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LOYOLA UNIVERSITY CHICAGO

PHYSIOLOGIC STUDIES OF IMMUNE CELL-DERIVED PEPTIDE MODULATION OF CHROMAFFIN CELL EPINEPHRINE SECRETION

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

BY JENNIFER CURRAN ROBERTS

CHICAGO, ILLINOIS

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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................ iii

LIST OF FIGURES ........................................ viii

LIST OF TABLES ........................................ xii

LIST OF ABBREVIATIONS ................................ xiii

Chapter

I. INTRODUCTION .......................................... 1

II. REVIEW OF RELATED LITERATURE ...................... 9
   A. The Catecholamines .................................. 9
      1. Physiologic Role in the Stress Response ... 9
      2. Sources and Secretion of the
          Catecholamines ................................ 10
   B. Effects of Catecholamines on
      Immune Cell Function .............................. 15
         1. Stress and Illness .......................... 15
         2. Sympathetic Innervation of Primary
             and Secondary Lymphoid Organs .......... 17
         3. Immune Cell Adrenoreceptors ............. 21
            a) Beta Adrenergic Modulation
                of Immune Cell Function ............. 23
            b) Alpha Adrenergic Modulation
                of Immune Cell Function ........... 27
   C. Immunomodulation of Neuroendocrine Function .. 30
      1. Immune Cell Secretion of
          Neuroendocrine Signalling Molecules .... 30
      2. Non-neurogenic Modulation of
          Chromaffin Cell Catecholamine Secretion . 35
   D. Rationale ........................................... 39

III. MATERIALS AND DETAILED METHODS .................. 40
   A. Cell Culture ....................................... 40
      1. Primary Cultures of Chromaffin Cells .... 40
         a) Isolation and Culture .................. 40
         b) Chromaffin Cell Culture Media ....... 40
         c) In Vitro Model of Secretion ........ 41
d) Data Analysis .................................. 43

2. Primary Cultures of Mononuclear Cells
from the Spleen .................................. 44
   a) Isolation and Culture ....................... 44
   b) Mononuclear Cell Culture Media .......... 45
   c) Epinephrine Co-Culture .................... 45

3. Hybridoma Cell Lines .......................... 46
   a) IL-A51/anti-CD8 ............................. 46
   b) IL-A11/anti-CD4 ............................. 46

B. Mononuclear Cell Separation and Purification 47
   1. Separation of Monocyte/Macrophage
      Populations .................................. 47
   2. T Cell/B cell Isolation and Separation
      Using Monoclonal Antibody ................ 48
         a) Isolation of Antibody
             from Hybridoma Cultures ............... 48
         b) Antibody Biotinylation and Coupling
             to Magnetic Microbead ................. 49
         c) Magnetic Based Cell Separation ....... 49

C. Peptide Purification and Isolation ............ 51
   1. G-25 Sephadex Column Gel Filtration ...... 51
   2. Polyacrylamide Gel Electrophoresis (PAGE) 52

D. Intracellular Calcium Measurements in
   Chromaffin Cells ............................... 52
   1. Solutions ................................ 52
      a) Fura-2 AM ................................ 52
      b) Modified Kreb's Buffer ................. 54
      c) Calcium Standard Buffer ............... 54
      d) Buffer "A" ................................ 54
      e) Buffer "B" ................................ 54
   2. Calibrations ................................ 55
      a) In Vitro ................................ 55
      b) In Situ ................................ 56
   3. Procedure ................................ 57
      a) Preparation of Chromaffin Cells ...... 57
      b) Loading of Fura-2 AM ................. 57
      c) Measurement of Intracellular
         Calcium Concentrations ............... 57
      d) Data Analysis ............................ 59

E. Antibody Production ........................... 60
   1. Carrier Protein ............................ 60
      a) Selection of Carrier Protein ....... 60
b) Coupling the Enriched Peptide Fraction to Carrier Protein 60

2. Polyvalent Antibody Production 60

3. Development of an ELISA 61

IV. SIGNAL TRANSDUCTION AND THE IMMUNE-DERIVED PEPTIDE: THE ROLE OF CALCIUM 64

A. Introduction 64

B. Materials and Methods 68

C. Results 74

D. Discussion 87

V. MONONUCLEAR CELL ORIGIN OF A NOVEL PEPTIDE MEDIATING PERIPHERAL EPINEPHRINE SECRETION 95

A. Introduction 95

B. Materials and Methods 100

C. Results 108

D. Discussion 116

VI. MONONUCLEAR CELL PEPTIDE MEDIATION OF CHROMAFFIN CELL EPINEPHRINE SECRETION 122

A. Introduction 122

B. Materials and Methods 126

C. Results 134

D. Discussion 151

VII. CHROMAFFIN CELL EPINEPHRINE SECRETION MEDIATED BY A MACROPHAGE PEPTIDE:

A ROLE FOR ENDOTOXIN 159

A. Introduction 159

B. Materials and Methods 162

C. Results 168

D. Discussion 178

VIII. SUMMARY AND CONCLUSIONS 184

Appendix 201

BIBLIOGRAPHY 211

VITA 230

PUBLICATIONS 231
## LIST OF FIGURES

### CHAPTER IV

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Effect of non-specific calcium channel blockers on conditioned media induced secretion</td>
<td>75</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of voltage-gated calcium channel blockers on conditioned media induced secretion</td>
<td>76</td>
</tr>
<tr>
<td>3.</td>
<td>Effect of non-specific and L-type calcium channel blockers on nicotine agonist induced secretion</td>
<td>77</td>
</tr>
<tr>
<td>4.</td>
<td>Effect of removal of intracellular calcium stores on conditioned media induced secretion</td>
<td>79</td>
</tr>
<tr>
<td>5.</td>
<td>Rise in chromaffin cell intracellular calcium in response to conditioned media stimulation</td>
<td>80</td>
</tr>
<tr>
<td>6.</td>
<td>Time course of conditioned media induced secretion</td>
<td>81</td>
</tr>
<tr>
<td>7.</td>
<td>Rise in chromaffin cell intracellular calcium in response to conditioned media stimulation in the presence of cadmium</td>
<td>84</td>
</tr>
<tr>
<td>8.</td>
<td>Rise in chromaffin cell intracellular calcium in response to conditioned media stimulation in the presence of cobalt</td>
<td>85</td>
</tr>
<tr>
<td>9.</td>
<td>Effect of thapsigargin pretreatment of initial conditioned media induced calcium spike</td>
<td>86</td>
</tr>
</tbody>
</table>

### CHAPTER V

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.</td>
<td>Evidence for diminished mononuclear cell viability as time in culture increases</td>
<td>109</td>
</tr>
</tbody>
</table>
11. Secretion experiment demonstrating that bioactivity of conditioned media decreases as mononuclear cell viability decreases ................. 110

12. Evidence that nonadherent T cells and/or B cells release the majority of bioactive peptide ................. 111

13. Secretion experiment providing evidence that T helper (CD4+) cell conditioned media possessed the majority of bioactivity . 114

14. SDS-PAGE demonstrating that both T helper and B cell populations are potential Sources of bioactive peptide . . . . . 115

CHAPTER VI

15. Conditioned media bioactivity comparable to that induced by maximal cholinergic stimulation or high potassium . . . . 135

16. Epinephrine content of chromaffin cells permeabilized with digitonin (20µM) for 20 minutes ................. 136

17. Lactate dehydrogenase activity in chromaffin cell media permeabilized with digitonin (20µM) for 20 minutes . . . . 137

18. Extracellular calcium dependence of conditioned media induced secretion .. 139

19. Effect of acid hydrolysis on conditioned media bioactivity . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 140

20. Secretion experiments using carboxypeptidase Y (CY) treated conditioned media .. 142

21. Secretion experiments from leucine aminopeptidase (LAP) treated conditioned media .................. 143
22. Bioactivity of G-25 Sephadex enriched fractions of conditioned media  . . . 144

23. Silver stain of a modified Schägger gel reveals a single peptide band in the bioactive fraction from G-25 purified conditioned media . . . . . . . . . . 145

24. Electroelution of conditioned media peptide reveals bioactivity is present only in the small molecular weight band . . 148

25. Evidence that conditioned media stimulates norepinephrine secretion from sympathetic ganglia cells . . . . . . . . . 149

26. Evidence of feedback inhibition by epinephrine on peptide production . 150

CHAPTER VII

27. Evidence that endotoxin stimulates an increase in conditioned media bioactivity . . . . . . . . . . . 169

28. Evidence that T cell and B cell populations are the source of bioactivity in non-mitogenically challenged cell cultures . . . . . . . . . 170

29. Macrophage conditioned media bioactivity increases after endotoxin incubation . 172

30. SDS-PAGE demonstrating that endotoxin stimulates the release of bioactive peptide from macrophages . . . . 173

31. Leucine aminopeptidase treatment yields bioactivity from non-bioactive conditioned media proteins . . . . 177
CHAPTER VIII

32. Schematic representation of possible physiologic loop of bioactive peptide-adrenal medullary interaction ........................................ 198

33. Extension of peptide-medullary loop .................................. 199

34. Schematic of potential calcium-dependent signal transduction mechanism of bioactive peptide ........................................... 200

Appendix

35. Examples of Enzyme Linked Immunoabsorbent Assays (ELISAs) .......................................................... 203

36. Polyvalent antisera inhibits conditioned media bioactivity .......................................................... 210
## LIST OF TABLES

### CHAPTER VII

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alterations in leucine aminopeptidase (LAP) activity in endotoxin (ETX) treated macrophage cultures and in endotoxic mouse serum</td>
<td>174</td>
</tr>
<tr>
<td>Appendix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Enzyme Linked Immunoabsorbent Assay (ELISA) provides evidence of a peptide specific antibody in polyvalent rabbit antisera</td>
<td>207</td>
</tr>
<tr>
<td>3.</td>
<td>Analysis using a competitive ELISA</td>
<td>209</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned Media</td>
<td></td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle's Balanced Salts Solution</td>
<td></td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salts Solution</td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>Chorionic Gonadotropin</td>
<td></td>
</tr>
<tr>
<td>NDV</td>
<td>Newcastle Disease Virus</td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin Releasing Hormone</td>
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</tr>
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<td>GH</td>
<td>Growth Hormone</td>
<td></td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
<td></td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol Trisphosphate</td>
<td></td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol Bisphosphate</td>
<td></td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
<td></td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral Blood Lymphocytes</td>
<td></td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed Lymphocyte Reaction</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immuno-absorbent assay</td>
<td></td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropin</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>Cluster Differentiation</td>
<td></td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
<td></td>
</tr>
<tr>
<td>EPI</td>
<td>Epinephrine</td>
<td></td>
</tr>
<tr>
<td>TSH</td>
<td>Thyrotropin</td>
<td></td>
</tr>
<tr>
<td>VIP</td>
<td>Intestinal Vasoactive Peptide</td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
<td></td>
</tr>
<tr>
<td>ETX</td>
<td>Endotoxin</td>
<td></td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer Cell</td>
<td></td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
<td></td>
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<tr>
<td>cAMP</td>
<td>Adenosine 3',5'-Cyclic Monophosphate</td>
<td></td>
</tr>
<tr>
<td>SEA</td>
<td>Staphylococcal Enterotoxin A</td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
<td></td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin Releasing Hormone</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine Tetrahydrochloride</td>
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</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

In vertebrates four major systems of intercellular communication exist: the neural, the endocrine, the neuroendocrine, and the immune systems. For many years, evidence has accumulated linking the neural, the endocrine, and the neuroendocrine systems, but the immune system was often viewed as essentially autonomous (Bateman et al., 1989). Current investigations, however, reveal information as to how the immune system is able to communicate with other systems. Evidence delineating the structural and functional connections between the central nervous system and the immune system has propelled research in this area to unprecedented expansion (Arnason, 1991; Harbour-McMenamin et al., 1986; Hiestand et al., 1986; Smith et al., 1982; Smith and Blalock, 1981). Identification of shared informational molecules such as cytokines, hormones, and neuropeptides provides molecular evidence for bidirectional communication
between these systems (Arnason, 1991; Carr and Blalock, 1991). One limb of this bidirectional communication may occur through the cytokines tumor necrosis factor-α, interleukin-1, and interleukin-6, which have all been shown to be potent modulators of the hypothalamic-pituitary-adrenal axis. More specifically, interleukin-1 has been shown to enhance sympathetic nerve activity (Haefli et al, 1993; Ichijo et al., 1994), modify the neuronal gene expression of molecules such as substance P (Jonakait, 1993), and is synthesized and released by cultured sympathetic neurons (Frieden et al, 1992). Studies indicate that immune-derived peptides may also play a role in modulating neural/endocrine function. In 1981, Blalock and Smith reported that human peripheral blood lymphocytes (PBL) infected with Newcastle disease virus (NDV) produced the proopiomelanocortin (POMC)-derived peptides, adrenocorticotropic (ACTH) and Beta-endorphin (Smith and Blalock, 1981). Interestingly, in studies with mice previously hypophysectomized to eliminate the pituitary as a source of ACTH, challenge with NDV significantly elevated plasma corticosterone levels (Smith et al., 1982). Furthermore, glucocorticoids released in response to
immunoreactive-ACTH were shown to negatively feedback on the immune cells to prevent further release of ACTH or endorphin. Thus, products of the immune system were found to affect output from the adrenal cortex.

More recent investigations have vastly expanded the list of peptide signalling molecules found to be synthesized and released by activated immune cells. These peptides include but are not limited to thyrotropin (TSH) (Smith et al., 1983), chorionic gonadotropin (CG) (Harbour-McMenamin et al., 1986), growth hormone (GH) and prolactin-related peptides (Hiestand et al., 1986). Evidence suggests that these peptides may serve an autocrine/paracrine function with cells of the immune system in addition to the traditional axes (Plaut, 1987). Preliminary studies suggest that there is yet another peptide of immune origin which, in contrast to those mentioned above, is able to stimulate catecholamine release from the adrenal medulla (Jones et al., 1993; Wang, 1993). Furthermore, preliminary experimental evidence in preparation for this dissertation work indicated that known peptide chromaffin cell secretagogues are not responsible for the observed phenomena. Such peptides include vasoactive intestinal
peptide (VIP), bradykinin, angiotensin II, and pituitary adenylyl cyclase activating polypeptide (PACAP) (Isobe, 1993; Livett and Marley, 1993; Marley, 1987). This previously uncharacterized peptide has also been shown to cause epinephrine secretion comparable to maximal neural stimulation but at an attenuated rate. Based on preliminary studies from this (Jones et al., 1993; Wang, 1993; Zhou and Jones, 1993) and other laboratories (Seidler and Mills, 1989) and the lymphoid-adrenal cortical interaction demonstrated by Smith and Blalock, a lymphoid-medullary interaction through a yet undescribed lymphocyte product is not surprising.

A variety of stressors, both physiologic and psychologic, activate a cascade of events which leads to the release of glucocorticoids and catecholamines from the adrenal gland by both neural and hormonal stimulations. The catecholamines, epinephrine and norepinephrine, are pivotal regulators of many physiologic events in man and have been shown to be potent modulators of the immune response. Immune cells have been shown to express both functional beta-2 (Krieger, 1983; Plaut, 1987) as well as alpha-1 adrenoreceptors (Sanders and Munson, 1985) which account for
the well-documented direct action of catecholamine on immune function (Malec et al., 1985; Mihran-Davis et al., 1991; Murray et al., 1993; Rinner et al., 1992). Catecholamine action on immune cells has long been viewed as being suppressive. This suppressive action ranges from decreases in T helper/T suppressor cell number ratio (Malec et al., 1989) to an inhibition of natural killer cell cytotoxicity (Whalen and Bankhurst, 1990). Interestingly, current literature indicates that catecholamine action may also act to enhance the immune response (Arnason, 1991). Data indicate that not only stress itself, but characteristics of the stressor such as duration (acute/chronic) and intensity/severity are also important variables modulating stress-induced immunologic alterations. For example, the same stressor can have a positive or negative relationship with immune responses depending on the intensity, such that less severe or acute stressors correlate with enhanced immune response, while potent/chronic stressors seem to correlate with depressed immunologic response (Rinner et al., 1992). Such immunoenhancement may serve to protect individuals for short periods of stress. Ultimately, chronic stress, such as that associated with long term
illness, and subsequent maintained elevations in plasma catecholamine appears to result in the well documented immunocompromising aspects of the stress response (Sanders and Munson, 1985). To date there have been no successful therapeutic approaches to control stress-induced immunosuppression. Identifying immune-derived signal molecule(s) that modulate stress hormone release may lead to therapeutic manipulation by suppressing or enhancing bioactive peptide release and/or activity.

This dissertation research has built upon previous studies which presented evidence for the non-neurogenic stimulation of adrenal medullary catecholamine release. Zhou and Jones (1993) demonstrated in conscious adrenal denervated rats that plasma epinephrine levels were significantly elevated 90 minutes after bacterial endotoxin challenge. This epinephrine elevation was approximately one-third that observed in non-denervated endotoxin challenged rats suggesting that a non-neural mechanism may comprise part of medullary epinephrine release. Related findings have been reported in newborn rats. Ganglionic blockade in endotoxin-challenged newborn animals was unable to prevent a rise in plasma catecholamine also suggesting a
non-neurogenic stimulation of adrenal medullary catecholamine release (Seidler and Mills, 1989). In vitro experiments have demonstrated that factor(s) released by immune cells may potentially act in vivo to modulate medullary catecholamine release (Jones et al, 1993; Wang, 1993). Experimental evidence indicates that these factor(s) are small molecular weight peptide(s) (<3,000) and are present and active in a variety of species (bovine, porcine, human).

Considering the clinical importance of stress-induced immunosuppression and the potential ramifications of immunomodulation of the stress response, understanding the role of various immune-derived signalling molecules in this process may provide pivotal information and a basis for future therapeutic intervention. This dissertation project was designed to begin to elucidate the components of the immune system which may play a role in communication between the immune system and the neuroendocrine system via the adrenal medulla. The hypothesis is that mononuclear cells release a novel bioactive peptide which stimulates adrenal medullary secretion in a calcium-dependent manner. The specific aims of the following studies include: 1) the
characterization of peptide-mediated secretion with an emphasis on the role of calcium in peptide-induced exocytosis, 2) the identification of the specific mononuclear cell subset(s) involved in release of the bioactive peptide and assessment of feedback inhibition by epinephrine on further peptide release, 3) the further isolation and characterization of this bioactive peptide using molecular biological and immunological techniques, and 4) the examination of the potential significance of this peptide in endotoxicosis and related shock states.
CHAPTER II

REVIEW OF RELATED LITERATURE

A. The Catecholamines

I. Physiologic Role in the Stress Response

The catecholamines, epinephrine and norepinephrine, are pivotal regulators of many physiological events in man. Increased circulating levels of catecholamine are known to mediate arteriolar constriction, elevation of blood glucose and free fatty acids, as well as positive chronotropic and inotropic effects. In 1929, Walter Cannon first associated these and other physiologic changes with increased circulating catecholamine in his discussion of a phenomenology referred to as the "fight or flight" response. This reaction occurs when animals, including man, perceive danger (cognitive stimuli) and prepares the animal to respond to the threat through either direct conflict ("fight") or escape ("flight"). An increased level of
circulating catecholamine, however, is also mediated by stimuli not recognized by the cerebral cortex (non-cognitive stimuli). Examples of non-cognitive stimuli include bacteria, tumors, and viruses. Thus, both psychological and physiological stimuli can evoke a classic stress response.

Documented actions of the catecholamines have been classified into four broad categories which include: 1) a peripheral excitatory action on certain smooth muscle types (i.e. blood vessels of the skin, mucous membranes) and on some sweat and salivary glands; 2) a peripheral inhibitory action on other types of smooth muscle (i.e. gut wall, bronchial tree); 3) a cardiac excitatory action responsible for positive chronotropic and inotropic effects; and 4) metabolic actions such as glycogenolysis and lipolysis. More recent investigations, however, have expanded this list of adrenergic actions to include modulation of immune function. The mechanisms and potential implications of this neuroendocrine-immune interaction are just beginning to be understood.

II. Sources and Secretion of the Catecholamines

The major sites of release of the catecholamines are
the postganglionic efferent sympathetic terminals and the adrenal gland. The adrenal gland, located in the abdominal cavity rostral to the kidneys, consists of two anatomically and embryologically distinct structures - the outer cortex and the inner medulla. While the glucocorticoid-secreting cortex is of mesodermal origin, the inner medulla, source of the catecholamines, is of neuroectodermal origin. During embryonic development, under influence of the corticosteroids, the sympathoadrenal progenitor cells of the neural crest migrate to the adrenal medullary cavity and differentiate into chromaffin cells (Anderson and Axel, 1986). Upon settling in the adrenal gland, the chromaffin cells are innervated by preganglionic sympathetic neurons of the splanchnic nerves and become part of the sympathetic nervous system.

These adrenal medullary chromaffin cells synthesize, store and secrete catecholamines in response to a variety of stimuli. While the list of chromaffin cell secretagogues has expanded, Feldberg et al. (1934) were the first to document that the release of acetylcholine from the splanchnic nerve was the immediate neurologic stimulus that induced adrenal medullary catecholamine secretion. This
group observed that stimulation of the splanchnic nerve led to the appearance of acetylcholine (Ach) in the blood of the feline adrenal vein and, ultimately, catecholamine (adrenaline/epinephrine) secretion from the adrenal gland. Furthermore, Feldberg and colleagues demonstrated both muscarinic and nicotinic agonists could stimulate catecholamine secretion indicating the presence of both cholinergic receptor types on chromaffin cells.

Depending on the animal model, activation of either the nicotinic or the muscarinic receptor can lead to chromaffin cell catecholamine secretion (Wakade and Wakade, 1983). Although knowledge of the exact mechanism of chromaffin cell exocytosis is poorly understood, one common feature of all models is that catecholamine secretion is dependent on an increase in intracellular calcium. The source of this calcium may be from internal stores, as is the case with muscarinic receptor stimulation, or from the influx of extracellular calcium, as with nicotinic receptor activation. Muscarinic receptor activation, the sole stimulus for chick adrenal medullary catecholamine secretion (Douglas, 1975) and non-functional in bovine chromaffin cells (Cheek and Thastrup, 1989), leads to the liberation of
diacylglycerol (DAG) and inositol trisphosphate (IP$_3$) from membrane phosphatidylinositol bisphosphate (phosphatidylinositol bisphosphate, PIP$_2$). The liberated IP$_3$ then binds to its intracellular receptor and, ultimately, stimulates calcium release from internal stores. Interestingly, chromaffin cells appear to possess two different, spatially distinct intracellular calcium stores - an IP$_3$ sensitive store and a caffeine sensitive store (Burgoyne et al., 1989). In bovine chromaffin cells, evidence indicates that the caffeine sensitive store is involved in calcium homeostasis rather than exocytosis (Cheek et al., 1990).

Unlike muscarinic receptor activation, the catecholamine secretion in response to nicotinic receptor stimulation requires the availability of extracellular calcium. Activation of nicotinic receptors is functionally important in cat (Douglas, 1975), dog (Tsujimoto and Ashidawa, 1975), and rat (Yoshizaki, 1973) and is the sole cholinergic stimulus for bovine chromaffin cell catecholamine secretion (Cheek et al., 1989). Nicotinic receptor activation increases cation conductance through the nicotinic receptor cation channel. The influx of cations, predominantly sodium, depolarizes the chromaffin cell and
activates voltage-gated sodium and calcium channels further depolarizing cells. The depolarization activates additional voltage-gated calcium channels which is followed by increased calcium influx through these channels. This increased intracellular calcium then triggers catecholamine secretion.

In addition to nicotinic and muscarinic receptor activation, chromaffin cell catecholamine secretion can also be induced by depolarizing concentrations of extracellular potassium. Such depolarization immediately activates voltage-dependent calcium channels thereby promoting calcium influx and subsequent catecholamine secretion. Thus, secretion in response to extracellular K⁺, like nicotine-induced secretion, is dependent on extracellular calcium. Further research with bovine chromaffin cells has indicated that calcium influx from extracellular sources is also essential for catecholamine secretion in response to other known chromaffin cell secretagogues (Cheek, 1991).
B. Effects of the Catecholamines on Immune Cell Function

I. Stress and Illness

The role of stress in illness has been debated for many years. Although numerous observations have been documented over the centuries which note a relationship between stressful periods in life and subsequent illnesses, definitive physiologic evidence of such a relationship has been difficult to obtain since both psychological (cognitive) and physical (non-cognitive) stimuli may contribute to physiologic stress. The idea that mental and physical health are interdependent can be found in early medicine in the works of Aristotle. This concept was further substantiated by Galen when he observed that melancholic women were more likely to develop cancer than their more confident and vital counterparts (Kort, 1994). Similar associations between emotional stress and health appear even in current studies. For instance, studies cite that job strain has a negative impact on health (Lerner et al., 1994). There is also evidence of a correlation between a positive mental state and better health in terminal AIDS patients (Kendall, 1994).
Early in this century Hans Selye expanded this notion of "mind-body" interaction when he noted that not only emotional stress but also physical stress may affect physiologic function. Selye described how "diverse noxious agents" produced nonspecific events that "sick people" share in common. Furthermore, Seyle explained that these "noxious agents" included both harmful physical agents (i.e. viruses, bacteria, excessive body weight) as well as how an individual reacted to a harmful physical agent (cognitive stimuli) (Selye, 1936).

One of the many difficulties in ascertaining a potential connection between stress and illness is in the definition of "stress". Unfortunately, as Selye would note, there is no reliable or accurate way to experimentally define stress or how it will affect an individual since not only the stressor itself but also the way an individual reacts to that stressor will effect the physiologic response (Selye, 1975). Typically, the term "stress" is defined in the context of research and pathophysiology as "the reactions of the body to forces of a deleterious nature, infections and various abnormal states that tend to disturb its normal physiological equilibrium (homeostasis)"
As Selye described in his early observations, this definition requires that the reactions of the body to physical/psychological stimuli as well as the stimuli themselves be considered the "stress". This is problematic when designing experimental protocols to determine the stress-illness relationship since both physical and psychological parameters are involved (Selye, 1980). Fortunately, experiments in animals as well as in humans indicate that the mechanism behind both physiological and psychological stress-induced dyshomeostasis may be attributed mostly to changes in the neuroendocrine system which modulates immune system function (Sheridan et al. 1994). Key factors of this neuroendocrine immunomodulation appear to be the pituitary and adrenal gland hormones, including the catecholamines.

II. Sympathetic Innervation of Primary and Secondary Lymphoid Organs

Investigations have demonstrated that adrenergic modulation of the immune system may occur at the earliest stages of the immune cell maturation process and may potentially dictate the functional capacity of immune cell
populations. Anatomical studies from several laboratories (Ackerman et al., 1987; Bellinger et al., 1988; Kendall and Al-Shawaff, 1991; Nohr and Weihe, 1991) show that autonomic nerve fibers, mainly noradrenergic, innervate primary, secondary as well as mucosal associated lymphoid tissue - organs which are the ultimate source of all cells of the immune system. These tissues include the bone marrow, thymus, spleen, lymph nodes and gut-associated lymphoid tissue (GALT). Distributed into specific compartments of these organs, these noradrenergic fibers are associated with various cellular components of the immune system including lymphocytes, macrophages, and mast cells (Felten and Olschowka, 1987; Felten and Felten, 1990).

Developmental studies of the noradrenergic innervation of lymphoid organs of the rat indicate that this sympathetic-immune association may occur post-gestationally and continue into adulthood (Ackerman et al., 1987; D.L. Felten et al., 1987). As the animals mature, however, there appears to be an age-dependent denervation of the spleen such that by age 27 months the total norepinephrine content of the rat spleen diminished by approximately 50% (S.Y. Felten et al., 1987; Bellinger et al., 1987). While this
diminished splenic norepinephrine content could be explained by decreased synthesis in existing splenic nerve terminals, further investigations indicated there was an actual retraction and loss of noradrenergic fibers in the aging spleen. Interestingly, sympathetic innervation in other organs of these aged animals, such as the heart, persisted and circulating catecholamine actually increased with age.

The functional role of noradrenergic innervation of lymphoid organs has yet to be fully understood. Studies examining central lymphoid compartments, the bone marrow and thymus, indicate that cell proliferation, differentiation, and emigration may be influenced by the local branches of the sympathetic nervous system (Webber et al., 1970; Byron, 1972, Singh, 1985). For instance, the catecholamines may inhibit hematopoiesis via alpha-1 adrenergic receptors on bone marrow cells (Maestroni and Conti, 1994). The effects of noradrenergic innervation on secondary lymphoid compartments, although more thoroughly examined, appear dependent on the animal model and experimental protocol. Several early studies (Besedovsky et al., 1979; Williams et al., 1981) suggested that the immune system was constitutively suppressed by noradrenergic innervation of
the secondary lymphoid organs. Chemical or surgical sympathectomy of the secondary lymphoid organs in these studies led to an augmentation of immunologic functions such as the antibody (Besedovsky et al., 1979) and the delayed hypersensitivity response (Braun et al., 1986). These investigations, however, utilized neonatal mice or rats and assessed immune cell function in these same animals after they reached adulthood. Interestingly, if the sympathectomy was not performed until the animal reached adulthood, the antibody response was either inhibited (Hall et al., 1982) or unaffected (Miles et al., 1981). Unlike earlier studies, these data suggested that in the adult animal the sympathetic nervous system maintained immune cell function in the secondary lymphoid organs. Although sympathectomy of the adult animal has been shown to suppress both T cell (Livnat, 1986; Madden et al., 1986) and B cell (Madden et al., 1986) function, natural killer cell and macrophage activity appears to be augmented when noradrenergic influences are removed from lymphoid organs (Koff et al., 1985; Madden et al., 1987). The variability of the noradrenergic influence on lymphoid function (i.e. age, organ/cell type) makes it difficult to designate
specific immunologic consequences to alterations in this neural-immune interaction at even the earliest stages of immune cell development.

III. Immune Cell Adrenoreceptors

The catecholamines exert their effects through two main classes of adrenergic receptors, α and β - a subdivision first proposed by Ahlquist (1948) to distinguish different adrenergic responses in various organ systems. Later studies (Arnold et al., 1966; Lands et al., 1967; Lager, 1974) revealed there are several different subtypes of each of these receptors, each appearing to be the product of a distinct gene family (Summers and McMartin, 1993). Subtypes of the beta subclass include type-1, type-2, and more recently the type-3 beta adrenoreceptor. Alpha adrenergic receptors are divided into type-1 and type-2 (Price et al., 1994).

The second messenger systems to which the adrenergic receptors are coupled are unique to each subtype. However, all sequenced adrenergic receptors have been shown to belong to the guanine nucleotide binding protein, or G-protein, linked superfamily of receptors (Cotecchia et al., 1990;
The α₁-adrenoreceptor, through G₉, stimulates phospholipase C (PLC) which initiates the hydrolysis of phosphatidylinositol bisphosphate (PIP₂) to produce the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP₃). These second messengers are responsible for the activation of protein kinase C (PKC) and the liberation of calcium from intracellular stores respectively (Berridge and Irvine, 1989). In contrast, the α₂-adrenoreceptor is coupled by G₁ to adenylyl cyclase or to ion channels. Activation of the α₂-receptor alters cellular activity by reducing intracellular adenosine 3', 5' - cyclic monophosphate (cAMP) or by directly modifying ion channel activity (e.g. Ca²⁺ or K⁺ channels) (Bylund, 1988). The β-adrenoreceptor subtypes are coupled through yet another G protein, G₉, to the membrane bound enzyme, adenylyl cyclase. Hence, β-adrenergic receptor activation alters cellular activity by increasing intracellular cAMP concentrations.

Characteristically, α receptors are more sensitive to norepinephrine while the β receptor subtype is more responsive to epinephrine stimulation (Goodman and Gilman, 1990). Similarly, the adrenergic blocking action of many
compounds is also selective for each receptor type. The imidazolines and haloalkylamines are selective for the α receptors whereas propranolol blocks only β receptors (Goodman and Gilman, 1990). More recently, compounds specific to receptor subtypes have been identified. For example, salbutamol and salmeterol are specific β2-receptor agonists while alpha-methylnorepinephrine stimulates the alpha-2 adrenoreceptor alone (Felsner et al., 1995; Muller-Wieland et al., 1994). The unique pharmacologic properties of these compounds make it possible to experimentally ascertain which adrenergic receptor type(s) are responsible for individual cellular or systemic alterations in response to catecholamine. Such pharmacologic agents were also utilized to determine that cells of the immune system possess functional α and β adrenergic receptors and are capable of responding to catecholamine.

A. Beta Adrenergic Modulation of Immune Cell Function

The β-adrenergic receptor function has been well characterized due to the availability of several high affinity labelled antagonists. Radioligand binding and pharmacologic studies have confirmed the presence of the β2-
adrenergic receptor on most cells of immune origin. Cell
types expressing functional \( \beta_2 \)-receptors include
granulocytes, natural killer cells, monocytes, B cells, and
T cells (Plaut, 1987). There is some discrepancy in the
literature as to which of these cell populations have the
highest receptor density. Some sources demonstrate the T
suppressor/cytotoxic (CD8+ phenotype) and the natural killer
cell populations as having the most \( \beta_2 \)-adrenoreceptors
(Murray et al., 1992) while others the B-cell population

The immunosuppressive properties of \( \beta_2 \)-receptor
activation were first demonstrated using human peripheral
blood lymphocytes (PBL) co-cultured with high concentrations
of the \( \beta \)-adrenergic agonists isoproterenol and epinephrine
(25 mM). Culture of these cells in the presence of the \( \beta \)-
agonists led to suppressed mitogen responsiveness (Hadden et
al., 1970). Even in the presence of a low, physiologically
relevant concentrations (1-100nM), addition of isoproterenol
for a brief period at the initiation or the end of the
culture period resulted in depressed response to the
mitogen. Conversely, in vitro studies demonstrated that
mitogenic stimulation led to a down-regulation of the \( \beta \)-
receptor on lymphocytes (Smith et al., 1971; Johnson et al., 1981; Cazaux et al., 1995). Among other actions, β-adrenergic agonists have also been shown to suppress IL-2 production, block expression of IL-2 receptors, inhibit T cell mediated proliferation, inhibit lymphocyte-mediated toxicity as well as human lung mast cell function, and depress NK cell function (Feldman et al., 1987; Plaut, 1987; Heilig et al., 1993; Benschop et al., 1994; Chong et al., 1995). β2-adrenoreceptor activation has also been shown to alter metabolic pathways in mononuclear leukocytes by inhibiting fatty acid synthesis (Muller-Wieland et al., 1994). In support of this suppressive property of the β2-adrenergic receptor, experimental evidence demonstrates that β-receptor antagonists tend to potentiate the immune response (Luecken and Lysle, 1992; Benschop et al., 1993; Gentilini et al., 1994).

While a trend emerges which indicates β-receptor activation suppresses immune cell function, the literature reveals this relationship is dependent on the cell population being studied and the experimental conditions. For instance, whereas T cytotoxic/suppressor cell function is depressed by β-receptor activation, Leu 3+8+ (Th
suppressor activator) cells responsiveness to PHA is enhanced in the presence of isoproterenol (10 µM) while Leu 3+8- (Th suppressor enhancer) cells are not effected (Khan et al., 1985). Therefore, one may not generalize that cell mediated immunity is inhibited by β-adrenergic receptor activation. Interestingly, the humoral immune response also may be enhanced by β-adrenergic receptor activation. β2-adrenergic receptor activation has been shown to lead to an enhanced antibody response (Burchiel and Melmon, 1979; Sanders and Munson, 1984a; Sanders and Munson, 1984b) through both direct and indirect mechanisms.

Investigations are addressing the role of immune cell β-adrenergic receptor activation in disease states such as juvenile type I diabetes mellitus (Schwab et al., 1993) or schizophrenia (Bondy et al., 1984). These studies demonstrate a decreased β-receptor responsiveness in the disease states. This is attributed to a diminished receptor density on the lymphocytes of diabetic children and a decreased receptor affinity in schizophrenic patients. In vitro models may potentially provide some insight into the mechanisms behind altered β-receptor responsiveness in these or other disease states. However, the literature
clearly demonstrates that to understand the clinical implications of disease-related diminished immune cell \( \beta \)-receptor responsiveness, variables such as the cell population(s) exhibiting the diminished response need to be considered.

**B. Alpha Adrenergic Modulation of Immune Cell Function**

Pharmacologic and radioligand binding studies demonstrate that both the \( \alpha_1 \)- and the \( \alpha_2 \)-adrenoreceptor are present on cells of immune origin. Leukocytes which have been shown to express functional \( \alpha \)-adrenergic receptors include T cells (Felsner et al., 1995), monocytes (Lappin and Whaley, 1982), neoplastic (chronic lymphocytic leukemia) B cells (Goin et al., 1991), and neutrophils (Hulin et al., 1985), although neutrophil expression of this receptor subtype has recently been brought into question (Musgrave and Seifert, 1994). Further examination of leukocyte \( \alpha \)-adrenoreceptor expression reveals that this receptor may not only differ among cell types but also may vary within a single cell population depending on its anatomical location. Experimental evidence demonstrates that while the \( \alpha \)-receptor is present on splenic lymphocytes (McPherson and
Summers, 1982), there is no evidence that the receptors are found on the circulating counterparts of these cells (Casale and Laliner, 1988).

The potential role of α-adrenergic receptor in modulation of immune cell function is just beginning to be understood. Several studies have employed α-adrenergic agonists and antagonists to determine the function of this receptor type in stress-induced immunomodulation (Hadden et al., 1979; Sanders and Munson, 1985). Some of the earliest work from Hadden et al. (1979) demonstrated that like epinephrine, high concentrations of norepinephrine (25 mM) in culture with human peripheral blood lymphocytes (PBL) led to an immune cell suppression. Interestingly, unlike the immunosuppressive β-receptor modulated response, more physiologic concentrations of norepinephrine (1-100nM) elicited an increased mitogen responsiveness in the cultured cells. Similar to the epinephrine-mediated β-response, the time and duration of exposure of the lymphocytes to the norepinephrine was important. Recent investigations show that this α-adrenergic immunopotentiating effect of the catecholamines may apply to the in vivo scenario as well. Carr et al. (1992) have demonstrated that in the mouse
model, cold stress consistently augmented total IgG and IgM production by splenic lymphocytes and this mediation was a result of activation of alpha-adrenergic pathways. However, since splenic lymphocytes were utilized in these studies, it is unclear which cell population(s) are responsible for this α-receptor mediated augmentation.

Much like β-adrenergic receptor, α-receptor activation not only has immunopotentiating (i.e. enhanced antibody response) but also immunosuppressive capabilities. Recently, Felsner et al. demonstrated that a 20h continuous treatment of rats with catecholamine markedly suppressed the in vitro reactivity of T lymphocytes. Using selective agonists, the group identified the relevant receptor to belong to the alpha-2 subtype (Felsner et al., 1995). Contrary to other experimental findings, Felsner and colleagues observed this alpha-mediated suppression on T cells isolated from peripheral blood but not in splenic T cells. This suggests that peripheral blood lymphocytes do possess functional alpha-adrenoreceptors. The suppressive action of alpha-adrenergic receptor activation is also prevalent when looking at metabolic pathways in mononuclear leukocytes. Muller-Wieland et al. (1994) demonstrated that
the specific alpha-2 receptor agonist alpha-methylnorepinephrine, but not the alpha-1 agonist phenylephrine, inhibited the fatty acid synthesis rate in isolated human mononuclear cells.

C. Immunomodulation of Neuroendocrine Function

I. Immune Cell Secretion of Neuroendocrine Signalling Molecules

A salient feature of immune system and neuroendocrine system interactions is their capacity to communicate with each other via common signal molecules and common receptors (Blalock, 1994). Central to this bidirectional communication is production of classic neuropeptide hormones by cells of immune origin. The first evidence that leukocytes could synthesize functional neuroendocrine hormones was obtained from studies which demonstrated that during the production of interferon alpha (IFNα), virally-challenged human leukocytes coexpressed a peptide which was antigenically related to the pituitary hormone, adrenocorticotropin (ACTH) (Smith and Blalock, 1981). This immune-derived ACTH was similar to its pituitary counterpart
with respect to its antigenicity, molecular weight, and retention time on high pressure liquid chromatography (HPLC). Furthermore, the leukocyte-derived ACTH could stimulate glucocorticoid secretion from adrenal cortical cells in vitro and, thus, was bioactive and potentially functional (Clarke et al., 1993). The potential biological relevance of this molecule was addressed in in vivo studies using hypophysectomized mice. Viral challenge of hypophysectomized animals led to a significant elevation in circulating glucocorticoids when compared to control mice even though the classic glucocorticoid stimulant, pituitary ACTH, was essentially eliminated. Splenocytes of these hypophysectomized mice also stained positive for both ACTH and additional POMC-derived peptides, the endorphins, while control mouse splenocytes did not (Smith et al., 1982). Ultimately, researchers demonstrated that this leukocyte-derived ACTH had the same amino acid sequence as pituitary-derived ACTH and originated from the same precursor, proopiomelanocortin (POMC), in immune cells as in the pituitary gland (Smith et al., 1990).

While immune cell production of the POMC-peptides was originally described in response to immunologic stimulation
of these cells, later studies revealed that immune cells synthesize and secrete bioactive ACTH and endorphin in response to corticotropin releasing hormone (CRH), the hypothalamic regulator of pituitary ACTH (Smith et al., 1985). Additionally, ACTH production was inhibited by the synthetic glucocorticoid, dexamethasone, much like pituitary cells. Interestingly, this immune cell-derived ACTH was also shown to suppress antibody and lymphokine production (Johnson et al., 1982; Koff and Dunegan, 1985) thereby potentially acting in an autocrine/paracrine manner. Thus, these reports served as a benchmark demonstrating that products of the immune system could produce and respond to functional neuroendocrine hormones.

Production of hormones by the immune system is not limited to the POMC-derived peptides but also includes thyrotropin (TSH) (Smith et al., 1983), chorionic gonadotropin (CG) (Harbor-McMennamin et al., 1986), growth hormone (GH) and prolactin-related peptides (Hiestand et al., 1986), somatostatin (Lygren et al., 1984; Goetzl et al., 1985), luteinizing hormone (LH) (Emanuele et al., 1990), and vasoactive intestinal peptide (VIP) (Lygren et al., 1984), among others. Experimental evidence indicates
that similar to the POMC peptides these immune-derived molecules may not only function to modulate neuroendocrine function but also may serve some local immunoregulatory function. For example, the opioid peptides have been shown to stimulate NK cell activity (Koff and Dunegan, 1985) while GH enhances function of the cytotoxic T cell as well as T cell colony formation (Mercola et al., 1981; Snow, 1985). In contrast, both somatostatin and VIP suppress immune function by inhibiting T cell proliferation (Payan et al., 1986).

In general, release of the neuroendocrine signalling molecules is positively regulated by an identified, specific stimulus. The hypothalamus, for example, releases CRH which directly stimulates the pituitary to release ACTH. In turn, this ACTH specifically stimulates adrenal cortical glucocorticoid secretion. Experimental evidence has accumulated which suggests that immune cell synthesis and release of hormone signalling molecules also occur in response to specific stimuli. Most of the aforementioned peptide hormones appear to be synthesized by leukocytes in response to both the classic neuroendocrine regulatory molecules and to specific immunologic stimuli. For
instance, TSH, the first de novo synthesized glycoprotein hormone found in the immune system, is synthesized and secreted by immune cells in response to the T cell mitogen staphylococcal enterotoxin A (SEA) (Smith et al., 1983) or thyrotropin releasing hormone (TRH) (Krueger et al., 1989). In contrast, a mixed lymphocyte reaction (MLR) is the only known stimulus for lymphocyte CG synthesis (Harbour et al., 1986).

Potentially, this information may be important when determining the physiologic significance of immune cell production of these peptides as well as provide insight into certain aspects of a pathophysiology related to an immune-derived neuroendocrine hormone. Such information has been utilized, for example, in current models of pregnancy. Lymphocyte-derived CG has been speculated to be an important suppressive factor necessary for a successful implantation of the blastocyst and, ultimately, a successful pregnancy (Blalock, 1992). Collectively, these results suggest that cells of the immune system should now be considered a physiologically relevant source of neuroendocrine signalling molecules.
II. Non-neurogenic Modulation of Chromaffin Cell Catecholamine Secretion

Modification of neuroendocrine function by signalling molecules from other systems such as the immune system extends to a variety of organs including the adrenal gland. As stated previously, the adrenal medulla secretes catecholamine during a typical stress response as a result of cholinergic stimulation of medullary chromaffin cell nicotinic and muscarinic receptors. Chromaffin cell secretagogues, however, are now known to include several additional signalling molecules. These mediators include but are not limited to angiotensin II (Powis and O'Brien, 1991), bradykinin (Owen et al., 1989), histamine (Bunn and Boyd, 1992), arachidonic acid (Negishi et al., 1990), as well as γ-aminobutyric acid (GABA) (Gonzalez et al., 1992).

Several studies have focused on characterizing the secretory mechanisms of each of these molecules. Catecholamine secretion in response to most of these secretagogues, like cholinergic catecholamine secretion, is a calcium dependent process (O'Sullivan et al., 1989; Cheek, 1991). The only exception appears to be a non-calcium dependent catecholamine release observed in response to high
concentrations (0.1-1.0mM) of histamine (Goh and Kurosawa, 1992). Interestingly, most of these mediators also utilize the phosphatidylinositol pathway to initiate chromaffin cell exocytosis. Similar to muscarinic receptor activation, the binding of arachidonic acid (Negishi et al., 1990), histamine (Noble et al., 1986), bradykinin (Owen et al., 1989), and angiotensin II (Sasakawa et al., 1989) to their respective chromaffin cell receptor stimulates phosphoinositide metabolism. Breakdown of membrane inositol phospholipids liberates the intracellular second messengers DAG and IP₃. Binding of the IP₃ molecule to its intracellular receptor, a receptor-gated channel, leads to release of calcium from internal stores and a subsequent intracellular calcium rise in the chromaffin cell. This initial increase in intracellular calcium is then followed by an influx of calcium from external stores (Owen et al., 1989; Negishi et al., 1990; Goh and Kurosawa, 1991; Powis and O'Brien, 1991). Apparently, influx of external calcium is essential for these secretagogues to stimulate catecholamine release since removal of extracellular calcium abolishes catecholamine secretion in all cases (Owen et al., 1989; Negishi et al., 1990; Goh and Kurosawa, 1991; Powis
Although histamine, bradykinin, angiotensin II, and arachadonic acid utilize the same second messenger system to increase intracellular calcium and stimulate catecholamine secretion, the efficacies of each of these chromaffin cell secretagogues differ considerably. Maximal histamine-induced catecholamine secretion from chromaffin cells is comparable to levels seen in cholinergic stimulation (Livett and Marley, 1986; Owen et al., 1989). Chromaffin cell stimulation by angiotensin and bradykinin, however, leads to the release of only a modest amount of catecholamine (Bonn and Marley, 1989; Owen et al., 1989). There are potentially several reasons for the observed differences in catecholamine secretion in response to these secretagogues. For example, the efficacious chromaffin cell secretagogue histamine stimulates a prolonged, more global calcium influx (Staudermann et al., 1990; Staudermann and Pruss, 1990; Cheek et al., 1993) when compared to other less efficacious secretagogues such as angiotensin II and bradykinin. In addition, these secretagogues appear to elicit different patterns of second messenger formation/breakdown in these cells (Staudermann and Pruss, 1990; Plevin and Boarder,
1988). Although knowledge of these systems is limited, the spatial and temporal variations in intracellular calcium as well as different patterns of second messengers may be reasons for the differences in catecholamine secreted in response to these molecules.
RATIONALE

Recent investigations from this laboratory provide evidence for yet another non-neural chromaffin cell secretagogue of immunologic origin. *In vitro* experiments have demonstrated that factor(s), like ACTH and the endorphins (Smith and Blalock, 1981; Smith et al., 1982), released by immune cells may potentially act *in vivo* to modulate adrenal medullary catecholamine release (Wang, 1992; Jones et al., 1993). Preliminary data indicate that these factor(s) are small molecular weight peptide(s) (<3,000) and are present and active in a variety of species (bovine, porcine, human). Similar to histamine, these unidentified factor(s) stimulate adrenal medullary chromaffin cell secretion to levels comparable to maximal cholinergic release (Jones et al., 1993). The following dissertation research was performed to further identify and characterize these immune-derived factor(s). Identification and characterization of immune-derived signalling molecules which may modulate neuroendocrine function (i.e. chromaffin cell epinephrine secretion) will provide insight to the potential homeostatic importance of intersystems communication. Results of these studies will provide a
foundation for future studies of immunomodulation of stress hormone release. As mechanisms of stress-associated immunologic changes and immune modulation of the stress response are understood, manipulation of these systems and improved patient outcome to stress and infectious challenge becomes possible.
A. Cell Culture

I. Primary Cultures of Chromaffin Cells

a. Isolation and Culture: Chromaffin cells were isolated from adult bovine adrenal glands according to the overall procedure described by Dahmer et al. (1990). In brief, adrenal glands were perfused with collagenase, incubated at 37°C and medullary tissue removed for further digestion. Digested cells were washed, counted, viability determined with trypan blue and purity assessed with neutral red. Cells were plated on collagen-coated 24-well plates at a density of $3 \times 10^5$ cells per cm$^2$ and media was changed after the first day and then as needed to maintain a normal pH.

b. Chromaffin Cell Culture Media: Primary cultures of chromaffin cells were maintained 4-7 days subsequent to
their isolation in media comprised of equal parts of F-12 Nutrient Mixture (Gibco BRL, Gaithersburg, MD) and Dulbecco's Modified Eagle's Medium (D-MEM, Gibco BRL, Gaithersburg, MD). Five hundred milliliters of this media contains 5 ml 1M HEPES, 55.6 mg penicillin, 220 µl gentamicin (50 mg/ml), 62.3 µl cytosine β-arabinofuranoside (Ara-C, 28 mg/ml), 53.4 µl 5-fluoro-2'-deoxyuridine (5-FDU, 24 mg/ml), and 2.76 ml Nystatin suspension (10,000 units/ml). Media is supplemented with fetal bovine serum (FBS, 10% v/v) and 5.6 ml filter-sterilized L-glutamine (200 mM) is added every thirty days.

c. In vitro Model of Catecholamine Secretion: Basic secretion experimental protocols are those described by Dahmer et al. (1990). In brief, growth media was removed and replaced with conditioned media (0.5ml, 90 min. incubation), serum-free RPMI-1640 (0.5ml, 90 min. incubation), Earle's Balanced Salts Solution (EBSS, 0.5ml, 90 min.), or secretagogue in EBSS (0.5ml, 10 min. incubation). After incubation, media was removed and saved. Catecholamine content in these samples represent that stimulated by conditioned media (CM), spontaneous/basal secretion (RPMI/EBSS), or that stimulated by a known
secretagogue. Cells were lysed by 1M perchloric acid (1.0ml) and saved to determine the remaining catecholamine content of the cells. The fraction of catecholamine (epinephrine) secreted into the media as a percent of total content was calculated by dividing the amount secreted by the total well content. Secretion experiments were routinely done in triplicate using three separate wells on any one plate which were derived from the same adrenal gland. Replicates of separate triplicate determinations were made on cells from different chromaffin cell preparations. Cell epinephrine content (ng/10^6 cells) ranged from 13.5±0.4 µg to 19.8±0.6 µg in all preparations. Epinephrine was quantified by HPLC using electrochemical detection. Mobile phase was 0.1M monochloroacetic acid with 0.5mM EDTA and 1.0mM sodium octyl sulfate adjusted to pH 3.0. Acetonitrile was added at 15ml/L. A BAS Liquid Chromatographic System including an LC 4C electrochemical detector and glassy carbon electrode was used for all analyses.

d. Data Analysis: All data are presented as epinephrine secretion as a percent of total cell content. Percent secretion of each experimental and control group(s) are
compared with every other group using an analysis of variance (ANOVA). A Tukey's follow-up test is utilized when statistically significant differences are found between groups. A p < .05 is considered statistically significant.

II. Primary Cultures of Mononuclear Cells from Spleen

a. Isolation and Culture: Whole bovine spleen was obtained from a local slaughter house. Inner spleen tissue, which includes both red and white pulp, was dissected out, cut into small pieces and forced through wire mesh in Hank's Balanced Salts Solution (HBSS) at room temperature. Crude cell suspensions were layered on density gradient media (Fico/Lite-LymphoH, Atlanta Biologicals), centrifuged and then separated cells were removed and washed. Isolated cells were transferred to flasks with RPMI media containing only essential amino acids and antibiotics at a density of 2x10^6 cells/ml. The growth media was isolated from the cells and either used promptly or frozen (-20°C) after at least 24 hours of culture. This media taken from mononuclear cell cultures containing all factors released by the mononuclear cell populations is referred to as conditioned media (CM). Secretion experiments utilized CM
filtered through Centricon filters (3,000 MW cutoff) to remove all factors greater than 3,000 MW.

b. Mononuclear Cell Culture Media: Isolated bovine splenocytes were maintained in serum-free RPMI-1640 (Gibco BRL, Gathersburg, MD). Five hundred milliliters of this medium contained 1 g NaHCO₃, 0.5 ml gentamicin (50mg/ml), 5 ml Dulbecco's Modified Eagle Medium (D-MEM), 5 ml penicillin-streptomycin (5,000 units/ml penicillin; 5,000 µg/ml streptomycin), and 25 µl 2-Mercaptoethanol (2-ME, 1M). L-glutamine (5.6 ml, 200mM stock) was added to media every thirty days.

c. Epinephrine Co-Culture: Isolated bovine mononuclear cells were counted and suspended in RPMI-1640 at 2X10⁶ cells/ml. Aliquots of cell suspension were then placed into 25 cm² tissue culture flasks with or without various concentrations of epinephrine (1nM - 1µM, Sigma Chemical Co, St. Louis, MO) and cells were allowed to incubate at 37°C for 24 hours. At that time, cell supernatant was harvested and then used in secretion experiments or frozen (-20°C) for future use. Epinephrine (1 µM), in the presence of the β-adrenergic receptor antagonist, propranolol (100 µM), was also incubated overnight with mononuclear cell cultures.
Since epinephrine may remain unoxidized in the supernatant of these cultures after the twenty-four hour culture period, experiments were performed concurrently to determine the amount of residual catecholamine. The various concentrations of epinephrine (1 nM - 1 µM, in RPMI-1640) were incubated at 37°C for 24 hours then analyzed using HPLC. Oxidation of epinephrine in cell cultures was minimized by the presence of either 0.2 mg NaSO₃/ml RPMI or 0.1% ascorbic acid.

III. Hybridoma Cell Lines

a. IL-A51/anti CD-8: The immortal cell line IL-A51, a murine hybridoma cell line producing monoclonal antibody (IgG1) to the bovine orthologue of human CD8, was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured at the concentration of 10⁶ cells/ml in a media comprised of RPMI-1640 supplemented with 10% (v:v) fetal bovine serum and 20 µM 2-Mercaptoethanol. Cultures were maintained by adding fresh media every 2-3 days as needed.

b. IL-A11/anti CD-4: The immortal cell line IL-A11, a murine hybridoma cell line producing monoclonal antibody (IgG2a) to the bovine orthologue of human CD4, was obtained
from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured at the concentration of 10⁶ cells/ml in RPMI-1640 supplemented with 10% fetal bovine serum and 50µM 2-Mercaptoethanol. Cultures were maintained by the addition of fresh media every 2-3 days as necessary.

B. Mononuclear Cell Separation/Purification

I. Separation of Monocyte/Macrophage Populations

The monocyte/macrophage populations were isolated from other splenocytes based on 1 hour adherence to plastic in culture flasks at 37°C (Clarke et al., 1993). Purity of the monocyte/macrophage cultures was assessed using two different staining methods. Wright-Giemsa staining was used to ensure no other adherent cell populations (i.e. dendritic cells, fibroblasts) were present in the culture. Non-specific esterase activity, unique to monocyte/macrophage mononuclear cells, was used to determine contamination of T and B cell populations in adherent cell cultures.
II. T cell/B cell Isolation and Separation Using Monoclonal Antibodies

In 1975, Kohler and Milstein described a technique for immortalization of B cells that produce a specific antibody. The method involves cell fusion between a normal antibody-producing B cell and a myeloma line, and selection of fused cells that secrete antibody of the desired specificity derived from the normal B cell. Such fusion-derived immortalized antibody-producing cells lines are referred to as hybridomas. Hybridomas producing monoclonal antibodies specific to the bovine orthologues of the human CD4 and CD8 molecules were obtained from American Type Culture Collection (Rockville, MD) and these cells were cultured according to given protocols.

a. Isolation of Antibody from Hybridoma Cultures:

Hybridoma cultures were allowed to grow until culture media was turbid. The hybridoma cell culture flask was then agitated, lysing cells and maximizing antibody concentration in the suspension. The cell suspension was then centrifuged at 2000g at 4°C. The supernatant was poured over a Proteins A/G column, bacterial products that bind the Fc portion of the antibody, and purified antibody was eluted from the
column using 0.1M glacial acetic acid (pH=2.0). Absorbance at 206 nm of column effluent was used to indicate antibody elution.

b. Antibody Biotinylation and Coupling to Magnetic Microbead: Antibody was conjugated with magnetic microbeads using the avidin-biotin system. Briefly, antibody was dialyzed against 0.1M NaHCO₃ (pH 8.4) overnight and concentration after dialysis determined via spectrophotometric analysis using the Bradford assay. Antibody was then concentrated or diluted to obtain a final concentration of 1 mg/ml. Immediately before use, 1.63 mg of NHS-LC-biotin (Pierce, Rockford, IL) was dissolved in 1 ml of dimethyl sulfoxide (DMSO) in a glass tube. This mixture was triturated and 50 µl was added to 1 ml antibody solution. This procedure results in a biotin substitution ratio of approximately 8:1. The reaction was allowed to proceed at room temperature for one hour. The reaction mixture was then dialyzed against 0.1 M NaHCO₃ for 48 hours. Sodium azide was not used since antibody labeled cells were maintained in culture for an extended period (24-48h).

c. Magnetic-based Cell Separation: Biotinylated antibody, anti CD4 or anti CD8, was added to the lymphocyte
cell suspension for 10 min to allow for adequate labeling with antibody and unbound/excess antibody then removed by three cold PBS washes (pH 7.4). Biotinylated antibody, bound to its respective cell surface marker, was coupled to a streptavidin linked magnetic microbead during a 10 min. incubation on ice, a reaction which occurs due to the high affinity of streptavidin for biotin ($K_d=10^{-19}$M). Cells were again washed in PBS and labeled cells passed over a magnetic column. In theory, all cells labeled with magnetically-conjugated antibody will be retained on the column while any unlabeled cells should pass through. Labeled cells, still bound to magnetic beads, are then be released from the column by removing the column from the magnetic field and washing several times in cold PBS. These cells, still labeled with antibody, may then be cultured according to standard conditions. This protocol was followed twice for each lymphocyte preparation, one passage to separate CD4 positive lymphocytes and a second to isolate CD8 positive lymphocytes. B cells were considered to be those populations which were not labeled by magnetically linked antibody and thus wash through the column. Purity of these cultures, as assessed using Fluorescence Activated Cell
C. Peptide Purification and Isolation

I. G-25 Sephadex Column Gel Filtration

G-25 Sephadex beads (10 g) were combined with deionized water at a ratio of 1 g per 2-3 ml water yielding a final volume of approximately 30 ml and equilibrated for 8-10 hours. This volume was placed in a 2 cm column which was attached to a continuous perfusion pump with a loop containing an injection valve and equilibrated with phosphate buffered saline (PBS, pH 7.2). Conditioned media (2 ml) was introduced using the loop. The molecular weight standard Blue Dextran (MW 2X10⁶) was totally excluded from the beads and provided a measure of the void volume. Potassium chromate (MW 194) totally penetrates the Sephadex beads and was used to determine the total volume. Fraction collection (2 ml each) began when the void volume was eluted and continued until the total volume was removed from the beads. Absorbance at 206 nm was measured as the fractions were eluted from the column and was used to indicate the presence of peptide.
II. Polyacrylamide Gel Electrophoresis (PAGE)

Basic methods were similar to those described by Schägger and von Jagow (1987). In brief, a 20% acrylamide separating gel was prepared containing 3.0 M Tris (pH 8.45) and glycerol. This was overlaid with a 10% spacer gel, followed by a 4% stacking gel (3.0 M Tris, pH 8.45) yielding a gel with approximate dimensions of 0.75mm X 6.5cm X 8.0cm. A discontinuous buffer system was utilized which included a Tris-base (pH 8.9) anionic buffer and a Tricine/Tris cationic buffer. Approximately 20 µg of sample was loaded into each lane and a constant voltage was applied to the gel (20V for 1 hour followed by 100V for 1-1.5 hours). The gel was removed from the apparatus once the dye front reached the anionic buffer-gel interface. The gel was then stained with Coomassie Blue or silver.

D. Intracellular Calcium Measurements in Chromaffin Cells

I. Solutions

a. Fura-2 AM: Permeability of the fura-2 molecule is achieved by addition of the acetoxyethyl (AM) ester group to the molecule, which masks the negatively charged carboxyl
groups rendering the molecule uncharged, hydrophobic, and able to cross the cell membrane. Once the fura-2 is internalized, nonspecific esterases cleave the AM group thus liberating the polycarboxylate form of the fura-2 molecule which, due to its charge, becomes trapped in the cell. Since AM esters are relatively insoluble in aqueous solutions, the mild nonionic surfactant Pluronic F127 is used as a dispersing agent. Additionally, serum proteins such as bovine serum albumin (BSA), which have been shown to improve loading efficiency possibly by acting as hydrophobic carriers of the AM ester, may also be used to maximize loading. The AM ester form of fura-2 was used in all analyses of intracellular calcium. A solution of BSA (1 mg/ml) was made and then triturated. In a plastic test tube, equal volumes of a 20% (weight:volume) solution of Pluronic F127 in DMSO and fura-2 AM (1mM in DMSO) were combined and vigorously mixed using the vortex. Approximately 2ml of BSA solution was added to the tube and contents were mixed once again. The contents of the test tube were then combined with additional BSA solution such that the final solution contained 1µM fura-2 AM, 0.1% BSA, and 0.02% Pluronic F127. The fura-2 concentration was kept
as low as possible to reduce overloading artifacts which
include incomplete hydrolysis, compartmentalization, and
toxicity from hydrolysis by-products (i.e., formaldehyde).

b. Modified Kreb's Buffer: A Modified Kreb's Buffer
Solution was used in all incubations and washes. This
solution contained NaCl 7.8 g/L (135 mM), KCl 0.44 g/L (5.9
mM), CaCl₂ 0.22 g/L (1.5 mM), MgCl₂ 0.24 g/L (1.2 mM), HEPES
2.76 g/L (11.6 mM), and D-glucose 2.07 g/L (11.5 mM). This
solution was used at a pH of 7.3.

c. Calcium Standard Buffer: A Calcium Standard Buffer
was used for both in vitro and in situ calibrations. This
buffer contained 3.728 g KCl/450 ml (110 mM), 1.046 g
MOPS/450 ml (11.1 mM), and 0.1015 g MgCl₂/450 ml (1.1 mM)
and was titrated to a pH of 7.2 with KOH.

d. Buffer "A": Used in both in vitro and in situ
calibrations, Buffer "A" is a 10mM K₂EGTA solution. This
solution was prepared by adding 3.804 g of the calcium
chelator EGTA (100mM) to 90 ml of Calcium Standard Buffer.
Once the EGTA was solubilized, the solution was titrated to
a pH of 7.2 with KOH and then to a final volume of 100 ml.

e. Buffer "B": Buffer "B", a 10mM CaEGTA solution, was
used in both in vitro and in situ calibrations. EGTA (3.804
g) and CaCO₃ (1.001 g) were added to 90 ml of Calcium Standard Buffer. This solution was then titrated to a pH of 7.2 with KOH and a final volume of 100 ml.

**II. Calibrations**

**a. In Vitro:** Measurement of fluorescence intensities at varying calcium concentrations under cell free conditions was achieved using fura-2 (free acid, 1 µM) addition to solutions of known calcium content and measurement of each respective fluorescence intensity. Free calcium concentrations were determined using the Maxchelator program. Fluorescence intensities were measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The solutions were as follows:

<table>
<thead>
<tr>
<th>Free Ca²⁺(nM)</th>
<th>Buffer&quot;A&quot;(ml/ml)</th>
<th>Buffer&quot;B&quot;(ml/ml)</th>
<th>FreeMg²⁺(mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.000</td>
<td>0</td>
<td>0.64</td>
</tr>
<tr>
<td>10</td>
<td>0.937</td>
<td>0.063</td>
<td>0.65</td>
</tr>
<tr>
<td>50</td>
<td>0.748</td>
<td>0.252</td>
<td>0.70</td>
</tr>
<tr>
<td>100</td>
<td>0.598</td>
<td>0.402</td>
<td>0.75</td>
</tr>
<tr>
<td>200</td>
<td>0.427</td>
<td>0.573</td>
<td>0.81</td>
</tr>
<tr>
<td>500</td>
<td>0.230</td>
<td>0.770</td>
<td>0.81</td>
</tr>
<tr>
<td>1000 (1µM)</td>
<td>0.130</td>
<td>0.870</td>
<td>0.93</td>
</tr>
<tr>
<td>2000 (2µM)</td>
<td>0.070</td>
<td>0.930</td>
<td>0.97</td>
</tr>
</tbody>
</table>
Intensity readings from the dual excitation wavelengths were obtained and divided (340/380) yielding ratio values. These ratios for each calculated calcium concentration were fit to a curve. This curve was then compared to in situ calibration results as well as experimental data.

**b. In Situ:** To account for cell-specific modifications in recorded fluorescence (i.e. intracellular buffering capacity) as well as any variation in cell preparation, in situ calibrations were performed at the end of each experiment. Briefly, cells were perfused with Buffer "A" containing 10 μM ionomycin, an ionophore, and 4 mg/L nigericin. Nigericin, a hydrogen-potassium exchanger, was used to compensate for any changes in intracellular pH due to the action of the calcium-hydrogen exchanging ionomycin. After 20 minutes of perfusion a fluorescence measurement was taken and recorded as $R_{\text{min}}$. At this point, the perfusing solution was switched to Buffer "B" containing 10μM ionomycin, 4 mg/L nigericin, and 10mM calcium chloride. The cells were perfused with this solution for 5-10 minutes at which time fluorescence readings were taken. The maximal fluorescence ratio at the end of the perfusion was recorded as $R_{\text{max}}$. Calibration software, incorporated into the Perkin
Elmer system utilized for these measurements, allowed for the measurement and automatic subtraction of all autofluorescence at both wavelengths.

III. Procedure

a. Preparation of Chromaffin Cells: Chromaffin cells were isolated as described previously (Cell Culture). Purified cells used in fura-2 analyses were plated on collagen-coated coverslips (9X22 mm) at the same density as those cells used in secretion protocols (3X10^5 cells per cm^2). Cells were maintained on coverslips in 60 mm tissue culture dishes. Media was changed after the first day in these cultures and then as needed to maintain normal pH.

b. Loading Fura-2 AM: Fura-2 AM solution (1µM) was added to 60 mm tissue culture dishes containing chromaffin cell coverslips and cells were incubated at room temperature for one hour. Dishes were covered with aluminum foil during the incubation period to inhibit exposure to light. Cells were washed twice in Modified Kreb's Buffer Solution prior to experimental use.

c. Measurement of Intracellular Calcium Concentration: Chromaffin cell coverslips were placed in a perfusion
apparatus which was subsequently placed in a disposable cuvette. Fluorescence intensity was measured using the Perkin-Elmer LS 50B Luminescence Spectrometer and accompanying software. Fluorescence scanning time ranged from 5 to 90 minutes. A baseline calcium measurement was obtained by fluorescence measurement for 40 to 60 seconds prior to the addition of secretagogue or drug. Secretagogues (2 ml) were added using a 3 ml syringe and 25 gauge needle while drugs (10µl) were injected using a Hamilton syringe (Hamilton Co., Reno, NE). The system was run via gravity feed (approximately 1 ml/min) and all effluent was collected in a vacuum flask. All experiments were performed at room temperature. Fluorescence intensities were obtained using excitation wavelengths of 340 and 380nm and measuring emission at 510 nm. Reported ratios represent fluorescence intensity at 340 nm excitation divided by that obtained with 380 nm excitation.

Intracellular calcium levels were determined by the equation:

\[
Ca_i = \frac{K_d \left( R - R_{min} \right)}{R_{max} - R} \frac{Sf2}{Sb2}
\]
Where \( K_d = 135 \text{nm (room temperature)} \), \( R_{\text{min}} \) and \( R_{\text{max}} \) are fluorescence ratios at zero and calcium levels which saturate the calcium binding capacity of fura-2 AM respectively, and \( \frac{S_f}{S_b} \) is the ratio of excitation efficiencies for free and bound fractions at 380nm (Gryniewicz, 1985). Ratioing not only results in measurement of intracellular calcium concentrations that are essentially independent of intracellular dye concentrations but also offsets the complications of leakage and photobleaching.

d. Data Analysis: Fluorescence intensity data from each experiment were converted to calcium concentrations utilizing a self-designed look-up table. This look-up table was constructed using the Gryniewicz and other mathematical equations and data obtained from in vitro and in situ calibrations. The final data are presented as the change in intracellular calcium concentration (ordinate) of the chromaffin cell population over time (abscissa) in response to CM or secretagogue.
E. Antibody Production

I. Carrier Protein

a. Selection of Carrier Protein: The production of antibody to concentrated peptide required the immunization of rabbits with peptide. For these studies, the concentrated peptide was conjugated with a carrier protein to increase antigenicity. The carrier protein that was employed was Keyhole Limpet Hemocyanin, a large immunogenic protein obtained from Sigma Chem. Co. (St. Louis, MO).

b. Coupling the Enriched Peptide Fraction to Carrier Protein: Glutaraldehyde was utilized which coupled peptide to carrier protein primarily through the N terminal alpha amino acid of the peptide. This procedure was described previously and requires the mixing of the carrier protein with the concentrated peptide followed by cross-linking with 0.3% glutaraldehyde (van Regenmortel et al., 1988). The peptide coupled carrier protein was then dialyzed against borate buffer (pH 8.5) for 48h.

II. Polyvalent Antibody Production

New Zealand White rabbits were bled prior to immunization to obtain a baseline and ensure no peptide-
specific antibody was present in the blood prior to immunization. 1 mg of the peptide/protein carrier complex was dissolved in 0.05 M phosphate buffered saline (PBS) and mixed with an equal volume of Freund's Adjuvant and injected into each rabbit subcutaneously near the axillary lymph nodes and intramuscularly in the hind leg. At two week intervals the animals were reinjected with the peptide/protein carrier complex in adjuvant. Following the second such injection, the animals were bled through the medial ear artery to obtain 5 ml of blood. The serum was separated and assessed for reactive antibody as described below (ELISA). Antisera was also tested in a bioassay to ensure antibody to a bioactive peptide is present.

**III. Development of an ELISA**

The enzyme linked immunoabsorbent assay (ELISA) is an important immunologic technique by which one can quantify antigen (e.g. peptide) concentration by using an indicator molecule (antibody) coupled covalently to an enzyme. Antigen/peptide is quantified by determining the initial rate at which enzyme converts a clear substrate in the solution to a colored product. Initially, an ELISA was
developed to determine peptide-antibody presence in both rabbit and mouse polyvalent antisera. Methods are well characterized (van Regenmortel et al, 1988) and are described below.

Briefly, a fixed quantity of concentrated peptide was attached to 96 well assay plates and incubated overnight at 4°C in phosphate buffered saline (PBS) at pH 7.2. Once the concentrated peptide was attached to the wells, the plastic plates were washed with PBS containing 0.05% Tween-20. The remaining active sites were blocked with 0.05% gelatin (Sigma Chem. Co., St. Louis, MO) for 2 hours at room temperature and wells washed again with PBS containing 0.05% Tween-20. Serial dilutions of antibody were prepared and incubated with a fixed amount of polyvalent antisera in the coated plastic wells overnight at 4°C. Wells were then washed at least three times with PBS containing 0.05% Tween-20. To measure the amount of specifically bound polyvalent antisera, each well was incubated with peroxidase coupled secondary antibody for 2 hr at 37°C (in this case a goat anti-rabbit secondary antibody, Accurate Chem. Corp., Westbury, NY). The plastic wells were then washed at least three times with PBS containing 0.05% Tween-20. 3,3'-
Diaminobenzidine tetrahydrochloride (DAB, 0.5 mg/ml), the enzyme substrate, was combined with PBS (pH=7.2) containing 0.3% H₂O₂ then 100µL of this solution was placed in each well and allowed to incubate for 1-3 hours at 37° C. Positive results, i.e. antibody binding specifically to peptide sequence, were indicated by a color change in the corresponding well. The intensity of the color would then be indicative of the amount of peptide bound in each well. Since the conversion of the chromagen, DAB, yields the color brown in a positive test, all plates were scanned at 492 nm absorbance to determine relevant peptide concentrations in each well.
CHAPTER IV

SIGNAL TRANSDUCTION AND THE IMMUNE-DERIVED PEPTIDE:
THE ROLE OF CALCIUM IN SECRETION

A. Introduction

The importance of stress in the etiology and prevention of disease is becoming increasingly apparent as the mechanics of stress-induced dyshomeostasis are understood. Extensive documentation demonstrates that humans can respond to both cognitive and non-cognitive stimuli through a stress response that results in the release of the stress hormone epinephrine into the circulation. This increased plasma epinephrine has a wide array of physiologic effects ranging from substrate mobilization supporting the hypermetabolism of fever to increased cardiovascular function. Interestingly, recent documentation indicates that epinephrine also may modify various immune cell functions.
thus potentially altering the immune response in times of stress (Sanders and Munson, 1985; Feldman et al., 1987; Plaut, 1987; Heilig et al., 1993). Typically, adrenal medullary release of epinephrine is attributed to neural stimulation of adrenal medullary chromaffin cells. Recent studies from this and other laboratories, however, indicate that medullary epinephrine release may also be mediated through a non-neural mechanism (Seidler and Mills, 1989; Zhou and Jones, 1993). Further experimental evidence indicates that one source of this regulation potentially lies in the immune system (Jones et al., 1993). Primary cultures of mononuclear cells from bovine, porcine and human sources have been shown to release a small (MW < 3,000) peptide which stimulates medullary epinephrine secretion to levels comparable to maximal cholinergic stimulation.

Although knowledge of the exact mechanism of chromaffin cell exocytosis is poorly understood, one common feature of all models is that catecholamine secretion is dependent on an increase in intracellular calcium. Initial reports documented that a rise in intracellular calcium is a sufficient stimulus to trigger chromaffin cell exocytosis (Dunn and Holz, 1983). Later studies employed the use of
calcium sensitive fluorescent dyes (e.g. fura-2) to temporally and spatially resolve the relationship between increased intracellular calcium and exocytosis (Kim and Westhead, 1989; O'Sullivan and Burgoyne, 1989; Cheek et al., 1989; Stauderman et al., 1990). Ultimately, investigations revealed that chromaffin cell secretion in response to known secretagogues, both cholinergic and non-cholinergic mediators, is a calcium dependent process (O'Sullivan et al., 1989; Cheek, 1991). The goal of the current study was to assess the basic signal transduction mechanism of this immune-derived peptide in chromaffin cell catecholamine secretion and to determine if the mechanism was similar to other known secretagogues. Central to this goal was elucidating the role of calcium in peptide-induced excitation-secretion coupling.

Multiple studies have documented the fact that adrenal medullary chromaffin cells secrete catecholamine in response to a rise in cytosolic calcium concentration ([Ca^{2+}]_i) (Cheek and Thastrup, 1989; O'Sullivan and Burgoyne, 1989; Stauderman et al, 1990). This rise in intracellular calcium can result from calcium influx, release of calcium from internal stores, or a combination of both. Bovine chromaffin
cells, the model system used in these experiments, appear to require the influx of extracellular calcium to induce catecholamine secretion since muscarinic agonists, coupled solely to internal calcium release, are ineffective secretagogues. Utilizing purified fractions of immune-derived peptide, experiments were conducted using non-specific as well as specific calcium channel blockers to determine if peptide-induced secretion was, indeed, dependent on calcium and, if so, to determine the importance of extracellular and/or intracellular stores. Further experiments were performed using the fluorescent calcium indicator, fura-2, to temporally resolve internal oscillations in calcium in response to immune-derived peptide stimulation.
B. Materials and Methods

**Primary Cultures of Chromaffin Cells**

**a. Isolation and Culture:** Adult bovine adrenal glands were used for chromaffin cell isolation according to the overall procedure described by Dahmer et al., (1990). In brief, adrenal glands were perfused with collagenase, incubated at 37°C and medullary tissue removed for further digestion. Digested cells were washed, counted, viability determined with trypan blue and purity assessed with neutral red. Cells were plated on collagen-coated 24-well plates at a density of $3 \times 10^5$ cells per cm$^2$ and media was changed after the first day and then as needed to maintain a normal pH.

**b. In vitro Model of Catecholamine Secretion:** Basic secretion experimental protocols are those described by Dahmer et al., (1990). In brief, growth media was removed and replaced with conditioned media (90 min. incubation), serum-free RPMI-1640 (90 min. incubation), Earle's Balanced Salts Solution (EBSS, 90 min.), or secretagogue in EBSS (10 min. incubation). After incubation, media was removed and saved. Catecholamine content in these samples represent that stimulated by conditioned media (CM), basal secretion
experiments), or that stimulated by a known secretagogue. Cells were lysed by 1M perchloric acid and acid saved to determine the remaining catecholamine content of the cells. The fraction of epinephrine secreted into the media as a percent of total content was calculated by dividing the amount secreted by the total well content. Secretion experiments were routinely done in triplicate using three separate wells on any one plate which were derived from the same adrenal gland. Replicates of separate triplicate determinations were made on cells from different chromaffin cell preparations. Catecholamines were quantified by HPLC using electrochemical detection. Mobile phase was 0.1M monochloroacetic acid with 0.5mM EDTA and 1.0mM sodium octyl sulfate adjusted to pH 3.0. Acetonitrile was added at 15mL/L. A BAS Liquid Chromatographic System including an LC 4C electrochemical detector and glassy carbon electrode was used for all analyses.

Primary Cultures of Mononuclear Cells from Spleen

Isolation and Culture: Whole bovine spleen was obtained from a local slaughter house. Inner spleen tissue, which includes both red and white pulp, was dissected out, cut into small pieces and forced through wire mesh in Hank's
Balanced Salts Solution (HBSS) at room temperature. Crude cell suspensions were layered on density gradient media (Fico/Lite-LymphoH, Atlanta Biologicals), centrifuged and then separated cells were removed and washed. Isolated cells were transferred to flasks with RPMI media containing only essential amino acids and antibiotics at a density of 2X10^6 cells/ml. The growth media was isolated from the cells and either used promptly or frozen (-20°C) after at least 24 hours of culture.

Data Analysis

All data are presented as epinephrine secretion as a percent of total cell content. Percent secretion of each experimental and control group(s) are compared with every other group using an ANOVA. A Tukey's follow-up test is utilized when statistically significant differences are found between groups. A p<0.05 is considered statistically significant.

Intracellular Calcium Measurements in Chromaffin Cells

The calcium sensitive fluorescent dye, fura-2, was utilized to temporally resolve changes in intracellular calcium concentrations in response to CM-peptide. Peptide-stimulated alterations in intracellular calcium were measured in
the presence and absence of non-specific calcium channel blockers to determine relative amounts of calcium influx as well as release from internal stores.

a. Preparation of Chromaffin Cells: Chromaffin cells were isolated as described previously (Cell Culture). Purified cells used in fura-2 analyses were plated on collagen-coated coverslips (9X22 mm) at the same density as those cells used in secretion protocols (3X10⁵ cells per cm²). Cells were maintained on coverslips in 60 mm tissue culture dishes. Media was changed after the first day in these cultures and then as needed to maintain normal pH.

b. Loading Fura-2 AM: Fura-2 AM solution (1µM) was added to 60 mm tissue culture dishes containing chromaffin cell coverslips and cells were incubated at room temperature for one hour. Dishes were covered with aluminum foil during the incubation period to inhibit exposure to light. Cells were washed twice in Modified Kreb's Buffer Solution prior to experimental use.

c. Measurement of Intracellular Calcium Concentration: Chromaffin cell coverslips were placed in a perfusion apparatus which was subsequently placed in a disposable cuvette. Fluorescence intensity was measured using the
Perkin-Elmer LS 50B Luminescence Spectrometer and accompanying software. Fluorescence scanning time ranged from 5 to 90 minutes. A baseline calcium measurement was obtained by fluorescence measurement for 40 to 60 seconds prior to the addition of secretagogue or drug. Secretagogues (2 ml) were added to the perfusion apparatus using a 3 ml syringe and 25 gauge needle while drugs (10µl) were injected using a Hamilton syringe (Hamilton Co., Reno, NE). All experiments were performed at room temperature (23°C). Fluorescence intensities were obtained using excitation wavelengths of 340 and 380nm and measuring emission at 510 nm. Reported ratios represent fluorescence intensity at 340 nm excitation/ that obtained with 380 nm excitation.

Intracellular calcium levels were determined by the equation:

\[ \text{Ca}_i = \frac{K_d (R - R_{\text{min}})}{R_{\text{max}} - R} S_f^2 \]

Where \( K_d = 135\text{nm} \) (room temperature), \( R_{\text{min}} \) and \( R_{\text{max}} \) are fluorescence ratios at zero and calcium levels which
saturate the calcium binding capacity of fura-2 AM respectively, and \( S_f/S_b \) is the ratio of excitation efficiencies for free and bound fractions at 380nm (Gryniewicz, 1985). Ratioing not only results in measurement of intracellular calcium concentrations that are essentially independent of intracellular dye concentrations but also offsets the complications of leakage and photobleaching.

Data Analysis

Fluorescence data from each experiment were converted to the appropriate calcium concentration utilizing a self-designed look-up table. This look-up table was constructed using the Gryniewicz and other mathematical equations and data obtained from in vitro and in situ calibrations. The final data are presented as the change in intracellular calcium concentration (ordinate) of the chromaffin cell population over time (abscissa) in response to CM or secretagogue.
C. Results

Extracellular Calcium is Essential for Conditioned Media-Induced Secretion.

Extracellular calcium influx in response to peptide stimulation was inhibited by the non-specific calcium channel blocker cobalt (CoCl₂, 2mM). As can be seen in figure 1, the channel blocker completely inhibited catecholamine secretion in response to conditioned media indicating that peptide-induced secretion requires the influx of extracellular calcium.

Blockers of voltage-gated calcium channels do not completely inhibit secretion in response to peptide.

Since bovine chromaffin cells contain the L, P, Q, and N-type voltage-gated calcium channels (Lopez et al., 1989; Lopez et al., 1994), specific blockers of these channels were used to assess the role of each channel type in peptide-induced calcium influx. Both the L- and P-type channel blockers, verapamil (1µM) and ω-Agatoxin VIA (100nM), significantly inhibited secretion in response to peptide. The N- and Q-type channel blockers, ω-Conotoxins GVIA (1µM) and MVIIC (3µM) respectively, did not effect peptide-induced
Figure 1. Extracellular calcium dependence of conditioned media (CM) induced secretion. Values are mean ± SEM. RPMI-1640 group represents basal chromaffin cell secretion. Triplicate determinations for all groups were made in each of 2 chromaffin cell preparations (n=6). *** = p<0.001 compared to CM alone using an ANOVA followed by Tukey's comparisons.
Figure 2. Effect of voltage-gated calcium channel blockers on conditioned media (CM)-induced secretion. Concentration of drug used are: verapamil (1µM), ω-agatoxin VIA (100nM), ω-conotoxin GVIA (1µM), and ω-conotoxin MVIIC (3µM). Values are mean ± SEM. RPMI-1640, basal chromaffin cell secretion, was 2-5% total cell content. Triplicate determinations for all groups were made in each of 3 chromaffin cell preparations (n=9). * = p<0.05 and *** = p<0.001 respectively compared to CM alone using an ANOVA followed by Tukey's comparisons.
Figure 3. Effect of non-specific and L-type calcium channel blockers on secretion in response to the nicotinic agonist dimethyl phenyl piperazinium (DMPP). Values are mean ± SEM. RPMI-1640 represents basal chromaffin cell secretion. Two triplicate determinations for all groups were made in 1 chromaffin cell preparation (n=6). *** = p<0.001 compared to all other groups using an ANOVA followed by Tukey's comparisons.
secretion (figure 2). The effect of non-specific and L-type calcium channel blockers on secretion in response to the nicotine agonist dimethyl phenylpiperazinium (DMPP, 10µM) can be seen in figure 3.

**Removal of Intracellular Calcium Sources does not Alter Peptide-Induced Secretion.**

The effect of removal of intracellular calcium stores on peptide-induced secretion was tested using dantrolene (30-50µM), a membrane-permeable drug which inhibits intracellular calcium mobilization, and thapsigargin (0.1-1µM), a pharmacologic agent which depletes internal calcium stores. As a control, the muscarinic agonist, methacholine, was used to stimulate fura-2 loaded chromaffin cells in the presence and absence of dantrolene or thapsigargin. Both dantrolene and thapsigargin completely inhibited the rise in intracellular calcium in response to methacholine. Conditioned media-induced secretion, however, was unaffected by either dantrolene or thapsigargin treatment (figure 4) indicating calcium release from intracellular stores is not essential for peptide induced secretion.
Figure 4. Effect of removal of intracellular calcium on conditioned media (CM) induced secretion. Values are mean ± SEM. RPMI-1640, basal chromaffin cell secretion, was 2-5% in all experiments. Triplicate determinations for all groups were made in each of 2 chromaffin cell preparation (n=6).
Figure 5. Rise in intracellular calcium in response to conditioned media (CM) stimulation is biphasic. Increased fluorescence ratio (F340nm:F380nm) indicates an increase in intracellular calcium. CM-stimulated increase in intracellular calcium includes an initial, fast calcium peak and a second, slow, sustained rise in calcium. Similar results were obtained in other experiments.
Figure 6. Time course of conditioned media induced secretion. Values are mean ± SEM. Basal secretion (RPMI-1640) was subtracted from each of the time points. Two triplicate determinations were made using one chromaffin cell preparation (n=6). Similar results were obtained on other chromaffin cell preparations.
Rise in intracellular calcium in response to peptide is biphasic.

Using the membrane-permeable, fluorescent calcium indicator, fura-2 AM, changes in chromaffin cell intracellular calcium in response to conditioned media peptide were recorded over a sixty minute period. Figure 5 demonstrates that CM produces an initial calcium spike followed by a slower, long term elevation in intracellular calcium levels. The biphasic nature of the intracellular calcium response to CM stimulation correlates temporally with the time course of conditioned media-induced secretion (figure 6).

Both intracellular and extracellular calcium are components of peptide-induced internal calcium rise.

Fura 2-AM loaded chromaffin cells were stimulated with conditioned media in the presence or absence of the non-specific calcium channel blockers cadmium (0.5mM) or cobalt (2.0mM). While the initial calcium spike was blunted and the long-term calcium increase abolished, there was still a significant increase in intracellular calcium in response to conditioned media (figures 7 & 8). Conversely, the initial intracellular calcium spike is significantly blunted in chromaffin cells incubated with thapsigargin prior to
chromaffin cells incubated with thapsigargin prior to stimulation with CM (figure 9). Taken together, these results indicate that CM-peptide induced secretion stimulates both calcium influx as well as calcium release from internal stores.
Figure 7. Cadmium blocks secondary, long term intracellular calcium increase in response to conditioned media (CM) stimulation. The trace is taken from the first 5 minutes of a 60 minute time period. No change occurred in intracellular calcium between 5 and 60 minutes. An increase in fluorescence intensity (ratio of F340nm:F380nm) indicates an increase in intracellular calcium. Compared to CM alone, cadmium attenuates the initial, fast calcium peak and
Figure 8. Cobalt blocks secondary, long term intracellular calcium increase in response to conditioned media (CM) stimulation. The trace is taken from the first 5 minutes of a 60 minute time period. No change occurred in intracellular calcium between 5 and 60 minutes. An increase in fluorescence intensity (ratio of F340nm:F380nm) indicates an increase in intracellular calcium. Similar to cadmium, cobalt (2.0mM) attenuates the initial, fast calcium peak and completely blocks the second, slow, long-term calcium rise. Similar results were obtained in other experiments.
Figure 9. Thapsigargin pretreatment blunts initial intracellular calcium spike. The trace is of the first 3 minutes of a 60 minute time period. No change occurred in intracellular calcium between 5 and 60 minutes. An increase in fluorescence intensity (ratio of F340nm:F380nm) indicates an increase in intracellular calcium. The initial calcium spike was significantly inhibited after thapsigargin pretreatment. Similar results were obtained in other experiments.
D. Discussion

Studies have demonstrated that chromaffin cells secrete catecholamine in response to a rise in cytosolic calcium concentration (Cheek and Thastrup, 1989; Cheek et al., 1990). This can result from an influx of extracellular calcium, release from intracellular stores, or a combination of both. For example, nicotinic agonists induce chromaffin cell depolarization which leads to the opening of voltage-gated calcium channels and subsequent calcium influx. Conversely, muscarinic agonists stimulate phosphoinositide metabolism which induces rapid accumulation of inositol phosphates that stimulate calcium release from intracellular stores. Finally, known peptide secretagogues (i.e. bradykinin, angiotensin II) stimulate chromaffin cell secretion via both extracellular calcium influx and inositol phosphate-activated intracellular calcium release. The goal of the present study was to determine if secretion in response to a novel, mononuclear cell-derived peptide was, indeed, dependent on calcium and, if so, to determine the importance of extracellular and/or intracellular stores.

Initial studies addressed the role of extracellular calcium in peptide-induced secretion. Several inorganic
ions (e.g. Cd\(^{2+}\), Co\(^{2+}\), La\(^{3+}\)) have been reported to block the various types of calcium channels. Evidence indicates that the binding site of these ions involves conserved glutamate residues in the proposed pore of the calcium channel (Hagiwara, 1981). In the present study, inhibition of calcium influx via one of these non-specific channel blockers (Co\(^{2+}\)) abolished conditioned media peptide-induced secretion (figure 1) indicating that extracellular calcium was essential for secretion in response to peptide stimulation. Further experiments were performed to assess the role of the voltage-gated calcium channels in peptide-induced secretion. Bovine chromaffin cells have been reported to contain the L-, P-, Q-, and N-type voltage gated calcium channels (Lopez et al., 1989; Lopez et al., 1994). These channels range from high conductance (22-28pS) channels which inactivate rather slowly (>500 msec time constant) (e.g., the L-type calcium channel) to channels of lesser conductance (12-22pS) whose inactivation time constants are rather rapid (50-100 msec) (e.g., the P-, N-, and Q-type channels). Specific blockers of these channel types were utilized to determine the relative importance of each channel type. While the L- and P-type channel blockers
(i.e. verapamil and ω-Agatoxin VIA) significantly inhibited peptide-induced secretion, the Q- and N-type calcium channel blockers (i.e. ω-Conotoxins GVIA and MVIIC) had no significant effect (figure 2). These results indicate that both the L- and P-type channel are involved in calcium influx in response to peptide but are not the only channels through which peptide-stimulated influx occurs. One potential mechanism by which these voltage gated channels are activated may be by depolarizing concentrations of calcium directly adjacent to the L- and P-type channels. Upon stimulation, chromaffin cell intracellular calcium has been shown to range from 300-1000 nM when measuring whole cell calcium content. It is estimated, however, that near the plasma membrane (near the voltage gated calcium channels), local calcium concentrations reach 10-100µM - potentially great enough to lead to a local depolarization and activation of L- and P-type channels. Alternate pathways responsible for the additional calcium influx include IP₃/IP₄ gated calcium channel(s), G-protein gated channel(s), a receptor gated calcium channel, or, potentially, activation of I_{CRAC} (Calcium Release Activated
Calcium current) in response to emptying of intracellular stores.

Although these data do not agree with recent reports (Lopez et al., 1994) which indicate that Q- and L-type channels are the predominant calcium channels involved in bovine chromaffin cell secretion, they are not surprising. First, the time course of CM-induced secretion (90 minutes) is obviously much longer than the time constants of inactivation for these voltage gated channels indicating a role for other channel types. Furthermore, studies performed by Lopez et al. (1994) utilized depolarizing concentrations of potassium to stimulate chromaffin cell secretion. Secretion in the present study, however, is in response to a mononuclear cell-derived peptide. Whereas the L-type channel blockers, such as verapamil, are well documented inhibitors of catecholamine release in response to high K+ (Boarder et al., 1987; Fonteriz et al., 1987), secretion in response to known chromaffin cell peptide secretagogues such as bradykinin have been shown to be unaffected by the presence of L-type calcium channel blockers (Owen et al., 1989). The difference between high K+ and bradykinin sensitivity to L-channel blockers indicate
that these agents stimulate calcium entry via separate routes (i.e., IP$_3$/IP$_4$ gated, receptor gated, or G-protein gated channels or I$_{CRAC}$). Potentially, the immune-derived peptide in this study stimulates calcium influx through non-L-type calcium channels similar to bradykinin.

The role of intracellular calcium stores in peptide-induced secretion was addressed in these studies via the use of two pharmacologic agents - dantrolene, which blocks calcium release from internal stores, and thapsigargin, which depletes intracellular calcium stores. Neither dantrolene nor thapsigargin altered chromaffin cell epinephrine secretion in response to peptide (figure 4). Although these results indicate that intracellular calcium is not essential for peptide-induced secretion, they do not exclude the release of calcium from these internal stores in response to peptide stimulation.

Further studies are presented which utilized the ratiometric fluorescent calcium dye, fura-2 AM, to temporally resolve the intracellular calcium rise in response to peptide. The kinetics of intracellular calcium changes as well as epinephrine secretion in response to peptide stimulation was shown to be biphasic in nature.
(figures 5 & 6) similar to the potent chromaffin cell secretagogue, histamine. Both antagonist and desensitization studies confirm, however, that the stimulatory effect of conditioned media cannot be attributed solely to histamine. While pretreatment with histamine (100 µM, 10 min.) did not effect CM-induced secretion from the same chromaffin cells, chromaffin cell histamine desensitization significantly inhibited secretion in response to a second challenge with histamine (CM=19.2±0.3, CM/histamine pretreatment=18.4±0.7, histamine=15.5±0.9, histamine/histamine pretreatment=2.4±0.2***, basal secretion=1.6±0.2; ***=p<0.001 vs histamine/no pretreatment).

Evidence is also presented in this study which demonstrates the non-specific calcium channel blockers Co^{2+} (2.0mM) and Cd^{2+} (0.5mM) used in conjunction with conditioned media diminish the initial calcium peak and completely abolished the delayed, maintained rise in intracellular calcium (figures 7 & 8). Conversely, the initial calcium spike in response to conditioned media was significantly reduced in chromaffin cells incubated with thapsigargin prior to stimulation with CM (figure 9). These
data indicate that while the secondary, maintained calcium elevation is due solely to extracellular influx, the initial calcium peak can be attributed to both calcium influx and release from internal stores. Calcium release from internal stores as well as influx from the extracellular environment in response to conditioned media peptide is characteristically similar to catecholamine secretion in response to other known peptides (e.g. bradykinin, angiotensin II, etc).

In summary, the data obtained in these studies strongly support current concepts of epinephrine secretion in response to peptide secretagogues from bovine chromaffin cells. Conditioned media peptide-induced secretion appears to utilize calcium regulated signal transduction mechanisms that are analogous to other known peptide secretagogues. Similar to bradykinin and angiotensin II (Owen et al., 1988; Plevin and Boarder, 1988), the peptide in the present study appears to stimulate both release of calcium from internal stores as well as extracellular influx. The dependence on extracellular calcium for secretion is common to both the conditioned media peptide and other peptide secretagogues. Additionally, the biphasic, maintained rise in intracellular
calcium in response to conditioned media peptide and the efficacious stimulatory effect of conditioned media peptide agree with studies which show that the more efficacious chromaffin cell secretagogues (e.g. histamine) stimulate long-term increases in intracellular calcium (Noble et al., 1988; Goh and Kurosawa, 1991).
CHAPTER V

MONONUCLEAR CELL ORIGIN OF A NOVEL PEPTIDE

MEDIATING PERIPHERAL EPINEPHRINE SECRETION

A. Introduction

The role of stress in illness has been debated for many years. Although numerous observations have been documented over the centuries which note a relationship between stressful periods in life and subsequent illnesses, definitive physiologic evidence of such a relationship has been difficult to obtain since both psychological (cognitive) and physical (non-cognitive) stimuli may contribute to physiologic stress. The idea that mental and physical health are interdependent can be found in early medicine in the works of Aristotle. This concept was further promoted by Galen when he observed that melancholic women were more likely to develop cancer than there more
confident and vital counterparts (Kort, 1994). Similar associations between emotional stress and health appear even in current studies. For instance, studies site that job strain has a negative impact on health (Lerner et al., 1994). There is also evidence of a correlation between a positive mental state and better health in terminal AIDS patients (Kendall, 1994).

One of the many difficulties in ascertaining a physiologic connection between stress and illness is in the definition of "stress". The term "stress" is defined in the context of research and pathophysiology as "the reactions of the body to forces of a deleterious nature, infections and various abnormal states that tend to disturb its normal physiological equilibrium (homeostasis)" (Burgess, 1987). This definition requires that not only the reactions of the body to physical/psychological stimuli but also the stimuli themselves be considered the "stress". This is problematic when designing experimental protocols to determine the stress-illness relationship since both physical and psychological parameters are involved (Selye, 1980). Fortunately, experiments in animals as well as in humans indicate that one of the prominent underlying causes of both
dyshomeostasis is changes in the neuroendocrine system which modulates immune system function (Sheridan et al., 1994). Key factors of this neuroendocrine immunomodulation appear to be the pituitary and adrenal gland hormones, including the catecholamines.

The catecholamines, epinephrine (released mainly from the adrenal medulla) and norepinephrine (released mainly from sympathetic nerve terminals), are pivotal regulators of many physiological events in man. Documented actions of the catecholamines include the aforementioned modulation of immune function, a cardiac excitatory action, metabolic actions, and peripheral actions specific to certain muscle groups. In 1929, Sir Walter Cannon first associated various physiologic changes with increased circulating catecholamine (largely epinephrine) in his discussion of the "fight or flight" response (Cannon, 1929). This reaction occurs when animals, including man, perceive danger (cognitive stimuli) and prepares the animal to respond through either direct conflict ("fight") or escape ("flight"). However, it is clear that stimuli not recognized directly by the central and peripheral nervous systems and for which there is no conscious perception also may lead to increased circulating
catecholamine and subsequent associated physiologic changes. Such stimuli are referred to as non-cognitive stimuli and include bacteria, tumors, viruses, and antigens.

Although there are many potential mediators, the exact mechanism by which non-cognitive factors modulate neuroendocrine functions such as catecholamine release is not yet clear. Hypothalamic-pituitary-adrenal axis activation occurs as a central response to non-neurogenic mediators. Equally important may be peripheral release of stress hormone in response to a local non-cognitive stimuli. Recent investigations provide evidence of one potential mechanism by which tumors, bacteria, etc, may elicit the release of the stress hormone epinephrine through immunologic mediators. An immune-derived peptide (MW<3,000) released from mononuclear cell populations has been shown to stimulate adrenal medullary chromaffin cell epinephrine secretion to levels comparable to maximal neural stimulation. The exact mononuclear cell source of this peptide, however, has yet to be determined. The present study was performed to determine which mononuclear cells may potentially release bioactive peptide. Such information may provide insight as to the potential role of this peptide and
subsequent immune-neuroendocrine interactions in both health and disease.
B. Materials and Methods

Primary Cultures of Chromaffin Cells

Isolation and Culture: Adult bovine adrenal glands were used for chromaffin cell isolation according to the overall procedure described by Dahmer et al., (1990). In brief, adrenal glands were perfused with collagenase, incubated at 37°C and medullary tissue removed for further digestion. Digested cells were washed, counted, and viability determined with trypan blue staining. Purity was assessed using neutral red as described by Role and Perlman (1980). Cells were plated on collagen-coated 24-well plates at a density of 3\times10^5 cells per cm^2 and media was changed after the first day and then as needed to maintain a normal pH.

In vitro Model of Catecholamine Secretion: Basic secretion experiments are similar to those described by Dahmer et al., (1990). In brief, growth media was removed from each well and replaced with conditioned media, serum-free RPMI-1640, Earle's Balanced Salts Solution (EBSS), or secretagogue in EBSS. After incubation, media was removed and saved. Catecholamine content in these samples represent that stimulated by conditioned media (90 minute incubation), spontaneous/basal secretion (RPMI/EBSS, 90 minute
incubation), or that stimulated by a known secretagogue (DMPP/55mM K⁺, 10 minute incubation) in the presence or absence of drug/channel blocker. Cells were then lysed by adding 1M perchloric acid and the acid saved for determination of the remaining cellular epinephrine content. Epinephrine secreted into the media as a percent of total content was calculated by dividing the amount secreted by the total well content. Secretion experiments were routinely done in triplicate using three separate wells on any one plate all of which were derived from the same adrenal gland. Replicates of separate triplicate determinations were made on cells from different chromaffin cell preparations. Analysis of epinephrine was accomplished with HPLC using electrochemical detection. Mobile phase contained 0.1M monochloroacetic acid with 0.5mM EDTA and 1.0mM sodium octyl sulfate adjusted to pH 3.0. Acetonitrile was added at 15ml/L. A BAS Liquid Chromatography System including an LC 4C amperometric detector and glassy carbon electrode was used in all analyses.

**Primary Cultures of Mononuclear Cells from Spleen**

**Isolation and Culture:** Whole bovine spleen was obtained from a local slaughter house. Inner spleen tissue,
which includes both red and white pulp, was dissected out, cut into small pieces and forced through wire mesh in Hank's Balanced Salts Solution (HBSS) at room temperature. Crude cell suspensions were layered on density gradient media (Fico/Lite-LymphoH, Atlanta Biologicals), centrifuged and then separated. Isolated cells were washed, viability assessed using Trypan Blue, and cells were then transferred to flasks with RPMI media containing only essential amino acids and antibiotics. The growth media was isolated from the cells after at least 24 hours of culture and either used promptly or frozen (-20°C).

Separation of Monocyte/Macrophage Populations: The monocyte/macrophage populations were isolated from other splenocytes based on 1 hour adherence to plastic in culture flasks at 37°C (Clarke et al., 1993). Purity of the monocyte/macrophage cultures was assessed using two different staining methods. Wright-Giemsa staining was used to ensure no other adherent cell populations (i.e. dendritic cells, fibroblasts) were present in the culture. Non-specific esterase activity, unique to monocyte/macrophage mononuclear cells, was used to determine contamination of T and B cell populations in adherent cell cultures.
Hybridoma Cell Lines

IL-A51/anti CD-8: The immortal cell line IL-A51, a murine hybridoma cell line producing monoclonal antibody (IgG1) to the bovine orthologue of human CD8, was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured at the concentration of 10^6 cells/ml in a media comprised of RPMI-1640 supplemented with 10% (v:v) fetal bovine serum and 20µM 2-Mercaptoethanol. Cultures were maintained by adding fresh media every 2-3 days as needed.

IL-A11/anti CD-4: The immortal cell line IL-A11, a murine hybridoma cell line producing monoclonal antibody (IgG2a) to the bovine orthologue of human CD4, was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured at the concentration of 10^6 cells/ml in RPMI-1640 supplemented with 10% fetal bovine serum and 50µM 2-Mercaptoethanol. Cultures were maintained by the addition of fresh media every 2-3 days as necessary.

T cell/B cell Isolation and Separation Using Monoclonal Antibodies

Isolation of Antibody from Hybridoma Cultures:

Hybridoma cultures were allowed to grow until culture media
was turbid. The hybridoma cell culture flask was then agitated, lysing cells and maximizing antibody concentration in the suspension. Cell suspension was then centrifuged at 2000g at 4°C to sediment large proteins and cell membrane. The supernatant was poured over a Proteins A/G column, bacterial products that bind the Fc portion of the antibody, and purified antibody was eluted from the column using 0.1M glacial acetic acid (pH = 2.0). Absorbance at 206nm of column effluent was used to indicate antibody elution.

**Antibody Biotinylation and Coupling to Magnetic Microbead:**

**Microbead:** Antibody was conjugated with magnetic microbeads using the avidin-biotin system. Briefly, antibody was dialyzed against 0.1M NaHCO₃ (pH = 8.4) overnight and concentration after dialysis determined via spectrophometric analysis using the Bradford assay. Antibody was then concentrated or diluted to obtain a final concentration of 1 mg/ml. Immediately before use, 1.63 mg of NHS-LC-biotin (Pierce, Rockford, IL) was dissolved in 1 ml of dimethyl sulfoxide (DMSO) in a glass tube. This mixture was triturated and 50 µl is added to 1 ml antibody solution. This procedure results in a biotin substitution ratio of approximately 8:1. The reaction was allowed to proceed at
room temperature for one hour. The reaction mixture was then dialyzed against 0.1M NaHCO₃ for 48 hours. Sodium azide, a cytotoxic substance which protects antibody integrity, was not used since antibody labeled cells will be maintained in culture for an extended period (24-48h).

**Magnetic-based Cell Separation:** Biotinylated antibody, anti CD4 or anti CD8, was added to the lymphocyte cell suspension for 10 min to allow for adequate labeling with antibody and unbound/excess antibody was removed by three washes in cold phosphate buffered saline (PBS) (pH=7.4). Biotinylated antibody, bound to its respective cell surface marker, was then coupled to a streptavidin linked magnetic microbead during a 10 min incubation on ice, a reaction which occurs due to the high affinity of streptavidin for biotin (Kd=10⁻¹⁹M). Cells were washed again in cold PBS and labeled cells passed over a magnetic column. In theory, all cells labeled with magnetically-conjugated antibody should have been retained on the column while any unlabeled cells would pass through. Labeled cells, still bound to magnetic beads, can then be extruded from the column by removing the column from the magnetic field and washing several times in cold PBS. These cells, still labeled with antibody, may
then be cultured according to standard conditions. This protocol was followed twice for each lymphocyte preparation, one passage to remove CD4 positive lymphocytes and a second to remove CD8 positive lymphocytes. B cells were considered to be those populations which were not labeled by magnetically linked antibody and thus washed through the column. The purity of the cell cultures was ascertained using FACS analysis and determined to be between 80% and 90%.

_G-25 Sephadex Column Gel Filtration_

G-25 Sephadex beads (10 g) were combined with deionized water at a ratio of 1 g per 2-3 ml water yielding a final volume of approximately 30 ml and equilibrated for 8-10 hours. This volume was placed in a 2 cm column which was attached to a continuous perfusion pump with a loop containing an injection valve and equilibrated with phosphate buffered saline (PBS, pH 7.2). Conditioned media (2 ml) from individual cell cultures was introduced using the loop. The molecular weight standard Blue Dextran (MW 2X10^6) was totally excluded from the beads and provided a measure of the void volume. Potassium chromate (MW 194) totally penetrates the Sephadex beads and was used to
reflect the total volume. Fraction collection (2 ml each) began when the void volume was eluted and continued until the total volume was removed from the beads. Absorbance at 206nm was measured as the fractions were eluted from the column and was used to indicate the presence of peptide.

**Polyacrylamide Gel Electrophoresis (PAGE)**

Basic methods were similar to those described by Schägger and von Jagow (1987). In brief, a 20% T separating gel was prepared containing 3.0 M Tris (pH 8.45) and glycerol. This was overlaid with a 10% spacer gel, followed by a 4% stacking gel (3.0 M Tris, pH 8.45) yielding a gel with approximate dimensions of 0.75mm X 6.5cm X 9.5cm. A discontinuous buffer system was utilized which included a Tris-base (pH 8.9) anionic buffer and a Tricine/Tris cationic buffer. Approximately 20μg of sample was loaded into each lane and a constant voltage was applied to the gel (20V for 1 hour followed by 100V for 1.5 hours). The gel was removed from the apparatus once the dye front reached the anionic buffer-gel interface and stained with silver.
C. Results

**Bioactive peptide is a product of viable mononuclear cells.**

Viability of mononuclear cells isolated from bovine spleen was determined at the time of isolation then at 24 and 48 hours post-isolation using trypan blue. Figure 10 demonstrates that over this time course viability decreases significantly at each time point. Over this same time course the bioactivity of conditioned media harvested from these mononuclear cell cultures also diminished (figure 11) indicating that the bioactive peptide was a viable cell product.

**Nonadherent T and/or B cell populations are the main mononuclear cell source of bioactive peptide.**

Mononuclear cells were separated based on 1 hour adherence to plastic at 37°C. Adherent monocyte/macrophage cell populations and nonadherent T cell and B cell populations were cultured separately for 24 hours then the CM was harvested and, using Centricon filters, molecules >3,000 MW were removed. Figure 12 shows that only the CM from the nonadherent cell cultures stimulated significant epinephrine release from primary chromaffin cell cultures
Figure 10. Mononuclear cell viability decreases between 24h and 48h post-isolation. The number of viable cells in culture were determined based on exclusion of trypan blue. Results shown are two separate splenocyte preparations. Similar results were obtained in three other preparations.
Figure 11. Conditioned media bioactivity decreases as mononuclear cell viability decreases. Values given are the mean ± SEM from the two different splenocyte preparations utilized in figure 1. Each bar represents 2 triplicates from one chromaffin cell preparation (n=6).
Figure 12. Nonadherent T cell and B cell conditioned media exhibits the majority of bioactivity. Values are the mean ± SEM from triplicates of two different chromaffin cell preparations (n=6). ***=p<0.01 compared to conditioned media (CM). Statistics utilized an ANOVA followed by a Tukey's follow up comparison.
indicating that these populations are the primary source(s) of bioactive peptide.

**CD4+ cell conditioned media exhibits the majority of bioactivity.**

Nonadherent cell populations were separated into CD4+ or CD8+ T cell populations and B cell populations using magnetic based cell separation. A bioassay of CM harvested from each of these populations revealed that only the CD4+ (T helper) cell CM exhibited any significant bioactivity (figure 13). Concurrently, to determine if contact between different cell populations was necessary to observe maximal bioactivity, cell populations were recombined and cultured in various combinations subsequent to separation. No significant difference was observed between the bioactivity of single cell population CM and CM from recombined cell cultures (data not shown).

**T helper and B cell populations are potential sources of bioactive peptide.**

Conditioned media from CD4+, CD8+, and B cell cultures was purified using a G-25 Sephadex column. Based on results from previous studies, fractions were pooled into groups which potentially contained bioactive peptide and those which did not. Samples from each pooled group (20µg peptide
which did not. Samples from each pooled group (20\(\mu\)g peptide in each) were run on a polyacrylamide gel to determine if bioactive peptide was present in CM from each population. Figure 14 shows that both the CD4+ and B cell populations CM contained bioactive peptide while no peptide was found in CD8+ cell CM.
Figure 13. T helper cell conditioned media possesses the majority of bioactivity. RPMI-1640 represents basal chromaffin cell secretion. Values are the mean ± SEM from 2 preparations from each of 2 splenocyte preparations (n=4). This media was then utilized in secretion experiments performed in triplicate on two chromaffin cell preparations (n=6). * = p<0.05 compared to conditioned media (CM) using an ANOVA and Tukey's follow up comparisons.
Figure 14. Bioactive peptide is produced by T helper (CD4+) and B cell populations. Samples in each lane are: Lane 1 = CD4+ cell CM, fractions MW<3,000; Lane 2 = CD8+ cell CM, fractions MW<3,000; Lane 3 = B cell CM; fractions MW<3,000; Lane 4 = Nonadherent Cell CM, Fractions MW <3,000; Lane 5 = enriched bioactive peptide isolated from total mononuclear cell cultures. The CD4+, B cell and nonadherent cell conditioned media all contain peptide of interest while there is no evidence of the band in CD8+ CM.
D. Discussion

As research in the area of neural-immune interaction progresses, it is becoming clear that the immune system and neuroendocrine system have the capacity to communicate with each other via common signal molecules and common receptors (Carr and Blalock, 1991). Central to this bidirectional communication is production of classic neuropeptide hormones by cells of immune origin. The first evidence that leukocytes could synthesize functional neuroendocrine hormones was obtained from studies which demonstrated that during the production of interferon alpha (IFNα), virally-challenged human leukocytes coexpressed a peptide which was antigenically related to the pituitary hormone, adrenocorticotropic (ACTH) (Smith and Blalock, 1981). This immune-derived ACTH was similar to its pituitary counterpart with respect to its antigenicity, molecular weight, and retention time on high pressure liquid chromatography (HPLC). This leukocyte-derived ACTH was shown to have the same amino acid sequence as pituitary-derived ACTH, originate from the same precursor, proopiomelanocortin (POMC), in immune cells as in the pituitary gland (Smith et al., 1982), and have similar biological activity (Clarke et
al., 1982), and have similar biological activity (Clarke et al., 1993).

Recent investigations have expanded the list of hormones and neuropeptides produced by immune cells to include thyrotropin (TSH) (Smith et al., 1983), chorionic gonadotropin (CG) (Harbor-McMennamin et al., 1986), growth hormone (GH) and prolactin-related peptides (Hiestand et al., 1986), somatostatin (Lygren et al., 1984; Goetzl et al., 1985), luteinizing hormone (LH) (Emanuele et al., 1990), and vasoactive intestinal peptide (VIP) (Lygren et al., 1984), among others. Although the biological relevance of immune cell production of such molecules is not yet fully understood, in vitro experimentation indicates these hormones and neuropeptides have the capacity to modulate both neuroendocrine and immune functions (Koff and Dunegan, 1985; Mercola et al., 1981; Snow, 1985; Payan et al., 1986). Previously, we have demonstrated that yet another immune-derived peptide may be added to this list of potential immune-neuroendocrine signalling molecules. This small peptide (MW<3,000) is released from mononuclear cells (MNC) and has been shown to stimulate adrenal medullary chromaffin cell epinephrine secretion to levels comparable to maximal
cholinergic stimulation (Jones et al., 1993).

Since all MNC used in these experiments are cultured in the absence of serum to omit serum-derived growth factors in the bioassay system, tests were performed to ensure that serum-free conditions did not alter MNC cell viability. Potentially, a decrease in viability might imply that the bioactive component of CM was a MNC death product. Figures 10 and 11 clearly show this is not the case. Although MNC viability does decrease as time in culture increases (figure 10), bioactivity of CM harvested over the given time course also decreases (figure 11). If the bioactive peptide were a product of MNC death, one would expect bioactivity to increase as viability decreased.

Further data are presented which demonstrate potential sources of this mononuclear cell peptide. Interestingly, just as cytokine production is unique to specific immune cell populations, investigations have revealed that not all immune cells are capable of producing all neuropeptides and hormones. For example, in cell cultures stimulated with endotoxin, B cells, but not T cells synthesize and release bioactive ACTH and endorphin (Harbour et al., 1991). Activated CD4+ T lymphocytes, however, appear to be the
source of opioid peptide in cultures stimulated with the pineal neurohormone melatonin (Maestroni and Conti, 1991). The present studies provide evidence that the immune cell source of the small bioactive peptide is also specific. Initial results demonstrate that when MNC populations are separated based on adherence to plastic, the non-adherent T cell and B cell populations exhibit the majority of bioactivity (figure 12). Further separation of these non-adherent cell populations using specific cell surface markers revealed that while the CD4+ (T helper) cell CM possessed the majority of bioactivity (figure 13), both the CD4+ cell and the B cell are potential sources of the bioactive peptide (figure 14). Peptide production by the CD4+ and B cell populations in the present study, unlike previously cited works, occurs without mitogenic stimulation. This finding is not surprising in that Kol et al., (1983) demonstrated that both mechanical stimulation and stimulation by density centrifugation media, as would occur during cell isolation, will activate mononuclear cells. This suggests that mononuclear cell isolation in the present study is a sufficient stimulus for mononuclear cell bioactive peptide production.
Identification of specific immune cell source(s) of the neuropeptides and hormones will provide insight into the physiologic significance of immune cell production of these substances. This information will also aid in understanding certain aspects of a pathophysiology or disease state based on an immune cell-specific peptide/hormone. For example, lymphocyte-derived CG has been speculated to be an important suppressive factor necessary for a successful implantation of the blastocyst and, ultimately, a successful pregnancy (Blalock, 1994). In this instance a deficiency of immune cells that normally produce this CG may be a causative factor contributing to infertility. Similarly, deficiency or dysfunction of CD4+ and/or B cell populations, as in cases of Acquired Immune Deficiency Syndrome (AIDS) or Severe Combined Immunodeficiency (SCID), may lead to subsequent alterations in bioactive peptide production. The lack or dysfunction of cell sources of the bioactive peptide in these patient populations may have serious ramifications in communications between the immune and neuroendocrine systems. Modifications in peptide release would potentially impact non-neurogenic catecholamine secretion. The implications of this altered neuroendocrine function,
however, remain to be elucidated. Further studies addressing the role of this peptide in homeostatic mechanisms may help explain specific physiologic abnormalities when the function of peptide-cell sources (CD4+, B cell) are impaired.
CHAPTER VI

MONONUCLEAR CELL PEPTIDE MEDIATION OF CHROMAFFIN CELL

EPINEPHRINE SECRETION

A. Introduction

Identification of informational molecules common to the central nervous system and the immune system such as cytokines, hormones, and neuropeptides provides molecular evidence for bidirectional communication between these systems (Weigent et al., 1990; Arnason et al., 1991; Epstein, 1993). Examples of signalling molecules include the cytokines tumor necrosis factor-α, interleukin-1, and interleukin-6 which have been shown to be potent modulators of the hypothalamic-pituitary-adrenal axis (Imura et al., 1991). Additionally, interleukin-1 has been shown to enhance sympathetic nerve activity (Haefli et al., 1993; Ichijo et al., 1994), modify the neuronal gene expression of molecules such as substance P (Jonakait, 1993), and is
synthesized and released by cultured sympathetic neurons (Frieden et al., 1992). Current literature indicates that immune-derived peptides may also play a role in modulating neural/endocrine function. In 1981, Blalock and Smith were the first to report that human peripheral blood lymphocytes (PBL) infected with Newcastle disease virus (NDV) produced the proopiomelanocortin (POMC)-derived peptides, adrenocorticotropic (ACTH) and Beta-endorphin (Smith et al., 1981; Smith et al., 1982; Clarke et al., 1993). Interestingly, in studies with mice previously hypophysectomized to eliminate the pituitary as a source of ACTH, challenge with NDV significantly elevated plasma corticosterone levels (Smith et al., 1981; Smith et al., 1982; Clarke et al., 1993). Furthermore, glucocorticoids released in response to immunoreactive-ACTH were shown to negatively feedback on the immune cells to prevent further release of ACTH or endorphin. Thus, products of the immune system were found to affect output from the adrenal cortex.

Although many details of immune cell-derived ACTH sequences and processing are recognized (Smith et al., 1990), such immune cell influences on classic stress hormones appear to be confined to the adrenal cortex. As of
this date there is little evidence that immune cells can influence the release of the other classic stress hormone epinephrine, from the adrenal medulla. In contrast, there is abundant clinical and experimental evidence that infectious challenge, which activates immune cells, results in profound epinephrine release which may serve important compensatory functions to maintain homeostasis (Benedict and Grahame-Smith, 1978; Jones et al., 1988). Although increased central sympathetic outflow is known to mediate such epinephrine release (Jones et al., 1988), recent evidence from this laboratory has shown that non-neural release of epinephrine during simulated infectious challenge can contribute approximately 30% of total circulating epinephrine (Zhou and Jones, 1993). In light of these facts, initial experiments were conducted to test the possibility that immune cells could mediate epinephrine release using an in vitro system (Jones et al., 1993). These experiments using conditioned media from mononuclear cells, suggested that a small molecular weight (<3,000) product(s) of mononuclear cells can cause epinephrine release from chromaffin cells and that classic nicotinic and muscarinic neural receptors were not involved. The goal of
the present study was to elucidate the physical and biochemical properties of the immune cell-derived product and determine if a feedback loop exists. The present study provides significant new information about this factor from mononuclear cells in regard to the specificity of immune cell-mediated epinephrine secretion. Most importantly, electrophoretic experiments suggest that this epinephrine secretion involves the action of a small molecular weight peptide. The present work clearly demonstrates the potential for circulating immune cells to produce a peptide which can cause epinephrine release from the adrenal medulla. This immune-derived peptide may play a role in the non-neurogenic epinephrine release in response to infectious challenge and other stressors.
B. Materials and Methods

Primary Cultures of Chromaffin Cells

Isolation and Culture: Adult bovine adrenal glands were used for chromaffin cell isolation according to the overall procedure described by Dahmer et al., (1990). In brief, adrenal glands were perfused with collagenase, incubated at 37°C and medullary tissue removed for further digestion. Digested cells were washed, counted, and viability determined with trypan blue staining. Purity was assessed using neutral red as described by Role and Perlman (1980). Cells were plated on collagen-coated 24-well plates at a density of \(3 \times 10^5\) cells per cm\(^2\) and media was changed after the first day and then as needed to maintain a normal pH.

In vitro Model of Catecholamine Secretion: Basic secretion experiments are similar to those described by Dahmer et al., (1990). In brief, growth media was removed from each well and replaced with conditioned media, serum-free RPMI-1640, Earle's Balanced Salts Solution (EBSS), or secretagogue in EBSS. After incubation, media was removed and saved. Epinephrine content in these samples represent that stimulated by conditioned media (90 minute incubation),
spontaneous/basal secretion (RPMI/EBSS, 90 minute incubation), or that stimulated by a known secretagogue (DMPP/55mM K+, 10 minute incubation) in the presence or absence of drug/channel blocker. Cells were then lysed by adding 1M perchloric acid and the acid saved for determination of the remaining cellular epinephrine content. Epinephrine secreted into the media as a percent of total content was calculated by dividing the amount secreted by the total well content. Secretion experiments were routinely done in triplicate using three separate wells on any one plate all of which were derived from the same adrenal gland. Replicates of separate triplicate determinations were made on cells from different chromaffin cell preparations. Analysis of epinephrine was accomplished with HPLC using electrochemical detection. Mobile phase contained 0.1M monochloroacetic acid with 0.5mM EDTA and 1.0mM sodium octyl sulfate adjusted to pH 3.0. Acetonitrile was added at 15mL/L. A BAS Liquid Chromatography System including an LC 4C amperometric detector and glassy carbon electrode was used in all analyses.

Primary Cultures of Superior Cervical Ganglion (SCG) Cells

Cell Isolation and Culture: Methods are as those
described previously (Wang et al., 1995). Briefly, superior cervical ganglia were dissected from fetal pigs (size: 15-20 cm, gestation age: 6.5-10 weeks) obtained from the local slaughterhouse. The surrounding tissue was carefully removed and the ganglia were transferred to a 0.2% collagenase-A solution. After gently stirring for 30 min at 37°C, the ganglia were transferred to a fresh collagenase solution. The cell suspension was centrifuged (200Xg; 4 min). The pellet, containing the dissociated cells was resuspended in culture medium (Nutrient Mix HAM F12) supplemented with 10% (v/v) horse serum, 1 µg/ml insulin, 1 µg/ml transferrin, 30 ng/ml nerve growth factor, and antibiotics. This procedure was repeated several times until all tissue was digested. The dissociated cells were submitted to a differential plating for 2 hours in order to remove most of the non-neuronal cells. The cells were counted in a Coulter counter and plated out at the density of 2X10⁴ cells/cm² in culture flasks or polyornithine/laminin coated 24 well plates. Cultures were maintained at 37°C in a humidified incubator under an atmosphere of 95% O₂ and 5% CO₂. The medium was changed every 72 hours.
In Vitro Model of Catecholamine Secretion: Superior cervical ganglia cells were loaded with [³H]norepinephrine for 2 hours, washed extensively (one 30 min. wash followed by three 15 min washes), then exposed to conditioned media or serum-free RPMI-1640 for 90 minutes. Media was harvested and supernatant radioactivity and the remaining radioactivity in each well was determined using the TRI-CARB 460 system (Packard, USA). Norepinephrine secreted into the media as a percent of cell content was calculated as radioactivity of the supernatant divided by total radioactivity of the cells in the well.

Digitonin Permeabilization of Chromaffin Cells:
Chromaffin cells were plated according to described methods in 24 well plates at 10%, 20%, 40%, 50%, 60%, 80%, 90%, and 100% of the normal/maximal density (6X10⁵ chromaffin cells/well). All experiments were performed in triplicate on each chromaffin cell preparation. Digitonin (20μM) was added to each of the wells and allowed to incubate for 20 minutes. Supernatant from each well was then harvested and assessed for catecholamine content using HPLC or for lactate dehydrogenase activity as described below.
Measurement of Lactate Dehydrogenase Activity in Cell Supernatant

Lactate dehydrogenase (LDH) activity of chromaffin cell supernatant was determined using the LDH procedure #500 from the Sigma Chemical Co. (St. Louis, MO). Briefly, 40 µl of supernatant was added to a cuvette containing 1 ml of the LDH substrates pyruvate and NADH in phosphate buffered saline (PBS). Absorbance of this solution at 340 nm was measured each minute for three minutes and the mean change in absorbance calculated. LDH activity (U/L) for each sample was calculated according to the manufacturer's instructions.

Primary Cultures of Mononuclear Cells from Spleen

Isolation and Culture: Whole bovine spleen was obtained from a local slaughter house. Inner spleen tissue, which includes both red and white pulp, was dissected out, cut into small pieces and forced through wire mesh in Hank's Balanced Salts Solution (HBSS) at room temperature. Crude cell suspensions were layered on density gradient media (Fico/Lite-LymphoH, Atlanta Biologicals), centrifuged and then separated. Isolated cells were washed, viability assessed using Trypan Blue, and cells were then transferred
to flasks with RPMI media containing only essential amino acids and antibiotics. The growth media was isolated from the cells after at least 24 hours of culture and either used promptly or frozen (-20°C).

**Characterization of Bioactive Components of Conditioned Media**

**Acid Hydrolysis:** Conditioned media was harvested from 24-hour mononuclear cell cultures then boiled in acid (3N HCl) for 30 minutes. After boiling, media was brought to physiologic pH, filtered (Centricon, 3,000 MW cutoff) then utilized in secretion experiments.

**Carboxypeptidase Y and Leucine Aminopeptidase:**
Conditioned media was harvested from 24-hour mononuclear cell cultures then incubated at 37°C either alone or with enzyme (final concentration of enzyme 25µg/ml). After the specified incubation time, media ± enzyme was centrifuged through a Centricon filter (3,000 MW cutoff) to remove peptidase enzyme or other molecules larger than 3,000 MW. Media was either used promptly in secretion experiments or frozen (-20°C).
Peptide Purification and Isolation

G-25 Sephadex Column Gel Filtration: G-25 Sephadex beads (10 g) were combined with 30 ml deionized water and equilibrated for 8-10 hours. This volume was poured into a 2 cm column which was attached to a continuous perfusion pump and equilibrated with Phosphate Buffered Saline (PBS, pH=7.2). A 2 ml injection loop was used to introduce conditioned media onto the column with an injection valve. Blue Dextran (MW 2X10^6) provided a measure of the void volume and potassium chromate (MW 194) was used to determine total volume. Fraction collection (2 ml each) began when the void volume was eluted and continued until the total volume was removed from the beads. Elution of peptide was determined by monitoring absorbance at 206nm as the effluent came off the column.

SDS-PAGE: Basic methods are similar to those described by Schägger and von Jagow (1987). In brief, a 20% T separating gel was prepared containing 3.0 M Tris (pH 8.45) and glycerol. This was overlaid with a 10% spacer gel, followed by a 4% stacking gel (3.0 M Tris, pH=8.45) yielding a gel with approximate dimensions of 0.75mm X 6.5cm X 9.5cm. A discontinuous buffer system was utilized which included a
Tris-base (pH 8.9) anionic buffer and a Tricine/Tris cationic buffer. Approximately 20µg of protein was loaded into each lane and a constant voltage was applied to the gel (20V for 1 hour followed by 100V for 1-1.5 hours). The gel was removed from the apparatus once the dye front reached the anionic buffer-gel interface and silver stained.
C. Results

Mononuclear Cell Conditioned Media (CM) Stimulates Chromaffin Cell Epinephrine Secretion to Levels Comparable to Maximal Cholinergic Stimulation.

Figure 15 shows that mononuclear cell-derived conditioned media (CM) stimulates chromaffin cell epinephrine secretion to levels comparable to maximal cholinergic stimulation and that induced by depolarizing concentrations of potassium. Mononuclear cells used in these experiments were cultured in the absence of serum to ensure that the bioactivity of MNC media (CM) was not a result of serum-derived factors.

CM - Induced Secretion Occurs through Specific, Typical Exocytotic Processes.

Lactate dehydrogenase (LDH) and modulation of external calcium were used to determine if CM - induced secretion occurred through stimulation of specific exocytotic processes. In LDH experiments, chromaffin cells were plated in 24 well plates in concentrations 10, 20, 40, 50, 60, 80, and 90 percent of the normal conditions ($6 \times 10^5$ cells/well). Cells were permeabilized with digitonin (20µM,
Figure 15. Conditioned media-induced catecholamine secretion comparable to maximal cholinergic stimulation. Values given are mean ±SEM from triplicates of 3 to 6 different chromaffin cell preparations (n=9 to 18). **p<0.001 when compared to CM, control, or high potassium using an ANOVA comparison with Tukey's follow-up test.
Figure 16. Epinephrine content in media of chromaffin cells permeabilized with digitonin (20µM) for 20 minutes. Maximal cells (100%) plated per well = 6 X 10^5 in a 24 well plate. Values given are the mean ± SEM from triplicates of 3 different chromaffin cell preparations (n=9).
Figure 17. Lactate dehydrogenase (LDH) activity in media of chromaffin cells permeabilized with digitonin (20µM) for 20 minutes. Maximal cells plated per well = 6 X 10⁵ in a 24 well plate. Values are mean ± SEM. LDH activity (U/L) defined as the amount of enzyme which catalyzes the formation of 1 µmol/L of NAD⁺ per minute under the conditions of the assay. ***=no detectable LDH activity. Results obtained from triplicates of 3 different chromaffin cell preparations (n=9).
20 min) then supernatant from each well was assessed for catecholamine content and LDH activity as previously described. With this information, the relationship between LDH and the catecholamine content was determined. As figures 16 and 17 demonstrate, the greater the concentration of digitonin-permeabilized cells the greater the epinephrine content and lactate dehydrogenase activity of the supernatant. This can be contrasted to the LDH activity and catecholamine content of cells exposed to CM. The average catecholamine released by CM exposure (90 min) was 2.1 µg per well (figure 16). However, there was no detectable LDH activity in CM supernatant (figure 17, ***) under the given experimental conditions indicating that CM stimulated specific secretory events. Similar results were obtained (no detectable LDH activity, significant catecholamine content) from chromaffin cell media after stimulation with the nicotinic agonist DMPP (data not shown).

Classic secretory pathways in chromaffin cells are intimately coupled to calcium and bovine chromaffin cells in particular are dependent upon the influx of extracellular calcium to observe secretagogue-induced secretion. Therefore, the availability of external calcium was
Figure 18. Extracellular calcium dependence of conditioned media (CM)-induced secretion. Values are mean ± SEM. RPMI-1640 group represents basal chromaffin cell secretion. Triplicate determinations for all groups were made in each of 2 chromaffin cell preparations (n=6). *** = p<0.001 compared to CM alone using an ANOVA followed by Tukey's comparisons.
Figure 19. Acid hydrolysis of conditioned media significantly reduces bioactivity. Values given are mean ± SEM from triplicate determinations in 3 chromaffin cell preparations (n=9). ***=p<0.001 compared to CM using an ANOVA and Tukey's comparisons.
modulated to determine if CM-induced secretion had a similar dependence on extracellular calcium. The influx of extracellular calcium was inhibited by the non-specific calcium channel blockers cadmium (0.1mM) and cobalt (2.0mM). Results presented demonstrate that both cadmium (figure 18) and cobalt (figures 2 & 18) reduced CM-induced secretion to levels near or at background indicating CM-induced secretion is dependent on extracellular calcium influx. Similar results were obtained using the nicotinic agonist 1,1-Dimethyl-4-Phenylpiperazinium (DMPP, 10µM), a secretagogue known to be dependent on external calcium to induce secretion (figure 3).

Evidence that Bioactive Component(s) of CM are Peptide(s)

Evidence that the bioactive component(s) of CM are peptide in nature is based on acid hydrolysis, enzymatic treatment, as well as polyacrylamide gel electrophoresis of purified fractions of CM. Acid hydrolysis (3N HCl, 100°C) of CM for 30 minutes significantly reduced the bioactivity of CM (Figure 19). Similarly, carboxypeptidase Y (CY, 25 µg/ml) treatment also significantly decreased CM bioactivity. As figure 20 illustrates, increasing incubation time with CY significantly decreased bioactivity
Figure 20. Secretion experiments using carboxypeptidase Y (CY)-treated conditioned media. Values are mean ± SEM from triplicates of 6 chromaffin cell preparations (n=18). Data represent epinephrine secretion from enzyme-treated CM as a percent of the time-matched control (incubated, non-enzymatically treated CM). * = p<0.05 when compared to time-matched control using an ANOVA and Tukey's follow up test.
Figure 21. Secretion experiments from leucine aminopeptidase (LAP)-treated conditioned media. Values given are mean ± SEM. Each bar represents triplicates on 2 different chromaffin cell preparations (n=6). ***=p<0.001 when compared to control using an ANOVA followed by Tukey's comparisons.
Figure 22. Bioactivity of G-25 Sephadex enriched fractions of conditioned media. Values are mean ± SEM taken from triplicates of each fraction of CM. RPMI-1640 represents basal chromaffin cell secretion. Filtered is bioactivity of Centricon filtered CM; unfiltered is CM bioactivity prior to filtration through the Centricon filter.
Figure 23. Silver stain of a modified Schägger gel reveals a single peptide band in the bioactive fraction from G-25 purified conditioned media. Lane 1 contains 20µg protein from total conditioned media (not filtered through Centricon filter), Lane 2 contains RPMI-1640 cell culture media, and Lane 3 the bioactive fraction from the G-25 column.
of CM when compared to time-matched controls. CY treatment did not, however, decrease CM bioactivity to background levels indicating that the enzymatic digestion was incomplete (see Discussion, pg 154). Treatment of CM with leucine aminopeptidase (LAP, 25µg/ml), unlike CY, significantly increased the bioactivity of CM when compared to time matched controls (Figure 21).

These data suggesting the peptide-nature of bioactive component(s) of CM are supported by SDS-PAGE electrophoresis of enriched CM fractions. Components of CM were separated on a G-25 column and individual fractions (2 ml each) were tested for bioactivity in secretion experiments (figure 22). Such bioactive fractions were also loaded and peptide(s) separated on modified Schagger gels via SDS-PAGE. These gels were subsequently stained with silver. Figure 23 shows a single peptide band in the lanes taken from bioactive fractions of CM. Electroelution of this band from similar gels also stimulated significant catecholamine release in secretion experiments when compared to other electroeluted bands (Figure 24). Mass spectroscopic analysis of this peptide determined the putative mass to be 627 Da.
Conditioned Media Peptide Stimulates Catecholamine Release from Superior Cervical Ganglia Cell Cultures

To determine if the bioactive peptide could stimulate catecholamine release from another secretory source, the sympathetic nerves, CM secretion experiments were performed on cells from porcine superior cervical ganglia. Cells exposed to CM released approximately 2 fold more $[^3]H$-norepinephrine as did those exposed to RPMI-1640 alone (Figure 25). This effect was concentration-dependent.

Feedback Inhibition by Epinephrine on Mononuclear Cell Peptide Production

Mononuclear cells were cultured in the presence of epinephrine to determine if the catecholamine could act in a classical negative feedback loop to inhibit further immune-cell peptide production. Data presented in figure 26 demonstrates that concentrations of epinephrine as low as 1nM may inhibit release of bioactive peptide and this inhibition is partially reversed by $\beta$-adrenergic blockade with propranolol.
Figure 24. Electroelution of conditioned media peptides reveals bioactivity is present only in peptide band of interest. Putative bioactive peptide (Band "B") was electroeluted and bioactivity tested alone; all other peptides were combined and tested for bioactivity in secretion experiments. Values are mean ± SEM from two triplicates on one chromaffin cell preparation (n=6). **=p<0.01 compared to basal chromaffin cell secretion (RPMI-1640) or secretion due to bands > 3,000 MW using an ANOVA and Tukey's follow up comparisons.
Figure 25. Conditioned media (CM) stimulates norepinephrine secretion from sympathetic ganglion cells. Basal refers to control secretion over 90 min with serum-free RPMI-1640. Conditioned media was diluted with serum-free RPMI-1640. Values are the mean ± SEM taken from 22 different ganglion cell preparations (n=22). *=p<0.05 compared to all other bars; **=p<0.05 compared to all other bars; ***=p<0.05 compared to all other bars. Statistics included an ANOVA with Tukey's follow-up comparisons.
Figure 26. Feedback inhibition by epinephrine (EPI) on mononuclear cell (MNC) peptide production. Values are the mean ± SEM from six samples of one chromaffin cell preparation (n=6). * = p<0.05 compared to CM alone; ** = p<0.01 and *** = p<0.001 compared to CM alone; # = p<0.05 compared to 1µM EPI+propranolol. Statistics utilized were an ANOVA with Tukey's follow up test. Similar results were obtained with MNC/EPI co-culture at concentrations between 1nM and 1µM.
D. Discussion

Extensive documentation demonstrates that humans respond to both cognitive and noncognitive stimuli through a stress response that results in the release of epinephrine into the circulation (Kjaer and Secher, 1992; Webber and MacDonald, 1993). This increased plasma epinephrine has a wide array of physiologic effects including various modifications of immune function (Malec et al., 1989; Mihran-Davis et al., 1991; Rinner et al., 1992; Murray et al., 1993). Results of the present study provide evidence that immune cells are able to synthesize and release a novel, small molecular weight peptide which modulates adrenal medullary chromaffin cell epinephrine secretion. These findings support the concept of bidirectional communication between the immune system and the adrenal medulla and suggest a novel mechanism by which circulating immune cells could mediate epinephrine secretion.

The nervous and immune systems share important informational molecules such as cytokines, hormones, and neuropeptides. These compounds serve as molecular signals which provide an important link between neural and immune
systems. Evidence is accumulating which clearly demonstrates that not only do the classic immune signalling molecules, the cytokines (i.e. TNF, IL-6, IL-1) (Haefli et al., 1993; Ichijo et al., 1994; Jonakait et al., 1993; Frieden et al., 1992; Imura et al., 1991), affect neuroendocrine function but also that immune cells are capable of synthesizing and releasing bioactive molecules which were previously believed to be exclusive to neuroendocrine systems (i.e. ACTH, β-endorphin, CGRP) (Smith et al., 1981; Smith et al., 1982; Smith et al., 1983; Harbor-McMenamin et al., 1986; Hiestand et al., 1986). Expansion of this list to include a lymphoid-adrenomedullary interaction through an undescribed lymphocyte product is not surprising.

The present study provides evidence that a single, novel small molecular weight peptide is released by MNC and stimulates adrenal medullary chromaffin cell epinephrine secretion to levels comparable to maximal cholinergic stimulation. Such conclusions are based upon evidence that isolated preparations of peptide retained bioactivity (figure 24). Evidence is also presented that excludes the potential impact of several extraneous variables in the
reported immune peptide-chromaffin cell interaction. For example, it is possible that the bioactive MNC peptide, rather than stimulating regulated catecholamine exocytosis, could have altered chromaffin cell integrity leading to the leak of catecholamine from cell cultures over the ninety minute CM incubation. Lactate dehydrogenase, a cytosolic enzyme which would leak concurrently with catecholamine if cell integrity was modified, was not detected in CM after chromaffin cell incubation (figure 17) while significant catecholamine was found in this same media (figure 16). Furthermore, CM-induced secretion was dependent on the availability of extracellular calcium (figure 18) indicating that CM stimulated chromaffin cell epinephrine secretion through specific, typical exocytotic mechanisms. Interestingly, the data presented here utilize conditioned media from MNC which were not stimulated with any mitogenic factors. The observed production of bioactive peptide in the present studies without mitogenic stimulation, however, is not surprising. Mechanical stimulation during isolation of MNC or the documented mitogenic effect of the density centrifugation media (Kol et al., 1983) may be a sufficient stimulus of the MNC for bioactive peptide production.
The peptide nature of the immune cell-derived molecule in the present study was determined using several different lines of evidence. Acid hydrolysis of bioactive CM significantly decreased the bioactivity (figure 19) presumably through hydrolysis of the peptide bond(s). The peptide nature of the molecule was also demonstrated using proteolytic digestion. Incubation of conditioned media with either carboxypeptidase Y (figure 20) or leucine aminopeptidase (figure 21) resulted in significant changes in bioactivity. However, CY digestion of CM resulted in incomplete inactivation while LAP treatment of CM significantly increased bioactivity. The incomplete inactivation by CY could be explained by restricted substrate specificity of the peptidase. CY activity has been shown to be limited when the amino acid to the left of the cleavage is glycine or asparagine or when the residue to the right of the cleavage site is histidine or lysine. Potentially, these amino acid residues may be present in the bioactive peptide and limit CY activity. The increased bioactivity of CM after LAP treatment is quite interesting and may provide a basis for in vivo experimentation. Conditioned media used in these experiments was not filtered
(Centricon, 3,000 MW cutoff) until after treatment with LAP was complete. The increase in bioactivity is hypothesized to be a result of LAP cleavage of a larger "parent" molecule into the small bioactive peptide. Current investigations are examining the possibility that endogenous LAP, known to be released by mononuclear cell populations (Nagaoka and Yamashita, 1984), could liberate bioactive peptide from a larger protein.

Additional evidence indicating the peptide nature of the bioactive factor involved G-25 Sephadex enrichment of CM and modified SDS-PAGE isolation/purification. Since peptides in solution have increased absorbance at 206 nm, experiments were conducted to determine if there was a correlation between an increase in absorbance and bioactivity. Fractions were obtained from the Sephadex G-25 column which showed increased absorbance at 206 nm as well as demonstrated high bioactivity (figure 22). Peptide-enriched fractions of CM were then analyzed using gel electrophoresis. Separation of peptide(s) using a modified Schägger gel stained with silver clearly show the presence of a small peptide (figure 23). Biological activity of samples removed from similar gels by electroelution revealed
that only fractions containing this and no other protein band(s) stimulated significant catecholamine secretion from primary chromaffin cell cultures (figure 24). Interestingly, CM peptide also stimulates catecholamine release from in vitro sympathetic ganglion cells. These results suggest that the peptide may also enhance norepinephrine release from peripheral sympathetic nerve terminals. These data are consistent with previously published research demonstrating increased norepinephrine release at constant nerve activity following a non-cognitive stimuli (endotoxin) (Jones et al., 1994). Additionally, epinephrine was shown to inhibit mononuclear cell peptide release and subsequent CM bioactivity (figures 25 & 26), thus, completing a potential physiologic axis.

The putative immune-adrenal medullary axis described in these studies may have several important clinical implications. Early in this century, Hans Selye noted that "diverse noxious agents" (stressors) produced nonspecific events that "sick people" share in common. These "noxious agents" included both physical agents (non-cognitive stimuli; i.e. viruses, bacteria, tumors) and emotional factors (cognitive stimuli). The immune-derived peptide
described in these studies may be one potential mechanism by which non-cognitive stimuli elicit the release of the stress hormone epinephrine. An increased systemic epinephrine in response to peptide would be beneficial during sickness supporting the hypermetabolism of fever by alterations in metabolic and cardiovascular function. Acute elevations in epinephrine are also associated with enhanced immune function (Murray et al., 1993; Sanders and Munson, 1985) which would be equally advantageous in combatting illness. Prolonged elevation of plasma catecholamine, however, has been shown to compromise immune function and, thus, would be detrimental (Malec et al., 1989; Mihran-Davis et al., 1991; Rinner et al., 1992; Murray et al., 1993). These studies suggest that increased epinephrine release as in response to bioactive peptide may negatively feedback on cells of the immune system and thus prevent further production of bioactive peptide. Removal of the peptide stimulus would limit further epinephrine secretion and regulate this immune-neuroendocrine axis.

The results of the present study provide further evidence for a physical link between the manifestations of stress and the immune system. These results also add to the
growing body of literature describing neuroendocrine-immune interactions and the potential importance of bidirectional communication between these systems in maintaining homeostasis. Potentially, the disruption of this putative immune-adrenal medullary axis may be a component of the pathophysiology of various chronic disease states. Understanding the capacity of the immune system to respond to a stressor through a compensatory mechanism and possibly alter the stress response itself via signalling molecules (e.g. bioactive peptide) may be useful in predicting disease onset and outcome.
CHAPTER VII

CHROMAFFIN CELL EPINEPHRINE SECRETION MEDIATED BY A
MACROPHAGE PEPTIDE: THE ROLE OF ENDOTOXIN

A. Introduction

The importance of stress in the etiology and prevention of disease is becoming increasingly evident as research in this area progresses (Sheridan et al., 1994). Traumatic injury, hemorrhage, as well as infection and its sequelae are known to create a severe stress on the body. A tightly regulated inflammatory/immune response to these types of stress provides protection from invading pathogens, limits injury, and promotes healing. However, in some patients this response is not regulated and leads to an uncontrolled systemic response commonly referred to as the systemic inflammatory response syndrome (SIRS). SIRS, which may occur in the presence or absence of infection, is manifest by changes in white blood cell (WBC) count (>12,000
cells/mm$^3$ or < 4,000 cells/mm$^3$) and maturity (> 10% immature bands), changes in body temperature (> 38°C or < 36°C), tachycardia, and tachypnea (Secor, 1994). Since the catecholamines, epinephrine and norepinephrine, have been shown to have profound effects on the cardiopulmonary system as well as on immune function, the marked elevations of plasma catecholamine that occur in response to stress may play an integral role in the onset and progression of SIRS. Catecholamines are well recognized to be released by both the sympathetic nervous system (mainly norepinephrine) and the adrenal medulla (epinephrine) during a stress response such as in trauma, hemorrhage or infectious challenge. While epinephrine secretion from the adrenal medulla is typically attributed to neural stimulation of medullary chromaffin cells, recent findings suggest that an immune cell-derived peptide released from mononuclear cells may also stimulate the secretion of medullary catecholamines (Jones et al., 1993). The present study was undertaken to test the possibility that endotoxin, a molecule which can induce SIRS, may stimulate the production/release of bioactive peptide from immune cells. Catecholamine secretion in response to endotoxin-induced release of the
immune cell-derived peptide may play a role in the altered cardiopulmonary and immune system function observed during SIRS and subsequent shock states.
B. Materials and Methods

Primary Cultures of Chromaffin Cells

Isolation and Culture: Adult bovine adrenal glands were used for chromaffin cell isolation according to the overall procedure as described by Dahmer et al., (1990). In brief, adrenal glands were perfused with collagenase, incubated at 37°C and medullary tissue removed for further digestion. Digested cells were washed, counted, and viability determined with trypan blue staining (>98% in all preparations). Purity was assessed using neutral red as described by Role and Perlman (1980) and was >92% in all cultures. Cells were plated on collagen-coated 24-well plates at a density of 3 X 10^5 cells per cm² and media (Dulbecco's Minimum Essential Media, F12, growth inhibitors, antibiotics, 10% FBS) was changed after the first day and then as needed to maintain normal pH.

In vitro Model of Catecholamine Secretion: Basic secretion experimental protocols are those described by Dahmer et al., (1990). In brief, growth media was removed and replaced with either conditioned media (90 min
incubation), RPMI-1640 (90 min, incubation), modified balanced salts solution (DMEM, 90 min incubation), or secretagogue in modified balanced salts solution (DMEM, 10 min incubation). After incubation, media was removed and saved. Catecholamine content in these samples represents either that stimulated by conditioned media, spontaneous/basal secretion (RPMI, salts solution with or without ETX/PHA), or that stimulated by a known secretagogue. Cells are then lysed by 1M perchloric acid and acid was saved to determine the remaining epinephrine content of the cells. Total epinephrine secreted into the media was calculated from the sum of the individual contents. Percent chromaffin cell secretion was calculated by dividing the amount secreted by the total. Secretion experiments were routinely done in triplicate using three separate wells on any one plate which were derived from the same adrenal gland. Replicates of separate triplicate determinations were made on cells from different chromaffin cell preparations. Analysis of epinephrine was accomplished with HPLC using electrochemical detection. Mobile phase contained 0.1M monochloroacetic acid with 0.5mM EDTA and 1.0mM sodium octyl sulfate adjusted to pH 3.0. Acetonitrile
was added at 15ml/L. A BAS Liquid Chromatography System including an LC 4C amperometric detector and glassy carbon electrode was used in all analyses.

**Primary Cultures of Mononuclear Cells from Spleen**

Whole bovine spleen was obtained from a local slaughter house. Inner spleen tissue, which includes both red and white pulp, was dissected out, cut into small pieces and forced through wire mesh in salts solution at room temperature. Crude cell suspensions were layered on density gradient media (Lymphoprep), centrifuged and then separated cells were removed and washed. Isolated cells were transferred to flasks with RPMI-1640 media containing no serum/growth factors. Macrophage/monocyte populations were isolated from other splenocytes based on 1 hour adherence to plastic in culture flasks at 37°C. Purity of cultures was determined by Wright-Giemsa staining and non-specific esterase activity. Cultures were either stimulated with LPS (S. enteritidis,1µg/ml) or phytohaemagglutinin (PHA, 50µg/ml) or left unstimulated. The growth media was isolated from the cells and either used promptly or frozen (-20°C) after at least 24 hours of culture.
Peptide Purification and Isolation

G-25 Sephadex Column Gel Filtration: G-25 Sephadex beads (10 g) were combined with 30 ml deionized water and equilibrated for 8-10 hours. This volume was poured into a 2 cm column which was attached to a continuous perfusion pump and equilibrated with Phosphate Buffered Saline (PBS, pH 7.2). A 2 ml by-pass loop connected to the column was used to introduce conditioned media onto the column with an injection valve. Blue Dextran (MW 2X10^6) provided a measure of the void volume and potassium chromate (MW 194) was used to determine total volume. Fraction collection (2 ml each) began when the void volume was eluted and continued until the total volume was removed from the beads. Elution of peptide was determined by monitoring absorbance at 206 nm as the effluent came off the column.

SDS-PAGE: Basic methods are similar to those described by Schägger and von Jagow (1987). In brief, a 20% T separating gel was prepared containing 3.0 M Tris (pH=8.45) and glycerol. This was overlaid with a 10% spacer gel, followed by a 4% stacking gel (3.0 M Tris, pH 8.45) yielding a gel with approximate dimensions of 0.75mm X 6.5cm X 9.5cm. A discontinuous buffer system was utilized which included a
Tris-base (pH 8.9) anionic buffer and a Tricine/Tris cationic buffer. Approximately 20μg of sample was loaded into each lane and a constant voltage was applied to the gel (20V for 1 hour followed by 100V for 1-1.5 hours). The gel was removed from the apparatus once the dye front reached the anionic buffer-gel interface. The gel was then silver stained.

**Leucine Aminopeptidase**

**Endotoxin Challenge of Mice:** Basic methods are those described by Gamelli et al., (1994). Briefly, adult male B₆D₂F₁ mice weighing 23-26g (Jackson Laboratory, Bar Harbor, Maine) were injected with a sublethal dose of lipopolysaccharide (10μg/ mouse, IP) from *Escherichia coli*. After three hours, animals were sacrificed and serum was obtained from blood.

**Activity of Conditioned Medias/ Mouse Serum:** Leucine aminopeptidase (LAP) activity of conditioned media and/or mouse serum was determined using the Leucine Aminopeptidase procedure number 251 from Sigma Diagnostics (St. Louis, MO). Briefly, media/sera were incubated in the presence of LAP substrate at 37°C for 1 hour. The reaction was terminated upon the addition of 2N hydrochloric acid. Sodium nitrite
(0.2% w:v, 3 minute incubation) and ammonium sulfate (0.5% w:v, 3 minute incubation) were added consecutively and the reaction was allowed to proceed at room temperature. Finally, samples were incubated with an alcoholic dye base solution (N-1-Naphthylethylenediamine in ethanol) for 45 minutes at room temperature. Each sample was transferred to a cuvette and absorbance (562nm) was read and recorded.

**Treatment of CM Fractions with LAP:** The peptide/protein content of G-25 enriched conditioned media fractions were characterized using SDS-PAGE. Fractions were divided into groups containing the peptide of interest (group "B") and those containing putative parent molecule(s) (> 3,000 MW, group "A"). Groups "A" and "B" were then each separated into samples of equal volume. These samples were incubated for 5 hours at 37°C either alone or with enzyme (LAP, 25µg/ml). After incubation, media ± enzyme was centrifuged through a Centricon filter (3,000 MW cutoff) to remove peptidase or other molecules larger than 3,000 MW. Media was either used promptly in secretion experiments or frozen (-20°C).
C. Results

**ETX enhances bioactivity of conditioned media (CM).**

Mononuclear cells isolated from bovine spleen were cultured 24h in the presence of either ETX (*S. enteritidis*, 1 µg/ml) or phytohemagglutinin (50 µg/ml). Media was harvested, filtered (Centricon) to remove all molecules greater than 3,000 MW, then used in secretion experiments. While PHA in culture had no significant influence on CM bioactivity, the presence of ETX in splenic MNC cultures significantly enhanced CM bioactivity (Figure 27). This enhanced chromaffin cell epinephrine release in response to ETX-stimulation, presumably, was due to increased synthesis/release of bioactive peptide.

**Bioactivity in unstimulated cell cultures come from T and/or B cell populations.**

To determine the mononuclear cell population(s) which significantly contributed to CM bioactivity in non-mitogenically challenged cell cultures, the mononuclear cell populations were divided based on adherence to plastic and their CM harvested after 24 hours. Purity of these cultures was assessed using Wright-Giemsa and a non-specific esterase stain and determined to be > 90%. CM from the nonadherent T
Figure 27. Endotoxin (ETX) stimulates an increase in conditioned media (CM) bioactivity. Basal secretion in serum-free RPMI-1640 ranged from 2-5% of total cell content in all experiments. Results are presented as the mean ± SEM taken from triplicates of one chromaffin cell preparation (n=3) for each bar. *** = p<0.01 compared to CM using an ANOVA and Tukey's follow up test. Similar results were obtained with other chromaffin cell preparations.
Figure 28. Nonadherent cell population(s) are the source of bioactivity in non-mitogenically challenged cell cultures. Basal secretion (serum-free RPMI-1640) ranged from 2-5% in all experiments. Values represent the mean ± SEM from 3 different spleen preparations. **=p<0.001 compared to CM from unseparated MNC cultures using an ANOVA followed by Tukey's comparisons.
cell and B cell populations had significantly greater bioactivity (figure 28) than adherent cell CM from monocyte/macrophage cell types.

**ETX stimulates monocyte/macrophage peptide production.**

Mononuclear cell populations were divided based on adherence to plastic then cultured either in the presence or absence of ETX (*S. enteritidis, 1µg/ml*) or PHA (50µg/ml). Neither ETX nor PHA significantly altered CM bioactivity from nonadherent cell populations (figure 29). Adherent monocyte/macrophage CM bioactivity, however, significantly increased after 24 hour culture with ETX (figure 29). This enhanced bioactivity could not be attributed to the action of ETX on chromaffin cell cultures.

**SDS-PAGE analysis of ETX stimulated cultures shows bioactive band present only in stimulated monocyte/macrophage cultures.**

To determine if bioactive peptide was present in conditioned media from ETX-treated and non-treated monocyte/macrophage cell cultures, purified CM peptides from these cultures were separated using SDS-PAGE. Each gel also included lanes containing G-25 enriched bioactive peptide which had been previously characterized for reference
Figure 29. Endotoxin (ETX) stimulates significant increases in bioactivity of monocyte/macrophage conditioned media (CM). Values presented are the mean ± SEM from 2 different MNC cultures. Secretion experiments were performed in triplicate on 2 chromaffin cell preparations (n=6). Basal chromaffin cell secretion (serum-free RPMI-1640) ranged from 2-4% in all experiments. Basal secretion was unaltered by the addition of ETX or PHA (see graph). ##=p<0.01 compared to non-stimulated monocyte/macrophage CM; **=p<0.01 compared to unstimulated, unseparated CM. Statistics utilized an ANOVA followed by Tukey's comparisons.
Figure 30. Endotoxin (ETX) stimulates the release of a novel, bioactive peptide from monocyte/macrophage populations. Samples in each lane are: Lane 1 = monocyte (-) ETX, fractions MW >3,000; Lane 2 = monocyte (-) ETX, fractions MW <3,000; Lane 3 = enriched peptide isolated from total mononuclear cell cultures; Lane 4 = monocyte (+) ETX, fractions MW >3,000; Lane 5 = monocyte (+) ETX, fractions MW <3,000. The previously characterized bioactive peptide is only detectable in media from monocytes cultured in the presence of ETX (Lane 5).
Table 1. Leucine aminopeptidase (LAP) activity is significantly increased in ETX-treated macrophage cultures and endotoxic mouse serum.

<table>
<thead>
<tr>
<th></th>
<th>- ETX</th>
<th>+ETX</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditioned Media</td>
<td>3.45 +/- .2</td>
<td>7.6 +/- .4 (***)</td>
<td>2.2</td>
</tr>
<tr>
<td>Macrophage CM</td>
<td>3.24 +/- .6</td>
<td>7.32 +/- 1.3 (**)</td>
<td>2.3</td>
</tr>
<tr>
<td>T/B Cell CM</td>
<td>9.36 +/- .3</td>
<td>9.035 +/- .7</td>
<td>1.0</td>
</tr>
<tr>
<td>Mouse Serum</td>
<td>9.2 +/- .3</td>
<td>13.8 +/- .2 (##)</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Data presented are leucine aminopeptidase activity expressed as units per million cells.

CM= conditioned media

***=p<0.001 compared to CM(-) ETX

**=p<0.01 compared to Macrophage (-) ETX

##=p<0.01 compared to normal serum (-) ETX
purposes. While the bioactive peptide band was not present in non-bioactive CM from unstimulated monocyte/macrophage cultures, the ETX-challenged monocyte/macrophage CM did contain the bioactive peptide (figure 30).

**Increased leucine aminopeptidase activity correlates with increased CM bioactivity.**

ETX-challenge of both the total MNC CM and the adherent cell CM increased the LAP activity of these cultures approximately 2 fold (table 1). Increased LAP activity in ETX-treated cultures was associated with significantly increased bioactivity compared to non-treated cultures. Nonadherent cell CM, which demonstrated significant bioactivity prior to stimulation, had significant LAP activity both in the presence or absence of ETX. The LAP activity of mouse serum also increased after ETX-challenge and was approximately 1.5 fold greater than control.

**LAP treatment of non-bioactive CM peptides >3,000 MW yields bioactivity.**

G-25 Sephadex enriched fractions of CM peptides were separated into two groups. Group "A" contained all CM peptides greater than 3,000 MW while group "B" contained only the bioactive peptide. While the bioactivity of group
"B" (bioactive peptide) was unaltered by LAP treatment, LAP digestion of group "A" yielded significant bioactivity in a previously non-bioactive media (figure 31).
Figure 31. LAP treatment yields bioactivity from non-bioactive CM proteins (>3,000 MW). G-25 purified fractions of CM were divided based on peptide content. Fractions containing bioactive peptide were pooled and labelled "B" while others with peptides/proteins >3,000 MW were combined and labelled "A". Values given are the mean ± SEM from two triplicates from one chromaffin cell preparation (n=6). **=p<0.01 compared to group "B" ± LAP using an ANOVA with Tukey's follow-up comparison. Similar results were obtained in two other chromaffin cell preparations.
D. Discussion

The inflammatory/immune response normally protects the body and limits the extent of injury. In the setting of critical illness and/or severe injury, however, regulation of this response is often lost for reasons that remain to be elucidated. An uncontrolled inflammatory/immune response combined with infection, inflammation, and/or ischemia often leads to the development of the Systemic Inflammatory Response Syndrome (SIRS), which is frequently associated with the development of septic shock, multiple organ dysfunction (MODS), and other complications of critical illness. Although multiple mediators are involved in the inflammatory/immune response, one of the most influential mediators of SIRS and subsequent septic shock appears to be endotoxin (Beutler, 1993). Endotoxin, through activation of phagocytic cells (e.g. the macrophage), stimulates the production and release of potent inflammatory/immune mediators which include tumor necrosis factor (TNF) and interleukin-1 (IL-1). Elevation of these factors leads to complications such as endothelial damage and metabolic abnormalities which are associated with SIRS. Interestingly, these same cytokines have been shown to
modify neuro/endocrine function via the hypothalamic-pituitary-adrenal axis (Imura et al., 1991; Haefli et al., 1993). Additionally, IL-1 has been shown to enhance sympathetic nerve activity (Ichijo et al., 1994), modify neuronal gene expression of substance P (Jonakait, 1993), and is synthesized and released by cultured sympathetic neurons (Frieden et al., 1992). Since the full manifestation SIRS and the sequelae that follow appears to be dependent on both inflammatory as well as hormonal mediators (Secor, 1994), the ability of immune-derived signalling molecules (TNF, IL-1 or even CM peptide) to modulate hormone release may have important clinical implications in SIRS or septic shock.

Among the hormonal mediators which contribute to SIRS/septic shock are the catecholamines, especially epinephrine - a hormone released from the adrenal medulla. The catecholamines have profound effects on cardiovascular function, cellular metabolic activity as well as immune cell function (Kjaer and Secher, 1992; Webber and Macdonald, 1993; Plaut, 1987). Recent evidence suggests that immune cells, however, may not only respond to changes in plasma catecholamine but also may modulate the release of
catecholamine from the adrenal medulla via a small peptide (Jones et al., 1993). This peptide-induced medullary catecholamine release may be an additional pathway by which plasma catecholamine is elevated during SIRS and subsequent septic shock.

The current study demonstrates that ETX, an influential mediator of SIRS/sepsis, enhances production of this peptide from mononuclear cell populations. Media (conditioned media, CM) taken from ETX-challenged MNC stimulated significantly more chromaffin cell epinephrine secretion than did CM from unstimulated or PHA-stimulated MNC cultures (figure 27). Although T and/or B cell populations are the primary source of peptide in cell cultures not stimulated with ETX (figure 28), ETX appears to stimulate bioactivity of CM by inducing peptide release from monocyte/macrophage cell populations (figures 29 & 30). Previously we have demonstrated that treatment of CM with the exopeptidase leucine aminopeptidase (LAP) significantly increased CM bioactivity (Jones et al, 1995). Interestingly, ETX has been shown to increase protease secretion from macrophage populations (Nathan, 1987). Equally noteworthy is the fact that monocyte/macrophages synthesize and release LAP
The premise of these studies was that ETX-challenge stimulated monocyte/macrophage LAP release and this LAP was cleaving some larger protein/peptide to liberate bioactive peptide in these cultures. Data presented in table 1 showing an increased LAP activity in ETX-treated monocyte/macrophage and total mononuclear cell cultures support this hypothesis. Potentially, this LAP may cleave the bioactive peptide from a larger parent compound released from the mononuclear cells. This idea is also supported by the data in figure 31 which demonstrates that previously non-bioactive CM peptides (>3,000 MW) exhibit significant bioactivity after 5 hour LAP treatment.

Much like TNF and IL-1, the bioactive peptide may be an additional signalling molecule released from ETX-stimulated monocyte/macrophage populations which is able to modulate hormone release. Production of this peptide via proteolytic cleavage of a larger parent compound is not surprising. Examples of enzymatic conversion of a promolecule to its bioactive compound include plasminogen activator, a serine-type proteinase, which converts inactive plasminogen to plasmin by cleaving a single peptide bond (Dano et al.,
Proteolytic cleavage of several promolecules are also involved in the activation of the complement cascade (Medicus et al, 1976; Müller-Eberhard, 1981), an integral part of host defense against infections. Endotoxin stimulation of monocyte/macrophage populations may stimulate the synthesis and release of both peptidases (e.g. LAP) and the parent molecule. These peptidases may then cleave the parent molecule at the amino terminus thereby liberating bioactive peptide which would stimulate adrenal medullary catecholamine release. Since LAP is an exopeptidase (i.e. LAP hydrolyzes peptide bonds ar or near the amino-terminus of the peptide), it is highly unlikely that LAP is liberating the bioactive peptide from the parent molecule. However, highly purified preparations of LAP have been shown to have traces of contaminating endopeptidases (Royer and Andrews, 1973; DeLange and Smith, ). Potentially, endopeptidase contaminants of the LAP utilized in these experiments may be responsible for cleavage of the parent compound and liberation of bioactive peptide.

The implications and potential applications of this knowledge are many fold. Since the imposition of a stressor (e.g. injury/infectious challenge) disrupts homeostasis, it
is important to understand how the immune system may compensate and function during such times of stress. In addition, the ability of the immune system to directly modify the stress response through epinephrine secretion may prove invaluable in understanding homeostatic mechanisms. As suggested by a growing body of literature, systems previously believed to be essentially autonomous (i.e. immune system) are now considered to share informational molecules such as cytokines, hormones, and neuropeptides with other (neural/endocrine/neuroendocrine) systems. Further evidence delineating other structural or functional connections between these systems, such as the presently described CM peptide, only strengthens the concept of systems interdependence and may potentially aid in understanding systemic dysfunction. Once the homeostatic role of signalling molecules are elucidated, recognizing the functional role of these molecules in a pathophysiology (e.g. SIRS/sepsis) may provide a basis for therapeutic intervention.
CHAPTER VIII

SUMMARY AND CONCLUSIONS

The present dissertation study further identified and characterized a small molecular weight (<3,000) peptide which stimulates adrenal medullary catecholamine secretion to levels comparable to maximal cholinergic stimulation. The first project in this study addressed the role of calcium in the signal transduction pathway of peptide-stimulated chromaffin cell secretion. Information obtained in these studies demonstrated that the calcium-dependent signalling mechanisms of bioactive peptide are similar to other known peptide secretagogues. The second project focused on the mononuclear cell source of this peptide. The data provided by these experiments may provide insight to the function of the peptide in local immunoregulation or in intersystems communication. The third project characterized the peptide-nature and other properties of the molecule as

184
well as isolated the bioactive peptide. This project was essential in obtaining a putative molecular weight, as well as potentially sequencing the isolated molecule. Finally, the fourth project examined a potential role for bioactive peptide in endotoxicosis and subsequent shock states. These final studies suggested a role for the peptide in vivo.

As can be integrated from the present study as well as previous works, a schematic representation of the significance of immunomodulation of adrenal medullary catecholamine release is presented in Figure 32. In response to non-cognitive stimuli, a large precursor molecule is released from mononuclear cell populations; specifically CD4+ T cells, B cells, and endotoxin-challenged monocyte/macrophage populations. Endogenous proteases (e.g. leucine aminopeptidase/LAP) cleave the bioactive peptide from this "parent" compound. In circulation, this peptide may reach the adrenal medulla and stimulate medullary chromaffin cell epinephrine secretion. The subsequent increase in plasma catecholamine may then feedback on the mononuclear cell populations to inhibit further peptide production. An extension of this working model may be found in figure 33. Since many regulatory molecules previously
thought to be limited to the immune system (e.g., IL-1, IL-6, TNF) are now known to be expressed by both (Krieger, 1983; Plaut, 1987; Carr and Blalock, 1991) it is possible that the parent molecule of conditioned media peptide may also be produced and processed in the CNS or in peripheral neuroendocrine structures. This provides a second potential source for conditioned media peptide modulation of catecholamine release during a typical stress response. A second possibility, as demonstrated in figure 33, is that CM-peptide may serve an autocrine/paracrine function and modulate cells of the immune system. As is depicted in the figure, studies have shown that peptide signalling molecules (e.g. ACTH, the endorphins, CG) common to both immune and neuroendocrine structures have immunoregulatory properties and are speculated to regulate immune function in vivo (Blalock, 1992, Blalock, 1994, Weigent et al., 1990). The immune cell-derived peptide characterized in this dissertation study may also serve in some immunoregulatory capacity.

A final schematic representation constructed from information provided in this dissertation study, in combination with current information in the literature, is
presented in Figure 34. This figure illustrates the potential calcium-regulated signalling mechanism of peptide-induced secretion. Bioactive peptide, similar to other known peptide-secretagogues (i.e. bradykinin and angiotensin II), binds to its cell surface receptor on the chromaffin cell. This receptor is coupled to a second messenger system, possibly via phophoinositide metabolism, which stimulates release of calcium from intracellular stores as well as extracellular calcium influx. Temporally, this rise in intracellular calcium in response to peptide would reach a peak quite quickly due to calcium influx as well as release from internal stores. A secondary, long term phase increase in internal calcium concentrations resulting from the influx of extracellular calcium would follow.

Evidence provided in this dissertation study in support of figures 32, 33, and 34 are enumerated as follows. The first project evaluated the signal transduction mechanism of this peptide. Specifically, the study utilized pharmacologic agents as well as a fluorescent dye indicator to address the role of calcium in peptide-induced secretion. Summary of results from the first project:
1. Extracellular calcium is essential for peptide-induced secretion.

2. Release of calcium from intracellular stores are not required for peptide induced-secretion.

3. Voltage-gated calcium channel blockers do not completely inhibit secretion in response to peptide stimulation.

4. The rise in intracellular calcium in response to peptide is biphasic.

5. Peptide-induced increase in intracellular calcium is due to release from internal stores as well as influx from the external environment.

Conclusions drawn from the first project:

1. The calcium dependence of peptide-induced secretion is similar to other known peptide secretagogues.

2. The biphasic, long term elevation in intracellular calcium may be one of the reasons for the substantial chromaffin cell epinephrine secretion, comparable to maximal cholinergic stimulation, in response to peptide.

The second project of this dissertation study identified potential mononuclear cell source(s) of bioactive
peptide. Specifically, mononuclear cell populations were separated utilizing cell-specific characteristics and immunologic methods. The presence of peptide in the conditioned media from each population was assessed using bioassay and polyacrylamide gel electrophoresis.

Summary of results from project two:
1. The bioactive peptide is a product of viable mononuclear cells.
2. T helper (CD4+) cell population conditioned media is significantly bioactive.
3. B cell populations produce bioactive peptide.

Conclusions from the second project:
1. Since the peptide is a product of viable cells in vitro it may be an immune cell product in vivo.
2. T helper cells release the majority of peptide, although B cells are a potential source.

The third project further isolated and characterized a single mononuclear cell peptide which possessed the majority of conditioned media bioactivity. Specifically, conditioned media was tested using several enzymatic and proteolytic
tests to determine the nature of the bioactive factor(s). Bioactive component(s) were further separated and isolated using SDS-PAGE.

Summary of results from the third project:
1. Lactate dehydrogenase is not present in conditioned media after exposure to bovine chromaffin cells while significant amounts of catecholamine are in the media.
2. Calcium is an essential component of peptide-induced secretion.
3. Acid hydrolysis of conditioned media significantly decreased bioactivity.
4. Peptidase treatment altered bioactivity of conditioned media.
5. SDS-PAGE of enriched fractions of conditioned media revealed the most bioactive fractions contained a single peptide band.
6. The single peptide band, electroeluted from the gel, exhibited bioactivity whereas other conditioned media peptides did not.
8. Mononuclear cell co-culture with epinephrine significantly reduces bioactivity of conditioned media.

Conclusions from project three:
1. Conditioned media-induced secretion is a discrete exocytotic event.
2. Bioactive factor in conditioned media is a peptide.
3. The majority of conditioned media bioactivity is due to a single, small molecular weight peptide (627Da).
4. Conditioned media peptide may stimulate both adrenal medullary epinephrine secretion as well as norepinephrine release from sympathetic nerve terminals.
5. Feedback regulation is suggested by significant decreases in CM bioactivity subsequent to mononuclear cell challenge with physiologically relevant concentrations of epinephrine.

The final project of this study addressed the potential role of this peptide in the long-term elevation of plasma catecholamine observed in endotoxosis. Specifically, in vitro techniques were utilized to determine conditioned media bioactivity and identify the presence of peptide in mononuclear cell cultures before and after challenge with
endotoxin. Significant bioactivity and presence of peptide were then correlated with the activity of a metalloprotease, leucine aminopeptidase.

Summary of results from the fourth project:
1. Conditioned media from monocyte/macrophage populations stimulates chromaffin cell epinephrine secretion only after challenge with endotoxin.
2. SDS-PAGE analysis of monocyte/macrophage conditioned media revealed that peptide band is present only in endotoxin-treated cultures.
3. Leucine aminopeptidase activity increased in endotoxin treated conditioned media from total mononuclear cell cultures and in monocyte/macrophage cultures.
4. Leucine aminopeptidase treatment of non-bioactive conditioned media peptides (MW>3,000) led to significant bioactivity.

Conclusions drawn from project four:
1. Monocyte/macrophage populations may be an important source of peptide in infectious/shock states.
2. Endotoxic stimuli may liberate proteases which cleave bioactive peptide from a non-bioactive parent molecule.

3. Endotoxin-liberated peptide may comprise part of the non-neurogenic release of catecholamine and may play a role in the long-term elevations in plasma catecholamines observed in septic shock.

For centuries a link has been noted between stressful periods in life, as a result of cognitive and non-cognitive factors, and illness (Selye, 1936; Selye, 1975; Kort, 1994). While many mechanisms are possible, a growing body of literature indicates that stress-induced dyshomeostasis is largely a result of interactions between the immune and neuroendocrine systems (Sheridan et al., 1994). Hormonal mediators of the stress response (i.e. the catecholamines and the glucocorticoids) have been shown to have profound effects on immunologic function. Both the corticosteroids and the catecholamines inhibit many functions of leukocytes and alter their trafficking patterns. In addition, these stress hormones modulate production of various cytokines and inflammatory mediators as well as modulate the effects of
these substances at various target tissues (Chrousos and Gold, 1992).

Interestingly, recent evidence suggests that not only do the stress hormones effect immunologic function, but also many immune-derived signalling molecules may modulate stress hormone release. For instance, cytokines such as interleukins-1 and -6 (IL-1, IL-6) and tumor necrosis factor (TNF) are all potent modulators of the hypothalamic pituitary adrenal axis (Imura et al., 1991; Haefli et al., 1993). Additionally, IL-1 has been shown to enhance sympathetic nerve activity (Ichijo et al., 1994). Finally, numerous endogenous, potentially immune-derived substances such as histamine (Bunn and Boyd, 1992), bradykinin (Owen et al., 1989), and angiotensin II (Powis and O'Brien, 1991) may stimulate catecholamine release from the adrenal gland.

The present dissertation study isolated and characterized a single, novel mononuclear cell-derived peptide which stimulates adrenal medullary catecholamine secretion to levels comparable to maximal cholinergic stimulation. This peptide, derived from T helper, B cell and endotoxin activated monocyte/macrophage cell populations, stimulates chromaffin cell epinephrine
secretion in a calcium dependent manner similar to other known peptide secretagogues. Furthermore, in vitro evidence suggests a role for this peptide in the elevation of plasma catecholamines seen in endotoxicosis. Finally, polyvalent antisera was obtained in this study which significantly inhibited conditioned media bioactivity (See Appendix) indicating the presence of an antibody specific for CM-peptide. This antisera was also used to develop methods for an enzyme-linked immunoabsorbent assay (ELISA) which may be utilized in future experimentation to screen for bioactive peptide (See Appendix).

Based on data obtained from the current work, further studies could be developed to determine the physiologic significance this peptide. The most important of these would be to sequence the isolated peptide as well as its parent molecule. Such information would provide a basis for production of peptide in large quantities to use in controlled in vitro and in vivo experimentation. For example, in vitro experiments could utilize pure peptide to assess the immunomodulatory properties, if any, of the molecule. Pure peptide may also be used to study, in greater detail, the calcium-dependent signal transduction
mechanism of the peptide. This sequence data could also be utilized to develop a Radioimmunoassay (RIA). An RIA is often utilized to quantitate small molecular weight peptides (e.g., bioactive peptide) since these small molecules lack the two non-overlapping epitopes required to perform commercially utilized sandwich ELISAs. This RIA, in conjunction with the already developed competitive ELISA, may be utilized in prospective whole animal studies to screen for and quantitate peptide in either the sera or various other compartments of animals during immunologic challenge. RIA/ELISA data which indicated that the peptide concentration increased in an animal population in response to a specific stimuli (e.g. endotoxin challenge) would then provide a basis for future in vivo experimentation. In vivo experimentation may include the use of anti-peptide antibody. Diminished plasma catecholamine in immunologically-challenged (e.g. endotoxin) animals treated with anti-peptide antibody versus endotoxic, untreated animals would indicate a cause-effect relationship between peptide and catecholamine release. Such specific action could be confirmed by infusing the peptide into animals and comparing plasma catecholamine levels to that in control
animals. These studies would add important new information as to how this peptide may play a role in homeostatic mechanisms. Knowledge of signalling molecules involved in intersystems communication and their role in homeostasis make therapeutic manipulation and improved patient treatment possible.
Figure 32. Potential in vivo mechanism of bioactive peptide production.
Figure 33. Extension of the current working model.
Chromaffin Cell

Figure 34. Potential calcium-dependent signal transduction mechanism of the bioactive peptide.
APPENDIX

EVIDENCE OF A POLYCLONAL ANTIBODY

SPECIFIC FOR BIOACTIVE PEPTIDE
APPENDIX

EVIDENCE OF A POLYCLONAL ANTIBODY

SPECIFIC FOR BIOACTIVE PEPTIDE

Antibodies (Ab), a family of structurally related glycoproteins produced/secreted by B cells, mediate the protective effects of humoral immunity by binding foreign substances referred to as antigens (Ag). Each B lymphocyte produces antibody of single specificity responsible for recognition and targeting of a single antigen. Several powerful experimental techniques have been developed based on the specificity of antibody-antigen interaction. One of these techniques is the enzyme linked immunoabsorbent assay (ELISA).

The ELISA is an immunologic technique (figure 35) by which one can quantify antigen (e.g. bioactive peptide) concentrations by using an indicator molecule coupled covalently to an enzyme. Antigen is quantified by determining the initial rate at which an enzyme converts a clear substrate in the solution to a colored product. Experiments in this study utilized anti-peptide antibody
**SANDWICH ELISA**

*secondary antibody specific for non-overlapping epitopes of antigen.

**increasing [antigen] = more secondary antibody bound = increased enzymatic color change.

= Primary antibody

= Secondary antibody

**enzyme (eg horseradish peroxidase) converts substrate placed in well (eg DAB) into colored product. Based on absorbance units, the intensity of the color change is proportional to [antigen] bound by antibody.

---

**COMPETITIVE ELISA**

*increasing [free antigen] competes with fixed amount of immobilized antigen for antibody; free antigen-antibody is washed away.

**increasing [free antigen] = less antibody-immobilized antigen binding = less color change.

= Free Antigen

= Immobilized Antigen

Figure 35. Diagram of Enzyme Linked Immunoabsorbent Assays (ELISA).
obtained from rabbit antisera to develop an ELISA specific for bioactive peptide.

Detailed methods for antibody production and development of ELISA may be found in Chapter III. Briefly, concentrated peptide, previously characterized using SDS-PAGE, was attached to a 96 well assay plate and incubated overnight at 4°C. Although a range of pH conditions were tested, physiologic pH (7.4) was sufficient to provide adequate peptide attachment to the plate. Concurrently, CM peptides >3,000 MW, Keyhole Limpet Hemocyanin (KLH) immunogen, KLH-linked peptide, bradykinin, angiotensin II, and rabbit antisera (primary/1° antibody were attached to plate wells using similar protocol. Large molecular weight CM proteins/peptides were attached to plates to screen for the presence of parent compound"s" reactive to antibody. Keyhole Limpet Hemocyanin (KLH) and KLH-linked peptide were used since this carrier-protein complex was used to challenge rabbits to produce antisera. Bradykinin and angiotensin II, small peptides unrelated to CM-peptide, were used as controls to determine background and antibody cross-reactivity. Finally, primary antibody (rabbit antisera) was
attached to plates to ensure that the secondary (2°) antibody (goat anti-rabbit IgG) bound primary.

After peptide/protein attachment, wells were washed (PBS/0.05% Tween-20) and active sites blocked with 0.05% gelatin (room temperature, 2h). Wells were washed again (PBS/Tween solution) then serial dilutions of rabbit antisera (50, 100, 250, 500, 1000, 5000 fold) were added to the wells and allowed to incubate (1h, 37°C; overnight, 4°C). To measure the amount of specifically bound antisera, each well was incubated with horseradish peroxidase (HRP) coupled secondary antibody (goat anti-rabbit antibody, Accurate Chem. Corp., Westbury, NY) for 2h at room temperature. Wells were washed at least three times with PBS/Tween then 0.5ml DAB Peroxidase substrate was added. The reaction between the peroxidase enzyme on the 2°-antibody and the substrate are as follows:

\[
\text{Peroxidase (HRP)} + \text{H}_2\text{O}_2 \quad \rightarrow \quad \text{O}_2 + 2\text{H}_2\text{O}
\]

\[
\text{O}_2 + \text{DAB} \quad \rightarrow \quad \text{insoluble, brown-black precipitate}
\]

Absorbance at 492nm was the indicator of the presence of reactive antibody.

Results obtained from 12 ELISA plates all indicated the
presence of anti-peptide antibody in the rabbit sera. An example of data taken from these studies appears in table 2. Similar studies were performed using the slot-blot technique. This technique involves blotting a membrane (i.e. nitrocellulose) with peptide/protein which adheres to the membrane. The membrane is then incubated with blocking solution, primary antibody, and secondary antibody in a protocol similar to that described for the ELISA. Data from the slot-blot experiments strongly support results obtained using the ELISA.

Interestingly, as can be seen in table 2, the antibody appears to bind a CM peptide/protein of MW>3,000 - a putative parent molecule. Limited studies utilizing Western blot analysis demonstrates that the antisera binds not only the peptide but also a larger CM peptide/protein. These same peptide bands are not detected by preimmune sera, further supporting antibody specificity.
Table 2. Enzyme Linked Immunoabsorbent Assay (ELISA) results indicate the presence of antibody to a bioactive peptide epitope. "A"=potential parent compound(s); "B"=peptide band of interest; ATII/Br= Angiotensin II/Bradykinin (indication of background absorbance). Results presented are absorbance units (492nm) from wells on a 96 well plate. Similar results were obtained in 11 other samples.
The specificity of this anti-peptide antibody was further confirmed using a competitive ELISA. Methods were similar to those described previously. In the competitive ELISA, however, a fixed quantity (0.10 mg peptide) was added to the diluted antisera prior to its addition to the wells. This peptide then "competed" against the peptide attached to the well for anti-peptide antibody. Table 3 demonstrates that specific binding diminished more than 80% in the presence of excess peptide.

Finally, direct proof of a relevant antibody was obtained via secretion experiments in the presence and absence of anti-peptide antibody. Antisera significantly inhibited secretion in response to conditioned media (Figure 36). This inhibition was not observed with a similar concentration of preimmune sera. Secretion in the presence of sera alone (in RPMI-1640) was not significantly different than basal secretion. These results indicate the presence of a relatively specific polyvalent antibody to CM-peptide in immunized rabbit sera.
Table 3. Bioactive peptide significantly reduces antibody binding to immobilized peptide/parent protein. Peptide/parent compound attached to 96 well plates was incubated in with rabbit antiserum (1:100 dilution) in the presence (+) or absence (-) of bioactive peptide (0.1mg). Antisera binding of immobilized peptide/protein was assessed according to ELISA protocol. Numbers mean absorbance (492nm) +/- SEM from two triplicate determinations (n=6).

<table>
<thead>
<tr>
<th></th>
<th>(-) Peptide</th>
<th>(+) Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;A&quot; Fractions</td>
<td>0.231+/-0.006</td>
<td>0.133+/-0.006</td>
</tr>
<tr>
<td>Bioactive Peptide</td>
<td>0.212+/-0.004</td>
<td>0.132+/-0.002</td>
</tr>
<tr>
<td>KLH-Peptide</td>
<td>0.243+/-0.004</td>
<td>0.148+/-0.003</td>
</tr>
</tbody>
</table>
Figure 36. Anti-peptide antibody significantly inhibits conditioned media (CM) bioactivity. Secretion experiments used filtered (Centricon, 3,000 MW cutoff) conditioned media in the presence or absence of rabbit antisera (1:100 dilution) or preimmune sera (1:100 dilution) over a 90 minute time course. RPMI-1640 indicative of basal secretion. Values presented are mean ± SEM from two triplicates from one chromaffin cell preparation (n=6). ***=p<0.001 compared to CM. Comparisons were made using an ANOVA with Tukey's follow-up test.
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VITA

Jennifer Curran Roberts, daughter of James and Mary Curran, was born on June 27, 1970 in Sandwich, Illinois. She attended elementary and secondary schools in the local public and parochial schools and graduated from Sandwich High School in June, 1988.

In August, 1988, she began her college education at College of St. Francis, Joliet, Illinois where she received a full scholarship for four years. She received a Bachelor of Science degree with high honors in Biology in May, 1992. In August of that year, Jennifer entered the graduate program in the Department of Physiology at Loyola University Stritch School of Medicine. Her dissertation work was completed under the direction of Dr. Stephen B. Jones. Jennifer was the recipient of an NIH Pre-doctoral Fellowship, a University Dissertation Fellowship and became a member of the Shock Society in 1995.

Jennifer married Joseph Roberts on June 21, 1992 and has one daughter, Paige Augusta.
A. Journals


B. Abstracts:


APPROVAL SHEET

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

\[\text{Date: } \text{Oct. 11, 1996}\]
\[\text{Director's Signature}\]