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Interaction of the immune system and enteric nervous system in guinea pig distal colon: Mediators causing release of acetylcholine and chloride secretion during intestinal anaphylaxis

Javed, Najma Hassan, Ph.D.
The Ohio State University, 1992
INTERACTION OF THE IMMUNE SYSTEM AND ENTERIC NERVOUS SYSTEM IN GUINEA PIG DISTAL COLON

Mediators Causing Release of Acetylcholine and Chloride Secretion During Intestinal Anaphylaxis

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Doctor of Philosophy Degree in the Graduate School of the Ohio State University

By

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* * * * *

The Ohio State University

1992

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To my family, friends and teachers
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<td>Description</td>
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<tr>
<td>Ach</td>
<td>Acetylcholine chloride</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Centigrade</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegration per minute</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>³H</td>
<td>Tritium</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>Isc</td>
<td>Short-circuit current</td>
</tr>
<tr>
<td>mA</td>
<td>Milliampere</td>
</tr>
<tr>
<td>mCi</td>
<td>Milli Curie</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
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<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>mS</td>
<td>Milli Siemen</td>
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<td>Abbreviation</td>
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<td>-----------</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>PD</td>
<td>Potential difference</td>
</tr>
<tr>
<td>RMCP</td>
<td>Rat mast cell protease</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SK&amp;F</td>
<td>Smith Kline &amp; French</td>
</tr>
<tr>
<td>μA</td>
<td>Microampere</td>
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<td>V</td>
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CHAPTER I
INTRODUCTION

NEUROIMMUNE MODULATION OF INTESTINAL ION TRANSPORT

The functions of the gastrointestinal system are controlled by three divisions of the autonomic nervous system. The parasympathetic or the craniosacral and sympathetic or thoracolumbar divisions constitute the extrinsic innervation of the gut (Figure 1). The third division, the enteric nervous system, comprises the local neural circuitry within the gastrointestinal tract. Subdivisions of the autonomic nervous system are based on the location of their ganglia and site of connections with the central nervous system. Although enteric nervous system is referred to as the "little brain" or "mini brain" in the gut, it is an independent, integrative nervous system (35, 173). Its activity is modulated by efferent inputs from the central nervous system via parasympathetic and sympathetic divisions (Figure 1). The neural activity in the intestinal tract coordinates the activity of effector systems (musculature, mucosal epithelium, endocrine cells and blood vessels) throughout the intestine. It programs
FIGURE 1. Schematic diagram of the extrinsic efferent innervation of the small and large intestine. DMN = dorsal motor nucleus; CG = celiac ganglia; SMG = superior mesenteric ganglia; IMG = inferior mesenteric ganglia. (Adopted from Cooke, H.J., 1986).
the motility patterns, blood flow and absorption or secretion by the mucosa.

The activity of the enteric nervous system is also modulated by mediators from the endocrine system and immune system. The concept of communication between the enteric nervous system and the intestinal immune system is rather new. The enteric nervous system which has as many neurons as the spinal cord, and the enteric immune system, with an equal number of immune cells as the rest of the body, interact to regulate motility and intestinal ion transport (Figure 2). The intestinal immune system has a detection and signaling function, whereas the enteric nervous system performs the integrative response function (173).

Recent studies have suggested that mast cells in the intestine are in direct contact with intestinal nerves (42, 145). Their close association with the intestinal nerves suggest a bidirectional communication between the two systems (Figure 2). There is growing evidence for morphological association between other immunocytes with intrinsic neurons, lamina propria elements, smooth muscle cells and epithelium, which further modify neuro-immune interactions (24).

Involvement of mast cells in neuro-immune communication is evident in certain pathophysiological conditions where
FIGURE 2. Schematic diagram of enteric neuroimmune communication. Direction of flow of information (arrows) suggests communication between mast cells and enteric nervous system; two-way communication between enteric nervous system and central nervous system; and one way communication between central nervous system and intestinal mast cells. (Adopted from Wood, J.D., 1991).
mast cell activation or proliferation is associated with type I hypersensitivity reactions (1, 3, 125). In these conditions, enteric immune system sensitization can occur in response to certain food antigens or antigens derived from procaryotic or eucaryotic parasites. Uptake of antigen across the epithelium triggers the formation of homocytotropic antibodies and development of immunity. Binding of the antibodies to receptors on the surface of the mast cells, results in sensitization. Subsequent exposure to the specific antigens crosslinks the antibodies, causing mast cell activation and degranulation. This leads to release of preformed chemical mediators and synthesis of new mediators from the mast cells, which can cause impaired absorption of salt and water in the intestine. Of these mediators, histamine has recently been recognized as an important signal substance for enteric neuro-immune communication (162, 163, 164 173).

Gastrointestinal symptoms resulting from type I hypersensitivity reactions are manifested as nausea, vomiting, abdominal pain and diarrhea. The specific mechanisms that give rise to alteration in intestinal transport function during type I hypersensitivity reactions are not completely defined. However, the close association
of mast cells, enteric nerves and intestinal epithelial cells suggest that mast cell mediators can stimulate secretion by acting directly on epithelial cells or by releasing intermediary messengers from enteric nerves or other cell types (1, 2, 3, 23, 24, 26, 125, 136, 162, 173). Direct and indirect evidence suggests that mast cell mediators induce secretion by activating cholinergic or peptidergic neurons or both in the submucosal plexus (42, 79, 96, 145, 163, 164).

Mast cells act as local signal detectors of luminal antigens in conjunction with the enteric nervous system to initiate a rapid flushing of the intestine with the consequence of eliminating the antigen. The central nervous system plays a role in mediating mast cell degranulation via input to the enteric nervous system. McQueen et al. (102) reported release of rat mast cell protease II, a marker of mast cell degranulation, after an audiovisual cue was administered to Pavlovian-conditioned animals sensitized to Nippostrongylus brasiliensis. These results imply that psychological factors may be involved as well in mediating intestinal anaphylaxis.

The exploration of interactions between the enteric neurons and immune system should provide a better
understanding of many idiopathic disorders of gastrointestinal tract, as well as advance our knowledge of the role of mast cells in control of normal physiological states. This understanding will provide us with a sound basis for future development and selection of appropriate therapeutic measures for intestinal allergic disorders.

This chapter will briefly focus on the role of enteric nervous system and intestinal immune system in regulation of epithelial ion transport. Interaction of these two systems, and mechanisms that have contributed to our understanding of the pathophysiology of gastrointestinal food hypersensitivity will also be reviewed.

THE ENTERIC NERVOUS SYSTEM

Functional Significance

The enteric nervous system consists of the myenteric plexus and the submucous plexus (Figure 3 and 4). Myenteric ganglia lying between the longitudinal and circular muscle layers regulate motility while submucous ganglia embedded in the submucosa control the epithelium and blood vessels. Myenteric neurons may participate in regulation of epithelial function by virtue of synaptic connections with submucous neurons.
FIGURE 3. Diagramatic representation of the wall of the intestine illustrating the ganglionated plexuses as they are seen in whole mount of intestine. (Reproduced from Furness and Costa, 1987).
FIGURE 4. Diagramatic representation of enteric plexuses as they are seen in transverse section. (Reproduced from Furness and Costa, 1987).
Morphology and Structure of the Enteric Nervous System

The enteric nervous system consists of ganglia, primary interganglionic fiber tracts and secondary and tertiary fiber projections to the effector systems. The interganglionic fiber tracts consist of unmyelinated fibers about 2.5 $\mu$m in diameter. The ganglia and interconnected nerve fibers extend from the esophagus to the anal canal. Enteric ganglia are compact structures consisting of cell bodies of neurons, glial cells and nerve cell processes (63, 65) (Figure 4). Unlike other autonomic ganglia, but like central nervous system, enteric ganglia are devoid of blood vessels, connective tissue and collagen fibrils. It has been suggested that blood vessels do not enter the ganglia, and a blood-ganglionic barrier analogous to the blood-brain barrier exists (67). The basal lamina between the elements of the ganglia is scanty. They are nonencapsulated and are located between the muscle layers or in the submucosa (63).

In the guinea pig, neuronal cell bodies are between 13 and 50 $\mu$m, and glial cells are about 5 $\mu$m in diameter. The enteric nerve plexuses in the small intestine have many features in common with plexuses in other regions of the bowel (63, 107).
Ganglionated and Aganglionated Plexuses of Enteric Nervous System

The enteric nervous system consists of two interconnecting ganglionated plexuses, the myenteric (Auerbach's) plexus located between the longitudinal and circular muscle layers and the submucosal (Meissner's) plexus located in the connective tissue of the submucosa (Figure 3 and 4). Myenteric neurons appear to be involved primarily in regulation of motility patterns, whereas submucosal neurons influence blood flow and ion and water transport (36, 63, 65). These ganglionated plexuses are continuous around the circumference and length of gastrointestinal tract. Although both myenteric and submucosal plexuses are spatially separated, neural connections between the two make them function as integrative unit in regulating the specific programmed behavioral patterns of the intestinal effector systems.

Myenteric Plexus

The myenteric plexus consists of two dimensional array of ganglia and interganglionic fiber tracts situated in close apposition to the longitudinal muscle. The cell bodies of the neurons in the myenteric ganglia project to the circular muscle, to other myenteric ganglia, to
submucosal ganglia, or directly to the epithelium. Myenteric ganglia vary in size, shape and orientation, and vary in different species and different regions of the same species (63, 68, 171). The myenteric plexus is the larger of the two ganglionated plexuses. In guinea pig ileum, the neuronal cell bodies are in a single layer in the myenteric ganglia and number from 5-160 with an average of 43/ganglion (64).

Three components of the myenteric plexus have been described. The primary plexus is composed of ganglia and internodal strands. Fine nerve strands running parallel to the circular muscle bundles, which do not link the ganglia, comprise the secondary plexus. The tertiary plexus with fine nerve fibers occupy spaces between the primary plexus and ramify over the longitudinal muscle fibers.

Other smaller aganglionated plexuses related to muscular activity are:

Circular Muscle Plexus

These are small nerve bundles which connect the primary and secondary plexus with the deep muscular plexus in the small intestine (63).
**Deep Muscular Plexus**

This plexus was first discovered by Cajal in 1895 and 1911 and named as plexus muscularis profundus. It forms a continuous meshwork and run parallel to the circular muscle separating a thin layer of muscle cells from the bulk of the circular muscle (63). Deep muscular plexus does not exist in the guinea pig colon; instead there is a submucosal plexus near the inner surface of the circular muscle.

**Submucous Plexus**

The submucous plexus is a smaller ganglionated plexus compared to the myenteric plexus. It is most prominent in the small and large intestine. It is absent from the terminal rectum, sparse in the stomach and does not exist as a ganglionated plexus in the esophagus (63, 65). The submucosal ganglia are small and less numerous than myenteric ganglia. The number of neurons per ganglion averages eight, with a range of 1 to 30 (64, 169). The variability in size along the length of the intestine in the same species seems to be less significant than for myenteric ganglia (63, 64). In most species, the submucous plexus contains two interconnected sets of ganglia forming an inner and outer submucous plexus (107).
Submucosal nerve fibers from submucosal neurons project to the epithelium, blood vessels, endocrine cells, muscularis mucosa, other submucous ganglia and myenteric ganglia (51, 63, 90, 91). The submucosa and mucosa of the small and large intestine contain myenteric fibers, sympathetic postganglionic axons, extrinsic sensory and afferent fibers, vagal and pelvic fibers and perivascular nerve fibers.

The mucosa consists of the muscularis mucosae, the lamina propria, into which simple tubular glands protrude (the intestinal crypts, or glands of Leiberkuhn), and villi or surface epithelium. The mucosal plexus is the dense network of fibers that surround the secreting crypt cells and project into the the subepithelial space associated with the absorbing cells. Transmitters released from vesicles in axon varicosities diffuse through the extracellular space to reach receptors on the target cells (30, 34, 35, 36, 171).

**REFLEX CONTROL OF INTESTINAL ION TRANSPORT**

Both the small and large intestines play an important role in body fluid homeostasis. About 8-9 liters of fluid enters the duodenum daily, and more than 80-85% is absorbed by the small intestine. Approximately 1.5 to 2 liters of
water containing sodium, potassium, chloride, and bicarbonate are delivered to the colon daily, where more than 90% is absorbed and only 1-2% excreted. Under normal conditions, the intestine has a large absorptive capacity which is dependent on the transport properties of surface (absorptive) cells and crypt (secretory) cells (34, 35, 36, 37). Absorption can shift to secretion if there is a functional defect in the intestinal mucosa, or if the mucosa is exposed to bacterial toxins or injurious chemicals. Fluid movement into the lumen results from osmotic gradients generated by either inhibition of sodium and chloride absorption in the villus or surface cells or stimulation of chloride secretion by the crypt glands or both (35, 36, 37). Excessive loss of salt and water in the feces, will give rise to symptoms of diarrhea (18, 35, 36, 53, 54).

Tonic activity of submucous neurons continuously suppress the absorptive capacity of the intestine (20). The specific neural pathways involved in reflex regulation of ion transport are not clearly delineated. Regulation of basal rates of ion transport appears to involve vasoactive intestinal peptidergic (VIP) secretomotor neurons in some species (20), and cholinergic secretomotor neurons in others (52, 84, 101).
Enteric reflex pathways regulate functions of the gastrointestinal effector systems including smooth muscle, fluid and electrolyte transport, blood flow, hormonal secretion and others (30, 34, 35, 36). The three functional categories of neurons involved in this reflex circuitry are sensory neurons, interneurons and motor neurons (35, 63, 171) (Figure 5).

**Sensory Neurons**

These are nerve cells with specialized endings that sense chemical, thermal, osmotic or mechanical alterations of the luminal contents and gut wall (30, 34, 35, 36) (Figure 5). Sensory information is encoded as action potentials that propagate along nerve processes to cell bodies located in the myenteric and submucosal ganglia, and is sent to the prevertebral ganglia, the spinal cord and the central nervous system. Release of neurotransmitters from sensory neurons activates interneurons within the neural circuitry. Intrinsic sensory neurons have not been identified unequivocally by either morphological or physiological techniques. Bornstein and Furness (13) suggested that sensory neurons in the submucous plexus are multipolar cholinergic neurons that are immunoreactive for substance P and calbindin (13). Kirchgessner et al. (93)
FIGURE 5. Reflex pathway in control of epithelial ion transport. Sensory neurons detect changes in mechanical, chemical or thermal conditions. Interneurons process and integrate information. Motor neurons release transmitters at neuro-epithelial junctions. Substance P, (Sub P); vasoactive intestinal peptide, (VIP); acetylcholine, (ACh).
provided evidence that these neurons which project to myenteric ganglia may be activated by 5-hydroxytryptamine released from enterochromaffin cells in response to mucosal distortion or luminal cholera toxin exposure (93). This finding raises the question of whether enterochromaffin cells are "taste cells" that sample the luminal contents and initiate neural reflex activity when 5-hydroxytryptamine is released.

**Interneurons**

These neurons form information links between enteric neurons (Figure 5). They are connected by synapses into networks that process sensory information and activate the motor neurons. Because of their multiple connections, interneurons form "micro" circuits that organize reflex responses to sensory inputs.

**Motor Neurons**

Motor neurons are the final outputs of neuronal control circuits in the enteric nervous system (Figure 5). Activation of motor neurons can change the behavior of effector systems (musculature, secretory and absorptive epithelium, vasculature, immune cells and enteroendocrine cells).
Neurochemical characterization suggests that there are two classes of secretomotor neurons in the submucosal plexus which influence epithelial ion transport (Figure 6). These are, 1) cholinergic secretomotor neurons which contain a plethora of peptides and, 2) VIP neurons in most species (62, 63). Cholinergic neurons are subdivided based on the presence of specific peptides (62, 63, 83) which are substance P, cholecystokinin-octapeptide, calcitonin gene related peptide, galanin, somatostatin, and neuropeptide Y (62, 63, 83). Another group of neurons contain acetylcholine without any one of the above peptides. VIP neurons contain peptide histidine isoleucine, galanin and dynorphin and possibly others (63, 84) (Figure 6). Table 1 lists the established and putative neurotransmitters present within the enteric nervous system.

Electrical field stimulation of muscle stripped submucosa/mucosa segments set up in Ussing flux chambers increase chloride secretion or decrease sodium chloride absorption (30, 31, 32, 74, 75, 97, 98). The secretory response to electrical field stimulation is reflected by an increase in short-circuit current that is dependent on frequency and stimulus strength (31, 77, 78, 84). This response differs in magnitude in different regions of the gut. The electrical field stimulation responses can be
FIGURE 6. Model of innervation of the epithelium. At least two types of submucosal secretomotor neurons have been identified which influence epithelial function; 1) cholinergic neurons with number of peptides and 2) VIP containing neurons with other peptides. Other inputs are from myenteric neurons and sympathetic extrinsic nerve fibers. Choline acetyltransferase, the synthetic enzyme for acetylcholine, (ChAT); cholecystokinin, (CCK); calcitonin gene-related peptide, (CGRP); galanin, (GAL); neuropeptide Y, (NPY); somatostatin, (SOM); vasoactive intestinal peptide, (VIP); dynorphin, (DYN); substance P, (SUB P); norepinephrine, (NE). (Adopted from Cooke, H.J., 1989).
### TABLE 1

**ESTABLISHED AND PUTATIVE NEUROTTRANSMITTERS PRESENT WITHIN THE ENTERIC NERVOUS SYSTEM.**

<table>
<thead>
<tr>
<th>Amines</th>
<th>Aminoacids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>Histamine</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>5-Hydroxytryptamine</td>
<td>Pituitary adenylate cyclase activating peptide</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Endothelin-1</td>
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</tbody>
</table>

<table>
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<tr>
<th>Purines</th>
<th>Pituitary adenylate cyclase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine 5'-triphosphate</td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Neuropeptides</th>
<th>Pituitary adenylate cyclase activating peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasoactive intestinal peptide</td>
<td></td>
</tr>
<tr>
<td>Pituitary adenylate cyclase activating peptide</td>
<td></td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>Substance P</td>
</tr>
<tr>
<td>Enkephalin</td>
<td>Enkephalin</td>
</tr>
<tr>
<td>Dynorphin</td>
<td>Dynorphin</td>
</tr>
<tr>
<td>Calcitonin gene-related peptide</td>
<td></td>
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<tr>
<td>Somatostatin</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>Bombesin</td>
<td>Bombesin</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>Neurotensin</td>
</tr>
<tr>
<td>Neurokinin A</td>
<td>Neurokinin A</td>
</tr>
<tr>
<td>Cholecystokinin</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>Galanin</td>
<td>Galanin</td>
</tr>
<tr>
<td>Gastrin-releasing peptide</td>
<td></td>
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<tr>
<td>Angiotensin</td>
<td>Angiotensin</td>
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<tr>
<td>Adrenocorticotropic hormone</td>
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<tr>
<td>Motilin</td>
<td>Motilin</td>
</tr>
<tr>
<td>Peptide histidine-isoleucine</td>
<td></td>
</tr>
<tr>
<td>Thyrotropin-releasing hormone</td>
<td></td>
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<tr>
<td>Nitric oxide</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
</tr>
</tbody>
</table>
abolished by a neurotoxin, tetrodotoxin, which blocks neuronal sodium channels and action potential-dependent release of neurotransmitters. Carey et al. (17) observed that electrical field stimulation of aganglionated tissue did not increase short-circuit current responses in guinea pig ileum when compared to ganglionated preparations. This suggests that the stimulus parameters activated cell bodies and not fine nerve processes that were still present after aganglionosis (17).

NEUROTRANSMITTERS AT EPITHELIAL JUNCTION

The neurons exert their influence on epithelial cells by release of neurotransmitters from vesiculated varicosities and subsequent diffusion of messenger to receptors on mucosal effectors. The potential transmitters at neuroepithelial junctions responsible for mediating secretory or antiabsorptive effects are known to be acetylcholine, VIP and substance P. Figure 6 shows a working model of the epithelial innervation and transmitters released at neuroepithelial junction as well as at the ganglionic synapses.
Acetylcholine

Submucosal cholinergic neurons have been identified by the presence of the enzyme choline acetyltransferase (ChAT), which is necessary for the synthesis of acetylcholine (84). Acetylcholine is released at ganglionic synapses as well as neuroepithelial junctions during neural stimulation (Figure 7). Neural stimulation of submucosal/mucosal segments of guinea pig colon evoked release of acetylcholine simultaneously with an increase in short-circuit current (78). Acetylcholine release was frequency dependent and was attenuated by tetrodotoxin. The response characteristics of submucosal neurons differ from myenteric neurons (11, 66, 78, 82, 152, 179, 180). (Details in chapter 3).

Acetylcholine acts at nicotinic receptors on neurons, or at muscarinic receptors either on neurons or on epithelial cells (19, 72, 96, 147). The muscarinic receptor on epithelial cells appear to be the M₃ type (131). Activation of M₃ receptors causes electrogenic chloride secretion or inhibits sodium and chloride absorption. Epithelial muscarinic receptors appear to be coupled to metabolism of phosphoinositides and changes in intracellular calcium (27). This effect is mimicked by exogenous acetylcholine or muscarinic cholinergic agonists (19, 36,
FIGURE 7. Schematic illustration of a neurohumoral transmission at cholinergic nerve endings. Choline is taken up into the nerve terminal by a sodium (Na\textsuperscript{+})-dependent carrier transport and is combined with activated acetate (AcCoA), released by mitochondria, to form acetylcholine (ACh). This reaction is catalysed by choline acetyltransferase. Synthesized ACh is transported into storage vesicles by a carrier which utilizes effluxing protons (H\textsuperscript{+}) as source of energy. Adenosine triphosphate (ATP) and proteoglycan (PG) are also stored in the vesicles. Influx of ionic calcium (Ca\textsuperscript{2+}) through Ca\textsuperscript{2+} channels causes fusion of the vesicular and neuronal membranes and liberation of ACh into the synaptic cleft. ACh will interact with muscarinic and nicotinic receptors on the postjunctional tissue and can be hydrolysed by acetyl cholinesterase (AChE) to acetate and choline. (Reproduced from Basic and Clinical Pharmacology, 2nd ed. Lange, 1984.)
Acetylcholine release is inhibited by the muscarinic receptor antagonist atropine (19, 78, 96).

The release of acetylcholine from myenteric neurons is regulated by autoreceptors on presynaptic terminals. Activation of autoreceptors inhibits release of acetylcholine (86, 87, 88, 89). Evidence for this comes from studies where release of acetylcholine was enhanced by the presence of muscarinic antagonists (88). This effect was not observed in colonic submucous neurons of the guinea pig (127). (Details in chapter 3).

There are other neurotransmitters released at neuroepithelial junctions. The evidence that muscarinic blockade reduces, but does not abolish the neurally-evoked secretory response suggests this possibility.

**Vasoactive Intestinal Peptide (VIP)**

Approximately one half of the neurons whose cell bodies are found in the submucosal ganglia of guinea pig ileum contain VIP (63, 84). This is a candidate transmitter for the neurally-evoked secretory response. VIP is colocalized with peptide histamine isoleucine and neuropeptide Y in rat intestine (63). In the guinea pig VIP is stored in the same neurons as peptide histidine isoleucine/dynorphin/galanin (30). VIP is released both by chemical and electrical
stimulation (105). VIP binds to receptors on the basolateral surface of the intestinal epithelial cells (30). The binding of VIP with its receptor activates adenylate cyclase and increases intracellular CAMP which inhibits sodium and chloride absorption and increases chloride secretion (105).

**Substance P**

Substance P is a peptide with 11 amino acids which belongs to the family of tachykinin's along with neurokinin A and B. Substance P can be found in enteric neurons, extrinsic sensory nerves and endocrine cells. Nerve terminals with substance P like immunoreactivity are found in circular muscle, muscularis mucosae, enteric ganglia and in the lamina propria adjacent to villus and surface cells and surrounding crypt cells (63). Its role as a transmitter at neuroepithelial junctions has not been clearly defined due to lack of specific substance P antagonists. The presence of substance P like immunoreactivity in nerve terminals within the submucous ganglia and in proximity to epithelial cells suggests a role in the regulation of mucosal function. Hubel (76) ruled out substance P as a transmitter important in regulation of secretion based on desensitization experiments. Others however, have suggested
that neural stimulation releases substance P which acts on epithelial cells to stimulate secretion (41).

Substance P receptors are present on neurons, because the response to substance P is partially blocked by tetrodotoxin (76, 83, 127). Multiple sites of action of substance P have been proposed. In the distal colon of guinea pig substance P evokes chloride secretion by activating cholinergic and noncholinergic neurons and by releasing histamine from mast cells (100). Keast et al. (83) suggested that two different receptors are involved in the substance P response in the guinea pig ileum. Their studies precluded identification of these receptors because of lack of specific substance P antagonists. Reddix et al. (130) in their recent studies have suggested that substance P acts at neurokinin-1 (NK-1) receptors on non-cholinergic neurons and possibly on epithelial cells to stimulate secretion in guinea pig ileum independent of histamine or prostaglandins.

Other Peptides

Neuropeptide Y and somatostatin are two other peptides found in cholinergic neurons of the submucosal plexus of the guinea pig ileum (63). Both these peptides act directly on epithelial receptors to cause an increase in sodium chloride
absorption and a decrease in chloride secretion (40, 44). Other peptides present in submucosal neurons are calcitonin gene-related peptide, cholecystokinin and galanin. Their roles in regulation of ion transport need to be elucidated.

**TONIC ACTIVITY OF SUBMUCOSAL NEURONS**

Tonic activity of submucosal neurons plays an important role in regulation of ion and water transport under resting conditions. It limits the absorptive capacity of the intestinal epithelium and aids in maintaining luminal fluidity and lubrication. Tonic activity of submucosal neurons is observed in submucosa/mucosa segments of ileum set up in Ussing flux chambers (20). Evidence that resting transport rates are under the influence of neural input comes from studies in which neural blockade reduced the basal ionic current, suggestive of decrease in active ion transport (20, 74).

**INTERACTION BETWEEN MYENTERIC AND SUBMUCOSAL NEURONS**

Submucosal neurons receive input from the myenteric plexus. Studies with extrinsically denervated segments suggest that interconnections between myenteric and submucous ganglia may be important for coordination of
motility patterns with ion transport. Immunoreactive nerve fibers containing 5-hydroxytryptamine, substance P, calcitonin gene-related peptide, thyrotropin-releasing hormone, enkephalin, somatostatin and norepinephrine have been detected in submucosal ganglia (62, 63, 92) (Figure 6). Many of these nerve fibers are of myenteric origin with the exception of norepinephrine-containing fibers which are from sympathetic neurons. Receptors for these transmitters are present on submucous motor neurons or interneurons. All of these putative transmitters can cause secretory responses except enkephalins and norepinephrine which induce inhibitory responses (30, 34, 36, 37).

EXTRINSIC INFLUENCE ON ION TRANSPORT

Both sympathetic and parasympathetic nerves modulate the activity of submucosal neurons.

Sympathetic Input

Stimulation of sympathetic fibers (via splanchnic nerves) to the intestine enhances the absorption of salt and water even in the absence of changes in hemodynamics (59). Sympathectomy removes this inhibition and causes an increase in fluid movement (secretion) in the intestine as seen in rat small intestinal transplants (166). Norepinephrine
released from sympathetic nerve terminals inhibits tonic activity of submucous neurons. Norepinephrine acts at adrenergic alpha-2 receptors on presynaptic terminals to suppress release of neurotransmitter and at postsynaptic sites to decrease neuronal excitability (148, 172). Norepinephrine also acts at adrenergic alpha-2 receptors on epithelial cells to enhance sodium chloride absorption (148).

Sympathetic input to the intestine plays an important role in body fluid hemodynamics. A decrease in extracellular volume, induced by hemorrhage or low sodium concentrations, triggers cardiopulmonary volume receptors and carotid sinus baroreceptors to increase sympathetic outflow to the gut and conserve water and electrolytes. Other circulating hormones complementing this effect are angiotensin II, aldosterone and antidiuretic hormone.

**Parasympathetic Input**

Not much is known about parasympathetic influence on ion transport. It has been observed in cats that stimulation of the sacral nerves decreases ion and water absorption in the colon by an action of acetylcholine at muscarinic cholinergic receptors (174). Electrical or physiologic
stimulation of vagus nerve reduces ion and water absorption in the small intestine (34).

IMMUNOLOGICAL REGULATION OF INTESTINAL ION TRANSPORT

Intestinal Immune System

One of the major components of the mucosal immune system is the gut associated lymphoid tissue (GALT). Twenty five percent of intestinal mucosa is lymphoid tissue. GALT is divided into three components based on morphology and anatomy. They are: (1) loose connective tissue lamina propria lymphocytes and plasma cells; (2) intraepithelial lymphocytes; and (3) Peyer's patches or scattered lymphoid follicles (24, 81). Other elements of immune system are macrophages, mast cells and myeloid derived granulocytes.

The first line of defense against uptake of foreign antigens involves gastric acid secretion and proteolytic enzymes which degrade antigens and proteins. Normal motility of the gut minimizes the mucosal contact of potential antigenic substances (158, 159). The local immune system, together with the mechanical barrier formed by the intestinal epithelial lining and tight junctions, the glycocalyx, and mucous coat of the intestinal mucosa, is the next line of defense to protect the host from any foreign antigen invasion through the intestinal gut wall.
It is currently believed that immunoglobulins produced by lymphoid cells and secreted by epithelial cells play an important regulatory role in the intestinal uptake of macromolecules (24, 81). These immunoglobulins (IgA, IgG and IgM) can block the entry of toxic macromolecules and infectious agents through the intestinal barrier (24, 81). Studies have shown that deficiencies in secreted immunoglobulins lead to abnormally high levels of antigen absorption which in turn stimulates the immune system and is the basis for many allergic inflammatory diseases (22, 24 81, 157).

The barrier function of intestine is not 100% efficient, although it prevents significant uptake of large molecules. When this barrier is damaged, as occurs in celiac disease, inflammatory bowel disease (IBD) or viral gastroenteritis, an increase in uptake of macromolecules is observed (146, 158, 159). Uptake of macromolecules is also higher during the first three months of neonatal life due to gut immaturity (153, 158). Functional gut closure to intact antigens occurs with intestinal maturity and production of IgA (153). Small amounts of macromolecule uptake can occur in adults as well (153). Selective IgA deficiency, preterm delivery, intestinal helminth infection and type of
feeding during the neonatal period may also influence antigen uptake by the intestinal epithelium (132).

The most common routes of antigen uptake across the intestine occurs at three sites; 1) the M cells (specialized cells without microvilli and lysosomal organelles,) overlying Peyer's patches, 2) the epithelial cells (transcellular), and 3) intercellular space (tight junctions) (118, 157).

The antigenic substances that penetrate the intestinal mucosa have the potential to stimulate intestinal lymphocyte cell populations and to interact with mucosal mast cells. Antigens encountered by the enteric route can stimulate local intestinal immune responses in the absence of a systemic immune response. One of the immune cell types which plays an important role in local hypersensitivity reactions in the gut is the mast cell. First exposure of these cells in the lamina propria results in sensitization and formation of specific antibodies (IgE, IgG classes) which bind to receptors on the surface of the mast cells. Subsequent exposure to the same antigen crosslinks homocytotropic antibodies and causes degranulation and release of mediators of these cells. These events occurring locally can lead to alteration in intestinal fluid and electrolyte transport. The details of these mechanisms will
be explained later in this chapter.

There are very few studies done on immediate hypersensitivity reactions in humans. Most of the current information has been obtained from studies on animal models of hypersensitivity reactions. This section will review these studies with special emphasis on food hypersensitivity reactions in the gut.

CLASSIFICATION OF HYPERSENSITIVITY REACTIONS

Immune mediated local hypersensitivity reactions can be classified into four types, using Coombs Gell Classification. Most of the studies have implicated immediate type I hypersensitivity reactions altering ion transport in the gut, but types II, III and IV can also occur depending upon the specific cells involved. Because of their rapid onset, types I, II, and III are labeled as immediate hypersensitivity reactions.

Type I Hypersensitivity: The type I hypersensitivity reaction, also called anaphylaxis type I, is dependent on the interaction of antigen with anaphylactic antibody (IgE or IgG subclasses) and amine containing cells, such as mast cells and basophils. This hypersensitivity can manifest itself as systemic or local anaphylaxis.
**Type II Hypersensitivity:** Antibodies directed against cell surface antigens interact with the complement pathways and effector cells to initiate cellular damage. Complement-dependent cytotoxic reactions result in lysis of target cells. Cytotoxic reactions are those in which the antibody combines with an antigen present in the tissues, either in the cells or in extracellular structures. The antigen can be a natural constituent of the tissues or it can be artificially bound to the cell surface.

**Type III Hypersensitivity:** Type III hypersensitivity is the arthus reaction characterized by inflammation due to formation of antigen-antibody complexes that activate complement and attract neutrophils and other immune cells. In this reaction large amounts of antigen-antibody complexes are formed in the blood and localized in the arteriolar networks throughout the organism. This type of reaction is common in persons injected with animal sera for prophylaxis or treatment of certain infections generating a syndrome called "serum sickness". It has also been recognized as a cause of many immunopathological effects in infectious and autoimmune diseases.
**Type IV Hypersensitivity:** Type IV hypersensitivity reaction involves antigen-activated T-Cells that release lymphokines. This type is also referred to as delayed hypersensitivity. Delayed hypersensitivity reactions appear 12-24 hours (or even several days) after contact with the antigen and are transferable by sensitized T lymphocytes, but not by the serum.

**MODELS OF TYPE I HYPERSENSITIVITY REACTIONS EFFECTING INTESTINAL ION TRANSPORT**

Experimental and clinical evidence suggests that the gastrointestinal tract can undergo local hypersensitivity reactions when antigens are transported across the intestinal epithelium in quantities sufficient to stimulate the immune system. These local hypersensitivity reactions in the gut produce nausea, vomiting, abdominal pain and diarrhea. Animals were sensitized by feeding or intraperitoneal injection of egg protein (ovalbumin), milk protein (β-lactoglobulin), and antigens from nematodes *Trichinella spiralis* and *Nippostrongylus brasiliensis* (1, 3, 23, 24, 26, 41, 43, 70, 125, 135, 136). Examination of immune mediated responses has involved experiments in which tissues (jejunum, ileum and colon) from immunized mice, rats and guinea pigs were exposed to antigens. Transport changes
induced by antigens have been measured electro-
physiologically by short-circuit current measurements, or
by tracing the transepithelial unidirectional fluxes of
radioactive ions (25, 125, 126, 164). Exposure to the
specific antigen results in a decrease in sodium and
chloride absorption and/or an increase in chloride secretion
or both (1, 3, 23, 24, 26, 41, 43, 70, 125, 135, 136).

The presence of food or nematode antigens in the lamina
propria, results in stimulation of reaginic antibodies (IgE
or IgG, in guinea pigs) which bind to the receptors on the
surface of the mast cells. Subsequent exposure to the
similar antigen results in crosslinkage of the homocytotropic
antibodies and triggers degranulation of the mast cells with
release of performed mediators and newly synthesized
mediators which can alter fluid and electrolyte transport in
the gut (4, 7, 22, 24, 41, 109, 134, 138) (Figure 8). Mast
cell mediators released include biogenic amines, purines,
lysosomal enzymes, chemotactic factors, proteoglycans,
neutral proteases, acid hydrolases, arachidonic acid
metabolites, platelet activating factors and many others (4,
7, 22, 24, 41, 109, 134, 138). Studies have shown that
mast cell mediators responsible for altered ion transport
are histamine, prostaglandins, 5-hydroxytyptamine, platelet
FIGURE 8. Model illustrating possible sites of action of mast cell mediators during type I hypersensitivity reactions. Mediators released from mast cells can act on other immune cells (1), on neurons (2) or directly on epithelial cells (3).
activating factor or others (4, 7, 22, 24, 41, 109, 134, 138).

It is interesting to note that although these studies differed in animal species, tissues, types of antigens, type of antibody production, method of immunization and specific mediators, there were some common findings. All immunological reactions induced type I hypersensitivity, and a chloride secretory response.

The mechanisms involved in chloride secretion are quite complex. However, due to the close association of mast cells with other immune cells, neurons, and epithelial cells in the lamina propria and submucosa, several mechanisms have been proposed by which mast cell mediators can alter transport function (Fig 8): 1) mast cell mediators could interact with receptors on the epithelial cells to initiate chloride secretion; 2) mast cell products could act directly on enteric neurons causing release of transmitters which influence the transporting cells; or 3) mast cell mediators could cause the release of other intermediary messengers from endocrine cells or cells of the immune system which could effect epithelial or neuronal function. Studies have suggested that all of these mechanisms may be involved in small intestinal or colonic chloride secretory mechanisms during intestinal anaphylaxis (1, 4, 23, 24, 25, 42, 43,
NEURONAL INVOLVEMENT DURING INTESTINAL ANAPHYLAXIS

Role of Enteric Nervous System in Altered Ion Transport

Most of the studies involving antigenic stimulation (ovalbumin, β-lactoglobulin and Trichinella spiralis) of chloride secretion have implicated submucosal neurons in mediating responses during intestinal anaphylaxis. The evidence comes from the studies involving use of tetrodotoxin which significantly reduced the antigen-induced secretory response in immune animals (1, 3, 4, 24, 42, 79, 129, 126, 162, 164). There are some conflicting reports on the type of neurons mediating anaphylactic responses. Indirect evidence obtained by using muscarinic antagonists has implicated cholinergic neurons in mediating the response to challenge with β-lactoglobulin and Trichinella spiralis antigen in the rat jejunum and guinea pig colon (23, 164). Other studies have failed to observe effects of muscarinic antagonists in guinea pig jejunum or colon in response to Trichinella spiralis antigen or β-lactoglobulin (1, 3, 23, 42, 129, 134, 136).

Javed et al. (79), have provided direct evidence for cholinergic neuronal involvement in β-lactoglobulin sensitized guinea pigs. They evoked release of
acetylcholine with a simultaneous increase in short-circuit current when \( \beta \)-lactoglobulin was administered to sensitized colonic tissues. This chloride secretory response was atropine and tetrodotoxin sensitive implicating cholinergic neurons that transfer information via muscarinic synapses (79).

Since muscarinic receptors are present on both neurons and epithelial cells, the results cannot distinguish whether cholinergic neurons involved are interneurons within the ganglionic circuitry or whether they are secretomotor neurons to the epithelium.

Cholinergic neural pathways with nicotinic transmission were not involved in epithelial secretory response, during type I hypersensitivity reactions. The evidence for this comes from studies in which mecamylamine, a nicotinic blocker, failed to effect Trichinella spiralis antigen and \( \beta \)-lactoglobulin-induced chloride secretion in guinea pig distal colon (79, 164).

The antigen-evoked secretory response may be mediated, in part, by peptidergic neurons. Studies in which the inhibitory effects of atropine on the secretory response to antigen were less than that caused by tetrodotoxin suggest the involvement of peptidergic neurons (79, 164). Further evidence for involvement of peptidergic nerves in intestinal
anaphylaxis comes from studies in which atropine failed to reduce the antigen-evoked secretory response (134). The transmitter responsible for non-cholinergically-mediated secretion in type I hypersensitivity reactions is unknown. One possibility that must be considered is substance P. Substance P has been shown to cause a greater increase in chloride secretory response in immune animals than in nonimmune animals (164). It can stimulate chloride secretion by activating both cholinergic and peptidergic submucosal neurons that innervate the epithelium and by releasing histamine from mast cells (164).

Studies by Stead et al. (145) have demonstrated that neurons containing substance P/calcitonin gene related peptide are in intimate contact with mucosal mast cells in nematode-infected rat intestine (145). Other studies have reported evidence of secretory response in immune rats by activation of extrinsic afferent nerve fibers containing substance P/calcitonin-gene-related peptide (42).

It has been observed that electrical field stimulation of submucosal neurons, evokes a chloride secretory response in immune animals which is greater than that seen in non-immune animals. The intimate contact of mast cells with peptidergic nerve fibers and close association with other
neurons, suggests that mast cell mediators may interact with endogenously released neurotransmitters to enhance secretion.

These studies suggest that neuro-immune interactions involving mast cell mediators alter ion transport directly or indirectly by activating cholinergic neurons and possibly other neuronal types that stimulate chloride secretion.

**Role of Central Nervous System and Mast Cell Communication**

Intestinal mast cells function as local antigen detectors that signal the enteric nervous system to activate the mucosal effectors. The central nervous system can modulate immune function and mast cell degranulation as well as alter the activity of the enteric nervous system. Evidence for its affects on mast cells is supported by studies of McQueen et al. (102), who reported that mast cells could be degranulated in Pavlovian-conditioned animals (102). Rats were sensitized to ovalbumin by subcutaneous injection, and to the parasite nematode *Nippostrongylus brasiliensis* to induce mast cell hyperplasia. Conditioned animals were exposed to an audiovisual cue for 15 minutes, injected with the antigen and then exposed to the cue once again. Protease specific to mucosal mast cells (RMCP II) was measured as a marker for mast cell degranulation (102).
Basal short-circuit current and RMCP II was elevated as a result of release of mast cell mediators in response only to the cue in conditioned rats (102). These studies suggest that psychological factors may be important determinants of type I hypersensitivity reactions.

Mast cells and specific mast cell mediators involved in these studies will be discussed briefly.

**Mast Cells and Role of Their Mediators in Intestinal Ion Transport**

The name "mast" is from a German word "masten," meaning "stuff" or "fatten" and was so named by Ehrlich in 1877 (49), who thought that granules were formed by uptake of extracellular material (49).

The physiologic role of mast cells is not well understood. However, its pathophysiologic role as an effector cell in immediate hypersensitivity is well established. Mast cells together with immunoglobulin molecules (IgE and IgG) are two important components of sensitization to antigens in type I hypersensitivity reactions (7, 24, 109, 135). Involvement of mucosal mast cells in antigen-induced changes in ion transport has been suggested by the, 1) specific release of mast cell-derived
amines, 2) application of mast cell mediators to tissues, which mimic the antigen-induced epithelial responses, 3) inhibition of antigen-induced responses by mast cell mediator antagonists, 4) preventing antigen-induced mast cell degranulation by stabilizing agents, 5) absence of antigen-induced changes in mast cell-depleted hosts.

Mast cells occur throughout the body, with special prevalence at sites which come into contact with the external environment such as the skin, lungs and gastrointestinal tract. The location of gastrointestinal mast cells is ideally suited to interact with antigens presented via an oral route.

Studies have suggested that there is marked variation in mast cell morphology, cytochemical and functional properties (5, 7). The best example of this heterogeneity is seen in gastrointestinal tract mast cells. In order to understand gastrointestinal diseases in which mast cell activation plays an important role, it is important to focus on some differences.

The origin of the mast cell in humans is not clear, but in rats, they originate from hemopoietic stem cells in the bone marrow. Although mast cells function like basophils, they differ with regard to size, stem cell of origin, nuclear and granular morphology, and cell surface
characteristics (5, 7, 24).

Electron microscopy reveals mast cell metachromatic granules which can vary in size and shape (Figure 9). Also seen are other subcellular structures with few mitochondria. Mammalian mast cells are generally ovoid or irregularly elongated in tissue and contain dense cytoplasmic granules which fills the cytoplasm.

Mast cells are present in all regions of the gastrointestinal tract. They decreases in number from the esophagus to the rectum. The duodenum contains 20,000 mast cells/mm³ as compared to skin which has 7000 mast cells /mm³ (116). They are distributed in the mucosa, lamina propria, submucosa, muscularis and serosa. Depending on staining characteristics and granular contents, mast cells in the rat are of two distinct types (Table 2). Connective tissue mast cells are more prevalent in outer layers of the intestinal tract and mucosal mast cells are present in the submucosa and lamina propria (5, 7, 50). The connective tissue mast cells are thymus independent and contain predominately heparin and the enzyme rat mast cell protease I (RMCP I). Mucosal mast cells are thymus dependent, found in the lamina propria and lung, characterized by predominance of chondroitin sulphate, and the enzyme RMCP II which is not
TABLE 2

PROPERTIES OF MUCOSAL AND CONNECTIVE TISSUE MAST CELLS
(RAT GASTROINTESTINAL TRACT)

<table>
<thead>
<tr>
<th>Mucosal Mast Cells</th>
<th>Connective Tissue Mast Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small variable shape, sparsely granulated</td>
<td>Large, uniform, densely granulated</td>
</tr>
<tr>
<td>Soluble granular proteoglycan matrix</td>
<td>Less soluble proteoglycan matrix</td>
</tr>
<tr>
<td>Chondroitin sulphate</td>
<td>Heparin</td>
</tr>
<tr>
<td>Low content of histamine and 5-hydroxytryptamine</td>
<td>High monoamine content</td>
</tr>
<tr>
<td>Proliferative, non-secretory polyamine response</td>
<td>Secretory polyamine response</td>
</tr>
<tr>
<td>Short life span</td>
<td>Long life span</td>
</tr>
<tr>
<td>Proliferative response to nematode infections</td>
<td>No proliferative response</td>
</tr>
<tr>
<td>IgE in cytosol</td>
<td>Non IgE in cytosol</td>
</tr>
<tr>
<td>Berberine negative</td>
<td>Berberine positive</td>
</tr>
<tr>
<td>Do not counterstain with safranin</td>
<td>Counterstain with safranin</td>
</tr>
<tr>
<td>Contain rat mast cell protease II</td>
<td>Contain rat mast cell protease I</td>
</tr>
<tr>
<td>Cromoglycate insensitive</td>
<td>Cromoglycate sensitive</td>
</tr>
</tbody>
</table>

found in other cells of the body (5, 9, 50). They react differently to pharmacological agents (mast cell stabilizing agents) and have different staining properties (50, 121).

Type I hypersensitivity reactions appear to be a common response to certain food antigens found in cow's milk, soy milk, shellfish and others or to parasitic infections (Trichinella spiralis or Nippostrongylus brasiliensis). Sensitization and subsequent exposure to these antigens can cause mast cell activation and release of chemical mediators as explained earlier. Most of these mediators are stored in the granules and are released on activation. Mast cells are also capable of synthesizing other mediators from membrane phospholipids by the action of phospholipases. The preformed and newly synthesized mast cell mediators are listed in Table 3. The mediators are usually categorized on the basis of their solubility (116).

Most of the studies mentioned earlier have implicated mast cell mediators in antigen-induced chloride secretion. The final epithelial response is a result of complex interaction of many mediators either directly on the epithelium or as a result of releasing intermediary messengers from the enteric nervous system or by other immune cell types. Some of the mast cell mediators that transduce these antigenic signals into epithelial responses
| TABLE 3 |
| MAST CELL MEDIATORS |

<table>
<thead>
<tr>
<th>Preformed mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Readily eluted following activation</td>
</tr>
<tr>
<td>Histamine</td>
</tr>
<tr>
<td>5-Hydroxytryptamine&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chemotactic factors for neutrophils &amp; eosinophils</td>
</tr>
<tr>
<td>Activators of kinin, complement, and clotting system</td>
</tr>
<tr>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>Prostaglandin-generating factor of anaphylaxis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Granule-associated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteoglycans: heparin, chondroitin sulfates</td>
</tr>
<tr>
<td>Proteolytic enzymes: tryptase, serine proteases</td>
</tr>
<tr>
<td>Inflammatory factor of anaphylaxis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mediators generated on activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandins</td>
</tr>
<tr>
<td>Hydroperoxyeicosatetraenoic acids (HPETEs)</td>
</tr>
<tr>
<td>Hydroxyeicosatetraenoic acids (HETEs)</td>
</tr>
<tr>
<td>Leukotrienes</td>
</tr>
<tr>
<td>Platelet-activating factor</td>
</tr>
</tbody>
</table>

<sup>a</sup> Demonstrated in rat mast cells.

include histamine, prostaglandins, serotonin, leukotrienes, platelet activating factor and many others (4, 7, 22, 24, 41, 109, 134, 138).

Studies have shown that endogenous histamine is one of the key mast cell mediators in altered gut function (1, 3, 23, 24, 43, 129). Histamine has been reported to stimulate ion and water secretion (1, 3, 23, 24, 43, 129). Studies by Baum et al. (8) have reported watery diarrhea associated with mast cell hyperplasia and microscopic colitis. Histamine was implicated as a mediator of altered fluid and electrolyte transport, because histamine H₁ receptor blockers abolished diarrhea (8).

Histamine also causes smooth muscle contraction and increases gastric acid secretion, gastric mucosal blood flow and mucus secretion, vasodilation and intestinal permeability (7). It can also act as chemotactic factor for eosinophils and neutrophils in late phase allergic response (7).

Mucosal mast cells are the main source of histamine in the gastrointestinal tract. Histamine is also present in enteroendocrine cells and enteric neurons in some species (119). Rat mucosal mast cell contains about 0.1 - 1 pg of histamine per cell (109). Histamine is formed by decarboxylation of L-histidine and degradation by
deamination with diamine oxidase or by methylation via histamine-N-methyl transferase. The biological responses of histamine are mediated by cell surface receptors designated as H₁, H₂, H₃ receptors (24, 33, 57, 139). Figure 10 explains the location of receptor subtypes mediating action of immunologically released histamine.

The role of histamine in anaphylactic responses is supported by the observations that histamine antagonists significantly reduced the secretory response to antigens. In these studies histamine acted at H₁ receptors on the epithelial cells to alter chloride secretion in the small and large intestine (1, 42, 43, 79, 126).

Histamine has been shown to alter chloride secretion by activating H₂ receptors on postsynaptic neurons and H₃ receptors on presynaptic axons (Fig 10) (79, 162, 163). Studies on normal guinea pig colon have established the H₁ effect as a transient increase in chloride secretion and H₂ effect as cyclical chloride secretion (162, 163). The release of acetylcholine evoked by β-lactoglobulin administered to sensitized colonic tissues was reduced by the H₂ antagonist cimetadine. There was a parallel decrease in short-circuit current as well. This suggested that endogenous histamine was one of the mediators of the
FIGURE 10. Location of receptor subtypes mediating the action of immunologically released histamine. Histamine acts at $H_1$ receptors on epithelial cells, and postsynaptic $H_2$ sites or presynaptic $H_3$ sites on submucosal neurons to influence chloride secretion.
anaphylactic response. Addition of exogenous histamine evoked release of acetylcholine in a concentration-dependent manner. These studies provide definitive evidence for involvement of histamine receptors in cholinergically mediated chloride secretion (79).

The presynaptic H₃ receptors are thought to be inhibitory receptors which regulate release of neurotransmitters. Evidence for this comes from studies in which the secretory response to exogenous histamine was smaller than the response to dimaprit, a selective H₂ agonist (79, 162, 163). Electrophysiological studies confirm that presynaptic H₃ receptors suppress the release of acetylcholine at nicotinic synapses (58). Histamine plays an important role in both physiological and allergic conditions in regulating intestinal transport function. The ability of histamine to evoke both transient and prolonged changes in intestinal chloride secretion (79, 162, 163, 164) suggest an important mechanism for host defense reactions. Osmotic driving forces cause fluid accumulation in the lumen. This may be important for altering the microenvironment to prevent establishment of parasites or to prevent the uptake of antigens by the gut wall. Both secretory and motor responses that result during type I hypersensitivity reactions may be important in flushing the lumen of its
It is well known that histamine may release intermediary messengers such as prostaglandins or neurotransmitters that regulate chloride secretion (1, 3, 23, 24, 43, 129). Prostaglandins are also synthesized and released from degranulating mast cells. In these studies piroxicam, a specific cyclooxygenase inhibitor, alone or in combination with cimetidine and pyrilamine attenuated the response to β-lactoglobulin in guinea pig distal colon (79, 163). (Details in chapter 4).

More direct evidence for a role for prostaglandins and histamine was seen when exogenous administration of these compounds evoked release of acetylcholine in parallel with an increase in short-circuit current (79). Anaplylactic responses to *Trichinella spiralis* antigen also have been associated with both histamine and prosaglandin release (23, 24, 136).

In addition to histamine and prostaglandins, 5-hydroxytryptamine has been implicated as one of the mediators for antigen-induced ion transport. Evidence for this comes from studies showing a significant reduction in short-circuit current in ovalbumin-challenged rats when cinancerin, 5-HT$_2$-receptor antagonist was administered (25).
Additional studies in sensitized animals exposed to parasitic antigens have demonstrated inhibition of secretion with 5-hydroxytryptamine receptor antagonist (23). Although these studies imply that 5-HT$_2$ receptors mediate in part, the secretory response to antigen in sensitized rats this may not be the case in other species. In $\beta$-lactoglobulin sensitized guinea pigs the ileal and colonic short-circuit current responses were reduced by ICS 205-930, a 5-HT$_3$ antagonist (1, 43). These observations are in conflict with study by Javed et al. (79) who reported that the 5-HT$_3$ antagonist ICS-205-930 failed to reduce the short-circuit current response to $\beta$-lactoglobulin in sensitized guinea pigs (79). The reasons for these differences are unclear.

Recent studies in models of gut anaphylaxis have implicated leukotrienes and platelet activating factor as well (126). Although various agents become active during local anaphylaxis, it is not clear, from which cells all mediators are derived. Some of them originate from mast cells. Other agents like leukotrienes and prostaglandins may be synthesized de novo by various cells under the influence of mast cell-derived factors (24).

Studies in non-immune animals have suggested a role for leukotrienes as mediators of chloride secretion in the colon. (Details in chapter 5). It is obvious from the
results of these studies that stimulation of chloride secretion by the intestinal epithelium during intestinal anaphylaxis is due to a combination of effects of mast cell mediators, and neurotransmitters, and other intermediary messengers.

**Intracellular Messengers in Epithelial Response**

Chloride hypersecretion due to bacterial and chemical agents is known to involve cyclic adenosine monophosphate (cAMP) as an intracellular messenger (43, 48, 74, 95). In hypersensitivity reactions where more than one mediator is involved, synergistic interactions are expected to occur. Histamine stimulates ion secretion by acting at H₁ receptors on epithelial cells as well as by increasing prostaglandin formation in rat and guinea pig colon (69, 176). Histamine and prostaglandins are reported to stimulate chloride secretion by calcium mediated and cAMP dependent mechanisms, respectively. Therefore the large secretory response during anaphylaxis may result from potentiating interactions of different intracellular transduction pathways (24, 136, 163, 164). Consistant with this possibility were the findings of elevated inositol triphosphate and cAMP in guinea pigs sensitized to *Trichinella spiralis* antigen (47, 134, 161).
In the rat jejunum the role of cAMP-signal transduction pathways in mediating secretion during anaphylaxis is unclear. Evidence for this comes from the findings that the mucosal increase in cAMP after ovalbumin challenge was transient and preceeded chloride secretion (125).

The alterations in ion transport during exposure to food antigens and parasites in different species is a complex mechanism involving many mediators and neurotransmitters. Therefore it is possible that secretory responses may involve interaction of more than one intraepithelial messenger system as well.

Mechanisms of Mast Cell Activation

Mast cells can be activated by number of immunologic and non-immunologic substances (Table 4). The immunologic signal for mast cell activation is the crosslinkage of high affinity receptors on the surface. Figure 11 shows a diagramatic representation of an immunologic mechanism of mast cell activation. The types of antibodies vary in different species. Antibody types can be measured and distinguished by passive cutaneous anaphylaxis (PCA) test (165). Antigen response can be passively transfered in
TABLE 4

SUBSTANCES CAUSING ACTIVATION OF MAST CELLS IN THE RAT

<table>
<thead>
<tr>
<th>Stimuli acting via the IgE receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
</tr>
<tr>
<td>Concanavalin A</td>
</tr>
<tr>
<td>Anti-IgE antibodies</td>
</tr>
<tr>
<td>Anti-IgE receptor antibodies</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other potential physiological stimuli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement anaphylatoxins</td>
</tr>
<tr>
<td>C3a, C4a, C5a</td>
</tr>
<tr>
<td>Neuropeptides</td>
</tr>
<tr>
<td>Substance P, gastrin, neurotensin, somatostatin</td>
</tr>
<tr>
<td>Cytokines</td>
</tr>
<tr>
<td>From neutrophils, lymphocytes, monocytes</td>
</tr>
<tr>
<td>ATP</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stimuli activating via putative receptors in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 48/80</td>
</tr>
<tr>
<td>Peptide 401 (MCDP from bee venom)</td>
</tr>
<tr>
<td>Polylysine</td>
</tr>
<tr>
<td>Dextran</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nonreceptor-mediated stimuli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium ionophores</td>
</tr>
<tr>
<td>Drugs</td>
</tr>
<tr>
<td>Contrast media</td>
</tr>
</tbody>
</table>

FIGURE 11. Diagrammatic representation of mechanism of mast cell activation and degranulation. After a second exposure to an antigen, antibody (IgE or IgG subclass) molecules bound to surface receptors are cross-linked by the antigen. This activates adenylate cyclase and results in the phosphorylation of certain proteins. At the same time, calcium (Ca$^{2+}$) enters the cell. These events lead to exocytosis of mast cell granules and release of stored mediators. In addition, phospholipases act on membrane phospholipids to synthesize new mediators e.g leukotrienes, prostaglandins and others. (Reproduced from Basic Histology, 5th ed., 1986).
guinea pigs and rats by serum transfer from β-lactoglobulin, *Trichinella spiralis* or ovalbumin sensitized animals (4, 43, 134). The homocytotropic antibodies involved are IgG₁ in guinea pig and IgE and IgG₂a in rats (24).

Cross linkage of the antibodies occupying receptors leads to calcium-influx and activation of cellular enzymes such as adenylate cyclase and methyl transferase. Calcium influx is facilitated by an increase in membrane phospholipid methylation. An increase in intracellular calcium leads to exocytosis (120). Calcium ionophores can directly allow calcium influx without receptor bridging. Some secretagogues, ATP and compound 48/80, can cause mast cell activation via their specific receptors on the surface of the mast cell (7).

An elevation of intracellular calcium, triggers granule movement to the cell surface. Fusion of granule membranes with the plasma membrane results in secretion into the extracellular space (Fig 11). Release of mediators depends on the kind of mediator and its solubility. *De novo* synthesis of new mediators can occur by mast cell activation.

**Inhibitors of Mast Cell Activation**

Mast cell activation and degranulation associated with release of mediators has been seen in many gastrointestinal
disorders. In order to identify potential therapeutic agents which can prevent mast cell degranulation and deleterious effects of mediators studies have attempted to find agents which can stabilize mast cell membranes.

Since mast cells are heterogenous, they respond differently to different pharmacological agents depending upon their site of location. Mucosal mast cells differ from connective tissue or peritoneal mast cells in their response (121). Drugs inhibiting peritoneal mast cells include \( \beta \)-adrenergic agents, methylxanthines, and sodium cromoglycate.

The use of cromoglycate in disorders of gastrointestinal mast cells have yielded both positive and negative results (154). This drug inhibits mast cell degranulation by mechanisms involving protein kinase C, cAMP or cAMP phosphodiesterases (29, 154). The flavinoid quercetin, which is structurally related to disodium cromoglycate, is a potent inhibitor of histamine release from mucosal mast cells (122, 164). Doxantrazole, an antiallergic drug, inhibits mediator release from rat mucosal mast cells but poorly from human mucosal mast cells (121). Other agents which have been useful and have been studied \textit{in vitro} are, \( H_1 \) and \( H_2 \) receptor antagonists,
calcium antagonists and corticosteroids (29).

These studies suggest the importance of understanding the biochemistry of mast cells for developing more specific therapeutic agents necessary to alleviate allergic response.

**Mast Cell Hyperplasia**

An increase in mast cell number has been observed in many gastrointestinal diseases in humans, like Crohn's disease, ulcerative colitis, and celiac disease (144, 146). Mast cell hyperplasia can also occur in response to parasitic infections in mice, rats and monkeys (6, 128, 133).

It is not well known how mast cell number is regulated in vivo. The information available suggests that mast cell proliferation may be dependent on integrity of the T-cell system (thymus derived T-lymphocytes) of the host. The evidence for this is the absence of mastocytosis in nude mice which are mast cell deficient and in T-cell-depleted rats (104, 133). T-cell-derived soluble factor is thought to be an important factor for in-vivo proliferation.

Both mucosal mast cell hyperplasia and factors controlling this process are best studied in parasitized rodents infected with the nematode *Nippostrongylus brasiliensis* (24, 128). Studies have shown that mucosal
Mast cell hyperplasia and activation plays an important role in host response to parasite rejection (24, 128, 164).

Infection with the enteric nematode, *Trichinella spiralis*, results in an immune response which triggers expulsion of the parasite from the intestine 14–21 days after the initial infection. When immunized animals are challenged with a secondary infection there is a rapid expulsion of the infected larvae within 15 minutes of the challenge. This response is initiated by an anaphylactic reaction involving mast cells, and results in altered motility and ion transport function necessary to flush the intestinal lumen (7, 22, 24, 129, 134, 135, 136).

There is indirect evidence that mast cell activation can indeed lead to parasite destruction and expulsion (85, 134, 136). Mast cells may be able to damage parasites indirectly by recruiting other inflammatory cells via chemotactic agents in that area (85, 110). Mast cell mediators, besides stimulating chloride secretion, can release goblet cell mucus and prevent worm attachment (110).

Some of the clinical disorders associated with mast cell number are given in table 5.
TABLE 5

CLINICAL DISORDERS ASSOCIATED WITH MAST CELLS

<table>
<thead>
<tr>
<th>I. Disorders of Mast Cell Proliferation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Localized mastocytosis</td>
<td></td>
</tr>
<tr>
<td>1. Cutaneous (Urticaria pigmentosa)</td>
<td></td>
</tr>
<tr>
<td>2. Solitary mastocytomas</td>
<td></td>
</tr>
<tr>
<td>B. Systemic mastocytosis</td>
<td></td>
</tr>
<tr>
<td>1. Mast cell infiltration of multiple organs</td>
<td></td>
</tr>
<tr>
<td>2. Mast cell leukemia (rare)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. Syndromes of Systemic Mast Cell Activation without increased number</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Food allergies</td>
<td></td>
</tr>
<tr>
<td>B. Exercise-induced anaphylaxis</td>
<td></td>
</tr>
</tbody>
</table>

FOOD HYPERSENSITIVITY

Food antigens are the most common antigens encountered by the gastrointestinal tract. It is estimated that the human gastrointestinal tract processes approximately 100 tons of food during a lifetime. About 45% of the population reports adverse reactions to food (10, 14, 39). It is estimated that immune-mediated food allergy affects at least 2-5% of the general population and up to 27% of children (10, 14, 16, 39).

Intestinal absorption of intact food antigens in macromolecular form was first suggested by Wilson and Walzer in 1935 (170). The intestinal uptake of food antigens is a necessary initial step in the development of a wide range of allergic diseases. There is ample evidence that in most animals and humans the intestinal uptake of macromolecules is maximal during the neonatal period and decreases thereafter, presumably because of the maturation of the intestinal mucosal barrier (106, 130, 167).

Gastrointestinal food allergies may be defined as clinical syndromes which are characterized by the onset of gastrointestinal symptoms following food ingestion. The underlying mechanism is an immunologically-mediated reaction within the gastrointestinal tract. The gastrointestinal symptoms associated with food antigens principally include
vomiting, diarrhea, abdominal colic and symptoms outside the alimentary tract (41, 42, 130, 155, 160). Food sensitive enteropathy may be seen as a sequel to acute gastroenteritis and may be found in association with immunodeficiency (24, 81). Several reports have clearly established the existence of food-sensitive colitis (108, 160).

Among food products, bovine milk constitutes a major source of dietary proteins for humans. Milk proteins consist of two major fractions: (1) caseins, which are enzymes, and (2) whey which contains \( \beta \)-lactoglobulin which is foreign to human and guinea pig milk (1, 39). \( \beta \)-lactoglobulin in the intestinal lumen is taken up into the cells by a transcellular pathway where 91-94% is degraded (103). Approximately 6-9% escapes cellular degradation and is probably transported intact by exocytosis across the basolateral membrane into the interstitial space (103). This intact form of \( \beta \)-lactoglobulin has been found to be the main factor responsible for milk protein immune reactivity and intolerance (95, 103). The exposure of the immune system to these intact forms of \( \beta \)-lactoglobulin results in formation of antibodies to the protein and development of immunity. When sensitized animals are challenged with \( \beta \)-
lactoglobulin, the antigens cross link homocytotropic antibodies on mast cells, and this results in degranulation and release of preformed mediators and synthesis of newly formed mediators which can then cause immediate type I hypersensitivity reactions.

The animal model sensitized to β-lactoglobulin typically involves an immediate type I hypersensitivity reaction common to all food allergens. In vitro challenge of intestinal tissues from immune mice, rats and guinea pigs with β-lactoglobulin, ovalbumin or the antigens from nematode parasites, results in characteristic chloride secretory response.

Despite innumerable observations it is still not possible to define specific neural pathways involved in altered ion transport during anaphylactic reactions in the intestine due to food antigens. More studies are required to understand the interactions of inflammatory mast cell mediators with intestinal neuronal circuitry. These studies will provide a sound basis for future development of therapeutic drugs for prevention of the underlying symptoms of colic and diarrhea during intestinal anaphylaxis.
STATEMENT OF THE PROBLEM

Several studies have provided indirect evidence for involvement of the enteric nervous system in immune regulation of epithelial ion transport. Many of these investigations have implicated submucous neurons in mediating secretion in immune tissues after antigenic challenge. These studies involved tissues from mice, rats, and guinea pigs sensitized to ovalbumin, milk protein (β-lactoglobulin), and Trichinella spiralis (1, 3, 24, 41, 42, 136). The evidence for this comes primarily from studies that demonstrate a reduction in antigen evoked secretion by the neurotoxin, tetrodotoxin (24, 41, 42). There are conflicting reports on the type of neurons involved. Indirect evidence obtained by using the muscarinic antagonist atropine implicated cholinergic neurons as mediators of the response to challenge with Trichinella spiralis antigen in the rat jejunum and guinea pig colon (9, 20). Other studies have failed to observe the effects of muscarinic antagonists in the guinea pig jejunum or colon in response to either Trichinella spiralis antigen or β-lactoglobulin (1, 3, 24, 41, 42, 134, 136).

The aim of this study was to determine whether cholinergic neurons in the submucous plexus mediated chloride secretion in colonic tissues from guinea pigs
sensitized to β-lactoglobulin. Since most of the earlier studies have indirectly implicated cholinergic neurons in immune and non-immune animals, as a first step to provide more direct evidence for the involvement of these neurons, a technique was developed to demonstrate an increase in acetylcholine release simultaneously with an increase in short-circuit current in response to electrical field stimulation, antigenic stimulation and in response to other secretagogues.

There is an abundance of studies on release of ACh from myenteric neurons (11, 82, 86, 152, 179), but little is known about the response characteristics of submucous neurons, particularly in colon (66, 175, 180). Therefore an additional aim of this investigation was to characterize ACh release from submucous neurons in the guinea pig distal colon.

In order to elucidate the regulatory function of the immune and neuronal systems, two major approaches have been used. First, antigens have been administered to sensitized tissues and in vitro changes in tissue function have been observed. Once antigen has evoked an epithelial response, pharmacological antagonists have been used to determine the immunological mediators or paracrine and neural factors
involved in transducing the antigenic signal into a functional change. The second approach has been, to apply known or putative chemical mediators of inflammation to gastrointestinal tissue and to evaluate their effects on function.

The specific mechanisms by which mast cell products can alter ion transport during type I hypersensitivity reactions are not completely understood. This study will focus on the role of the mast cell mediators, histamine, prostaglandins and leukotrienes on secretion during intestinal anaphylaxis. It will examine the interaction of the mucosal immune system with the enteric nervous system. It will examine the role of cholinergic neurons in anaphylactically mediated chloride secretion in an animal model of milk hypersensitivity.

This study was designed to answer four testable hypotheses:

**Hypothesis I:** That $\beta$-lactoglobulin stimulates acetylcholine release from submucosal neurons leading to increase in chloride secretion in distal colon of guinea pigs sensitized to cow's milk.

**Hypothesis II:** That endogenous histamine activates cholinergic neurons to influence colonic chloride secretion.
Hypothesis III: That endogenous prostaglandins activate cholinergic neurons during intestinal anaphylaxis.

Hypothesis IV: That leukotrienes modulate or alter colonic electrolyte transport by activating cholinergic neurons during intestinal anaphylaxis.

Development of a new technique will enable us to demonstrate the increase in acetylcholine release simultaneously with an increase in short-circuit current, which is crucial evidence for direct involvement of cholinergic neurons. The characterization of ACh will enable us to understand the response characteristics of submucous neurons as compared to myenteric neurons in the colon and other regions of the intestine.

This study will be the first to demonstrate acetylcholine release from submucous neurons in response to antigen challenge with β-lactoglobulin in guinea pigs sensitized to bovine milk. The determination of the mechanisms of action of mast cell products in mediating allergic responses will provide a sound basis for neuro-immune interactions, and future development of therapeutic drugs for prevention of the underlying symptoms of colic and diarrhea during intestinal anaphylaxis.
CHAPTER II
MATERIAL AND METHODS

DEVELOPMENT OF METHODOLOGY: SIMULTANEOUS ASSESSMENT OF ACETYLCHOLINE RELEASE AND ION TRANSPORT

Acetylcholine is a neurotransmitter which acts at ganglionic synapses and at neuroepithelial junctions to induce chloride secretion in the guinea pig distal colon. This project is designed to investigate the role of submucosal cholinergic neurons in mediating the intestinal allergic response to cow's milk protein, β-lactoglobulin. The protocols developed were as follows:

Preparation and Dissection of Submucosa/mucosa Sheets

Male albino Hartley guinea pigs (Harlam Sprague Dawley, Indianapolis, IN) (350-600 g) were allowed food and water ad libitum prior to the experiments.

Guinea pigs were killed by a stunning blow to the head and exsanguination. The gastrointestinal system was exposed by an abdominal incision. The distal colon 5-10 cm proximal to the anus was removed, opened along the mesenteric borders, and rinsed free of intestinal contents by washing
with fresh Krebs-Ringer buffer solution. The colon was pinned to sylgard 184 encapsulating resin (Dow Corning, Midland, MI) in a culture dish. Under microscopic visualization, the longitudinal and circular muscle layers along with the myenteric plexus were removed from the submucosa/mucosa by blunt dissection. In the guinea pig, this dissection method effectively removed the myenteric ganglia and left the submucosal ganglia behind. The submucosa/mucosa was divided into 4-8 segments and mounted between two halves of Ussing flux chambers (Figure 12). Submucosa/mucosa preparations were stretched among 6 anchoring pins in one half of the chamber which contained a circular milipore filter to support the tissue. Two semicircular aluminum foil ribbon electrodes were placed 5 mm apart on the serosal or submucosal surface of the stretched piece of intestine. The surface area of the submucosal side was 0.78 cm², and weighed 34.5±0.8 mg wet weight (n = 5). The mucosal and serosal sides of the tissues were bathed separately by identical Krebs-Ringer buffer solutions recirculating from a reservoir maintained at 37°C, buffered at pH =7.2-7.4 and gassed with 95% O₂ and 5% CO₂. The composition of the solution (in mM) was: 120 NaCl, 6 KCl, 1.2 MgCl₂·6H₂O, 1.3 NaH₂PO₄·H₂O, 14.4 NaHCO₃, 2.5 CaCl₂·2H₂O and 12.5 glucose. The tissues were
FIGURE 12. Schematic diagram of a flux chamber to illustrate the position of ribbon electrodes for stimulating enteric neurons. Top: Flux chamber halves with ribbon electrodes on one chamber half. Bottom: Flux chamber halves clamped together. The ribbon electrodes are juxtaposed to the serosal or submucosal surface of the intestine and attached to the output of an electronic stimulator. a,b: Flux chamber halves; c: aluminum foil or silver-silver chloride ribbon electrodes; d: stainless steel anchoring pins; e: port for Ringer-agar bridges; f: port for silver-silver disc electrodes; g: port for perfusing the tissue; h: leads to an electronic stimulator. Reproduced from Physiology of the Gastrointestinal Tract, 2nd. ed., 1987.
equilibrated for at least 25 or 30 minutes before the
beginning of each experiment.

MODIFICATION OF THE USSING FLUX CHAMBER SHORT-CIRCUIT
CURRENT TECHNIQUE

Ussing chambers and voltage clamp techniques were used
to study intestinal ion transport in vitro (Fig. 15 and 35).
The foil electrodes were attached by alligator clips and
leads to an electronic stimulator which delivered bipolar
electrical pulses. A pair of silver-silver chloride disk
electrodes were connected to a voltage-clamp apparatus
(Physiological Instruments, VC600, Houston, TX) that
compensated for the solution resistance between the voltage-
sensing bridges. Throughout the experiment, the tissues
were short circuited with a voltage clamp apparatus to
abolish or null out the spontaneous PD (Figure 15 and 35).
The short-circuit current necessary to change the
transepithelial PD by 8 mV was used to calculate tissue
conductance according to Ohm's law. Tissues from the same
animal were paired on the basis of similar conductances.
Both surfaces of the tissues were bathed with an identical
buffered solutions of Krebs-ringer. When both surfaces of
the tissues were bathed by solutions of identical
composition under short-circuit current conditions, the
electrochemical driving forces were zero. Short-circuit current is a direct measure of the total flux of ionic charge across the membrane in the absence of any electrochemical gradients and can be calculated as follows:

\[ J^{sc} = z \Delta J^{Na} + z \Delta J^{Cl} + z \Delta J^{R} \]

Where \( J^{sc} \) is the net ion flux, \( \Delta J \) is the net flux of sodium (Na), chloride (Cl), or residual (R) flux in \( \mu \text{Eq/cm}^2/\text{hr} \), and \( z \) is the ionic valance.

Most of the current is attributed to sodium chloride transport. Residual current is due either to anion secretion or cation absorption. Most of the residual flux is thought to reflect bicarbonate secretion.

Short-circuit current (\( \mu \text{A} \)) can be converted to units of flux, \( \mu \text{Eq/cm}^2/\text{hr} \)

\[ J_{sc} = \text{Isc (\( \mu \text{A} \))} \times 3600 \text{ sec/hr} \div A \text{ (cm}^2) \]

\[ J_{sc} = \text{Isc} \times 0.047523 \]

Earlier studies have implicated cholinergic neurons in regulation of chloride secretion (30, 34, 35, 36, 74, 75, 97, 98). However to obtain more direct evidence for their involvement it was necessary for us to demonstrate an
increase in acetylcholine release simultaneously with an increase in short-circuit current. We therefore developed a method for simultaneous assessment of acetylcholine release and ion transport in sheets of colonic submucosa/mucosa. This method was utilized to characterize acetylcholine release and to study the response characteristics of submucosal cholinergic neurons in the guinea pig distal colon. These protocols will be described later in this chapter.

**ELECTRICAL FIELD STIMULATION OF SUBMUCOUS NEURONS**

Submucous neurons were stimulated by an electrical current. The current was passed from a Grass SD-9 electronic stimulator (Grass Instruments, Quincy, MA) parallel to the plane of the tissue via a pair of aluminum foil ribbon electrodes juxtaposed between the flux chamber halves and submucosal surface (97, 98). Bipolar stimulus pulses [0.5-10 Hz, 35V amplitude (6-8 mA), 0.5-2 ms] were applied continuously for 1.5, 2 or 5 min periods. Changes in short-circuit current were continuously monitored with a Kipp and Zonen recorder and IBM-XT computer. Stimulus-evoked changes in short-circuit current were calculated by subtracting the maximum current during stimulation from basal levels prior to stimulation.
CHEMICAL STIMULATION OF SUBMUCOUS NEURONS

This was achieved by adding chemicals or drugs to the serosal surface either in the superfusate or in the form of injection into the inflow tubing to the chamber. In the latter, the volume of injectate was either 10 µl or 20 µl. Short-circuit current responses and acetylcholine release from submucous neurons were examined in parallel during electrical field stimulation or during chemical stimulation. Results from studies where drugs were injected were always compared to tissues injected with the vehicle in which the drug was dissolved.

CHARACTERIZATION OF ACETYLCHOLINE RELEASE FROM SUBMUCOSAL NEURONS

Release of \(^3\)H-acetylcholine was determined in the absence of cholinesterase inhibitors as the stimulus-evoked outflow of \(^3\)H from preparations preloaded with \(^3\)H-choline in response to electrical field stimulation and chemical stimulation. Experiments were performed according to the following time schedule.

(i) Equilibration period

Tissue preparations were mounted in flux chambers as described earlier. The mucosal and submucosal surface were
bathed with 4 ml Krebs-Ringer buffer recirculated from reservoir maintained at 37°C and allowed to equilibrate for 25-30 minutes. Solutions were continuously bubbled with a mixture of 95% O₂ and 5% CO₂. Special attention was paid to tissue conductances to monitor tissue viability.

(ii) **Uptake of ³H-choline by submucous neurons in flux chambers**

The serosal surface of the tissues were loaded with 10µCi/ml with specific activity 86.7 Ci/mmol ³H-choline chloride (New England Nuclear) with or without 5 µM atropine (Sigma Chemical Co., St Louis, MO). The submucosal medium also contained 1µM non-radioactive choline chloride (Sigma chemical Co., St Louis, MO). During this labelling period submucous neurons were stimulated continuously at 1Hz, 35V (7-8 mA), 2ms for 30 minutes to maximize the uptake of ³H-choline. Tissues were incubated for additional 30 minute in the presence of ³H-choline without electrical field stimulation.

(iii) **Washout period**

After the labelling period each segment of tubing that connected the inner and outer ports of the flux chamber with the glass reservoir was clamped (Figure 15, horizontal bars) and the submucosal medium containing ³H was discarded. A catheter was inserted into the outer port to perfuse the
submucosal surface at a rate of 1 ml/min with oxygenated Krebs-Ringer solution warmed by a heat exchanger (Figure 15C). The inner port contained a catheter for collection of samples (Figure 15E). Excess $^3$H-choline was washed out by perfusion for 60 minutes with Krebs-Ringer buffer containing 10μM hemicholinium-3 and 1μM choline chloride. Hemicholinium-3 was included in the perfusate until the end of the experiment in order to prevent re-uptake of $^3$H-choline. Sixty minutes of washout was sufficient for the basal outflow or radioactive $^3$H to reach constant rate of decline. Cholinesterase inhibitors were not present in the perfusion fluid.

(iv) Collection of Samples with $^3$H-ACh

After washout, one ml samples were collected continuously every minute throughout the duration of the experiment. Two 1-minute collections were made before each stimulus to establish basal $^3$H release. This was followed by collection of two samples during a 2-minute stimulation period and an additional six samples before this sequence was repeated twice.

In the experiments where drugs were added, infusion of the drug was timed so that it reached the chamber simultaneously with a 5 or 10μl injection of the drug. This
combination of perfusion and single injection served to increase the concentration of the submucosal medium rapidly and to maintain it at that concentration for the duration of the experiment.

For analysis of $^3$H-ACh after collection, 0.9 ml of each sample was transferred to scintillation vials containing 5 ml of scintillation cocktail Pico-Flour 40 or Ultima Gold (Packard Instrument Co., Downers Grove, IL). These counting media were biodegradable and had the highest counting efficiency. Radioactivity of samples was quantified for spontaneous (basal) and evoked (stimulated) release of $^3$H-ACh in a liquid scintillation spectrometer Tri-Carb 2000CA (Packard Inst. Co., Downer's Grove, IL). At the end of each experiment the flux chambers were opened and the tissues were carefully transferred to scintillation vials containing 1 ml of soluene-100 a tissue solubilizer containing 0.5 N quaternary ammonium hydroxide in toluene (Packard Instrument Co., Downers Grove, IL) and kept overnight. Radioactivity of solubilized tissues was then determined to calculate the total tissue stores of $^3$H.

The experimental approach to study response characteristics of submucosal neurons was as follows:

The effects of stimulating submucosal neurons on ACh release are unknown in guinea pig distal colon. Therefore
in this part of the research the characteristics of submucosal neurons in response to electrical field stimulation were studied. The experimental protocols are as follows:

1. **Effect of Electrical Field Stimulation on ACh Release and Short-Circuit Current.**

   In this series of experiments, uptake of $^3$H-choline and the subsequent synthesis and release of $^3$H-acetylcholine was studied in submucosa/mucosa preparations of guinea pig distal colon. Tissues were set up in Ussing flux chambers modified to assess the release of ACh simultaneously with changes in short-circuit current, as described earlier in this chapter. The tissues were electrically stimulated at parameters of 0.5, 1, 3, 5 and 10 Hz, 35V and 2 ms duration for three 2 minute periods.

   The time course of both the responses were compared and plotted. These effects were studied in the presence and absence of the muscarinic antagonist, atropine (10 µM). The relationship between stimulus evoked release of ACh and change in short-circuit current were assessed in the presence and absence of atropine in guinea pig distal colon. Stimulus parameters used were 0-10 Hz, 35V and 2ms.
2. **Effect of frequency on stimulus evoked $^3$H-ACh release and short-circuit current.**

In order to see whether electrically stimulated release of ACh from submucosal neurons was frequency dependent we studied the release of ACh as a function of 0.5, 1, 3, 5 and 10 Hz. Initially most of these experiments were carried out in the presence of 5 μM atropine as mentioned earlier in this section. Since atropine did not effect ACh release, all experiments where atropine was not present in the incubation media were pooled. However, it was not possible to pool the data for Isc responses as atropine significantly reduced the epithelial response (Chapter 3).

ACh release at different frequencies were compared during the first 2 minute period of stimulation (S1) as well as two additional 2 minute periods of stimulation (S2) and (S3). The results of this study suggest that high frequencies of stimulation are necessary to evoke maximal release of ACh and chloride secretion. These characteristics differentiate colonic submucous neurons from myenteric neurons which respond at much lower frequencies (71, 93, 177, 178, 179).
3. **Effect of time on stimulus evoked release of $^3$H-ACh and short-circuit current.**

Since ACh release peaked at 5 Hz, our subsequent experiments were done at a frequency of 5 Hz, with other parameters being 35 V, and 2 ms for 2 minutes. At this frequency, the effect of time on ACh release for three consecutive periods of electrical field stimulation (S1, S2, S3) of 2 minute duration, were examined. Short-circuit current responses were recorded simultaneously in parallel with collection of samples for ACh release. Both basal and stimulated ACh release levels were compared during the 25 minute time period.

4. **Effect of tetrodotoxin on stimulus-evoked $^3$H-ACh release and short-circuit current.**

These experiments were done in order to verify that $^3$H was released from submucous neurons during stimulation. Tetrodotoxin 0.2 μM (Sigma Chemical Co., St. Louis, MO) was used to block neurotransmitter release. Tetrodotoxin is a sodium channel blocker which prevents action potential dependent release of neurotransmitters. Tissues were perfused continuously with Krebs-Ringer with glucose. Two basal samples were collected followed by first period of electrical field stimulation (S1) for 2 minutes. Stimulus
parameters were 5Hz, 35V and 2ms. The perfusion fluid was combined with 2 μM tetrodotoxin and timed to reach the serosal bath at the same time that 5 μl of 95 μM tetrodotoxin was injected into the submucosal medium, 2 minutes before the second 2 minute stimulation (S2). This combination of perfusion and injection served to increase the concentration rapidly in the submucosal compartment and was sustained throughout the remainder of the experiments. Six minutes after the termination of S2 a third electrical stimulation (S3) was applied to the tissues for a period of 2 minutes. Control tissues were treated with vehicle exactly the same way as the experimental tissues. Acetylcholine release and short-circuit current were assessed in parallel in both control and experimental tissues.

5. Effect of atropine and stimulus evoked\(^3\)H-ACh release and short-circuit current.

In this series of experiments the effect of atropine a specific cholinergic muscarinic antagonist were assessed on: The release of ACh and change in Isc in response to electrical field stimulation (5 Hz, 35V, 2 ms) before and after the treatment with atropine.
Perfusion of atropine (5 \( \mu M \)) solution was timed to reach the tissue simultaneously with 10 \( \mu l \) of 1 mM atropine injected into the submucosal medium, 2 minutes before S2. These responses were compared with vehicle-treated control tissues.

Additional experiments were done to investigate whether atropine would alter \( ^3H \)-ACh release at different frequencies of stimulation. The frequencies tested ranged from 0-10 Hz.

The results of these experiments are further explained and discussed in chapter 3.

The release of \( ^3H \)-ACh was verified by chromatographic analysis of radiolabelled samples in response to electrical field stimulation.

**SEPARATION OF \( ^3H \)-ACETYLCOLINE AND \( ^3H \)-CHOLINE BY CATION EXCHANGE COLUMN CHROMATOGRAPHY**

Chromatographic separation of radiolabelled samples was analyzed before and after electrical field stimulation by a modification of the method reported by Wikberg (171). The incubation medium contained 50 \( \mu M \) physostigmine salicylate (Sigma Chemical Co, St. Louis, MO) to prevent degradation of \( ^3H \)-ACh by cholinesterase. The columns were packed with cation exchange resin Bio-Rex 70, sodium form, mesh 200-400 (Bio-Rad, Richmond, CA) to separate \( ^3H \)-ACh from \( ^3H \)-choline
and other products derived from $^3$H-choline. Bio-Rex 70 resin is a weakly acidic cation exchanger. This resin is used for the purification and fractionation of peptides, proteins, antibiotics and other cationic molecules. Before packing the columns and application of samples, this resin was equilibrated to the initial buffer conditions. The following equilibration procedure was followed:

1. Bio-Rex 70 resin was weighed and soaked in Na$_2$HPO$_4$ buffer. The volume of the buffer was 4-5 times the volume of the resin.

2. Resin was allowed to equilibrate for at least 30 minutes. The pH was tested and adjusted with HCL (acid) or NaOH (base). It was reequilibrated until the pH was stable. The operating pH range for this resin is 5-14.

3. After the pH was stable the buffer was decanted off. Step 1 and 2 were repeated using fresh buffer until no change was observed when fresh buffer was added.

4. The excess buffer was decanted off and the resin was poured into the columns.

The columns were equilibrated with 0.1 M Na$_2$HPO$_4$ buffer (pH 7.0). About 2-3 bed volumes of buffer were passed through the columns. Both pH and conductivity were monitored. When
the pH and conductivity of the effluent were the same as that of the inflow media, the columns were washed with 5 ml of distilled water. The resin and columns were then considered fully equilibrated and ready for sample application.

Three ml samples from unstimulated (basal) and electrically stimulated tissues were placed on cation-exchange resin columns, followed by 5 ml of distilled water. The columns were eluted with Na$_2$HPO$_4$ buffer (0.1M, pH7) at a flow rate of 3 ml/hr. The effluent was collected directly into scintillation vials containing 5 ml of scintillant (Pico-Flour 40). The radioactivity of the effluent was analyzed in a liquid scintillation spectrometer Tri-Carb 2000 CA (Packard, Downer's Grove, IL).

In some samples $^{14}$C-ACh, specific activity 1 to 5 mCi/mmol (New England Nuclear), was added to identify the $^3$H-ACh peak. The increase in $^3$H occurring after stimulation was in the form of $^3$H-ACh, as disintegrations per minute (dpm). Tritium and $^{14}$C counting efficiency of the scintillation counter was 68 % and 90 %, respectively.

The results of chromatographic analysis are discussed in chapter 3 and illustrated in Fig 16.

Next series of experiments were designed to provide evidence for the interaction of immune system with the
enteric nervous system.

NEURO-IMMUNE INTERACTIONS: ROLE FOR CHOLINERGIC NEURONS IN INTESTINAL ANAPHYLAXIS

These series of experiments were based on the hypothesis: That $\beta$-lactoglobulin (a milk protein antigen) stimulates acetylcholine release from submucosal neurons leading to an increase in chloride secretion in the distal colon of guinea pigs sensitized to cow's milk.

The steps involved in preparation and dissection of tissues, setting them up in modified Ussing flux chambers, equilibration period, loading of tissues with $^3$H-choline chloride, washing period, collection of samples and analysis of samples in scintillation spectrometer are the same and have been explained earlier in this chapter.

This study utilized submucosal/mucosal preparations from guinea pig model of bovine milk hypersensitivity to $\beta$-lactoglobulin.

Development of an animal model of milk hypersensitivity

Two-week old male albino guinea pigs of the Hartley strain (Harlan-Sprague-Dowley, Inc, Indianapolis, IN) were kept in central animal facility quarters at Ohio State
University medical school, under controlled temperature. At the time of arrival these guinea pigs were separated into two groups. One group of non-immune animals were given food and water *ad libitum* for 3-4 weeks. In the second immune group, bovine milk was substituted for water for a 3-4 week period and food was given *ad libitum*. Three days prior to the experiments, cow's milk was removed and replaced by water. During this period, guinea pigs given cow's milk developed immunity to β-lactoglobulin (4, 95). About 5% of the animals in our study, failed to develop immunity to β-lactoglobulin based on the unresponsiveness of the tissue to β-lactoglobulin. The non-immune, age matched guinea pigs were used as controls for this study.

The protocols developed for this part of the study are explained in chapter 4 and are briefly listed in this section.

**BETA-LACTOglobULIN STUDIES**

1. **Effect of exogenous β-lactoglobulin on $^3$H-ACh release and short-circuit current.**

Two basal samples were collected, followed by 20 μl injection of bovine milk β-lactoglobulin (Sigma chemical Co., St Louis, MO) in the serosal side of the chamber as a bolus. Beta-lactoglobulin with a molecular weight of 36,000
was available as a lyophilized powder containing β-lactoglobulin A and B. Following the injection, tissues were continuously perfused and samples were collected for another 25-30 minute time period. Non-cumulative concentration-response curves were generated by addition of a single concentration of β-lactoglobulin to the submucosal solutions bathing each immune or non-immune tissue. The concentrations tested were 5 nM, 0.02 μM, 0.2 μM, and 20 μM respectively. Only one concentration (20 μM) was tested in the age matched non-immune animals. Short-circuit current responses were recorded in parallel during collection of samples for ACh release.

2. Comparison of the time course of ACh release and Isc in response to β-lactoglobulin.

These studies were done by utilizing 20 μl injection of β-lactoglobulin on the serosal side of the chamber to achieve an effective transient concentration of 20 μM. Utilizing the same procedure as above, the time course of ACh release and short-circuit current were compared and analysed.

3. Effects of tetrodotoxin and cholinergic receptor antagonists, atropine and mecamylamine and serotonin
receptor antagonists on Isc response to β-lactoglobulin.

These series of experiments were done in our laboratory by Dr. Y.Z. Wang. Since they are related studies and have implications for this study, they are included.

These studies were done to determine if ACh released from submucous neurons could be prevented by tetrodotoxin. The types of neurons and specific receptors involved in initiating the response to β-lactoglobulin in immune animals was investigated. The results of these experiments are discussed in chapter 4.

HISTAMINE STUDIES

The next series of experiments were designed to determine the interaction of mast cell mediator, histamine, with enteric nerves and the epithelium.

These experiments were based on the hypothesis:

That the endogenous mast cell mediator histamine, will activate cholinergic neurons to stimulate acetylcholine release, which is associated with Cl⁻ secretion in the guinea pig distal colon.

To determine whether cholinergic neurons were involved in mediating chloride secretion by histamine, similar methodology was used as previously described. The
protocols designed, were as follows:

1. **Effect of histamine blockers on ACh release and short-circuit current in response to β-lactoglobulin in immune animals.**

Histamine blockers, 10 μM cimetidine (H₂), 10 μM pyrilamine (H₁) (Sigma Chemical Company, St. Louis, MO) alone and in combination were perfused continuously through the serosal side of the chamber. The response to 2 μM β-lactoglobulin injection in the presence and absence of histamine blockers was examined. Acetylcholine release and change in short-circuit current were assessed simultaneously in other control and experimental tissues. These concentrations of antagonists are known to block the effects of histamine in guinea pig distal colon (162, 163).

Since the maximum response to β-lactoglobulin was significantly reduced by tetrodotoxin, atropine and histamine blockers, (pyrilamine and cimetidine) it suggested that histamine and cholinergic neurons were involved in mediating the antigen response. Therefore our next set of experiments were designed to study the:
2. Effect of exogenous histamine on ACh release and short-circuit current.

Two different concentrations of histamine (Sigma Chemical Company, St. Louis, MO) were used to establish a concentration response relationship. Ten µl of 10 µM or 1 mM histamine was injected on the serosal side of the chambers with the tissues. Both ACh release, and change in Isc were assessed simultaneously in the distal colon of immune guinea pigs.

3. Effect of H₂ receptor agonist on ACh release and short-circuit current.

Dimaprit is an H₂ receptor agonist, which is known to activate H₂ receptors on submucous neurons to cause cyclical patterns of chloride secretion in guinea pig distal colon (162). Five basal samples were collected. Dimaprit was infused so that it reached the chamber simultaneously with a 20 µl injection of the drug. This combination served to increase the concentration of the submucosal medium to a final concentration of 10 µM, which was maintained for the duration of the experiment. The results of these experiments are explained in chapter 4.
4. **Histamine assays.**

Quantification of histamine was done by assaying the samples. The samples were collected and frozen at -70°C and sent to Dr. Kim Barrett's laboratory at the University of California Medical Center, San Diego, where they were analyzed for total tissue stores of histamine in colonic submucosa/mucosa, antigen-evoked histamine release after treatment of tissues with β-lactoglobulin and vehicle, and tissue stores of histamine after treatment with vehicle and β-lactoglobulin.

Total of 216 samples (103 from non-immune and 113 from immune tissues of guinea pig distal colon) were collected.

**Protocols for tissue preparation and sample collection for histamine assays.**

Segments of distal colon from immune and non-immune (control) guinea pigs were dissected by the method described earlier in this chapter. Two 3 centimeter segments of submucosa/mucosa preparations were cut into small pieces and were placed in plastic vials containing 1.5 ml of fresh oxygenated Krebs-Ringer solution. Vials were weighed before and after adding the tissue segments.

Vials with Krebs and tissues were then incubated at 37°C for 10 minutes. After incubation, fluid was discarded
and 1.5 ml of fresh buffer was added. Tissues were incubated for an additional 10 minutes. Beta-lactoglobulin 15 µl (10^{-4} M) was added to one set of vials. The same quantity of vehicle was added to another set of vials as controls. After 10 minutes, supernatants were carefully aspirated into separate vials. The tissues were washed with 2 ml Krebs-ringer solution followed by addition of 1.5 ml fresh solution.

Supernatants and tissues with Krebs-Ringer were incubated at 100°C for 10 minutes. Vials were capped to avoid evaporation of fluid. Tissues were homogenized by a small, hand tissue homogenizer. Both tissue samples and vials with supernatants were centrifuged at 5°C at 2000g for 5 minutes. Supernatants were aspirated carefully for all samples, and total volume of the aspirate was measured. One ml of supernatants were then transferred into vials and frozen at -70°C.

For total tissue stores, tissue segments were placed in the vials containing 1.5 ml Krebs-ringer solution. Tissue weights were determined, followed by incubation of tissues at 100°C for 10 minutes. Tissues were then homogenized and centrifuged at 5°C, 2000g for 5 minutes. Supernatants were aspirated and total volume was determined. One ml aliquots
were then frozen and stored at -70°C. Samples were then sent on dry ice to San Diego, for histamine analysis.

PROSTAGLANDIN STUDIES

The next set of experiments were based on the hypothesis:

*That endogenous prostaglandins E₂ activates cholinergic neurons during intestinal anaphylaxis.*

Studies of intestinal anaphylaxis in guinea pigs have implicated postaglandins as mediators in intestinal secretion (1, 3, 23, 24, 43, 129). To determine whether prostaglandins can activate cholinergic neurons in response to β-lactoglobulin the following investigations were done:

1. **Effect of β-lactoglobulin on ACh release and short-circuit current response in presence and absence of piroxicam.**

   Piroxicam is a specific inhibitor of cyclooxygenase pathway of arachidonic acid metabolism. The serosal side of the tissues from immune animals were perfused with 10 μM piroxicam (Sigma Chemical Company, St. Louis, MO), 15 minutes prior to the injection of twenty μl of 2 μM β-lactoglobulin. Acetylcholine release and short-circuit
current changes were examined in parallel.

In the presence of piroxicam, there was a significant reduction in ACh release and short-circuit current in response to β-lactoglobulin. This suggested that endogenous prostaglandins were involved in mediating ACh release. Based on these findings our next series of experiments were done to examine the:

2. **Effect of exogenous prostaglandins on ACh release and short-circuit current.**

   Ten μl of 0.5 μM or 5 μM PGE₂ (Sigma Chemical Company, St. Louis, MO) were injected into the chamber during continuous perfusion. Equal volumes of vehicle were injected in the control tissues. Samples were collected for ACh release while short-circuit current was being monitored to assess the secretory response.

   Since studies from 1 and 2 implicated both histamine and prostaglandins, we investigated:

3. **The effect of β-lactoglobulin on ACh release in presence and absence of histamine antagonists and piroxicam in combination.**

   To assess the interactive effect of histamine and prostaglandins, a combination of 10 μM cimetidine, 10 μM
pyrilamine and 10 µM piroxicam were perfused through the serosal side of the chamber, 15 minutes prior to 20 µl of β-lactoglobulin injection to achieve the final effective concentration of 2 µM on the serosal side. In the control tissues the same concentration of β-lactoglobulin was injected in the absence of these blockers for comparison of the response. Both ACh release and short-circuit current were examined in parallel. The results of these experiments are explained in chapter 4.

LEUKOTRIENE STUDIES

During this phase of the project the experimental protocols were based on the hypothesis:

That leukotriene D₄ modulates colonic electrolyte transport by activating cholinergic neurons during intestinal anaphylaxis.

Leukotrienes constitute a distinct family of arachidonic acid metabolites produced via the lipoxygenase pathway, and are a potent mediators of hypersensitivity and inflammation. Increased mucosal synthesis of leukotrienes has been reported in inflammatory bowel disease and ulcerative colitis (140, 181). Although leukotrienes are released from mast cells during intestinal anaphylaxis, their role in mediating chloride secretion are unknown. The
aim of this study was to investigate the effects of leukotriene D₄ (LTD₄) on distal colonic ion transport in guinea pigs sensitized to β-lactoglobulin and in age-matched, non-immune animals.

The methods and results of this study are discussed in chapter 5, and are briefly listed in this section.

Muscle stripped segments of guinea pig distal colon were setup in conventional type of Ussing flux chambers (Fig. 13). Our investigation was carried out according to the following experimental protocol sequence:


2. Effect of 25 μM leukotriene antagonist SK&F 102922 on 0.15 μM exogenous LTD₄ response.

3. Effect of LTD₄ on change in short-circuit current in immune and non-immune tissues and establishment of concentration response relationship. Concentrations tested were, 0.15 μM, 0.5 μM, and 1 μM LTD₄.

4. Effect of 2Cl/Na/K cotransporter blocker bumetanide on 0.5 μM LTD₄ evoked short-circuit current response.

5. Effect of LTD₄ in presence and absence of tetrodotoxin.


8. Effect of carbachol in presence of LTD$_4$ with and without tetrodotoxin.

9. Effect of carbachol on monolayers of T84 colonic cell line treated with and without LTD$_4$. This part of the study was done by other colleagues in our laboratory and was included because of its implications to this study.

For complete information of above studies please refer to chapter 5 of this document.

HISTOLOGICAL STUDY FOR MAST CELL POPULATION IN THE TISSUES

This study was carried out with the help of a histochemist in our laboratory to verify the number of mast cells per unit area in the submucosal layer of the distal colon. Tissues were obtained from 9 β-lactoglobulin sensitized immune guinea pigs and 9 non-immune age matched control animals. The segments of distal colon were opened along the mesenteric border, and rinsed free of intestinal contents. The colon was pinned to Sylgard 184 encapsulated resin (Dow Corning, Midland, MI) in a culture dish, with the
mucosal side facing up. The mucosa was scraped off with a
glass slide and the muscle layer was gently teased off from
the submucosal layer with a pair of fine forceps. The
dissection was carried out at room temperature under a
dissecting microscope (Bausch and Lomb). The tissues were
continuously perfused with chilled oxygenated Krebs-Ringer
buffer solution by a perfusion pump. The dissected
submucosal layer was fixed in barium solution for 7 hours at
4°C followed by washout in 70% ethanol at 4°C overnight.
After rehydration the preparations were stained with alcian
blue. The preparations were mounted on a gelatinized glass
slide and held flat by filter paper and a cover slide for
dehydration through graded alcohol solutions. After removal
of the filter paper and the cover slide the preparations
were cleared in xylene solution and then mounted in
permount.

Mast cells were counted with an eyepiece square grid
(Fisher Scientific, IL). This grid covered a field area of
0.05 mm² at a 40 X objective magnification. The cells were
counted in 300 random fields for each animal tissue. Only
stained cells with at least identifiable nuclei were counted.
The number of mast cells were determined per mm² area of
the submucosal layer. The results obtained for each group
of animals are presented as means ± SEM and compared by the Student's t-test.

**CALCULATION OF DATA**

Methods adopted for the calculation of the data have been discussed in chapters 3, 4, and 5 of this document.

Submucosa/mucosa segments of distal colon were setup in Ussing flux chambers. For experiments where drugs (10 or 20 µl) were injected, evoked release was estimated from weights of areas under the curve of the stimulus-evoked responses. These were converted to dpm/cm² after comparison with known standard weights of defined areas under the curve. The standard weights of areas under the curve were determined by injecting a different range of concentrations of ³H-choline chloride (0.1-0.8 µCi/ml) (specific activity 86.7 Ci/mmol) in 10 or 20 µl to the serosal side of the flux chambers. A factor was determined by linear regression analysis for 10 and 20 µl injections of drugs. The weights of the areas under the curves in all studies with the drugs was multiplied by the factor to determine the change in radioactivity as dpm/cm². Final results were expressed as % of average basal ³H or % of control. The linear regression curves are shown in figure 13 and 14. The factors for 10 and 20 µl injections were 719.85 and 677.69
FIGURE 13. Standard curve for $^3$H-ACh release calculation for 10 μl injections with 0.1, 0.2, 0.5, and 0.8 μCi/ml $^3$H-choline (specific activity 86.7 Ci/mmol) (standards). Graph shows linear regression analysis through zero for converting weights of defined areas under the curve into disintegrations per min (dpm). $Y = dpm/cm^2$; $X = weight$ of area in mg. ($Y = X \times 719.846$).
FIGURE 14. Standard curve for $^3$H-ACh release calculation for 20 μl injection with 0.1, 0.2, 0.5 and 0.8 μCi/ml $^3$H-choline (specific activity 86.7 Ci/mmol) (standards). Graph shows linear regression analysis through zero for converting weights of defined areas under the curve into disintegrations per min (dpm). $Y = \text{dpm/cm}^2; X = \text{weight of}$
respectively.

**STATISTICS**

All results are presented as mean ±S.E.. Statistical significance of difference between control and experimental tissues was analysed by Students t test. A paired t test was used to determine the statistical significance of differences between experimental manipulations of the same tissue. An unpaired t test was used to determine the significance of differences between different tissues or animals. For comparison of three or more means, a one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple range test was used to determine the level of significance. Values of p < 0.05 were considered to be statistically significant. A single data point was excluded if it was greater than 2 S.D.s from the mean calculated with the parameter included.
CHAPTER III

ACETYLCHELINE RELEASE FROM COLONIC SUBMUCOUS NEURONS
ASSOCIATED WITH CHLORIDE SECRETION IN THE GUINEA PIG

INTRODUCTION

Cholinergic neurons comprise approximately 50% of the total population of submucous neurons in the guinea pig small intestine (13, 62, 63). They are a heterogeneous population and have been classified into three groups based on their neurochemical coding and functional properties (13, 62, 63). They receive synaptic inputs from myenteric neurons, synapse with other submucous neurons, or project to the epithelium or blood vessels. The proximity of cholinergic nerve terminals to epithelial cells suggests a role for these neurons in regulation of epithelial transport.

Functional studies have demonstrated an increase in short-circuit current and chloride secretion during electrical field stimulation of submucous neurons in the guinea pig and rat small intestine or colon (17, 19, 96, 97). The stimulus-evoked response was reduced by muscarinic and nicotinic cholinergic blockers and tetrodotoxin (17, 19,
96, 97). On this basis, cholinergic neurons were implicated in regulation of chloride secretion in the guinea pig colon (96, 97). More direct evidence for their involvement would necessitate a demonstration of an increase in acetylcholine (ACh) release simultaneously with an increase in short-circuit current.

The purpose of this study was to develop a method for the simultaneous assessment of ACh release and ion transport. Although there is an abundance of literature on release of ACh from myenteric neurons (71, 82, 92, 152, 179), little is known about the response characteristics of submucous neurons, particularly in the colon (66, 175, 180). An additional aim was to characterize ACh release from submucous neurons in the guinea pig distal colon. The results demonstrate a highly significant correlation of acetylcholine release and short-circuit current indicative of chloride secretion. The results on colonic submucous neurons suggest that high frequencies of stimulation are necessary to evoke maximal release of ACh and chloride secretion. These characteristics distinguish colonic submucous neurons from myenteric neurons in this and other regions of the intestine.
METHODS

Preparation of Submucosa/Mucosa Sheets

Male albino Hartley (Harlan-Sprague-Dawley, Inc., Indianapolis, IN) guinea pigs (350-600 g) were allowed food and water ad libitum. They were killed by a stunning blow to the head and exsanguination. The distal colon, approximately 5-10 cm proximal to the anus was removed, opened along the mesenteric border and rinsed free of intestinal contents. The colon was pinned to Sylgard 184 encapsulating resin (Dow Corning, Midland, MI) in a culture dish and the longitudinal and circular muscle layers were removed by blunt dissection. This resulted in a flat sheet of submucosa/mucosa which was divided into 2 segments and mounted in flux chambers. The surface area of the submucosal side was 0.78 cm² which was equivalent to 34.5 ± 0.8 mg (n = 5) wet weight.

The chambers were equipped with a pair of Krebs-Ringer/agar bridges connected to calomel half cells for measurement of transepithelial potential difference (17, 19, 96, 97). A pair of silver-silver chloride disk electrodes were connected to a voltage clamp apparatus that compensated for the solution resistance between the voltage-sensing bridges. The short-circuit current necessary to change the transepithelial PD by 8 mV was used to calculate tissue
conductance according to Ohm's law. Tissues from the same animal were paired on the basis of similar conductances.

**Electrical Field Stimulation of Submucous Neurons**

Submucous neurons were stimulated by an electrical current which was passed from an electronic stimulator parallel to the plane of the tissue via a pair of aluminum foil ribbon electrodes juxtaposed between the flux chamber halves and the submucosal surface (93, 97). Bipolar stimulus pulses (0.5-10 Hz; 35 V amplitude (6-8 mA); 2 ms) were applied continuously for 2 or 5 min periods. Short-circuit current was recorded on a chart recorder and responses to electrical stimulation were compared to basal short-circuit current prior to the beginning of stimulation. Stimulus-evoked changes in short-circuit current were calculated by subtracting the maximum current during stimulation from basal levels.

**Uptake of $^3$H-Choline by Submucous Neurons in Flux Chambers**

The mucosal and submucosal surfaces were bathed for 30 min by 4 ml Krebs-Ringer buffer recirculated from a reservoir maintained at 37°C. The submucosal media also contained 1 µM choline chloride, 10 µCi/ml (86.7 Ci/mmol)
$^3$H-choline with or without 5 μM atropine. Submucous neurons were stimulated continuously at 1 Hz, 35 V, 2 ms for 30 min during the period of uptake of $^3$H-choline. Tissues were incubated for an additional 30 min in the presence of $^3$H-choline without electrical field stimulation.

After this period, each segment of tubing which connected the inner and outer ports of the flux chamber with the glass reservoir was clamped (Figure 15, horizontal bars) and the submucosal media containing $^3$H was discarded. A catheter was inserted into the outer port in order to perfuse the submucosal surface at a rate of 1 ml/min with oxygenated Krebs-Ringer solution warmed by an heat exchanger (Figure 15, C). The inner port contained a catheter for collection of samples (Figure 15, E). Excess $^3$H-choline was washed out by perfusion for 60 min with Krebs-Ringer buffer containing 10 μM hemicholinium-3 and 1 μM choline chloride. Cholinesterase inhibitors were not present in the perfusion fluid.

**Collection of Samples with $^3$H-ACh**

One ml samples were collected continuously every min throughout the duration of the experiment. Three 1 min collections were made before each stimulus to establish basal $^3$H release. This was followed by collection of 2
FIGURE 15. Diagram of a flux chamber modified for perfusion of the submucosal surface of guinea pig distal colon (A). Both mucosal and submucosal surfaces were bathed in warmed oxygenated 4 ml buffer recirculated from a water-jacketed reservoir (B). The tubing on the submucosal side of the tissue was clamped (horizontal bars), and perfusion and collection cathetors were inserted into the outer and inner ports. Oxygenated fluid, which was warmed by a heat exchanger (C), was perfused by a pump (D). Samples were collected at 1-min intervals (E). Electrical potential difference (mV) and short-circuit current (μA) were monitored continuously by a voltage-clamp apparatus.
samples during a 2 min stimulation period and an additional 6 samples before this sequence was repeated twice.

In the experiments with tetrodotoxin or atropine, infusion of the drug was timed so that it reached the chamber simultaneously with a 5 or 10 µl injection of the drug. This combination of perfusion and single injection served to increase the concentration of the submucosal media rapidly and to maintain it at 0.2 µM (tetrodotoxin) or 5 µM (atropine) for the duration of the experiment.

**Calculation of Data**

Release of $^3$H-ACh was measured in the absence of cholinesterase inhibitors as the outflow of $^3$H from submucosa/mucosa preparations preloaded with $^3$H-choline. Radioactivity in each 0.9 ml aliquot of sample was calculated as total DPM/cm$^2$ for a 2 or 5 min period of electrical field stimulation. Basal release was determined by fitting a linear regression through all basal values measured before and after stimulation. Basal levels prior to and after stimulation were averaged. Evoked release was measured as weights of areas of the stimulus-evoked responses and converted to DPM/cm$^2$ after comparison with known standard weights of defined areas (Figure 13 and 14). (Details in chapter 2). Results were expressed as % above
average basal $^3$H or as a % of total tissue stores.

**Chromatographic Separation of Radiolabeled Samples**

For these experiments, 50 $\mu$M physostigmine was included in the incubation media to prevent degradation of $^3$H-ACh by cholinesterase. Three ml samples from unstimulated and electrically-stimulated tissues were placed on a cation exchange resin (BioRex 70, Bio-Rad Labs., Richmond, CA) to separate $^3$H-ACh from other $^3$H products (168). The column was eluted with Na$_2$HPO$_4$ buffer (0.1M) into vials containing 5 ml of scintillant and the radioactivity of the effluent was analyzed in a liquid scintillation spectrometer (Packard 2000CA, Downer's Grove, IL). In some cases, $^{14}$H-ACh was added to the sample to identify the $^3$H-ACh peak. The increase in $^3$H occurring after stimulation was in the form of $^3$H-ACh. For detailed description of the methodology see chapter 2.

**Statistics:**

Paired or unpaired Student's t test or one way analysis of variance followed by Neumann Keuls multiple range test was used to determine statistical significance. Values of $P<0.05$ were considered to be statistically significant.
RESULTS:

Electrical Properties

Viability of tissues set up in flux chambers modified for perfusion of the submucosal surface was assessed by examining basal Isc, total tissue conductance and the change in Isc evoked by a 2 min period of electrical field stimulation (10 Hz, 35 V, 2 ms). Basal short-circuit current and tissue conductance were \(-35\pm3 \, \mu A/cm^2\) and \(8.9\pm0.3 \, mS/cm^2\) (N=8), respectively, after the 2 hour experimental period. The average stimulus-evoked change in Isc after the experimental period was \(288\pm14 \, \mu A/cm^2\) (n=8). These values are well within the range of values reported previously for basal short-circuit current, total tissue conductance and neurally-evoked changes in ion transport in the guinea pig colon set up in conventional flux chambers (96, 97). These similarities provide evidence of the feasibility of using the perfusion system for simultaneous measurement of ACh and short-circuit current.

Column Chromatography

Chromatographic analysis of basal and stimulated samples revealed three peaks, (i) \(^3\text{H}\)-labeled metabolite, (ii) \(^3\text{H}\)-ACh and (iii) \(^3\text{H}\)-choline. Basal samples demonstrated a small peak corresponding to \(^3\text{H}\)-ACh. The
increase in $^3$H during electrical field stimulation corresponded to an increase in $^3$H-ACh release which was verified by comparison with a standard containing $^{14}$C-ACh (Figure 16 A,B,C and D). This was consistent with other reports (168, 175, 179).

**Effect of Electrical Field Stimulation on $^3$H-ACh Release in Submucosa/Mucosa Sheets**

Figure 17 illustrates DPM/cm$^2$ of $^3$H in each 1 min sample of perfusate released from the submucosal side during a 25 min period. The tissues were electrically stimulated (10 Hz, 35 V, 2 ms) for three 2 min periods as indicated by the solid bars. The stimulus-evoked increase in $^3$H-ACh release reached a maximum by 2 min and then declined toward basal levels within the next 6 min. At this frequency of stimulation, there was a significant decrement in the stimulus-evoked release of $^3$H-ACh and Isc responses during subsequent periods (S2 and S3). This effect was investigated and the findings are reported below.

Electrical field stimulation for 2 min (10 Hz, 35 V, 2 ms) evoked an $78 \pm 4\%$ (n=14) increase in $^3$H-ACh above basal levels of $^3$H (4792$\pm$517 DPM/cm$^2$) during period S1 (Figure 17). A 5 min period of stimulation caused a greater increase in $^3$H-ACh outflow above basal $^3$H levels of 3345$\pm$369
FIGURE 16. Chromatographic separation of radiolabelled samples to verify that the increase in $^3$H during electrical field stimulation of submucosal neurons in the guinea pig distal colon corresponds to an increase in $^3$H-ACh release. A and B: Verification of ACh and choline peaks by radiolabelled standards. C and D: Spontaneous (basal) and electrically evoked (stimulated) samples from experiments demonstrating peaks for $^3$H-metabolite, $^3$H-ACh, and $^3$H-choline. These studies included, 50 $\mu$M physostigmine in the incubation medium to prevent ACh breakdown. The increase in $^3$H occurring after stimulation was in the form of $^3$H-ACh (D).
FIGURE 17. Representative trace to illustrate effect of electrical field stimulation (EFS) on $^3$H-ACh release in flat sheets of submucosa/mucosa set up in flux chambers. Stimulus parameters were 10 Hz, 35 V, 2 ms pulses for 2 min. Each period of EFS (horizontal bars) was followed by a 6-min rest period.
DPM/cm² $(119\pm21\% \text{ n=5; p<0.05})$ (Figure 18). The stimulus-evoked $^3$H-ACh release expressed as a fraction of total stores of $^3$H in the tissue ranged from 0.12% to 0.26%.

Effect of Frequency on Stimulus-Evoked $^3$H-ACh Release

Electrical stimulation of submucous neurons evoked a frequency-dependent increase in release of $^3$H-ACh over basal $^3$H (S1, Figure 19). Submaximal release of $^3$H-ACh occurred at frequencies of 0.5, 1 and 3 Hz. Maximal release rates were attained at frequencies of 5-10 Hz.

To investigate whether attenuation of the response occurred with successive stimulations, three consecutive periods of electrical field stimulation of 2 min duration designated by S1, S2 and S3, (Figure 19), were separated in time by a 6 min rest period. At frequencies of 0.5, 1 and 3 Hz, $^3$H-ACh release was similar during periods S1, S2 and S3. At frequencies of 5 and 10 Hz, the release was greatest during period S1 and declined during S2 and S3 (Figure 19). At frequencies of 5 and 10 Hz, $^3$H-ACh release during S1 differed statistically from release during S2 and S3. Likewise, release during S2 differed from release at S3 at frequencies of 5 and 10 Hz.
FIGURE 18. Comparison of $^3$H-ACh release for 2 minute and 5 minute of electrical field stimulation (EFS). Stimulus parameters were 10 Hz, 35 V, 2 ms; $n = 14$ for 2 min and 5 for 5 min stimulation (* $p < 0.05$).
FIGURE 19. Effect of stimulus frequency on $^3$H-ACh release in guinea pig distal colon. S1, S2, and S3 represent three 2-min stimulation periods separated by a 6-min interval. Stimulus parameters were 0-10 Hz, 35 V, 2 ms for 2 min. Numbers at the top are frequencies of stimulation; n=5 animals for 0 and 3 Hz, 10 for 0.5 Hz, 9 for 1 Hz, 41 for 5 Hz, and 14 for 10 Hz. Studies were done in the presence and absence of 10 μM atropine; however, because there were no significant differences between control and atropine-treated tissues, the results were pooled. S1, S2, and S3 values for 0.5, 1, and 3 Hz are not statistically significant. At 5 and 10 Hz, S1 differed from S2 and S3; S2 differed from S3 (p < 0.05).
Effect of Tetrodotoxin on Stimulus-Evoked $^3$H-ACh Release

Tetrodotoxin was used to prevent neurotransmitter release and to verify that $^3$H was released from submucous neurons during stimulation. This neurotoxin selectively blocks sodium channels, thus blocking depolarization and action-potential dependent release of neurotransmitter. Tetrodotoxin had no significant effect on basal $^3$H release ($4032\pm518$ DPM/cm$^2$, n=7) compared to vehicle-treated control tissues ($3624\pm392$ DPM/cm$^2$). Likewise, tetrodotoxin did not alter baseline short-circuit current ($-12\pm6$ $\mu$A/cm$^2$, n=7) compared to vehicle treatment ($-8\pm6$ $\mu$A/cm$^2$).

Three periods (S1, S2, S3) of electrical field stimulation at 5 Hz evoked an increase in release of $^3$H-ACh above basal levels of $^3$H in control (S1,4350±409; S2, 4016±444; S3, 3624±391 DPM/cm$^2$) and experimental groups (S1, 5167±534; S2, 4602±543; S3, 4032±518 DPM/cm$^2$), (Figure 20A). The perfusion solution containing tetrodotoxin (2 $\mu$M) was timed to reach the serosal bath at the same time that 5 $\mu$l of 95 $\mu$M tetrodotoxin was injected into the submucosal media. This occurred at approximately 2 min before S2. This ensured that the concentration of tetrodotoxin was elevated rapidly in the submucosal compartment and was sustained throughout the remainder of the experiment. Tetrodotoxin significantly reduced the release of $^3$H-ACh in period S3.
FIGURE 20. Effect of tetrodotoxin on $^3$H-ACh release and short-circuit current in guinea pig distal colon. A: effect on $^3$H-ACh release. B: effect on short-circuit current (Isc). Arrow indicates perfusion of solutions with vehicle in control or tetrodotoxin (2 μM) in experimental tissues. Perfusion was timed to reach the tissue at the same time that 5 μl of vehicle or 100 μM tetrodotoxin was injected into the submucosal medium. This occurred at 2 min before S2. Open bars, Control; Hatched bars, Experimental; n = 7. (* p < 0.05).
compared to vehicle-treated control tissues (Figure 20A).

The response to tetrodotoxin on short-circuit current followed the same pattern as that of $^3$H-ACh release (Figure 20B). Tetrodotoxin significantly reduced the Isc response after the third period of stimulation (S3). These responses with tetrodotoxin suggest that release of $^3$H-ACh occurred from nerves, and this action is dependent on activation of sodium channels.

**Effect of Atropine on Stimulus-Evoked $^3$H-ACh Release and Short-Circuit Current**

Atropine had no significant effect on basal $^3$H levels (7458±880 DPM/cm$^2$) compared to vehicle-treated controls (7476±589 DPM/cm$^2$). Likewise atropine did not alter basal Isc (26±7 μA/cm$^2$, n=6) compared to vehicle-treated tissues (24±6 μA/cm$^2$, n=6).

Three periods (S1, S2, S3) of electrical field stimulation at 5 Hz, 35 V, 2 ms evoked an increase in $^3$H-ACh above basal levels in control and experimental groups (Figure 21A). Perfusion of atropine (5 μM) solutions was timed to reach the tissue at the same time that 10 μl of 1 mM atropine was injected into the submucosal media. This occurred approximately 2 min before S2. Atropine had no effect on $^3$H-ACh release during S2 or S3 (Figure 21A). This
FIGURE 21. Effect of atropine on $^3$H-ACh release and short-circuit current in guinea pig distal colon. A: effect on $^3$H-ACh release. B: effect on short-circuit current Isc). Arrow indicates perfusion of solutions with vehicle in control tissues or atropine (10 μM) in experimental tissues. Perfusion was timed to reach the submucosal bath at the same time that 5 μl of vehicle or 1 mM atropine was injected into the bath. This occurred 2 min before S2. Stimulus parameters were 5 Hz, 35 V, 2 ms. Open bars, Control; Hatched bars, Experimental; n = 7 (* p < 0.05).
contrasted to the effect of atropine on Isc which was reduced significantly during periods S2 and S3 (Figure 21B).

Additional experiments were done to investigate whether atropine would alter $^3$H-ACh release at different frequencies of stimulation. Atropine did not alter significantly the stimulus-evoked release of $^3$H-ACh (filled triangles, Figure 22A), but significantly reduced the stimulus-evoked increase in short-circuit current (filled circles, Figure 22B). The difference between the short-circuit current curves in the presence and in the absence of atropine reflects the chloride secretory response due to activation of muscarinic cholinergic receptors (Figure 22B). Approximately 50% of the maximal short-circuit current response to neural stimulation was due to an action of ACh at muscarinic receptors. Since muscarinic receptors are present on epithelial cells as well as on submucous neurons (96, 156), the secretory response must be attributed to either activation of cholinergic motor neurons, or cholinergic interneurons synaptically coupled via muscarinic synapses to motor neurons.

**Correlation of Stimulus-Evoked $^3$H-ACh Release with Short-Circuit Current**

In Figure 23, the stimulus-evoked increase in $^3$H-ACh plotted as a % above basal $^3$H levels is shown as a function
FIGURE 22. Comparison of the effect of atropine on $^3$H-ACh release and change in short-circuit current (Isc) in guinea pig distal colon. A: effect on $^3$H-ACh release. B: effect on Isc. Stimulus parameters were 35 V, 0-10 Hz, 2 ms for 2 min. ± SE are indicated by vertical bars; n = 5 at 0 and 0.5 Hz, 4 at 1 Hz, and 8 at 10 Hz; Filled tringles and open circles, Control; Open triangles and filled circles, Atropine (10 μM).
FIGURE 23. Relationship between stimulus-evoked $^3$H-ACh release and change in short-circuit current in the absence of atropine in guinea pig distal colon. Stimulus parameters were 35 V, 0-10 Hz, 2 ms for 2 min; n = 33. Correlation coefficient (r) is 0.86.
of the change in short-circuit current in the absence of atropine. The correlation coefficient was 0.86, indicating a highly significant correlation between the two parameters.

DISCUSSION:

Electrical field stimulation of submucous neurons in the guinea pig distal colon evoked an increase in short-circuit current due entirely to an increase in chloride secretion (96, 97). Since the response was attenuated by cholinergic antagonists and tetrodotoxin, cholinergic neurons were postulated to play a role in regulation of epithelial function (96, 97). The results of this study provide direct evidence for the involvement of cholinergic neurons in the micro-circuits that influence the colonic mucosa by demonstrating simultaneously short-circuit current responses associated with increases in ACh release. The demonstration of a significant correlation between stimulus-evoked short-circuit current responses and ACh released, in addition to a reduction in short-circuit current responses caused by muscarinic blockade provide strong evidence for a role of ACh in regulation of chloride secretion.

The stimulus-evoked release in $^3$H was attributed to an increase in $^3$H-ACh based on separation of radiolabeled samples by column chromatography in this study and in other
reports (168, 175, 179). The ability of tetrodotoxin, which blocks neural sodium channels and prevents action potential-dependent transmitter release, to attenuate the response to electrical field stimulation is evidence of the neuronal source of \( ^3 \text{H}-\text{ACh} \). The inability of tetrodotoxin and atropine to alter significantly basal \( ^3 \text{H} \) release and short-circuit current suggests that there is no intrinsic activity in cholinergic neurons of the submucous plexus when connections from the myenteric plexus are severed. This is consistent with previous observations (96, 97). In intact preparations, submucous neurons are likely to receive numerous synaptic inputs from tonically active neurons (171). Even though no ongoing activity was evident in our studies, a small amount of \( ^3 \text{H}-\text{ACh} \) was detected by column chromatography in basal samples treated with cholinesterase inhibitors. These observations suggest that if there is some ongoing release of ACh in the resting state, it is probably rapidly degraded and is insufficient to evoke changes in ion transport.

The enteric neurons responsible for \( ^3 \text{H}-\text{ACh} \) release were predominantly submucous neurons. The muscle-stripped preparations contained submucous neurons with intact axon varicosities in the lamina propria. The circular muscle was
not present and therefore a large source of myenteric terminals was removed. Complete elimination of all myenteric terminals was not possible, however, since some myenteric axons are present in submucous ganglia and in the mucosa and lamina propria (13, 62, 63). Nevertheless, previous reports suggested that electrical field stimulation at the stimulus strengths used in this study depolarized cell somas, and did not evoke release of neurotransmitters from fine axons that were severed from their cell somas (17). Therefore, $^3$H-ACh released by electrical field stimulation is likely to have resulted from depolarization of cell somas of submucous neurons, resulting in action potential discharge and release of neurotransmitter.

The release of $^3$H-ACh was dependent on stimulation frequency over a wide range. A significant increase in $^3$H-ACh and short-circuit current occurred at the lowest frequency of 0.5 Hz was near maximal at 10 Hz. Yau et al (180) reported that electrical stimulation of circular muscle/submucosa preparations of guinea pig ileum evoked release of $^3$H-ACh at even lower frequencies of 0.1 Hz. They did not measure Isc responses and consequently it was uncertain whether this level of ACh would have resulted in a secretory response. Precautions are necessary in making comparisons between this study and that of Yau et al. (180),
because of differences in regions of intestine studied, the differences in numbers of myenteric and submucous axon terminals present, and the use of different stimulus strengths.

The results suggest that there is a decline with time in the amount of $^{3}$H-ACh released in response to the same electrical stimulus. Stimulation at a frequency of 5 Hz resulted in $^{3}$H-ACh release which was 1.5 times larger than at the subsequent two stimulations. Whether depletion of ACh stores or desensitization is responsible for the attenuated response is unknown. The results imply that if drugs are to be used in future experiments to determine their stimulatory or inhibitory effects on electrically-evoked release of $^{3}$H-ACh, they should be administered between the second and third periods when the amount of release is comparable. This characteristic of submucous neurons was reminiscent of the responses in myenteric neurons (92).

Colonic submucous neurons displayed markedly different frequency-response characteristics from myenteric neurons (93, 180). In colonic submucous neurons, $^{3}$H-ACh release at 10 Hz was near maximal, whereas release from ileal myenteric neurons was only 14-15% of maximal (180). Frequencies of
0.1 Hz were sufficient to evoke maximal release of $^3$H-ACh from myenteric neurons. The finding that high frequencies of stimulation were necessary to evoke maximal ACh release from submucous neurons in the colon is similar to the observations of Yau et al. (180) in submucous neurons in the guinea pig ileum. At stimulus frequencies of 10 Hz, the increase in $^3$H-ACh release was estimated to be $3.8 \times 10^{-13}$ mol/g/min in submucous neurons in the colon compared with $4.2 \times 10^{-13}$ mol/g/min in the ileum (180).

North et al. (117) reported that presynaptic muscarinic receptors regulated the release of ACh in submucous neurons of the guinea pig small intestine. Although a thorough examination of the role of muscarinic autoreceptors in regulation of transmitter release from submucous neurons was not the focus of this study, results with atropine provide some initial observations. Atropine did not increase $^3$H-ACh release over a wide range of stimulus frequencies as would be expected if it prevented inhibition of release by presynaptic autoreceptors (82, 92, 93, 117). This observation raises the possibility that presynaptic muscarinic autoreceptors may not play a significant role in the synaptic circuitry regulating chloride secretion in the colon. Although our results would appear to support this conclusion, other interpretations must be considered.
Because muscarinic receptors are present on postsynaptic neurons as well as at presynaptic sites, it is possible that increased release of ACh from presynaptic neurons in the presence of atropine was masked by reduced release when muscarinic receptors on postsynaptic neurons were blocked (46, 60, 61, 96, 156).

Previous studies of $^3$H-ACh release from submucous neurons were done on strips of tissues placed in baskets, or in strips suspended between stimulating electrodes in organ baths (66, 171, 180). It was not possible to determine whether changes in $^3$H-ACh release correlated with changes in ion transport in those studies. The method of perfusing tissue in flux chambers has the advantage of a direct comparison between the two parameters. Furthermore, this technique allows an assessment of the tissue viability which was not possible in previous studies. These results demonstrate the feasibility of measuring $^3$H-ACh release simultaneously with Isc responses. They provide new information to suggest that the stimulus response characteristics of colonic submucous neurons differ from myenteric neurons in this and other regions.
INTRODUCTION

Colic, diarrhea, nausea and vomiting are symptoms often associated with allergic reactions to milk products (42). These symptoms result from type I hypersensitivity reactions triggered by mast cells when milk protein is absorbed intact by the gastrointestinal tract of sensitized animals (24, 42, 162). Bovine milk is a major source of the protein, β-lactoglobulin, which is foreign to human or guinea pig milk (1). Approximately 6-9% of ingested and absorbed β-lactoglobulin escapes cellular degradation and is transported intact across the epithelium (95, 103). The presence of the foreign protein in the lamina propria stimulates production of homocytotropic antibodies which occupy receptors on the surface of mast cells. Subsequent challenge of sensitized animals with the antigen crosslinks the antibodies and triggers degranulation of mast cells and synthesis of new mediators (24, 109, 138). Mast cell products released include biogenic amines, purines, enzymes,
chemotactic factors, proteoglycans, neutral proteases, acid hydrolases, arachidonic acid metabolites, platelet activating factor and many others (24, 42, 109, 138).

In vitro challenge of intestinal tissues from immune mice, rats and guinea pigs with ovalbumin, β-lactoglobulin or the antigens from the nematode parasites, Trichinella spiralis or Nippostrongylus brasiliensis, results in a characteristic chloride secretory response (1, 3, 23, 24, 26, 43, 125, 136). Mast cell mediators responsible for chloride secretion are histamine, prostaglandins, 5-hydroxytryptamine, platelet activating factor or others (1, 3, 23, 24, 26, 43, 125, 136).

Several mechanisms have been proposed for mast cell products to alter transport function: 1) mast cell mediators could interact with receptors on the epithelial cells to initiate chloride secretion, 2) mast cell products could act directly on enteric neurons causing release of transmitters which influence the transporting cells or 3) they could cause release of other intermediary messengers from endocrine cells or cells of the immune system which could affect epithelial or neuronal function. Evidence suggests that all three mechanisms may play a role in the secretory response during intestinal anaphylaxis (24, 42,
Submucous neurons have been implicated in mediating secretion in immune tissues after antigenic challenge. The evidence for this comes primarily from studies that demonstrate a reduction in antigen-evoked secretion by the neurotoxin, tetrodotoxin, in the mouse, rat and guinea pig (24, 42, 129). There are conflicting reports on the types of neurons involved. Indirect evidence obtained by using muscarinic antagonists implicated cholinergic neurons as mediators of the response to challenge with *Trichinella spiralis* antigen in the rat jejunum and guinea pig colon (23, 164), whereas other studies have failed to observe effects of muscarinic antagonists in the guinea pig jejunum or colon in response to either *Trichinella spiralis* antigen or β-lactoglobulin (1, 3, 24, 42, 129, 136).

The aim of this study was to determine whether cholinergic neurons in the submucous plexus mediated chloride secretion in colonic tissue from guinea pigs sensitized to β-lactoglobulin. The results provide both indirect and direct evidence to show that histamine and prostaglandins, probably released from mast cells during antigenic challenge, stimulate chloride secretion by activating cholinergic neurons. The observations suggest
communication between the immune system and the enteric nervous system in regulation of epithelial function (58, 173).

METHODS

Preparation of Submucosa/Mucosa Sheets

Two week old male albino guinea pigs of the Hartley strain (Harlan-Sprague-Dawley, Inc., Indianapolis, IN) were allowed food ad libitum. One group of non-immune animals was given water ad libitum for 3-4 weeks. In the immune group, bovine milk was substituted for water for a 3-4 week period. Three days prior to the experiments, cow's milk was removed and replaced by water. During this period, guinea pigs given bovine milk developed immunity to β-lactoglobulin (4, 95).

Guinea pigs were killed by a stunning blow to the head and exsanguination. The distal colon approximately 5-10 cm proximal to the anus was removed, opened along the mesenteric border and rinsed free of intestinal contents. The colon was pinned to Sylgard 184 encapsulating resin (Dow Corning, Midland, MI) in a culture dish and the longitudinal and circular muscle layers were removed by blunt dissection. This resulted in a flat sheet of submucosa/mucosa which was divided into 2-8 segments and mounted in flux chambers
(Figure 15). The surface area of the submucosal side was 0.78 cm². The tissues were perfused with a chilled Krebs-Ringer solution (in mM): 120 NaCl, 6KCl, 1.2 MgCl₂, 6H₂O, 1.3 NaH₂PO₄H₂, 14.4 NaHCO₃, 2.5 CaCl₂, 12.5 glucose. Solutions were maintained at 37°C and were bubbled with 95% O₂ and 5% CO₂.

The chambers were equipped with a pair of Krebs-Ringer/agar bridges connected to calomel half cells for measurement of transepithelial potential difference (PD). A pair of silver-silver chloride disk electrodes were connected to a voltage clamp apparatus that compensated for the solution resistance between the voltage-sensing bridges. The short-circuit current necessary to change the transepithelial PD by 8 mV was used to calculate tissue conductance according to Ohm's law. Tissues from the same animal were paired on the basis of similar conductances.

**Electrical Field Stimulation of Submucous Neurons**

Submucous neurons were stimulated by an electrical current which was passed from an electronic stimulator parallel to the plane of the tissue via a pair of aluminum foil ribbon electrodes juxtaposed between the flux chamber halves and the submucosal surface (Figure 12) (78, 96).
Bipolar stimulus pulses (10 Hz; 35 V amplitude (8 mA); 0.5 ms) were applied continuously for 90 s periods. Short-circuit current was recorded on a chart recorder and responses to electrical stimulation were compared to basal short-circuit current prior to the beginning of stimulation. Stimulus-evoked changes in short-circuit current were calculated by subtracting the maximum current during stimulation from basal levels.

**Uptake of $^3$H-Choline by Submucous Neurons in Flux Chambers**

The mucosal and submucosal surfaces were bathed for 30 min by 4 ml Krebs-Ringer buffer recirculated from a reservoir maintained at 37°C. The submucosal media also contained 1 μM choline chloride and 10 μCi/ml (86.7 Ci/mmol) $^3$H-choline. Submucous neurons were stimulated continuously at 1 Hz, 35 V, 2 ms for 30 min during the period of uptake of $^3$H-choline as previously described (78). Tissues were incubated for an additional 30 min in the presence of $^3$H-choline without electrical field stimulation.

After this period, each segment of tubing which connected the inner and outer ports of the flux chamber with the glass reservoir was clamped and the submucosal media containing $^3$H was discarded (Figure 15 chapter 3, horizontal bars) (78). A catheter was inserted into the
outer port in order to perfuse the submucosal surface at a rate of 1 ml/min with oxygenated Krebs-Ringer solution warmed by an heat exchanger (Figure 15C). The inner port contained a catheter for collection of samples (Figure 15E). Excess $^{3}$H-choline was washed out by perfusion for 60 min with Krebs-Ringer buffer containing 10 $\mu$M hemicholinium-3 and 1 $\mu$M choline chloride. Cholinesterase inhibitors were not present in the perfusion fluid.

Drugs were perfused at least 10 min prior to injection of $\beta$-lactoglobulin. A single concentration of $\beta$-lactoglobulin or vehicle for each immune or nonimmune tissue was injected into the submucosal compartment of the chamber and the time course of ACh release was examined. Injection of vehicle served as a control for pressure-induced changes in ACh release. One ml samples were collected continuously every min throughout the duration of the experiment. Two 1 min collections were made before each stimulus to establish basal levels of $^{3}$H release (Figure 24). This was followed by collection of 25-30 samples after addition of a single concentration of $\beta$-lactoglobulin to each tissue (Figure 24). The concentrations used to assess the concentration-response relation for $\beta$-lactoglobulin ranged between 5 nM to 20 $\mu$M.
FIGURE 24. Schematic diagram of the steps involved in ACh release studies. After 1 hr of loading with $^3$H-choline, the submucosal surface of the tissues was continuously washed and samples collected at 1 min intervals. Two basal samples were collected prior to addition of $\beta$-lactoglobulin ($\beta$-LG) or other drugs. An additional samples were collected after injection of the antigen or other drugs.
Calculation of ACh Release

Release of $^3$H-ACh was measured in the absence of cholinesterase inhibitors as the outflow of $^3$H from submucosa/mucosa preparations preloaded with $^3$H-choline (78). Radioactivity in each 0.9 ml aliquot of sample was calculated as total dpm/cm$^2$. Basal release was determined by fitting a linear regression through all basal values measured before and after addition of drug. Evoked release was measured as weights of areas under the curve of the stimulus-evoked responses and converted to dpm/cm$^2$ after comparison with known standard weights of defined areas under the curve (Figure 14, chapter 2). Results were expressed as % above average basal $^3$H or % of control. Previous studies with column chromatography have validated the release of $^3$H-ACh (78). Methods and results of chromatographic analysis are illustrated in figure 16 and are discussed in chapter 3.

Effect of Inhibitors of Mast Cell Mediators on Short-Circuit Current Response to $\beta$-Lactoglobulin

Non-cumulative concentration-response curves were generated by addition of a single concentration of $\beta$-lactoglobulin to the submucosal solutions bathing each immune or non-immune tissue. Planimetry was used to
estimate areas under the curves and to compare changes in Isc evoked by β-lactoglobulin during a 5 min period.

Experiments were designed to determine whether blockade of arachidonic acid metabolism or of histamine receptors would alter the secretory response to β-lactoglobulin. Tissues were preincubated for at least 10 min with 1 μM piroxicam, 10 μM pyrilamine or 10 μM cimetidine prior to adding 50 nM or 5 μM β-lactoglobulin. The concentrations were chosen for their ability to cause maximal suppression of histamine responses or bradykinin-induced prostaglandin release (162, 163). The results were expressed as a percent of the response to β-lactoglobulin in the absence of blocking drugs.

Effects of Tetrodotoxin and Cholinergic or Serotonin Receptor Antagonists on Short-Circuit Current Response to β-Lactoglobulin

Tissues were perfused with vehicle, 0.15 μM tetrodotoxin, 5 μM atropine, 50 μM mecamylamine or 1.7 μM ICS 205-930 at least 10 min prior to addition of 5 μM β-lactoglobulin to the serosal bathing solution. The results were expressed as a percent of the response to β-lactoglobulin in tissues without blocking drugs.
Concentrations of these drugs were chosen on the basis of their ability to cause maximal suppression of neurally-evoked secretion or of secretory responses to cholinergic agonists.

**Effects of Blocking Drugs on ACh Release in Response to β-Lactoglobulin**

Immune tissues were continuously perfused with 10 μM cimetidine, 10 μM piroxicam, 10 μM cimetidine with 10 μM pyrilamine or a combination of cimetidine, pyrilamine and piroxicam at least 10 min prior to injection of β-lactoglobulin or vehicle into the chamber. Twenty μl of β-lactoglobulin calculated to give an effective concentration of 2 μM was injected into the chamber which was continuously perfused. Consequently, in these studies the contact time of β-lactoglobulin with mast cells was less than when the antigen was continuously present in the bath. Results were normalized to control values and expressed as % of β-lactoglobulin response in vehicle treated, immune tissues.

**Effects of Histamine and prostaglandin E₂ on ACh Release**

Ten to 20 μl of histamine, or the H₂ receptor agonist, dimaprit, prostaglandin E₂ or vehicle was injected into the chamber during continuous perfusion. The concentrations of
the drugs were calculated to give an effective concentration of 5 μM or 1 mM for histamine, 10 μM for dimaprit and 0.5 μM or 5 μM for PGE₂. Samples were collected for 28-38 min or until basal release was attained. ACh release was normalized to control and expressed as a % of basal ³H.

Chemicals

Unless otherwise stated, drugs were purchased from Sigma Chemical Co. (St. Louis, MO). Mecamylamine HCl was obtained from Merck Sharp and Dohme Research Lab (Rahway, NY). Dimaprit and cimetidine were gifts from Smith, Kline and Beecham (Hertfordshire, England).

Statistics

Paired or unpaired Student's t test or one way analysis of variance followed by Neumann Keuls multiple range test was used to determine statistical significance. Values of P<0.05 were considered to be statistically significant.

RESULTS

Electrical Parameters

In 10 immune guinea pigs, basal Isc and tissue conductance averaged -23±7 μA/cm² and 11±1 mS/cm²,
respectively. In 7 non-immune animals, basal $I_{sc}$ and tissue conductance were $-22 \pm 4 \, \mu A/cm^2$ and $10 \pm 1 \, mS/cm^2$, respectively, and did not differ from that in immune animals (Table 6).

Effect of $\beta$-Lactoglobulin on Short-Circuit Current

In immune animals, addition of $\beta$-lactoglobulin to the serosal bath evoked an increase in short-circuit current within 30 s (Figure 25). Often large oscillations in short-circuit current were observed prior to decay of the short-circuit current toward baseline within 10 or 15 min (Figure 25A). Changes in short-circuit current were observed at concentrations as low as 1 nM and reached a maximum at 1-10 $\mu M$ (Figure 26). $\beta$-Lactoglobulin had no effect when administered to the submucosal bath of tissues from non-immune animals (Figure 26). Previous studies have demonstrated that the change in short-circuit current in the guinea pig colon in response to $\beta$-lactoglobulin was due to stimulation of chloride secretion, and consequently these studies were not repeated (1, 3, 43).

Effects of Blockers of Mast Cell Mediators on the Short-Circuit Current Response to $\beta$-Lactoglobulin

In immune animals, blockade of prostaglandin synthesis with either 1 $\mu M$ or 10 $\mu M$ piroxicam significantly reduced
**TABLE 6**

**ELECTRICAL PARAMETERS OF GUINEA PIG DISTAL COLON**

*(IMMUNE AND NON-IMMUNE GROUP)*

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Baseline Isc (µA/cm²)</th>
<th>Gₜ (mS/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMMUNE (10)</td>
<td>-27±7</td>
<td>11±1</td>
</tr>
<tr>
<td>NON-IMMUNE (7)</td>
<td>-22±4</td>
<td>10±1</td>
</tr>
</tbody>
</table>

Values are ± SE. Isc, short-circuit current; Gₜ, total tissue conductance. Nos. in parenthesis are no. of tissues.
FIGURE 26. Change in short-circuit current (Isc) in the colon of immune and non-immune quinea pigs in response to β-lactoglobulin. Means ± SEM are indicated by the vertical bars. Filled circles = immune; Filled triangles = non-immune. n = 3-6.
the response to β-lactoglobulin to 59±5% of control values of 204±42 μA/cm², (n=5; Figure 27A) and 59±18% of control (n=4), respectively.

In immune animals, 10 μM pyrilamine, an histamine H₁ receptor antagonist, significantly reduced the response to 5 μM β-lactoglobulin from control responses of 311±28 μA/cm², n=13 (Figure 27A). To determine whether histamine H₂ receptors were also involved in the β-lactoglobulin-induced secretory response, 10 μM cimetidine was added to the serosal bath. Cimetidine significantly reduced the 50 nM β-lactoglobulin response to 73±12% of control values of 117±25 μA/cm², n=8 (not shown).

A combination of histamine antagonists (10 μM pyrilamine plus 10 μM cimetidine) and 5 μM piroxicam was used to determine whether blockade of histamine receptors and prostaglandin synthesis simultaneously could alter the response to 5 μM β-lactoglobulin. The combined drug cocktail nearly abolished the response to β-lactoglobulin (8±3% of control responses of 158±40 μA/cm²; n=12) (Figure 27A).

**Effect of Tetrodotoxin and Cholinergic and Serotonergic Receptor Antagonists on the Short-Circuit Current Response to β-Lactoglobulin**
FIGURE 27. Effect of drugs on β-lactoglobulin-induced increase in short-circuit current expressed as a % of response in colonic mucosa of immune guinea pigs. A. Effect of 1 µM piroxicam (PIR), 10 µM pyrilamine (PYR) and 5 µM PIR, 10 µM PYR and 10 µM cimetidine (CIM) combined. n = 6, 13, 12, respectively. B. Effect of 0.15 µM tetrodotoxin (TTX), 5 µM atropine (ATR), 50 µM mecamylamine (MEC), 1.7 µM ICS 205-930. n = 6, 21, 7, 8, respectively. β-lactoglobulin concentration was 5 µM except for studies with cimetidine where it was 50 nM. (*, p<0.05).
In immune animals, 0.15 μM tetrodotoxin significantly reduced the response to β-lactoglobulin by approximately 75% from control levels of 355±48 μA/cm², n=6 (Figure 27B). This concentration was sufficient to block neurotransmitter release, because it reduced the response to electrical field stimulation to 5±0.3% of control values of 399±38 μA/cm². Blockade of muscarinic receptors with 10 μM atropine reduced the response to β-lactoglobulin, but to a lesser degree than did tetrodotoxin (Figure 27B). This concentration of atropine was maximally effective in abolishing the secretory responses to muscarinic agonists, or in reducing the response to electrical field stimulation to 29±4% of control values of 259±33 μA/cm². Atropine abolished the oscillatory changes in Isc that were superimposed on the elevated baseline current (Figure 25B).

Blockade of nicotinic receptors with 50 μM mecamylamine had no significant effect on the β-lactoglobulin response which averaged 328±38 μA/cm², n=7 (Figure 27B). This concentration of mecamylamine reduced the response to the nicotinic agonist, 0.1 mM dimethylpiperazinium, from 230±35 μA/cm² to 51±27 μA/cm² (n=7; P<0.05).

To assess the possibility that 5-hydroxytryptamine was released from myenteric axons in the submucosa in response
to β-lactoglobulin, 1.7 μM ICS 205-930 was added to the submucosal bath prior to addition of β-lactoglobulin. The 5-HT₃ receptor antagonist had no effect on the β-lactoglobulin response of 201±35 μA/cm² (Figure 27B). This concentration of ICS 205-930 was shown previously to maximally inhibit 5-HT₃ receptors on submucous neurons of the guinea pig colon (38, 59).

**Effect of β-Lactoglobulin on ACh Release**

In 12 tissues from non-immune animals, basal release of ³H was 5746±517 dpm/cm² and was similar to that in 12 immune tissues (4473±361 dpm/cm²). β-Lactoglobulin did not alter ACh release significantly in non-immune animals (Figure 28). β-Lactoglobulin evoked a concentration-dependent increase in ACh release in immune animals (Figure 28). Figure 29 illustrates a linear change in ACh release and short-circuit current, at β-lactoglobulin concentrations over a range of 5 nM to 20 μM. Changes in ACh release occurred at concentrations as low as 5 nM and reached a maximum at 10μM.

**Comparison of the Time Course of ACh Release and Short-Circuit Current in Response to β-Lactoglobulin**

In these studies, ACh release was measured simultaneously with Isc when 20 μl of β-lactoglobulin was
FIGURE 28. Effect of β-lactoglobulin on acetylcholine (ACh) release above basal in guinea pig distal colon. Means ± SEM are given by the vertical lines for 6-7 immune (filled circles) and 6 non-immune guinea pigs (filled triangle). n = 6-7 for immune and 6 for non-immune animals.
FIGURE 29. Effect of β-lactoglobulin (β-LG) on acetylcholine (ACh) release and short-circuit current (Isc) response in immune tissues. Diagram showing a linear relationship between ACh release (Y axis) and Isc (X axis) in response to β-LG (5 nM - 20 μM). ACh release is expressed as % above control and Isc in μA/cm²; n = 6-7.
injected into the perfusion chamber to achieve an effective transient concentration of 20 \( \mu M \). ACh release occurred within 30 sec of administration of \( \beta \)-lactoglobulin to immune tissues (Figure 30). It reached a peak within 2 min and then decayed toward baseline by 10 min. The time course for the Isc response closely paralleled ACh release (Figure 30). The short-circuit current values were approximately 8-10 times less than maximal responses shown in Figure 26, because of the relatively short contact time of \( \beta \)-lactoglobulin with mast cells.

**Effects of Histamine Antagonists and Piroxicam on Ach Release in Response to \( \beta \)-Lactoglobulin**

Release of ACh was examined in tissues preincubated with 10 \( \mu M \) cimetidine, or a combination of 10 \( \mu M \) cimetidine and 10 \( \mu M \) pyrilamine and compared with control tissues. Cimetidine alone, or a combination of cimetidine and pyrilamine significantly reduced the response to \( \beta \)-lactoglobulin (Figure 31). This finding implicated endogenous histamine as a chemical messenger in the anaphylactic response.

To determine if release of ACh in response to \( \beta \)-lactoglobulin was altered by blockade of prostaglandin synthesis, 10 \( \mu M \) piroxicam was added 10 min prior to
FIGURE 30. Comparison of the time course of β-lactoglobulin-induced increase in short-circuit current (Isc) and acetylcholine (ACh) release in distal colon from immune guinea pigs. Filled circles = ACh release; Open circles = Isc. Concentration of β-lactoglobulin was 20 μM. Means ± SEM are given by the vertical lines; n = 6.
FIGURE 31. Effect of cimetidine, pyrilamine and piroxicam on acetylcholine (ACh) release in distal colon of immune guinea pigs. 10 μM cimetidine (CIM, n = 5); 10 μM pyrilamine (PYR, n = 5), 10 μM piroxicam (PIR, n = 5). Combinations of CIM/PYR, N = 6; and PIR/CIM/PYR, N = 6. Means ± SEM are indicated by the vertical bars. (*, p < 0.05).
addition of antigen. Piroxicam reduced the release of ACh to 37±11% (n=5) of control values (Figure 31). Although the reduction in ACh release with piroxicam appeared to be greater than the reduction in the presence of a combination of cimetidine, pyrilamine and piroxicam, this was not statistically significant (Figure 31).

Effects of Histamine Agonists and Prostaglandin E<sub>2</sub> on ACh Release in Immune Tissues

The studies with histamine antagonists suggested that endogenous histamine was one of the mediators for the increase in ACh in response to β-lactoglobulin. Additional studies were done to determine whether exogenous histamine could evoke release of ACh. Histamine evoked a concentration-dependent increase in ACh release in immune tissues (Figure 32). Histamine at 10 μM and 1 mM evoked 71±12% and 251±69% increase in <sup>3</sup>H-ACh release above basal levels of <sup>3</sup>H of 5917±409 dpm/cm<sup>2</sup> and 4276±339 dpm/cm<sup>2</sup>, respectively (p<0.05). Associated with the increase in <sup>3</sup>H-ACh release was an increase in chloride secretion of 58±18 μA/cm<sup>2</sup> and 236±23 μA/cm<sup>2</sup> (p<0.05), respectively (Figure 33). To verify that H<sub>2</sub> receptors are involved, effect of dimaprit, an H<sub>2</sub> receptor agonist was assessed. Ten μM dimaprit evoked an increase in <sup>3</sup>H-ACh release that was
FIGURE 32. Effect of histamine, dimaprit and prostaglandin E$_2$ on acetylcholine release in distal colon from immune guinea pigs. 10 μM and 1 mM histamine (HIS, n = 7, 8); 10 μM dimaprit (DIM, n = 7); 0.5 μM and 5 μM prostaglandins E$_2$ (PGE$_2$, n = 4, 5). (*, p < 0.05).
FIGURE 33. Effect of exogenous histamine on $^3$H-ACh release and short-circuit current (Isc). Histamine evoked a concentration-dependent (10 $\mu$M and 1 mM) increase in Ach release (left panel) associated with parallel increase in short-circuit current (right panel), n = 7-8. (*, p<0.05).
482±37% above basal values of 5376±407 dpm/cm² (immune tissues) and 914.2±226% above basal values of 4812±477 dpm/cm² (non-immune tissues). This release in ACh evoked by dimaprit was greater than that evoked by histamine. The concentrations of histamine (0.1mM) and dimaprit (5 µM) have been previously shown to elicit a maximal Isc response (162, 163).

Blockade of cyclooxygenase pathways of arachidonic acid metabolism suggested that endogenous prostaglandins were involved in mediating release of ACh. To show that exogenous prostaglandins could mimic the response to β-lactoglobulin, PGE₂ was injected. PGE₂ evoked a significant concentration-dependent (0.5 µM and 5 µM) increase in ACh release (Figure 32). The increase in ACh release was associated with a parallel increase in short-circuit current of 77±6 µA/cm² and 38.2±8.2 µA/cm² (p<0.05), respectively (Figure 34).

Analysis of Histamine Stores and Release From Submucosa/Mucosa of Guinea Pig Distal Colon
Submucosal/mucosal segments of distal colon from immune and guinea pigs were examined for histamine release in response to vehicle and β-lactoglobulin and for total tissue stores (Figure 35 and 36). Analysis of supernatants from tissues
FIGURE 34. Effect of exogenous prostaglandin E₂ (PGE₂) on \(^{3}H\)-ACH release and short-circuit current (Isc). PGE₂ evoked a concentration dependent (0.5 and 5 \(\mu\)M) increase in ACh release (left panel) associated with parallel increase in short-circuit current (right panel), \(n = 4-5\). (*, \(p<0.05\)).
FIGURE 35. Histamine release from submucosa/mucosa of guinea pig distal colon (non-immune and immune) treated with vehicle (VEH) and $\beta$-lactoglobulin ($\beta$-LG). There was a significant increase in histamine release in response to $\beta$-lactoglobulin from immune tissues. (*, $p < 0.05$). Number of tissues are indicated as $n$ values.
FIGURE 36. Total tissue histamine stores from submucosa/mucosa of guinea pig distal colon (non-immune and immune animals). The histamine stores in both non-immune and immune animals were not different (mg/ng). Number of tissues are indicated as n values.
incubated with vehicle revealed a basal release of histamine in immune and non-immune animals, 0.023±0.009 ng/mg/10 min; n=22 and 0.026±0.006 ng/mg/10 min; n=16 respectively. There was a significant increase in histamine release from immune tissues treated with β-lactoglobulin (0.13±0.04 ng/mg/10 min; n=22) as compared to vehicle treated immune and non-immune controls. However the total tissue stores in both immune and non-immune animals were similar (immune, 5.3±0.7 ng/mg; n=23 and non-immune, 5.7±0.8 ng/mg; n=11).

These results suggest that histamine release is significantly increased in immune animals in response to antigen β-lactoglobulin.

**Histological Examination of Mast Cells and Their Distribution in Submucosal Layer of Guinea Pig Distal Colon**

Mast cells in the submucosal layer of the guinea pig colon were visible with alcian blue staining. The staining appeared less intense in the immune animals as compared to that of the non-immune controls. The average distribution of the mast cells per mm$^2$ of the immune tissues was 22.0±3.0 mm$^2$ (n=9), and non-immune tissues was 25.0±5.0 mm$^2$ (n=9) (Table 7). These numbers of mast cells in two groups of animals were statistically not different.
<table>
<thead>
<tr>
<th>Tissues</th>
<th>n</th>
<th>Mast Cells / mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Immune</td>
<td>9</td>
<td>25.0 ± 5.0</td>
</tr>
<tr>
<td>Immune</td>
<td>9</td>
<td>22.0 ± 3.0</td>
</tr>
</tbody>
</table>
DISCUSSION

Our studies demonstrate that endogenous histamine is one of the mediators of the secretory response in immune animals challenged with β-lactoglobulin. The source of histamine is likely to be sensitized mucosal mast cells which release their products in response to the immunologic stimulus, β-lactoglobulin (1, 3, 4, 43, 129). The role of histamine in anaphylactic responses is supported by the observation that histamine antagonists significantly reduced, but did not abolish, the secretory response. The ability of pyrilamine to reduce the Isc response to β-lactoglobulin confirms previous reports that implicated H₁ receptors as mediators of secretion (1, 42, 43). Our findings extend previous reports by suggesting an involvement of H₂ receptors as well. This conclusion is based on the ability of cimetidine to reduce the Isc response to β-lactoglobulin in sensitized animals. It is unlikely that the concentrations of antagonists used had non-specific effects based on previous reports from this and other laboratories (33, 162, 163). Studies from our laboratory have established in non-immune guinea pigs that histamine acts at H₁ receptors to mediate a transient increase in chloride secretion and at H₂ receptors to induce cyclical chloride secretion by activating submucous
neurons which project to the epithelium (162, 163).

These studies provide definitive evidence that prostaglandins is another mediator of the secretory response to β-lactoglobulin. This is supported by the observation that piroxicam reduced, but did not abolish, the secretory response. Since piroxicam is a specific inhibitor of cyclooxygenase without effects on phospholipase, thromboxane or prostacyclin synthetase or lipoxygenase steps, its ability to attenuate the β-lactoglobulin-induced Isc provides strong evidence for the involvement of prostaglandins as mediator (21, 163).

Our observations are consistent with the reports that histamine and prostaglandins are involved in anaphylactic responses in Trichinella spiralis parasitized hosts (23, 24, 136). The conclusion that prostaglandins mediate changes in Isc in response to β-lactoglobulin confirms the reports of Cuthbert et al. (43). Despite their reports that the β-lactoglobulin-induced secretory response could be completely abolished with prostaglandin synthesis inhibitors, this was not found in our studies. Therefore our results suggest the possibility that additional mediators such as histamine are released from mast cells or other cells in the lamina propria.
Both direct and indirect evidence suggests that mast cell mediators induce secretion in part by activation of cholinergic neurons. Our results are the first to demonstrate the release of ACh from submucous neurons in response to β-lactoglobulin in guinea pigs sensitized to bovine milk and to show a correlation with the time course of secretion. The finding that ACh release so closely paralleled the time course of the Isc response to β-lactoglobulin suggests that ACh is an important neurotransmitter mediating the tetrodotoxin-sensitive portion of secretion during anaphylaxis. Furthermore, the ability of histamine antagonists and blockers of prostaglandin synthesis to reduce ACh release after β-lactoglobulin challenge provides evidence for the involvement of both histamine and prostaglandins in this response. This was substantiated by demonstrating an increase in ACh release in response to exogenous application of either histamine analogs or PGE₂.

Activation of cholinergic neural pathways in this response was further substantiated by the findings that atropine significantly reduced the Isc response to β-lactoglobulin. This contrasts to the observation that blockade of nicotinic receptors did not alter the β-lactoglobulin response. The results suggest that nicotinic
transmission is not necessary for transfer of information to the epithelium in type I hypersensitivity reactions. This finding in milk sensitized animals is similar to our findings in the guinea pig sensitized to *Trichinella spiralis* antigen (164). The implications of these findings are: 1) Mast cell mediators act directly on motor neurons to release acetylcholine which interacts with muscarinic receptors on the epithelium to initiate chloride secretion, or, 2) they activate interneurons that are synaptically coupled via muscarinic, peptidergic or serotonergic synapses with motor neurons. The presence of muscarinic receptors on neurons and epithelial cells precludes distinguishing between these two possibilities (96).

It is possible that peptidergic neurons also mediate the secretory response to β-lactoglobulin. Evidence for this conclusion comes from the findings that the reduction in the secretory response by atropine was less than that by produced by tetrodotoxin. Identification of the peptidergic neurotransmitter was outside the scope of this study. However, consistent with the possibility that peptidergic neurons are involved in secretory responses to β-lactoglobulin are the studies of Stead et al. (145). They reported that neurons containing substance P/calcitonin
gene-related peptide were in intimate contact with mucosal mast cells in normal and nematode-infected rat intestine (145). Other studies have suggested that extrinsic afferent neurons containing substance P/calcitonin gene-related peptide may be necessary to elicit the full secretory response to ovalbumin in sensitized rats (42).

It is unlikely that mast cell mediators stimulate serotoninergic neurons which are known to be synaptically coupled to cholinergic neurons in the guinea pig colon. This conclusion is based on the failure of ICS 205-930, a 5-HT₃ receptor antagonist, to reduce the short-circuit current response to β-lactoglobulin. Failure of the antagonist to reduce the β-lactoglobulin response could not be attributed to insufficient blockade of 5-HT₃ receptors, because this concentration was shown to abolish nearly completely the response to 10 μM 5-hydroxytryptamine in the guinea pig colon (59). Our results contrast to those of Baird and Cuthbert (3) who reported that ICS 305-930 inhibited the anaphylactic response to β-lactoglobulin. They did not measure 5-hydroxytryptamine release directly to confirm the involvement of serotoninergic neurons. Reasons for differences between the two studies are unclear.

The results emphasize the importance of cholinergic neurons in mediating chloride secretion during intestinal
anaphylaxis. They suggest that interactions between the immune system and the enteric nervous system underlie intestinal malaise associated with food allergies.
CHAPTER V

NEURAL PATHWAYS MEDIATING THE CHLORIDE SECRETORY RESPONSE TO LEUKOTRIENE D₄ IN GUINEA PIG DISTAL COLON

INTRODUCTION

In the gastrointestinal tract, mast cells occur in the epithelium, mucosa, submucosa, muscle layers and serosa (109). Activation or degranulation of mast cells by immunologic and non-immunologic stimuli has long been associated with immediate type I hypersensitivity reactions initiated when appropriate antigen binds to and cross links the homocytotropic antibodies on the surface of mast cells. This triggers the release of preformed mediators stored in secretory granules and synthesis of newly formed mediators (109, 136, 138). Products released include biogenic amines, purines, lysosomal enzymes, chemotactic factors, proteoglycans, neutral proteases, acid hydrolases, arachidonic acid metabolites, platelet activating factors and many others (109, 136, 138). Among newly generated mediators, arachidonic acid metabolites play a major role in both local and systemic anaphylaxis and inflammation. These metabolites are generated by both cyclooxygenase and
lypoxigenase pathways resulting in prostaglandins, thromboxanes, and prostacyclins as well as hydroperox-eicosatetraenoic acid (HPETEs), hydroxyeicosatetraenoic acid (HETEs) and leukotrienes (LTS) which appear to be involved in various pathological processes, such as inflammation, asthma and other hypersensitivity reactions (12, 15, 55, 124, 137). Biological activities of leukotrienes have been explored in several in vivo and in vitro systems.

Enhanced synthesis of leukotrienes have been reported in response to antigen challenge and calcium ionophore A23187 exposure (114, 115, 140, 181). Tissue levels of this lipid are increased in inflammatory bowel disease (140, 181). The role of peptidoleukotrienes LTC₄, LTD₄ and LTE₄ in mediating the inflammatory response is not clearly understood. Only a few studies have examined the role of leukotrienes on intestinal ion transport. Sulfidopeptide leukotrienes LTC₄, LTD₄ and LTE₄ stimulate electrogenic chloride secretion in ileum, and colon of rats and rabbits and inhibit sodium and chloride absorption in rat ileum (80, 142, 143). Leukotriene C₄ stimulates chloride secretion in rabbit ileum by a mechanism that is independent of its metabolism to LTD₄ and LTE₄.

The effects of the sulfidopeptide leukotrienes are mediated by distinct LTD₄/LTE₄ and LTC₄ receptors on
intestinal epithelial cells (143). There are significant species differences in effects of leukotrienes. The purpose of this study was to examine the effects of LTD₄ on ion transport in guinea pig colon. Furthermore, an additional aim was to determine whether endogenous leukotrienes mediate secretion during intestinal anaphylaxis.

The results suggest that LTD₄ plays a neuromodulatory role to augment cholinergically-mediated chloride secretion in both immune and non-immune animals.

METHODS

Animals

Male albino guinea pigs (Hartley Strain, Harlan-Sprague Dawley, Indianapolis, IN), approximately 14 days old, were allowed food ad libitum. One group of age-matched control animals was given water to drink throughout for 3-4 week period. The second group was given pasturized cow's milk to drink in place of water for 3-4 weeks. At least 3 days before the experiment, water was substituted for milk. Guinea pigs given milk develop serum antibodies to β-lactoglobulin indicative of immunity to the protein (4, 95).

Guinea pigs were killed by a stunning blow to the head and exsanguination. The distal colon 5-10 cm proximal to
the anus was removed, opened along the mesenteric border, and rinsed free of intestinal contents with buffer solution. The longitudinal and circular muscle layers were removed by blunt dissection to give sheets of submucosa/mucosa, which were mounted in Ussing flux chambers (area 0.78 cm²) (Figure 37). The mucosal and serosal sides were bathed separately with 10 ml of Krebs/Ringer buffer with 0.1% gelatin. The solution was maintained at 37°C, gassed with 95% O₂, 5% CO₂ and buffered at pH of 7.2 - 7.4. The composition of the solution in mM was 120 NaCl, 6 KCl, 1.2 MgCl₂.6H₂O, 1.3 NaH₂PO₄O, 14.4 NaHCO₃, 2.5 CaCl₂.2H₂O and 10 - 12.7 glucose. All recirculating glass reservoirs and flux chambers were washed with alcohol and coated with gelatin before each experiment.

**Electrical Measurements**

Transepithelial electrical potential difference (PD) was measured with a pair of Krebs/Ringer agar bridges connected to calomel half-cells. A pair of Ag/AgCl disk electrodes were connected to a voltage clamp that compensated for the solution resistance between the PD sensing bridges by delivering a short-circuiting current that nulled out the spontaneous PD (Figure 37). The short-circuit current necessary to change the transepithelial PD
FIGURE 37. Schematic diagram of a conventional flux chamber with water-jacketed, bubble lift perfusion system for in vitro studies on the intestine. Buffer solutions were gassed with 95% O₂ and 5% CO₂. (a,b) Mucosal and submucosal halves; (c) Flat sheets of colon; (d) Water-jacketed perfusion chambers; (e) Ringer/agar bridges and calomel electrodes for measurement of transmural potential difference (PD); (f) Silver/silver chloride disk electrodes for passing short-circuit current across the tissues.
by 8 mV was used to calculate tissue conductance according to Ohm's law. Tissues from the same animal were paired on the basis of similar conductances.

Submucous neurons were electrically stimulated by passing current parallel to the plane of the tissue via a pair of aluminum foil ribbon electrodes placed between the flux chamber halves and the submucosal surface. Bipolar rectangular stimulus pulses of 0.5 ms duration, 10-35 V (7-8 mA) amplitude, and 10 Hz frequency were applied before adding a drug to the serosal side. The change in Isc after electrical field stimulation or addition of drug was recorded on a chart recorder and compared to basal Isc before the start of stimulation. Drugs were always added to the serosal surface of the preparation.

**Experimental Protocols**

After a 45 minute equilibration period, submucous neurons were electrically stimulated once for 90 seconds at maximal stimulus parameters (35 V, 10 Hz, 0.5 ms) or twice at submaximal stimulus parameters (10 V, 10 Hz, 0.5 ms). Ten or 15 minutes later, three different concentrations (0.15, 0.5, and 1 μM) of leukotrienes were added to different tissues.
In studies to determine the effects of 0.1 μM tetrodotoxin, 5 μM atropine or 5 mM bumetanide, at least 10 - 15 minute after the last period of electrical field stimulation, each of the drugs was added to a separate tissue. Fifteen minutes later, 0.5 μM leukotriene D₄ or vehicle was added to the serosal bath and this was followed by another period of electrical field stimulation. Prior to the end of the experiment 10 μM carbachol was added to the serosal bath. The responses to leukotriene D₄ were analysed as the peak response during a five minute period.

Epithelial Cell Cultures

Epithelial cells from the T₈⁴ human colonic carcinoma cell line were obtained at passage 41 from Dr. Michael J. Welsh (Howard Hughes Medical Institute and Department of Medicine, University of Iowa, IA). Cells from passages 43-45 were used. T₈⁴ cells were maintained in culture as described previously (45). Cells were plated at a density of 3.5 x 10⁵ cells on Millicell-HA (0.45 μm, 12 mm diameter) culture plate inserts (Millipore Co., Bedford, MA). Inserts containing confluent monolayers (7-8 days after plating) were mounted in modified Ussing chambers which had been washed with alcohol and siliconised. Cells were bathed on
mucosal and serosal sides with 5 ml oxygenated Krebs/Ringer solution at 37°C. Either 1 μM leukotriene D₄ or vehicle were added to the serosal side. After 10 minutes 100 μM carbachol was added to the serosal side followed by 1 μM prostaglandin E₂.

**Chemicals**

Unless otherwise stated, drugs were purchased from Sigma Chemical Co. (St. Louis, MO). Leukotriene antagonist SK&F 102922 was obtained from Smith, Kline and Beecham (Welwyn Garden City, UK).

**Statistics**

Paired or unpaired Student's t test or one-way analysis of variance followed by Newman-Keuls multiple range test was used to determine statistical significance. Values of p<0.05 were considered to be statistically significant.

**RESULTS**

**Basal Electrical Parameters**

For tissues set up in Ussing flux chambers short-circuit current and total tissue conductance were recorded throughout the experiments to monitor the tissue viability. Basal Isc and tissue conductance were -40±3.7 μA/cm² and
9.9±0.8 mS/cm² (n = 39), respectively. These values are well within the range of values reported previously for guinea pig colon (14).

**Effect of Leukotriene Antagonist SK&F 102922 on \( \beta \)-Lactoglobulin-Induced short-circuit current Response**

Mediators released from mast cells in immune animals challenged with \( \beta \)-lactoglobulin evoked an increase in short-circuit current to 284±52.4 \( \mu \)A/cm² (n = 6). This \( \beta \)-lactoglobulin-induced short-circuit current response was significantly reduced to 210±65.2 \( \mu \)A/cm² or 63% of control (n = 6) after addition of 25 \( \mu \)M SK&F 102922, a leukotriene antagonist (P<0.05). This suggests that endogenous leukotrienes are released from mast cells in response to antigen challenge (Figure 38).

**Effect of Exogenous and Endogenous LTD₄ on short-circuit current Response in Guinea Pig Distal Colon**

In colonic tissues from both non-immune and immune animals, the response to LTD₄ appeared to vary depending on the level of ongoing activity. Consequently, the effects of LTD₄ were analysed according to the presence or absence of ongoing neural activity. Ongoing neural activity was
FIGURE 38. Effect of leukotriene, antagonist on β-lactoglobulin-induced short-circuit current (Isc) response. Beta-lactoglobulin induced Isc response in immune animals was reduced to 63% of control after addition of 25 μM SK&F 102922 (n = 6) (*, P < 0.05). Control indicates maximum β-lactoglobulin response. This suggests that leukotrienes mediate, in part, secretory response when mast cell mediators are released.
defined as oscillations in baseline short-circuit current at least 10-12 μA/cm² or more which were abolished by 0.1 μM tetrodotoxin. Figure 39 illustrates a typical short-circuit current tracing in two groups of animals: A) Non-immune or immune, no ongoing activity (Figure 39A); B) Non-immune or immune, ongoing activity (Figure 39B). Addition of LTD₄ evoked an increase in short-circuit current that was greatest in amplitude in tissues with ongoing neural activity (Figure 39B).

**Effect of LTD₄ on Short-Circuit Current in Immune and Non-Immune Guinea Pig Distal Colon**

In tissues with no ongoing activity, the short-circuit current responses to increasing concentrations of LTD₄ were relatively small (Figure 40A). Responses were similar in non-immune and immune animals at 0.15 μM leukotriene D₄, but were statistically different at higher concentrations (p<0.05).

In tissues with ongoing neural activity, leukotriene D₄ evoked larger changes in short-circuit current in both non-immune and immune animals (Figure 40B) than in those without ongoing activity (Figure 40A). There was a trend for a greater short-circuit current response to 0.15 μM leukotriene D₄ in immune tissues, but this was not
FIGURE 39. A representative trace of short-circuit current (Isc). Effect of LTD₄ on tissues with no-ongoing activity (-) and with ongoing activity (+). A. When there is no ongoing activity (no change in basal Isc), addition of LTD₄ causes a small Isc response. B. In presence of ongoing activity (change in basal Isc) addition of LTD₄ causes a significantly higher Isc response.
FIGURE 40. Effect of LTD₄ on short-circuit current (Isc). A. In tissues with no ongoing activity (−), the Isc responses to increasing concentrations of LTD₄ (0.15 - 1 μM) were relatively small. B. In tissues with ongoing activity (+), LTD₄ evoked larger changes in Isc in both non-immune and immune animals than in those without ongoing neural activity (n = 5-6; *, P < 0.05).
statistically different from responses in non-immune animals. Higher concentrations of LTD\textsubscript{4} evoked larger responses in immune tissues compared to non-immune tissues.

**Effect of Leukotriene Antagonist SK&F 102922 on Exogenous LTD\textsubscript{4} Induced Short-Circuit Current Response**

In immune tissues with ongoing activity, exogenous 0.15 \(\mu\text{M} \) LTD\textsubscript{4} evoked an increase in short-circuit current to 42±10 \(\mu\text{A/cm}^2\) \((n = 13)\). This response was significantly reduced to 15±4 \(\mu\text{A/cm}^2\) \((n = 13)\) \((P<0.05)\) when the tissues were pretreated with the leukotriene antagonist SK&F 102922 \((25 \mu\text{M})\) (Figure 41).

**Effect of Bumetanide on LTD\textsubscript{4}-Induced Changes in Short-Circuit Current**

Leukotriene D\textsubscript{4} \((0.5 \mu\text{M})\) increased short-circuit current by 86±23 \(\mu\text{A/cm}^2\) in immune animals with ongoing neural activity. Bumetanide \((50 \mu\text{M})\), known to block chloride uptake by the basolateral Na/2Cl/K cotransporter, significantly increased baseline short-circuit current by 54±9 \(\mu\text{A/cm}^2\) \((n = 7)\) as reported previously \((14)\). Bumetanide significantly reduced the LTD\textsubscript{4} response to 25±10 \(\mu\text{A/cm}^2\) \((n = 6; P<0.05)\) (Figure 42). The reduction in LTD\textsubscript{4}-induced short-circuit current response was attributed to a
FIGURE 41. Effect of SK&F 102922 (leukotriene antagonist) on LTD$_4$ response expressed as change in short-circuit current ($I_{sc}$). Leukotriene D$_4$ (0.15 $\mu$M) induced an increase in $I_{sc}$ (control) which was significantly reduced by pretreatment of the tissues with 25 $\mu$M SK&F 102922 (n = 13; *, $P < 0.05$).
FIGURE 42. Effect of bumetanide on LTD$_4$-induced short-circuit current (Isc) response. Leukotriene D$_4$ (0.5 µM) evoked an increase in Isc response (control) which was significantly reduced by bumetanide (5 mM) (n = 6; *, P < 0.05). This reduction was attributed to reduction in chloride secretion.
reduction in chloride secretion.

**Effect of Electrical Field Stimulation of Submucous Neurons on Chloride Secretion**

Electrical field stimulation of submucosa/mucosa preparations evoked an increase in short-circuit current that was nearly completely abolished by tetrodotoxin in this and previous studies (97).

The response to neural stimulation was shown to be due entirely to an increase in chloride secretion (97). Magnitudes of the maximal neurally-evoked secretory responses were compared in non-immune \((n = 42)\) and immune \((n = 36)\) tissues (Figure 43). Each group was further divided on the basis of presence or absence of ongoing neural activity. Immune animals were characterized by a greater neurally evoked secretory rate than non-immune animals irrespective of the presence of ongoing neural activity (Figure 43). In both non-immune and immune animals, the stimulus-evoked secretory rate was greater in tissues with ongoing activity (Figure 44).

Because the secretory rates were higher in non-immune and immune animals with ongoing neural activity, the effects of \(\text{LTD}_4\) on short-circuit current were reexamined, and the results were normalized to the maximal chloride secretory
FIGURE 43. Effect of electrical field stimulation (EFS) of submucous neurons on chloride secretion (immune vs. nonimmune). Immune animals (n = 63) had a larger neurally-evoked secretory rate than did nonimmune (n = 42) animals irrespective of the presence of ongoing neural activity, (*, P < 0.05 for immune animals). Nonimmune = open bar. Immune = hatched bar.
FIGURE 44. Comparison of effect of electrical field stimulation (EFS) of submucous neurons in tissues with no ongoing activity vs. tissues with ongoing activity. In both non-immune and immune animals the stimulus evoked secretory rate was greater in tissues with ongoing neural activity for number of tissues indicated. (*, p < 0.05).
response. When the results were normalized, similar trends as described for non-transformed short-circuit current were observed. In general immune and non-immune tissues with ongoing neural activity exhibited greater responses to LTD$_4$ than did tissues that had no ongoing activity.

Thus 1 μM LTD$_4$ evoked an increase in short-circuit current that was 34±9% of the maximal neurally-evoked response in non-immune animals with ongoing activity and 41±9% in immune animals with ongoing activity. This contrasted to only 3.4±0.7 - 6.9±1.3% of the neurally evoked response in non-immune and immune animals, respectively, with no ongoing activity (Figure 45, 46).

**Effects of Tetrodotoxin and Atropine on LTD$_4$-Evoked Response**

In immune tissues with ongoing activity, 0.5 μM LTD$_4$ evoked an increase in short-circuit current (Figure 47). Atropine and tetrodotoxin reduced, but did not abolish, the response by 67% and 81% respectively. In the same tissues, atropine and tetrodotoxin reduced the neurally-evoked response to electrical field stimulation by 44%, and tetrodotoxin by 94% (Figure 47).
FIGURE 45. Effect of LTD$_4$ on short-circuit current (Isc), immune tissues expressed as percent of electrical field stimulation (EFS). Tissues with ongoing neural activity exhibited greater responses to LTD$_4$ (0.15 - 1 µM) in both immune and non-immune animals. Data is expressed as percent of maximal secretory response (EFS) (n = 5-6; *, p < 0.05)
FIGURE 46. Effect of LTD$_4$ on short-circuit current (Isc), in nonimmune tissues expressed as percent of electrical field stimulation (EFS). Tissues with no ongoing neural activity exhibited much smaller responses to LTD$_4$ (0.15 - 1 μM) as compared to tissues with ongoing neural activity. Data is expressed as percent of maximum secretory response (EFS) (n = 5-6; *, p < 0.05).
FIGURE 47. Effects of tetrodotoxin (TTX) and atropine on LTD4-evoked short-circuit current (Isc) responses. In immune tissues with ongoing neural activity, LTD4 (0.5 μM) evoked an increase in Isc. This response was significantly reduced, but not abolished by atropine and tetrodotoxin by 67% and 81% respectively (n = 5-6, *, P < 0.05).
Effect of Blocking Ongoing Neural Activity on Carbachol-Induced Short-Circuit Current in the Presence and Absence of LTD₄

Carbachol (50 μM) evoked an increase in Isc in immune tissues with ongoing activity. When tissues were pretreated with 0.15, 0.5 and 1 μM LTD₄, carbachol evoked an increase in short-circuit current that was 2 times greater than in its absence (Figure 48). To investigate further if the amplification of the carbachol response, was due to release of neurotransmitters by LTD₄, tetrodotoxin was added to block ongoing neural activity and release of neurotransmitters. If LTD₄ was added first, carbachol evoked a greater increase in short-circuit current as shown before (Figure 49, LTD₄). When LTD₄ was present, addition of tetrodotoxin did not prevent the augmented carbachol response (Figure 49, LTD₄ + TTX). However, if tetrodotoxin was added prior to LTD₄, the augmentation of the carbachol response was reduced (Figure 49, TTX + LTD₄) compared to tissues in which tetrodotoxin was added after LTD₄. Furthermore the carbachol response in tissues in which tetrodotoxin was added prior to LTD₄ was similar to the response to carbachol in tetrodotoxin treated tissues (Figure 49, TTX + LTD₄ compared to TTX + Vehicle). These results suggest that leukotriene D₄ enhanced the carbachol
FIGURE 48. Effect of LTD$_4$ on carbachol response in immune animals. Carbachol 50 µM evoked an increase in short-circuit current (Isc) response in immune tissues with ongoing activity. In tissues pretreated with LTD$_4$ (0.15 - 1 µM), carbachol evoked an increase in Isc that was two fold greater than in absence of LTD$_4$ (VEH) (n = 6; * p < 0.05).
FIGURE 49. Amplification of carbachol response by LTD₄ in presence and absence of tetrodotoxin. When tissues were pretreated with tetrodotoxin to block the on-going neural activity and release of neurotransmitters, LTD₄ (0.5 μM) did not significantly enhance the carbachol (50 μM) response. However, if tetrodotoxin was added after LTD₄ already had its effect, the amplified carbachol response was enhanced (LTD₄ + TTX) and LTD₄) (n = 5-7; *, p < 0.05).
response by releasing a neurotransmitter which amplified the carbachol response.

**Effect of Leukotriene D₄ on Carbachol and Prostaglandin E₂-**
**Evoked Changes in Short-Circuit Current in T₈₄ Cells**

T₈₄ cells were used to determine whether LTD₄ had direct effects on epithelial cells to amplify carbachol and prostaglandins responses. LTD₄ 1 μM did not alter short-circuit current in 10 monolayers (1.7±0.7 μA/cm²) compared to 18 vehicle-treated controls (1.4±0.7 μA/cm²). Likewise, there was no change in tissue conductance with LTD₄, and vehicle treated monolayers.

Carbachol 0.1 mM increased Isc by 27.2±4.6 μA/cm² (n = 10) in the presence of LTD₄. This response was not different from an increase of 28.7±6.2 μA/cm² (N = 18) evoked by carbachol in the absence of LTD₄ (Figure 50). Prostaglandin E₂ evoked an increase in short-circuit current of 41±4.0 μA/cm² in monolayers treated with LTD₄, and 35.8±4.9 μA/cm² in vehicle treated monolayers.

**DISCUSSION**

The results suggest that sulfidopeptide leukotrienes are one of the mediators of secretory response during intestinal anaphylaxis. Evidence for this comes from the
FIGURE 50. Effect of carbachol on LTD$_4$ treated T$_{84}$ colonic cell monolayers. LTD$_4$ did not alter the carbachol (100 μM)-induced increase in short-circuit current (Isc) (n = 8). (Control = carbachol response in untreated monolayers of T$_{84}$ cells).
observation that leukotriene antagonists reduced the secretory response evoked by immunologic challenge of milk-sensitized colonic tissue. Previous studies have shown that prostaglandins and histamine are other key mediators of the anaphylactic-induced secretory response (43, 79, 136, 164). The mechanisms by which leukotrienes mediate secretion were investigated in both immune and non-immune animals. The results indicate that leukotrienes may either have direct effects on epithelial cells as well as modulating neural activity or may release an intermediary messenger from other cells in the submucosa.

Even during neural blockade, leukotrienes still evoked small response. Although the failure of leukotrienes to evoke secretion by T_{84} would argue against direct effects on epithelial cells, other interpretations are possible. Since T_{84} cells are derived from a human colon carcinoma, they may have differentiated and lost their receptors. Their membrane receptors may be different from those in the guinea pig colon.

The change in Short-circuit current evoked by leukotriene is due to chloride secretion. The response was relatively transient and precluded measurement of ionic fluxes. Nevertheless, the ability of bumetanide which blocks the sodium/potassium/2 chloride cotransporter, to
reduce the leukotriene response is suggestive of chloride secretion. It is likely that leukotrienes modulate spontaneous activity of submucous neurons.

In colonic tissues from both immune and non-immune animals the response to LTD$_4$ appeared to vary depending on the level of spontaneous ongoing activity. The ongoing activity was reflected by oscillatory changes in Short-circuit current and were abolished by atropine and tetrodotoxin, suggesting their neuronal origin.

The amplitude of short-circuit current responses evoked by LTD$_4$ were greater in tissues with ongoing neural activity in both immune and non-immune animals than in tissues without such activity. These observations suggested that the level of ongoing activity is a major determinant of the magnitude of the leukotriene-evoked change in short-circuit current. The results also suggest that tissue responsiveness to leukotriene is enhanced in immune animals compared to non-immune animals regardless of the level of ongoing neural activity. This implies that there already may be an increased release of mast cell mediators present in sensitized tissues. Support for this comes from studies in cow's milk sensitized guinea pigs where there was increase in the spontaneous firing patterns in sensitized
tissues compared to non-immune tissues (57). Approximately 33% of neurons were spontaneously active compared to only 5% in non-immune controls. Although basal short-circuit current was not altered in the two groups, this may reflect lack of synchronous firing of neurons (57). Thus leukotrienes seem to be more effective in immune than in non-immune animals.

Another interesting aspect of tissue responsiveness was demonstrated in experiments with carbachol. If neural blockade occurred prior to administration of leukotrienes, the carbachol induced secretory response was small. However, if leukotrienes were administered first followed by tetrodotoxin, then the carbachol-induced secretory response was much greater. This observation suggests that leukotrienes stimulate neurons to release neurotransmitters that interact synergistically with carbachol to augment secretion. Whether this is a direct effect of leukotrienes on neural receptors or whether it is due to release of an intermediary messenger is uncertain. Smith et al. suggested that leukotrienes released prostaglandins in the rabbit colon (142). This was not tested in our study.

Our results with tetrodotoxin are consistent with studies of Bloomquist and Kream who have shown LTD_{4} responses in guinea pig ileum to be partially blocked by tetrodotoxin (12).
Their studies suggest that effects of tetrodotoxin are due to inhibition of release of substance P in response to stimulation with LTD$_4$ (12). Others have demonstrated the role of indomethacin sensitive pathway for LTD$_4$, and LTB$_4$ responses in rabbit distal colonic muscularis mucosae and distal colonic motility (15, 124). They suggest the direct effect of these leukotrienes on the specific receptors.

In summary, the results of the present study demonstrate that LTD$_4$ augments chloride secretion by increasing cholinergic neural activity, rather than by acting directly on epithelial cells. It further stresses that the level of ongoing neuronal activity is the major determinant of the magnitude of the changes in Isc responses by LTD$_4$. The fact that the β-lactoglobulin evoked increase in Isc was reduced significantly by SK&F 102922 a LT antagonist supports the role for endogenous peptidoleukotrienes together with other mediators in mediating colonic chloride secretion during anaphylaxis.
CHAPTER VI
SUMMARY AND CONCLUSION

A number of diseases involving the gastrointestinal tract are thought to be caused by immunologically mediated reactions to food antigens. Food antigens are the most common antigens encountered by the gastrointestinal tract. The intestinal uptake of food antigens is the important initial step in the development of wide range of food sensitive enteropathies. Colic, diarrhea, nausea and vomiting are the symptoms associated with allergic reactions to many of the food antigens which are manifested as type one hypersensitivity reactions. This local hypersensitivity occurs in susceptible individuals when antigens are transported across the intestinal epithelium in an intact form in sufficient quantities.

Among food products, bovine milk constitutes a major source of dietary proteins for humans. Milk proteins include $\beta$-lactoglobulin which is foreign to human and guinea pig milk. Although it comprises only 10% of the total milk proteins it is the most antigenic and heat resistant protein. Small amounts (6-9%) of $\beta$-lactoglobulin, which
escapes cellular degradation, are transported intact through the epithelial cells via a transcellular pathway into the lamina propria (103). Within the lamina propria, the presence of foreign protein stimulates the production of homocytotropic antibodies (IgE and IgG subclass), from B lymphocytes with the help of T lymphocytes. The antibodies bind to the receptors on the surface of mast cells. On subsequent exposure to the antigen the antibodies are crosslinked and degranulation of mast cells occur with release of chemical mediators which can alter fluid and electrolyte transport in the gut. Very little is known about the specific mechanisms responsible for the alteration in transport function. Because mast cells appear to be associated with neurons within the intestinal wall, it is likely that the mediators released from mast cells stimulate neurons which release transmitters that effect the absorbing and secreting cells or they can act directly on the epithelial cells (Figure 51).

Several studies have provided indirect evidence for involvement of the enteric nervous system in immune regulation of epithelial ion transport. The evidence for this primarily comes from studies that demonstrate a reduction in antigen-evoked intestinal secretion by the neurotoxin, tetrodotoxin (24, 41, 42, 129). Most of these
FIGURE 51. A model of proposed mechanism for type I hypersensitivity reactions in guinea pig colon. (1) When guinea pigs are fed with cow's milk, most of the β-lactoglobulin (β-LG) is degraded within the epithelial cells. Small amount (6-9%) of β-LG escapes the degradation and is absorbed intact across the intestinal epithelium; (2) The presence of β-lactoglobulin in the lamina propria triggers an immune response causing antibody production (IgG) by B-lymphocytes with the help of T-lymphocytes; (3) Antibodies occupy receptors on the mast cells leading to sensitization; (4) On subsequent exposure to the antigen β-LG results in crosslinkage of the antibodies; (5) This results into mast cell degranulation and release of mediators; (6) Mast cell mediators can act on neurons (a) or directly on epithelial cells (b); (7) Chloride (Cl) secretion results from either direct actions of mast cell mediators, or in response to neurotransmitters. Acetylcholine = ACh.
studies involved tissues from mice, rats and guinea pigs sensitized to ovalbumin, milk protein (β-lactoglobulin) or the antigens from the nematode parasites, Trichinella spiralis or Nippostrongylus brasiliensis (1, 3, 23, 24, 41, 42, 136). In these studies there are conflicting reports on the types of neurons involved during anaphylactic reactions. Results provide indirect evidence for cholinergic neuronal involvement by the use of muscarinic antagonists (23, 164). Some studies failed to observe effects of muscarinic antagonists in the guinea pig jejunum and colon in response to either β-lactoglobulin or Trichinella spiralis antigen (1, 3, 24, 42, 129, 136) and this suggests that non-cholinergic neurons may be involved as well.

Most of the earlier studies have used indirect methods to implicate involvement of cholinergic neurons in immune responses. As a first step to provide more direct evidence for the involvement of these neurons, a technique was developed to measure changes in ACh release simultaneously with changes in ion transport reflected by short-circuit current measurements.

Acetylcholine is a major transmitter in the enteric nervous system and controls muscle, epithelial and vascular function of the gut. It has been implicated as a neurotransmitter at both ganglionic synapses and at
neuroepithelial junctions to induce chloride secretion in a wide variety of species including the guinea pig, rat, rabbit and human (74,75,76,96,97,99,175,180) (Fig 52). There is an abundance of studies on ACh release from myenteric neurons (71, 82, 92, 152, 171), but little is known about the response characteristics of submucous neurons, particularly in the colon (66, 175, 180). Our results provide new information on response characteristics of submucous neurons in the colon which distinguish these neurons from myenteric neurons in the colon and other regions. They demonstrate that colonic submucous neurons require higher frequencies of stimulation to evoke release of ACh. Furthermore they demonstrate that presynaptic muscarinic autoreceptors do not appear to play a major role in regulation of ACh release and epithelial secretion.

Histamine is one of the mediators in mast cells that could interact with neuronal elements in the milk sensitized animals. Histamine stores and mast cell numbers were similar in immune and non-immune animals. Therefore the milk-sensitized guinea pig colon is not characterized by mast cell hyperplasia that is often observed in animals with parasitic infections (23, 24, 134,135). Nevertheless, histamine release was significantly increased only in immune
FIGURE 52. A working model illustrating the mechanism of action of blockers affecting ACh release and chloride secretion. (1) Tetrodotoxin (TTX) blocks sodium channels and prevents action-potential-dependent release of neurotransmitters from all neurons. If chloride secretory response to electrical field stimulation is not abolished, it may suggest the involvement of nonneuronal mediators; (2) Muscarinic receptors are present on neurons and on epithelial cells. Blockade by atropine (ATR) can occur at either or both sites effecting acetylcholine (ACh) release. Attenuation and inability to completely block the secretory response suggests the involvement of noncholinergic mechanisms; (3) Mecamylamine (MEC) blocks nicotinic receptors which are present on neurons and not on epithelial cells. Nicotinic blockade can cause attenuation of chloride secretion by decrease in ACh release at neuroepithelial junctions. M = Muscarinic receptor.
tissues treated with β-lactoglobulin, and it consequently must be considered as a potential mediator of the secretory response either by acting directly on epithelial cells or on neurons innervating them.

This study is the first to demonstrate the antigenically-evoked release of ACh simultaneously with an increase in chloride secretion. It shows that chloride secretory response evoked by β-lactoglobulin was attenuated by the neurotoxin, tetrodotoxin suggesting that the neurons mediated the response. Attenuation of the response by atropine suggested that cholinergic neurons were involved during antigenic stimulation of chloride secretion. The ability of histamine antagonists and prostaglandin synthesis inhibitors alone, or when used in combination, to attenuate the antigen-induced ACh release implicated histamine and prostaglandins as mediators. Evidence that exogenous application of histamine, and prostaglandin E2 evoked a concentration-dependent increase in ACh release and short-circuit current response in parallel suggest the involvement of these mediators. Dimaprit evoked a greater increase in ACh release than histamine, suggesting the involvement of H₂ receptors during the antigen-induced chloride secretory response in immune animals. These studies provide both indirect and direct evidence to show that histamine and
prostaglandins, probably released from mast cells during antigenic challenge, stimulate chloride secretion by activating cholinergic neurons. These observations suggest communication between the immune system and the enteric nervous system in regulation of epithelial function (58, 173).

The role of peptidoleukotriene D₄, a mast cell mediator was assessed to examine its effect on ion transport in guinea pig colon. A significant reduction in β-lactoglobulin and LTD₄-induced secretion by a leukotriene antagonist provided strong evidence for sulfidopeptide leukotrienes as one of the mediators of the anaphylactic response in milk sensitized guinea pig. The secretory response to LTD₄ in both immune and non-immune animals was influenced by the presence of ongoing neuronal activity. In these studies the level of ongoing activity seemed to be the major determinant of the magnitude of the leukotriene evoked short-circuit current response. It is likely that leukotrienes modulate spontaneous activity. The ongoing activity in both immune and non-immune tissues was abolished by tetrodotoxin and atropine, which is suggestive of their neuronal origin. The sensitivity of the secretory response to LTD₄ with bumetanide provides evidence for increase
short-circuit current being due to chloride secretion.

The fact that LTD$_4$ response in submucosal/mucosal segments was not abolished by neural blockade suggests the possibility of direct epithelial effects as well. Evidence for this was obtained from the observations that leukotrienes failed to evoke secretion in T$_{84}$ human colonic carcinoma cell line. This does not completely exclude the possibility of direct effects of LTD$_4$, because differentiation process in this cell line could result in loss of receptors or they may have different receptors than in distal colon of guinea pig.

The increased tissue responsiveness to LTD$_4$ in immune animals is consistent with electrophysiological findings which revealed increased number of spontaneously firing neurons in immune guinea pig as compared to non-immune animals. The increased responsiveness of carbachol in presence of LTD$_4$ and its attenuation when tetrodotoxin was added prior to LTD$_4$ suggests that leukotrienes stimulate neurons to release neurotransmitters that interact synergistically with carbachol to augment secretion. It is not clear whether this effect is a direct effect of leukotrienes on neuronal receptors or it is due to release of an intermediary messengers.
The overall results of this study demonstrate that LTD₄ augments chloride secretion by increasing cholinergic neural activity, rather than by acting directly on epithelial cells. Leukotriene D₄ plays a neuromodulatory role to augment cholinergically mediated chloride secretion in immune animals.

CONCLUSION

These results provide evidence for the involvement of cholinergic neurons in anaphylactic responses. Three mediators, histamine, prostaglandins and leukotrienes, all influence intestinal secretion by their interactions with enteric neurons. This study provides important new information on the interactions of the immune system and the enteric nervous system. The determination of the mechanism of action of mast cell products in mediating allergic responses will provide a sound basis for future development of therapeutic drugs for prevention of the underlying symptoms of colic and diarrhea during intestinal anaphylaxis. In order to accomplish this goal, additional research is necessary to define specific neural pathways, as well as important genetic, cellular and humoral mechanisms that govern responses to intestinal antigens.
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