EFFECTS OF ABLATION OF THE MYENTERIC PLEXUS ON THE PHARMACOLOGICAL RESPONSES AND ELECTRICAL ACTIVITY OF RAT JEJUNAL MUSCLE

by

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EFFECTS OF ABLATION OF THE MYENTERIC PLEXUS ON THE PHARMACOLOGICAL RESPONSES AND ELECTRICAL ACTIVITY OF RAT JEJUNAL MUSCLE

By Deborah Ann Fox

Under the supervision of Professor Paul Bass

Studies were undertaken to determine (1) the effects of selective chemical ablation of the myenteric plexus on rat jejunal motility in vitro and in vivo and (2) the involvement of the myenteric plexus in the longitudinal muscle responses to putative enteric neurotransmitters as well as adrenergic agonists. Application of benzalkonium chloride to the serosal surface of the rat jejunum was used to ablate the myenteric plexus.

Isolated rat jejunum devoid of myenteric neurons does not respond to ganglionic stimulants, nerve-selective electrical stimulation or physostigmine. Responsiveness to carbachol and maximum tension development in response to barium chloride were not altered, indicating that contractility was not impaired. Since this preparation behaves as though it is aganglionic despite the presence of submucosal neurons, the data suggest that the motor neurons innervating the longitudinal muscle are in the myenteric plexus.

Myoelectric activity of normal and myenteric neuron-ablated jejunum was measured. These studies revealed that ablation of the

myenteric plexus disrupts the basic electric rhythm but not the propagation of the migrating myoelectric complex.

The effects of putative enteric neurotransmitters on longitudinal muscle responses of normal and myenteric neuron-ablated jejunum were compared. These studies suggest that substance P and norepinephrine elicit their mechanical responses by a direct action on the smooth muscle, while adenosine triphosphate, 5-hydroxytryptamine, cholecystokinin-octapeptide and vasoactive intestinal peptide act indirectly through the myenteric plexus. Acetylcholine has both direct and indirect actions.

The mechanical responses of normal and myenteric neuron- ablated jejunum produced by alpha and beta adrenergic receptors were examined. Results suggest that (1) beta receptors mediate relaxation and are located on the smooth muscle (2) alpha-1 receptors mediate relaxation and are located on both the smooth muscle and the myenteric plexus and (3) alpha-2 receptors mediate contraction and are located on the myenteric plexus.

Approved: _____

Paul Bass
Professor of Pharmacology

INTRODUCTORY REVIEW

THE ENTERIC NERVOUS SYSTEM: ANATOMY AND NEURONAL TYPES

The earliest intimation that the gut had its own nerve supply came from Remak in 1847. He described unmyelinated nerves to the gut running in the mesentery and ending in the muscle layers. Ten years later, Meissner (1857) described ganglion cells and a plexus of nerves in the submucosal layer of the intestine. It was Auerbach who made the first complete description of the myenteric plexus. Though his experiments were conducted with very primative equipment, in his own home, at his own expense, he described ganglion cells connected by unmyelinated nerve fibers located between the external muscle layers of the gut (Auerbach, 1864). He knew that the plexus extended throughout the gastrointestinal tract, and postulated that its function was to initiate peristaltic contractions.

The enteric nervous system as described more recently, is comprised of the two major plexuses of Meissner and Auerbach (submucosal and myenteric plexuses, respectively), as well as several subsidiary aggregations of nerve fibers. These subsidiary nerve fibers are found in the circular muscle, muscularis mucosa and the loose areolar connective tissue of the lamina propria of the mucosa (Schofield, 1968; Gabella, 1976; Furness and Costa, 1980; Gershon, 1981). Figure 1 shows a diagrammatic representation of the enteric nervous system.

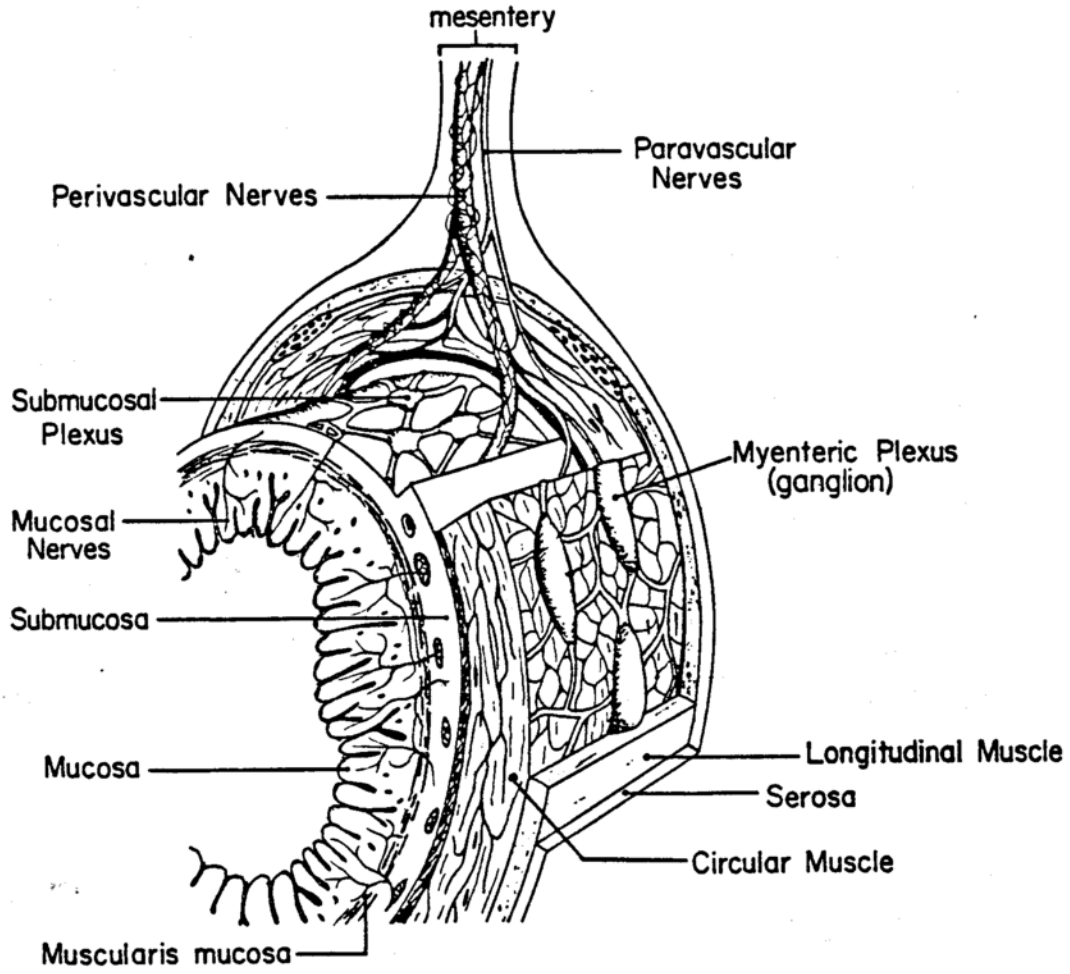


Figure 1. A diagram of a section of small intestine showing the distribution of nerve elements in the various layers of the intestine. (Adapted from Gershon and Erde, 1981)

The enteric nervous system, defined as the intrinsic innervation of the gastrointestinal tract, is part of the autonomic nervous system. In fact Langley (1921), who first described the organization of the autonomic nervous system, originally classified the enteric nervous system as the third division of the autonomic nervous system. The autonomic nervous system now is usually considered to contain only two divisions, parasympathetic and sympathetic (Kuntz, 1953), which are defined by their connections to the central nervous system (CNS). The parasympathetic division of the autonomic nervous system is associated with a craniosacral preganglionic outflow, while the sympathetic division is associated with a thoracicolumbar preganglionic outflow from the CNS. Parasympathetic innervation to the gut is furnished by the vagus nerves to the stomach, small intestine and proximal portion of the large intestine, and by the pelvic nerves to the remainder of the large intestine and rectum. The preganglionic fibers of the vagus and pelvic nerves synapse with neurons of the myenteric and submucosal plexuses. Postganglionic fibers from these plexuses innervate the smooth muscle and secretory cells. Preganglionic sympathetic fibers innervating the gut arise from neurons of the intermediolateral columns of the spinal cord, and pass through the sympathetic chain without synaptic relay to the celiac, superior mesenteric and inferior mesenteric ganglia. Postganglionic fibers from the celiac ganglia

innervate the stomach and proximal duodenum. Fibers from the superior mesenteric ganglia innervate the remainder of the small intestine and the proximal large intestine; while fibers from the inferior mesenteric ganglia innervate the remainder of the large intestine. Most of the postganglionic sympathetic fibers to the gut terminate in the myenteric and submucosal plexuses. Therefore, efferent nerve fibers from both parasympathetic and sympathetic divisions of the autonomic nervous system are interspersed throughout the enteric nerve network and comprise the extrinsic innervation of the gastrointestinal tract.

Though Langley's description of the autonomic nervous system is not in vogue with modern pharmacology textbooks, his implication that the enteric nervous system is autonomous is supported by the anatomical independence of the enteric nervous system. Ultrastructurally, the myenteric plexus possesses characteristics dissimilar to other regions of the peripheral nervous system. In fact, many of the structural features of the myenteric plexus resemble the central nervous system (CNS) (Komuro et al., 1982; Gabella, 1972). For instance, there is a paucity of extracellular space and an absence of perineurial or endoneurial sheaths surrounding myenteric axons. The myenteric plexus completely lacks collagen. The supporting cells of the myenteric plexus more closely resemble the astroglia of the CNS than the Schawnn cells of the peripheral nervous sytems (Gabella,

1971, Cook and Burnstock, 1976, Gabella, 1981). The interior of the myenteric plexus is also unusual in that is completely avascular, with capillaries that supply the neural tissue lying outside of the enclosing glial sheath of the plexus. These outlying capillaries are different from ordinary enteric capillaries in that they are thick-walled and nonfenestrated and have impermeable junctions that prevent the passage of intravascular tracer molecules between endothelial cells (Gershon and Bursztajn, 1978). There is therefore a "blood-myenteric plexus barrier" to macromolecules which may be functionally analogous to the blood-brain barrier.

Perhaps the most striking resemblance of the enteric plexuses to the CNS is in the diversity of neuron and axon terminal types found in the plexuses. The neuronal types in the enteric ganglia have been classified utilizing criteria such as morphology, electrophysiology or neurotransmitter types. Dogiel first described three types of neurons (types I, II and III) based on the morphology of axonal processes. (Dogiel, 1895, 1896, 1899). Electrophysiologically, two types of enteric neurons have been identified on the basis of their differing membranous properties. These two types of neurons are known as the S and AH cells (Hirst et al., 1974) or type 1 and type 2 (Nishi and North, 1973) cells, respectively. Neuronal cell bodies have been classified into nine different types using the criteria of neuronal size, distribution of organelles, location of

the neurons and their relationship to satellite cells (Cook and Burnstock, 1976). Enteric axon terminals have been classified into eight (Cook and Burnstock, 1976) or ten (Furness and Costa, 1980) types using size, shape and content of synaptic vesicles seen in the profile of axonal varicosities as the identifying criteria. Neuronal types based on content of transmitter are discussed below.

Cholinergic Neurons

Acetylcholine formation by the gut was demonstrated by Dikshit in 1938. Later it was shown that acetylcholine was released from the gut spontaneously (Feldberg and Lin, 1949; Chujyo, 1953) and by transmural electrical stimulation (Paton, 1957). However, it was not until 1968 that acetylcholine was unequivocally shown to be synthesized and released from the myenteric plexus (Paton and Zar, 1968). Paton and Zar demonstrated that acetylcholine was neither contained in, nor released by electrical stimulation of strips of guinea pig longitudinal muscle without the adherent myenteric plexus.

The actual distribution of cholinergic cell bodies and fibers in the intestine has only recently been established. Though data indicative of cholinergic nerve distribution have been obtained with acetylcholinesterase histochemistry (Koelle et al., 1949), this enzyme cannot be relied upon as a cholinergic marker because it is associated with both cholinergic and non-cholinergic peripheral

neurons (Silver, 1974). Furness and coworkers, however, were able to detect cholinergic nerves in the enteric plexuses using antibodies against choline acetyltransferase (ChAT) (Furness et al., 1983; Furness et al., 1984). ChAT-immunoreactive nerve cell bodies were found in both myenteric and submucosal plexuses of the stomach, small intestine, and colon of the guinea pig, and small intestine of the mouse. Varicose ChAT-containing nerve fibers were found in the ganglia, circular smooth muscle and mucosa of the small intestine of both guinea pig and mouse. It was estimated that at least 20% of all myenteric and 50% of all submucosal ganglion cells contain ChAT. The demonstration of ChAT-containing neurons in the enteric plexuses provides a basis for determining the circuitry of enteric cholinergic neurons at a structural level.

Norepinephrine Neurons

Sympathetic nerve stimulation has long been known to relax nonsphincteric regions of the mammalian small intestine (Finkleman, 1930). This effect was attributed to the release of an adrenergic substance (Finkleman, 1930) that was presumed to be norepinephrine (von Euler, 1951). The localization of norepinephrine-containing nerves in the gut by formaldehyde-induced fluorescence was first accomplished by Norberg in 1964 and then a year later by Jacobowitz (1965). These researchers found adrenergic nerve fibers but no cell bodies, within the gut wall. In fact, the only part of the gut of

any mammal known to contain a substantial number of adrenergic cell bodies is the proximal colon of the guinea pig (Costa et al. 1971; Furness and Costa, 1971). Otherwise, most of the cell bodies which give rise to the adrenergic fibers innervating the gastrointestinal tract are located in the prevertebral ganglia of the celiac, superior mesenteric, and inferior mesenteric plexuses. In accordance with the extrinsic nature of adrenergic cell bodies, extrinsic denervation depletes norepinephrine detected biochemically or histochemically from the wall of the gut (Hamberger and Norberg, 1965; Jacobwitz, 1965; Ahlman et al., 1973; Furness and Costa, 1978; Juorio and Gabella, 1974).

Most adrenergic nerves ramify amongst neurons of the myenteric and submucosal ganglia or form plexuses around submucosal arteries (Norberg, 1964; Jacobowitz, 1965). In comparison with the enteric plexuses, adrenergic innervation of the external muscle layers is sparse. There are essentially no adrenergic fibers within the longitudinal muscle and a sparse innervation of the circular muscle of both the small and large intestine (Norberg 1964; Jacobowitz, 1965; Gabella and Costa, 1967). Noradrenergic nerve fibers also innervate the mucosa and are found close to the central lacteals of the villi and mucosal glands (Thomas and Templeton, 1981; Keast et al., 1984).

Serotonin (5-Hydroxytryptamine, 5-HT)

Most of the 5-HT found in the gut is contained within the

enterochromaffin cells of the mucosa (Erspamer, 1966), with only a relatively small amount of detectable 5-HT remaining after the mucosa has been removed (Feldberg and Toh, 1953; Robinson and Gershon, 1971). Using histofluorescence methods, non mucosal 5-HT has been detected in enteric neurons in situ and in culture (Dreyfus et al., 1977). The presence of 5-HT in enteric neurons has been confirmed with immunohistochemical methods using antibodies prepared against tryptophan hydroxylase (Gershon et al., 1977) and against 5-HT itself (Costa et al., 1982; Furness and Costa, 1982b). The projections and ramifications of 5-HT containing neurons in the intestine have been studied by making microsurgical lesions of intestinal nerves and examining consequent changes in the distribution of 5-HT-immunoreactive neurons (Furness and Costa, 1982b). These studies have shown that varicose 5-HT-immunoreactive fibers which occur around the ganglion cells of both plexuses arise from nerve cell bodies in the myenteric plexus which send their axons in an anal direction. Since the varicose terminals are mainly located around other enteric neurons in both the myenteric and submucosal plexuses, it is conceivable that these neurons act as interneurons in descending pathways within the intestinal wall.

Adenosine Triphosphate (ATP)

ATP or a related purine nucleotide has been suggested as a possible nonadrenergic inhibitory transmitter in gastrointestinal

smooth muscle (Burnstock et al., 1970; Burnstock, 1979). The evidence both for and against ATP as an inhibitory transmitter has been recently reviewed by Furness and Costa (1982c) and will not be discussed here.

Though it has been shown that enteric nerve terminals contain and release ATP upon stimulation, the specific distribution of ATP-containing neurons in the enteric nervous system has not been established. However, it has been suggested that neurons which fluoresce in the presence of quinacrine may be ATP-containing neurons. Quinacrine-positive cell bodies and fibers have been found in enteric ganglia (Crowe and Burnstock, 1981).

Vasoactive Intestinal Peptide (VIP)

In addition to ATP, VIP is another possible transmitter in enteric inhibitory nerves. Unlike ATP, the distribution of enteric VIP-containing neurons has been extensively studied in the gastrointestinal tract of many mammalian species (Furness et al., 1980; Schultzberg et al., 1980; Furness et al., 1981; Hutchinson et al., 1981; Costa and Furness, 1982a; Furness and Costa, 1982a; Costa and Furness, 1983; Tange, 1983). VIP immunoreactivity has been found in nerve cell bodies and axons throughout the gastrointestinal tract. The nerve cell bodies are located in the myenteric and submucosal plexuses, while the axons are found in the muscle layers, mucosa, ganglia of the plexuses, and around small blood vessels. Costa and

Furness (1983) have mapped the origins and projections of VIP-containing neurons in the guinea pig small intestine and have found that: (1) VIP-immunoreactive neurons in the myenteric ganglia project anally to other myenteric ganglia, anally to the submucosal ganglia, to the underlying circular muscle, and to prevertebral ganglia; (2) VIP-immunoreactive neurons in the submucosal ganglia project to the submucosal arteries and to the mucosa. The circuitry of VIP-containing nerves in the intestine is consistent with VIP's involvement in intestinal motility, blood flow and transepithelial movement of water and electrolytes.

Substance P

Substance P was first detected in intestinal extracts by its spasmogenic action on the intestine by von Euler and Gaddum (1931). It was suggested that substance P was contained in nerves when reduced concentrations of substance P were found in the aganglionic segments of colon from patients with Hirschsprung's disease (Ehrenpreis and Pernow, 1952). Following elucidation of its chemical structure (Chang and Leeman, 1970), substance P was detected throughout the gastrointestinal tract by radioimmunoassay (Powell et al., 1973) and specifically in enteric neurons by immunohistochemistry (Pearse and Polak, 1975; Nilsson et al., 1975; Schultzberg et al., 1980; Furness et al., 1980; Furness et al., 1981; Costa et al., 1981; Tange, 1983). Substance P immunoreactivity is found in cell

bodies and axons of both myenteric and submucosal plexuses. Axons from cell bodies in the myenteric plexus project to other myenteric ganglia, to the circular muscle, to the submucosal ganglia and to the villi. Projections are both oral and anal. Axons from cell bodies in the submucosa project to submucosal blood vessels and the mucosa. Not all substance P axons are of intrinsic origin. Some substance P-containing axons in the submucosa disappear if extrinsic nerves are cut (Costa et al., 1981).

Somatostatin

Somatostatin immunoreactivity has been found in the nerve plexuses of the gastrointestinal tract (Costa et al., 1977; Schultzberg et al., 1980; Furness et al., 1981, Tange, 1983). Varicose processes of enteric somatostatin neurons have been mapped and found to ramify in the myenteric and submucosal ganglia. Lesioning studies indicate that axons arising from somatostatin nerve cell bodies in the myenteric plexus project in an anal direction. Somatostatin axons in the submucosal ganglia and the mucosa appear to arise from cell bodies in the submucosa. Somatostatin neurons do not supply the muscle layers. Therefore, it has been suggested that enteric somatostatin neurons function as interneurons in the enteric plexuses (Furness et al., 1981).

Enkephalins

The enkephalins are pentapeptides first extracted from the

brain in 1975 by Hughes and colleagues (Hughes, 1975; Hughes et al., 1975). Since that time, the enkephalins have been found in enteric neurons of many mammalian species (Alumet et al., 1978; Schultzberg et al., 1980; Furness et al., 1981; Tange, 1983). The distribution of enkephalin-containing neurons has been most extensively studied in the guinea pig. In this species, about 25% of the total number of nerve cell bodies in the myenteric plexus demonstrate enkephalin immunoreactivity. Varicose axons in the myenteric plexus form a network around the neurons in the ganglia and in the circular muscle. Axons in the myenteric plexus project in both the oral and anal directions. In contrast to the distribution of other peptides in the guinea pig intestine, enkephalin nerve cell bodies were not found in the submucosal plexus. Axons were rare in the submucosa and were not found in the mucosa. Enkephalin cell bodies were, however, found in the submucosal plexus of the dog (Tange, 1983). The distribution of enkephalin-containing nerves in the guinea pig small intestine suggests that these nerves are involved in the control of motility, but not directly involved with intestinal blood flow or secretion.

Neurotensin

Neurotensin was first isolated from the hypothalamus by Carraway and Leeman in 1973. However, the major source of neurotensin appears to be the gastrointestinal tract, with approximately 85%

of total neurotensin in the rat located in the gut (Carraway and Leeman, 1976). Neurotensin-immunoreactive nerve fibers are found in the myenteric and submucosal plexuses and in the muscle layers of the dog (Tange, 1983), guinea pig (Schultzberg et al., 1980; Leander et al., 1984) and rat (Schultzberg et al., 1980). No immunoreactive neurotensin cell bodies have been detected in the intestine of these species, and the origin of enteric neurotensin fibers has not been determined.

Cholecystokinin/Gastrin

The distribution of cholecystokinin (CCK) and gastrin in the enteric nervous system is usually discussed together because the antisera used in immunohistochemical studies recognize the COOH-terminal pentapeptide common to both gastrin and CCK, and therefore do not discriminate between the two peptides (Schultzberg et al., 1980; Leander et al., 1984). CCK/gastrin-immunoreactive cell bodies were observed in the submucosal and myenteric plexuses of the guinea pig and rat colon, and guinea pig duodenum. CCK/gastrin-immunoreactive fibers were observed in small numbers in most areas of the gastrointestinal tract of both rat and guinea pig. Projections of CCK/gastrin-containing nerves have not been determined.

Bombesin/Gastrin Releasing Peptide

Bombesin is a tetradecapeptide first isolated from amphibian skin (Anastasi et al., 1971). Several years after the isolation of

bombesin, gastrin releasing peptide (GRP) (comprised of 27 amino acid residues), so named because of its intense effect of elevating blood gastrin levels, was isolated from porcine nonantral gastric mucosa (McDonald et al., 1979). The COOH-terminal decapeptides of GRP and bombesin differ by only one amino acid residue. Because of these similarities in structure, many antisera used in immunohistochemical studies of these peptides can cross react. Therefore, resulting immunoreactivity is often referred to as bombesin/GRP-like. Bombesin/GRP-like immunoreactivity has been reported in the enteric neurons of the mammalian gut (Dockray, 1979; Iwanaga, 1983; Costa et al., 1984). Immunoreactive fibers were found in the myenteric and submucosal plexuses and the circular muscle. Bombesin/GRP-immunoreactive cell bodies were found in the myenteric but not the submucosal plexus. The axons from the immunoreactive cell bodies in the myenteric plexus project annally to other myenteric ganglia, submucosal ganglia and circular muscle. These fibers also project to the celiac ganglia.

Co-localization of Enteric Neuronal Substances

In recent years, the idea that a given neuron stores and releases only one chemical transmitter has been challenged. Evidence for coexistence of established transmitters with biological peptides has been presented for both central and peripheral nervous systems (Burnstock, 1976; Gilbert and Emson, 1983). Examples of co-

localization of putative transmitters in the enteric nerves have been cited. Schultzberg and coworkers (1980) have shown that some neurons are immunoreactive for both cholecystokinin and somatostatin. Choline acetyltransferase and some peptides have been located in the same population of submucosal neurons. (Furness et al., 1984). Myenteric neurons containing both 5-HT and substance P have been found in the guinea pig and rat (Legay et al., 1984). The function of enteric neurons containing more than one substance has not been determined.

NEUROMUSCULAR TRANSMISSION IN THE INTESTINE

Transmission from both intrinsic and extrinsic nerves to intestinal muscle has been extensively studied (for review, see Costa and Furness, 1982b). Transmission from intrinsic nerves can be elicited by electrical stimulation, drugs, or by evoking reflexes (i.e., distention of the intestine). Extrinsic neurotransmission has been studied by electrical stimulation of the vagus, splanchnic, mesenteric and pelvic nerves. A brief discussion of the types of responses elicited by the intestine from intrinsic nerve stimulation will be discussed below. Though there is a vast body of data on the responses to stimulation of extrinsic nerves, this literature will not be reviewed here.

The Neuromuscular Junction in the Intestine (For review, see Costa and Furness, 1982b)

The layers of the intestine are composed of individual muscle fibers (2-5 μm in diameter, 500 μm long) which are connected both electrically and mechanically and are loosely arranged in muscle bundles of different sizes. These muscle bundles are separated by connective tissue septa into larger units which are also interconnected. The nerves which are present in the muscle layers consist of bundles of axons partly enveloped by Schwann cells. Within these bundles, individual axons are varicose for distances of several millimeters. It is generally believed, though not proven, that transmitter is released from varicosities along the length of the axon. Because axons traverse spaces between several muscle cells, and their varicose portions are considerably longer than the muscle cells, each axon probably influences several muscle cells directly, and because of the interdependence of muscle cells, influences many muscle cells indirectly. In contrast to skeletal muscle, there are no structurally identifiable synaptic specializations to identify points of neurotransmission in the intestine.

Transmission from Intrinsic Nerves to Intestinal Muscle

By using different conditions of stimulation and agonist and antagonist drugs, it has been possible to distinguish transmission via cholinergic nerves, noncholinergic excitatory nerves and enteric

inhibitory nerves. Paton (1955), using his technique of coaxial electrical stimulation, first described transmission from myenteric cholinergic neurons to the longitudinal muscle of the guinea pig ileum. He demonstrated that single electrical pulses elicited brief contractions which were abolished by atropine, hyoscine and tetrodotoxin, but not by hexamethonium. The complete abolition of contractions by antimuscarinic drugs, the lack of a detectable effect by ganglionic blocking agents, and the observation that relaxation was not produced by this type of electrical stimulation, suggested that the response was due to stimulation of excitatory cholinergic neurons without involvement of cholinergic interneurons or other nerve types. Since the work of Paton, several researchers have described cholinergic excitatory responses in the muscle layers of different intestinal preparations (Day and Vane, 1963; Bennett, 1966; Day and Warren, 1968; Furness, 1969; Bennett and Stockley, 1975; Anuras et al., 1977; Yokoyama and Ozaki, 1978).

In addition to cholinergic excitatory neurons, there is evidence for release of other excitatory substances from enteric nerves. Ambache and coworkers (Ambache and Freeman, 1968; Ambache et al., 1970) showed that electrical stimulation of the guinea pig small intestine could elicit atropine-resistant contractions. These contractions were subsequently shown to be due to the release of substance P from intrinsic neurons (Franco et al, 1979 a,b).

Evidence for excitatory noncholinergic transmission has also been found in the proximal colon of the guinea pig (Costa and Furness, 1972). Since transmural stimulation of the guinea pig proximal colon produced prolonged contractions of the longitudinal muscle which were resistant to hyoscine, but blocked by tetrodotoxin, methysergide, and exposure to 5-HT, it has been suggested that an indoleamine might be released from these nerves.

The existence of intrinsic inhibitory neurons in the intestine was suggested at the turn of the century when it was realized that the intestine possessed reflexes involving relaxations (Mall, 1896; Bayliss and Starling, 1899; see pages 29-30). The nature of such neurons remained unstudied for decades until Ambache (1951) showed that nicotine caused intestinal relaxation instead of contraction when cholinergic nerves were paralyzed by botulinum toxin. Since the relaxations were blocked by hexamethonium, this suggested the presence of local inhibitory ganglion cells in the enteric plexuses, interpreted at the time to be short intrinsic adrenergic neurons. However, with the use of adrenergic neuron blocking drugs and adrenergic receptor antagonists, inhibitory responses elicited by nicotine were shown <u>not</u> to be mediated by adrenergic neurons (Burnstock et al., 1964). Evidence for electrical stimulation of enteric inhibitory neurons supplying both layers of the small and large intestines of many mammalian species is available (for review, see Costa and

Furness, 1982). The type of transmitter released from enteric inhibitory neurons is unknown at the present time. However, data supportive of VIP (Furness and Costa, 1982a) and/or ATP (Burnstock et al., 1970, Burnstock, 1979) as the inhibitory transmitter in these nerves have been presented.

ADRENERGIC RECEPTORS IN THE GUT

The fact that epinephrine and stimulation of sympathetic pathways to the intestine produced similar effects on intestinal movements was noted in the early twentieth century. It was generally assumed that epinephrine or a similar substance was the transmitter from postganglionic sympathetic nerves (Elliot, 1904, 1905; Starling, 1906; Cannon and Rosenblueth, 1937 and Dale, 1938). Actual release of an epinephrine-like substance was demonstrated by Finkleman in 1930. He found that stimulation of the mesenteric nerves supplying an isolated segment of rabbit duodenum liberated a substance which relaxed a second, unstimulated piece of intestine. The idea that epinephrine acts via receptors stems from Langley (1905) who postulated that epinephrine and other pharmacologically active agents exerted their effects by interaction with "receptive substances." Langley also suggested that different types of "receptive substances" existed in the same tissue, and determined whether an organ would be stimulated or inhibited by a drug. The classical

studies of Dale (1906) also support the idea of distinct types of adrenergic receptors. Dale found that excitatory responses to sympathetic nerve stimulation and epinephrine were blocked by ergot alkoloids, whereas the inhibitory responses were not. However, it was not until 1948, when Ahlquist proposed the existence of two types of adrenergic receptors designated alpha and beta, that this concept became recognized by the scientific community. Ahlquist's studies, subsequently supported by a large body of experimental evidence, were based on the demonstration of a difference in the orders of potency for several sympathomimetic amines on various organ systems.

Ahlquist had originally designated the adrenergic receptors in the gut to be of the alpha type. However, this conclusion was revised when it was found that a combination of alpha and beta adrenergic receptor antagonists was necessary to abolish the inhibitory effects of catecholamines on the canine ileum in vivo (Ahlquist and Levy, 1959). The mediation of the inhibitory action of catecholamines on intestinal nonsphincter smooth muscle by both alpha and beta adrenergic receptors has been reported by others (Furchgott, 1960; Bucknell and Whitney, 1964; van Rossum and Mujic, 1965; Furchgott, 1967; Reddy and Moran, 1968; Andersson and Mohme-Lundholm, 1969; Bowman and Hall, 1970; Kosterlitz et al., 1970).

The location of the alpha and beta adrenergic receptors involved in intestinal relaxation has been the object of many

studies. In general, intestinal alpha receptors have been associated with the neural elements, while the locus of beta receptors is assumed to be the smooth muscle. Lum and coworkers (1966), demonstrated that cold storage of isolated rabbit jejunum abolishes the relaxation mediated by alpha, but not beta, adrenergic receptor stimulation. Since cold storage of intestinal segments (1-8°C for 1-5 days) eliminates nerve-mediated responses (Ambache, 1946; Innes, 1957), these data suggested that alpha receptors were located somewhere on intestinal nerves while beta receptors were located on smooth muscle cells. There is much evidence that the "neuronal" effects of sympathomimetic amines producing intestinal relaxation are due to an inhibition of acetylcholine release from cholinergic nerves. This mode of action of sympathomimetic amine-induced relaxation was suggested by Jacobj in 1892. He found that sympathoadrenal discharge blocked the excitatory influence of the vagus nerve on gastrointestinal smooth muscle. This implied that the sympathomimetic amines might be exerting their action indirectly by inhibiting the excitatory nerves of the gut.

There is now considerable evidence that sympathomimetic amines reduce acetylcholine output from intrinsic intestinal nerves by stimulating alpha adrenergic receptors. For example, Kosterlitz and coworkers (1970) demonstrated that the inhibitory effects of epinephrine, norepinephrine and isoproterenol on acetylcholine-

induced contractions were antagonized by propranolol but not phenoxybenzamine, indicating mediation by smooth muscle beta receptors. However, the inhibitory responses to electrical stimulation and the output of acetylcholine were diminished by epinephrine and norepinephrine but not by isoproterenol. Further, the depressant effect on acetylcholine release was antagonized by phenoxybenzamine but not propranolol, suggesting mediation by alpha adrenergic receptors. Wikberg (1977), utilizing innervated and mechanically denervated guinea pig longitudinal smooth muscle strips, suggested that alpha receptors mediating relaxation were located on cholinergic neurons, whereas beta receptors were located on smooth muscle. Wikberg, however, suggested the presence of alpha receptors on both smooth muscle and cholinergic neurons in the rabbit jejunum. Evidence that the adrenergic receptors mediating the inhibition of acetylcholine release in the intestine are of the alpha type are supported by others (Paton and Vizi, 1969; Gillespie and Khoyi, 1977; Del Tacca et al., 1970).

After the realization of the existence of two subtypes of alpha adrenergic receptors (Langer, 1974), research was initiated to characterize the type of alpha receptor located on cholinergic neurons in the gut. Through the use of potency ratios of alpha adrenergic receptor agonists and selective alpha-1 and alpha-2 antagonists, it has been generally accepted that alpha-2 adrenergic receptors

mediate intestinal inhibition of acetylcholine release (Wikberg, 1978; Drew, 1978; Wikberg, 1979; Andrejak et al., 1980; Kilbinger and Wessler, 1979). Direct evidence that intestinal alpha-2 receptors are located on the neurons of the myenteric plexus was provided by Wikberg and Lefkowitz (1982). They found that longitudinal muscle strips without adherent myenteric plexus did not bind [^{3}H]-clonidine.

The existence of two subtypes of beta adrenergic receptors (beta-1 and beta-2) was suggested by Lands and coworkers (1967a,b). They assigned the beta adrenergic receptors mediating relaxation of the rabbit jejunum to the beta-1 category. Since that time, using beta-1 and beta-2 selective agonists and antagonists, others have subclassified gut beta receptors. It has been suggested that beta-1 adrenergic receptors mediate inhibitory responses in guinea-pig ileal preparations (Levy and Apperley, 1978; Williams and Broadly, 1982; Grassby and Broadly, 1984; Aquleem Mian et al., 1984), while the presence of both beta-1 and beta-2 adrenergic receptors have been suggested in the rat gastric fundus (Lefebvre et al., 1985) and cat colon strip (Ek and Lundgren, 1982). Sim and Lim (1983) suggested that the beta adrenergic receptors mediating relaxation in the rabbit small intestine are mainly of the beta-1 subtype, while those in the large intestine are mainly of the beta-2 type. It appears that the type of beta receptor mediating inhibition of gut

smooth muscle may be dependent upon species or area of the gut, and both beta-1 and beta-2 receptors may be present in the same tissue. However, it should be noted that the potency of beta antagonists in gut tissues is considerably lower than that observed in non-gut tissues (i.e., heart, vasculature). Therefore, classifying gut beta receptors into beta-1 or beta-2 types using antagonist potencies may be subject to criticism.

ENTERIC NERVOUS CONTROL OF INTESTINAL MOTILITY

Because an understanding of the basic properties of intestinal smooth muscle is needed to discuss its control by nerves, these subjects are reviewed briefly below. Intestinal smooth muscle exhibits two types of electrical activity: the basic electric rhythm (BER) and spike potentials.

Basic Electric Rhythm (BER)

The basic electric rhythm (BER) represents cyclic changes in membrane potential. This phenomenon is also referred to as "slow waves," "pacesetter potentials," and "electrical control potentials." These membrane oscillations are omnipresent, propagative and are not associated with a mechanical event (for review, see Bass, 1968).

The origin of the BER is controversial. It has been postulated that the BER is initiated in the longitudinal muscle and spreads

electronically to the circular muscle (see Bortoff, 1976, Daniel and Sarna, 1978). Other evidence suggests that the BER is generated by circular smooth muscle cells (Taylor et al., 1975). A more complex theory suggests the initiation of low amplitude BER in the longitudinal muscle which spreads to the circular muscle where it is amplified and spreads back to reinforce the longitudinal muscle BER (Kobayasi et al., 1966; Connor et al., 1977). Recently it has been suggested that the BER may originate from the Interstial Cells of Cajal, connective tissue type cells which are intimately associated with the intrinsic nerve plexuses (Thuneberg, 1982; Thuneberg et al., 1983).

The frequency of the BER varies with species and region of the intestine. For example, the BER frequency in the duodenum is 17 cycles/min in the human, 18-19 cycles/min in the dog, 36-40 cycles/min in the rat. In the small intestine, the frequency of the BER decreases from duodenum to ileum in a stepwise fashion such that frequency plateaus occur along areas of intestine. Each frequency plateau is separated from the next by an area in which the amplitude of the BER waxes and wanes, (for review, see Costa and Furness, 1982b).

The BER has been shown to propagate around the circumference of the intestine through the circular muscle (Bass et al., 1961; Kobayashi et al., 1966), as well as anally along the longitudinal

axis of the small intestine. The velocity of BER propagation decreases along the length of the intestine (i.e., in the dog, the BER propagates at a velocity of 14 cm/sec in the duodenum and 3 cm/sec in the distal jejunum) (McCoy and Baker, 1969).

Spike Potentials

The spike potential is the myoelectric correlate to intestinal smooth muscle contraction. Spike potentials are superimposed upon the BER and represent rapid depolarizations and repolarizations of the smooth muscle membrane. The frequency of occurrence of spike potentials, synonomous with the rate at which the intestine can contract, is governed by the BER frequency (for review, see Bass, 1968).

When the GI tract is in the interdigestive (fasted) state, spike potentials coordinate themselves in a distinct pattern which propagates in an aboral manner down the GI tract (for review see Wingate, 1981). This group of spike potentials is referred to as the migrating myoelectric complex (MMC). Since the spike potentials elicit contractions, the complex is also referred to as the migrating motor complex. This phenomenom was first described by Boldyreff in the beginning of the twentieth century (Boldyreff, 1911), though reference to his work is usually neglected. By the end of the 1960's with the advent of extraluminal recording devices, the complex had been "rediscovered" by numerous investigators (Reinke et

al., 1967; Szurszewski, 1969; Carlson, 1970; Code and Marlett, 1975).

The MMC, which propagates from the stomach to the distal ileum, consists of essentially three cycling phases. For example, in the dog, a 60-minute basal period essentially devoid of contractions is followed by approximately 30 minutes of preburst or intermediate contractile activity, which terminates in a 10 minute burst of activity consisting of contractions of maximal amplitude and frequency (Carlson et al., 1970). These phases have also been referred to as phase I, II and III, respectively (Code and Marlett, 1975) and have been observed in several species.

The frequency of occurrence of the MMC and the velocity of its caudal migration vary with species (for reviews see Wingate, 1981; Costa and Furness, 1982b). As with the BER, the MMC exhibits a gradient drop in propagation velocity along the intestine. The MMC is interrupted by food in carnivores and omnivores but not herbivores. Functionally, the MMC is thought to sweep interdigestive secretory products from the intestine and has sometimes been referred to the "housekeeper" of the gut.

Intrinsic Intestinal Reflexes

The importance of the intrinsic innervation of the gastrointestinal tract has been recognized since the latter half of the nineteenth century. By this time, investigators realized that the intestine could transport its contents aborally even when it was

disconnected from the central nervous system (see Cannon, 1911). Further, they suggested that the caudal propulsion of intestinal contents was due to intrinsic nerve reflexes. The first disclosure that the intestine was capable of a polarity of responses came from Nothnagel (1882). He showed that exposure of the rabbit intestinal mucosa to sodium chloride crystals resulted in a contraction which spread from the stimulated region upwards, whereas quiescence prevailed below the stimulated region. This was later confirmed by Mall (1896). A more complete characterization of the intrinsic nerve reflexes involved in peristalsis is contained in the classical articles by Bayliss and Starling (1899, 1900, 1901). Their work has often been summarized simplistically on the basis of their major conclusion, which is referred to as the Law of the Intestine: "Local stimulation of the gut produces excitation above and inhibition below the excited spot. These effects are dependent on the activity of the local nervous mechanism." This dual polarized reflex response was named the "myenteric reflex" by Cannon (1911). In 1917, Trendelenburg demonstrated similar reflexes in isolated intestinal segments. However, the validity of the Law of the Intestine did not go without question. Some investigators were unable to detect relaxation below the advancing bolus (See Alvarez, 1948).

Since the early description of enteric reflexes, research

toward elucidating the nervous pathways involved in these reflexes has flourished. Upon stimulation of the intestinal mucosa, the reflex associated with contraction oral to the stimulation has been referred to as the "enteric ascending excitatory reflex." The reflex associated with relaxation anal to the point of stimulation has been referred to as the "enteric descending inhibitory reflex" (Costa and Furness, 1976). Both reflexes are abolished by tetrodotoxin (Fukuda, 1968; Crema et al., 1970; Frigo and Lecchini, 1970; Costa and Furness, 1976; Furness and Costa, 1976) and nicotine (Bayliss and Starling, 1899; Nakayama, 1962; Crema et al., 1970; Costa and Furness, 1976; Furness and Costa, 1976). Atropine and hyoscine antagonize the ascending excitatory reflex (Raiford and Mulinos, 1934a,b; Hukuhara and Miyake, 1959; Kosterlitz and Lees, 1964; Crema et al., 1970; Costa and Furness, 1976; Furness and Costa, 1976), suggesting that the final neuron in this pathway is cholinergic and stimulates the circular muscle via muscarinic receptors. The type of inhibitory neuron involved in the descending reflex pathway is unknown. None of the antagonists to known transmitter substances selectively antagonizes the descending inhibitory arc of the enteric reflex (Fukuda, 1968; Crema et al., 1970; Costa and Furness, 1976).

Enteric Nervous System in Coordinated Intestinal Movements

After the realization that the nerve plexuses embedded within

the gut wall were involved in intestinal reflexes, whether or not these nerves were necessary for normal coordinated movements of intestinal smooth muscle was still an unanswered question. With more sophisticated techniques for the study of motility in conscious animals, and the establishment of normal intestinal myoelectric and contractile patterns (BER and MMC), research efforts focused on determining whether enteric neurons were involved in the generation and/or maintenance of these patterns. It was hoped that this line of work would yield information on the involvement of the enteric nervous system in the normal physiological processes of intestinal smooth muscle.

Approaches used to examine the contribution of the enteric neurons in intestinal motor functions include the following techniques: transection of extrinsic nerves, antagonism of nerve transmission with drugs, transection and reanastomosis of the gut wall, and formation of Thiry Vella loops (involves transection of a segment of intestine with blood vessels and nerves intact and adapting the ends of the segment to the outside through two stomas; intestinal continuity of the remainder of the intestine is established by end-to-end anastomosis). Several researchers agree that extrinsic innervation of the gut is not critical to the initiation of the MMC or its caudal migration. Vagotomy does not alter the appearance of the MMC or its ability to migrate down the intestine (Marik and

Code, 1975, Weisbrodt et al., 1975; Ruckebusch and Bueno, 1977; Waterfall, 1983). However, the temporal relationship of the different phases of the MMC, and the rate of propagation of the complex, may vary after vagotomy (Marik and Code, 1975). Sympathetic outflow does not appear to be essential for initiation or propagation of the MMC. Cutting the splanchnic nerve (Ruckebusch and Bueno, 1975) or celiac and superior mesenteric ganglionectomy (Marlett and Code, 1979) does not alter the properties of the complex.

Intrinsic nervous control of the MMC has been postulated. Experiments in which intestinal continuity was disrupted by transection and reanastomosis (Sarna et al., 1983; Heppell et al., 1983) or formation of Thiry Vella Loops (Bueno et al., 1979; Ormsbee et al., 1981) demonstrated a disruption of the coordinated propagation of the MMC. In addition, close intrarterial injections of atropine, hexamethonium and tetrodotoxin block the MMC and its caudal migration (Sarna et al., 1981).

It should be noted that in addition to the suggested neural control of the initiation and propagation of the MMC, hormonal factors may also play a role. The peptide motilin had been shown to initiate migrating motor complexes indistinguishable from those occurring spontaneously (Lux et al., 1980). Also, plasma motilin levels increase at the time of appearance of spontaneous migrating myoelectric complexes (Peeters et al., 1980).

More direct studies aimed at demonstrating intrinsic nervous control of motility involve destroying the enteric ganglion cells and studying consequent changes in the electrical and mechanical activities of the intestine. Szurszewski and Steggerda have shown that destruction of enteric ganglion cells in a segment of canine intestine in vivo by anoxia (method first described by Hukuhara et al., 1961, see page 41) reduced the BER and contractile frequency in the anoxic segment of intestine and in the intestinal segment caudad to it. The usual caudal migration of the BER was also disrupted. Quantitative studies on the degree of ganglion cell death were not performed in these studies (Szurszewski and Steggerda, 1968a,b). Khin Kyi Kyi and Daniel (1970) conducted experiments similar to those of Szurszewski and Steggerda, but argued that anoxia did not produce complete denervation, and suggested that changes in the BER were attributed to smooth muscle damage.

Another direct approach used to study the interaction of enteric nerves in motility is described by Wood, 1973. Wood examined myoelectric activity in the colon of the Piebald mouse (hereditary megacolon, see pages 37-38). He observed an increase in the discharge of spike potentials which was associated with uncoordinated phasic contractions superimposed upon tonic contractions in the aganglionic colon. His data suggest that enteric neurons have an inhibitory influence on the colon.

PATHOLOGY OF THE ENTERIC NERVOUS SYSTEM

The enteric nervous system is subject to both congenital and acquired anomolies. Mention of these disorders is pertinent because they provide insight into the importance of the enteric nervous system in the maintenance of normal gut muscle physiology.

Hirschsprung's disease

Hirschsprung's disease is a congenital defect of the distal colon characterized by an absence of enteric nerve ganglia (Howard, 1972), a reduction in neuronal peptides (Bishop et al., 1981) and an increase in acetylcholinesterase (Hamoudi et al., 1982) and adrenergic nerve activity (Touloukian, 1973). The aganglionic segment of colon is constricted, while the segment of colon orad to the aganglionic area is dilated. There is usually an increase in the thickness of the smooth muscle of the dilated colonic segment and occasionally of the aganglionic region. This pathological condition presents itself at a very early age. It is clinically characterized by constipation and often by intestinal obstruction which may require surgical removal of the aganglionic area of the colon.

The pathogenesis of Hirschsprung's disease is obscure, but is thought to be due to incomplete craniocaudal migration of ganglion cells from the neural crest during gestation (Okamoto and Ueda, 1967). The causative factors which might interupt this migration

have not been identified. Recently, an alternative hypothesis to the pathogenesis of Hirschsprung's disease has been offered by Taguchi and coworkers (1985). They found fibromuscular displasia of arteries in the aganglionic colonic segment in 8 out of 25 Hirschsprung's patients they studied, and suggested that intestinal ischemia may be involved in the absence of colonic ganglion cells in Hirschsprung's disease. The authors postulated that ischemia may disrupt the craniocaudal migration of ganglion cells during development, or alternatively, cause atrophy of ganglion cells after their migration. The latter alternative is consistent with studies showing that ischemia or anoxia is disruptive to intestinal ganglion cells (see page 41-42).

Achalasia (for review see Smith, 1972; Goyal, 1983)

Achalasia is a motor disorder of the esophageal smooth muscle in which the lower esophageal sphincter does not relax properly during swallowing, and normal peristalsis of the esophageal body is replaced by abnormal contractions. Clinically, patients complain of dysphagia, chest pain, and regurgitation. Barium x-ray shows a persistant narrowing of terminal portion of the esophagus, representing the nonrelaxing lower esophageal sphincter, with dilation proximal to the narrowed segment.

The underlying pathology of achalasia is defective innervation of the esophagus. Histopathological studies have demonstrated a

marked reduction in myenteric neurons in the esophagi of achalasic patients. Experimental destruction of esophageal neurons produces an achalasia-like condition in animals (See page 42).

Chronic Idiopathic Intestinal Pseudo-obstruction

Chronic idiopathic intestinal pseudo-obstruction (CIIP) is a term used to describe a clinical syndrome in which patients exhibit symptoms that mimic those of organic intestinal obstruction, although an obstructing lesion cannot be found (Schuffler et al., 1981). The patients complain of abdominal pain, bloating and a marked alteration of bowel habits. Ineffective intestinal propulsion and motility are characteristics of this disorder.

The pathogenesis of CIIP is not well defined. Disorders of both the smooth muscle and the myenteric plexus have been described. Smooth muscle abnormalities are characterized by degeneration and fibrosis, which may be familial or sporadic in occurrence (Schuffler et al., 1977; Schuffler et al., 1981). Myenteric plexus lesions are characterized by a reduction in neurons and nerve fibers, fragmentation of axons, and replacement of nerve tracts with Schwann cells (Dyer et al., 1980; Schuffler and Jonak, 1982). Treatment of the disease often requires surgical intervention (Schuffler and Deitch, 1980).

Chagas Disease (for review, see Smith, 1972)

Chagas disease, which is confined to South America, is caused

by infection with Trypanosoma cruzi. The condition is of interest because there is a specific damage to the myenteric plexus and the cause is known. The trypanosome is spread by an insect vector, the Reduviid bug, which is infected by biting animals which carry the parasite. Human infection occurs when the Reduviid bug bites and simultaneously deficates on the victim. The trypanosome, contained in the feces is then taken into the bloodstream of the victim through the bite. The target organs for infection are the heart and the GI tract, resulting in cardio- and enteromegaly. The GI tract regions involved are usually the esophagus and colon, where disruption of the myenteric plexus and thickening of the smooth muscle occur. Clinical symptoms are similar to those seen in achalasia and Hirchsprung's Disease (see above).

Congenital Aganglionosis in Animals (Bolonde, 1975; Ikadai et al., 1981).

Congenital absence of enteric ganglion cells occur in some rodent strains. In the Spotting Lethal Mutant Rat strain and the Piebald Lethal and Lethal Spotting mouse strains, megacolon appears to be a recessive trait inherited in a Mendelian fashion. These animals also have a deficiency in hair pigmentation. There is embryological evidence to indicate that the aganglionic distal colon in these animals develops as a consequence of delayed migration of neuroblasts from the neural crest along the gut. Since melanoblasts

are also derived from the neural crest, the association of a deficiency in colonic ganglion cells with a deficiency in coat pigmentation is not surprising. Like Hirchsprung's patients, these animals have a constricted aganglionic segment of colon with marked orad dilation (megacolon). The animals exhibit marked stool impaction and retarded growth and eventually die within the first four to five weeks of life.

METHODS USED TO STUDY INTRINSIC NERVES

The realization that the gut was able to exhibit rhythmic contractions and reflexive activity without connections to the central nervous system provided the early impetus for the study of intrinsic intestinal nerves. Later, when it was demonstrated that the etiology of disease states such as Hirschsprung's disease and achalasia was an absence or alteration of intrinsic nerves, many investigators were interested in developing animal models for the study of these conditions. Therefore, many experimental approaches have been utilized either to abolish the action of or completely destroy intrinsic gut nerves. These methods are discussed below.

Bayliss and Starling (1899), after demonstrating that intestinal reflex activity occurred even when extrinsic gut nerves were transected, state: "It becomes now important to inquire how the motor functions of the intestine will be modified if we abolish

or paralyze the action of its local nervous system." To execute this task, Bayliss and Starling painted the surface of an exposed coil of intestine with a 2.5% solution of cocaine or injected the animal with nicotine. With both of these techniques, they observed the maintenance of rhythmic contractions and a transient abolition of reflex activity. The apparent myogenic nature of rhythmic contractions prompted Magnus (1904) to dissect the longitudinal muscle from circular muscle of the dog intestine in an attempt to obtain a denervated muscle preparation. He demonstrated that virtually all of Auerbach's plexus adhered to the longitudinal muscle upon separation. To eliminate any traces of the plexus in the circular muscle, Magnus seared the outer surface of the muscle with silver nitrate. Initially, since the longitudinal muscle with attached plexus exhibited rhythmic contractions, and the denervated circular muscle did not, Magnus assumed that these contractions were neurogenic. Later, when he was able to induce the circular muscle to contract by certain chemicals such as barium chloride, he revised his conclusion. Other investigators, interested in the nature of rhythmic contractions or the action of certain drugs, dissected inner circular muscle strips (i.e., those closest to the submucosa) in order to be certain that the muscle strips were totally denervated (Gunn and Underhill, 1914; Alvarez and Mahoney, 1922; Evans and Underhill, 1923; Gasser, 1926; Eura, 1927; Van Esveld, 1928; and Evans and

Schild, 1953). These investigators were also able to demonstrate intestinal contractions in the absence of nerves.

Interested in the activity of denervated longitudinal muscle and the source of acetylcholine in the intestine, Paton and Zar (1965; 1968) developed a denervated guinea pig longitudinal muscle preparation. They showed that upon separation of the longitudinal from the circular smooth muscle, the myenteric plexus adhered to only portions of the longitudinal muscle. By careful dissection, plexus-free longitudinal muscle strips could be separated from the remainder of the longitudinal muscle. The completeness of denervation was substantiated by histological and pharmacological methods. This technique has been subsequently cited by several investigators in a variety of experiments.

Functionally denervated intestinal segments with muscle layers intact have been prepared by cold storage and anoxia. Storing the intestine at 0-8°C for 1-5 days or lowering tissue bath temperature reduces the responsiveness to electrical stimulation, physostigmine and nicotine, but not acetylcholine (Ambache, 1946; Innes et al., 1957; Day and Vane, 1963; Ochillo et al., 1978). In addition, cold storage of intestinal segments can actually reduce the levels of acetylcholine, cholinesterase and choline acetyltransferase in the tissue (Ochillo et al., 1978). Anoxic intestinal segments, produced by stopping the flow of oxygen, replacing oxygen with nitrogen, or

adding cyanide, reduces the responsiveness of the intestine to physostigmine, nicotine, 5-HT, electrical stimulation, epinephrine and pilocarpine, but not potassium, barium or acetylcholine (Gross and Clark, 1923; West et al., 1951; Day and Vane, 1963). It should be noted that in the experiments with anoxia and cold storage, the intrinsic nerves are still present, although functionally impaired.

Anoxia has been employed in in vivo preparations for the destruction of enteric neurons. Cannon and Burket (1913) found that if they arrested blood flow to the intestine by compressing the intestinal wall for 3.5 hours, nerve cells were absent in the compressed area 16 days later. Hukuhara et al.,(1961) showed that if the blood supply to a loop of canine intestine was replaced by Tyrode's solution for four or more hours, variable destruction of the myenteric plexus occurred, and enteric reflexes were abolished. This procedure was confirmed by Nagata and Steggerda (1963) who reported that most of the ganglion cells in the myenteric plexus were completely destroyed, while the submucosal plexus was less sensitive to the anoxia. Okamoto and coworkers (1967), modified the Hukuhara preparation by adding 0.002% mercuric choride to the Tyrode's solution, and found destruction of the myenteric plexus after only one hour of perfusion. They performed their experiments on the distal colon, pylorus and esophagus of the dog. In all cases, they observed stenosis of the anoxic area with proximal dilation and

muscular hypertrophy characteristic of both achalasia and Hirschsprung's disease. The Hukuhara preparation was subsequently utilized by other researchers to investigate mechanical and myoelectric activity of denervated intestine in vivo (Szursewski and Steggerda, 1966; 1968a,b; Khin Kyi Kyi and Daniel, 1970; see page 33).

Several chemical approaches have been utilized to ablate intrinsic intestinal nerves in vivo. As an animal model for achalasia, Deloyers and coworkers (1957) injected 2 ml of a 5% solution of phenol between the muscle layers of the cat esophagus. The animals exhibited diminished appetite and megaesophagus, characteristic of achalasia. Histologically, there were variable lesions of the ganglion cells of the myenteric plexus without evidence of fibrosis or modification of the muscle. This technique was tried in the colon by Johnson and coworkers (1960). However, they observed lysis of the muscle layers along with the nerves and concluded that it was not an adequate model to produce selective ganglion cell damage in the gut. Another method employed to destroy ganglion cells in the colon was repeated injections of Urokon (sodium acetrizoate) into the inferior colic artery (McElhannon, 1959). Six weeks to eight months after injections of 35-60% Urokon, there was evidence of colonic constriction at the injection site with dilation and hypertrophy of the muscle proximal to the constriction. Virtually a

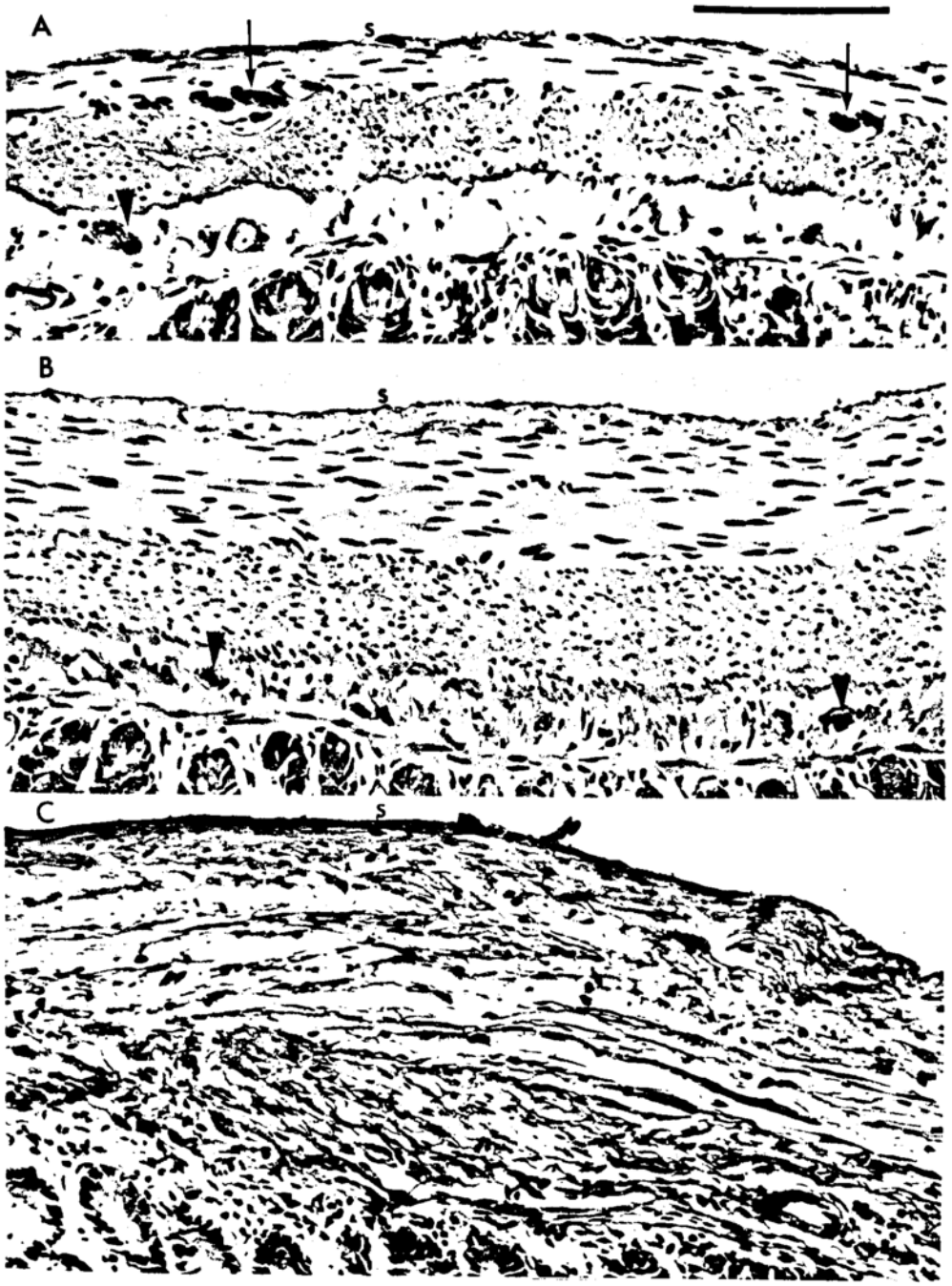
complete lack of enteric ganglion cells was observed, and those that did remain possessed pyknotic nuclei and vacuolization of cytoplasm. Mucosa, submucosa and muscle were observed to be comparable to those of control animals. Higher concentrations of Urokon produced perforation of all layers of the colon, peritonitis, and death.

In the latter half of the 1970's, research aimed at developing a Hirschprung's model in rats was initiated. Maintenance of a 0.01% mercuric chloride solution in the colon of the rat under a pressure of 50 mm Hg for 1 hour produced a Hirschsprung's-like pathology (Imamura et al., 1975). Within 5 days, a narrowed colonic segment with proximal megacolon was evident. Histologically, 1-3 months after treatment, atrophy or complete disappearance of the enteric ganglia was apparent. In 1978, Sato and coworkers demonstrated that serosal application of benzalkonium chloride (BAC) to the colon and anorectum of the rat destroyed the enteric ganglia. Denervation was qualitatively confirmed by Nissl, Bodian, supervital methylene blue, acetylcholinesterase and catecholamine staining procedures. They also showed that the aganglionic colonic segments were incapable of reflex activity induced by mechanical distension or pinching, but were able to contract in the presence of barium chloride. Electron microscopic observation did not reveal any marked changes in the smooth muscle after BAC treatment (Sakata et al., 1979).

Our laboratory became interested in the study of the enteric

nervous system approximately five years ago. We were concerned primarily with the contribution of the intrinsic intestinal nerves in small intestinal motility. Our first task in this line of research was to choose or develop a technique to destroy the enteric nervous system. Ideally, we wanted a technique which was simple, inexpensive, applicable to both in vivo and in vitro study, and chronic in nature. We decided to modify the technique of Sato and coworkers, 1978) for use on the small intestine of the rat. Our data has been published (Fox et al., 1983) and are summarized below.

We found that a 30 minute serosal application of the cationic surfactant, BAC, to the jejunum of the rat reduced, in a concentration-dependent manner (0.015 to 0.25% BAC), the number of ganglion cells in the myenteric plexus without affecting the number of ganglion cells in the submucosal plexus (figures 2 and 3). Maximum reduction in the number of ganglion cells in the myenteric plexus was greater than 90%. Observation by light microscopy revealed that the smooth muscle layers were thickened, though normal in appearance. Serosal application of concentrations of BAC greater than 0.25% were disruptive to the musculature and caused perforation and death of the animals. The reduction in myenteric ganglion cell number was evident as early as 5 days after the initial treatment, and was maximal 10 or more days post-treatment. We analyzed immunohistochemically the distribution of enkephalin, somatostatin, VIP,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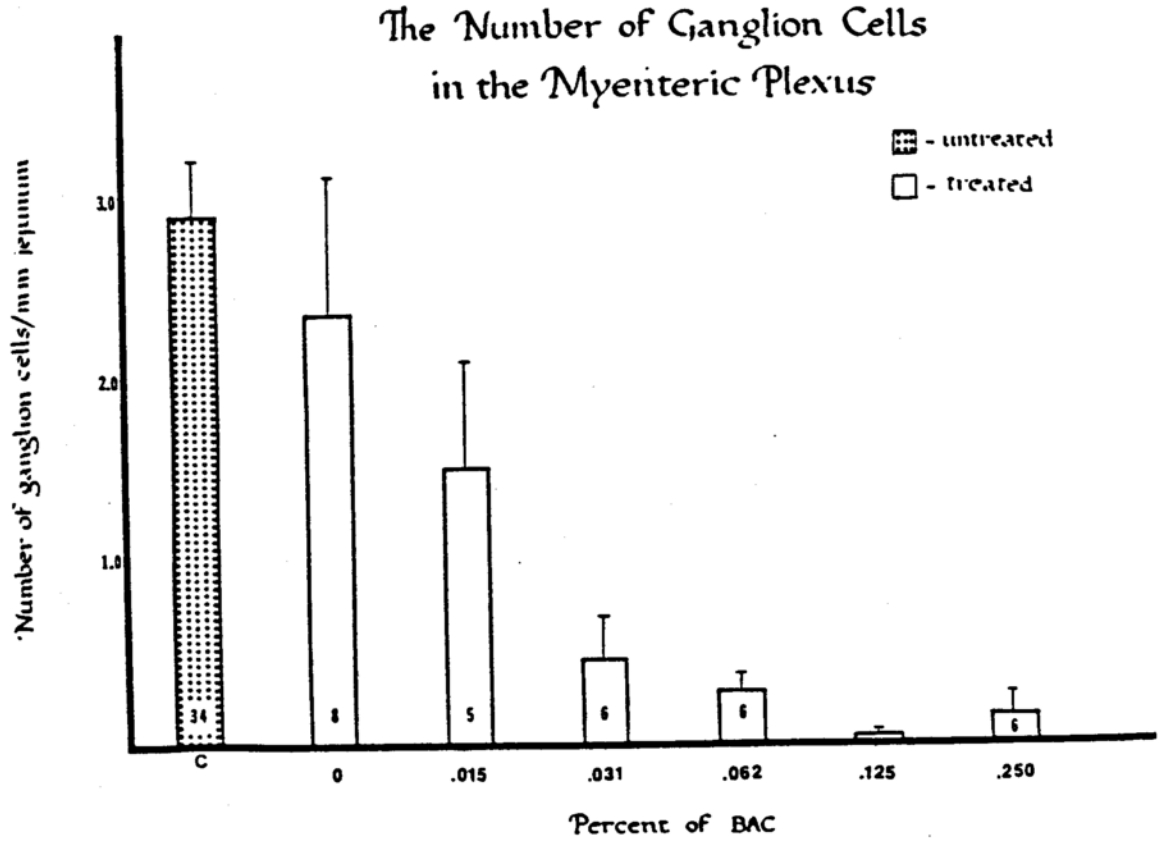
The Number of Ganglion Cells
in the Myenteric Plexus
- untreated
- treated
Number of ganglion cells/mm jejunum
3.0
2.0
1.0
34
8
5
6
6
6
C
0
.015
.031
.062
.125
.250
Percent of BAC

and substance P (endogenous peptides found in the enteric plexuses, see pages 6-15) in the BAC-treated intestine, and observed a reduction in peptide immunoreactivity in the myenteric plexus and adjacent muscle layers. Serosal application of other surfactants of varying charges (benzethonium chloride, cationic; dioctyl sodium sulfosuccinate, sodium lauryl sulfate, and sodium ricinoleate, anionic; Triton X-100, nonionic) produced similar results to those obtained with BAC (i.e., reduction in the number of myenteric but not submucoal neurons). Collectively, our data show that by manipulating the concentration of surfactant we apply to the serosal surface of the rat jejunum, we can selectively eliminate greater than 90% of the neurons in only one component of the enteric nervous system - the myenteric plexus. Because elimination of myenteric neurons occurs without alteration of the remainder of the intestinal wall, it is a useful technique to study the contribution of the myenteric plexus in several intestinal functions. None of the aforementioned methods used in the study of intrinsic intestinal nerves are selective for the myenteric plexus. Also, the chronic nature of our preparation makes it applicable to the study of compensatory changes which may occur after removal of myenteric neurons.

Statement of the Problem

The enteric nervous system is a complex nerve network consisting primarily of two ganglionated plexuses: the myenteric (Auerbach's) plexus, which is situated between the two external muscle layers, and the submucosal (Meissner's) plexus, which lies in the submucosa. Involvement of the enteric nervous system in intestinal motility has been suggested by investigators employing a host of pharmacological and surgical techniques. However, the methods employed thus far in the study of enteric nerve function have not been able to clearly discern the individual contributions of the myenteric and submucosal plexuses in the regulation of intestinal contractility.

Fox and coworkers (1983) have described a method by which selective ablation of the myenteric plexus is achieved without alteration of the remainder of the intestine. This technique is unique in that only one component of the enteric nervous system, the myenteric plexus, is destroyed. The primary aim of the present investigation is to employ this technique to assess the role of the myenteric plexus in intestinal motility both in vivo and in vitro.

Many enteric neuronal substances influence intestinal motility. However, whether these compounds act directly upon intestinal smooth muscle or indirectly through the enteric nerves to produce their effects has not been clearly defined. Therefore, another aim of the present study is to determine the contribution of the myenteric plexus in the responses of the intestinal muscle to enteric neuronal

substances.

Adrenergic agents evoke responses of the intestinal muscle by interaction with both alpha and beta adrenergic receptors. It had been previously proposed that within the intestine, alpha receptors are located on neurons, while beta receptors are present on the muscle. The present investigation will determine whether the myenteric plexus is the site of one or both of these receptor types which mediate mechanical responses of intestinal muscle.

The specific goals of this investigation can be summarized as follows:

1. Pharmacological characterization of the effect of ablation of the myenteric plexus on intestinal contractility in vitro.

2. Examination of the myoelectric activity of jejunum which is devoid of myenteric neurons in the conscious rat.

3. Determination of the contribution of the myenteric plexus in the intestinal muscle responses produced by several putative enteric neurotransmitters.

4. Determination of the location of adrenergic receptors which mediate mechanical responses of intestinal smooth muscle.

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CHAPTER 1

Pharmacological Characterization of Rat Jejunal Contractility After Chronic Ablation of the Myenteric Plexus

Abstract

We have pharmacologically characterized longitudinal muscle contractility of the isolated rat jejunum chronically devoid of myenteric neurons. The myenteric plexus of a 3-4 cm segment of rat jejunum was ablated by serosal application of the cationic surfactant benzalkonium chloride (BAC). Fifteen days after treatment, both BAC-treated and untreated control jejunal segments were removed from each animal for pharmacological studies. BAC-treated tissues did not respond to the ganglionic stimulants nicotine, DMPP and McN-A-343, or the acetylcholinesterase inhibitor, physostigmine. Short pulse duration transmural electrical stimulation (0.1 msec) caused a frequency-dependent, tetrodotoxin-sensitive contraction in control but not BAC-treated tissues. Long pulse duration transmural electrical stimulation (5.0 msec) contracted both control and BAC-treated jejunum. These contractions were partially reduced but not completely blocked by tetrodotoxin. To assess smooth muscle function, the mechanical responses to the muscarinic receptor agonist carbachol were examined. Carbachol contracted both control and BAC-treated tissues with maximum responses and ED_{50} values which were not significantly different. In addition, the maximum tension development produced by 10^{-2} M barium chloride in similar lengths of control and BAC-treated jejunal segments was not significantly different. These studies demonstrate that chronic ablation of the myenteric plexus by serosal application of benzalkonium chloride

eliminates neuronally-mediated responses without a concomitant alteration in longitudinal muscle contractility. Since nerve-mediated longitudinal muscle responses are lost despite the presence of an intact submucosal plexus in the BAC-treated jejunum, our data suggest that the motor neurons innervating the rat jejunal longitudinal muscle are located in the myenteric plexus.

Introduction

The enteric nervous system consists of two ganglionated plexuses: the myenteric (Auerbach's) plexus, which is situated between the two external muscle layers; and the submucosal (Meissner's) plexus, which lies in the submucosa. Intrinsic nerve fibers connect the two plexuses and also extend into the muscle and the mucosa. In addition, extrinsic nerves from both the parasympathetic and sympathetic divisions of the autonomic nervous system are interspersed throughout the enteric nerve network (Jessen et al., 1980; Gershon, 1981; Llewellyn-Smith et al., 1983).

The precise role of the enteric nervous system in the regulation of intestinal smooth muscle contractility has been difficult to ascertain due to the location of the nerve plexuses. Because enteric neurons are contained within the gut wall, the classical denervation techniques of ganglionectomy and nerve transection are difficult to perform and require disruption of the muscle layers. Nevertheless, several experimental approaches have been devised to remove or obliterate intrinsic intestinal nerves from the intestine. Paton and Zar (1965, 1968) stripped the myenteric plexus away from guinea pig longitudinal muscle strips to obtain nerve-free muscle preparations. Denervated circular muscle strips have been prepared by virtue of the myenteric neurons' ability to adhere to the longitudinal muscle upon separation of the muscle layers (Evans and Schild, 1953). Anoxia elicits degenerative changes in

intramural ganglion cells (Hukuhara et al., 1961). Cold storage of intestinal segments reduces the levels of acetylcholine, acetylcholinesterase and choline acetyltransferase, as well as the responsiveness to electrical stimulation and ganglionic stimulating drugs (Ambache, 1946; Innes et al., 1957; Ochillo, et al, 1978). The aforementioned methods utilize acutely denervated muscle strips or involve a functional loss of all nerve activity in the intestine. Hence, the effect of chronic denervation of the enteric nervous system on intestinal smooth muscle function, or the individual contributions of the myenteric and submucosal plexuses in the regulation of intestinal contractility have not been examined.

Recently, our laboratory has shown that serosal application of surfactants to the rat jejunum reduces, in a dose-dependent manner, the number of ganglion cells in the myenteric plexus without affecting the neurons of the submucosal plexus (Fox et al., 1983). In addition, the presence of somatostatin, substance P, met-enkephalin and VIP immunoreactivity (endogenous peptides found in enteric nerves) is markedly reduced in the area of the myenteric plexus and adjacent muscle layers after surfactant treatment. Our method offers the advantage of producing a selective, chronic denervation of only one component of the enteric nervous system, the myenteric plexus. Thus, it is a useful technique to study the contribution of the myenteric plexus in intestinal functions.

The present investigation was undertaken to characterize the role of the myenteric plexus in the regulation of longitudinal smooth muscle contractility. This was accomplished by comparing the mechanical responses produced by muscle and neuronal stimulation in normal and myenteric neuron-ablated jejunal segments in vitro.

Methods

Animal Treatment

In all experiments, the cationic surfactant benzalkonium chloride (BAC) was utilized to ablate the myenteric plexus. This method has been previously described by Fox et al., 1983. Briefly, male albino rats (Sprague-Dawley Inc., Madison, WI) weighing 200-225 gm were anesthetized by an i.p. injection of a combination of pentobarbital and chloral hydrate. A midline incision was made, and a portion of the jejunum was brought outside the peritoneal cavity. A 3-4 cm jejunal segment was delineated by serosal suture tags beginning approximately 8 cm from the ligament of Treitz. A 0.062% solution of BAC was applied to the serosal surface of the delineated jejunal segment every 5 minutes for 0.5 hr. Following this treatment, the intestine was thoroughly rinsed with saline and returned to the peritoneal cavity. The midline incision was closed, and the animals were allowed to recover. Fifteen days after treatment, animals were sacrificed by a blow to the back of the head, and the treated jejunal segment and an untreated control segment approximately 3 cm orad to this were removed for experimentation. Therefore, in all

experiments, each animal served as its own control. This treatment regimen has been shown to destroy greater than 90% of the ganglion cells in the myenteric plexus of the BAC-treated jejunal segments (Fox et al., 1983).

Pharmacological Studies

After BAC-treated and control jejunal segments were removed (≈ 2 cm each) and freed from mesenteric attachments, they were suspended along their longitudinal axis in water-jacketed 25 ml tissue baths containing Krebs bicarbonate buffer (NaCl, 118; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 2.5; $MgCl_2 \cdot 6\ H_2O$, 0.5; NaH_2PO_4, 1.0; $NaHCO_3$, 25.0; and glucose, 11.0 mM). The solution was aerated with a mixture of oxygen (95%) and carbon dioxide (5%) and maintained at a constant temperature of 37°C by a HAAKE D1 immersion circulator. Mechanical responses were recorded isometrically on a Grass model 5D polygraph with Grass transducers (FT 03) (Grass Instrument Co. Quincy, Mass.). One gram of resting tension was applied to the tissues, and they were allowed to equilibrate for 60-90 minutes before initiation of experiments. Buffer was changed every 15 minutes during the equilibration period.

Drug responses were obtained in a non-cummulative fashion. Immediately after the peak response to a given concentration of drug was obtained, the tissue was washed three times with Krebs buffer. The time interval between addition of increasing concentrations of drug was 10 minutes. Contractile responses were expressed as a

percentage of the maximum contraction obtainable for a given tissue in the presence of 10^{-2} M barium chloride. We have found that 10^{-2} M barium chloride maximally contracts both control and BAC-treated tissues, so this concentration of barium chloride was added to the bath at the end of the experiment for determination of maximum tissue contraction.

Transmural Electrical Stimulation

Jejunal segments were mounted on a plexiglass holder containing two vertical silver electrodes, 1.5 cm in height, positioned 0.8 cm from one another. The jejunal segments were placed over one electrode and fixed to the holder such that one electrode was positioned within the lumen, while the other electrode was outside the serosa. The other end of the tissue was attached to the Grass force transducer for the measurement of isometric tension. Experimental conditions such as buffer, resting tension and equilibration period were the same as that described under pharmacological studies.

Tissues were stimulated by electrical square wave pulses from a Grass Model S48 stimulator. The stimulator was connected to a voltage divider unit for simultaneous electrical stimulation of both BAC-treated and control tissues. The effect of stimulation of pulses of short (0.1 msec) and long (5.0 msec) duration was studied over a frequency range of 0.5-64 Hz. These parameters of pulse duration were chosen because short pulse duration stimuli have been reported to stimulate only nerves, while long pulse duration

eletrical stimulation stimulates both nerve and muscle (Gershon, 1967). A pulse height of 25V was used throughout the experiments. This voltage elicited a maximum response regardless of the frequency. The stimulus was applied for 2 seconds for short and 30 seconds for long pulse duration stimulation experiments. The maximum response was not altered if the stimulus was applied for longer periods than those designated. Stimuli were delivered no less than every 4 minutes.

In order to assess blockade by tetrodotoxin, after one frequency-response curve was obtained on a given tissue, the tissue was washed three times with buffer, then incubated with tetrodotoxin (3×10^{-7} M) for 15 minutes before a second frequency-response curve was obtained. Preliminary experiments have shown that the responses of 2 consecutive frequency-response curves spaced 15 minutes apart were not significantly different. All electrically-induced contractions were expressed as a percentage of the maximum contraction produced by 10^{-2} M barium chloride.

Drugs

Nicotine, 1,1-dimethyl-4 phenyl-piperazinium iodide (DMPP), carbamylcholine chloride (Carbachol), physostigmine sulfate (Eserine sulfate), papavarine HCl, tetrodotoxin, and hexamethonium bromide were obtained from Sigma Chemical Co., St. Louis, MO. Barium chloride was obtained from Mallinckrodt Chemical Works, St. Louis, MO. McN-A-343 was a gift from McNeil Pharmaceutical, Spring House, PA.

Benzalkonium chloride (Zephiran chloride®) was a gift from Winthrop Laboratories, Inc., New York, N.Y. Drug solutions were freshly prepared on the day of the experiment. All drugs were dissolved in 0.9% sodium chloride except barium chloride and papavarine HCl, which were dissolved in distilled water because the high concentrations used were insoluable in saline. Final drug concentrations are expressed as molar strengths using the weight of the salt.

Results

Effect of Ganglionic Stimulants

The mechanical responses elicited by three ganglionic stimulants were examined on control and BAC-treated jejunum. As shown in figure 1, nicotine, DMPP and McN-A-343 produced a biphasic response in control tissues only. The response was characterized by an initial relaxation followed by a contraction. Some tissues exposed to DMPP exhibited relaxation followed by a return to baseline tension instead of a contraction. If control tissues were exposed to a high dose of nicotine (3×10^{-4} M) without prior exposure to lower doses, relaxation was not observed, and the contractile response was more prominent.

In all cases, the three ganglionic stimulants did not alter the baseline activity in the BAC-treated tissues. The BAC-treated tissues, however, were able to relax in the presence of papavarine HCl and contract in response to barium chloride.

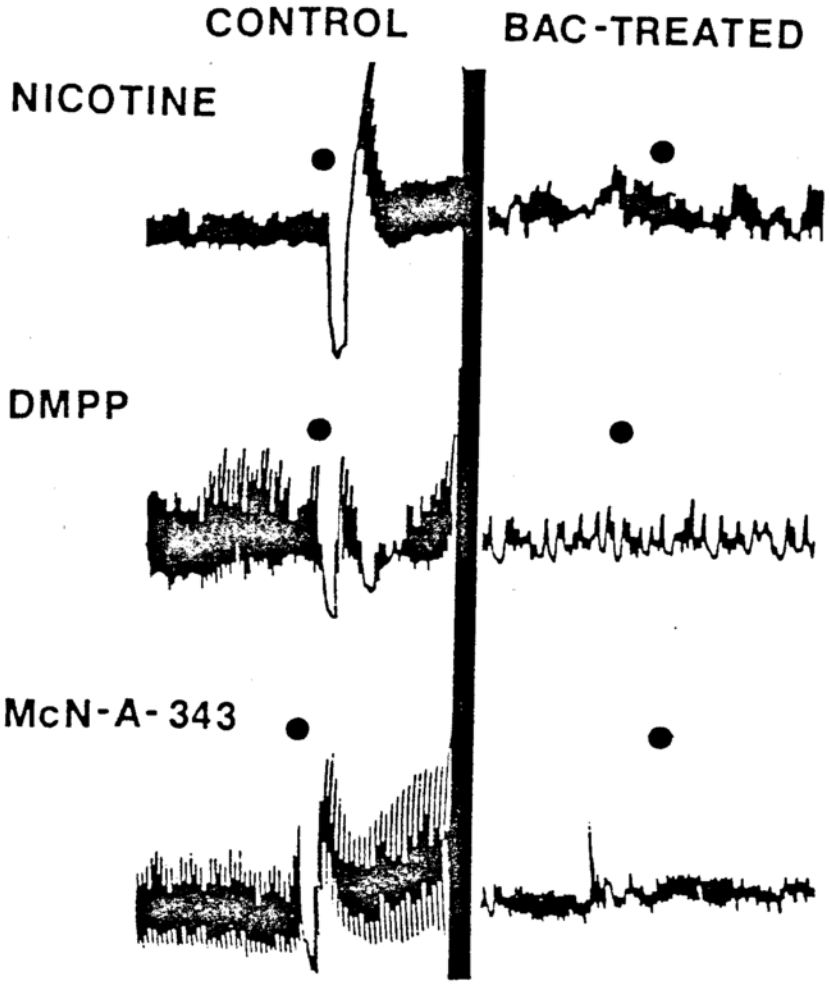


Figure 1. Representative tracings of the effect of three ganglionic stimulants on control (left panel) and BAC-treated (right panel) jejunum. For each agent, the control and BAC-treated responses were from the same animal. The dot depicts the addition of drug to the bath. The concentrations of drug added were nicotine, 3×10^{-4} M; DMPP, 1×10^{-4} M; and McN-A-343, 3×10^{-5} M. Note the lack of response on the BAC-treated tissues.

Effect of Physostigmine

Physostigmine, an acetylcholinesterase inhibitor, produced a concentration-dependent contraction in control jejunum which was markedly greater than the response observed in the BAC-treated jejunum (fig. 2). The contractions elicited by physostigmine were characterized by a slow increase in tension which took 3-4 minutes to reach a maximum at each concentration. Once the maximum effect for physostigmine in the dose-response curve had been observed, increasing concentrations produced submaximal contractions. BAC-treated tissues responded minimally or not at all to physostigmine but contracted in the presence of barium chloride.

Effect of Transmural Electrical Stimulation

The effect of both short (0.1 msec) and long (5.0 msec) pulse duration transmural electrical stimulation was examined on control and BAC-treated jejunum. As shown in figure 3, an electrical stimulus with a pulse duration of 0.1 msec and a frequency of 12 Hz caused a slight relaxation followed by a contraction in control jejunum (upper left panel) but produced no response in BAC-treated jejunum from the same animal (upper right panel). The inital relaxation was more pronounced at lower frequencies (not shown). The contractile response produced by short pulse electrical stimulation in control jejunum was frequency-dependent (0.5-32 Hz) and completely blocked by 3×10^{-7} M tetrodotoxin (fig. 4). However, regardless of frequency, electrical stimulation with a pulse

duration of 0.1 msec, did not alter baseline activity in the BAC-treated jejunum (fig. 4).

In contrast, electrical square wave pulses of 5.0 msec duration produced a response in both control and BAC-treated jejunum (fig. 3, bottom two panels). In control jejunum, the response was characterized by a relaxation followed by a contraction. The BAC-treated jejunum exhibited the contractile response only. In both control and BAC-treated jejunum, the contractile response produced by 5.0 msec pulse duration electrical stimulation was frequency-dependent (0.5-32 Hz) and significantly reduced but not completely blocked by 3×10^{-7} M tetrodotoxin (fig. 5). The percentage of the contractile response produced by 5.0 msec pulse duration electrical stimulation which was blocked by tetrodotoxin was similar in both control and BAC-treated jejunum (Table 1). Tetrodotoxin (3×10^{-7} M) was the lowest concentration which completely blocked the contractions produced by 0.1 msec pulse duration electrical stimulation in the control tissues.

Effect of Carbachol

To demonstrate that the nonresponsiveness of the BAC-treated jejunum to pharmacological and electrical nerve stimulation was due to a neuronal loss rather than a deficit in smooth muscle function, the response to the muscarinic receptor agonist carbachol was compared on both control and BAC-treated jejunum. Carbachol produced a concentration-dependent contractile response in both control and

BAC-treated jejunum (fig. 6). The maximum responses, expressed as of percentage of the maximum contraction produced by 10^{-2} M barium chloride, (99.6%, control; 100%, BAC-treated) and the ED_{50} values (3.06×10^{-7} M, control; 3.58×10^{-7} M, BAC-treated) for carbachol in control and BAC-treated tissues were not significantly different (Student's t-test for paired values, $p < 0.05$). There was, however, a slight delay (< 5 sec) in onset of the response produced by carbachol in the BAC-treated tissues compared to controls.

The maximum tension development (produced by 10^{-2} M barium chloride) for similar lengths ($\approx$ 2 cm) of control and BAC-treated jejunal segments was measured in order to determine if the absolute amount of tension produced by these preparations differed. There was not a significant difference in the maximum tension development between control and BAC-treated jejunum (4.1 ± 0.2 g, control; 4.7 ± 0.4 g, BAC-treated). (Student's t-test for paired values, $p > 0.05$, n=28). Therefore, expression of carbachol responses as a percentage of the maximum contraction does not mask the actual contractile ability of the muscle.

Because the BAC-treated jejunum was chronically denervated, we looked for the possible appearance of supersensitivity to carbachol. Consistent with the phenomenon of denervation supersensitivity, we expected the BAC-treated carbachol dose-response curve to be shifted to the left of the control carbachol dose-response curve. This leftward shift was not observed as shown in figure 6.

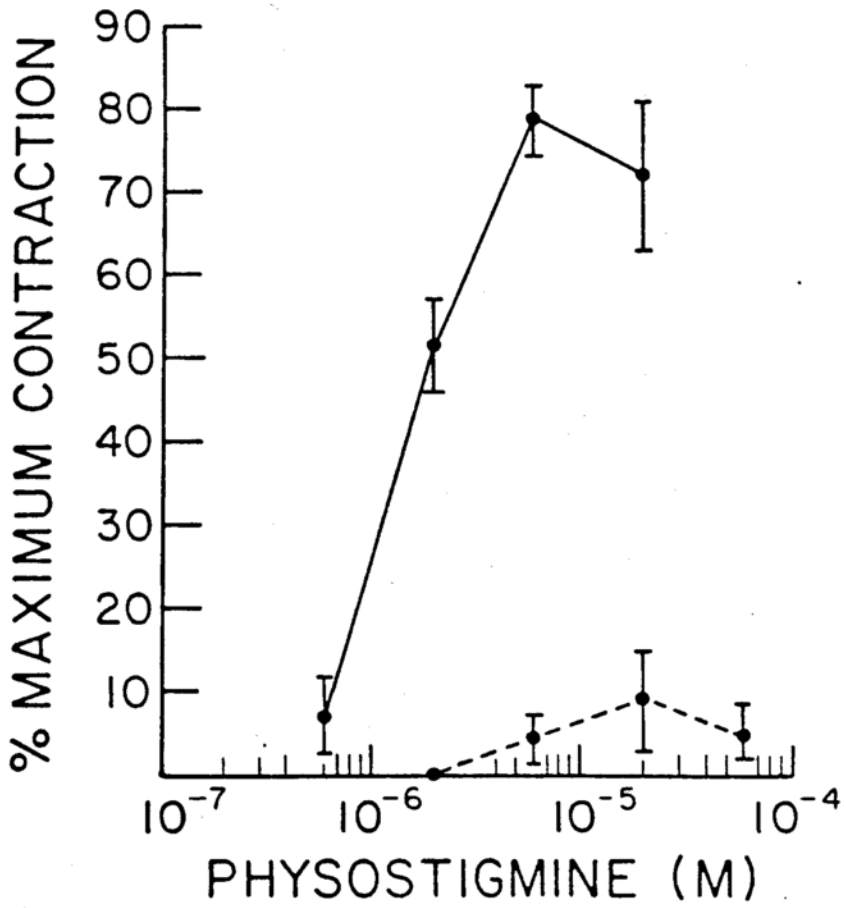


Figure 2. Log dose-response curves for the contractile response for physostigmine on control (——) and BAC-treated (---) jejunal segments. Vertical bars represent S.E.M. (n = 5).

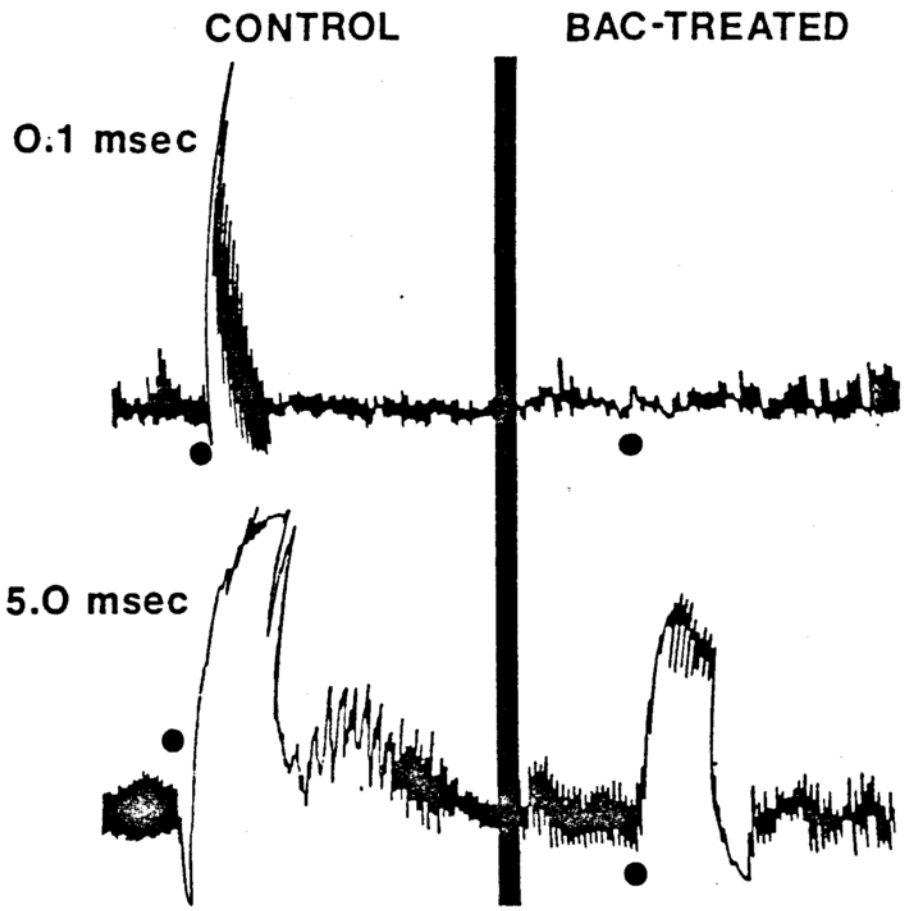
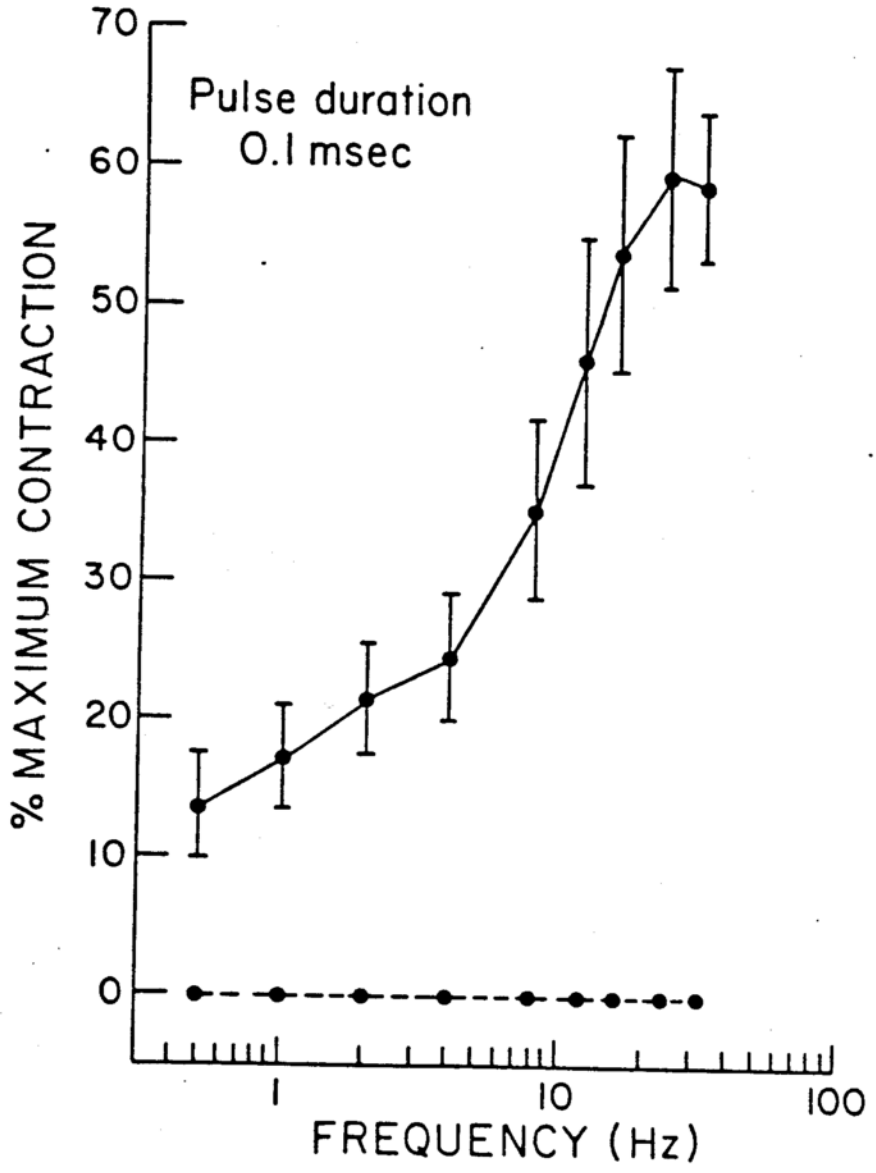


Figure 3. Representative tracings of the responses to short (0.1 msec) and long (5.0 msec) pulse duration transmural electrical stimulation on control (left panel) and BAC-treated (right panel) jejunum. Dots depict the initiation of the electrical stimulus. The 0.1 msec pulse duration tracings were performed at a frequency of 12 Hz. The 5.0 msec pulse duration tracings were performed at a frequency of 2 Hz.


70
60
50
40
30
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10
0
% MAXIMUM CONTRACTION
Pulse duration
0.1 msec
1
10
100
FREQUENCY (Hz)

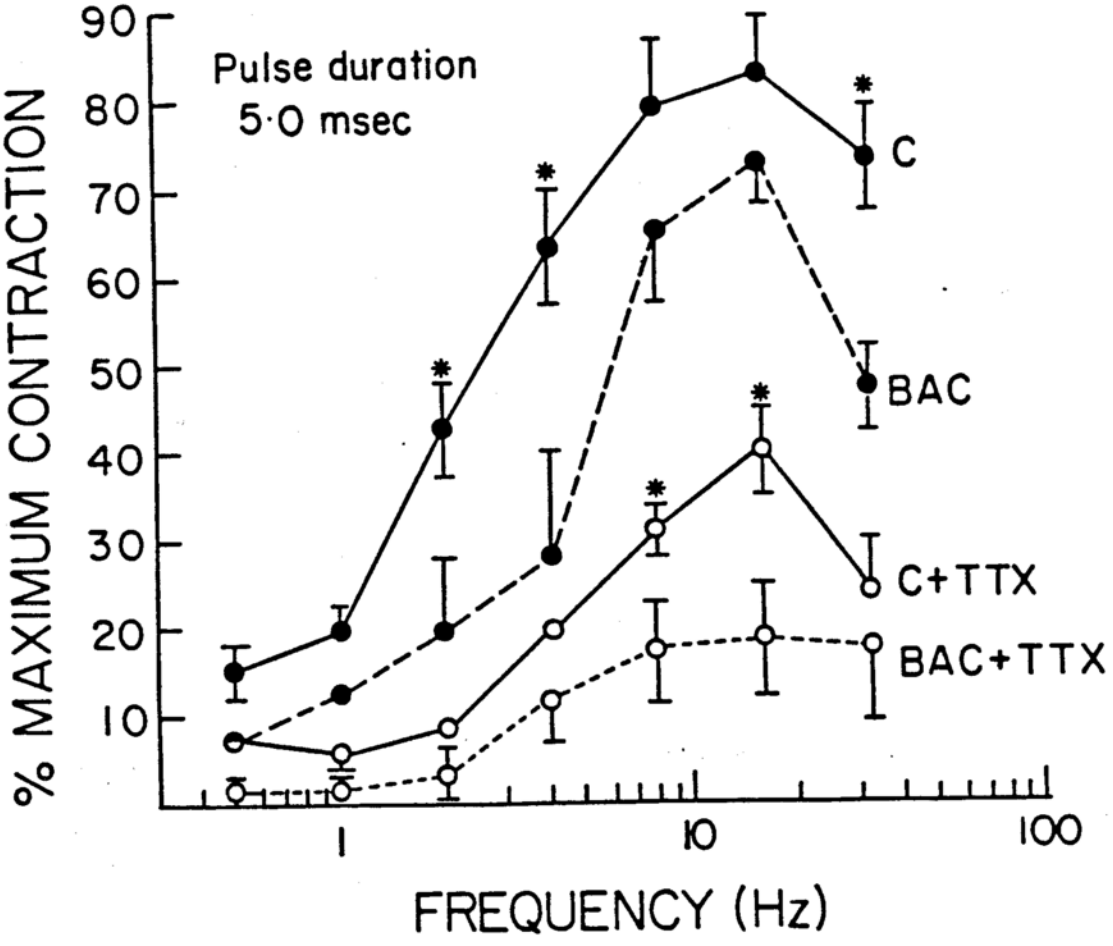
Pulse duration
5·0 msec
% MAXIMUM CONTRACTION
90
80
70
60
50
40
30
20
10
1
10
100
FREQUENCY (Hz)
C
BAC
C+TTX
BAC+TTX
*

TABLE 1 Comparison of the percentage of the contractile response produced by long-pulse duration (5.0 msec) stimulation which is blocked by tetrodotoxin (TTX) in control and BAC-treated rat jejunum. (Data adapted from figure 5).

	Percentage of contractile response blocked by TTX[a,b]	
Frequency	Control	BAC-Treated
0.5	69.1 ± 20.8	83.3 ± 16.7
1.0	76.2 ± 6.5	91.3 ± 5.4
2.0	77.3 ± 8.5	88.9 ± 7.9
4.0	68.5 ± 7.7	68.7 ± 7.6
8.0	57.8 ± 6.6	75.1 ± 7.3
16.0	51.3 ± 4.8	73.4 ± 8.9*
32.0	68.0 ± 5.8	68.0 ± 10.5

[a] Percentage of response which is blocked by TTX is calculated as:

$$100 \times \left[1 - \left(\frac{\%\text{ maximum contraction with TTX}}{\%\text{ maximum contraction without TTX}}\right)\right]$$

[b] Numbers represent mean ± SE of the percent response blocked by TTX.

* Significantly different from control (Student's t-test for paired values, $p < 0.05$)

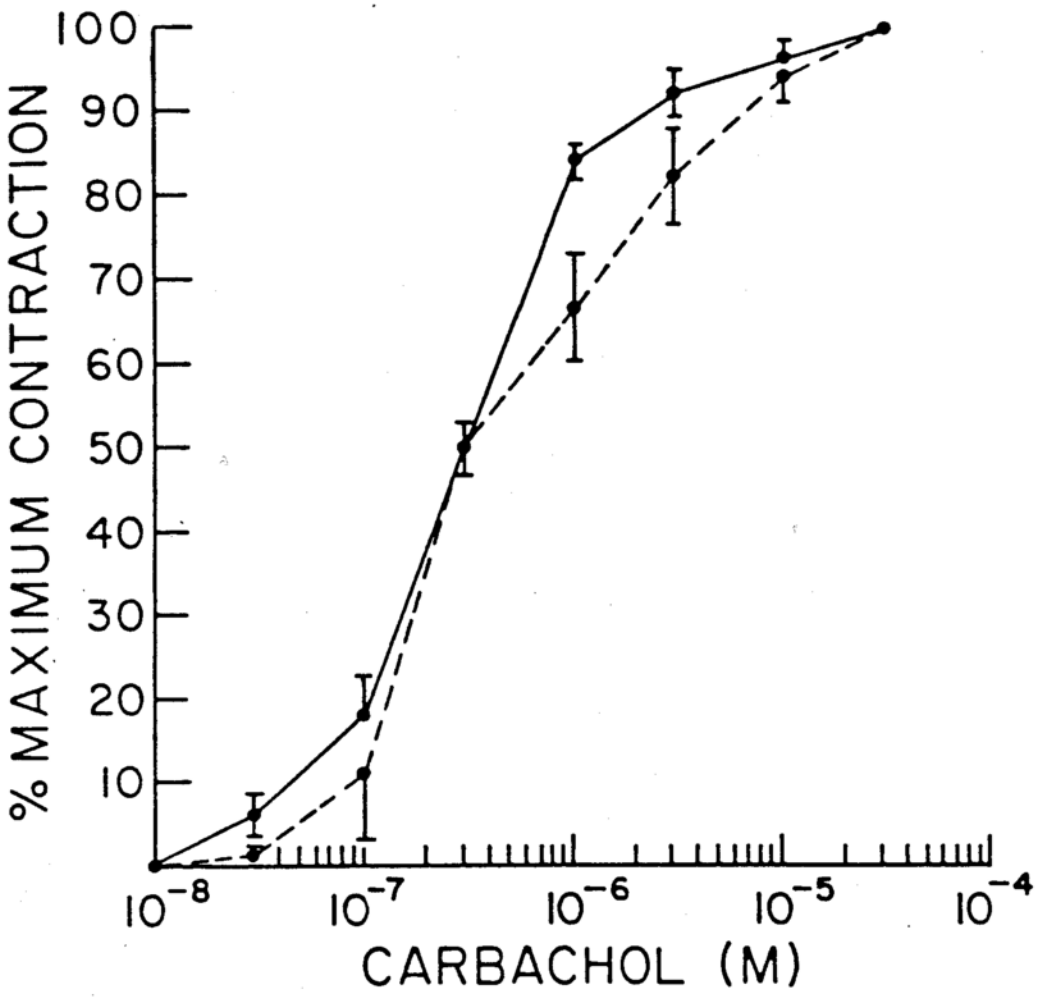

Figure 6. Log dose-response curves for the contractile response of carbachol on control (——) and BAC-treated (---) jejunal segments. Vertical bars represent S.E.M. (n = 6).

To pursue further the possibility of denervation supersensitivity with carbachol, we repeated carbachol dose-response curves in the presence of 3×10^{-5} M hexamethonium (n=2). This was performed to block possible carbachol stimulation of nicotinic receptors which could occur in control but not BAC-treated tissues. No leftward shift of the carbachol dose-response curve was observed in the presence of hexamethonium. In addition, carbachol dose-response curves were repeated in tissues removed from animals 30 days (instead of the usual 15 days) after the initial BAC-treatment. There was also not a significant difference between control and BAC-treated jejunum in the ED_{50} values or maximum responses for carbachol when tissues were studied 30 days after BAC treatment (n=3). Therefore, BAC-treated jejunum did not exhibit denervation supersensitivity to a cholinergic agonist.

Tissue Characteristics

BAC-treated tissues were slightly larger in diameter than control jejunum. Also, similar lengths of BAC-treated jejunum removed 15 days after treatment were significantly heavier than control tissues (0.18 ± .01 g/cm, BAC-treated; 0.09 ± .004 gm/cm; control; Student's t-test for paired values; n = 6).

Many of the BAC-treated jejunal segments exhibited periods of spontaneous contraction (fig. 7). These contractions ranged from sharp spikes of phasic activity to sustained increases in tension with amplitudes occasionally as high as those obtained with 10^{-2} M

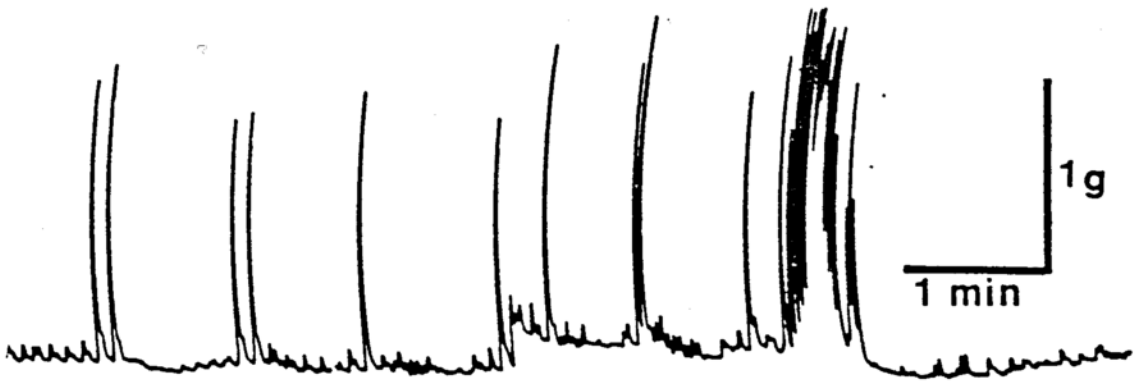

Figure 7. A representative tracing of the spontaneous contraction exhibited by some BAC-treated jejunal segments.

barium chloride. Spontaneous contractions were observed in the presence of 3×10^{-7} M tetrodotoxin indicating a myogenic origin to the spontaneous activity. Persistent spontaneous contraction made approximately 10% of the BAC-treated tissues unusable for experimentation. Spontaneous high amplitude contraction was not seen in the untreated control jejunal segments.

Discussion

We have previously shown by histological and immunohistochemical techniques that serosal application of BAC to the rat jejunum selectively destroys greater than 90% of the myenteric neurons without affecting the number of submucosal neurons (Fox et al., 1983). The effects of BAC are dependent both on concentration and time of sacrifice after initial treatment. The present investigation pharmacologically demonstrates a loss in neurally-mediated responses without a concomitant deficit in longitudinal smooth muscle contractility in preparations lacking myenteric neurons.

BAC-treated jejunum behaves like ganglion-free muscle strips (Gasser, 1926; Evans and Schild, 1953; Paton and Zar, 1965; Paton and Zar, 1968), aganglionic segments of colon from patients with Hirschsprung's disease (Beleslin et al., 1980), cold stored (Ambache, 1946; Innes et al., 1947; Day and Vane, 1963) and anoxic (West et al., 1951; Day and Vane, 1963) intestine. These preparations as well as the BAC-treated jejunum are not responsive to nicotine, physostigmine or nerve-selective electrical stimulation,

indicating a loss in neuronal function. The above preparations are, however, responsive to muscarinic receptor agonists (i.e., acetylcholine and carbachol) which act primarily but not exclusively on intestinal smooth muscle. An elaboration of the responsiveness of the BAC-treated jejunum to both nerve and muscle stimulation is presented below.

BAC-treated tissue was unable to respond to the ganglionic stimulants, nicotine and DMPP, which stimulate nicotinic receptors, or McN-A-343 purported to act on muscarinic receptors at the level of the ganglia (Roszkowski, 1961). BAC-treatment has apparently eliminated the site of action of these three ganglionic stimulants. It is noteworthy that even though the submucosal plexus is present in the BAC-treated jejunum, these ganglionic stimulants do not produce any mechanical response. The implications of this observation are twofold. The myenteric plexus is the site of action of ganglionic stimulants to elicit mechanical responses in the isolated intestine. Secondly, the motor neurons innervating longitudinal muscle of the rat jejunum are located in the myenteric plexus.

The slow increase in tension produced by physostigmine in control jejunum was drastically reduced in BAC-treated tissue. There may be two mechanisms by which physostigmine causes contraction in the intestine. By virture of its ability to block acetylcholinesterase, it may cause an accumulation of spontaneously released acetylcholine from cholinergic nerves (Johnson, 1963). In addition,

physostigmine has been shown to release acetylcholine in a guinea pig tracheal preparation (Carlyle, 1963) and in the guinea pig ileum (Cox and Lomas, 1972) independent of its acetylcholinesterase blocking properties. Since BAC-treated jejunum is devoid of myenteric ganglion cells, acetylcholine released spontaneously or by inducement would be diminished. Therefore, either mechanism of physostigmine-induced contraction in the rat jejunum would explain the greatly reduced response in BAC-treated jejunum.

Transmural electrical stimulation has been a standard method to assess intrinsic neuronal activity in the gastrointestinal tract. Most researchers agree that mechanical or functional denervation abolishes the responses to transmural stimulation (Day and Vane, 1963; Paton and Zar, 1965, 1968). However, certain parameters of transmural stimulation are critical to selective neuronal stimulation. Gershon (1967) has observed that contractions of transmurally stimulated guinea pig ileum and rabbit jejunum are blocked by tetrodotoxin. Even if the stimulus frequency or amplitude were increased, tetrodotoxin was still able to block the contractions. However, if pulses of long duration were utilized (1-10 msec, rabbit jejunum; 0.3-0.5 msec, guinea pig ileum), tetrodotoxin, 10^{-7} gm/ml was not able to completely block the responses. Likewise, Bennett and Stockley (1973) have described contractions induced by electrical stimulation of the guinea pig ileum (1 msec pulse duration, 30 sec train, 0.2-64 Hz) which were reduced but not abolished by 5 x

10^{-7} gm/ml tetrodotoxin. Both groups of researchers have attributed tetrodotoxin-resistant contractions to direct electrical excitation of smooth muscle cells. In the present investigation, we have also concluded that the pulse duration of the electrical stimulus is crucial to neuronal selectivity. Short pulse duration (0.1 msec) electrical stimulation caused a frequency-dependent, tetrodotoxin-sensitive contraction in control jejunum only. BAC-treated jejunum did not respond to short pulse duration stimulation regardless of the frequency employed. Conversely, long (5.0 msec) pulse duration electrical stimulation contracted both control and BAC-treated jejunum. These contractions were significantly reduced but not abolished by tetrodotoxin (3×10^{-7} M). Since both nerve and muscle contribute to the contraction produced by long pulse duration stimulation in control jejunum, and predominantly muscle is responding in BAC-treated tissue, comparable tetrodotoxin-sensitive responses in these two tissues is perplexing. One would have expected little or no reduction in the contractile response by tetrodotoxin in the BAC-treated jejunum. The concentration of tetrodotoxin utilized in long pulse duration stimulation experiments was the lowest concentration which completely blocked the contractions produced by the low pulse duration electrical stimulation in control tissues. Because lower concentrations of tetrodotoxin did not completely block the low pulse duration stimulation, we did not attempt to use any lower concentrations in the high pulse experiments to unmask the

discrepancies of the tetrodotoxin-sensitive contractions. Four possibilities are offered in explanation of our unexpected results. (1) A small number of myenteric neurons (< 10%) may still exist in BAC-treated tissues to contribute a contractile response with long pulse duration electrical stimulation. (2) Though not consistant with results obtained with ganglionic stimulants, it is possible that the submucosal neurons, which are still intact in the BAC-treated jejunum, were stimulated at the long pulse duration electrical stimulation. (3) Tetrodotoxin may nonspecifically reduce smooth muscle responses in the rat jejunum. (4) Perhaps serosal treatment with BAC changes the relative distribution of ion channels in the jejunal smooth muscle such that there is an increase in the number of Na^+ channels making the muscle more sensitive to tetrodotoxin.

It is noteworthy that unlike control jejunum, BAC-treated jejunum did not exhibit relaxation prior to contraction in response to long (5.0 msec) pulse duration electrical stimulation. This suggests the absence of inhibitory neurons in the BAC-treated tissue.

In order to substantiate that the lack of neuronal responses in the BAC-treated jejunum was not due to poorly functioning smooth muscle, the response to carbachol, a muscarinic agonist, was assessed. Both BAC-treated and control jejunum were equally responsive to this agonist as evidenced by nearly superimposable dose-response curves. This, in conjunction with the fact that both control

and BAC-treated tissue exhibit similar degrees of maximum contraction to barium chloride, suggests that BAC-treatment does not result in a deficit in smooth muscle contractility.

Since this preparation was chronically denervated, one might have expected to observe a denervation supersensitivity response to a muscarinic agonist. Denervation supersensitivity is characteristic of other denervated smooth muscle preparations. For instance, the vas deferens, iris sphincter (Fleming et al., 1973) and detrusor muscle (Ekström and Malmberg, 1984) display a significant shift to the left in the dose-response curve to muscarinic agonists after denervation. Although supersensitivity to the inhibitory effects of adrenergic agonists has been demonstrated after chronic sympathetic denervation of the intestine (Frigo et al., 1984), supersensitivity to a muscarinic agonist in a denervated intestinal preparation has not been reported. The guinea pig ileum, however, exhibited a non-specific supersensitivity to acetylcholine, histamine and potassium after chronic treatment with the ganglionic blocking agent chlorisondiamine (Fleming et al., 1973).

The cholinergic innervation to the smooth muscle of the intestine differs from previously mentioned smooth muscle preparations which exhibit denervation supersensitivity to muscarinic agonists, in that the intestinal ganglia are contained within the gut wall rather than outside of the organ. Therefore, the paucity of references dealing with denervation supersensitivity in intestinal smooth

muscle is probably due to the lack of a technique of removal of the intramural ganglia in a chronic preparation. Though we have been able to successfully ablate the myenteric plexus, we have not observed a supersensitivity response to carbachol in rat jejunal longitudinal muscle even 30 days after the initial treatment with BAC. Denervation supersensitivity in other smooth muscle preparations usually manifests itself approximately one week after denervation (Fleming et al., 1973). Our inability to observe a supersensitivity response to carbachol in BAC-treated jejunum may be due to the presence of an intact submucosal plexus or a small number of remaining myenteric neurons.

Some of the physical properties of the BAC-treated jejunum, such as increases in weight and muscle thickness, are characteristic of other denervated smooth muscle preparations. For instance, the rat urinary bladder becomes distended, markedly increases in weight, and the muscle thickens after autonomic denervation (Ekström and Uvelius, 1981). The Piebald mouse, which has a congenital ganglionic aplasia of the terminal segment of the large intestine, displays hypertrophy of the muscularis externa (Brann et al., 1977). Denervated smooth muscle of the rat urinary bladder exhibits both hyperplasia and hypertrophy (Ekström et al., 1984). Smith (1972) claims that hypertrophy and probably hyperplasia of the intestinal smooth muscle are important features common to all conditions, whether congenital or acquired, in which there is chronic damage to

the myenteric plexus. Though we have observed an increase in the thickness of both longitudinal and circular muscle layers in the BAC-treated jejunum (Fox et al., 1983), we have not determined whether the increase in muscle thickness is due to a hypertrophy or hyperplasia of the smooth muscle cells. Based upon the observations of other denervated smooth muscle preparations, it appears that the increases in muscle thickness and weight of the BAC-treated tissue are a consequence of the denervation and not a result of some nonspecific effect of the surfactant treatment.

Spontaneous contractions displayed by the BAC-treated tissue are probably myogenic and a result of denervation. Both phasic contractions and sustained tonic contractions were observed in BAC-treated jejunal segments. These contractions were observed in the presence of tetrodotoxin indicating a myogenic origin. In vivo, measurement of myoelectric activity in the BAC-treated jejunum revealed much longer duration spike potentials than in control jejunum, suggesting sustained contractions (Fox and Bass, 1984). Similar to isolated BAC-treated jejunum, muscle strips from denervated rat urinary bladder exhibit marked spontaneous contractions which are unaffected by tetrodotoxin (Ekström and Uvelius, 1981). Evans and Schild (1953) observed that ganglion-free circular muscle preparations of cat jejunum became progressively contracted in the course of an experiment. Similarly, the circular muscle of the aganglionic region of colon in the Piebald mouse has an increased

incidence of discharge of action potentials associated with uncoordinated phasic contractions superimposed upon tonic contracture (Wood, 1973). It has been suggested that one function of enteric ganglia is the maintenance of a tonic state of inhibition in the inherently excitable intestinal musculature (Wood, 1972; Ohkawa and Prosser, 1972). Our data are consistent with this suggestion. Several types of myenteric neurons are ablated in the BAC-treated jejunum (Fox et al., 1983), and lack of a neuronal influence which is primarily inhibitory would explain the tissue's propensity towards contraction.

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CHAPTER 2

Effect of Ablation of the Myenteric Plexus on the Myoelectric Activity of the Rat Jejunum

ABSTRACT

Serosal application of benzalkonium chloride (BAC), a cationic surfacant, was utilized to selectively ablate the myenteric neurons of the rat jejunum. The myoelectric activity of the BAC-treated area and the areas both orad and caudad to it were assessed. In the jejunal segment devoid of myenteric neurons, the basic electric rhythm (BER) pattern was erratic and the amplitude of the BER attenuated. The BER frequency of the jejunal area caudad to the BAC-treated area was 30.7 ± 0.73 cycle/min, which was significantly reduced from the normal BER frequency (37.7 ± 0.64 cycle/min). Spike potentials were normal in areas both orad and caudad to the treated area, existing in regular cycling bursts (migrating myoelectric complex; MMC). Spike potentials in the treated area did not appear until approximately 13-17 days post BAC treatment. These spikes, though of greater duration and lower frequency than the adjacent areas, always appeared after an orad burst and were followed by a caudad burst. In addition, the following motility parameters of the regions orad and caudad to the treated area were not significantly different from control animals: burst duration, period, and burst propagation velocity. In conclusion, the present investigation has demonstrated that BAC-induced ablation of the myenteric neurons in the rat jejunum disrupts the BER but not the MMC propagation. This

suggests that the myenteric neurons play a modulatory role in the generation and propagation of the BER while the submucosal neurons may be more important in the control of the MMC.

INTRODUCTION

Two forms of electrical activity have been recorded from the muscle of the gastrointestinal tract of various mammalian species. One form, called the basic electric rhythm (BER) or "slow waves," consists of propagating cyclic oscillations of smooth muscle membrane potential. The BER is omnipresent and its frequency varies with different portions of the GI tract as well as with different species (Bass, 1968). At the present time, the genesis of the BER is controversial. Based on in vitro experimentation, it was postulated that the BER is initiated in the longitudinal muscle and spreads electronically to the circular muscle (Connor et al., 1977). In contrast, other evidence suggests that the BER is generated by circular muscle cells (Taylor et al., 1975). Recently, it has been suggested that the BER may originate from the Interstitial Cells of Cajal, connective tissue type cells, which are intimately associated with the intrinsic nerve plexuses (Thuneberg, 1982).

The other form of intestinal myoelectric activity, called spike potentials, is characterized by rapid depolarizations and repolarizations of the smooth muscle membrane. Spike potentials, when they occur, are superimposed upon the BER and elicit smooth muscle contractions. Spike potentials are thought to originate in the circular smooth muscle. A distinct pattern of electrical spike potentials, referred to as the migrating myoelectric complex (MMC),

occurs in the fasted state in carnivores and omnivores and in both fed and fasted states in herbivores (for review, see Costa and Furness, 1982). The MMC consists of regularly cycling stages (Carlson et al., 1970) which begin in the upper portion of the GI tract and transverse the entire small intestine (Szurszewski, 1969). The rate of propagation of the MMC is slow compared to the propagation velocity of the BER. For example, in the dog duodenum, the MMC and BER travel at the rate of 5.7-11.7 cm/min (Code and Marlett, 1975) and 15 cm/sec (Bass and Wiley, 1965a), respectively.

Based on indirect evidence, intrinsic nerves have been implicated in the propagative control of the BER and MMC. Ligation (Bass and Wiley, 1965a) as well as transection and reanastomosis (Bass and Wiley, 1965b) of the small bowel decreases BER frequency caudad to the ligature and transection sites, respectively. MMC propagation has been altered pharmacologically utilizing local injections of atropine, tetrodotoxin and hexamethonium (Sarna et al., 1981) and surgically by sequential transections and reanastomoses (Sarna et al., 1983). In contrast to these indirect studies, it has been shown that disruption of the myenteric neurons produced by 4 hours of hypoxia (Hukuhara et al., 1961) decreases both the BER and contractile frequency in the dog small intestine (Szurszewski and Steggerda, 1968a,b; Khin Kyi Kyi and Daniel, 1970). However, the degree of hypoxia-induced damage to the myenteric neurons was not adequate-

ly quantified, and different experimenters yielded conflicting results.

We have shown that benzalkonium chloride (BAC), when applied to the serosal surface of the rat jejunum for 30 minutes, selectively reduces the number of ganglion cells in the myenteric plexus without affecting those of the submucosal plexus (Fox et al., 1983). In addition, a loss in neuronal function, without a concomitant deficit in smooth muscle contractility has been demonstrated in the isolated rat jejunum 15 days after BAC treatment. (Fox and Bass, 1985). The results of these studies suggest that the motor neurons innervating the longitudinal muscle of the rat jejunum are located in the myenteric plexus.

We undertook the present study to examine the effects of selective myenteric neuronal ablation on jejunal myoelectrical activity in the unanesthetized, unrestrained rat with emphasis on BER and MMC propagation.

METHODS

Electrodes and connectors, utilized in the rat were a modification of those of Weisbrodt et al., 1980 (see Appendix for details). Construction of the electrodes differed in that the silver wire embedded in the epoxy was filed flat so that it was flush with the serosa of the intestine instead of protruding into it. Also, the dental acrylic encapsulated socket to which the electrodes were soldered was held in place by a rat jacket instead of being implanted subcutaneously.

Male albino rats (Sprague Dawley, Madison, WI) weighing 350-375g were anesthetized by an i.p. injection of .25-.3 ml/100 g of a pentobarbital-chloral hydrate mixture previously described (Fox et al., 1983). A 1-2 cm skin incision was made in the midscapula and also in the left flank. Through the use of a trocar, the electrode leads were tunneled from scapula to flank. The scapular wound was closed around the wires, and the connector was secured and held in place by a rat jacket (made by us; fashioned after those of Alice King, Medical Arts, Los Angeles, CA). A midline laparotomy was made, and electrodes were brought into the peritoneal cavity through a stab wound in the left abdominal muscles. The reference electrode was implanted subcutaneously in the lower abdominal region.

In several rats, the myenteric plexus of a segment of jejunum was chemically ablated by serosal application of benzalkonium chlo-

ride (BAC) as previously described (Fox et al., 1983). Briefly, a solution of 0.062% BAC was applied to the serosal surface of a 2 cm segment of jejunum every 5 minutes for one-half hour. This concentration of BAC was shown to destroy greater than 90% of the myenteric neurons in the rat jejunum. After treatment, the serosa was thoroughly rinsed with saline, and a monopolar electrode was sutured to the midportion of the treated jejunal segment. Two other electrodes, one 3 cm orad and one 3 cm caudad to the treated area were also sutured to the jejunum. The orad electrode was situated approximately 3 cm distal to the Ligament of Treitz. The midline muscle and skin were closed in two layers by single interrupted sutures. In addition to the BAC-treated animals, control animals were prepared with 3 electrodes spaced 3 cm apart on the jejunum in a similar location to the treated animals.

After surgery, animals were housed singly in cages with wire meshed bottoms. Animal quarters were maintained at a constant temperature on a 12 hour light/dark cycle. Intestinal myoelectric recordings were made periodically with a Beckman Pen Recorder Model 411 beginning 2 days after surgery. Rats were unanesthetized, unrestrained, and fasted for at least 20 hours prior to recordings. The duration of recording sessions were one to two hours.

Several parameters of intestinal myoelectric activity were assessed in both control animals and those with myenteric neuron-ab-

lated jejunal segments. These were: the burst (phase 3) duration, period, velocity of propagation of the burst, and BER frequency. In addition, BER propagation velocity was recorded in control animals.

The burst duration was characterized as the time interval when spike potentials were superimposed on at least 95% of the BER. Period refers to the time interval of occurrence of MMCs (i.e., burst duration + time interval between bursts). In control rats, burst propagation velocity in cm/min was calculated by determining the elapsed time between the beginning of a burst on two consecutive electrodes and dividing this time value into the distance between the electrodes. In treated rats, the elapsed time between the electrodes orad and caudad to the treated region was utilized in this calculation. BER frequency was determined by counting the number of individual BERs in one minute intervals randomly at a given electrode site in a given record. The BER propagation velocity (cm/sec) was determined in control animals in a similar manner to the burst propagation velocity above. Because of the nature of the electrical activity in the treated animals, BERs on consecutive electrode sites could not be evaluated. Therefore, BER propagation velocity could not be determined in these animals.

For purposes of data analysis, a single record was selected from each animal. Within this given record, each parameter was

quantified 3-6 times. Statistical analyses consisted of Student's t-test and one-way analysis of variance (ANOVA).

RESULTS

Within 2 days after surgery, the control rats (those not treated with BAC) exhibited normal BER patterns and frequencies at all 3 electrode sites. Spiking was intermittant at day 2 but progressed to normal propagated bursts (MMC) by day 5-7 (Fig. 1 - Control). Values for the various myoelectric parameters tested appear in Table 1. These values are in agreement with those reported by others (Ruckebush and Fioramonti, 1975; Fioramonti and Bueno, 1980; Weisbrodt et al., 1980; Hutton and Wingate, 1981; Wright et al., 1981). In control animals, the propagation velocity of the BER was 2.25 ± 0.14 cm/sec.

In discussing the results of the treated animals, reference must be made to the jejunal segment devoid of myenteric neurons (i.e., the BAC-treated area) and the regions both orad and caudad to this treated area. In the orad jejunal area, the BER was of a normal frequency of 37.7 ± 0.64 cycle/min (Table 1) which was evident two days after surgery and persisted for the duration of the animal preparation. Spiking in the orad area appeared in bursts beginning 3-4 days post surgery. The BER frequency of the caudad area was reduced to 30.7 ± 0.73 cycle/min, which was significantly

different from the orad area (Student's t-test for paired values, $p < .01$). Similar to the orad region, the smooth muscle in this area elicited spiking in bursts beginning 3-4 days after surgery.

The myoelectric activity of the myenteric neuron-ablated jejunal area was markedly altered. The BER, when present, was erratic with a reduced frequency and attenuated amplitude (see Fig. 2). The BER frequency of the jejunal area devoid of myenteric neurons could not be quantified because of its irregularity. Spike potentials were absent in this area for approximately 2 weeks after treatment. On days 13-17, spikes appeared, but as depicted in Fig. 1 - BAC-treated, and Fig. 2, they were of greater duration and lower frequency than the areas orad and caudad to it, and were not always associated with BER.

In the treated animals, propagation of orad bursts began between days 6-9. From this time, until approximately day 13, the MMC started on the orad electrode and skipped to the caudad electrode, without any spiking activity on the myenteric neuron-ablated region. On days 13-17 when irregular spiking on the myenteric neuron-ablated segment appeared, its incidence coincided with the orad burst and was followed by a caudad burst (Fig. 1 - BAC-treated). There appeared to be an apparent propagation through the myenteric neuron-ablated region. Once this pattern was initiated in an animal, its

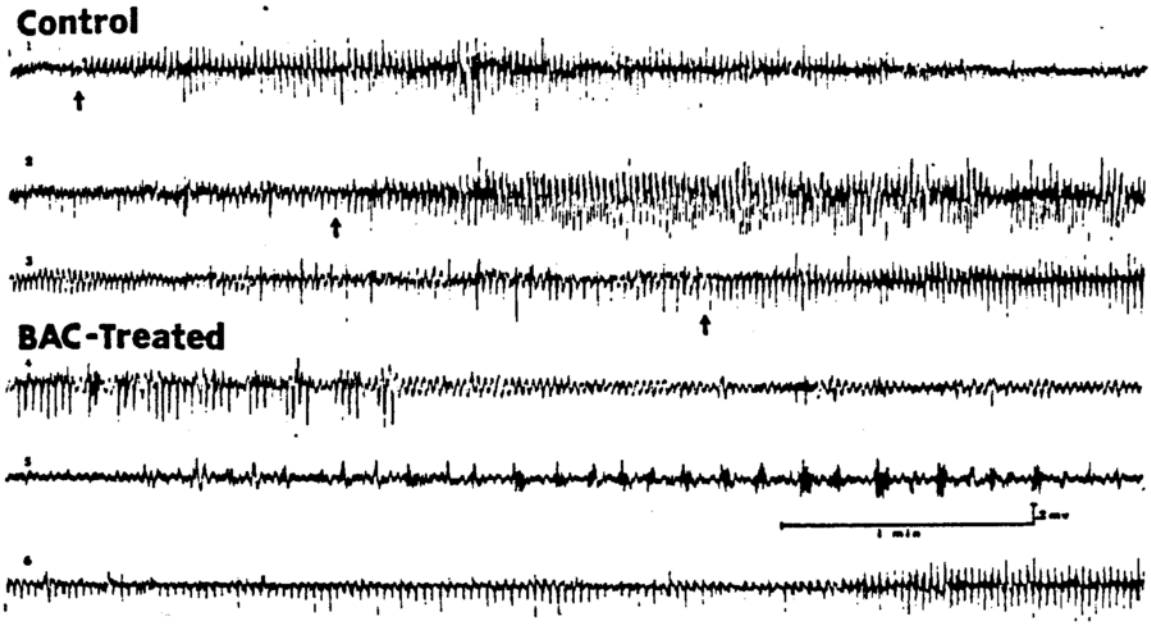

Figure 1. Electromyograms of the jejunal migrating myoelectric complexes (MMCs) of both control and BAC-treated rats. In control animals, electrode sites 1, 2, and 3 are spaced 3 cm apart beginning 3 cm from the Ligament of Treitz. Arrows depict the start of the MMC at each electrode site. The electromyograms of BAC-treated animals were obtained 14 days after treatment. Sites 4, 5 and 6 refer to the jejunal segment orad, at, and caudad to, respectively, the jejunal segment devoid of myenteric neurons. The long duration, low frequency spikes of the BAC-treated area (5), are in sequence with the end of the burst at the orad site (4) and the beginning of the burst at the caudad site (6).

Table 1: Jejunal Myoelectric Parameters in Control and BAC-Treated Animals

		BAC-TREATED ANIMALS	
	CONTROL ANIMALS	ORAD	CAUDAD
Burst duration (min)	2.98 ± 0.17	3.39 ± 0.47	3.69 ± 0.25
Period (min)	12.29 ± 0.84	13.07 ± 1.28	15.06 ± 2.28
BER frequency (cycle/min)	38.20 ± 0.41	37.7 ± 0.64	30.70 ± 0.73[a]
Burst propagation velocity (cm/min)	2.06 ± 0.18	1.53 ± 0.19[b]	

Numbers represent means ± SEM (control, n=6 means with 3 independent observations within each mean; BAC-treated, n=4 means with 3-6 independent observations within each mean).

[a] Significantly different from orad jejunal area ($P < .01$)

[b] Based on the distance and time lapse between orad and caudad electrode sites.

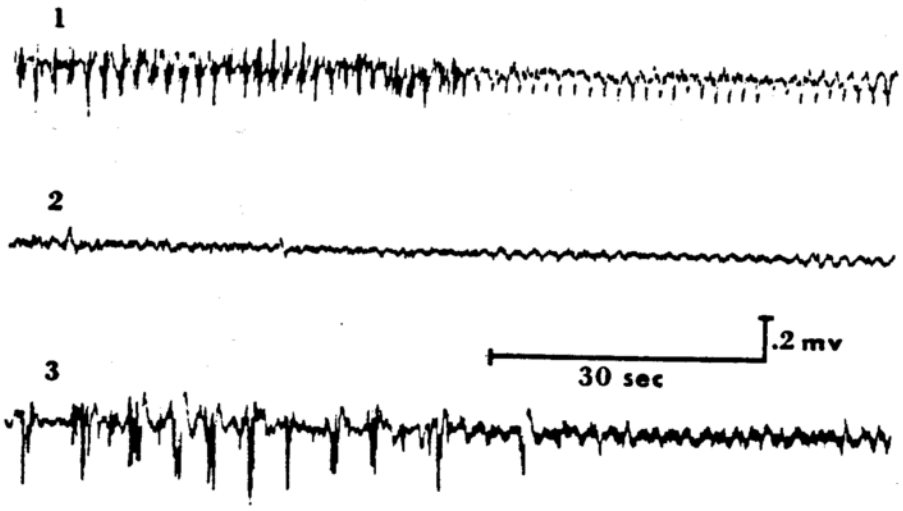

Figure 2. Electromyograms of the jejunum in the same rat, 5 and 35 days after BAC treatment. Five days after BAC treatment, tracings 1 and 2 depict the myoelectric activity orad and at the site of treatment, respectively. Note the regularity of the spiking and BER in the orad region (1) in contrast to the undefined, nonspiking pattern in the BAC-treated area. (2). Tracing 3 represents the myoelectric activity of the same BAC-treated area taken 35 days after treatment. Note the presence of spikes which are of a longer duration and lower frequency than controls. Distinctive BER was not present in the treated area for the duration of the experiment.

regularity persisted until the animal was sacrificed (as long as 2 months).

Parameters of myoelectrical activity for the areas orad and caudad to the myenteric neuron-ablated area also appear in Table 1. As analyzed by a one-way ANOVA, there was not a significant difference among control animals and the orad and caudad areas of BAC-treated animals with respect to burst duration, period, or propagation velocity of the MMC (Student's t-test for unpaired values). In some of the animals, there was an occasional orad burst which was not propagated or an extra distal burst.

DISCUSSION

A chemically induced, enteric neuronal ablation of the colon and anorectum of the rat has been demonstrated with local application of the cationic surfactant benzalkonium chloride (BAC) (Sato et al., 1978; Sakata et al., 1979). In addition, we have shown that serosal application of BAC as well as other cationic, anionic, and nonionic surfactants to the rat jejunum selectively reduces the number of ganglion cells in the myenteric plexus (Fox et al., 1983). The number of ganglion cells in the submucosal plexus and the contractility of longitudinal smooth muscle (Fox and Bass, 1985) were not altered by this treatment. Also, the serosal application of BAC reduced the vasoactive intestinal peptide-, somatostatin-, met-enke-

phalin-, and substance P-like immunoreactivity only in the myenteric plexus, further demonstrating selectivity (Fox et al., 1983). This is a simple, selective method for ablating myenteric neurons.

The present investigation has demonstrated that chemical ablation of myenteric neurons disrupts BER but not MMC propagation in the rat. Regarding the BER, it has been shown that 4 hours of hypoxia in a loop of canine bowel destroys or causes vacuolization of the ganglion cells in the myenteric plexus. Meissner's (submucosal) plexus was less sensitive to the hypoxia (Hukuhara et al., 1961; Nagata and Steggerda, 1963). Szurszewski and Steggerda (1968a,b), utilizing this technique, have demonstrated a decrease in BER and contractile frequency associated with the myenteric neuronal disruption at and below the site of treatment. In addition, the caudad direction of conduction of the BER was disrupted in the treated area. Khin Kyi Kyi and Daniel (1970), however, concluded that hypoxia-induced damage to myenteric neurons was not complete, and changes in BER and contractile activity may be attributed to smooth muscle alterations. The present study, utilizing BAC-induced ablation of the myenteric plexus in the rat, indicates a disruption of BER in the jejunal area devoid of myenteric neurons, and a reduced frequency caudad to the myenteric neuron-ablated area. Our results in the rat are in agreement with Szurszewski, as well as with ligation (Bass and Wiley, 1965a) and transection and reanasto-

mosis (Bass and Wiley, 1965b) studies in the dog in which the BER frequency was decreased caudad to the ligature and transection site, respectively. Caution, however, must be exercised when interpreting the latter results because both muscle and nervous connections are disrupted.

The Interstitial Cells of Cajal (ICC) are connective tissue-type cells, located in 4 networks which are closely associated with both the nerve and muscle elements of the gut. Through both morphological and topographical characterization, it has been postulated that these cells may be the source of the intestinal basic rhythm (Thuneberg, 1982). Since one network of these cells are intermingled with the myenteric plexus, it is possible that our serosal treatment with BAC ablated the ICC along with the myenteric neurons. Therefore, our results cannot preclude the possibility that the ICC is the controlling factor in BER generation and propagation.

The role of both the extrinsic and intrinsic nerves in the initiation and propagation of the MMC has been previously examined. It appears as though extrinsic innervation is not crucial to the generation or propagation of the MMC. Neither vagotomy (Marik and Code, 1975), celiac and superior mesenteric ganglionectomy (Marlett and Code, 1979) nor complete mesenteric transection with vascular reanastomosis (Heppell et al., 1983) alters the MMC's appearance in the intestine or its caudal migration. However, the timing of the com-

plexes and lengths of different phases of the cycle may be changed by extrinsic denervation.

Intrinsic nervous control of the MMC has been postulated. Sarna and coworkers (1981) have shown that close intra-arterial mesenteric injections of atropine, hexamethonium and tetrodotoxin block the propagation of the MMC in the dog. Also, it has been demonstrated that after a series of intestinal transections and reanastomoses the onset of phase 3 (burst phase) of the MMC in the resulting intestinal segments are independent of one another (Sarna et al., 1983). These studies, as well as those of Heppell et al. (1983), which also utilize intestinal transection, support the notion that enteric nerves are instrumental in the initiation and propagation of the MMC.

In contrast to the studies above, the present investigation suggests that the myenteric plexus is not involved in the control of the MMC in the rat. After chemical ablation of the myenteric neurons, there is a normal propagation of the MMC. In addition, the burst duration and burst propagation velocity in intestinal areas both orad and caudad to the aganglionic area are not different from control animals. The discrepancy between our results and those of others may be due to the indirect or nonselective methods of the latter. Transection and reanastomosis disrupts enteric nerve as well as muscle continuity making interpretation questionable. The

results obtained by pharmacological studies are indirect since the intrinsic nerve elements are still intact. More importantly, it is possible that both the myenteric and submucosal plexuses must be altered to affect the MMC or that the submucosal plexus is more important in its control. Our method selectively ablates the myenteric neurons, whereas the methods employed in the above studies would alter both intrinsic nerve plexuses.

The delayed appearance of spike potentials on the BAC-treated jejunal area may be indicative of initial muscle damage with subsequent recovery. Spike potentials, the electrical correlates to smooth muscle contraction, did not appear on the BAC-treated area until approximately 2 weeks after the initial treatment. Our data on the isolated rat jejunum support normal muscle contractility 15 days after BAC treatment (Fox and Bass, 1985). This was evidenced by the fact that both control and BAC-treated jejunum exhibited similar degrees of maximum tension development and displayed similar responsiveness to carbachol. However, preliminary <u>in vitro</u> data at early time points after BAC treatment are suggestive of an impairment of muscle contractility (unpublished results). Therefore, in a functional sense, the neuronal selectivity of BAC is only apparent 15 days or greater after treatment.

When spike potentials occur on the BAC-treated area, they are of a longer duration and decreased frequency than the areas both

orad and caudad to the treated area. These spikes are not usually associated with the remnants of BER found in the BAC-treated area. This pattern is not unlike that seen after i.v. administration of 100 μg/kg morphine in the unanesthetized dog. This morphine treatment initiates duodenal spasm accompanied by complete disruption of the BER (Bass, 1968). Perhaps the function of the BER is to prevent the gut from going into contracture.

The concept of the MMC and BER possessing differential control mechanisms is not unique if one examines their normal propagative properties. For instance, we have shown that in the unanesthetized rat the propagation velocity of the BER and MMC is 2.25 ± 0.14 cm/sec and 2.06 ± 0.18 cm/min, respectively. The magnitude of the difference in propagation rates is also comparable in the dog (Szurszewski, 1969; Code and Marlett, 1975). The burst or phase 3 of the MMC, in which spike potentials are superimposed on virtually all the BER, propagates caudally down the length of the small bowel. The frequency of the spike potentials composing the burst is governed by the BER frequency. However, while the BER propagates caudally, the individual spikes superimposed on the BER within the burst do not (Bass et al., 1961). Rather, the entire burst of spikes propagates at a velocity significantly different than the BER. Therefore, even though there is a relationship between the spike frequency within a burst and the BER frequency, the propaga-

tive nature of these two forms of gut electrical activity appear to be controlled by different mechanisms.

In conclusion, the present investigation has demonstrated that BAC-induced ablation of myenteric neurons in the rat jejunum disrupts the BER but not the MMC propagation. This suggests that the myenteric neurons play a modulatory role in the generation and propagation of the BER, while the submucosal neurons may be more important than the myenteric neurons in the control of the MMC.

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CHAPTER 3

The Myenteric Plexus as a Site of Action of Several Putative Enteric Neurotransmitters in the Isolated Rat Jejunum

ABSTRACT

The contribution of the myenteric plexus in the mechanical responses of rat jejunal longitudinal muscle produced by several enteric nerve substances was evaluated. The myenteric plexus of a 3-4 cm segment of rat jejunum was destroyed by serosal application of 0.062% benzalkonium chloride (BAC). Fifteen days after BAC treatment, both the BAC-treated and an orad control jejunal segment were removed and the mechanical responses of the longitudinal muscle produced by the following substances were examined: substance P, acetylcholine (ACh), 5-hydroxytryptamine (5-HT), cholecystokinin octapeptide (CCK-8), norepinephrine, vasoactive intestinal peptide (VIP), bombesin, leu-enkephalin, and somatostatin. Substance P produced a concentration-dependent contraction in both control and BAC-treated jejunum, in which the -log molar ED_{50} values and maximum responses were not significantly different. ACh also elicited concentration-dependent contractions in both tissues. However, the ACh dose-response curve obtained in the BAC-treated jejunum was shifted 5.4-fold to the right of the control curve, without a reduction in the maximum response. The concentration-dependent contractile response elicited by 5-HT in control jejunum was greater than the response observed in BAC-treated jejunum. CCK-8 produced a concentration-dependent

contraction only in control jejunum. Norepinephrine produced a concentration-dependent relaxation in both control and BAC-treated jejunum in which the -log molar ED_{50} values and maximum responses were not significantly different. VIP and ATP produced a concentration-dependent relaxation in control jejunum which was markedly greater than the response observed in BAC-treated jejunum. Neurotensin elicited a concentration-dependent relaxation in control jejunum, but a variable response in BAC-treated jejunum. Variable responses to bombesin were observed in both control and BAC-treated jejunum. Leu-enkephalin failed to produce a response in BAC-treated jejunum, and the response in control jejunum was variable. Somatostatin did not alter baseline tension of either control or BAC-treated jejunum. We conclude that: (1) substance P and norepinephrine produce their mechanical responses by acting predominatly on the longitudinal smooth muscle; (2) 5-HT, CCK-8, ATP and VIP act predominantly through the myenteric plexus; (3) ACh possesses both direct and indirect actions; and (4) because the responses to neurotensin, leu-enkephalin, bombesin, and somatostatin were equivocal, a conclusion as to their site of action could not be made in this preparation.

INTRODUCTION

A myriad of substances have been detected in the enteric ganglia of the gastrointestinal tract. These include the classical neurotransmitters acetylcholine and norepinephrine, as well as 5-hydroxytryptamine, adenosine triphosphate, and a host of neuronal peptides (Schultzberg et al., 1980; Gershon, 1981; Llewellyn-Smith et al., 1983). This heterogeneous group of endogenous substances have in common the ability to alter gastrointestinal motility. However, the effects of these compounds on intestinal motor activity can vary with species, region of the GI tract, and experimental conditions. Also, the site of action of many of these enteric neuronal substances with regard to their influence on smooth muscle contractility is unknown or equivocal. Therefore, the physiological roles of several of these putative enteric neurotransmitters have not been fully described.

Recently, our laboratory has developed a technique to selectively ablate the myenteric plexus of the rat jejunum (Fox et al., 1983). We have demonstrated that serosal application of benzalkonium chloride (BAC) to the rat jejunum eliminates greater than 90% of myenteric neurons without affecting the number of submucosal neurons. In addition, VIP-, substance P-, somatostatin-, and enkephalin-like immunoreactivity is markedly

reduced in the area of the myenteric plexus and in adjacent muscle layers. Pharmacological assessment of longitudinal muscle contractility in isolated BAC-treated jejunum (i.e. jejunum devoid of myenteric neurons) revealed that BAC-treated jejunal muscle lacks neuronal activity as evidenced by its nonresponsiveness to ganglionic stimulants, nerve-selective electrical stimulation, and physostigmine (Fox and Bass, 1985a). However, the responsiveness of the BAC-treated smooth muscle to the muscarinic receptor agonist carbachol, and the maximum tension developed in response to barium chloride, were not altered (Fox and Bass, 1985a). These data suggest that ablation of the myenteric plexus eliminates the motor neurons to the longitudinal muscle of the rat jejunum. This loss in neuronal activity occurs without a concomitant deficit in smooth muscle contractility. Therefore, in an intact intestinal segment, this technique can be used as a model to determine whether an agonist which affects intestinal smooth muscle contractility acts directly on the smooth muscle or indirectly through the myenteric plexus.

The aim of the present investigation was to assess the site of action of several putative enteric neurotransmitters. This was accomplished by comparing the mechanical responses they produced in normal and myenteric neuron-ablated (BAC-treated) rat jejunum.

METHODS

Application of benzalkonium chloride (BAC) to the serosal surface of the rat jejunum was used to ablate the myenteric plexus. This method has been previously described by Fox and co-workers (1983). In summary, male albino rats (Sprague-Dawley Inc., Madison, WI) weighing 200-225 gm were anesthetized by an i.p. injection of a pentobarbital-chloral hydrate combination. A midline incision was made, and a portion of the jejunum was brought outside the peritoneal cavity. A 3-4 cm jejunal segment was delineated by serosal suture tags beginning approximately 8 cm from the ligament of Treitz. A 0.062% solution of BAC was applied to the serosal surface of the delineated jejunal segment every 5 minutes for 0.5 hr. After this treatment, the intestine was thoroughly rinsed with saline and returned to the peritoneal cavity. The midline incision was closed, and the animals were allowed to recover. Fifteen days after treatment, animals were sacrificed by a blow to the back of the head and the treated jejunal segment and an untreated control segment approximately 3 cm orad to this were removed for experimentation. Therefore, in all experiments, each animal served as its own control. This treatment regimen has been shown to destroy greater than 90% of the ganglion cells in the myenteric plexus of the BAC-treated jejunal segments (Fox et

al., 1983) and eliminate neurally mediated responses of the longitudinal muscle (Fox and Bass, 1985a).

After BAC-treated and control jejunal segments were removed (≈ 2 cm each) and freed from mesenteric attachments, they were suspended along their longitudinal axis in water-jacketed, 25-ml tissue baths containing Krebs bicarbonate buffer (NaCl, 118; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 2.5; $MgCl_2 \cdot 6\ H_2O$, 0.5; NaH_2PO_4, 1.0; $NaHCO_3$, 25.0; and glucose, 11.0 mM). The solution was aerated with a mixture of oxygen (95%) and carbon dioxide (5%) and maintained at a constant temperature of 37°C by a Haake D1 immersion circulator. Mechanical responses were recorded isometrically on a Grass model 7D polygraph with Grass force transducers (FT-03). One gram of resting tension was applied to the tissues, and they were allowed to equilibrate for 60-90 minutes prior to addition of any drugs. Buffer was changed every 15 minutes during the equilibration period.

Drug responses, with the exception of norepinephrine, were obtained in a noncumulative fashion. Immediately after the peak response to a given concentration of drug was obtained, the tissue was washed three times with Krebs buffer before the addition of an increased concentration of drug to the bath. Different drug concentrations were added at 10-minute intervals. Norepinephrine responses were obtained in a cumulative manner by increasing its concentration by a factor of about 3

while the previous concentration remained in contact with the tissue (van Rossum, 1963). Drug responses, whether contraction or relaxation, were expressed as a percentage of the maximum response for a given tissue. Maximum contraction of both control and BAC-treated jejunum was produced by exposure to 10^{-2}M barium chloride. Papavarine (2×10^{-3}M) produced maximal relaxation of both control and BAC-treated jejunum. Differences between BAC-treated and control jejunum with regard to the mean -log molar ED_{50} values and maximum respones obtained for the various agonists used in this study were compared using Student's t-test for paired values.

Drugs

The following drugs were obtained from Sigma Chemical Co., St. Louis, MO: substance P, 5-hydroxytryptamine creatinine sulfate complex (5-HT), (-)-norepinephrine bitartrate, vasoactive intestinal peptide (VIP), neurotensin, bombesin, acetylcholine chloride (ACh), adenosine 3'-triphosphate disodium salt (ATP), somatostatin, leucine-enkephalin (leu-enk), bacitracin, and papavarine hydrochloride. Barium chloride was obtained from Mallinckrodt Chemical Works, St. Louis, MO. Sodium metabisulfite was obtained from Matheson, Coleman & Bell, Norwood, OH. Sulfated cholecystokinin octapeptide (CCK-8) was a gift from the Squibb Institute for Medical Research. Benzalkonium

chloride (BAC) was a gift from Winthrop Laboratories, New York, NY.

All peptides were dissolved in 0.9% saline containing the peptidase inhibitor bacitracin (2×10^{-5}M). Norepinephrine solutions were prepared in 0.9% saline containing 0.05% of the antioxidant sodium metabisulfite. Neither bacitracin nor sodium metabisulfite in the concentrations used affected baseline tension of the tissues. Solutions of 5-HT, ATP, and ACh were prepared in 0.9% saline. Barium chloride and papavarine were dissolved in distilled water. All drug solutions were freshly prepared on the day of the experiment. All peptide solutions were kept on ice for the duration of the experiments. Final bath concentrations of the drugs were expressed as molar concentrations using the weight of the salts.

RESULTS

A summary of the effects of the agonists on which quantitative data could be compiled are shown in Table 1. A detailed description of the effects of all of the compounds are described below.

Substance P

Substance P produced a concentration-dependent contraction (3×10^{-9} - 10^{-5}M) in both control and BAC-treated jejunum

Table 1

Summary of Effects of Several Agonists on Control and BAC-Treated Rat Jejunum

	-Log Molar ED_{50} with SEM		% Maximum Response with SEM[a]		
Agonist	Control	BAC-Treated	Control	BAC-Treated	n[b]
Substance P	6.67±0.08	6.45±0.06	84.4±2.8	76.4±1.9	7
ACh	6.65±0.10	6.00±0.10*	96.7±1.7	92.9±3.8	7
5-HT	6.70±0.09	-	80.3±3.5	13.6±7.1*	7
CCK-8	7.79±0.30	-	30.0±4.0	0±0*	5
Norepinephrine	6.38±0.19	6.40±0.17	73.9±2.2	80.3±2.8	9
VIP	7.30±0.10	-	42.9±5.1	7.5±4.8*	6
ATP	6.27±0.12	-	44.1±6.2	6.2±3.0*	5
Neurotensin	8.85±0.19	-	54.4±3.8	-	7

[a]Maximum effect of each agonist calculated as a percentage of the maximum response. The contractile responses of substance P, ACh, 5-HT, and CCK-8, were expressed as a percentage of barium chloride-induced contraction. Relaxant responses produced by norepinephrine, VIP, ATP, and neurotensin were expressed as a percentage of papavarine-induced relaxation.

[b]n = number of observations.

*Statistically significant difference between control and treated values, Student's t-test, $p < 0.05$.

Note: A -log molar ED_{50} with SEM value could not be obtained for 5-HT, CCK-8, VIP or ATP in the BAC-treated jejunum because 1 or more of the tissues were nonresponsive to the agonists, and an ED_{50} value could not be obtained. For neurotensin, neither -log molar ED_{50} with SEM or % maximum response with SEM could be calculated in the BAC-treated tissue. This was due to the variable nature of the neurotensin response in the BAC-treated tissues - see text.

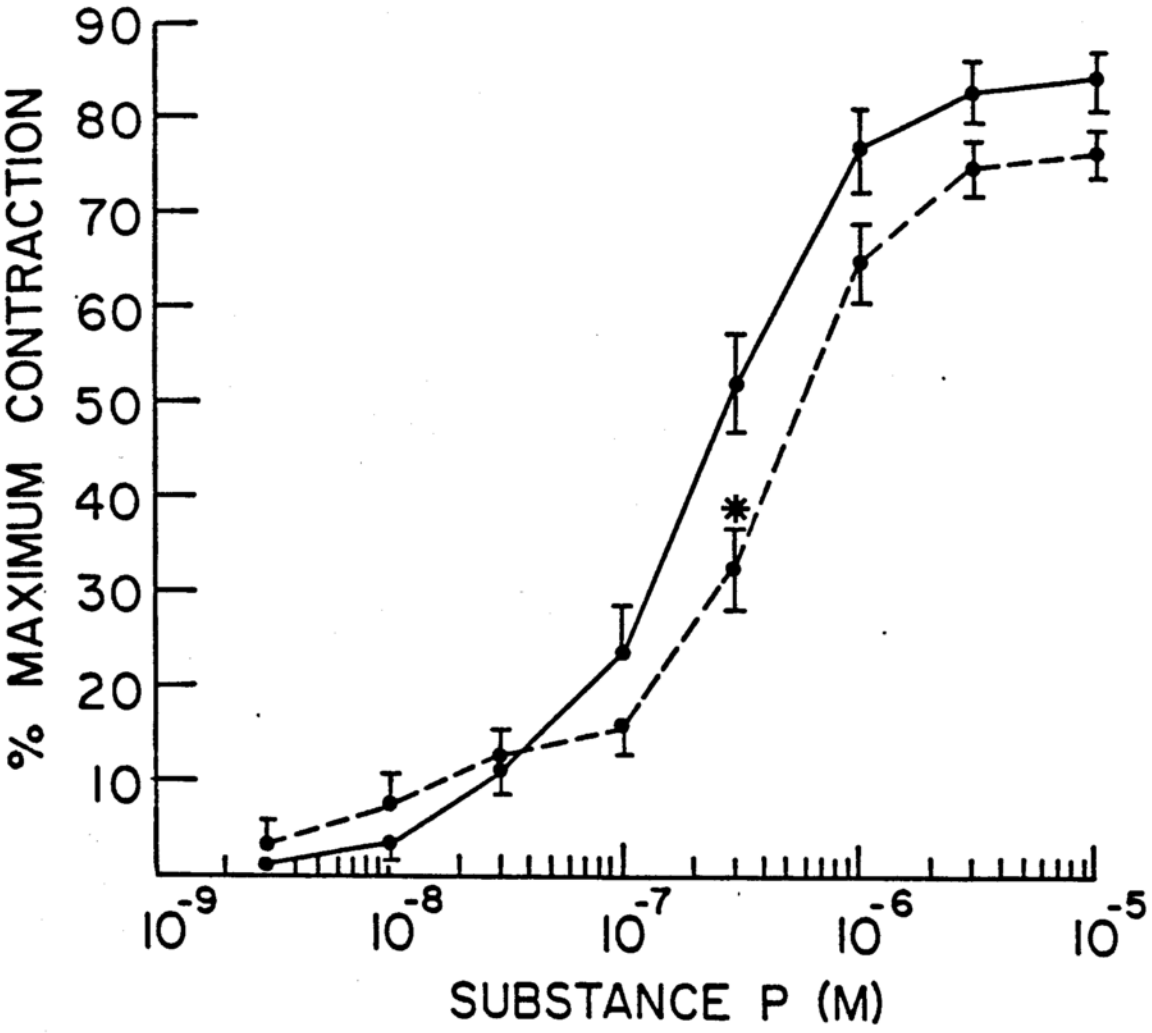

Figure 1. Log dose-response curves for substance P in contracting control (——) and BAC-treated (– – –) rat jejunum. Vertical lines represent ± S.E.M.. Data taken from these curves are summarized in Table 1. An asterisk denotes a statistically significant difference ($p < 0.05$) between treated and control values ($n = 7$).

(fig. 1). There was not a significant difference between control and BAC-treated tissues in either the maximum response or -log molar ED_{50} value for substance P (Table 1).

ACh

ACh produced concentration-dependent contractions in both control and BAC-treated jejunum (fig. 2). There was a significant difference between control and BAC-treated jejunum in the -log molar ED_{50} value but not in the maximum response obtained for ACh (table 1). The ACh dose-response curve obtained in BAC-treated jejunum was shifted 5.4-fold to the right of the control curve.

5-HT

5-HT produced a concentration-dependent contraction (10^{-8} - 10^{-5}M) in control jejunum which was markedly greater than that observed in BAC-treated jejunum (fig. 3). The maximum response for 5-HT in BAC-treated tissues was only 16.9% of that observed in control tissues (Table 1). Three of the seven BAC-treated tissues tested did not respond to 5-HT even at concentrations of 10^{-4}M.

CCK-8

CCK-8 produced a concentration-dependent contraction (3 x 10^{-10} - 10^{-6}M) in control jejunum only (fig. 4). CCK-8 did not

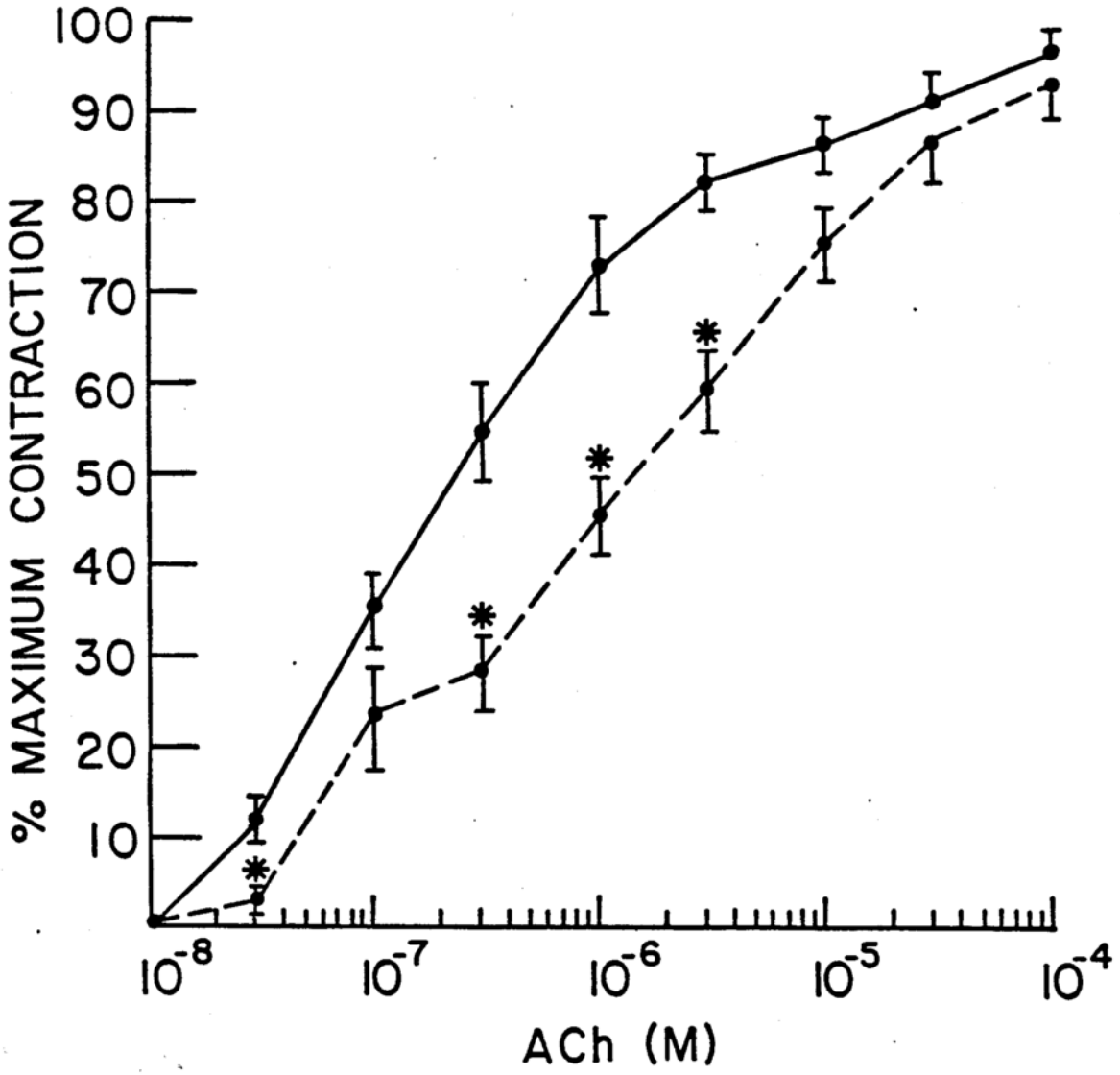

Figure 2. Log dose-response curves for ACh in contracting control (——) and BAC-treated (— — —) rat jejunum. Vertical lines represent ± S.E.M. Data taken from these curves are summarized in Table 1. An asterisk denotes a statistically significant difference ($p < 0.05$) between treated and control values ($n = 7$).

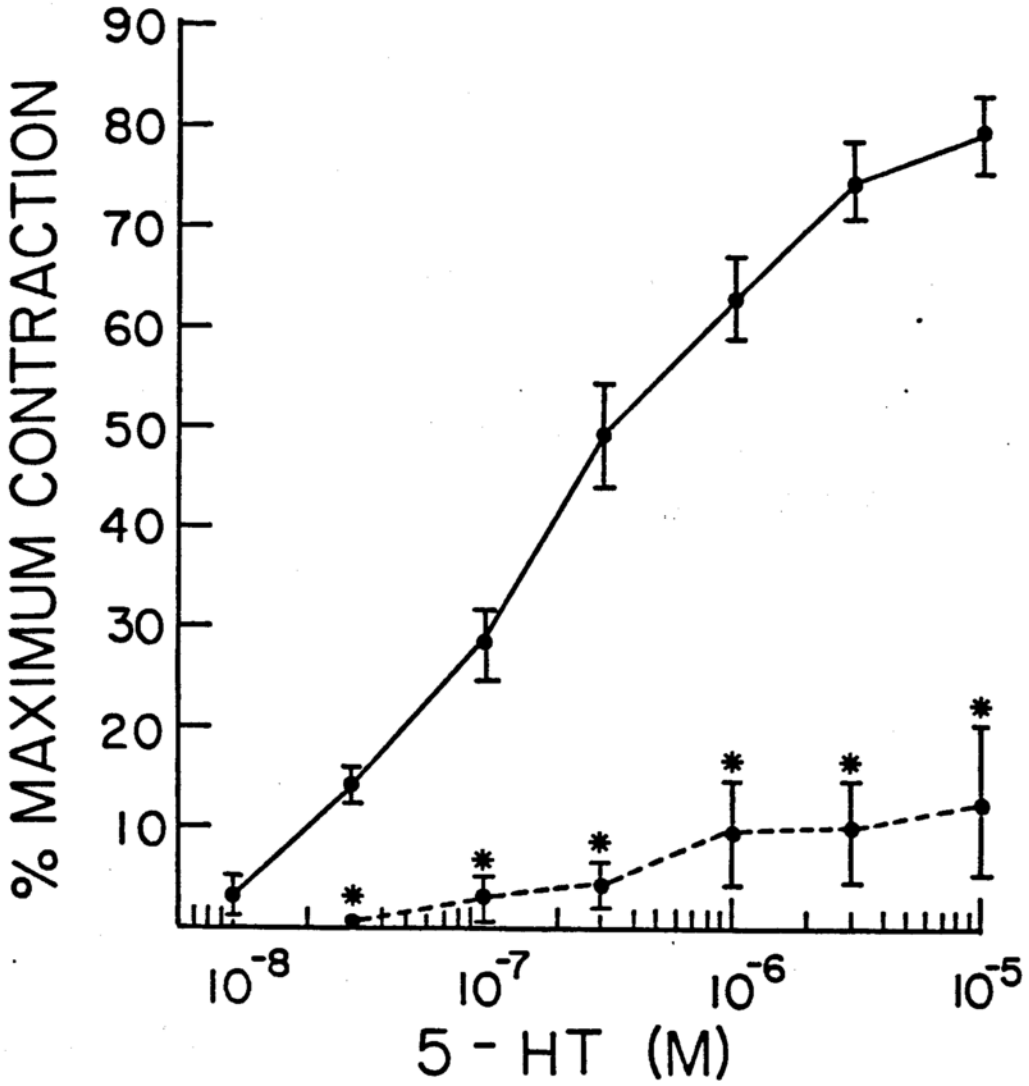

Figure 3. Log dose-response curves for 5-HT in contracting control (——) and BAC-treated (— — —) rat jejunum. Vertical lines represent ± S.E.M. Data taken from these curves are summarized in Table 1. An asterisk denotes a statistically significant difference ($p < 0.05$) between treated and control values ($n = 7$).

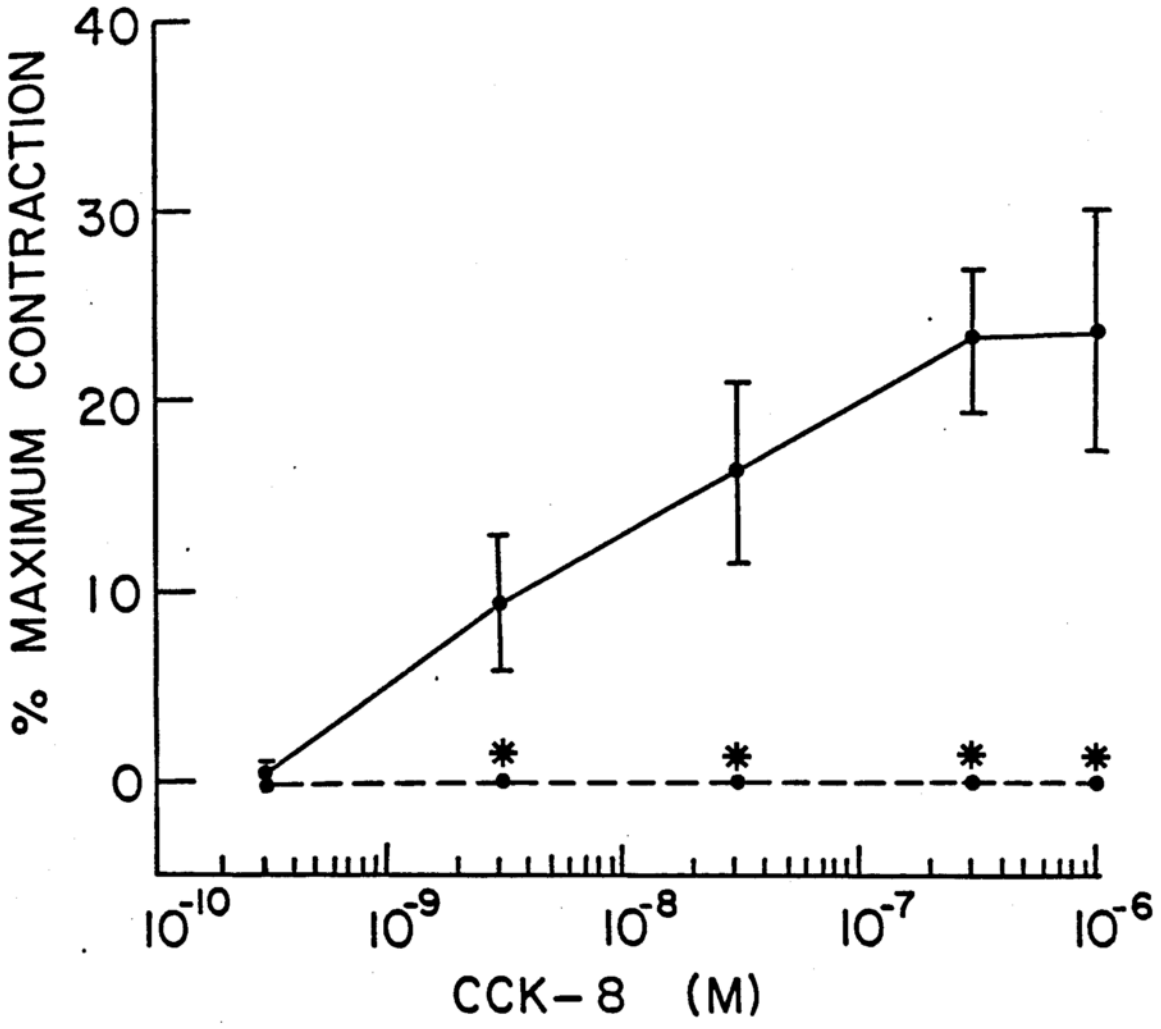

Figure 4. Log dose-response curves for CCK-8 in contracting control (——) and BAC-treated (— — —) rat jejunum. Vertical lines represent ± S.E.M.. Data taken from these curves are summarized in Table 1. Note lack of response to CCK-8 in the BAC-treated jejunum (n = 5).

alter baseline activity of BAC-treated jejunum at concentrations up to 3×10^{-6}M. The maximum response and -log molar ED_{50} value for CCK-8 in control jejunum is shown in table 1.

Norepinephrine

A concentration-dependent relaxation (3×10^{-9} - 3×10^{-4}M) was observed in both control and BAC-treated jejunum in the presence of norepinephrine (fig. 5). There was not a significant difference between control and BAC-treated tissues in either the -log molar ED_{50} value or the maximum response for norepinephrine (Table 1).

VIP

VIP elicited a concentration-dependent relaxation (10^{-8} - 10^{-6}M) in control jejunum which was markedly greater than that observed in BAC-treated tissues (fig. 6). The maximum response produced by VIP in BAC-treated jejunum was only 17.5% of that obtained in control tissues.

ATP

ATP produced a concentration-dependent (10^{-7} - 3×10^{-5}M) relaxation in control jejunum which was markedly greater than that observed in the BAC-treated jejunum (fig. 7). The maximum response for ATP in BAC-treated jejunum was only 14% of that observed in control tissues (Table 1).

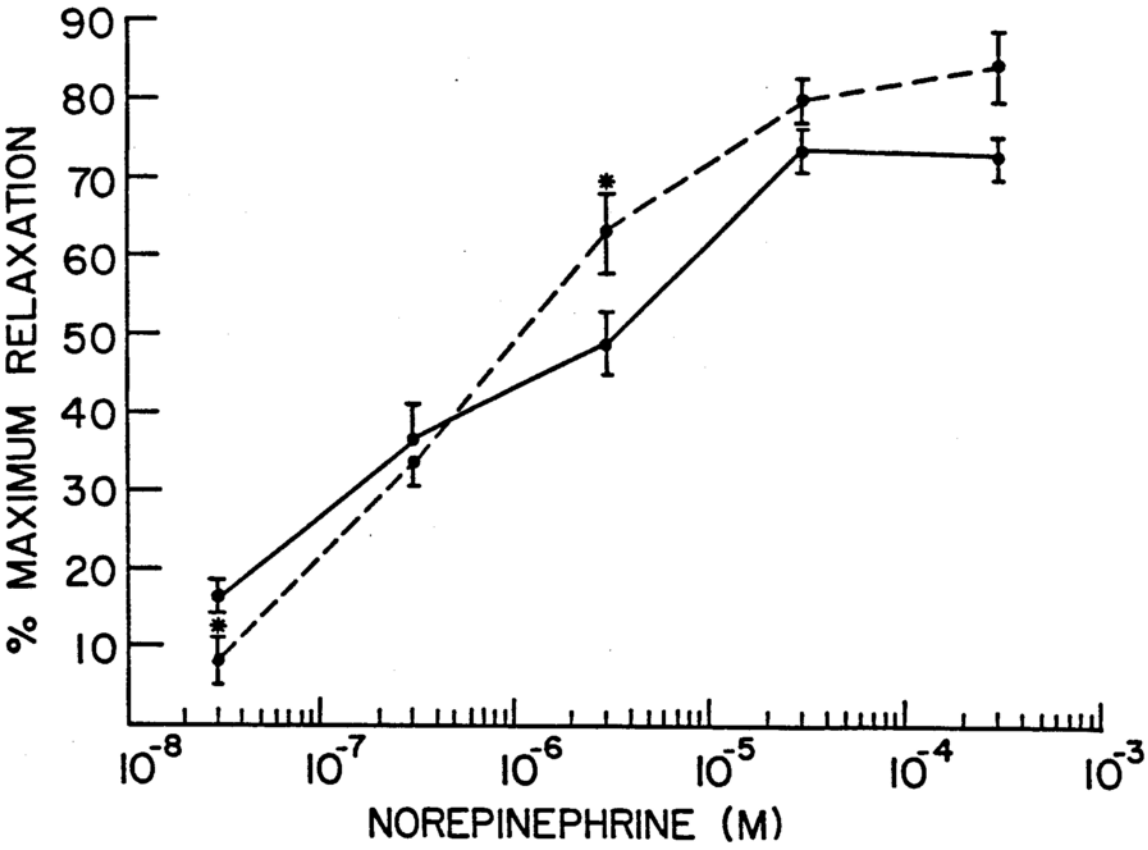

Figure 5. Log dose-response curves for norepinephrine in relaxing control (——) and BAC-treated (— — —) rat jejunum. Vertical lines represent ± S.E.M. Data taken from these curves are summarized in Table 1. An asterisk denotes a statistically significant difference ($p < 0.05$) between treated and control values ($n = 9$).

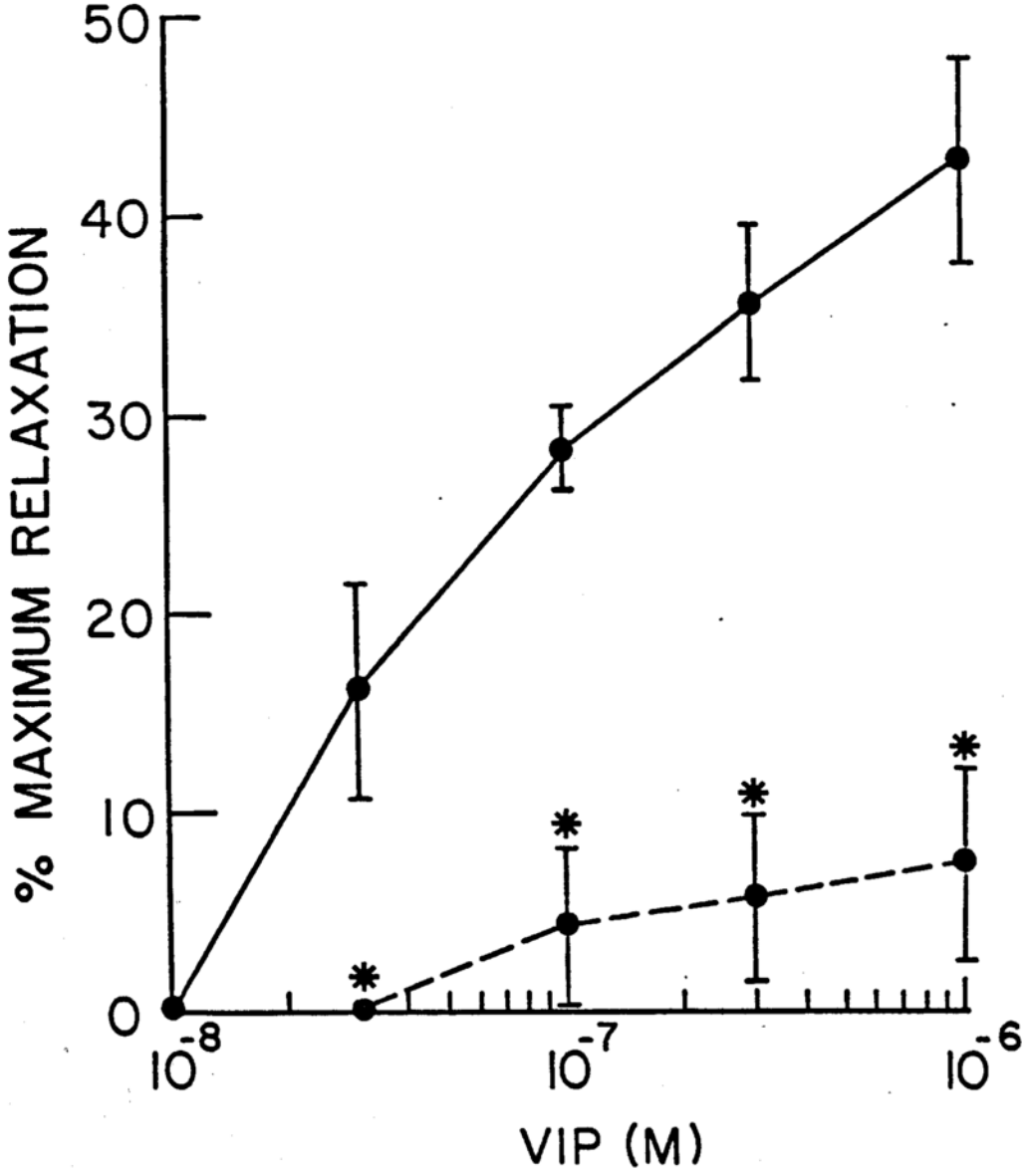

Figure 6. Log dose-response curves for VIP in relaxing control (——) and BAC-treated (– – –) rat jejunum. Vertical lines represent ± S.E.M. Data taken from these curves are summarized in Table 1. An asterisk denotes a statistically significant difference between treated and control values ($p < 0.05$) ($n = 6$).

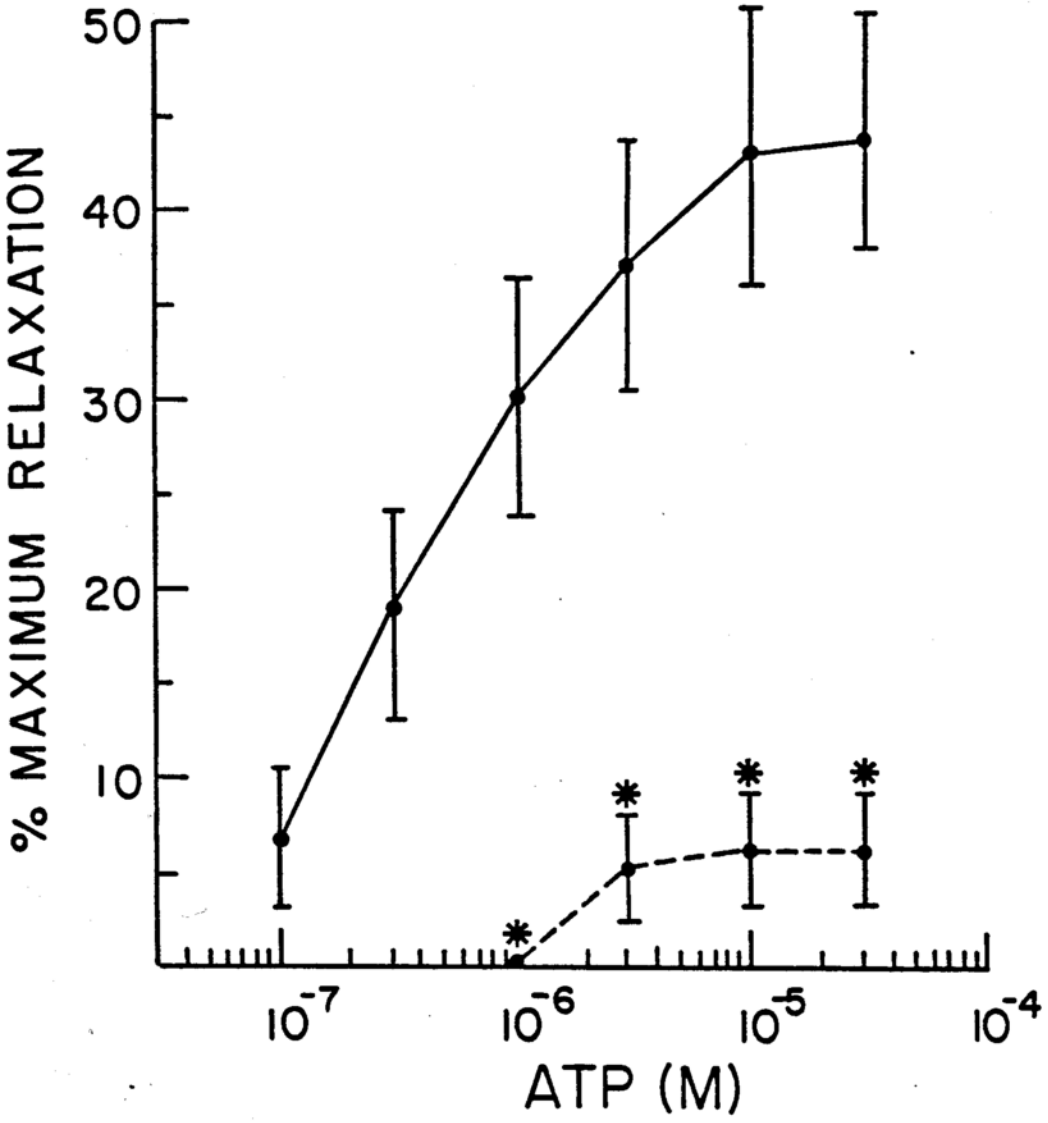

Figure 7. Log dose-response curves for ATP in relaxing control (——) and BAC-treated (– – –) rat jejunum. Vertical lines represent ± S.E.M. Data taken from these curves are summarized in Table 1. An asterisk denotes a statistically significant difference ($p < 0.05$) between treated and control values ($n = 5$).

Neurotensin

Neurotensin caused a concentration-dependent relaxation (3 x 10^{-10} - 10^{-7}M) in control jejunum with a -log molar ED_{50} value of 8.85 and maximum response of 54.4% (Table 1). In the BAC-treated jejunum, low neurotensin concentrations (3 x 10^{-10} - 10^{-9}M) caused no observable effect. Higher concentrations of neurotensin appeared to induce contractions similar to those which have been observed spontaneously in some BAC-treated tissues (Fox and Bass, 1985a). This effect is characterized by periods of high amplitude contractions, either phasic or tonic, generated by some BAC-treated tissues without the addition of any drugs. The contractions exhibited by the BAC-treated tissue in the presence of neurotensin were not concentration-related and were very similar in nature to those which were generated spontaneously.

Bombesin

Variable responses to bombesin were observed in both control and BAC-treated jejunum. Concentrations of bombesin in 10-fold increments from 3 x 10^{-10} to 3 x 10^{-6}M were examined in 7 animals. In control jejunum, no effect was observed at 3 x 10^{-10}M. At concentrations of 3 x 10^{-9} and 3 x 10^{-8}M, contractions were observed. Higher concentrations of bombesin produced a biphasic response characterized by relaxation followed

by contraction. Examining the effects of bombesin on the BAC-treated jejunum, 2 tissues exhibited a concentration-related contraction, 1 tissue relaxed slightly to concentrations of 3×10^{-8} and 3×10^{-7}M, and the remainder of the tissues (4) were not affected by any concentration of bombesin.

Leu-enkephalin

BAC-treated jejunum did not respond to leu-enk (3×10^{-10} - 10^{-5}M, n = 8). The response to leu-enk in the control jejunum was variable. Leu-enk produced a slight contraction in 2 tissues, relaxation in 3 tissues, and no effect in 3 tissues.

Somatostatin

No response to somatostatin (10^{-9} - 2×10^{-6}M) was observed in either control or BAC-treated jejunum (n = 3).

DISCUSSION

The results of the present investigation suggest that substance P and norepinephrine produce their mechanical responses by acting predominantly on the longitudinal smooth muscle; whereas 5-HT, CCK-8, VIP, and ATP act indirectly through the myenteric plexus. ACh possesses both direct and indirect actions. Inferences as to the site of action of neurotensin, leu-enkephalin, bombesin, and somatostatin could not be made because of the equivocal nature of their responses. The data

supportive of these conclusions are discussed in detail below.

Substance P

The undecapeptide substance P was discovered in extracts of intestine and brain by Von Euler and Gaddum in 1931. Since that time, through the use of immunohistochemistry, intestinal substance P has been localized to the enteric neurons (Pearse and Polak, 1975; Nilsson et al., 1975; Schultzberg et al., 1980; Costa et al., 1981), and release of substance P from these neurons has been demonstrated (Franco et al., 1979).

It has long been recognized that substance P is a potent stimulator of gastrointestinal smooth muscle. However, the site of action of substance P in eliciting intestinal contraction has not been firmly established (for review, see Bertaccini, 1982a). Many investigators believe that substance P directly stimulates intestinal smooth muscle because its contractions are unaffected by tetrodotoxin, ganglionic blockers, indomethacin, or agents which block the action of acetylcholine, histamine, or 5-HT (Bertaccini, 1982a). Also, Paton and Zar (1968) have shown that guinea pig longitudinal muscle strips with and without adherent myenteric plexus responded similarly to substance P. In addition to a direct action on smooth muscle, other researchers suggest that substance P activates cholinergic neurons innervating the smooth muscle

(Hedqvist and von Euler, 1975; Holzer and Lembeck, 1980). The results of the present study, in which both control and BAC-treated jejunum responded similarly to substance P, support the contention that substance P contracts longitudinal muscle by acting directly on smooth muscle. Similar to the results of Paton and Zar, our data suggest that the myenteric plexus is probably not involved in the stimulatory effect of substance P on intestinal longitudinal muscle.

ACh

It is well known that exogeneous application of ACh and other muscarinic agonists to the smooth muscle of the gastrointestinal tract elicits contractions (for review, see Daniel, 1982). After acute denervation of intestinal smooth muscle (i.e. mechanical removal of the myenteric plexus from longitudinal muscle, anoxia or storage of intestinal segments at low temperatures), ACh is still able to contract the intestine, indicative of a direct action on the smooth muscle (Paton and Zar, 1968; Ochillo et al., 1978, Day and Vane, 1963). In the present investigation, BAC treatment (i.e., chronic ablation of the myenteric plexus) resulted in a 5.4-fold shift to the right of the ACh dose-response curve, without a reduction in the maximum response. This suggests that lack of the myenteric plexus has altered the responsiveness of the muscle to ACh. Since

others who studied the effect of ACh on acutely denervated preparations did not construct dose-response curves, we cannot compare our results with theirs.

In a previous study, we have shown that there was not a significant difference between control and BAC-treated jejunum in either maximum response or the -log molar ED_{50} value for the contractile response to carbachol (Fox and Bass, 1985a). The differences in the effects observed with ACh and carbachol may be explained on the basis of the relative selectivities of carbachol and ACh for muscarinic and nicotinic receptors. Carbachol is more selective for muscarinic receptors than ACh. Stated another way, ACh is less discriminant in its action, and has more nicotinic activity than carbachol (Day, 1979). The fact that BAC-treated jejunum does not respond to nicotine and DMPP (Fox and Bass, 1985a) is suggestive of a lack of nicotinic receptors in this tissue. Therefore, because ACh has more nicotinic receptor activity than carbachol, it follows that the ACh but not the carbachol dose-response curve would be shifted to the right after BAC treatment. These results suggest that ACh has both direct and indirect actions, while carbachol has primarily direct effects on the longitudinal muscle of the rat jejunum.

5-HT

5-HT contracts both the circular and longitudinal smooth muscle of the intestine of several species (for review, see Furness and Costa, 1982a). Experiments to determine the site of action of 5-HT have been conducted mainly in longitudinal muscle preparations of the guinea pig ileum, and have produced contradictory results. Gaddum and Picarelli (1957) demonstrated that 5-HT contracted the guinea pig ileum by both a direct action on the muscle and by an indirect action characterized by the stimulation of acetylcholine release from intrinsic nerves. Brownlee and Johnson (1963) and Day and Vane (1963) found that 5-HT contracted the guinea pig ileum by stimulating receptors in nervous tissue and that the smooth muscle receptors were of negligible importance in eliciting the 5-HT response. Costa and Furness (1979), by systematically studying the effects of 5-HT on various portions of the longitudinal muscle of the guinea pig intestine, found that there were regional differences in the site of action of 5-HT. They showed that the effects of 5-HT on the most orad ileal segments were mediated almost entirely by cholinergic nerves, while in the distal ileum, there was a prominent direct muscle component to the action of 5-HT. Our data, which demonstrate a greatly diminished 5-HT response in BAC-treated jejunum, is consistent with a neural site of action of 5-HT. The small 5-HT response

which remains in the BAC-treated tissues may be the result of a direct action of 5-HT on the smooth muscle. However, since a small percentage of myenteric neurons may persist after BAC treatment (< 10%), the possibility of an entirely neurally mediated response in the rat jejunum cannot be precluded.

CCK-8

The carboxy terminal 8 amino acid residues of cholecystokinin (CCK-8) has the full range of biological activity and is even more potent than the parent compound. CCK-8 affects the smooth muscle of most regions of the gastrointestinal tract, though specific effects of CCK-8 are dependent on area of the gut, species, and *in vivo* vs. *in vitro* nature of experiments (for review, see Bertaccini, 1982b). For comparison to BAC-treated jejunum, the discussion will be limited to *in vitro* studies involving the effects of CCK-8 on rodent intestine.

CCK-8 has been shown to contract both the longitudinal and circular muscle layers of the guinea pig ileum in a concentration-dependent manner. The contractile response of CCK-8 was completely inhibited independently by atropine and tetrodotoxin (Hedner and Rorsman, 1968; Hedner, 1970). Later, it was demonstrated by Vizi and co-workers that CCK-8 contracts longitudinal muscle strips by releasing acetylcholine from the myenteric

plexus (Vizi, 1973; Vizi et al., 1973). Direct evidence for involvement of the myenteric plexus in CCK-8-mediated contraction of the intestine was provided by Hutchison and Dockray (1981). They demonstrated that guinea pig ileal longitudinal muscle strips without adherent myenteric plexus did not respond to CCK-8. Also, the CCK-8 response in the innervated strips was abolished by tetrodotoxin and reducing the bath temperature to 15°C. In the present study, BAC-treated jejunal segments, which lack myenteric neurons, failed to contract in the presence of CCK-8. Therefore, our results support the contention that the myenteric plexus mediates the contractile response of CCK-8 in the intestine.

Norepinephrine

Sympathomimetic amines, in most circumstances, have an inhibitory influence on the tone of nonsphincter smooth muscle of the gastrointestinal tract. This relaxation has been shown to be mediated by both alpha and beta adrenergic receptors (Alquist and Levy, 1959; Furchgott, 1960; Andersson and Mohme-Lundholm, 1969; Bowman and Hall 1970). It has been suggested that alpha adrenergic receptor-mediated relaxation, depending on the intestinal preparation, can be the result of either an inhibition of acetylcholine release from intrinsic intestinal nerves or a direct action on the smooth muscle (Wikberg, 1979).

In contrast, beta receptors responsible for intestinal relaxation are probably located only on intestinal smooth muscle (Lum et al., 1966; Bowman and Hall, 1970).

In the present investigation, norepinephrine relaxed both control and BAC-treated jejunum to a similar extent. This observation suggests that the myenteric plexus is probably not involved in the relaxant effect of norepinephrine in the rat jejunum, and that the smooth muscle is more likely the site of norepinephrine's action. These results are similar to those obtained by us with beta, but not alpha adrenergic receptor agonists (Fox and Bass, 1985b), suggesting that norepinephrine may be stimulating smooth muscle beta receptors in this preparation.

VIP

VIP is found in the intrinsic intestinal nerves of many species (Schultzberg et al., 1980; Furness and Costa, 1982b). The pharmacological action of VIP on gastrointestinal smooth muscle has been studied in many species and is characterized primarily by relaxation (for review, see Furness and Costa, 1982a). Where it was tested, VIP-induced relaxation was resistant to tetrodotoxin, which is suggestive of an action on muscle rather than on inhibitory nerves. However, the guinea pig small intestine has been shown to contract in the presence

of VIP, and these contractions were antagonized by atropine and tetrodotoxin. In the present investigation, the relaxant response to VIP was markedly diminished after BAC treatment. This result suggests that VIP-induced relaxation is primarily mediated through the myenteric plexus, though a slight direct effect on the longitudinal muscle may also occur.

ATP

The purine nucleotide ATP relaxes most gastrointestinal smooth muscle preparations, presumably by a direct action on the smooth muscle (Burnstock et al., 1970). Burnstock and co-workers (1970) have demonstrated that tetrodotoxin abolishes electrically induced relaxation in atropine-pretreated taenia coli without affecting the relaxation produced by ATP itself. This is in direct contrast to the observations of the present investigation, in which there was a markedly reduced ATP response in BAC-treated jejunum in comparison to innervated jejunal segments. Our results suggest that a substantial portion of the relaxant response to ATP is mediated by myenteric neurons. These discrepant results as to the site of action of ATP may be explained in part by the different methodologies employed in the studies. Burnstock's use of tetrodotoxin alone to negate a neural component of ATP's relaxant effect may be misleading. The action of many neurally acting

drugs such as DMPP and nicotine are not entirely blocked by tetrodotoxin (Burks, 1973; Goldenberg, 1971). Gershon (1967), who made an extensive study of the effects of tetrodotoxin on innervated smooth muscle preparations, suggests that "since only the nerve action potential is abolished by tetrodotoxin and transmitter stores remain intact, it is certainly possible that part of the response to any given drug in the presence of tetrodotoxin might be due to transmitter release through an action of the drug not involving a conducted action potential." Burnstock does in fact entertain the possibility that ATP may have a "tyramine-like" action (i.e., the release of an inhibitory transmitter by displacement from transmitter stores). Therefore, a neurally mediated action of ATP on intestinal smooth muscle should be considered.

Neurotensin

Neurotensin is a tetradecapeptide originally isolated from the hypothalamus (Carraway and Leeman, 1973). However, the major source of neurotensin appears to be the GI tract, as approximately 85% of total neurotensin immunoreactivity in the rat has been localized to the gut (Carraway and Leeman, 1976). The action of neurotensin on the gut is dependent upon species and region of the GI tract. For instance, neurotensin contracts the guinea pig ileum (Carraway and Leeman, 1973; Kitabgi

and Freychet, 1978) and taenia coli (Kitabgi and Freychet, 1978; Leander et al., 1984), but relaxes the rat duodenum (Carraway and Leeman, 1973) and rat ileum (Kitabgi and Freychet, 1978). If the tone is raised in the guinea pig ileum, a biphasic response (relaxation followed by contraction) occurs. Regarding site of action, tetrodotoxin did not affect the relaxant response to neurotensin in the rat ileum or the neurotensin-induced contraction in the guinea pig taenia coli. Tetrodotoxin abolished the contraction and the contractile phase (but not the relaxation phase) of the biphasic response induced by neurotensin in the guinea pig ileum. These results suggest that neurotensin may act on intestinal smooth muscle both directly (relaxation of the rat and guinea pig ileum; contraction of guinea pig taenia) and through a neurally mediated, partly cholinergic process (contraction of the guinea pig ileum). However, it should be noted that resistance of a drug response to tetrodotoxin may not necessarily eliminate the possibility of a neural component to the drug's action (Gershon, 1967; Goldenberg, 1971; Burks, 1973).

In the present study, neurotensin produced a concentration-dependent relaxation in control but not BAC-treated jejunum. In BAC-treated jejunum, neurotensin elicited high amplitude contractions which were not concentration-dependent. These contractions were similar to those generated spontane-

ously by some BAC-treated tissues (Fox and Bass, 1985a). Failure of the BAC-treated jejunum to relax in the presence of neurotensin is suggestive of a myenteric involvement in the response. However, since we cannot explain the high amplitude contraction produced by neurotensin in the BAC-treated jejunum, we hesitate to make a definitive conclusion.

Bombesin

Bombesin, a peptide first isolated from amphibian skin, is found in the mammalian enteric nervous system (Costa et al. 1984; Hutchison et al., 1981). Its pharmacological action on most intestinal preparations is characterized by contraction of the smooth muscle (Bertaccini, 1982a). However, the effects of bombesin on the rat large intestine and duodenum are quite variable (Erspamer et al. 1972). The rat large intestine responded to bombesin with an increase in tone, though a dose-response relationship was not established. The response to bombesin in the rat duodenum was variable, with contractions at some doses and relaxations at others. Our results, have shown a variable response to bombesin in both control and BAC-treated rat jejunum. Thus, conclusions regarding the site of action of bombesin in the rat jejunum could not be made. The rat does not appear to be an acceptable species to study the effects of bombesin on intestinal smooth muscle.

Leu-Enkephalin

Enkephalins are opiate peptides which are located primarily in the myenteric plexus of the intestine (Schultzberg et al., 1980; Furness and Costa, 1980). The pharmacological action of opiates and opiate peptides on the intestine is species dependent. They increase tone in most mammalian species (for review, see Furness and Costa, 1982a). However, in the guinea pig, opiate agonists suppress electrically induced contractions of the isolated ileum (Lord et al., 1977), but do not affect the basal tone of the taenia coli (Cocks and Burnstock, 1979). In conscious rats, Burks (1976) found that morphine produced a dose-related, naloxone-sensitive tonic and phasic increase in intestinal motility, while Weisbrodt and co-workers (1980) reported an inhibition of myoelectrical activity. In isolated rat intestinal preparations, slight contractions have been observed with superfusion of opioid peptides in the upper small intestine, while stronger contractions appeared in the rectum and colon (Nijkamp and Van Ree, 1980). Both the inhibitory and stimulatory actions of opiates appear to be neurally mediated. Inhibitory actions of opiates on transmurally stimulated guinea pig ileum are due to an inhibition of acetylcholine release (Lord et al., 1977). Stimulatory actions of opiates on both rat and dog intestine appear to be due to a release of acetylcholine and 5-HT (Burks, 1973; 1976). In our

system, leu-enkephalin produced a variable response on the basal tone of the normal rat jejunum; however, no effect on BAC-treated jejunum was noted. Because of the equivocal response to enkephalin on control rat jejunum, the intestinal site of action of leu-enkephalin on the intestine could not be determined in this preparation.

Somatostatin

The tetradecapeptide somatostatin has been identified by immunohistochemical methods in the enteric nerves of the intestine (Hökfelt et al., 1975; Schultzberg, 1980; Costa et al., 1980). Lesioning studies indicate that somatostatin-containing nerve cell bodies give rise to axons which project anally and terminate around other neurons. These data imply that somatostatin-containing neurons probably function as interneurons in descending pathways (Costa et al., 1980). Pharmacological studies show that somatostatin inhibits electrically (Guillemin, 1976; Furness and Costa, 1979) and peptide-induced acetylcholine release from the intestine (Teitelbaum et al., 1984), suggesting an inhibitory action on cholinergic nerves.

Somatostatin produces variable responses on the basal tone of intestinal smooth muscle. Somatostatin did not affect the basal tone of the guinea pig taenia coli (Cocks and Burnstock, 1979), but relaxed the longitudinal muscle of the guinea pig

ileum and distal colon (Furness and Costa, 1979). In the present study, somatostatin did not alter the basal tone of either control or BAC-treated jejunum; therefore, the site of action of this peptide could not be ascertained.

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CHAPTER 4

Chemical Ablation of the Myenteric Plexus Impairs Alpha But Not Beta Adrenergic Receptor Mediated Mechanical Responses of Rat Jejunal Longitudinal Muscle

ABSTRACT

The mechanical responses produced by alpha and beta adrenergic receptor agonists were evaluated on control and myenteric neuron-ablated rat jejunal longitudinal muscle. The myenteric plexus of the jejunum was destroyed by serosal application of benzalkonium chloride (BAC). The beta adrenergic receptor agonists isoproterenol and sulfonterol produced a concentration-dependent relaxation of both control and BAC-treated jejunum. Dose-response curves obtained in control and BAC-treated jejunum were nearly superimposable regardless of the beta agonist used. Isoproterenol was 160 and 180-fold more potent than sulfonterol in producing relaxation in control and BAC-treated jejunum, respectively. Isoproterenol-induced relaxation was antagonized by the beta receptor antagonists propranolol and practolol but not by butoxamine. The alpha-1 selective agonists phenylephrine and methoxamine produced a concentration-dependent relaxation in control jejunum which was greater than that observed in the BAC-treated jejunum. BAC treatment resulted in a reduction in the maximum response and a shift to the right of the phenylephrine and methoxamine dose-response curves. The relaxant responses of methoxamine and phenylephrine in control jejunum were blocked by prazosin but not yohimbine. The alpha-2 selective agonist clonidine also produced a concentration-dependent, prazosin-sensitive, yohimbine-resistant relaxation which was markedly greater in control than

BAC-treated jejunum, consistent with alpha-1 receptor stimulation. The alpha-2 selective receptor agonists UK-14,304, M-7, and B-HT 920 produced a concentration-dependent contraction in control but not BAC-treated jejunum. Clonidine in the presence of prazosin also produced contractions in control jejunum only. The contractile response produced by UK-14,304 was antagonized by yohimbine but not by atropine.

Our results suggest that in rat jejunal longitudinal muscle: (1) beta adrenergic receptors mediate relaxation and are located on the smooth muscle; (2) alpha-1 adrenergic receptors mediate relaxation and are located on both the smooth muscle and myenteric plexus; (3) alpha-2 adrenergic receptors mediate contraction and are located on the myenteric plexus.

INTRODUCTION

The inhibitory effects of catecholamines on the intestine of many mammalian species are mediated by both alpha and beta adrenergic receptors (Ahlquist and Levy, 1959; van Rossum and Mujic, 1965; Reddy and Moran, 1968, Andersson and Mohme-Lundholm, 1969, Weisbrodt et al., 1969; Bowman and Hall, 1970). However, the location of these adrenergic receptor types and their mechanism of mediating intestinal relaxation appear to be different. Many researchers support the view that alpha receptors mediate intestinal relaxation by an action on nerves, while the beta adrenergic receptor mediated response is elicited by a direct effect on the smooth muscle cells (Lum et al., 1966; Kosterlitz et al., 1970; Wikberg, 1977, 1979). It has been suggested that alpha adrenergic receptor stimulation evokes intestinal relaxation by inhibiting acetylcholine release from cholinergic neurons (Kosterlitz et al., 1970; Gillespie and Khoyi, 1977). These alpha receptors have been shown to be related to the alpha-2 subtype (Drew, 1978; Wikberg, 1978, 1979; Andrejak, 1980) Direct evidence that intestinal alpha-2 adrenergic receptors are located on neurons of the myenteric plexus was provided by Wikberg and Lefkowitz (1982). They demonstrated that guinea pig longitudinal muscle strips without adherent myenteric plexus did not bind (^{3}H)-clonidine.

Our laboratory has developed a technique to selectively ablate

the myenteric plexus of the rat jejunum. We have demonstrated that application of benzalkonium chloride (BAC) to the serosal surface of the rat jejunum eliminates greater than 90% of myenteric neurons without affecting the number of submucosal neurons in intact intestinal segments (Fox et al., 1983). In addition, BAC-treated jejunal longitudinal muscle does not respond to either ganglionic stimulants, nerve selective electrical stimulation, or physostigmine, but does respond to the muscarinic receptor against carbachol (Fox and Bass, 1985). These results suggest that BAC treatment results in a loss in neuronal activity without a concomitant deficit in longitudinal muscle contractility. Therefore, this technique can be used as a model to determine whether an agonist which affects intestinal smooth muscle activity acts directly on the smooth muscle or indirectly through the myenteric plexus.

The present investigation was undertaken to determine the location of alpha and beta adrenergic receptors mediating mechanical responses of the longitudinal muscle of rat jejunum.

METHODS

Animal Treatment

In all experiments, the cationic surfactant benzalkonium chloride (BAC) was utilized to ablate the myenteric plexus. This method has been previously described by Fox et al., 1983. In

summary, male albino rats (Sprague-Dawley Inc., Madison, WI) weighing 200-225 gm were anesthetized by an i.p. injection of a combination of pentobarbital and chloral hydrate. A midline incision was made, and a portion of the jejunum was brought outside the peritoneal cavity. A 3-4 cm jejunal segment was delineated by serosal suture tags beginning approximately 8 cm from the ligament of Treitz. A 0.062% solution of BAC was applied to the serosal surface of the delineated jejunal segment every 5 minutes for 0.5 hr. Following this treatment, the intestine was thoroughly rinsed with saline and returned to the peritoneal cavity. The midline incision was closed, and the animals were allowed to recover. Fifteen days after treatment, animals were sacrificed by a blow to the back of the head, and the treated jejunal segment and an untreated control segment, approximately 3 cm orad to this, were removed for experimentation. Therefore, in all experiments, each animal served as its own control. This treatment regimen has been shown to destroy greater than 90% of the ganglion cells in the myenteric plexus of the BAC-treated jejunal segments (Fox et al., 1983) and eliminate neurally-mediated mechanical responses of the longitudinal muscle (Fox and Bass, 1985).

Pharmacological Studies

After BAC-treated and control jejunal segments were removed (≈ 2 cm each) and freed from mesenteric attachments, they were

suspended along their longitudinal axis in water-jacketed 25 ml tissue baths containing Krebs bicarbonate buffer (NaCl, 118; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 2.5; $MgCl_2 \cdot 6\ H_2O$, 0.5; NaH_2PO_4, 1.0; $NaHCO_3$, 25.0; and glucose, 11.0 mM). The solution was aerated with a mixture of oxygen (95%) and carbon dioxide (5%) and maintained at a constant temperature of 37°C by a HAAKE D1 immersion circulator. Mechanical responses were recorded isometrically on a Grass model 7D polygraph with Grass force transducers (FT 03) (Grass Instrument Co. Quincy, Mass.). One gram of resting tension was applied to the tissues, and they were allowed to equilibrate for 60-90 minutes before commencement of experiments. The buffer was changed every 15 minutes during the equilibration period.

Responses to beta adrenergic receptor agonists were obtained in a cumulative manner (van Rossum, 1963). Noncumulative dose-response curves were obtained for alpha adrenergic receptor agonists because recovery of the responses occurred despite continued presence of agonist in the tissue bath. Immediately after the peak response to a given concentration of alpha agonist was obtained, the tissue was washed three times with Krebs buffer before the addition of an increased concentration of agonist to the bath. Concentrations of alpha agonists were added at 10 minute intervals. High concentrations of alpha agonists produced similar responses with and without prior exposure to the same agonist, indicating that desensitization

of the tissues to alpha agonists did not occur.

In experiments to determine the effects of antagonists on agonist responses, a pair of contiguous jejunal segments were utilized. Antagonist was added to the buffer of one segment of the pair such that the antagonist was present throughout the equilibration period and agonist exposure period. Antagonist was in contact with the tissues at least one hour before agonist dose-response curves were generated. The ability of the antagonists to cause a rightward shift of the agonist dose-response curve was determined by calculating the dose ratio as antilog [(-log ED_{50} of agonist without antagonist) - (-log ED_{50} of agonist with antagonist)]. Then apparent dissociation constants (K_B) for antagonists were calculated by the following standard equation:

$$K_B = \frac{[\text{antagonist}]}{(\text{dose ratio} - 1)}$$

In initial experiments, precaution was taken to avoid effects which may alter directly or indirectly potency determinations of adrenergic agonists and antagonists. Rats were pretreated with reserpine 5 mg/kg, 20-24 hours prior to experiments to avoid the release of endogenous catecholamines. Cocaine (3×10^{-5}M) and hydrocortisone (10^{-4}M) were added to the buffer to block neuronal and extraneuronal uptake, respectively. Phentolamine (10^{-5}M) was added to

the buffer to block alpha receptors when the effects of beta agonists were studied, whereas propranolol (3×10^{-6}M) was used to block beta receptors when alpha agonists were studied. Unfortunately, this pretreatment regimen produced a high incidence of spontaneous contractions in the BAC-treated tissues. Spontaneous contraction, a previously described characteristic of some BAC-treated tissues (Fox and Bass, 1985), usually prohibits the utilization of approximately 10% of the BAC-treated tissues for drug studies. However, the treatment regimen described above increased the frequency of spontaneous contractions to such an extent that greater than 50% of the BAC-treated tissues were unusable for drug studies. Because of this problem, in experiments with alpha agonists, the only pretreatment used was 3×10^{-6}M propranolol to block beta responses; and in studies with beta agonists, phentolamine (10^{-5}M) and hydrocortisone (10^{-4}M) were used to block alpha receptors and extraneuronal uptake, respectively.

Drug responses, whether contraction or relaxation, were expressed as a percentage of the maximum response for a given tissue. Maximum contraction of both control and BAC-treated jejunum was produced by exposure to 10^{-2}M barium chloride. Papavarine (2×10^{-3}M) produced maximum relaxation of both control and BAC-treated jejunum.

Drugs and Solutions

The following drugs were used: papavarine HCl, yohimbine HCl, atropine sulfate, (-)-isoproterenol-(+)-bitartrate, (-)-phenylephrine HCl, reserpine, cocaine HCl, clonidine HCl, (±)-propranolol HCl, hydrocortisone 21-hemisuccinate sodium (Sigma Chemical Co., St. Louis, MO); barium chloride (Mallinckrodt Chemical Works, St. Louis, MO); methoxamine HCl, butoxamine HCl (Burroughs Wellcome Co., Research Triangle Park, NC); (±)-sulfonterol, M-7, B-HT 920 (Smith Kline and French Laboratories, Philadelphia, PA); UK-14,304-18 (Pfizer Central Research, Sandwich, England); prazosin (Pfizer Laboratories Division, New York, NY); phentolamine HCl (Ciba-Geigy Corp., Summit, NJ); practolol (Ayerst Laboratories Inc., New York, NY).

All drug solutions were prepared on the day of each experiment. Dilutions of isoproterenol were made from 10^{-2}M refrigerated stock solutions prepared in 0.9% sodium chloride with 0.05% sodium metabisulfite. Solutions of M-7, B-HT 920 and UK-14,304 were prepared in distilled water and kept on ice for the duration of the experiment. Reserpine was prepared according to the method described in Martindale (1977) and stored in amber bottles under refrigeration. All other drug solutions were prepared in 0.9% sodium chloride. Final drug concentrations were expressed in molar strengths using the weight of the salt.

RESULTS

Effects of beta agonists and antagonists

Isoproterenol and sulfonterol produced a concentration-dependent relaxation of both control and BAC-treated jejunum (fig. 1). There was not a significant difference between control and BAC-treated jejunum with regard to the maximum responses and -log molar ED_{50} values obtained in the presence of either isoproterenol or sulfonterol (Table 1). Isoproterenol was approximately 160-fold and 180-fold more potent than sulfonterol in producing relaxation of control and BAC-treated jejunum, respectively.

The influence of beta adrenergic receptor antagonists on the responses to beta agonists was studied. Initial experiments showed that antagonists produced similar effects on both control and BAC-treated tissues. Therefore, quantified data are presented for control jejunum only. Butoxamine (10^{-4}M) did not alter either isoproterenol or sulfonterol relaxation dose-response curves. Practolol (10^{-5}M) and propranolol (10^{-5}M) both produced parallel shifts to the right of the isoproterenol dose-response curve without altering the maximum response (fig. 2). The magnitude of the rightward shift of the isoproterenol dose-response curve produced by practolol and propranolol and their respective apparent K_B and pA_2 values are summarized in Table 2.

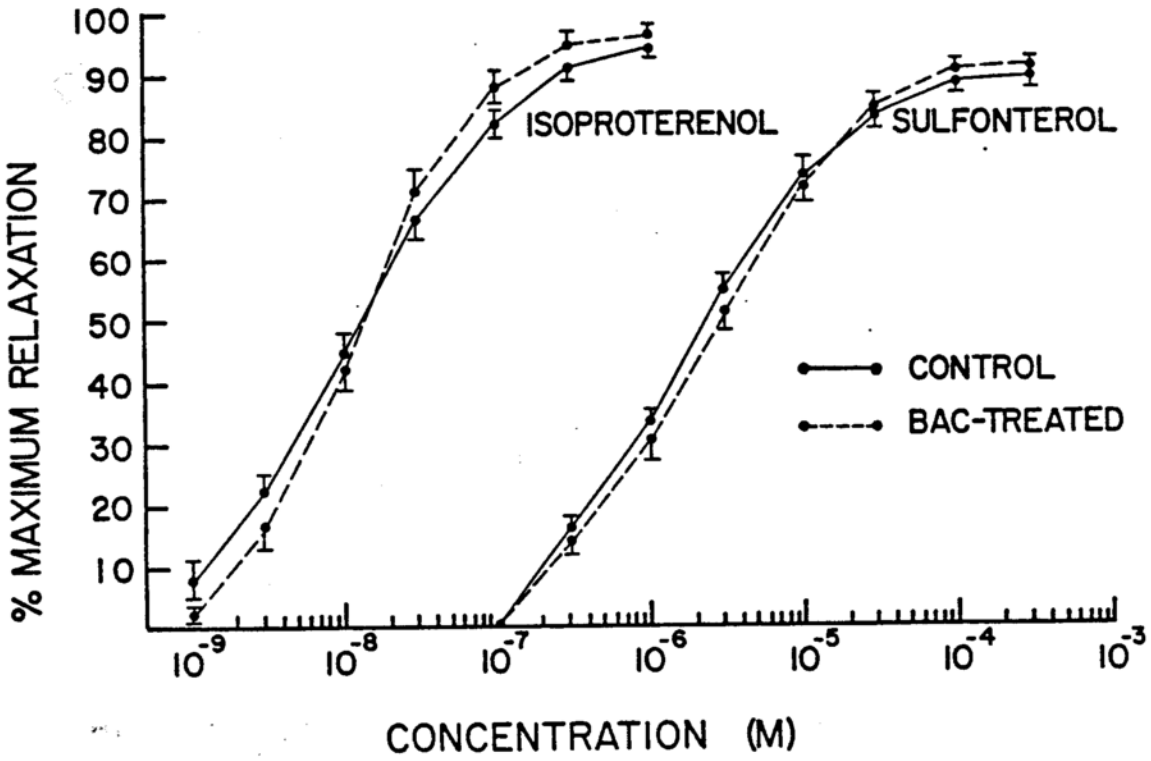

Figure 1. Log dose-response curves for isoproterenol and sulfonterol in producing relaxation in control (•——•) and BAC-treated (•----•) jejunum. Vertical lines represent S.E.M. Data taken from these curves are summarized in Table 1.

TABLE 1: Relaxant Effects of Adrenergic Receptor Agonists on Control and BAC-treated Jejunum.

Agonist	-Log molar ED_{50}[a] with S.E.M.		Maximum relaxation with S.E.M. (%)[b]		n[c]
	Control	Bac-Treated	Control	Bac-Treated	
isoproterenol	7.95 ± 0.07	7.88 ± 0.06	94.6 ± 1.7	96.5 ± 1.3	7
sulfonterol	5.75 ± 0.05	5.62 ± 0.06	89.4 ± 2.0	90.8 ± 0.9	9
phenylephrine	7.00 ± 0.07	5.77 ± 0.10*	59.5 ± 4.4	23.2 ± 3.8*	7
methoxamine	6.10 ± 0.05	5.74 ± 0.12*	59.5 ± 3.9	31.1 ± 6.7*	7
clonidine[d]	5.91 ± 0.10	---	55.9 ± 4.8	3.4 ± 2.2*	6

[a]Negative logarithm of the molar concentration of agonist required to produce 50% of its maximum response.

[b]Maximum degree of relaxation produced by the agonists. (Expressed as a percentage of relaxation produced by 2×10^{-3} M papavarine.)

[c]Number of paired observations.

[d]No response to clonidine was observed in 50% of the BAC-treated tissues, so a mean -log molar ED_{50} value could not be determined in the BAC-treated jejunum.

*Statistically significant difference between control and BAC-treated values ($p < 0.05$).

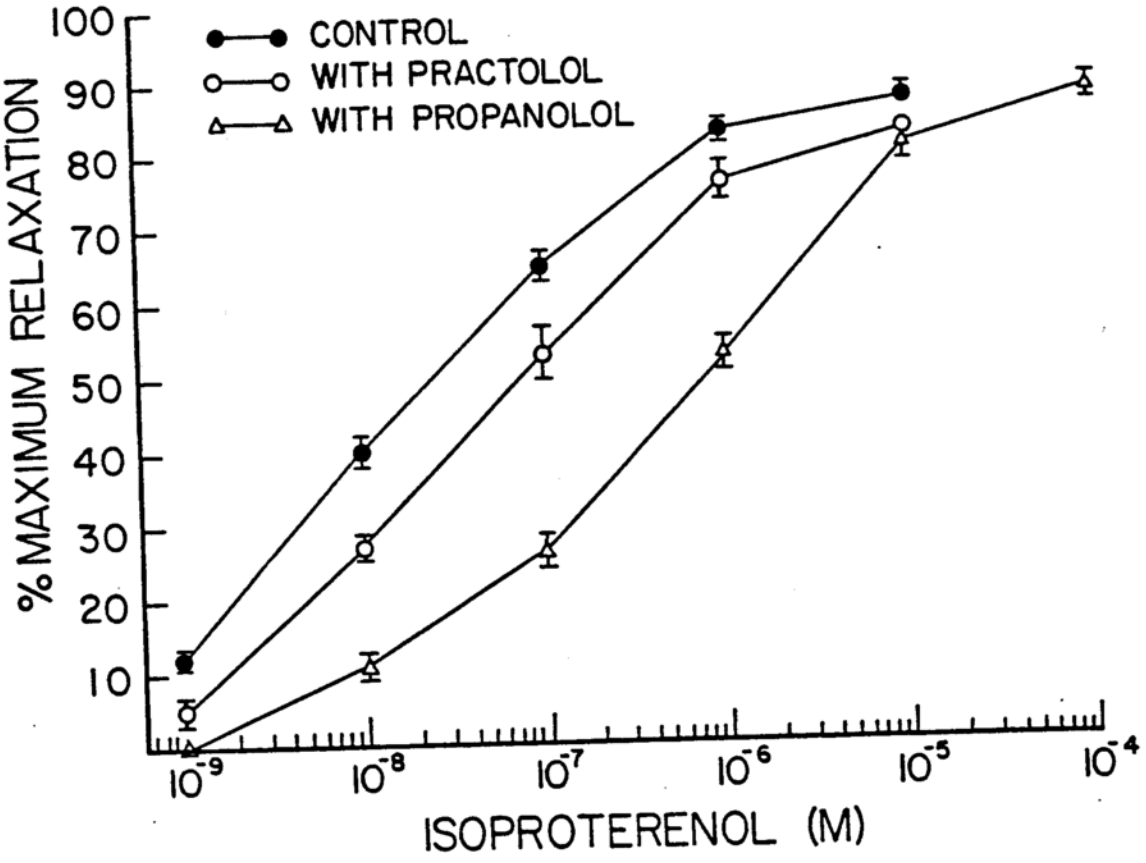

Figure 2. Log dose-response curves for isoproterenol in relaxing normal rat jejunum in the absence (•) and presence of 10^{-5}M practolol (o) or 10^{-5}M propranolol (Δ). The control curve is a composite of curves obtained in experiments testing both antagonists. Vertical lines represent S.E.M. Data taken from these curves are summarized in Table 2.

TABLE 2. Effects of practolol and propranolol on isoproterenol-induced relaxation in normal rat jejunum.

Beta Antagonist	Log Shift[a] with S.E.M.	Dose Ratio[b] with S.E.M.	Apparent K_B (M)[c]	PA_2[d]
Practolol (10^{-5}M)	0.50 ± 0.03	3.16 ± 0.21	4.85×10^{-6}	5.3
Propranolol (10^{-5}M)	1.45 ± 0.02	28.1 ± 1.5	3.74×10^{-7}	6.4

[a]Calculated as (-log molar ED_{50} of isoproterenol without antagonist) - (-log molar ED_{50} of isoproterenol with antagonist). (n = 6, practolol; n = 5, propranolol)

[b]Antilog of log shift

[c]Calculated as [antagonist]/(dose ratio - 1)

[d]-log K_B

Effects of alpha agonists and antagonists

The alpha agonists phenylephrine, methoxamine and clonidine produced a concentration-dependent relaxation in control jejunum which was greater than that observed in BAC-treated jejunum (figures 3, 4, and 5, respectively). The maximum responses and -log molar ED_{50} values obtained for these agonists in control and BAC-treated jejunum are summarized in Table 1. BAC treatment resulted in a reduction of the maximum responses to phenylephrine, methoxamine and clonidine, and a shift to the right of the phenylephrine and methoxamine dose-response curves. A mean -log molar ED_{50} value for clonidine in BAC-treated jejunum could not be obtained because half of the tissues did not respond at all to this agonist.

The relative potencies of phenylephrine and methoxamine changed after BAC treatment. In control jejunum, phenylephrine was 8-fold more potent than methoxamine in evoking relaxation. However, in BAC-treated jejunum, there was not a significant difference in the -log molar ED_{50} values obtained for these agonists. In order to try to explain the change in relative potencies of phenylephrine and methoxamine which occurred after BAC treatment, separate experiments were performed on normal jejunum. It was found that there was not a significant difference in the log shifts in the phenylephrine and methoxamine dose-response curves after separate exposure to 3 x 10^{-5}M cocaine, 10^{-4}M hydrocortisone or after pretreatment of the

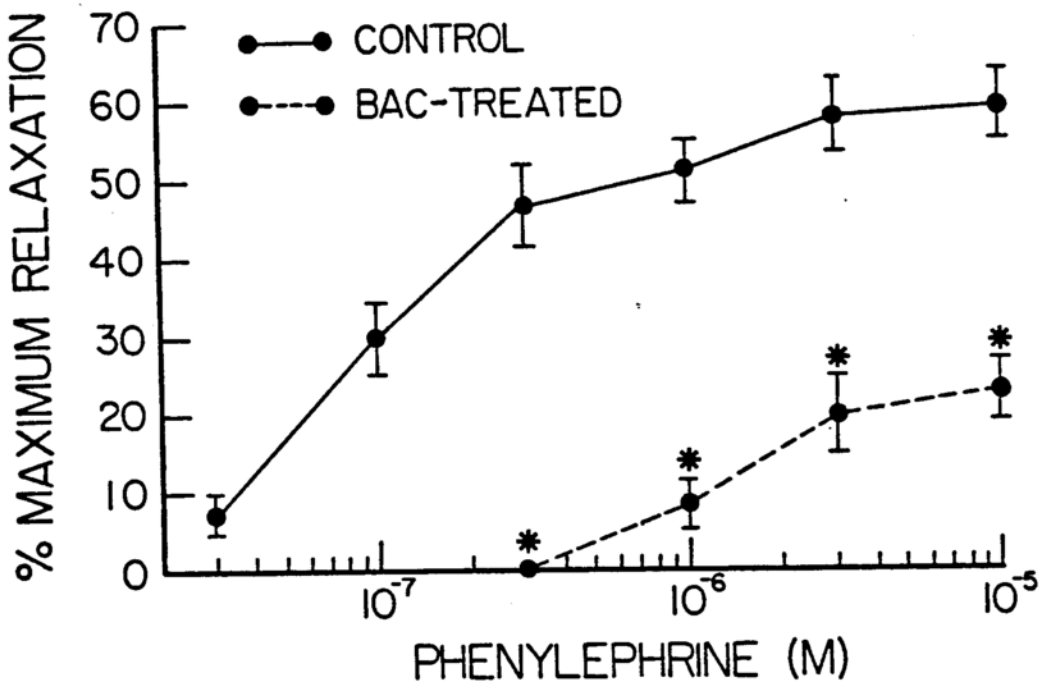

Figure 3. Log dose-response curves for phenylephrine in producing relaxation in control (•——•) and BAC-treated (•----•) jejunum. Vertical lines represent S.E.M. Data taken from these curves are summarized in Table 1. Asterisk denotes statistically significant difference between control and treated values, $p < 0.05$.

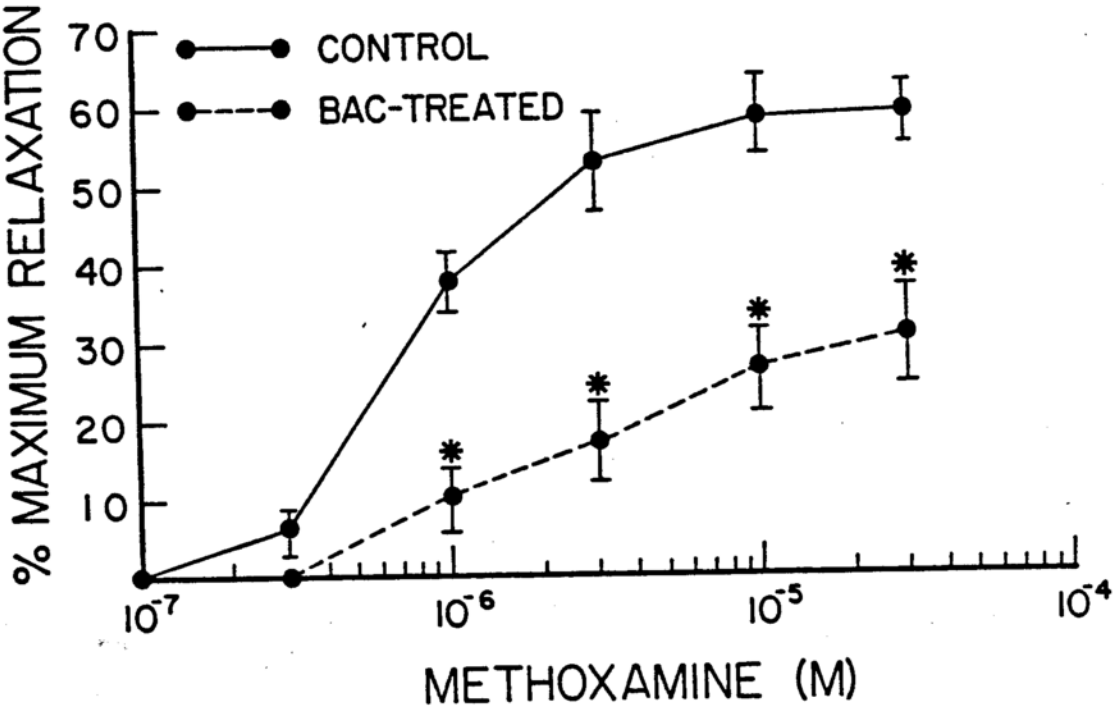

Figure 4. Log dose-response curves for methoxamine in producing relaxation in control (•——•) and BAC-treated (•----•) jejunum. Vertical lines represent S.E.M. Data taken from these curves are summarized in Table 1. Asterisk denotes statistically significant difference between control and treated values, $p < 0.05$.

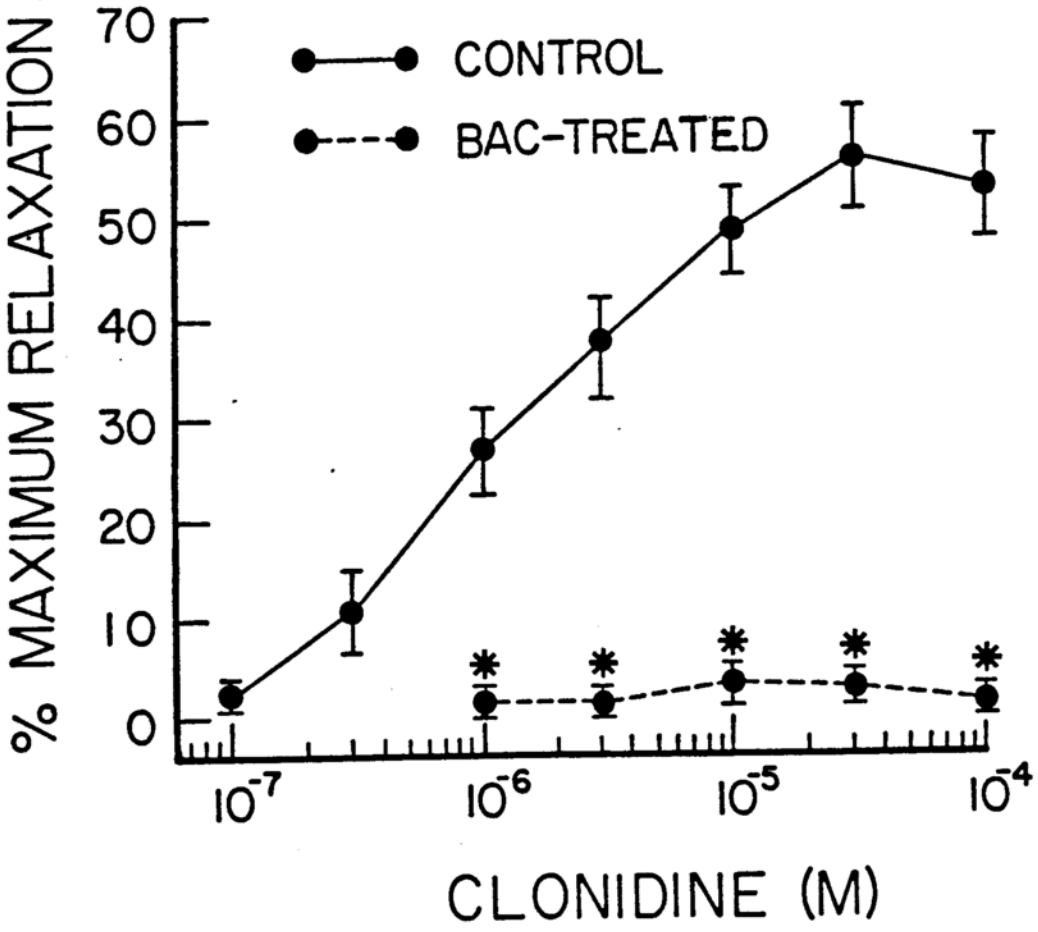

Figure 5. Log dose-response curves for clonidine in producing relaxation in control (•——•) and BAC-treated (•----•) jejunum. Vertical lines represent S.E.M. Data taken from these curves are summarized in Table 1. Asterisk denotes a statistically significant difference between control and treated values, $p < 0.05$.

animal with reserpine 5 mg/kg, 20-24 hours before experiments. This indicates that differences in neither neuronal uptake, extraneuronal uptake nor release of endogenous catecholamines could explain the change in relative potencies of phenylephrine and methoxamine which occurred after BAC treatment.

In normal rat jejunum, prazosin (2×10^{-7}M) antagonized the relaxation produced by methoxamine and phenylephrine (n=2). This concentration of prazosin completely blocked methoxamine-induced relaxation, whereas phenylephrine was able to relax only at concentrations greater than 3×10^{-5}M. In the presence of prazosin, methoxamine elicited contractions at concentrations greater than 10^{-5}M. Yohimbine (10^{-6}M) did not cause a significant shift in either the methoxamine or phenylephrine dose-response curves (n=2).

The alpha-2 selective agonists UK-14,304, M-7 and B-HT 920 produced a concentration-dependent contraction of control jejunum (fig. 6). These agonists failed to produce a response in the BAC-treated jejunum. Clonidine in the presence of 2×10^{-7}M prazosin also produced concentration-dependent contractions in control jejunum only (fig. 6). The data from figure 6 are summarized in Table 3. Clonidine was 20 times more potent in producing contraction in the presence of prazosin than relaxation without prazosin. The maximum responses of the alpha-2 agonists were not significantly different (ANOVA, $p > 0.05$). Yohimbine (10^{-6}M) caused a 39-fold

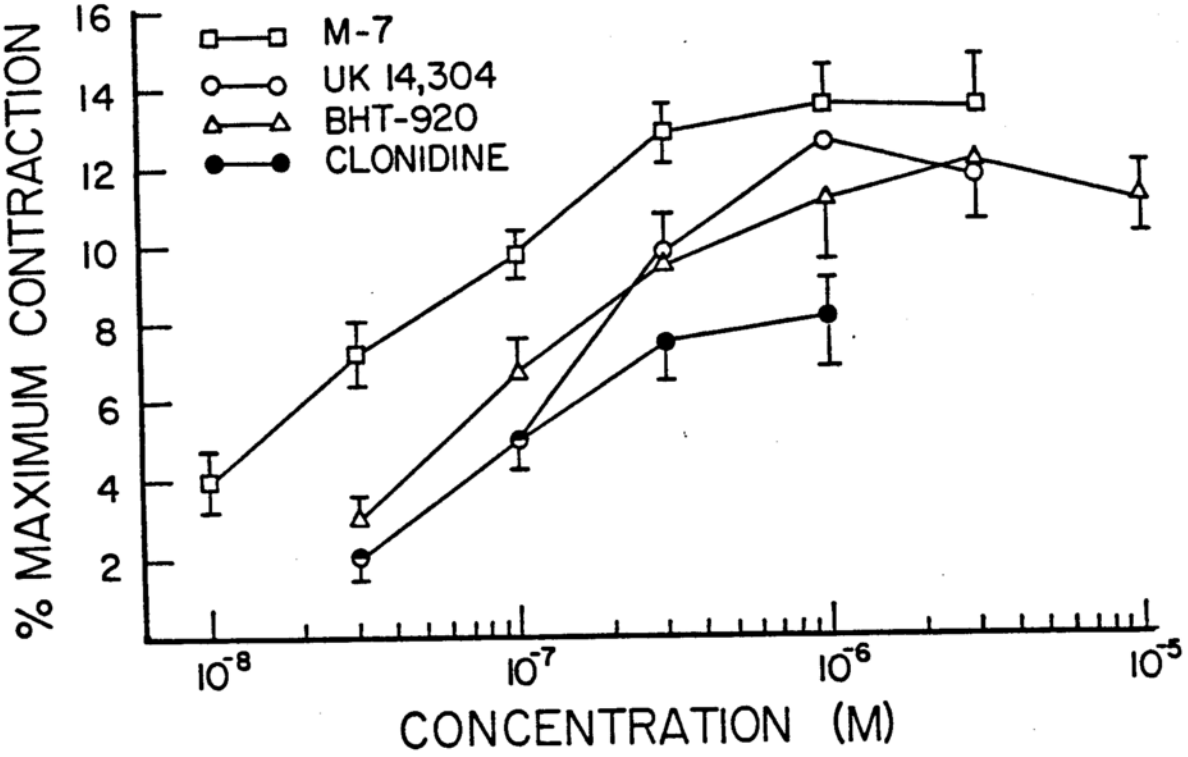

Figure 6. Log dose-response curves for the alpha-2 selective agonists M-7 (□), UK-14,304 (o), B-HT 920 (Δ) and clonidine (•) in producing contraction of control jejunum. Clonidine dose-response curve was generated in the presence of prazosin (2×10^{-7}M). Vertical lines represent S.E.M. Data taken from these curves are summarized in Table 3.

TABLE 3. Contractile response of alpha-2 selective agonists on the normal rat jejunum.

alpha-2 selective agonists	-Log molar ED_{50}[a] with S.E.M.	Maximum Response (%)[b]	n[d]
UK-14,304	6.92 ± 0.08	12.7 ± 1.3	6
M-7	7.49 ± 0.09	13.7 ± 0.9	7
B-HT 920	7.05 ± 0.09	12.1 ± 1.3	8
Clonidine[c]	7.22 ± 0.09	8.1 ± 1.1	4

[a]Negative logarithm of the molar concentrations of α_2 agonist required to produce 50% of its maximum response.

[b]Maximum degree of contraction produced by the α_2 agonists. (Expressed as a percentage of the maximum contraction produced by 10^{-2}M barium chloride.)

[c]Response obtained in the presence of 2×10^{-7}M prazosin.

[d]Number of observations.

rightward shift in the UK-14,304 dose-response curve (fig. 7). The apparent K_B and pA_2 values for yohimbine were 4.19×10^{-8}M and 7.5, respectively. The contractile response produced by UK-14,304 was not blocked by 10^{-5}M atropine sulfate.

DISCUSSION

The results of the present study suggest that in the rat jejunum: (1) beta adrenergic receptors mediate longitudinal muscle relaxation and are located on the smooth muscle; (2) Alpha-1 adrenergic receptors mediate relaxation of the longitudinal muscle and are located on both the smooth muscle and the myenteric plexus and (3) alpha-2 adrenergic receptors mediate contraction of the longitudinal muscle and are located on the myenteric plexus. The evidence for these conclusions is discussed below:

Beta adrenergic receptors

A smooth muscle location for beta adrenergic receptors mediating relaxation of rat jejunal longitudinal muscle is based on the observation that there was not a significant difference between control and BAC-treated jejunum in the relaxant response evoked by the beta receptor agonists isoproterenol and sulfonterol. Since BAC-treatment destroys the myenteric plexus (Fox et al., 1983) and eliminates neurally-mediated responses of the longitudinal muscle (Fox and Bass, 1985), the mechanical responses produced by the beta

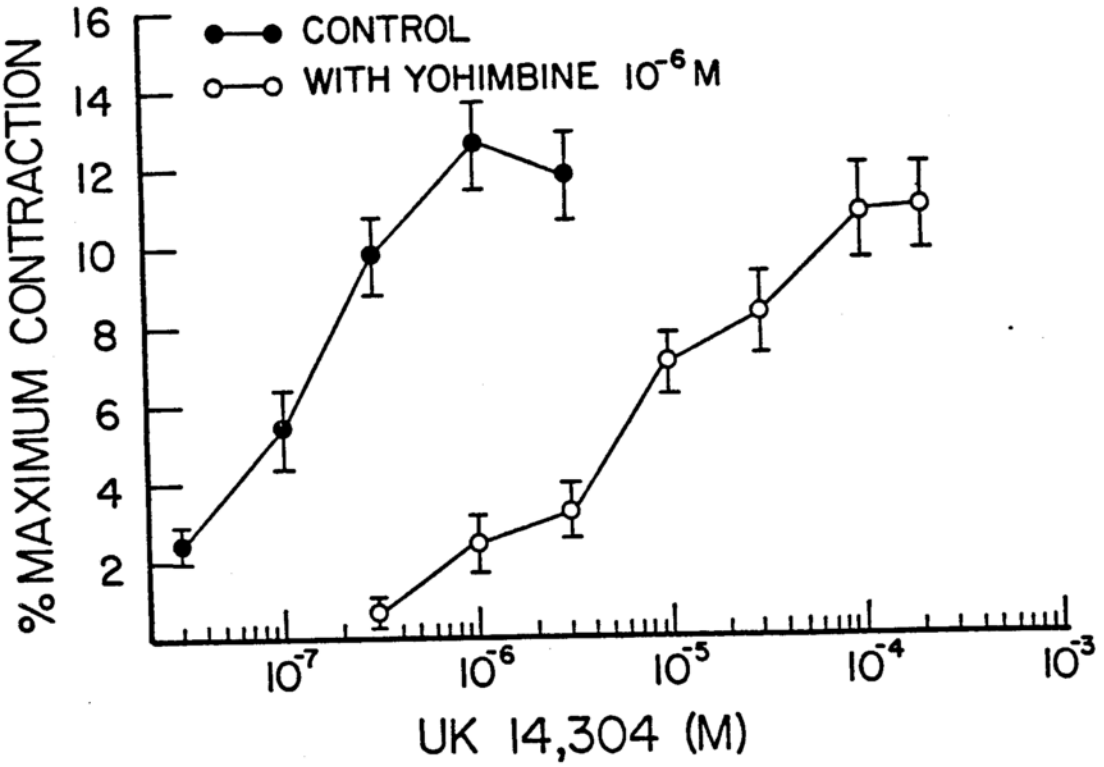

Figure 7. Log dose-response curves for UK-14,304 in producing contraction of control jejunum in the absence (•) and presence (o) of 10^{-6}M yohimbine. Vertical lines represent S.E.M. Dose ratio, apparent K_B and pA_2 value are 39 ± 8, 4.19 x 10^{-8}M and 7.5, respectively.

agonists in BAC-treated jejunum may be attributed to an effect on the smooth muscle membrane. Also, since the relative potencies of isoproterenol and sulfonterol were similar in control and BAC-treated jejunum, the same receptor type probably mediates the relaxant response to beta agonists in these tissues.

The data obtained with the beta antagonists used in this study suggest that the beta agonists evoke relaxation of the rat jejunum by activating receptors which may be unlike the typical beta-1 and beta-2 receptors first described by Lands and coworkers (1967a,b). This conclusion is based on the observation that the potencies of the beta antagonists in the rat jejunum are considerably lower than the potencies of these antagonists in non-gut tissues. For example, the pA_2 value for propranolol in a number of tissues is about 9 (Furchgott, 1972), compared to a value of only 6.4 in rat jejunal longitudinal muscle. The potencies of the beta-2 selective antagonist butoxamine (Levy, 1966) and the beta-1 selective antagonist practolol (Dunlop and Shanks, 1968) in the rat jejunum were also low, and may invalidate subclassification of beta receptors in this tissue. Though the beta receptors in the rat jejunum may bear resemblance to the beta-1 subtype because propranolol and practolol, but not butoxamine, shifted the isoproterenol relaxation dose-response curve, the high concentrations of these antagonists used in comparison to other tissues are inconsistent with this conclusion.

The potency determined for practolol in the rat jejunum (pA_2, 5.3) is more similar to that reported in the trachea (pA_2, 5.5), a tissue presumed to contain mostly beta-2 type receptors, than that found in the atria (pA_2, 6.4) a tissue presumed to contain mostly beta-1 type receptors (Buckner and Patil, 1971). Also, given that the pA_2 value for butoxamine is 5.1 in the guinea pig atria (O'Donnell and Wanstall, 1979), a butoxamine concentration of 10^{-4}M should have shifted the isoproterenol dose-response curve in the rat jejunum if "typical" beta-1 receptors were involved. For these reasons, classification of the receptors in the rat jejunum into the beta-1 or the beta-2 subtype may be subject to question.

The low potency of beta antagonists is not peculiar to the rat jejunum and appears to be a common phenomenon in gastrointestinal smooth muscle. For propranolol, reported pA_2 values were 6.9 in the circular muscle of the rabbit stomach fundus (Bristow et al., 1970), 6.8 in the longitudinal muscle of the rabbit small intestine, (Furchgott, 1972) and 6.4 in the rat esophagus (Buckner and Christopherson, 1974). A pA_2 value for practolol of 5.4 has been reported in the guinea pig ileum (Grassby and Broadley, 1984). Therefore, the low pA_2 values are not restricted to a single species or region of the gastrointestinal tract. The reason for the apparent resistance of gastrointestinal smooth muscle to the action of beta antagonists remains to be determined, though different barriers

governing access to receptors in different tissues may be considered.

Alpha receptors

Our conclusion that alpha-1 receptors mediate relaxation of the rat jejunum and are located on both the smooth muscle and the myenteric plexus is based on the observation that the alpha-1 selective agonists phenylephrine and methoxamine were more potent and efficacious in producing relaxation in control than BAC-treated jejunum. Since BAC-treated jejunum does not exhibit neurally-mediated responses (Fox and Bass, 1985), the relaxation produced by phenylephrine and methoxamine in the BAC-treated jejunum appears to be the result of an action on smooth muscle alpha-1 receptors. The greater magnitude of the response in control tissues suggests that phenylephrine and methoxamine are capable of stimulating both neural and muscle alpha-1 receptors. Wikberg (1977,1979) has shown that relaxation of the rabbit jejunum is also mediated by both neural and smooth muscle alpha receptors.

The relaxant response produced by the alpha-2 selective agonist clonidine appears to be mediated by alpha-1 receptors, since the response was blocked by prazosin but not by yohimbine. The minimal relaxant response to clonidine in the BAC-treated tissues suggests that the smooth muscle alpha-1 receptors are of negligible importance in mediating the relaxant response of clonidine. Also, there

was a difference in the relative potencies of phenylephrine and methoxamine in control and BAC-treated jejunum which could not be explained on the basis of differences in neuronal or extraneuronal uptake, ability to release catecholamines or alpha-2 agonist properties. Therefore, it appears as though alpha-1 agonists differ in their ability to gain access to the neural and smooth muscle alpha-1 receptors.

The alpha-2 selective agonists UK-14,304 (Cambridge, 1981), M-7 (Shepperson and Langer, 1981), B-HT 920 (Timmermans and van Zwieten, 1982) and clonidine in the presence of the alpha-1 selective antagonist prazosin (Davey, 1980), elicited contractions in control but not BAC-treated jejunum. The fact that contractions were not observed in the BAC-treated tissue suggests that the alpha-2 adrenergic receptors mediating contraction are located on the myenteric plexus. A myenteric plexus locus for alpha-2 receptors is consistent with the findings of Wikberg and Lefkowitz (1982) in the guinea pig ileum. The pA_2 value for yohimbine in antagonizing the contractile response of UK-14,304 (7.5) was in close agreement with a value of 7.58 reported for yohimbine in blocking the inhibitory effect of clonidine on the transmurally stimulated guinea pig ileum (Shebuski and Zimmerman, 1985). This suggests that UK-14,304 is indeed stimulating alpha-2 receptors.

In our system, clonidine appears less selective for alpha-2

receptors than M-7, UK-14,304 and B-HT 920. Clonidine-induced contractions of the rat jejunum were apparent only after blockade of alpha-1 receptors with prazosin, whereas the other three alpha-2 selective agonists elicited contractions without blockade of alpha-1 receptors. In the absence of prazosin, clonidine produced relaxation which was not blocked by yohimbine, indicating action at alpha-1 receptors. However, clonidine was more potent on the alpha-2 than the alpha-1 receptor. The nonselectivity of clonidine relative to M-7, B-HT 920 and UK-14,304 has been reported in other systems (Timmermans and van Zwieten, 1982). Our studies also indicate that the alpha-1 selective agonist methoxamine, which produced concentration-dependent relaxation, can stimulate alpha-2 receptors at high concentrations, manifested by contraction. The alpha-2 receptor mediated contractile response of methoxamine was revealed only after blockade of alpha-1 receptors with prazosin.

The contractions evoked by alpha-2 receptor stimulation in the rat jejunum may be due to the release of an excitatory transmitter, or the inhibition of an inhibitory transmitter from the myenteric plexus. Because the contractions elicited by UK-14,304 were not blocked by atropine, it does not appear as though acetylcholine release can account for the contractions. Other putative enteric neurotransmitters which elicit contraction of the rat jejunal longitudinal muscle include: 5-HT, substance P and cholecystokinin-

octapeptide (Fox et al., 1985). Alternatively, it is possible that alpha-2 receptor stimulation inhibits the release of VIP, ATP, neurotensin, or norepinephrine, enteric neuronal substances which relax the rat jejunum (Fox et al., 1985). A systematic examination of separate blockade of these excitatory and inhibitory substances on alpha-2 agonist-induced contraction would be needed to determine the mechanism of the alpha-2 mediated contractile response. Unfortunately, specific antagonists are not available for many of these compounds.

In general, the effect of adrenergic agonists on non-sphincteric smooth muscle of the gut is inhibitory. However, a number of reports have described stimulatory effects of adrenergic agonists in some preparations. (For review, see Burnstock and Wong, 1981). Where characterized, the stimulatory effects were shown to be due to excitatory alpha receptors. For example, in the isolated rat colon, catecholamine-induced relaxation was reversed to contraction after beta adrenergic receptor blockade (Gagnon and Belisle, 1970). However, besides the rat jejunum, we are unaware of another gut muscle preparation in which alpha receptors mediating contraction were shown to be of the alpha-2 subtype.

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Summary of Findings

It has been recognized for more than a century that the enteric nervous system is involved in the regulation of the movements of the intestinal musculature. However, the individual contributions of the different components of the enteric nervous system (i.e. the myenteric and submucosal plexuses) in the control of intestinal motility have not been clearly defined. The difficulties encountered in the study of enteric nerve components and their relationship to intestinal functions stem from the intrinsic nature of the nerve plexuses. Because the enteric ganglia are contained within the gut wall, ganglionectomy or nerve transection cannot be performed without disruption of the intestinal muscle. Hence, functions associated with the individual nerve plexuses have not been determined.

Fox and coworkers (1983), by applying benzalkonium chloride (BAC) to the serosal surface of the rat jejunum, have been able to destroy the myenteric plexus without altering the remainder of the intestine. The present investigation has utilized this technique to obtain information concerning the role of the myenteric plexus in the regulation of intestinal motility. Evidence has been presented which suggests that the myenteric plexus contains the motor neurons which innervate the longitudinal muscle of the rat jejunum. It appears as though the submucosal plexus may not have a major regulatory influence on longitudinal muscle contractility.

The role the myenteric plexus plays in the normal coordinated

movements of the intestine in the conscious rat was examined by measuring the myoelectric activity of BAC-treated jejunum. It was found that the basic electric rhythm was disrupted after BAC treatment, indicating that the myenteric plexus plays a modulatory role in this smooth muscle phenomenon. In contrast, the migrating myoelectric complex (MMC) was able to propagate through the area of the intestine devoid of myenteric neurons, suggesting that the myenteric plexus does not greatly influence the propagation of the MMC. Other researchers have shown that the propagation of the MMC is interrupted by tetrodotoxin, hexamethonium or transection and reanastomosis of the intestine. These methods disrupt both the myenteric and submucosal plexuses. The combination of the present results and those of others suggest that the submucosal plexus may exert some influence in the propagative control of the MMC. However, this suggestion is speculative since hormonal factors may also influence the MMC. Selective ablation of the submucosal plexus or destruction of both intestinal nerve plexuses would provide insight into this possibility.

Studies examining myoelectric activity of the intestine revealed a potential limitation of the BAC-treated preparation. Delayed appearance of spike potentials in the BAC-treated jejunum was indicative of initial damage to the smooth muscle and subsequent recovery. It is possible that BAC was initially disruptive to both nerve and muscle membranes. The selectivity of BAC for nerves may have arisen from the failure of enteric ganglion cells to regenerate, whereas the musculature was apparently capable of self-repair. In order to assure

normal contractility after BAC treatment, experiments should be conducted at least 2 weeks after BAC treatment.

The mediators of enteric nerves have a profound influence on intestinal motility. Precisely where they act to exert their effects on intestinal movements is not clear. The present study has provided fundamental information regarding the site of action of several putative enteric neurotransmitters. The question of whether or not the myenteric plexus was involved in the mediation of the longitudinal muscle responses to these substances was explored. It was concluded that the myenteric plexus was instrumental in the longitudinal muscle responses to 5-hydroxytryptamine, cholecystokinin-octapeptide, vasoactive intestinal peptide and adenosine triphosphate, but not substance P or norepinephrine. The latter two compounds exerted their effects by direct interaction with the smooth muscle. It was revealed that the neurotransmitter acetylcholine had both a direct and indirect action, consistent with its known effect on both muscarinic and nicotinic receptors. Because of the equivocal nature of the responses of the longitudinal muscle to neurotensin, enkephalin, somatostatin and bombesin, the site of action of these compounds could not be established. Determination of the specific location within the myenteric plexus where neuronal mediators exert their effects would require additional pharmacological studies as well as ablative techniques selective for specific nerve types (i.e. 6-hydroxydopamine for sympathetic nerves; capsaicin for substance P containing nerves).

The present studies also contributed to our knowledge of the

the location of adrenergic receptors in the gut. These studies have shown that beta and alpha-1 receptors mediate relaxation of the longitudinal muscle and are located on the smooth muscle. Stimulation of alpha-1 receptors located on the myenteric plexus can also elicit relaxation of the longitudinal muscle. A unique finding of this investigation was that alpha-2 receptors on the myenteric plexus can produce contraction of the longitudinal muscle. Neuronal alpha-2 receptors which mediate contraction of the longitudinal muscle have not been previously identified in gut muscle preparations. The precise mechanisms by which adrenergic receptors mediate responses of the longitudinal muscle of the rat jejunum remain to be determined.

Experiments aimed at determining the relative distribution of cholinergic neurons in the myenteric and submucosal plexuses (Appendix B) by measuring choline acetyltransferase (ChAT) activity in BAC-treated jejunum resulted in provocative data concerning the interactions between myenteric and submucosal neurons. The data suggest that ablation of myenteric neurons may cause compensatory increases of ChAT activity in the submucosal plexus over time. This suggests that a neurotransmitter from one plexus may exert feedback control over transmitter production in the neurons of another plexus in the gut.

Further studies which would compliment the preliminary ChAT data presented in the Appendix include: (1) immunocytochemical studies to determine the actual distribution of ChAT-containing nerves in the intestine. (2) determination of ChAT activity in BAC-treated intes-

tinal segments in which the mucosa and submucosa have been removed (Difficulties in dissection may limit this type of experimentation), and (3) determination of possible compensatory increases in levels of the neuronal peptides.

An important function of the gastrointestinal tract is absorption and secretion of water and electrolytes. The enteric nervous system has been postulated to be involved in the regulation of mucosal transport, though the individual contributions of the myenteric and submucosal plexuses have not been determined. The BAC-treated preparation appeared to be a potentially useful technique for deciphering the contribution of the myenteric plexus in mucosal transport properties. However, the increase in smooth muscle thickness which occurs as a consequence of BAC-treatment prohibited the measurement of transport properties by conventional techniques (Appendix C). For this line of research to be fruitful, other techniques must be devised which will destroy the myenteric plexus without increasing the thickness of the muscle. The possibility exists however, that any method which destroys the myenteric plexus will result in an increase in intestinal muscle thickness.

The conclusions of the present investigation are summarized as follows:

1. The longitudinal muscle of the isolated rat jejunum in which the myenteric but not the submucosal plexus is absent responds as though it is aganglionic. This suggests that the motor neurons inner-

vating the longitudinal muscle are located in the myenteric plexus.

2. Myoelectric studies conducted in the conscious rat suggest that ablation of the myenteric plexus disrupts the basic electric rhythm but not the propagation of the migrating myoelectric complex in rat jejunum.

3. Substance P and norepinephrine elicit mechanical responses of the rat jejunal longitudinal muscle by acting directly on the smooth muscle. Cholecystokinin-octapeptide, 5-hydroxytryptamine, vasoactive intestinal peptide and adenosine triphosphate exert their effects by an indirect action through the myenteric plexus. Acetylcholine has both direct and indirect effects.

4. Beta adrenergic receptors mediate relaxation of the longitudinal muscle and are located on the smooth muscle. Alpha-1 receptors mediate relaxation and are located both on the muscle and the myenteric plexus. Alpha-2 receptors mediate contraction and are located on the myenteric plexus.

APPENDIX A

Construction and Implantation of Electrodes for Measurement of Intestinal Myoelectric Activity in the Rat

A. Recording Electrode (modification of Weisbrodt et al., J. Pharmacol. Exp. Ther. 214:333-338, 1980).

A 4 mm piece of silver wire was bent to form a 90° angle and was soldered to a 15 cm piece of 36 gauge stranded silver-plated copper wire with Teflon insulation (Micro-measurements). The solder junction was coated first with rosin solvent, (Micro-measurements) and then with isopropyl alcohol, and allowed to air dry. The silver wire attached to the insulated wire was slipped into a 6-8 mm piece of silastic tubing (ID, 1.5 mm), such that the silver wire was protruding out from the middle of the tubing. Via a syringe, the piece of tubing (with enclosed wire) was filled with epoxy resin. After the epoxy resin was allowed to dry for 24 hours, the tubing was removed with a scalpel blade. The silver wire protruding from the hardened epoxy resin was filed such that it was almost level with the epoxy resin. Two notches were made in the epoxy resin close to the silver wire, but on the opposite side of the electrode. RTV sealant was placed on the wire at end of electrode (see Figure 1, A).

B. Reference Electrode

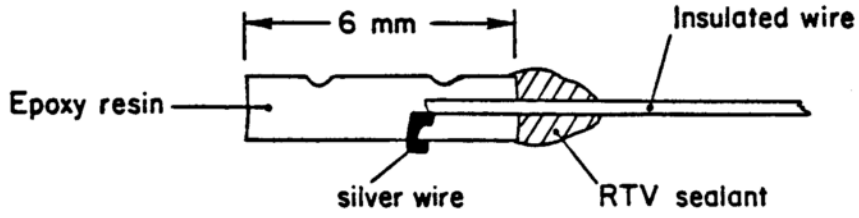
A piece of silver wire was curled a few times into a spiral and soldered onto Micro-measurement insulated wire. Rosin solvent, then isopropyl alcohol were applied to the solder

junction and allowed to air dry. A drop of epoxy resin was then applied to the solder junction and allowed to dry for 24 hours. A 1.5 cm piece of silastic tubing (ID, .0/2"; OD, 0.060") was placed in xylene for about 5 minutes to expand the tubing for placement over the solder junction. A drop of RTV sealant was placed at both ends of tubing (see Figure 1, B).

C. Construction of Back Plug (see Figure 2)

Three electrodes and one reference electrode were soldered to a socket, (socket, from local electronics store). RTV sealant was placed on solder junctions and allowed to dry for 24 hours. Socket, with attached electrodes, was positioned on a 1.5 cm piece of Tygon tubing (diameter appropriate for size of socket). Dental acylic was then placed around the junction of the socket and the tubing. Two pieces of dental acylic were formed into flaps and placed around the tubing. Two holes were placed in the flaps while the dental acylic was soft. A header (from local electronics store), was placed into the socket to keep the plug free from debris. The header was also used as a portion of the connector to the recording unit.

A. Recording Electrode



B. Reference Electrode

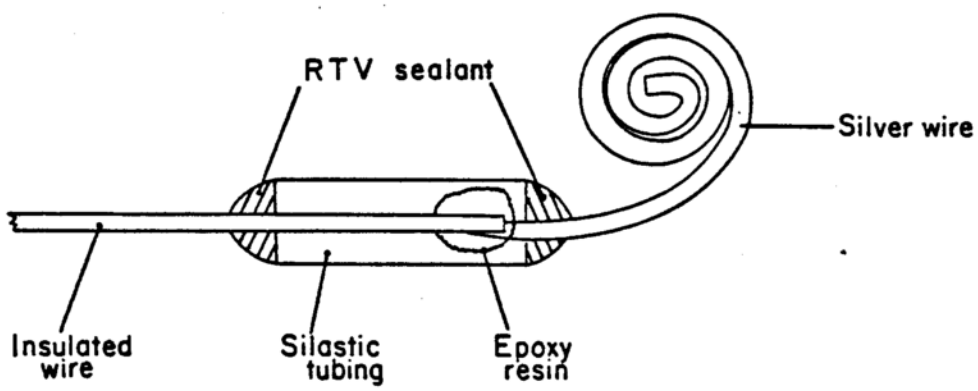


Figure 1. Diagrammatic representation of recording (A) and reference (B) electrodes used in measuring myoelectric activity from the intestine of the unanesthetized rat (see text for details of construction).

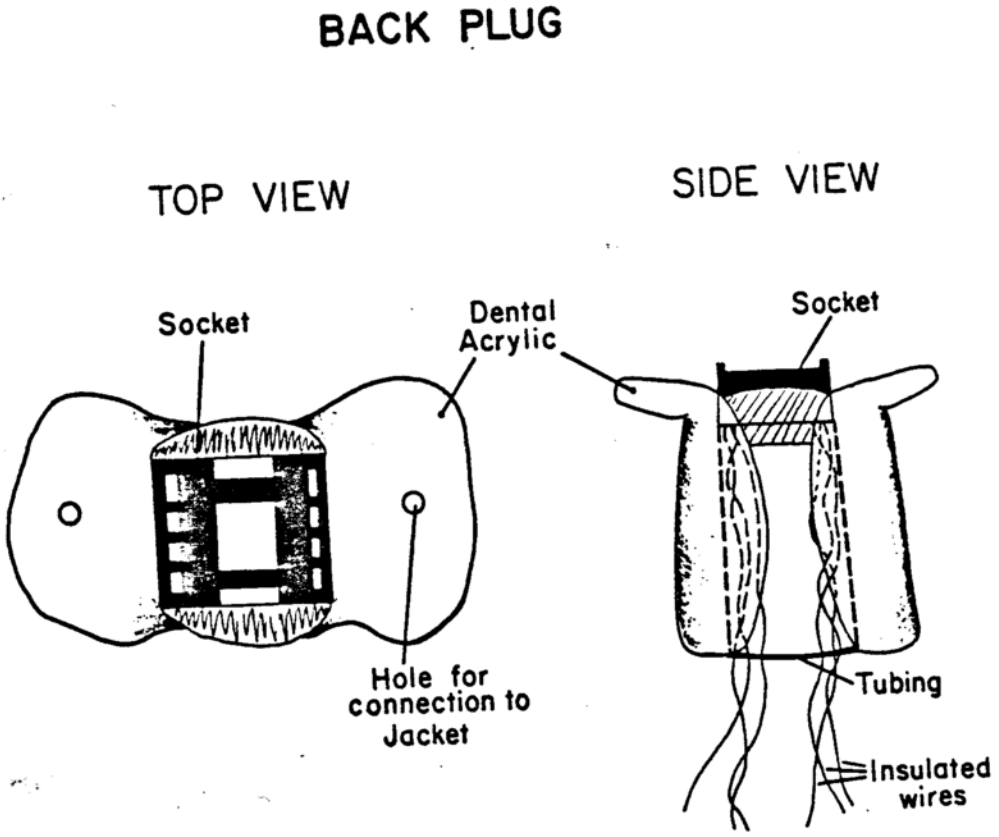


Figure 2. Diagrammatic representation of back plug (see text for details of construction).

D. Surgical Procedure

The electrodes of the back plug were slipped through the hole in the Velcro strip portion of a rat jacket (made by us; fashioned after those of Alice King, Los Angeles, CA), such that the back plug was situated on top of the Velcro strip.

The rats were anesthetized, and a 1.0 cm longitudinal skin incision was made between the scapula, and also in the flank area. A trocar was passed subcutaneously from the incision in the scapula to that in the flank. Electrodes were passed through the trocar such that when the trocar was removed, electrodes were protruding through flank wound, and the Velcro strip with back plug was situated on top of the scapular wound. The skin wound in the scapular region was sutured with 3-0 silk (single interrupted sutures) such that last suture was juxtaposed to the wires. The Velcro strip with plug was positioned on top of the sutured wound. The rat's front legs were placed through the holes in the other part of the rat jacket such that the two Velcro strips on this part of jacket were secured to the Velcro strip already situated in the scapular area. A wire was threaded through the holes in the back plug and secured to the hooks of the rat jacket.

The animal was then placed on its dorsal surface and a midline abdominal incision was made. A stab wound was made

through the abdominal wall at the point the electrodes were protruding through the flank wound. The electrodes (excluding reference) were brought through the stab wound into the abdominal cavity. The reference electrode was implanted subcutaneously in the flank area, and the skin incision was closed. Electrodes were sutured to the serosal surface of the intestine using 6-0 ethibond suture. This was performed by passing two pieces of suture through the serosa, and tying them around the two notches in the electrode. After all electrodes were in place, the midline incision was closed in two layers using a single interrupted suture pattern. The muscle was closed with 3-0 chromic gut; the skin was closed with 3-0 silk (see Figure 3).

Sources of Materials Used

RTV Silicone Rubber Adhesive Sealant
RTV 112 White self-leveling
General Electric Company
Silicone Products Department
Waterford, NY 12188

M-Line Rosin Solvent
336 FTE 50 ft. std. silver plated
copper twisted wire
Micro-measurements
P.O. Box 27777
Raleigh, NC 27611

Epoxy Resin
Epoxylite #211, Midget Kit
from: The Epoxylite Corp.
1235 S. State College Blvd.
Anaheim, CA 92806

Rat Jackets
Alice King
5043 Ona Knoll Avenue
Los Angeles, CA 90043
(213) 292-3680

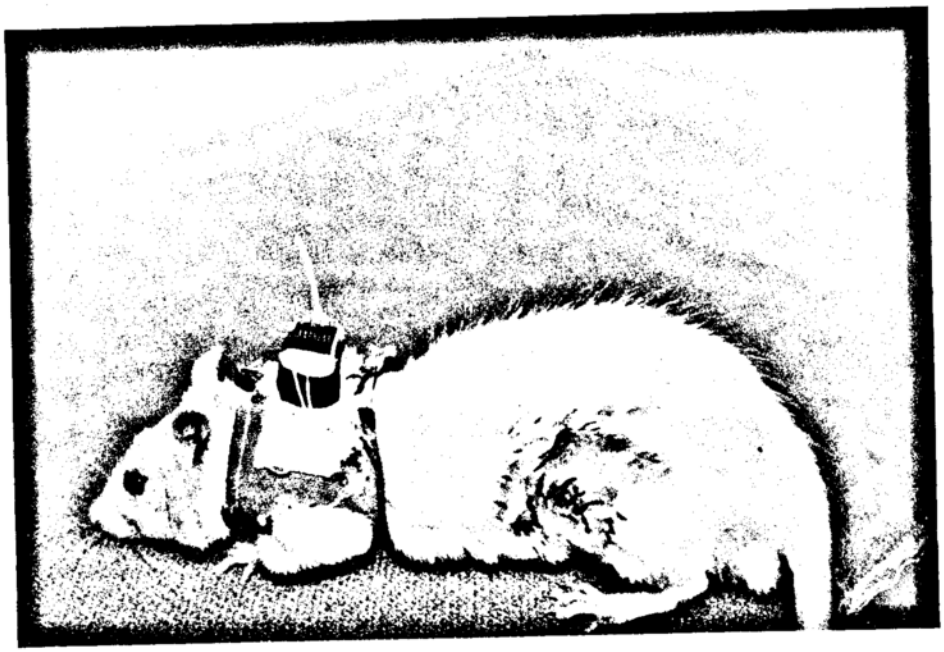


Figure 3. Photograph of conscious rat shown with back plug and rat jacket.

APPENDIX B

Changes in Choline Acetyltransferase Activity in Myenteric Neuron-Ablated Rat Jejunum

Note: ChAT assays were performed under the direction of Dr. June Dahl by Deborah Blume.

Introduction

Serosal application of the cationic surfactant benzalkonium chloride (BAC) to the rat jejunum destroys over 90% of myenteric neurons without altering the number of submucosal neurons. In addition, immunocytochemical studies showed that VIP, substance P, somatostatin, and enkephalin immunoreactivity was markedly reduced in the area of the myenteric plexus and adjacent muscle layers (Fox et al., 1983). With an interest in the relative distribution of cholinergic neurons in the myenteric and submucosal plexuses, we undertook the present study to determine the activity of choline acetyltransferase (ChAT) in control and BAC-treated jejunum. ChAT was used as a specific marker for cholinergic neurons.

Methods

A 0.062% solution of BAC was applied to the serosal surface of a 3 cm segment of rat jejunum as previously described (Fox et al., 1983). Groups of 6-8 animals were sacrificed by a sharp blow to the back of the head, 2, 5, 15, 22, and 45 days after BAC treatment. The BAC-treated jejunal segment and an untreated control segment 4-8 cm orad to it, were removed, weighed, and lengths measured. The tissues were frozen immediately in liquid nitrogen and stored at -50°C until the time of assay.

Tissues were homogenized in 10 volumes of 25 mM phosphate

buffer, pH 7.4, containing 0.1 mM mercaptoethanol, 1 mM EDTA, and 3 mM phenylmethylsulfonylfluoride. The homogenates were centrifuged at 27,000 x g for 30 minutes. ChAT activity in the resultant supernatant solutions was measured essentially as described by Fonnum (1975). The reaction mixture contained 50 mM phosphate, pH 7.4, 300 mM NaCl, 20 mM EDTA, 0.1 mM neostigmine, 8 mM choline, 0.2 mM acetyl-CoA, and 40,000 cpm [^{3}H]acetyl-CoA. One hundred microliters of reaction mixture were added to a 10 µl aliquot of supernatant contained in a 20 ml scintillation vial and the reaction allowed to proceed at 37°C for 30 min. The reaction was terminated by the addition of 5 ml of 10 mM sodium phosphate, pH 7.4. Then 2 ml of 5 mg/ml tetraphenylboron in acetonitrile and 10 ml of toluene scintillation fluid were added, the contents of the vial gently swirled and then counted in a liquid scintillation counter. Protein was determined by the assay of Bradford (1976) using bovine plasma gamma globulin as a standard.

Results and Discussion

Tissue Parameters

The total weight per centimeter and total protein per centimeter was significantly increased in BAC-treated segments of jejunum by 15 days post BAC treatment. As shown in Figure 1, the values for gm wet weight/cm and mg protein/cm in BAC-treated jejunum were significantly greater than control on days 15, 22, and

45, but not on days 2 and 5 post BAC treatment. Values for mg protein/gm wet weight were calculated for control and BAC-treated jejunum and were found not to be significantly different at any time tested after treatment with BAC. This indicates that the time-related weight increases observed in BAC-treated tissue were associated with concomitant increases in total protein and not merely an increase in water content of the tissues. The most probable source of the increases in weight and protein content of BAC-treated jejunum is the smooth muscle. Previous histological examination revealed an increase in the thickness of both longitudinal and circular smooth muscle layers of rat jejunum after BAC treatment (Fox et al., 1983).

ChAT Activity

Because of the increases in weight and total protein observed in the BAC-treated tissues, ChAT activity was expressed per centimeter length of jejunum. The time-related changes in total units of ChAT in control and BAC-treated jejunum are shown in table 1. Total units of ChAT were significantly lower in BAC-treated segments than in control segments at 2 and 5 days after BAC treatment. On days 15 and 22 post BAC treatment, units of ChAT/cm were higher in BAC-treated than control segments, though this increase was not statistically significant. Forty-five days after BAC treatment, ChAT levels were significantly higher than those found

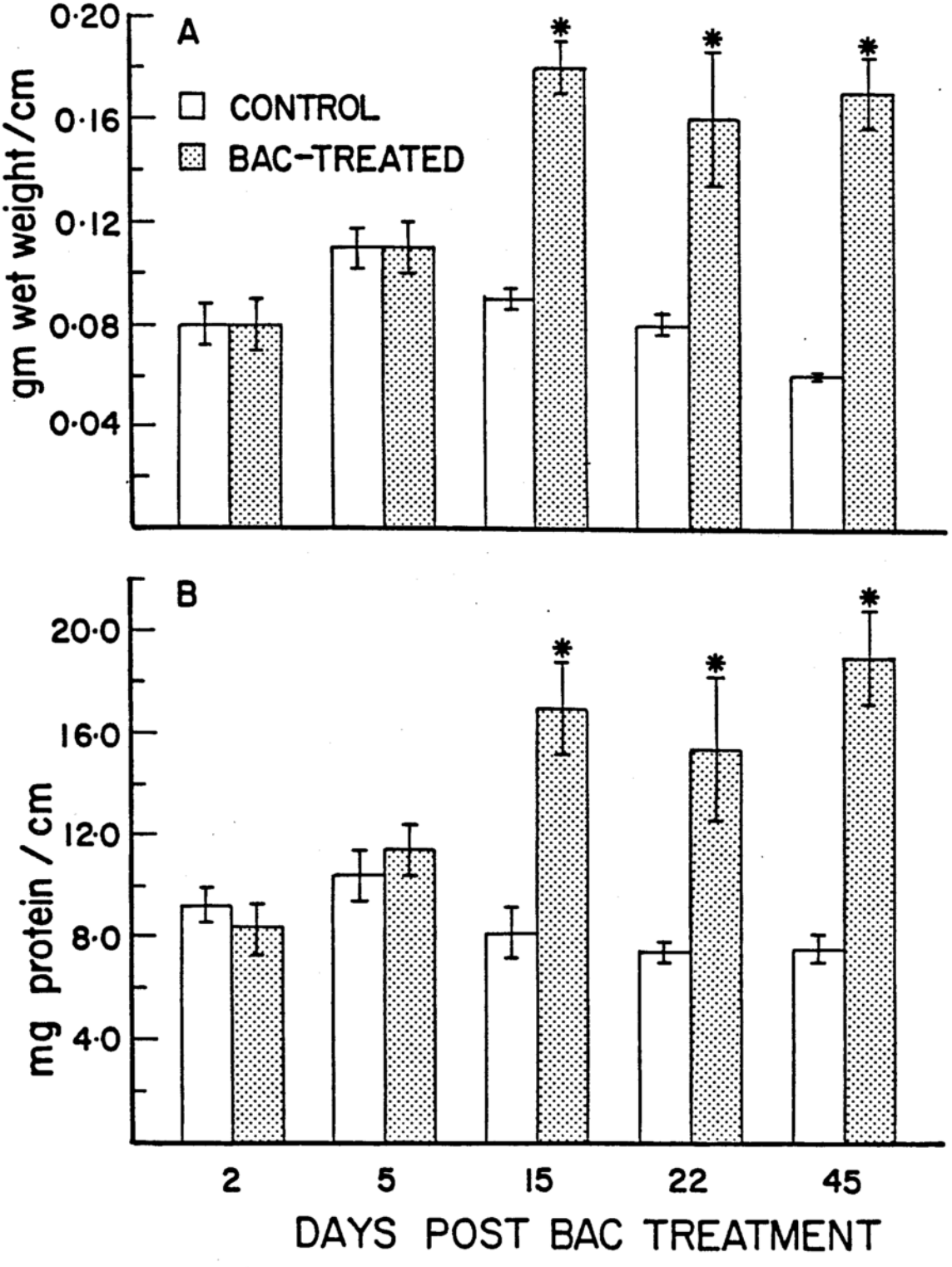

A
CONTROL
BAC-TREATED
gm wet weight/cm
0·20
0·16
0·12
0·08
0·04
B
mg protein / cm
20·0
16·0
12·0
8·0
4·0
2
5
15
22
45
DAYS POST BAC TREATMENT

Table 1. Time-related changes in ChAT activity in the rat jejunum after serosal application of BAC.

Days post BAC Treatment	Units[a] of ChAT activity/cm length		
	Control	BAC-Treated	n[b]
2	343 ± 24	209 ± 29*	6
5	363 ± 44	254 ± 19*	6
15	492 ± 35	614 ± 78	6
22	633 ± 72	774 ± 59	7
45	439 ± 27	778 ± 78*	8

[a]Unit is defined as pmoles of ACh formed per minute

[b]Number of paired observations

*Denotes a statistically significant difference between control and BAC-reated values ($p < 0.05$).

in control tissues.

These results suggest that: (1) A substantial portion of intestinal ChAT is found in the submucosal plexus. This suggestion is based on the observation that despite ablation of 90% of the myenteric neurons, approximately 60% of the ChAT activity is still present in the first few days after BAC treatment. (2) A decrease in cholinergic neurons in the myenteric plexus elicits a compensatory increase in ChAT activity possibly from the submucosal plexus. Given that the total number of neurons in the myenteric plexus is reduced by greater than 90%, the time related increases in ChAT activity observed in the treated jejunum relative to control probably result from a source other than the myenteric plexus. The likely source of the increase in ChAT activity is the submucosal plexus, which is not altered by treatment with BAC. Others have shown that there are nerve fibers connecting the myenteric and submucosal plexus (Jessen et al., 1980). Perhaps some neurons in the myenteric plexus have an inhibitory influence on the ChAT containing neurons in the submucosal plexus. Removal of this inhibitory influence by ablating the myenteric plexus may account for the increases in ChAT activity observed in the BAC-treated jejunum over time.

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APPENDIX C

Effect of Chronic Ablation of the Myenteric Plexus on Mucosal Transport in the Rat Jejunum

INTRODUCTION

The intestinal epithelium is capable of both absorbing and secreting water and electrolytes. Several lines of evidence implicate enteric neuron involvement in mucosal transport. Electrical field stimulation (EFS) of guinea pig (Cooke et al., 1983), rabbit (Hubel, 1978), and human (Hubel, 1982) isolated ileum causes an increase in mucosal transmural potential difference (PD), short circuit current (I_{sc}) and chloride secretion. Scorpion venom, which evokes neurotransmitter release, elicits similar effects as EFS with regard to PD, I_{sc} and chloride ion secretion (Hubel and Renquist, 1981; Cooke, 1983). In addition, putative enteric neurotransmitters alter mucosal activity. Substances which cause the mucosa to secrete include: acetylcholine (Cooke, 1984), substance P (Kachur et al., 1982), serotonin (Donowitz et al., 1980), ATP (Kohn et al., 1970), VIP (Schwartz et al., 1974), gastrin (Bynum, 1971), cholecystokinin (Matuchansky et al., 1972) and neurotensin (Kachur et al., 1982). Catecholamines (Field and McColl, 1973), somatostatin (Dltarmsathaphorn et al., 1980) and enkephalins (Kachun et al., 1980) enhance absorption. Thus, it is evident that enteric nerves and their mediatiors can alter transport of the intestinal mucosa.

The results of the above studies regarding enteric neurons and mucosal function, are based on a variety of techniques. With the

methods utilized, the separate involvement of the two components of the enteric nervous system (myenteric and submucosal plexuses) in intestinal mucosal transport cannot be ascertained.

The present study was initiated to determine the contribution of the myenteric plexus in intestinal mucosal transport. Our aim was to compare jejunal mucosal transport characteristics of normal and benzalkonium chloride (BAC)-treated jejunum exposed to nerve and enterocyte stimulants. Since BAC treatment destroys the myenteric plexus while leaving the remainder of the intestine intact (Fox et al., 1983), it is a novel approach to dissect the components of the enteric nervous system as they affect mucosal transport.

The method chosen to examine mucosal transport was the Ussing technique. In this system, the intestine is mounted as a flat sheet in a chamber in which both surfaces are perfused by warmed oxygenated Ringer's solution. Transmural potential difference (PD), short circuit current (I_{sc}) and ion flux can be measured using this technique. We were forced to discontinue this project because the increase in muscle thickness of the BAC-treated jejunum made measurement of the electrical parameters questionable. The methods employed and results obtained which prompted the decision to abandon this work are presented below.

METHODS

The method used to ablate the myenteric plexus was the same as that previously described (Fox et al., 1983; Fox and Bass, 1985). Sprague Dawley rats (Madison, WI) weighing 200-225 gms were anesthetized, and the serosal surface of a 3-4 cm segment of jejunum was treated with a solution of 0.062% BAC every 5 minutes for 0.5 hours. After treatment, the intestine was thoroughly rinsed with saline and the animals were allowed to recover. After 15 days, both the BAC-treated and an untreated control jejunal segment were removed from each animal for experimentation.

The excised intestinal segments were opened along the mesenteric border and rinsed with ice cold Ringer's solution. The flat sheets of intestine were mounted between the two halves of an Ussing flux chamber exposing a surface area of 0.66 cm^2. Both surfaces of the intestine were perfused separately with 10 ml of Ringer's solution which was oxygenated and maintained at 37°C by a water-jacketed, gaslift circulating system. The gas employed (95% O_2, 5% CO_2) was first bubbled through water to minimize evaporative water loss. The ionic composition of the bicarbonate-Ringer's buffer in millimoles per liter was: Na, 143; K, 5; Mg, 1.1; Ca, 1.25; HCO_3, 25; Cl, 123.7; H_2PO_4, 0.3; and HPO_4, 1.65. The mucosal side of the tissue contained 40 mM mannitol and the serosal side contained 40 mM glucose. The chambers were equipped with a pair of

Ringer-agar bridges connected to calomel half cells for measurements of transmural electrical potential difference (PD). A second pair of bridges was placed in Ringer's solution containing Ag-AgCl electrodes which were connected to an automatic voltage clamp apparatus which compensated for solution resistance between the PD sensing bridges and measured short circuit current (I_{SC}). PD and I_{SC} were recorded periodically throughout experiments.

Tissue resistance (R_T) was measured by taking advantage of Ohm's Law:

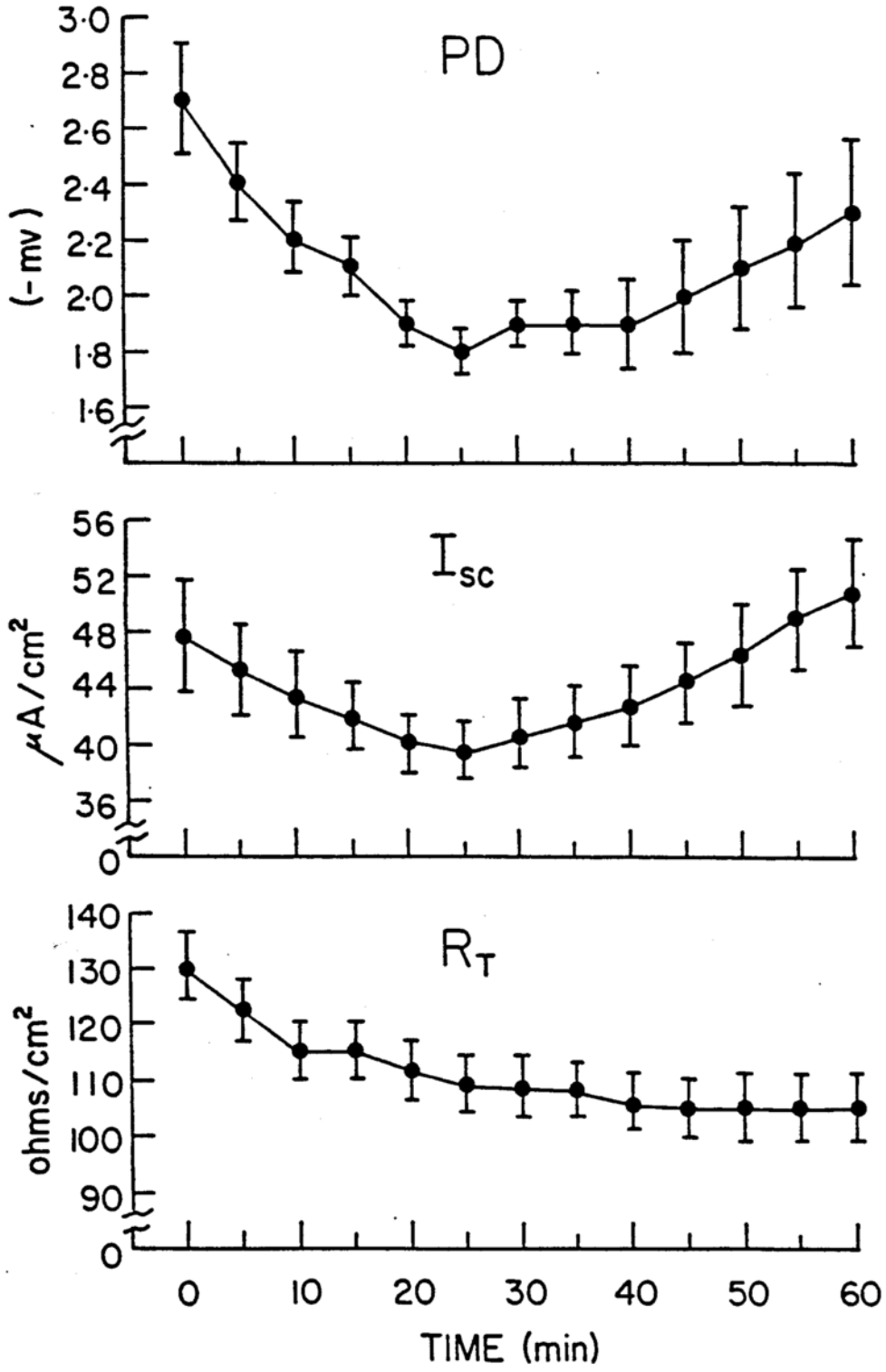
$$\Delta V = \Delta I \times R$$

Resistance was calculated by obtaining the voltage change (ΔV) after passing a 100 μ amp current (ΔI) through the tissue and applying it to the above formula.

RESULTS AND DISCUSSION

Before performing experiments using BAC-treated animals, baseline electrical parameters in normal rat jejunum were measured. PD, I_{SC} and R_T were recorded every 5 minutes for 1 hour after mounting the tissue in the chamber. These results are shown in figure 1. The time-related fluctuations in PD, I_{SC} and R_T are similar to those observed by others (Cooke et al., 1983; Field and McColl, 1973; Hubel, 1982).

In experiments using BAC-treated animals, electrical


PD
(-mv)
3·0
2·8
2·6
2·4
2·2
2·0
1·8
1·6
I_{sc}
μA/cm²
56
52
48
44
40
36
0
R_T
ohms/cm²
140
130
120
110
100
90
0
0
10
20
30
40
50
60
TIME (min)

parameters were measured in the BAC-treated intestinal segment as well as an untreated control jejunal segment ≈ 4-8 cm orad to the BAC-treated area. (Preliminary experiments demonstrated that electrical parameters in control jejunal segments orad and caudad to the treated area were not significantly different from one another, so the orad segment was chosen as the control). R_T in BAC-treated tissue was 240 ± 31 ohms/cm^2 compared to only 122 ± 6 ohms/cm^2 in corresponding control jejunum (n = 6 pairs). This 2-fold increase in tissue resistance observed in the BAC-treated jejunum correlates well with the 2-fold increase in weight of the BAC-treated tissue reported previously (Fox and Bass, 1985).

PD and I_{sc} in BAC-treated tissues were much lower than corresponding control jejunal segments (figures 2 and 3, pre-drug values). In many cases these values approached zero over time. Possibilities for low PD and I_{sc} values include: (1) The increased thickness of BAC-treated jejunal muscle may hinder adequate oxygenation of the mucosa from the serosal side. This may cause the BAC-treated mucosa to deteriorate earlier than controls. (2) The increased muscle thickness of the BAC-treated jejunum may not allow adequate distribution of ions across the intestine to generate a potential difference.

The BAC-treated tissue was not responsive to either carbachol or norepinephine (figures 2 and 3, respectively), which supposedly

act directly on the enterocyte (Cooke, 1984; Field and McColl, 1973). As shown in figure 2, carbachol elicited a secretory response characterized by an increase in PD and I_{sc} in control jejunum only. Norepinephrine produced an antisecretory or absorptive response characterized by a decrease in PD and I_{sc} (figure 3). However, no effect was observed in the BAC-treated tissue.

BAC-treated jejunum did not exhibit the characteristic secretory responses produced by theophylline 1 mM added to the serosal side of the tissues, or 20 mM glucose added to both sides of the tissue.

In summary, values of PD, I_{sc} and R_T in BAC-treated jejunum were markedly different than control. In addition, BAC-treated jejunum was non-responsive to carbachol, norepinephrine, theophylline and glucose, stimulants which presumably act directly on the enterocyte. Because of these results, further experiments designed to explore the contribution of the myenteric plexus in mucosal transport using nerve stimulation of both control and BAC-treated jejunum would be futile. Therefore, this line of research has been abandoned until other techniques can be devised to destroy the myenteric plexus with minimal changes in tissue thickness.

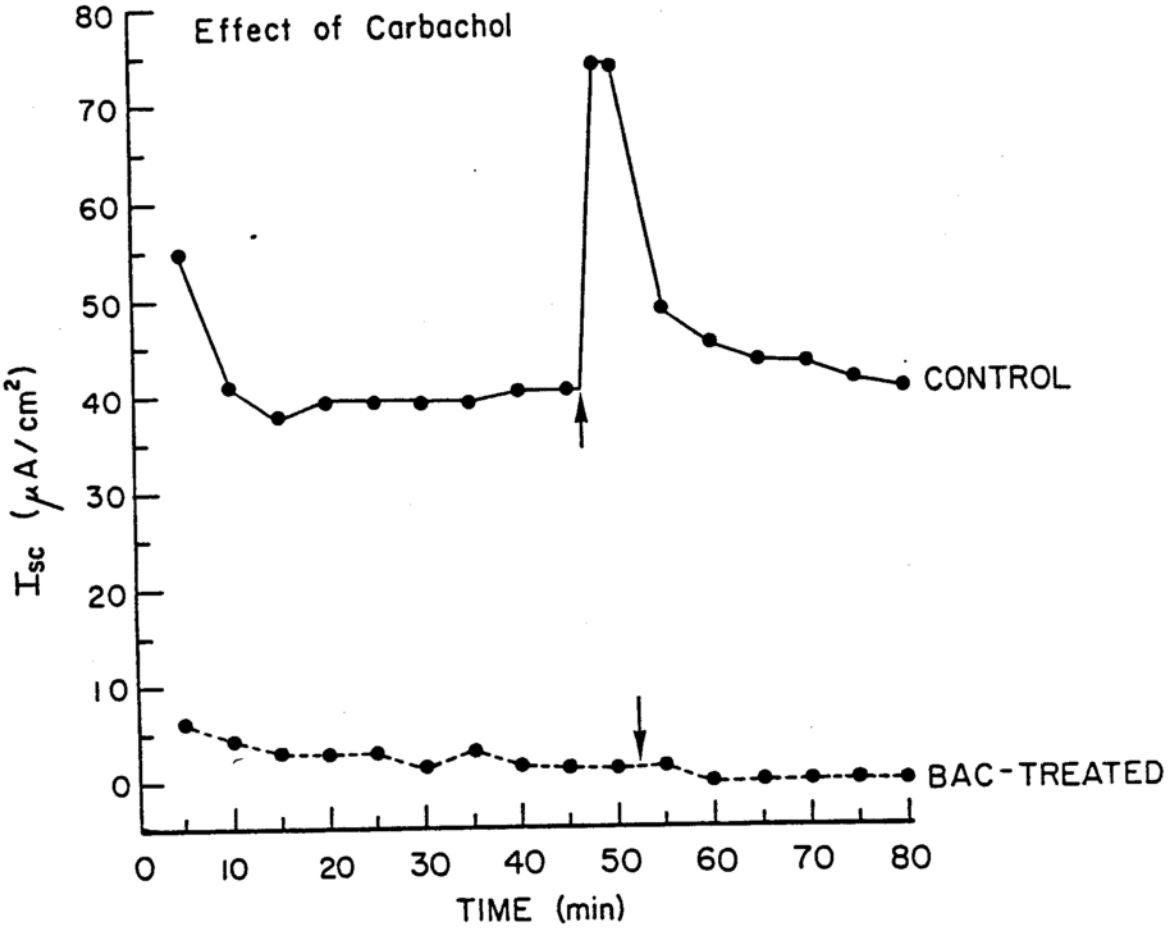


Fig. 2. A representative experiment of the effect of carbachol on short circuit current (I_{sc}) in control (•——•) and BAC-treated (•-----•) rat jejunum. Carbachol was added to the serosal side of the tissue at the times indicated by the arrows. PD values (not shown) paralleled those of I_{sc}. Note lack of response in BAC-treated jejunum.

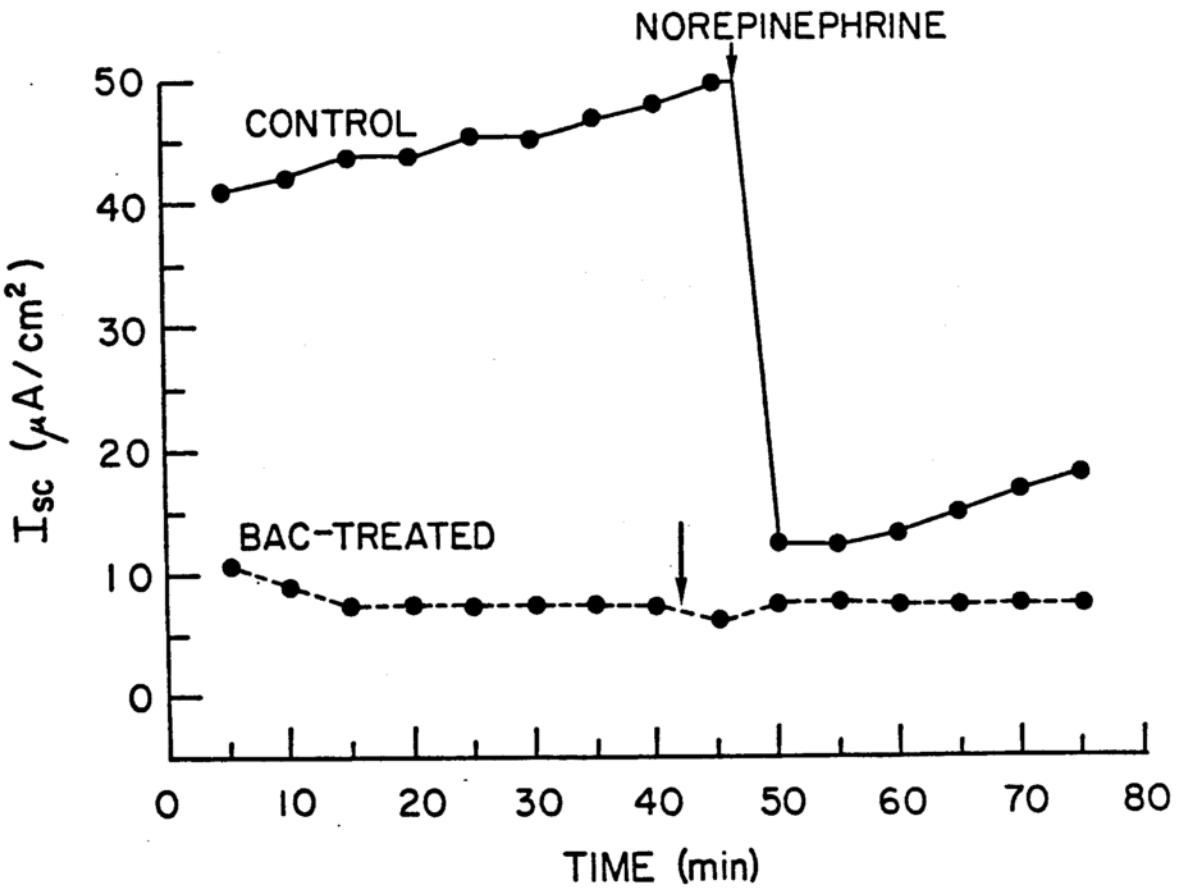


Fig. 3. Representative experiment of the effect of norepinephine on short circuit current (I_{sc}) in control (•——•) and BAC-treated (•-----•) rat jejunum. Norepinephine was added to the serosal side of the tissue at the times indicated by the arrows. PD values (not shown) paralleled those of I_{sc}. Note lack of response on BAC-treated jejunum.

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APPENDIX D

Effect of Exposing the Mucosa of the Rat Jejunum to Benzalkonium Chloride

Introduction

Serosal application of benzalkonium chloride (BAC) to the rat jejunum destroys the ganglion cells in the myenteric but not the submucosal plexus (Fox et al., 1983). Selectivity for myenteric neurons may be explained in part if one postulates that serosal application of BAC gains access to these neurons by an inward diffusion. To reach the myenteric plexus, BAC would have to traverse the serosa and longitudinal muscle. An additional barrier of the circular muscle would have to be overcome for BAC to reach the submucosal plexus. Considering these diffusional barriers, destruction of the submucosal plexus was attempted by exposing the mucosal surface of the jejunum to BAC. This route of administration would position the drug in closer proximity to the submucosal plexus.

Methods

Rats were anesthetized, and an abdominal midline incision was made. A section of jejunum was brought outside the peritoneal cavity, and a 3 cm segment was delinated by two serosal suture tags. The jejunal segment was clamped at the level of the two tags to occlude the lumen at these points. A 1% solution of BAC was injected via a syringe and a 27 gauge needle into the lumen of the jejunal segment between the two clamps. The solution was

allowed to remain in the intestinal segment for 30 minutes. After this period of time, the distal clamp was removed, and copious amounts of saline were injected into the lumen at the level of the remaining orad clamp to dilute and wash the BAC through the intestine. Following this treatment, the midline incision was closed and the animals were allowed to recover. After 20 days, both the treated segment and a control segment approximately 6 cm orad to the treated area were removed and processed for histological examination. The number of ganglion cells in both the myenteric and submucosal plexuses was counted and expressed per millimeter length of jejunum (see Fox et al., 1983, for details of histological processing and counting procedure).

Results and Discussion

At the light microscopic level, the morphology of the muscle and mucosa of the treated jejunum did not appear to be altered by exposing the mucosa to 1% BAC. Using this high surfactant concentration, one would have expected a disruption of the villi. However, since these tissues were examined 20 days after the initial insult, renewal of mucosal epithelial cells would have occurred in this period of time (Tier and Madara, 1981).

The number of ganglion cells in the myenteric and submucosal plexuses of the rat jejunum was not altered by exposing the mucosa to 1% BAC (Table 1). Previous studies (Fox et al., 1983) have

Table 1. Number of ganglion cells/mm in the myenteric and submucosal plexuses of the rat jejunum after exposing the mucosa to 1% BAC for 30 minutes.

	Mean Number of Ganglion Cells/mm Jejunum	
	Myenteric Plexus	Submucosal Plexus
Control	3.80 ± 0.32	3.18 ± 0.25
BAC-Treated	3.63 ± 0.27	3.26 ± 0.42

Numbers refer to the mean number of ganglion cells per millimeter of jejunum ± S.E.M. (n = 6).

BAC-treated and control samples were taken from the same group of animals.

shown that a concentration of BAC as low as 0.015% applied to the serosal surface of the intestine caused a significant reduction in the number of ganglion cells in the myenteric plexus. Serosal application of 1% BAC to the rat jejunum caused intestinal perforation and death of the animals within 2 days after treatment. In contrast, we have just shown that 1% BAC applied to the mucosal surface of the rat jejunum did not alter the number of ganglion cells in either plexus. The lack of a mucosal effect of BAC may be due to the presence of tight junctions found between both absorptive and crypt cells of the villi (Trier and Madara, 1981). Since the mucosal surface of the intestine is normally exposed to food and drugs, it is fortunate that these agents do not gain access to the intestinal nerves.

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