UNIVERSITY OF CALGARY

THE ROLE OF CALCITONIN GENE-RELATED PEPTIDE (CGRP) IN COLONIC INFLAMMATION AND SECRETION IN THE RAT DISTAL COLON

BY

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF NEUROSCIENCE AND GASTROINTESTINAL SCIENCES

CALGARY, ALBERTA

JUNE, 1999

ABSTRACT

Calcitonin gene-related peptide (CGRP) is a neuropeptide located in intrinsic and extrinsic neurons of the GI tract. This study was done to examine the contribution of CGRP to colonic inflammation and secretion in the trinitrobenzenesulfonic acid (TNBS) model of colitis in the rat, by using available antagonist and agonists of the CGRP receptor subtypes in vivo and in vitro. In vitro studies in Ussing chambers showed that CGRP caused a concentration-dependent Cl^- secretion by acting on a novel receptor located directly on the colonic epithelium. The response to CGRP was preserved in inflamed tissues. CGRP increased conductance and permeability. In the in vivo experiments, administration of CGRP increased the microscopic damage score at 3 days post-TNBS, while a $CGRP_1$ receptor antagonist reduced the macroscopic damage score 7 days post-TNBS. These results suggest that CGRP is a neuropeptide capable of modulating both the inflammatory response and the epithelial function of the inflamed rat colon.

ACKNOWLEDGEMENTS

I wish to acknowledge my supervisors Dr. Wallace K. MacNaughton and Dr. Keith A. Sharkey for their guidance and support during the last two years. I would also like to thank my friends Winnie Ho and Cathy MacNaughton not only for their technical assistance but also for their friendship which is very precious to me. I also thank Kelly Cushing for her technical support.

DEDICATION

This thesis is dedicated to my husband and parents who always supported me.

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AC	adenylate cyclase
ACh	acetylcholine
ADM	adrenomedullin
Ala	alanine
Asn	arginine
Asp	aspartic acid
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
Ca^{2+}	calcium ion
C. difficile	*Clostridium difficile*
CaMKII	Ca^{+}/calmodulin-dependent protein kinase II
cAMP	adenosine 3',5'-cyclic monophosphate
cGMP	guanosine 3',5'-cyclic monophosphate
CGRP	calcitonin gene-related peptide
ChAT	choline acetyltransferase
Cl^-	chloride ion
CO_2	carbon dioxide
^{51}Cr-EDTA	^{51}Chromium-labeled ethylenediamine tetraacetic acid
CRLR	calcitonin-receptor-like receptor
Cys	cystine
[Cys(ACM)^{2,7}]CGRP	[acetamidomethyl-cysteine^{2,7}] calcitonin gene-related peptide
[Cys(Et)^{2,7}]hαCGRP	[ethionamide-cysteine^{2,7}] calcitonin gene-related peptide
D	dalton
EC_{50}	effective concentration of the half maximal effect
ENS	enteric nervous system
G	conductance
Gly	glycine
h	hour

H^+	hydrogen ion
HCO_3^-	bicarbonate ion
His	histidine
5-HT	5-hydroxytryptamine
IBD	inflammatory bowel disease
ID_{50}	inhibitory dose of the half-maximal effect
IFN γ	interferon γ
IL	interleukin
Ile	isoleucine
IL-1Ra	interleukin-1 receptor antagonist
i.p.	intraperitoneal
i.v.	intravenous
I_{SC}	short circuit current
K^+	potassium ion
kDa	kiloDalton
kg	kilogram
LT	leukotriene
Leu	leucine
Lys	lysine
Met	methionine
min	minute
ml	milliliter
mM	millimolar
MPO	myeloperoxidase
NK	neurokinin
NKA	neurokinin A
Na^+	sodium ion
NaCl	sodium chloride
nM	nanomolar
NO	nitric oxide

O_2	oxygen
PD	potential difference
PGs	prostaglandins
Phe	phenylalanine
PI	phosphatidylinositol
PKA	protein kinase A
PLC	phospholipase C
Pro	proline
RAMPs	receptor activity modifying proteins
RCP	receptor component protein
RDC1	canine orphan receptor 1
ROMs	reactive oxygen metabolites
Ser	serine
SP	substance P
TGF-β	transforming growth factor β
Th	T helper
TNBS	trinitrobenzene sulphonic acid
Thr	threonine
Tyr	tyrosine
TTX	tetrodotoxin
μA	microamps
μM	micromolar
UC	ulcerative colitis
V	volt
Val	valine
VIP	vasoactive intestinal polypeptide

CHAPTER 1

OVERVIEW

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract characterized by periods of remission and exacerbation. Although no specific cause has been recognized as yet, several important factors involved in this disease are known. Genetic, environmental, microbiological, immune and neuronal factors all may have contributory roles (1).

Diarrhea is one of the common signs and symptoms of IBD and other inflammatory diseases of the intestine. Diarrhea in general is considered a protective mechanism of the intestine. In inflammatory disorders of the intestine, normal regulatory mechanisms of electrolyte transport and epithelial cell function are believed to be altered (2). In addition, increased intestinal permeability may play a role in the pathogenesis of diarrhea in patients with Crohn's disease (1). In addition to increased permeability in patients themselves, increased permeability has been reported in symptom-free, first-degree relatives (3). Several studies have confirmed this observation, although some have failed to find altered permeability in relatives (1;3-6). Many factors are involved in the regulation of electrolyte transport and intestinal permeability. Alterations in the structure and function of the ENS in inflammation may contribute to the dysfunction of electrolyte transport and intestinal permeability.

The nervous system exerts the greatest regulatory influence over the intestine, and may participate in intestinal inflammation. The first piece of evidence suggesting the involvement of the nervous system in IBD was the histological observation of an increased number of myenteric ganglion cells in chronic ulcerative colitis (7). This has led to further studies examining the distribution of neuropeptides and neurotransmitters in IBD (8). Whilst, some abnormalities have been documented, the role that nerves play in

IBD is still unclear. Neurotransmitters released by nerve fibers in the gastrointestinal tract or elsewhere can produce local inflammatory reactions by interacting with the immune system in this way they may contribute to the pathogenesis of IBD and its symptoms(9). The local anesthetic, lidocaine, has anti-inflammatory effects in ulcerative proctitis in human and in the TNBS model of rat colitis, suggesting a neuronal involvement in IBD and experimental colitis. Although all of the anti-inflammatory effects of lidocaine are not the result of its neuronal effect (10). Primary afferent nerves have been suggested to play an important role in gastrointestinal mucosal defense and their ablation by systemic administration of capsaicin exacerbates colitis induced by acetic acid, dextran sodium sulfate, formalin and TNBS in animals (1). Sympathetic nerves have also been demonstrated to play a role in an animal model of colonic inflammation (11). Two possible ways that nerves are involved in IBD have been proposed. First, nerves may play a role in the development and maintenance of inflammation through local release of certain neurotransmitters. These neurotransmitters, including substance P and calcitonin gene-related peptide, have been shown to modulate many aspects of mucosal function, including blood flow and secretion. They may play a role in the recruitment of granulocytes and lymphocytes and in the modulation of mast cell activation (12;13). Lymphocyte function may also be modulated by neurons within the colonic mucosa, since these cells have receptors for a number of neuropeptides and norepinephrine and in cell culture neuropeptides can influence cytokine secretion, apoptosis and differentiation of immune cells (14;15). Second, once initiated (by whatever means), the process of inflammation may disrupt the normal pattern of innervation and interactions of nerves and their target tissues (16). Such an example is the work showing that the inflammatory

mediator. IL-1β is responsible for reduced [^{3}H]norepinephrine release from the myenteric plexus, of the TNBS-induced inflamed rat distal colon (17).

Of the many neuropeptides present in the intestine, we have chosen to study calcitonin gene-related peptide (CGRP). The reason is that different experimental models of intestinal inflammation in rabbits, rats and ferrets have revealed reduced CGRP-immunoreactivity as well as decreased intestinal content in a time-dependent fashion, suggesting CGRP release in animal models of intestinal inflammation (18). However, the experiments examining at the effect of this neuropeptide in experimental colitis are few. CGRP is a 37 amino acid peptide found in both central and peripheral nervous systems as well as non-neuronal tissues such as thyroid, pancreas and kidney (19). CGRP is a neurotransmitter in the enteric nervous system, both in the submucosal and myenteric plexuses, and is also found in primary afferent neurons innervating the gastrointestinal tract (31).

The vasodilatory and immunomodulatory (such as mast cell degranulation and neutrophil accumulation) effects of CGRP are known in contributing to neurogenic inflammation in human skin (20). Axonal reflexes induced in primary afferent fibers can generate certain responses such as mast cell degranulation, granulocyte infiltration and edema. Substance P and CGRP are two important neurotransmitters in spinal primary afferents (9;21). These fibers have a dual afferent-efferent function, meaning that both limbs of a reflex pathway (afferent or sensory and efferent or motor limbs) are mediated through fibers of the same neuron (9). The release of CGRP caudally from primary afferent fibers as well as enteric neurons may contribute to gastrointestinal inflammation.

CGRP is able to excite myenteric neurons and release acetylcholine in the guinea pig ileum (22;23).

CGRP also induces electrolyte transport in the gastrointestinal tract. Different mechanisms are involved in CGRP-induced water and electrolyte secretion in different species. In the guinea pig colon *in vitro*, CGRP-induced ion transport was through a neuronally mediated pathway, by releasing acetylcholine from the myenteric plexus. The cholinergic myenteric neurons can either activate submucosal secretomotor neurons or directly project to the mucosa of the guinea pig distal colon causing chloride secretion (23). In the rat colon studied *in vitro*, secretion occurred only when the myenteric plexus was removed (24).

In this project, we were interested in investigating the role of the nervous system in an animal model of IBD, with a focus on CGRP. **The hypothesis we tested was that CGRP is a proinflammatory and secretory peptide involved in colonic inflammation and secretion in the rat distal colon.** We investigated the pathophysiological role for CGRP in the TNBS model of colitis in the rat, using pharmacological tools. Furthermore, we tried to determine the mechanisms underlying electrolyte secretion induced by CGRP in the rat distal colon and to identify the CGRP receptor(s) involved in this process in both normal and inflamed tissues. Finally, we also examined epithelial responses to CGRP in inflamed tissues.

CHAPTER 2

INTRODUCTION AND LITERATURE REVIEW

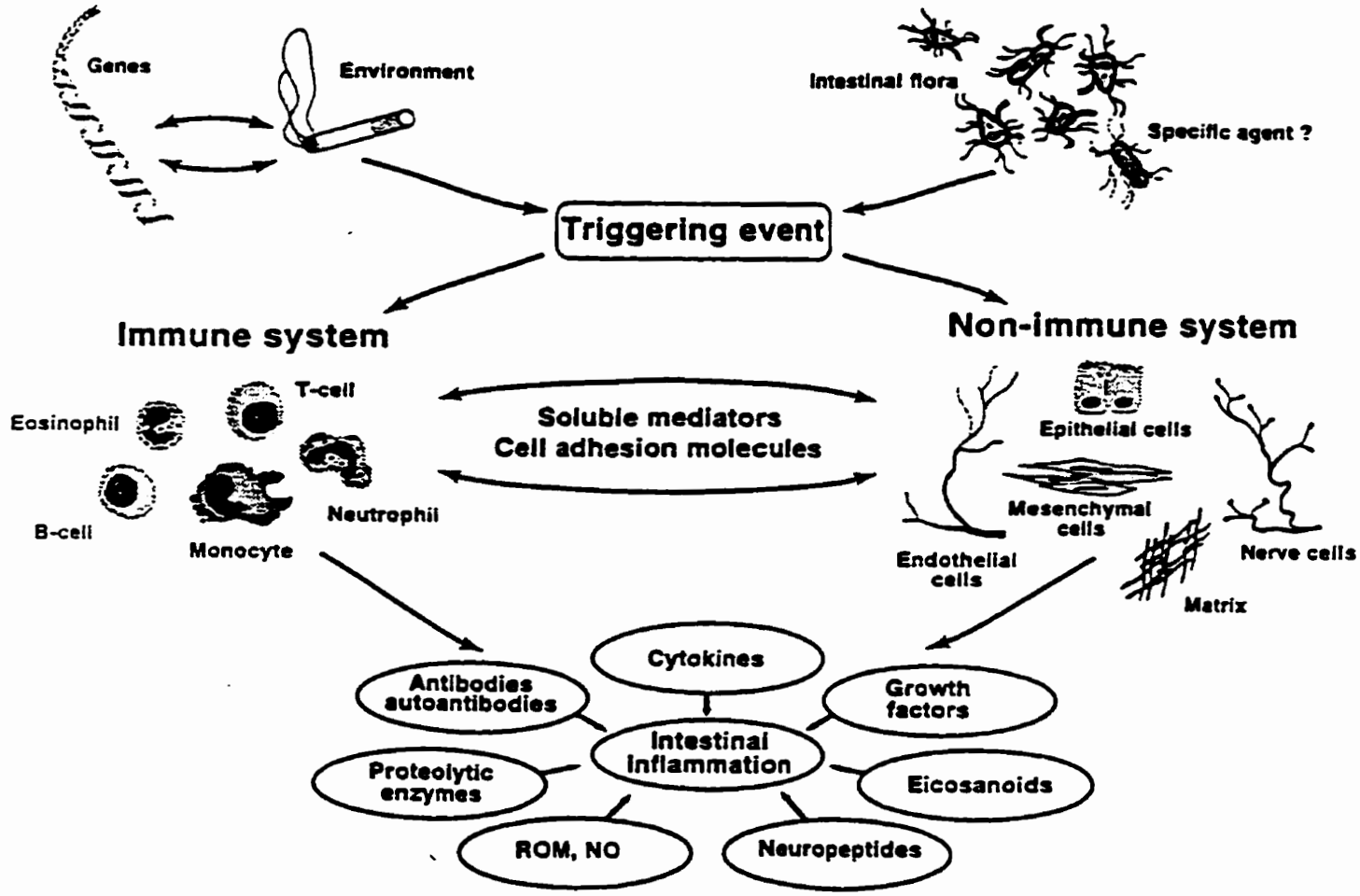
2-1 INFLAMMATORY BOWEL DISEASE (IBD)

Inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis, is a recurrent inflammatory condition of the gastrointestinal tract in humans. No single agent or distinct mechanism has yet been found to explain the pathogenesis of IBD. Several factors may contribute to the development of IBD. These include a genetic predisposition, environmental factors, microbial agents, cell-mediated immunity, humoral immunity, cytokines and nervous system abnormalities (Figure 1) (1).

In view of the limitations of experimental studies of human disease, animal models reproducing many, if not all, of the characteristics of IBD are extremely valuable. Spontaneous colonic lesions reported in a few different animals are sporadic and impractical to use experimentally. Mutant animals (rats and mice) have been generated in which specific targeted genes have been deleted. Examples are knockout mice lacking interleukin 2 (IL-2), interleukin 10 (IL-10), transforming growth factor β (TGF-β), N-cadherin. These models are very important but are expensive, hard to produce in quantities sufficient for extensive experiments.

Experimental colitis can also be induced by a variety of chemical or natural substances such as acetic acid, trinitrobenzene sulfonic acid, indomethacin and carrageenan (1). The most widely used animal model first developed by Morris et al. is trinitrobenzene sulfonic acid (TNBS) model. TNBS dissolved in ethanol produces a chronic colonic inflammation when administered intracolonically. Ethanol breaks the integrity of the colonic epithelium, so that the hapten (TNBS) can enter and couple to a substance of high molecular weight, probably a tissue protein, to elicit an immunological response. The inflammatory response to TNBS/ethanol is dose-dependent. TNBS-

Figure 1. Diagram showing the components and events involved in IBD etiopathogenesis. Interacting environmental and genetic factors in combination with the microbial intestinal flora or a still unidentified microorganism trigger an event that activates intestinal immune and non-immune systems. Through secretion of soluble mediators and expression of adhesion molecules, immune and non-immune cells exchange signals, resulting in further cell activation and amplification of the production of antibodies and autoantibodies, cytokines, growth factors, eicosanoids, neuropeptides, nitric oxide, reactive oxygen metabolites, and proteolytic enzymes culminating in inflammation and tissue damage (25).


Genes
Environment
Intestinal flora
Specific agent ?
Triggering event
Immune system
Non-immune system
T-cell
Eosinophil
B-cell
Monocyte
Neutrophil
Soluble mediators
Cell adhesion molecules
Epithelial cells
Mesenchymal cells
Endothelial cells
Nerve cells
Matrix
Cytokines
Antibodies autoantibodies
Growth factors
Intestinal inflammation
Proteolytic enzymes
Eicosanoids
ROM, NO
Neuropeptides

induced inflammation in the rat persists for 6-8 weeks and it is possible to induce relapse by repeated administration of TNBS (26;27). TNBS-induced colitis is a self-limited model of colonic inflammation and does not relapse as does human IBD, but colitis can be reactivated by low doses of intravenously administered TNBS. The clinical manifestations of Crohn's disease, diarrhea, weight loss, are similar in this animal model (28). Transmural inflammation with granuloma, skip-segment ulceration and inflammation, cobblestone-like appearance of the mucosa, lymphoid infiltration and crypt distortion are similar to Crohn's disease (29).

The involvement of the nervous system in the pathogenesis of IBD was proposed during the 1950s (7). Further studies examined the distribution of neuropeptides and neurotransmitters in IBD (8). Whilst, some abnormalities have been documented, the role that nerves play in IBD is still unclear. Neurotransmitters released by nerve fibers in the gastrointestinal tract can produce local inflammatory reactions by interacting with the immune system as well as inducing vasodilation or secretion (9;16). Another possibility is that the normal pattern of innervation and interactions of nerves and their target tissues may be disrupted by inflammatory cells and mediators. An increased number of myenteric ganglion cells in chronic ulcerative colitis as well as axonal degeneration and necrosis have been observed in IBD, indicating the disruption of the normal innervation of intestine in IBD (7).

Studies have suggested that intestinal secretion is impaired after colitis, reducing the capacity of the intestinal mucosa to mount a full response to luminal pathogens (30;31). The intestinal ion transport response to histamine was reported to be smaller in magnitude in IBD compared with non-IBD tissues from both the small and large intestine

of humans (30). In mitomycin-induced colitis in the rat, Kachur et al. (1995) showed that the maximal increases in ion transport stimulated by bradykinin, prostaglandin E_1, carbachol, substance P and serotonin were depressed at three and/or seven days after colitis (31). In this model, mitomycin-C injected intraperitoneally produced inflammation with histological similarities to ulcerative colitis in humans. Asfaha et al. (1999) reported depressed secretory responses to electrical field stimulation, isobutylmethylxanthine and carbachol even 6 weeks post-inflammation in the rat colon. At this time point there was no histological evidence of colonic inflammation and the basal electrolyte transport parameters and permeability were not different from controls (32). Interestingly, some evidence indicates that inflammation not only disturbs intestinal function at the site of inflammation, but also at remote, non-inflamed, regions of the gut. For example, there was an altered myenteric nerve function, as measured by [^{3}H]norepinephrine release from the myenteric plexus preparations of the inflamed and non-inflamed regions of the intestine preloaded with [^{3}H]norepinephrine in the TNBS-induced distal colitis in the rat (17).

Increased intestinal permeability has been documented in different animal models of intestinal inflammation as well as in IBD patients and a subpopulation of their relatives, suggesting that this may play a pathogenic role in IBD (4), although this is controversial (1). It has been shown that distal colitis in the rat induced an increase of tight junction permeability at remote sites such as duodenum and ileum. Altered permeability was accompanied by alterations in the tight junction structural protein occludin (33). The transmembrane protein occludin has a functional role in forming

junctional seals, transepithelial resistance and the size-selective solute permeability barrier of tight junction (34).

2-2 CALCITONIN GENE-RELATED PEPTIDE (CGRP)

It is almost 2 decades since the 37 amino acid peptide, α-CGRP was discovered as a product of the alternative mRNA splicing of the calcitonin gene (35). A few years later, another gene, encoding β-CGRP was discovered (Figure 2) (36). α-CGRP and β-CGRP are different in three and one amino acids in human and rat, respectively. Both forms are widely distributed in the peripheral and central nervous systems and so far it appears that their biological profiles are similar (19). In the gastrointestinal tract, CGRP is found in the neurons and nerve fibers. In the colon, CGRP is present in both the extrinsic and intrinsic nerve fibers, while in the stomach CGRP is present only in extrinsic nerves. Primary afferent neurons express both α- and β-CGRP, but predominantly α-CGRP (37).

A. Biological activity: CGRP has many biological activities including vasodilatory, neuromodulatory, immunomodulatory and secretory effects. It is one of the most potent vasodilators known. CGRP immunoreactive nerve fibers are widely distributed around blood vessels. The density of CGRP-containing fibers around arteries is in general higher than around veins (19). In the mammalian digestive system, CGRP immunoreactive vascular fibers are associated mostly with small arteries and arterioles (37). The vasodilatory effect of CGRP is either directly mediated on the vascular smooth muscle or indirectly via the endothelium, depending on the tissue studied. CGRP relaxes vascular smooth muscle either by increasing cAMP in smooth muscle cells or by the opening of glibenclamide-sensitive, ATP-sensitive potassium channels (39). In certain

Figure 2. Amino acid sequences of human calcitonin (CT), αCGRP, βCGRP, [Cys(Acm)^{2,7}]*h*αCGRP and [Cys(Et)^{2,7}]*h*αCGRP, adrenomedullin (ADM), and amylin as well as rat αCGRP and βCGRP (38).

***h*Calcitonin:** Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2

***h*αCGRP:** Ala-Cys-**Asp**-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-**Val**-Val-Lys-**Asn**-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2

***h*βCGRP:** Ala-Cys-**Asn**-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-**Met**-Val-Lys-**Ser**-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2

ratαCGRP: Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-**Glu**-Ala-Phe-NH_2

ratβCGRP: Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-**Lys**-Ala-Phe-NH_2

[Cys(Et)^{2,7}] ***h*αCGRP:** Ala-Cys(Et)-Asp-Thr-Ala-Thr-Cys(Et)-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2

[Cys(Acm)^{2,7}] ***h*αCGRP:** Ala-Cys(Acm)-Asp-Thr-Ala-Thr-Cys(Acm)-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2

***h*Amylin:** Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-NH_2

***h*Adrenomedullin:** Tyr-Arg-Gln-Ser-Met-Asn-Asn-Phe-Gln-Gly-Leu-Arg-Ser-Phe-Gly-Cys-Arg-Phe-Gly-Thr-Cys-Thr-Val-Gln-Lys-Leu-Ala-His-Gln-Ile-Tyr-Gln-Phe-Thr-Asp-Lys-Asp-Lys-Asp-Asn-Val-Ala-Pro-Arg-Ser-Lys-Ile-Ser-Pro-Gln-Gly-Tyr-NH_2

blood vessels, vasorelaxation by CGRP is endothelium-mediated via nitric oxide production (19).

As a neurotransmitter, CGRP is co-localized and co-released with substance P from primary afferent fibers, which have their cell bodies in the dorsal horn of the spinal cord. It has an algesic (painful) effect in certain models of nociception and modulates the acute antinociceptive action of opioid agonists. The development of tolerance to the analgesic effects of morphine was dose-dependently reversed by intrathecal administration of a $CGRP_1$ receptor antagonist (into the subarachnoid space of the spinal cord) (40). It has been suggested that primary sensory neurons play a dual role in the response to acute injury, where the central terminals transmit information set up by noxious event to the central nervous system, and the peripheral terminals mediate a local inflammatory response via an axon reflex (9;21). CGRP immunoreactivity is decreased in rabbit colitis, ferret jejunitis/ileitis and rat colitis which might be due to its release from nerve terminals of the intestine during inflammation (41;42). In the periphery CGRP can potentiate edema formation and in the spinal cord it is involved in the transmission of presumed nociceptive information (43).

CGRP also possesses neuromodulatory activities. For example, CGRP increases acetylcholine release in the myenteric plexus of the guinea pig colon and excites myenteric neurons (23). CGRP might have immunomodulatory effects on the GI tract. Substance P and CGRP can increase lymphocyte output from the mesenteric lymphatic duct of the rat through the gastrointestinal tract (12). CGRP receptors have been found on lymphocytes, bone marrow cells, thymocytes and splenocytes and CGRP induces apoptosis in thymocytes (44). In terms of its effect on lymphocytes, CGRP as well as

substance P, neuropeptide Y and somatostatin can directly induce secretion of interleukin 2, interferon-gamma (IFN γ), interleukin 4 and interleukin 10, from T cells. CGRP drives the Th_1 lymphocyte population toward Th_2 cytokine profile, and Th_2 lymphocyte population to the IL-2 and IFN γ secreting Th_1 profile. Such a phenomenon cannot be induced by classical antigenic stimulation (14). CGRP is also able to release histamine from rat peritoneal mast cells and human connective tissue mast cells (20). Approximately two-thirds of intestinal lamina propria mucosal mast cells are associated with neuron-specific enolase-containing nerves, which contain substance P and CGRP (45). Rabbit leptomeningeal granular cells which, resemble mast cells, release serotonin when CGRP is administered (46).

CGRP causes ion secretion in the gastrointestinal tract, through different mechanisms in different species. CGRP causes chloride secretion through a neuronally mediated pathway by releasing acetylcholine from the myenteric plexus of the guinea pig colon, while in the rat colon, secretion evoked *in vitro* is evident when the myenteric plexus has been removed (23;47). In the rat colon, CGRP-induced chloride secretion was not inhibited by a $CGRP_1$ receptor antagonist. On the other hand, significant inhibition of CGRP-induced secretion was observed in the primary human colonic adenocarcinoma (HCA) cell line (A-7), but not in colony-29 (Col-29) cells, a subpopulation of cells derived from this line (47). This evidence suggests different receptors are involved in CGRP-induced chloride secretion.

B. Subtypes, receptors and analogues: The CGRP superfamily includes 3 other peptides: amylin, adrenomedullin and calcitonin (48). They share some structural homology as well as some common biological effects. All of these peptides have a

conserved N-terminal ring structure linked by a disulfide bridge and an amidated C-terminus (38). The CGRP family of receptors belongs to the seven transmembrane domain G-coupled receptor family (49). Several receptors have been cloned and proposed as receptors for members of this family of peptides (48). Using pharmacological and radiochemical binding approaches, at least 3 distinct receptors in different tissues have been identified. The best-characterized pharmacological receptor for CGRP is the so-called $CGRP_1$ receptor. This is typified by the receptor found in the heart (atria), which is activated by CGRP and antagonized by a CGRP analogue, human (h)$CGRP_{8-37}$ (50). The N-terminal part of the CGRP molecule including the cyclic N-terminal hexapeptide, is essential for stimulation of CGRP receptors, while the C-terminal sequence is important for high affinity binding but is not sufficient to activate the receptor. Shorter C-terminal fragments such as $CGRP_{12-37}$, $CGRP_{19-37}$ and $CGRP_{23-37}$ also act as competitive $CGRP_1$ receptor antagonists, although their potency progressively decreases with shortening of their sequence (51). Two receptors have been cloned and appear to have characteristics of a $CGRP_1$ receptor. These two receptors are canine orphan receptor (RDC1) and calcitonin-receptor-like-receptor (52-54).

Another CGRP receptor found in peripheral tissues, the $CGRP_2$ receptor, is only weakly antagonized by $hCGRP_{8-37}$ but is potently activated by the linear CGRP analogues, [$Cys(Acm)^{2,7}$]hCGRPα and [$Cys(Et)^{2,7}$]hCGRPα (55;56). Finally, there is evidence from radioligand binding studies for an atypical CGRP receptor in the brain (nucleus accumbens) that is distinct from both the $CGRP_1$ or $CGRP_2$ receptors. This unique population of CGRP receptors has similar affinity to salmon calcitonin, rat amylin and CGRP (55).

Amylin or amyloid fibril protein has 50% sequence homology to CGRP and shares some common biological activity with CGRP, such as inhibition of insulin secretion and glucose transport into skeletal muscles (Figure 2). Amylin interacts at high concentrations (ID_{50} 240-fold higher than for CGRP), with a bona fide CGRP receptor characterized in the SK-N-MC cell neuroblastoma line, and also with calcitonin receptors (ID_{50} 200-fold higher than for salmon calcitonin) in the T47D human breast carcinoma cell line (57).

Adrenomedullin (ADM), a 52 amino acid peptide with 21% homology with hα-CGRP, exhibits potent hypotensive effects (Figure 2). Interestingly, the $CGRP_1$ receptor antagonist inhibited the vasodilatory effect of adrenomedullin in the rat mesenteric and coronary vessels (58;59). Despite these common sites of activity for CGRP family members, specific binding sites have been recognized for each peptide in different tissues (55).

One of the important issues related to the function of the CGRP receptors is the existence of accessory proteins necessary for the receptor activity. Receptor component protein (RCP) is an intracellular membrane-associated protein, which was found to be necessary for conferring CGRP receptor activity to Xenopus oocytes and NIH3T3 cells (48). A second type of accessory protein of importance is the receptor activity modifying proteins (RAMPs). The RAMP family members (RAMP1, RAMP2 and RAMP3) are transmembrane proteins. RAMP1 is not a CGRP receptor in its own right, but when co-expressed with calcitonin-receptor-like receptor (CRLR) confers CGRP receptor activity to oocytes, and co-transfection of CRLR and RAMP1 into HEK293T (a subset of human embryonic kidney) or Swiss 3T3 cells is required for CGRP receptor responses. Neither

RAMP1 nor CRLR alone induced significant response to CGRP when cells were transfected with cDNA of each component, but expression of both together produced cells that responded to CGRP. Surprisingly, the coexpression of RAMP2 and CRLR results in adrenomedullin receptor activity. RAMPs are required to transport CRLR to the plasma membrane. A possible mechanism of action of RAMPs involves controlling the glycosylation of CRLR; RAMP1 presents CRLR at the cell surface as a mature glycoprotein and CGRP receptor; RAMP2 transported receptors are core glycosylated and are adrenomedullin receptor (Figure 3) (48;60).

2-3 ENTERIC NERVOUS SYSTEM

The enteric nervous system (ENS) is recognized as an independent integrative system that behaves like a "minibrain" positioned close to the effector systems it controls. It consists of two interconnected ganglionated plexuses: the submucosal and myenteric plexuses (figure 4). The submucosal plexus is located between the submucosa and mucosa, and is responsible for the majority of the neuronal regulation of electrolyte transport. The myenteric plexus is sandwiched between the circular and longitudinal muscle layers and is predominantly responsible for the control of motility. The ENS includes distinct types of neurons (secretomotor, motor, sensory neurons and interneurons) and can display reflex activity without input from the central nervous system. It also interacts with an extrinsic innervation from efferent (sympathetic and parasympathetic) and afferent (spinal and vagal) neurons, which are operative in coordinating its activity. The ENS is organized with the neural elements and integrated circuitry necessary for independent processing of sensory information and the programming of the organized behavior of the effector systems in response to the

Figure 3. The role of RAMPs 1 and 2 and CRLR in generating CGRP or ADM receptors (60).

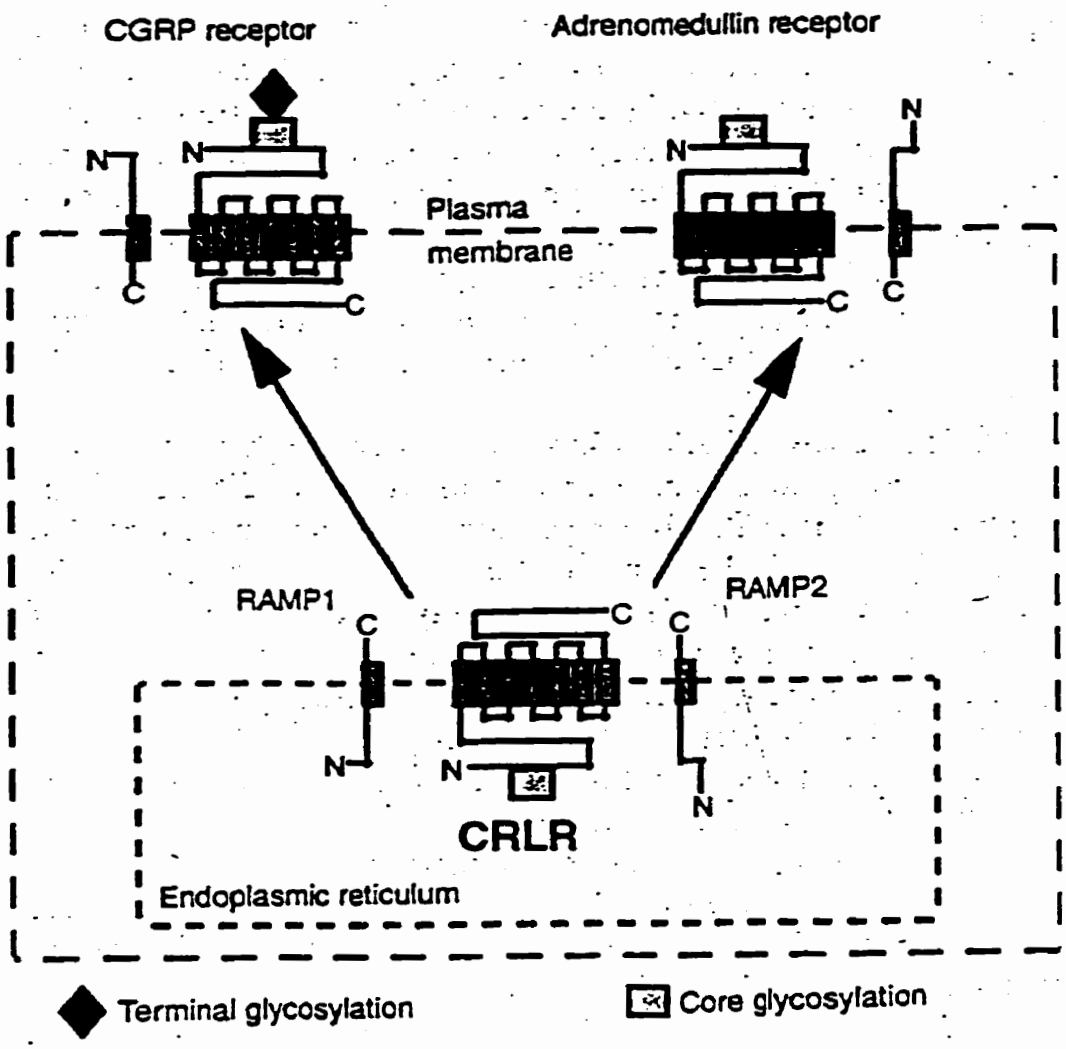
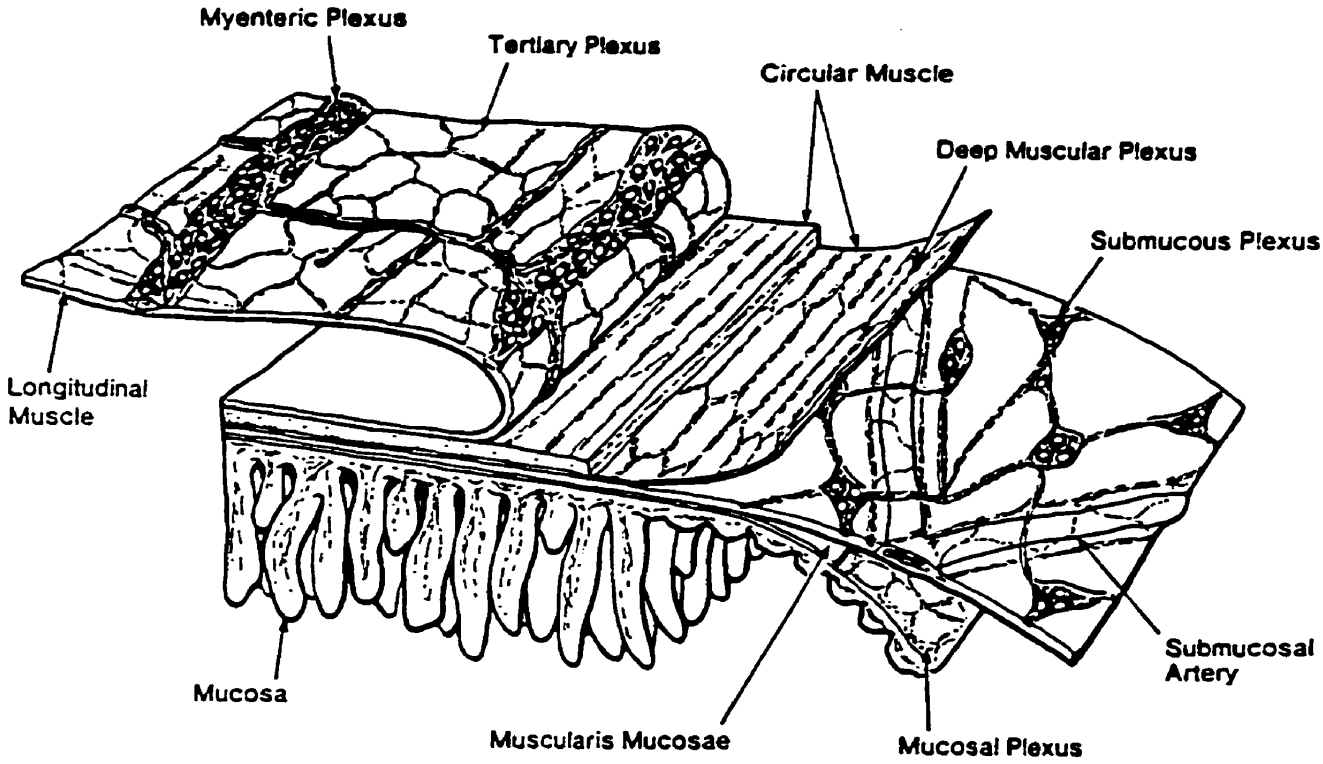

CGRP receptor
Adrenomedullin receptor
N
C
Plasma
membrane
RAMP1
RAMP2
CRLR
Endoplasmic reticulum
Terminal glycosylation
Core glycosylation

Figure 4. The enteric nervous system (61).


Myenteric Plexus
Tertiary Plexus
Circular Muscle
Deep Muscular Plexus
Submucous Plexus
Longitudinal Muscle
Submucosal Artery
Mucosa
Muscularis Mucosae
Mucosal Plexus

intraluminal environment of the bowel (16;62). The ENS is also likely to be important in the regulation of the structure of the intestinal tract. It has been documented that myenteric plexus ablation by benzalkonium chloride (BAC) had a direct effect on intestinal structure. Myenteric plexus ablation in the rat jejunum increased villus height, crypt depth, and muscle thickness in the BAC-treated segment and proximal segment, but only muscle thickness was increased in the distal segment (63).

A. The ENS in inflammation: There is evidence suggesting the involvement of enteric neurons and neural pathways during intestinal inflammation. Vasoactive intestinal peptide (VIP)- and substance P-immunoreactivity in enteric nerves were decreased in severe inflammatory lesions of patients with active ulcerative colitis. Even in resolving and quiescent ulcerative colitis, VIP-immunoreactive nerves were still decreased. In the uninvolved mucosa of ulcerative colitis, there was no change in the distribution of these neuropeptides. In Crohn's disease, the distribution abnormality of both neuropeptides resembled that of ulcerative colitis (8). Recently, it was shown that the expression of c-Fos in calbindin-immunoreactive neurons was correlated with enhanced neuronal electrical and metabolic activity during nematode-induced intestinal inflammation, suggesting that excitation-transcription coupled changes occur in enteric neural microcircuits in inflammation (64). In TNBS-induced colitis in the rat, lidocaine administration had the greatest attenuation on colitis after removal of the proinflammatory role of sympathetic nerves by chemical sympathectomy (11).

B. CGRP innervation of the GI tract: Two distinct populations of CGRP-containing neurons innervate the digestive system: intrinsic enteric, and extrinsic, primary afferent neurons. Enteric CGRP immunoreactive neurons are confined to the

myenteric and submucosal plexuses of the small and large intestines and express β-CGRP. They innervate the intestinal plexuses, the muscle layers, the submucosa and mucosa. Extrinsic primary afferent CGRP-containing neurons, the majority of which are located in the spinal ganglia, express both α- and β-CGRP. Spinal ganglia are the only source of the CGRP innervation of the stomach. CGRP-containing ganglion cells are found only in the enteric plexuses of the small and large intestine but not the upper GI tract (37). These different patterns of CGRP innervation along the length of the gastrointestinal tract may result in a different role of CGRP in the pathogenesis of GI diseases. In the stomach, CGRP is well known for its direct and indirect anti-ulcer activity. By direct action on blood vessels and decreased acid output or indirectly, through the release of somatostatin, CGRP-containing primary afferent neurons play a role in gastroprotection (65-67). In the TNBS model of colitis in the rat, primary afferent neurons have a protective effect (11). Blockade of intestinal neurons by lidocaine also has a protective effect in this model, although the non-neuronal effect of lidocaine can not be ruled out (10). In the large and small intestine, CGRP released during inflammation from primary afferent neurons, as well as enteric neurons, may contribute to inflammatory processes by immunomodulation and neuromodulation. CGRP is also an important secretory neuropeptide. CGRP-induced secretion is mediated through the adenyl cyclase pathway in the guinea pig and rat colon (23;47).

2-4 Mechanisms of Intestinal Water and Ion Transport

The gastrointestinal epithelium regulates the transport of fluid, electrolytes, nutrients and macromolecules between the external (luminal) and internal environments.

Water transport is coupled with solute movement and is passive. Water and ion transport occur by two routes: transcellular and paracellular (68).

A. Transcellular pathway

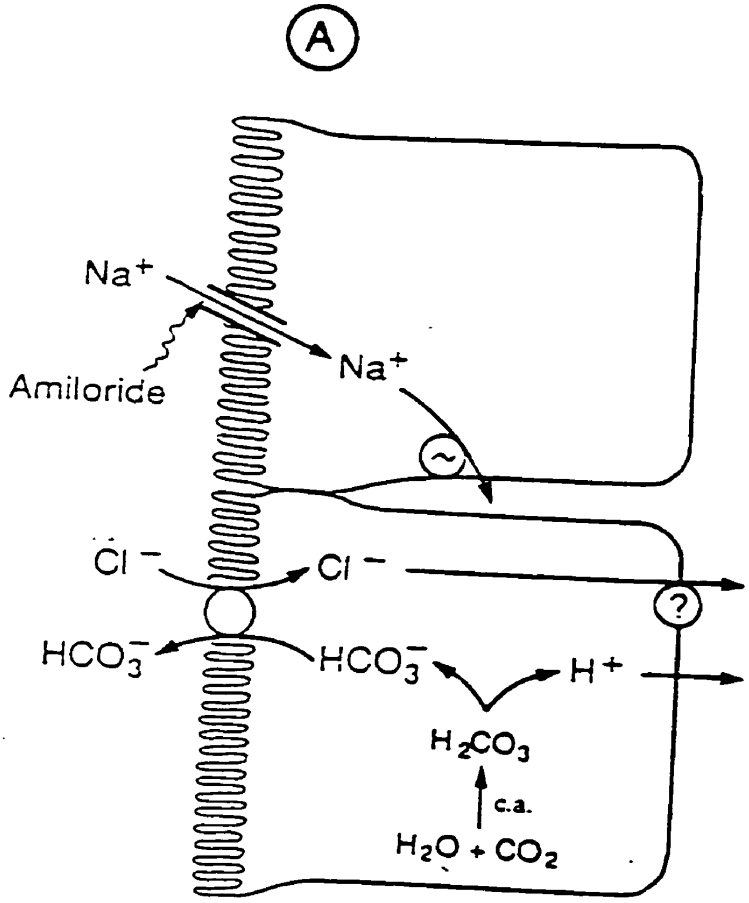
The transcellular pathways of ion and water transport include specific cellular mechanisms responsible for electrolyte transport: ion channels, transporters and pumps located on intestinal epithelial cells (34).

Absorptive pathways: There are several routes for Na^+ absorption. In the small intestine, Na^+ is absorbed by solute-dependent Na^+ co-transporters. Non nutrient-dependent Na^+ absorption mainly occurs via luminal-membrane, amiloride-sensitive Na^+/H^+ exchangers in the duodenum/jejunum. In the ileum and colon, the Na^+/H^+ exchanger is coupled to a Cl^-/HCO_3^- exchanger. Consequently, Na^+ absorption is accompanied by HCO_3^- absorption. Potential difference (PD)-dependent Cl^- absorption occurs along the length of small and large intestine. In the distal small intestine and proximal colon, Na^+ and Cl^- absorption are coupled and electroneutral. In the ileum and colon, Cl^- absorption is HCO_3^--dependent (Figure 5) (69).

Secretory pathways: Intestinal secretion is largely driven by active secretion of Cl^- and HCO_3^- (68). Cl^- secretion involves four membrane proteins. Briefly, Cl^- enters the cell by the basolateral Na^+-K^+-$2Cl^-$ co-transporter and is secreted into the lumen via the Cl^--selective channel. Na^+ and K^+, which accompany Cl^- entry, are recycled across the basolateral membrane by K^+-selective channels and Na^+, K^+ ATPase. Therefore the driving force for Cl^- secretion is based on the activity of the Na^+ pump (Figure 6) (70).

HCO_3^- secretion is dependent on, or associated with various factors, which makes it more difficult to measure and interpret. HCO_3^- accumulation in the intestinal lumen can

Figure 5. Model of active Na^+ and Cl^- absorption in distal colon of rabbit (**A**) and rat (**B**). Na^+ absorption in the rabbit distal colon is electrogenic without evidence of Na^+-H^+ exchange. In contrast, electroneutral Na^+-H^+ absorption is present in the rat distal colon without evidence of Na^+-conductive channels (69).


A
Na+
Amiloride
Na+
Cl −
Cl −
HCO3−
HCO3−
H+
H2CO3
c.a.
H2O + CO2
?

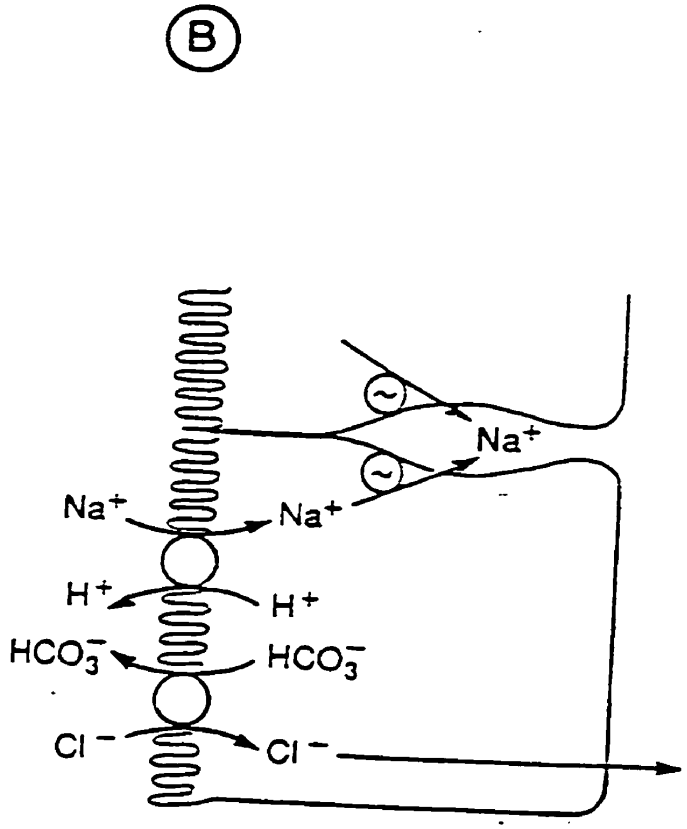
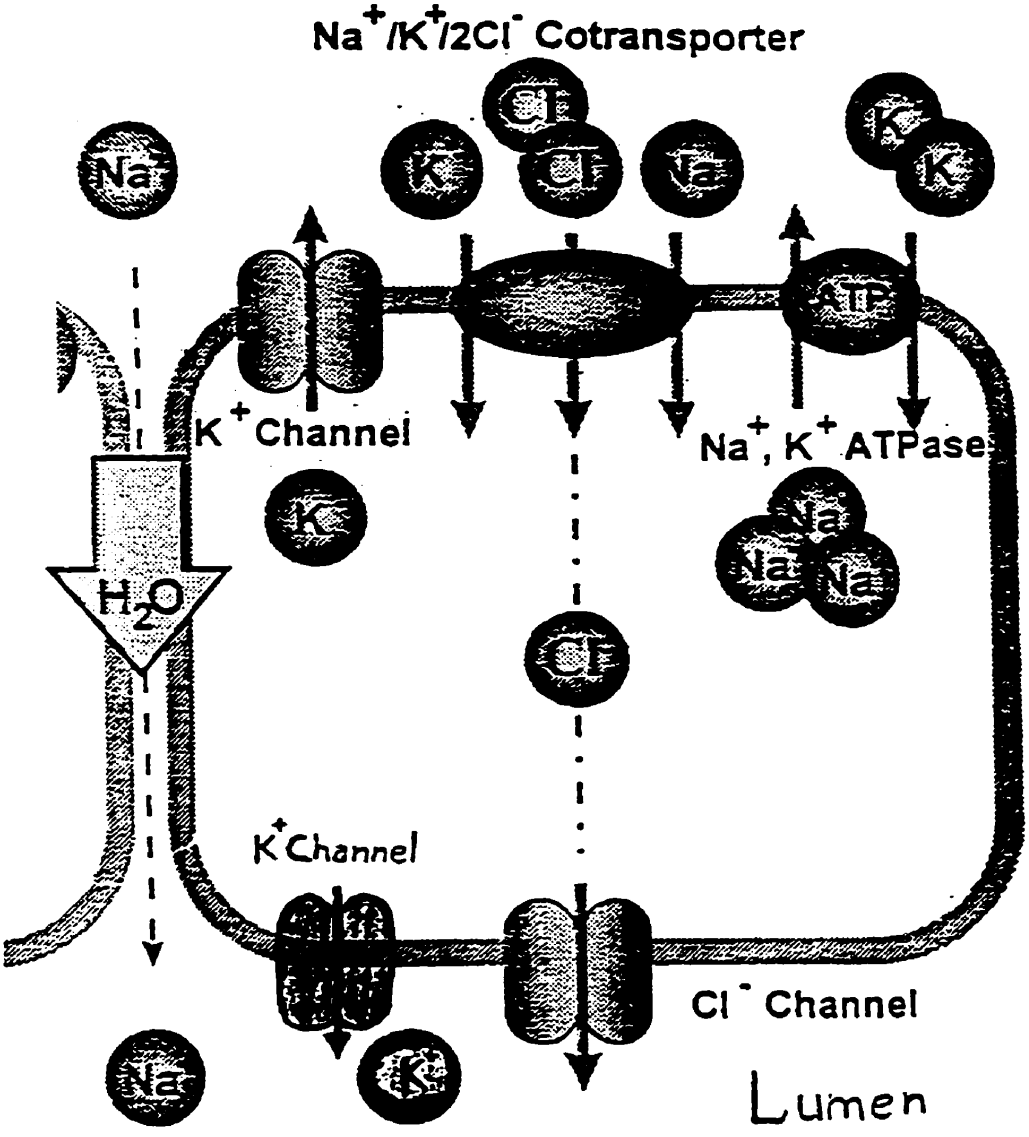

B
Na+
Na+
Na+
H+
H+
HCO3−
HCO3−
Cl −
Cl −

Figure 6. Ion transport pathways in secretory epithelia. This cell shows the four essentials ion transport mechanisms involved in fluid and electrolyte movement in Cl^- secreting epithelia: basolateral Na^+, K^+ ATPase with a stoichiometry of 3 Na^+:2 K^+, the electroneutral Na^+-K^+-2Cl^- cotransporter located in the basolateral membrane and the basolateral membrane K^+ channel and apical Cl^- channel. Transepithelial Cl^- movement drives the fluid and electrolyte secretion process (70). K^+ secretion involves the basolateral Na^+, K^+ ATPase pump and the apical barium-sensitive K^+ conductance channels.


Na+/K+/2Cl- Cotransporter
Cl
K
Cl
Na
K
K
ATP
Na
K+ Channel
Na+, K+ ATPase
K
Na
Na
Na
H2O
Cl
K+ Channel
Cl- Channel
Na
K
Lumen

result from the absorption of associated H^+, the secretion of OH^-, and/or the secretion of HCO_3^-. There are multiple routes for HCO_3^- transport, via the paracellular pathway, a Cl^-/HCO_3^- exchanger, Na^+HCO_3 co-transporters and conductive HCO_3^- pathways (Figure 7) (71).

K^+ secretion occurs throughout the colon, mediated by a barium-sensitive K^+ conductance in the apical membrane of the enterocytes. K^+ enters the basolateral membrane of the enterocyte via the Na^+, K^+ ATPase pump and by the Na^+-K^+-$2Cl^-$ cotransporter (Figure 6) (70).

B. Paracellular Pathways and Intestinal Permeability

Paracellular pathways allow a passive flow of ions, followed by water, down concentration and electrochemical gradients. It is defined as the space between epithelial cells made up of the tight junction and the lateral intercellular space in series. It is now abundantly clear that the tight junction is not a simple gasket-like barrier (Figure 8) (34). The tight junction demonstrates ion selectivity, varies significantly in permeability among different tissues, may be subject to physiological regulation and undergoes dynamic modulation (72). Tight junctions contribute to the maintenance of the potential difference across the intestinal epithelium. Paracellular permeability demonstrates a certain degree of selectivity with respect to the direction of the ion movement, as permeability in the serosal to mucosal direction is greater than the opposite direction. It has been hypothesized that the tight junction contains size- and charge-selective aqueous channels or pores formed by integral membrane proteins that otherwise act to occlude the extracellular space.

Three assays are commonly used to investigate paracellular permeability to ions

Figure 7. Model for duodenal surface epithelial secretion of HCO_3^- based on experiments with the proximal bulfrog duodenum. Transport involves: (a) Cl^-/ HCO_3^- exchange stimulated by some gastrointestinal hormones and inhibited by furosmide; (b) transport independent of luminal Cl^-, stimulated by prostaglandins and insensitive to furosmide; (c) an anion carrier which under some conditions displays affinity also for Cl^-; and (d) passive migration of HCO_3^- through shunt pathways which are sensitive to variations in transmucosal hydrostatic pressure. Similar mechanisms for secretion of HCO_3^- by duodenal surface epithelium probably operate in mammalian duodenum (71).

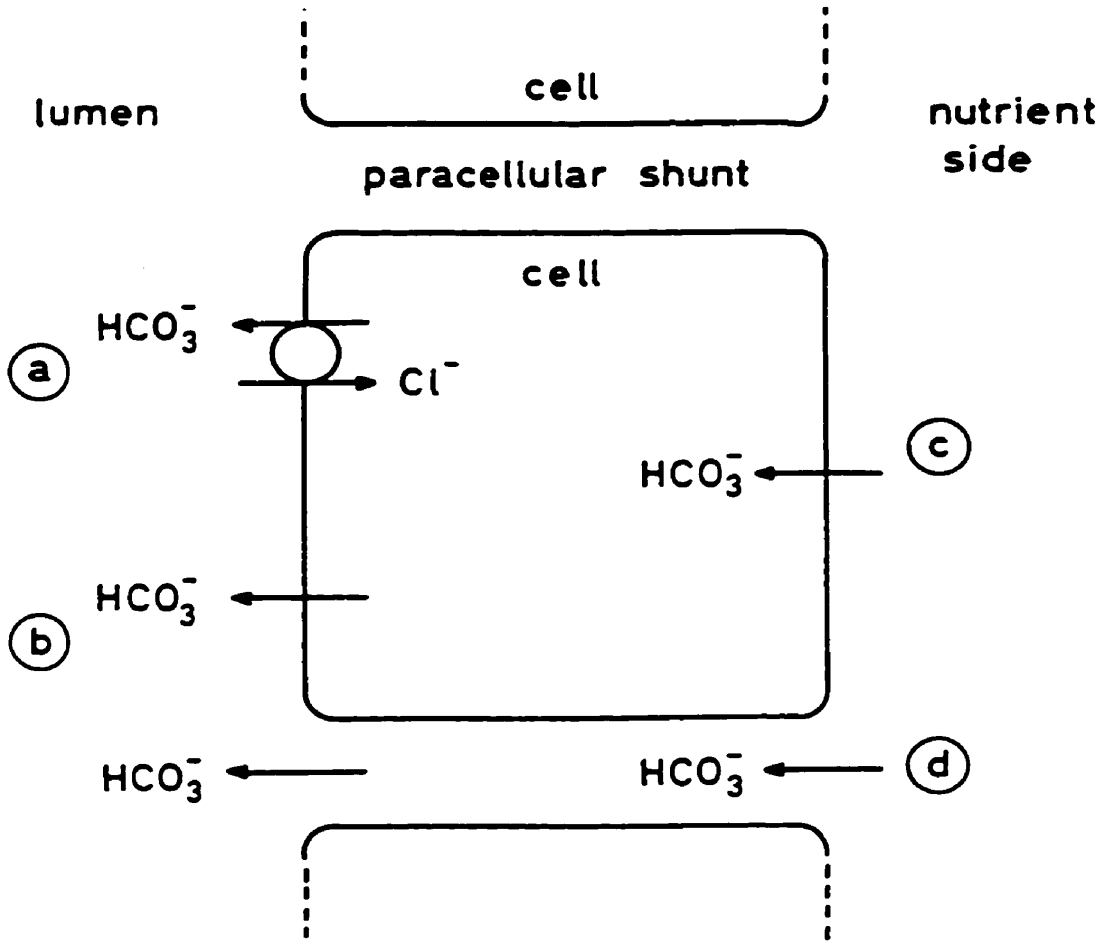
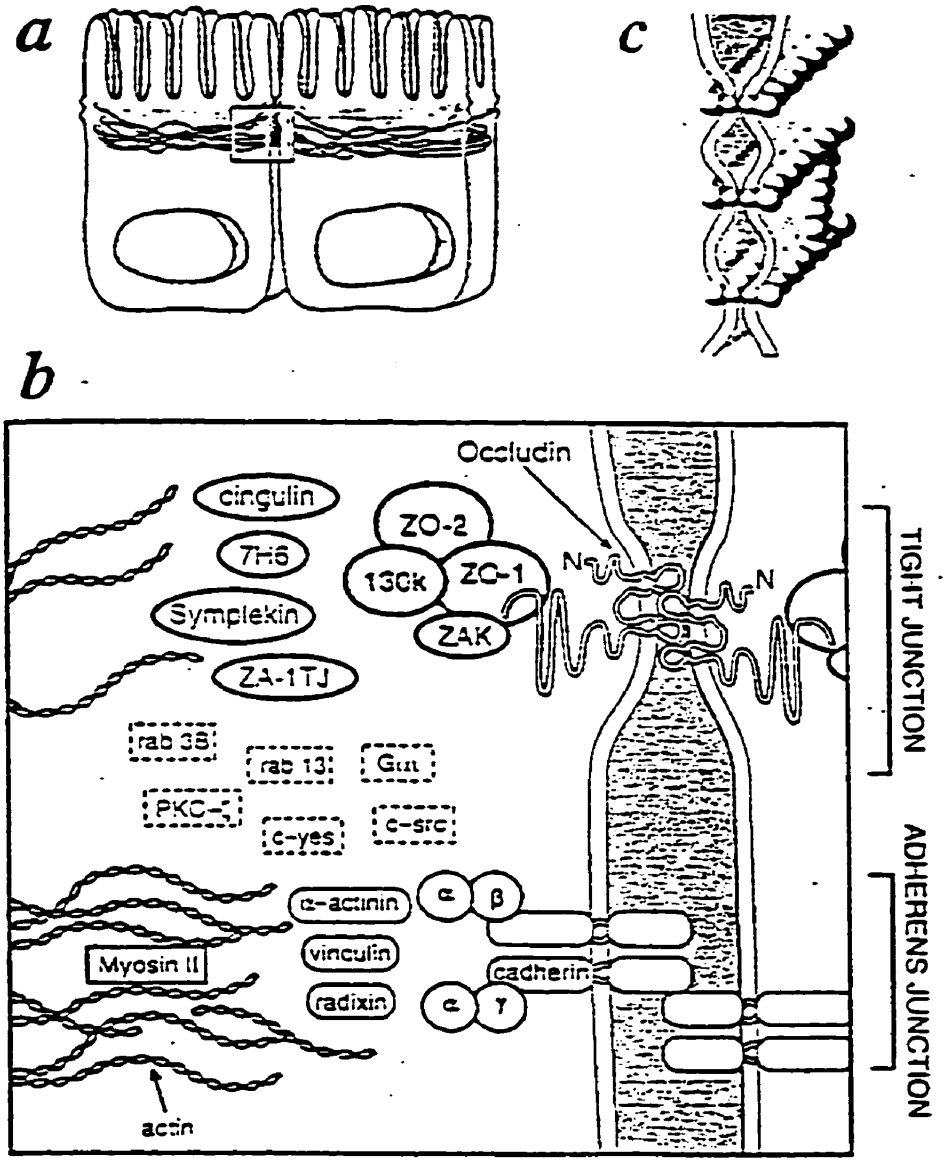

lumen
cell
nutrient side
paracellular shunt
cell
HCO_3^-
Cl^-
a
c
HCO_3^-
HCO_3^-
b
HCO_3^-
HCO_3^-
d

Figure 8. Model depicting the protein components of the apical junction complex, the tight and adherens junction, in a highly polarized epithelial cells. (a) Continuos cell-cell contact is made at the apical end of the lateral interspace where a ring of bidirectional actin filaments is concentrated. (b) The boxed region is depicted at higher magnification, showing identified proteins and their protein associations. Some proteins are specific to one junction, others may be shared (broken boxes). (c) Interpretation of freeze-fracture EM images showing barriers formed by intercellular contact of branching linear polymers of occludin and its cytoplasmic scafold (34).


a
c
b
Occludin
cingulin
ZO-2
7H6
130k
ZO-1
Symplekin
ZAK
ZA-1TJ
N
N
rab 3B
rab 13
Gut
PKC-ζ
c-yes
c-src
α-actinin
α
β
Myosin II
vinculin
cadherin
radixin
α
γ
actin
TIGHT JUNCTION
ADHERENS JUNCTION

and uncharged hydrophylic molecules. Passage of electron-dense dyes, transepithelial electrical resistance and transepithelial flux of substances without affinity for the membrane transporters, like polyethylene glycol or various methylated dextrans with molecular weights as high as 2×10^6 D (72).

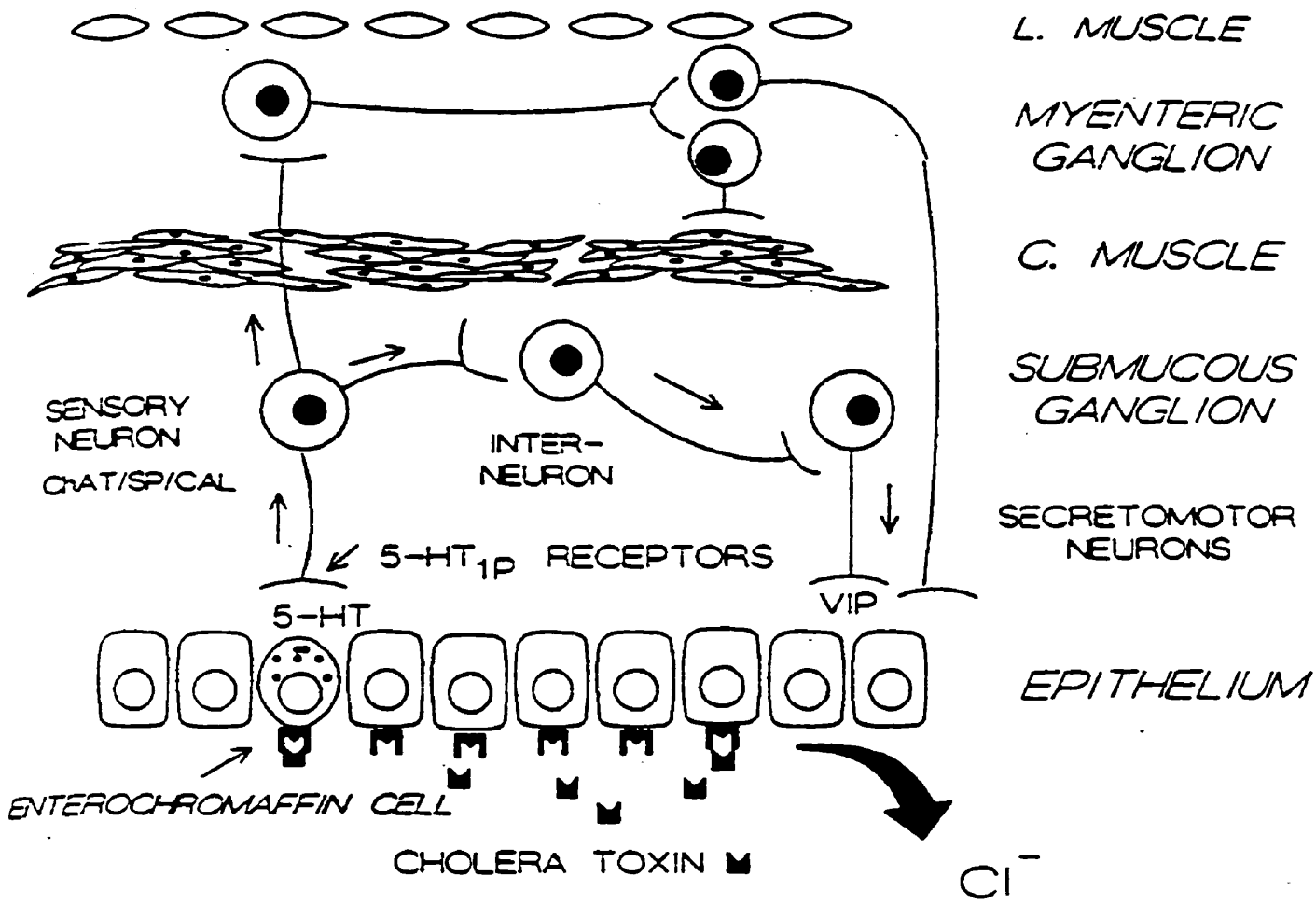
2-3 Regulation of Electrolyte and Water Transport

A. Neuronal regulation

Microcircuits in the intestine incorporate reflex circuitry for initiating and sustaining propulsive motility as well as functional submucosal microcircuits that program mucosal secretion, absorption and local blood supply to the mucosa. Reflexes are activated by luminal contact of chemical or noxious agents or by distension or mucosal stroking (73). Cooke et al., showed that mucosal stroking of the stripped guinea pig small intestine causes chloride secretion by activating neuronal reflexes in the submucosal plexus (Figure 9) (73). There is debate about the identity of cells that transduce the environmental signals in the intestine. Sensory receptors may be extrinsic or intrinsic afferent nerve endings that respond to the perturbation in the environment.

Of the large array of neurotransmitters found in submucosal secretomotor neurons, substance P, acetylcholine and VIP are the most studied neurotransmitters in reflex control of secretion. Cholinergic secretomotor neurons are distinguished by the large variety of peptides they contain as well as the use of muscarinic synapses for transmission of signals to the epithelium. VIP-ergic secretomotor neurons also contain several other peptides (73). Mucosal stroking of guinea pig ileum causes enterochromaffin cells to release 5-hydroxytryptamine (5-HT), which activates 5-HT_{1P} receptors on submucosal primary afferent neurons containing acetylcholine/substance P.

Figure 9. Reflex regulation of ion transport in the guinea pig small intestine. Mucosal stroking or cholera toxin stimulates the release of 5-hydroxytryptamine from enterochromaffin cells. 5-hydroxytryptamine binds to 5-HT_{1P} receptors on afferent neurons in submucose plexus and activates a reflex that involves VIP secretomotor neurons. The afferent neuron contains substance P and is also immunoreactive for calbindin (Cal). Based on other studies, it is likely that this neuron is cholinergic, because it contains choline acetyltransferase (ChAT), which is necessary for synthesis of ACh. L and C, longitudinal and circular muscle, respectively (73).


L. MUSCLE
MYENTERIC GANGLION
C. MUSCLE
SUBMUCOUS GANGLION
SENSORY NEURON
ChAT/SP/CAL
INTER-NEURON
SECRETOMOTOR NEURONS
5-HT1P RECEPTORS
5-HT
VIP
EPITHELIUM
ENTEROCHROMAFFIN CELL
CHOLERA TOXIN
Cl⁻

Then, VIP or cholinergic submucosal secretomotor neurons transmit the signal to the epithelial cells, causing Cl^- secretion (74-76).

The gastrointestinal tract receives extrinsic innervation from the central nervous system via both sympathetic and parasympathetic divisions of the autonomic nervous system. The autonomic and enteric nervous systems coordinate the regulation of motility, mucosal blood flow and epithelial ion and water transport. Most command signals from the central nervous system to the gut interface with the ENS first, although there is some direct innervation of the epithelium by the sympathetic nervous system. In general, the sympathetic nervous system stimulates electrolyte absorption and inhibits intestinal motility, whereas the parasympathetic nervous system stimulates both intestinal secretion and motility (73).Sympathetic neurons can inhibit secretion by presynaptic inhibition of fast (nicotinic) and postsynaptic inhibition of slow (non-cholinergic) neuronal excitation of enteric neurons (77).

Classical reflexes require a primary afferent neuron, an interneuron and a motor neuron for transmission to effector tissues. The primary afferent neuropeptides, substance P and CGRP, play important roles in the maintenance and spread of peripheral inflammation (78). Extrinsic reflexes within the prevertebral ganglia, spinal cord and brain also play role in the regulation of intestinal function. Neurogenic tone of active ion transport in *in vitro* preparations of the gut suggests that reflexes within the gut wall alone can control ion transport (73). One way in which the ENS regulates intestinal absorption and secretion is via input from primary afferent neurons. In response to noxious stimuli within the gut lumen, extrinsic and intrinsic afferent neurons generate action potentials leading to reflex activation. Primary afferent neurons activated by

capsaicin induce chloride secretion in the mucosa-submucosal preparations of the guinea pig ileum (79). Substance P and CGRP released from primary afferent nerve endings are the primary candidates for this phenomenon. The released neuropeptides activate submucosal secretomotor neurons through a neurokinin-1 receptor (NK-1 receptor). The $CGRP_1$ receptor antagonist, $CGRP_{8-37}$, did not block the capsaicin-induced chloride secretion (79;80). CGRP does contribute to the secretory and inflammatory effects of *Clostridium difficile* toxin A in the rat ileum. Pretreatment of rats with capsaicin dramatically inhibited fluid secretion and inflammation in the ileal loops exposed to *C. difficile* toxin A. Administration of *C. difficile* toxin A increased the CGRP content in dorsal root ganglia and in the ileal mucosa. In this study, pretreatment of rats with the $CGRP_1$ antagonist, $CGRP_{8-37}$, before instillation of *C. difficile* toxin A into ileal loops significantly inhibited toxin-mediated fluid secretion, mannitol permeability and histological damage (81).

It is likely that the same afferent neuronal pathways or neurotransmitters which stimulate smooth muscle contraction also stimulate epithelial secretion. Both intestinal contraction and secretion are coordinated in response to mucosal stroking as well as intestinal distension (73;82;83). Substance P, neurokinin A (NKA) and CGRP are expressed by enteric neurons and primary afferent neurons and probably influence motility, electrolyte and fluid secretion, vascular and immune functions in a peptide- and region-specific fashion and are candidates for distension induced reflexes (82;84). The released neurontansmitters can interact. NKA can induce duodenal motility and increase mucosal permeability and bicarbonate secretion in the in situ perfused duodenum in anesthetized rats. In a study by Hallgren et al. (1998), the NKA-induced increased

permeability was potentiated by luminal perfusion with lidocaine and diminished by VIP. Elevation of intraluminal pressure, however, potentiated the stimulatory effect of NKA on bicarbonate secretion. In contrast, the tachykinin decreased the rate of alkalinization in rats subjected to elevated intraluminal pressure and treated with indomethacin. Similarly, NKA partially inhibited the VIP-stimulated bicarbonate secretion. Luminal lidocaine did not affect the secretory response to NKA. It was concluded that the NKA-induced increase in duodenal mucosal bicarbonate secretion is independent of neurons and possibly mediated by prostanoids. The increase in mucosal permeability in response to NKA may be suppressed by mucosal nerves, perhaps utilizing VIP as one of the transmitters (85).

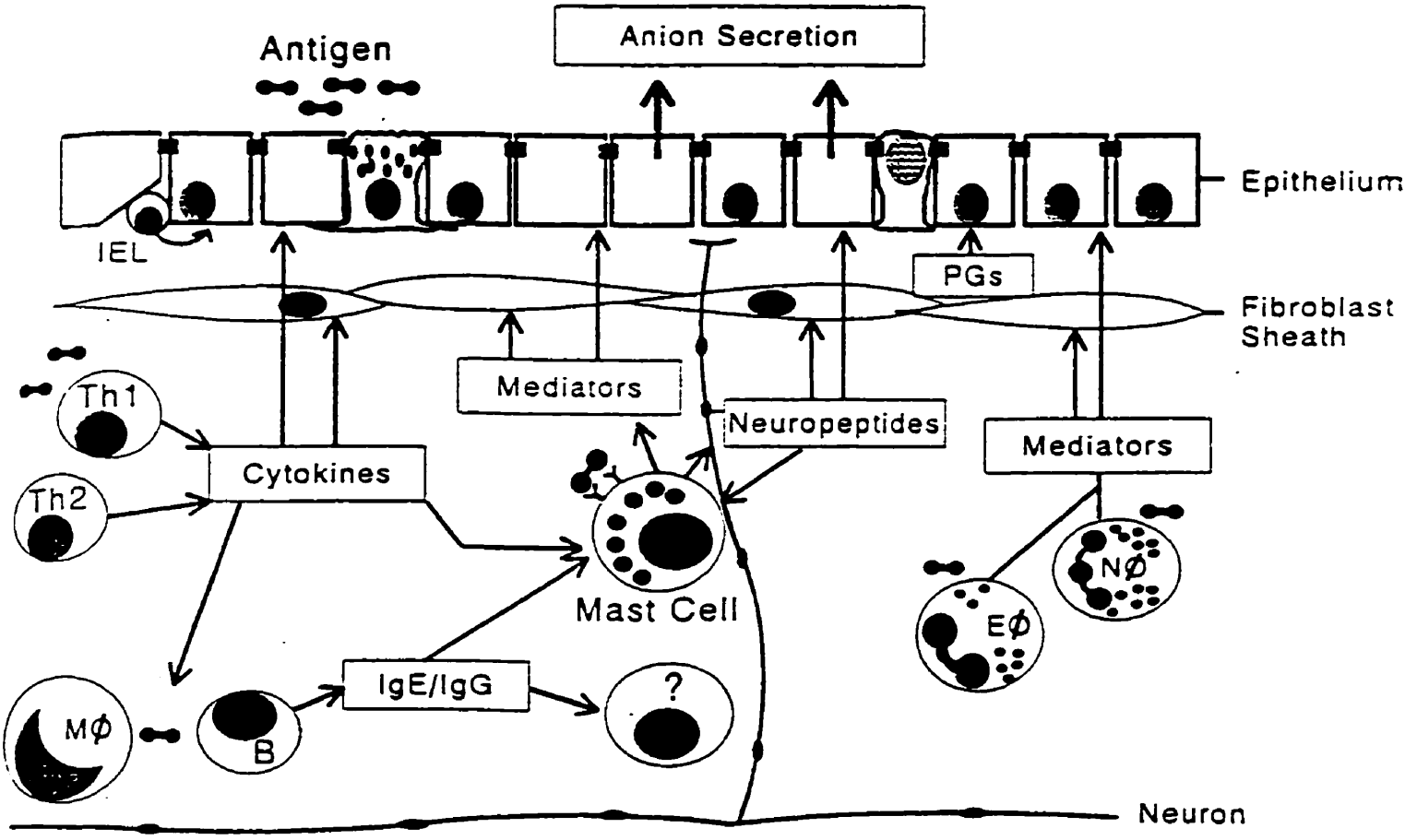
B. Autocrine and paracrine regulation

Classical effector cells of immune reactions (lymphocytes, eosinophils, mast cells, neutrophils and macrophages) are normally present in or near the intestinal epithelial layer where intimate associations with nerve endings occur (86;87). This anatomical association has important functional implications. Thus, upon activation most of these cells are able to secrete a wide array of mediators that influence intestinal physiology by acting directly on enterocytes or indirectly through secondary activation of the enteric nervous system (2). Besides immune cells, other cells such as mesenchymal (fibroblasts, myofibroblasts, smooth muscle cells), endothelial cells and also acellular components such as the extracellular matrix are able to influence electrolyte transport (88;89). These cells are capable of secreting a variety of inflammatory and secretory mediators as well as responding to such mediators. These mediators include arachidonic acid metabolites, cytokines, nitric oxide, reactive oxygen metabolites, enzymes and growth factors. Most

of these molecules stimulate chloride secretion and inhibit sodium absorption. The mediators released by resident cells can locally affect the cell (autocrine) or indirectly target other adjacent cells (paracrine) (Figure 10) (73). A study of monolayers of human T84 epithelial cells showed altered ion transport and permeability after co-culture with superantigen-activated immune cells, where interferon γ (IFN γ) and tumor necrosis factor α were key mediators in the response. The transport and barrier abnormalities evoked by superantigen-activated immune cells were inhibited by IL-10 but not IL-4 (90). Epithelial cells, themselves, play an active role in immunological and inflammatory events, acting as an antigen-presenting system and releasing cytokines and chemokines that participate in the trafficking of immune cells to the gut (88). The released mediators may potentiate or eliminate the effects of other mediators. For example, IFN γ mRNA can be induced in human lamina propria T lymphocytes by IL-12. In preactivated lymphocytes, IL-7 enhanced IFN γ release induced by both IL-12 and anti-CD2, whereas it had no effect alone (91).

Bi-directional interactions between the immune system and the ENS play an important role in gut inflammation. The neuronal Na^+ channel blocker, tetrodotoxin, partly blocked antigen-induced secretory responses in the intestine of sensitized rats (45). In addition, neurotransmitters can modulate cytokine production by intestinal smooth muscle. Interleukin-1β (IL-1β) significantly increased IL-6 mRNA expression and IL-6 protein in rat cultured intestinal smooth muscle cells. This effect was dose-dependently enhanced by a VIP, CGRP and norepinephrine and inhibited by VIP antagonist and propranolol, respectively. The protein kinase A inhibitor, 6-22 amide, abolished the actions of VIP, CGRP, and norepinephrine (92).

Figure 10. Many inflammatory mediators are known to stimulate intestinal salt and water secretion. They derive from numerous sources, including fibroblasts, macrophages, neutrophils, eosinophils and mast cells. Some inflammatory mediators can activate secretion by directly stimulating mucosal receptors or by activating other cells of the lamina propria which play a role in regulation of intestinal water and electrolyte transport, such as myofibroblasts or enteric neurons. IEL, intraepithelial leukocyte; EØ, eosinophil; NØ, neutrophil; PGs, prostaglandins: Th1 and Th2, T-helper cells type 1 and 2 (2).


Antigen
Anion Secretion
Epithelium
IEL
PGs
Fibroblast Sheath
Th1
Th2
Cytokines
Mediators
Neuropeptides
Mediators
Mast Cell
NØ
EØ
MØ
B
IgE/IgG
?
Neuron

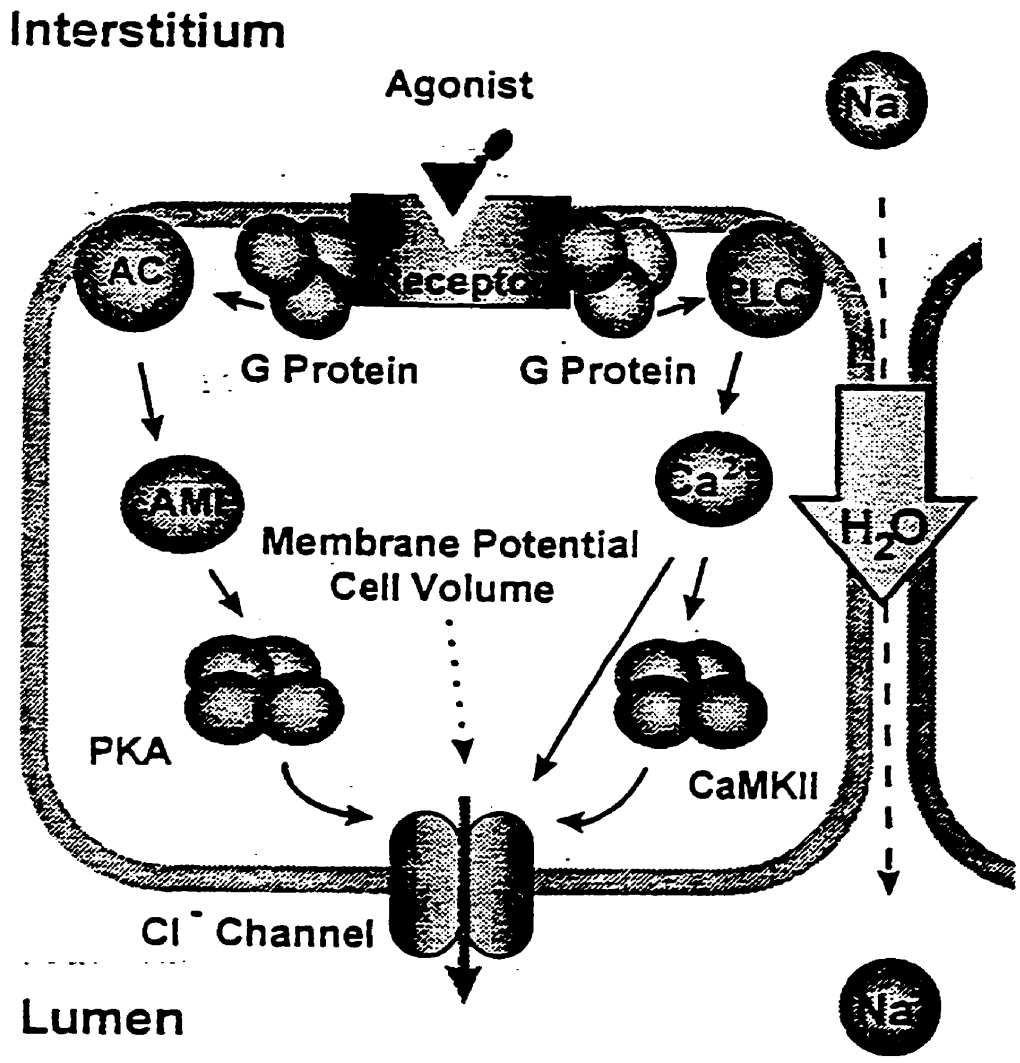
A neuro-immune link has been identified between the ENS and intestinal lymphoid tissues, where axonal fibers containing acetylcholine (ACh), norepinephrine, CGRP and SP all have synaptic terminals in the lymphoid tissues of the lamina propria (93). Prostaglandins (PGD_2, PGE_2, PGI_2, and $PGF_2\alpha$) and leukotriene C_4 (LTC_4), are capable of evoking chloride secretion in guinea pig colon through activation of submucosal cholinergic neurons (94).

The regulation of electrolyte and water transport in the mammalian intestine is regulated by neuronal input from both the CNS and the ENS which impinge upon a number of cell types in the intestinal mucosa. These neuronal inputs can act directly on epithelial cells to influence transport or they may act via stimulation of immune and other cell types. Mast cells in particular play a major role in the modulation of electrolyte and water transport through direct stimulation of epithelial cells as well as neuronal inputs (2).

C. Intracellular regulation of Cl^- secretion

A number of signal transduction pathways are involved in the regulation of ion transport. The second messengers involved in the regulation of secretory/absorptive pathways are: cAMP, cyclic guanosine monophosphate (cGMP), Ca^{2+} and phosphatidylinositol (PI) (Figure 11). The final result is activation of protein kinases that phosphorylate proteins involved in ion secretion. VIP, prostaglandin E_2 and cholera toxin cause Cl^- secretion through an increase in intracellular cAMP. Carbachol and serotonin (5-HT) act through inositoltriphosphate resulting increased intracellular Ca^{2+}. CGRP-induced Cl^- secretion in the epithelial monolayers (HCA-7 and Col-29) is accompanied by the accumulation of cAMP (47).

Figure 11. Activation mechanism for Cl^- channels in secretory epithelial cells Binding of an agonist to its receptor activates G proteins which in turn stimulate either adenyl cyclase (AC) or phospholipase C (PLC) to increase the intracellular concentration of cAMP and free Ca^+, respectively. Phosphorylation of channel proteins by protein kinase A (PKA) or Ca^+/calmodulin-dependent protein kinase II (CaMKII) results in the opening of cAMP or Ca^+-activated Cl^-channels, respectively. In some types of epithelial cells Ca^+ may directly activate Cl^- channels. Agonist induced changes in volume and membrane potential have been shown to activate additional classes of Cl^- channels (volume-sensitive and voltage-gated channels) independent of intracellular concentrations of cAMP and free Ca^+ (70).


Interstitium
Agonist
Na
AC
G Protein
G Protein
Membrane Potential
Cell Volume
PKA
CaMKII
Cl⁻ Channel
Lumen

2-4 Hypothesis and Objectives

Diarrhea and increased permeability of the intestinal mucosa are prominent features of IBD. Involvement of the nervous system as well as other factors in the pathogenesis of IBD have been documented although the exact etiology of IBD is unknown.

CGRP causes a chloride secretion accompanied by an increase in intracellular cAMP in epithelial cells. There are species differences in the way CGRP induces electrolyte secretion. In the guinea pig colon, CGRP excites myenteric neurons and releases acetylcholine which is responsible for inducing chloride secretion. In the rat colon, the action of CGRP has been suggested to be directly on the epithelium. The receptor mediating the secretory effect of CGRP has not been determined, though in the rat colon, it has been reported that $hCGRP_{8-37}$ did not modify the dose-response curve to CGRP.

The release of CGRP and other neuropeptides during intestinal inflammation has been documented by immunohistochemistry and radioimmunoassay. CGRP has important effects on many different cell types, such as neurons, mast cells, immune cells, intestinal epithelium, smooth muscle cells and vasculature. The identification of the mechanisms underlying CGRP-induced secretion and the total effect of CGRP in inflammatory processes are crucial to clarifying the role, if any, of CGRP in the pathogenesis of IBD.

Therefore, we hypothesized that **CGRP contributes to colonic inflammation and altered secretion in the TNBS model of colitis in the rat.**

It has been suggested that CGRP induces chloride secretion by acting directly on the colonic epithelium in the rat, but the mechanisms and receptors involved are not clear. The secretory effect of CGRP in inflamed tissues has not been studied. Finally, there is some controversy about the role of CGRP in the TNBS model of rat colitis. The aims of this thesis were to address the following:

1. To examine the effect of CGRP on Cl^- secretion in the normal and inflamed rat colon.
2. To establish the mechanism(s) and receptor(s) responsible for CGRP-induced electrolyte secretion in normal and inflamed rat colon.
3. To determine the effect of CGRP on epithelial permeability of inflamed rat colon.
4. To investigate the role of CGRP in colonic inflammation by the use of a $CGRP_1$ receptor antagonist and *h*αCGRP.

CHAPTER 3

MATERIALS AND GENERAL METHODS

3-1 Animals

Male Wistar rats (180-200 g) were obtained from a breeding colony maintained by the University of Calgary Animal Care Service or Charles River Breeding colony (Montreal, QC, Canada). The animals were housed under controlled environmental conditions (23-24°C, light from 7:00 to 19:00 h, food and water *ad libitum*) for at least two days before being used to ensure that they were acclimatized to their conditions. The experimental protocols used in this study were approved by the University of Calgary Animal Care Committee and conform to the guidelines established by the Canadian Council on Animal Care.

3-2 Induction of colitis

Trinitrobenzene sulfonic acid (TNBS) dissolved in ethanol when administered intracolonically produces chronic colonic inflammation resembling Crohn's disease (26). Ethanol breaks the integrity of the colonic mucosa, so that the hapten, TNBS, can enter and couple to a substance of high molecular weight such as tissue protein to elicit an immunological response. TNBS-induced inflammation in the rat persists for 6-8 weeks and it is possible to induce relapse by repeated luminal administration of TNBS alone (26;27). The inflammatory response to TNBS/ethanol is dose dependent. It is a self-limited model of colonic inflammation and does not spontaneously relapse as human IBD, but colitis can be reactivated by low doses of intravenous administered TNBS and chronicity can be driven by repeated administration of TNBS. Some of the clinical manifestations of Crohn's disease including diarrhea and weight loss, are similar in this animal model (28;29). Transmural inflammation with granuloma, skip-segment

ulceration and inflammation, cobblestone-like appearance of the mucosa, lymphoid infiltration and crypt distortion are similar to Crohn's disease (29).

In these experiments, rats were anesthetized with halothane (2-2.5% in oxygen), that allowed a prompt return to consciousness. While anesthetized, 0.5 ml of 2,4,6-trinitrobenzensulfonic acid (TNBS, 60 mg/ml) dissolved in 50% (vol/vol) ethanol was instilled into the lumen of the colon through a polyethylene catheter inserted rectally such that the tip was approximately 8 cm proximal to the anus. Control animals received 0.5 ml of physiological saline as described above. After recovery from anesthesia, the animals were placed in the cage and kept under the controlled environmental conditions. One week after treatment, animals were killed by an overdose of sodium pentobarbital (>60mg/kg, i.p.) and the colon was removed.

3-3 Experimental procedures

A. Assessment of colitis

The severity of colitis was assessed by macroscopic damage scoring, histological damage scoring of the effected tissue and by measurement of myeloperoxidase (MPO) activity. The method of macroscopic damage scoring takes into account the presence and absence of diarrhea (0-1) and a score based on the extent of mucosal damage from normal (score of 0) to a score of 10 depending on the presence and extent of ulceration and the extent of hyperemia. In addition to diarrhea (0-1) and mucosal damage score (0-10), adhesion (0-2) and the maximal bowel wall thickness (mm) were also taken into account in assessing macroscopic damage score (Table 3.1). The severity of macroscopic damage was determined by an observer unaware of the treatments the rats received.

Table 3.1 Criteria for "macroscopic damage score"

Feature	Score
Ulcerations	
Normal appearance	0
Localized hyperemia, no ulcer	1
Ulceration without hyperemia or bowel wall thickening	2
Ulceration with inflammation at one site	3
Two or more sites of ulceration and inflammation	4
Major sites of damage extending > 1 cm along length of colon	5
Major sites of damage extending >2 cm along length of colon, with score increasing by 1 for each additional cm	6-10
	plus
Adhesion	
No adhesions	0
Minor adhesions (colon can be easily separated from other tissue)	1
Major Adhesions	2
Diarrhea	
No	0
Yes	1
Thickness	
The maximum bowel wall thickness (X) in mm, was added to the above score	X
	Total score

Colonic samples for microscopic damage scoring were fixed overnight by immersion in a solution containing 1% formalin, 1% glacial acetic acid and 80% methanol at 4° C, dehydrated, embedded in paraffin, cut into 12 μm thickness and stained with haematoxylin and eosin. Microscopic scoring was based on a semi-quantitative scoring system in which the following features were considered and scored as follows: extent of destruction of normal mucosal architecture (0, normal; 3, maximal damage), presence and degree of cellular infiltration (0, normal; 3, maximal infiltration), extent of muscle thickening (0, normal; 3, maximal thickness), presence or absence of crypt abscess (0, absent; 1, present) and the presence or absence of goblet cell mucus depletion (0, absent; 1, present). In each case a numerical score was given with a maximum score of 11. All histological assessments were performed using coded slides to prevent observer bias. MPO activity as a quantitative index of inflammation was determined using an assay described by Krawisz et al. (1984) (95). Briefly, segments of colon taken from the piece of tissue used in the functional studies, weighing about 100 mg were homogenized (50 mg/ml) in 50mM potassium phosphate buffer containing 5 g/l hexadecyltrimethylammonium bromide. Samples were centrifuged (13,000 g in a microcentrifuge; Beckman Canada, Mississauga, ON, Canada) for 2 min. Aliquots (7 μl) of each sample supernatant were added to a 96 well microtitre plate. A 200 μl aliquot of o-dianisidine reagent was added to each well. This reagent was prepared by adding 16.7 mg O-dianisidine to 90 ml distilled water, 10 ml potassium phosphate buffer and 50 μl 1% hydrogen peroxide. The plate was immediately read at 450 nm using a Molecular Devices UV Max kinetic plate reader (Molecular Devices, Sunnyvale, CA, USA). Three

readings were taken and the activity was calculated using SoftMax software (Molecular Devices).

B. Study of electrolyte transport

Study of electrolyte transport in the flat sheet of mucosal-submucosal preparation of the rat distal colon was carried out in Ussing-type diffusion chambers *in vitro*. This method provided a good method for pharmacological intervention of tissues in assessing the role of CGRP by addition of different blockers or antagonist to the chambers. Also it enabled us to study the effect of CGRP while eliminating the effect of the higher neuronal centers on electrolyte transport in the studied tissues. The chambers are equipped with silver-silver chloride electrodes for measurement of potential differences and passing a transmural short circuiting current. The electrical potential difference across the tissue can be held (voltage-clamped) at zero by passing current or "short circuiting", through an external circuit connected between electrodes. When the tissue is bathed by solutions of identical composition under short circuit conditions, the short circuit current is a measure of active ion transport. The short circuit current is related to ion fluxes in the following manner:

$$J^{SC} = z\Delta J^{Na} + z\Delta J^{Cl} + z\Delta J^{R}$$

Where J^{SC} is the net ion flux, ΔJ is the net ion flux of sodium (Na^+), chloride (Cl^-), or residual (R) flux in $\mu A/cm^2$ and z is ionic valance (z is 1 for Na^+ or Cl^-) (69).

3-4 Drugs and reagents

Human αCGRP, [Cys $(Acm)^{2,7}$]hαCGRP, [Cys $(Et)^{2,7}$] hαCGRP and $hCGRP_{8-37}$ were synthesized and purified by a collaborator Dr. S. St. Pierre (Department of Chemistry, University of Quebec in Montreal, Montreal, Quebec, Canada H3C 3P8)

using standard solid-phase methods. Rat adrenomedullin and rat amylin were obtained from Bachem (Torrance, CA, USA). Doxantrazole [3-(1*H*-Tetrazol-5-yl)–9*H*-thioxanthen-9-one 10,10-dioxide monohydrate], tetrodotoxin, and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the peptides were dissolved in 0.25% acetic acid for *in vitro* study, whereas they were dissolved in distilled water for *in vivo* experiment. Doxantrazole and indomethacin were dissolved in dimethyl sulphoxide. Control tissues received appropriate vehicles in all cases.

3-5 Statistics

Data are expressed as mean ± SEM. Multiple groups were compared using a one-way analysis of variance with post hoc analysis (Newman Keuls test). Unpaired Student's t tests were used to compare two different groups. Probability values of < 0.05 were considered statistically significant. EC_{50} was calculated from non-linear regression analysis of the line by the appropriate software (GraphPad InStat, Version 3).

CHAPTER 4

THE RECEPTORS AND MECHANISMS UNDERLYING CGRP-INDUCED CHLORIDE SECRETION IN THE NORMAL AND INFLAMED RAT COLON

4.1 Introduction

The ENS plays an important role in the regulation of electrolyte transport. Neurogenic tone of active ion transport in *in vitro* preparations of the gut suggests that reflexes within the gut wall alone can control ion transport (73). One way in which the ENS regulates intestinal absorption and secretion is via activation of the neuronal reflex circuit, directly by the input from primary afferent neurons or indirectly through communication between ENS and mast cells/enterochromaffin cells (74-76). Of the large array of neurotransmitters found in submucosal secretomotor neurons, substance P, acetylcholine and VIP are the most studied neurotransmitters in reflex control of secretion (73). In response to noxious stimuli within the gut lumen, extrinsic and intrinsic afferent neurons generate action potentials leading to reflex activation (79). Substance P and CGRP released from primary afferent nerve endings are the primary candidates for this phenomenon (79;80).

Studies have suggested that intestinal secretion is impaired after colitis, reducing the capacity of the intestinal mucosa to mount a full response to luminal pathogens (30-32). Alterations in the structure and function of the ENS in inflammation may contribute to the dysfunction of electrolyte transport. In this study, the hypothesis "CGRP is a proinflammatory and secretory peptide involved in colonic inflammation and secretion in the rat distal colon" was tested. The effect of CGRP on electrolyte transport was studied in the normal and inflamed rat distal colon, since the exact mechanisms and receptors involved in CGRP-induced secretion are not well understood.

4.2 Methods

A. Tissue preparation

Animals were studied 7 days after intrarectal administration of saline or TNBS. The distal colon was excised and placed in ice cold oxygenated Krebs' buffer without glucose of the following composition (mM): NaCl 115.0, KH_2PO_4 2.0, $MgCl_2$ 2.4, $CaCl_2$ 1.3, KCl 8.0, and $NaHCO_3$ 25.0. A thin glass rod was inserted into the colon and the *muscularis externa* and associated myenteric plexus were removed from the underlying submucosa by blunt dissection. The tissues were then cut open along the mesenteric border. Four adjacent pieces of mucosa/submucosal preparations were routinely obtained from each saline-treated or untreated rat. Samples from TNBS-treated animals were obtained from the colon immediately adjacent to the most inflamed area. The maximally inflamed region was not used, as removal of the muscle was not possible. Two adjacent pieces of mucosa/submucosal preparations were routinely obtained from each TNBS-treated rat.

B. Electrolyte transport

Ion transport was studied in a standard Ussing-type diffusion-chamber apparatus. Mucosal preparations were placed between two halves of Ussing chambers (0.6 cm^2 exposed area; Navicyte Inc., Sparks, NV, USA). Each side was bathed in 4 ml oxygenated (95 % O_2- 5 % CO_2) Krebs-glucose (glucose 10 mM) solution, pH 7.35 – 7.45, at 37°C. In some experiments, normal Krebs was replaced with chloride-free Krebs solution of the following composition (mM): sodium isethionate 115.0, magnesium gluconate 2.4, calcium gluconate 1.3, potassium gluconate 8.0, KH_2PO_4 2.0, and $NaHCO_3$ 25.0. Tissues were voltage-clamped to zero potential difference (PD) by

passing short circuiting current from a voltage clamp apparatus (EVC-4000, World Precision Instruments, Sarasota, FL, USA). Short circuit current (Isc, $\mu A/cm^2$) was measured as an indicator of net active ion transport across the tissue with a digital data acquisition system (MP100, Biopac Systems, Santa Barbara, CA, USA) and analysis software (AcqKnowledge V3.03, Biopac Systems). Tissue conductance (G, mS/cm^2), an indicator of epithelial resistance, was calculated from the current and potential difference values using Ohm's law:

Tissue conductance (G, mS/cm^2) = Short circuit current (I_{SC}, $\mu A/cm^2$) / Potential difference (PD, mV)

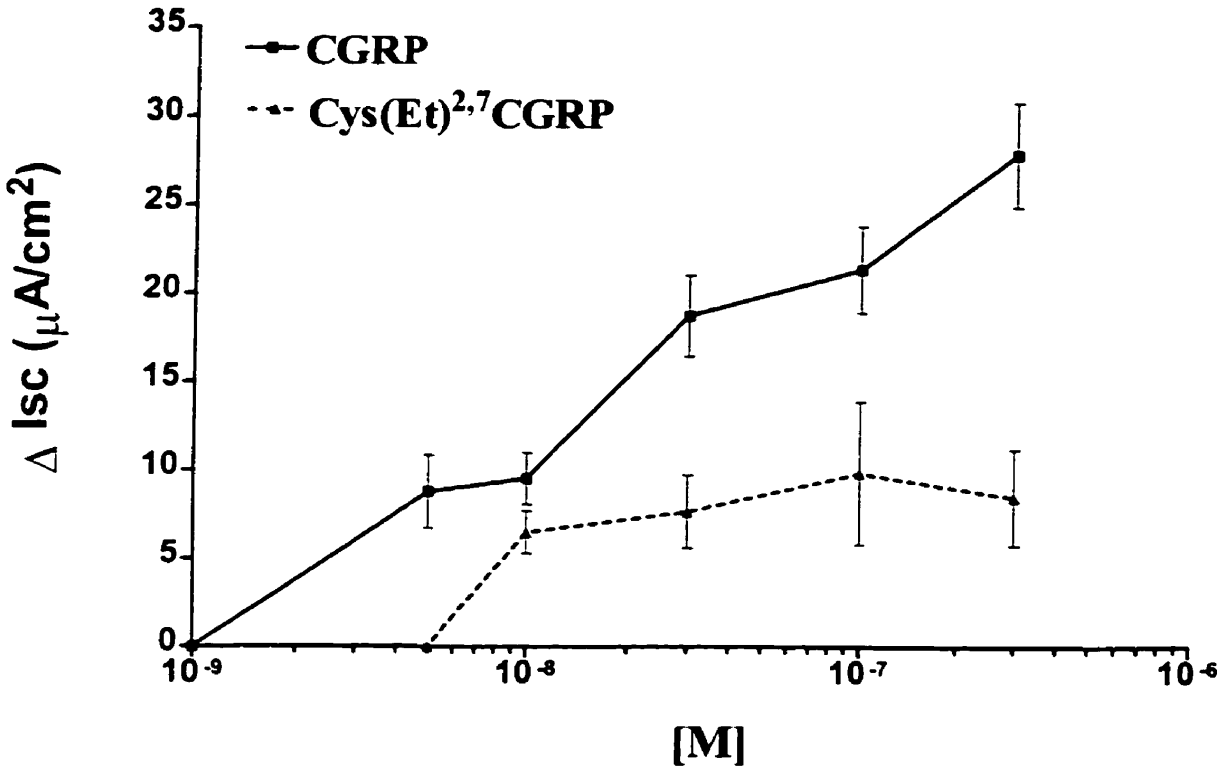
After a 20 min equilibration period, peptides or drugs were added to the serosal bathing fluid. When different drugs or peptides were added consecutively, 10-15 min interval was allowed between applications. In all cases, tissues were paired so that baseline conductances were within 20% of each other. One member of the pair was exposed to the drug or peptide and the other received an equivalent volume of the vehicle.

4.3 Results

A. Electrolyte transport in response to CGRP and related peptides

When added to the serosal side of the preparations, CGRP caused a rapid increase in I_{SC} that peaked and then slowly returned to baseline levels within 20 min. CGRP induced a concentration-dependent increase in I_{SC} in normal colonic tissues with EC_{50} value of 40 ± 2 nM (Figure 12). In contrast to CGRP, the response to the $CGRP_2$ receptor agonist [Cys $(Et)^{2,7}$] hαCGRP was considerably smaller in magnitude (Figure 12). We

Figure 12. Concentration-response curves to CGRP (-●-) and [Cys(Et)^{2,7}]*h*αCGRP (-▲-) in the normal tissues. Each concentration of CGRP or [Cys(Et)^{2,7}]*h*αCGRP was added as a single dose to separate tissues. Data shown represent mean ± SEM, n=4-9.


CGRP
Cys(Et)2,7CGRP
Δ Isc (μA/cm2)
35
30
25
20
15
10
5
0
10-9
10-8
10-7
10-6
[M]

also examined a single concentration (300nM) of another putative $CGRP_2$ receptor agonist, [Cys $(Acm)^{2,7}$] hαCGRP. At this concentration [Cys $(Acm)^{2,7}$] hαCGRP had a smaller effect (4 ± 2 $\mu A/cm^2$, n=4) than that produced by an equimolar concentration of [Cys $(Et)^{2,7}$] hαCGRP (9 ± 3 $\mu A/cm^2$, n=4). Animals treated with TNBS developed colitis as previously described (26). Typical features included hemorrhage, edema, ulceration and adhesions. The macroscopic damage score and MPO levels in vehicle-treated and inflamed tissues are shown in Table 4.1. The macroscopic damage score of each group of animals receiving a different concentration of CGRP revealed no significant differences among groups, suggesting that comparable tissues were used in every group studied. Baseline I_{SC} was similar in normal and inflamed distal colon (-96 ± 15 $\mu A/cm^2$, vehicle; -89 ± 19, inflamed). CGRP caused a concentration-dependent increase in I_{SC} in inflamed tissues (Figure 13). EC_{50} value of inflamed tissues was 19 ± 9 nM. There were no qualitative differences in the response to CGRP between normal and inflamed animals and the EC_{50} values were not found to be significantly different.

As in normal tissues, the $CGRP_2$ receptor agonist, [Cys $(Et)^{2,7}$] hαCGRP caused a smaller response than CGRP (Figure 13). In inflamed tissues, there appeared to be a shift to the right in the concentration-response curve to [Cys $(Et)^{2,7}$] hαCGRP, since at 10 nM there was no response whereas controls had a maximal response. In contrast to CGRP, addition of the cholinergic agonist, carbachol (5 μM), to the serosal side of the preparation caused a smaller increase in I_{SC} in TNBS-treated animals compared to non-inflamed tissues (Figure 14). Replacement of the normal buffer with chloride free buffer almost abolished the responses to both CGRP and [Cys $(Et)^{2,7}$] hαCGRP (Figure 15).

Table 4.1 Macroscopic damage score and myeloperoxidase activity of the normal and TNBS-induced inflamed tissues were used to assess the degree of inflammation of the tissues.

	Saline-treated	**TNBS-treated**
	n = 25	n = 31
Macroscopic Damage Score	0	6 ± 1*
MPO Activity (mU/mg/min)	6 ± 1	76 ± 9*

* $p<0.05$ compared to saline-treated animals.

Figure 13. Concentration-response curves to CGRP (◆) and [Cys(Et)^{2,7}]*h*αCGRP (◆) in the inflamed tissues. Each concentration of CGRP or [Cys(Et)^{2,7}]*h*αCGRP was added as a single dose to separate tissues. Data shown represent mean ± SEM, n=4-9.

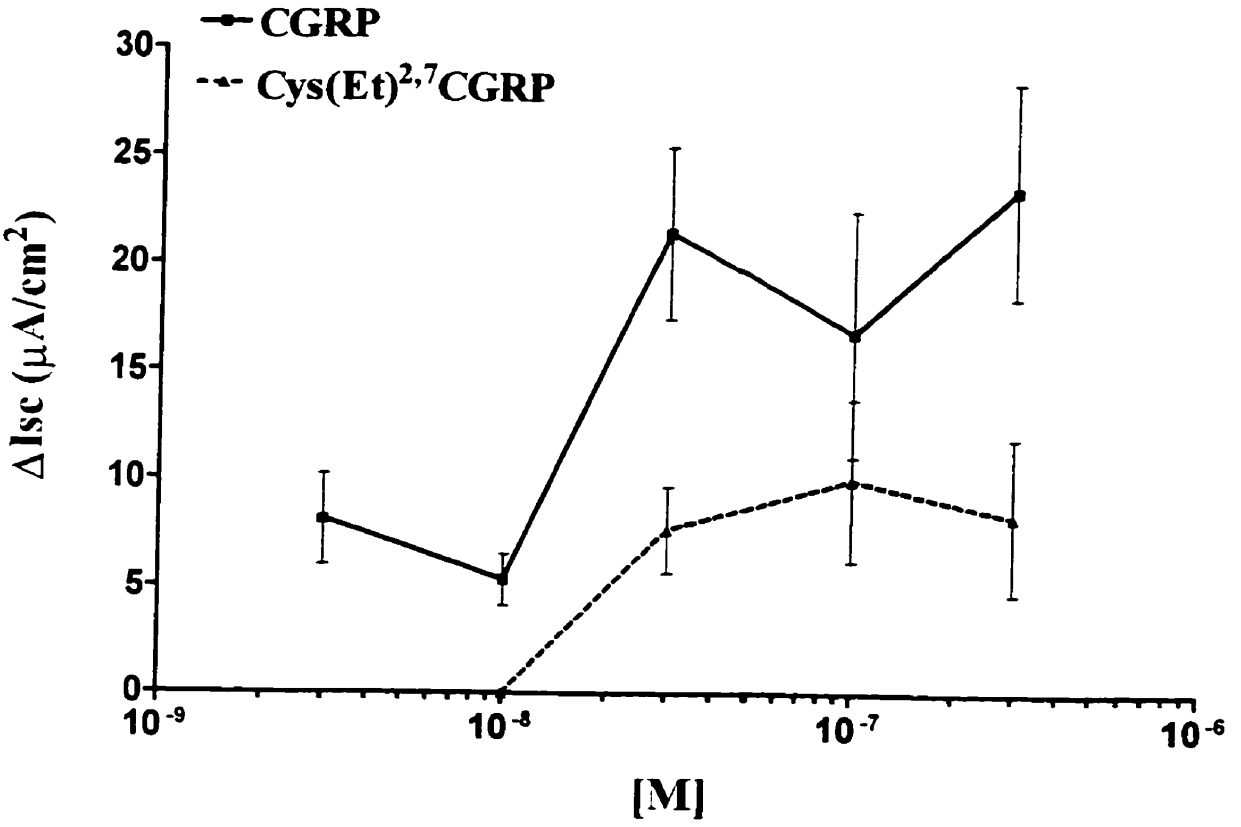

CGRP
Cys(Et)2,7CGRP
ΔIsc (μA/cm2)
30
25
20
15
10
5
0
10-9
10-8
10-7
10-6
[M]

Figure 14. Increases in the short circuit current (ΔI_{SC}) following administration of carbachol (5 μM) to normal and inflamed colonic preparations, n=29-33 per group. Each column represents mean ± SEM and * $p<0.05$ compared to control.

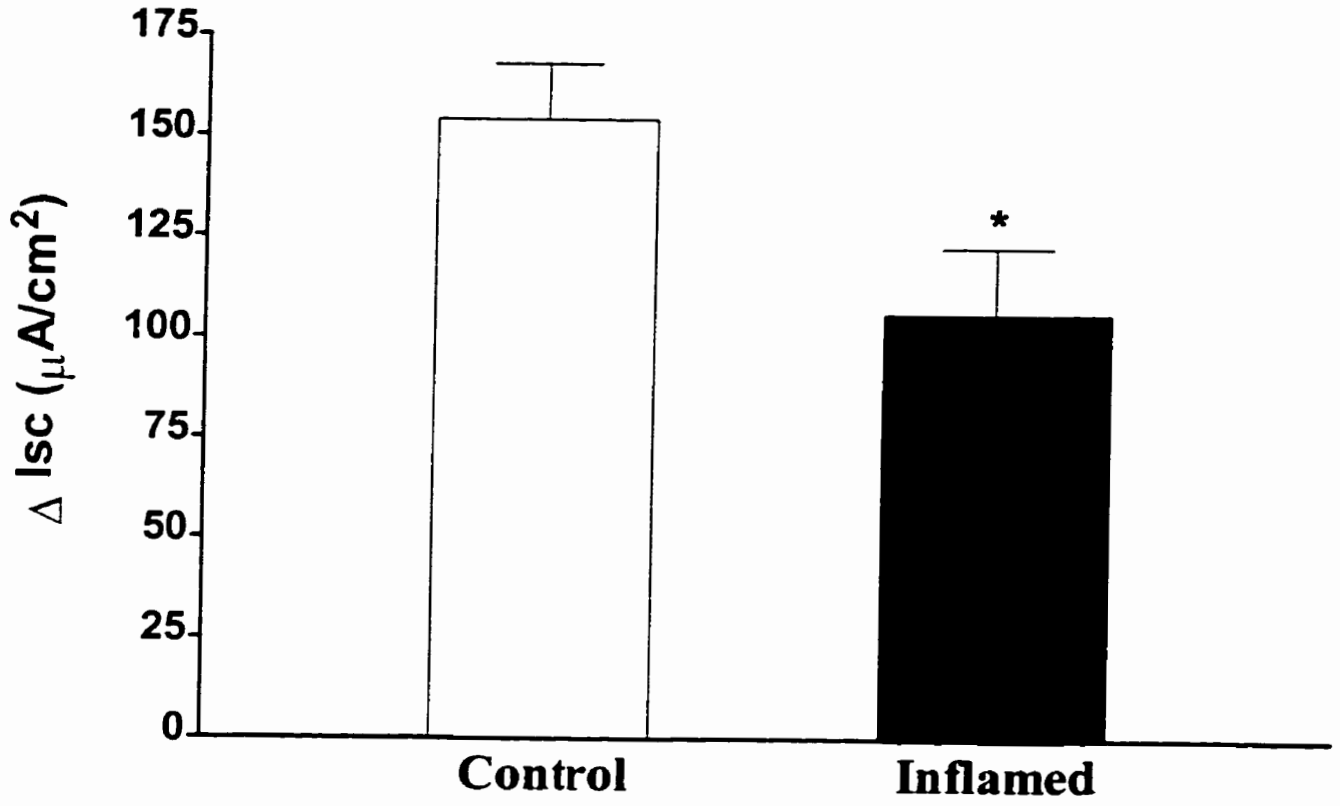
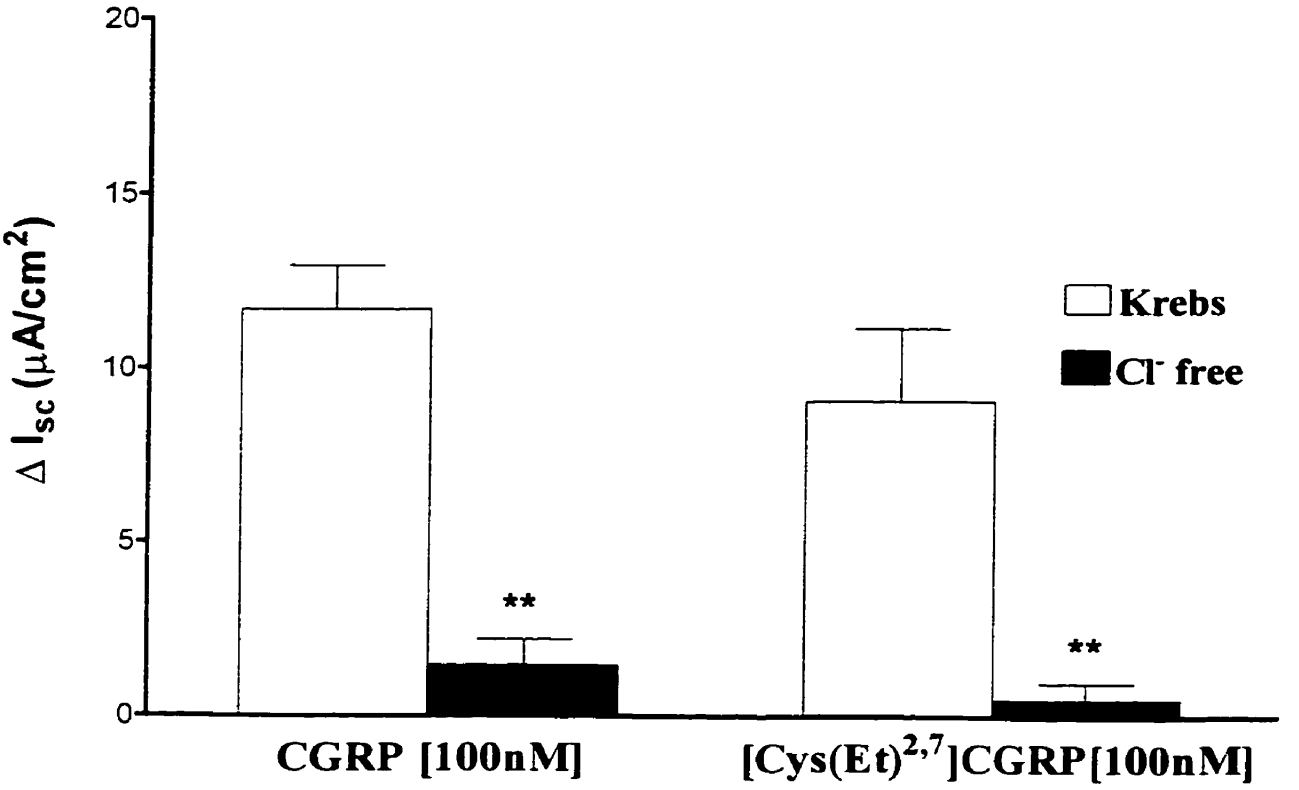

175
150
125
100
75
50
25
0
Δ Isc (μA/cm²)
*
Control
Inflamed

Figure 15. The effect of chloride removal on CGRP [100nM] and [Cys $(Et)^{2,7}$] hαCGRP [100nM] response of the distal colon preparations of the rat. CGRP or [Cys $(Et)^{2,7}$] hαCGRP was added as a single dose to separate tissues and the increase in short circuit current (Isc) was recorded, n=4 per group, ** $p<0.01$ compared to tissues bathed in Krebs' buffer.


20
15
10
5
0
Δ Isc (μA/cm2)
Krebs
Cl- free
**
**
CGRP [100nM]
[Cys(Et)2,7]CGRP[100nM]

To assess whether other members of the CGRP family caused electrolyte secretion in the rat colon, the effects of amylin (300nM) and adrenomedullin (300nM) were examined in normal and inflamed rat colon. Amylin (7 ± 1 $\mu A/cm^2$, n=4 vehicle; 9 ± 2 $\mu A/cm^2$, n=3 inflamed) and adrenomedullin (5 ± 2 $\mu A/cm^2$, n=4 vehicle; 6 ± 1 $\mu A/cm^2$, n=4 inflamed) only caused a modest ion transport at high concentrations in the normal and inflamed rat colon.

B. The effects of antagonists on CGRP-induced electrolyte transport

In order to examine the receptors involved, the effect of a competitive and reversible $CGRP_1$ receptor antagonist was assessed on CGRP-induced chloride secretion. The $CGRP_1$ receptor antagonist, $CGRP_{8-37}$ (1μM), pretreatment (10-15 min) did not change the baseline I_{SC} in either normal or inflamed tissues, nor did it significantly alter the I_{SC} response to CGRP in either groups (Figure 16). This dose of $CGRP_{8-37}$ has shown to inhibit Cl^- secretion by human adenocarcinoma cell line HCA-7 in the Ussing chamber (47).

Similarly, the cholinergic muscarinic antagonist, atropine (1μM), the mast cell stabilizer, doxantrazole (1μM), and the cyclooxygenase inhibitor, indomethacin (1μM), were without effect on the response to CGRP or $CGRP_2$ agonist (Table 4.2). However, the neural blocker tetrodotoxin (TTX, 1μM) significantly reduced the response to the $CGRP_2$ agonist, [Cys $(Et)^{2,7}$] hαCGRP (30nM), while having no effect on CGRP response (Figure 17).

Since the action of CGRP could not be blocked by any of the inhibitors or antagonists tested above, we examined the ability of CGRP to desensitize its own

Figure 16. The short circuit current response to CGRP in the presence of $CGRP_{8-37}$ (1μM) in normal and TNBS-induced inflamed tissues. Control tissues received vehicle instead. Each column represents mean ± SEM. n=4-5 per group.

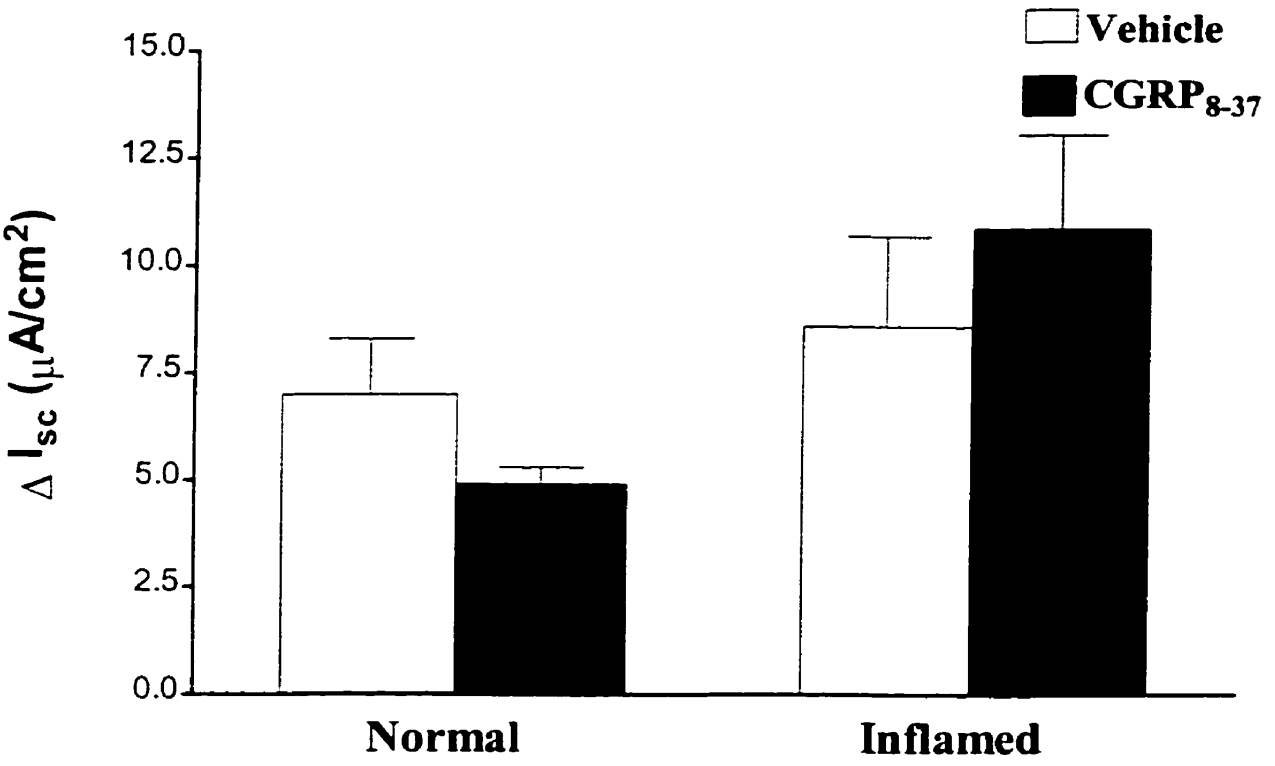
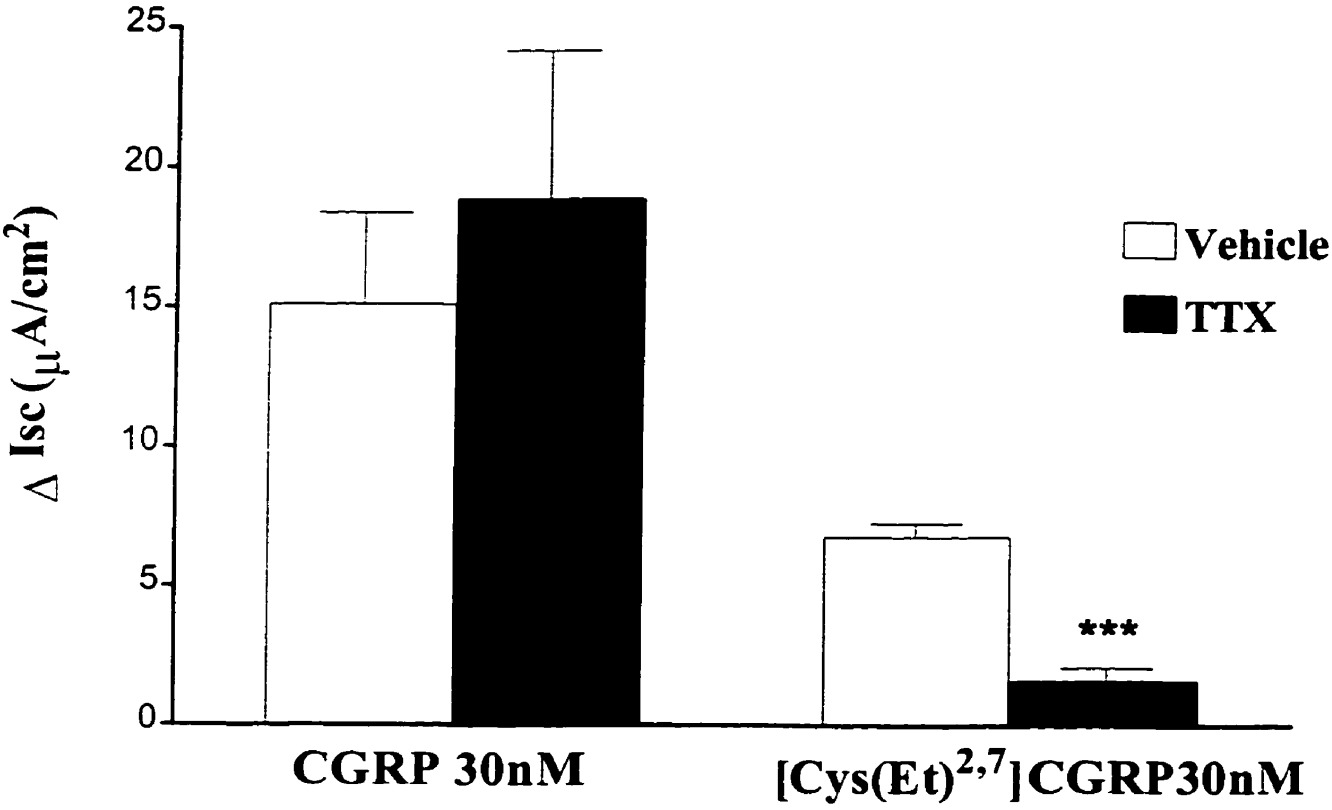

Vehicle
CGRP8-37
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Δ Isc (μA/cm2)
Normal
Inflamed

Table 4.2 The effect of indomethacin (1 μM), doxantrazole (1 μM) or atropine (1 μM) pretreatment of colonic preparations on CGRP- or [Cys $(Et)^{2,7}$] hαCGRP-induced increase in short circuit current.

	CGRP [30nM] (Mean ± SEM, n=4) $\mu A/cm^2$	**[Cys $(Et)^{2,7}$] hαCGRP [30nM]** (Mean ± SEM, n=4-5) $\mu A/cm^2$
Vehicle	13 ± 7	4 ± 1
Indomethacin	20 ± 7	6 ± 1
Vehicle	11 ± 4	3 ± 1
Doxantrazole	13 ± 4	7 ± 2
Vehicle	6 ± 2	4 ± 1
Atropine	7 ± 3	5 ± 2

Figure 17. The effect of neuronal Na^+ blocker, tetrodotoxin (TTX), on short circuit response of colonic preparations to CGRP (30 nM) or the $CGRP_2$ agonist, [Cys $(Et)^{2,7}$] hαCGRP, (30 nM). Preparations were pretreated with TTX (1 μM) or vehicle 10 min prior to the peptide application. Each column represents mean ± SEM. n=4-5 per group and *** $p<0.001$ compared to vehicle.


25
20
15
10
5
0
Δ Isc (μA/cm²)
Vehicle
TTX
***
CGRP 30nM
[Cys(Et)2,7]CGRP30nM

receptor as a way to examine further the site of action of CGRP agonists. Repeated application of CGRP at a high concentration (300 nM x 2) caused a significant attenuation in the response to this agonist (Figure 18). Subsequent administration of the $CGRP_2$ agonist, [Cys $(Et)^{2,7}$] hαCGRP (30 nM) to desensitized tissues resulted in a response which was indistinguishable from that of control tissues not exposed to CGRP (Figure 18). Unlike CGRP, repeated administration of [Cys $(Et)^{2,7}$] hαCGRP (300nM) did not cause desensitization and application of CGRP (30nM) after repeated administration of [Cys $(Et)^{2,7}$] hαCGRP was indistinguishable from vehicle treated tissues not exposed to the [Cys $(Et)^{2,7}$] hαCGRP (Figure 19).

4.4 Discussion

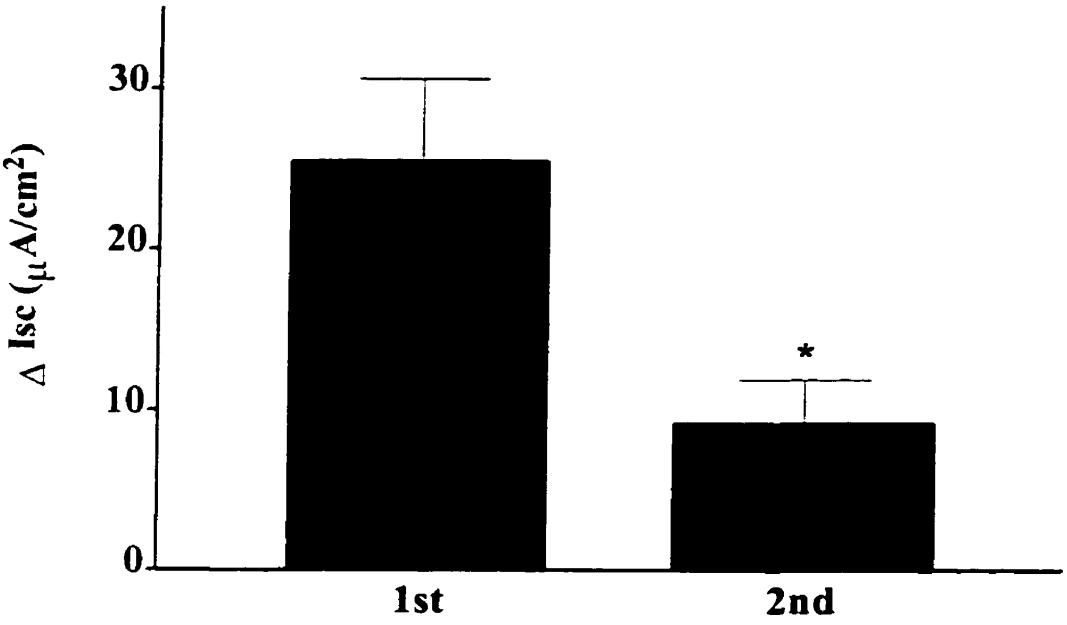
A. Responses in normal tissues

CGRP and the $CGRP_2$ receptor agonists, [Cys $(Et)^{2,7}$] hαCGRP and [Cys $(Acm)^{2,7}$] hαCGRP, caused increased I_{SC} in the rat colon. I_{SC} peaked within 5 minutes and gradually returned to the baseline within 20 minutes. CGRP (3 to 300 nM) and [Cys $(Et)^{2,7}$] hαCGRP (10 to 300 nM) caused a chloride-dependent secretory response. Lower concentrations (<3 nM for CGRP and <10 nM for [Cys $(Et)^{2,7}$] hαCGRP) had no effect on short circuit current.

In the present study we showed that the change in I_{SC} in response to CGRP and [Cys $(Et)^{2,7}$] hαCGRP was due to chloride secretion in the rat colon as their effects were totally abolished in chloride-free buffer. $CGRP_2$ agonists had lower efficacy than CGRP in inducing chloride secretion. The response to a maximal dose of [Cys $(Et)^{2,7}$] hαCGRP was lower than CGRP. This result shows that the $CGRP_2$ receptor cannot be responsible

Figure 18. The effect of CGRP-desensitization of colonic preparations upon the secretory response to [Cys (Et)^{2,7}] hαCGRP. **A.** A second application of CGRP (300 nM) caused a significantly reduced secretory response to CGRP compared to the first application. **B.** Tissues that were pretreated with CGRP (300 nM x 2, CGRP-desensitized tissues). [Cys (Et)^{2,7}] hαCGRP [10nM] was then added as a single dose. Control tissues were pretreated with vehicle instead of CGRP. Results are shown as mean ± SEM for n=5-7 per group. * $p<0.05$ compared to the first response.

A.


30
20
10
0
Δ Isc (μA/cm²)
*
1st
2nd

B.

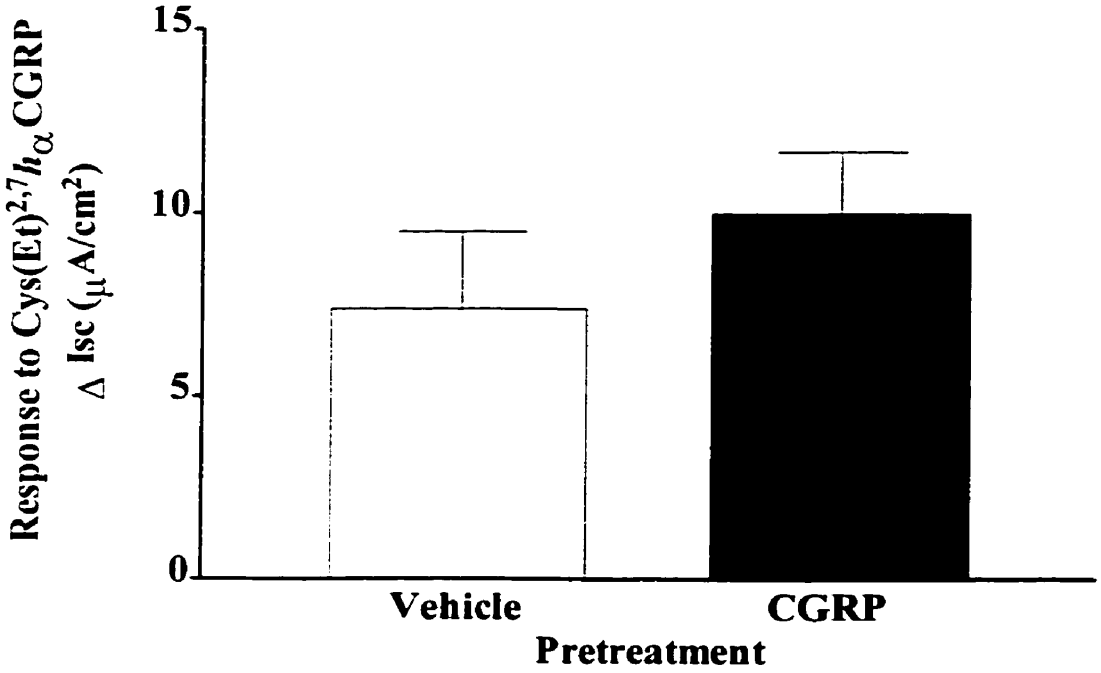
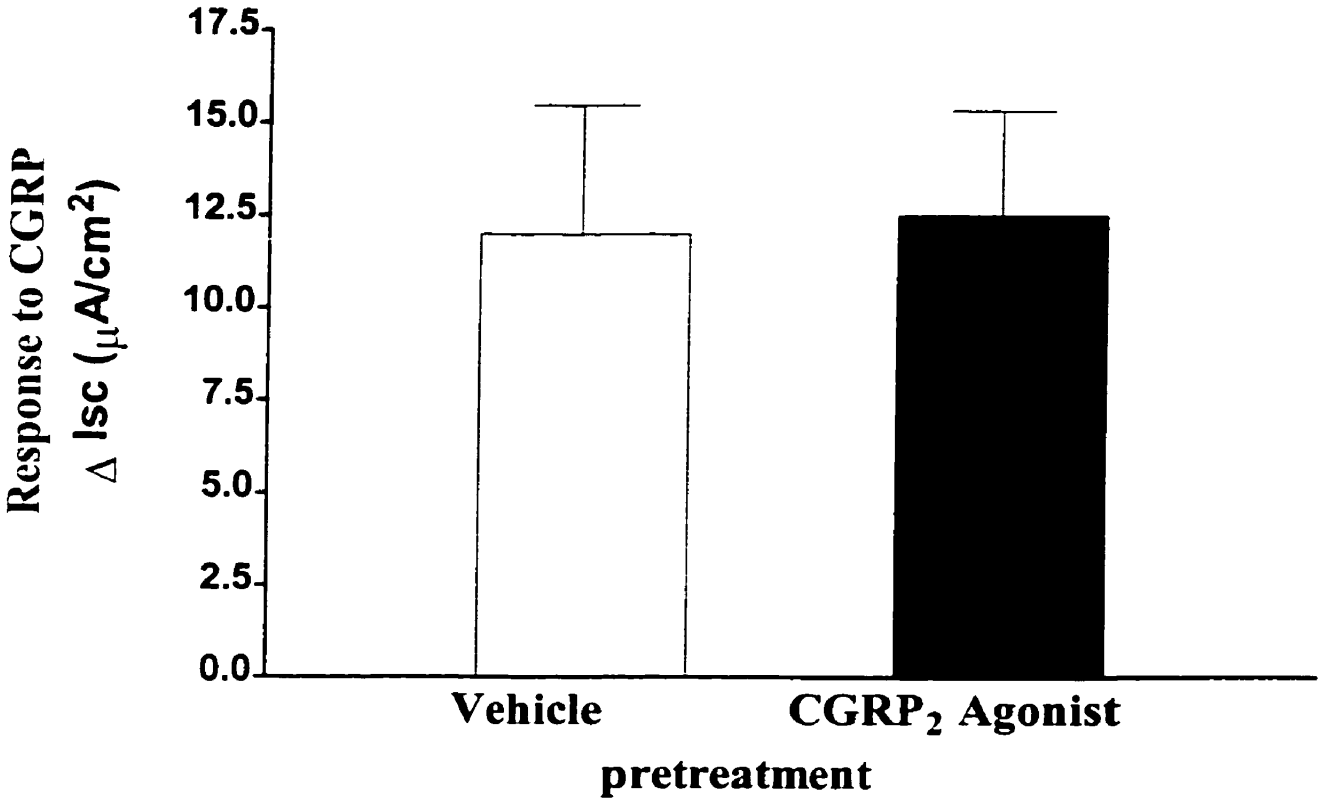

15
10
5
0
Response to Cys(Et)²,⁷hα CGRP
Δ Isc (μA/cm²)
Vehicle
CGRP
Pretreatment

Figure 19. The effect of [Cys(Et)^{2,7}]*h*αCGRP-pretreatment of colonic preparations upon the ecretory response to CGRP. CGRP [30nM] was added as a single dose after second application of a $CGRP_2$ agonist, [Cys (Et)^{2,7}] hαCGRP. The second application of [Cys (Et)^{2,7}] hαCGRP [300 nM] caused no difference in secretory response of tissues compared to the first application. Control tissues received vehicle each time instead of [Cys (Et)^{2,7}] hαCGRP. Results are shown as mean ± SEM for n=5 per group.


Response to CGRP
Δ Isc (μA/cm²)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Vehicle
CGRP2 Agonist
pretreatment

for the whole effect of CGRP.

The results of this study are consistent with a previous study which showed that CGRP was resistant to the actions of the $CGRP_1$ receptor antagonist, $hCGRP_{8-37}$ in the rat distal colon (47). These results suggest that another type of CGRP receptor other than type 1 and 2 exists and is responsible for the effect of CGRP on chloride secretion. So far 3 types of receptors have been proposed. CGRP type 1 is responsible for most of the cardiovascular effects of CGRP. The CGRP type 2 receptor, which cannot be blocked by the $CGRP_1$ antagonist, $CGRP_{8-37}$, and the CGRP type 3 receptor in the nucleus accumbens in the brain is responsive to salmon calcitonin and amylin as well as CGRP (55). In the present study, the response to amylin was very small, suggesting that the secretory response to CGRP was not mediated by the $CGRP_3$ receptor. Using colonic adenocarcinoma cell lines in addition to a preparation similar to our own, Cox and her colleagues studied both α and β forms of CGRP on chloride secretion. Interestingly, one colonic adenocarcinoma cell line (Col-29) had characteristics similar to rat colon, with no sensitivity to $hCGRP_{8-37}$, but another (HCA-7) was sensitive to the $CGRP_1$ antagonist. Based on these data, it was speculated that another CGRP receptor in addition to type 1 receptor is able to mediate colonic secretion in normal and adenocarcinoma cell lines, most likely the $CGRP_2$ receptor (47). The existence of a CGRP receptor other than type 1 has been proposed in the gastric mucosa. Using isolated gastric mucosal cells, Tu and Kang found that CGRP conferred protection against cytotoxic injury and this response was not blocked by $hCGRP_{8-37}$ (96). They did not examine a $CGRP_2$ agonist in that study, so firm conclusions cannot be drawn. We have now shown that the $CGRP_2$

receptor is responsible for only a small component of the response to CGRP in the rat colon.

The response to CGRP in the rat colon appeared to be receptor-mediated, since it rapidly desensitized while [Cys $(Et)^{2,7}$] hαCGRP application did not desensitize the tissues. Furthermore, the response to [Cys $(Et)^{2,7}$] hαCGRP was not affected by prior desensitization of the CGRP receptor and vice versa, which indicates different receptors being involved. CGRP receptors belong to the family of G-protein-coupled receptors (GPRs). Multiple mechanisms exist to control the signaling and density of GPRs. Upon agonist binding and receptor activation, a series of reactions participate in the turn off or desensitization of GPRs. Many GPRs are phosphorylated by protein kinases and consequently uncoupled from G proteins. In addition, many GPRs are sequestered from the cell surface and become inaccessible to their activating ligands. However, recent studies suggest that GPRs might be regulated by different mechanisms (97).

Our work rules out an indirect effect of CGRP on secretion through nerves, mast cells, and prostaglandin. Our data are also consistent with a CGRP receptor located directly on the colonic epithelium, and which has the characteristics of neither $CGRP_1$ nor $CGRP_2$ receptors. We suggest that this novel receptor and the $CGRP_2$ receptor are differentially localized, since the effects of [Cys $(Et)^{2,7}$] hαCGRP were blocked by TTX, supporting a neuronal localization, whereas the action of CGRP was TTX resistant.

In the guinea pig, the CGRP receptors mediating chloride secretion are localized on enteric nerves. Interestingly in this species, the effects are not mediated at the level of the submucosal plexus, but require an intact myenteric plexus as well. The site of action of CGRP appeared to be at the level of the myenteric plexus, which then acts through the

release of acetylcholine to cause secretion through submucosal secretomotor neurons. In support of a physiological role for CGRP is the observation that it causes acetylcholine release from the myenteric plexus. Thus, at least in the guinea pig, CGRP-containing nerves may act at the myenteric plexus to modulate cholinergically mediated secretion (23).

It is known that the activation of capsaicin sensitive primary afferent neurons in the guinea pig ileum causes a biphasic chloride secretion (79;80). Capsaicin treatment releases neuropeptides from primary afferent neurons. The second phase of capsaicin-induced chloride secretion in the guinea pig ileum can be blocked by NK_1 receptor antagonist. This result shows the functional role for substance P in the secretory events of the intestine. Not only is CGRP colocalized with SP and coreleased from primary afferent neurons, but also it exists in the ENS. In that study, MacNaughton et al. (1997) showed that unlike NK_1 receptor antagonist. The $CGRP_1$ receptor antagonist was not able to block capsaicin-induced chloride secretion in the submucosal sheets of guinea pig ileum. There are 3 possibilities to account for these observations. First, CGRP released from primary afferent neurons may play no physiological role in the intestinal secretion. Second, CGRP in the guinea pig colon needs an intact myenteric plexus to mediate chloride secretion, while the experiment by MacNaughton et al. performed was using stripped guinea pig ileum. It is possible that in the ileum as well as in the colon, CGRP mediated chloride secretion requires an intact myenteric plexus. Third, other CGRP receptors apart from type 1 mediate CGRP activity, which cannot be blocked by $CGRP_{8-37}$.Furhter studies are required to establish which of these possibilities are correct.

Recent studies have suggested that other members of the CGRP family of peptides may act through common receptors. For example some of the effects of adrenomedullin are mediated through the $CGRP_1$ receptor (58). It appears to be a common calcitonin-receptor-like-receptor (CRLR). Recent evidence suggests that a family of three receptor-activity-modifying proteins (RAMPs) differentially regulate the transport and glycosylation of the CRLR to confer the properties of either the CGRP or the adrenomedullin receptor (48;60). We examined the effects of adrenomedullin and amylin and neither peptide caused a significant change in I_{SC} except at very high doses. Similarly, Cox found that salmon calcitonin was inactive in the rat colon and had little effect on adenocarcinoma cell lines (47). Taken together these pharmacological data support the conclusion that CGRP acts at a putative novel receptor on the rat colonic epithelium. That this is a novel CGRP receptor requires verification by cloning, immunohistochemistry and receptor binding studies. Nevertheless, the characteristics of the current receptor are distinct from those of other previously reported even from the third type of CGRP receptors in the CNS.

B. Responses in inflamed tissues

In inflamed tissues, CGRP as well as the $CGRP_2$ agonist, [Cys(Et)^{2,7}] hαCGRP, caused a qualitatively similar responses. There was a slight shift to the right in the concentration-response curve in the inflamed tissues, but the EC_{50} values were not different. The shift to the right in the [Cys (Et)^{2,7}] hαCGRP concentration-response curve might be due to increased or altered level of proteases in the inflamed tissues, degrading the peptide faster than normal tissues (98). Structural changes of the inflamed tissues as well as changes in the receptor distribution and the secondary messengers are other

possibilities for the shift of concentration-response curve in the TNBS-treated tissues. As in normal tissues the $CGRP_1$ antagonist, $CGRP_{8-37}$, did not block the secretory effect of CGRP in inflamed colonic tissues.

Colitis appears to render the colonic epithelium less responsive to certain agonists, including those that act intracellularly. A hyporesponsive epithelium has also been described in the inflamed small bowel in animal and human tissues. The responsiveness to both Ca^{++}-dependent and cAMP-dependent secretagogues is reduced in mouse colon 1 week after induction of colitis (99), and in rat colon 6 weeks after the induction of colitis (32). In rat colitis, Kachur at al. (1995), showed that maximal increases in the net ion transport to bradykinin, prostaglandin E_1, carbachol, substance P and serotonin were depressed at 3 and/or 7 days after colitis, but the Isc response to theophylline was not affected. Theophylline activates an intracellular pathway instead of interacting with membrane-associated proteins (31). On the other hand, CGRP activates adenylate cyclase as an intracellular pathway. cAMP is also used for signaling by prostaglandin E_2, while carbachol and serotonin use the inositol triphosphate signaling pathway (47;68). The mechanism for the decreased Isc may relate to an uncoupling of receptors from second-messenger systems or a desensitization of receptor-linked secretory mechanisms. In this experiment, we found that the effects of CGRP, [Cys $(Et)^{2,7}$] hαCGRP, adrenomedullin and amylin were essentially identical in inflamed and normal tissues. This result was interesting in light of the fact that the response to 5 μM carbachol was reduced in the same preparations. The mechanism underlying the preservation of the response to CGRP is not clear, and needs further investigation. Receptors for CGRP, calcitonin, amylin and adrenomedullin are believed all to be G_s-

coupled receptors activating adenylate cyclase. It is possible that inflammation-induced hyporesponsiveness is dependent upon the upstream activation sequence of the adenylate cyclase pathway, and that this differs in CGRP-induced adenylate cyclase activity, compared to other activators. Functionally, the retention of responsiveness to CGRP may be important as part of the homeostatic mechanisms of mucosal defense, in which fluid secretion plays an integral part.

Another possibility to explain the normal response to CGRP in inflamed tissues is upregulation of CGRP receptors during inflammation. Inflammatory bowel disease is associated with NK1 receptor upregulation in intestinal blood vessels and lymphoid structures (84). Human epithelial cells constitutively express the intracellular interleukin 1 receptor antagonist (IL-1Ra) type I, which is up-regulated in patients with Crohn's disease or ulcerative colitis and also in *in vitro* studies using phorbol myristate acetate and interleukin 1β (100). The responsiveness to CGRP may, therefore, reflect an adaptive response in an attempt to preserve secretory capacity of the colon, should the lumen of the bowel be exposed to potentially harmful agents following inflammation.

CHAPTER 5

CGRP AND COLONIC EPITHELIAL PERMEABILITY:

AN *IN VITRO* STUDY

5.1 Introduction

Increased intestinal permeability may play a role in the pathogenesis of patients with Crohn's disease. Tight junction structure and the intracellular regulation of tight junction permeability have been the subjects of recent comprehensive reviews (34;72). In addition to patients themselves, increased permeability can be found in symptom-free first-degree relatives (3). Several studies have confirmed this observation, although some have failed to find altered permeability in relatives (1;3;5;6).

The concept that nerves may regulate epithelial barrier function is supported by a report describing increased blood-toward-lumen transport of horseradish peroxidase (MW 40,000) through the intestinal epithelial basolateral space into the tight junctions in rats treated with intravenous carbachol. Additionally, nerves have been shown to regulate epithelial permeability in the respiratory tract under baseline conditions and in relation to antigen challenge of sensitized animals (45). There is also evidence that neurotransmitters, including neuropeptides, may regulate tight junction structure and permeability. For example, duodenal epithelial permeability is increased by neurokinin A, an effect which is decreased by VIP (85).

In this study, the hypothesis "CGRP is a proinflammatory and secretory peptide involved in colonic inflammation and secretion in the rat distal colon" was tested and the effect of CGRP on electrical conductance and permeability to ^{51}Cr-EDTA of the normal and inflamed colonic tissues was investigated.

5.2 Methods

A. Tissue preparation and electrical conductance

In these experiments, the TNBS model of colonic inflammation was used as described in Chapter 3. Briefly, 7 days after TNBS or saline treatment, rats were sacrificed and the distal colon was removed. Mucosal-submucosal preparation of the rat distal colon was mounted in Ussing chambers as described in the previous chapter. Electrical conductance was obtained from Ohm's law in the following way:

Electrical conductance (G, mS/cm^2) = short circuit current (I_{SC}, $\mu A/cm^2$)/potential difference (PD, mV)

B. Epithelial permeability

Transepithelial transport of ^{51}Cr-EDTA, a large pore permeability marker (MW 342 Daltons), was used to assess CGRP-induced changes in mucosal permeability in Ussing chambers. ^{51}Cr-EDTA (7.3mCi/ml) was obtained from Mandel Scientific (Guelph, ON, Canada).

After a 10 min equilibration time, an equal volume of buffer was removed and replaced with 24 μCi ^{51}Cr-EDTA in each chamber. Control tissue received saline instead of ^{51}Cr-EDTA. At the same time that isotope or vehicle was added, samples were taken from the mucosal side to indicate the background activity of the chambers. ^{51}Cr-EDTA equilibrated within 15 min in the serosal side, then every 5 min samples were taken from mucosal side to calculate the baseline permeability of the inflamed tissues. Twenty-five min after the addition of label, CGRP (300nM) or vehicle (saline) was added to the serosal side and samples were taken every 5 min for 20 min. 400 μl mucosal samples and 40 μl serosal samples were counted for 300 sec in a gamma counter (1480 WIZARD,

Wallac OY, Turku, Finland). The ^{51}Cr-EDTA permeability of tissues is expressed as the percentage of the label in the mucosal chamber after addition of CGRP or vehicle at each time point compared to the average baseline permeability of the tissues during the 20 min prior to addition of CGRP or vehicle.

After performing several experiments, it became clear that permeability did not increase in some animals after CGRP. It is not clear why, but this may be due to the intrinsic variability of this method, or of the inflamed tissues. Therefore, we reported results from 2 groups of animals separately: those that responded to CGRP and those that did not.

Permeability of the label was calculated in the following way:

$$\%\ ^{51}\text{Cr-EDTA permeability} = \Delta A_{t\,m} / \text{Average } A_{(\Delta\,20\ \text{min})\,m} \times 100$$

Where: t is time and m is mucosal side

$\Delta A_{t\,m}$ Change in ^{51}Cr activity in the time t (min) in the mucosal side =

$$[A_{(t-5\ \text{min})\,m} + (A_{t\,m} + A_{(t-5\ \text{min})\,m} + \ldots) / (V_{Ch} / V_S)] - [A_{t\,m} + (A_{(t-5\ \text{min})\,m} + A_{(t-10\ \text{min})\,m} + \ldots) / (V_{Ch} / V_S)] / A_{Ts}$$

$A_{t\,m}$ = the ^{51}Cr activity measured on the mucosal side at time t (min)

A_{Ts}= the ^{51}Cr activity measured on the serosal side at 25 minutes (for measurement of baseline permeability before CGRP addition) or at 45 minutes (for measurement of CGRP-induced permeability).

V_{Ch} = Chamber volume (4000 µl)

V_S = sample volume (mucosal 400 µl, serosal 40 µl)

5.3 Results

The Effect of CGRP on Colonic Epithelial Conductance and Permeability

The effect of CGRP on epithelial resistance was assessed in vehicle-treated and inflamed tissues. Baseline conductance, an indicator of epithelial resistance, was 15.4 ± 3 mS/cm^2 in vehicle-treated animals and 19.0 ± 3 mS/cm^2 in TNBS-treated rats. In control tissues, the addition of CGRP (10-300 nM) had little effect on conductance suggesting that it was not modifying epithelial permeability. In inflamed tissues, however, there was a marked increase in conductance as shown in Figure 20.

To investigate the effect of CGRP on colonic permeability in inflamed tissues further, we examined epithelial permeability directly using a well-established marker of permeability ^{51}Cr-EDTA (101). Labeled EDTA added to the serosal side of the preparation was measured in the mucosal side before and after the addition of CGRP (300nM) or vehicle in inflamed tissues. Our results were expressed as the percentage change in permeability after the addition of vehicle or CGRP compared to the average basal permeability of tissues during the 25 minutes prior to their addition. Some animals clearly responded (5/10 rats) with an increased permeability, while others showed no change whatsoever (Figure 21). In those animals that appeared to respond to CGRP, there was an initial increase in permeability, that was sustained for 20 min. Experiments were not carried beyond a total time of about 60 minutes because after that time we observed a net increase in conductance in all tissues as a result of degradation of the tissues in the Ussing chambers.

Figure 20. Increases in the electrical conductance (ΔG; mS/cm^2) following administration of CGRP in normal and inflamed colonic preparations, n=4-9 per group. Each column represents mean ± SEM. * $p<0.05$ compared to saline group.

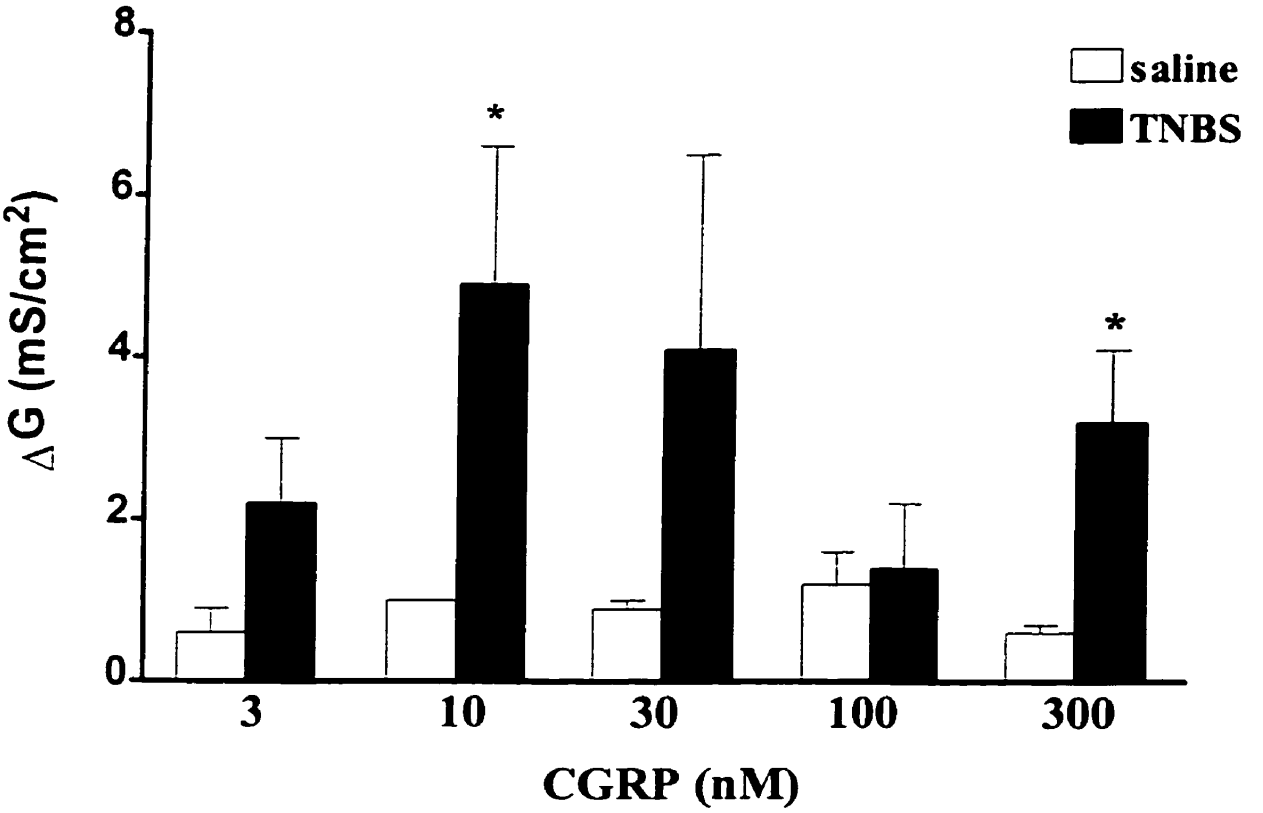
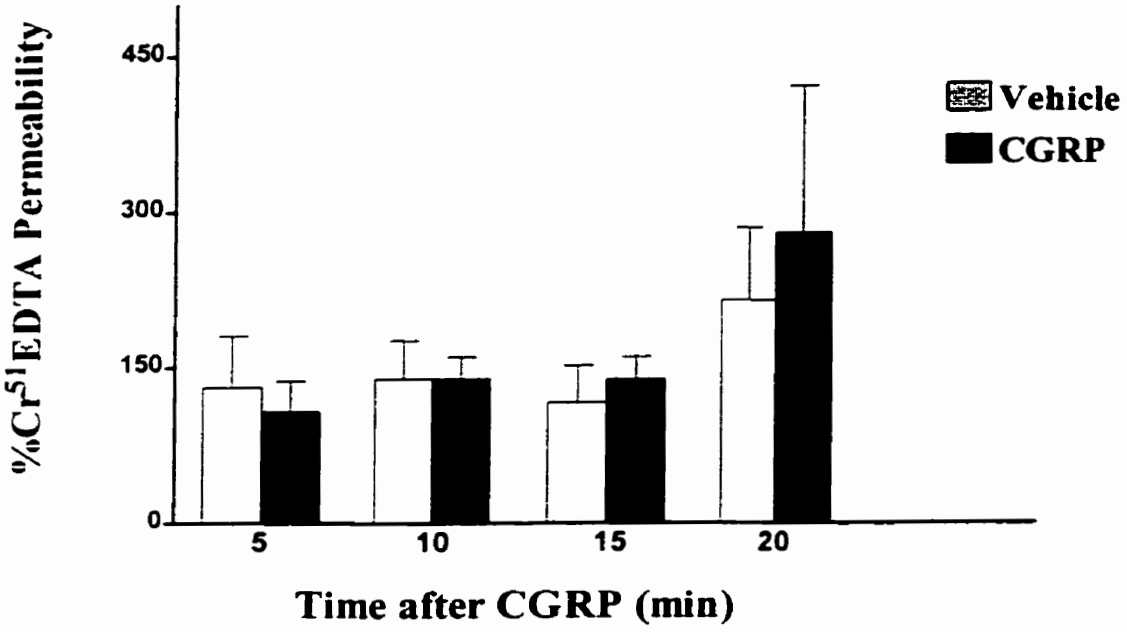
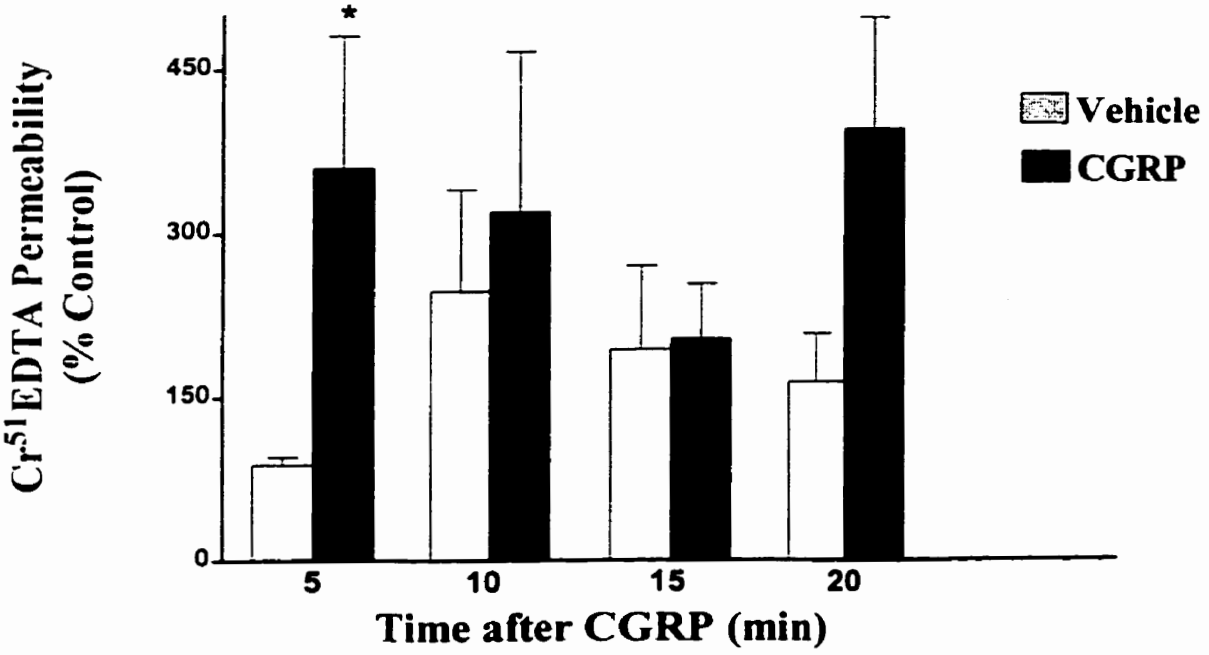

8
6
4
2
0
ΔG (mS/cm²)
saline
TNBS
*
*
3
10
30
100
300
CGRP (nM)

Figure 21. Permeability of inflamed tissues to ^{51}Cr-EDTA in response to CGRP [300 nM] or vehicle, n=5 per group. **A.** Non-respondent group. **B.** Respondent group. Data are expressed as percentage of the permeability at each time point compared to the permeability of tissues during the 25 min time before addition of CGRP or vehicle. Each column represents mean ± SEM and * $p<0.05$ compared to vehicle.

A.


%Cr51EDTA Permeability
450
300
150
0
5
10
15
20
Time after CGRP (min)
Vehicle
CGRP

B.


Cr51EDTA Permeability
(% Control)
*
450
300
150
0
5
10
15
20
Time after CGRP (min)
Vehicle
CGRP

5.4 Discussion

The major finding of this study was the ability of CGRP to increase conductance and epithelial permeability acutely in inflamed, but not normal tissues. Paracellular conductance of ions and probes such as ^{51}Cr-EDTA is regulated by the tight junctions between enterocytes.

The concept that nerves may regulate epithelial barrier function is supported by different reports (45;85). This experiment provides evidence to suggest that CGRP is another neuropeptide which can alter epithelial permeability, but only in the inflamed colon. The ENS in fact may mediate the majority of the secretory response induced by enterotoxins or phagocytes (102). An example for such an interaction is the involvement of nerves in antigen-induced increase in epithelial permeability of the jejunum of the sensitized rats (101).

The mechanism underlying the observation that the effect of CGRP on permeability occurs only in inflamed tissue is not clear. It is possible that the microenvironment to which the enterocyte is exposed in inflamed gut alters intracellular signaling pathways regulating tight junction assembly, structure or selectivity. Another possibility is that during inflammation, inflammatory mediators cause alterations in tight junction structure. It has been shown that there was an increase of tight junction permeability at remote sites such as the duodenum and ileum in rats with distal colitis. This permeability increase was accompanied by alterations in the tight junction protein occludin (33). Such alterations in tight junction structure may render the epithelium more susceptible to the modulatory effects of the neurotransmitters such as CGRP. In an *in vivo* experiment, the neuronal blocker, TTX, inhibited the antigen-induced increase in

^{51}Cr-EDTA permeability and the uptake of the antigen in the intestine of sensitized rats, but tetrodotoxin had no effect on the basal permeability of the tissues in the sensitized rats which was higher than normal. This result suggests that neurons were involved in modulating responses to altered homeostasis in the sensitized but not the basal state (101). It seems that the neuronal factors in the intestine are more than just a relaying system for information. They probably have direct effects on epithelial architecture. In the aforementioned experiment, TTX also significantly reduced villus shortening after antigen challenge, but had no effect on unchallenged sensitized animals (101). It has been documented that myenteric plexus ablation by benzalkonium chloride had a direct effect on intestinal morphology. Myenteric plexus ablation increased villus height, crypt depth, and muscle thickness in the treated and proximal segments, but only muscle thickness was increased in the distal segment (63).

In inflammation, therefore, activation of a CGRP receptor may result in effects on tight junction permeability not observed in normal tissue. Interestingly, the effect of CGRP on electrical conductance of the inflamed tissues were not concentration-dependent and CGRP increased the permeability of only half of the inflamed tissues. Why this was the case is not clear. The answer to these issues need additional studies to reveal the mechanism of increased permeability of inflamed tissues by the neuropeptide CGRP.

CHAPTER 6

THE ROLE OF CGRP IN THE DEVELOPMENT OF TNBS-INDUCED COLITIS: AN *IN VIVO* STUDY

6.1 Introduction

The nervous system exerts the greatest regulatory influence over the intestine, and may participate in intestinal inflammation. The first piece of evidence suggesting the involvement of the nervous system in IBD was the histological observation of an increased number of myenteric ganglion cells in chronic ulcerative colitis (7). This has led to further studies examining the distribution of neuropeptides and neurotransmitters in IBD (8). Whilst, some abnormalities have been documented, the role that nerves play in IBD is still unclear. Neurotransmitters released by nerve fibers in the gastrointestinal tract or elsewhere can produce local inflammatory reactions by interacting with the immune system in this way they may contribute to the pathogenesis of IBD and its symptoms(9). The anti-inflammatory effect of lidocaine in ulcerative proctitis in human and in the TNBS model of rat colitis suggests a neuronal involvement in IBD and experimental colitis (10). Primary afferent nerves play an important role in gastrointestinal mucosal defense and their ablation by capsaicin exacerbates colitis in several different animal models(1). Sympathetic nerves have also been demonstrated to play a role in an animal model of colonic inflammation (11). Two possible ways that nerves are involved in IBD have been proposed. First, nerves may play a role in the development and maintenance of inflammation through local release of certain neurotransmitters such as substance P and CGRP. Substance P and CGRP have modulatory effects on intestinal blood flow, electrolyte secretion, mast cell activation, recruitment of granulocytes and lymphocytes, and cytokine secretion, apoptosis and differentiation of immune cells (12-15,19). Second, once initiated (by whatever means), the process of inflammation may disrupt the normal pattern of innervation and interactions of nerves and their target tissues (16). Different

experimental animal models of intestinal inflammation have revealed reduced CGRP-immunoreactivity, suggesting the possibility of CGRP release in animal models of intestinal inflammation (18). However, the experiments examining at the effect of this neuropeptide in experimental colitis are few. In this study, we examined a pathophysiological role for CGRP in the TNBS model of colitis in the rat, using pharmacological tools. The hypothesis testd was that "CGRP is a proinflammatory and secretory peptide involved in colonic inflammation and secretion in the rat distal colon".

6.2 Methods

A. Insertion of osmotic minipumps

Under general anesthesia using halothane (2-2.5% in oxygen), osmotic minimumps (Alzet, model 1001, 1003 or 1007, Alzet Pharma., CA, USA) were placed subcutaneously under the skin of the neck of rats through a 1 cm incision. Just before the operation, the minipumps were filled with CGRP (1 μg/h), CGRP $_{8-37}$ (0.5, 1 or 5 μg/h), or saline (as control, 1 μg/h). Twelve hours after pump insertion, rats received TNBS or saline intrarectally. Rats were divided into groups and studied 1, 3 or 7 days after the induction of colitis. The effect of CGRP $_{8-37}$ (1 μg/h) was compared to saline in the 1 day experiment (n= 9-10 per group). In the 3 day experiment, the rats were administered either saline or CGRP (1 μg/h) (n=4-5 per group). In the 7 day study, rats were divided into 4 groups, controls received saline (1 μg/h) or CGRP $_{8-37}$ (0.5, 1 and 5 μg/h).

B. Intravenous administration of the peptides

In this experiment, different routes and dosages for CGRP or CGRP $_{8-37}$ were used. The effect of I.V. treatment with CGRP or CGRP $_{8-37}$ in the TNBS model of colitis was assessed and compared to control animals that received saline. Animals were divided

into 3 groups (n=3 per group). Saline, CGRP or CGRP $_{8-37}$ (25 μg/kg) were injected twice per day intravenously through the tail vein using a 30 gauge needle, starting one day before intrarectal administration of TNBS. The rats were examined 1 and 4 days later.

6.2 Results

The effect of CGRP or CGRP$_{8-37}$ on TNBS colitis

One day after TNBS-induced inflammation, CGRP $_{8-37}$ (1μg/h) administered via pump or intravenously (25 μg/kg/q12h) or intravenous CGRP (25 μg/kg/q12h) had no significant effect on any of the inflammatory criteria: myeloperoxidase activity, macroscopic and microscopic damage scores (Table 6.1).

Three days after TNBS treatment, CGRP treated animals (1μg/h, pump) had significantly more colonic microscopic inflammation than the control group (saline pump). Other parameters of the inflammation were not significantly different. In the 4 day experiment, intravenous administration of CGRP or CGRP $_{8-37}$ (25 μg/kg/q12h) did not change colonic damage or weight gain in the TNBS model (Table 6.2). CGRP $_{8-37}$ treated rats had the same extent of colonic inflammation as the control.

Seven days after the induced of colitis, the effect of CGRP$_1$ receptor antagonist was assessed. In these experiments, three doses of CGRP $_{8-37}$ (0.5, 1 and 5 μg/h) were administered via minipump. Table 6.3 shows the result of different doses of the antagonist on body weight change post-TNBS compared to the control group that received saline. Seven days after TNBS, animals gained weight but there was no difference among groups. Total macroscopic damage score (adhesion + diarrhea + the length of inflamed colon + thickness) are shown in Figure 22. Animals treated with CGRP $_{8-37}$ (1 μg/h) had lower damage scores. Interestingly, the higher dose of CGRP $_{8-37}$

Table 6.1 The effect of CGRP and a $CGRP_1$ receptor antagonist on body weight change (g), macroscopic damage score, microscopic damage score and myeloperoxidase activity of inflamed tissues (24 hours post-TNBS). Rats received $CGRP_{8-37}$ or saline via osmotic minipumps (1 μg/h, n=9-10 per group) or saline, CGRP and $CGRP_{8-37}$ via intravenous injections (25 μg/kg/q12h, n=3 per group). Data represent mean ● SEM.

	Saline pump	**$CGRP_{8-37}$ pump**	**Saline iv**	**CGRP iv**	**$CGRP_{8-37}$ iv**
Δ Body weight (g)	-18 ± 4	-17 ± 3	-21 ± 4	-17 ± 1	-20 ± 3
Macroscopic Damage Score	9 ± 1	10 ± 1	10 ± 1	9 ± 1	10 ± 1
Microscopic inflammation	8 ± 1	8 ± 1	6 ± 1	6 ± 1	6 ± 1
†MPO Activity (mU/mg/min)	25 ± 7	27 ● 3	155 ± 27	256 ± 38	148 ± 24

† Note: The MPO activity was measured at different time. Only data within each study, which were measured at the same time, are compared.

Table 6.2 The effect of CGRP and a $CGRP_1$ receptor antagonist on body weight change (g), macroscopic damage score, microscopic damage score and myeloperoxidase activity of TNBS-inflamed tissues. Rats received saline or $CGRP_{8-37}$ via osmotic minipumps (1 μg/h, n=4-5 per group) for 3 days, or CGRP or $CGRP_{8-37}$ via intravenous injection (25 μg/kg/q12h n=3 per group) for 4 days. Data represent mean ± SEM.

	Saline Pump	**CGRP pump**	**Saline iv**	**CGRP iv**	**$CGRP_{8-37}$ iv**
Δ Body weight (g)	-7 ● 9	-1 ± 3	1 ± 9	-10 ± 7	-9 ± 6
Macroscopic Damage Score	11 ± 0	10 ± 1	8 ± 3	12 ± 3	8 ± 2
Microscopic inflammation	7 ± 0.3	9 ± 0.4 *	10 ± 1	10 ± 0	10 ± 1
†MPO Activity (mU/mg/min)	95 ● 18	152 ± 32	77 ± 34	162 ± 91	199 ± 73

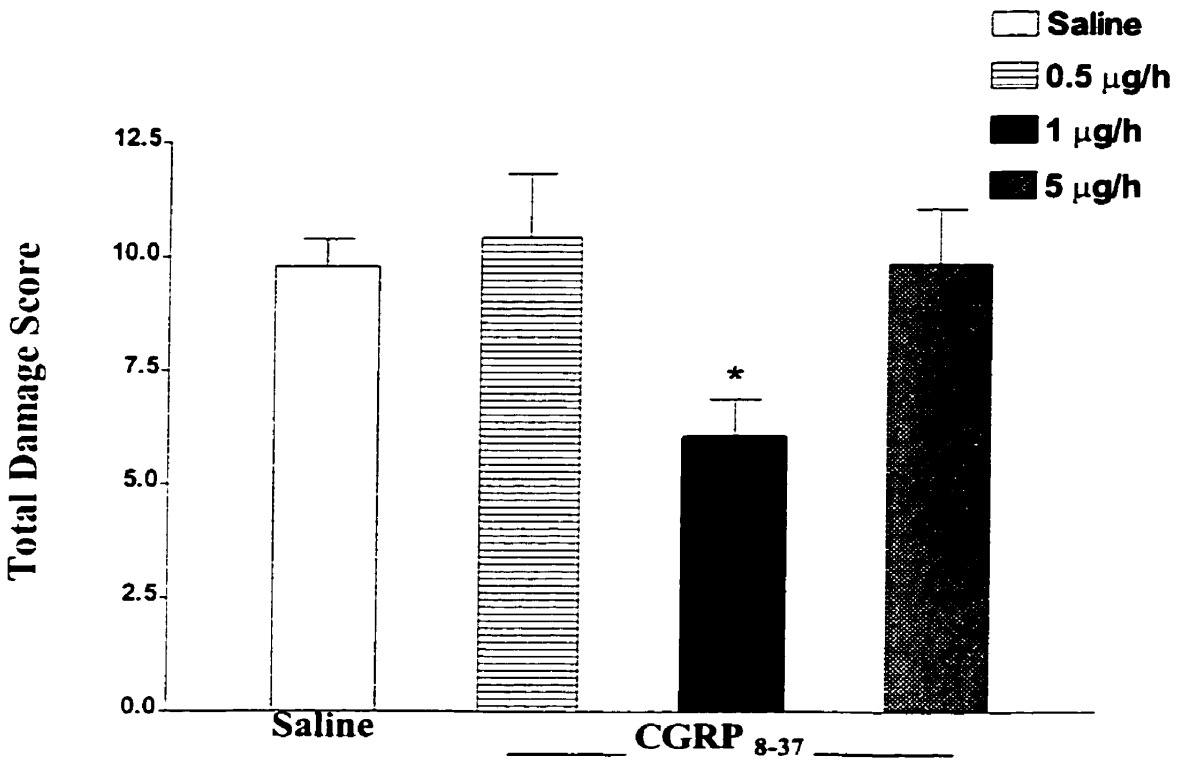
*$p<0.05$ compared to saline pump.

† Note: The MPO activity was measured at different time. Only data within each study, which were measured at the same time, are compared.

Table 6.3 The effect of $CGRP_1$ receptor antagonist on body weight changes of TNBS-induced inflamed rats. Rats received $CGRP_{8-37}$ (0.5, 1, 5 μg/h) via osmotic minipumps. Controls received saline. Each column represents mean ± SEM and n=8-17 per group.

	Δ Body weight (g) **mean ± SEM**
Saline	10 ± 6
$CGRP_{8-37}$ (0.5 μg/h)	12 ± 7
$CGRP_{8-37}$ (1 μg/h)	29 ± 8
$CGRP_{8-37}$ (5 μg/h)	19 ± 5

Figure 22. The effect of $CGRP_1$ receptor antagonist on colonic total damage score. Rats received $CGRP_{8-37}$ (0.5, 1, 5 μg/h) via osmotic minipumps. Controls received saline. Each column represents mean ± SEM, n=6-9 per group. * $p<0.05$ compared to animals receiving saline pump.


Saline
0.5 μg/h
1 μg/h
5 μg/h
Total Damage Score
12.5
10.0
7.5
5.0
2.5
0.0
*
Saline
CGRP 8-37

(5 μg/h) had no greater beneficial effect in the inflammatory score compared to the lower dose of CGRP $_{8-37}$ (0.5 μg/h). Microscopic damage score and MPO activity were not reduced in animals treated with CGRP $_{8-37}$ (Figure 23 and 24).

6.3 Discussion

This experiment was done at 3 different time points to assess the effect of a $CGRP_1$ receptor antagonist and/or CGRP in different stages of acute inflammation in TNBS-induced colitis in the rat. Since the pharmacokinetics of CGRP and CGRP $_{8-37}$ are not known, two different routes of drug administration were used. Peptides were administered by continuous subcutaneous infusion using osmotic minipumps, or intravenous injection twice per day.

The colitis induced by intracolonic administration of TNBS in a vehicle of ethanol is in part due to the caustic properties of this mixture and in part to the immune/inflammatory response (27). In this model, as well as IBD, there is evidence for involvement of the nerves. Primary afferent, capsaicin-sensitive, nerves have been suggested to play an important role in gastrointestinal mucosal defense, primarily through regulation of the hyperemic response to luminal irritants (11). In the TNBS model of colitis in the rat, primary afferent nerves have an important protective function in the first 7 days of inflammation but do not seem to play a significant role in the later stages of inflammation (103). Although capsaicin-treated rats had lower macroscopic damage score, but there was no difference between them and the control group with respect to MPO activity or mucosal destruction as measured by microscopic damage score at 7 days post-TNBS. Furthermore, capsaicin treatment had no effect on colonic inflammation in the rats treated with capsaicin as neonates (11). CGRP and substance P are believed to be

Figure 23. The effect of $CGRP_1$ receptor antagonist on colonic microscopic damage score. Rats received $CGRP_{8-37}$ (0.5, 1, 5 μg/h) via osmotic minipumps. Controls received saline. Each column represents mean ± SEM and n=8-17 per group.

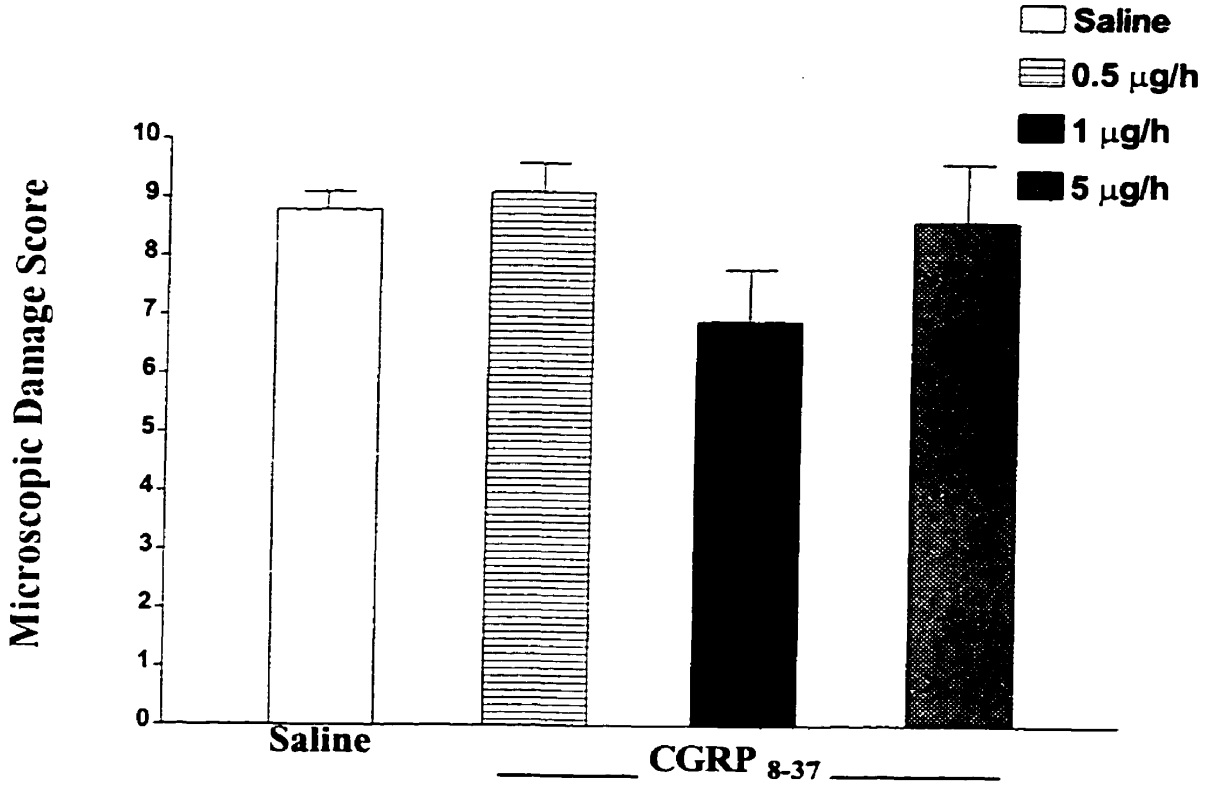
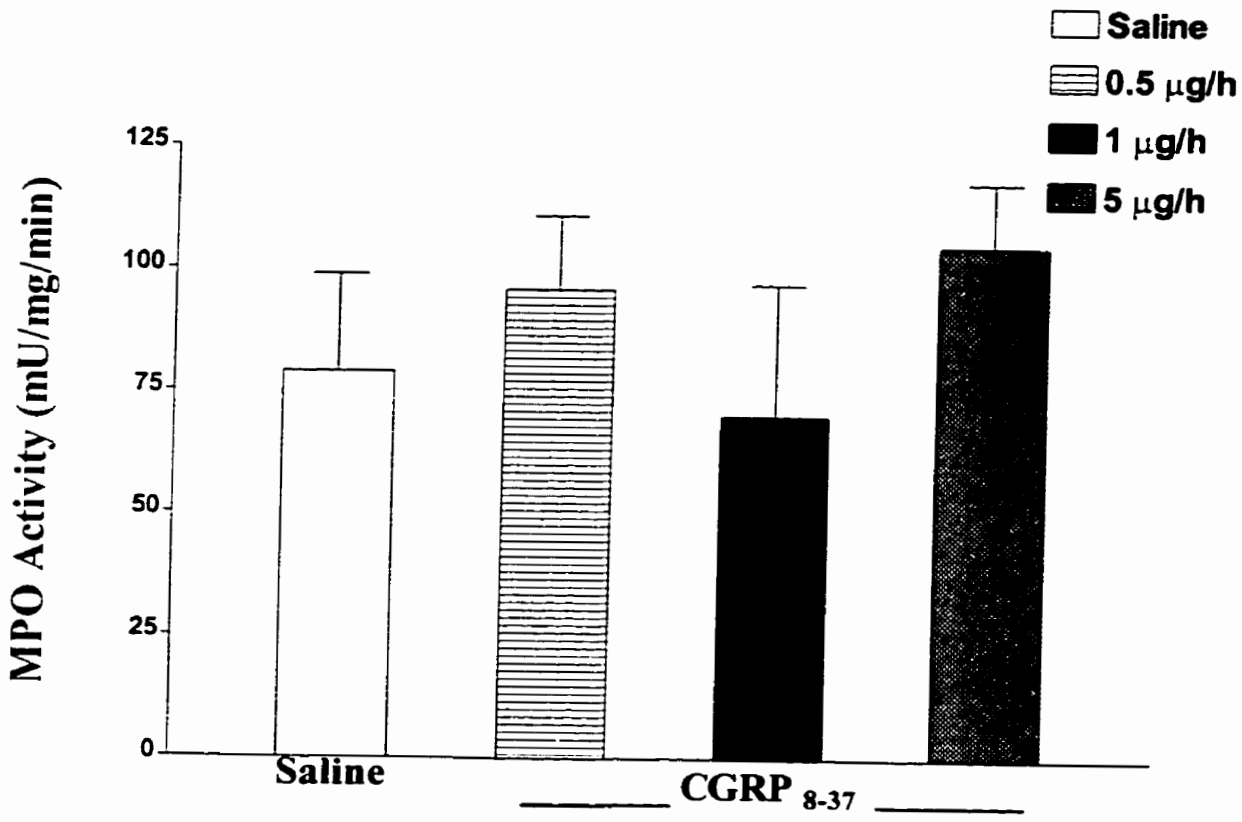

Saline
0.5 μg/h
1 μg/h
5 μg/h
Microscopic Damage Score
10
9
8
7
6
5
4
3
2
1
0
Saline
CGRP 8-37

Figure 24. The effect of $CGRP_1$ receptor antagonist on colonic myeloperoxidase activity. Rats received $CGRP_{8-37}$ (0.5, 1, 5 μg/h) via subcutaneously inserted pumps. Controls received saline. Each column represents mean ± SEM and n=8-17 per group.


Saline
0.5 μg/h
1 μg/h
5 μg/h
MPO Activity (mU/mg/min)
125
100
75
50
25
0
Saline
CGRP 8-37

the neurotransmitters which mediate the vasodilatory effect of primary afferent neurons (19). The CGRP content of the rat colon reduced by 50% after capsaicin pretreatment, suggesting that a major portion of CGRP in the intestine is of intrinsic origin (41). In addition to primary afferent nerves, sympathetic nerves have been implicated in inflammation. Sympathetic nerves have a detrimental role in the TNBS model of colonic inflammation in the rat (11). At the same time the local anesthetic agent lidocaine plus chemical sympathectomy lowered damage score even more than sympathectomy alone. It is suggested that in TNBS induced colitis lidocaine is acting on enteric nerves and that these nerves have a net proinflammmatory effect in inflammation. Removal of the proinflammatory effect of sympathetic nerves by sympathectomy and inhibition of the release of the neurotransmitters from enteric nerves reduces damage score further (11).

Vasodilation is not the only effect of CGRP and substance P. They have neuromodulatory and immunomodulatory effects which contribute to the proinflammatory activity (104). They can increase lymphocyte movement through the gastrointestinal tract and activate lymphocytes (12). CGRP induced marked secretion of cytokines such as IL-2, IL-4, IL-10 and INF γ from T cells (29). These neurotransmitters are also able to degranulate mast cells. Despite the suggested proinflammatory effect of CGRP and substance P, the results of experiments using antagonists are confusing, mainly because of different receptors subtypes involved in mediating the responses to these peptides and the different antagonists used in the experiments. In TNBS treated rats, daily administration of NK_1 and NK_2 receptor antagonists (SR 140333 and SR 48968, respectively) reduced colonic inflammation evaluated by measuring gut permeability, myeloperoxidase activity, macroscopic and microscopic damage scores

(105). In another experiment, while NK_1 receptor antagonists (RP 67580 and CP-96,345-1) significantly reduced the infiltration of the granulocytes into colonic tissue during the first 12 hours after induction of colitis in the rat, repeated administration of RP 67580 over a period of 3 day period failed to significantly affect the severity of the tissue injury (106).

In this study, we demonstrated that at one day post-TNBS, neither CGRP nor $CGRP_{8-37}$ had any effect on colonic inflammation. Probably at this time point, the caustic effect of TNBS/ethanol on the colonic mucosa is the major cause of inflammation or CGRP does not appear to be an early inflammatory mediator. Another possibility is that the $CGRP_1$ receptor is not the only receptor involved in mediating the effect of CGRP on the colon *in vivo*. Therefore $CGRP_{8-37}$, might have little or no effect on colonic inflammation. Our *in vitro* study showed that different CGRP receptor subtypes mediate the effect of CGRP on mucosal-submucosal preparations of the rat colon and the $CGRP_2$ receptor is located on neurons.

At day 3 of TNBS-induced colitis CGRP, when released constantly by subcutanous pump, increased colonic microscopic inflammation. This was not observed when CGRP was administered intravenously. It seems that the intravenous route or the schedule and/or dosage is not as effective as continuous release of the peptide by the pump. The increased inflammation was evident on only microscopic damage score. The macroscopic appearance of the tissues or MPO activity were not different from controls and the inadequate dosage of the administered CGRP might be an explanation. Another explanation is that the endogenous CGRP may have already been released and may have had its effects on TNBS-induced colonic inflammation. Therefore, the exogenous CGRP

may cause more microscopic inflammation (mucosal architecture, inflammatory cell infiltration and muscle changes) but this effect is not so great as to be able to change the macroscopic appearance of the inflamed colon.

Seven days after TNBS, $CGRP_{8-37}$ (1 μg/h, through pump) decreased only macroscopic damage score. A lower concentration of the antagonist had no effect. Interestingly, a higher concentration of $CGRP_{8-37}$ (5 times more) did not lower inflammatory indices. This result might be explained by the fact that $CGRP_{8-37}$ is a competitive antagonist which shares the 8-37 C-terminal of the CGRP molecule. Thus, there is the possibility for acting as a partial agonist at higher doses. In this experiment, a $CGRP_1$ receptor antagonist was used, whilst the data from the *in vitro* study show the existence of $CGRP_2$ receptor on the neurons and a novel CGRP receptor on the epithelium. Therefore, the answer to the net effect of CGRP in colonic inflammation depends on the recognition of the exact receptors involved. The development of specific antagonists or blockers for receptor subtypes other than $CGRP_1$ is required to explain this process.

Reinshagen et al, used a similar method as we did to investigate the neuropeptide responsible for the protective effect of primary afferent neurons. $CGRP_{8-37}$ (1 μg/h) was administrated by minipumps. CGRP antibody (ip) and NK_1 receptor antagonist (RP 67580) were used. Results suggests that NK_1 receptor blockade had no protective effect in the TNBS model of colitis in the rat (TNBS; 30 mg/kg) and macroscopic damage score, ulcer index and MPO activity were claimed to be significantly higher in $CGRP_{8-37}$ and CGRP antibody-treated rats (107). These results are in contrast to ours. We observed only an increased macroscopic damage score in animals treated with $CGRP_{8-37}$, but there

were no data suggesting a protective effect for CGRP in the TNBS model of inflammation. The differences may be related to the dose of TNBS used. In our study, TNBS, 150 mg/kg, was used, which was 5 times more than Reinshagen's study. The number of animals in our study was higher (8-17 per group in 7 day experiment) than Reinshagen's study (6 per group), since the SEMs of the groups were overlapping in some instances in Reinshagen's study. Microscopic appearance of the control tissues in our experiment was more inflamed than Reinshagen's study. Although, the exact cause of the difference between two experiment is not known,

Further experiments are needed to investigate the characteristics of the new receptors in colonic tissues of the rat. The development of specific antagonists for CGRP receptor subtypes are important for investigating the total effect of CGRP in TNBS and other models of intestinal inflammation.

CHAPTER 7

CONCLUSION

In conclusion, the results of the *in vitro* study that CGRP has a role in mediating the epithelial secretion and the altered permeability of the inflamed colonic preparations of the rat. The study revealed that CGRP causes chloride secretion in the rat colon by acting at a novel receptor located directly on the colonic epithelium. The secretory effect is well preserved in inflamed tissues in spite of the epithelium being hyporesponsive to other agonists. The mechanism underlying the preservation of the response to CGRP is not clear, and needs further investigation. This mechanism may represent an adaptive phenomenon in preserving the secretory capacity of the inflamed colon and may be due to the upregulation of CGRP receptors or to changes in the epithelium during inflammation, which make it more susceptible to the secretory effect of CGRP than carbachol. The mechanism of preservation of CGRP-induced secretory response of inflamed tissues seems an interesting subject for further investigation in the future.

Studying the mechanisms of CGRP-induced secretion revealed that the location and pathways underlying the novel CGRP receptor and the $CGRP_2$ receptor are clearly different. While the effect of the new receptor is not neuronally mediated, the $CGRP_2$-induced chloride secretion involves neuronal activation. Although, it is believed that CGRP receptors belong to the G-coupled-receptor family, it seems there are different pathways downstream that regulate receptor inactivation since the novel CGRP receptor but not $CGRP_2$ receptor was partially desensitized by repeated application of CGRP.

One of the objectives of this study was to assess the effect of CGRP in epithelial permeability of the inflamed colon. Inflamed tissues showed increased paracellular permeability in response to CGRP. This result is consistent with one other study indicating the modulatory effects of neuronal factors on the permeability of the sensitized

rats challenged with antigen. Taken together, these data suggest that CGRP may play an important role in modulating tight junction function and/or structure, in inflamed but not in normal, colonic epithelium. This study did not address the receptor subtypes and the mechanism involved in the CGRP-induced increased permeability of inflamed tissues and further experiments are clearly needed.

Although we showed that at least two CGRP receptors are involved in CGRP-induced chloride secretion in the stripped rat colon, more experiments are needed to assess the pathophysiological effect of CGRP. Does CGRP released from capsaicin-sensitive primary afferent neurons play a functional role? This question could be answered pharmacologically, once appropriate agonists and antagonists are discovered, or by the use of a specific potent CGRP neutralizing antibody. Nevertheless, full characterization and confirmation of the new receptor will require that it be cloned, an endeavor which was beyond the scope of this study.

The results of the *in vivo* study (Chapter 6) showed that CGRP is able to modulate the colonic damage in the TNBS model of colonic inflammation. Interpreting the *in vivo* study, one should consider the existence of different CGRP receptor subtypes in the gastrointestinal tract. The limitation of our study was the lack of appropriate antagonists of all CGRP receptor subtypes. However, neither CGRP nor the $CGRP_1$ receptor antagonist had any effect on colonic inflammation at 24 hours post-TNBS. This observation can be explained by the caustic effect of TNBS/ethanol on the mucosa or the involvement of other initiatory inflammatory mediator at this time point. At day 3, continuous administration of CGRP increased microscopic damage of inflamed tissues, suggesting the proinflammatory effect of CGRP in this model of colonic inflammation.

Consistent with our hypothesis, a $CGRP_1$ antagonist decreased the macroscopic damage of the inflamed colon at day 7 post-TNBS. In the *in vitro* experiment, the $CGRP_1$ receptor was not involved in the secretory response of neither normal nor inflamed colon to CGRP. Therefore, it is possible that different CGRP receptors are involved in regulating the effects of CGRP on the rat colon. The existence of distinct receptors might explain why $CGRP_{8-37}$ only decreased macroscopic damage but had no effect on neutrophil infiltration or microscopic damage of the inflamed colon.

These studies have provided new knowledge of the role of CGRP in the regulation of colonic mucosal function, and have implicated CGRP in the development of colonic inflammation. The exact role of CGRP in colonic inflammation needs more precise knowledge of the functional CGRP receptors. Further studies require the development of specific pharmacological tools to facilitate a proper assessment of this important enteric and secretory neurotransmitter.

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