THE IMMUNOMODULATION OF THE ENTERIC NERVOUS SYSTEM:

The effect of cytokines on neurotransmitter release and content

BY

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For my father and mother:

Graham and Valerie Didcote.

ABSTRACT

Inflammatory conditions of the gut, such as ulcerative colitis and Crohn's disease are associated with an alteration in intestinal motility. The mechanisms underlying altered motor function is unknown, but may be the result of alteration in smooth muscle and/or enteric nerve function. Recent studies using *Trichinella spiralis* (*T.spiralis*) infection of rats, as a model of intestinal inflammation, have shown that the accompanying inflammatory response in these animals is associated with an altered enteric nerve function. These changes included a suppression of noradrenaline and acetylcholine release and an increased substance P content within the neuromuscular layer of the inflamed jejunum. Furthermore, there was an expression of inflammatory cytokines within the mucosa and the deeper muscular layers of the jejunum within 12 hrs from the onset of *T.spiralis* infection. This elevation in cytokine expression occurred prior to the changes in myenteric neurotransmitters. It is therefore possible that inflammatory cytokines within the neuromuscular layer cause a change in neurotransmitter release and content, and thus contribute to the altered gut motility observed in the nematode-infected rats.

Addition of exogenous IL-1β or TNFα to isolated longitudinal muscle-myenteric plexus (LM-MP) preparations caused a suppression of stimulated noradrenaline release, which was time and concentration dependent. This suppressive action was biphasic in manner, displaying an early protein synthesis independent

effect and a delayed protein synthesis dependent effect. Furthermore, the delayed suppressive action of these cytokines on noradrenaline release was mediated by endogenous IL-1 and both the early and delayed suppressive actions were dependent on prostanoid synthesis. On examination of a putative direct interaction between these cytokines and adrenergic nerves using a nerve varicosity preparation, only IL-1β was found to suppress the evoked noradrenaline release. This suppressive action by IL-1β was time- and concentration-dependent, and in part, mediated by prostanoids. Although incubation of TNFα alone with the varicosities was unable to induce a response, the presence of TNFα did potentiate IL-1β-induced suppression of noradrenaline release. The conclusions drawn from this study are that TNFα causes a suppression of noradrenaline release from myenteric nerves which is evident only in a multicellular preparation and likely involves intermediary cells and their products including prostaglandins and/or thromboxanes. This contrasts with IL-1β, which in addition to an indirect effect, also suppressed noradrenaline release by directly interacting with adrenergic nerve terminals.

IL-1β also caused an increase in substance P content within the myenteric plexus. The increase in this neuropeptide was time and concentration dependent, and could be depleted by scorpion venom. Immunohistochemical studies indicated that substance P was only found present within myenteric nerves. The IL-1β-induced increase in substance P was considered to be due to increased synthesis, since cycloheximide prevented the cytokine-stimulated increase substance P content induced by IL-1β. Moreover, the increase in substance P content was mediated by

prostanoid synthesis, but not nerve growth factor. The conclusion drawn from these experiments is that IL-1β stimulates the synthesis of substance P within intrinsic nerves of the myenteric plexus.

A putative role of endogenous IL-1 in the alteration of neurotransmitters in the *T.spiralis* model of intestinal inflammation was examined using a selective IL-1 receptor antagonist (IL-1ra). Treatment of the animals with IL-1ra prior to *T.spiralis* infection attenuated the suppressed noradrenaline release and increased substance P content observed in the inflamed LM-MP preparations. Therefore the results from these experiments and those performed using exogenous IL-1β and TNFα support the notion that inflammatory cytokines alters myenteric neurotransmitters, resulting in a disruption of enteric nerve function and thereby contributing to alterations in intestinal motility.

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2. Hurst,S.M., A.M.Stanisz, K.A.Sharkey and S.M.Collins. Interleukin-1β induced increase in substance P in rat myenteric plexus. Gastroenterology 1993;105:1754-1760.
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4. Hurst,S.M. and Collins,S.M. Interleukin-1β suppression of norepinephrine release from rat myenteric varicosities. Am.J.Physiol. (In revision, 1993).

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1. Collins,S.M. McHugh,K., Hurst,S. and Weingartin,H. Neuro-immune interactions in the pathophysiology of inflammatory bowel disease. Chapter 24, In: Innervation of the gut, edited by Tache,Y., Wingate,P.L. and Burke,T.F. (In Press, 1993).
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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

NA	Noradrenaline
IL-1β	Interleukin-1β
TNFα	Tumour necrosis factor-α
IL-1ra	Interleukin-1 receptor antagonist
^{3}H	Tritium
T.spiralis	*Trichinella spiralis*
EFS	Electrical field stimulation
KCl	Potassium chloride
IR-SP	Immunoreactive substance P
UV	Ultra violet
TLC	Thin layer chromatography
SE	Standard error
PGE_2	Prostaglandin E_2
DNP	Dinitrophenol
NADH	Nicotinamide adenine dinucleotide (reduced form)
LDH	Lactate dehydrogenase
EDTA	Ethylenediaminetetraacetic acid (disodium salt)

EGTA	Ethylene glycol-bis (β-amino-ethyl ether) N,N,N',N'-tetra acetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	Inhibition concentration$_{50}$
^{125}I	^{125}Iodine
^{35}S	^{35}Sulphur
HPLC	High pressure liquid chromatography
RIA	Radioimmunoassay
BSA	Bovine serum albumin
PBS	Phosphate buffered saline
NGF	Nerve growth factor
IgG	Immunoglobin G
Kd	Dissociation constant
P	Probability

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction.

Conditions such as ulcerative colitis and Crohn's disease, are accompanied by changes in physiological function, such as altered gut motility, secretion and absorption (for review see Podolsky, 1991). Changes in these physiological events could be a direct result of the inflammatory process, since treatment with steroids alleviates some of the physiological alterations associated with these diseases (Rachmilewitz and Zimmerman, 1990; McDermott, 1988). The altered physiology may reflect not only changes in target tissues, such as epithelium or smooth muscle, but may also be the result of inflammation-induced changes in enteric nerves, which regulate intestinal physiology.

Recent studies in intestinal inflammation induced by nematode infection in rats have shown changes in acetylcholine and noradrenaline release, as well as in the substance P content in the longitudinal muscle-myenteric plexus (LM-MP) of the jejunum (Collins et al.,1989; Swain et al.,1991; Swain et al.,1992). Furthermore, the alteration in noradrenaline release observed in the chemical colitis induced by 2,3,4 trinitrobenzene sulphonic acid (TNB) administration was observed not only in the

inflamed region of the colon but also in un-inflamed regions of the gut (Jacobsen et al.,1993$_a$). In the nematode, *Trichinella spiralis* (*T.spiralis*) infection model of intestinal inflammation, cytokines IL-1α, IL-1β, TNFα and IL-6 levels were increased not only in the mucosal layer, but also within the neuromuscular layer of jejunum from infected animals (Khan and Collins, 1992). These observations, taken in conjunction with the alterations in nerve function observed in this model, raise the possibility of a causative relationship of these cytokines with nerve dysfunction (Collins et al.,1989; Swain et al.,1991; Swain et al.,1992). Therefore the purpose of this study was to explore the interactions between cytokines and enteric nerve function in the rat small intestine.

1.2. Specific aims of the study.

The specific aims of the study were:

1. To investigate the effects of interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) on noradrenaline release from myenteric nerves of the small intestine.
2. To investigate the effect of IL-1β on substance P content in the myenteric plexus of the small intestine.
3. To examine the role of endogenous IL-1 in the alteration of noradrenaline release and substance P content observed in the *T.spiralis* infection model of intestinal inflammation.

1.3 Enteric nervous system.

1.3.1. General introduction.

The enteric nervous system (ENS) is considered to be the third branch of the autonomic system (for review see Furness and Costa, 1987; Gabella,1987). It is composed of extrinsic and intrinsic (enteric) nerves, which, together with their support cells, are classified into several anatomically distinct networks or plexuses (figure 1.1). There are two major nerve plexuses, myenteric and between the circular muscle and lamina propria, submucous, starting in the oesophagus and extending distally. The myenteric plexus, but not the submucous plexus, extends throughout the digestive tract. The intramural nerves of both plexuses can be visualized as a mesh-like laminar structure, with axonal processes innervating not only target cells (eg. smooth muscle or secretory epithelium), but also interneurons connecting sensory receptors to motor and secretory nerves. Also there are interconnecting nerve fibres which run within and between the myenteric and submucous plexuses, allowing integrated multi-synaptic activities to occur entirely within the ENS. The network appearance of the ENS has prompted researchers in the past to refer to it as the "CNS of the gastrointestinal tract" (Gabella,1987).

Extrinsic input to the ENS is supplied by both branches of the autonomic nervous system. Parasympathetic innervation of the ENS is primarily by the vagus and pelvic nerves. Pre-ganglionic axons arise from cell bodies within the medulla and the sacral regions of the spinal cord and enter various organs of the gut where they synapse mainly with nerve cells in the alimentary canal. Sympathetic innervation of

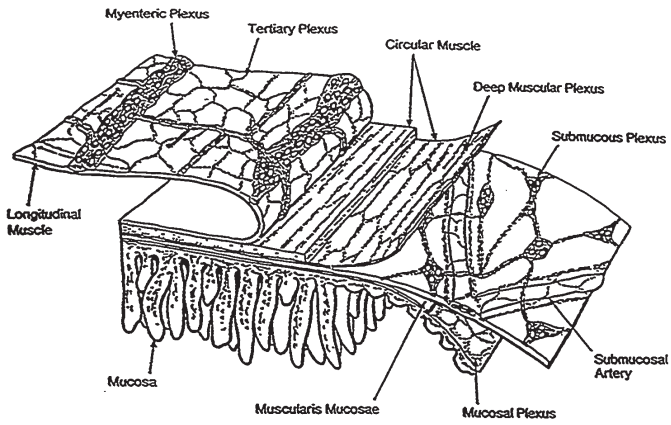


Figure 1.1: Diagrammatic representation of enteric nerve plexuses in the small bowel. (reproduced from Furness and Costa, 1980).

the ENS consists of afferent and efferent nerve combinations that run between the spinal cord and the prevertebral ganglia, eventually connecting with ganglia of the intestine. Pre-ganglionic nerve fibres arise within the spinal cord and synapse with ganglia that innervate elements of the ENS. A few nerve axons from these ganglia end directly on secretory and absorptive cells, as well as muscle cells in non-sphincter regions. Afferent fibres present within the sympathetic neuron system project back to the prevertebral ganglia and/or the spinal cord.

1.3.2. Myenteric plexus.

The myenteric plexus consists of a network of small fibres and ganglia which form a distinct layer between the longitudinal and circular muscle of the intestine. Myenteric ganglia vary in size, shape and orientation from species to species, as well as within the various regions of the gut. Furthermore, the neural network, consisting of ganglia and nerve axons of various sizes, may be distorted by the movement of the elaborate *muscularis externa*, which has to be taken into account when assessing the frequency and thickness of the various components of the myenteric plexus (Gabella and Trigg, 1984). In some species, for example, rat myenteric ganglia are not well defined but appear circumferentially arranged within the plexus (Gabella,G.,1987). Also varicosities within the myenteric plexus are not true anatomical endings of the nerves, but appear at regular intervals along the nerve fibre (Dahlstrom and Haggendal, 1966). A unique feature of the myenteric plexus is that large surface areas of the perikaryon and the large dendrites are directly in contact with the basal

lamina. The neuronal surface is thus directly exposed to the extracelluar space and connective tissue. This feature of the myenteric plexus may allow it to regulate and be regulated by the surrounding microenvironment. Small blood vessels have been identified in close proximity to the ganglia of the myenteric plexus (without penetrating them), however in some species the vascular architecture appears to be in direct contact with the ganglia (eg. guinea-pig) (Gabella, 1987). It has been suggested that a blood-myenteric barrier exists which prevents diffusion of small protein molecules, analogous to the blood-brain barrier. The major evidence supporting this notion is the absence of fenestration in the capillaries of the muscularis externa (Gershon and Bursztajn, 1978).

1.3.3.Adrenergic neural input.

Apart from a few exceptions (eg. intrinsic, noradrenaline containing myenteric ganglia shown in the guinea pig rectum), the main sympathetic input to the gut comes from extrinsic nerve fibres, releasing noradrenaline, which have been detected within the ENS using fluorescence microscopy (figure 1.2). The basis of the fluorescence is that the phenol ring present within the structure of the noradrenaline causes the molecule to fluoresce under ultra violet light (Gabella, 1979). The distribution of varicosities along adrenergic axons is fairly regular in those nerves running intra-muscularly or within the connecting mesh of either the myenteric or submucous plexus. The noradrenaline content of the intestine has also been assessed using radioimmunoassay. In different layers of the small intestine, noradrenaline is

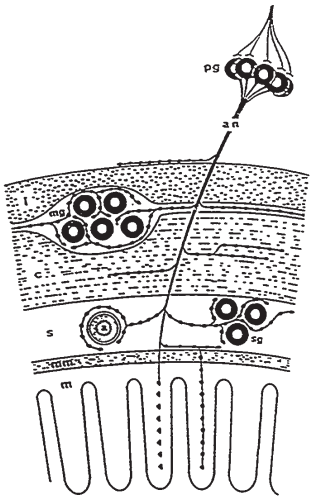


Figure 1.2: The distribution of the terminals of adrenergic nerves within the wall of the intestine. Most of the terminals (drawn as varicose fibres) ramify amongst neurons of the myenteric (mg) and submucous (sg) ganglia or form plexuses around arteries (a). A few fibres innervate the circular muscle and the *muscularis mucosae* and some axons extend into the villi. A few adrenergic axons contribute to the serous plexus when present, adrenergic nerve trunk (an), circular muscle (c), longitudinal muscle (l), mucosa (m), *muscularis mucosae* (mm), prevertebral ganglion (pg) and submucosa (s). (Taken from Costa and Furness, 1974).

concentrated in the myenteric plexus and submucosal areas, where nerves are the most prevalent. However, noradrenaline has also been found within the muscle and mucous layers of the intestine as well (Furness and Costa, 1974)

Release of noradrenaline-like substances was demonstrated by Finkleman, (1930), who found that stimulation of the mesenteric nerve supplying segments of rabbit duodenum liberated a substance which relaxed smooth muscle. This was the first direct demonstration of the release of an intestinal transmitter. The relaxation caused by nerve stimulation was mimicked by exogenous adrenaline and was antagonized by ephedrine or desensitization of the muscle to adrenaline (Astrom, 1949). Noradrenergic nerves influence motility, mucosal transport and intestinal blood flood. The noradrenergic nerves which run into the myenteric ganglia of the intestine are usually inactive in the resting individual; their discharge is evoked through reflex pathways originating both within and outside the digestive tract. Furthermore, noradrenergic reflexes arising from within the intestine act as a buffer to regulate gastric emptying and inhibit intestinal movement allowing for digestion and absorption of nutrients (Furness and Costa, 1987).

1.3.4. Neuropeptide - Substance P.

A number of biologically active peptides have been located in the wall of the small intestine (table 1.1). Peptide-containing neurons are present in sympathetic ganglia, in dorsal root ganglia and in various regions of the central nervous system. With the availability of a selective antibody, peptidergic nerves can be detected in the

SUBSTANCE	LOCATION AND ROLE
Adenosine triphosphate (ATP)	Probably contributes to transmission from enteric inhibitory muscle motoneurons.
γ-aminobutyric acid (GABA)	Present in different populations of neurons, depending on species and region. Does not appear to be a primary neurotransmitter.
Calcitonin gene-related peptide (CGRP)	Present in some secromotoneurons and interneurons.
Dynorphin (DYN) and dynorphin-related peptides	Present in secromotoneurons, interneurons and motoneurons to muscle. Does not appear to be a primary neurotransmitter.
Enkephalin (ENK) and enkephalin-related peptides	Present in interneurons and muscle motoneurons in most regions these substances probably provide feed-back inhibition of transmitter release.
Galanin	Present in secromotoneurons, descending interneurons and inhibitory motoneurons in human intestine. Role unknown.
Gastrin-releasing peptide (GRP)	Excitatory transmitter to gastrin cells. Also found in nerve fibres to muscle and in interneurons, where its role are unknown.
Neuropeptide Y	Present in secretomotoneurons, where it appears to inhibit secretion of water and electrolytes. Also present in interneurons and inhibitory muscle motoneurons.
Serotonin	Appears to participate in excitatory neuro-neuronal transmission.
Somatostatin	Despite its wide spread distribution in enteric neurons no clearly defined roles have been established.
Tachykinins (substance P, neurokinin A, neuropeptide K and neuropeptide γ	Excitatory transmitters to muscle; and are co-transmitters with acetylcholine. May contribute to excitatory neuro-neuronal transmission.
Vasoactive intestinal peptide (VIP)	Excitatory transmitter from secretomotoneurons. Possibly a transmitter of enteric vasodilator neurons. Contributes to transmission from enteric inhibitory muscle motoneurons

Table 1.1. Location and function of putative neuropeptides in the gastrointestinal tract. (Adapted from Furness et al., 1992).

intestine by immunohistochemical techniques. There is also evidence to suggest that these neuropeptides are co-localized with either cholinergic or adrenergic nerves in the intestine. For example, neuropeptide Y is found in adrenergic nerves (Koch et al.,1987) and substance P is found together with acetylcholine in parasympathetic input to the gut (Holzer and Maggi, 1992).

Substance P is a member of the tachykinin family of peptides (these include substance P, neurokinin A and neuropeptide-γ) The primary preprotachykinin messenger RNA transcript is differentially processed into three mature preprotachykinin (PTT) messenger RNA isoforms (α, β and γ). α- PPT messenger RNA encodes substance P alone, the β-PPT messenger RNA encodes both substance P and neurokinin A while γ-PPT messenger RNA encodes substance P, neurokinin A and neuropeptide γ. (Jonassen and Leeman, 1991).

Von Euler and Gaddum, (1931) first demonstrated that substance P occurs in extracts of the intestine and that it was a powerful stimulant of intestinal smooth muscle. In the gut, substance P has been located not only in intrinsic and extrinsic nerves in the myenteric and submucous plexuses (Costa et al.,1981, Sharkey et al.,1984), but also in the substance P-containing enteroendocrine cells located in the epithelium of the intestine in certain species (Roth et al.,1992). In the intestine of all species, substance P axons supply the myenteric and submucosal ganglia, circular muscle and mucosa. The majority of nerve fibres appear to be of intrinsic origin: a dense network of substance P-containing nerves, as well as immune reactive substance P nerve cell bodies have been located within the myenteric and submucous

plexuses (Llewllyn-Smith et al.,1984). However, substance P containing nerves also originate from external sources such as the dorsal root ganglia. Substance P in these ganglia has been implicated in both the afferent transmission of nociceptive information and in the regulation of inflammation (Lembeck and Holzer, 1979). In this case, substance P is thought to be synthesized in the cell bodies of dorsal root ganglia and transported to peripheral and central terminations (Brimijoin et al.,1980). Furthermore, substance P has also been located within intrinsic and extrinsic cholinergic nerves suggesting that acetylcholine and substance P are co-released upon stimulation. Substance P and acetylcholine co-localization in the myenteric and submucous plexus ganglia has been shown in guinea-pig ileum (Holzer and Maggi, 1992 and Keast et al.,1985). High concentrations of substance P and neurokinin A have been found in the myenteric plexus, and submucous plexus of the intestine (Furness et al.,1992). Moreover, significant concentrations of neurokinin A, together with substance P, have also been found in nerves located in guinea-pig ileum, suggesting that these two tachykinins may be co-released upon stimulation.

Pharmacological and histochemical studies in several systems have led to the identification of three tachykinin receptors: NK_1, NK_2, and NK_3 (Regoli et al.,1988). Although substance P displays structural homology with other tachykinins, its C-terminal amino acids is distinctly different from the other peptides (Inverson, 1982) and binds with high affinity to the NK_1 receptor (Regoli, 1988). Recently, a functional cDNA clone for rat substance P receptor (NK_1) has been successfully isolated and characterized and its function assessed electrophysiologically (Hershey

et al.,1991). Furthermore, substance P receptors have been located on a variety of cells within the intestine, including smooth muscle, epithelium and nerves.

The endogenous biological activity of substance P is usually produced by the intact undecapeptide (11 animo acids) form of the neuropeptide, however, truncated C-terminus analogues of this form have also been shown to exert a biological effect (Blumberg and Teichberg, 1979). Substance P has many effects on a variety of intestinal functions. It alters motility by affecting smooth muscle cells and neurotransmission (Daniel et al.,1989) and the regulation of ion secretion. This may be the consequence of either a direct action of substance P on epithelial cells or an indirect effect on the release of acetylcholine and other neurotransmitters that in turn modulate ion secretion. (Perdue et al.,1987; Kuwahara and Cooke, 1990).

1.4. Inflammation

1.4.1 General introduction.

The role of the inflammatory process is to first contain and then eliminate a noxious stimulus, such as an infection or injury, and to initiate the healing and repair process. The clinical signs of inflammation are defined as heat, edema, pain and redness (for review see Robins et al.,1988).

As a result of tissue damage, a number of immediate local and systemic events occur simultaneously. At the local level, dilation of the blood vessels coupled with extravascular leakage of cells and plasma components give rise to the redness (vasodilation and increased blood flow) and swelling (plasma fluid) within tissues.

There is an influx of inflammatory/immune cells (neutrophils, monocytes / macrophages) into the tissue, resulting in accumulation and activation of these cells. The release of digestive proteinases and cytokines along with metabolites of arachidonic acid, results in direct damage and/or altered function of local tissue cells. Systemic events occur in parallel with local events. These include the mobilization of bone marrow precursor cells and leucocytosis, fever, the modulation of carbohydrate metabolism and the synthesis of acute phase proteins in the liver. Both the local and systemic events that occur during the acute inflammatory response serve to contain tissue damage or infection and promote the subsequent healing process, in order to restore both the structure and function of the particular tissue involved.

1.4.2. Role of cytokines.

The release of a variety of cytokines is a consequence of tissue damage or infection. Interleukin-1 (IL-1) and tumour necrosis factor-α (TNFα) are recognized as the principal inducers of the acute inflammatory response, resulting in the production of a series of secondary mediators that act either locally or systemically (Manlovani and Dejana, 1989).

Interleukin-1 (IL-1): IL-1 was previously known by several names, relating to a particular property. These include endogenous pyrogen and leucocyte endogenous mediator. It appears in two distinct forms, IL-1α and IL-1β (Dinarello, 1991). Both forms of IL-1 are initially synthesized as 31kDa precursor (pro-IL-1). In order to

produce the biologically active cytokines, the pro-IL-1 is cleaved by an interleukin-1 converting enzyme (ICE) generating a carboxyl terminus 17KDa peptide, called "mature IL-1". Mature IL-1α and IL-1β are fully biologically active forms, whereas, pro-IL-1 has been reported to be only partially active (Jobling et al.,1988; Rosenwasser et al.,1986). The amount of IL-1β messenger RNA found in stimulated human peripheral blood monocytes is usually 25 to 50 fold greater than the α form (Demczuk et al.,1987). The two forms of IL-1 appear to be under separate transcriptional control (Turner et al.,1989). With endotoxin stimulation in a variety of cells, expression of IL-1β rapidly occurs in macrophages (Fenton et al.,1988), endothelium (Libby et al.,1986$_a$), smooth muscle (Libby et al.,1986$_b$) and blood monocytes (Schindler et al.,1990). Endotoxin-stimulated IL-1β messenger RNA is observed within 15 minutes, with peak expression seen at 3-4 hours continuing for up to 8 hours and then decreases rapidly. Transcription and translation of IL-1 are distinct events; cells containing untranslated IL-1 messenger RNA are considered "primed" and small amounts of another stimulus (endotoxin or IL-1 itself) rapidly trigger translation and usually result in more IL-1 synthesis than in un-primed cells. Cellular sources of IL-1 include a variety of haemopoietic cells, such as blood monocytes and tissue macrophages (both are considered the primary source), neutrophils, T and B lymphocytes and mast cells. However, IL-1 production is also observed in non-haemopoietic cells, including endothelium, epithelium, smooth muscle cells, fibroblasts, microglia and nerves (Dinarello, 1991).

There are two IL-1 receptors, IL-1RI and IL-1RII, corresponding to 80kDa

and 68kDa IL-1 binding proteins on T and B cells, respectively (Dinarello and Thompson, 1991). However, these two receptors forms have been located on other cells. IL-1α and IL-1β mediate their biological activities via both IL-1 receptors, although IL-1β has a greater affinity for IL-1RII, whereas IL-1α has a higher affinity for IL-1RI.

Naturally occurring inhibitors of IL-1 have been reported in a variety of biological fluids, including serum, synovial exudate and urine (Dinarello and Thompson, 1991). Many of these agents are non-specific, since in addition to preventing IL-1 activity they also suppress or bind to other cytokines, particularly, IL-2 or TNFα. An IL-1 specific inhibitory activity in monocyte conditioned medium was purified by Hannum and colleagues (1990). Expression of the complementary DNA encoding for the inhibitor in *Escherichia coli* (*E.coli*), yielded an IL-1 inhibitory protein which was shown to prevent the biological effects of IL-1α and IL-1β by binding to the IL-1 receptors, but displayed no agonists properties of its own. The generic term IL-1 inhibitor was replaced by the more specific term, IL-1 receptor antagonist (IL-1ra). To date, there are no examples of the receptor antagonist failing to block the biological response to IL-1 in cultured cell systems (Dinarello, 1991). A 100 to 1000 fold excess of the IL-1ra has also been shown to prevent the *in vivo* physiological effect of adding exogenous IL-1β in the brain (Kent et al.,1992).

Tumour necrosis factor-α (TNFα): The other pro-inflammatory cytokine, TNFα, which until 1985, was known as macrophage factor, causes necrosis of experimental animal tumours (Balkwill, 1989). TNFα possesses a wide range of cell regulatory, immune

and inflammatory properties that overlap with the properties of other cytokines, such as IL-1 and interferon-γ. TNFα is beneficial to the host in controlling immunity, inflammation and repair, but it can be lethal when high levels of the cytokine are maintained and enter the general circulation.

The TNF gene is closely linked to another cytokine, with almost identical properties, lymphotoxin, and both genes map within the major histocompatibility complex (MHC) region on the short arm of chromosome 6 (Pennica et al.,1984). The biologically active TNFα exists as a trimer composed of mature 17kDa subunits. A pre-TNFα protein (26KDa) is produced in the cell, which is then cleaved to produce the mature 17KDa TNFα protein. This pre-TNFα protein has also been shown to possess biological activity within the cell membrane (Fiers et al.,1987; Kriegler et al.,1988). Originally, monocytes/macrophages were thought to be the sole producers of TNFα, but, as with IL-1, there is increasing evidence of its production by both haemopoietic and non-haemopoietic cells.

TNFα exerts its biological effect primarily by interacting with specific cell surface receptors displaying high and low affinity binding sites (Tartaglia and Goeddal, 1992). However, there are studies which show that TNFα is capable of inducing a physiological response by creating a pore-like structure within the cell membrane (Kagan et al.,1992). The multiple biological activities of TNFα (Schultze et al.,1992) have led to the development of the concept that this cytokine is primarily responsible for the wasting that is observed in the course of chronic disease (Cerami and Beutler, 1988). However, IL-1 has also been shown to possess similar properties

(McHugh et al.,1993).

A specific inhibitor of TNFα has been described in the urine of febrile patients (Seckinger et al, 1988) and in the supernatants of cells cultured from rheumatoid synovial fluid (Roux-Lombard et al.,1988). This TNFα inhibitor is a 31-33kDa protein that differs from IL-1 inhibitor and appears to possess an amino acid structure with no homology to any known protein (Engelmann et al.,1989; Olsson et al.,1989). This TNFα inhibitor is specific, failing to prevent IL-1α, IL-1β or lymphotoxin-stimulation of cells. Studies show that the inhibitor may represent a stable version of cell-surface TNFα receptor (Novick et al.,1989).

In conclusion, IL-1 and TNFα, although structurally quite distinct, regulate many of the same biological effects that occur during the acute inflammatory response. In fact, IL-1 and TNFα not only induce each other's synthesis but also regulate each other's biological activity (for review see Chaplin and Hoquist,1992). These two cytokines have been shown to act synergistically in immunocytes to up-regulate the expression of IL-2 receptors (Scholz and Altman,1989). In other cells, TNFα has been shown to potentiate IL-1- induced prostaglandin synthesis in cultured synovial fibroblasts (Meyer et al.,1990) and granulocyte colony-stimulating factor (GC-SF) expression in vascular smooth muscle cells (Zolliner et al.,1992).

1.5. Nerve changes in inflammatory bowel disease (IBD).

1.5.1 Nerve structure.

Extensive morphological studies using both the light and electron microscope

show degeneration (Oehmichen and Reifferscherd, 1977; Dvorak et al.,1980; Strobach et al.,1990) and proliferation (Davis et al.,1953; Storesteen et al.,1953; Nadorra et al.,1986) of enteric nerves of inflamed intestinal tissues from Crohn's disease patients. These have been described as forms of hyper-plastic ganglion cells and prominent nerve bundles (Shepherd and Jass, 1987). Early studies (Davis et al.,1953; Storesteen et al.,1953) demonstrated that alteration in neural structure was also present in non-inflamed colon. Tissue preparations from ulcerative colitis patients showed that hyperplastic changes occurring in the myenteric plexus were also seen in uninflamed regions of the gut. These groups individually concluded that altered nerve structure in both inflamed and uninflamed gut segments might be a basis for the extensive changes in intestinal physiology seen and that these changes in apparently un-inflamed parts of the bowel may possibly play a permissive role in the extension of the inflammatory disease (McInerney et al.,1964). These observations were subsequently confirmed by Okamato et al (1964) and Dvorak et al (1980). In addition, it has been speculated that a gradient of neural changes occurs in the myenteric plexus, ranging from hyperplasia associated with a relatively mild form of IBD, to gross structural damage observed in inflamed tissue in the active form of the disease.

There are limited and somewhat conflicting data concerning changes in neurotransmitter content reported in inflamed gut segments removed from IBD patients (Koch et al.,1991). Decreases in vasoactive intestinal peptide (VIP) and peptide histidine-methionine content in colonic mucosal layers from IBD patients

and in muscularis externa in tissues from patients with Crohn's disease have been observed (Bishop et al.,1980; Koch et al.,1988). Both neuropeptide Y, which appears co-localized with noradrenaline in post-ganglionic sympathetic nerves, and substance P content are elevated in gut segments from IBD patients (Koch et al.,1987). In contrast, mucosal concentrations of neuropeptide Y and somatostatin, which have been detected in enteric nerves (Furness et al.,1992) as well as endocrine sources, are decreased in IBD patients compared to tissues obtained from control individuals (Koch et al.,1991).

Location of neuropeptides within the gut is determined using immunohistochemical techniques (Furness and Costa, 1987), whereas the content is measured mainly by radioimmunoassay. The use of this approach, has led to discrepancies in the recorded levels of substance P in IBD. Koch and colleagues (1987) report a 50% increase in substance P content in extracts of the mucosa-submucous layer of the descending colon but not in the inflamed ascending or transverse colon. A 240% increase in substance P levels was reported by Goldwin et al (1989) in rectal biopsies of inflamed mucosa compared to uninflamed segments, whereas Sjolund et al (1983) showed a 76% decrease in substance P in inflamed colon with an insignificant change in the ileal mucosa. Contradictory levels of substance P reported in the literature may be due to the method of calculation in substance P content, which varies between studies. Although neuropeptide level may be more correctly expressed when the number of nerves are taken into consideration, in the inflamed bowel there is not only a change in nerve structure but also the

presence of inflammatory cells that also have been shown to contain neuropeptides. For example, substance P has been located in eosinophils (Weinstock et al.,1992). Not only are there differences in the amount of substance P reported but also its neural location. A study by Sjolund et al. (1983), examining substance P distribution in nerve fibres in patients with Crohn's disease, reported diffuse areas with hyper-innervation of the lamina propria with an increase in substance P observed in distal but not proximal colon of these patients. However, the available data is unable to distinguish clearly which axons participate in the inflammatory response within the various regions of the gut. Moreover if hyperplasia and necrosis leads to increased tissue weight or protein content, then changes unrelated to substance P content in nerves can lead to apparent alterations (Eysselein et al.,1991).

Although the substance P levels together with their neural source is unclear, there appears to be alteration in the surface expression of substance P receptors in various tissues within the inflamed bowel from IBD patients. Using autoradiography, Mantyh et al. (1989) quantitatively analyzed NK_1 receptor expression. Inflamed tissues showed a significant elevation in these receptors on lymph nodules and arterioles as well as circular and longitudinal muscle layers. These changes in receptor expression may be the result of either the inflammatory process or the availability of substance P in a particular region of the gut. There are no reports of changes occurring in other neurotransmitter/neuropeptide receptors in inflamed tissues from IBD patients to date. If however, the changes in substance P-containing nerves and receptors are indicative of neural changes in the gut then one would

expect this also to occur with other neurotransmitter/neuropeptides within the gut.

1.5.2. Changes in nerve function.

Majority of the information available on changes in enteric nerve function has been extrapolated from structural analysis on isolated gut specimens from IBD patients. No study to date has directly shown alterations in nerve function in patients with IBD.

Changes in gut motility in IBD patients probably reflect alterations in the function of smooth muscle and enteric nerves, although the effect of inflammation on nerve function in IBD has yet to be studied directly (for review see Collins,S.M.,1993). *In vivo* studies of colonic motility using patients with ulcerative colitis correlated diarrhoea with sigmoidal hypomotility which may be the direct result of loss in inhibitory neural input to the smooth muscle (Kern et al.,1951, Rao et al, 1988). The prominence of adrenergic nerves in inflamed bowel (Penttila, 1975) led to the speculation that alteration in the sympathetic input to the circular muscle may alter the contractility of the muscle layer and therefore, motility.

The existence of changes in visceral afferent properties during inflammation has been shown in several systems (for review see Raybould and Mayer, 1991). Inflammation may increase sensory perception in the gut resulting in abdominal pain or discomfort leading to altered physiology, such as an exaggeration of reflex responses normally triggered during digestion. Furthermore, sensory nerves may also play a role in maintaining the inflammatory state of the bowel, possibly via the

release of neuropeptides such as substance P or calcitonin-related peptide (CGRP) following antidromal excitation of the nerves (Payan, 1989).

1.6. Nerve changes in experimental models of inflammatory bowel disease (IBD).

Experimental animal models of IBD provide insights into the impact of inflammation on enteric nerve function. A recent study using TNB-induced colitis in rat revealed that there was not only a decrease in the release of noradrenaline from the myenteric plexus of inflamed distal colon, but also a similar decrease in noradrenaline release in uninflamed segments of the colon and terminal ileum (Jacobsen et al.,1993_b). These results may have a bearing on the changes in nerve structure reported in uninflamed tissues from IBD patients (Davis et al.,1953; Storesteen et al.,1953). Treatment with either systemic or topical steroids attenuated the inhibition of noradrenaline release indicating that the changes in neural function were attributable to the inflammatory response. Furthermore, the authors (Jacobsen et al.,1993_b) concluded that inflammation at one site in the gut alters neural function at uninflamed sites and that this occurs as a result of the inflammatory process. Similar changes in neurotransmitter release were reported using *T.spiralis* infection in rat, as a model of small bowel inflammation. In this situation there was a suppression in both acetylcholine (Collins et al.,1989) and noradrenaline (Swain et al.,1991) release from the jejunal myenteric plexus. These effects were attributed to the inflammatory process, since treatment with systemic steroids removed the inhibitory effect on neurotransmitter release. However, there was no alteration in

neurotransmitter release in uninflamed tissue segments distal to the site of inflammation (terminal ileum).

An increase in the level of substance P has been reported in a variety of inflamed tissues and implicated in the development of neurogenic inflammation in several systems, including the gut (Payan, 1989). This consequently led to its being labelled as a proinflammatory neurotransmitter. Measurement of immunoreactive substance P in inflamed longitudinal muscle-myenteric plexus preparations from *T.spiralis* infected rats (Swain et al.,1992) showed an 8 fold increase over the substance P levels measured in similar preparations from uninfected rats. This increase in substance P was abolished when the rats were treated with steroid prior to *T.spiralis* infection, thus suggesting that the increase in the neuropeptide's level were secondary to the inflammatory process. The increase in substance P could be attenuated by 80% with capsaicin treatment of rats *in vivo* prior to infection with the nematode, suggesting that the location of the increased control is primarily in unmyelinated sensory afferent nerves. However, the capsaicin-insensitive component (approx. 20%) indicates that there may be also an increase in substance P levels within other sources, such as intrinsic nerves of the myenteric plexus or inflammatory cells. In contrast, acute colitis in rabbits induced by formalin and immune complexes, was accompanied by a decrease in immune reactive substance P content in mucosal-submucosal and muscle layers of the inflamed colon (Eysselein et al.,1991). These changes may be explained by an increase in release of the peptide from enteric and sensory nerves of the gut or alternatively by a diminished peptide synthesis within

these nerves or by nerve damage. However, the formalin-immune complex model is a harsher, more penetrating injury which could have resulted damage to the myenteric plexus causing a decrease in substance P content.

1.7. Role of cytokines in inflammatory bowel disease (IBD).

Although the causes of IBD remain unclear, inflammatory mediators, such as cytokines, undoubtedly play a major role in immunological responses of the gut. Among these compounds, IL-1 and TNFα are considered the primary inducers of the acute inflammatory response and serve to amplify the immune response (for review see Cominelli, 1993).

Peripheral blood monocytes (PBM) and intestinal macrophages appear to be the principal source of these cytokines in IBD. The amount of IL-1 produced in PBM from patients with IBD compared to healthy control subjects was originally examined using bioassay, but the results from these studies are conflicting. A study by Miura and Hatinatashi, (1988) showed no significant difference in spontaneous or stimulated (endotoxins) IL-1 activity in monocyte cultures from Crohn's disease patients compared to healthy volunteers. In contrast, an elevated spontaneous IL-1 activity was measured in monocytes collected from Crohn's patients by Stsangi et al. (1987). However, because the results from the bioassay used in these studies can be affected by either endogenous inhibitory molecules or the synergistic effects of other endogenous cytokines, it is difficult to interpret these findings. Sartor et al (1988) reported that tissue levels of immunoreactive IL-1 are increased 16 fold in active

ulcerative colitis and 6 fold in active Crohn's disease compared to control and quiescent ulcerative colitis tissues. Similarly, Luimsky et al. (1990) reported enhanced IL-1 levels in supernatants of colonic mucosal biopsies from patients with active IBD.

The recent discovery of a native IL-1 receptor antagonist (IL-1ra) has stimulated research examining the role of IL-1 in intestinal inflammation. IL-1 and IL-1ra gene expression and translation are regulated differently; IL-1 expression usually precedes IL-1ra expression. In some clinical conditions the ratio of IL-1ra to IL-1 is increased (Arend et al.,1991; Poulsia et al.,1991). This may serve to limit the IL-1-induced amplification of the inflammatory response. Therefore, a relative lack of IL-1ra may be a basis for an exaggerated or poorly contained inflammatory response observed in IBD. The balance between IL-1 and IL-1ra expression in intestinal tissue and cell preparations from patients with IBD has recently been investigated. Tissues from healthy volunteers showed little IL-1α or IL-1β compared to the levels of IL-1ra. In contrast, in inflamed tissue segments from Crohn's patients, although similar amounts of IL-1ra were seen compared to the control group, there was a significant elevation in the amount of IL-1α and IL-1β observed (Cominelli et al.,1992). Consequently, the ratio of IL-1ra to IL-1 was decreased in grossly inflamed tissues from Crohn's patients compared to uninflamed tissues from controls or even from the same patients, suggesting that it is an elevation in IL-1 levels and not a decrease in IL-1ra levels that exaggerates the inflammatory response.

Studies measuring the amount of TNFα in IBD are still in their infancy. Detectable levels of TNFα have been observed in serum (Yagita and Oruma, 1990),

supernatants from cultured PBM (Masda et al.,1990) and in stools (Braegger et al.,1992) from patients with IBD, whilst others report no difference in TNFα production by lamina propria mononuclear cells derived from IBD and intestinal mucosal biopsies from healthy individuals (Mahida et al.,1989). With the increasing availability of immunological assays for measurement of selective cytokines, more recent studies have adopted this approach rather than relying on the bioactivity of a particular cytokine. However as cytokine levels are difficult to measure accurately using immunological assay (eg. radioimmunoassay or ELISA) in small mucosal biopsies, some researchers have turned to measuring the expression of these cytokines by molecular techniques. This may involve the measurement of messenger RNA of a particular cytokine. While some studies comparing inflamed and uninflamed mucosal biopsies (Stevens et al.,1990; McCarbe et al.,1993) report an increased TNFα messenger RNA expression, another study indicated no increased expression of this cytokine (Isaac et al.,1990). However, investigation of the cytokine messenger RNA levels without measuring protein levels does not give a clear indication of the amount of cytokine in the preparation, as often cytokine synthesis is regulated at the post-transcriptional level (Wodnar-Filipowicz and Moroni, 1990).

1.8. Role of cytokines in experimental models of inflammatory bowel disease (IBD):

Animal models of gut inflammation have been achieved using a variety of methods, including special diets, chemical irritants and manipulation of the intestinal immune response. None of these models closely mimics Crohn's or ulcerative colitis

in terms of the natural history of the disease. However, they provide a basis for determining the role of immune and inflammatory responses in changes in intestinal physiology. Moreover, these models can also be applied in the investigation of novel pharmacological therapies aimed at preventing the resulting physiological changes.

Examination of inflamed tissue segments from nematode infected animals showed increased messenger RNA expression of IL-1α, IL-1β, TNFα and IL-6 by day 6 post infection (Khan et al., 1992). The increase in these cytokines suggests a possible causal role in the physiological changes in small bowel function of this model. The specific role of IL-1 as a putative mediator of IBD has also been determined directly by blocking its effects using an IL-1 receptor antagonist (IL-1ra). Pretreatment of rabbits with IL-1ra prior to the induction of colitis by formalin and immune complexes resulted in a marked decrease in tissue inflammatory cell infiltration, edema and tissue necrosis (Cominelli et al.,1991). These data demonstrate that blockade of IL-1 prevents the onset and development of the inflammation in this model of immune complex-induced colitis. Similar results have been observed by McCafferty (1992) using TNB-induced colitis in rats, and by Sartor et al (1991), using a model of enterocolitis induced by serosal injection of bacterial peptidoglycans. The latter model employed by Sartor may be of particular relevance to IBD because of the chronic nature of the inflammatory response observed using this model.

The role of TNFα in IBD is not clear. Its role has been examined in an animal model of graft versus host disease (GVHD). Although this model has only

a limited resemblance to human IBD, it is useful in that it allows the involvement of specific cytokines in intestinal inflammation to be explored. In mice undergoing GVHD where the animals were pretreated with specific TNFα neutralizing antibodies, the results showed almost a complete prevention of gut dilation, flattening of the villi, crypt cell necrosis and mucosal inflammation and in addition, a significant reduction in mortality (Piguet et al.,1987). A direct measure of possible involvement of TNFα in bowel injury was shown in studies in which rats displayed ischemic necrosis upon systemic administration of the recombinant cytokine (Sun and Hsueh, 1988).

1.9. Effect of cytokines on nerve function in other tissues.

Acute phase reaction of the inflammatory process in response to tissue damage or infection is accompanied by systemic changes, including changes in neural function within the CNS (Alder et al.,1990; Blatteis, 1990). The induction of fever, malaise and alteration in ACTH secretion has been shown to be mediated by cytokines. In particular, IL-1, when injected into the brain, causes fever, ACTH secretion and synthesis of acute phase proteins in the liver. Also acute administration of IL-1, peripherally or centrally, suppresses appetite (Chance and Fischer, 1991; Hellerstein et al.,1989), and chronic peripheral administration of IL-1 (Mrosovsky et al.,1989; Otterness et al.,1988) results in anorexia. Injection of IL-1ra into specific areas of the brain prevented anorexia seen in rats undergoing acute chemical colitis (McHugh et al.,1993).

The relationship between cytokines released in the periphery and the presence of these inflammatory mediators within the CNS is unclear. Bank et al. (1991) suggests the presence of active transport mechanisms that transport cytokines across the blood-brain barrier. Alternatively, since most cytokines, including IL-1 and TNFα are believed (due to their molecular size) not to cross the blood-brain barrier (Blatteis, 1990; Bluthe et al.,1991), they may enter the CNS where the barrier is interrupted, i.e. the organum vasculosum lateral terminals.

Immunological analysis of the location of IL-1 in the human brain shows its presence in neural elements within the hypothalamus, suggesting that it may act as a neuromodulator in the central component of acute phase reaction (Breeder, 1988). Further studies of the location of cytokine receptors in the CNS, have shown the presence of IL-1 receptors expressed on the surface of a variety of cells within the CNS (Dinarello, 1989). IL-1 receptors have been located on both neuronal and non-neuronal tissues in the brain through autoradiography analysis (Farrar et al.,1987; Haour et al.,1990).

The cellular sources of brain cytokines remain uncertain and although blood monocytes have been shown to enter the brain after certain types of trauma (Oehmichen, 1983), it is thought that the source of cytokines is within the CNS itself. Cells intrinsic to the brain have been implicated as a source of the several cytokines; astrocytes, microglia and neurons (Fontana et.al.,1982; Guilian et al.,1986) have been shown to produce cytokines *in vitro*. However, it is glial cells, which have been shown to play an important role in nurturing the growth and repair of neurons, that may

be the primary source of cytokine production in the CNS (Guilian et al.,1986). Glial cells are capable of secreting growth regulatory factors or of producing extracelluar matrix proteins that influence neuronal growth as well as control neuronal development and recovery from CNS injury. Microglia IL-1 stimulates astroglia proliferation *in vitro, evidence* supporting a role for IL-1 in the regulation of neuron axon growth (Guilian et al.,1986; Blatties, 1990).

The role of cytokines in the alteration of central nerve function in the presence of an inflamed intestine remains unclear. No comprehensive picture has evolved as to the effect of inflammatory mediators on nerve function in the CNS. IL-1β is shown to stimulate both noradrenaline and dopamine release from the hypothalamus (Palazzol and Quadri, 1990), and decrease acetylcholine release from the hippocampus (Rada et al.,1991). *In vitro* studies show that addition of TNFα to sympathetic neurons induced a suppression of evoked (but not basal) release of noradrenaline (Soliven and Albert, 1992), whereas other cytokines, including IL-1, appear to have no effect. The effect of cytokines on neurotransmitter content in the CNS has been studies by Scarborough and co-workers who reported that both IL-1β and TNFα induced somatostatin synthesis in the hypothalamus (for review see Scarborough, 1991). In studies examining the action of cytokine on nerve activity, local application of IL-1 and TNFα in the vertebrate brain causes a decrease in the firing rates of neurons (Plata-Salaman et al.,1988).

Using abdominal ganglia of *Aplysia kurodai*, the mechanism of cytokines' effect on neuronal activity has been examined (Sawada et al.,1991). Extracelluar

injection of IL-1 induced a slow outward current associated with a decrease in sodium conductance causing membrane hyperpolarization. Other studies, examining the effect of cytokines on specific neurons in the brain showed that IL-1 decreases the activity of warm-sensitive neurons in rat hypothalamus (Hori et al.,1988) and both TNFα and IL-1β suppressed the activity of glucose-sensitive neurons in the rat lateral hypothalamic area and increased neuronal activity of glucose receptor bearing neurons in the rat ventromedial hypothalamic nuclei (Oomura et al.,1974; Plata-Salaman et al.,1988). These results suggest that cytokines have selective effects on specific neuron's activities and that the overall physical effect of these inflammatory mediators on nerve function is very complex and varies among the different regions of the brain (Blatteis, 1990).

CHAPTER 2

MEASUREMENT OF NORADRENALINE RELEASE FROM LONGITUDINAL MUSCLE-MYENTERIC PLEXUS (LM-MP).

2.1. Introduction.

The presence of adrenergic nerve fibres in the various layers of the gut has been shown using fluorescence histochemical techniques (Furnesss and Costa, 1974), but, this technique did not provide an insight into the functional role of noradrenaline in the gut. This prompted Wu and Gaginella (1981_a), to examine the functional aspect of noradrenaline in the gut initially by measuring noradrenaline uptake by the mucosal-submucous layer of the colon, using ^{3}H-noradrenaline as a marker. This group then went on to characterize the release of noradrenaline from these tissue preparations demonstrating neural release of ^{3}H-noradrenaline upon stimulation with KCl (Wu and Gaginella, 1981_b). These experiments were performed using a "superfusion" technique, in which the tissue preparations were initially loaded with ^{3}H-noradrenaline and then placed in a superfusion apparatus allowing the release of ^{3}H-noradrenaline to be measured in the superfusate. Furthermore, these studies also demonstrated the application of this method in examining the effect of

inflammatory mediators on noradrenaline release, showing that prostaglandin E_2 caused a suppression of evoked ^{3}H-noradrenaline release from the mucous-submucous layer of the colon.

Later studies by Collins and colleagues, adopted the superfusion technique, this time characterizing noradrenaline release from longitudinal muscle-myenteric plexus (LM-MP) preparations of rat jejunum. The method was subsequently applied to the investigation of putative changes in noradrenaline release during inflammation of the small bowel using the nematode *T.spiralis* infection of rats (Swain et al.,1991). In these studies a suppression of noradrenaline release was observed in the inflamed LM-MP preparations from rat jejunum in comparison with tissue preparations from uninfected animals.

The aim of my initial experiments was to confirm the superfusion technique involving measuring the release of noradrenaline and to subsequently apply it to investigate the effect of cytokines on noradrenaline release.

2.2. Methods.

2.2.1 Preparation of the longitudinal muscle - myenteric plexus layer (LM-MP):

The isolation of LM-MP preparations from the rat small intestine has been described previously by Collins and colleagues (Collins et al.,1989). Male Sprague Dawley rats (200-250g) were sacrificed by a blow to the head, followed quickly by cervical dislocation. A 2-3cm segment was cut from the proximal jejunum and placed over a plastic rod. After the removal of any excess mesentery, the surface of the

tissue was lightly scored. The LM-MP layer was gently removed and placed in 2ml of Krebs buffer containing (in mM): NaCl,120.9; KCl,5.9; $CaCl_2$,2.5; $MgCl_2$,1.2; $NaHCO_3$,15.5; NaH_2PO_4,1.2; glucose,11.1; EDTA,0.004; ascorbic acid,0.11; and pargyline,0.03, and bubbled with 95% O_2:5% CO_2.

2.2.2. Measurement of ^{3}H-noradrenaline (^{3}H-NA) release:

LM-MP preparations were preincubated in oxygenated Krebs buffer containing 0.5µM (15 Ci/mM) ^{3}H-noradrenaline (^{3}H-NA) for 40 mins in a 37°C water bath (Swain et.al,1991). The tissue segments were then washed with Krebs buffer, suspended in superfusion chambers maintained at 37°C and superfused with Krebs buffer at a flow rate of 1ml/min using a peristaltic pump. The superfusate was collected every 2 mins throughout the 40 mins experiment using a Altrorac 7000 fraction collector. The tritium content in the collected superfusate fractions was counted using a Beckman liquid scintillation counter at a counting efficiency of 35%. Stimulation of ^{3}H release was performed with either 50mM KCl or by electric field stimulation (30 volts, 0.5 msec, 10Hz for 1 min). The tissue preparations at the end of the experiment were blotted dry, weighed and solubilized in 1ml protosol (tissue solubilizer). This mixture was then neutralized with 100µl glacial acetic acid and 3ml aqueous scintillant and counted for tritium.

2.2.3. Calculation of stimulated ^{3}H-NA release:

Calculation of stimulated release of ^{3}H-NA was first calculated by a method

described by Kilbinger and Wessler (1980). The difference between the total ^{3}H release (R) during the stimulation period and the estimated ^{3}H baseline (S) (using calculated linear regression analysis) was measured. This value was then expressed as a percentage fraction (F) of the total tritium (T) presence in the tissue. The T value was calculated as the summation of ^{3}H in all the superfusate fractions plus the residual counts remaining in the tissue at the end of the experiment:

$$F = \frac{R-S}{T} \times 100$$

2.2.4. Measurement of noradrenaline release.

Superfusate fractions and the varicosity supernatants collected in response to stimuli were also analyzed for noradrenaline content by thin layer chromatography (TLC), using a technique previously described by Lehman (1983). Briefly, both samples together with external standards: ^{3}H-NA and noradrenaline were applied quickly (to avoid oxidization of the samples) to a silica gel TLC plate. This was then placed in an enclosed apparatus saturated with a solvent system consisting of; n-butanol:acetic acid:water (25:4:10). The presence of noradrenaline was observed under ultra violet (UV) lamp and selected sections of the TLC plate were counted for radioactivity.

2.2.5. Electron microscopy analysis of the LM-MP preparation.

This technique was performed by Ms. Irene Berezin from the Dept.

Biomedical Sciences (McMaster University, Ontario). The LM-MP preparations were initially placed in a fixing solution containing 2% glutaraldehyde and then prepared for electron microscopy analysis by a method described in appendix 2.

2.2.6. Materials.

Sprague Dawley rats were supplied by Charles River Farms (Montreal, Quebec); ^{3}H-NA (sp.act.13.3Ci/mM) was from New England Nuclear (Boston, MA) and the remaining chemicals used in this study were supplied by Sigma Chemicals, (St.Louis, MO).

2.3. Results.

Electron microscopic assessment of the LM-MP preparation of rat jejunum (figure 2.1) was used to demonstrate the presence of myenteric ganglia in close association with a macrophage-like cells. Also shown (figure 2.1) in close proximity to the ganglion are smooth muscle cells and cells known as the interstitial cell of Cajal.

The marker of noradrenaline release used to monitor the stimulated release, was ^{3}H-noradrenaline (^{3}H-NA). Previous studies have shown that optimum ^{3}H-NA loading of the LM-MP preparation is achieved by 40 mins (Swain et al.,1991). The typical experiments show an average ^{3}H-NA uptake of 11200 dpm/mg tissue wt. after a 40 mins incubation period. Figure 2.2A portrays one experiment with a typical washout curve for the release of ^{3}H-NA from the tissues over 80 mins. Electrical

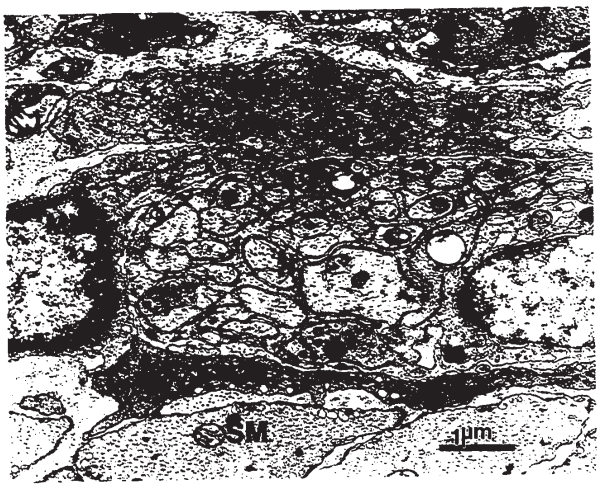


Figure 2.1: Electron micrograph of the LM-MP preparation. This micrograph shows a cross section through the myenteric plexus of rat jejunum showing close apposition contacts between a macrophage-like cell (MLC) and a nerve bundle (N). The MLC posses well developed golgi (G) and rough endoplasmic reticulum (rER), which are a characteristic feature of many rat jejunum MLC. There are also interstitial cells of Cajal (IC) and smooth muscle cells (SM) present in close proximity to the nerve ganglion. Bar = 1μm.

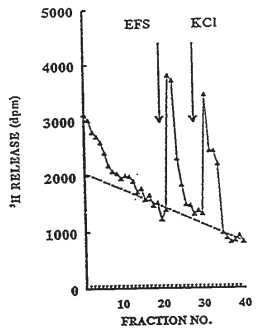
field stimulation (EFS) of the tissues was carried out after 40 mins (Fraction # 20) and resulted in a fractional release of 1.76% and after 60 mins (fraction # 30) with 50mM KCl, 1.7%, (figure 2.2B).

To determine whether the stimulated ^{3}H counted in the superfusate was actually ^{3}H-NA, the fluorescent property of noradrenaline was used. As shown in figure 2.3., the location of ^{3}H peak on the TLC plate correlated with the observed fluorescence. These results indicate that the stimulated ^{3}H release measured in either the superfusate or varicosity supernatants represents ^{3}H-noradrenaline release. The numbers in brackets refer to the percentage of ^{3}H-NA originally added to the TLC plate and therefore an indicator of whether the ^{3}H is still bound to the noradrenaline molecule. The Rf value is the ratio between the distance travelled by the standard/sample versus the solvent front. The results shown in figure 2.3. indicate that the Rf values for the samples were the same as those measured for noradrenaline standard; 0.86. Measurement of the radioactivity in 5 sections of the TLC plate (between the origin and the solvent front) showed that the highest counts recorded for either ^{3}H-NA (96%) or samples from superfusion (63.5%) and varicosity (71%) experiments coincided with the location of the noradrenaline standard. Therefore these results demonstrate that the ^{3}H collected following superfusion and supernatants from nerve varicosities consists primarily of ^{3}H-NA.

2.4. Conclusion.

These results demonstrate the feasibility of measuring stimulated ^{3}H release

A



B

STIMULI	^{3}H-NA RELEASE [Fractional Release (%)
EFS (30V, 0.5msec, 10Hz, 1 min)	1.76
KCL (50mM)	1.70

Figure 2.2: ^{3}H-NA release from LM-MP preparations. Panel A shows a typical washout curve for the release of ^{3}H over 80 mins. At fraction 20 (40 mins) and 30 (60 mins) the tissues are stimulated with EFS and KCl, respectively. The data are expressed as ^{3}H (dpm/2ml superfusate fraction). Panel B indicates the stimulated (EFS or KCl) release of ^{3}H-NA expressed as % fractional release of total ^{3}H.

NA (1mM) (10µl x 4)	^{3}H-NA (0.1µCi/ml) (10µl x 4)	Varicosity supernatant (10µl x 4)	Superfusion supernatant (10µl x 4)	
Fluorescence	64581 dpm's (93.5%) No fluorescence	256 dpm's (48.9%) Fluorescence	50 dpm's (20%) Fluorescence	Section 5 Solvent front
Fluorescence	1834 dpm's (2.6%) No fluorescence	115 dpm's (22.4%) Fluorescence	112 dpm's (43.5%) Fluorescence	Section 4
Nofluorescence	1521 dpm's (2.2%) No fluorescence	88 dpm's (17.6%) No fluorescence	47 dpm's (9.1%) No fluorescence	Section 3
Nofluorescence	232 dpm's (0.33%) No fluorescence	36 dpm's (7%) No fluorescence	24 dpm's (9.3%) No fluorescence	Section 2
Nofluorescence	290 dpm's (0.42%) No fluorescence	21 dpm's (4%) No fluorescence	27 dpm's (10.5%) No fluorescence	Section 1 sample origin
Nofluorescence	17 dpm's No fluorescence	22 dpm's No fluorescence	17 dpm's No fluorescence	Background

Figure 2.3: Diagrammatic representation of noradrenaline release. This figure shows the correlation between ^{3}H (indicated by dpm's) and noradrenaline (represented as fluorescence) in the superfusate or varicosity supernatants. The results in brackets represent a percentage of the radioactivity added to the TLC plates.

from LM-MP preparations using the superfusion technique and nerve varicosities can therefore be applied to investigate the effect of inflammatory mediators on noradrenaline release from the myenteric plexus.

CHAPTER 3

EFFECT OF IL-1β AND TNFα ON NORADRENALINE RELEASE FROM LONGITUDINAL MUSCLE - MYENTERIC PLEXUS PREPARATIONS

3.1. Introduction.

It is becoming increasingly clear that the nervous system is subject to the influence of the immune system. Several cytokines, including interleukin-1 (IL-1) and tumour necrosis factor-α (TNFα), have been associated with altered neural functions, ranging from changes in feeding behaviour to tissue maintenance in the CNS (Alder et al.,1990; Blatties, 1990; McHugh et al.,1993). Both interleukin-1β (IL-1β) and TNFα have been implicated in the alteration of neurotransmission; IL-1β has been shown to decrease the acetylcholine content of rat hippocampus (Rada et al.,1991), whereas TNFα is shown to suppress noradrenaline release from sympathetic neuron cultures (Soliven and Albert, 1992). However, less is known about the ability of these cytokines to modulate the enteric nervous system.

Recent demonstrations of altered enteric nerve function in animal models of intestinal inflammation raise the possibility that enteric nerves may be targets for inflammatory cytokines. Inflammation induced by *T.spiralis* infection in the rat caused a marked suppression of noradrenaline (Swain et al.,1991) and acetylcholine (Collins

et al.,1989) release from the jejunal myenteric plexus in those studies. Furthermore, preliminary data using the same model revealed the expression of several cytokines, among them, IL-1β and TNFα, in the longitudinal muscle - myenteric plexus (LM-MP) layers during the early stages of the enteric infection by the nematode (Khan et al., 1992). These studies prompt consideration of both IL-1β and TNFα as putative mediators of altered neural function in the myenteric plexus of *T.spiralis*- infected rat.

In this study, the ability of both IL-1β and TNFα to alter ^{3}H-Noradrenaline (^{3}H-NA) release from the rat LM-MP preparation has been examined. The multicellular longitudinal muscle-myenteric plexus preparation (LM-MP) was initially used in order to incorporate the range of putative target cells of cytokine actions.

3.2. Methods.

3.2.1. Measurement of noradrenaline release from LM-MP preparations.

See chapter 2.2.

3.2.2. Effect of human recombinant interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) on ^{3}H-noradrenaline (^{3}H-NA) release from LM-MP preparations.

Immediate effect of IL-1β and TNFα.

Initial experiments were designed to assess the ability of IL-1β and TNFα to induce a change in ^{3}H-NA release when added directly to the superfusate. To

examine an immediate effect of the cytokine, 10ng/ml IL-1β or 50ng/ml TNFα was added either to the superfusate alone or simultaneously with EFS or 50mM KCl. An equal volume of saline was added in control experiments These concentrations of cytokines were taken from existing literature (Rada et al.,1991, Soliven and Albert,1992).

Preincubation effect of IL-1β and TNFα.

To evaluate a delayed effect of the cytokines, LM-MP preparations were preincubated with either IL-1β (0.01-100ng/ml) or TNFα (0.01-100ng/ml) for specified periods (0-150 mins). Within these experiments, a 40 mins ^{3}H-NA loading period at the end of the preincubation was included, in the presence of either cytokine. Thus, the stated preincubation time represents the total exposure of the tissue to either IL-1β or TNFα.

Specificity of IL-1β and TNFα.

The specificity of the IL-1β or TNFα was assessed by either neutralizing the cytokine with rabbit polyclonal anti-IL-1β or anti-TNFα antibodies or by boiling the cytokines, which eliminated the possibility of an effect due to contaminating endotoxin. In experiments involving the neutralization of the cytokines, IL-1β (10ng/ml) was preincubated with an equal volume of rabbit polyclonal anti-human IL-1β antibody (in 1:10 dilution) for 20 mins at 37°C, prior to addition to the incubation mixture. Similarly, TNFα (50ng/ml) was preincubated with an equal volume of rabbit polyclonal anti-human TNFα antibody (1 unit/ml) for 20 mins at 37°C before addition to the LM-MP preparations. In control experiments, hrIL-1β

or hrTNFα was incubated with saline for 20 mins at 37°C before adding to the tissues. In experiments involving boiling of the cytokines, either IL-1β or TNFα were boiled for 20 mins prior to preincubation with the LM-MP preparations.

Induction of protein synthesis by IL-1β and TNFα.

Measurement of ^{35}S-methionine uptake by LM-MP preparations. The induction of protein synthesis was estimated by measuring ^{35}S-methionine uptake (Debolois et al.,1988) by the LM-MP preparations. Tissue segments (20-30mg) were placed in 1ml RPMI (Roswell Park Memorial Institute) medium supplemented with 10% fetal calf serum and 10mM glutamine. This mixture was then incubated with a final concentration of 10μCi/ml ^{35}S-methionine in the presence of a proteinase inhibitor, trasylol (100 Kallikrein Inactivating Units/ml) for specified times in a CO_2 incubator (Forma Scientific) at 37°C. The tissues were then blotted, washed in phosphate buffer saline (PBS) x 3, solubilized in 1ml tissue solubilizer (protosol) and counted in a Beckman scintillation counter at a 35% counting efficiency.

Effect of IL-1β and TNFα on ^{35}S-methionine uptake. LM-MP preparations were preincubated with either saline, IL-1β (10ng/ml) or TNFα (50ng/ml) in the presence of ^{35}S-methionine for specific times (0-150 mins). Co-incubation of the tissues with the protein synthesis inhibitor, cycloheximide (100μg/ml) in the presence of saline, IL-1β (10ng/ml) or TNFα (50ng/ml) was used to prevent the induction of protein synthesis after a 30 or 120 mins incubation period in experiments with IL-1β and 45 or 120 mins incubation in experiments using TNFα.

Effect of cycloheximide on ^{3}H-NA release. The same concentration of cycloheximide

(100μg/ml) that successfully blocked both IL-1β and TNFα induction of protein synthesis was then used to examine the role of induced proteins in the inhibitory effect of both cytokines on evoked ^{3}H-NA release. Tissues were preincubated with saline or 10ng/ml IL-1β for 30 or 120 mins in the presence or absence of 100μg/ml cycloheximide. Subsequently, the LM-MP preparations were measured for ^{3}H-NA release.

Effect of IL-1 receptor antagonist:

To establish that the suppressive effect of either IL-1β or TNFα on evoked ^{3}H-NA involved IL-1, a selective, competitive IL-1 receptor antagonist (IL-1ra) was used. In these experiments, the LM-MP preparations were preincubated with 10μg/ml IL-1ra, a concentration 1000 x greater than IL-1β, which has previously been shown adequate to block the effect of exogenous addition of IL-1β in *in vivo* studies (Kent et al.,1992), for 15 mins prior to addition of either saline, IL-1β or TNFα (50ng/ml); then further incubated for either 30 and 120 mins in experiments involving IL-1β and 45 and 120 mins in TNFα experiments.

In experiments where the role of endogenous IL-1 was examined in the suppression of evoked ^{3}H-NA release after 120 mins exposure to IL-1β, the tissues were preincubated with either saline or IL-1β (10ng/ml) for 90 mins, before the addition of either IL-1ra (10μg/ml) or the anti-human IL-1β antibody (1:10) to the medium. The incubation then proceeded for another 30 mins (total 120 mins), before the ^{3}H-NA release studies. In all experiments, control tissues received an equal volume of saline in place of IL-1ra or the IL-1β antibody.

Role of prostaglandins:

Measurement of prostaglandin E_2. In order to assess the ability of IL-1β to induce prostaglandin synthesis in the LM-MP preparations: Prostaglandin E_2 (PGE_2) was measured in the incubation medium by Dr. Paul Stetsko from Dept. Pathology (McMaster University, Ontario,), as described in appendix 1. In these experiments, the tissues were preincubated with either saline, IL-1β (10ng/ml) or TNFα (50ng/ml) in the presence or absence of cyclo-oxygenase inhibitors, piroxicam (1μM) or indomethacin (10μM) for 30 or 120 mins in experiments using IL-1β and 45 and 120 mins incubation in experiments using TNFα. Control experiments received an equal volume of saline in place of the cyclo-oxygenase inhibitor.

Effect of cyclo-oxygenase inhibitors on ^{3}H-NA release. The same concentrations of cyclo-oxygenase inhibitors were then applied to determine the role of prostaglandins in the cytokine's suppressive action on evoked ^{3}H-NA release. 1μM piroxicam or 10μM indomethacin were preincubated with LM-MP preparations with either saline, IL-1β (10ng/ml) or TNFα (50ng/ml) for 30 or 120 mins in IL-1β experiments and 45 and 120 mins in experiments using TNFα, prior to ^{3}H-NA release studies. In control experiments, an equal volume of saline was used in place of the cyclo-oxygenase inhibitor.

3.2.3. Statistical analysis.

All studies involved at least 4 separate experiments and results are expressed

as mean ± S.E. (standard error). The Student's paired t test was used to compare two means within each experiment, and a one way analysis of variance was applied when comparing more than two means. Statistical significance was inferred from a P value < 0.05.

3.2.4. Materials.

Sprague Dawley rats were supplied by Charles River Farms (Montreal, Quebec); ^{3}H-NA (sp.act. 13.3Ci/mM) was from New England Nuclear (Boston, MA); IL-1β was from Sclavo (Sienna, Italy) and Upstate Biotechnology Incorporated (Lake Placid, NY); Rabbit anti-human IL-1β antibody was a generous gift from Dr.R.Newton of DuPont Pharmaceuticals (Glenolden, PA); the IL-1 receptor antagonist was a generous gift from Dr.R.Thompson of Synergen (Denver, CO); RPMI medium and fetal calf serum were from GIBCO (Grand Island, NY); Proteinase inhibitor, Trasylol was from Miles (Ontario, Canada); L-^{35}S-methionine (sp.act.1261 Ci/mM) was from Amersham Canada Ltd. (Oakville, Ontario); TNFα was from R&D systems (Minneapolis, MN); Anti-human TNFα antibody was from Genzyme (Inter-Medico, Ontario, Canada); PGE_2 Kit was from Amersham In.plc (Amersham, U.K.) and the rest of the chemicals were supplied by Sigma Chemical (St.Louis, MO).

3.3. Results.

3.3.1.Effect of IL-1β on ^{3}H-NA release from LM-MP preparations.

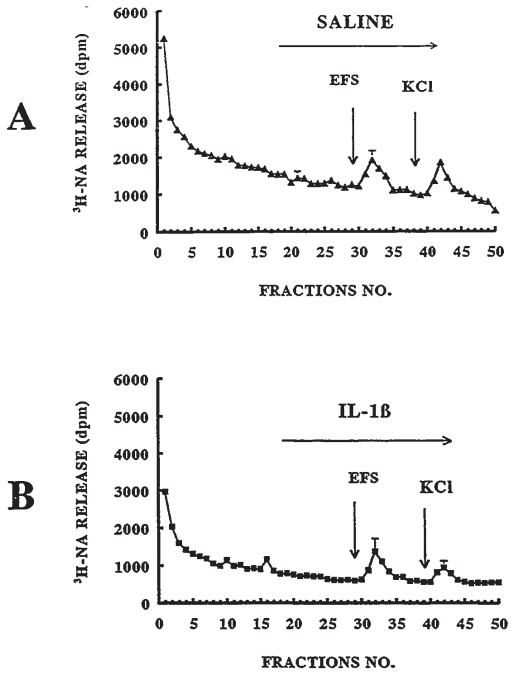


Figure 3.1: Immediate effect of IL-1β on ^{3}H-NA release. Release of ^{3}H-NA from the tissues into the superfusate. A: after stimulation by EFS or 50mM KCl in the presence of saline. B: after stimulation by EFS or KCl in the presence of 10ng/ml IL-1β as shown. Each panel is a combination of 5 experiments; error bars represent mean ± S.E. of ^{3}H-NA release; dpm/2ml superfusate fractions.

Immediate effect of IL-1β on ^{3}H-NA release.

The addition of IL-1β to the superfusate did not significantly alter the basal release of ^{3}H-NA (figure 3.1). The content of ^{3}H-NA in the superfusate after the addition of saline (figure 3.1A) or 10ng/ml IL-1β (figure 3.1B) was 0.02±0.025 and 0.01±0.01%, respectively (P>0.05). The addition of the same concentration of IL-1β to the superfusate during stimulation of the tissue with either EFS or KCl had no effect on evoked ^{3}H-NA release obtained in the presence of saline. The evoked release of ^{3}H-NA by EFS or KCl in the presence of saline was 1.3±0.3 and 0.66±0.23%, respectively (P>0.05), whereas the stimulated release in the presence of IL-1β was 0.76±0.39 and 0.83±0.45%, EFS or KCl, respectively.

Preincubation of IL-1β with LM-MP preparations.

^{3}H-NA uptake. Although there was variation in amount of ^{3}H-NA taken up by the saline-treated tissue (during the 40 mins loading period) between the specified preincubation time points, the addition of IL-1β (10ng/ml) to the incubation medium was not significantly (11677±3818 dpm/mg tissue wt.) differ from the uptake of ^{3}H-NA by saline-treated tissues (13621±4301 dpm/mg tissue wt.) after 150 mins incubation.

^{3}H-NA release. After 150 mins preincubation of LM-MP preparations with 10ng/ml IL-1β there was no significant alteration in the basal release of ^{3}H-NA observed in the superfusion experiment; 0.05±0.01% vs 0.05±0.01%, saline vs IL-1β, respectively. However, preincubation of the tissues with IL-1β did cause a suppression of the evoked release of ^{3}H-NA. As illustrated in figure 3.2, the suppression was biphasic;

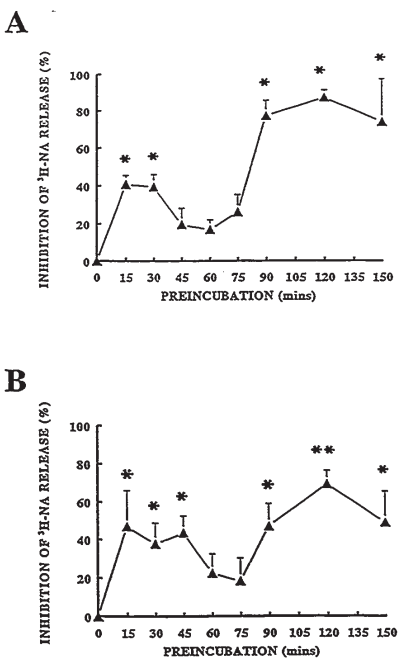


Figure 3.2: Time-dependence of IL-1β effect on ^{3}H-NA release. Tissues were preincubated with 10ng/ml IL-1β for 0-150 mins before stimulation of ^{3}H-NA release by EFS (A) or KCl (B). Inhibition of ^{3}H-NA release are expressed as percentage inhibition of corresponding values obtained in absence of IL-1β. Results are the mean ± S.E. of 4 experiments. *P<0.05, **P<0.001. for difference from control.

the first phase of suppression was evident after 15 mins preincubation with the cytokine and persisted for at least 30 mins. During the first phase of suppression, EFS- (figure 3.2A) and KCl- (figure 3.2B) induced ^{3}H-NA release was suppressed by 49.3±1.04 ($P<0.05$) and 33.6±15.6% ($P<0.05$), respectively. No suppression of ^{3}H-NA release was evident after 60 mins preincubation, but further exposure of the tissues to IL-1β revealed a second phase of inhibition that was evident by 90 mins. Maximum suppression of EFS- or KCl-induced ^{3}H-NA release in the second phase was 89.3±4 ($P<0.05$) or 69.2±7.1% ($P<0.001$), respectively, and was observed after 120 mins.

Concentration dependence. As shown in figure 3.3A, the suppression of EFS- induced ^{3}H-NA release by IL-1β was concentration dependent over a range of 0.1-100ng/ml IL-1β, with the maximum suppressive effect on evoked ^{3}H-NA release observed using 10ng/ml IL-1β (89.3±3.9%, $P<0.01$). In contrast, the suppression of KCl- (figure 3.3B) induced ^{3}H-NA release was concentration dependent over a range of 0.01-10ng/ml IL-1β. The concentration-response relationship for IL-1β suppression of KCl-induced ^{3}H-NA release was biphasic with the first peak of suppression occurring with 0.1ng/ml IL-1β; no inhibition was seen using 1ng/ml in any experiment involving KCl-induced ^{3}H-NA release after 120 mins preincubation with the cytokine. However, increasing the concentration of IL-1β resulted in further suppression of ^{3}H-NA release that was maximal at 10ng/ml and declined thereafter. Unless stated otherwise, optimum conditions of IL-1β-induced suppression were used in subsequent experiments and consisted of a preincubation time of 120 mins with 10ng/ml IL-1β.

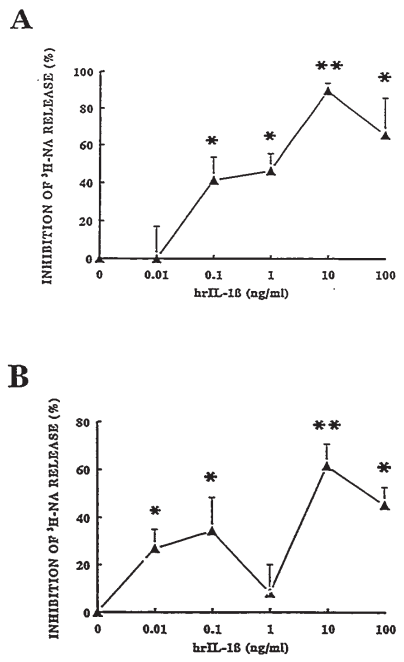


Figure 3.3: Concentration-dependence of IL-1β effect on ^{3}H-NA release. Tissues were preincubated with identified concentrations of IL-1β (0.01-100ng/ml) for 120 mins before stimulation of ^{3}H-NA release by EFS (A) or KCl (B). The data are expressed as a percentage inhibition of control (saline) values. Results are the mean ± S.E. of 4 experiments. *$P<0.05$, **$P<0.001$.

Table 3.1: Specific effect of IL-1β on ^{3}H-NA release from LM-MP preparations.

STIMULI	EFS (% ^{3}H-NA RELEASE)	KCl (50mM) (% ^{3}H-NA RELEASE)
CONTROL	3.12±0.6	1.71±0.1
IL-1β	0.9±0.21*	0.56±0.1*
IL-1β + IL-1β ANTIBODY	2.8±0.1**	1.3±0.2**
BOILED IL-1β	3.1±0.3**	3.9±0.9**

LM-MP preparations were preincubated for 120 mins with saline, 10ng/ml IL-1β alone, IL-1β neutralized with anti-human IL-1β antibody, or boiled IL-1β before stimulation of ^{3}H-NA release by EFS or 50mM KCl. Results are expressed as the mean ± S.E. from 4 separate experiments, where * represents a significant ($P<0.05$) difference from control (saline), and ** indicates a significant ($P<0.001$) difference from the values obtained with IL-1β alone.

Specificity of IL-1β effect.

As shown in table 3.1, combining the IL-1β with a polyclonal rabbit anti-human IL-1β antibody (1:10) for 20 mins at 37°C before preincubation with the tissue neutralized the inhibitory effect of IL-1β on both EFS- and KCl-induced ^{3}H-NA release by 90% ($P<0.01$) and 74% ($P<0.01$), respectively. In addition, the inhibitory properties of IL-1β on evoked ^{3}H-NA release could be abolished by boiling the cytokine for 20 mins before incubation with the tissue. The absence of the IL-1β-induced suppression of evoked noradrenaline release after boiling, indicates that endotoxins, which are heat stable (Freuenberg and Galanos, 1990), are not responsible for the suppressive action of the recombinant cytokine.

Induction of protein synthesis by IL-1β.

^{35}S-methionine uptake. ^{35}S-methionine uptake by the LM-MP preparations was used to estimate the overall protein synthesis occurring in response to 10ng/ml IL-1β (figure 3.4). There was no increase in ^{35}S-methionine uptake by the tissues (figure 3.4A) observed until 90 mins. Exposure of the LM-MP preparations to IL-1β for 120 mins induced a 148% increase in ^{35}S-methionine uptake. This increase in ^{35}S-methionine uptake was blocked by the co-incubation of the LM-MP preparations with IL-1β plus 100μg/ml of the protein synthesis inhibitor, cycloheximide (figure 3.4B). Statistical comparison of the results showed there to be no significant ($P>0.05$) difference in ^{35}S-methionine uptake between saline, cycloheximide or IL-1β plus cycloheximide- treated tissues; 305.4±76.6, 330.4±83.7 or 317±97.1 cpm/mg tissue, respectively. This results indicate that IL-1β is capable of inducing protein

synthesis in the LM-MP preparation after 90 mins incubation with the cytokine.

^{3}H-NA release. We next examined the role of protein synthesis in the inhibitory effect of IL-1β on evoked ^{3}H-NA release, at incubation time points, 30 and 120 mins (figure 3.5). The presence of 100μg/ml cycloheximide failed to alter the suppression of either EFS- (figure 3.5A) or KCl- (figure 3.5B) induced ^{3}H-NA release observed after 30 mins exposure to 10ng/ml IL-1β. The failure of cycloheximide to prevent the inhibitory effect of the cytokine on noradrenaline release, was expected since no ^{35}S-methionine uptake was observed in the tissues after this incubation time (Fig.3.4). Cycloheximide alone had no significant effect on either EFS- or KCl-induced ^{3}H-NA release. In contrast, the protein synthesis inhibitor prevented the IL-1β suppressive effect seen after 120 mins on both EFS- (Fig.3.5A) and KCl- (Fig.3.5B) induced ^{3}H-NA release by 97% ($P<0.001$) and 74% ($P<0.01$). Since IL-1β was shown to induce protein synthesis in the tissues after 120 mins incubation, the attenuation of stimulated noradrenaline release with the cycloheximide indicates that the delayed suppressive effect of IL-1β on evoked noradrenaline release is dependent on the synthesis of endogenous proteins. Preincubation of the tissues for 120 mins with cycloheximide alone did not alter either EFS- (4.07±0.59%) or KCl- (2.12±0.2%) induced ^{3}H-NA release compared to values seen for saline-treated tissues; 3.55±0.58% or 2.86±0.96%, EFS or KCl, respectively.

Effect of IL-1 receptor antagonist.

Co-incubation of 10ng/ml IL-1β plus 10μg/ml IL-1ra with the LM-MP preparations attenuated the inhibitory effect of IL-1β on evoked ^{3}H-NA release after

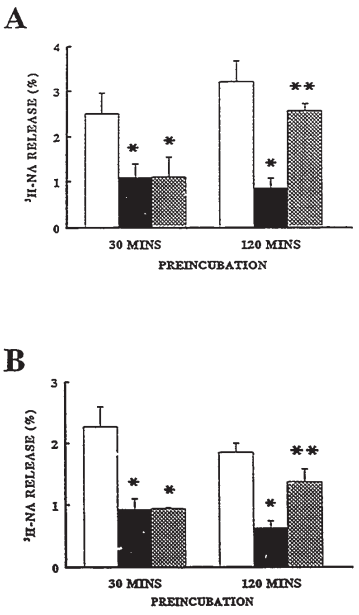


Figure 3.5: Effect of cycloheximide and IL-1β on evoked release of ^{3}H-NA. Tissues were preincubated for 30 or 120 mins with buffer (open bars); IL-1β(solid bars); or IL-1β plus cycloheximide (crossed bars) before stimulation of ^{3}H-NA release by EFS (A) or KCl (B). Results are the mean ± S.E. of 5 separate experiments. *P<0.05, represents significant difference from control (saline), whereas, **P<0.01, indicates a difference from the effect of IL-1β alone.

both 30 and 120 mins preincubation periods (figure 3.6) in the presence of the antagonist. Suppression of EFS- (figure 3.6A) induced ^{3}H-NA release by IL-1β was attenuated by 90% ($P<0.001$) and 82% ($P<0.001$), at 30 and 120 mins, respectively., Similar inhibitory effects by the IL-1ra was observed on KCl- (figure 3.6B) induced ^{3}H-NA release; 85% ($P<0.01$) and 87% ($P<0.01$); 30 and 120 mins, respectively. There was no significant ($P>0.05$) difference between evoked ^{3}H-NA values observed in tissues preincubated with either saline or IL-1β plus IL-1ra for both the 30 and 120 mins incubation times. Furthermore, preincubation of the tissue with the 10μg/ml receptor antagonist alone did not alter EFS- or KCl-induced ^{3}H-NA release after either 30 mins or 120 mins, compared to saline-treated tissues (data not shown).

^{35}S-methionine uptake was recorded after 30 mins incubation of the tissue with IL-1β. However the suppression of ^{3}H-NA release was attenuated in the presence of the IL-1ra. These results suggest that suppression of ^{3}H-NA release induced by IL-1β involves IL-1 receptors.

Role of endogenous IL-1. The role of endogenous IL-1 in IL-1β- induced suppression of evoked ^{3}H-NA release observed after 120 mins preincubation was investigated using the receptor antagonist (figure 3.7). The antagonist (10μg/ml of IL-1ra) was added to the medium 90 mins after incubation of the tissues with IL-1β. By 90 mins an increase in ^{35}S-methionine uptake was recorded (figure 3.4), suggesting the induction of endogenous protein, which may include IL-1. The aim of this experiment was to block the suppressive action of IL-1β on noradrenaline release

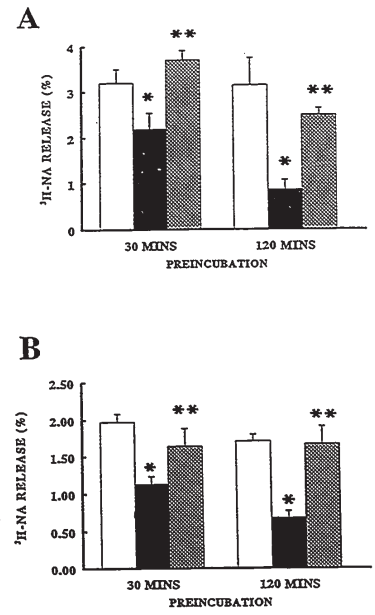


Figure 3.6: Effect of IL-1 receptor antagonist (IL-1ra) on evoked release of ^{3}H-NA. Tissues were preincubated with saline alone (open bars); IL-1β (solid bars); IL-1ra (15 mins, 37°C) then IL-1β (crossed bars) before stimulation of ^{3}H-NA release by EFS (A) or KCl (B). Results are the mean ± S.E. of 5 separate experiments. *P<0.05, represents a difference from control (saline), whereas,P<0.0, indicates a difference from the effects of IL-1β alone.**

after its induction of *de novo* proteins. The delayed addition (after 90 mins) of the receptor antagonist attenuated the suppressive effect of the cytokine on both EFS- (figure 3.7A) and KCl- (figure 3.7B) induced ^{3}H-NA release by 98% ($P<0.001$) and 85% ($P<0.001$), respectively. The effect of adding IL-1ra at 90 mins could not be mimicked by adding anti-human IL-1β antibody (1:10) under similar conditions, suggesting that the suppressive effect of IL-1β on noradrenaline release after a 120 min preincubation period involved endogenous (ie. rat) IL-1 and was not due to the exogenous human recombinant IL-1β initially added. Comparison between EFS- (figure 3.7A) or KCl (figure 3.7B) induced ^{3}H-NA release from the tissues with either preincubation with IL-1β alone or in the presence of anti-human IL-1β antibody (after 90 mins) were not significantly ($P>0.05$) different.

Role of prostaglandins.

To elucidate the role of prostaglandins in the IL-1β- induced suppression of evoked ^{3}H-NA release, I first examined the levels of prostaglandin E_2 (PGE_2) following exposure of the tissue to IL-1β. As shown in figure 3.8A, after a short incubation period (30 mins) there was a 2 fold increase ($P<0.05$) in PGE_2 levels measured in the tissues in response to 10ng/ml IL-1β, compared to corresponding control tissues treated with saline. This increase in PGE_2 was reduced to within control (saline) concentrations by the presence of cyclo-oxygenase inhibitors in the incubation medium. As shown in figure 3.8B, prolonged exposure of the tissues to IL-1β similarly caused a 2 fold increase in PGE_2 levels in comparison with corresponding controls ($P<0.05$). The IL-1β-induced increase in PGE_2 after 120 mins

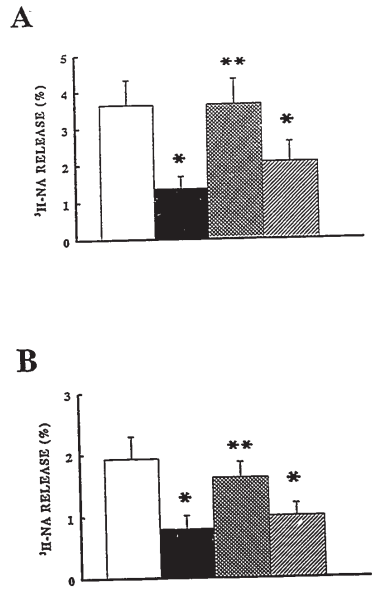


Figure 3.7: Effect of endogenous IL-1 on evoked release of ^{3}H-NA. Tissues were preincubated with saline alone (open bars) for 120 mins; IL-1β alone for 120 mins (solid bars); IL-1β for 90 mins before adding IL-1ra and incubating for a further 30 mins (crossed bars); IL-1β alone for 90 mins before adding anti-human IL-1β antibody and incubating for a further 30 mins (hatched bars). tissues were then stimulated with either EFS (A) or KCl (B) and ^{3}H-NA release was measured. Results are shown as the mean ± S.E. of 5 separate experiments, with *$P<0.05$, representing a difference from control (saline) alone and **$P<0.05$, indicating a difference from the effect of IL-1β alone.

incubation with the tissues was blocked by either 1μM piroxicam or 10μM indomethacin. These results indicate that IL-1β is capable of activating cyclo-oxygenase and inducing metabolites. The prevention of the increase in PGE_2 by both piroxicam and indomethacin, indicates that these concentrations are adequate in preventing IL-1β activation of the cyclo-oxygenase enzyme. This has also been shown in other systems (Brurch et al.,1983), therefore, these concentrations of the inhibitors were subsequently used to examine the role played by endogenous prostaglandins in IL-1β- induced suppression of evoked ^{3}H-NA release.

The presence of either piroxicam or indomethacin in the incubation medium reduced the inhibitory effect on evoked ^{3}H-NA release after 30 and 120 mins preincubation of the tissues with the cytokine (figure 3.9). IL-1β- induced suppression of EFS- (figure 3.9A) stimulated ^{3}H-NA release observed after 30 mins was attenuated by 48% ($P<0.01$) and 65% ($P<0.01$) in the presence of indomethacin and piroxicam, respectively; however, it failed to return the evoked ^{3}H-NA responses to within control levels, suggesting that the IL-1β-induced suppression of EFS-stimulated ^{3}H-NA release only partially involves cyclo-oxygenase metabolites. In contrast, results seen in response to KCl- (figure 3.9B) stimulation indicated an 88% ($P<0.001$) and 98% ($P<0.001$) (indomethacin and piroxicam, respectively) reduction in the IL-1β suppressive action, indicating a putative role of prostanoids in the suppression of KCl-stimulated noradrenaline release induced by IL-1β. No significant ($P>0.05$) difference was observed in the values for either EFS- or KCl-induced ^{3}H-NA release between tissues incubated with IL-1β plus piroxicam or indomethacin

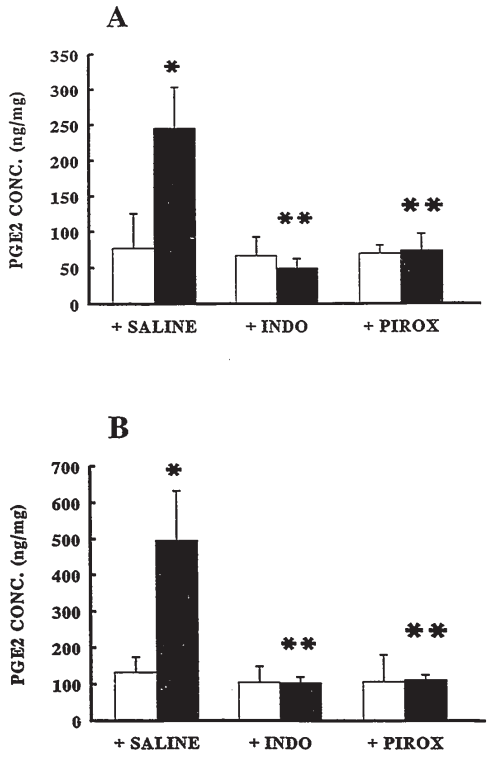


Figure 3.8: The effect of IL-1β on PGE_2 levels in LM-MP preparations. Tissues were preincubated for either 30 (A) or 120 (B) mins with saline (open bars) or IL-1β (solid bars) in the presence of either saline, piroxicam or indomethacin. Results are expressed as ng/mg tissue wt. and are the mean ± S.E. of 3 experiments. *$P<0.05$, represents a difference from control (saline), whereas **$P<0.05$, indicates a difference from IL-1β alone.

versus saline alone.

Exposure of the LM-MP preparations to IL-1β plus either indomethacin or piroxicam for 120 mins (Fig.11) caused an attenuation in the cytokine suppression of EFS-induced ^{3}H-NA release by 53% ($P<0.01$) and 90% ($P<0.01$). The presence of piroxicam and indomethacin in the incubation medium attenuated, although to different extent (not significant), the IL-1β-induced suppression of EFS stimulated noradrenaline after 120 mins. Similar results were observed in the attenuation of KCl-induced ^{3}H-NA release by IL-1β; 92% ($P<0.01$) and 99% ($P<0.01$), indomethacin and piroxicam, respectively. Furthermore, there was no significant ($P>0.05$) difference observed between the values for either EFS- or KCl-induced ^{3}H-NA release obtained from tissues incubated with IL-1β plus either piroxicam or indomethacin or saline alone.

3.3.2. Effect of TNFα on ^{3}H-NA release from LM-MP preparations.

Immediate effect of TNFα on ^{3}H-NA release.

As shown in figure 3.10, there was no change in either the basal or evoked ^{3}H-NA release from LM-MP preparations in response to TNFα. There was no significant ($P>0.05$) alteration in the basal release of ^{3}H-NA upon the addition of 50ng/ml TNFα (0.025±0.12%) to the superfusate when compared to corresponding saline (0.046±0.17%) control values. Simultaneous addition of TNFα (figure 3.10A) with either EFS- or KCl-stimulation also failed to show a significant change ($P>0.05$) in the evoked ^{3}H-NA release in the presence of TNFα (1.23±0.7% and 1.16±0.25%)

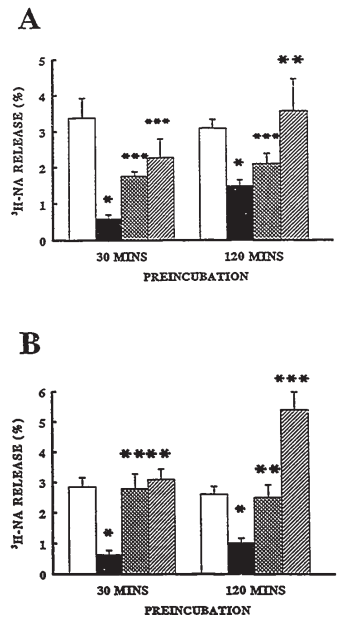


Figure 3.9: The effect of cyclo-oxygenase inhibitors and IL-1β on evoked release of ^{3}H-NA. Tissues were preincubated for either 30 or 120 mins with saline (open bars); IL-1β (solid bars); IL-1β plus piroxicam (crossed bars); or IL-1β plus indomethacin (hatched bars) before stimulation of ^{3}H-NA release with either EFS (A) or KCl (B). Results are the mean ± S.E. of 6 separate experiments. *P<0.01, represents a difference from the effect of control (saline), whilst **P<0.05, indicates a significant difference from IL-1β alone and ***P<0.05, represents a significant difference from both control and IL-1β values.

from those values observed from control preparations (figure 3.10B): 1.24±0.74% and 0.98±0.19%, EFS and KCl, respectively.

Effect of preincubation with TNFα on ^{3}H-NA release.

^{3}H-NA uptake. There were variations in the uptake of ^{3}H-NA observed at specified time points in response to preincubation of the tissues with saline (data not shown), however after a 150 mins preincubation of the tissue with the cytokine does not alter the uptake of ^{3}H-NA uptake; 17466±1308 vs 17265±284 dpm/mg tissue wt.; saline-treated and TNFα- treated tissues, respectively.

^{3}H-NA release. There were no changes in the basal release of ^{3}H-NA seen after 150 mins preincubation of the tissues in the presence or absence of 50ng/ml TNFα; 0.038±0.01% vs 0.039±0.013%, saline vs TNFα, respectively. In contrast, preincubation of LM-MP preparations with TNFα (50ng/ml) caused a marked suppression in evoked release of ^{3}H-NA (figure 3.11). Suppression of either EFS- (figure 3.11A) or KCl- (figure 3.11B) induced ^{3}H-NA release was evident after an incubation period greater than 30 mins, and upon longer incubation of the tissues with the cytokine a biphasic suppressive effect of TNFα was observed. The initial suppression in evoked release was seen after 30 mins exposure of the tissues to TNFα, and was maximal by 60 mins, which disappeared after 75 mins exposure of the tissue to the cytokine. However, after prolonged incubation of the cytokine (>90 mins) a recurrence of the suppressive effect of TNFα on evoked ^{3}H-NA release was found; EFS- and KCl-induced ^{3}H-NA release.

Concentration dependence. As shown in figure 3.12A, the smallest concentration of

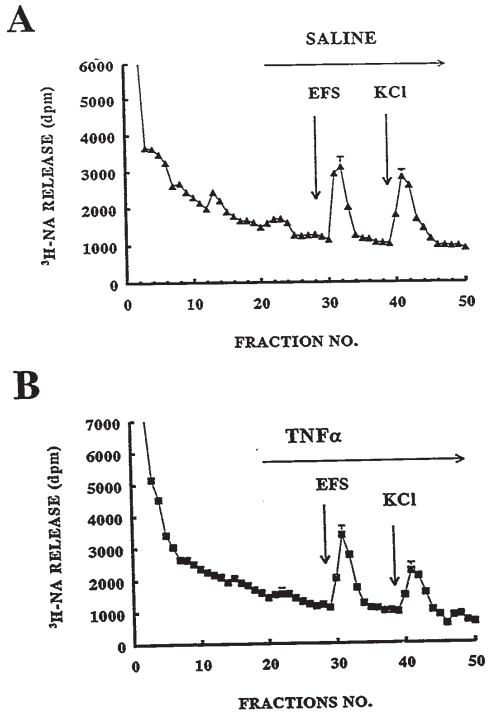


Figure 3.10: Immediate effect of TNFα on ^{3}H-NA release. Release of ^{3}H-NA from the tissues into the superfusate. A: after stimulation of EFS or 50mM KCl in the presence of saline. B: after stimulation by EFS or KCl in the presence of 50ng/ml TNFα. Each panel is a combination of 5 experiments; error bars represents a mean ± S.E. of ^{3}H-NA release; dpm/2ml superfusate fraction.

TNFα that suppressed EFS- induced ^{3}H-NA release was 0.1ng/ml, whereas, a maximal effective TNFα concentration of 50ng/ml, caused an 86.9±3.8% suppression of ^{3}H-NA release. In respect to KCl- (figure 3.12B) induced ^{3}H-NA release, the smallest effective TNFα concentration was 1ng/ml with the maximal effective TNFα concentration being 50ng/ml. Higher concentrations of TNFα produced sub-maximal suppressions of both EFS- and KCl-induced ^{3}H-NA release. Unless stated otherwise, future experiments examining the effect of 50ng/ml TNFα on ^{3}H-NA release involved a 120 min preincubation period.

Specificity of TNFα effect.

As shown in table 3.2, preincubation of the cytokine with a rabbit polyclonal anti-human TNFα antibody (1 unit/ml) for 20 mins at 37°C, prior to the 120 mins preincubation with tissues, successfully neutralized the suppressive effect of TNFα (50ng/ml) on EFS- (96%, P<0.001) and KCl- (>99%, P<0.001) induced ^{3}H-NA release. When the TNFα was boiled for 20 mins the cytokine's suppression of either EFS- or KCl-induced ^{3}H-NA release was also attenuated; 76% (P<0.01) and 93.9% (P<0.001), EFS and KCl, respectively, indicating that the presence of endotoxins in the recombinant cytokine were not responsible for the suppression of stimulated noradrenaline release.

Induction of protein synthesis by TNFα.

Preincubation of the LM-MP preparations with TNFα (50ng/ml) for short periods (<60 mins) was not associated with an increase in ^{35}S-methionine uptake. (figure 3.13A). However, after 90 mins incubation of the tissues with the cytokine there was

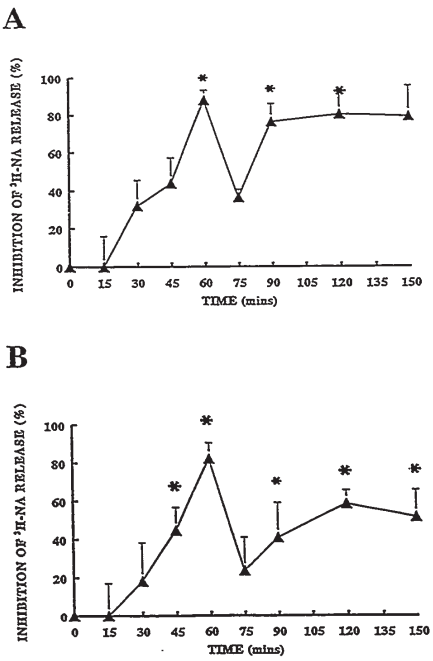


Figure 3.11: Time-dependence of TNFα effect on ^{3}H-NA release. Tissues were preincubated with 50ng/ml TNFα for 0-150 mins before stimulation of ^{3}H-NA release by EFS (A) or KCl (B). Inhibition of ^{3}H-NA release is expressed as a percentage inhibition of corresponding values obtained in absence of TNFα. Results are the mean ± S.E. of 4 experiments. *P<0.05

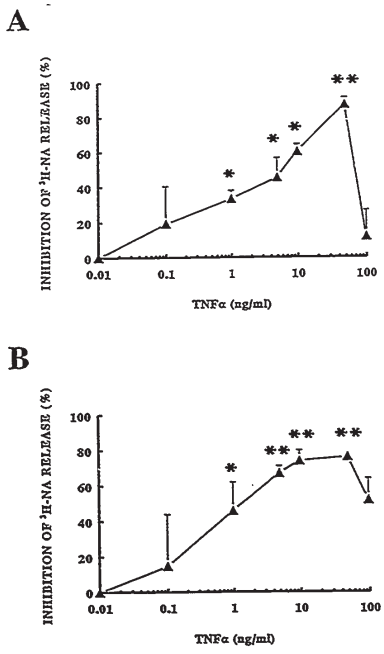


Figure 3.12: Concentration-dependence of TNFα effect on ^{3}H-NA release. Tissues were preincubated with identified concentrations of TNFα (0.01-100ng/ml) for 120 mins before stimulation of ^{3}H-NA release by EFS (A) or KCl (B). The data are ± S.E. of 4 experiments. *P<0.05, **P<0.001.

an 80% increase in ^{35}S-methionine uptake compared with saline-treated tissues. An 144% increase was seen after a 120 mins exposure of the LM-MP to the cytokine. The effects of this protein synthesis inhibitor cycloheximide on ^{35}S-methionine uptake and ^{3}H-NA release was examined (figure 3.13B). Co-incubation of the tissues with 100μg/ml cycloheximide plus TNFα for 120 mins caused an 80% reduction in the cytokine-induced uptake of ^{35}S-methionine. These results demonstrated the ability of TNFα to induce protein synthesis in LM-MP preparation from rat jejunum after 90 mins incubation. The ability of both IL-1β and TNFα to initiate rapid induction of protein synthesis has been shown in other systems (Dinarello, 1991).

The same concentration of cycloheximide was then used to examine the role of protein synthesis in TNFα-induced suppression of evoked ^{3}H-NA release. Co-incubation of LM-MP preparations with TNFα plus cycloheximide for 45 mins failed to alter the cytokine-induced suppression of either EFS- (figure 3.14A) or KCl- (figure 3.14B) stimulated ^{3}H-NA release. Since TNFα also failed to induce an increase in ^{35}S-methionine uptake, it was expected that the early suppressive effect of TNFα on stimulated noradrenaline release would be insensitive to cycloheximide (figure 3.13). Furthermore, incubation of the tissues with cycloheximide alone had no effect on EFS- or KCl-induced ^{3}H-NA release compared to saline controls at this time point.

In contrast, the late (120 mins) suppressive effect of TNFα on EFS- (figure 3.14A) induced ^{3}H-NA, was attenuated by 79±7.6%, P<0.001). Similarly, the effect of TNFα on KCl- (figure 3.14B) induced ^{3}H-NA release was reduced by 70.5±13.5%

Table 3.2: Specific effect of TNFα on ^{3}H-NA release from LM-MP preparations.

STIMULI	EFS (% ^{3}H-NA RELEASE)	KCl (50mM) (% ^{3}H-NA RELEASE)
CONTROL	3.21±0.17	2.07±0.4
TNFα	0.7±0.18*	0.66±0.1*
TNFα + TNFα ANTIBODY	3.07±0.77**	4.8±1.4**
BOILED TNFα	2.45±0.26**	1.9±0.4**

Tissue preparations were preincubated for 120 mins with saline, 50ng/ml TNFα alone, TNFα neutralized with anti-human TNFα antibody, or boiled TNFα prior to stimulation of ^{3}H-NA release by EFS or 50mM KCl. Results are the mean ± S.E. of 4 separate experiments, where * represents a significant (P<0.012) difference from control (saline) and ** indicates a significant (P<0.01) difference from the response to TNFα alone.

(P<0.01) in the presence of the protein synthesis inhibitor. Since there was an increase in protein synthesis after 120 mins induced in the LM-MP preparations by TNFα, these results suggest that the delayed (120 mins) suppressive action of this cytokine on evoked noradrenaline release is dependent on *de novo* protein synthesis. Furthermore cycloheximide incubated with tissues alone had no effect on EFS- (4.07±0.59%) or KCl- (2.87±1.08%) induced ^{3}H-NA release compared to saline-treated tissues (3.58±0.21% or 2.21±0.23%, EFS or KCl, respectively).

Effect of IL-1 receptor antagonist.

A selective IL-1 receptor antagonist was used to elucidate the involvement of endogenous IL-1 in the suppression of evoked ^{3}H-NA release caused by TNFα. As shown in figure 3.15, co-incubation of the receptor antagonist (10μg/ml) with TNFα for 45 mins had no effect on the ability of the cytokine to suppress ^{3}H-NA release by EFS (figure 3.15A). A similar result profile was obtained for KCl- (figure 3.15B) induced ^{3}H-NA release. However, upon examination of the delayed (120 mins) suppressive effect of TNFα on evoked ^{3}H-NA release, IL-1ra markedly attenuated the ability of TNFα to suppress both EFS- (figure 3.15A) and KCl- (figure 3.15B) induced ^{3}H-NA release by 98% (P<0.001) and 69% (P<0.001), respectively. These results suggests that the protein-dependent suppression of noradrenaline release observed after 120 mins in the presence of TNFα involves endogenous IL-1. Incubation of LM-MP preparations with IL-1ra alone failed to alter EFS- (2.61±0.11%) or KCl- (1.5±0.15%) induced ^{3}H-NA release when compared to saline-treated tissues; 3.21±0.3% or 2.29±0.1%, EFS or KCl, respectively.

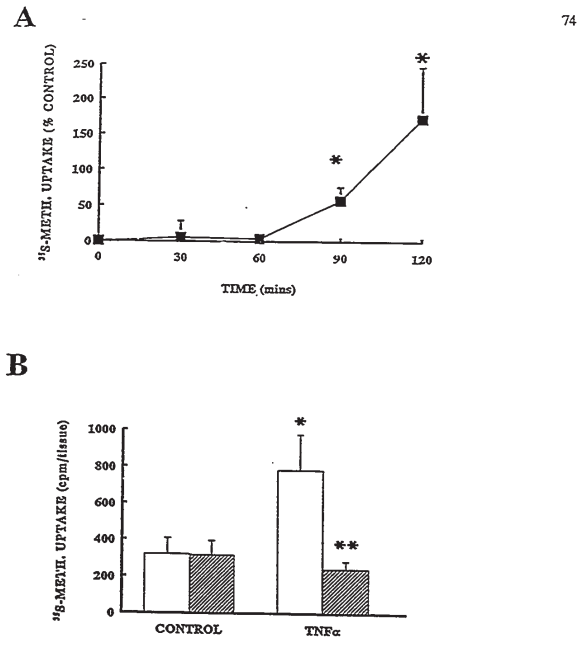


Figure 3.13: Effect of TNFα on ^{35}S-methionine uptake by LM-MP preparations. Panel A represents the time-dependent effect of TNFα (50ng/ml) on ^{35}S-methionine uptake of corresponding values obtained in the absence of TNFα (control). Results are the mean ± S.E. of 6 experiments, *P<0.05 indicates a difference from controls. Panel B represents the effect of cycloheximide on ^{35}S-methionine uptake. ^{35}S-methionine incorporation was measured in the presence (hatched bars) or absence (open bars) of cycloheximide. The data are expressed as cpm/mg tissue wt., where the results are the mean ± S.E. of 6 experiments. *P<0.01 indicates a difference from control (saline), whereas, **P<0.01 represents a difference from TNFα.

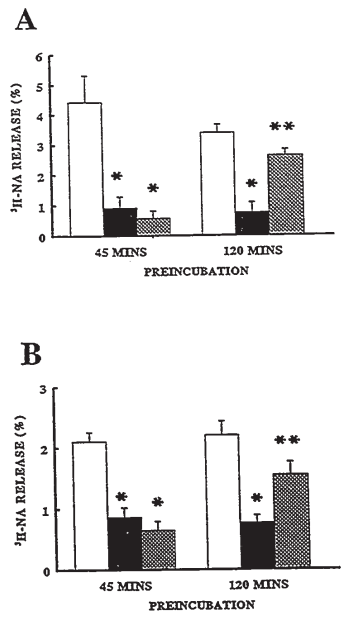


Figure 3.14: Effect of cycloheximide and TNFα on evoked release of ^{3}H-NA. Tissues were preincubated for 45 or 120 mins with buffer (open bars); TNFα (solid bars); or TNFα plus cycloheximide (crossed bars) before stimulation of ^{3}H-NA release by EFS (A) or KCl (B). Results are the mean ± S.E. of 5 separate experiments. *P<0.05, represents significant difference from control (saline), whereas, **P<0.01, indicates a difference from the effect of TNFα alone.

Role of prostaglandins.

In order to determine the role of prostaglandins in the TNFα suppressive effect on evoked ^{3}H-NA release (figure 3.16), we initially measured the levels of PGE_2. After a short incubation period (45 mins) of the LM-MP preparations with TNFα (50ng/ml), there was a significant ($P<0.05$) increase in PGE_2 concentrations compared to control levels (figure 3.16A). The elevated levels of this prostaglandin were completely abolished in the presence of either 1μM piroxicam or 10μM indomethacin. This early induction of the synthesis of such cyclo-oxygenase induced by TNFα implies that this process is independent of protein synthesis, since ^{35}S-methionine uptake by the LM-MP preparation was induced by TNFα only after 90 mins. Prolonged (120 mins) exposure of the LM-MP preparations to the cytokine caused a significant increase in PGE_2 levels (figure 3.16B). This increase in PGE_2 production induced by TNFα was prevented by the presence of either 1μM piroxicam or 10μM indomethacin in the medium. The induction of prostanoids by TNFα after 120 min incubation may be due to a protein synthesis dependent and independent mechanisms.

The concentrations of cyclo-oxygenase inhibitors, piroxicam (1μM) and indomethacin (10μM) used in this study prevented the increase in prostanoid levels in the LM-MP preparations after both a 45 min and 120 min incubation period with the TNFα. Therefore these concentrations of cyclo-oxygenase inhibitors were used to investigate the putative role of prostaglandins in TNFα-induced suppression of ^{3}H-NA release (figure 3.17). Both piroxicam and indomethacin attenuated the early (45

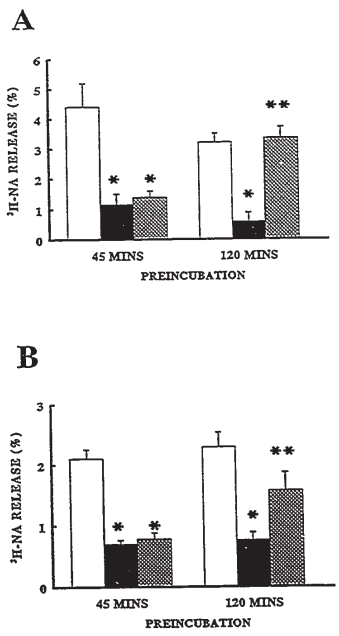


Figure 3.15: Effect of IL-1 receptor antagonist (IL-1ra) on evoked release of ^{3}H-NA. Tissues were preincubated with saline alone (open bars); TNFα (solid bars); IL-1ra (15 mins, 37°C) then TNFα (crossed bars) before stimulation of ^{3}H-NA release by EFS (A) or KCl (B). Results are the mean ± S.E. of 5 separate experiments. *P<0.05, represents a difference from control (saline), whereas,**P<0.0, indicates a difference from the effects of TNFα alone.

mins) TNFα- induced suppression of EFS- (figure 3.17A) induced ^{3}H-NA release by 65.3% ($P<0.01$) and 62.4% ($P<0.01$). These inhibitors also significantly reduced the cytokine's suppressive effect on KCl- (figure 3.17B) induced ^{3}H-NA release: 63.2% ($P<0.01$) and 74.3% ($P<0.01$), piroxicam and indomethacin, respectively. These results demonstrate that the early suppression of evoked noradrenaline release induced by TNFα is partly dependent on prostanoid synthesis. Furthermore, incubation of the tissues for 45 mins with either piroxicam or indomethacin in the absence of TNFα had no effect on EFS- or KCl-induced ^{3}H-NA release compared to saline controls (data not shown).

The effect of these cyclo-oxygenase inhibitors revealed a similar pattern with regards to the suppression of evoked ^{3}H-NA release following 120 mins incubation with TNFα (50ng/ml). Suppression of EFS- (figure 3.17A) induced ^{3}H-NA release by TNFα was completely blocked (>99%, $P<0.001$) in the presence of 1μM piroxicam and attenuated by 80% ($P<0.001$) with 10μM indomethacin added to the incubation mixture. Similarly, TNFα suppression of KCl- (figure 3.17B) induced ^{3}H-NA release was also attenuated by these cyclo-oxygenase inhibitors: 60% ($P<0.001$) by piroxicam and 97%, ($P<0.001$) by indomethacin, thus clearly indicating the involvement of prostanoid synthesis in TNFα-induced suppression of noradrenaline release from LM-MP layer of rat jejunum. EFS- or KCl-induced ^{3}H-NA release from tissues treated with piroxicam or indomethacin alone, respectively, was not significantly different from saline-treated tissues. Furthermore, the magnitude in the reduction of TNFα-induced suppression of evoked ^{3}H-NA release by both cyclo-

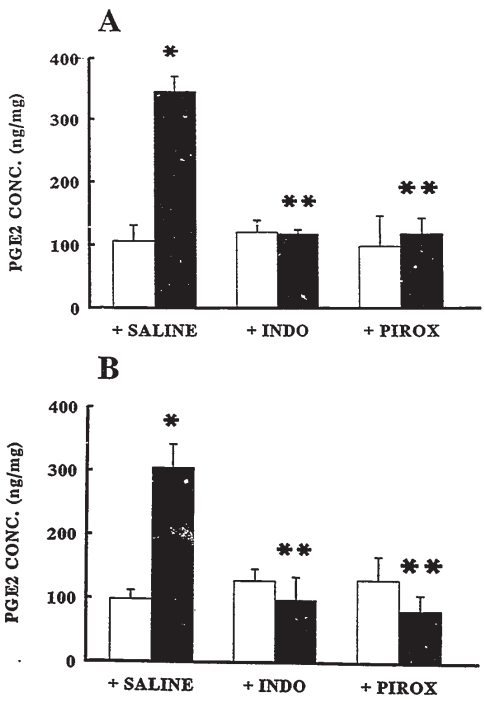


Figure 3.16: The effect of TNFα on PGE_2 levels in LM-MP preparations. Tissues were preincubated for either 45 (A) or 120 (B) mins with saline (open bars) or TNFα (solid bars) in the presence of either saline, piroxicam or indomethacin. Results are expressed as ng/mg tissue wt. and are the mean ± S.E. of 3 experiments. *P<0.05 represents a difference from controls (saline), whereas **P<0.05 indicates a difference from TNFα alone.

oxygenase inhibitors was not significantly different at either time point investigated.

3.4. Summary.

The LM-MP layer, a multicellular preparation, provides a model in which to explore the effect of inflammatory mediators on myenteric nerve function, where the interactions between exogenous cytokine administration and several cell types (including nerves) may be studied.

Both IL-1β and TNFα failed to alter either baseline or stimulated ^{3}H-NA release upon addition to the superfusate and had no effect on either the basal release or uptake of ^{3}H-NA over 150 mins exposure to each cytokine. However, following preincubation with the cytokines, both IL-1β and TNFα induced a biphasic suppression of evoked ^{3}H-NA, which resulted in an early (<60 mins) suppressive effect that was protein synthesis independent and a delayed (>90 mins) suppressive effect that was dependent on protein synthesis.

The suppressive actions of both IL-1β and TNFα were not due to endotoxin contamination of the recombinant protein (Stanley et al.,1992), since their inhibitory effects were removed upon boiling the cytokines for 20 mins prior to addition to the tissue (Freuenberg and Galanos, 1990). Removal of the cytokine's inhibitory effect with neutralizing antibodies also prevented both IL-1β and TNFα from suppressing stimulated ^{3}H-NA release, illustrating the specificity of the responses.

Examination of the underlying mechanism revealed that co-incubation of the tissues with either IL-1β or TNFα plus IL-1 receptor antagonist completely reversed

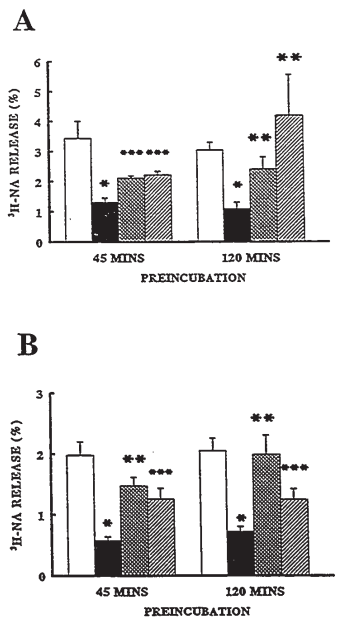


Figure 3.17: The effect of cyclo-oxygenase inhibitors and TNFα on evoked release of ^{3}H-NA. Tissues were preincubated for either 45 or 120 mins with saline (open bars); TNFα (solid bars); TNFα plus piroxicam (crossed bars) or TNFα plus indomethacin (hatched bars) before stimulation of ^{3}H-NA release with either EFS (A) or KCl (B). Results are the mean ± S.E. of 6 experiments. *P<0.01 represents a difference from the effect of control (saline), whilst **P<0.05 indicates a significant difference from TNFα alone and ***P<0.0 represents a significant difference from both control and TNFα values.

the inhibitory effect of both cytokines after 120 mins incubation, whereas only the IL-1β suppressive effect was abolished after a short exposure (30 mins). Furthermore, both IL-1β and TNFα were capable of inducing piroxicam- and indomethacin-sensitive prostaglandin synthesis after either short and prolonged incubation with the LM-MP preparations. The presence of these cyclo-oxygenase inhibitors in the incubation medium together with either IL-1β or TNFα caused a reduction in their ability to suppress stimulated ^{3}H-NA release.

3.5. Conclusion.

These results indicate that both IL-1β and TNFα suppress stimulated noradrenaline release from noradrenergic nerves. The data observed after a short exposure of the LM-MP to both cytokines suggests that the underlying mechanisms may involve either a direct interaction with noradrenergic nerves or more probably the induction of mediators, such as prostaglandins, which in turn alter noradrenergic nerve function. However, on prolonged exposure of the cytokines, it appears that endogenous IL-1 mediates the changes in noradrenaline release induced by exogenous addition of either IL-1β or TNFα.

The results from these experiments raise the question as to whether IL-1β and possibly TNFα alter noradrenaline release by directly interacting with noradrenergic nerves located within the myenteric plexus of the rat small bowel ?

CHAPTER 4

MEASUREMENT OF NORADRENALINE RELEASE FROM MYENTERIC NERVE VARICOSITIES FROM RAT SMALL INTESTINE

4.1 Introduction.

The release of neurotransmitters from nerve terminals was originally explored using synaptosomal preparations from various regions of the brain (Blaustein, 1975, 1979; DeLangen et al.,1979). Isolation of synaptosomes was achieved with relative ease and proved to be a most successful approach for monitoring neurotransmitter release from the nerve terminals.

Isolated nerve varicosities from the myenteric plexus have been used to characterize the release of several neurotransmitters (Jonakait et al.,1979; Dowe et al.,1980; Briggs and Cooper,1981, McDonald et al.,1989), including the co-release of neurotransmitters / neuropeptides (Hammond et al.,1988;). A popular technique for measuring neurotransmitter release from synaptosomes, involves the superfusion of radio-labelled neurotransmitter loaded synaptosomes (DeLangen et al.,1979). The application of this technique to the measurement of neurotransmitter release from the nerve terminals in the gut has not been used extensively. Most groups have developed other methods, determined by the type of neurotransmitter measured

(Briggs and Cooper,1981; Hammond et al.,1988; Jonakait et al.,1979). Such studies by Hammond and co-worker have measured the release of noradrenaline from nerve varicosities from the guinea pig ileum using a technique which involves loading the varicosities with tritiated noradrenaline and measuring the neural stimulated release of ^{3}H-noradrenaline into the supernatant (Hammond et al.,1988).

In this study, the aim was to monitor the uptake and release of noradrenaline from nerve varicosities in response to cytokine administration. In these experiments, a crude preparation of nerve varicosities of the rat small bowel myenteric plexus was used. Although the effect of inflammatory mediators on neurotransmitter release has been previously studies in synaptosomes prepared from the hypothalamus (Wedel et al.,1978), their effect on neurotransmitter release from the nerve terminals of myenteric plexus of the small intestines has not been examined.

4.2. Methods.

4.2.1. Development of the myenteric nerve varicosity preparation.

A crude nerve varicosity preparation was employed in my studies as in our experience and that of others (Hammond et al.,1988; Briggs and Cooper, 1981), a more refined preparation is associated with loss of biological activity. Longitudinal muscle-myenteric plexus preparations were isolated from the rat small intestine, as described by Collins et al (1989), and placed into 0.32M sucrose on ice. Nerve varicosities were prepared from the myenteric plexus by a method adapted from that of Hammond and colleagues (1988). The tissue was minced with scissors and

homogenized for 3 x 5 secs, then centrifuged for 10 mins at 1,000xg, 4°C. The supernatant was stored on ice and the pellet washed with 0.32M sucrose. The supernatants were pooled, filtered through gauze and centrifuged for 50 mins at 20,000xg, 4°C. The pellet containing the varicosities was resuspended in Locke's buffer containing (in mM): NaCl,140; KCl,5; $NaHCO_3$,5; $MgCl_2$,1; $CaCl_2$,2.5; glucose,10; HEPES,10; EDTA,0.004; ascorbic acid,0.11; and pargyline (a monoamine oxidase inhibitor),0.03; at a protein concentration of 1mg/ml (Lowry et al,.1951). The varicosities were allowed to rehydrate at room temperature (24°C) for 30 mins with oxygen "blowing" gently over the surface.

4.2.2. ^{3}H-NA uptake by the varicosity preparation.

Optimal ^{3}H-NA uptake. The optimal conditions required for maximal ^{3}H-NA uptake by the nerve varicosities were determined by calculating the active ^{3}H-NA uptake over time at either 37°C or 24°C. The ^{3}H-NA uptake was measured in the presence or absence of a metabolic inhibitor, dinitrophenol (DNP), which provided passive or total ^{3}H-NA uptake by the varicosities, respectively. DNP prevents ATP production, required for the active transport mechanism, by uncoupling the electron transport chain from the oxidative phosphorylation pathway in the mitochondria, which is the primary source of ATP in this preparation (Stryer, 1988). The nerve varicosity preparation was initially preincubated for 5 mins at either 37°C or room temperature (24°C) prior to the measurement of ^{3}H-NA uptake. The preparation was then divided in two, where one half was pre-treated with dinitrophenol (50μM) and the

other treated with the equivalent volume of saline, followed by the addition of 0.1μCi/ml ^{3}H-NA. Next the varicosity preparations were then incubated at either 37°C or 24°C. At specified times over a 60 min incubation period, 100μl aliquots of the preparation were removed and immediately filtered. The pellet collected on the filters was then solubilized with protosol and counted for ^{3}H in a scintillation counter.

The results were calculated as percentage ^{3}H-NA uptake of baseline uptake occurring immediately after the addition of ^{3}H-NA to the varicosity preparation, at specified temperatures examined, and expressed as either total or active ^{3}H-NA uptake. Active uptake was calculated as follows:

ACTIVE UPTAKE = TOTAL UPTAKE - PASSIVE UPTAKE.

Specific ^{3}H-NA uptake. Specific uptake of ^{3}H-NA by adrenergic nerves in the varicosity preparation was determined by the presence of a noradrenaline uptake inhibitor, desipramine. Varicosities were incubated in either the presence or absence of 50μM desipramine for 20 mins at 37°C, 100μl aliquots of the varicosities were then filtered. The pellets collected on the filters were then solubilized and counted for ^{3}H.

The data were expressed as dpm/mg protein, with the specific ^{3}H-NA uptake calculated as the difference between values obtained from ^{3}H-NA uptake in the presence and absence of desipramine during the incubation period.

4.2.3. Release of ^{3}H-NA from myenteric nerve varicosities.

After the nerve varicosity preparation was loaded with ^{3}H-NA, it was washed twice with Locke's buffer and resuspended in the same buffer containing 50μM desipramine; this prevented the re-uptake of ^{3}H-NA. Prior to the ^{3}H-NA release studies, the varicosities were allowed to recover from the washing procedure at room temperature (24°C) for 20 mins.

Optimal ^{3}H-NA release. To determine the optimal stimulated release of ^{3}H-NA, stimuli, KCl (75mM) and ionomycin (10μg/ml) were used. 10μl of saline or stimulant was added to 500μl of the varicosity preparation, this mixture was then incubated at either 37°C or room temperature (24°C) for specified times (0-30 mins). Duplicate 200μl aliquots were centrifuged using a bench top centrifuge at 11,500xg for 5 mins. 150μl of the supernatants were aspirated and added to 2ml of aqueous counting solution and counted for ^{3}H in a scintillation counter. The presence of noradrenaline in the supernatant was detected by thin layer chromatography (TLC), as described by Lehman (1983), see figure 2.3.

The release of ^{3}H-NA was calculated as dpm/mg protein and the stimulated release of ^{3}H-NA expressed as a percentage over basal release (saline-induced ^{3}H-NA release).

Selective ^{3}H-NA release. KCl- (0.5-75mM) and ionomycin- (0.1-10μg/ml) stimulated ^{3}H-NA release were used to determine:

(1) specific ^{3}H-NA release from adrenergic nerve varicosities. 50μM desipramine was added to the preparation prior to the loading of varicosities with ^{3}H-NA and left in the medium throughout the rest of the experiment.

(2) dependence of ^{3}H-NA release on calcium. 2mM EGTA (chelating agent) was added to the buffer after the varicosities were loaded with ^{3}H-NA.

Scorpion venom (0.1-100μg/ml) in the presence or absence of tetrodotoxin (1μM) was used to demonstrate the role of sodium channel activation.

4.2.4. Measurement of lactate dehydrogenase (LDH) activity.

The activity of a cytoplasmic enzyme, lactate dehydrogenase (LDH), was used as a marker of intact membranes within the varicosity preparation. LDH activity was measured by using a technique reported by White et al (1982). Briefly, 100μl 22.7mM sodium pyruvate (in phosphate buffer) was added to a silicon coated cuvette containing (in final concentrations): 2.8ml 100mM potassium phosphate buffer; 50mM sodium chloride and 0.5mM NADH. To start the reaction, 100μl of the varicosities supernatant was added to the cuvette and the decrease in absorbance at 340mm was monitored in a visual/UV dual spectrophotometer (Model-Response, Gilford Institute) against a phosphate buffer blank. The LDH (unit activity) activity was calculated as the change in absorbance per min x 1000, and expressed as unit LDH activity / mg of protein. The LDH activity was also recorded after disruption of the membranes in the preparation with the addition of 10μl 10% triton X100. The "occluded" (amount contained within the cytoplasm) LDH activity was calculated as the difference between activity in the varicosity preparation before and after the addition of triton X100.

4.2.5. Electron microscopy analysis of the nerve varicosity preparations.

This technique was performed by Ms. Sarka Lhotak from the Intestinal Diseases Research Program (McMaster University, Ontario) and is described in appendix 2.

4.2.6. Statistical analysis.

All studies involved at least 4 separate experiments and are expressed as mean ± S.E. The actual number of experiments is indicated in the figure legend for each experiment. Statistical significance was inferred at $P<0.05$ using the Student's t test or by applying a one way analysis of variance in experiments comparing more than two means.

4.2.7. Materials.

Sprague Dawley rats (200-250g) were obtained from Charles River Breeding Farms (Montreal, Quebec); ^{3}H-NA (sp.act. 13.3Ci/mM) was obtained from New England Nuclear (Boston, MA); and the rest of the chemicals were supplied by Sigma Chemical (St.Louis, MO).

4.3. Results.

4.3.1. Analysis of the myenteric nerve varicosity preparation.

Electron microscopic analysis of the crude varicosity preparation demonstrated the presence of neural vesicles of various sizes (figure 4.1). As expected, since this

was purposely a crude preparation, mitochondria were present, both within the vesicles and also free in the medium. Electron lucent membranes were also present, varying in size and shape. Furthermore, the presence of enclosed membranes was also determined biochemically, by measuring the occluded activity of lactate dehydrogenase (LDH) after disruption of the membranes with the detergent, triton-X100. As shown in table 4.1, the baseline LDH activity increased by 150% after the addition of triton-X100 to the varicosity preparation. Occluded LDH was calculated (Table 1) as the difference between LDH activity before and after the addition of triton-X100.

4.3.2 ^{3}H-NA uptake by the myenteric varicosity preparation.

Uptake of ^{3}H-NA by the myenteric varicosity preparation was time-dependent and significantly ($P<0.05$) greater at 37°C than 24°C. As shown in figure 4.2A, incubation of the varicosities at 24°C resulted in an overall small increase in the total ^{3}H-NA, with maximum uptake observed after 30 mins. In contrast, incubation of the preparations at 37°C caused a rapid time-dependent increase in the total ^{3}H-NA uptake which showed a 148.2±23.9% ($P<0.01$) increase above control after 15 mins, and remained elevated over the next 50 mins (159.7±16.4%, $P<0.05$).

Subtraction of the values observed for passive ^{3}H-NA uptake from total ^{3}H-NA uptake within one experiment determined active ^{3}H-NA uptake. Expression of the results as active ^{3}H-NA uptake in experiments performed at 37°C, the first 20 mins showed a rapid uptake in noradrenaline by the varicosities (figure 4.2B),

whereas after this time, ^{3}H-NA uptake by the varicosities was mainly due to passive diffusion. Examination of the active uptake calculated for varicosities incubated at 24°C failed to show an increase in ^{3}H-NA uptake, indicating that uptake was mainly passive (figure 4.2B). Therefore, in the subsequent experiments, ^{3}H-NA uptake by the varicosity preparation was performed at 37°C for 20 mins to obtain maximal loading.

The total uptake of ^{3}H-NA by the preparation consisted of a non-specific component plus adrenergic-specific, desipramine-sensitive uptake. In the presence of the noradrenaline uptake inhibitor, desipramine, the total ^{3}H-NA uptake of 2440±80.2 dpm/mg protein was reduced to 1106±188.6 dpm/mg protein, indicating that 58% ($P<0.01$) of the total uptake was specific for adrenergic nerves. This was expected since other membranes; for example smooth muscle membranes and mitochondria presence within the varicosity preparation are also capable of taking up ^{3}H-NA.

4.3.3. ^{3}H-NA release by the myenteric nerve varicosity preparation:

Considerable variation has been seen in the expression of stimulated noradrenaline release from intestinal nerve varicosities by other groups (Hammond et al.,1988; Briggs and Copper, 1981; Jonakait et al.,1979). In this study I also found considerable variation in the basal ^{3}H-NA release between individual experiments and this range varied between 1200-16,000 dpm/150μl supernatant. Since there was a linear relationship between basal release and the amount of protein in the

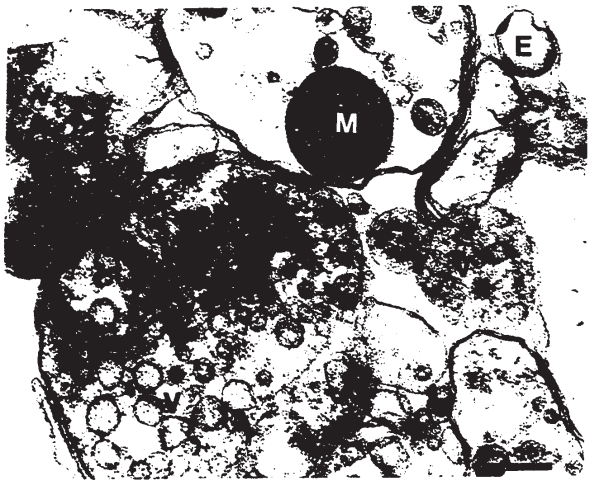


Figure 4.1: Electron micrograph of the rat myenteric varicosity preparation. Examination of the crude myenteric varicosity preparation showed terminal varicosities containing vesicles of different morphologies (V). Other structures present include mitochondria (M), which are seen free and within some nerve varicosities and electron-lucent membranes (E). Bar = 0.2μm.

preparation (data not shown), I decided to express stimulated ^{3}H-NA release as a percentage increase above the basal ^{3}H-NA release obtained from individual experiments.

Although there was no significant difference in stimulated ^{3}H-NA release ($P<0.05$) when measured at either room temperature or 37°C after short (<10 mins) incubations, the values obtained for KCl-induced ^{3}H-NA release after this time were significantly ($P<0.05$) greater at 24°C than those observed at 37°C (figure 4.3A). Similarly, ionomycin- (figure 4.3B) induced ^{3}H-NA release after 20 mins was greater when the varicosities were incubated at room temperature (24°C). In the following experiments stimulated ^{3}H-NA from this myenteric nerve varicosity preparation was measured after a 20 mins incubation period at 24°C.

All stimuli (KCl, ionomycin and scorpion venom) caused a concentration-dependent increase in ^{3}H-NA release. Maximum KCl-induced ^{3}H-NA release was observed using 75mM (figure 4.4A). This concentration of KCl was then used to examine the selectivity of evoked ^{3}H-NA release. As shown in figure 4.4B, KCl-induced ^{3}H-NA release was completely blocked in the presence of desipramine (98%, $P<0.001$) in the incubation medium, and reduced by 88% ($P<0.001$) in the absence of calcium. The calcium ionophore, ionomycin, caused a dose dependent (0.001-100μg/ml) increase in evoked ^{3}H-NA release. Maximum ^{3}H-NA release induced by ionomycin was observed with 10μg/ml (figure 4.5A). This concentration of ionomycin was then applied to determine the selectivity of ^{3}H-NA release. In agreement with the results obtained for KCl-induced ^{3}H-NA release, ionomycin-

Table 4.1: Measurement of occluded lactate dehydrogenase (LDH) activity from myenteric varicosity preparation.

	LDH ACTIVITY (UNITS/mg/ml protein)
BASAL ACTIVITY (Pre-Triton-X100)	88.2±24
FINAL ACTIVITY (Post-Triton-X100)	222.9±31
OCCLUDED ACTIVITY	134.7±27.6

LDH activity was measured before and after the addition of Triton-X100 to the varicosity preparation. Occluded LDH activity refers to the amount of LDH activity within enclosed membranes and is calculated as the difference between pre and post Triton-X100 treatment. LDH activity is expressed as units/mg protein, with results portrayed as the mean ± S.E. of 5 separate experiments.

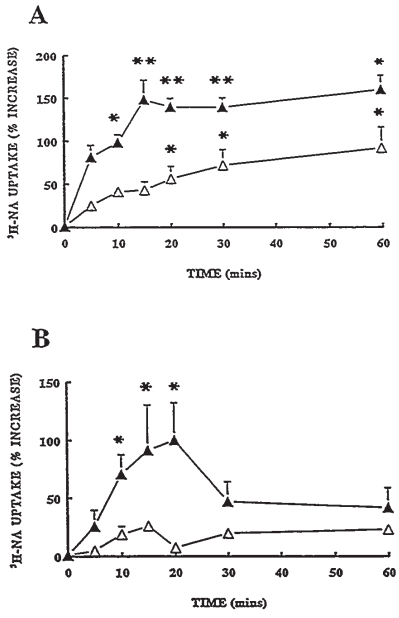


Figure 4.2: Uptake of ^{3}H-NA by the myenteric nerve varicosity preparation. ^{3}H-NA uptake by the varicosity preparation was measured at specified times over 60 mins at either 37°C (▲-▲) or 24°C (Δ-Δ). Panel A represents the total ^{3}H-NA uptake by the preparation, whereas Panel B shows active ^{3}H-NA uptake by the preparation. The data are expressed as percentage ^{3}H-NA uptake over ^{3}H-NA uptake occurring at time zero for each temperature examined. The results are the mean ± S.E. of 4 separate experiments, where *P<0.05 and ** P<0.01, represent a difference from the values obtained a time zero.

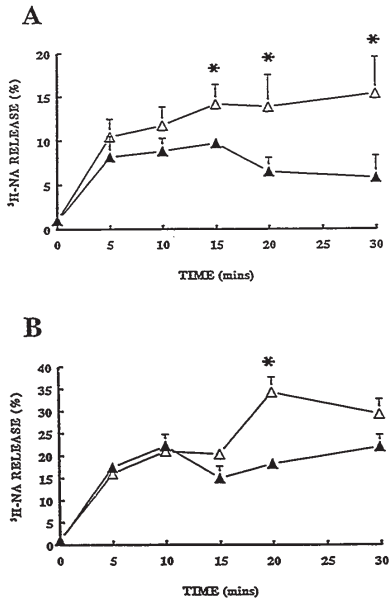


Figure 4.3: Release of ^{3}H-NA by the myenteric nerve varicosity preparation. KCl- (A) or ionomycin (B) induced ^{3}H-NA release was measured at specified times over 30 mins at either 37°C (▲-▲) or 24°C (Δ-Δ). The data are calculated as percentage ^{3}H-NA release above basal release and are the mean ± S.E. of 4 experiments separate experiments. *P<0.05, represents the difference in stimulated ^{3}H-NA release values seen at a particular time point.

(figure 4.5B) induced ^{3}H-NA release was completely blocked by both the presence of desipramine (90%, P<0.001) and absence of calcium (95%, P<0.001) in the incubation medium.

Another stimulus investigated was the neurotoxin, scorpion venom which opens sodium channels. This stimulant caused a concentration-dependent increase in ^{3}H-NA release from the varicosity preparation, as shown in figure 4.6A. Maximum evoked release was not reached, even with 100μg/ml scorpion venom, so the specific action of the stimulus was investigated using a concentration, 10μg/ml scorpion venom, showing similar values in evoked ^{3}H-NA release compared to those observed with KCl (75mM). To determine the specificity of scorpion venom effect on sodium channels, another toxin, tetrodotoxin, (which closes sodium channels) was used. As shown in figure 4.6B, 10μg/ml scorpion venom-induced ^{3}H-NA release was completely abolished in the presence of 1μM tetrodotoxin.

4.4. Summary.

The myenteric nerve varicosity preparation from rat small intestine was examined with electron microscopy. Biochemical examination of the preparation was limited to the measurement of "occluded" LDH activity, which although crude, did indicate the presence of enclosed plasma membranes within the varicosity preparation.

Uptake of noradrenaline by the varicosity preparation was maximum after 20 mins incubation with ^{3}H-NA at 37°C. The use of a selective noradrenaline uptake

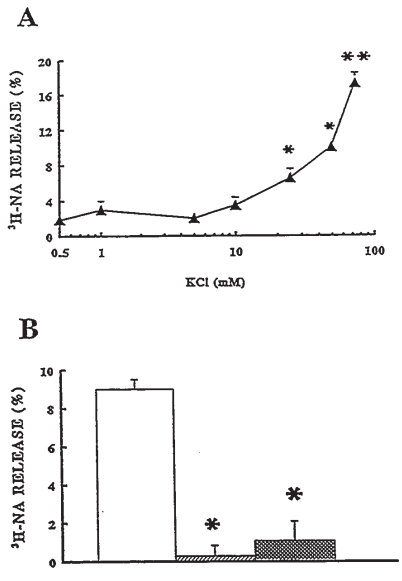


Figure 4.4: KCl-stimulated ^{3}H-NA release from myenteric nerve varicosity preparation. Panel A represents the concentration-dependence of KCl- (0.5-75mM) induced ^{3}H-NA release from the varicosities. Results are expressed as percentage ^{3}H-NA release above basal release and are the mean ± S.E. of 8 separate experiments. * and ** represents a significant (<0.05 and <0.01, respectively) difference from basal ^{3}H-NA release. Panel B shows KCl- (75mM) induced ^{3}H-NA release from varicosities following simultaneous incubation with saline (open bar); 50μM desipramine (hatched bar); or 2mM EGTA (crossed bar). Results are expressed as percentage ^{3}H-NA release above basal release and are the mean ± S.E. of 5 experiments. * indicates significant (P<0.001) difference from saline controls.

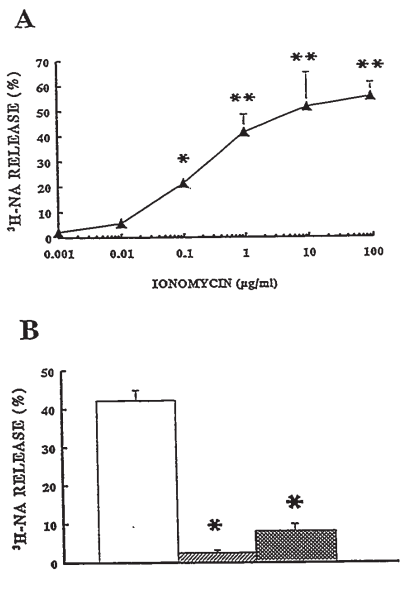


Figure 4.5: Ionomycin-stimulated ^{3}H-NA release from myenteric nerve varicosities. Panel A represents the concentration-dependence of ionomycin- (0.001-100μg/ml) induced ^{3}H-NA release from the myenteric varicosities. The results are calculated as percentage ^{3}H-NA release above basal (saline) release and are the mean ± S.E. of 7 experiments. * and ** indicates significant (P<0.05 and P<0.001, respectively) difference from basal ^{3}H-NA release. Panel B shows ionomycin- (10μg/ml) induced ^{3}H-NA release from varicosities following simultaneous incubation with saline (open bar); 50μM desipramine (hatched bar); or 2mM EGTA (crossed bar). Results are expressed as percentage ^{3}H-NA release above basal release and are the mean ± S.E. of 6 experiments. * represents a significant (P<0.001) difference from control (saline).

inhibitor, desipramine, demonstrated that the specific uptake of ^{3}H-NA by adrenergic varicosities was only 58%. However, stimulated ^{3}H-NA release, performed at 24°C, was completely blocked in the presence of this inhibitor, indicating the ^{3}H-NA was released selectively from adrenergic varicosities.

A significant increase in ^{3}H-NA release when stimulated with either KCl, ionomycin or scorpion venom was shown when the data were expressed as a percentage increase over the basal ^{3}H-NA release. Maximum evoked ^{3}H-NA release was observed after 20 mins incubation of the stimulant with the loaded varicosity preparation; KCl, ionomycin and scorpion each caused a concentration-dependent increase in ^{3}H-NA from the varicosity preparation.

4.5. Conclusion.

Although the varicosity preparation used in this study was crude, the results clearly demonstrate the presence and integrity of nerve varicosities capable of taking up and specifically releasing noradrenaline from nor-adrenergic varicosities, following activation of sodium channels by scorpion venom, or following the calcium-dependent stimulation by KCl or ionomycin. This varicosity preparation was therefore applied to investigate the question of whether the IL-1β and TNFα altered noradrenaline release from the LM-MP preparation of rat small bowel involved a direct interaction with adrenergic nerve varicosities.

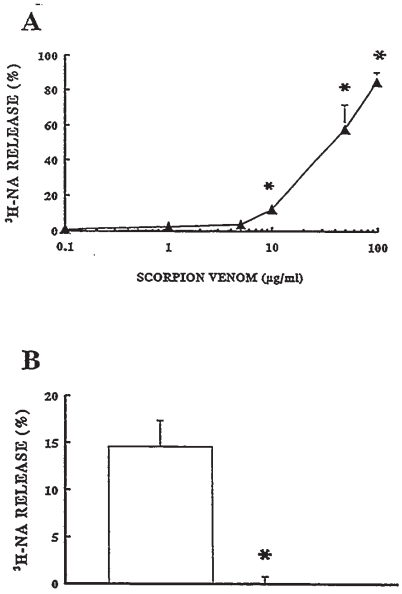


Figure 4.6: Scorpion venom-induced ^{3}H-NA release from the varicosity preparation. Panel A shows the concentration-dependence of scorpion venom- (0.1-100µg/ml) induced ^{3}H-NA release from varicosity preparation. The results are expressed as percentage ^{3}H-NA release above basal release and are the mean ± S.E. of 4 experiments. * indicates a significant (P<0.01) difference from basal ^{3}H-NA release. Panel B represents scorpion venom- (10µg/ml) induced ^{3}H-NA release from varicosities following simultaneous incubation with saline (open bar) or tetrodotoxin (filled bar). The data are expressed as percentage above basal release and are the mean ± S.E. of 5 separate experiments. * represents a significant (P<0.001) difference from saline controls.

CHAPTER 5

EFFECT OF INTERLEUKIN-1β (IL-1β) AND TUMOUR NECROSIS FACTOR-α (TNFα) ON NORADRENALINE RELEASE FROM MYENTERIC NERVE VARICOSITIES

5.1. Introduction.

The results from chapter 3, clearly demonstrate the ability of both IL-1β and TNFα to suppress noradrenaline release from the longitudinal muscle-myenteric plexus layer of non-infected rats. The suppressive effect of either cytokine on noradrenaline release was biphasic in nature with the appearance of an early suppressive effect occurring within 60 minutes, which was independent of protein synthesis. The late suppressive actions (>90 mins) on noradrenaline release of IL-1β and TNFα appeared to involve the induction of endogenous IL-1. On the basis of these observations it can be hypothesised that IL-1β and possibly TNFα may independently interact with adrenergic nerve axons of the myenteric plexus causing a direct suppression of noradrenaline release. This hypothesis is supported by the previously demonstrated presence of IL-1 and TNFα receptors on neural tissue in other systems (Farrar et al.,1987; Dinarello, 1989; Haour et al.,1990; Hart et

al.,1993). However, because of the multicellular nature of the LM-MP preparation the possibility that the suppression of noradrenaline release reflected the action of an unidentified mediator on adrenergic nerves cannot be excluded. Also in the gut there is also the possibility that there are receptors for these cytokines on nerve cell bodies, where the cytokines may modulate not only the release of neurotransmitters, but also their synthesis.

This study was concerned with evaluating the possibility that IL-1β and TNFα act on myenteric adrenergic nerves within the preparation to suppress noradrenaline release. A preparation of nerve varicosities from the myenteric plexus, as used by other groups, was adopted to investigate the possibility that IL-1β and TNFα interact with noradrenergic nerves to cause a suppression of noradrenaline release.

5.2. Methods.

5.2.1. Measurement of ^{3}H-NA release from myenteric nerve varicosities.

See chapter 4.2.

5.2.2. Effect of IL-1β and TNFα on ^{3}H-NA release from myenteric nerve varicosities.

Immediate effect of IL-1β and TNFα.

Basal (saline) or stimulated (75mM KCl or 10µg/ml scorpion venom) ^{3}H-NA release from varicosities was measured after the addition of either saline, IL-1β

(10ng/ml) or TNFα (50ng/ml) to the varicosity preparation.

Preincubation of varicosities with IL-1β or TNFα.

Myenteric nerve varicosities were incubated with IL-1β (0.01-10ng/ml) or TNFα (50ng/ml) for specified times prior to stimulation of ^{3}H-NA release. Included within the specified preincubations was a 20 min period in which the varicosities were loaded with ^{3}H-NA. At the end of the preincubation, the varicosities were washed twice with Locke's buffer, containing 10μM desipramine, at 4°C and centrifuged for 20 mins at 20,000xg. The varicosities were then resuspended in the Locke's buffer at a protein concentration of 1mg/ml and measured for ^{3}H-NA release in the presence of saline, KCl (75mM) or scorpion venom (10μg/ml) at 24°C.

Specific action of IL-1β.

The purity of the recombinant cytokine with respect to possible endotoxin contamination was tested by boiling for 20 mins prior to incubation with the varicosity preparation. The specificity of the action of IL-1β was determined by preincubating the cytokine with a polyclonal rabbit anti-human IL-1β antibody (1:10 dilution) at 37°C for 20 mins, before the subsequent incubation with the varicosities. Control experiments involved preincubation of the varicosities with either saline or IL-1β.

Assessment of IL-1β and TNFα toxic effect.

Measurement of LDH activity in the varicosity preparation has previously been described in chapter 3.2.

Effect of IL-1β and TNFα. In initial experiments, the LDH activity of the preparation was measured immediately before and after the addition of either IL-1β (10ng/ml) or TNFα (50ng/ml) to the varicosities. LDH activity was also measured in the varicosity supernatant after 50 mins incubation with either saline, IL-1β (10ng/ml) or TNFα (50ng/ml).

Presence of IL-1 receptors.

To determine whether the effect of IL-1β was receptor mediated, the varicosities were preincubated with 10μg/ml IL-1 receptor antagonist (IL-1ra) at 37°C for 15 mins, before addition of IL-1β to the incubation mixture. Control experiments involved incubation of the varicosities with equal volumes of saline, IL-1β or IL-1ra alone.

Role of prostaglandins.

Inhibition of prostaglandin synthesis was achieved by using cyclo-oxygenase inhibitors, piroxicam (1μM) or indomethacin (10μM) in the incubation medium. These concentrations of cyclo-oxygenase inhibitors were then used to examine the role of prostaglandins in the effect of the cytokine effect on ^{3}H-NA release. The varicosity preparation was simultaneously preincubated with either 1μM piroxicam or 10μM indomethacin together with IL-1β for 50 mins. Control experiments received equal volumes of saline, IL-1β, piroxicam or indomethacin alone.

5.2.3. Statistical analysis.

All studies involved at least 4 separate experiments and are expressed as mean

± S.E. The actual number of experiments is indicated in the figure legend for each experiment. Statistical significance was inferred at $P<0.05$ using the Student's t test or by applying a one way analysis of variance in experiments comparing more than two means.

5.2.4. Materials.

Sprague Dawley rats (200-250g) were obtained from Charles River Breeding Farms (Montreal, Quebec); ^{3}H-NA (sp.act. 13.3Ci/mM) was obtained from New England Nuclear (Boston, MA); IL-1β was from Sclavo (Sienna,Italy) and Upstate Biotechnology Incorporation (Lake Placid, NY); Rabbit anti-human IL-1β was a generous gift from Dr.R.Newton of DuPont (Glenolden, PA); the IL-1 receptor antagonist was generous gift from Dr.R.Thompson of Synergen (Denver, CO); human recombinant TNFα was supplied by R&D systems (Minneapolis, MN); and the rest of the chemicals were supplied by Sigma Chemical (St.Louis, MO).

5.3. Results.

5.3.1. Effect of IL-1β on ^{3}H-NA release from myenteric nerve varicosities.

In order to monitor changes in stimulated ^{3}H-NA release from myenteric nerve varicosities in the presence of IL-1β or TNFα, 75mM KCl and 10μg/ml scorpion venom were used as stimuli, since they showed similar values in evoked ^{3}H-NA release.

Immediate effect of IL-1β on ^{3}H-NA release.

As shown in table 5.1, the basal ^{3}H-NA release was unaltered following addition of IL-1β (10ng/ml) to pre-loaded varicosities. Simultaneous addition of 10ng/ml IL-1β together with either KCl (75mM) or scorpion venom (10μg/ml) failed to significantly alter the evoked release of ^{3}H-NA from the respective stimuli.

Preincubation of IL-1β with the myenteric nerve varicosity preparation.

^{3}H-NA uptake. Preincubation of the varicosity preparation with IL-1β for 50 mins had no effect on the uptake of ^{3}H-NA. However, after this time, a decrease in the ability of the varicosities to take up and release noradrenaline was diminished, so the effects of either IL-1β or TNFα on noradrenaline uptake or release were not examined after this time.

^{3}H-NA release. Basal ^{3}H-NA release from preparations pretreated with IL-1β was not significantly different from varicosities treated with saline (figure 5.1A). However, preincubation of the varicosities with the cytokine caused a suppression of the subsequent evoked ^{3}H-NA release. KCl-induced ^{3}H-NA release (figure 5.1B) was suppressed by 55.4±6.3% after 20 mins ($P<0.01$), and by 63.8±8.1% after 50 mins ($P<0.01$) exposure to IL-1β. The time course of IL-1β-induced suppression of ^{3}H-NA release by scorpion venom (figure 5.1C) showed a small, statistically insignificant suppression of 21.1±6.7% ($P=0.08$) after 10 mins, however, a significant suppression was only seen after 50 mins.

Table 5.1: Effect on basal and stimulated ^{3}H-NA release after the immediate addition of IL-1β on the varicosity preparation.

STIMULI	CONTROL (% ^{3}H-NA RELEASE)	IL-1β (10ng/ml) (% ^{3}H-NA RELEASE)
BASAL RELEASE	1.0±0.3	1.0±0.83
KCL (75mM)	21.5±3.5	23.9±4.6
SCORPION VENOM (10µg/ml)	18±3	20.9±4.4

Basal (saline) or stimulated (75mM KCl or 10µg/ml scorpion venom) release of ^{3}H-NA is measured after the addition of either saline or 10ng/ml hrIL-1β to the nerve varicosity preparation. Results are expressed as percentage ^{3}H-NA release above basal (saline) ^{3}H-NA release and are the mean ± S.E. of 7 separate experiments.

Concentration dependence. IL-1β caused a concentration-dependent suppression of KCl-induced ^{3}H-NA release, as shown in figure 5.2A. Suppression was maximal using 10ng/ml IL-1β). IL-1β did not suppress ^{3}H-NA release by scorpion venom (figure 5.2B) at concentrations of 0.1ng/ml or less. Maximum suppression occurred at 1ng/ml IL-1β and any further increase of the cytokine concentration caused submaximal suppression of ^{3}H-NA release. The IL-1β concentration required to cause a 50% suppression (IC_{50}, calculated using probit analysis, Bliss, 1931) of the maximal suppressive effect on KCl-induced ^{3}H-NA release was 5ng/ml, which was significantly greater than the corresponding value of 0.6ng/ml calculated for the suppression on scorpion venom-stimulated release of neurotransmitter.

In the subsequent experiments, unless otherwise stated, a concentration of 10ng/ml IL-1β was used to explore the mechanisms underlying IL-1β-induced suppression of stimulated ^{3}H-NA release.

Specificity of IL-1β effect.

In order to exclude an effect due to endotoxin contamination, we boiled the IL-1β for 20 mins. As shown in table 5.2, this abolished the cytokine's inhibitory effect on KCl- or scorpion venom-induced ^{3}H-NA release. In addition, preincubation of IL-1β with a rabbit polyclonal anti-human IL-1β antibody for 20 mins at 37°C, prior to incubation with the varicosities, neutralized the suppressive effect of IL-1β on both KCl- and scorpion venom-induced ^{3}H-NA release. There was no significant difference observed between the values of stimulated ^{3}H-NA release of saline-treated varicosities and those treated with boiled IL-1β or with the neutralized IL-1β.

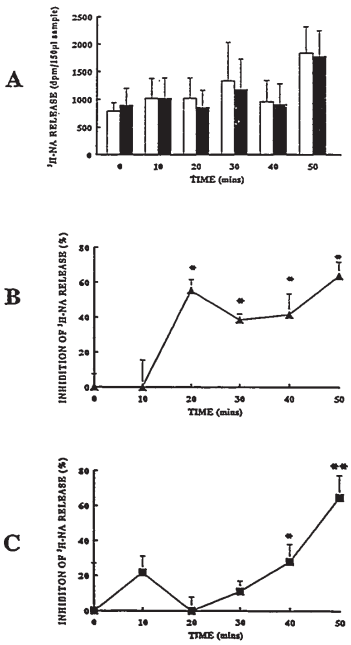


Figure 5.1: Time-dependence of IL-1β effect. The varicosity preparation was preincubated with IL-1β (10ng/ml) for the times indicated, prior to stimulation of ^{3}H-NA release by saline (A); 75mM KCl (B); or 10µg/ml scorpion venom (C). The data in panel A are expressed as dpm/150µl of supernatant, whereas, the data in panels B & C are calculated as percentage inhibition of corresponding control (saline) ^{3}H-NA release at indicated times. The results are the mean ± S.E. of 5 separate experiments, where * and ** represent P values (<0.05 and <0.001, respectively) for significant differences from respective control values.

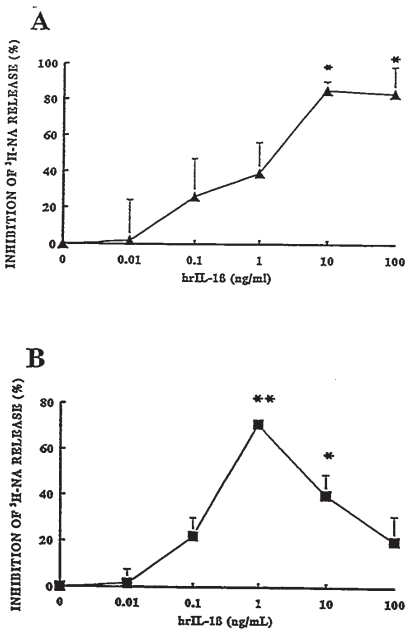


Figure 5.2: Concentration-dependence of IL-1β effect. The varicosity preparation was preincubated with IL-1β at the concentrations indicated for 50 mins prior to stimulation of ^{3}H-NA release by KCl (A) or scorpion venom (B). Results are expressed as percentage inhibition of control (saline) ^{3}H-NA release and are the mean ± S.E. from 6 separate experiments. * and ** represents P values of <0.05 and <0.001, indicating a significant difference from control stimulated ^{3}H-NA release.

Toxic effect of IL-1β.

Measurement of LDH activity was used to assess whether the effect of IL-1β on noradrenaline release was the result of a loss in membrane integrity. Adding IL-1β (10ng/ml) directly to the varicosities did not significantly alter the basal release of LDH activity (P=0.76). Preincubation of the varicosity preparations with IL-1β for 50 mins also failed to alter the basal LDH activity (P=0.8). As shown in table 5.3, the occluded LDH activity (the amount of LDH present with the cytoplasm) revealed no significant (P>0.05) change occurring between varicosity preparations treated with saline or 10ng/ml IL-1β.

Presence of IL-1 receptors.

To determine whether IL-1β-induced suppression of ^{3}H-NA release was receptor mediated, we used a selective receptor antagonist (IL-1ra). As shown in figure 5.3, preincubation of the varicosities with 10μg/ml receptor antagonist for 15 mins before adding IL-1β (10ng/ml) abolished the cytokine's suppressive effect on KCl- (figure 5.3A) or scorpion venom- (figure 5.3B) induced ^{3}H-NA release. There was no significant difference between the values obtained from evoked ^{3}H-NA release when the varicosities were treated with saline, IL-1β plus IL-1ra or IL-1ra alone: KCl, 8.88±1.53% vs 8.78±2.72% vs 10.1±2.38% or Scorpion venom, 14.4±2.63% vs 9.54±1.4% vs 13.7±3.0%, (saline vs IL-1β plus IL-1ra vs IL-1ra alone, respectively).

Role of prostaglandins.

Table 5.2: Specific action of IL-1β on ^{3}H-NA release from myenteric varicosities.

STIMULI	KCl (75mM) (% ^{3}H-NA RELEASE)	S.VENOM (10μg/ml) (% ^{3}H-NA RELEASE)
CONTROL	12.39±1.89	.20±2.84
IL-1β	2.50±0.86*	6.18±1.07*
IL-1β + IL-1β ANTIBODY	12.7±1.3**	17.1±2.92**
BOILED IL-1β	15.22±1.59**	22.54±3.77**

Myenteric nerve varicosities were preincubated for 50 mins with saline, 10ng/ml hrIL-1β alone, hrIL-1β neutralized with anti-human IL-1β antibody or boiled hrIL-1β before stimulation of ^{3}H-NA release by KCl or scorpion venom. Results are expressed as percentage ^{3}H-NA release above basal release and are the mean ± S.E. from 4 separate experiments. * indicates significant (P<0.01) difference from control (saline), whereas, ** represents a significant (P<0.01) difference from the response seen with hrIL-1β alone.

Table 5.3: Assessment of a toxic effect by IL-1β on the myenteric varicosity preparations: measurement of LDH.

	IMMEDIATE EFFECT LDH ACTIVITY (UNITS/mg/ml protein)		DELEAYED EFFECT LDH ACTIVITY (UNITS/mg/ml protein)	
	CONTROL	IL-1β	CONTROL	IL-1β
BASAL ACTIVITY (Pre-Triton-X100)	88.2±24	54.5±7.7	64.3±21.5	82.5±21.7
FINAL ACTIVITY (Post-Triton-X100)	222.9±31	220.1±31	221.0±34.6	242.8±30.6
OCCLUDED ACTIVITY	134.8±27.6	165.6±28.7	156.7±23.4	160.4±20.5

LDH activity was measured immediately after the addition of either saline or 10ng/ml hrIL-1β or after a 50 mins preincubation period with the varicositiy preparation. Occluded LDH activity was calculated as the difference between the LDH activity recorded before and after the addition of Triton-X100 to the preparations. The results are expressed as units/mg protein and are the mean ± S.E. of 6 separate experiments.

Co-incubation of the varicosity preparation with 10ng/ml IL-1β plus piroxicam (1μM) or indomethacin (10μM) caused a significant reduction in the IL-1β-induced suppression of KCl-induced ^{3}H-NA release of 56% ($P<0.05$) and 65% ($P<0.05$), respectively (figure 5.4A). There was no significant difference between the values obtained from the incubation of varicosities with IL-1β plus piroxicam or IL-1β plus indomethacin. Although both inhibitors caused a significant reduction in the suppressive effect of IL-1β on KCl-induced ^{3}H-NA, there was also a significant ($P<0.05$) difference seen from the values obtained in saline control experiments (figure 5.4A). This suggests that IL-1β-induced suppression of KCl-evoked ^{3}H-NA release involves prostaglandin-dependent and -independent mechanisms. In contrast, the presence of either piroxicam or indomethacin abolished the IL-1β-induced suppression of ^{3}H-NA release evoked by scorpion venom (figure 5.4B), suggesting that the inhibitory effect on scorpion venom-induced ^{3}H-NA release by IL-1β is entirely dependent on the activation of cyclo-oxygenase pathways. Preincubation of the varicosities with either piroxicam or indomethacin alone did not significantly alter KCl- (8.2±1.3% or 7.8±1.2%) or scorpion venom- (11.62±1.76% or 13.78±2.91%) induced ^{3}H-NA release from varicosities treated with saline; 12.5±1.8% or 19.6±2.02%, KCl and scorpion venom, respectively.

5.3.2. Effect of TNFα on ^{3}H-NA release from myenteric nerve varicosities.

Immediate effect of TNFα.

Addition of TNFα (50ng/ml) to ^{3}H-NA loaded varicosities did not significantly

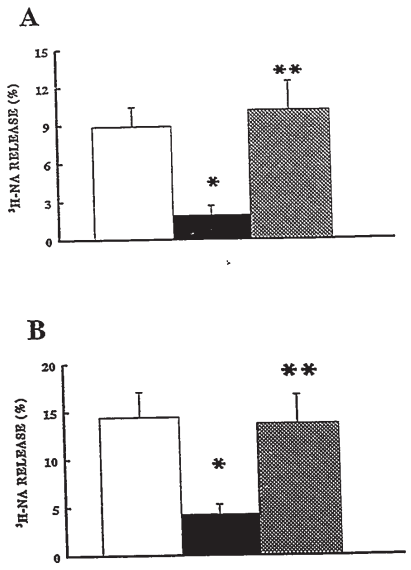


Figure 5.3: Presence of IL-1 receptors. The varicosities were preincubated for 50 mins with saline (open bars); 10ng/ml IL-1β (solid bars); or IL-1β in the presence of IL-1ra (crossed bars) prior to stimulation of ^{3}H-NA release by KCl (A) or scorpion venom (B). The results are expressed as percentage ^{3}H-NA above basal release and are the mean ± S.E. of 5 separate experiments. * represents a significant ($P<0.01$) difference from control, whereas ** indicates a significant ($P<0.01$) difference from the response observed with IL-1β alone.

alter the basal release of ^{3}H-NA (table 5.4). Simultaneous incubation of TNFα with stimuli, KCl (75mM) or scorpion venom (10μg/ml) also failed to cause an alteration in evoked ^{3}H-NA release when compared to corresponding saline controls.

Preincubation of TNFα.

Since TNFα (50ng/ml) caused a significant suppression of evoked ^{3}H-NA release after 45 mins in experiments using LM-MP preparations, this time point was initially used to assess the effect of TNFα (50ng/ml) on myenteric nerve varicosities. As shown in table 5.5, there was no significant alteration observed in basal or evoked ^{3}H-NA release after 45 mins incubation with the cytokine compared to saline controls.

Toxic effect of TNFα.

Measurement of LDH activity was also used to asses whether the inability of TNFα to alter the stimulated release of noradrenaline involved a loss in membrane integrity. TNFα (50ng/ml) when added directly to the varicosities did not significantly alter the basal release of LDH activity ($P=0.89$). Preincubation of the varicosity preparations with TNFα for 45 mins failed to alter the basal LDH activity. As shown table 5.6, the occluded LDH activity was not altered when the varicosity preparations were treated with either TNFα or saline.

5.4. Summary.

The results from this study demonstrate that IL-1β and TNFα do not alter

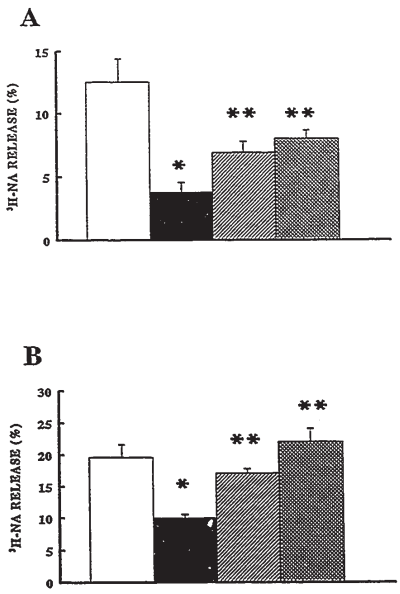


Figure 5.4: Role of prostaglandins in IL-1β effect on ^{3}H-NA release. The varicosities were preincubated for 50 mins with saline (open bars); 10ng/ml IL-1β alone (solid bars); IL-1β plus piroxicam (hatched bars); or IL-1β plus indomethacin (crossed bars) prior to KCl- (A) or scorpion venom- (B) induced ^{3}H-NA release. Results are expressed as percentage ^{3}H-NA release above basal release and are the mean ± S.E. of 4 separate experiments. * represents a significant ($P<0.001$) difference from control, whereas, ** represents a significant ($P<0.05$) difference from the response observed with IL-1β alone.

Table 5.4: Immediate and simultaneous effect of TNFα on ^{3}H-NA release from myenteric varicosity preparation.

STIMULI	CONTROL (% ^{3}H-NA RELEASE)	IL-1β (10ng/ml) (% ^{3}H-NA RELEASE)
BASAL (SALINE) RELEASE	1.0±0.43	1.94±0.5
KCL (75mM)	19.5±2.44	17.34±3.2
SCORPION VENOM (10μg/ml)	19.7±3.1	16.52±3.9

Basal (saline) or stimulated (75mM KCl or 10μg/ml scorpion venom) release of ^{3}H-NA measured after the addition of either saline or 50ng/ml TNFα to the nerve varicosity preparation. Results are expressed as percentage ^{3}H-NA release above basal (saline) ^{3}H-NA release and are the mean ± S.E. from 6 separate experiments.

basal or stimulated ^{3}H-NA release following immediate addition to the nerve varicosity preparation. Furthermore, prolonged exposure of the membrane preparation to both cytokines failed to produce any alteration in the uptake or basal release of ^{3}H-NA. However IL-1β caused a suppression of evoked ^{3}H-NA release after prolonged incubation of the cytokine with the nerve varicosities. The inability of TNFα to alter the evoked ^{3}H-NA release may be due to the absence of an intermediate cell and /or factors, which was present in the intact LM-MP preparation. A disruption of membrane integrity was ruled out, since TNFα did not alter the LDH activity after 45 mins incubation, when compared to varicosities treated with saline.

A suppression of KCl-induced ^{3}H-NA release was seen after 20 mins, whereas an inhibition of scorpion venom-induced release was not observed until 40 mins. This difference in the effect of IL-1β on stimulated noradrenaline release may indicate differences in the underlying mechanisms. The specific effect of IL-1β was shown by removal of its biological response by neutralization with an anti-IL-1β antibody or by boiling the recombinant cytokine for 20 mins. This specific effect was mediated by IL-1 receptors. Furthermore IL-1β did not cause a significant difference in the LDH activity after immediate addition to the varicosity preparations or after 50 mins incubation. Since occluded LDH activity is a marker for intact membranes, the effect does not appear to be mediated by disruption of the integrity of membrane vesicles.

In the presence of cyclo-oxygenase inhibitors, the IL-1β suppressive effect on scorpion venom- induced ^{3}H-NA release was completely reversed, whereas the effect

on KCl-induced ^{3}H-NA release was only partly attenuated, suggesting the presence of least two different mechanisms mediating the action of IL-1β on noradrenaline release from nerve varicosities.

5.5. Conclusion.

The underlying mechanism of the IL-1β and TNFα effect on noradrenaline release from LM-MP preparations, indicates that prostaglandin synthesis mediates the early suppressive effect of both cytokines. Examination of the action of IL-1β using the nerve varicosity preparation demonstrated that IL-1β suppresses noradrenaline release partly through prostaglandin production. The apparent inability of TNFα to suppress noradrenaline release in the nerve membrane preparations, in light of its ability to induce prostaglandin synthesis in the LM-MP after a short incubation period (45 mins), suggests that unlike IL-1β, the TNFα receptor is not linked to prostaglandin synthesis.

Table 5.5: Effect of TNFα preincubation (45 mins) on ^{3}H-NA release from myenteric varicosity preparation.

STIMULI	CONTROL (% ^{3}H-NA RELEASE)	TNFα (50ng/ml) (% ^{3}H-NA RELEASE)
BASAL (SALINE)	1.0±0.8	2.4±5.5
KCL (75mM)	20.0±4.99	19.86±9.94
SCORPION VENOM (10μg/ml)	16.7±4.23	18.8±8.66

The myenteric nerve varicosity preparation was preincubated with either saline or 50ng/ml TNFα for 45 mins. Release of ^{3}H-NA is measured in response to saline, 75mM KCl or 10μg/ml scorpion venom. Results are expressed as percentage ^{3}H-NA release above basal release and are the mean ± S.E. of 6 separate experiments.

Table 5.6: Assessment of a toxic effect by TNFα on the myenteric varicosity preparation: measurement of LDH activity.

	IMMEDIATE EFFECT LDH ACTIVITY (UNITS/mg/ml protein)		DELEAYED EFFECT LDH ACTIVITY (UNITS/mg/ml protein)	
	CONTROL	TNFα	CONTROL	TNFα
BASAL ACTIVITY (Pre-Triton-X100)	88.2±24	62±4.3	64.3±21.5	72±5.8
FINAL ACTIVITY (Post-Triton-X100)	222.9±31	243±51	221.0±34.6	207.9±42
OCCLUDED ACTIVITY	134.8±27.6	180.9±14	156.7±23.4	135±17.7

LDH activity was measured immediately after the addition of either saline or TNFα (50ng/ml) or after a 45 mins preincubation period with the varicositiy preparation. Occluded LDH activity was calculated as the difference between the LDH activity recorded before and after the addition of Triton-X100 to the preparations. The results are expressed as units/mg protein and are the mean ± S.E. of 6 separate experiments.

CHAPTER 6.

INTERACTIONS BETWEEN IL-1β AND TNFα ON NORADRENALINE RELEASE FROM THE MYENTERIC PLEXUS.

6.1. Introduction.

The results so far indicate that both IL-1β and TNFα are capable of suppressing noradrenaline release in the LM-MP layer of the small intestine, although only IL-1β appears to cause suppression of noradrenaline release from isolated nerve varicosities. In other tissues, especially within the immune system, IL-1β and TNFα have a synergistic effect on the physiological response in specific tissues (for review see Chaplin and Hoquist,1992); for example, IL-1β and TNFα act synergistically in up-regulating the expression of IL-2 receptors on thymic lymphoma cells (Scholz and Altman, 1989). In other cells, TNFα has been shown to potentiate IL-1β- induced production of prostaglandin E_2 in cultured synovial fibroblasts (Meyer et al.,1990) and granulocyte colony stimulating factor expression in arterial smooth muscle cells (Zolliner et al.,1992). A synergistic action in these situations can be defined as the ability of a sub-threshold substance concentrations, to induce a physiological response to another substrate greater than the sum of the individual responses to each substance when added concomitantly to a particular tissue/cell

(Berenbaum, 1989).

The simultaneous increase in IL-1β and TNFα expression in the jejunal LM-MP layer from *T.spiralis* infected rats (Khan et al, 1992), together with their individual suppressive effects on noradrenaline release after short exposure (<60 mins) prompted an investigation into a possible interaction occurring between IL-1β and TNFα on myenteric nerves to suppress noradrenaline release.

6.2. Methods.

6.2.1. Effect of IL-1β plus TNFα on ^{3}H-NA release from LM-MP preparations.

Measurement of noradrenaline release from LM-MP preparations.

The method used to measure ^{3}H-NA release from LM-MP preparations has been described in chapter 2.2.

Immediate effect of IL-1β plus TNFα.

These initial experiments were set up to examine the ability of IL-1β plus TNFα to induce a change in the ^{3}H-NA release when added to the superfusate. Saline, 0.01ng/ml IL-1β, 1pg/ml TNFα or IL-1β plus TNFα was added to the superfusate simultaneously with either saline (basal) or EFS.

Preincubation of IL-1β plus TNFα.

An incubation period of 45 mins was employed to investigate the effect of IL-1β plus TNFα on noradrenaline release.

IL-1β concentration-dependence. In order to determine a putative synergistic action

between IL-1β and TNFα on ^{3}H-NA release, the concentration of the TNFα remained constant (1pg/ml). LM-MP preparations were preincubated for 45 mins with specified concentrations of IL-1β (10^{-14}-10^{-8}g/ml) in the presence or absence of 1pg/ml TNFα, prior to stimulation of ^{3}H-NA release by EFS or 50mM KCl. Within this incubation time there was a 40 mins loading of the tissues with ^{3}H-NA, which occurred simultaneously with the presence of the cytokines.

Specificity of IL-1β plus TNFα effect.

The specific effect of IL-1β plus TNFα was assessed by using either a selective IL-1 receptor antagonist (IL-1ra) or a neutralizing anti-human TNFα antibody. In experiments involving the IL-1ra, the LM-MP preparations were preincubated with 1μg/ml IL-1ra for 15 mins in a 37°C water bath prior to the 45 mins incubation with 0.01ng/ml IL-1β plus 1pg/ml TNFα. Neutralization of the TNFα was achieved by preincubating 0.01 units/ml of a polyclonal rabbit human TNFα antibody with the TNFα prior to the incubation with the tissues in the presence of 0.01ng/ml IL-1β. Control tissues were treated with either equal volumes of one of the following: 0.01ng/ml IL-1β; 1pg/ml TNFα; 1μg/ml IL-1ra or 0.01 units/ml anti-human TNFα antibody alone.

6.2.2. Effect of IL-1β plus TNFα on ^{3}H-NA release from myenteric nerve varicosities.

Measurement of ^{3}H-NA release from varicosity preparation.

The method used to measure ^{3}H-NA release has been described in chapter

4.2.

Immediate effect of IL-1β plus TNFα.

Basal (saline) or stimulated (75mM KCl or 10μg/ml scorpion venom) ^{3}H-NA release was measured after the addition of one of the following: 0.01ng/ml IL-1β; 1pg/ml TNFα or IL-1β plus TNFα to the myenteric nerve varicosity preparation.

Preincubation of IL-1β plus TNFα.

The effect of IL-1β plus TNFα was examined using a 50 min incubation period. The nerve varicosities were preincubated with either saline; 0.01ng/ml IL-1β; 1pg/ml TNFα or IL-1β plus TNFα for 50 min prior to the stimulation of ^{3}H-NA release by either saline (basal), 75mM KCl or 10μg/ml scorpion venom.

Specificity of IL-1β plus TNFα effect.

The specific action of IL-1β plus TNFα on evoked ^{3}H-NA release was assessed using either a IL-1 receptor antagonist or a neutralizing anti-human TNFα antibody. In experiments using the IL-1ra, the varicosity preparations were preincubated with 1μg/ml IL-1ra for 15 min in a 37°C water bath, before the 50 min incubation with 0.01ng/ml IL-1β plus 1pg/ml TNFα. For the experiments involving neutralizing the TNFα, 0.01 units/ml of a rabbit polyclonal anti-human TNFα antibody was incubated with the TNFα for 20 min in a 37°C water bath, prior to the cytokine's incubation with the varicosities in the presence of 0.01ng/ml IL-1β. Control preparations received equal volumes of one of the following: 0.01ng/ml IL-1β; 1pg/ml TNFα; 1μg/ml IL-1ra or 0.01 units/ml of anti-human TNFα antibody.

Role of prostaglandins.

The role of prostaglandins in the effect of IL-1β plus TNFα on ^{3}H-NA release was investigated using cyclo-oxygenase inhibitors, piroxicam and indomethacin. The myenteric nerve varicosity preparations were preincubated with 0.01ng/ml IL-1β plus 1pg/ml TNFα in the presence of either 1μM piroxicam or 10μM indomethacin for 50 mins prior to stimulating ^{3}H-NA release with either 75mM KCl or 10μg/ml scorpion venom. Control tissues were treated with either saline, 0.01ng/ml IL-1β, 1μM piroxicam or 10μM indomethacin alone.

6.2.3. Statistical analysis.

All experiments involved at least 5 separate experiments and are expressed as mean ± S.E. (standard error). The actual number of experiments is indicated in the figure legend for each experiment. Statistical significance was inferred at $P<0.05$ using the Student's t test or by applying a one way analysis of variance in experiments comparing more than two means.

6.2.4. Materials.

Sprague Dawley rats (200-250g) were from Charles River Breeding farms (Montreal, Quebec); ^{3}H-NA (sp.act.13.3Ci/mM) was obtained from New England Nuclear (Boston, MA); IL-1β was from Upstate Biotechnology Incorporation (Lake Placid, NY); human recombinant TNFα was from R&D systems (Minneapolis, MN); the IL-1 receptor antagonist was a generous gift from Dr.R.Thompson of Synergen (Denver, CO); rabbit anti-human TNFα antibody was from Genzyme (Iner-Medico,

Ontario, Canada); and the remaining chemicals were supplied by Sigma Chemicals (St.Louis, MO).

6.3. Results.

6.3.1. Effect of IL-1β plus TNFα on ^{3}H-NA release from LM-MP preparations.

Immediate effect of IL-1β plus TNFα.

Addition of either 0.01ng/ml IL-1β; 1pg/ml TNFα or IL-1β plus TNFα did not cause a significant change in basal (saline) or electrical field stimulated ^{3}H-NA release from the LM-MP preparations when compared to the respective values observed upon the addition of saline (Table 6.1).

Preincubation of IL-1β plus TNFα.

In this study a preincubation period of 45 mins was chosen, in order to explore a putative direct interaction between IL-1β and TNFα and nerve varicosities. A 1pg/ml concentration of TNFα caused no alteration in ^{3}H-NA release stimulated by either EFS or KCl, when compared to saline controls; 2.9±0.51% vs 3.3±0.7% or 3.37±0.27% vs 3.29±0.52%, EFS or KCl, respectively.

Concentration-dependence. As shown in figure 6.1A, the suppression of EFS- induced ^{3}H-NA release by IL-1β alone was concentration-dependent over a range 0.001-10ng/ml IL-1β, with the maximum suppression in ^{3}H-NA release observed using 10ng/ml IL-1β. When 1pg/ml TNFα was added to the incubation medium, there was a shift of the IL-1β concentration-dependent suppression of ^{3}H-NA release to the

left. A significant increase in the suppression of EFS stimulated ^{3}H-NA by 0.001ng/ml IL-1β was seen in the presence of 1pg/ml TNFα. The suppressive effect on EFS-induced ^{3}H-NA release observed for 0.01ng/ml IL-1β was increased by 50% ($P<0.01$) in the presence of 1pg/ml TNFα. The lowest concentration of IL-1β that induced suppression of KCl- (figure 6.1B) stimulated ^{3}H-NA release was 0.001ng/ml, with the maximum inhibitory effect of the cytokine observed using 0.1ng/ml IL-1β. Co-incubation of the tissues with 0.01ng/ml IL-1β plus 1pg/ml TNFα caused a statistically significant greater suppression of KCl-induced ^{3}H-NA release than incubation with 0.01ng/ml IL-1β alone. The suppression of stimulated noradrenaline release induced by IL-1β concentrations greater than 0.1ng/ml were not significantly altered in the presence of 1pg/ml TNFα (figure 6.1A).

In subsequent experiments, a concentration of 0.01ng/ml IL-1β together with 1pg/ml TNFα was employed to explore the underlying mechanisms of the potentiating effect of TNFα on IL-1β suppression of ^{3}H-NA release.

Specificity of IL-1β plus TNFα effect in LM-MP preparations.

The approach used to examine the specific action of IL-1β plus TNFα involved the removal of the physiological activity of each cytokine independently. In experiments where the tissues were preincubated with IL-1 receptor antagonist, the suppressive effect of IL-1β plus TNFα on EFS- (figure 6.2A) and KCl- (figure 6.2B) induced ^{3}H-NA release was attenuated by 86% and 75% ($P<0.01$), respectively. The values observed in these experiments were not significantly different from those seen from treatment of the tissues with IL-1β alone. The neutralization of the TNFα with

Table 6.1: Immediate and simultaneous effect of IL-1β plus TNFα on ^{3}H-NA release from LM-MP preparations.

STIMULI	BASAL RELEASE ^{3}H-NA RELEASE (%)	EFS (30v, 0.5msec, 10Hz, 1min) ^{3}H-NA RELEASE (%)
SALINE	0.14±0.11	0.85±0.25
hrIL-1ß (0.01ng/ml)	0.06±0.01	0.72±0.38
TNFα (1pg/ml)	0.07±0.05	0.81±0.23
hrIL-1ß + TNFα	0.09±0.06	0.52±0.10

Basal (saline) or stimulated (EFS) release of ^{3}H-NA is measured simultaneously with the superfusion of either saline, 0.01ng/ml hrIL-1β, 1pg/ml TNFα or hrIL-1β plus TNFα over the LM-MP preparations. Results are calculated as percentage release of total ^{3}H-NA content and are the mean ± S.E. of 4 separate experiments.

a rabbit polyclonal anti-human TNFα antibody prior to the incubation with the tissues plus IL-1β attenuated the potentiating inhibitory effect of IL-1β plus TNFα on both EFS- (figure 6.2A) or KCl- (figure 6.2B) induced ^{3}H-NA release by 86% or 75%, respectively. There was no significant difference observed between the values obtained for tissues incubated with IL-1β plus TNFα in the presence of anti-human TNFα antibody and those from incubation with IL-1β alone. There was also no significant difference observed between IL-1ra, anti-human TNFα antibody and IL-1β observed in either EFS- (3.75±1.38% vs 2.3±0.5% vs 3.61±0.61%) or KCl- (2.41±1.2% vs 2.47±0.5% vs 3.7±0.15%) induced ^{3}H-NA release.

6.3.2. Effect of IL-1β plus TNFα on ^{3}H-NA release from myenteric nerve varicosities.

Immediate effect of IL-1β plus TNFα.

As shown in table 6.2, incubation of the varicosity preparation with either saline, 0.01ng/ml IL-1β, 1pg/ml TNFα or IL-1β plus TNFα simultaneously with saline (basal), 75mM KCl or 10μg/ml scorpion venom failed to significantly alter the release of ^{3}H-NA.

Preincubation of IL-1β plus TNFα.

The possibility of TNFα potentiating the IL-1β suppressive effect on evoked ^{3}H-NA in nerve varicosities was examined using a 50 mins preincubation period, by applying a concentration of IL-1β (0.01ng/ml) that previously failed to alter ^{3}H-NA release after this exposure period.

^{3}H-NA release. Preincubation of the nerve varicosities with either 0.01ng/ml IL-1β,

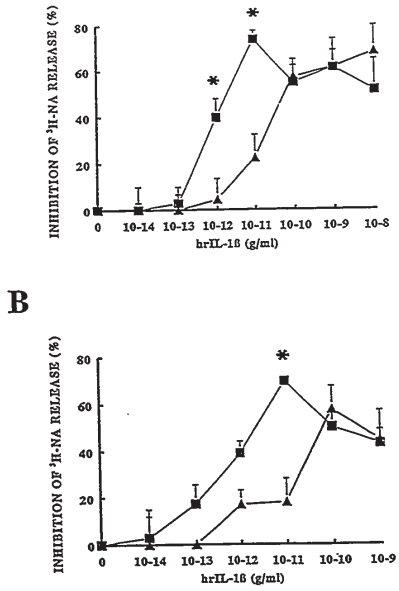


Figure 6.1: Concentration-dependence of IL-1β and IL-1β plus TNFα effect on evoked ^{3}H-NA release. LM-MP preparations were preincubated with specified concentrations of IL-1β (10^{-14}-10^{-8}g/ml) alone (▲) or together with 1pg/ml TNFα (■) for 45 mins prior to stimulation of ^{3}H-NA release with EFS (A) or KCl (B). The data are calculated as percentage inhibition of saline control values and the results are represented as a composite where the mean ± S.E. is taken from 5 separate experiments. * represents a significant ($P<0.05$) difference between the values obtained for IL-1β plus TNFα against IL-1β alone at a particular concentration.

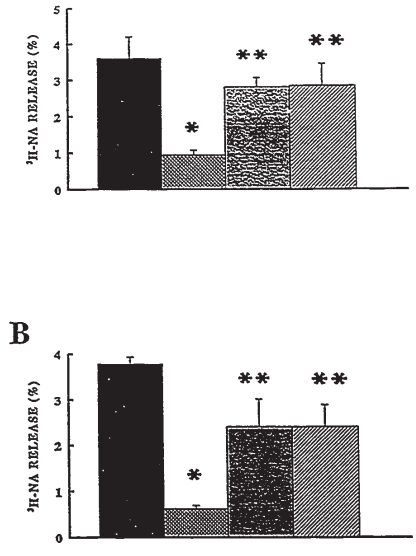


Figure 6.2: Specificity of IL-1β plus TNFα effect. LM-MP preparations Tissues were preincubated for 45 mins with either 0.01ng/ml IL-1β (solid bars); 0.01ng/ml IL-1β plus 1pg/ml TNFα (crossed bars); or IL-1β plus TNFα in the presence of either 1μg/ml IL-1ra (dotted bars) or 0.01 unit/ml anti-human TNFα antibody (hatched bars) prior to stimulation of ^{3}H-NA release by either EFS (A) or KCl (B). The results are expressed as percentage ^{3}H-NA release of the total content and are the mean ± S.E. of 4 separate experiments. * represents a significant ($P<0.01$) difference from IL-1β alone, whereas, ** indicates a significant ($P<0.01$) difference from values obtained from tissues incubated with IL-1β plus TNFα.

1pg/ml TNFα or IL-1β plus TNFα for 50 mins failed to alter significantly the basal release of ^{3}H-NA. However, co-incubation of the varicosities with IL-1β in the presence of TNFα caused a significant suppression of both KCl- (figure 6.3A) and scorpion venom- (figure 6.3B) induced ^{3}H-NA release by 48% and 52%, ($P<0.01$), respectively. Examination of the values obtained from preincubation of the varicosities with either saline, 0.01ng/ml IL-1β or 1pg/ml TNFα revealed no significant difference in either KCl- or scorpion venom-stimulated ^{3}H-NA release.

Specificity of the IL-1β plus TNFα effect using nerve varicosities.

The nerve varicosity preparations incubated with IL-1β (0.01ng/ml) alone showed no significant difference in evoked ^{3}H-NA when compared to saline and therefore was used as the control in examining the specificity of the potentiating effect of TNFα and IL-1β on suppression of evoked ^{3}H-NA release. The specific interaction of IL-1β with TNFα was explored by removing the biological function of either IL-1β or TNFα. In experiments where the varicosities were preincubated with 10μg/ml of the IL-1 receptor antagonist, the potentiation effect of TNFα of IL-1β suppression of both KCl- (figure 6.4A) or scorpion venom- (figure 6.4B) induced ^{3}H-NA release was attenuated by 93% and >99%, respectively to within control levels of stimulated noradrenaline release. The values observed in these experiments were not significantly ($P>0.05$) different from those seen for IL-1β treated tissues. With the use of a rabbit polyclonal anti-human TNFα antibody (0.01 units/ml), the TNFα was neutralized prior to incubation with the nerve varicosity preparations in the presence of IL-1β. This attenuated the inhibitory effect of IL-1β plus TNFα on both

Table 6.2: Effect on basal and stimulated ^{3}H-NA release after the immediate addition of IL-1β plus TNFα to the myenteric varicosity preparation.

STIMULI	KCl (75mM) ^{3}H-NA RELEASE (%)	SCORPION VENOM (10μg/ml) ^{3}H-NA RELEASE (%)	BASAL RELEASE (dpm/mg protein)
SALINE	7.01±2.61	21.15±5.62	9872±1874
hrIL-1ß 0.01ng/ml	8.05±0.97	22.95±5.37	9797±1878
TNFα 1pg/ml	7.72±2.65	23.0.5±5.59	9868±1836
hrIL-1B + TNFα	8.77±1.53	24.65±6.50	9880±1895

Basal (saline) or stimulated (75mM KCl or 10μg/ml scorpion venom) release of ^{3}H-NA was measured after the addition of either saline, 0.01ng/ml hrIL-1β, 1pg/ml TNFα or hrIL-1β plus TNFα to the nerve varicosities. Results are expressed as percentage ^{3}H-NA release above basal (saline) release and are the mean ± S.E. of 4 separate experiments.

KCl- (figure 6.4A) or scorpion venom- (figure 6.4B) induced ^{3}H-NA release by 93% and 73%, respectively. There was no significant ($P>0.05$) difference observed between the values obtained for tissues incubated with IL-1β plus TNFα in the presence of anti-human TNFα antibody and those from incubation with IL-1β alone (10.68±1.18% vs 11.52±2.11% or 9.45±1.81% vs 12.77±2.17%, KCl or scorpion venom, respectively). Furthermore, no significant change in the values observed between IL-1ra, anti-human TNFα antibody and IL-1β effects on either KCl- (10.2±4% vs 9.95±0.35% vs 11.52±2.11%) or scorpion venom- (9.8±2.7% vs 11.82±1.2% vs 12.77±-2.17%) stimulated ^{3}H-NA release.

Role of prostaglandins.

Co-incubation of the nerve varicosity preparations with 0.01ng/ml IL-1β plus 1pg/ml TNFα in the presence of 1μM piroxicam or 10μM indomethacin induced an attenuation in KCl- (figure 6.5A) induced ^{3}H-NA release by 48.5% and 53.5%, piroxicam and indomethacin, respectively. This was not significantly ($P>0.05$) different from the values for IL-1β plus TNFα. In contrast, the presence of either piroxicam or indomethacin in the incubation medium with IL-1β plus TNFα caused a significant attenuation in scorpion venom- (figure 6.5B) stimulated ^{3}H-NA release (87.8% and 93.3%, piroxicam and indomethacin, respectively), which was not significantly ($P>0.05$) different from the values observed from IL-1β treated varicosities; 13.56±-2% vs 14.56±2.2% vs 16.4±2.8%. Preincubation of the varicosities with either piroxicam or indomethacin alone did not cause a difference in KCl- or scorpion venom-induced ^{3}H-NA release from varicosities treated with

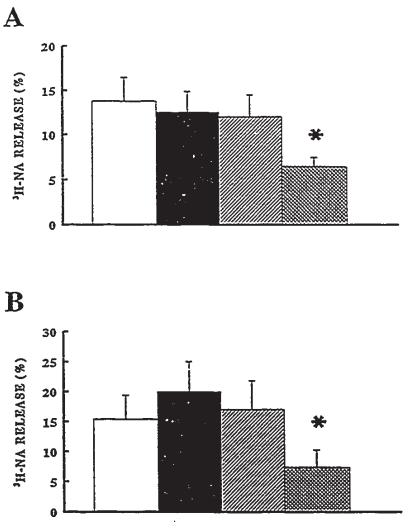


Figure 6.3: Effect of IL-1β plus TNFα on ^{3}H-NA release from myenteric nerve varicosities. The nerve varicosity preparation was preincubated for 50 mins with either saline (open bars); 0.01ng/ml IL-1β (solid bars); 1pg/ml TNFα (hatched bars) or IL-1β plus TNFα (crossed bars) prior to stimulation of ^{3}H-NA release by either KCl (A) or scorpion venom (B). The results are expressed as percentage ^{3}H-NA release above basal release and are the mean ± S.E. of 7 separate experiments. * represents a significant ($P<0.01$) difference from values obtained from incubation with saline, IL-1β or TNFα alone.

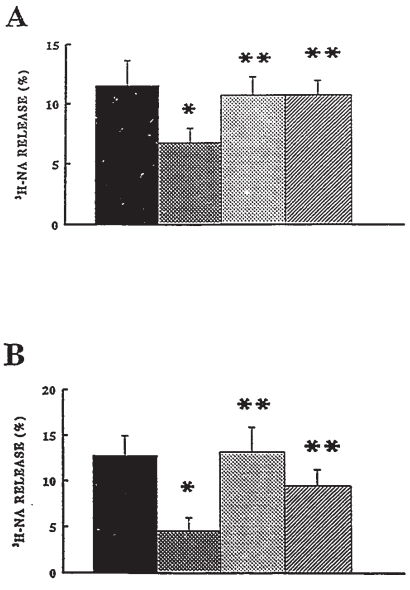


Figure 6.4: Specificity of the IL-1β plus TNFα effect. The myenteric nerve varicosities were preincubated for 50 mins with either IL-1β (solid bars); IL-1β plus TNFα (crossed bars); or IL-1β plus TNFα in the presence of either IL-1ra (dotted bars) or anti-human TNFα antibody (crossed bars) prior to stimulation of ^{3}H-NA release by either KCl (A) or scorpion venom (B). Results are expressed as percentage ^{3}H-NA above basal release and are the mean ± S.E. of 4 separate experiments. * represents a significant (P<0.01) difference from IL-1β alone and ** represents a significant (P<0.01) difference from the values observed when the varicosities were preincubated with IL-1β plus TNFα.

saline.

6.4. Summary.

Addition of IL-1β (0.01ng/ml) plus TNFα (1pg/ml) to the superfusate showed no alteration in the basal or immediate evoked ^{3}H-NA release from LM-MP preparations. However, preincubation of these cytokines for 45 mins with the tissues caused a significant suppression in stimulated ^{3}H-NA release. The potential "synergistic interactions" between IL-1β and TNFα are dependent on the biological activity of the individual cytokines, as removal of either TNFα with a neutralizing antibody or the IL-1β effect, by using an IL-1 receptor antagonist, reversed the suppression of evoked ^{3}H-NA release observed. Examination of the underlying mechanism using the varicosities showed that 1pg/ml TNFα and 0.01ng/ml IL-1β had no effect on basal or evoked ^{3}H-NA release upon immediate addition to the varicosity preparations, but displayed a suppressive effect on evoked (not basal) ^{3}H-NA release after 50 min incubation with the varicosity preparations. The potentiating effect of TNFα on IL-1β suppression of evoked ^{3}H-NA was reversed by removing either the IL-1β or TNFα biological effect. Further exploration of the underlying mechanism revealed that the suppression of scorpion venom-induced ^{3}H-NA release by TNFα plus IL-1β was completely abolished in the presence of cyclo-oxygenase inhibitors, piroxicam and indomethacin, whilst KCl-induced ^{3}H-NA was only slightly attenuated.

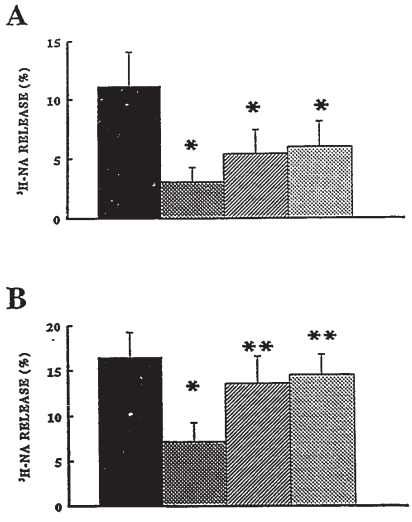


Figure 6.5: Role of prostaglandins in the IL-1β plus TNFα effect. The nerve varicosities were preincubated for 50 mins with IL-1β alone (solid bars); IL-1β plus TNFα (crossed bars); or IL-1β plus TNFα in the presence of either piroxicam (hatched bars) or indomethacin (dotted bars) prior to stimulation of ^{3}H-NA release by either KCl (A) or scorpion venom (B). Results are expressed as percentage ^{3}H-NA release above basal release and are the mean ± S.E. of 5 separate experiments. * represents a significant (P<0.01) difference from IL-1β values and ** indicates a significant (P<0.05) difference from those values obtained from varicosities preincubated with IL-1β plus TNFα.

6.5. Conclusion.

The potentiation of IL-1β suppression of noradrenaline release by TNFα occurs at the neural level and appears to partly involve prostaglandin synthesis. Although the presence of TNFα receptors has not been demonstrated, it is possible that IL-1β and TNFα may act on the same cell, eg. myenteric varicosities, using different receptors, and because these receptors appear to involve different aspects of the same signalling mechanisms may induce a synergistic effect on the suppression of noradrenaline release.

CHAPTER 7

EFFECT OF IL-1β ON SUBSTANCE P CONTENT IN LONGITUDINAL MUSCLE - MYENTERIC PLEXUS (LM-MP) OF THE RAT SMALL INTESTINE.

7.1 Introduction.

Substance P is a widely distributed neuropeptide, which has been shown to accumulate within inflamed tissues, where it is believed to contribute to the inflammatory process (Payan, 1989). The impact of inflammation on intestinal function using *T.spiralis*-infected rats as a model, are accompanied by changes in cholinergic (Collins et al.,1989), adrenergic (Swain et al.,1991) and peptidergic (Swain et al.,1992 nerves in the inflamed jejunum. Furthermore, within 24 hrs of the initial infection with the nematode the expression of several cytokines was increased (Khan et al., 1992), including IL-1β, which has been shown to alter substance P synthesis in sympathetic ganglia (Freidin and Kessler, 1991; Jonakait et al.1991). In the *T.spiralis* model, there is a 5 fold increase in immunoreactive substance P (IR-SP) in the LM-MP layers of the inflamed jejunum; the increase in IR-SP could be depleted by treatment with scorpion venom, indicating that the neuropeptide was

contained in nerves. Furthermore, the elevation in IR-SP content was attenuated in tissues isolated from animals that were treated with corticosteroid during the *T.spiralis* infection (Swain et al.,1992). These findings led the authors to conclude that the increased neuronal IR-SP content was a result of the inflammatory process.

In this particular study, the effect of IL-1β on substance P content in the LM-MP layer was investigated. Although a previous study, using capsaicin-treated rats, showed that the increase in substance P observed in myenteric plexus of nematode-infected animal is attenuated by 80%, suggesting an extrinsic sensory nerve origin, it is the aim of this study to examine changes in intrinsic substance P content. The LM-MP layer provided us with a multicellular model containing intact intrinsic myenteric ganglia but only axons of sensory afferent nerves.

7.2. Methods.

7.2.1. Incubation of LM-MP preparations.

Male Sprague Dawley rats (200-250g) were sacrificed by a blow to the head followed by cervical dislocation. The small intestine was removed and placed in Krebs buffer, containing (in mM) NaCl,120.9; KCl,5.9; $CaCl_2$,1.2; $NaHCO_3$,15.5; NaH_2PO_4,1.2; and glucose,11.1; bubbled with 95% oxygen and 5% carbon dioxide. LM-MP segments (20-30mg) were dissected from the jejunum. Each LM-MP segment was placed in a well of a 24-well tissue culture plate, together with 1ml of RPMI medium supplemented with 10% fetal calf serum and 10mM glutamine and the proteinase inhibitor, trasylol (100 Kallikrein Inactivating Units/ml). The tissue

preparations were incubated for specified times (2-24 hrs)at 37°C in a CO_2 incubator, then blotted and washed x 3 with phosphate buffered saline (PBS). Finally, each tissue segment was weighed and prepared for measurement of immunoreactive substance P (IR-SP) by radioimmunoassay or immunohistochemical analysis.

7.2.2. Assessment of immunoreactive substance P (IR-SP) in the LM-MP preparations.

Measurement of IR-SP by radioimmunoassay (RIA).

Preparations of LM-MP preparations. LM-MP preparations were prepared for IR-SP measurement using the method described by Ferri et al (1989). The tissues were placed in 2ml acetic acid (0.05M) on ice and homogenized 3 x 5 secs at setting 5 using a Kinematica polytron (Model-CH06010). The homogenates were then incubated for 30 mins in a 37°C water bath, followed by centrifugation for 5 mins at 11,500xg in a bench top centrifuge (Beckman Model Microfuge B). The supernatants were aspirated and stored at -70°C until the measurement of IR-SP.

Measurement of IR-SP by RIA. The levels of IR-SP was measured by Dr. Andrei Stanisz from the Intestinal Diseases Research Program (McMaster University, Ontario). IR-SP was determined using an inhibition-type solid phase RIA using ^{125}I-substance P and a polyclonal rabbit anti-human substance P antibody, directed against the C-terminus. The anti-substance P antibody used in the assay was reported (Instar Corp., Still, MN) not to cross react with any neuropeptide of similar structure, including neurokinin A or smaller biological fragments of the substance

P molecule. The sensitivity of the assay lay between 30 (lower limit) and 2000 pg/ml (upper limit). Limitation of the RIA analysis of substance P failed to distinguish between the oxidized (biologically inactive) and un-oxidized (biologically active) forms of substance P released by the LM-MP preparations. However in this study, the aim was to examine changes in the substance P content. Previous studies using high pressure liquid chromatography (HPLC) as a tool for the analysis of substance P revealed that the releasable form of substance P from nerves of the intestine was mainly of the undecapeptide form (Akagi et al.,1980). Furthermore this technique could be used to distinguish the oxidized form of the neuropeptide and the biologically active substance P.

The protocol of IR-SP measurement is outlined below:

A polystyrene 96-well plate was coated with 150µl of polyclonal rabbit anti-substance P antibody (1:5000 dilution in PBS) and incubated at room temperature for 12 hrs. The antibody was aspirated off and excess 1% BSA (bovine serum albumin) in PBS was added and incubated at room temperature for 1 hr, after which the wells were then emptied, dried by blotting with paper towels. 150µl of 1% BSA was added to the wells, followed by 50µl of substance P standards (0.3-1350pg/100µl) or samples (1:10 dilution), leaving the last row of the plates empty (totals). Radioactive substance P (^{125}I-substance P) was then added to all wells, except blanks, in a concentration that produced approximately 10-15 x 1000 cpm/well, this mixture was then covered with plastic film and incubated at room temperature for 12 hrs. The liquid was then aspirated and the plates washed gently under running

water, after drying the plates by blotting with paper towels, the individuals wells were cut from the plates and counted for radioactivity using a gamma counter (LKB Wallac Model).

Calculation of IR-SP levels. The concentration of substance P in the sample were expressed as pg/100µl and calculated from a standard curve included within the experiment (A). These results were then multiplied by 10 to give IR-SP (pg/ml). These values were corrected for dilution factor of 20 and finally, IR-SP were expressed as pg/mg tissue wt.:

$$\frac{(A \times 10) \times 20}{\text{tissue wt. (mg)}} = \text{IR-SP (pg/mg tissue wt.)}$$

7.2.3. Immunohistochemical analysis of IR-SP.

Histochemical analysis of substance P in the LM-MP preparations was performed by Dr. Keith Sharkey from the Dept. Medicine (University of Calgary, Alberta) and is described in appendix 3.

7.2.4. Effect of IL-1β on IR-SP content.

Time dependence. In the initial experiments examining the effect of IL-1β on IR-SP content, the LM-MP preparations were incubated with either saline or 10ng/ml IL-1β for 2, 4, 6 or 12 hrs in the presence of the proteinase inhibitor, trasylol.

Concentration dependence. In these experiments the tissue segments were incubated with specified concentrations of IL-1β (0.01-100ng/ml) for 6 hrs prior to the

measurement of IR-SP content.

Specificity of IL-1β effect.

To exclude an effect due to endotoxin contamination of the recombinant protein, the IL-1β was boiled for 20 mins prior to incubation with the LM-MP preparations for 6 hrs. The specific action of the human recombinant IL-1β was examined using a selective IL-1 receptor antagonist (IL-1ra) or a neutralizing polyclonal rabbit anti-human IL-1β antibody. In experiments involving IL-1ra, tissue segments were preincubated with 10µg/ml IL-1ra alone for 15 mins at 37°C before adding 10ng/ml IL-1β and incubating for a further 6 hrs. In experiments involving neutralizing the IL-1β effect, 10ng/ml IL-1β was preincubated with a 1:10 dilution of the polyclonal rabbit anti-human IL-1β antibody for 20 mins at 37°C prior to the 6 hrs incubation with the LM-MP preparations. Control tissues received equal volumes of saline, IL-1ra, IL-1β antibody or IL-1β alone.

Experiments aimed at depleting IR-SP.

Human recombinant IL-1β- (10ng/ml) or saline-treated tissues were placed in 1ml Krebs buffer with either saline, scorpion venom (10µg/ml) or scorpion venom plus tetrodotoxin (1µM) and incubated for 20 mins in a 37°C water bath with oxygenation. The tissue segments were alternately blotted and washed with PBS x 3, before being weighed and prepared for IR-SP analysis by RIA.

Role of protein synthesis.

Initially, the ability of IL-1β to induce protein synthesis was examined by measuring the uptake of ^{35}S-methionine in the LM-MP preparations (Chapter 3.2).

The tissues were incubated with saline or 10ng/ml IL-1β together with 10µCi/ml ^{35}S-methionine in the presence or absence of 100µg/ml cycloheximide.

The same method was applied to investigating the role of protein synthesis in IL-1β effect on IR-SP content. In this situation, LM-MP preparations were incubated for 6 hrs with either saline or 10ng/ml IL-1β in the presence of absence of 100µg/ml cycloheximide prior to the measurement of IR-SP content. Control tissues received saline, IL-1β or cycloheximide alone.

Role of nerve growth factor - 7S (NGF).

In experiments examining the effect of NGF on IR-SP levels, tissues were incubated for 6 hrs with indicated concentrations of mouse NGF-7S (10^{-13}-10^{-9}g/ml). The tissues were then processed for IR-SP measurement by RIA.

The role of NGF in IL-1β effect on IR-SP content was also examined directly by utilizing the ability of two different sources of anti-mouse NGF antibodies to neutralize the IL-1β effect. Tissue preparations were incubated for 6 hrs with saline, 10ng/ml IL-1β or 0.1ng/ml NGF in the presence of either affinity purified rabbit mono-specific anti mouse NGF factor IgG (10µM) or affinity purified sheep mono-specific anti-mouse NGF factor IgG (10µM), prior to the measurement of IR-SP content by RIA. Control tissues received, saline, IL-1β, NGF, rabbit anti-NGF or sheep anti-NGF alone.

Role of prostaglandins.

In order to determine the role of prostaglandins in the IL-1β effect on IR-SP content, the ability of cyclo-oxygenase inhibitors, piroxicam and indomethacin, to

selective block the activation of the cyclo-oxygenase (Brurch et al.,1983). Either 1µM piroxicam or 10µM indomethacin were incubated with the LM-MP preparations in the presence of 10ng/ml IL-1β for 6 hrs prior to the measurement of IR-SP content. Control tissues received either saline, IL-1β, piroxicam or indomethacin alone.

7.2.5. Statistical analysis.

All studies involved at least 4 separate experiments and results are expressed as mean ± S.E. (standard error). The Student's paired t test was used to compare two means within each experiment, and a one way analysis of variance was applied when comparing more than two means. Statistical significance was inferred from a P value < 0.05.

7.2.6. Materials:

Sprague Dawley rats were supplied by Charles River Breeding Farms (Montreal, Quebec); RPMI medium and fetal calf serum were from Gibco (Grand Island, NY); Tissue culture plates were from Becton-Dickinson Labware (Lincoln Park, NJ); the proteinase inhibitor, trasylol, was from Miles Canada Inc. (Ontario, Canada); human recombinant IL-1β was a generous gift from Sclavo (Sienna, Italy) and from Upstate Biotechnology In. (Lake Placid, NY); the IL-1 receptor antagonist was a generous gift from Dr.R.Thompson of Synergen (Denver, CO); anti-human IL-1β antibody was a generous gift from Dr.R.Newton of DuPont (Glenolden PA); mouse NGF (7S) was from Chemicon Int. Inc. (Temecula, CA); the affinity purified

rabbit and sheep anti-mouse NGF antibodies were a generous gift from Dr.J.Diamond from Dept. Biomedical Science (McMaster University, Ontario, Canada); the radioactive isotopes ^{125}I-substance P (sp.act.200Ci/mM) and ^{35}S-methionine (sp.act.1000Ci/mM) as well as the tissue solubilizer, protosol, were supplied by Amersham Canada Ltd. (Oakville, Canada); polyclonal rabbit anti-substance P antibody was from Incstar Corp. (Stillwater, MN); goat anti-rabbit IgG conjugated to FITC was from Dako, Dimension Labs (Ontario, Canada); and the rest of the chemicals were supplied by Sigma Chemicals (St.Louis, MO).

7.3. Results.

7.3.1. Preincubation of IL-1β with LM-MP preparations.

Time-dependence. Incubation of LM-MP preparations with 10ng/ml IL-1β for 4 hrs or less produced no significant change in IR-SP content compared to saline-treated controls. However, after 6hrs there was a significant increase in IR-SP content from 285.9±34.7 to 999±128 pg/mg tissue wt. ($P<0.001$). Prolonged exposure of the tissues to the cytokine produced no further increase in IR-SP; therefore in subsequent experiments a 6 hrs incubation period was used.

Concentration-dependence. In control tissues, IR-SP content was 159.9±13.3 pg/mg tissue wt. As shown in Fig.7.1, preincubating the tissues with IL-1β over a concentration range of 0.01-100ng/ml caused a dose-dependent increase in IR-SP content. The lowest IL-1β concentration that produced a significant increase in substance P was 0.1ng/ml (252.5±20 pg/mg tissue wt, $P<0.05$), whereas maximal IR-

SP increase was observed using 10ng/ml IL-1β (399.2±15.7 pg/mg tissue wt., $P<0.001$). A further increase in IL-1β concentration by 10 fold produced a sub-maximal increase in IR-SP content (301±17.6 pg.mg tissue wt., $P<0.05$).

In the following experiments we explored mechanisms underlying IL-1β-induced increase in substance P content using a 6 hrs incubation period and a IL-1β concentration of 10ng/ml.

Specificity of IL-1β effect.

To exclude an endotoxin effect of the human recombinant cytokine preparations, we boiled IL-1β for 20 mins. As shown in table 7.1, boiling abolished the increase in IR-SP content. The specificity of the IL-1β effect was further examined using a neutralizing anti-human IL-1β antibody or a selective IL-1 receptor antagonist (table 7.1). Preincubating the cytokine with a specific IL-1β antibody produced a 65% ($P<0.001$) attenuation of the increased IR-SP, whereas incubation of the tissue with IL-1ra before the addition of IL-1β attenuated the cytokine-induced increase in IR-SP content by 78% ($P<0.001$). The IR-SP content in the LM-MP preparations incubated with either IL-1ra or the anti-human IL-1β antibody alone was not altered from values observed from tissues treated with saline (control); 124±15.1, 158±36.6 and 132.6±13.4 pg/mg tissue wt., IL-1ra, IL-1β antibody and saline, respectively.

Localization of IR-SP.

A functional and morphological approach was used to determine the source of the increased IR-SP induced by IL-1β. As shown in figure 7.2, incubation of the

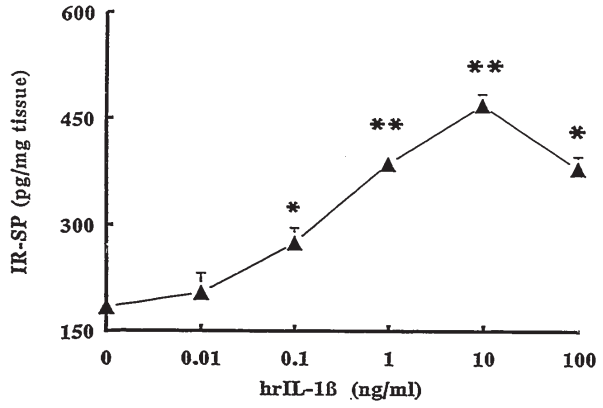


Figure 7.1: Concentration-dependence of IL-1β effect on IR-SP content. LM-MP preparations were incubated for 6 hrs with the indicated concentrations of IL-1β (0.01-100ng/ml), tissues were washed and IR-SP content measured by RIA. Results are expressed as IR-SP (pg/mg tissue wt.) and are the mean ± S.E. of 6 separate experiments. * and ** represent P values of <0.05 and <0.01, respectively.

tissues with IL-1β caused a significant increase in IR-SP levels. When the IL-1β-treated tissues were subsequently incubated in the presence of 10μg/ml scorpion venom, there was a 90% decrease in the IR-SP content to 186.5±46.8 pg/mg tissue wt. ($P<0.01$): the residual IR-SP levels in the tissue was similar to that observed in saline-treated controls. When the sodium channel blocker, tetrodotoxin (1μM) was present in the medium, the subsequent addition of scorpion venom failed to deplete the IL-1β- induced increase in IR-SP content. The small amount of IR-SP presence in the tissues after stimulation with scorpion venom, indicates its source is probably inaccessible to neural stimulation, or is present within a non-excitable source. However, the latter possibility is unlikely, since immunohistochemical analysis of the preparation only found substance P within neural tissues. LM-MP preparations incubated with saline for 6 hrs prior to adding scorpion venom, in the presence or absence of tetrodotoxin, showed no alteration in IR-SP content when compared to saline-treated controls. Immunohistochemical analysis of saline-treated preparations revealed substance P-like immunoreactivity localized predominantly in varicose nerve fibres, terminals in the myenteric plexus and internodal strands, and within nerve cell bodies in the plexus (figure 7.3, top panel). IL-1β-treated tissues showed a similar distribution of IR-SP to that observed in saline-treated LM-MP preparations (figure 7.3, bottom panel).

Role of protein synthesis.

Human recombinant IL-1β caused a 3.8 fold increase (figure 7.4A) in ^{35}S-methionine uptake by the LM-MP preparations after 6 hrs exposure. Co-incubation

Table 7.1: Specific effect of IL-1β on immunoreactive substance P (IR-SP) content in the LM-MP preparations.

STIMULI	IR-SP (pg/mg tissue wt.)
CONTROL	132.6±13.4
IL-1β	2155±244*
IL-1β + IL-1β ANTIBODY	626.5±60**
BOILED IL-1β	169.7±18**
IL-1β + IL-1ra	896±147**

LM-MP preparations were preincubated for 6 hrs with saline, 10ng/ml IL-1β alone, IL-1β neutralized with anti-human IL-1β antibody; boiled IL-1β or IL-1β plus IL-1ra, before measurement of IR-SP content by radioimmunoassay. The data are calculated as IR-SP pg/mg tissue wt. and are the mean ± S.E. from 5 separate experiments. * represents a significant ($P<0.05$) difference from control (saline), whereas, ** indicates a significant ($P<0.001$) difference from the values obtained with IL-1β alone.

of 10ng/ml IL-1β with cycloheximide completely prevented the cytokine-induced increase in ^{35}S-methionine uptake ($P<0.001$). There was no significant difference observed in ^{35}S-methionine uptake in tissues incubated with saline or cycloheximide. As shown in figure 7.4B, incubation of the tissues with 10ng/ml IL-1β in the presence of cycloheximide attenuated the cytokine-induced IR-SP content by 88% ($P<0.001$). There was no significant difference in IR-SP levels in tissues incubated with either saline or cycloheximide alone. These results indicate that the increase in substance P induced by IL-1β is dependent on protein-synthesis. This has been shown in sympathetic ganglia, where IL-1β induces expression of the preprotachykinin messenger RNA responsible for substance P (Freidin and Kessler, 1991; Jonakait et al.,1991).

Role of nerve growth factor.

Preincubation of the LM-MP preparations with specified concentrations of mouse NGF-7S (0.0001-1ng/ml) caused a dose-dependent increase in the IR-SP content. As shown in figure 7.5A, the lowest NGF concentration to induce an increase in IR-SP levels compared to saline controls was 0.01ng/ml; 141±6.6 vs 317±30 pg/mg tissue wt., saline vs NGF, respectively ($P>0.05$). Maximal IR-SP increase was seen using 0.1ng/ml NGF; 1097±109 pg/mg tissue wt., $P<0.01$), a higher concentration of NGF (1ng/ml) did not further increase the substance P content in the tissue preparations (1112±167 pg/mg tissue wt., $P<0.01$). NGF has been shown to induce substance P synthesis in sensory nerves (Vedder et al.,1993). In turn, IL-1β has been shown to induce NGF synthesis in a variety of tissues (for review see

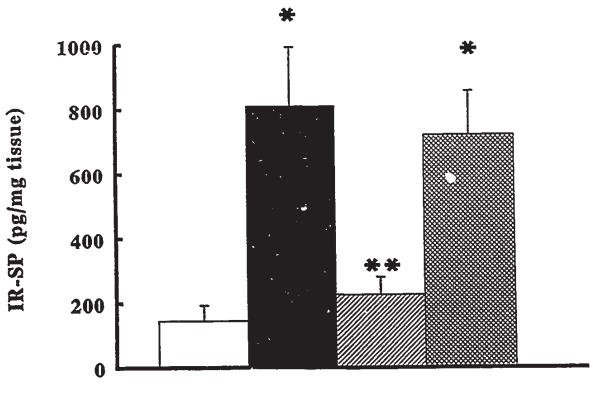


Figure 7.2: Effect of scorpion venom on residual IR-SP content. LM-MP preparations were incubated for 6 hrs with saline (open bar); 10ng/ml IL-1β (solid bar); or IL-1β for 6 hrs followed by stimulation with either scorpion venom alone (hatched bar); or scorpion venom plus tetrodotoxin (crossed bar). Tissues were washed and IR-SP content measured by RIA. The results are expressed as IR-SP (pg/mg tissue wt.) and are the mean ± S.E. of 5 experiments. * represents a significant ($P<0.05$) difference in IR-SP content from saline controls, where ** indicates a significant ($P<0.01$) difference from IR-SP values in response to IL-1β alone.

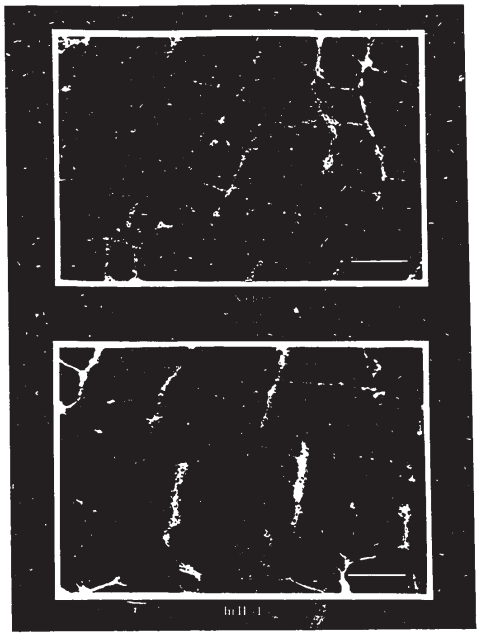


Figure 7.3: Fluorescence micrographs of immunoreactive substance P in the LM-MP preparations. The top panel illustrates the IR-SP distribution observed within the intrinsic nerves of saline-treated tissues. IR-SP was predominantly localized in the myenteric plexus and internodal strands. After treatment with 10ng/ml IL-1β (bottom panel), the immunoreactive fibres containing substance P displaying the same distribution as saline-treated tissues. Scale bar = 100μm.

Bartfai and Schultberg, 1993). Therefore it is possible that the IL-1β-induced increase in substance P is mediated by NGF. This hypothesis was explored using two separate sources of anti-mouse NGF antibodies, which has been shown to cross react with rat nerve growth factor (J.Diamond, personal communication). Co-incubation of the tissues with 0.1ng/ml NGF plus either rabbit anti-mouse NGF or sheep anti-mouse NGF IgG caused a significant ($P<0.01$) decrease in NGF-induced IR-SP levels in the LM-MP by 90% and 95% ($P<0.01$), respectively. The presence of either antibody returned the levels of IR-SP to within control IR-SP levels (figure 7.5B). In contrast, the increase in IR-SP content induced by IL-1β ($P<0.01$) remained unaltered when the tissues were co-incubated with 10ng/ml IL-1β plus either neutralizing NGF antibody (figure 7.5B). Tissues incubated with rabbit anti-mouse NGF alone showed no significant difference in IR-SP levels compared to saline-treated tissues. IL-1β-induced increase in substance P was not mediated by endogenous NGF production, since the neutralizing NGF antibodies failed to prevent the increase in substance P in response to IL-1β, but successfully blocked the NGF-induced increase in substance P.

Role of prostaglandins.

Co-incubation of the tissues with 10ng/ml IL-1β plus either 1μM piroxicam or 10μM indomethacin for 6 hrs caused an attenuation in the cytokine-induced IR-SP increase in content by 94% and 95% ($P<0.01$), respectively, returning IR-SP levels to within those values observed for tissues treated with saline alone (figure 7.6). Incubation of LM-MP preparations with piroxicam or indomethacin alone did

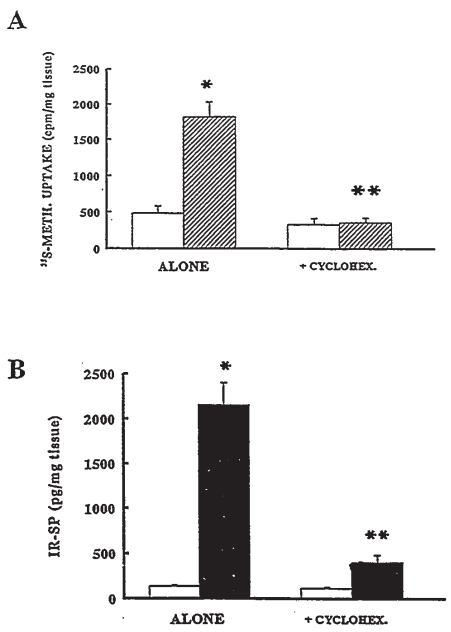


Figure 7.4: Protein synthesis dependence of IL-1β increase in IR-SP content. Panel A shows the effect of IL-1β on ^{35}S-methionine uptake by the LM-MP preparations. Tissues were incubated for 6 hrs with saline (open bars) or 10ng/ml IL-1β (hatched bars) in the presence of either saline or cycloheximide. The LM-MP preparations were then washed and ^{35}S-methionine counted. The results are calculated as ^{35}S-methionine cpm/mg tissue wt. and are the mean ± S.E. from 5 experiments. * represents a significant ($P<0.001$) difference from saline controls, whereas, ** indicates a significant ($P<0.001$) difference from values obtained from tissues treated with IL-1β alone. Panel B represent the role of protein synthesis in IL-1β effect on IR-SP content. The tissues were incubated for 6 hrs with saline (open bars) or with IL-1β alone (solid bars) or in the presence of cycloheximide. LM-MP preparations were washed and measured for IR-SP content using RIA. The results are expressed as IR-SP pg/mg tissue wt. and are the mean ± S.E. from 5 separate experiments. * represents a significant ($P<0.01$) difference from saline control, whereas ** indicates a significant ($P<0.01$) difference from IL-1β alone.

not significantly ($P>0.05$) alter the IR-SP levels compared to saline- (control) treated tissues; 150.3±14.8, 118.7±15.7 or 134±10.7 pg/mg tissue wt., respectively. These results indicate the IL-1β-induced substance P increase in the myenteric plexus involves the production of cyclo-oxygenase metabolites. Although studies have demonstrated the ability of substance P to induce prostaglandins (Brurch et al.,1983), there have been no previous studies showing the opposite effect; prostaglandin induction of substance P synthesis.

7.4. Summary.

The results from this study indicate that IL-1β caused a time- and concentration-dependent increase in IR-SP content in the LM-MP preparations. The action of IL-1β was specific in that it could not be attributed to contamination with a heat stable endotoxin contamination as the effect was abolished by boiling the cytokine (Fruenberg and Galanos, 1990), and could be blocked by using an anti-IL-1β antibody. In addition, the cytokine effect was receptor mediated, as the increase in substance P induced by IL-1β was prevented by a selective receptor antagonist (Dinarello and Thompson, 1991).

The neural origin of the increased substance P was ascertained by demonstrating the release of the neuropeptide by neural stimulation and morphologically by immunohistochemical analysis; 90% of the increased IR-SP content induced by IL-1β in the LM-MP preparation could be released by the sodium channel agonist, scorpion venom, whose action was blocked in the presence of a sodium channel blocker, tetrodotoxin.

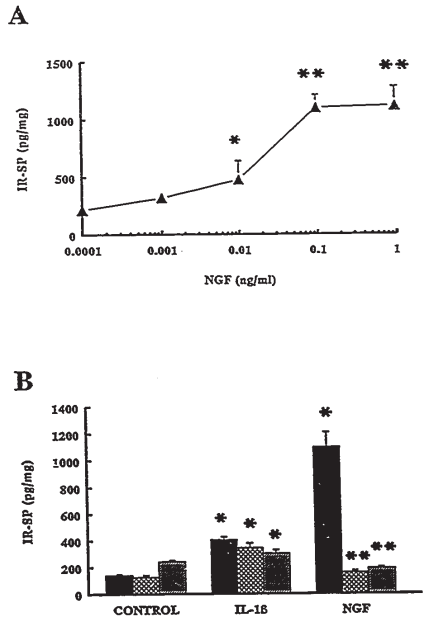


Figure 7.5: Role of nerve growth factor (NGF) in IL-1β effect on IR-SP content. Panel A represents the effect of NGF on IR-SP content in the LM-MP preparations. Tissues were incubated for 6 hrs with specified concentrations of NGF-7S (0.0001-1ng/ml) prior to the measurement of IR-SP content in the tissues. Results are expressed as IR-SP pg/mg tissue wt. and are the mean ± S.E. of 6 experiments. * and ** indicate a significant ($P<0.05$ and $P<0.01$,respectively) difference from saline controls. Panel B represents the effect of neutralizing NGF antibodies on IL-1β and NGF-7S effect on IR-SP content. Tissues were incubated for 6 hrs with either saline (solid bars); rabbit anti-mouse NGF antibody (crossed bars) or sheep anti-mouse NGF antibody (double hatched bars) in the presence of saline, IL-1β or NGF prior to measurement of IR-SP content by RIA. The data were calculated as IR-SP pg/mg tissue wt. and are the mean ± 5 separate experiments. * represents a significant ($P<0.01$) difference from saline controls, whilst ** indicates a significant ($P<0.01$) difference from values obtained from tissue responses to IL-1β or NGF-7S alone.

The results using cycloheximide, suggests that the IL-1β induced an increase in substance P content involves synthesis of a protein that could reflect an intermediary factor, such as NGF. However IL-1β could cause an increase in synthesis of substance P within the intrinsic nerves of the myenteric plexus. Examination of the mechanism underlying the increase in IR-SP content in LM-MP induced by IL-1β shows that it was not mediated by NGF, although NGF alone increased the IR-SP content in the LM-MP preparations, but did appear to involve prostaglandin synthesis.

7.5. Conclusion.

IL-1β causes an increase in substance P content in the intrinsic nerves of the myenteric plexus. The localization of the IL-1β site of action has not been established, it is possible that there is a direct interaction with receptors on substance P- containing nerves. However, recent studies using sympathetic ganglia indicate that IL-1β increases substance P synthesis via the induction of intermediatory substances from non-neuronal cells (Freidin and Kessler, 1991). Examination of the putative underlying mechanisms involved in the increase of substance P induced by IL-1β, suggests that it is protein synthesis-dependent and appears to involve lipid mediators derived from cyclo-oxygenase activation, eg prostanoids and thromboxanes, but not nerve growth factor, even though NGF was shown to increase substance P in the LM-MP.

These results may have bearing on the recent observation (Khan and Collins, 1993) that the increase in substance P synthesis in the jejunal neuromuscular layer

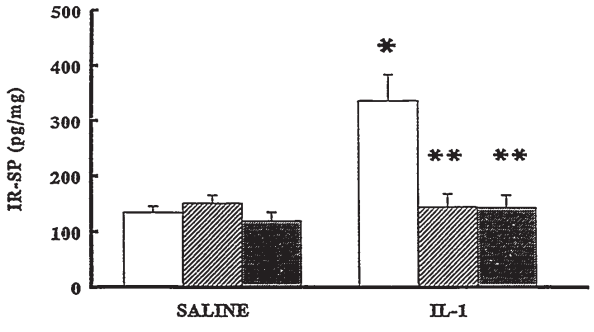


Figure 7.6: Role of prostaglandins in IL-1β effect on IR-SP content. Tissues were incubated for 6 hrs with either saline or 10ng/ml IL-1β in the presence of either saline (open bars); piroxicam (hatched bars); or indomethacin (double hatched bars) before the measurement of IR-SP content by RIA. Results are expressed as IR-SP pg/mg tissue wt. and are the mean ± S.E. of 4 separate experiments. Where * represents a significant ($P<0.01$) difference from saline controls and ** indicates a significant ($P<0.05$) difference from values obtained from tissues treated with IL-1β alone.

from the *T.spiralis* infected rat was attenuated when the animals were pretreated with IL-1 receptor antagonist prior to the nematode infection (Swain et al.,1992). This suggests that the induction of substance P in the intrinsic ganglia of the myenteric plexus is caused by an elevation in endogenous IL-1 produced in this model of inflammation.

CHAPTER 8

THE ROLE OF ENDOGENOUS IL-1 IN CHANGES OF NORADRENALINE RELEASE AND SUBSTANCE P CONTENT OBSERVED IN MYENTERIC PLEXUS FROM *T.SPIRALIS* INFECTED RATS.

8.1. Introduction.

T.spiralis is a nematode that inhabits the epithelial cells of the proximal small intestine of small rodents (Wakelin and Blackwell, 1988). Primary infection with the parasite is associated with an inflammatory infiltration of the mucosa and sub-mucosa of the jejunum. The enteric stage of the infection is marked by accelerated small bowel transit (Castro et al.,1979) and changes in intestinal myoelectrical activity (Palmer et al.,1984), which may be the result of changes in smooth muscle or enteric nerve function (for review see Collins, 1993). Collins and co-worker adopted this animal model of small bowel inflammation and have shown changes in acetylcholine (Collins et al.,1989) and noradrenaline (Swain et al.,1991) release and substance P content (Swain et al.,1992). The changes in myenteric nerve function were attenuated by systemic steroid treatment prior to the infection with the parasite, therefore the alteration in nerve function was attributed to the inflammatory response.

A number of animal models have been developed and applied to the

investigation of putative treatments of inflammatory bowel disease. Studies by Cominelli and colleagues have explored the potential therapeutic values of an IL-1 receptor antagonist. In a colitis model in rabbit induced by formalin and immune complexes, this groups demonstrated an attenuation of the inflammatory response in the colon when the animals were treated with the IL-1ra prior to the induction of colitis (Cominelli et al.,1991). This observation, together with the recent evidence supporting an increase in IL-1 levels within the LM-MP layer of *T.spiralis*-infected rats (Khan et al.,1992), prompted a study in examining the role of endogenous IL-1 in myenteric nerve changes that occur in the *T.spiralis* infected animals.

8.2 Methods.

8.2.1. Infection of rats with T.spiralis.

Infections of Sprague Dawley rats with *T.spiralis* was performed by Ms. Patricia Blennerhassett (Intestinal Disease Research Program). Briefly, serial infections of male CD1 mice were maintained through oral intubation of 400-500 larvae per mouse. Larvae were retrieved from infected mice 30-90 days after the infection. Male Sprague Dawley rats (200-250g) each received 7500 larvae suspended in 1ml PBS administered by gavage. Rats were examined on the sixth day post infection. Non-infected age-matched rats were used as controls.

8.2.2. Experiments involving IL-1 receptor antagonist.

An IL-1 receptor antagonist (Dinarello and Thompson, 1991) was used to

examine the role of endogenous interleukin-1 in the suppression of ^{3}H-NA release or the increase in substance P content observed previously in inflamed tissue from *T.spiralis*-infected rats. Male Sprague Dawley rats received either saline or IL-1ra (10mg in 250µl of saline) delivered by a 7 day osmotic pump implanted subcutaneously 36 hrs before infection with *T.spiralis*. In the absence of a specific Kd value for IL-1 receptor in the LM-MP layer, the therapeutic concentration of IL-1ra used in this study gave a plasma IL-1ra concentration that was a 100-1000 fold greater (Kent et al.,1992) than the IL-1 protein levels measured in the LM-MP preparations (McHugh et al.,1993). On day 6 after the initial infection with the nematode, the animals were sacrificed and the jejunum removed. LM-MP segments (20-30mg) were gently isolated and measured for either noradrenaline release or substance P content.

8.2.3. Measurement of noradrenaline release from LM-MP preparation.

The method used for the measurement of ^{3}H-noradrenaline release has been previously described in chapter 2.2.

8.2.4. Measurement of IR-SP in LM-MP preparations.

The method used for the measurement of immune reactive-substance P has been previously described in chapter 7.2.

8.2.5. Statistical analysis.

All studies involved at least 3 animal, although the exact numbers used are recorded in the legend section. When comparing two groups the Student's t test was used; a one way analysis of variance was used for comparison of more than two groups. The results are expressed as mean ± S.E. (standard error) and statistical significance was achieved if the P value was <0.05.

8.2.6. Materials.

Male Sprague Dawley rats were supplied by Charles River Breeding Farms (Montreal, Quebec); ^{3}H-NA (sp.act.13.3Ci/mM) was from New England Nuclear (Boston, MA); ^{125}I-substance P (sp.act.200Ci/mM) were from Amersham Canada Ltd. (Oakville, Canada); polyclonal rabbit anti-substance P antibody was from Incstar Corp. (Stillwater, MN); IL-1 receptor antagonist was a generous gift from Dr.R.Thompson of Synergen (Denver, CO); Osmotic minipumps were from Alza (CA); and the remaining chemicals were supplied by Sigma Chemicals (St.Louis, MI).

8.3. Results.

8.3.1. The effect of IL-1ra on ^{3}H-NA release.

^{3}H-NA release from the LM-MP layer was expressed as percentage ^{3}H-NA released of total ^{3}H taken up by the tissue. Examination of the tissues showed differences in the weight of tissue; a 4cm tissue segment from control- or IL-1ra-treated animals were lighter than an equal length of tissue from *T.spiralis*-infected

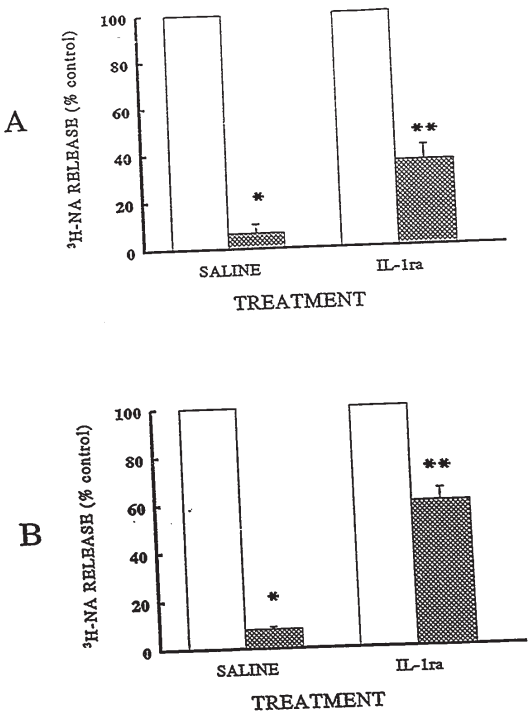


Figure 8.1: Role of endogenous IL-1 in ^{3}H-NA release. LM-MP preparations were removed from saline control (open bars) or *T.spiralis*-infected (crossed bars) rats treated with saline or IL-1 receptor antagonist, and stimulated for ^{3}H-NA release by either EFS (A) or KCl (B). Results are expressed as a percentage of respective control; saline or IL-1ra, and are the mean ± S.E. of 3 separate experiments. * represents a significant (P<0.01) difference from values observed in saline control rats, whereas, ** indicates a significant (P<0.05) difference from saline-treated *T.spiralis*-infected animals.

rat treated with either saline or the receptor antagonist. The increase in weight is considered to be the result of hypertrophy of the smooth muscle layer (Blennerhassett et al.,1992), however, changes in nerve content cannot be excluded. The calculations of the data therefore do not take into consideration the changes in nerve content which might occur during the infection period and due to the effect of the IL-1ra treatment. To partly compensate for this inaccuracy, a comparison between changes in either ^{3}H-NA release or IR-SP content in the inflamed tissue in the presence or absence of IL-1ra was carried out.

As shown in figure 8.1, LM-MP preparations removed from *T.spiralis*-infected rats showed a 92% (P<0.01) or 90% (P<0.01) suppression in EFS- (A) or KCl- (B) induced ^{3}H-NA release compared to tissue removed from un-infected animal; EFS, 0.370.14% vs 5.0±0.89% or KCl, 0.15±0.08% vs 1.62±0.16%, infection vs control, respectively. In animals treated with IL-1 receptor antagonist, an attenuation of the suppressive effect was seen in both EFS (figure 8.1A) and KCl (figure 8.1B) stimulated ^{3}H-NA release. There was no significant (P>0.05) difference observed in stimulated ^{3}H-NA release from un-infected animals treated with saline or IL-1ra; 5.0±0.89% vs 3.9±1.1% or 1.6±0.16% vs 2.3±0.6%, EFS or KCl, respectively.

8.3.2. The effect of IL-1ra on IR-SP content.

In tissue preparations removed from animals infected with *T.spiralis* the IR-SP content measured by RIA was 6 fold greater than the levels observed in un-infected rats; 41.87±7.5 vs 299±141 ng/g tissue wt. (P<0.01), infected vs un-infected,

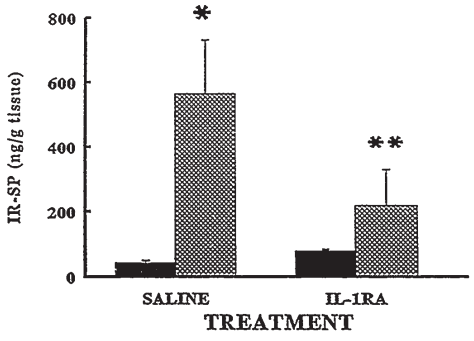


Figure 8.2: Role of endogenous IL-1 in IR-SP content. LM-MP preparations were removed from non-infected (solid bars) or *T.Spiralis*-infected (crossed bars)rats treated with either saline or IL-1ra, and measured for IR-SP content by RIA. Results are expressed as IR-SP ng/g tissue wt. and are the mean ± S.E. from 3 separate experiments. * represents a significant (P<0.01) difference from values observed from uninfected rats, whereas, ** indicates a significant (P<0.05) difference from saline-treated *T.spiralis*-infected rats.

respectively. As shown in figure 8.2, treatment of the *T.spiralis* infected animals with IL-1ra caused a 72.6±13.2% ($P<0.05$) reduction in the elevation of IR-SP levels in the LM-MP layer (117±94 ng/g tissue wt.) in infected rats. Examination of the IR-SP concentrations in tissues removed from un-infected animals treated with either saline or IL-1ra failed to show an increase in IR-SP levels (41.8±7.5 vs 79±5 ng/g tissue wt., saline vs IL-1ra, respectively).

8.4. Summary.

Treatment of the rats with IL-1 receptor antagonist during the infection with *T.spiralis* caused a marked attenuation in the suppression of ^{3}H-NA release observed in the inflamed tissue. Similarly, the substantial increase in IR-SP measured in the inflamed LM-MP preparations was reversed in tissues removed from animals treated with the receptor antagonist. The delivery of the IL-1ra using the mini-pumps was 90% effective (based on the differences in the initial and final weight).

8.5. Conclusion.

Although the increase in weight of the LM-MP preparations of the jejunum of the *T.spiralis* rat was not affected by IL-1ra treatment, the changes in enteric nerve function, in respects to noradrenaline release and substance P content, were partly reversed by the presence of IL-1ra during the infection with the nematode. The partial attenuation in the noradrenaline release in neuromuscular layer from infected animals treated with IL-1ra, suggests that other inflammatory mediators are involved

in alteration of adrenergic nerve function, eg. exogenous TNFα has been shown to suppress noradrenaline release in LM-MP layer after a short exposure (<60 mins). In terms of substance P levels, a dramatic decrease in substance P content was observed in *T.spiralis*-infected rats treated with IL-1ra, thus illustrating a role for endogenous IL-1 in the increased substance P observed in this model.

CHAPTER 9

DISCUSSION

The rationale for studying the effect of cytokines on enteric nerves; altered intestinal physiology in the nematode-infected rat.

Nematode infection of small mammals is accompanied by an alteration in gut physiology. Infection with the nematode *Trichinella Spiralis* (*T.spiralis*) initiates an inflammatory response in the proximal small intestine. This, in turn, influences the physiology of other tissues in the wall of the intestine, including nerves, epithelium and smooth muscle, eventually causing widespread disruption of gut function (Castro, 1991). The *T.spiralis*-induced inflammatory response is reproducible and has proven to be a valuable "*in vivo*" model in which to explore immunophysiological interactions in the inflamed gut.

Among the changes in intestinal physiology that occur during *T.spiralis* infection are alterations in gut motility. Studies by Castro et al. (1979) showed an accelerated transit through the small bowel in rats infected with *T.spiralis*. Since transit reflects gut motility, this alteration in transit time implies a change in intestinal motility. Mechanisms underlying these changes are not fully understood but

may be the consequence of a variety of events, including changes in neural, hormonal or other (endogenous) factors occurring in the animal during *T.spiralis* infection.

The alterations in intestinal motility observed in the *T.spiralis* infected rats are, in part, attributable to functional changes within the gut wall. Studies using extrinsically denervated gut segments from *T.spiralis*-infected rats also showed increased propulsive properties (Alizadeh et al.,1987). However, those results cannot exclude the involvement of extrinsic factors contributing to the changes in motility. Nevertheless, the data do support the hypothesis that alterations in smooth muscle and/or enteric nerves contribute to the alterations in transit through the small bowel during *T.spiralis* infection and, hence, intestinal motility.

Changes in intestinal smooth muscle function occur during *T.spiralis* infection. Studies using longitudinal muscle strips revealed an increased smooth muscle contractility in the inflamed jejunum (Vermillion et al.,1988), but a decreased contractile response in the un-inflamed ileum (Marzio et al.,1990), creating an aboral gradient of contractility down the small intestine. Therefore, it is possible that changes in smooth muscle function may contribute to the propulsive properties of the inflamed intestine, and in turn contribute to the accelerated transit through the small bowel reported in the rat during *T.spiralis* infection.

Experiments utilizing denervated gut segments also implicate intrinsic enteric nerves in altered gut motility seen during *T.spiralis* infection. The precise role of specific neural populations is difficult to assess, since the actions of the many enteric neurotransmitters on motor function are varied. Palmer et al. (1984) showed that in

addition to accelerated transit through the gut in *T.spiralis* infected rats, there was also a general increase and lack of organization in spike activity. These alterations in myoelectrical properties may reflect a disruption of control mechanisms in the enteric nervous system originating from underlying changes in enteric nerve function. Recent work investigating the alterations in enteric nerve function during *T.spiralis* infection has demonstrated changes in neurotransmitter function and content in the myenteric plexus. Studies by Collins et al. showed altered regulation of at least two neurotransmitters, noradrenaline and substance P. A decrease in noradrenaline release from the longitudinal muscle-myenteric plexus (LM-MP) preparations of *T.spiralis*-inflamed jejunum was shown 6 days post infection (Swain et al.,1991). The suppression of noradrenaline release may result in a loss of its modulatory actions on other nerves, leading to a disruption of neural control. An increase in the substance P content of the myenteric plexus was also demonstrated in the *T.spiralis*-inflamed jejunum (Swain et al.,1992), which may contribute to the inflammatory process and possibly to exaggerated release of this neuropeptide. Further studies revealed that the altered enteric nerve function was a consequence of the inflammatory response induced by *T.spiralis* infection. Treatment of rats with steroids prior to and during the course of *T.spiralis* infection, abolished the suppression of noradrenaline release and the increase in substance P content in the LM-MP of the jejunum 6 days post infection (Swain et al.,1991, 1992). Inflammatory cytokines may mediate the changes in enteric nerve function in the inflamed intestine of *T.spiralis* infected rats. The nematode infection induces an inflammatory response that results

in the expression of several cytokines, including IL-1β and TNFα, in the muscle layers of the small bowel within 12 hrs from the onset of *T.spiralis* infection (Khan et al.,1992). Although the effect of cytokines on nerve function is unclear, both IL-1 and TNFα have been shown to alter neural excitability or neurotransmitter properties in studies involving both the central and peripheral nervous system. For example, IL-1β has been shown to suppress acetylcholine (Rada et al.,1991) and catecholamine (Palazzol and Quadri, 1990) release in hippocampus slices. TNFα has been reported to inhibit the secretion of noradrenaline release in sympathetic neurons (Soliven and Albert, 1992). It is, therefore possible that these cytokines have similar effects on neurotransmitter activity in the LM-MP layer of the infected small bowel and that they may play a causal role in the development of the altered noradrenaline release and substance P content observed in the *T.spiralis* model.

Therefore, the aim of this thesis was to characterize the actions of IL-1β and TNFα on noradrenaline release and substance P content in LM-MP preparations from healthy rats, as a prelude to investigating the role of IL-1 as a mediator of the neural changes observed in the *T.spiralis* infected rat. The results demonstrate that exogenous IL-1β causes a suppression of noradrenaline release and an increase in substance P content in the LM-MP of the jejunum. In addition, some of these effects were shared by TNFα, which suppresses noradrenaline release through the release of endogenous IL-1. Furthermore, treatment of *T.spiralis* infected rats with a selective IL-1 receptor antagonist attenuated the suppression of noradrenaline release, and the increase in substance P content. These results implicate IL-1 as a

mediator of neurotransmitter changes seen in the LM-MP of the inflamed intestine.

PART I:

Rationale for using longitudinal muscle-myenteric plexus (LM-MP) preparation; direct and indirect effects of cytokines on adrenergic nerves.

Initial experiments were aimed at examining the mechanisms underlying the effect of IL-1β and TNFα on noradrenaline release, and the results of these experiments indicated the involvement of both indirect and direct pathways. Since the actions of these cytokines may involve intermediate cells, it was decided in the first instance to use a multicellular LM-MP preparation, rather than a preparation derived from a single cell type. The LM-MP preparations were loaded with tritiated noradrenaline, superfused and the release of the neurotransmitter was measured in the superfusate. Although this method does not reveal the endogenous levels of noradrenaline, it does provide a means by which to quantify the release of noradrenaline from the enteric nerves following cytokine exposure. Time course experiments revealed that both IL-1β and TNFα suppressed stimulated noradrenaline release in a biphasic manner, via both distinctive early and delayed components. The results raise the possibility that both IL-1β and TNFα suppress noradrenaline release via a direct pathway involving an interaction between these cytokines and adrenergic nerves accounting for the early component, and an indirect pathway dependent on other events accounting for the delayed effect.

A case for the role of endogenous IL-1 in the suppression of noradrenaline release.

The IL-1β- and TNFα-induced delayed suppression of evoked noradrenaline release from enteric nerves was dependent on protein synthesis. Exposure of the LM-MP preparations to either IL-1β or TNFα over 120 mins stimulated ^{35}S-methionine incorporation, which was successfully blocked by an inhibitor of protein synthesis, cycloheximide. The presence of this inhibitor in the incubation medium also prevented the suppression of ^{3}H-noradrenaline release induced by either IL-1β or TNFα. Therefore, the delayed suppression of noradrenaline by either IL-1β or TNFα involves the induction of intermediary protein(s), that may affect adrenergic nerves, suppressing neurotransmitter release.

My findings also support the notion that the delayed suppression of noradrenaline release by both cytokines is dependent on the induction of endogenous IL-1. The presence of a selective IL-1 receptor antagonist in the incubation medium blocked the TNFα-induced delayed suppression of ^{3}H-noradrenaline release. This result taken in conjunction with protein synthesis, suggests the possibility that TNFα induces the synthesis of endogenous IL-1, which, in turn, may mediate the suppression of noradrenaline release. Prevention of the suppressive effect of IL-1β using the IL-1ra does not distinguish the activity of exogenous human recombinant IL-1β from that of endogenous rat IL-1. Therefore, this result alone does not prove that the delayed suppression of noradrenaline by IL-1 is mediated by endogenous IL-1. When the same experiment was repeated using a specific anti-human IL-1β neutralizing antibody, the human recombinant IL-1β-induced suppression of noradrenaline release was unaltered. Taken together, the fact that the delayed (after

90 mins) addition of IL-1ra blocked the release while anti-human IL-1β antibody failed to do so supports the role of endogenous (rat) IL-1 in the observed suppression of noradrenaline release. Since endogenous IL-1 was not measured in the LM-MP preparations, this conclusion is based on the selectivity of both IL-1ra and the anti-human IL-1β neutralizing antibody.

The source of the endogenous IL-1 in the LM-MP preparation from healthy rats is presently unknown. However structural evidence suggests that macrophage-like cells are a likely source. Macrophages from several systems have been shown to be a rich source of IL-1. Transmission electron microscopy of LM-MP preparations from healthy rats shows the presence of macrophage-like cells in the plexus lying in close proximity to myenteric ganglia, thereby providing a structural basis for consideration of this cell as a putative source of IL-1. Although their location alone does not necessarily imply a function, the location of these putative immune cells in the neuromuscular layer supports the hypothesis that the macrophage-like cell is a source of IL-1 in the LM-MP. Further support for such a role comes from studies on macrophage-like cells in the neuromuscular layer of the mouse colon which exhibited phagocytic properties upon stimulation (Mikkelsen et al.,1988). Also recent studies, using smooth muscle cells or glial cells isolated and cultured from the neuromuscular layer of the rat intestine, point toward the possibility of additional cells being the source of IL-1. Exogenous IL-1β and TNFα also induced IL-1 synthesis in to either smooth muscle cells or glial cells (Warner et al.,1987; Guilian et al.,1986). These cells may also be sources of endogenous IL-1 in the LM-MP

preparation.

The development of a nerve varicosity preparation to study a direct effect of cytokines on nerves.

In contrast to the delayed effect of IL-1β and TNFα, my results with the LM-MP suggest that their early suppressive action (< 60 mins) reflects a possible direct neural effect of the cytokines. Since the early suppressive effect of these two cytokines was independent of protein synthesis, the early suppression of noradrenaline release by IL-1β or TNFα in the intact tissue preparation may involve either intermediary substrate(s) independent of protein synthesis which could be another cell type in the tissue and/or a direct interaction between the cytokines and adrenergic nerves.

Myenteric nerve varicosities from the LM-MP preparation were used to investigate a direct effect of either IL-1β or TNFα on adrenergic nerves. This involved the development of a varicosity preparation capable of selective uptake and release of noradrenaline. My findings show the ability of this crude varicosity preparation to take up and release ^{3}H-noradrenaline in a calcium-dependent and desipramine-sensitive manner, thereby providing a model in which to monitor the uptake and release from adrenergic nerve varicosities. The data obtained from this vesicle preparation were reproducible and showed less variation in stimulated noradrenaline release results those obtained using the superfusion method of release analysis. While the crudity of the preparation remains a concern, purification of the

preparation could only be achieved at the expense of its ability to release ^{3}H-noradrenaline. Nevertheless, this preparation does provide a model in which to explore a putative direct effect of the cytokines with adrenergic nerve terminals.

Evidence in favour of the hypothesis that cytokines may interact with enteric nerves.

My studies suggest that IL-1β interacts with adrenergic nerve terminals to suppress noradrenaline release. The conclusion is based on studies showing that addition of IL-1β, but not TNFα, to myenteric varicosity preparations suppressed stimulated noradrenaline release. A disruption of the membrane integrity by IL-1β was ruled out since occluded lactate dehydrogenase activity was similar in IL-1-treated and control preparations. IL-1β results also showed that its suppression of noradrenaline release in the varicosities was specific and mediated via IL-1 receptors. Therefore, it may be that the IL-1β causes early suppression of noradrenaline by a direct interaction of this cytokine with its receptor on adrenergic nerve varicosities.

The suppression of noradrenaline release by IL-1β potentially involves a heterogenous population of IL-1 receptors on nerves linked to different effector mechanisms. This conclusion is considered in light of the results from experiments involving kinetic and stoichiometry analysis of stimulated ^{3}H-noradrenaline release: In studies examining the concentration-response relationship, different IC_{50} values were obtained for IL-1β suppression of either KCl- or scorpion venom-induced ^{3}H-noradrenaline release; the concentration required to suppress scorpion venom-induced noradrenaline release was greater than that for KCl-stimulated release. In

addition, the time course of the effect of IL-1β on noradrenaline release in the varicosity preparation varied according to the stimuli applied. IL-1β caused a significant suppression of KCl-induced ^{3}H-noradrenaline release after 20 mins, whereas scorpion venom-stimulated noradrenaline release was not significantly suppressed until 40 mins.

Another line of evidence in support of IL-1β suppression of noradrenaline by different effector pathways comes from studies examining the dependence on prostaglandin or thromboxane synthesis for its action. The presence of a cyclo-oxygenase inhibitor, indomethacin or piroxicam, in the medium, partially inhibited the IL-1β-induced suppression of KCl-stimulated ^{3}H-noradrenaline release, but completely abolished the cytokine's suppression of noradrenaline release induced by scorpion venom. Therefore it is possible that KCl-stimulated ^{3}H-noradrenaline release, which is insensitive to tetrodotoxin, and scorpion venom-stimulated release of noradrenaline, which is abolished by tetrodotoxin (Collins et al.,1989), act via sub-populations of IL-1 receptors on nerves coupled to different effector pathways.

The role of prostaglandins or thromboxanes in cytokine-mediated effects on noradrenaline release.

IL-1 receptors are expressed on the surface of several cell types, whose membranes may be present on the varicosity preparation; glial cells, smooth muscle cells and nerve cells (Gottschall et al.,1991; French et al.,1993; Hart et al.,1993). It is possible, therefore, that membrane bound factors could be released by IL-1 and

contribute to its suppression of noradrenaline release. In this study, the putative involvement of prostaglandins as an intermediary substrate in the suppression of noradrenaline release by either IL-1β or TNFα was considered. Studies in other systems demonstrate the ability of prostaglandins to alter the release of neurotransmitters. For example, in colonic mucosa preparations, exogenous prostaglandin E_2 (PGE_2) suppresses noradrenaline release (Wu and Gaginella, 1981_b). My observations support the involvement of prostaglandins in both the early and delayed suppression of noradrenaline release induced by either IL-1β or TNFα.

Moreover, the early suppressive effect of IL-1β on noradrenaline release in the varicosity preparation was, in part, mediated by prostaglandins. The delay in onset of the action of IL-1β in this preparation most likely reflects the time required to generate sufficient prostaglandins, as well as the time required for the prostanoids to suppress noradrenaline release. For example, in hypothalamic synaptosomes, the action of prostaglandins on ^{3}H-noradrenaline release is delayed in onset by 30 mins (Wedel et al.,1978). This delay may signal the involvement of other events in the suppression of noradrenaline release, such as activation of second messenger systems. The prostaglandin independent suppression of KCl-stimulated ^{3}H-noradrenaline release in the varicosity preparation may reflect a sub-class of IL-1 receptors that regulate membrane potential, as identified in *Aplysia* neurons, and are not linked to prostanoid or thromboxane synthesis (Sawada et al.,1991; Szucs et al.,1992).

The precise source of cytokine-induced prostaglandin production in the LM-MP preparations cannot be determined. Among the variety of cells that have been

shown to produce prostanoids upon exposure to either IL-1β or TNFα are glial cells, fibroblasts, smooth muscle cells and nerves, all of which are present in the tissue preparation (for review see Wallace, 1993). Each of these cell types might produce prostaglandins in response to cytokine exposure.

In conclusion, although the favoured model is that IL-1β directly interacts with adrenergic nerve terminals, I cannot exclude the possibility that the initial action of IL-1β is on the membrane of other cell types which liberate mediators (eg. prostaglandins) which, in turn, interact with adrenergic nerves to suppress noradrenaline release. The major contaminant of the varicosity preparation, however, is muscle. A recent study (Bortolami et al.,1993) reveals that there are very few (approx. 45) IL-1 receptors per smooth muscle cell taken from non-inflamed rabbit gut. If a similar number exist on rat smooth muscle cells it is unlikely that sufficient prostaglandins could be synthesized to have a profound effect on noradrenaline release in the varicosity preparation. The interpretation of the presence of an IL-1 receptor on neural membrane is supported by the findings in other systems where IL-1 receptors have been identified on nervous tissue in the brain (Farrar et al.,1987; Haour et al.,1990) and on sympathetic neurons (Hart et al.,1993).

The putative direct effect of TNFα on enteric nerves.

TNFα causes an early, as well as a delayed suppression of stimulated ^{3}H-noradrenaline from LM-MP preparations. This early suppressive effect was independent of protein synthesis, and therefore raises the possibility that TNFα may

suppress noradrenaline release via a direct interaction with enteric nerves. However, such an effect could not be demonstrated using the varicosity preparations, where addition of TNFα failed to alter stimulated ^{3}H-noradrenaline release. To explain these data, one may speculate that the actions of TNFα involves an intermediary cell which either releases a pre-formed protein mediator or involves the synthesis and release of a non-protein mediator, which then suppresses noradrenaline release.

In other systems, TNFα has been shown to alter nerve activity through its receptor on the surface of the nerve membrane (Soliven and Albert, 1992). In this study the evidence for a direct interaction of TNFα with enteric nerves was suggested by the ability TNFα to potentiate IL-1-induced suppression of noradrenaline release in the nerve varicosity preparation. The results showed that the presence of TNFα in the incubation medium potentiated the IL-1β-induced early suppression of stimulated ^{3}H-noradrenaline release using both the LM-MP preparation and nerve varicosities. The simplest interpretation of these data is that TNFα receptors on nerve varicosities initiate a post-receptor event that potentiates IL-1β suppression of noradrenaline release from the nerve varicosities but is, itself, unable to trigger an alteration in noradrenaline release. However, a recent study has shown that TNFα may alter the physiology of a cell independent of its surface receptor (Kagan et al.,1992). Since a TNFα receptor-mediated suppression of noradrenaline release was not examined in this study, this possibility cannot be excluded. Other possible interpretations of these data include the action of TNFα on membranes of a different cell type to produce a membrane derived mediator which in turn

potentiates the action of IL-1β. However, the results also suggest that TNFα interacts with neural membranes to suppress noradrenaline release when used in conjunction with the effect of IL-1β.

A model for the actions of IL-1β and TNFα.

A model summarizing the effects of IL-1β and TNFα on noradrenaline release is shown in figure 9.1. Further studies are required to clarify the putative interaction of cytokines with enteric nerves to alter neurotransmitter release. Such approaches could include the direct identification of IL-1 and TNFα receptors on enteric nerves, by radio-ligand binding studies using purified nerve varicosity preparations, or by immunohistochemical analysis of the intact LM-MP preparations. Furthermore, the development of myenteric nerve cultures may clarify the interaction between cytokines and nerves in the alteration of neurotransmitter release. However, a major disadvantage of this system is the heterogeneity of the nerve population in the myenteric plexus that makes direct interactions between cytokines and a specific nerve difficult to assess. The feasibility of this approach is supported by work in other systems. For example, TNFα suppresses noradrenaline release in sympathetic nerve cultures (Soliven and Albert, 1993).

On the involvement of endogenous IL-1 in the suppression of noradrenaline release in T.spiralis-infected rats.

Having shown that IL-1 is capable of suppressing noradrenaline release from

nerves within the LM-MP layer of the small intestine from healthy rats, I then examined whether endogenous IL-1 contributes to the changes in noradrenaline release observed in tissue from the *T.spiralis* infected animal. Treatment of the rats with IL-1 receptor antagonist during the period *T.spiralis* infection attenuated the suppression of noradrenaline release from the neuromuscular preparation by approximately 50%. The interpretation of these data rely on the previously demonstrated ability of the receptor antagonist to selectively block the biological activity of IL-1 *in vivo* (Kent et al.,1992). The partial efficacy of IL-1ra in the infected intestine, suggests that either inadequate amounts of the receptor antagonist were utilized or that other mediators are involved in the suppression of stimulated ^{3}H-noradrenaline release in the *T.spiralis* infected rat. Nevertheless, the main conclusion drawn from this series of experiments is the involvement of endogenous IL-1 in the suppression of noradrenaline release, supporting the conclusions drawn from the *in vitro* experiments using exogenous IL-1β and TNFα. That is, IL-1 potentially interacts with enteric nerves to suppress noradrenaline release.

Pathophysiological implications.

My results suggest that IL-1 is a major factor involved in the suppression of noradrenaline release observed within the gut of *T.spiralis* infected animals. The evidence to support this statement comes from the results using the *in vivo* model, as well as *in vitro* experiments (LM-MP and varicosity preparations), which provide an explanation of how IL-1 suppresses noradrenaline release, implicating both direct

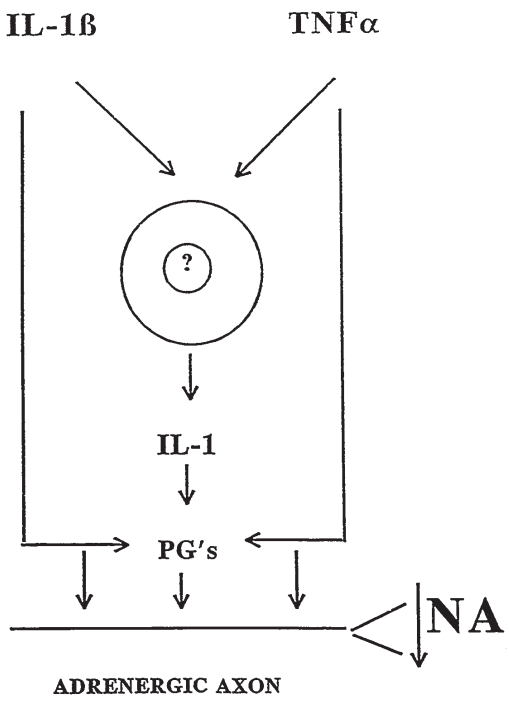


Figure 9.1: A model describing the underlying suppressive action of IL-1β and TNFα on noradrenaline release in the myenteric plexus.

and indirect interactions of IL-1 with enteric nerves. Noradrenaline is the major neurotransmitter of the sympathetic nervous system. Sympathetic stimulation usually inhibits motility, therefore it is possible that a decrease in the bio-availability of noradrenaline could promote an increase in motor activity. Therefore, one may speculate that the suppression of noradrenaline release seen in this study results in a loss of a fine-tuning of the neural control of motility. This speculation is supported by the increased myoelectrical activity observed by Palmer et al. (1984) in the intestine of rats infected with *T.spiralis*.

PART II:

Rationale for studying mechanisms underlying changes in substance P in the inflamed intestine.

Also investigated in this study were putative alterations in neuropeptide content, which may influence enteric nerve function and hence neural regulation of gut motility during *T.spiralis* infection. Substance P is an excitatory neurotransmitter that affects a variety of cellular functions, including smooth muscle contractility (Wali, 1985) and growth (Greenwood et al.,1990). In addition, substance P is considered to be a neurotransmitter of sensory nerves (Raybold and Meyer, 1991) in the gut and is known to possess pro-inflammatory properties (Payan, 1989). Studies have shown that *T.spiralis* infection of rats causes an increase in substance P levels in the neuromuscular layer of the inflamed jejunum (Swain et al.,1992). The neural source of the increased substance P is unclear, but possible sources include:

primary afferent fibres with the cell bodies in the dorsal root ganglia; intrinsic nerves whose ganglia are located within the enteric nervous system; and inflammatory cells. For example, substance P has been located within eosinophils during *T.spiralis* infection (Weinstock, 1992). Changes in substance P content within the neuromuscular layer of *T.spiralis* infected rats may have an impact on the inflammatory processes and exhibit an exaggerated physiological function.

The increased levels of substance P were shown to be a result of the inflammatory response since treatment of rats with steroids prior to and during the course of infection abolished the increase in substance P content in the LM-MP layer of the small bowel (Swain et al.,1992). This result is supported by work in other systems, such as asthma (Nadel, 1991) and rheumatoid arthritis (Matucci-Cerinic et al.,1993), which have also linked inflammation to an increase in substance P. Although the mediators involved in this elevation of substance P levels have yet to be identified, studies using cultured sympathetic ganglia have shown that IL-1 can stimulate increases in substance P (Freidin and Kessler, 1991; Jonakait et al.,1991). These results, together with the evidence that IL-1 messenger RNA levels increase in the neuromuscular layer of small bowel during *T.spiralis* infection (Khan et al.,1992), suggest that IL-1 may be involved in the inflammation-induced increase in substance P content in this tissue. My goal was first to investigate the ability of exogenous IL-1β to influence substance P content in LM-MP preparations from healthy rats and, later, to use the IL-1ra to examine the role of endogenous IL-1 in the elevated substance P content observed in the *T.spiralis* infected animals.

Effect of exogenous IL-1β on substance P levels.

The increase in substance P content was selective for IL-1β, since the increase could be prevented by either boiling the cytokine, or by neutralizing the recombinant cytokine with specific antibodies prior to incubation with the tissue preparation. Moreover, the presence of the IL-1 receptor antagonist in the incubation medium also blocked the IL-1β-induced increase in this neuropeptide thus indicating that IL-1 receptors mediate the increase in substance P content. The results using exogenous IL-1β is similar to that found in tissue from *T.spiralis*-infected animals. These results are consistent with IL-1 being a mediator involved in the increase of substance P content observed in the inflamed intestine of the nematode-infected animal.

The IL-1β-induced increase in substance P content is present in nerves of the LM-MP preparation. Incubation of IL-1β-treated LM-MP preparations with scorpion venom, an inhibitor of sodium channel activation (Catterall, 1980), caused a 90% depletion of substance P from the tissue. This effect was blocked by the co-incubation with tetrodotoxin, a toxin that prevents activation of fast sodium channels (Catterall, 1980). These observations indicate that substance P is situated within the nerves of the neuromuscular layer of the small bowel. This was supported by subsequent immunohistochemical analysis of IL-1β-treated tissue preparations. The residual amount of substance P remaining after scorpion venom treatment may reflect an inadequate concentration of scorpion venom. However, increasing the concentration of this neurotoxin beyond 10μg/ml may lead to non-specific toxic effects because the venom is a complex mixture of several toxins. I cannot exclude

the possibility that substance P is present in other cells of the neuromuscular layer, including occasional inflammatory cells resident in the neuromuscular layer, macrophage-like cells, or glial cells. Nevertheless, evidence from two distinct approaches argue that the major source of IL-1β-induced increase in substance P content of LM-MP occurs in nerves.

The increase in substance P content was a reflection of IL-1β-induced synthesis of the neuropeptide in the LM-MP preparation. First, I have shown that IL-1β stimulated an increase in ^{35}S-methionine incorporation by the tissues. Moreover, the presence of cycloheximide in the incubation medium prevented IL-1β-induced increase in substance P; evidence which supports IL-1β induction of substance P synthesis. Direct support for this hypothesis (eg. by demonstrating increased transcription of substance P message) has not been obtained in this study. Since primary afferent nerves have their cell bodies outside the LM-MP preparation, it is concluded that the IL-1β induced substance P synthesis likely occurs in intrinsic nerves which have their cell bodies in ganglia of the enteric nervous system.

It is possible that the apparent increase in substance P content could be due to cross-reactivity of IL-1 or IL-1-induced mediators with the anti-substance P antibody used in the radioimmunoassay. Furthermore, there is also the possibility that the increased substance P could be due to IL-1 inhibiting the degradation of the neuropeptide, ie. it could be hypothesized that IL-1 decreases neutral endopeptidase (NEP) activity. However, this eventuality was excluded on the grounds that IL-1-induced increased substance P was contained in nerves and no NEP activity has been

demonstrated inside of nerves (Hwang et al.,1993). Although my experimental findings were limited, the hypothesis that IL-1β causes an increase in substance P levels in intrinsic nerves of the myenteric plexus is favoured, similar results have been reported in other systems (Jonakait et al.,1991; Freidin and Kessler,1991).

On the potential role of other factors in the action of IL-1β.

The precise mechanism underlying the IL-1β-induced increase in substance P content is presently unknown. It is possible that IL-1β directly interacts with intrinsic nerves of the LM-MP preparation causing a direct up-regulation of synthesis at the pre- and/or post transcriptional levels. However, the heterogenous nature of the tissue makes it difficult to prove a direct interactions of IL-1β with substance P containing nerves; an intermediary cell may be involved. Interestingly, recent studies using cultured sympathetic ganglia have shown that IL-1β causes an increase in substance P synthesis only in the presence of non-neuronal cells (Freidin and Kessler, 1991; Jonakait et al.,1991). This finding, together with the lack of evidence to support a direct effect of IL-1β on substance P synthesis in nerves raises the possibility that IL-1β causes an increase in substance P content via an intermediary cell.

I examined the role of putative mediators, such as nerve growth factor (NGF) and prostaglandins, in the IL-1β-induced increase in substance P content of the neuromuscular layer of the small bowel. In other systems, such as superior cervical and dorsal root ganglia, NGF has been shown to cause an increase in substance P synthesis (Vedder et al.,1993). Furthermore, IL-1β has been shown to induce NGF

synthesis in several cell types, including glial and smooth muscle cells (Bartfai and Schultberg, 1993). I, therefore, examined the putative involvement of NGF in the IL-1β-induced increase in substance P content by using LM-MP preparations. Initial experiments showed that addition of exogenous NGF caused an increase in substance P content in the tissue preparation, and this effect was selectively blocked by anti-NGF neutralizing antibodies. However, the presence of these neutralizing antibodies in incubation medium failed to prevent the IL-1β-induced increase in substance P content. These observations are supported by others who indicate that endogenous NGF is not involved in the increased substance P synthesis by IL-1β in sympathetic ganglia (Jonakait et al.,1991). Since both IL-1β and NGF are capable of increasing substance P levels in the LM-MP preparation, it may be of interest to explore the underlying mechanism(s) to determine whether there is a common mediator that modulates substance P synthesis in intrinsic nerves of the myenteric plexus. Furthermore, since substance P is considered promote and maintain an inflammatory response (Payan, 1987) and NGF has been shown to exert a protective action under inflammatory conditions (Amico-Roxas et al.,1989), it may be interesting to investigate the possibility of an interaction between the two substances in the neuromuscular layer during intestinal inflammation.

Since I had already found that prostanoid synthesis is a common element of IL-1- and TNFα-induced suppression of noradrenaline release, I examined the role of these mediators in the IL-1β-induced increase in substance P content. Incubation of the tissue preparation with IL-1β in the presence of either indomethacin or

piroxicam attenuated the cytokine-induced increase in substance P content by approximately 50%. The ability of exogenous prostanoids to increase substance P content in this tissue preparation was not examined in this study, and to date no study has shown a direct link between prostaglandins and substance P synthesis. However, the involvement of prostaglandins has been shown indirectly by Jonakait et al. (1991). This group showed that the presence of indomethacin in the incubation medium blocked IL-1β induced synthesis of substance P in sympathetic ganglia, implicating prostaglandins as mediators. Since the concentrations of the inhibitors used in this study have been shown to selectively block cyclo-oxygenase activity, it may be concluded that prostanoids are among the putative mediators involved in IL-1β-induced increase in substance P content in the neuromuscular layer of the small bowel.

Other possible intermediates, such as leukaemia inhibitory factor (LIF) and endogenous neurotransmitters, involved in the IL-1β-induced increase in substance P have not been considered in this study. However these are of potential importance and will be discussed briefly. LIF, also called cholinergic differentiation factor, belongs to a series of proteins known as "neurokines" (Rao and Landis, 1992). These mediators have been shown to alter the neurotransmitter phenotype of certain nerves during development and during pathophysiological conditions. Recent work has reported that LIF induces substance P synthesis in neuronal cultures (Freidin and Kessler, 1991). It has also been shown that IL-1β-induced substance P synthesis in sympathetic ganglia is mediated by LIF (Shadiack et al.,1993). It would therefore be

of interest to examine the role of LIF and other neurokines, such as interleukin-6 and ciliary neurotrophic factor, in the IL-1β-induced increase in substance P content in the neuromuscular layer of the small bowel.

Although the initiation of changes in nerve function is the result of the inflammatory process, there could be other regulatory mechanisms occurring within the microenvironment surrounding enteric nerves which, in turn, modulate nerve function. Kessler and Freidin (1991) have suggested that there is a hierarchy among regulatory influences of cytokine actions on nerves . For example, the presence of scorpion venom or KCl in the incubation medium prevented the induction of substance P synthesis by IL-1β in sympathetic ganglia (Jonakait et al.,1991). Therefore the local availability of native neurotransmitters in the microenvironment may influence the phenotypic expression of the nerve. Since IL-1β alters the release of neurotransmitters, such as acetylcholine (Main et al.,1993) and noradrenaline, it is possible that these changes in local neurotransmitter release in sufficient concentrations may influence the effect of IL-1β on substance P content in the neuromuscular layer.

On the role of IL-1 as a mediator of increased substance P content in the inflamed intestine.

The results from experiments using exogenous IL-1β showed a similar increase in substance P content to that observed in LM-MP preparations from *T.spiralis* infected rats. I then examined the putative role of endogenous IL-1 in infected

animals. Treatment of the rats with IL-1 receptor antagonist during *T.spiralis* infection attenuated the increase in substance P content by over 80%. These results therefore suggest that endogenous IL-1 is a major contributor to the substance P increase in the neuromuscular layer of the nematode-infected animals. As stated previously, the inability of IL-1ra to completely prevent the increase in substance P content may be a consequence of an inadequate dose of IL-1ra. Alternatively it may be due to involvement of other endogenous factors. *In vitro*, NGF alone was shown to increase substance P content in the myenteric plexus, but my studies demonstrate that NGF but did not mediate the IL-1β-induced increase in substance P content in the LM-MP preparation. It has been shown recently that NGF levels are elevated in the neuromuscular layer of jejunum in day 6 post *T.spiralis* infection (Blennerhassett and Davis, 1991). Although it is possible that IL-1 causes an increase in NGF levels, as indicated in other systems (Bartfai and Schultberg, 1993), it remains possible that any increase in substance P independent of IL-1 may involve NGF.

The site of action of endogenous IL-1 in the intact animal, resulting in elevated levels of this neuropeptide in the LM-MP preparation, is unknown. Endogenous IL-1 may influence the levels of substance P content in either extrinsic sensory nerves, infiltrating inflammatory cells and/or intrinsic nerves present within the neuromuscular layer during *T.spiralis* infection. Using the *in vitro* model I have produced evidence in support of endogenous IL-1 causing an increase in substance P levels in intrinsic nerves. This is endorsed by recent studies measuring preprotachykinin messenger RNA expression selective for substance P, which showed

an increased expression of substance P in the LM-MP preparation during *T.spiralis* infection. This increased expression was subsequently abolished upon treatment with the IL-1 receptor antagonist (Khan and Collins, 1993). These result do not, however, exclude an action of endogenous IL-1 on extrinsic nerves or other cells in the intact animal.

Pathophysiological implications.

The consequence of the increase in substance P content on intestinal physiology is unclear. Substance P is known to have a profound effect on a variety of cell types in the wall of the intestine. An increase in substance P content within the neuromuscular layer of the small intestine may be relayed to the nerve terminals. Stimulation of these nerves cause an increase in substance P release and therefore may exaggerate its physiological activity on surrounding tissues. In terms of motility, an increase in releasable substance P will alter smooth muscle function, causing increased contractility (Wali, 1985). Such increased muscle contractility may contribute to the accelerated transit through the small bowel occurring during *T.spiralis* infection. In contrast, there is also the possibility that the increase in substance P content measured in the LM-MP layer of inflamed small intestine may be the result of a decrease in neuropeptide release.

In addition, the increase in substance P content within intrinsic nerves of the neuromuscular layer may act as a modulator of myenteric ganglion function. Using animal models, it has been shown that inflammation at one site alters neural function

and muscle function at non-inflamed remote sites (Jacobsen et al, 1993$_b$; Marzio et al.,1990). An increase in substance P within myenteric ganglia might relay the message of a changed microenvironment (ie. inflammation) to unaffected areas of the myenteric plexus (figure 7.2). Therefore, it is possible that cytokine-induced changes in nerve phenotype may contribute to this extension of altered physiological function.

Finally, recent studies have suggested that an increase in substance P contributes to the maintenance of the inflammatory response during intestinal damage or infection (McCafferty et al., personal communication). Treatment of mice with a specific antibody against substance P during *T.spiralis* infection attenuated the inflammatory response as monitored by myeloperoxidase activity. The same antibody treatment also prevented lymphocyte proliferation in the intestinal mucosa (Agro and Stanisz, 1993). These observations demonstrate that an increase in substance P levels in the gut may lead not only to the exaggerated physiological effects of the neuropeptide, but also to the maintenance of inflammation during either intestinal damage or infection.

Clinical implication.

The effect of inflammation on nerve function in patients with inflammatory bowel (IBD) disease is poorly understood. Alterations in nerve structure have been reported in inflamed as well as non-inflamed intestinal segments from patients with IBD (Davis et al.,1953; Storesteen et al.,1953). Specifically there is evidence of

increased noradrenaline (Penttila et al.,1975) and substance P (Koch et al.,1987) content in gut segments from patients with ulcerative colitis. However, the significance of the changes in neurotransmitter content is unknown. Since reference to an alteration in nerve function comes only from indirect sources, such as demonstration of abnormal gut motility and sensory perception (for review see Collins, 1993), future studies could investigate the implication of changes in neurotransmitter content on enteric nerve function in patients with IBD and determine whether there are similar changes in neurotransmitter properties as those observed in the *T.spiralis* model of intestinal inflammation.

An interesting observation from my thesis is that treatment of animals with an IL-1 receptor antagonist during *T.spiralis*-induced inflammation attenuated the changes in both noradrenaline release and substance P content in the neuromuscular layer of the small bowel. Perhaps the attenuation of altered neurotransmitter properties with selective antagonists of inflammatory mediators, eg., IL-1 may alleviate the adverse physiological changes occurring during inflammatory diseases of the gut. For example, transit time through the small intestine might be slowed by such treatments. Therefore, this thesis work provides not only insights into changes in enteric nerve function underlying the pathophysiology of gastrointestinal inflammatory diseases but it also identifies possible treatments aimed at blocking selective inflammatory mediators.

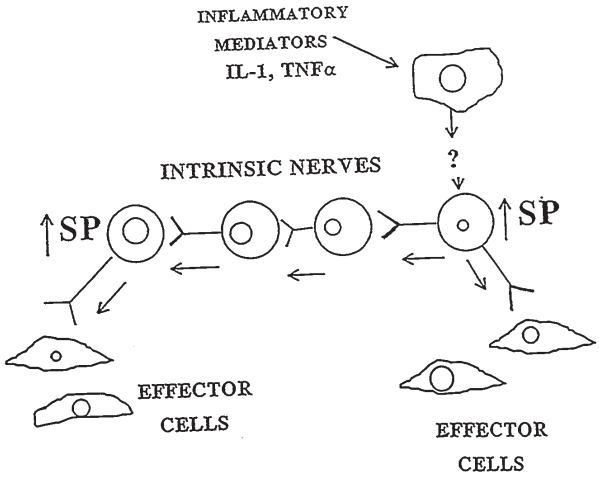
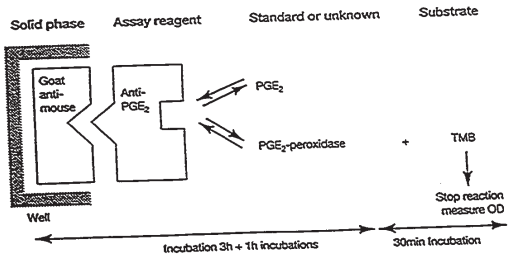


Figure 9.2: Diagram displaying a putative function of the IL-1β-induced increase of substance P in the myenteric plexus.

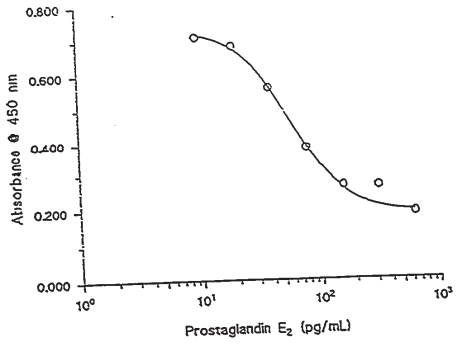
APPENDIX 1.

Measurement of prostaglandins E_2 (PGE_2).

The measurement of PGE_2 was based on the competition between unlabelled PGE_2 and a fixed quantity of peroxidase labelled PGE_2 for a limited number of binding sites on a PGE_2 specific antibody. With fixed amounts of antibody and peroxidase labelled PGE_2, the amount of peroxidase labelled ligand bound by the antibody was inversely proportional to the concentration of added unlabelled ligand. A schematic representation of the protocol is shown below:



The samples measured using this kit were diluted 1:100 in order to produce reading within the linear part of the dose response curve (1-32pg/ml). The sensitivity of the assay upper limits of the assay was 16pg/ml. Although the cross reactivity of anti-PGE_2 observed with PGE_1 is 7%, cross reactivity with other prostaglandins is <1%. The results were calculated directly from the standard curve and then corrected for the dilution factor. An example of a standard curve obtained using this kit is shown below:



APPENDIX 2

Electron microscopy analysis of LM-MP preparations and nerve varicosities.

Initially, the varicosities or the LM-MP preparations was placed in a fixative solution consisting of 2% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) and allowed to stand for 1 hr at 4°C. This suspension was then re-spun for 5 mins at 11,500 xg and the pellet resuspended in 1% O_5O_4 in 0.1M sodium cacodylate buffer, fixed for 1 hr at 4°C. This mixture was then re-centrifuged and passed through a series of dehydration steps with an ascending ethanol series, then a 100% propylene oxide and finally embedded in Spurr's epoxy resin. The embedded pellet was then cut into thin sections with a diamond knife and stained with uranyl acetate and lead citrate. The sections were then viewed using a JOEL 1200EX Biosystem (Tokyo, Japan) electron microscope and photos were taken using Kodak TMax 400 ASA film.

APPENDIX 3

Immunohistochemical analysis of LM-MP preparations for immunoreactive substance P.

LM-MP preparations were pinned flat on cork and submerged in Zamboni's fixative for 12 hrs at 4°C before rinsing with DMSO (dimethyl-sulphoxide) for 10 mins x 3, followed by PBS for 10 mins x 3. The tissues were then stored in PBS/0.01% sodium azide at 4°C prior to immunohistochemical analysis. Detection of substance P- like immunoreactivity was carried out as previously described by Sharkey et al. (1984). Briefly, the fixed LM-MP preparations were incubated with a rabbit anti-substance P antibody (1:1000) for 48 hrs at 4°C. The antibody was then washed off and the tissues further incubated in goat anti-rabbit IgG conjugated to florescein isothiocyanate (FITC) for 1 hr at room temperature. Finally, the tissues were washed again in PBS containing 0.1% triton X100 and mounted on glass slides using bicarbonate-buffered glycerol (pH 8.6). Photomicrographs were taken using a Zeiss Axioplan microscope with epifluorescence using Kodak TMax 400 ASA film.

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