated whether the VPP analog is an antagonist to VP at the level of the pituitary gland. We determined that VPP is an agonist rather than an antagonist to VP at the level of the pituitary. Figure 7 represents a comparison of VP with two analogs of VP (i.e., VPP and VPB; see Materials and Methods for details). Relative to Control, medium concentration of ACTH increased for cells treated with 10^{-7} M VP, VPP and VPB. At equilmolar concentration (10^{-7} M), VP and VPB were more potent than VPP in terms of stimulating secretion of ACTH. At 10^{-6} M, the VPP analog tended to be less effective ($P > .05$) than 10^{-6} M VP.

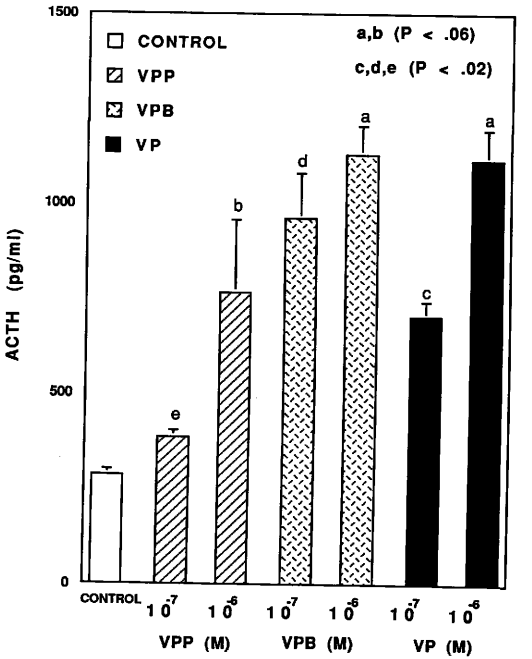

Figure 7. Stimulatory action of VP and two VP analogs at various concentrations in steer anterior pituitary cell cultures. Cells (400,000 viable cells/well) were challenged on Day 5 of culture for 3 h (n = 6 wells/treatment/culture).

medium concentration of ACTH relative to Control ($P > .10$). At 10^{-7} M, the stimulatory action of VP was apparent with a 2.5-fold increase ($P < .0001$) over Control (284.9 $\pm$ 14.9 pg/ml). At an equimolar concentration of 10^{-7} M CRF was more potent than VP in

Interestingly, the results obtained by treating the cells with the VPB analog do not concur with the potency report for this analog as compared to VP (Schwartz et al., 1991) in sheep. Schwartz demonstrated that VPB was only 1/36 as potent as VP in terms of stimulating secretion of ACTH in sheep. The present results, however, demonstrate that in cultured bovine corticotrophs the ACTH stimulating ability of VPB is equivalent to VP at 10^{-7} M. The dissimilarity in the activity of VPB in these two studies is especially interesting given that VP is more potent in terms of stimulating secretion of ACTH by ovine corticotrophs. The discrepancy in the action of this analog may stem from the existence of receptor subtypes (e.g., V1, V1b and V2; Jard et al., 1988). It is conceivable that in the pituitary of the sheep there is a greater concentration of the V1b subtype receptor, compared to the bovine pituitary.

Time Course of CRF- and VP-induced ACTH Secretion. To determine the temporal aspect of CRF- and VP-induced secretion of ACTH by cultured bovine anterior pituitary corticotrophs, the cells were challenged for 15, 30 or 120 min with medium alone, each neurohormone alone or a combination of the two neurohormones (Figure 8). By 15 min post-challenge, VP (10^{-6} M), CRF (10^{-7} M) and the combination of CRF + VP each enhanced ($P < .05$) medium

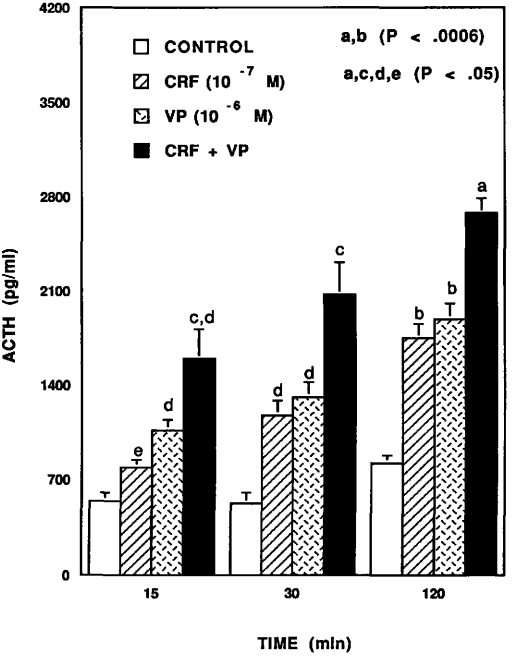

Figure 8. Temporal profile of ACTH secretion induced by CRF, VP and the combination of CRF + VP in steer anterior pituitary cell culture (400,000 viable cells/well). Cells were challenged on Day 5 of culture for either 15, 30 or 120 minutes.

cells treated with either CRF or VP alone. By 30 min post-challenge, secretion of ACTH induced by CRF (10^{-7} M) and VP (10^{-6} M) were equivalent ($P > .10$). However, medium concentration of ACTH for cells treated with the combination of CRF and VP was greater ($P < .05$) than the medium concentration of ACTH for cells that were treated with either CRF or VP alone.

By 120 min post-challenge, medium concentration of ACTH for the cells treated with the combination of CRF and VP increased 3.3-fold relative to Control and was greater than ($P < .001$) medium concentration of ACTH for cells treated with CRF and VP alone. There was no difference ($P > .10$) in medium concentration of ACTH for cells treated with CRF or VP alone. The 180 min post-challenge secretion of ACTH is represented in Figure 5 for CRF and Figure 6 for VP. By 180 min post-challenge, the stimulatory action of VP had reached a plateau whereas CRF continued to increase medium concentration of ACTH and surpassed ($P < .001$) the stimulatory action of VP.

These results demonstrate that the onset of VP action or response to VP is more acute than that of CRF and tends to plateau after 120 min. The stimulatory action of CRF however is slow initially but continues to increase medium concentration of ACTH beyond the stimulatory action of VP. Similar temporal profiles of plasma concentration of ACTH were observed when the two neurohormones were evaluated *in vivo* (see Figures 14 and 15). Recently, other investigators (Cantor et al., 1993) have also reported similar temporal profiles for CRF- and VP-induce B-endorphin in

concentration of ACTH relative to Control (541.6 ± 61.8 pg/ml). Medium concentration of ACTH was greater ($P < .05$) at 15 min for VP-treated cells relative to the CRF-treated cells. At 15-min, the medium concentration of ACTH was greater ($P < .05$) for the cells treated with the combination of CRF and VP relative to those perifused ovine anterior pituitary cells. There are at least two factors that could account for these temporal differences: 1) CRF and VP activate separate and different second messenger systems (i.e., protein kinase A and protein kinase C, respectively) and 2) the mechanism(s) involved in transport of these two neurohormones is different.

Dex-induced Inhibition of ACTH Secretion. The ability of the synthetic glucocorticoid, DEX, to suppress protein kinase A (CRF) mediated secretion of ACTH is represented by Figure 9. Addition of 10^{-6} M DEX alone to the cultured cells did not affect ($P > .10$) medium concentration of ACTH relative to Control (284.9 ± 14.9 pg/ml). Addition of CRF increased secretion of ACTH ($P < .0001$) 5.5-fold relative to Control. Concurrent treatment with DEX decreased CRF-induced secretion of ACTH by 52%.

The ability of DEX to inhibit protein kinase C mediated secretion of ACTH is represented by Figures 10 and 11. Medium concentration of ACTH was increased ($P < .0001$) by treating cells with VP, VPB and VPP (10^{-6} M each). Medium concentration of ACTH was enhanced 3.9-fold each for VP and VPB, and 2.2-fold for VPP relative to Control (284.9 ± 14.9 pg/ml). Concurrent treatment with

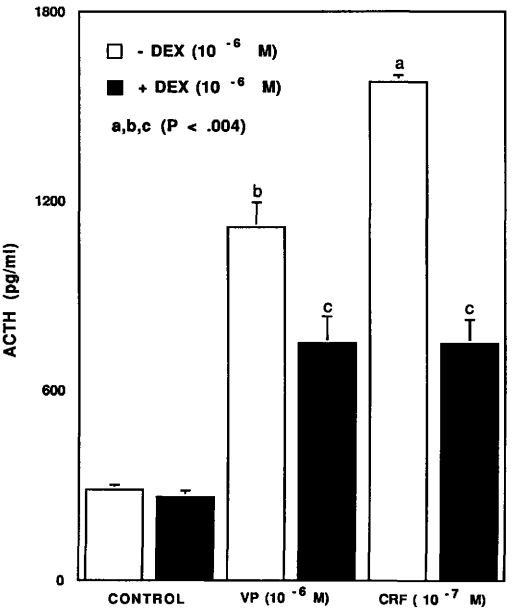

Figure 9. Dexamethasone's inhibition of CRF- and VP-induced ACTH secretion by cultured steer anterior pituitary cells (400,000 viable cells/well). Cells were challenged with CRF, VP and/or DEX for 3 h on Day 5 of culture (n = 6 wells/treatment/culture).

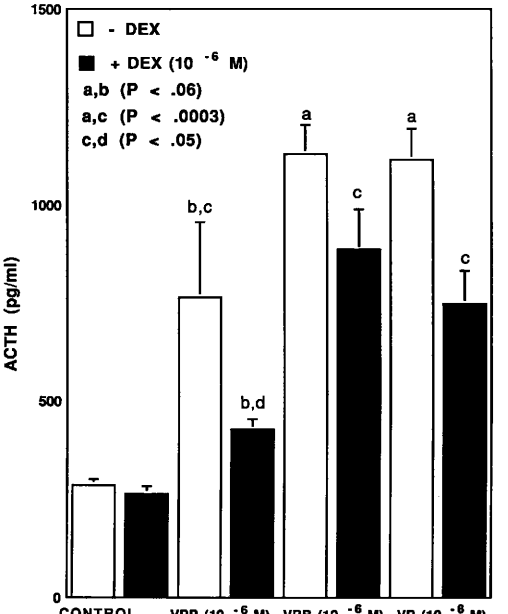

Figure 10. Ability of DEX to suppress protein kinase C mediated ACTH secretion induced by VP and the two VP analogs (i.e., VPP and VPB) in steer anterior pituitary cell cultures. Cells (400,000 viable cells/well) were challenged on Day 5 of culture for 3 h (n = 6 wells/treatment/culture).

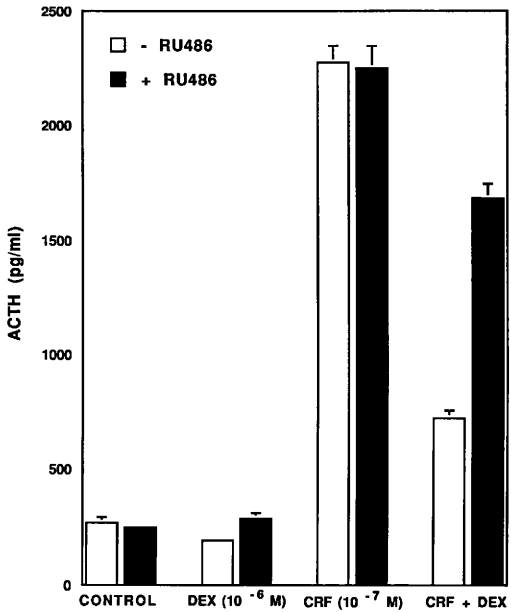

Figure 11. Ability of the antiglucocorticoid RU486 to negate the inhibitory effect of DEX on CRF-induced ACTH secretion in steer anterior pituitary cell culture. Cells (400,000 viable cells/well) were challenged on Day 5 of culture for 3 h (n = 6 wells/treatment).

DEX decreased VP-, VPB-and VPP-induced secretion of ACTH by 32%, 21% and 44%, respectively.

It has been demonstrated previously that CRF action is mediated via the protein kinase A system and VP action is mediated via the protein kinase C system (Abou-Samra et al., 1987; Wagner, 1987). Present results indicate that the inhibitory action of the synthetic glucocorticoid (e.g., DEX) is expressed in both the protein kinase A and protein kinase C mediated ACTH secretion by cultured bovine corticotrophs. However, total suppression of the stimulatory action of CRF and VP in terms of ACTH secretion is not achieved with DEX. Although the mode of action of DEX remains elusive, it appears to block futher secretion of ACTH, but does not negate the previous stimulatory effects on the cell.

RU486 Negation of the Inhibitory Effects of DEX. The ability of the antiglucocorticoid (RU486) to block the inhibitory effect of DEX on CRF-induced ACTH secretion by *bovine* and *porcine* anterior pituitary corticotrophs is presented in Figures 11 and 12. Addition of 10^{-6} M RU486 or co-treatment with 10^{-6} M DEX did not affect ($P > .10$) medium concentration of ACTH relative to Control (267.5 ± 25.8 pg/ml). An 8.5-fold increase ($P < .0001$) in medium concentration of ACTH occurred in response to treatment with 10^{-7} M CRF. Co-treatment with CRF plus DEX suppressed medium concentration of ACTH by 68.1%. However, when RU486 was added to the combination treatment of CRF plus DEX, RU486 was able to negate 62% of the inhibitory effect of DEX.

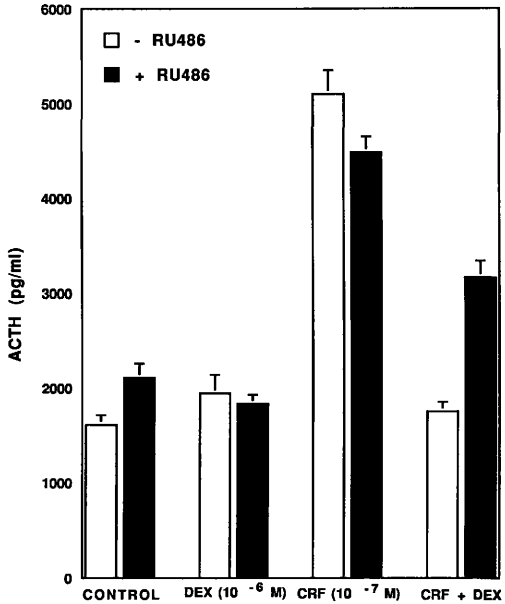

Figure 12. Ability of RU486 to negate the inhibitory action of DEX on CRF-induced ACTH secretion in porcine anterior pituitary cell culture. Cells (200,000 viable cells/well) were challenged for 4 h on Day 5 of culture (n = 6 wells/treatment).

The mechanism of action for RU486 at the glucocorticoid receptor remains elusive (Spitz and Bardin, 1993). The ability of RU486 to negate the suppressive action of DEX in porcine anterior pituitary corticotrophs is presented in Figure 12. Addition of 10^{-6} M RU486 or co-treatment with 10^{-6} M DEX did not affect ($P > .10$) medium concentration of ACTH relative to Control (1605.6 $\pm$ 103.6 pg/ml). A 3.2-fold increase ($P < .0001$) in medium concentration of ACTH occurred in response to treatment with 10^{-7} M CRF relative toControl. Co-treatment with CRF plus DEX suppressed medium concentration of ACTH by 66%. Forty-two percent of this suppression was negated however when RU486 was added to the combination treatment of CRF plus DEX.

The ability of RU486 to block the inhibitory action of DEX on CRF-induced secretion of ACTH in the *bovine* and *porcine* clearly demonstrates its antiglucocorticoid activity on anterior pituitary cells *in vitro*. Subsequent *in vitro* studies are in progress to evaluate the action of RU486 on the hypothalamus and infundibulum for both *bovine* and *porcine* tissues. Also, *in vivo* studies are being designed to investigate the ability of RU486 to inhibit the effects of DEX in the *bovine*.

Experiment 2: Activation of the Bovine HPA Axis by bCRF and VP In Vivo

In Vivo Experimental Design. There was no interaction ($P > .05$) between effects of day of experiment and treatment; therefore,

values reported represent the means and S.E.M. for treatments pooled over all four days of the experiment. Standard error of the means are not represented to enhance the clarity of the graphs depicting the *in vivo* response to CRF and VP. There were no differences ($P > .10$) among treatment means for any of the discrete time periods during the PRE period. Therefore only 1 h of the 4 h PRE period is illustrated in graphs along with representatives for the 6 h POST period.

Control (Saline) Group. There was no difference ($P > .05$) in plasma concentration of ACTH among cows ($n = 8$ cows) that received the saline alone treatment during the 4-h PRE period. At Time 0, cows were given a 5 ml injection of physiological saline. Saline injection had no affect ($P > .05$) on plasma concentration of ACTH during the 6-h POST period (Figure 13). Therefore, the remaining treatment groups will be compared to Control. The mean plasma concentration of ACTH for all cows pooled over PRE and POST periods averaged 28.2 ± 1.6 pg/ml.

bCRF Treatment Group. During the PRE period plasma concentration of ACTH did not differ ($P > .10$) among cows assigned to the bCRF treatment group (Figure 14). At Time 0, the bCRF treatment group did not differ ($P > .05$) from Control. At 1 min POST, plasma concentration of ACTH was increased 2.2-fold over Control (32.7 ± 7.2 pg/ml) in cows challenged with bCRF. At 5 min POST, plasma concentration of ACTH was further increased 5-fold relative to Control. Maximal observed plasma concentration of ACTH (324.8 ± 126.2 pg/ml) was at 15 min POST. After 15 min POST, plasma

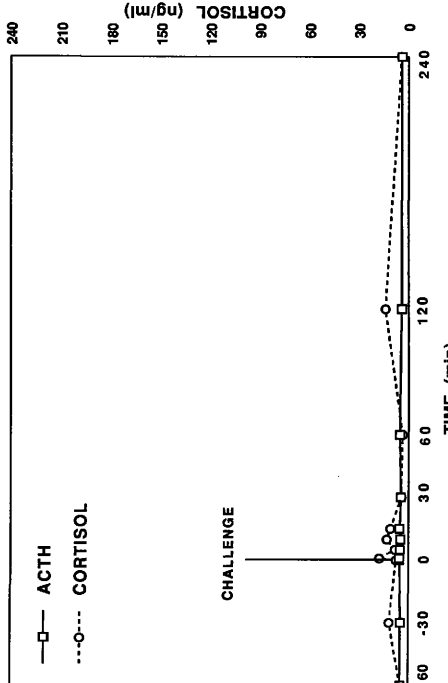

Figure 13. The temporal aspect of the responsiveness of endogenous ACTH (solid line) and cortisol (dashed line) following saline infusion (n = 8 cows). Mean plasma concentration of ACTH did not differ ($P > .10$) during the entire period of blood sampling for the Control group (28.2 ± 1.6 pg/ml).

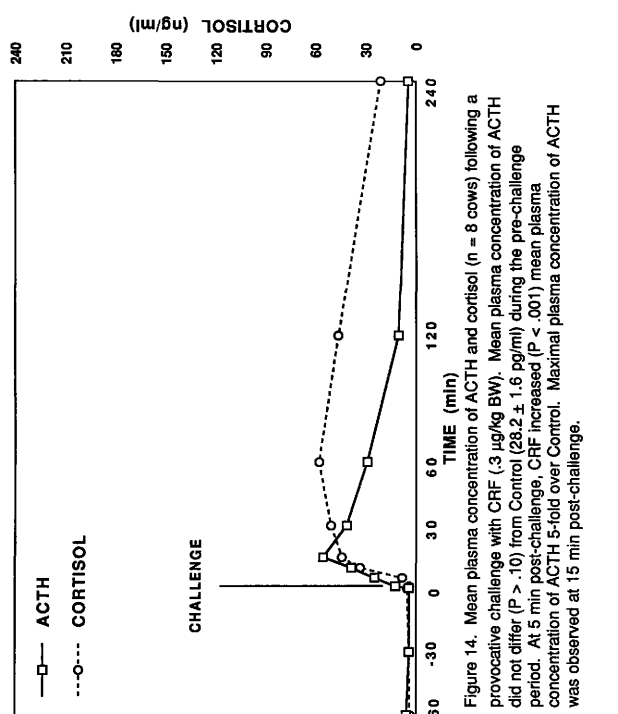

Figure 14. Mean plasma concentration of ACTH and cortisol (n = 8 cows) following a provocative challenge with CRF (.3 µg/kg BW). Mean plasma concentration of ACTH did not differ (P > .10) from Control (28.2 ± 1.6 pg/ml) during the pre-challenge period. At 5 min post-challenge, CRF increased (P < .001) mean plasma concentration of ACTH 5-fold over Control. Maximal plasma concentration of ACTH was observed at 15 min post-challenge.

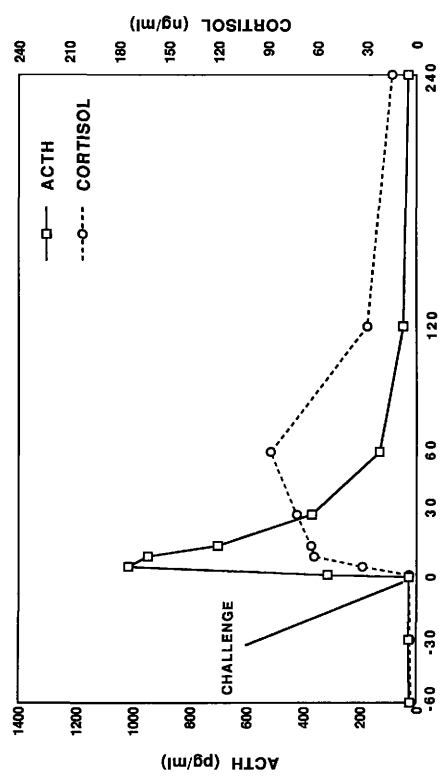

Figure 15. Mean plasma concentration of ACTH and cortisol (n = 8 cows) following stimulatory challenge with VP (1 µg/kg BW). VP acutely activated the pituitary-adrenal axis with a 9.6-fold increase ($P < .01$) over Control (32.7 ± 7.2 pg/ml) at 1 min post-challenge. Maximal plasma concentration of VP-induced ACTH secretion was observed at 5 min post-challenge.

concentration of ACTH continually declined until 60 min POST at which time plasma concentration of ACTH for cows challenged with bCRF did not differ ($P > .05$) from that observed for Control cows.

VP Treatment Group. At Time 0, the VP treatment group did not differ ($P > .05$) from Control. Although an acute difference ($P < .01$) resulting from the VP challenge (9.6-fold increase) was detected as early as 1 min POST relative to Control (Figure 15). At 5 min POST, plasma concentration of ACTH was increased 35.5-fold relative to Control. Maximal plasma concentration of VP-induced ACTH secretion was observed at 5 min POST. The decline in VP-induced ACTH secretion was continuous after 5 min POST, however, it did not decline as rapidly as it had increased. At 60 min POST, the stimulatory effects of VP were no longer evident and plasma concentration of ACTH did not ($P > .05$) differ from Control.

bCRF plus VP Treatment Group. At Time 0, the bCRF plus VP treatment group did not differ ($P > .05$) from Control. Plasma concentration of ACTH for cows challenged with a combination of bCRF plus VP reflected not only an acute (within 1 min) response but also a prolonged (greater than 120 min) elevated plasma concentration of ACTH (Figures 16 and 17). At 1 min POST, plasma concentration of ACTH was increased 22.4-fold relative to Control (32.7 ± 7.2 pg/ml). Maximal plasma concentration of ACTH (1399.8 ± 260.1 pg/ml) occurred at 10 min POST. Although the decline in plasma concentration of ACTH was continuous after 10 min POST, it remained elevated above Control for an additional 110 min.

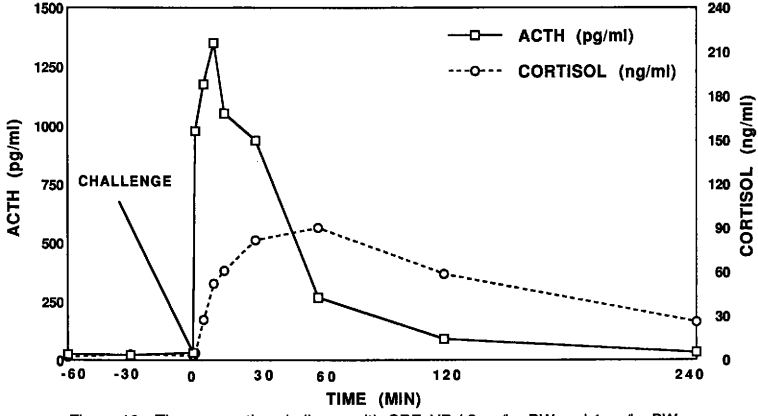

Figure 16. The provocative challenge with CRF+VP (.3 μg/kg BW and 1 μg/kg BW, respectively) (n = 8 cows) increased ($P < .001$) plasma concentration of ACTH 22.4-fold over Control (32.7 ± 7.2 pg/ml) at 1 min post-challenge. Maximal plasma concentration of ACTH occurred at 10 min post-challenge and remained above Control ($P < .01$) for an additional 110 min.

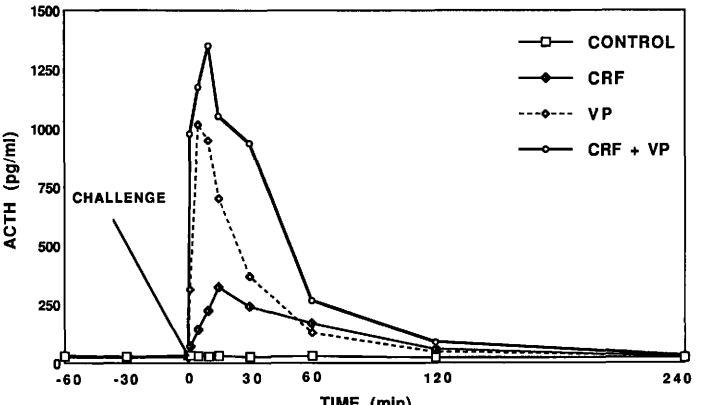

Figure 17. Mean plasma concentration of ACTH is increased by both CRF and VP. VP-induced ACTH secretion is more acute with a greater magnitude than CRF-induced ACTH secretion (1017.7 ± 219.9 pg/ml and 324.7 ± 126.2 pg/ml, respectively). The duration of response, however, is sustained (120 min post-challenge; $P < .05$) compared to the individual challenge responses alone.

The enhancement in plasma concentration of ACTH due to treatment with bCRF plus VP is consistent with the synergistic reports by Liu et al. (1983) in humans, Rivier et al. (1984) in the rat and Pradier et al. (1986) in sheep. The temporal aspects associated with plasma concentrations of ACTH observed for cows during the *in vivo* portion of this study are undoubtly supported by the aforementioned *in vitro* results. Also, these data correspond to the real-time observations reported by Cantor et al. (1993) for ovine tissue. Other *in vivo* studies evaluating VP-induced secretion of ACTH in the bovine are not available at this time for direct independent comparisons of the *in vivo* action of VP.

Plasma Concentration of Cortisol. An increase in plasma concentration of ACTH preceded the increase in plasma concentration of CS (Figures 13 through 16) irrespective of secretagogue. Areas under the hormone response curves (AUC) were calculated for plasma concentration of both ACTH and CS. Areas were utilized for correlation analysis during the PRE (PRE AUC) and POST (POST AUC) periods. Responsiveness of ACTH and CS (i.e., POST AUC) were tightly coupled ($r = .71$, $P < .0001$). Cortisol POST AUC was positively correlated with CS PRE AUC ($r = .59$, $P < .0003$) but not with PRE AUC for ACTH ($r = -.31$, $P < .08$). Plasma concentration of CS at 0 min was lowly correlated with POST AUC for CS ($r = .32$, $P < .08$) and peak CS ($r = .23$, $P < .21$).

These data provide insight regarding the dynamics of ACTH and CS responsiveness to CRF and VP. These two neurohormones, CRF and VP, may be used to assess the pituitary-adrenal capacity in cattle.

However, due to the acute, transient response of ACTH and CS to the VP challenge, it is necessary that the sample collection window be narrower. Also, these hypothalamic neurohormones may be used in provocative tests to predict and(or) rank cattle with respect to relative secretion of ACTH and CS during stress-free periods.

IMPLICATIONS

The pituitary-adrenal axis in the cow can be stimulated by the independent action of the endogenous neurohormones CRF and VP. Although VPP and VPB have been reported to be antagonists of VP in peripheral tissue, they both exert agonist activity on the bovine anterior pituitary. Our *in vitro* studies confirm that CRF is more potent than VP in terms of stimulating secretion of ACTH in the bovine corticotroph; however, direct *in vivo* comparisons of potency have not been reported at this time for cattle.

There is a consistent and profound difference in acute and protracted ACTH response to CRF and VP *in vitro* and *in vivo.* Although CRF and VP mediate secretion of ACTH via two separate second messenger systems (protein kinase A and protein kinase C, respectively) the ACTH response induced by the combination of CRF and VP has both an acute phase and an extended response. Therefore, an animal's response to an exteroceptive stimulus, (e.g., an acute response versus an extended response) is the consequence of integrated crosstalk between the protein kinase A and protein kinase C pathways.

Further *in vivo* studies need to be conducted to confirm that bCRF rather than VP is the more potent stimulator of ACTH secretion in cattle. The present results from *in vitro* experiments suggest that CRF is the more potent stimulator of ACTH secretion *in vivo ;* however, this remains to be established. It would also be relevant to

evaluate both VPP and VPB *in vivo* given the dual agonist/antagonist activities of these VP analogs.

The initial results obtained with the antiglucocorticoid, RU486, need to be expanded to gain further insight into the hormonal mechanism(s) by which this antisteriod blocks DEX-induced suppression of CRF-induced secretion of ACTH. The ongoing studies evaluating the activity of RU486 at the levels of the hypothalamus and infundibulum should provide further insight into the sites of action of this antiglucocorticoid.

Additional studies to quantitate the concentration of CRF and VP in portal blood of cattle during a physiologically stressful situation, such as during transportation, need to be undertaken. Also, there needs to be further investigation of the V1b subtype receptor and interactions between CRF and VP involved in the regulation of VP and CRF receptors. Finally, evaluation of genetic and gender diffences in the HPA axis of cattle needs to be addressed.

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APPENDIX A

Pars Distalis Weights

Species	Sex Class	Slaughter Weight (lbs)	Pars Distalis (g)
bovine	steer	1580	1.5003
bovine	steer	1524.5	1.4479
bovine	steer	1500	1.4248
bovine	steer	1830.5	1.7392
bovine	steer	1550.5	1.4730
bovine	steer	1431	1.3594
bovine	steer	1134.5	1.0494
bovine	steer	1281	1.1544
bovine	steer	1573.5	1.6149
bovine	steer	1163.5	1.4403
bovine	steer	1175.5	1.1845
bovine	steer	1371	1.5351
bovine	steer	1088	1.3179
bovine	steer	1221	1.7578
bovine	steer	1359	1.3121
bovine	steer	1119.5	1.2902
bovine	steer	1123.5	1.6933
bovine	steer	1424	1.7670
bovine	steer	1133	1.4389
bovine	steer	1050	1.1380
bovine	steer	1168	1.3961
bovine	steer	1221	1.1960
bovine	steer	975	1.0852
bovine	steer	1175	1.2297
bovine	steer	855	0.6521
bovine	steer	855	0.6317
bovine	steer	787	0.8693
bovine	steer	914	1.0036
bovine	steer	918	0.8872
bovine	steer	811	0.8941
bovine	cow	1100	1.2080
bovine	cow	1060	0.9740
porcine	boar	167	0.1879
porcine	boar	210	0.1720
porcine	boar	203	0.1780

APPENDIX B

PITUITARY CELL CULTURE PROCEDURES

A. *Cell Culture Methodology*

1. Animals are humanely stunned and decapitated. After exsanquitation, the head is removed at the occipitoatlantal joint and transferred to a processing room where a band saw is used to expose the brain.

2. The brain is removed to expose the *neurocranium* to expose the pituitary gland. The pituitary gland is removed with a sterile scalpel and hemostats, placed in a sterile plastic bag, and transferred immediately to the tissue culture room.

3. The pituiary gland is spit mid-sagitally and separated under sterile conditions in a laminar flow hood into the pars distalis of the adenohypophysis and neurohypophysis (with pars intermedia).

4. The pars distalis is weighed and then sliced into 1 mm thick sections with a Stadie-Riggs tissue slicer. The slices are placed into a 1X solution of Dulbecco's Modified Eagle Medium (DMEM) tissue culture medium until further processing.

5. The slices are minced into 1mm cubic portions with the blade of the tissue slicer and then washed two times in 1X DMEM.

6. Pieces are placed in sterile 125-ml Erelenmyer flasks (approximately 2 pars distalis/flask) containing 75 ml DMEM with .3% collagenase (350 units/mg tissue). The flasks are immersed into a 37°C water bath and agitated with a magnetic stir bar for 1 hour.

7. After dispersal, the solution is filtered through a single layer of sterile gauze. The filtrate is then dispensed into

50-ml centrifuge tubes and centifuged at 200 xg for 15 min to precipitate the cells.

8. The liquid is aspirated and the pellet of cells is resuspended in 50-ml of1X DMEM and then centrifuged. This washing procedure is repeated three times.

9. After the third wash, the pellet of cells is resuspended in 20 ml of 1X DMEM. 100 µl of the cell suspension is pipetted into a sterile polypropylene tube containing 100 µl of trypan blue stain. The stained cell solution is then pipetted into a hemacytometer chamber.

10. Concentration of viable cells is determined by counting the number of non-stained cells on the grid and inserting this number into the following equation:

$$\frac{(\text{\# of cells/grid}) \times 2 \times 10{,}000 \times (\text{mls of cell suspension})}{(\text{desired concentration of cells/ml})}$$

11. The cells are the diluted to a concentration of 400,000 viable cells/ml (200,000 each for heifers and boars) in 1X DMEM containing 1.0 M L-glutamine and 10% fetal calf serum.

12. The cell suspension is then dispensed in 1-ml fractions into 35 mm x 10 mm polystyrene 6-well tissue culture dishes and placed into a 37°C incubator (95% humidified air and 5% carbon dioxide.

13. The day of culture establishment is considered to be Day 0 and spent medium is replaced with 1 ml fraction of supplemented 1X DMEM on Days 2, 3 and 4 (medium is added gently to the inner side wall to prevent dislodging of the cells).

14. On Day 5, the cells are washed twice with serum-free medium and appropriate treatments added. The volume serum-free medium added is 1 ml minus 50 ml multiplied by the number of treatments added to a particular well. The final total volume in each well is 1 ml.

15. After the appropiate incubation period, 3-h for steers and 4-h for cows and boars, the medium is collected in plastic pipette tips, dispended into polypropylene tubes and stored at -20°C until medium concentration of ACTH is determined by RIA.

B. *Cell Culture Medium, Collagenase and Viocase*

1. Cell Culture Medium
   a. To prepare 1 liter of 5X DMEM, add the contents of 5 one-liter packages of DMEM to approximately 800 mls of doubled distilled water.
   b. Then add 50 ml each of 50X MEM amino acids with L-glutamine and 100X MEM non-essential amino acids 10 mM.
   c. To this solution add 5 ml each of Funfizone (Amphotericin-B 250 mg/ml) and penicillin-streptomycin (10,000 U/ml penicillin, 10,000 mg/ml streptomycin).
   d. Dissolve 18.5 g each of sodium bicarbonate and HEPES 25 mM and then pH solution to 6.8. After appropriate pH is obtained, adjust final volume of solution to 1 liter.
   c. Solution is then sterilized by passing through a filtration system equipped with a .2 micron filter and store at 4°C. Filtering solution will raise the final pH to approximately 7.0).
   d. To prepare 500 ml of 1X DMEM, add 100 ml of 5X DMEM to 400 ml sterile double distilled water and store at 4°C until day of culture at which time the medium should be heated in a water bath to 37°C.
   e. The supplemented cell culture medium is prepared by adding 5 ml of L-glutamine and 50 ml of fetal calf serum to 445 ml of the 1X DMEM. This solution may also be stored at 4oC, but should be heated to 37oC before using in the cell culture.

2. Preparation of Collagenase
   a. To prepare 150 ml of collagenase solution, dissolve 450 mg of collagenase in 150 ml of 1X DMEM and stir with a magnetic stir bar until completely dissolved.
   b. Filter solution by passing through a .45 micron filter system attached to a vaccum.

c. The solution can then be sterilized by passing through a .2 micron filter attached to a vaccum. This will provide enough collagenase solution to dissociate approximately 4 grams of tissue.

3. Preparation of Viocase solution
   a. To prepare 100 ml of the viocase solution, add 20 ml of double distilled water to a bottle of pancreatin 10X (25g/liter) and vortex.
   b. Add 10 ml of the pancreatin solution to 90 ml of 1X DMEM.
   c. To sterilize, pass solution through a .2 micron filter system attached to a vaccum.

APPENDIX C

ACTH ASSAY PROCEDURES

A. *Assay Methodology*

1. Day 1 (During the entire procedure keep all tubes on ice or at 4°C).
   a. Add 200 µl Buffer B to N tube in triplicate.
   b. Add 100 µl Buffer B to O tube in triplicate.
   c. Add 100 µl of standard or unknown to appropriately labelled polypropylene tubes.
   d. Add 100 µl of first antibody to all tubes except the N and T tubes and vortex.
   e. Incubate for 24 hours at 4°C.

2. Day 2
   a. Add 100 µl of ^{125}I-ACTH to all tubes, T tubes in triplicate and vortex.
   b. Incubate for 72 hours at 4°C.

3. Day 5
   a. Add 100 µl of second antibody to all tubes except the T tubes.
   b. Incubate for 4 hours at 4°C.
   c. Add 1.6 ml of cold separation buffer to each tube except T tubes and centrifuge immediately for 20 min at 6000 x g.
   d. Decant supernatant and blot tubes on absorbent paper. Make sure that tubes are maintained at 4°C or in ice water bath during this portion of the procedure.
   e. Count precipitate for 2 min on gamma counter.

B. *Assay Solutions*

1. Buffer A
   a. For 1 liter of Buffer A, dissolve 16.84 g of Na_2HPO_4, 4.74 g of Na_2EDTA and .2 g NaN_3 in approximately 800 ml of deionizied distilles water.
   b. Adjust volume to 1 liter and pH with 1 M NaOH to 7.4.

2. Buffer B
   a. For 400 ml of Buffer B, combine 400 µl of Triton x-100 and 10 ml of Trasylol with 400 ml of Buffer A. This will provide enough Buffer B for approximately 800 tubes.

3. Buffer C
   a. Buffer C is used for diluting the first antibody, therefore the amount needed depends on the number of assay tubes. Buffer C is made by adding 2% normal rabbit plasma to Buffer B.

4. Buffer D
   a. Buffer D is used for reconstituting the first antibody and is made by adding 1% normal rabbit plasma to Buffer A.

5. Separation buffer
   a. To make 2 liters of Separation buffer, add 50 g of 2.5% bovine serum albumin to 2000 ml of Buffer A.

APPENDIX D

CORTISOL ASSAY PROCEDURES

A. *Assay Methodology*

1. Sample preparation
   a. Pipet 50 µl of sample into appropriately labelles glass tubes.
   b. Add 450 µl of PBSG to each tube and vortex.
   c. Cork tubes and place in 70°C water bath for 1 hour.
   d. Allow samples to cool down to room temperature and then proceed with setting up the assay.

2. Setting up assay
   a. Pipet 500 µl of standard into appropriately labelled glass tubes in triplicate.
   b. Add 100 µl of antibody to all tubes except the T and N tubes.
   c. Add 100 µl of trace to all tubes and vortex.
   d. Allow to incubate at 4°C for 12 to 18 hours. An alternative method is to allow to incubate at room temperature for 1 hour then transfer to 4°C for 3 hours.

3. Day 2
   a. Add 5 ml of Ecolume cocktail to mini-vials and label caps for vials.
   b. This portion of the procedure should be carried out in the cold room at 4°C. Make sure charcoal is thoroughly mixed and add 200 µl of the charcoal to all tubes except the T tubes. Shake racks of tubes and allow to set for 15 min.
   c. Centrifuge for 10 min at 2282 x g. Put tubes on ice or place in cold room immediately after centrifuging.
   d. Decant supernatant into the mini-vials and count each vial for 1 min in beta counter.

B. *Assay Solutions*

1. PBSG
   a. To make 1 liter of PBSG, dissolve the following chemicals in 900 ml of double distilled water: 8.17 g of NaCl, .856 g of NaH_2PO_4, .54 g of Na_2HPO_4, 3.72 g EDTA and .1 g thimersal.
   b. Adjust pH to 7.4.
   c. Add 1 g of gelatin to a 1 liter bottle and then add the EDTA-PBS from part b to the bottle.
   d. Dissolve gelatin mixture in a magnetic stir/hot plate while heating at a low to moderate temperature for approximately 2 hours.
   e. After cooling to room temperature the PBSG is ready to use. Store remaining PBSG at 4°C.

2. Charcoal solution
   a. Combine .0625 g of Dextran Pharmacia T-70 with .625 g of Charcoal Norit SPXX and 100 ml of PBSG in a 200 ml beaker and mix with a magnetic stir bar. Store at 4°C for up to two weeks.

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AWARDED RESEARCH GRANTS AND FUNDING

1993: In vitro regulation of CRF and ACTH by an anti-glucocorticoid (RU486) in Cattle. Texas A&M University Faculty Mini-Grant Program. P.G. Harms (PI), J.A. Carroll and S.T. Willard. $1,200.

TEACHING EXPERIENCE

Animal Science 434: Artificial Breeding of Livestock