REGULATION AND SITE OF ACTION OF EXOGENOUS AND ENDOGENOUS OPIOIDS ON GROWTH HORMONE AND PROLACTIN SECRETION IN HOLSTEIN CALVES

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(ABSTRACT)

Four studies were conducted to investigate the effect and site of action of exogenous and endogenous opioids on pituitary growth hormone (GH) and prolactin (PRL) secretion in Holstein calves. In the first study, the effect of the opioid agonist DAMME (D-Ala^2,N-Me-Phe^4,Met(O)^5-ol enkephalin) on plasma GH and PRL secretion was measured in Holstein calves in fall season. Plasma concentrations of both GH and PRL increased in response to DAMME injection. Pre-treatment with either the lipid soluble opioid antagonist naloxone (NAL), which readily penetrates the blood brain barrier (BBB), or the peripherally acting antagonist methyl levallorphan mesiltate (MLM), blocked the PRL response to DAMME. Naloxone, but not MLM, negated the GH response to DAMME. In spring, the experiment was repeated with similar results.

In the second experiment, the opioid antagonists NAL and MLM were administered alone to determine whether endogenous opioids mediate basal GH and PRL secretion, and the site of action of any of opioid-mediation of basal GH and PRL. In fall, NAL administration increased both plasma GH and PRL secretion. Methyl levallorphan mesilate did not affect PRL, but increased plasma GH concentrations. In spring, a second trial using 5 different doses of each antagonist was conducted. Naloxone did not affect GH levels at any dose in spring, but decreased plasma PRL at the same dose which increased plasma PRL in fall. Plasma PRL was again unaffected by MLM, but plasma GH was increased by 3 seperate doses of MLM.

The third experiment was designed to determine if the increases in plasma PRL seen after DAMME administration were mediated via dopaminergic mechanisms. Plasma PRL in calves again increased in response to DAMME injection alone. In calves pre-treated with the long-acting

dopamine agonist 2-Br-&alp. ergocryptine (CB154), plasma PRL was unresponsive to DAMME injection. The pituitaries of calves treated with CB154 in this experiment were able to respond to thyrotropin-releasing hormone (TRH) injection with increased PRL secretion.

In the final experiment, the role of growth hormone-releasing hormone (GRH) in facilitating GH release after DAMME injection was investigated, and whether endogenous opioidergic mechanisms play a role in mediating the effects of exogenous GRH on GH secretion. Plasma GH concentrations increased in calves recieving either DAMME or D-ala^2, fragment 1- 29 amide, a synthetic GRH. The immediate increase in plasma GH concentrations after GRH injection in calves pre-treated with DAMME was approximately 5 fold less than that in calves not pre- treated with DAMME. Calves recieving DAMME and GRH in combination also produced a GH response curve with greater area under it than either compound alone, indicating possible synergism between the synthetic GRH and a DAMME-sustained release of endogenous GRH. Naloxone administration concommitantly with synthetic GRH did not alter the abiltiy of the synthetic GRH to increase GH secretion overall, compared to synthetic GRH alone.

In conclusion, these studies are the first to indicate that dairy breeds are able to respond to exogenous opioids with increased secretion of pituitary GH and PRL, as is known to occur in other mammalian species. Also, they indicate that opioid receptors mediating pituitary GH secretion to exogenous opioids in Holstein calves are located somewhere within the BBB, and those mediating PRL secretion are at a site outside the BBB. It appears from these studies that endogenous opioids within the BBB play a role in regulating basal PRL secretion, and that this regulation differs in fall and spring. A role for endogenous opioids in the regulation of GH secretion in Holstein calves may exist also, at least in fall, but the results are less conclusive. The peripheral opioid antagonist MLM alone may facilitate increased GH secretion in Holstein calves via an agonistic, not antagonistic, mechanism. These studies indicate that the increased PRL secretion seen following opioid administration in Holstein calves is mediated through a dopaminergic mechanism. It appears that endogenous opioids do not mediate the pituitary response to exogenous GRH in Holstein calves, and that GH increases after DAMME injection are facilitated, at least in part, by increased release of endogenous GRH.

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INTRODUCTION

The mammary glands are modified skin glands that are characteristic of all mammals. They are unique among glands, in that their function is transferring food from parent to offspring in a utilizable form. In this respect, the dairy cow is unique as well. Bred and selected for many generations to produce quantities of milk far in excess of that needed by the calf, this species has the largest mammary development as a % of body weight.

The ability of the mammary gland to produce milk undergrids the entire dairy industry. For a dairy cow to undergo a successful lactation , several stages of mammary growth must occur from the time of birth until first calving and lactation. From birth to puberty, growth of the mammary gland is essentially isometric (Anderson, 1985), but at puberty (6-8 months in dairy cattle) lengthening and branching of the mammary duct system occurs, with differentiation of the alveoli (Delouis et al., 1980). Upon successful breeding (at approximatly 15 months), the major portion of mammary growth begins, which seems logical, as mammary gland growth should correspond to fetal growth.

The primary physiological mediators that stimulate all of these stages of mammary growth, and initiate and maintain lactation during the peri-partum and post-partum period are hormones (see Tucker, 1981, for review). Endocrinology therefore, especially that pertaining to mammary growth and lactation, is an extremely important area of research in the dairy cow. In this regard, the pituitary hormones prolactin (PRL) and growth hormone (GH) are critical for a successful lactation in this species.

Prolactin is essential for development of the mammary gland in most mammals, including dairy cattle (Elias, 1980). Structural differentiation of alveolar epithelium and ontogeny of key biochemical steps in the milk secreting cells that facilitate maximal milk production in the post-partum period both require PRL (Akers et al., 1981a and 1981b). Alpha-lactalbumin, a major milk protein which also is believed to be the rate limiting component of lactose synthetase (Kuhn et al., 1980), requires PRL for its synthesis (Akers, 1985). This implies that PRL is a major promoter

of lactose synthesis, the principle carbohydrate in milk and the milk component which is most rate-limiting in milk secretion, due to its osmolality (Larson, 1985).

Growth hormone is considered essential for maximal mammary development in cattle as well (Tucker, 1981). Injections of exogenous GH have been shown to increase mammary parenchymal tissue in post- pubertal heifers (Sejrsen et al., 1986). Circulating concentrations of GH are inversely related to energy balance (Sejrsen, 1978), and overfeeding at or near puberty and post- puberty is thought to decrease plasma GH concentrations. This has been shown to result in decreased mammary parenchymal tissue and lowered milk yields in all subsequent lactations (Little and Kay, 1979; Harrison et al., 1983).

Lactation also is affected by GH concentrations. Growth hormone was first demonstrated to be important in milk secretion over 50 years ago when it was shown that injection of crude pituitary gland extracts increased milk production in cows (Asimov and Krouze, 1937). At the time, researchers were unaware of what fraction of the extracts actually caused the increased production. However, during the 1940's, researchers in the United Kingdom further purified these extracts and found that the galactopoietic effect was due to somatotropin, or GH (Young, 1947). Long term studies with large groups of animals were impossible at the time due to lack of large amounts of the hormone.

In 1981, this problem was overcome when the first experiment utilizing recombinant methionyl bovine somatotropin indicated it was as effective as pituitary derived GH in enhancing milk production (Bauman et al., 1982). Since that time, a number of long and short term experiments utilizing recombinant bovine growth hormone have verified this (see Peel and Bauman, 1987, for review).

Growth hormone and PRL secretion from the anterior pituitary have been known for some time to be under hypothalamic control. Krulich et al. (1968) first demonstrated the presence of a substance in sheep hypothalamic extracts which could inhibit basal release of pituitary GH from rats in vitro. After extensive purification and characterization of these extracts, a 14 amino acid peptide was isolated and determined to be the putative physiological GH release-inhibiting factor, subsequently named somatotropin release-inhibiting factor, or SRIF (Brazeau et al., 1973; Burgus

et al., 1973). Some 9 years later, Guillemin et al. (1982) reported the discovery of the putative hypothalamic GH-releasing factor, isolated from a pancreatic tumor in a human cancer patient who had developed acromegaly. This peptide was subsequently named somatocrinin, or growth hormone-releasing factor (GRF).

While regulation of GH release from the anterior pituitary appears to be mediated primarily by 2 hypothalamic peptides (ie, somatostatin and somatocrinin), pituitary release of PRL in mammals is controlled predominately via chronic inhibition by the catecholamine dopamine (DA). Other factors are known to inhibit PRL release (Schally et al., 1976) and prolactin-releasing factors also play a role in its regulation (del Pozo and Brownell, 1979), but DA, secreted into hypophysial portal blood from tuberoinfindibular neurons (TIDA) originating in the arcuate nucleus and terminating in the median emminence, appears to be the primary antagonist (Hokfelt and Fuxe, 1972; Moore, 1987).

A new chapter in regard to neuroendocrine regulation of GH and PRL was introduced in the late 1970's, shortly after the existance of the once putative endogenous opioid peptides was confirmed (Hughes, 1975; Hughes et al., 1975). Since that time, there have been a multitude of studies on the numerous physiological functions that appear to involve these compounds. These include endocrine and exocrine secretion from the pancreas (Watkins et al., 1980), gastrointestinal tract regulation (Orwoll and Kendall, 1980), adrenal medulla regulation of catecholamine secretion (Lewis et. al, 1980), and an as yet uncertain role in gonadal function (Tsong et al., 1982). Opioids may also play some role in the placenta, as they are found here as well (Liotta et al., 1982). The opioids also are of great interest in human behavioral studies, particularly in the areas of food consumption, learning and memory, and mental illness (see Olson et al., 1985, for review).

However, one of the most interesting findings of the endogenous and exogenous opioids to be realized was their profound effect on secretion of several pituitary hormones, including GH and PRL. The plant-derived opiate alkaloids (particularly morphine) were already known to increase the secretion of pituitary GH and PRL (Meites, 1966; Kokka et al., 1973). In the first few years after their discovery, several laboratories reported that administration of natural or synthetic versions of the endogenous opioids also could increase GH and PRL secretion dramatically. Studies with spe-

cific opioid antagonists confirmed that endogenous opioids were involved in regulation of basal secretion of GH and PRL as well (see Morley, 1981, and Pfieffer and Herz, 1984, for review).

One of the first clues to the existance of endogenous opioids was the observation that the electrically stimulated contractions of the isolated guinea pig ileum and mouse vas deferens in vitro could be inhibited by the known opiate analgesics (Henderson et al., 1972). Interestingly, the order of potencies in these smooth muscle models correlated well with the analgesic potencies of the drugs used. Observations like this led to the belief that the site of action of opiates included a specific receptor, in the true pharmacological sense. This proved to be the case, and in 1973, three seperate laboratories reported stereospecific opioid binding to receptors in homogenates of rat brain and guinea pig intestine (Terenius, 1973; Pert and Snyder, 1973; Simon et al., 1973).

It had been suspected for many years prior to the discovery of specific opiate receptors that endogenous opioids might exist in mammals, primarily due to the behavorial consequences of the opiate alkaloids. The dramatic effects of morphine on pain tolerance and its ability to produce physical dependance suggested some underlying common mechanism of action, such as a specific receptor. Now, with the demonstration of such a receptor, it seemed unlikely to many workers in opiate research that selective endogenous receptors for plant alkaloids would have been developed by nature in mammals. The idea that opiate receptors might be for "endogenous humoral substances" was first proposed by Collier (1972).

The first attempts to isolate these putative endogenous ligands for the opiate receptors had been unsuccessful, due to the mistaken assumption of chemical similarity to morphine. Then, Hughes (1975) reported the isolation of a compound from pig brains which was peptide in nature and produced morphine- like responses in the in vitro model systems. He named this compound enkephalin (meaning "in the head"), and later that year demonstrated that this compound was actually a mixture of two pentapeptides (Hughes et al., 1975). They subsequently named these compounds Met- and Leu- enkephalin, as they differed only in their N-terminal amino acid residues, methionine and leucine, respectively.

The following year, Li and Chung (1976) isolated and identified a unique peptide from the pituitaries of camels which was extremely potent in opioid pharmacological assays. The same year,

Bradbury et al. (1976) found that the C-terminal fragment of beta lipotrophin (β-LPH), a 91 amino acid pituitary peptide with lipolytic activity (Li, 1964), also had potent opioid activity. This C-fragment of β-LPH (β-LPH 61-91), in agreement with Li and Chung (1976), was named beta-endorphin (β-END). Ling et al. (1976) isolated two other fragments of β-LPH with opioid activity that same year (β-LPH 61-76 and 61-77) which were subsequently named α- and γ-endorphin, respectively.

In 1982, thanks to molecular biological techniques, a complete picture of the structural relationships between the known endogenous opioid peptides emerged. It was learned that three seperate mRNAs (and three seperate genes) code for three opioid precursors. Opioid peptides are currently classified into three groups based on these biosynthetic pathways (Imura et al., 1983; Akil et al., 1988). The group 1 opioids consist of those peptides which arise from enzymatic processing of the 267 amino acid protein proopiomelanocortin or "POMC" molecule. The group 2 molecules, which include Met- and Leu-enkephalin, are derived from a common precursor known as pro-enkephalin. The amino acid sequence for Met-enkephalin is the same as the first 5 amino acids in β-END (β-LPH 61-65), and it was once thought that this peptide was a cleavage product from β-END. However, this is now known to be false (Hughes, 1979). The group 3 molecules, which includes the dynorphins and recently discovered peptides rimorphin and leumorphin, all have Leu-enkephalin at their N-terminus, and all are derived from the pro-dynorphin molecule.

Before discussing the three principal opioid families further, some mention should be made of the nomenclature used in the literature when addressing these compounds , as it can be confusing at times. The term "endorphin", meaning an endogenous morphine-like peptide, was originally proposed by Dr. Eric Simon of the New York University Medical Center in 1975, at a time when it was thought that there were few naturally occurring peptides with opioid activity. However, it later became clear that enkephalins were not produced by cleavage of βEND, and the term "endorphin" is used increasingly to describe only those opioids derived from the POMC molecule.

Natural or synthetic enkephalins and dynorphins, or members of these families are generally referred to simply as enkephalins or dynorphins, which distinguishes them from endorphins. When a general descriptor indicating no particular group is required, the phrase "endogenous opioid

peptides" is often used. Similarly, the term "opioid" should be used when referring to any of the endogenous or synthetic opioid peptides, while "opiate" is appropriate when referring to any of the alkaloid deriviatives of opium (Cox, 1982). A consistent nomenclature for amino acid derivatives was recommended by IUPAC, however this occurred before the discovery of the endogenous opioids.

Group 1 opioids are all derived from the β-LPH molecule, which itself is the termininal 91 amino acids of the POMC molecule. As discussed, amino acid residues 61-91 of β-LPH represent β-END. Depending on the tissue it is produced in, β-END cleaved from the β-LPH molecule may itself may be further cleaved to δ-END (β-LPH 61-87), as well as the previously mentioned α- and γ-END residues, or an acetylated version of any or all 3 (Imura et al. 1983). The acetylated endorphins are generally devoid of any opioid activity, whereas the α-, γ-, and δ-endorphins have opioid activity, but are not nearly as potent as β-END in most instances.

The POMC molecule is found in the pituitary in scattered cells of the anterior lobe, and in practically every cell in the intermediate lobe (Akil et al., 1988). Beta lipotrophin, β-END, and adrenocorticotrophic hormone (ACTH), all cleavage products of the POMC molecule, have been found co-localized in the same cells (Pelletier et al., 1977) and in the same storage granules (Weber et al., 1978). In addition to containing β-LPH and ACTH, the POMC molecule also contains three copies (α, β, and γ) of melanocyte stimulating hormone (MSH). In the central nervous system (CNS), POMC is synthesized primarily in the arcuate nucleus of the hypothalamus (Watson et al., 1977), and smaller amounts are found in the nucleus tractus solitarius (Schwartzberg and Nakane, 1983).

Shortly after the discovery of β-END, several laboratories developed assays to detect its presence in blood (Guillemin et al., 1977; Hollt et al., 1978). Concentrations of blood-borne β-END have now been measured in several species, and generally they parallel changes in blood ACTH (Vuolteenaho et al., 1982). The source of this blood-borne β-END is thought to be the anterior pituitary, as selective removal of this structure markedly reduces basal concentrations of plasma β-END (Przewlocki et al., 1982), although other peripheral sites are possible sources (see Autelitano et al., 1986, for review).

Because β-END is secreted from the pituitary concommitantly with ACTH and is much less sensitive to aminopeptidases in plasma than enkephalins (Meek et al., 1977), it has been speculated that β-END in plasma may be a hormone (McKnight and Kosterlitz, 1980). However, it has been contended (Cox and Baizman, 1982) that plasma concentrations of β-END are much lower (picomolar range) than the levels required for in vitro binding (nanomolar range). Finally, a temporal relationship between plasma β-END and some physiological function has not yet been demonstrated with absolute certainty (Malven, 1987).

The group 2 peptides are the enkephalin-like peptides, and include the aforementioned Met-enkephalin and Leu-enkephalin, as well as Met-enkephalin with a C-terminal extension of arginine and phenylalanine, or arginine, glycine and leucine (Met- enkephalin-Arg-Phe and Met-enkephalin-Arg-Gly-Leu, respectively; Imura et al., 1983). Three other compounds thought to belong to this group are Peptides E, F, and B, all of which have been isolated from the adrenal medulla, and are believed to be intermediates in the processing of proenkephalin (Kilpatrick et al., 1981).

The enkephalins are less restricted in their distribution in the CNS than endorphins, are found throughout the brain and spinal cord, and are located peripherally in the autonomic nervous system, the adrenal glands, and the intestines (Akil et al., 1988). Although the ratio of contents of Met- and Leu- enkephalin varies from region to region, Met-enkephalin is always present in greater amounts (Hughes et al., 1977). Due the previously mentioned high sensitivity of enkephalins to degradation by aminopeptidases, and because immunohistochemical fluorescence of enkephalins in the CNS is mainly associated with nerve fibers and terminals, a possible neurotransmitter role for enkephalins has been suggested (Hokfelt et al., 1977; Simantov et al., 1977).

The group 3 peptides comprise dynorphin, α- and β neo- endorphin, and the recently discovered peptides rimorphin and leumorphin. As discussed previously, all arise from a common precursor, prodynorphin. The initial discovery of the dynorphins came when an opioid-like peptide was found in porcine and bovine pituitary glands that had a lower molecular weight and was more basic than β-END (Cox et al. 1975). This compound exhibited very potent opioid activity in the guinea pig myenteric plexus and mouse vas deferens bioassays, but was not derived from POMC.

The first 13 amino acids of this material were subsequently isolated and characterized from porcine pituitary extracts by Goldstein et al. (1979). Because of its previously described high potency in bioassays, this opioid was given the name dynorphin (from Greek dynamis = power).

Subsequently, Goldstein et al. (1981) determined that the full sequence of this peptide consisted of 17 amino acids, and that its high potency in the guinea pig ileum bioassay was due to the first 13 amino acids, which they had reported earlier. The following year, a 4000 dalton peptide was isolated from porcine pituitary glands which contained the structure of dynorphin previously characterized by Goldstein et al. (1981), followed by another peptide with Leu-enkephalin at its N-terminus (Fischli et al. 1982). The two Leu-enkephalin containing peptides were seperated by a lysine-arginine residue, which suggested that the 4000 dalton peptide was cleaved into two seperate Leu-enkephalin peptides. This was indeed the case, and the 17 amino acid peptide, characterized previously by Goldstein et al. (1981) was termed "dynorphin A", and the 13 amino acid peptide following it was called "dynorphin B". An octapeptide that had been previously extracted from porcine hypothalami (Minamino et al., 1980) is thought to be a product of further cleavage of dynorphin A, resulting in the existance of two dynorphin A's: 1-17 and 1-8.

Another Leu-enkephalin containing peptide, α-neo-endorphin, was first isolated from porcine hypothalami (Kangawa et al., 1979). Kangawa et al. (1981) later reported the complete amino acid sequence of this peptide, which was found to be a decapeptide. This compound was similar to dynorphin in that the Leu-enkephalin sequence is followed by two basic amino acids, but the remaining carboxylic extension is different from both dynorphin A and B. A fragment of α-neo-endorphin lacking the carboxyl terminal lysine residue was also found by Kangawa and coworkers (1981) and named β-neo-endorphin.

Pro-dynorphin distribution in the mammalian body is intermediate in complexity compared to POMC and pro-enkephalin. The highest concentrations are found in the posterior pituitary and the hypothalamus, and of particular note is the dynorphin system found in the magnocellular nuclei of the hypothalamus by Watson et al. in 1981. Pro-dynorphin has been shown to co-exist in magnocellular neurons with the vasopressin pre-cursor, propressophysin (Watson et al., 1982), which sends axons to the posterior lobe of the pituitary. In addition to their putative role in

vasopressin release, dynorphins also are thought to be involved in regulation of follicle stimulating hormone and luteinizing hormone release, as they are co-localized with the gonadotrophs in the pituitary as well (Khachaturian et al., 1986).

Matching the structural complexities of the endogenous opioids is the multiplicity of opioid receptors. The first evidence of the existance of multiple receptors came from the experiments of Martin and co-workers (1976) in chronic spinal dogs. Striking differences in pharmacological responses to different narcotic analgesics and their inability to substitute for one another in suppressing withdrawal symptoms in addicted dogs led them to postulate the existance of at least three types of receptors, which were named for the prototype drug used: μ (mu) for morphine, κ (kappa) for ketocyclazocine, and σ (sigma) for compound SKF 10047 (N-allylnorphenazocine).

Following the discovery of the enkephalins, Lord et al. (1977) provided evidence for another receptor type. They determined that electrical stimulation of the guinea pig ileum was much more sensitive to inhibition by morphine and related alkaloids than by the enkephalins, whereas the opposite was observed for the mouse vas deferens. It was suggested that these two tissues contained different types of opioid receptors, and these new receptors in the vas deferens were designated δ (delta) receptors. In vitro binding competition studies in guinea pig brain homogenates supported the existance of this new receptor. An ε (epsilon) receptor has also been postulated, for which the primary ligand is β-END, as well as receptor subtypes for the established μ and κ receptors (see Wood, 1982, and Simon, 1984, for review).

In light of the previously discussed importance of GH and PRL on milk secretion in dairy cattle, the primary purpose of this investigation was to elucidate the role of both endogenous and exogenous opioids in regulation of pituitary GH and PRL in Holstein calves. There is a paucity of information concerning opioid effects on GH and PRL release in ruminants, and no published information on this subject in dairy animals that we are aware of.

This dissertation will be presented in four chapters, each of which has been or will be submitted as a research paper or technical note. The first chapter examines the regulation of GH and PRL secretion in Holstein calves by exogenous opioids in both fall and spring. The likely site of action (peripheral vs central) of these compounds was postulated by using opioid antagonists which

are either able or unable to cross the blood brain barrier (BBB), in conjunction with an opioid agonist. Chapter two examines the physiological regulation of basal plasma GH and PRL in Holstein calves by endogenous opioids in fall and spring, and the probable site of action of these effects as well. In Chapter three, the mechanism of action of an opioid agonist to increase pituitary PRL secretion was studied to determine if it was dopaminergically mediated. Finally, Chapter four examines whether increases in plasma GH seen in Holstein calves after injection of an opioid agonist can be accounted for by increased hypothalamic GRF secretion, and if the increases in plasma GH in Holstein calves seen after exogenous GRF administration are mediated by pituitary opioids or opioid receptors.

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CHAPTER 1

THE EFFECT AND SITE OF ACTION OF OPIOID AGONISTS ON PLASMA GROWTH HORMONE AND PROLACTIN SECRETION IN HOLSTEIN CALVES

INTRODUCTION

Since the isolation and identification of the endogenous opioid peptides by Hughes et al. (1975) and Li and Chung (1976), peripheral administration of natural or synthetic analogues of endgenous opioids has been demonstrated to increase circulating plasma levels of growth homone (GH) and prolactin (PRL) in several mammals, including rats (Bruni et al., 1977; Dupont et al., 1977; Rivier et al, 1977; Shaar and Clemens, 1980) humans (Catlin et al., 1980; Delitala et al., 1984), and ruminants (Hart and Cowie, 1978; Bolton et al., 1983; Armstrong and Spears, 1988).

Several studies have indicated that opioids increase PRL secretion by affecting hypothalamic dopamine (DA) turnover in both the hypothalamus and the median emminence (ME), and decreasing DA released into hypophysial portal blood (Gudelsky and Porter, 1979; Haskins et al., 1981). Increases in plasma GH seen after exogenous opioid or opiate injection are thought to be mediated primarily by increased secretion of hypothalamic growth hormone-releasing hormone (GRH; Miki et al., 1984; Wehrenburg et al., 1985). The observation that incubation of dispersed pituitary cells with opioids or opiates generally does not affect hormone secretion also suggests a hypothalamic or ME site of action (Rivier et al., 1977; Shaar et al., 1977; Grandison et al., 1980).

Because opioids are water soluble peptides, and because some of the studies mentioned above describe centrally-mediated changes in plasma hormones, particularly GH, after peripheral opioid administration, a question that needs to be considered is how these compounds penetrate the blood brain barrier (BBB), if indeed they actually do. The capillary endothelium of most blood vessels is a monocellular layer specialized for rapid exchange of solutes between blood and interstitial fluid (Meisenberg and Simmons, 1983). In most tissues, this exchange is attained, in addition to active transport processes, by passive diffusion through "pores" in the endothelial cell layer. Tight junctions between cells are lacking, allowing for diffusion of water-soluble solutes between the blood and interstitial compartments.

The capillaries in more than 99% of the brain parenchyma, in contrast, possess tight junctions between adjacent endothelial cells (Pardridge et al., 1981). The cells themselves appear to lack pores for the diffusion of water soluble molecules, and pinocytotic vesicles are largely absent. The brain capillary endothelium behaves like a continious lipid bilayer towards small molecules, and circulating molecules can gain access to the brain interstitium by one of two mechanisms: lipid mediation through the endothelium, or carrier mediation via specific, enzyme-like carriers located within the endothelial luminal membranes (Pardridge, 1983).

The literature concerning peptide penetration of the BBB is conflicting. Many of the earlier studies using radiolabelled tracers assumed that radioactivity accumulating in the brain after peripheral injection of these compounds was peptide. Later, chromatographic assay of tracer was required to distinguish peptide from radioactive breakdown products. The elegant technique of Oldendorf (1971) used to determine the brain uptake index (BUI) considered these difficulties, yet studies using the BUI index showed little or no passage of peptides, including enkephalins, across the BBB (Cornford et al., 1978). It was eventually realized, however, that this technique was not sensitive enough to rule out the possibility that very small amounts of peptides were crossing (Fenstermacher et al., 1981), and because of the potency of these peptides, such small amounts could be biologically significant.

One route whereby peripherally administered opioids or other peptides might enter the central nervous system (CNS) is via the circumventricular organs (CVO's), small areas of the brain that line

the ventricles that have no BBB (Weindl, 1973). The CVO's include the choroid plexus, the area prostrema, the ME, and the lamina terminales of the hypothalamus, which, as discussed previously, is the putative site of action of some peripherally administered opioids on pituitary GH, and possibly PRL release. Neuronal connections between the CVO's and the remainder of the brain could create a link between the periphery and the brain (Epstein, 1982). Regardless of how they enter, the fact is there are studies that have directly demonstrated opioid peptide penetration into the hypothalamus or other areas of the CNS after peripheral injection (Pezalla et al. 1978; Merin et al., 1980; Rappaport et al., 1980).

Another factor to consider when measuring changes in hormone secretion or any other physiological parameter after opioid or opiate agonist injection, is whether or not the observed differences were indeed the result of specific activation of opioid receptors. In this regard, the highly specific opioid antagonist naloxone (NAL) is very useful. Naloxone is a pure narcotic antagonist, and its high specificity led early investigators to adopt as a criterion of an opioid effect that it be blocked and reversed by this compound (Goldstein, 1984). However, antagonism of an opioid-mediated effect by NAL provides no evidence in regard to the above cited discrepancies concerning peripheral vs central site of action of the opioid. This is because NAL is able to antagonize opioid effects at either location, a result of its lipid solubility which allows it to readily cross the BBB (Blumberg and Dayton, 1973).

One approach to elucidating the site of action of a peripherally injected opioid is the use of congeners of NAL and other opioid antagonists which have an additional alkyl substituent on the nitrogen atom in the ring structure, producing a quaternary amine (Brown and Goldberg, 1985). These compounds were designed to traverse the BBB less readily than their tertiary counterparts, due to their greater polarity. Thus, blockage of an opioid-mediated effect by these compounds indicated the opioid was producing its effect somewhere outside the BBB. However, use of these quaternary opiate antagonists is complicated by their much lower affinity for the opioid receptor relative to their tertiary form (Opheim and Cox, 1976). Therefore, these compounds must be administered at much higher doses to achieve degrees of antagonism similar to the parent forms (Brown and Goldberg, 1985).

This problem has been alleviated to some extent with the synthesis of N-methyl levallorphan (MLM), the quaternary analog of the opioid antagonist levallorphan. This compound was developed to antagonize the peripherally constipating effects of opioid and opiate analgesics, while not interfering with the centrally-mediated anti-nociceptive effect. It is virtually unable to cross the BBB, is a relatively pure opioid antagonist, and maintains good antagonistic potency after quaternization. Therefore, MLM has been proposed as a pharmacologically useful tool in selectively differentiating between peripherally or centrally mediated effects of exogenous or endogenous opioids (Bianchetti et al., 1985 and 1989).

The objectives of this study were to measure plasma GH and PRL responses of Holstein calves to an i.v bolus of the synthetic enkephalin DAMME (Roemer et al., 1977) in fall and spring, and secondly, to determine if changes in plasma GH and PRL after DAMME administration are mediated via opioid receptors within or outside the BBB, by pre-treating calves prior to DAMME injection with either saline, the lipid soluble opioid antagonist NAL, or the peripherally selective opioid antagonist MLM.

MATERIALS AND METHODS

Animals. Eighteen Holstein calves, ranging from 4 to 6 months of age and averaging 158 kg BW, were acquired from the university herd in fall (1988). In spring (1989), a second group of 18 calves, also 4 to 6 months of age and ave BW, were obtained. In both fall and spring studies, all calves were randomly assigned to 1 of 3 treatments (6 calves/treatment): 1) Pre-treatment with saline (SAL), and subsequent injection of DAMME (D-Ala^2,N-Me-Phe^4,Met(O)-ol^5 enkephalin, 6 ug/kg BW, Sigma Chemical Co., St.Louis, Mo.) 30 min later, or 2) Pre-treatment with NAL, 1.0 mg/kg BW (Sigma Chemical Co., St. Louis, Mo.), and injection of DAMME (6 ug/kg) 30 min later, or 3) Pre-treatent with MLM, 1.0 mg/kg BW (SANOFI Research Center, Milan, Italy) and injection of DAMME (6ug/kg BW) 30 min later. All calves had been maintained in sheltered pens at the university calf barn since birth.

The day prior to administration of treatments, calves were haltered and weighed, and subsequently tied into individual stanchions with free access to water and alfalfa hay. On day of treatment, indwelling jugular cannulae were installed in all calves at 0600h. Clotting of blood in cannulation tubing was prevented by infusion of a 3.5% citrate solution after cannulation, and immediately after each sample was drawn. External tubing with sample ports were securely taped to the side of each calve's neck for easy access. At 1100h, serial bleeding every 15min commenced. The initial 1 ml of each sample was discarded, and a 10 ml sample then collected through sample ports and placed into collection tubes containing 200ul of a 3.5% EDTA solution. Animals were preinfused with either NAL, MLM, or SAL through cannula ports after the 1300h sample was drawn. All calves in all groups were subsequently injected with DAMME through cannula ports immediately after the 1330h sample was drawn. Sampling continued every 15min until 1730h. Blood samples were placed in ice immediately after withdrawal, and centrifuged for 30 min at 1800 x g. Plasma was decanted and frozen at -20 C until assayed for GH, PRL, or insulin (INS).

Hormone Determination. All hormones were quantified by a double antibody radioimmunoassay according to the procedure of Barnes et al. (1985). Growth hormone intra- and interassay coefficients of variation (CV) were 7.7% and 8.3%, respectively. For PRL, intrassay CV averaged 7.4%, and interassay 9.3%. Intra- and interassay CV for INS radioimmunoassays were 8.6% and 13%, respectively.

Statistical Analysis. Blood hormone data in both seasons were analyzed by least-squares analysis of variance with the GLM procedure of the Statistical Analysis Systems (SAS), SAS Institute, Cary, North Carolina. Samples were assigned to one of 3 periods. Period 1 represented samples taken from 1100h to 1330h (basal period); period 2 represented samples taken after DAMME administration (from 1345h to 1600h; treatment period); period 3 represented samples taken from 1615h to 1730h, at which time sampling was terminated.

The statistical model contained treatment, calf within treatment, period, treatment x period, calf x period within treatment, sample within period, and treatment x sample within period. Treatment was tested using calf within treatment as the error term; period and treatment x period were tested using calf x period within treatment as the error term. Non-orthogonal contrasts were

used to compare least squares means for periods 1 vs 2, 1 vs 3, and 2 vs 3, within each treatment. All critical F values to determine significance of contrasts were adjusted using the Improved Bonferroni F test (Games, 1977).

RESULTS

Prolactin increased more than 5 fold ($P<.01$) from period 1 to period 2 in SAL/DAMME calves in the fall study (Table 1). This increase was blocked in calves pre-treated with both MLM and NAL (Table 1). Results in spring were very similar, with a more than 6 fold increase ($P<.01$) occurring in plasma PRL in SAL/DAMME calves from period 1 to period 2 (Table 1). Pre-treatment with MLM did not block an increase ($P<.01$) in PRL from period 1 to period 2 after DAMME injection in MLM/DAMME calves in spring (Table 1), but this increase was not nearly as great as that seen in SAL/DAMME calves (an approximate 100% increase vs an approximate 650% increase, respectively). Calves pre-treated with NAL in spring had similar PRL concentrations in all periods (Table 1).

In the fall study, mean plasma GH concentration more than doubled ($P<.01$) from period 1 to period 2 in the SAL/DAMME calves and increased ($P<.05$) more than 55% in MLM/DAMME calves from period 1 to period 2 (Table 2). Plasma GH returned to near pre- treatment levels by period 3 in both groups. Plasma GH did not change significantly between periods in NAL/DAMME calves (Table 2).

Growth hormone results in spring were similar, but less conclusive. Plasma GH rose nearly 60% from period 1 to period 2 in SAL/DAMME calves (Table 2), which approached significance ($P<.06$). Plasma GH concentrations remained elevated in period 3, in contrast to fall results. In MLM/DAMME spring calves, plasma GH increased ($P<.05$) more than 90% from period 1 to period 2 (Table 2). Growth hormone was not different between periods in NAL/DAMME spring calves (Table 2).

DISCUSSION

In both fall and spring, an immediate and acute increase and subsequent gradual decline in plasma PRL after DAMME injection in SAL/DAMME calves was observed (Figures 1 and 2). The GH response to DAMME injection in the same group was not immediate, particularly in spring, and was characterized by 2 or 3 pulses before returning to period 1 levels (Figures 3 and 4). This is in agreement with the reported response of GH and PRL in sheep after peripheral DAMME injection (Bolton et al., 1983). This difference in response of GH and PRL to DAMME injection may be due to modulation of GH and PRL by different opioid receptors. A number of different opioid receptor types are known to exist (Wood, 1981), and in the rat, it appears that opioid stimulated PRL release is mediated via mu receptors and GH release occurs through the delta receptors (Koenig et al., 1984; Panerai et al., 1985; Leadem and Yagenova, 1987).

The data in this study support a similar concept in calves. DAMME will stimulate delta receptors, but is predominately a mu receptor agonist (Rothman et al., 1987). This is in contrast to the action of natural enkephalins, which have a greater affinity for delta receptors than mu receptors. If opioid-mediated release of PRL occurs via mu receptors in calves, as in rats, then this could account for the sharply acute release of pituitary PRL seen in SAL/DAMME calves in both fall and spring, as these receptors would be the most responsive to the mu preferring agonist DAMME.

Further evidence to support different opioid receptor activation by DAMME for mediation of GH and PRL release is seen by examining the attenuation of DAMME induced PRL release by NAL and MLM in Figures 1 and 2. Naloxone and MLM are both mu preferring opioid antagonists, with NAL being approximatley 2.5 times more potent a mu receptor antagonist than MLM (Bianchetti et al., 1985). Although PRL increased significantly after DAMME injection in the MLM pre-treated calves in spring (Table 1), it did not increase nearly as much as in the saline pre- treated calves, indicating that the response is being attenuated to a large extent by MLM.

Examination of Figures 1 and 2 shows that the peak in PRL after DAMME administration in MLM pre-treated calves was approximately 2 times as great as the PRL peak in the NAL pre-treated calves, particularly in fall, which could be explained by the receptor specificty data mentioned above if indeed the PRL response to DAMME was being mediated through mu receptors. Further studies using more highly specific mu and delta agonists would test this hypothesis.

Exact physiological mechanisms which facilitate the acute surge in plasma PRL in response to DAMME cannot be derived from this study. However, pituitary PRL release appears to be controlled primarily by chronic inhibition by dopamine, released into hypophysial portal blood from tuberoinfindibular (TIDA) neurons terminating in the ME (Gudelsky and Porter, 1979), or possibly norepinephrine being released into portal blood from the ME (Thomas et al.,1989). The results of this study indicate that the receptors mediating the DAMME effect on PRL are located at a site outside the BBB since both MLM and NAL were able to attenuate the effect of DAMME on PRL release observed in saline pre-treated calves. This is in agreement with Bianchetti et al. (1988), but in contrast to Panerai et al. (1981). One hypothesis would be that DAMME inhibits dopamine or norepinephrine release into hypophysial portal vessels from catecholaminergic neurons terminating in the ME, a structure known to be rich in catecholamines, opioids, opioid receptors, and which lies outside the BBB (Kiser et al., 1976; Finley et al.,1981; Leshin et al., 1988). This appears to at least partly explain how PRL increase is facilitated by exogenous opioids and opiates in rats (Deyo et al., 1979; Gudelsky and Porter, 1979).

As mentioned, GH increased after DAMME injection in SAL/DAMME calves in fall, and tended to increase in spring (Table 2). In both seasons, GH increased after DAMME in the MLM/DAMME calves, but did not change significantly between periods in the NAL/DAMME calves (Figures 3 and 4), indicating opioid receptors mediating this increase were located somewhere within the CNS. This agrees with results of a similar study conducted in rats (Panerai et al., 1981).

As stated previously, pituitary GH secretion in response to exogenous opioid peptides appears to be modulated by the delta opioid receptors in rats, which in turn seem to produce their effect in this species primarily by facilitating increased secretion of growth hormone releasing hormone (GRH) from the hypothalamus (Wehrenberg et al., 1985). Indeed, the increase in GH

secretion seen in rats (Murakami et al., 1985) and gilts (Armstrong et al., 1990) after DAMME administration can be blocked with antibodies to GRH. Furthermore, increased GH secretion in response to exogenous opioids is not blocked in rats immunized against somatostatin (Chihara et al.,1978).

This study does not prove that GH response to DAMME in calves is mediated through increased GRH secretion. However, Figures 3 and 4 show distinct GH peaks following DAMME injection in saline and MLM pre-treated calves, unlike the large single peak and subsequent gradual decline of PRL seen in SAL/DAMME calves (Figures 1 and 2). In light of this, as well as the aforementioned GRH and somatostatin studies, and because GH secretion in cattle is asynchronous and pulsatile (Anfinson et al., 1975), it seems reasonable to hypothesize that the generation of distinct spikes of GH in SAL/DAMME and MLM/DAMME calves in Figures 3 and 4 are due to periodic bursts of hypothalamic GRH.

Exogenous opioid and opiate administration has been shown to affect basal pancreatic insulin secretion in dogs (Ipps et al., 1980) and humans (Reid and Yen, 1981), while other studies have been unable to show opioid effects on basal secretion (Schusdziarra et al., 1983). Basal insulin secretion was measured in the SAL/DAMME calves in spring, and although mean insulin concentration was higher in period 2 (Table 3), the change was not statistically different. These findings are in agreement with those of Bolton et al. (1983) in which plasma insulin was unchanged in sheep after DAMME administration, and in contrast to Hart and Cowie (1978), who reported reduced insulin secretion in goats treated with morphine and an enkephalin analogue.

Rectal temperatures were measured in all groups in spring, (every hour before DAMME injection, and every 30 min after). Although opioids can have profound effects on body temperature depending on dose and route of administration (Clark, 1981) no differences in body temperature were observed between periods in this study. The overall average body temperature for calves in this study was 101.5 F.

In the final analysis, these results indicate that, in the case of GH at least, it appears that the opioid peptide DAMME is able to penetrate the BBB and bind opioid receptors in the CNS. As discussed previously, there are many factors to consider regarding if and how water soluble peptides

are able to penetrate the BBB and affect the CNS. To prove peptide penetration of the BBB with absolute certainty would require sacrifice of animals, removal of the brain, and positive identification of the peptide in question in the brain tissue. This was not possible in this study.

However, opioid peptides similar in structure to DAMME have been shown to enter the brain parenchyma after peripheral administration (Rapoport et al., 1980), and DAMME has a long half-life in ruminants which might help facilitate its entry into the CNS (Bolton et al., 1982). Furthermore, DAMME is one of the rare examples of a peptide showing pronounced biological activity after oral administration, and is able to produce pronounced analgesia after peripheral administration, which is a centrally-mediated effect (Hill et al., 1978). Still more evidence that this peptide penetrates the BBB was the observation in this study of a mild to moderate catatonic-like state that occurred in several calves approximately 1 minute after injection of DAMME, and lasted, on average, 30 minutes.

In conclusion, this study demonstrates that peripheral administration of the synthetic enkephalin DAMME increases circulating concentrations of GH and PRL in Holstein calves in both fall and spring. These results also suggest that DAMME is able to penetrate the BBB, and that the opioid receptors mediating GH release in Holstein calves are located somewhere within the CNS. Those receptors which modify PRL secretion, in contrast, appear to be located at a site somewhere outside the BBB. We have forwarded hypotheses as to the possible physiological mechanism and type of opioid receptor involved in the DAMME-mediated GH and PRL secretion observed in Holstein calves, but more specific studies are required to validate these hypotheses.

SUMMARY

Two experiments, one in fall and one in spring, were conducted to determine the effects of peripheral administration of the synthetic enkephalin DAMME (D-Ala^2,N-Me-Phe^4,Met(O)-ol enkephalin) on growth hormone (GH) and prolactin (PRL) release in Holstein calves, and whether the effect of DAMME on secretion of these 2 pituitary hormones was mediated within or outside the blood brain barrier (BBB). In the fall, DAMME significantly increased both plasma PRL and GH in Holstein calves. The lipid- soluble opioid antagonist naloxone (NAL), which readily crosses the BBB, and the peripherally selctive opioid antagonist N-methyl levallorphan mesilate (MLM) were able to attenuate the effect of DAMME on plasma PRL in calves. Naloxone, but not MLM, was able to block the effect of DAMME on plasma GH release in fall.

In spring, DAMME significantly increased plasma PRL in calves, and plasma GH rose to near signifcance after DAMME injection. Prolactin was unchanged after DAMME injection in NAL pre-treated calves, but rose signifcantly in MLM pre-treated animals. However, the PRL response to DAMME in MLM pre-treated calves in spring was not nearly as great as the PRL response to DAMME in saline pre-treated calves, indicating that the response was attenuated to a large extent by MLM, but the dose of MLM may not have been sufficient to block it completely.

Plasma GH increased significantly after DAMME injection in MLM treated calves again in spring. Naloxone once again blocked the GH response to DAMME. Rectal temperatures were measured in all calves in spring, and plasma insulin (INS) was quantified in a subset of saline pre-treated, DAMME injected calves. Rectal temperatures did not change in this study, and plasma INS was unaffected by DAMME treatment.

These results demonstrate that Holstein calves are capable of significant GH and PRL responses to an exogenous opioid, a phenomena previously demonstrated in other species but heretofore not demonstrated in dairy animals. Collectively, both fall and spring studies indicate that the synthetic opioid peptide DAMME is able to penetrate the BBB, and that opioid receptors medi-

ating the GH response to DAMME are somewhere within the BBB, and those receptors mediating the PRL response are at a site somewhere outside the BBB.

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Table 1. PLASMA PROLACTIN CONCENTRATIONS* OF HOLSTEIN CALVES PRETREATED WITH OPIOID ANTAGONISTS OR SALINE 30 MIN PRIOR TO OPIOID AGONIST INJECTION.

Group	No. calves	Period	Plasma Prolactin concentration (ng/ml) (fall)	(spring)
SAL/DAMME	6	1	1.8 ± .7[a]	4.1 ± 1.5[a]
		2	10.2 ± .7[b]	26.9 ± 1.5[b]
		3	1.8 ± .8[a]	4.6 ± 1.8[a]
MLM/DAMME	6	1	2.3 ± .7	6.3 ± 1.5[a]
		2	4.3 ± .7	13.1 ± 1.5[b]
		3	1.3 ± 1.3	3.3 ± 1.8[a]
NAL/DAMME	6	1	3.6 ± .7	7.1 ± 1.5
		2	4.6 ± .7	10.5 ± 1.5
		3	3.8 ± .8	4.9 ± 1.8

* = Least squares means, ± sem.
[a,b] Means with different superscripts in same group and season differ ($P<.01$).
DAMME = D-Ala^2,N-Me-Phe^4,Met(O)^5-ol enkephalin (6 ug/kg).
LMS = Methyl levallorphan mesilate, (1.0 mg/kg)
NAL = Naloxone, (1.0 mg/kg).
SAL = Saline.

Table 2. PLASMA GROWTH HORMONE CONCENTRATIONS* OF HOLSTEIN CALVES PRETREATED WITH OPIOID ANTAGONISTS OR SALINE 30 MIN PRIOR TO OPIOID AGONIST INJECTION.

Group	No. calves	Period	Plasma Growth Hormone concentration (ng/ml) (fall)	(spring)
SAL/DAMME	6	1	12.9 ± 2.1[a]	16.1 ± 2.8
		2	28.4 ± 2.1[c]	25.7 ± 2.8
		3	13.7 ± 2.4[a]	27.5 ± 3.4
MLM/DAMME	6	1	14.9 ± 2.1[a]	17.2 ± 2.8[a]
		2	23.3 ± 2.1[b]	33.2 ± 2.8[b]
		3	17.5 ± 2.1[a,b]	23.9 ± 3.4[a,b]
NAL/DAMME	6	1	15.8 ± 2.1	13.0 ± 2.8
		2	21.6 ± 2.1	16.1 ± 2.8
		3	15.4 ± 2.4	20.9 ± 3.6

* = Least squares means, ± sem.
[a,b] Means with different superscripts in same group and season differ (P<.05).
[a,c] Means with different superscripts in same group and season differ (P<.01).
DAMME = D-Ala^2,N-Me-Phe^4,Met(O)^5-ol enkephalin (6 ug/kg).
LMS = Methyl levallorphan mesilate (1.0 mg/kg).
NAL = Naloxone (1.0 mg/kg).
SAL = Saline

Table 3. PLASMA INSULIN CONCENTRATIONS* OF HOLSTEIN CALVES PRETREATED WITH SALINE 30 MIN PRIOR TO OPIOID AGONIST INJECTION (SPRING).

Group	No. calves	Period	Plasma insulin concentration (ng/ml)
SAL/DAMME	6	1	0.5 ± .5
		2	1.3 ± .5
		3	0.5 ± .6

* = Least squares means, ± sem.
DAMME = D-Ala^2,N-Me-Phe^4,Met(O)^5-ol enkephalin (6 ug/kg).
SAL = Saline.

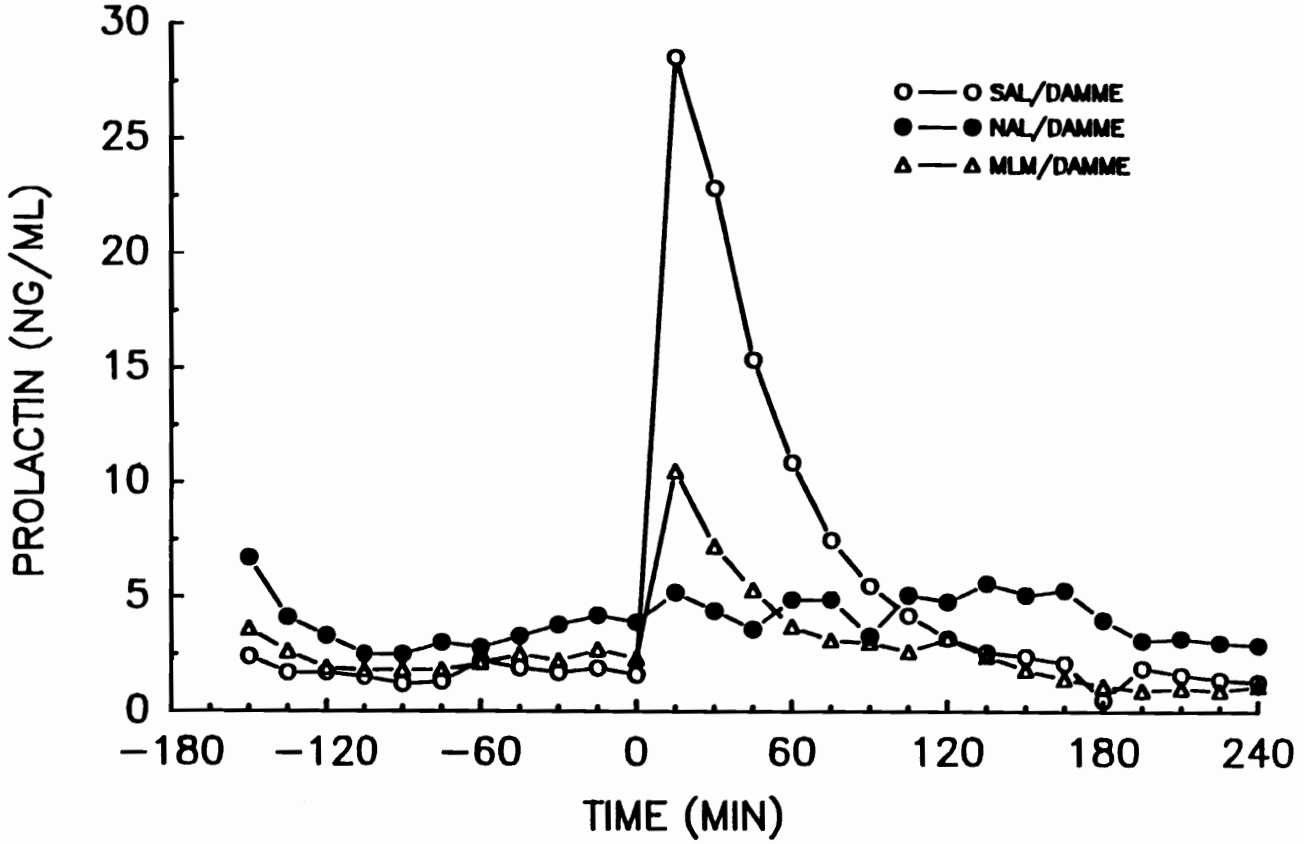

Figure 1. Plasma prolactin concentrations before (period 1) and after (periods 2 and 3) DAMME injection (time=0) in Holstein calves pre-treated with saline (SAL), naloxone (NAL), or methyl levallorphan mesilate (MLM) 30 min prior to DAMME in fall season. Period 1= -150 to 0 minutes; period 2= 15 to 150 minutes; period 3= 165 to 240 minutes.

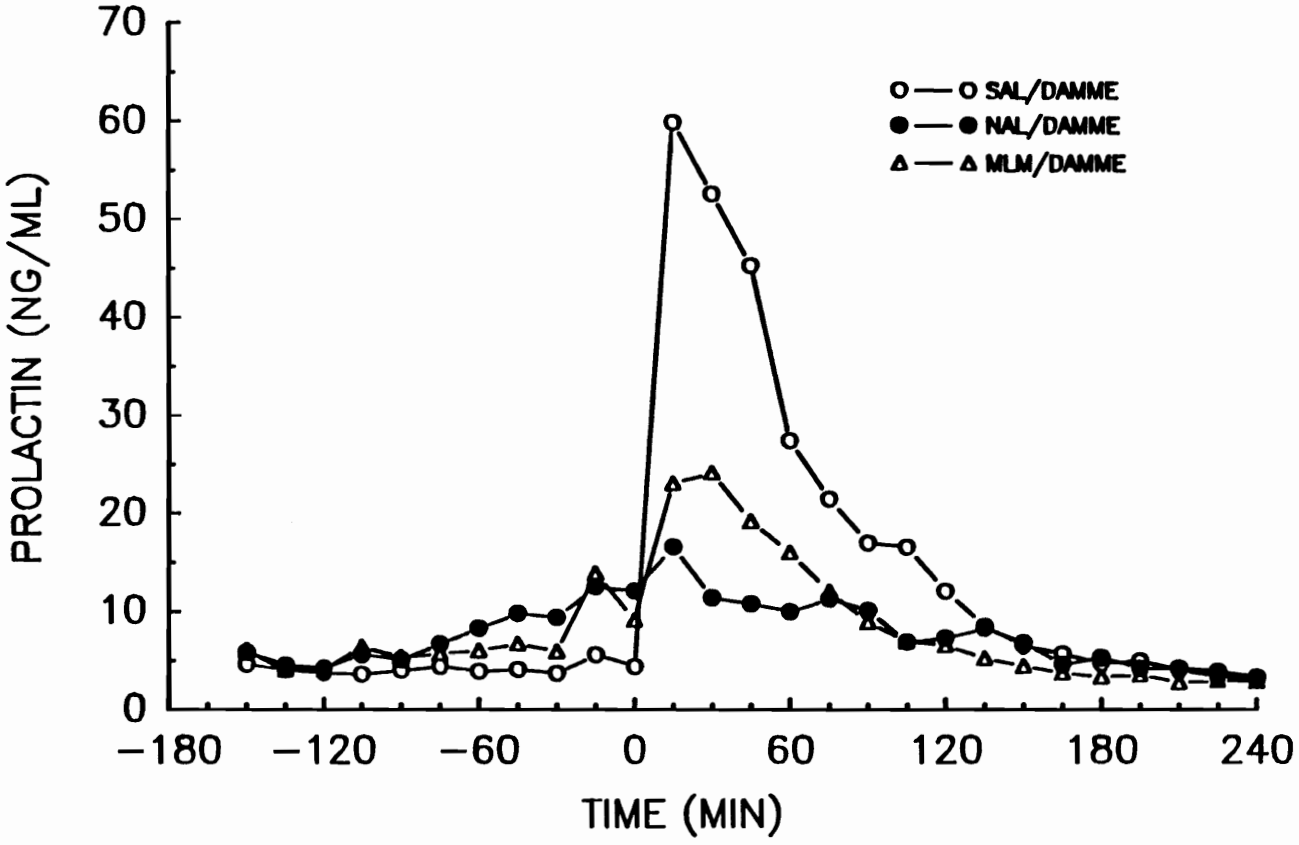

Figure 2. Plasma prolactin concentrations before (period 1) and after (periods 2 and 3) DAMME injection (time=0) in Holstein calves pre-treated with saline (SAL), naloxone (NAL), or methyl levallorphan mesilate (MLM) 30 min prior to DAMME in spring season. Period 1= -150 to 0 minutes; period 2= 15 to 150 minutes; period 3= 165 to 240 minutes.

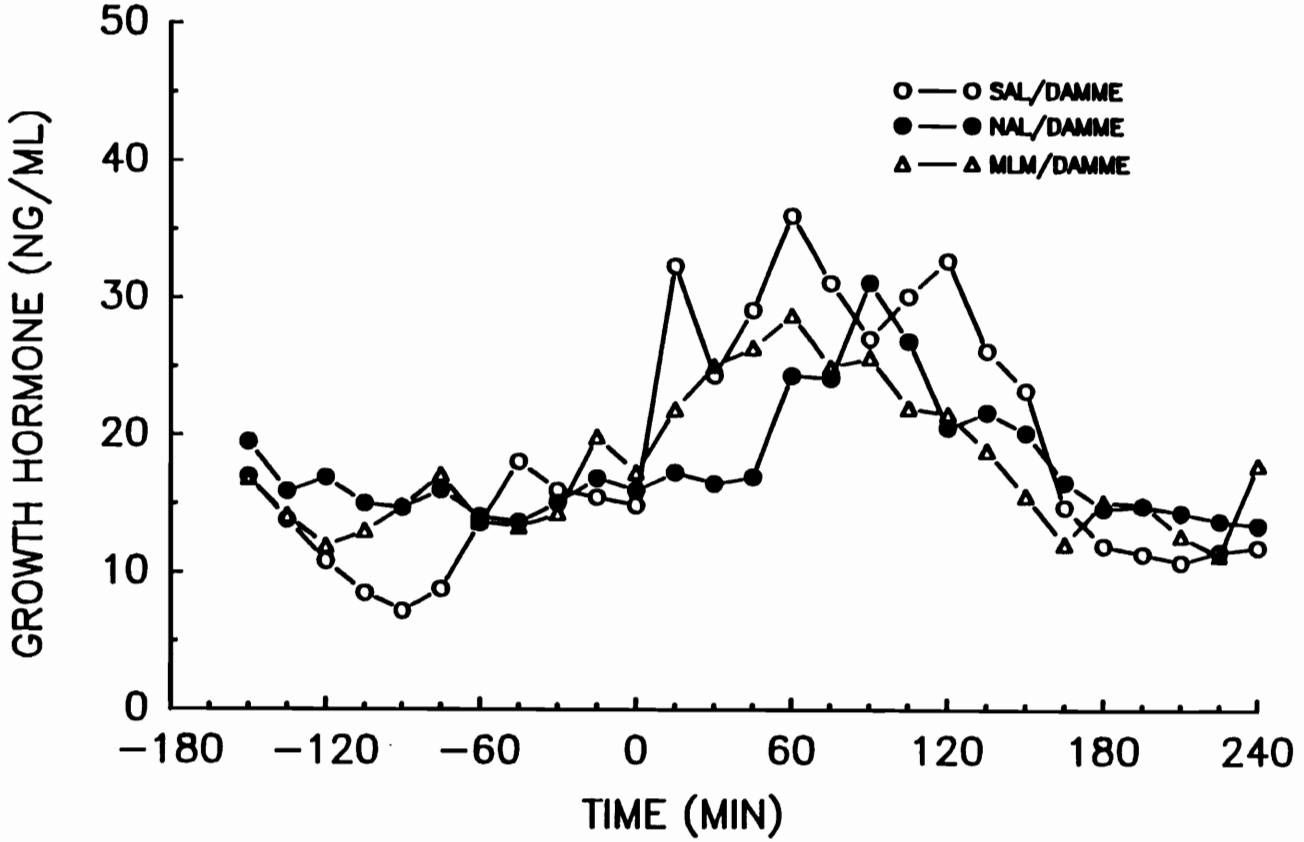

Figure 3. Plasma growth hormone concentrations before (period 1) and after (periods 2 and 3) DAMME injection (time=0) in Holstein calves pre-treated with saline (SAL), naloxone (NAL), or methyl levalorphan mesilate (MLM) 30 min prior to DAMME in fall season. Period 1= -150 to 0 minutes; period 2= 15 to 150 minutes; period 3= 165 to 240 minutes.

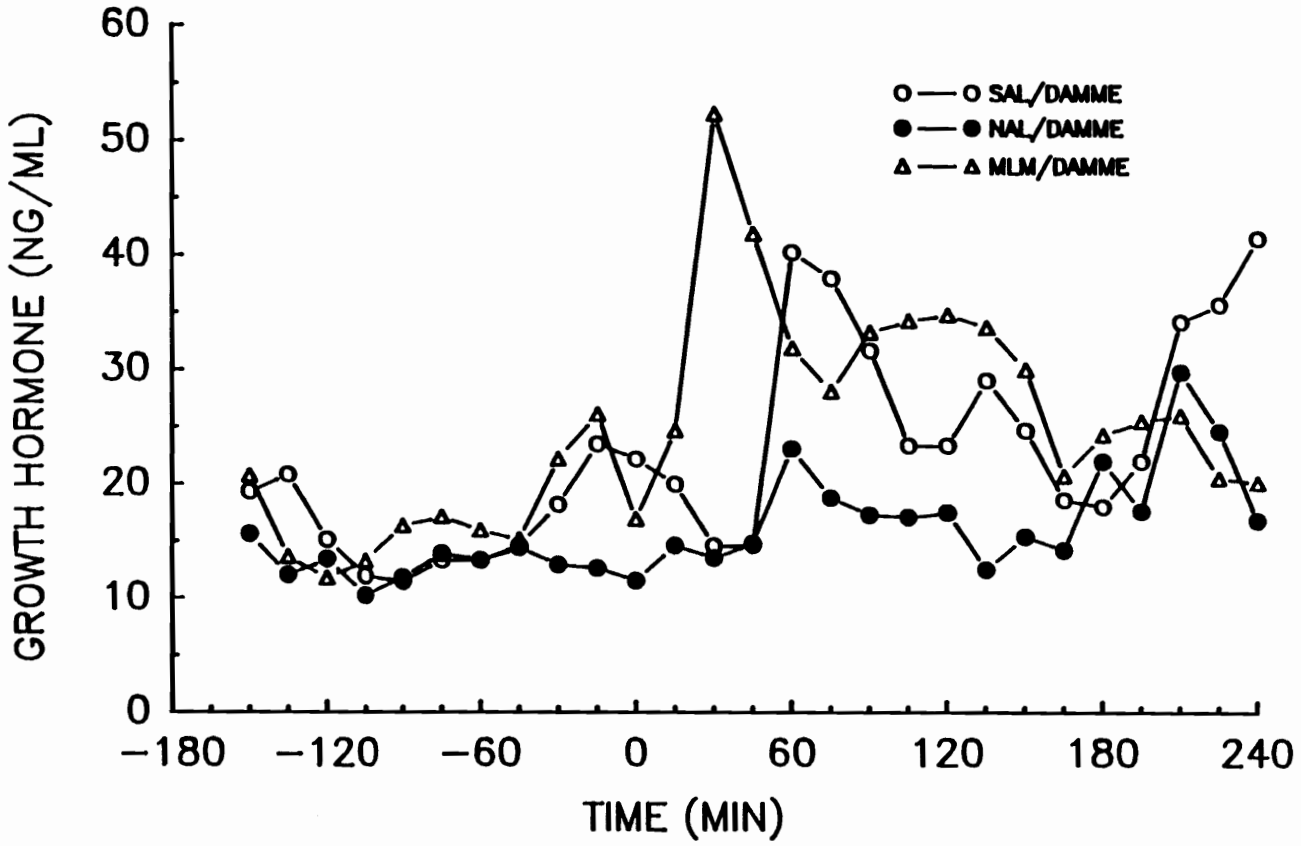

Figure 4. Plasma growth hormone concentrations before (period 1) and after (periods 2 and 3) DAMME injection (time=0) in Holstein calves pre-treated with saline (SAL), naloxone (NAL), or methyl levallorphan mesilate (MLM) 30 min prior to DAMME in spring season. Period 1= -150 to 0 minutes; period 2= 15 to 150 minutes; period 3= 165 to 240 minutes.

CHAPTER 2

THE EFFECT AND SITE OF ACTION OF ENDOGENOUS OPIOIDS ON PLASMA GROWTH HORMONE AND PROLACTIN IN HOLSTEIN CALVES

INTRODUCTION

As described previously in chapter 1, a number of studies have demonstrated the ability of natural or synthetic analogues of the endogenous opioids to affect pituitary secretion of growth hormone (GH) and prolactin (PRL) in several mammalian species. However, changes in hormone secretion after administration of these compounds is best described as a pharmacological effect, as opposed to a physiological effect (McKnight and Kosterlitz, 1980).

Also discussed in Chapter 1 was the concept that when changes in the plasma concentration of a pituitary hormone after opioid agonist administration can be negated or attenuated by the opioid antagonist naloxone (NAL), this is strong evidence that the agonist is indeed working through endogenous opioid receptors. This is because NAL is a highly specific opioid antagonist that is thought to bind only opioid receptors (see Blumberg and Dayton, 1973, for review). However, the observation that administration of opioid antagonists alone (ie, not in the presence of an agonist) alters pituitary GH and PRL secretion is the most convincing evidence of an actual physiological role for endogenous opioids in the regulation of pituitary hormones (McKnight and Kosterlitz, 1980).

To date, several studies in which NAL by itself has been administered to various species have indicated endogenous opioids may be involved in physiological regulation of basal pituitary growth hormone (GH) and prolactin (PRL) secretion. Examples of this for both GH and PRL have been

demonstrated in rats (Bruni et al., 1977; Rivier et al.,1977; Shaar and Clemens, 1980) ruminants (Gregg et al., 1986; Hart and Cowie, 1978; McMillen and Deayton, 1989; Leshin et al., 1990) and for PRL in swine (Mattioli et al., 1986; Armstrong et al., 1988). However, as reviewed in Chapter 1, a physiological effect produced by NAL itself provides no information as to whether the opioid receptors responsible for mediating detected changes are located within the blood brain barrier (BBB) or at a site outside of it, as NAL is lipid soluble and readily diffuses across biological membranes, including the BBB (Berkowitz et al., 1976). A peripherally selective opioid antagonist, methyl levallorphan mesilate (MLM), has been developed which readily antagonizes opioid receptors, maintains good antagonistic potency after quaternization, and is virtually unable to cross the BBB (Bianchetti et al., 1985 and 1989).

The objectives of the present study were to determine if endogenous opioids are involved in mediation of basal PRL and GH secretion in Holstein calves in both spring and fall, and to determine the site of action (peripheral vs central) of any such regulation by injection of both the lipid soluble antagonist NAL and the peripherally selective antagonist MLM.

MATERIALS AND METHODS

Animals. In the fall (1988) study, eighteen Holstein calves, ranging from 3 to 5 months of age and averaging 151 kg BW, were acquired from the university herd. Animals were randomly assigned to 1 of 3 treatments (6 calves/treatment): 1) Saline injection or 2) MLM injection (1.0 mg/kg, Sanofi Research, Milan, Italy) or 3) NAL injection (1.0 mg/kg, Sigma Chemical, St.Louis, Mo.). All animals were kept in sheltered pens at the university calf barn. The day prior to administration of treatments, calves were haltered and weighed, and subsequently tied into individual stanchions with free access to water and alfalfa hay.

On day of treatment, indwelling jugular cannulas were installed in all calves at 0600h. Clotting of blood in cannulation tubing was prevented by infusion of a 3.5% citrate solution after cannulation, and immediately after each sample was drawn. External tubing with sample ports

was securely taped to the side of each calve's neck for easy access. At 1100h, serial bleeding every 15 min commenced. The initial 1 ml of each sample was discarded, and a 10 ml sample was then drawn and placed into collection tubes containing 200ul of a 3.5% EDTA solution. Animals were injected with either NAL, MLM, or SAL through cannulae ports immediately after the 1330h sample was drawn. Sampling continued every 15 min until 1715h. Blood samples were placed in ice immediately after withdrawal, and centrifuged for 30 min at 1800 x g. Plasma was decanted and frozen at -20 C until assayed for GH and PRL.

In the spring (1989) study, 20 Holstein calves, ranging from 4 to 6 months of age and averaging 163kg BW were randomly assigned to 1 of 5 doses (4/dose): Injection of .10, .25, .50, 1.0, or 2.0 mg/kg of MLM. All animals were subsequently crossed over 1 week later and injected with NAL at the same dose. Protocol for animal cannulation, feeding, plasma collection, storage, and GH and PRL assay was identical to fall. Blood samples from 2 calves receiving .10, 1.0, and 2.0 mg/kg MLM and NAL were randomly selected and plasma insulin (INS) quantified. Rectal temperatures were measured in all calves in spring, every hour before treatment administration, and every 30 min after treatments for 2 hrs.

Hormone Determination. All hormones were quantified by a double antibody radioimmunoassay according to the procedure of Barnes et al. &lpar1985). Growth hormone intra- and interassay coefficients of variation (CV) were 6.9% and 8.2%, respectively. For PRL, intrassay CV averaged 7.0%, and interassay 8.2%. Intra- and interassay CV for INS radioimmunoassays in spring were 8.2% and 11.1% respectively.

Statistical Analysis. Blood hormone data from both seasons were analyzed by least-squares analysis of variance with the GLM procedure of the Statistical Analysis Systems (SAS), SAS Institute, Cary, N.C. All samples were assigned to 1 of 3 periods. Period 1 represented samples taken from 1100h to 1330h (basal period); period 2 represented samples taken after treatment administration (from 1345h to 1600h; treatment period); and period 3 represented samples taken from 1615h to 1715h, at which time sampling was terminated.

The statistical model used to analyze fall data contained treatment, calf within treatment, period, treatment x period, period x calf within treatment, sample within period, and treatment x

sample within period. Treatment was tested using calf within treatment as the error term; period and treatment x period were tested using period x calf within treatment as the error term. Fixed effects were treatment and period. In the spring study, the statistical model used contained treatment, dose, treatment x dose, calf within dose, treatment x calf within dose, period, treatment x period, dose x period, treatment x dose x period, period x calf within dose, and treatment x period x calf within dose. Dose was tested using calf within dose as the error term, treatment x dose was tested using treatment x calf within dose as error, and dose x period was tested using period x calf within dose as error. Fixed effects were dose, period, and treatment.

Non-orthogonal contrasts were used to compare least squares means for periods 1 vs 2, 1 vs 3, and 2 vs 3 within each treatment in fall, and within treatment/dose in spring. All critical F values to determine significance of contrasts were adjusted using the Improved Bonferroni F test (Games, 1977).

RESULTS

Plasma PRL concentration increased ($P<.05$) from period 1 to period 2 in response to NAL injection in fall season (Table 1). Plasma PRL began to rise immediately after NAL infusion, peaked approximately 45 min later, began increasing again at 60 min post-injection, and declined to pre-treatment concentrations 3 hr after NAL administration (Figure 1). In contrast, the same dose of NAL in spring (1.0 mg/kg) caused a decline ($P<.05$) in PRL from period 1 to period 3 (Table 2, Figure 3).

Methyl levallorphan mesilate administration did not affect plasma PRL in fall or spring (Tables 1 and 2). A single calf in the MLM group in fall had plasma PRL samples 8 to 10 fold higher throughout the sampling period than any other animal on study. Re-analysis of PRL data for the MLM group after removing samples from this calf resulted in more uniform pre-treatment means overall among groups, and results of contrasts of period means for all groups were the same. Prolactin data from this calf is therefore omitted in Table 1 and Figure 1.

The large spike of plasma PRL at approximately 90 min after treatment injections in the SAL group in fall (Figure 1) resulted from 2 samples from a single animal in this group in which PRL concentrations were 6 to 7 fold higher than the next highest sample produced from this animal.

Plasma GH increased ($P<.05$) from period 1 to period 3 in fall in NAL injected animals (Table 1). The increase was not immediate, and plasma GH in this group nearly paralleled that of the saline group for 3 hrs post NAL injection. At this time, plasma GH began to increase abruptly, and peaked approximately 30 minutes later (Figure 2). Plasma GH was unaffected by NAL at any dose in spring (Table 3).

Plasma GH increased abruptly ($P<.05$) in response to MLM injection in fall (Table 1, Figure 2), and remained elevated for approximately 4 hrs until sampling was terminated. Plasma GH increased ($P<.05$) in spring after MLM injection at .25, .50, and 2.0 mg/kg (Table 3, Figure 4). No differences in plasma insulin were detected after treatments at any of the doses analyzed (Table 4). Rectal temperatures averaged approximately 101.5 F in spring, and did not change after administration of treatments in any animals.

DISCUSSION

The increase in plasma PRL after NAL injection in fall (Table 1) is unique, as all studies of which we are aware wherein NAL was administered alone to rodents (Bruni et al., 1977; Cocchi et al., 1977; Shaar et al., 1977; Lien et al., 1979; Martin et al., 1979; Shaar and Clemens, 1980; Van Vugt and Meites, 1980;) swine (Mattioli et al., 1986; Armstrong et al., 1988), ruminants (Hart and Cowie, 1978; Schillo et al., 1985; Gregg et al., 1986; Whisnant et al., 1986b; McMillen and Deayton, 1989; Leshin et al., 1990) or primates (Gold et al., 1979; Morley et al., 1980) and plasma PRL quantified, the result was either no change or decreased plasma PRL.

Because of this unusual finding, the same dose of NAL and four other doses which incorporated the range of antagonist doses used in the above studies were chosen for the spring trial, to

determine if the fall PRL results were repeatable at the same or different doses. As seen in Table 2 and Figure 3, only the same dose (1.0 mg/kg) used in fall affected basal PRL in spring, but produced the opposite effect. This decrease in plasma PRL concentration in Holstein calves after NAL injection in spring is in agreement with other ruminant studies (Hart and Cowie, 1978; Gregg et al., 1986; McMillen and Deayton, 1989; Leshin et al., 1990).

An explanation for the increase in plasma PRL after NAL injection in fall is elusive. Pituitary PRL release appears to be controlled primarily through chronic inhibition by dopamine released into hypophysial portal blood from tuberoinfindibular (TIDA) neurons which terminate in the median emminence (Gudelsky and Porter, 1979; Moore, 1987). The majority of research has indicated endogenous opioids control the release of basal PRL from the pituitary by chronically inhibiting these or other dopaminergic neurons, the cell bodies of which lie within the hypothalamus (Grandison and Guidotti, 1977; Guidotti and Grandison, 1978). Also, opioid-mediated release of both PRL and GH is not thought to occur directly at the pituitary, which is outside the BBB, as addition of opioid agonists or antagonists to cultured pituitary cells does not alter hormone secretion from these cells (Rivier et al., 1977; Grandison and Fratta, 1980), further suggesting a hypothalamic site of action of endogenous opioids on PRL secretion.

It seems logical, therefore, that peripheral administration of an opioid antagonist capable of crossing the BBB, such as NAL, could block endogenous opioidergic mechanisms at the hypothalamic level which inhibit dopamine release, thereby allowing increased secretion of dopamine into hypophysial portal blood, and a concommitant drop in pituitary PRL release. In the present study, both fall and spring results agree with the above postulated central nervous system site of action of endogenous opioids on PRL secretion, due to the failure of the peripheral opioid antagonist MLM to affect PRL secretion in either season.

The NAL effect of increasing plasma PRL in fall does not agree with the previously described mechanism of opioid mediation of PRL release. Since the site of action of NAL on PRL secretion is likely within the central nervous system, a possible explanation of this increased PRL secretion might be that endogenous opioids in Holstein calves during fall season are stimulatory to dopaminergic neurons in the hypothalamus, as opposed to inhibitory. Antagonism of these

endogenous opioid receptors by NAL would therefore decrease dopaminergic activity, thereby decreasing dopamine release into hypophysial portal blood, which would allow for increased PRL secretion at the pituitary level.

A second possible explanation may be that in fall season, endogenous opioids in Holstein calves inhibit one of several hypothalmic peptides known to act as PRL-releasing factors (Leong et al., 1983), and antagonism of these opioids by NAL at the opioid receptor facilitates increased secretion of one or more of these peptides into hypophysial portal blood. Although the majority of evidence suggests a hypothalamic-dopaminergic mechanism for endogenous opioid mediation of pituitary PRL secretion, at least 3 studies have demonstrated that exogenous opioids are capable of affecting PRL secretion in rats by some mechanism independant of a dopaminergic pathway (Arita and Porter, 1983; Shin et al., 1988; Yogev et al., 1989). Also, "disinhibition" by NAL of endogenous opioid-mediated inhibition of a hypothalamic peptide controlling pituitary hormone release is known to exist for gonadotrophin-releasing hormone (Pfieffer and Herz, 1984).

The increased plasma GH which occurred approximately 3 hrs after NAL injection in the fall study (Table 1, Figure 2) is also uncharacteristic of plasma GH responses to NAL in all species tested. In rodents, NAL administration alone affects plasma GH similarly to plasma PRL, decreasing or not changing circulating levels (Bruni et al., 1977; Cocchi et al., 1977; Shaar et al., 1977; Lien et al., 1979; Martin et al., 1979; Tannenbaum et al., 1979; Shaar and Clemens, 1980; Van Vugt and Meites, 1980). Naloxone also has been shown to decrease plasma GH in sows (Armstrong et al., 1990), and in ruminants, plasma GH was decreased (Leshin et al., 1990) or did not change (Hart and Cowie, 1978) following a single injection of NAL. Interestingly, increases in plasma GH concentration after NAL administration have been reported in humans (Janowsky et al., 1978; Volavka et al., 1980).

Whether NAL could be responsible for the increased plasma GH in this study 3 hrs after its administration is unclear. Blumberg and Dayton (1973) reported that the duration of action of NAL is 2-4 hrs after a single dose. A single i.v. dose of NAL was reported to reduce plasma PRL in goats up to 7 hours (Hart and Cowie, 1978), and 4-6 hours in sows (Mattioli et al., 1986). In contrast, Nagi et al. (1976) reported a half-life for NAL of 30 min in rats, and 64 min in humans

after i.v. injection. Also, NAL increases plasma luteinizing hormone (LH) in ruminants, but plasma LH returns to pre-NAL levels within 1-2 hours (Whisnant et al., 1986a; Rund et al., 1989). The failure of NAL to increase plasma GH at any dose in spring casts further doubt on whether the increased GH secretion in fall was NAL-mediated.

The effects of MLM on plasma GH are more apparent. Plasma GH more than doubled after MLM injection in fall (Table 1) and increased in spring at the .25, .50, and 2.0 mg/kg doses (Table 3). Although MLM acts as an antagonist in the presence of an opioid agonist (Bianchetti et al., 1989), particularly at the "mu" opioid receptors (Bianchetti et al., 1985), the action of MLM alone on pituitary GH secretion in Holstein calves in this study was most likely agonistic.

There are two reasons to support this contention. First, in the guinea-pig ileum and mouse vas deferens test, NAL is more than twice as potent an opioid antagonist as MLM (Bianchetti et al., 1985). Yet, the aforementioned controversial effects of NAL on GH release in fall (Table 1) were not as dramatic as the effects of MLM in both fall and spring (Tables 1 and 3), and NAL had no affect on plasma GH at any dose in spring. Also, NAL is thought to be a "pure" opioid antagonist with no agonist properties (Blumberg and Dayton, 1973). Therefore, if the MLM-mediated increase in plasma GH seen in this study were via an opioid antagonistic action, it seems likely NAL would have produced similar results, by virtue of its superior antagonistic properties.

Secondly, the parent tertiary amine of MLM, the narcotic antagonist levallorphan, is thought to be a partial agonist of GH secretion, as administration of levallorphan alone (ie, not in the presence of an opioid agonist) results in large increases in plasma GH in rats (Lien et al., 1979). This study suggests that levallorphan is able to retain some of its ability to agonize GH secretion after methylation of the chiral nitrogen to produce MLM, despite the fact that this type of methylation is thought to remove any agonistic effects of antagonists such as levallorphan, while retaining its antagonistic properties (Bianchetti et al., 1983). It is also worth noting that, to our knowledge, this drug (MLM) has not been used in any species other than rodents, and the GH response in Holstein calves may be a species effect.

Because MLM crosses the BBB very poorly, it is likely that its ability to increase GH secretion in this study was peripherally mediated. However, as described in Chapter 1, endogenous opioids are though to control secretion via release of growth hormone releasing factor (GRF) from the hypothalamus (Wehrenberg et al., 1985), which lies inside the BBB. As previously mentioned also, opioid agonists and antagonists are not thought to regulate pituitary hormone secretion by any direct action at the pituitary. These facts considered, the most logical mechanism and site of action of MLM on GH secretion would be mediation of GRF release from the median emminence, a structure which lies outside the BBB and contains opioids, opioid receptors, as well as several hypothalamic peptide releasing factors (Kiser et al., 1976; Finley et al., 1981; Leshin et al., 1988). Further studies, however, are required to prove this hypothesis.

The spring study demonstrated no effect of MLM or NAL on basal plasma INS (Table 4). Although NAL has been shown to alter post- prandial secretion of INS in dogs and humans after carbohydrate or fat-rich test diets (Rewes et al., 1983; Schusdziarra et al., 1984), the failure of either NAL or MLM to alter basal INS secretion in spring is in agreement with the effects of opioid antagonists on basal INS secretion in rats (Tannenbaum et al., 1979). Endogenous opioids are also thought to play a physiological role in temperature regulation (Clark, 1981). However, neither NAL nor MLM affected rectal temperatures in these calves.

In retrospect, when considering these results or the results of any study in which opioid antagonists alone are used to implicate involvement of endogenous opioids in mediation of a physiological parameter, it is prudent to consider the following facts. Blockage or removal of an opioid agonist-mediated effect by NAL has been adopted as a criterion to prove the agonist truly works via opioid receptors. Changes in a physiological function by NAL alone, however, implies endogenous opioid involvement in that function. Present knowledge of the relatively poor NAL affinity for the delta (δ) and kappa (κ) receptors (15 times poorer than for mu receptors) however, implies that all negative in vivo results may be inconclusive, because dosages could have been inadequate (Goldstein, 1984). In contrast, a strongly positive response to NAL implies that a mu receptor-mediated process has been exposed. This may well have implications in the present study

due to fact that, modification of PRL release is thought to be mediated primarily through the mu opioid receptor (Koenig et al. 1984; Panerai et al. 1985).

The above cited ambiguities concerning negative results with NAL have been partially resolved with the development of type- specific opioid antagonists (Kosterlitz and Paterson, 1981; Jackson et al., 1989), which will at least be useful in elucidating which endogenous opioid-mediated functions are controlled by which receptor type. There will still be questions as to whether the antagonist is acting through opioid receptors specifically, or through some non-opioid pathway. Finally, even if the effect is known to be mediated through opioid receptors, the conclusion drawn is only that endogenous opioids, somewhere, participates in mediating the overall response seen. Findings such as these, however, are useful as starting points to further elucidate on mechanisms involved.

Also, it must be considered that the peripheral opioid antagonist MLM has not been used previously in any species other than rodents that we are aware of. It is not known with absolute certainty that this compound does not cross the BBB in the bovine. Direct studies demonstrating its absence in the brain after peripheral administration in cattle, or indirect studies showing blockage of a peripherally mediated opioid effect, such as constipation, while simultaneously not blocking a centrally- mediated effect such as anti-nociception would be required to prove its inability to cross the BBB in this species with certainty. Studies such as the latter would be difficult due to the lack of behavioral models in cattle that exist for rodents to quantitatively measure pain response.

In conclusion, the present study represents the first information regarding a physiological role for endogenous opioids in dairy cattle. The results indicate that endogenous opioids play a role in regulation of basal PRL secretion in Holstein calves, and this regulation differs with season. The effect of endogenous opioids on basal PRL release appears to be mediated within the BBB, as the peripheral antagonist MLM had no effect on basal PRL secretion. This is in contrast to the opioid agonist DAMME, which, as reported in Chapter 1, appears to increases PRL secretion by occupying receptor sites somewhere in the periphery. This may indicate different regulation of PRL release by exogenous opioids, which is generally a pharmacological dose of opioids, as compared to regulation of PRL release by endogenous opioids. The pharmacologically elevated plasma levels

of a peripherally administered opioid may allow activation of opioid receptors at a peripheral site, such as the ME. In regard to this apparent differential site of action between exogenous and endogenous opioids on facilitating PRL secretion, the dose of MLM used in these studies should also be considered, due to the previously described differences between MLM and NAL in potency for the mu opioid receptor (ie, NAL being more potent), which, as also discussed previously, appears to be the primary opioid receptor involved in PRL release.

Endogenous opioids may also be involved in regulation of basal GH secretion in Holstein calves in fall, however the reported discrepancies over the half-life of NAL and the failure of NAL to affect GH release at five different doses in spring make this finding inconclusive. The peripheral opioid antagonist MLM significantly elevated plasma GH in Holstein calves in fall and spring. The mechanism of action of MLM on pituitary GH secretion appears to be agonistic, rather than antagonistic, and the opioid receptors mediating this effect are likely located somewhere in the periphery, due to the inability of this drug to readily cross the BBB.

SUMMARY

Two studies were carried out, in fall and spring, to determine if endogenous opioids are involved in basal secretion of growth hormone (GH) and prolactin (PRL) in Holstein dairy calves, and if so, whether the endogenous opioid receptors involved in mediating basal secretion of of these pituitary hormones were located within or outside the blood brain barrier (BBB).

In fall, the lipid soluble opioid antagonist naloxone (NAL), which readily crosses the BBB, increased secretion of both basal GH and PRL at a dose of 1.0 mg/kg. The effect of NAL on GH secretion, however, did not occur untill some 3 hrs after NAL administration, making this result suspect due to controversy over the half-life of NAL. The peripherally selective opioid antagonist methyl levallorphan mesilate (MLM), also at 1.0 mg/kg, did not affect PRL secretion in fall, but increased GH secretion. These results contradicted GH and PRL responses to opioid antagonists seen in other species. Therefore, the spring study incorporated a range of antagonist doses (.10, .25, .50, 1.0, and 2.0 mg/kg) similar to those used in other studies of this type, to determine if fall results were repeatable.

In spring, NAL treatment affected PRL secretion only at the same dose used in fall (1.0 mg/kg), but this time decreased plasma PRL concentrations. Naloxone did not affect plasma GH secretion at any dose in spring. Plasma PRL concentrations were again unchanged by MLM at all doses in spring, but plasma GH concentrations in Holstein calves in spring were increased by MLM at .25, .50, and 2.0 mg/kg. However, the physiological mechanism whereby MLM affected GH secretion in this study was believed to be through agonism, not antagonism, of the endogenous opioid receptors regulating plasma GH secretion.

In conclusion, these studies provide the first evidence of endogenous opioid regulation of basal GH and PRL in dairy animals. Results indicate that opioids play a role in basal PRL secretion somwhere within the BBB in Holstein calves, and this regulation is different in fall and spring. Prior

studies in rodents have indicated that changes in PRL secretion by opioids are most likely mediated through the mu opioid receptor. Because NAL has its greatest affinity for the mu receptor, and due to the fact NAL had a more dramatic and conclusive effect on basal PRL release than basal GH, it is possible that opioid-mediated changes in basal PRL release occur through mu receptors in this species as well.

As described, the effect of antagonists on basal GH release in this study are less conclusive. Endogenous opioids may be involved with basal GH secretion somewhere within the central nervous system in fall, but questions as to the half-life of NAL, and failure of NAL to significantly affect GH secretion at any dose in spring makes this finding suspect. Similarly the profound effect on GH secretion in Holstein calves by the peripheral opioid antagonist MLM in this study indicates agonism, not antagonism, of endogenous opioid receptors involved in GH secretion, at a site somewhere outside the BBB.

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TABLE 1. PLASMA PROLACTIN AND GROWTH HORMONE CONCENTRATIONS* IN HOLSTEIN CALVES BEFORE AND AFTER INFUSION WITH OPIOID ANTAGONISTS (1.0 MG/KG) OR SALINE (FALL).

Group	No. calves	Period	Prolactin (ng/ml)	Growth Hormone (ng/ml)
Saline	6	1	1.9 ± .4	10.8 ± 1
		2	2.6 ± .5	14.3 ± 1
		3	1.7 ± .2	13.4 ± 2
Naloxone	6	1	1.6 ± .4[a]	7.7 ± 1[a]
		2	3.6 ± .4[c]	12.5 ± 1[a,b]
		3	1.7 ± .6[a]	14.4 ± 2[b]
MLM[1]	5	1	1.8 ± .3	10.1 ± 1[a]
		2	2.3 ± 1	22.0 ± 1[c]
		3	1.9 ± .5	19.9 ± 1[c]

* = Least squares means, ± sem. [a,b] Means with different superscripts within groups differ (P<.05).
[a,c] Means with different superscripts within groups differ (P<.01).
[1] Calf 519 omitted. MLM = Methyl levallorphan mesilate.

TABLE 2. PLASMA PROLACTIN CONCENTRATIONS* IN HOLSTEIN CALVES BEFORE AND AFTER INFUSION WITH OPIOID ANTAGONISTS (SPRING).

Group	No. calves	Period	Dose (mg/kg) .10	.25	.50	1.0	2.0
			ng/ml				
Naloxone	20(4/dose)	1	9.9 ± 1.2	9.7 ± 1.2	11.9 ± 1.2	12.5 ± 1.2[a]	10.2 ± 1.2
		2	11.2 ± 1.3	10.7 ± 1.3	8.8 ± 1.3	10.3 ± 1.3[a,b]	9.9 ± 1.3
		3	8.8 ± 2.1	8.4 ± 1.7	8.2 ± 1.7	6.9 ± 1.7[b]	8.6 ± 1.7
MLM	20(4/dose)	1	12.9 ± 1.2	9.8 ± 1.2	11.3 ± 1.2	12.1 ± 1.2	12.4 ± 1.2
		2	11.2 ± 1.3	10.1 ± 1.3	11.4 ± 1.3	8.6 ± 1.3	9.0 ± 1.3
		3	9.9 ± 1.7	8.0 ± 1.8	8.7 ± 1.7	7.6 ± 1.7	8.6 ± 1.7

* = Least squares means, ± sem.
[a,b] Means with different superscripts within groups and dose differ (P<.05).
MLM = Methyl levallorphan mesilate.

TABLE 3. PLASMA GROWTH HORMONE CONCENTRATIONS* IN HOLSTEIN CALVES BEFORE AND AFTER INFUSION WITH OPIOID ANTAGONISTS (SPRING).

Group	No. calves	Period	Dose (mg/kg) .10	.25	.50	1.0	2.0
			ng/ml				
Naloxone	20(4/dose)	1	14.7 ± 1.9	17.2 ± 1.9	19.2 ± 1.9	18.8 ± 1.9	27.4 ± 1.9
		2	19.2 ± 2.1	17.4 ± 2.1	24.7 ± 2.1	20.8 ± 2.1	27.4 ± 2.1
		3	18.5 ± 3.3	20.1 ± 2.7	22.0 ± 2.7	23.7 ± 2.7	29.3 ± 2.7
MLM	20(4/dose)	1	16.1 ± 1.9	15.5 ± 1.9[a]	18.1 ± 1.9[a]	17.8 ± 1.9	21.5 ± 1.9[a]
		2	21.7 ± 2.1	23.1 ± 2.1[b]	25.8 ± 2.1[b]	23.3 ± 2.1	29.2 ± 2.1[b]
		3	16.2 ± 2.7	20.6 ± 2.7[a,b]	20.3 ± 2.7[a,b]	23.7 ± 2.7	30.3 ± 2.7[b]

* - Least squares means, ± sem.
[a,b], Means with different superscripts within groups and dose differ (P<.05 or less).
MLM - Methyl levallorphan mesilate.

TABLE 4. PLASMA INSULIN CONCENTRATIONS* IN HOLSTEIN CALVES BEFORE AND AFTER INFUSION WITH OPIOID ANTAGONISTS (SPRING).

Group	No. calves	Period	Dose (mg/kg) .10	1.0	2.0
Naloxone	6	1	.83 ± .3	.88 ± .3	.66 ± .3
	(2/dose)	2	.75 ± .3	.79 ± .3	.71 ± .3
		3	.80 ± .4	.88 ± .4	.71 ± .4
MLM	6	1	.84 ± .3	.71 ± .3	.72 ± .3
	(2/dose)	2	.79 ± .3	.63 ± .3	.76 ± .3
		3	.72 ± .4	.72 ± .4	.63 ± .4

* = Least squares means, ± sem.
MLM = Methyl levallorphan mesilate.

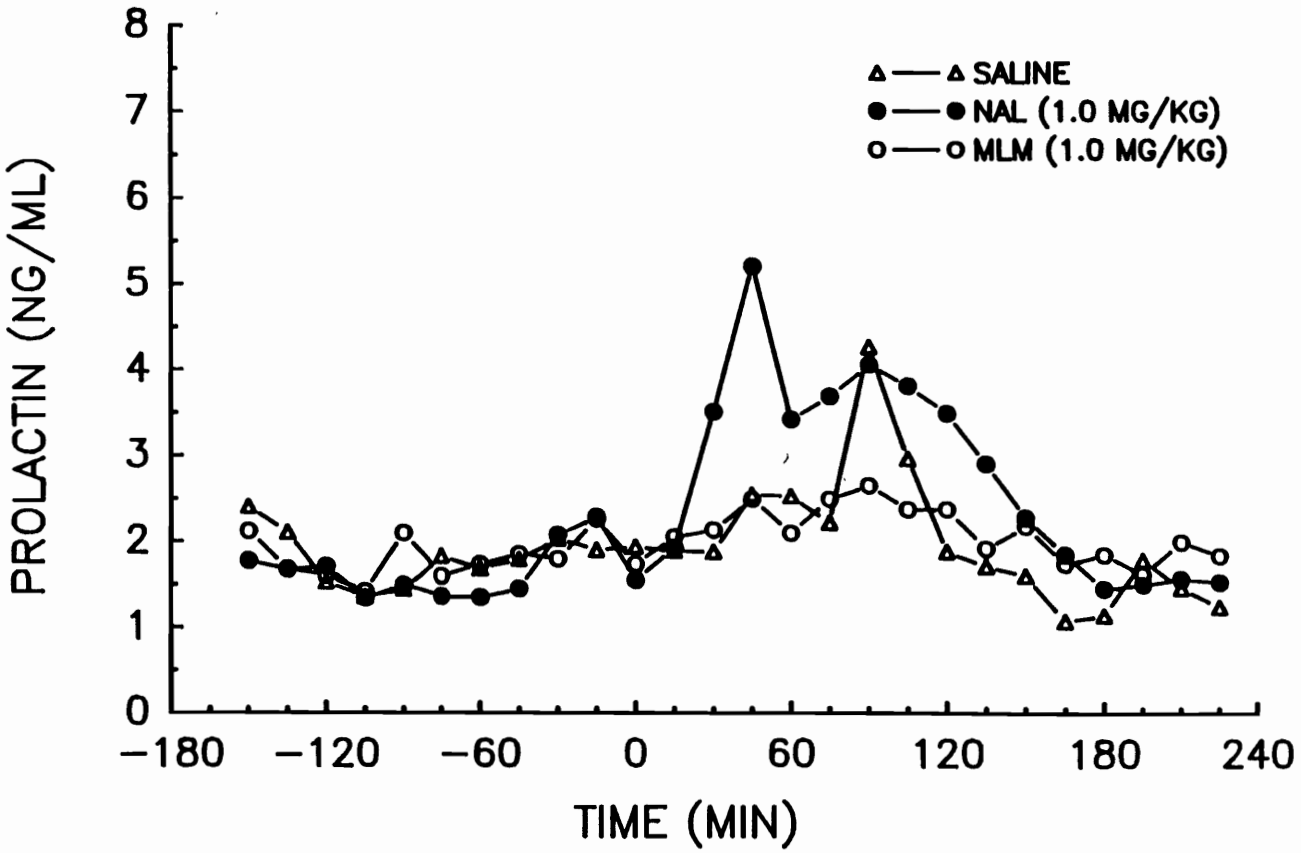

Figure 1. Plasma prolactin concentrations in Holstein calves before (period 1) and after (periods 2 and 3) naloxone (NAL), methyl levallorphan mesilate (MLM), or saline injection in fall season. All treatments injected at time=0. Period 1= -150 to 0 minutes; period 2= 15 to 165 minutes; period 3= 180 to 225 minutes.

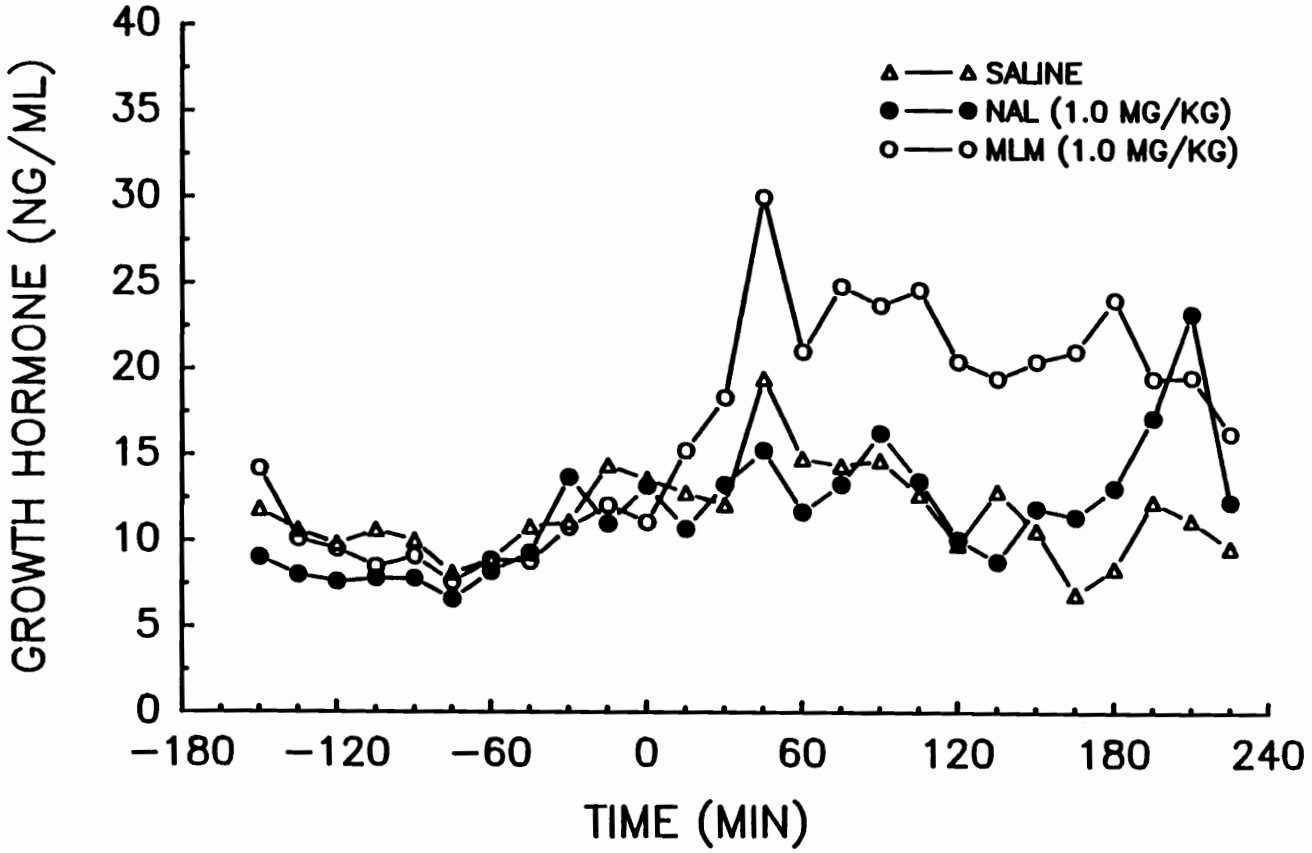

Figure 2. Plasma growth hormone concentrations in Holstein calves before (period 1) and after (periods 2 and 3) naloxone (NAL), methyl levallorphan mesilate (MLM), or saline injection in fall season. All treatments injected at time=0. Period 1= -150 to 0 minutes; period 2= 15 to 165 minutes; period 3= 180 to 225 minutes.

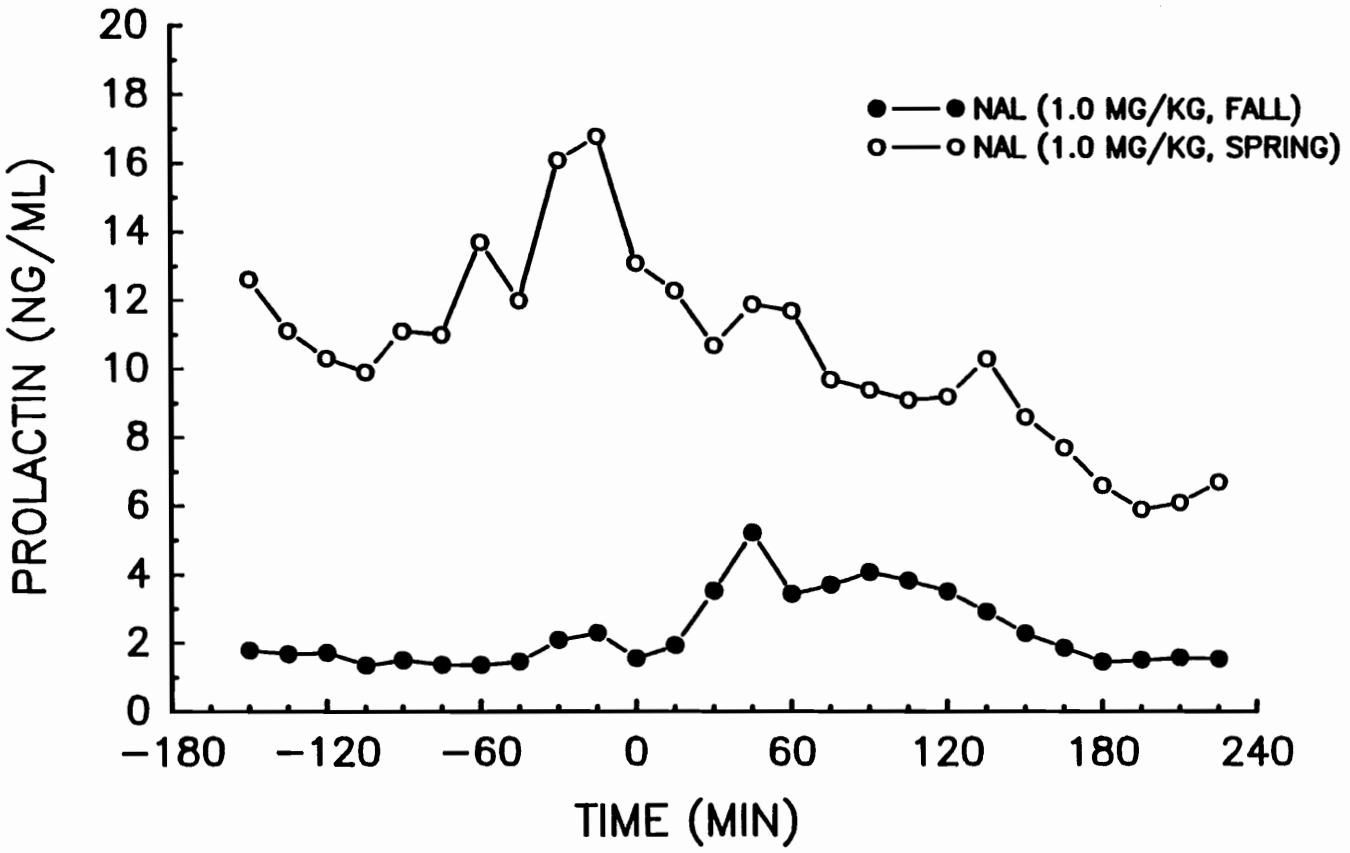

Figure 3. Plasma prolactin concentrations in Holstein calves before (period 1) and after (periods 2 and 3) naloxone (NAL) injection in both spring and fall season. Naloxone injected at time=0. Period 1= -150 to 0 minutes; period 2= 15 to 165 minutes; period 3= 180 to 225 minutes.

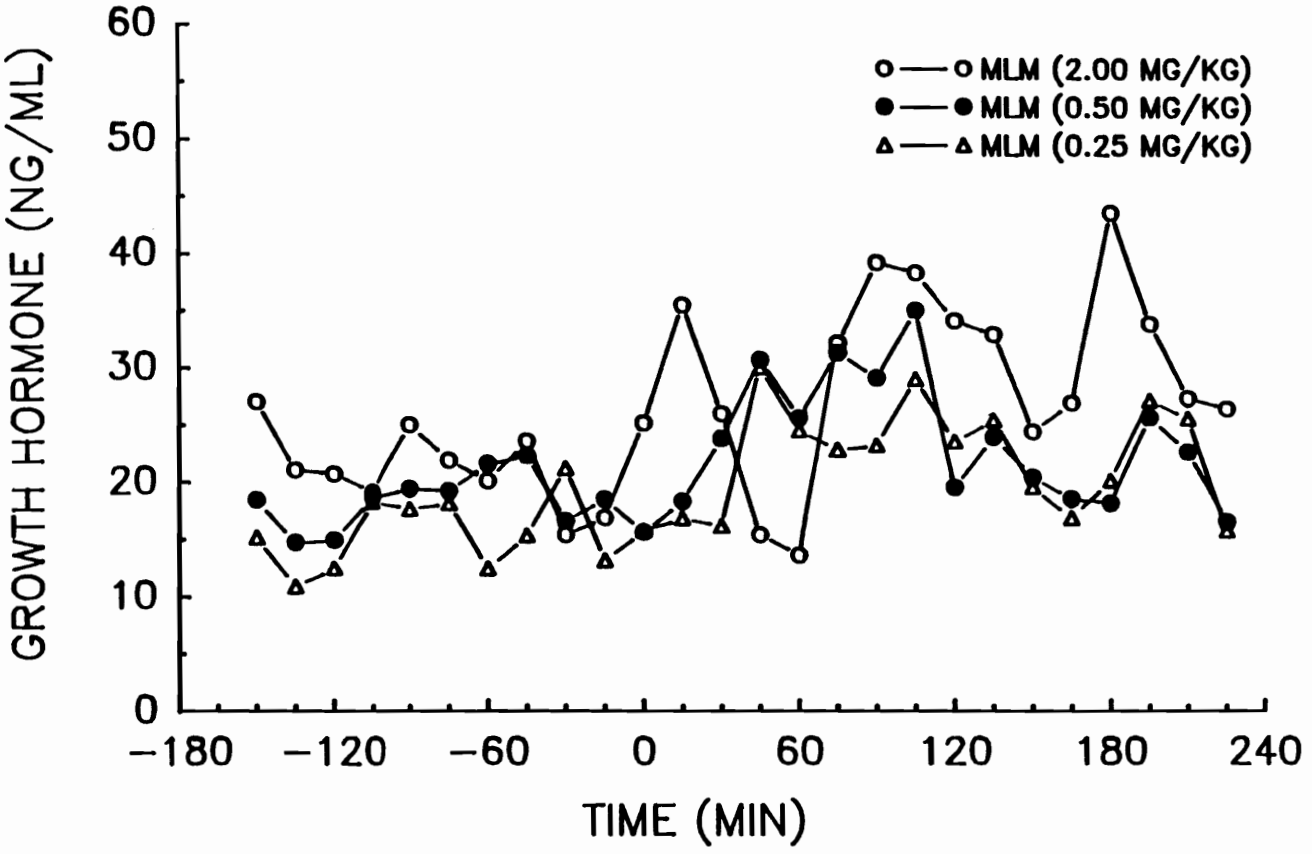

Figure 4. Plasma growth hormone concentrations in Holstein calves before (period 1) and after (periods 2 and 3) different doses of methyl levallorphan mesilate (MLM) injection in spring season. All doses injected at time=0. Period 1= -150 to 0 minutes; period 2= 15 to 165 minutes; period 3= 180 to 225 minutes.

CHAPTER 3

EXOGENOUS OPIOIDS INCREASE PLASMA PROLACTIN IN HOLSTEIN CALVES PRIMARILY VIA A DOPAMINERGIC MECHANISM

INTRODUCTION

As discussed in Chapter 1, since the identification of the first endogenous opioids in 1975 (Hughes et al.), one of the most commonly observed endocrine changes after opioid or opiate agonist administration in rats (Bruni et al., 1977; Rivier et al., 1977; Shaar and Clemens, 1980), primates (Graffenreid et al., 1978; Gold et al., 1979; Catlin et al., 1980) or ruminants (Hart and Cowie, 1978; Bolton et al., 1983; McMillen and Deayton, 1989) is increased circulating plasma prolactin (PRL) concentrations.

The secretion of adenohypophysial hormones in general is regulated by releasing and inhibiting factors or hormones from the hypothalamus. Regulation of pituitary PRL release appears to be mediated primarily via chronic inhibition by the catecholamine dopamine (DA), and the source of this DA is the tuberoinfindibular neurons (Hokfelt and Fuxe, 1972). The cell bodies of these neurons are located primarily in the paraventricular and arcuate nuclei of the hypothalamus, and their axons project down to the external layer of the median emminence (ME), where they terminate near perivascular spaces of the primary capillary loops of the hypophysial portal system (Moore, 1987). Dopamine released from these neurons is therefore able to travel directly to the adenohypophysis, where it activates receptors on lactotrophs and thereby inhibits PRL release.

The mechanism of action whereby opioids or opiates affect PRL secretion remains somewhat controversial. As also described in Chapter 1, opioids and opiates administered peripherally

probably do not affect PRL secretion directly, as addition of these compounds to pituitary cell cultures is generally without effect on release of this hormone (Rivier et al., 1977; Shaar et al., 1977; Besser et al., 1979; Grandison et al., 1980).

Several studies have indicated that exogenous opioids or opiates increase circulating plasma PRL by affecting hypothalamic DA turnover, and/or decreasing the level of DA in hypophysial portal blood (Gudelsky and Porter, 1979; Deyo et al., 1979; Van Loon et al., 1980; Haskins et al., 1981; Reymond et al., 1983). In contrast, other investigators have shown that changes in plasma PRL concentrations after opioid or opiate administration cannot be completely accounted for by dopaminergic mechanisms (Arita and Porter, 1983; Shin et al., 1988; Yogev et al., 1989).

In Chapter 1, it was demonstrated that the synthetic enkephalin DAMME (Roemer et al., 1977) significantly increases plasma PRL concentrations in Holstein calves immediately after peripheral injection. The objective of the present study was to determine if DAMME increases plasma PRL in Holstein calves by decreasing the amount of DA in the environment of the anterior pituitary or hypothalamus, by measuring plasma PRL after DAMME injection alone, and after DAMME or thyrotropin releasing hormone (TRH) administration in animals pre-treated with the long-acting dopamine agonist CB154.

MATERIALS AND METHODS

Animals. This study was conducted at the Virginia Tech dairy research facility on the campus of Virginia Tech in spring, 1990. Twenty four Holstein calves ranging from 5 to 7 months of age and averaging 161 kg BW, were acquired from the university herd and randomly assigned to 1 of 4 treatments (6 calves/treatment): 1) Saline injection or 2) DAMME injection (D-Ala^2,N-Me-Phe^4,Met(O)^5-ol enkephalin, 6.0 ug/kg in saline, Sigma Chemical Co., St. Louis, Mo.) or 3) DAMME (6.0 ug/kg) injection after pre-treatment with CB154 (2-Br-α ergocryptine, .20 mg/kg in 80% ethanol, Sigma Chemical, St.Louis, Mo.), or 4) thyrotropin-releasing hormone

(TRH) injection (200ug in saline, Sigma Chemical Co., St.Louis, Mo) following pre-treatment with CB154.

All animals were housed in sheltered pens at the university heifer barn. The day prior to establishment of baseline PRL levels, calves were haltered and weighed, and subsequently tied into individual stanchions with free access to water and alfalfa hay. On the following day (day 1), indwelling jugular cannulae were installed in all calves at 0600h. Clotting of blood in cannulation tubing was prevented by infusion of a 3.5% citrate solution after cannulation, and immediately after each sample was drawn. External tubing with sample ports was securely taped to the side of each animals neck for easy access. At 1100h, serial bleeding every 15 min commenced. The initial 1 ml of each sample was discarded, and a 10 ml sample was then drawn and placed into collection tubes containing 200ul of a 3.5% EDTA solution. Blood samples were placed in ice immediately after withdrawal, and centrifuged for 30 min at 1800 x g. Plasma was decanted and frozen at -20 C until assayed for PRL. Animals were sampled for a period of 2 hr, and sampling terminated after the 1300h sample was drawn. All calves assigned to recieve CB154 were injected s.c 3hr after the last sample was drawn on day 1.

On day 2, serial bleeding through jugular cannulae every 15 min was again commenced at 1100h. Sampling continued for 2 hrs, at which time all treatments were administered (immediately after the 1300h sample was drawn). Treatments (saline, DAMME, and TRH) were administered as a single bolus through the cannula ports. Sampling every 15 min was continued until 1730h. Protocol for plasma collection, storage, and PRL assay was identical to day 1.

Hormone Determination. Prolactin was quantified by a double-antibody radioimmunoassay according to the procedure of Barnes et al. (1985). Prolactin intra- and interassay coefficients of variation (CV) were 6.3% and 8.0%, respectively.

Statistical Analysis. Blood hormone data were analyzed by least-squares analysis of variance with the GLM procedure of the Statistical Analysis Systems (SAS), SAS Institute, Cary, N.C. Two data sets were used in the analysis. The first set contained data from both day 1 and day 2. Samples taken from 1100h to 1300h on day 1 were designated as period 1; samples taken from 1100h to 1300h (basal period) on day 2 represented period 2; samples collected from 1315 to 1500h on day

2 were designated period 3, while samples taken from 1515h to 1730h on day 2 were designated period 4. The second data set contained only samples taken on day 2. Samples taken from 1100h to 1300h (basal period) were designated period 1 samples. Period 2 represented samples taken over a 1 hr and 45 min period after treatment administration (from 1315h to 1500h; treatment period), and period 3 represented samples taken from 1515h to 1730h, at which time sampling was terminated.

The statistical model used to analyze all data contained treatment, calf within treatment, period, treatment x period, period x calf within treatment, sample within period, and treatment x sample within period. Treatment was tested using calf within treatment as the error term; period and treatment x period were tested using period x calf within treatment as the error term. Fixed effects were treatment and period. In the first data set, non-orthogonal contrasts were used to compare least squares means for periods 1 vs 2, within treatments, to determine the effectiveness of CB154 on basal PRL levels. Non- orthogonal contrasts were used in the second data set to compare periods 1 vs 2, 1 vs 3, and 2 vs 3, all within treatments. All critical F values to determine significance of contrasts were adjusted using the Improved Bonferroni F test (Games, 1977).

RESULTS

Calves injected with CB154 after sampling was terminated on day 1 had lower ($P<.01$) plasma PRL levels on day 2 (Table 1). Plasma prolactin in calves not receiving CB154 were not different between day 1 and day 2 (Table 1). The synthetic enkephalin DAMME increased ($P<.01$) plasma PRL in calves not pre-treated with CB154 (Table 2). This increase was characterized by an immediate surge in plasma PRL, followed by a gradual decline back to or below pre-treatment levels over a 3 hr period (Figure 1). This response is similar to the DAMME-mediated PRL response seen in other studies with ruminants (Bolton et al., 1983; Johnson et al., 1989). Plasma PRL was not different after DAMME injection on day 2 in calves pre-treated with CB154 on day 1 (Table 2).

Therefore, the presence of the DA agonist was able to block the pituitary response to the synthetic enkephalin (Figure 2).

Interestingly, the tri-peptide TRH was able to increase ($P<.05$) plasma PRL in calves pre-treated with CB154 (Table 2), which is in agreement with the effects of TRH on lactating cows pre-treated with CB154 (Beck et al., 1979). This PRL response was similar to that of calves receiving only DAMME injection, being characterized by an abrupt increase, and gradual decline to near pre-treatment levels over a 3 to 4 hr period (Figure 3).

DISCUSSION

The effects of CB154 on plasma PRL in Holstein calves here is in agreement with other cattle studies (Beck et al., 1979; Akers et al., 1981). A decrease in plasma PRL in after injection of a DA agonist seems logical, as release of PRL from the anterior pituitary is thought to be controlled primarily by chronic inhibition at the pituitary level by DA released into hypophysial portal blood from tuberoinfindibular neurons (TIDA), the cell bodies of which lie primarily in the arcuate nucleus, with axons terminating near perivascular spaces of the primary capillary loops of the hypophysial portal system (Hokfelt and Fuxe, 1972; Moore, 1987). The existance of DA receptors on the pituitary gland, the association of DA with PRL secretory granules, and the observation that concentrations of DA in hypophysial portal blood is sufficient to inhibit PRL secretion in vivo are supportive of this concept (Gibbs and Neal, 1978; Goldsmith et al., 1979; Nansel et al., 1979).

As mentioned previously, exogenous opioids probably do not affect PRL secretion directly at the pituitary, but instead, they likely increase PRL secretion by increasing hypothalamic DA turnover, and decreasing DA secreted into hypophysial portal blood. For example, Gudelsky and Porter (1979) measured DA in basal pituitary stalk blood in saline-treated ovarioectomized rats, and in rats receiving morphine, β-endorphin, and a synthetic enkephalin analogue. Morphine and the opioid peptides produced an 85 to 95% reduction in DA concentration in pituitary stalk blood, compared

to rats receiving the saline. Pre-treatment with the opioid antagonist naloxone (NAL) prevented the opioid- mediated reduction in hypophysial portal blood DA.

Van Loon et al. (1980) found that after catecholamine synthesis inhibition with α-methyltyrosine, β-endorphin inhibited the decline in hypothalamic DA concentration, and concluded that β-endorphin produced this effect by inhibiting the release of hypothalamic DA from nerve endings. Haskins et al. (1981) demonstrated a 70 to 80% reduction of DA concentration in the hypophysial portal blood of rats after ejection of morphine ions iontophoretically into the arcuate nucleus. Reymond et al. (1983) administered morphine intracerebroventricularly to rats and measured a 90% reduction in the rate of release of hypothalamic DA into hypophysial portal blood over a subsequent 60 min period. With regard to the DA agonist used in this study, McLeod and Lehmeyer (1974) demonstrated that CB154 inhibits PRL release from pituitary cell cultures, and that this action could be blocked by the DA receptor antagonist haloperidol. This strongly suggests that CB154 can act as a DA agonist directly at pituitary DA receptors. Therefore, if one accepts the theory that opioids increase PRL secretion by decreasing DA reaching the anterior pituitary, the ability of CB154 to agonize pituitary DA receptors would explain the inability of DAMME to significantly increase PRL in the presence of CB154 in this study. Our results are in agreement with those of Takahara et al. (1978) in which male rats injected intracerebroventricularly with βendorphin had significant increases in plasma PRL concentrations, but these increases were completely blocked by pre-treatment with CB154.

In contrast to the above physiological explanation for opioid-mediated increases in plasma PRL, other studies have shown that the decreased level of DA in hypophysial portal blood seen after opiate administration cannot completely account for subsequent increases in PRL. Arita and Porter (1983) demonstrated that morphine increased PRL and decreased DA in hypophysial portal blood of rats, but that infusion of DA to raise portal blood DA levels to pre-treatment concentrations could only suppress the morphine-evoked PRL increase by 52 to 75%. Shin et al. (1988) found that morphine was still able to increase PRL in rats which were treated with large doses of pimozide, and were therefore without functional dopaminergic receptors. Yogev et al. (1989) reported that administration of pimozide to rats increased PRL secretion, but injection of the opioid

antagonist naltrexone in pimozide-treated rats reduced the PRL response to pimozide, indicating that endogenous opioid pathways may affect pituitary PRL secretion through some hypothalamic neurotransmitter other than DA.

These studies together suggest that exogenous opioids likely increase PRL secretion by at least 2 mechanisms, ie. by the known effect of decreasing hypophysial DA levels, and some action which does not involve a dopaminergic mechanism. One possible alternative mechanism is opioid-mediated release of one or more of the known hypothalamic PRL-releasing factors (del Pozo and Brownell 1979). Some evidence for this is shown by the fact that serotonergic neurons stimulate the release of PRL-releasing factors (Clemens, 1978), and interruption of serotonergic neurotransmission has been found to antagonize the morphine- induced release of PRL (Koenig et al., 1979; Spampinato et al., 1979).

Assuming the dose of CB154 used in this study (.2 mg/kg) was pharmacological, then it is likely all pituitary DA receptors were exposed to this DA agonist. Yet, there was a transient, albeit insignificant, increase in plasma PRL following DAMME injection in calves pre-treated with CB154 (Figure 2). In light of the above studies, it seems possible that the opioid agonist DAMME may increase plasma PRL in Holstein calves not only by decreasing DA concentrations reaching the pituitary (as indicated by blockage by CB154), but less significantly, via stimulation of release of one or more hypothalamic PRL-releasing factors, which might account for the small post-DAMME increase in PRL in CB154-treated calves. The fact that TRH, a known hypothalamic PRL-releasing factor, increased PRL in calves treated with CB154 in this study demonstrates that the pituitary is able to respond to hypothalamic peptides while under strong dopaminergic stimulation.

In conclusion, it appears that the opioid agonist DAMME facilitates increased PRL secretion in Holstein calves primarily by decreasing the amount of DA reaching the pituitary. It is possible, however, that a secondary, physiologically less significant mechanism of action may involve DAMME-induced release of one or more hypothalamic PRL-releasing factors, although further studies are required to confirm this.

SUMMARY

A study was undertaken to determine whether previously seen increases in plasma prolactin (PRL) concentrations in Holstein calves after administration of the opioid agonist DAMME could be accounted for by a decreased amount of endogenous dopamine (DA), the primary PRL-releasing antagonist, in the pituitary environment.

Three hours after completion of sampling on day 1 to establish baseline plasma PRL concentrations, calves selected to recieve the long acting dopamine agonist CB154 were injected with this compound. On day 2, calves not previously treated with CB154 were injected with saline or DAMME. Calves treated on day 1 with CB154 recieved either DAMME or thyrotropin releasing-hormone (TRH) injection on day 2.

The DA agonist CB154 significantly decreased plasma PRL concentrations from day 1 to day 2 in calves receiving this compound. Calves injected on day 2 with DAMME which had not been previously injected on day 1 with CB154 responded to the opioid agonist with significant increases in plasma PRL concentrations. Calves injected with DAMME on day 2 which had recieved CB154 on day 1 did not respond to DAMME with increased plasma PRL. A second group of calves which had been injected on day 1 with CB154, recieved TRH injection on day 2, and responded to TRH with significant increases in plasma PRL concentrations.

In conclusion, the inability of calves pre-treated with a long acting DA agonist to respond to the opioid agonist DAMME with increased plasma PRL concentrations, as did calves not recieving CB154, indicates that DAMME facilitates increased plasma PRL primarily by decreasing DA reaching the pituitary. The fact that TRH was able to increase PRL secretion in the presence of CB154 indicates that the pituitary glands of Holstein calves are capable of responding to PRL-releasing factors while under strong dopaminergic stimulation.

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TABLE 1. COMPARISON OF BASAL PLASMA PROLACTIN CONCENTRATIONS ON DAY 1 (PRIOR TO CB154 INJECTION) WITH BASAL PLASMA PROLACTIN CONCENTRATIONS ON DAY 2 (AFTER CB154 INJECTION, BUT PRIOR TO SALINE, DAMME, OR TRH INJECTIONS).

Group	No. calves	Day 1	Day 2
SALINE	6	13.4 ± 2.3	15.8 ± 2.3
DAMME	6	18.7 ± 2.2	16.7 ± 2.3
CB154/DAMME	6	13.3 ± 2.2[a]	.81 ± 2.3[b]
CB154/TRH	6	13.7 ± 2.2[a]	2.4 ± 2.3[b]

* = Least squares means, ± sem.
[a,b] Means with different superscripts in same row differ (P<.01).
DAMME = D-Ala^2,N-Me-Phe^4,Met(O)^5-ol enkephalin (6 ug/kg).
CB154 = 2-Br-alpha-ergocryptine (.20 mg/kg).
TRH = Thyrotropin-releasing hormone (200 ug).

TABLE 2. PLASMA PROLACTIN CONCENTRATIONS* IN HOLSTEIN CALVES BEFORE (PERIOD 1) AND AFTER (PERIODS 2 AND 3) SALINE, DAMME OR TRH INJECTION (DAY 2).

Group	No. calves	Period	Prolactin (ng/ml)
SALINE	6	1	15.8 ± 2.2
		2	13.3 ± 1.9
		3	14.2 ± 2.4
DAMME	6	1	16.7 ± 2.2[a]
		2	36.0 ± 1.9[b]
		3	10.8 ± 2.4[a]
CB154/DAMME	6	1	.8 ± 2.1
		2	1.1 ± 1.9
		3	.9 ± 2.4
CB154/TRH	6	1	2.4 ± 2.1[a]
		2	16.6 ± 1.9[b]
		3	5.6 ± 2.4[a]

* = Least squares means, ± sem.
[a,b] Means with different superscripts differ (P<.01).
DAMME = D-Ala^2,N-Me-Phe^4,Met(O)^5-ol enkephalin (6 ug/kg).
CB154 = 2-Br-alpha ergocryptine (.20 mg/kg).
TRH = Thyrotropin-releasing hormone.

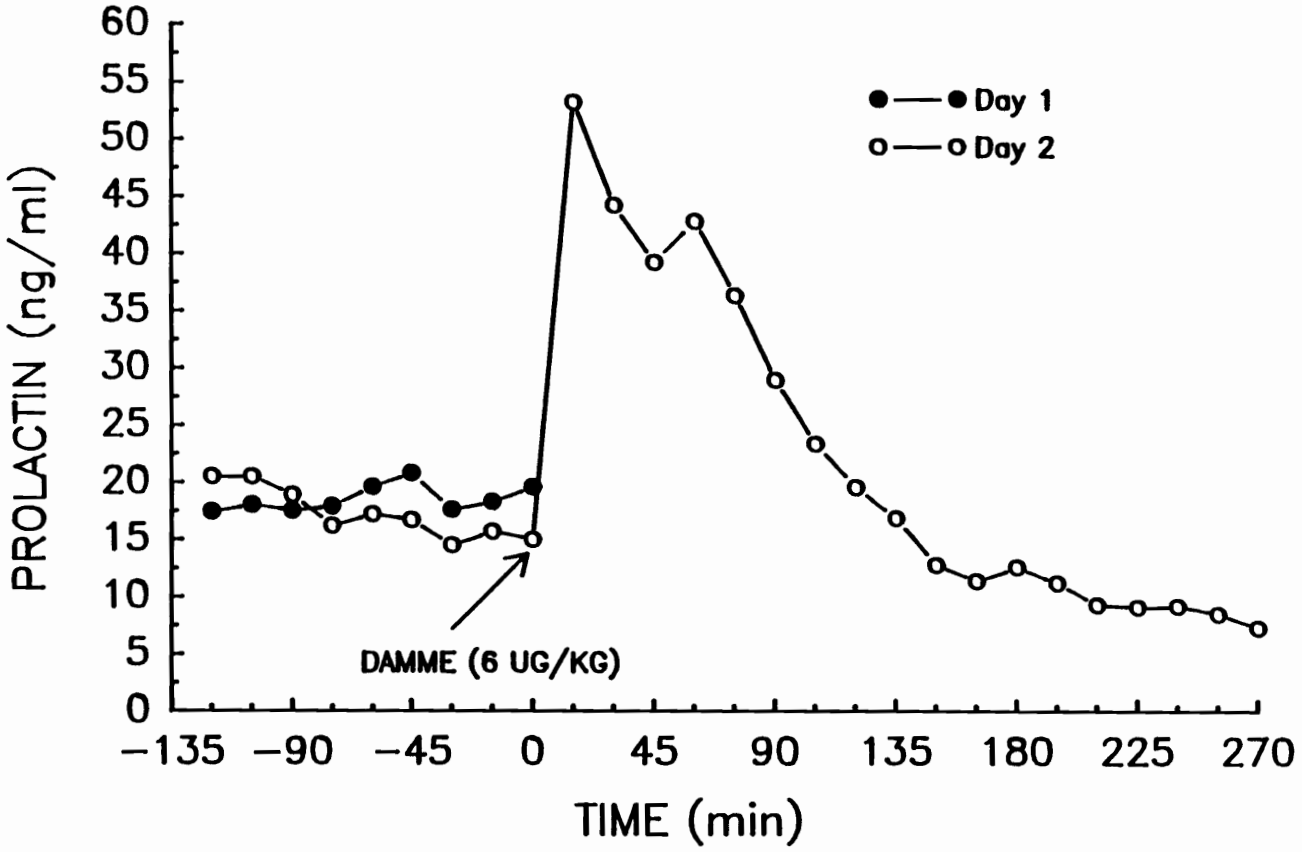

Figure 1. Comparison of day 1 and day 2 basal prolactin levels, and the effect of DAMME injection (time=0) on prolactin release in Holstein calves on day 2. Period 1= -120 to 0 minutes; period 2= 15 to 120 minutes; period 3= 135 to 270 minutes.

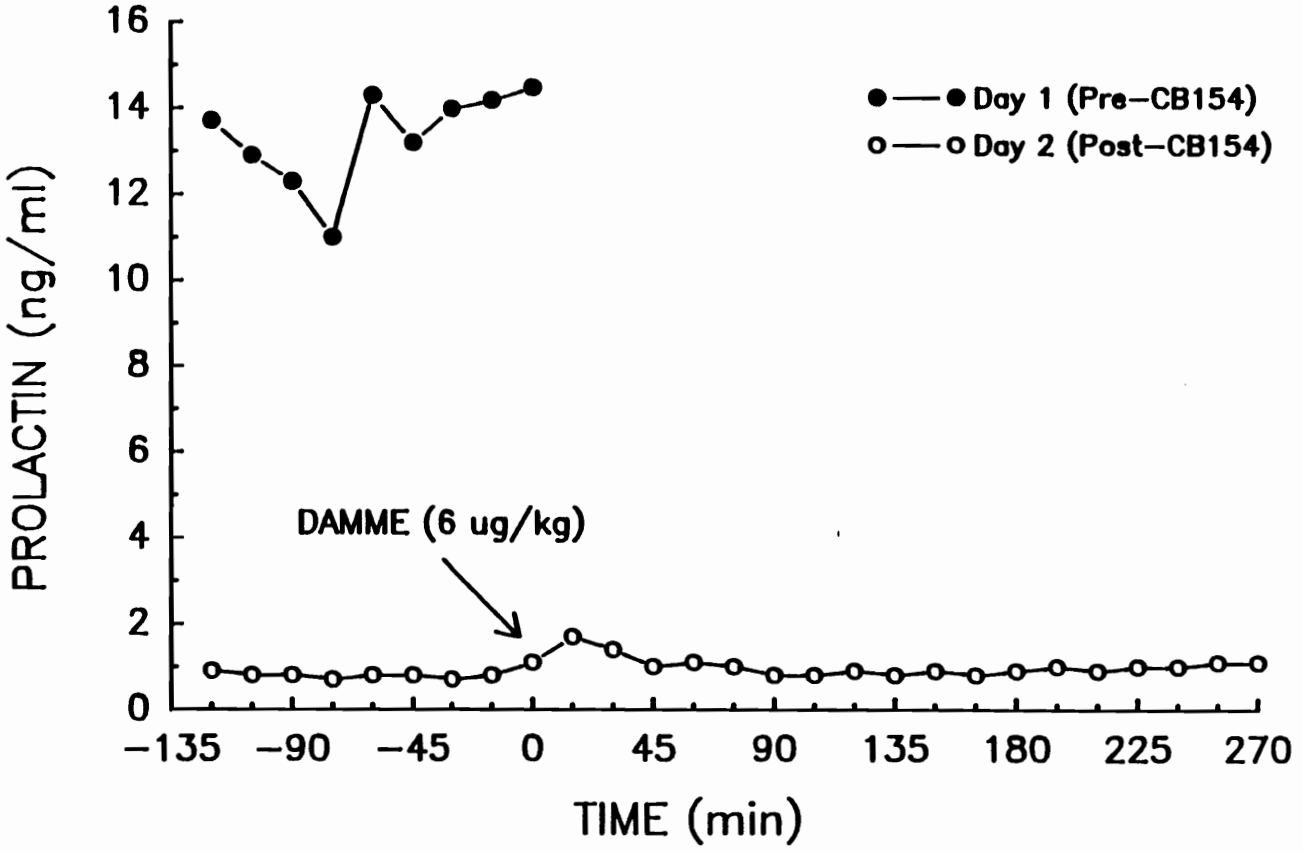

Figure 2. Plasma prolactin concentrations in Holstein calves before (day 1) and after (day 2) CB154 injection, and the effect of DAMME injection (time=0) on prolactin release on day 2. Period 1= -120 to 0 minutes; period 2= 15 to 120 minutes; period 3= 135 to 270 minutes.

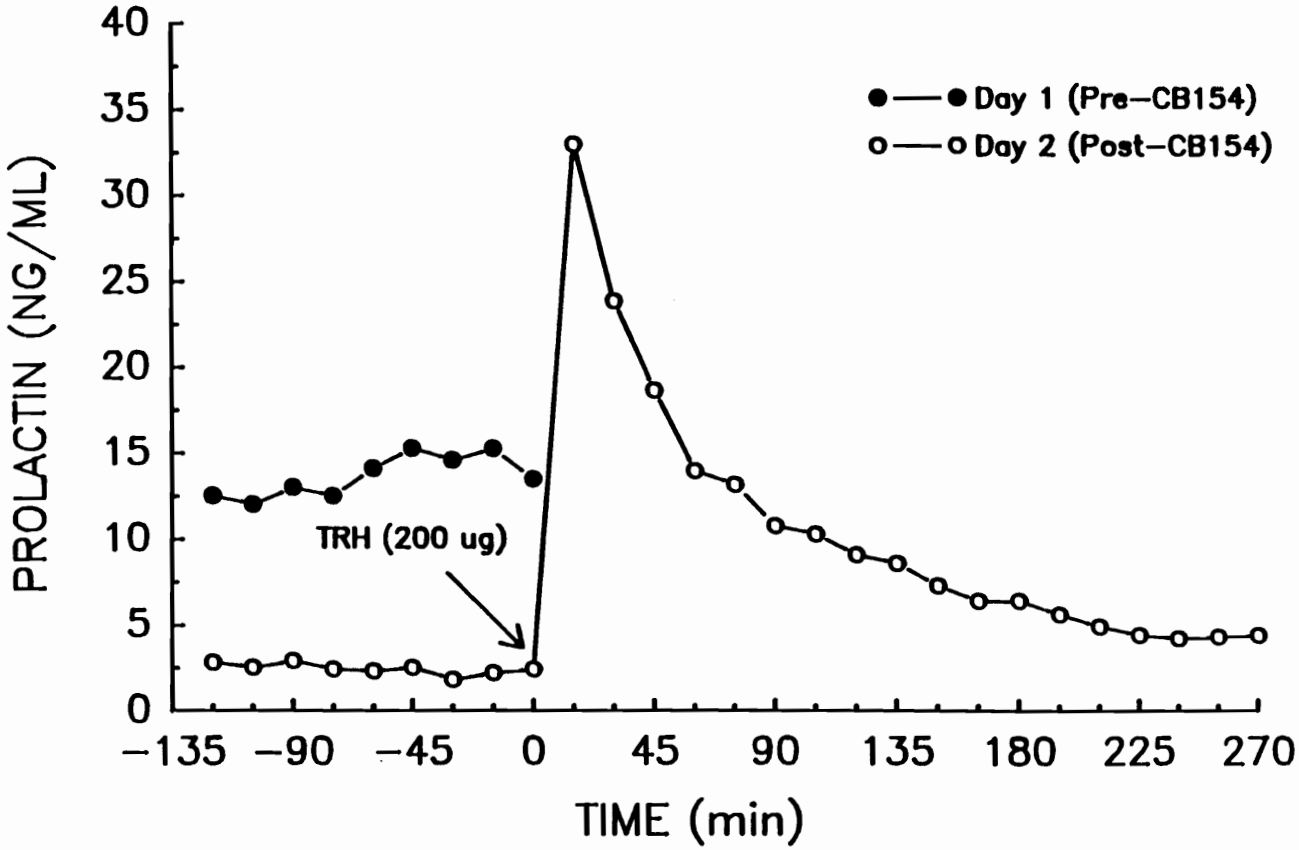

Figure 3. Plasma prolactin concentrations in Holstein calves before (day 1) and after (day 2) CB154 injection, and the effect of thyrotropin-releasing hormone injection (time=0) on prolactin release on day 2. Period 1= -120 to 0 minutes; period 2= 15 to 120 minutes; period 3= 135 to 270 minutes.

CHAPTER 4

EVALUATION OF THE ROLE OF GROWTH HORMONE-RELEASING FACTOR IN OPIOID-MEDIATED GROWTH HORMONE RELEASE IN HOLSTEIN CALVES

INTRODUCTION

As discussed in chapter 1, one of the endocrine-related phenomena realized soon after the discovery of the endogenous opioids was that these compounds were able to increase pituitary growth hormone (GH) secretion when administered peripherally or centrally. However, the opioidergic mechanism responsible for this GH increase was and is still not known with absolute certainty. With respect to this, it was also mentioned earlier (Chapters 1 and 3) that the mechanism whereby opioids affect secretion of any pituitary hormone is probably not a direct effect, as incubation of pituitary cells with opioids or opiates generally produces no change in hormone secretion from these cells. Therefore, a hypothalamic site of action has been hypothesized (see Pfieffer and Herz, 1984, for review).

Most studies have indicated that GH release from the pituitary of mammals is regulated predominately by two hypothalamic peptides, somatotropin-release inhibiting factor or SRIF, and somatotropin-releasing factor, also called growth hormone releasing hormone, or GRH (Wehrenberg et al., 1982; Tannenbaum and Ling, 1984). The role of these two peptides in mediating opioid- induced increases in GH is somewhat controversial. Somatostatin had been reported to antagonize morphine-induced increases in GH (Meites et al. 1979). Also, Hollander et al. (1978) and Drouva et al. (1980), observed decreases in potassium-stimulated somatostatin release from hypothalamic organ cultures after incubation with opioids.

More recently, evidence has shifted toward increased GRH secretion, not decreased SRIF, as the more likely mediator of elevated GH levels seen after opioid administration, at least in rats. This hypothesis is based on studies wherein antibodies to GRH blocked the effects of opioids or opiates on GH release in this species (Miki et al., 1984; Murakami et al., 1985; Wehrenberg et al., 1985), whereas antibodies to somatostatin did not (Chihara et al., 1978). A similar result is seen after opioid administration in swine immunized against GRH (Armstrong et al., 1990).

When GRH binds the GH receptor on the pituitary somatotroph, cyclic adenosine monophosphate (cAMP) levels in the somatotroph increase in a dose-dependant manner (Harwood et al., 1984), resulting in phosphorylation and dephosphorylation of a distinct population of protein kinase substrates, and culminating in increased GH secretion from the somatotroph. It had been known for some time that endogenous opioids can interact with adenyl cyclase, the enzyme catalyzing cAMP production, and suppress production of cAMP (see Ronai and Szekely, 1982, for review). However, in early studies using the opiates (prior to discovery of the endogenous opioids), data obtained by various groups on this subject had been conflicting.

For this reason, neuroblastoma x glioma hybrid cells were developed as a model for studying in vitro specific opioid actions (Klee and Nirenburg, 1974). These cells contained large amounts of opioid receptors which remained coupled to adenyl cyclase, and the large availability of these "immortal" cells made results obtained in this cultured line more reproducible. Subsequently, it was determined that opiates, as well as opioids, inhibit both basal and prostaglandin-stimulated adenyl cyclase activity in these cells, in an opioid-receptor mediated process (Goldstein et al., 1977; Hamprecht, 1978; Klee, 1979). In this regard, the pituitary gland, including the pars distalis where the somatotrophs are located, contains both opioids and opioid receptors (Bloom, 1977; Simantov and Snyder, 1977). To our knowledge, however, it has not been demonstrated that exogenous or endogenous opioids are involved specifically in pituitary GH release at any point after GRF binding to its receptor.

In chapter 1, the GH-releasing effect of the synthetic enkephalin DAMME in Holstein calves was described, and it was concluded that DAMME facilitates GH release at a site within the blood brain barrier (BBB), such as the hypothalamus. The objectives of this study were to 1) determine

if the centrally- mediated increases in plasma GH seen after DAMME administration in Holstein calves are mediated by increased hypothalamic GRH secretion, as appears to be the case in rats, and 2) to determine whether endogenous opioids in the pituitary mediate any post- exogenous GRH binding events which facilitate increased GH secretion, such as increased cAMP production, by administering the highly specific opioid antagonist naloxone (NAL) concommitantly with exogenous GRH.

MATERIALS AND METHODS

Animals. This study was conducted at the Virginia Tech dairy research facility on the campus of Virginia Tech in Winter, 1989. Twenty Holstein calves ranging from 4 to 7 months of age and averaging 157 kg BW, were acquired from the university herd and randomly assigned to 1 of 4 treatments (5 calves/treatment): 1) DAMME injection (D-Ala^2, N-Me-Phe^4, Met (0)^5-ol enkephalin), 6 ug/kg in saline; Sigma Chemical Co., St. Louis, Mo.), or 2) GRH injection (D-ala^2, fragment 1-29 amide, human, .1 ug/kg), in saline; Sigma Chemical Co., or 3) DAMME injection (6 ug/kg) plus GRH injection (.1 ug/kg) 1 hr later, or 4) GRH injection (.1 ug/kg) simultaneously with NAL (2.0 mg/kg, in saline; Sigma Chemical Co.), plus NAL injections (2.0 mg/kg) at two consecutive 90 min intervals, following the initial simultaneous injection of NAL and GRH.

All animals were housed in sheltered pens at the university heifer barn. The day prior to insertion of jugular cannulas and serial bleeding, calves were haltered, weighed, and subsequently tied into individual stanchions with free access to water and alfalfa hay. On the following day, indwelling jugular cannulas were installed in all calves at 0600h. Clotting of blood in cannulation tubing was prevented by infusion of a 3.5% citrate solution after cannulation, and immediately after each sample was drawn. External tubing with sample ports was securely taped to the side of each animals neck for easy access. At 1100h, blood sampling every 15 min commenced. The initial 1 ml of each sample was discarded, and a 10 ml sample was then drawn and placed into collection tubes containing 200ul of a 3.5% EDTA solution. After the 1300h sample was taken,

all initial treatments were infused as a single bolus through cannula ports. Subsequent treatments in groups 3 and 4 were also administered as a single bolus, at the times described above. Blood samples were placed in ice immediately after withdrawal, and centrifuged for 30 min at 1800 x g. Plasma was decanted and frozen at -20 C until assayed for GH.

Hormone Determination. Growth hormone was quantified by a double-antibody radioimmunoassay according to the procedure of Barnes et al. (1985). Growth hormone intra- and interassay coefficients of variation (CV) were 5.9% and 8.3%, respectively.

Statistical Analysis. Blood hormone data were analyzed by least-squares analysis of variance with the GLM procedure of the Statistical Analysis Systems (SAS), SAS Institute, Cary, N.C. Samples were assigned to 1 of 3 periods. Period 1 represented samples taken from 1100h to 1300h (basal period). Period 2 represented samples taken over a 1 hr period after initial treatment administration (from 1315h to 1400h), and period 3 represented samples taken from 1415h to 1730h, at which time sampling was terminated.

The statistical model used to analyze all data contained treatment, calf within treatment, period, treatment x period, period x calf within treatment, sample within period, and treatment x sample within period. Treatment was tested using calf within treatment as the error term; period and treatment x period were tested using period x calf within treatment as the error term. Fixed effects were treatment and period. Non- orthogonal contrasts were used to compare least squares means for periods 1 vs 2, 1 vs 3, and 2 vs 3 within each treatment. All critical F values to determine significance of contrasts were adjusted using the Improved Bonferroni F test (Games, 1977).

RESULTS

DAMME injection increased ($P<.05$) plasma GH levels in calves recieving DAMME only (Table 1). This increase was characterized by an immediate rise in plasma GH, which returned to near pre- treatment levels after approximately 2 hrs, then peaked once more 3 hours after the initial DAMME injection (Figure 1). Plasma GH concentrations in calves in the DAMME/GRH group

more than doubled in the the first 60 min after DAMME injection (period 2; Table 1), but this increase was not statistically different from period 1 GH levels. The profile of GH increase in these calves differed from calves receiving DAMME only, in that the rise in plasma GH was not as great immediately (Figure 1). However, mean plasma GH levels in this group abruptly increased at 60 min post-DAMME injection, and at this time were at levels comparable to those in calves receiving DAMME only (Figure 1). Immediately after the 60 minute sample was obtained, GRH was injected.

Plasma GH was higher ($P<.01$) in period 3 after GRH injection in the DAMME/GRH calves when compared to basal levels in period 1 (Table 1). Although plasma GH rose approximately 50% from period 2 to period 3 in this group (Table 1), this increase was not statistically significant. Samples collected from DAMME/GRH calves at 60 minutes post-DAMME (immediately prior to GRH injection) had an average GH concentration of approximately 50 ng/ml (Figure 1). The next samples taken from this group (15 min post-GRH) averaged approximately 115 ng/ml of assayable GH. This was the peak response, with levels gradually declining until they reached pre- treatment levels nearly 3 hrs later (Figure 1).

The growth hormone-releasing hormone analogue used in this study increased ($P<.01$) mean GH concentrations nearly 5 fold overall in period 2 in calves recieving only this peptide (Table 2). This synthetic GRH had been shown to be very effective in facilitating GH release in Holstein calves (Scarborough et al., 1988). Once again, the GRH-mediated increase in plasma GH was immediate, had peaked by the next sample taken, and gradually declined to near pre-treatment concentrations 3 hrs later, at which time GH levels began to rise again (Figure 2). Growth hormone concentrations increased more than 4 fold overall from period 1 to period 2 ($P<.01$) in calves recieving GRH and NAL simultaneously (Table 2). The pattern of increase was similar to that of calves recieving only GRH, except the immediate increase was not as large, and a second GH surge occurred 90 min post-GRH/NAL injection, similar in magnitude to the secondary GH increase seen at 3 hrs post-GRH injection in calves recieving only GRH (Figure 2).

DISCUSSION

It was our assumption in this study that the DAMME-mediated increases in plasma GH seen previously in Holstein calves (Chapter 1) were the result of increased hypothalamic GRH reaching the pituitary. Therefore, our goal was to inject exogenous GRH at a time when endogenous GRH levels, and therefore GH levels, were already elevated by DAMME, and to compare the response to exogenous GRH in these calves to the GRH response of calves with baseline GH levels. Previous work (Johnson et al., 1989) indicated that DAMME-induced GH increases in Holstein calves peaked at approximately 1 hour after injection of DAMME. Therefore, one hour post-DAMME treatment was chosen as the injection time for GRH in this study. As seen in Figure 1, results obtained in this study were similar to those obtained previously, with GH concentrations again peaking at approximately 1 hour after DAMME injection in calves receiving only DAMME, and surging to their peak level immediately prior to GRH injection in calves receiving both DAMME and GRH.

Examination of Tables 1 and 2 shows that GH did not increase significantly immediately after GRH injection in DAMME/GRH calves as it did in calves not pre-treated with DAMME. Also, as noted above, plasma GH concentrations in this study averaged approximately 50 ng/ml in DAMME-injected calves just prior to GRH injection (Figure 1). Immediately after GRH was administered, GH concentrations increased to approximately 115 ng/ml, where the response peaked. Therefore, the immediate increase in GH concentration after GRH was approximately 65 ng/ml. This increase was likely due exclusively to the exogenous GRH, and not DAMME, as calves recieving only DAMME did not exceed the GH concentrations in the DAMME/GRH calves measured just prior to GRH injection (Figure 1).

Calves recieving only GRH had average plasma GH concentrations of approximately 15 ng/ml just prior to GRH injection (Figure 2). Plasma GH concentrations peaked immediately after GRH administration at just over 100 ng/ml, an approximate 660% increase, and began to decline from

that point. Conversely, plasma GH concentrations in DAMME pre-treated calves only increased approximately 130% immediately after GRH injection (Figure 1).

Two plausible theories would seem to best explain the percent differences in response to exogenous GRH in calves pre-treated or untreated with DAMME. First, it was stated previously that GRH acts at the pituitary somatotroph to increase cAMP levels, which results in a cascade of events that eventually leads to GH release from the somatotroph. It was also noted that opiates and opioids have been shown to interact with adenyl cyclase to suppress production of cAMP. Therefore, it is possible that the increase in GH concentration after DAMME injection was due to some factor other than increased endogenous GRH, and the blunted GH response to exogenous GRH in DAMME pre-treated calves is due to suppression of exogenous GRF-mediated cAMP production by the opioid DAMME.

The second possible explanation for the smaller immediate increase in GH concentration in response to GRH in DAMME pre- treated calves is that, as hypothesized, the DAMME-stimulated increase in plasma GH concentrations during the 1 hour prior to exogenous GRH injection was due to increased release of endogenous GRH. Therefore, there was already a higher concentration of endogenous GRF occupying pituitary GRF receptors in DAMME pre-treated calves, making fewer viable receptors available for activation by exogenous GRH.

Of these two hypotheses, the latter would seem to be the more realistic and accurate. First, even though opioids and opiates have been shown to suppress cAMP production, the vast majority of these studies were in vitro, and a large amount of conflicting data was published. With the advent of neuroblastoma x glioma hybrid cells to use as a model, results were more uniform and conclusive, and opioids did generally inhibit cAMP production, via adenyl cylase, in these cells. However, effects of opioids and opiates in vitro in malignant hybrid cells may not be representative of in vivo responses of pituitary cells to opioids, and we are unaware of any studies, in vitro or in vivo, which demonstrate that opiates directly inhibit cAMP production by hypothalamic peptides binding to receptors in pituitary cells.

Furthermore, of the four studies cited previously in which GH responses to opioids were blocked by antibodies to GRH, three of these investigators (Miki et al., 1984; Murakami et al.,

1985; Armstrong et al., 1990) used the same opioid agonist used in the present study, namely DAMME. And, even though the immediate GH response to exogenous GRH was some 5 fold smaller in magnitude in DAMME pre-treated calves, the overall GH response to exogenous GRH is much more similar between the two groups upon examining the area under the GRH response curves (Figures 1 and 2). Indeed, the greater shift to the right seen in the DAMME/GRH curve may indicate synergism between the exogenous GRH and a DAMME-mediated sustained release of endogenous GRH.

The immediate GH response to GRH in calves which recieved the opioid antagonist NAL concommitantly, and at two 90 min intervals subsequent to GRH injection was somewhat lower in magnitude than that in calves receiving GRH only (Figure 2). Both the GRH and NAL/GRH groups had plasma GH concentrations of approximately 15 ng/ml in the samples taken immediately prior to treatment injections. As described above, calves receiving only GRH had GH concentrations averaging just over 100 ng/ml in samples collected immediately after GRH injection. Calves receiving the opioid antagonist NAL concommitantly with GRH peaked at approximately 70 ng/ml in the samples collected immediately after treatments were administered.

However, Table 2 indicates that the percent increase overall in average GH concentrations from the basal period (period 1) to the response period (period 2) for both groupsis similar (an approximately 460% increase in GRH only calves, vs an approximate 425% increase in NAL/GRH calves). This seems to indicate that the pituitaries of calves in both groups responded similarly to this dose of GRH, relative to their baseline GH levels. Therefore, evidence that NAL is modifying the GH response to exogenous GRH release is lacking. Furthermore, if endogenous pituitary opioids were indeed able to modify the response to exogenous GRH in this study, then the expected result of co-administering NAL with GRH, if any, would be one of facilitating a greater, not lesser, pituitary GH response to GRH, at least compared to the same dose of GRH alone. This is because NAL should "disinhibit" any endogenous pituitary opioid inhibition of GRH binding-induced cAMP formation in the somatotroph, allowing for greater proliferation of post-receptor binding events.

It should be noted that the logic for injecting NAL twice more at 90 min intervals after the initial injection concommitantly with GRH was that the GRH analogue used in this study is known to be capable of elevating plasma GH levels for several hours in Holstein calves (Scarborough et al., 1988). In contrast, the half-life of NAL is controversial, with reports ranging from only 90 minutes to 5 or 6 hours (see Chapter 2). Therefore, if a modified response to the synthetic GRH used in this study by NAL was to be demonstrated, subsequent NAL injections after the initial treatments seemed neccessary.

In conclusion, these results demonstrate that the synthetic GRH analogue D-ala^2 amide fragment 1-29, as well as the opioid agonist DAMME, are potent releasers of pituitary GH in Holstein calves. In this study, exogenous GRH was not able to significantly increase plasma GH concentrations immediately in DAMME pre-treated calves, as it did in calves not recieving the opioid agonist. It appears, therefore, that DAMME increases pituitary GH secretion in Holstein calves at least partly by increasing the amount of endogenous GRH reaching the adenohypophysis, which other investigators have shown is the GH releasing-mechanism of opioids in rodents. Furthermore, it does not appear from the results of this study that endogenous opioids modify the response of exogenous GRH in Holstein calves, at least not at the doses of NAL and GRH used here.

SUMMARY

The mechanism of opioid-mediated growth hormone (GH) release and the role of endogenous opioids in modifying exogenous growth hormone-releasing factor (GRH) in Holstein calves was investigated. Twenty Holstein calves allotted to four treatment groups (5 calves/treatment) were used in this study: 1) injection of the opioid agonist DAMME, or 2) injection of both DAMME and a synthetic GRH analogue, or 3) injection of GRF analogue only, or 4) injection of both GRH and the specific opioid antagonist naloxone (NAL).

Both DAMME and GRH injected alone increased plasma GH release in Holstein calves. The synthetic GRH used in this study, however, failed to significantly increase plasma GH concentrations in the period following its injection in calves pre- treated with DAMME, as it did in calves not receiving DAMME prior. A shift to the right in the GH response curve in the DAMME/GRH group, compared to the GRH group, indicated possible synergism between DAMME-induced release of endogenous GRH with exogenous GRH in sustaining pituitary GH release longer than either peptide alone.

The immediate plasma GH response in calves recieving both NAL and GRH, although significant, was not of as great a magnitude as that in calves recieving GRH alone. However, the percent change in plasma GH concentration averaged between pretreatment and treatment periods were very similar between these two groups (a 425% increase vs a 460% increase, respectively).

These results indicate that the mechanism of action of the opioid agonist DAMME in facilitating increased GH secretion in Holstein calves is mediated at least partly through increased release of endogenous GRH, which previous work by other investigators has indicated is the mechanism of opioid-induced GH secretion in rodents. It is known that GRH increases cyclic AMP (cAMP) production in somatotrophs after binding its receptor, and in vitro studies have indicated that opioids are able to antagonize adenyl cyclase-induced cAMP production. Although opioids and opioid receptors are present in the pituitary gland, this study indicates that, at the doses used,

endogenous opioids do not modify the response of plasma GH to exogenous GRH in Holstein calves.

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TABLE 1. PLASMA GROWTH HORMONE CONCENTRATIONS* IN HOLSTEIN CALVES BEFORE (PERIOD 1) AND AFTER (PERIOD 2) DAMME INJECTION (GRH INJECTED AT BEGINNING OF PERIOD 3 IN DAMME/GRH GROUP).

Group	No. calves	Period	Growth Hormone (ng/ml)
DAMME	5	1	15.4 ± 5.9^{a}
		2	43.4 ± 8.8^{b}
		3	$23.7 \pm 4.6^{a,b}$
DAMME/GRH	5	1	13.5 ± 5.9^{a}
		2	$29.0 \pm 8.8^{a,c}$
		3	44.8 ± 4.6^{c}

* = Least squares means, ± sem.
^{a,b} Means with different superscripts in same group differ ($P<.05$).
^{a,c} Means with different superscripts in same group differ ($P<.01$).
DAMME = D-Ala^{2},N-Me-Phe^{4},Met(O)^{5}-ol enkephalin (6 ug/kg).
GRH = Growth hormone-releasing hormone (.1 ug/kg).

TABLE 2. PLASMA GROWTH HORMONE CONCENTRATIONS* IN HOLSTEIN CALVES BEFORE (PERIOD 1) AND AFTER (PERIOD 2) GRH INJECTION (NAL INJECTED SIMULTANEOUSLY WITH GRH IN GRH/NAL GROUP, AND TWICE AFTERWARDS AT 90 MIN INTERVALS).

Group	No. calves	Period	Growth Hormone (ng/ml)
GRH	5	1	15.6 ± 5.9^{a}
		2	72.1 ± 8.8^{b}
		3	23.0 ± 4.8^{a}
GRH/NAL	5	1	12.8 ± 5.9^{a}
		2	54.4 ± 8.8^{b}
		3	22.5 ± 4.8^{a}

* - Least squares means, ± sem.
^{a,b} Means with different superscripts in same group differ ($P<.01$).
GRH - Growth hormone-releasing hormone (.1 ug/kg).
NAL - Naloxone (2.0 mg/kg)

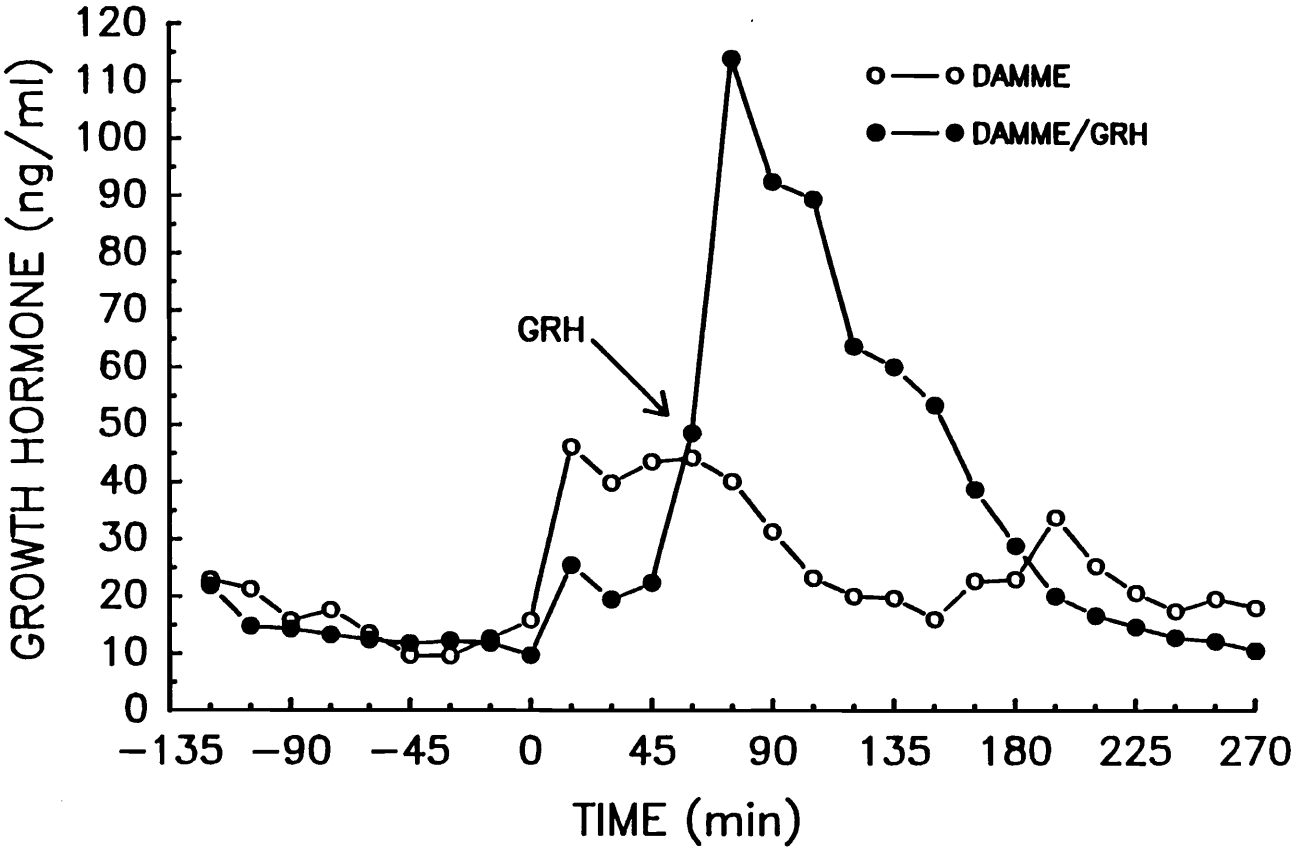

Figure 1. The effect of DAMME or DAMME/GRH (growth hormone-releasing hormone) on plasma growth hormone concentrations in Holstein calves on day 2. DAMME injected at time=0 in both groups. GRH administered 1 hr post-DAMME in DAMME/GRH group. Period 1= -120 to 0 minutes; period 2= 15 to 60 minutes; period 3= 75 to 270 minutes.

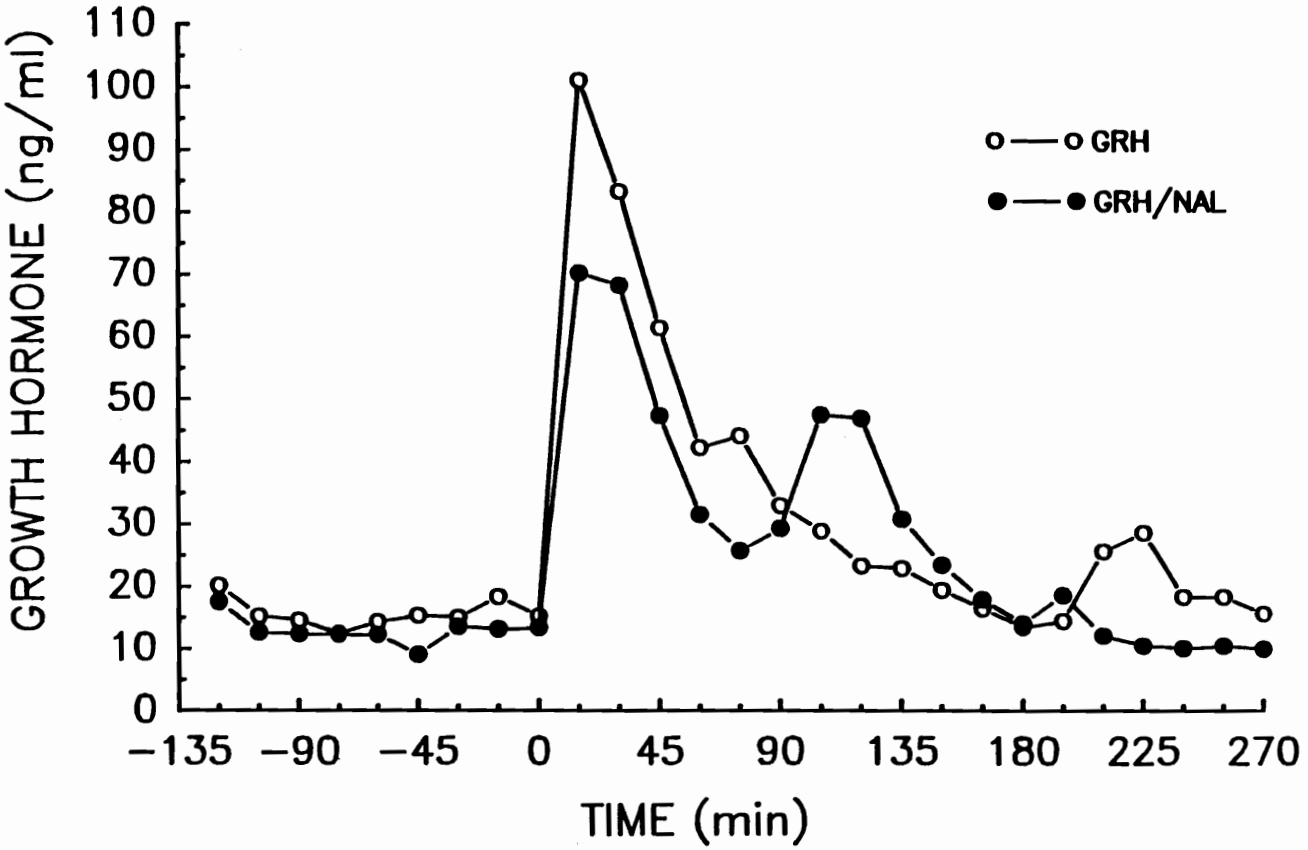

Figure 2. The effect of growth hormone-releasing hormone (GRH) or GRH/NAL (naloxone) in combination on plasma growth hormone concentrations in Holstein calves. GRH injected at time=0. NAL injected concommitantly with GRH, and at 2 subsequent 90 minute intervals in GRH/NAL group. Period 1= -120 to 0 minutes; period 2= 15 to 60 minutes; period 3= 75 to 270 minutes.

GENERAL SYNTHESIS

The four studies which comprise this dissertation provide the first information as to the effect of endogenous and exogenous opioids on release of pituitary growth hormone (GH) and prolactin (PRL) in the dairy cow, the site of action of these effects in regard to whether they occur within or outside the central nervous system (CNS), and preliminary information concerning the physiological mechanisms which are primarily involved in producing the increases seen in GH and PRL after opioid agonist administration.

Collectively, these studies provide evidence that, as in other species, dairy animals respond to exogenous opioids with increased secretion of GH and PRL. They also demonstrate that the opioid receptors to which agonists bind to facilitate PRL release are outside the blood brain barrier (BBB), and those mediating GH release are somewhere within the BBB. Results also indicate that basal secretion of PRL, and possibly GH, is regulated to some extent by endogenous opioidergic systems in dairy calves, and this system likely lies within the BBB, at least for PRL. It appears from these results that dopaminergic mechanisms are primarily responsible for the increased PRL secretion seen in Holstein calves after opioid agonist injection, and that hypothalamic growth hormone-releasing factor (GRH) is at least partly responsible for mediating GH increases seen in response to exogenous opioid injection.

Although GRH is known to increase GH secretion from the somatotroph at least partly via increasing cyclic AMP (cAMP) production in the cell, and opioids are known to inhibit cAMP production, it appears that endogenous opioids do not modify the GH response to exogenous GRH. The use of pre-pubertal heifers for these studies eliminates the confounding effects of fluctuating gonadal steroids on opioid-mediated changes in pituitary hormone secretion (Wilkinson et al., 1981; Bhanot and Wilkinson, 1983).

All information gained concerning site of action of exogenous or endogenous opioids (Chapters 1 and 2) was inferred from use of peripherally vs centrally acting opioid antagonists alone or in conjunction with an opioid agonist. Administration of opioid agonists and antagonists directly into the CNS would provide more direct evidence concerning site of action of these compounds, but the use of cannulas to facilitate injection of drugs directly into specific nuclei or ventricles of the brain is often not practical, due to the special stereotaxic apparatus and x- ray equipment required, as well as extensive surgical skills (Leshin et al., 1990). Similarly, in Chapters 3 and 4, cannulation of hypophysial portal vessels to directly measure changes in portal blood concentrations of dopamine, somatostatin, or GRH after opioid administration, or pituitary stalk-section to rule out hypothalamic involvement in response to exogenous opioids, would have been very useful in determining mechanism of action of exogenous opioids on pituitary GH and PRL secretion in Holstein calves. Again, however, the equipment and surgical skills required make these procedures impractical in many instances (Gordon et al. 1987).

As with nearly all scientific research, these studies have provoked new questions which provide ample opportunity for future endeavors. In regards to the increases in GH and PRL seen in Holstein calves after administration of the opioid agonist DAMME, what types of opioid receptors are being activated? Although DAMME is known to be most active at the mu receptor (Rothman et al. 1987), these studies do not prove that the mu receptors were responsible for mediating changes in GH and PRL seen after DAMME injection. Indeed, the different plasma profiles for GH and PRL seen in Chapter 1 after DAMME injection indicates at least 2 different receptor types may be mediating these responses. Studies with rats have shown that the mu opioid receptors are the primary mediators of agonist-induced PRL release, while the delta receptors mediate GH release (Koenig et al., 1984; Leadem and Yagenova, 1987). The administration of more receptor-specific opioid agonists (Bianchetti et al., 1985) and measurement of GH and PRL responses would provide evidence as to which receptor types are involved in mediating these hormone changes in dairy cattle.

With regard to Chapter 2, injection of more specific opioid antagonists (Kosterlitz and Paterson, 1981; Jackson et al., 1989) would also be useful in elucidating on receptor types involved in physiological GH and PRL secretion in Holstein calves. Also, it should be considered that the

failure of methyl levallorphan mesilate (MLM) to affect plasma PRL at any dose in the spring trial (Chapter 2) may have been a result of inadequate dose. Both MLM and naloxone (NAL) are mu receptor-preferring antagonists, with NAL being some 2.5 times as potent a mu antagonist (Bianchetti et al., 1985). In Chapter 1, results indicated that opioid receptors mediating PRL secretion in response to an opioid agonist are outside the BBB. As noted above, opioid agonist-mediated PRL secretion appears to occur through the mu opioid receptors, so perhaps higher doses of MLM than the highest dose used (2.0 mg/kg) would affect basal PRL secretion, as did NAL. The fact that the parent compound of MLM (levallorphan) has been shown to decrease basal secretion of PRL in rats when administered alone at doses similar to those used in spring in Chapter 2 (Lien et al., 1979) is justification for further investigation with higher doses of MLM.

APPLICATION

Bauman et al. (1984) discussed the major sources of variation and prospects for improvement in the dairy cow. They identified four major sources of variation, and pinpointed one, nutrient partitioning, as the area in which individual cows differed most substantially. The importance of GH and PRL in nutrient partitioning has been known for some time (see Bauman et al., 1982, and Peel and Bauman, 1986, for reviews). Although increases in PRL are important in initiating lactogenesis and supporting the subsequent lactation in the dairy cow (Akers et al., 1981), administration of exogenous PRL after calving is probably not beneficial in terms of milk production (Plaut et al., 1987). Growth hormone, conversely, is particularly important in galactopoeisis in the dairy cow and it is now well known that exogenous GH can enhance milk production in this species (Peel and Bauman, 1986).

The study of differences in hormonal regulation of nutrient partitioning and metabolism in high- and low-yielding cows is the natural basis in a search for predictors of milk production (Land, 1981), and differences between high- and low-yielding cows are likely expressed during lactation when cows are stressed and in energy deficit. It is well established in other species that stress causes increased secretion of pituitary β-endorphin, a potent opioid as well as the primary blood-borne opioid, into the plasma (Rivier et al., 1982; Young et al., 1986). Although there are several valid assays now established for the measurement of plasma β-endorphin, as well as commercial RIA

kit availability, to our knowledge, plasma levels of this opioid have not been quantified in dairy cows at different physiological time periods throughout a lactation.

This study has established that the hypothalamo-pituitary axis of dairy calves is responsive to exogenous opioids, and that basal pituitary secretion of these hormones is regulated to some extent by endogenous opioids. Some of the questions which arise are, does secretion of endogenous opioids such as β-endorphin into plasma change during the lactation of a dairy cow, especially during the stress of early lactation? If so, are changes in plasma opioids like β-endorphin characterized by changes in food intake, as they appear to be in rats (Davis et al., 1983)? Since we have established in this study that GH secretion in dairy cows is increased by opioids, it may be possible that feeding or stress-induced changes in plasma βendorphin during early lactation modifies GH secretion, which in turn may modify nutrient partitioning, the major source of variation identified in dairy cows.

Long term studies in which exogenous opioids are administered to large groups of lactating dairy cows is generally not feasible due to the high cost of these peptides. Research has shown (Barnes et al., 1985), however, that dairy cows selected for milk production respond differently to insulin challenge than do cows randomly selected. Also, physiological GH concentrations are higher in selected cows than in cows not selected for milk production, and selected cows are able to respond with greater secretion of GH to a metabolic challenge than non-selected cows (Kazmer et al. 1986). Therefore, future studies using select and non-select cows to measure basal plasma βendorphin levels concommitantly with levels of important metabolic hormones at different physiological time periods throughout lactation, or, if costs permit, measurements of hormone changes in lactating cows in response to opioid challenge at different stages of lactation, may be useful in establishing whether or not there is a place for endogenous or exogenous opioids as predictors of productive merit in dairy cows.

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APPENDICES

Appendix A. Table 1. Analysis of variance for the effect of DAMME on plasma prolactin secretion in calves pre-treated with saline, naloxone, or methyl levallorphan mesilate in fall.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	2	246.9697	2.31	0.1332
Calf(Tmt)	15	800.7771	9.54	0.0001
Period	2	1655.9911	25.39	0.0001
Tmt*Period	4	1230.0043	9.43	0.0001
Calf*Period(Tmt)	30	978.2018	5.82	0.0001
Sample(Period)	24	2676.8641	19.92	0.0001
Tmt*Sample(Period)	48	2533.7383	9.43	0.0001

Appendix A. Table 2. Analysis of variance for the effect of DAMME on plasma growth hormone secretion in calves pre-treated with saline, naloxone, or methyl levallorphan mesilate in fall.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	2	71.9099	0.09	0.9147
Calf(Tmt)	15	6011.8382	3.98	0.0001
Period	2	9920.2779	18.27	0.0001
Tmt*Period	4	1909.3629	1.76	0.1635
Calf*Period(Tmt)	30	8145.6137	2.69	0.0001
Sample(Period)	24	3396.2069	1.40	0.1000
Tmt*Sample(Period)	48	2916.3332	0.60	0.9831

Appendix A. Table 3. Analysis of variance for the effect of DAMME on plasma prolactin secretion in Holstein calves pre-treated with saline, naloxone, or methyl levallorphan mesilate in spring.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	2	1978.4350	2.15	0.1510
Calf(Tmt)	15	6900.5922	21.41	0.0001
Period	2	15191.8918	57.79	0.0001
Tmt*Period	4	7059.7424	13.43	0.0001
Calf*Period(Tmt)	30	3942.9047	6.12	0.0001
Sample(Period)	24	14948.9787	28.98	0.0001
Tmt*Sample(Period)	48	9503.9019	9.21	0.0001

Appendix A. Table 4. Analysis of variance for the effect of DAMME on plasma growth hormone secretion in Holstein calves pre-treated with saline, naloxone, or methyl levallorphan mesilate in spring.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	2	5643.6699	3.19	0.0702
Calf(Tmt)	15	13277.4397	6.48	0.0001
Period	2	9546.5041	9.72	0.0006
Tmt*Period	4	3743.1120	1.91	0.1353
Calf*Period(Tmt)	30	14728.9621	3.60	0.0001
Sample(Period)	24	6123.2707	1.87	0.0086
Tmt*Sample(Period)	48	10789.8068	1.65	0.0063

Appendix A. Table 5. Analysis of variance for the effect of DAMME on plasma insulin secretion in Holstein calves in spring.

Source of Variation	df	Sum of Squares	F value	P>F
Calf(Tmt)	5	74.5424	0.93	0.4640
Period	2	25.4971	0.97	0.4135
Calf*Period(Tmt)	10	131.9884	0.82	0.6066
Sample(Period)	24	385.3590	1.00	0.4695

Appendix B. Table 1. Analysis of variance for the effect of naloxone, methyl levallorphan mesilate, or saline on basal prolactin secretion in Holstein calves in fall.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	2	93.4207	0.57	0.5756
Calf(Tmt)	15	1222.4345	51.90	0.0001
Period	2	187.8825	7.40	0.0024
Tmt*Period	4	31.8054	0.63	0.6475
Calf*Period(Tmt)	30	380.9068	8.09	0.0001
Sample(Period)	23	61.7374	1.71	0.0235
Tmt*Sample(Period)	46	84.3578	1.17	0.2215

Appendix B. Table 2. Analysis of variance for the effect of naloxone, methyl levallorphan mesilate, or saline on basal prolactin secretion in Holstein calves in fall (data from calf 519 omitted).

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	2	7.7880	0.24	0.7871
Calf(Tmt)	14	223.8857	19.90	0.0001
Period	2	105.1673	10.66	0.0004
Tmt*Period	4	39.1586	1.99	0.1242
Calf*Period(Tmt)	28	138.0840	6.14	0.0001
Sample(Period)	23	48.6457	2.63	0.0001
Tmt*Sample(Period)	46	37.2842	1.01	0.4627

Appendix B. Table 3. Analysis of variance for the effect of naloxone, methyl levallorphan mesilate, or saline on basal growth hormone secretion in Holstein calves in fall.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	2	2505.5327	2.67	0.1017
Calf(Tmt)	15	7033.4000	13.50	0.0001
Period	2	4549.9082	17.86	0.0001
Tmt*Period	4	1317.3350	2.59	0.0570
Calf*Period(Tmt)	30	3821.1364	3.67	0.0001
Sample(Period)	23	1575.1843	1.97	0.0056
Tmt*Sample(Period)	46	965.0085	0.60	0.9805

Appendix B. Table 4. Analysis of variance for the effect of 5 different doses of naloxone or methyl levallorphan mesilate on plasma prolactin secretion in Holstein calves in spring.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	1	35.4043	2.95	0.0864
Dose	4	155.1541	0.13	0.9710
Tmt*Dose	4	113.7854	0.27	0.8953
Calf(Dose)	15	4640.4107	25.74	0.0001
Tmt*Calf(Dose)	15	1604.4404	8.90	0.0001
Period	2	1252.8675	52.13	0.0001
Tmt*Period	2	48.6118	2.02	0.1329
Dose*Period	8	402.8190	0.72	0.6712
Tmt*Dose*Period	8	303.4416	3.16	0.0016
Calf*Period(Dose)	30	2093.4972	5.81	0.0001
Tmt*Calf*Period(Dose)	30	2132.6785	5.92	0.0001

Appendix B. Table 5. Analysis of variance for the effect of 5 different doses of naloxone or methyl levallorphan mesilate on plasma growth hormone secretion in Holstein calves in spring.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	1	8.0025	0.10	0.7489
Dose	4	11000.2813	1.01	0.4342
Tmt*Dose	4	198.8571	0.05	0.9943
Calf(Dose)	15	40924.0505	34.96	0.0001
Tmt*Calf(Dose)	15	14234.0194	12.16	0.0001
Period	2	4935.3931	31.62	0.0001
Tmt*Period	2	1053.9411	6.75	0.0012
Dose*Period	8	852.6043	0.61	0.7658
Tmt*Dose*Period	8	627.7607	1.01	0.4300
Calf*Period(Dose)	30	5280.4113	2.26	0.0001
Tmt*Calf*Period(Dose)	30	7123.8036	3.04	0.0001

Appendix B. Table 6. Analysis of variance table for the effect of 3 doses of naloxone and methyl levallorphan mesilate on plasma insulin.

Source of variation	df	Sum of squares	F value	P>F
Tmt	1	0.2403	9.98	0.5361
Dose	2	0.4635	9.62	0.6780
Tmt*Dose	2	0.4934	10.24	0.6623
Period	2	0.0812	1.69	0.2256
Tmt*Period	2	0.1010	2.10	0.1647
Dose*Period	4	0.3044	3.16	0.0540
Tmt*Dose*Period	4	0.0569	0.59	0.6750

Appendix C. Table 1. Analysis of variance for data set 1 analyzing the effect of CB154 on basal plasma prolactin secretion from day 1 to day 2 in Holstein calves.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	3	29321.8626	5.58	0.0060
Calf(Tmt)	20	35011.2133	107.34	0.0001
Period	3	11193.7784	15.87	0.0001
Tmt*Period	9	18133.9176	8.57	0.0001
Calf*Period(Tmt)	60	14109.9469	14.42	0.0001
Sample(Period)	32	5349.3650	10.25	0.0001
Tmt*Sample(Period)	96	6298.2442	4.02	0.0001

Appendix C. Table 2. Analysis of variance for data set 2 analyzing the effect of saline, DAMME, DAMME/CB154, or TRH/CB154 on plasma prolactin secretion on day 2 in Holstein calves.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	3	35358.5022	10.58	0.0002
Calf(Tmt)	20	22281.1514	70.33	0.0001
Period	2	9509.9685	20.51	0.0001
Tmt*Period	6	14136.9072	10.16	0.0001
Calf*Period(Tmt)	40	9273.8966	14.64	0.0001
Sample(Period)	24	4514.2528	11.88	0.0001
Tmt*Sample(Period)	72	4801.7741	4.21	0.0001

Appendix D. Table 1. Analysis of variance for the effect of DAMME, growth hormone-releasing factor (GRF), DAMME/GRF, and naloxone/GRF on plasma growth hormone secretion in Holstein calves.

Source of Variation	df	Sum of Squares	F value	P>F
Tmt	3	4644.7464	0.53	0.6687
Calf(Tmt)	16	46818.6157	8.30	0.0001
Period	2	70879.0329	23.42	0.0001
Tmt*Period	6	38902.0374	4.28	0.0028
Calf*Period(Tmt)	32	48421.1569	4.29	0.0001
Sample(Period)	24	55831.3047	6.60	0.0001
Tmt*Sample(Period)	72	61413.1840	2.42	0.0001

VITA

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