Physiology and Pharmacology of Rat Jejunal Smooth Muscle Following Chemical Ablation of Myenteric Neurons

by

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Abstract

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Under the supervision of Professor Paul Bass

Studies were undertaken to determine (1) the physiologic and pharmacologic responses of myenterically denervated rat jejunal longitudinal and circular smooth muscle and (2) the structure-activity relationship of individual alkyldimethylbenzylammonium chloride homologs which comprise the benzalkonium chloride mixture used to ablate myenteric neurons.

Active tension generation by longitudinal and circular muscle is initially impaired after denervation. Return of normal function occurs within seven days and is dependent on replacement of necrotic smooth muscle. Denervated longitudinal muscle is capable of generating greater active tension than control tissue 15 days after denervation, probably due to hyperplasia of this muscle layer. Increased resting tension and active stress generation by denervated

circular and longitudinal muscle also occur after ablation of myenteric neurons, suggesting that normal innervation is necessary for the maintenance of normal contractile mechanics of smooth muscle.

The responses to carbachol of longitudinal and circular muscle were altered after myenteric denervation. These alterations were apparently due to changes in the nicotinic (neuronal) component of the action of carbachol. Longitudinal muscle was less sensitive to carbachol apparently due to the loss of local excitatory innervation which contributes to the carbachol-induced contractile response. The muscarinic responses of control and denervated circular muscle were equal, indicating that the initial supersensitive response of denervated circular muscle to carbachol was due to the loss of nicotinically-mediated inhibition of contraction. With time, normal, then subsensitive responses of myenterically denervated circular muscle to carbachol were observed. These responses were apparently due to nicotinically-mediated inhibition of contraction by submucosal neurons.

The effect of alkyl chain length on myenteric neuron ablative activity of a series of alkyldimethylbenzylammonium chlorides was investigated. These studies indicate that the tetradecyl homolog, which comprises 50% of the commercial benzalkonium chloride mixture used to ablate myenteric

neurons, was the most effective ablative agent.

Approved: _____

Paul Bass
Professor of Pharmacology

Dedication

To Debora L. Herman, without whose love and support this thesis would not have been possible.

To Jeffrey, Jessica and Katherine, our pride and joy.

To my parents, Robert and Carolina Herman, who always encouraged me to pursue my academic goals.

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Introductory Review

Anatomy and Physiology of the Enteric Nervous System

Historical Perspective. The enteric nervous system consists of the neuronal and glial elements contained within the wall of the gastrointestinal tract. The presence of nerve cells in the gut was first reported by Remak in 1840. Later, a description of the ganglionated plexus located in the submucosa was provided by Meissner (1857). Auerbach (1862, 1864) first described in detail the ganglionated plexus found between the layers of external muscle. These early morphologic studies identified the major components of the enteric nervous system.

The enteric nervous system is part of the autonomic nervous system. Langley (1900, 1921) suggested that the enteric nervous system, the sympathetic and the parasympathetic nervous systems, be considered as the three components of the autonomic nervous system. He differentitated the enteric nervous system from the other two systems since 1) enteric ganglion cells were histologically different from other autonomic ganglion cells, 2) the connections of the enteric ganglion cells to the central nervous system (CNS) differ from those of other

peripheral ganglia to the CNS, and 3) complete reflex pathways existed within the enteric nervous system.

Components of the Enteric Nervous System. A schematic representation of the enteric nervous system is shown in Figure 1. The myenteric, or Auerbach's plexus, is a ganglionated plexus found between the outer longitudinal and inner circular smooth muscle layers. The deep muscular plexus is a collection of nerve fibers within the inner portion of the circular muscle. The primary source of these fibers is believed to be cell bodies located within the myenteric plexus (Wilson, et. al., 1987). However, submucosal neurons may project a few fibers to the deep muscular plexus (Ekblad, et. al., 1987). The submucosal, or Meissner's plexus is a ganglionated plexus located within the submucosal region of the gastrointestinal tract. Submucosal neurons are believed to provide the majority of fibers found in the periglandular and villus plexuses.

Cellular Morphology. As previously mentioned, the morphology of cells within the enteric nervous system differs considerably from that of other peripheral nervous elements.

Dogiel (1895, 1896, 1899) provided the earliest

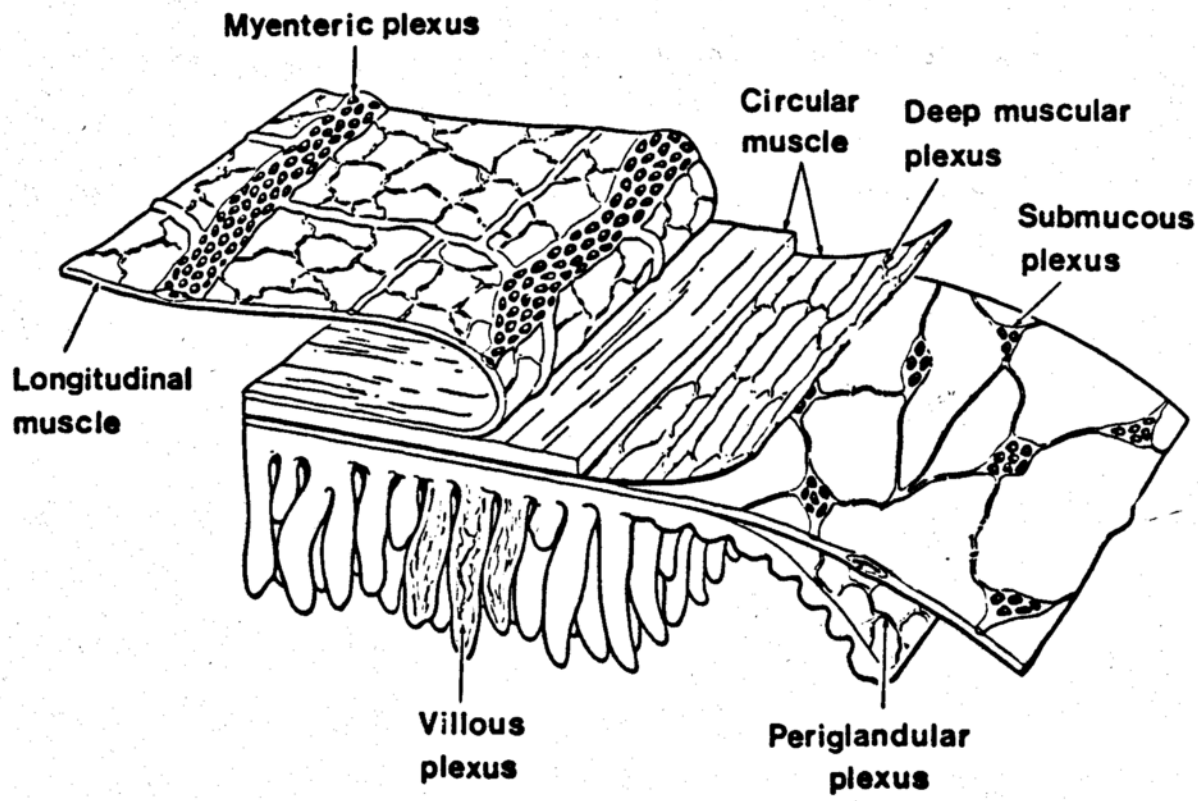

Figure 1. Diagrammatic representation of elements of the enteric nervous system. (Adapted from Costa et. al., 1987)

morphologic description of the types of enteric neurons. Based on methylene blue stained enteric ganglia from several species, he classified enteric neurons into three types. Type I cells had one long axon and many short irregular dendrites. Type II cells had a short axon with long dendrites. Type III cells had a long axon and intermediate length dendrites. This somewhat arbitrary classification scheme is widely used today.

Enteric glial cells exhibit cellular morphology which more closely resembles glia of the central rather than peripheral nervous system (Gabella, 1971, 1981; Komuro, et. al., 1982). In addition, enteric glial cells contain both S-100 protein (Ferri, et. al., 1982) and glial fibrillary acidic protein (Jessen and Mirsky, 1980; Bjorklund, et. al., 1984), two proteins found in astrocytes in the CNS but not in peripheral Schwann cells. Kumoro et. al. (1982) have presented a classification scheme which describes three types of enteric glia based on size and presence of glial filaments.

Enteric Transmitter Neurochemistry. (for review, see Fox, 1985; Furness and Costa, 1987). A large number of chemicals have been identified or implied to be neurotransmitters in the enteric nervous system. A list of these chemicals is presented in Table 1. Inclusion of a candidate transmitter on the list is based on localization

Table 1

Known and Putative Enteric Neurotransmitters

acetylcholine (ACh)

angiotensin

calcitonin gene related peptide (CGRP)

cholecystokinin (CCK)

dynorphin (DYN)

enkephalin (methionine and leucine) (ENK)

galanin

gamma-aminobutyric acid (GABA)

gastrin releasing peptide (GRP)

5-hydroxytryptamine (5-HT)

neuropeptide Y (NPY)

neurotensin

norepinephrine

peptide HI

somatostatin (SOM)

Substance P (SP)

vasoactive intestinal peptide (VIP)

(adapted from Furness and Costa, 1987)

(immunohistochemical and/or biochemical) within enteric neurons, and/or production of physiologic responses which mimic neuronal stimulation after exogenous application of the chemical.

Burnstock (1976) provided evidence for the existence of multiple neurotransmitters within a single enteric neuron. Recent studies (Schultzberg et. al., 1980; Ekblad et. al., 1984; Legay et. al., 1984; Furness et. al., 1984, Furness et. al., 1985; Costa et. al., 1985; Costa et. al., 1986) indicate that this is the rule rather than the exception for enteric neurotransmitters. Table 2 lists the known transmitter colocalization combinations in myenteric and submucosal neurons.

Neuronal Circuitry. (for review, see Furness and Costa, 1987). Early morphological studies on enteric neuronal projections (Dogiel, 1899; Kuntz, 1913, 1922; Hill, 1927) were hampered by techniques which were variable with respect to staining of cell bodies and processes. With variations in staining, it proved difficult to trace neuronal projections with certainty.

Antisera raised against neurotransmitters of the enteric nervous sytem, which have become available within the last ten years, have provided powerful tools to assess enteric neuronal projections. Through the combined use of immunocytochemistry, and surgical lesioning techniques first

Table 2

Neurochemical Colocalization in Enteric Neurons[1]

Plexus	Neurochemical definition
myenteric	DYN/ENK/VIP/NPY/GRP
	DYN/VIP/GRP/CCK/ENK
	DYN/VIP/GRP/CCK/SP
	SOM/SP/CCK
	VIP/DYN
	ACh/CGRP/CCK/NPY/SOM
	DYN/ENK/GRP/VIP ± NPY
	SP/ENK ± DYN
	DYN/GRP/VIP ± NPY
submucosal	DYN/VIP
	ACh/CCK/CGRP/NPY/SOM
	ACh/SP

[1]see Table 1 for abbreviations

employed by Temereskasi (1955), various investigators have been able to trace the neuronal projections of immunohistochemically defined neurons within the enteric nervous system. An exhaustive account of these studies will not be presented here (see Costa et. al., 1986 for detailed review), but the results can be summarized as follows. In general, the majority of neurons in the myenteric plexus project circumferentially or caudally into the musculature and to the submucosal plexus. A few fibers from myenteric neurons also project into the mucosa. The majority of the submucosal neurons appear to project orally into the submucosa or mucosa. There is limited evidence that some submucosal neurons may project into the circular muscle (Ekblad et. al., 1987). These results, in combination with those from the electrophysiological studies reviewed below, have provided valuable insight into enteric neuronal circuitry and control of intestinal function.

It was known from the early studies of intestinal motility performed by Bayliss and Starling (1899, 1901) that there is polarity of enteric neuronal circuitry. These authors found that a bolus placed in the lumen of the small intestine always moved caudally, never orally. They observed excitation (muscle contraction) orad to the bolus and inhibition (muscle relaxation) caudad to the bolus. These responses could also be elicited by gentle pinching of

the musculature. This lead Bayliss and Starling to formulate the "law of the intestine", namely that "excitation at any point of the gut excites contraction above, inhibition below" (Bayliss and Starling, 1899). It was also noted that sectioning of extrinsic nerves had no effect on these reflexes, but local application of cocaine, nicotine or muscarine abolished the responses. From these observations, Bayliss and Starling correctly concluded that the reflexes were mediated by nerves intrinsic to the gastrointestinal tract.

Costa and Furness (1976) performed the most recent detailed analyses of the "law of the intestine". They confirmed the "law", and found that tetrodotoxin or removal of the myenteric plexus abolished the ascending and descending responses. Through the use of specific antagonists, they also found that the ascending excitatory pathways apparently involve neurons which release 5-hydroxytryptamine and acetylcholine, and that the descending inhibition was apparently mediated by non-adrenergic, non-cholinergic neurons.

A number of electrophysiological studies (Hirst and McKirdy, 1974; Hirst et. al., 1975; Yokoyam and Ozaki, 1978; Ozaki, 1979; Bornstein et. al., 1986) have also demonstrated the polarity of enteric neuronal pathways. These authors have used intracellular microelectrodes to stimulate

myenteric neurons and record muscle responses orad and caudad to the site of stimulation. In general, stimulation of myenteric neurons produces excitatory junctional potentials in muscle orad to the site of stimulation and inhibitory junctional potentials in muscle caudad to the site of stimulation.

A combination of immunohistochemical, electrophysiological and pharmacological techniques should provide further insight into enteric neuronal circuitry and allow a better understanding of the control of gastrointestinal function by the enteric nervous system.

The Enteric Nervous System and Intestinal Motility

Coordinated movement of the small intestine is necessary for mixing, absorption and net caudal transport of luminal contents. The role of the enteric nervous system in intestinal motility has been reviewed elsewhere (Fox, 1985; Furness and Costa, 1987). It is generally believed that neurons located in the myenteric plexus are responsible for the control of longitudinal muscle function (Fox and Bass, 1984; Wood, 1987). Motor control of circular muscle is also generally attributed to neurons within the myenteric plexus (Wood, 1987). Both inhibitory and excitatory junctional

potentials have been recorded in circular muscle after stimulation of myenteric neurons (Hirst et. al., 1975; Bornstein et. al., 1986). Surgical removal of longitudinal muscle with attached myenteric plexus in the guinea pig resulted in the loss of nerve fibers in the circular muscle (Furness and Costa, 1979; Wilson et. al., 1987) as well as the loss of the response of circular muscle to nerve selective electrical stimulation (Bornstein et. al., 1986).

There is, however, physiologic and morphologic evidence which suggests a direct role for submucosal neurons in the motor control of circular muscle. Electrical field stimulation of submucosal neurons produced inhibitory junctional potentials in canine colonic circular muscle in the presence or absence of the myenteric plexus. Microinjection of acetylcholine onto submucosal neurons mimicked the effect of electrical field stimulation (Sanders and Smith, 1986). Surgical lesioning studies in rat intestine demonstrated that most, but not all nerve fibers disappeared from circular muscle after removal of longitudinal muscle with attached myenteric plexus. The authors suggested that the source of the remaining fibers may be neuronal cell bodies located in the submucosal plexus (Ekblad et. al., 1987). Thus, the possibility that submucosal nerves can directly affect circular muscle functions has some experimental support.

Ablation of Enteric Neurons

One approach to the study of enteric nervous control of intestinal function is the removal, either acutely or chronically, of the influence of such neurons. Through a comparison of intestinal function in the presence and absence of enteric neurons, one can draw inferences about the role of such neurons. To this end, many techniques have been utilized to abolish the action of or ablate enteric neurons. These methods have been reviewed in detail by Fox (1985) in her thesis introduction.

The surgical (Magnus, 1904; Gunn and Underhill, 1914; Alvarez and Mahoney, 1922; Evans and Schild, 1953; Paton and Zar, 1965), physical (Ambache, 1946; Innes et. al., 1957; Hukuhara et. al., 1961; Day and Vane, 1963; Ochillo et. al., 1978), chemical (Deloyers et. al., 1957; McElhannon, 1959; Frantzides et. al., 1987) and pharmacological methods (Bayliss and Starling, 1899; Gershon, 1967) all have disadvantages which limit their usefulness in the study of the enteric nervous control of intestinal function. Surgical methods are generally useful for acute, <u>in vitro</u> experiments. In addition, traumatization of tissue by surgery may confound the interpretation of results. The physical, chemical and pharmacological techniques employed

may be technically difficult to perform, produce variable degrees of ablation, and most importantly, lack selectivity for specific enteric plexuses.

Sato et. al. (1978) demonstrated that serosal application of the cationic surfactant benzalkonium chloride to the anorectum of the rat destroyed "intramural nerve elements" without affecting the remainder of the bowel. This method provided the basis for the development of a selective myenterically denervated model of rat jejunum by Fox et. al. (1983).

Myenterically Denervated Rat Jejunal Model

Fox et. al. (1983) demonstrated that a variety of surfactants (cationic, anionic or non-ionic), when applied serosally, were capable of selectively ablating neurons of the myenteric plexus in the rat jejunum. Of the surfactants examined, the cationic surfactant benzalkonium chloride (BAC) was found to be the most potent ablative agent. BAC, in a concentration-dependent manner, reduced the number of neurons in the myenteric plexus without affecting the number of neurons in the submucosal plexus. Immumohistochemical examination of BAC-treated jejunum indicated a loss of immunoreactivity to vasoactive intestinal peptide, somatostatin, met-enkephalin and substance P in the

myenteric region. The only other remarkable histologic change noted was a thickening of both longitudinal and circular muscle layers.

Mucosal exposure to BAC at a 500-fold higher concentration than is effective serosally had no effect on the number of enteric neurons (Fox (1985), Appendix D).

See et. al. (1988) examined in detail the thickening of intestinal muscle following myenteric neuron ablation with BAC. Through the use of light and electron microscopic techniques, these investigators found that initially after BAC treatment virtually all of the longitudinal and a portion of the circular muscle was necrotic. Within 24 hours of BAC treatment, the remaining smooth muscle cells began cell division, as evidenced by ^{3}H-thymidine uptake and the presence of mitotic figures. Both muscle layers exhibited normal thicknesses and cell numbers five days after treatment. Cell division continued, and the muscle layers exhibited significant thickening, which was maximum 15 days posttreatment, due to hyperplasia of smooth muscle. The hyperplasia of circular muscle gradually declined until, by day 45 posttreatment, the number of cells in the circular muscle was comparable to that in naive tissue. The number of smooth muscle cells in BAC-treated longitudinal muscle remained elevated, even 165 days after treatment.

The contractile capability of longitudinal smooth

muscle following myenteric denervation with BAC was examined by Fox and Bass (1986a). BAC-treated tissues failed to contract in response to ganglionic stimulants (nicotine, 1,1-dimethyl-4-phenylpiperazinium iodide or McN-A-343), physotigmine, or short-pulse nerve selective electrical stimulation. However, long-pulse electrical stimulation, carbachol and barium chloride initiated contractile activity in BAC-treated longitudinal muscle which was not significantly different from control tissue. From these results, the authors concluded that 1) motor neurons innervating longitudinal muscle are located in the myenteric pus, and 2) myeeric plexus ablation by serosal application of BAC does not alter longitudinal muscle contractility. While the first conclusion appears true, the second may not be entirely valid given data presented in Chapters 1 and 2 of this thesis. Nonetheless, smooth muscle is capable of responding to pharmacological and electrical stimuli following myenteric plexus ablation with BAC.

The myenteric denervation produced by serosal application of BAC has several advantages over previous attempts of enteric denervation. First, because the number of submucosal neurons is unaffected by treatment, the role of the myenteric plexus in intestinal function can be evaluated. Second, both <u>in vivo</u> and <u>in vitro</u> acute or chronic studies can be performed. Finally, the technique is

simple, inexpensive, reproducible and applicable to small experimental animals.

The myenterically denervated model has some limitations. The thickened smooth muscle may prevent the use of the tissue in certain experiments. Fox (1985, Appendix C) believed that the thickened muscle was responsible for the lack of tissue response in experiments with the Ussing chamber. Changes in length-stress properties of the smooth muscle (Chapter 1), if not taken into account, may confound pharmacological experiments. Recent evidence (N. See, unpublished observations) suggest that in addition to ablation of myenteric neurons, extrinsic nerve fibers in treated jejunum may also be ablated. These limitations, however, do not preclude the use of the model in the study of the enteric nervous system and intestinal function.

Fox and Bass (1984) used the myenterically denervated jejunal model to ascertain the role of the myenteric plexus in the control of the basic electric rhythm (BER) and migrating myoelectric complex (MMC), two stereotypic forms of electrical activity of the gastrointestinal tract. The authors found that in the denervated segment, the BER pattern was erratic, and the amplitude of the BER greatly attenuated. The spiking activity of the MMC was slightly altered, but the MMC appeared to propagate through the

denervated segment. From these results, it was concluded that myenteric neurons may play a modulatory role in the generation and propagation of the BER, but submucosal neurons or humoral factors may be more important in the control of the MMC.

By examining the isometric contractile responses of control and myenterically denervated longitudinal muscle, Fox et. al (1986) were able to determine the site(s) of action of several putative enteric neurotransmitters. Their results indicated that substance P and norepinephrine produced their mechanical effects by acting predominantly on longitudinal muscle. In contrast, 5-hydroxytryptamine, cholecystokinin-octapeptide, adenosine triphosphate, vasoactive intestinal peptide and neurotensin produced their mechanical effects on longitudinal muscle indirectly via the myenteric plexus. Acetylcholine was found to have both direct and myenteric plexus mediated actions on longitudinal muscle.

A similar study (Fox and Bass, 1986b) was performed with control and myenterically denervated rat jejunum to determine the location of alpha and beta adrenergic receptors which mediate mechanical responses of longitudinal muscle. Through the use of selective agonists and antagonists, the authors were able to demonstrate that 1) beta receptors which mediate relaxation are located on

longitudinal muscle, 2) alpha-1 receptors also mediate relaxation but are located on both smooth muscle and myenteric neurons, and 3) alpha-2 receptors are located on myenteric neurons and mediate contraction of rat jejunal longitudinal muscle.

The effect of myenteric denervation on the levels of neurotransmitters or synthetic enzyme activity was investigated by Dahl and coworkers (1987). Within two days of treatment, reductions in choline acetyltransferase (ChAT) activity and vasoactive intestinal peptide (VIP) were observed. In contrast, there was no reduction in the concentration of leucine-enkephalin (leu-ENK). By 15 days after treatment, the ChAT activity and VIP levels were comparable to control, while 45 days after treatment, ChAT acitivity, VIP and leu-ENK levels were all at least twice control values. These results suggest a compensatory increase in transmitter production by submucosal neurons following myenteric denervation, and that the myenteric neurons normally inhibit transmitter production by submucosal neurons.

Pathophysiology of Myenteric Denervation

A number of human diseases, such as Hirschsprung's disease and achalasia, are associated with lesions of the

myenteric plexus. Krishnamurthy and Schuffler (1987) have reviewed in detail many of these conditions. One variant of Hirschsprung's disease that these authors did not fully address is that of zonal aganglionosis or Hirschsprung's disease with skip lesions. Consideration of this condition is warranted here because the myenterically denervated model described by Fox et. al. (1983) may prove to be an excellent experimental model of zonal aganglionosis since the length of denervated bowel can be easily manipulated.

Classic Hirschsprung's disease is a congenital defect characterized by the absence of myenteric and submucosal ganglia in the terminal colon. The extent of aganglionosis is limited to the rectum and sigmoid colon in 80 - 90 % of all cases, but occasionally may include the entire large and portions of the small intestine (Sprinz et. al., 1961; Howard, 1972). The patients generally present with intestinal obstruction, which can be life threatening. Often, surgical removal of the affected area is necessary.

A number of reports have appeared in the literature in which patients present with symptoms of classic Hirschsprung's disease, but the area of aganglionosis is localized (Tiffin et. al., 1940; Sprinz et. al., 1961; MacIver and Whitehead, 1972; Kadair et. al., 1977; Martin et. al., 1979; de Chadarevian et. al., 1982; Taguchi et. al., 1983; Seldenrijk et. al., 1986). The earliest such

report was probably that of Tittel (1901). He observed no ganglia in the jejunum, normal ganglia in the ileum and only sparse ganglia in the colon of a 15 month old infant. He speculated that these abnormalities of the myenteric plexus may alter peristaltic activity. Other authors have found regions of normal ganglia which were interrupted by zones of aganglionosis.

The study of intestinal function associated with zonal aganglionosis has been limited by the lack of an appropriate experimental model. As Tiffin et. al. pointed out in 1940,

> "The nature of the disturbance of control of intestinal motility...from localized absence of...the myenteric plexus is difficult to discover...We have found no experimental work which is accurately comparable to the condition under consideration."

The myenterically denervated rat model developed by Fox et. al. (1983) may be useful in the study of intestinal zonal aganglionosis.

The existence of skip lesions of myenteric neurons casts some doubt on the generally accepted etiologies of Hirschsprung's disease. Okamoto and Ueda (1967) proposed that aganglionosis of the terminal colon was due to incomplete caudal migration of neuroblasts to this region of

the colon during development. Rothman and Gershon (1984) claim that neural crest cells can migrate normally, but fail to enter the terminal colon due to abnormalities of the microenvironment of the embryonic tissue. Since the majority of neural crest cells which migrate into the intestine enter at the esophagus and migrate caudally, the proposed etiologies mentioned above could not account for the presence of zonal aganglionic variants.

Recently, another theory on the cause of Hirschsprung's disease which could explain the existence of zonal variants has been proposed (Taguchi et. al., 1985). These investigators found fibromuscular dysplasia of mesenteric arteries in 8 of 25 children with Hirschsprung's disease. These authors speculate that ischemia of the bowel during development may be responsible for both the fibromuscular dysplasia and the absence of enteric ganglia in these patients. If true, localized ischemia during development could account for the observed cases of zonal aganglionosis. This proposal deserves further consideration.

Smooth Muscle

The structure and physiology of smooth muscle differs considerably from that of skeletal muscle. Because the gastrointestinal tract contains three layers of smooth

muscle, a consideration of the structure and contractile properties of smooth muscle is in order.

Cellular characteristics. (for review, see Gabella, 1987). Smooth muscle cells are small spindle-shaped cells approximately 500 to 700 microns long. These cells have a high surface-to-volume ratio (approximately 1.5 square microns per cubic micron) and the presence of caveolae, or membrane invaginations, further increases the surface available for exchange with the extracellular space.

Like skeletal muscle, smooth muscle contains both actin and myosin. However, the ratio of actin to myosin in smooth muscle is several times higher than that in skeletal muscle, primarily due to the low content of myosin in smooth muscle (Murphy et. al., 1974; Tregear and Squire, 1973). Smooth muscle does not contain troponin C, but does contain more intermediate filaments than does skeletal muscle. In smooth muscle, these filaments, along with actin and myosin, make up two distinct fibrillar domains (Small, 1985; Small et. al., 1986; Furst et. al., 1986). One domain is made up of caldesmon, tropomyosin, actin and myosin and forms longitudinal arrays within the cells. The other domain, the F-A-D domain, consists of filamin, actin and desmin, whose longitudinal arrays are anchored at the surface of the cells in dense plaques. These dense plaques, which are rich in vinculin, plectin and filamen, and are in turn linked to

dense bodies rich in alpha-actinin.

Tissue structure of intestinal smooth muscle. Smooth muscle cells in the small intestine are oriented in three distinct muscle layers. The outer longitudinal and inner circular muscle layers make up the muscularis externa. The muscularis mucosae is a thin, longitudinally oriented layer of smooth muscle located between the submucosa and mucosa. The structure of each of these layers is similar. Muscle cells lie approximately parallel to each other and are surrounded by basal lamina. The cells appear to be linked by collagen and elastin, two components of the extracellular matrix. It appears that microfibrils connect the basal lamina of the smooth muscle to collagen fibrils (Gabella, 1984). These connections are most obvious at the sites of dense bands on the cell surface. It has been suggested that the extracellular connective tissue constitutes a "tendon" that allows the transmission of force generated by contracting muscle cells.

There is another junctional specialization that links smooth muscle cells in the small intestine. The nexus, or gap junction, is a small oval-shaped area which lies in close apposition to an identical area on an adjacent cell. These areas of membrane are linked by transcellular protein subunits which provide channels for ion exchange. Thus, the smooth muscle cells are metabolically and electrically

coupled. In most species, gap junctions are found only in circular muscle. However, gap junctions have been observed in both muscle layers in the cat small intestine.

Neuromuscular transmission. (see Furness and Costa, 1987, for review). In contrast to skeletal muscle, there are no specialized junctional regions for neuromuscular transmission in intestinal smooth muscle. In fact, in the rat and guinea pig, longitudinal muscle is devoid of nerve fibers. Neurotransmission to longitudinal muscle in these species is believed to be due to diffusion of neurotransmitters from nerve fibers located at the surface of myenteric ganglia. In circular muscle (and longitudinal muscle of larger species) nerve axons run parallel to smooth muscle cells and exhibit varicosities from which neurotransmitters are released. The transmitters then diffuse the 20 to 40 nm to exert actions on the smooth muscle cells.

Smooth Muscle Contraction

General characteristics. Contraction of smooth muscle, like that of skeletal muscle, is initiated by an increase in intracellular free calcium. Activated smooth muscle, in contrast to skeletal muscle, can maintain tension with little oxygen and adenosine triphosphate utilization (Paul

and Peterson, 1975; Krisanda and Paul, 1984). High levels of intracellular free calcium are not required for tension maintenance by smooth muscle (Morgan and Morgan, 1984). The contractile characteristics, along with the unique structural features already mentioned (pp. 21 - 24), imply that the mechanism of contraction of smooth muscle is markedly different from that of skeletal muscle.

Contractile mechanism. The molecular mechanism by which smooth muscle contracts was initially thought to be similar to the sliding filament mechanism proposed for skeletal muscle (Huxley, 1957; Huxley, 1974). While actin-myosin cross bridge formation and cycling are important in smooth muscle contraction, the sliding filament theory cannot account for the morphological, biochemical and physiological properties of smooth muscle contraction.

Initiation and maintenance of contraction in smooth muscle are considerably different than in skeletal muscle. In skeletal muscle, membrane depolarization and release of calcium from the sarcoplasmic reticulum initiate contraction. The muscle maintains contraction as long as intracellular free calcium remains elevated and adenosine triphosphate is available. These conditions are presumably necessary for continued cross bridge cycling.

The initiation of contraction in smooth muscle is considerably more complex (Dillon et. al., 1981; Kamm and

Stull, 1985). A flow diagram of the process is presented in Figure 2. After an appropriate stimulus elevates the intracellular concentration of calcium (Ca), calcium binds with calmodulin (CaM). The calcium-calmodulin complex then activates myosin light chain kinase (MLCK). The activated form of MLCK then phosphorylates two 20 kD light chains of myosin. This phosphorylation allows the interaction of actin and myosin, which activates myosin ATPase activity , and ultimately leads to cross bridge cycling and contraction. When myosin is dephosphorylated (by a specific phosphatase), ATPase activity is lost, actin dissociates from myosin and relaxation occurs.

If this mechanism is to account for the sustained phase of smooth muscle contraction, then it must be assumed that the intracellular level of free calcium remains high and myosin light chain remains phosphorylated. Neither of these assumptions has proven to be correct (Morgan and Morgan, 1984; Silver and Stull, 1984; Askoy et. al, 1983). This lead to the formulation of the "latch bridge hypothesis" by Dillon et. al. (1981). In this theory initiation of contraction is as shown in Figure 2, but the authors propose that there is a change in the nature of the cross bridges

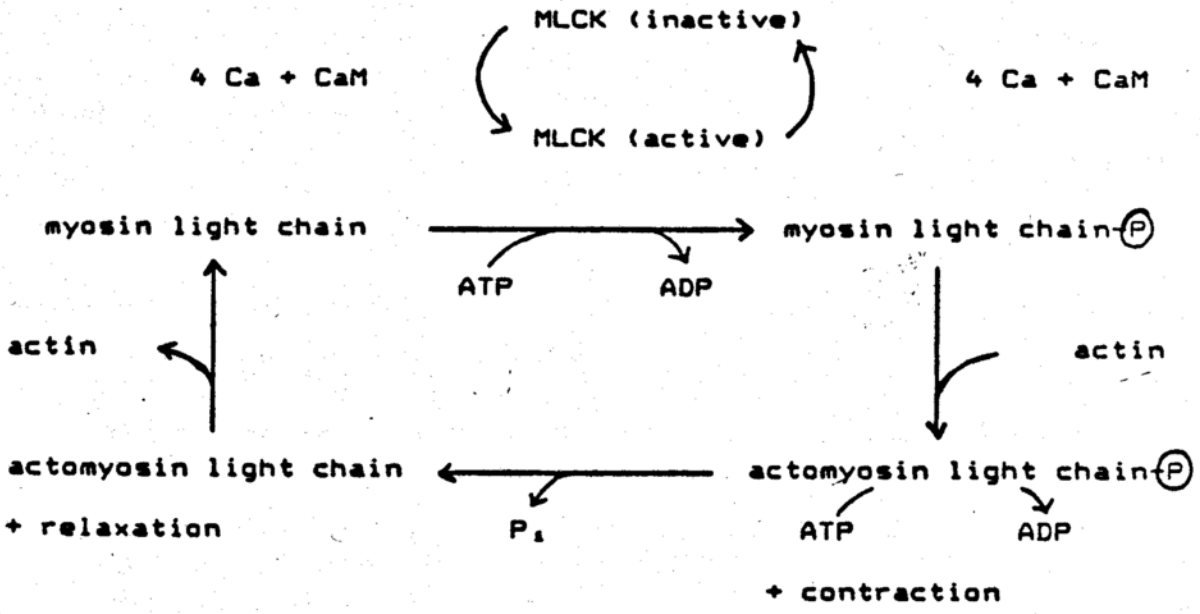

Figure 2. Initiation of smooth muscle contraction. See text for details.

after myosin is dephosphorylated. This new, long-lasting form of actin-myosin bridge is what is referred to as the latch bridge. Cycling of this cross bridge is slow and exquisitely sensitive to calcium, hence contraction is maintained in the absence of high calcium with little energy expenditure.

Recently, the latch bridge hypothesis has come under attack by Rasmussen and coworkers (1987). As these authors point out, there is no direct evidence for the existence of latch bridges. Also, it is possible to induce slow developing, sustained contractions without myosin light chain phosphorylation (Park and Rasmussen, 1985). Finally, the latch hypothesis ignores potential contributions of intermediate filaments.

As an alternative to the latch bridge hypothesis, Rasmussen et. al. (1987) propose a two-phase (cooperative) model of smooth muscle contraction. In this model, initiation of contraction is the result of the activation of myosin light chain kinase (Figure 2). The maintenance of contraction, according to these authors, is due to activation of C-kinase and resulting phosphorylation of intermediate filament proteins. By restructuring the domains of the intermediate filaments (primarily the F-A-D domain, p. 22) in a process called solation, the length of individual cells can be altered, and tension maintained in

accord with the known energetics of the process. Although this is an attractive hypothesis, there is currently little evidence in support of this model.

Static mechanical properties. (Murphy, 1976; Murphy, 1980; Paul, 1987). Although it contains only about 10 -20 % of the myosin found in skeletal muscle, smooth muscle can generate as much or more isometric force per unit area as can skeletal muscle. The amount of isometric force generated by smooth muscle varies with the length of the muscle. The relationship between force and muscle length has been well studied in smooth and skeletal muscle, and was critical in the genesis of the sliding filament theory of skeletal muscle contractions (Gordon et. al., 1966).

A simplified model for the mechanical properties of smooth muscle is shown in Figure 3. The first element of the model is the contractile element, or smooth muscle proper, which behaves as a simple viscous element in the resting state and offers little resistance to stretch. When stimulated, the contractile element can shorten, or develop force if constrained. The second element, the series elastic element, estimates the compliance of the contractile element, and is necessary since there is some shortening of the contractile element even under isometric conditions. This component is in series with the third component, the parallel elastic element, which represents the elastic

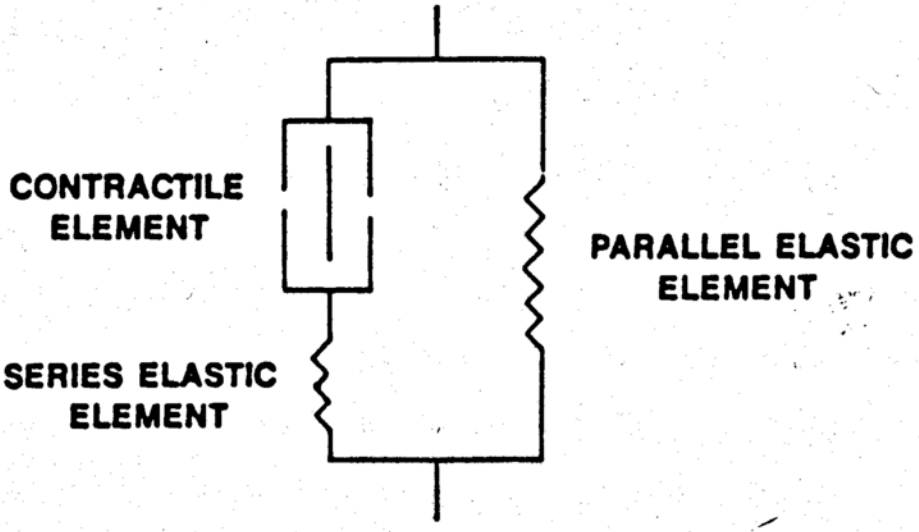

Figure 3. Model for the mechanical properties of smooth muscle. See text for details.

component of inactivated muscle. The extracellular matrix components collagen and elastin probably account for the majority of the parallel elastic element. In contrast to what the name implies, the parallel elastic element is relatively inelastic.

Smooth muscle is capable of generating force over a wide range of lengths. An idealized length-tension diagram is shown in Figure 4. Some investigators prefer comparing the length-stress (force/area) properties of smooth muscle since differences in cross sectional area can be normalized. Total force is the sum of active (due to the contractile element) and passive (due primarily to the parallel elastic element) force. At short lengths, total force and active force are virtually identical. As the muscle is stretched toward its optimum length for active tension development (L_o), passive force increases. Beyond L_o, active force generation declines, due to less efficient actin-myosin interaction, but total force continues to increase due to increases in passive force. The increase in passive force is the result of attempting to stretch the elements of the extracellular matrix.

Knowledge of the length-tension relationship, in addition to providing information about contractile filament interactions, is also necessary for the proper conduct of

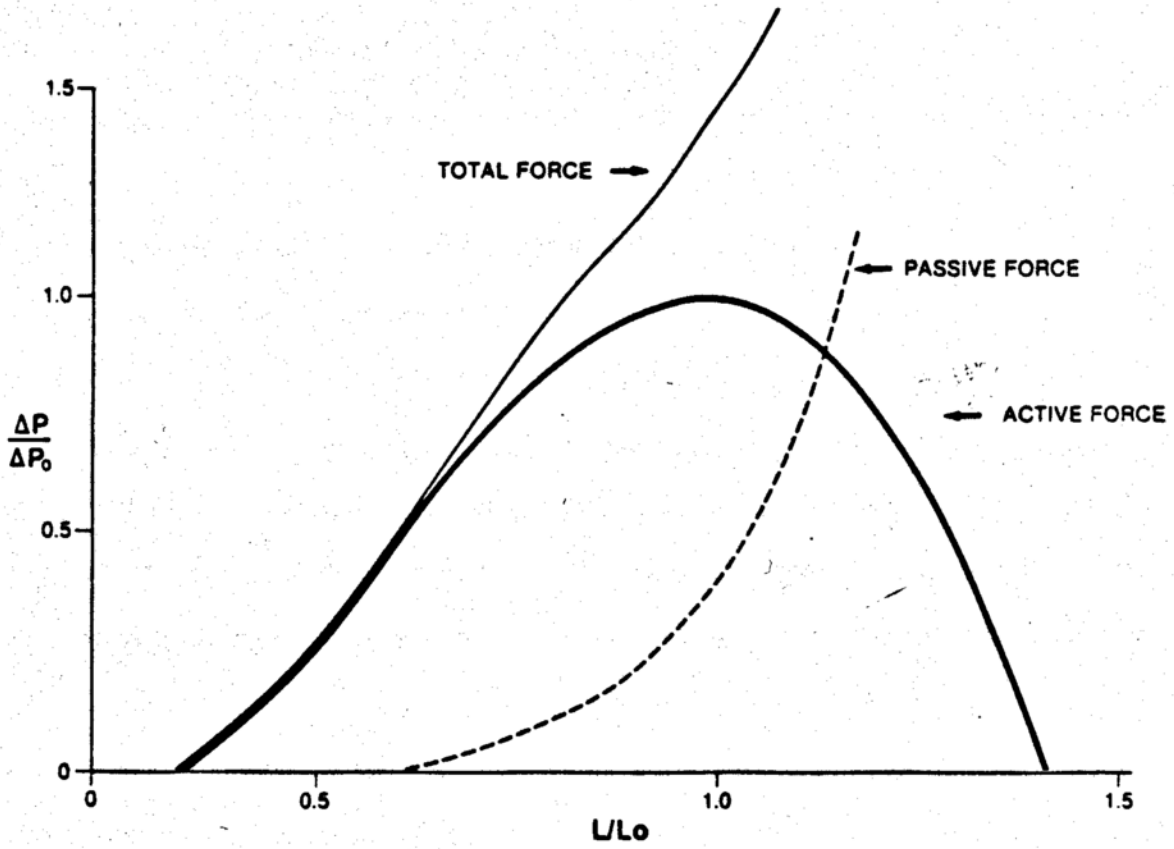

Figure 4. Relationship between isometric force and muscle length. Ordinate: isometric force normalized to maximum active isometric force. Abscissa: tissue length normalized to optimal length for tension development. See text for details.

pharmacologic studies with smoothmuscle. A number of investigators (Tallarida et. al., 1974; Price et. al., 1981; Price et. al., 1983; Foley, 1986; Herlihy and Berado, 1986; Coskinas and Price, 1987) demonstrated that the pharmacologic sensitivity of smooth muscle is length dependent. It was shown that muscles which were not at L_o (either shorter or longer) were less sensitive to pharmacologic stimulation than muscles set at L_o. Presumably, this phenomenon is the result of less than optimum interactions between contractile elements. These studies point out the need to examine the length-tension properties of smooth muscle following experimental manipulations (denervation, toxicant exposure, surgery) which may alter smooth muscle structure or function.

Mechanical properties of denervated smooth muscle. Denervation or decentralization of the rat urinary bladder results in hypertrophy (Ekstrom and Uvelius, 1983) and hyperplasia (Ekstrom et. al., 1984) of bladder smooth muscle. Ekstrom and Ulevius (1981) examined the length-tension properties and maximal force output of the denervated bladder. Maximal force output at L_o of denervated muscle was not significantly different from that of innervated muscle. When tension was plotted as a function of length divided by <u>in situ</u> length, the length-tension curve of denervated muscle was shifted to the right

of the control curve. However, when tension was plotted as a function of length divided by L_o, there were no differences between control and denervated tissues. The passive tension at L_o of denervated smooth muscle was twice that of control tissue. This finding suggests that there were changes in tissue structure which affect the parallel and/or series elastic elements of denervated bladder smooth muscle. The authors suggest that changes in the orientation of the smooth muscle cells or increases in collagen and elastin may account for the increase in passive tension observed following denervation. Ekstrom et. al. (1982) reported similar changes in the decentralized urinary bladder of the rat.

Alterations in contractile mechanics of smooth muscle have also been observed following surgical interruption of normal innervation. Seidel et. al. (1984) have demonstrated that venous smooth muscle exhibits decreased force per cross sectional area following venous autograft or arterial graft. Interestingly, the changes observed in the autograft were reversible while those in the arterial graft were not. The reestablishment of normal innervation in the autograft, if it occurred, may account for the return of normal contractile activity.

Weisbrodt et. al. (1985) have demonstrated that transection and reanastomosis of the rat small intestine

results in significant thickening of both longitudinal and circular muscle layers. In addition, the ability of operated circular muscle to generate active stress (force/area) was significantly greater than that of unoperated muscle. Increases in contractile protein content per cell or rearrangement of circular muscle cells were believed to account for the observed mechanical changes.

While tissues examined by Seidel et. al. (1984) and Weisbrodt et. al. (1985) were not classically denervated, interruption of normal innervation may have had a role in producing the changes observed in smooth muscle mechanical properties.

One additional study which demonstrated changes in the length-tension properties of intestinal longitudinal muscle deserves mention. Vermillion and Collins (1988) reported that longitudinal muscle from rats infected with Trichinella spiralis exhibited the ability to generate more active tension at shorter tissue lengths than did control tissue. These changes were noted early after infection and were reversible by 23 days after infection. It is not known, however, if T. spiralis infection alters enteric nervous system function.

Pharmacologic responses of denervated smooth muscle. Altered responsiveness of denervated smooth muscle to exogenously applied pharmacologic agents was first observed

by Anderson in 1904. Application of eserine to denervated cat nictitating membrane resulted in significant pupillary dilation of the denervated eye; the innervated eye was unaffected. After studying numerous denervated tissues, Cannon (1939) formulated the "law of denervation" which states

> "When in a series of efferent neurons a unit is destroyed, an increased irritabilty to chemical agents develops in the isolated structure..."

Although Cannon's law states that denervated structures exhibit increased irritability (supersensitivity), decreased responsiveness of denervated tissue (subsensitivity) has also been noted. The phenomenon of altered sensitivity following denervation has been observed in a variety of tissues. Several excellent reviews on the subject have been published (Fleming et. al., 1973; Fleming, 1976; Westfall, 1981). This discussion will be limited to selected studies of gastrointestinal smooth muscle.

Smooth muscle of the gastrointestinal tract has exhibited both super- and subsensitive responses to agonists after alterations in innervation. Luco (1937) and Youmans (1938) demonstrated that sympathetic denervation of loops of bowel in rabbits or dogs increased the responsiveness of

denervated tissue to norepinephrine. More recently, Frigo et. al. (1984) demonstrated that sympathetic denervation induced supersensitivity to both alpha- and beta-adrenergic agonists in the guinea pig colon. Fleming (1968) demonstrated that chronic treatment with ganglionic blocking agents induced a non-specific supersensitivity in the guinea pig ileum. However, chronic treatment with reserpine had no effect on the responsiveness of guinea pig ileum to acetylcholine, histamine or potassium. Ueki et. al. (1985) demonstrated cholinergic supersensitivity in colonic muscle from Piebald mice, which lack enteric ganglia in the terminal colon.

Alterations in the sensitivity of gastrointestinal smooth muscle have also been noted in clinical studies. Kramer and Ingelfinger (1951) reported hypersensitivity of esophageal smooth muscle to the injection of methacholine in patients with achalasia, a condition in which myenteric neurons are absent in the esophagus. Kamijo et. al. (1953) and Wright and Shepard (1965) have demonstrated decreased sensitivity to acetylcholine of colonic muscle from patients with Hirschsprung's disease.

There have been a number of mechanisms proposed to account for the changes in sensitivity of denervated smooth muscle (Westfall, 1981). Changes in receptor number, affinity or efficacy could account for the altered

sensitivity, but little experimental evidence in support of receptor alterations has been found to date. Alterations in membrane potential and calcium metabolism have also been proposed to account for sensitivity changes. At this time, however, there is no generally accepted mechanism to explain altered sensitivity of denervated smooth muscle.

One final note regarding sensitivity changes. It has been observed that reinnervation of previously denervated nictitating membrane results in the return of the supersensitive response of the muscle toward normal (Simeone, 1937; Vera et. al., 1957; Luco and Vera, 1964). A similar phenomenon had been observed in the denervated-reinnervated parotid gland (Ekstrom and Emmelin, 1974). Evidence presented in Chapter 3 of this thesis suggests that reinnervation may occur in myenterically denervated circular muscle.

The Chemistry of Benzalkonium Chloride

Benzalkonium chloride (BAC; Figure 5) is a homologous mixture of N-alkyldimethylbenzylammonium chlorides with N-alkyl groups varying from C_8 to C_{18}. BAC is prepared commercially by refluxing coconut oil or tallow and benzyl chloride (Linfield, 1970). Individual alkyl derivatives can be prepared by refluxing the appropriate dimethylamine and

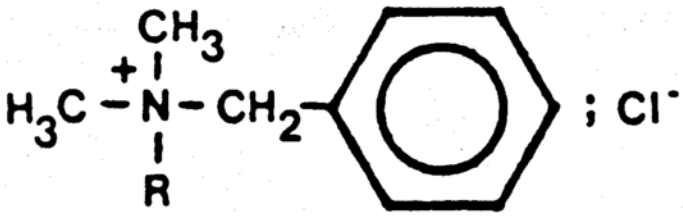

Figure 5. Structure of benzalkonium chloride (BAC). R = N-alkyl groups from C_8 to C_{18}.

benzyl chloride in an appropriate solvent (Cutler et. al., 1967a; Abidi, 1980). Selected physical properties of these compounds are presented in Table 3.

Domagk (1935) first recognized the antimicrobial activity of these compounds, and this discovery lead to the formulation of many commercial antimicrobial products. Because of their excellent surfactant properties (Table 3), these compounds are used today in a variety of commercial applications. BAC is used in the formulation of cosmetics, textiles, lubricants, points and soil stabilizers.

The Biologic Effects of Benzalkonium Chloride

Antimicrobial activity. Since the original observations of Damagk (1935), numerous investigations on the antimicrobial activity of BAC have been performed. Armstrong and Froelich (1964) demonstrated that Zephiran chloride, a commercial preparation of BAC, had significant antiviral activity against the Rous sarcoma virus, yellow fever virus, myxoviruses and the SV-40 virus. However, Zephiran was inactive against polio and Cocksackie viruses. BAC also possesses high protozoacidal activity (Lawrence, 1946).

BAC possesses significant antibacterial activity against both Gram-positive and Gram-negative bacteria, but

Table 3

Physical Properties of BAC Homologs

N-alkyl	Melting point (°C)	Surface tension[1]	CMC[2]
8	71.4 - 72.4	67.5	220
10	41.5 - 43.8	60.6	37
12	44.9 - 46.8	47.6	6.9
14	50.5 - 52.5	43.6	1.2
16	54.0 - 56.8	43.5	0.24
18	65.0 - 66.8	43.0	0.033

[1]surface tension (dynes/cm) of a 0.1% solution at 25 °C.

[2]critical micelle concentration, x 10^3 mole/l.

adapted from Cutler et. al. (1967a)

Gram-negative organisms are less susceptible to the actions of BAC (Ross et. al., 1953; Cutler et. al., 1967b; Tomlinson et. al., 1977; Daoud et. al., 1983). There are also significant differences in the antimicrobial potencies of the individual alkyldimethylbenzylammonium chloride homologs. The differences in activity can be summarized as follows. The dodecyl (C_{12}) homolog is most potent against yeast and fungi. The tetradecyl (C_{14}) homolog is most effective against Gram-positive bacteria, while the hexadecyl (C_{16}) homolog is most potent against Gram-negative organisms. Shorter and longer chain homologs are considerably less potent than the dodecyl, tetradecyl and hexadecyl homologs. Consequently, these three homologs , in varying proportions, make up the majority of homologs in commercial preparations of BAC. The ratio of these homologs in commercial mixtures has been shown to affect the antimicrobial activity of the mixtures (Fichards and Mizrahi, 1978).

The structure-antibacterial activity relationship of the BAC homologs can be explained on the basis of their mechanism of bactericidal activity and the physical-chemical properties. BAC homologs are believed to exert antimicrobial activity by non-specific solubilization of cell membranes. In bacteria, this is thought to be a multistage process characterized by adsorption onto and

penetration into the cell wall, and subsequently, the cell membrane (Blois and Swarbuck, 1972b). It was suggested that the initial absorption step is the result of electrostatic interactions between negatively charged surface proteins and the positively charged nitrogen group of the homolog. This interaction is believed to facilitate penetration of the hydrocarbon moiety into the cell wall and membrane. The membrane perturbation caused by penetration results in increased cellular permeability which ultimately leads to bacterial lysis.

Given the non-specific mechanism of action of BAC, it is probable that the activity of a given homolog is related to its concentration at and ability to interact with the cell membrane. These factors are related to aqueous solubility and relative surface activity of the homolog. Within the BAC homologous series, it is known that as alkyl chain length increases, aqueous solubility of monomer decreases (Daoud et. al., 1973) and relative surface activity increases (Blois and Swarbuck, 1972). Also, as aqueous solubility of monomer decreases, micelle formation occurs at lower surfactant concentrations. Given these facts, an explanation for the structure-activity relationship can be proposed. If the aqueous solubility of a homolog is high (i.e., C8), it will exist in the monomeric form capable of membrane interaction (Helenius and Simon,

1975). However, because of low surface activity, the ability to disrupt the membrane will be relatively low. If the aqueous solubility of a homolog is low (i.e., C_{18}), much of the homolog will be present in micelles and little monomer will be available for membrane interaction. Minimal membrane disruption will occur even though the homolog possesses relatively high surface activity. The tetradecyl homolog apparently possesses the optimum combination of aqueous solubility and surface activity for membrane interaction and is thus the most effective antimicrobial agent.

There are several lines of evidence which suggest that BAC produces myenteric denervation by a similar non-specific mechanism as that proposed to account for its antimicrobial activity. First, Fox et. al. (1983) observed that a variety of surfactants (both ionic and nonionic) were capable of ablating myenteric neurons. Of those tested, however, BAC was the most potent. Second, the demonstration of specific binding of BAC homologs to albumin (Perrin and Nelson, 1974) is consistent with the concept of protein-homolog interaction. Interestingly, the tetradecyl homolog appeared to have the highest binding affinity of the homologs tested. Finally, See et. al. (1988) have observed necrosis of virtually all longitudinal, a portion of the circular muscle, and all myenteric neurons initially after serosal

exposure to BAC. Subsequent division of smooth muscle, but not myenteric neurons, is apparently responsible for the generation of the myenterically denervated model.

Pharmacologic activity. BAC has been shown to bind to bovine serum albumin *in vitro* (Perrin and Nelson, 1974; Lien and Perrin, 1976). The binding of the BAC homologs varies widely, depending on alkyl chain length. Short chain homologs (octyl and decyl) exhibit little protein binding (less than 20% at 1mM) while longer chain homologs are highly bound (greater that 95%) at equimolar concentrations. Based on estimated binding constants, the tetradecyl and hexadecyl homologs exhibit the highest binding affinity. The significance of this *in vitro* binding, if any, to *in vivo* biological effects of BAC is not known.

BAC can inhibit the polyamine-induced release of histamine from mast cells, or at higher concentrations, can cause histamine release (Read and Kiefer, 1979; Read et. al., 1982; Read and Lagunuff, 1986). The mechanism of inhibition appears to be competitive antagonism at the polyamine receptor on the mast cell surface since increasing the concentration of the polyamine can overcome inhibition. The mechanism of release is thought to be non-specific membrane disruption. As in previous studies of antimicrobial activity, the tetradecyl homolog was found to be the most potent in antagonizing and causing histamine

release.

Toxicology. (for review, see Cutler and Drobeck, 1970). Intraperitoneal or intravenous LD50 (dose lethal to 50% of the test population) of BAC ranges from 10 - 300 mg/kg body weight in rats, guinea pigs and rabbits. Acute oral LD50 values in mice range from 500 mg/kg for the C_8 homolog to 3500 mg/kg for the C_{18} homolog (Cutler et. al., 1967b). Signs of toxicity in acute oral studies were observed in the gastrointestinal tract (hemorrhage, mucosal necrosis); depression of respiratory and CNS function were also observed. In general, odd-chain homologs were slightly more toxic than even-chain homologs in the mouse oral LD50 studies. Dermal and eye irritation studies indicated that BAC was a mild to moderate irritant (Finnegan et. al., 1953; Duprey and Hoppe, 1960).

Several chronic feeding studies in rats (Fitzhugh and Nelson, 1948; Alfredson et. al., 1951) and dogs (Alfredson et. al., 1951) have been reported. Animals fed levels of 0.5% BAC or higher in the diet died within 3 months of the start of the study. Growth rate was reduced in rats, even at the lowest dose used (0.063%). Pathologic changes included diarrhea, abdominal distention, and focal necrosis of mucosa. No increase in tumor incidence was observed.

Three cases of fatal human intoxication have been reported. Two of the deaths occurred following oral

ingestion (Adelson and Sunshine, 1952; Wolff, 1961), the other following uterine instillation (Arnold and Krefft, 1952). Death occurred rapidly (within 30 minutes), but the apparent cause of death was not identified.

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Statement of the Problems

The research described in the first three chapters which follow focuses on the physiologic and pharmacologic responses of myenterically denervated rat jejunal smooth muscle. Interruption of normal innervation of smooth muscle has been shown to alter various mechanical properties of the muscle. In particular, alterations in length-stress properties have been observed, and these changes are thought to reflect changes in smooth muscle morphology. Alterations in length-stress properties have also been shown to affect the pharmacologic sensitivity of smooth muscle. Therefore, the aim of the research presented in the first chapter was to assess the length-stress properties and active tension generation in myenterically denervated rat jejunal longitudinal and circular muscle. In addition to providing information on muscle mechanics and morphology, this information is necessary for the proper conduct of subsequent pharmacologic studies.

Changes in the pharmacologic sensitivity of excitable tissues, including smooth muscle, have been observed following denervation. Therefore, the sensitivity of myenterically denervated rat jejunal longitudinal and circular muscle to carbachol, a non-selective cholinergic agonist, was determined various times after denervation.

Interruption of continuous nerve pathways within the small intestine by localized myenteric plexus ablation might be expected to alter the neuronal pathways which mediate ascending and descending intestinal reflexes. Therefore, another aim of this investigation was to determine the responses to carbachol of jejunal muscle orad and caudad to a localized region of myenteric denervation.

Another aim of the present investigation was to determine if the changes with time in the carbachol-induced contractile responses of myenterically denervated circular muscle reflect changes in the muscarinic or nicotinic component of carbachol's action.

The biologic activity of the individual alkyldimethylbenzylammonium chloride homologs which make up benzalkonium chloride vary greatly. Another aim of the present investigation was to examine the structure-activity relationship of the homologous series and 1) determine which of the individual homologs possess the ability to ablate myenteric neurons, and 2) identify, for use in future studies, the homolog which is most effective in ablating myenteric neurons.

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

1. Characterization of the mechanical changes induced

in jejunal smooth muscle at various times after myenteric denervation.

2. Characterization of the responses of jejunal smooth muscle to carbachol at various times after myenteric denervation.

3. Determination of the contribution of nicotinic and muscarinic components in the carbachol-induced contractile activity of circular muscle at various times after denervation.

4. Evaluation of the structure-activity relationship of individual alkyldimethylbenzylammonium chloride homologs with respect to myenteric neuron ablative activity.

Chapter 1

Temporal Changes in Mechanical Properties of Rat Jejunal Smooth Muscle After Myenteric Plexus Ablation

(see Appendix A)

Chapter 2

Altered Carbachol-Induced Contractile Responses of Rat Jejunal Smooth Muscle Following Local Myenteric Plexus Ablation

ABSTRACT

Alterations in smooth muscle responsiveness and enteric reflexes may occur after myenteric denervation. These changes may lead to obstruction. We determined the *in vitro* carbachol-induced contractile responses of control and myenterically denervated longitudinal and circular rat jejunal smooth muscle 15, 30 and 45 days after myenteric plexus ablation. Denervated longitudinal muscle exhibited decreased responsiveness to carbachol at all times examined. Denervated circular muscle was initially supersensitive, but with time became subsensitive. The nature of these changes probably reflects the loss of the nicotinic (neuronal) component of the action of carbachol. Tissue orad and caudad to the myenterically denervated segment also exhibited altered responses to carbachol. This suggests that there are alterations in ascending excitatory and descending inhibitory neuronal pathways. Alterations in smooth muscle responses, both at and beyond the site of myenteric plexus ablation may account, in part, for altered intestinal motility which may lead to obstruction.

INTRODUCTION

Transit through the intestinal tract requires normal intestinal motility, which in turn requires integrity of the myenteric plexus. Pathologic changes in the myenteric plexus, such as the loss of neuronal cell bodies or processes, can have serious consequences (for review, see Smith, 1982; Krishnamurthy and Schuffler, 1987), the most severe of which is life-threatening intestinal obstruction (Dyer et. al., 1969; Bughaighis and Emery, 1971; Tanner et. al., 1976; Krishnamurthy et. al.,1986). The clinical presentation (obstruction) is similar whether the lesion involves the entire small and/or large intestine or is localized (zonal or skip lesions, Tifflin et. al., 1940; Sprinz et. al., 1961; MacIver and Whitehead, 1972; Kadair et. al., 1977; Martin et. al., 1979; de Chadarevian et. al., 1982; Taguchi et. al., 1983; Seldenrijk et. al., 1986). Alterations in motility which lead to obstruction may be caused by changes in enteric reflexes or motor patterns (Bayliss and Starling, 1899; Costa and Furness, 1976; Furness and Costa, 1987) responsible for normal intestinal motility. Additionally, alterations in smooth muscle responsiveness following denervation (Cannon, 1939; Cannon and Rosenblueth, 1949) might be expected to contribute to altered motility.

The ability to ablate myenteric neurons in a defined area of rat jejunum by serosal application of benzalkonium chloride has been described by our laboratory (Fox et. al., 1983). Comparison of control and myenterically denervated tissues in vitro has proven useful in the determination of the site(s) of action of putative enteric neurotransmitters (Fox et. al., 1986) and the distribution of adrenergic receptor subtypes in the rat jejunum (Fox and Bass, 1986a). In addition, in vivo recording of electrical activity of control and myenterically denervated portions of the rat jejunum has shown that the migrating myoelectric complex propagates through a region of denervated gut in the absence of the basic electric rhythm (Fox and Bass, 1984). These studies have provided insight into the role of the myenteric plexus in intestinal function. The myenterically denervated model can be used to determine if changes in smooth muscle responses occur following destruction of the myenteric plexus and whether such changes extend beyond the boundaries of the lesion.

In this study, we used the myenterically denervated (MD) rat jejunal model to determine the carbachol-induced contractile responses of control and MD longitudinal and circular muscle at various times following myenteric plexus ablation. In addition, the responses of tissue orad and caudad to the MD segment were also determined.

Materials and Methods

Myenteric Neuron Ablation. Myenteric neurons were ablated as previously described (Fox et. al., 1983). Male rats, 200 - 225 g (Sprague-Dawley, Madison, WI) were anesthesized with a pentobarbital- chloral hydrate anesthetic (0.3 ml/ 100 g body wt). A 3 - 4 cm portion of jejunum, 6 - 8 cm caudad to the ligament of Trietz, was exteriorized through a small midline incision and delineated with two serosal sutures placed at the mesenteric border. Benzalkonium chloride (BAC, 0.062% solution in saline) was applied to the serosal surface of the delineated segment every 5 min for 30 min (6 applications). After treatment, the bowel was rinsed with saline, returned to the peritoneal cavity, and the animals allowed to recover. This treatment protocol has been shown to ablate at least 90 % of the myenteric neurons in the rat jejunum with no effect on the number of submucosal neurons (Fox et. al., 1983); selective histologic examination of treated tissue in this study (N = 4) confirmed the effectiveness of treatment. Ten animals were treated similarly with saline alone. Animals were housed in stainless steel wire mesh-bottomed cages, allowed access to food and water ad libitum, and maintained on a

12-h light:dark cycle.

Muscle Responses. The *in vitro* isometric contractile responses of longitudinal and circular muscle strips from age-matched naive, saline-treated and myenterically-denervated (MD) rats were determined 15, 30 and 45 days after treatment. The responses of tissue 2 cm orad and 2 cm caudad to the serosal sutures that delineated the MD segment were also determined. Carbamylcholine chloride (carbachol) was used to elicit contractile responses. Carbachol stimulates both muscarinic and nicotinic cholinergic receptors, but is not susceptible to hydrolysis by acetylcholinesterase (Molitor, 1936). Animals were sacrificed by a blow to the head followed by cervical dislocation. The jejunal segment was opened along the mesenteric border and the mucosa removed by scraping with a microscope slide. Longitudinal and circular muscle strips (0.5 X 1 - 2 cm) were cut parallel and perpendicular, respectively, to the mesenteric border. Strips were then suspended in water-jacketed tissue baths which contained Krebs-bicarbonate buffer [(in mM) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2 \cdot 2H_2O$, 0.5 $MgCl_2 \cdot 6H_2O$, 1.0 NaH_2PO_4, 25.0 $NaHCO_3$, and 11.0 glucose]. The solution was maintained at 37 °C, and was continuously aerated with 95 % O_2, 5 % CO_2. One end of the muscle strip was attached to an immobile support, the other to a force transducer (model FT-03, Grass Instruments,

Quincy, MA) coupled to a polygraph (model 7D, Grass Instruments). Tissue responses were recorded under isometric conditions. Tissues were allowed to equilibrate 60 min prior to the addition of carbachol. During the equilibration period, tissues were gently stretched to obtain appropriate resting tensions for optimal tension development. Resting tensions for naive and saline-treated longitudinal and circular muscle strips were 1.0 and 0.5g, respectively (Herman and Bass, 1987). Preliminary studies indicated these values were appropriate for tissue orad and caudad to the MD segment. Resting tensions for MD longitudinal and circular muscle at 15 days were 3.0 and 1.5g, respectively, while at 30 and 45 days after myenteric denervation, appropriate tensions were 1.0 and 1.5g, respectively (Herman and Bass, 1987). Dose-response curves were obtained by exposing the tissues to stepwise increments of carbachol concentrations. After the maximal steady-state response to a concentration was obtained, the bathing solution was changed 3 times, and 10 min elapsed before the addition of a higher concentration. After the maximal tissue response to carbachol was obtained, 10^{-2} M barium chloride was added to the baths. Barium interacts directly with the contractile elements or postreceptor regulatory cascade (Hansen et. al., 1984) to produce, at this concentration, the maximum contraction attainable in a given

tissue (Fox and Bass, 1986b).

Histologic examinations. Randomly selected tissue samples were obtained from naive (untreated) and MD animals (orad, denervated, caudad) and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4). Tissues were then dehydrated through a graded series of ethanols, embedded in glycol methacrylate embedding media (JB-4, Polysciences, Warrington, PA) and sectioned (2 or 3). Sections were then stained with methylene blue-basic fuchsin and the number of myenteric neurons per mm jejunum determined in three separate sections per animal.

Drugs. BAC (Zephiran chloride) was purchased from a local pharmacy. Carbachol was obtained from Sigma Chemical Co. (St. Louis, MO), and barium chloride was purchased from Mallinckrodt Chemical Works (St. Louis, MO). Drugs were dissolved in 0.9 % saline and prepared freshly on the day of the experiment. Carbachol and barium chloride were added to the tissue baths in a volume equal to 1 % of the bath volume.

Analyses of Results. All data are presented as mean ± SEM. Carbachol-induced contractile data was expressed as a percent of the maximum contraction produced by barium (= 100 %) and plotted against carbachol concentration. An EC_{50} value, the concentration which produced 50 % of the maximal response to carbachol, was determined from the individual

dose-response curves. Geometric (- log EC_{50}) means (Fleming et. al., 1972) were then calculated and compared statistically. Longitudinal and circular muscle responses from age-matched naive and saline-treated rats 15, 30 and 45 days after myenteric denervation were analyzed with a one-way analysis of variance (ANOVA). No significant differences were observed between naive and saline-treated tissue in the respective muscles at any time, so all such data for each layer were pooled. Contractile data from orad, MD and caudad tissue 15, 30 and 45 days after myenteric denervation, as well as neuronal cell counts, were compared to the respective pooled controls with an ANOVA and Newman-Keuls procedure (Tallarida and Murray, 1987), and p values < 0.05 were considered significant.

Results

In-life, gross and microscopic observations. Animals tolerated the BAC treatment well; mortality was less than 1%. There were no observed differences in body weight gain or general appearance between untreated and MD animals. Viscous, granular chyme was noted in the MD and orad portions of jejunum in many of the MD animals. Such chyme was not observed in control animals. The number of neurons/mm jejunum in control tissue (4.9 ± 0.3, $N = 14$) was not different from the number in tissue orad (4.2 ± 0.4,

N = 6) or caudad (4.6 ± 0.3, N = 6) to the MD segment.

Myenterically denervated (MD) tissue. Dose-response curves of control and MD longitudinal and circular muscle 15, 30 and 45 days after myenteric denervation are presented in Figure 1 and 2, respectively. The curves of MD longitudinal muscle all lie to the right of the control curve, suggesting subsensitivity to carbachol. Analyses of the - log EC_{50} values (Figure 3) indicated that these shifts were significant. The magnitude of the shift relative to control (- log EC_{50} = 6.47 ± 0.09, N = 35) was approximately 3-fold 15 (6.03 ± 0.12, N = 13) and 30 (5.94 ± 0.05, N = 6) days after myenteric denervation, and 6-fold (5.67 ± 0.14, N = 11) 45 days after myenteric denervation.

The dose-response curve of MD circular muscle at 15 days (- log EC_{50} = 6.05 ± 0.11, N = 16) lies to the left of the control curve (5.61 ± 0.09, N = 27), indicating supersensitivity to carbachol. In contrast, at 30 (5.32 ± 0.13, N = 15) and 45 (5.05 ± 0.21, N = 5) days, the dose-response curves from MD circular muscle lie to the right of the control curve, indicating subsensitivity to carbachol. Analyses of the - log EC_{50} values (Figure 4) indicated that the shifts 15 and 45 days after myenteric denervation were significant, and 3- and 4-fold, respectively, in magnitude. MD circular muscle at day 30 exhibited a trend towards subsensitivity ($0.05 < p < 0.10$).

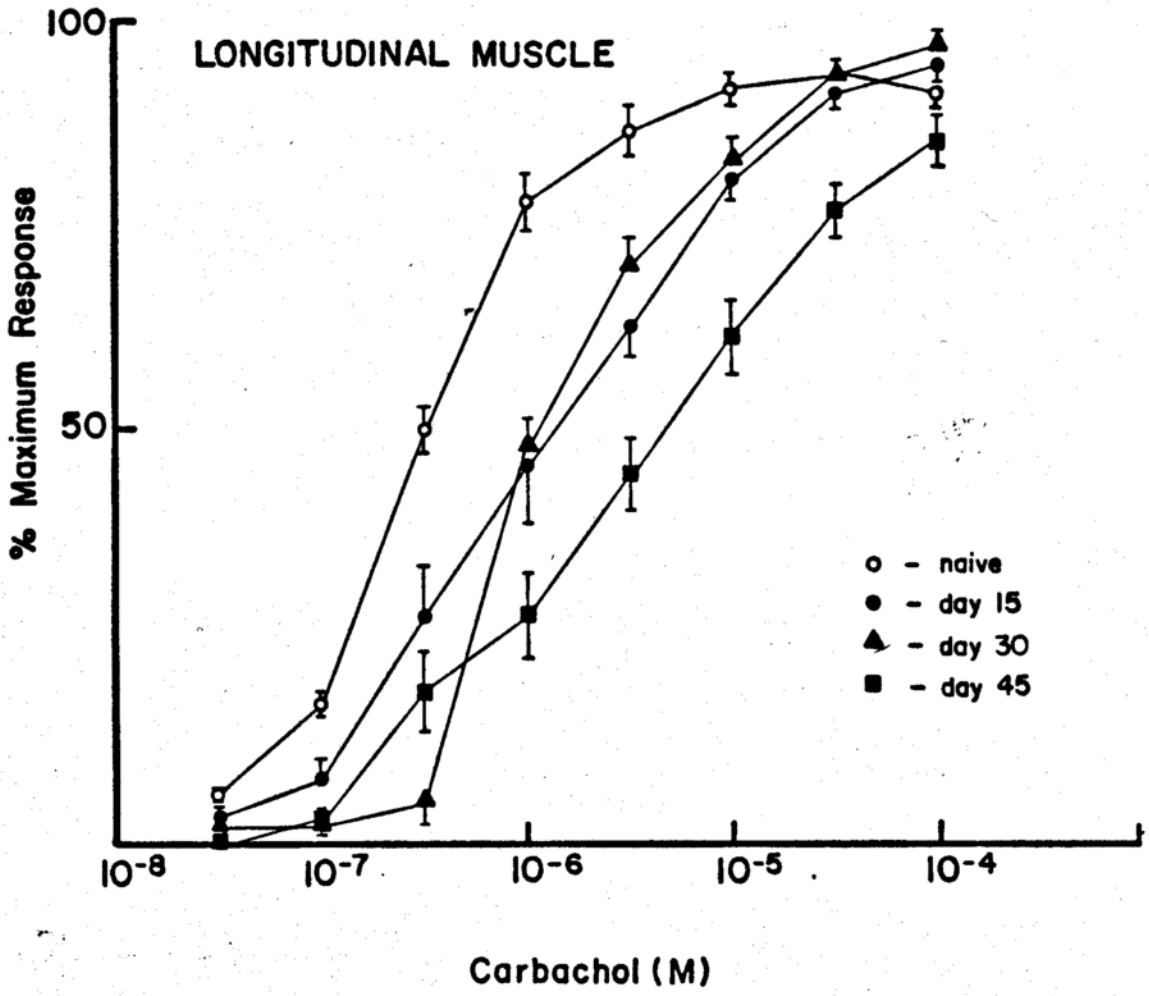


Figure 1. Responses of longitudinal jejunal muscle to carbachol various times after myenteric plexus ablation. Control (○), 15- (●), 30- (▲) and 45-days (■) after ablation. Each data point represents the mean ± SEM of responses of 6 - 35 animals.

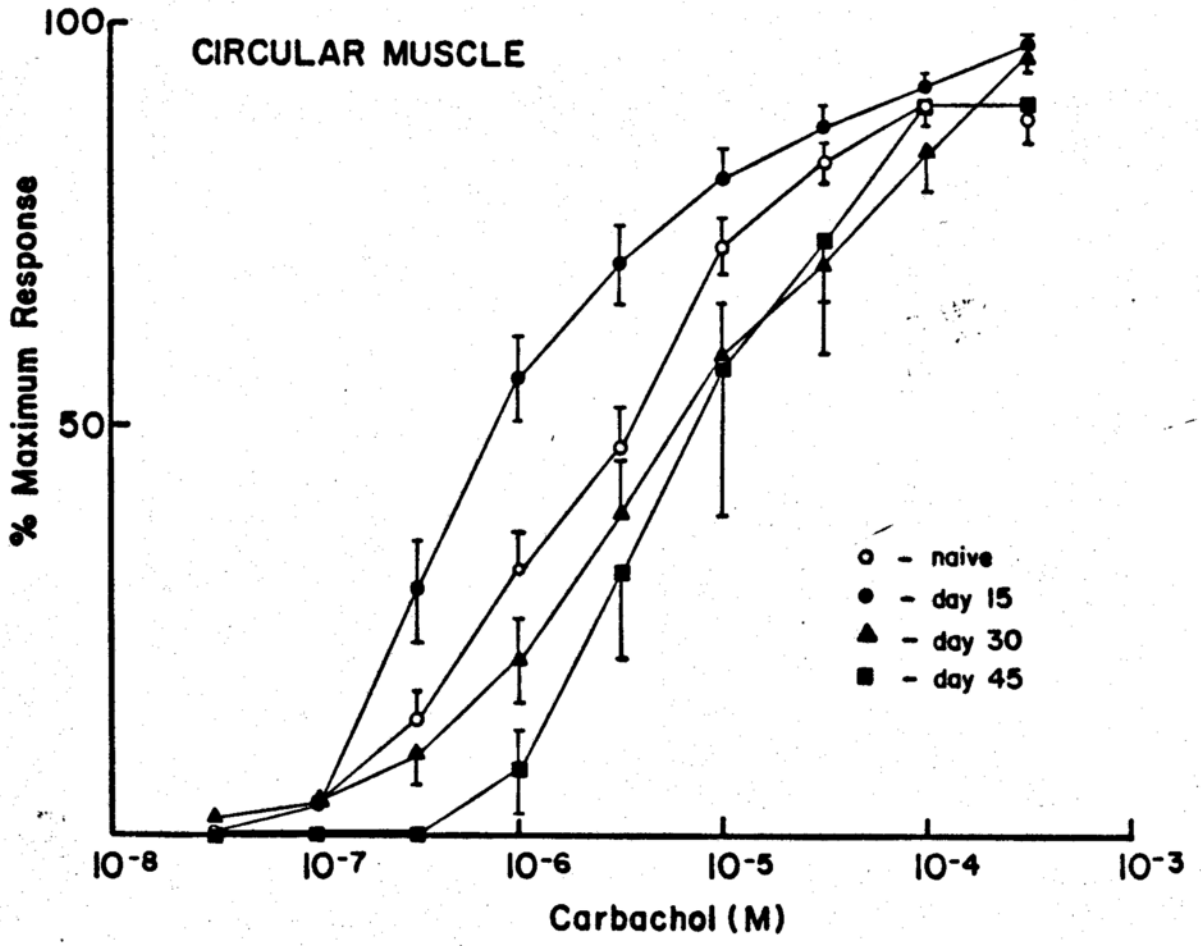


Figure 2. Reponses of circular jejunal muscle to carbachol at various times after myenteric plexus ablation. Control (○), 15- (●), 30- (▲) and 45-days (■) after ablation. Each data point represents the mean ± SEM of responses of 6 - 27 animals.

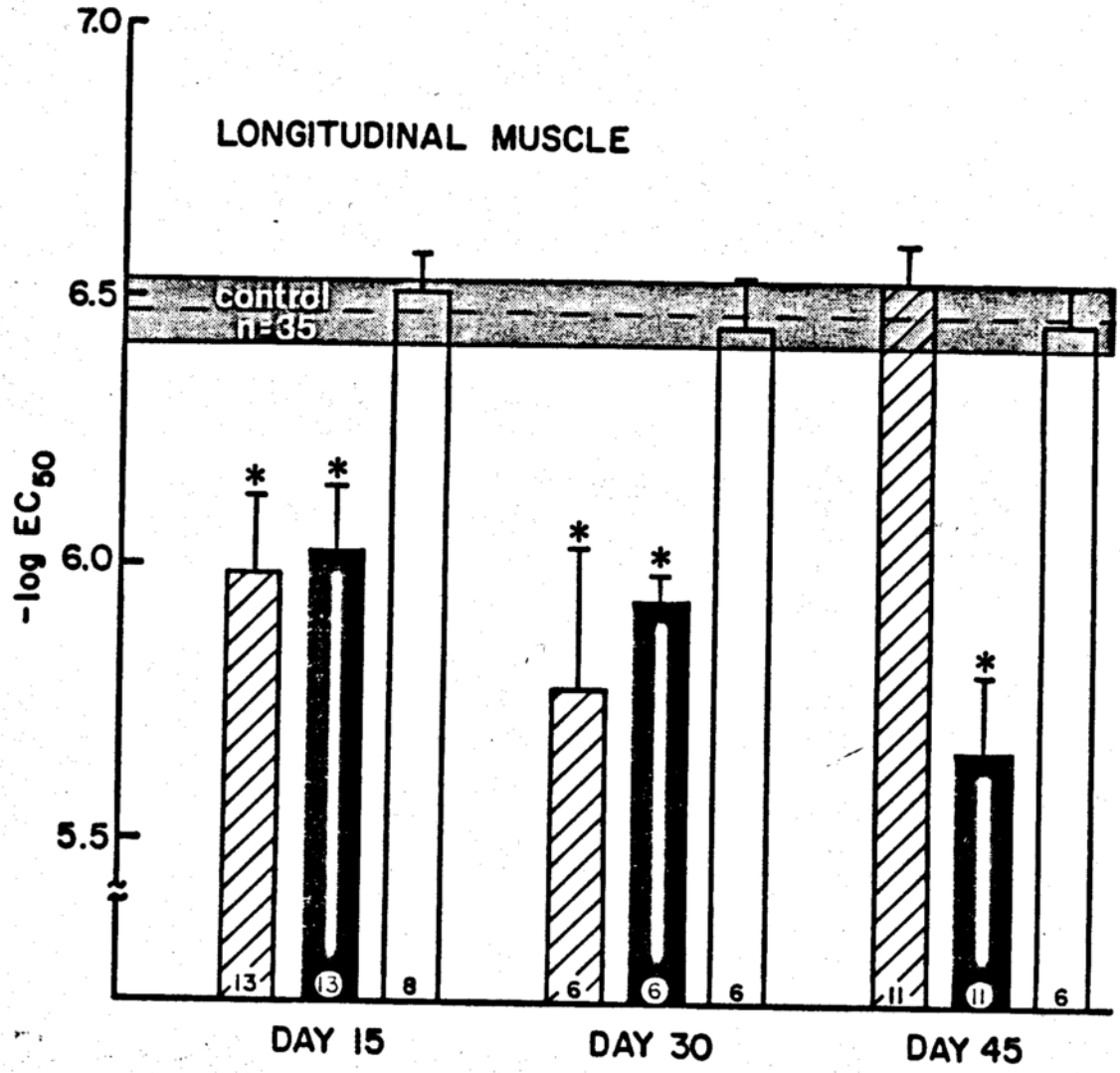


Figure 3. Responsiveness (- log EC_{50}) to carbachol of longitudinal jejunal smooth muscle orad (hatched bars) and caudad (open bars) to myenterically denervated muscle (closed bars) various times after denervation. Values are means ± SEM from the number of animals indicated in the bars. * indicates significant difference from control mean, $p < 0.05$.

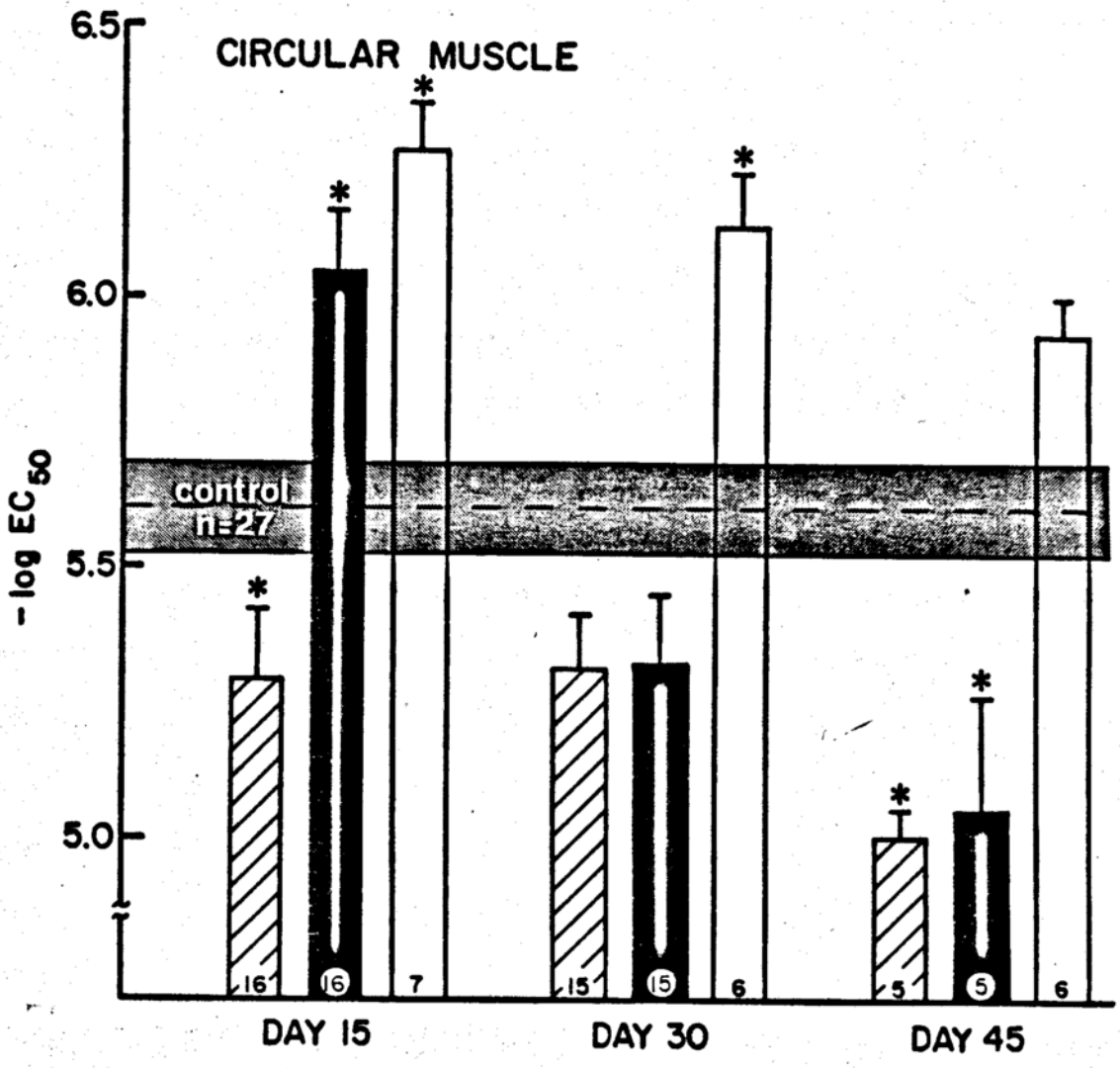


Figure 4. Responsiveness (- log EC_{50}) to carbachol of circular jejunal muscle orad (hatched bars) and caudad (open bars) to myenterically denervated muscle (closed bars) various times after denervation. Values are means ± SEM from the number of animals indicated in the bars. * indicates significant difference from control mean, $p < 0.05$.

Tissue orad, caudad to MD segment. Longitudinal muscle 2cm orad to MD jejunum exhibited signficantly reduced responses to carbachol 15 and 30 days after myenteric denervation, but was comparable to control at 45 days (Figure 3). The magnitude of the reductions 15 and 30 days after treatment were 3- and 5-fold, respectively.

Circular muscle 2cm orad to MD jejunum (Figure 4) also exhibited reduced responses (2 - 4 fold shifts in - log EC_{50} values) to carbachol (significant 15 and 45 but not 30 days after myenteric denervation), but unlike longitudinal muscle, a return of normal responses of circular muscle orad to the MD segment was not observed.

Circular muscle 2 cm caudad to the MD segment exhibited enhanced responses (3 - 5-fold) to carbachol 15, 30 and 45 days after myenteric denervation, but only the responses at 15 and 30 days were statistically significant. In contrast, longitudinal muscle 2 cm caudad to MD jejunum was comparable to control at all times studied.

DISCUSSION

Our results demonstrate that the carbachol-induced contractile responses of MD muscle are altered, and that the nature of the changes differ in longitudinal and circular muscle. MD longitudinal muscle exhibited subsensitive

responses at all times examined, while the responses of MD circular muscle varied with time. The subsensitivity of the longitudinal muscle was unexpected since the "law of denervation" (Cannon, 1939) states that the loss of innervation results in supersensitivity of effectors to agonists. There are two possible explanations for the paradoxical response seen in MD longitudinal muscle. It is known that following BAC treatment, the longitudinal muscle layer is destroyed and subsequently replaced with new muscle cells (See et. al., 1988). It is possible that these new cells express fewer muscarinic receptors, or receptors with lower affinity than those of control cells. Such changes could account for the observed subsensitivity.

Alternatively, the subsensitive responses can be explained on the basis of both the pharmacologic properties of carbachol and myenteric innervation of the longitudinal muscle. Carbachol is a cholinergic agonist which stimulates both muscarinic and nicotinic receptors (Molitor, 1936). Stimulation of muscarinic receptors results in muscle contraction, while stimulation of nicotinic receptors produces either muscle contraction or relaxation. The nature of the response produced by nicotinic stimulation is dependent on whether excitatory or inhibitory innervation is predominant in a given muscle layer. In general, myenteric innervation of longitudinal muscle is excitatory (Anuras et.

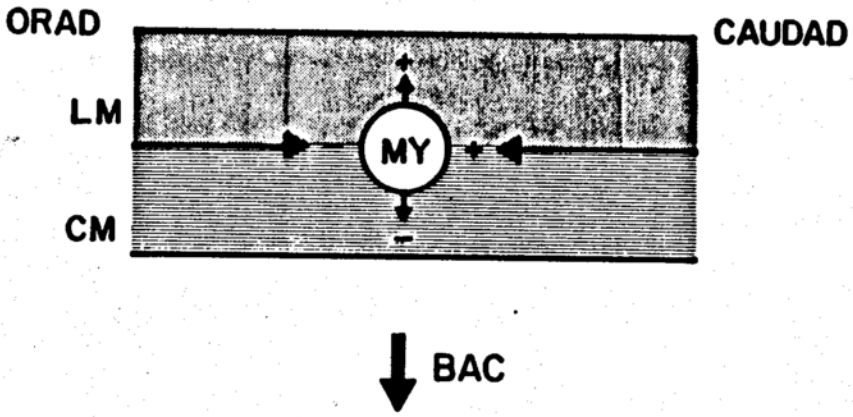
al., 1977; Yokoyama and Ozaki, 1978; Wood, 1987; Figure 5A), presumably due to the release of acetylcholine (ACh). Thus, in control (innervated) longitudinal muscle, carbachol produced contractions by direct action on the muscle and by release of ACh from myenteric neurons. In MD longitudinal muscle, the carbachol-induced contraction is due exclusively to direct action on the muscle since myenteric neurons are absent (Figure 5B). Thus, the lack of carbachol-induced ACh release from myenteric neurons may account for the subsensitive responses of MD longitudinal muscle.

In contrast to the responses of MD longitudinal muscle, the nature of the response of MD circular muscle varied with time. Fifteen days after myenteric denervation, MD circular muscle exhibited significant supersensitivity to carbachol. This is consistent with the law of denervation, and could be due to an increased number of muscarinic receptors in circular muscle. Increases in receptors in skeletal (McConnell and Simpson, 1976) and smooth muscle (Powers and Colucci, 1985) have been associated with supersensitive responses to cholinergic and adrenergic agonists. Alternatively, the supersensitivity can be explained, as in the case of MD longitudinal muscle, on the basis of the action of carbachol and intrinsic innervation of circular muscle. As in longitudinal muscle, carbachol produces contraction of both control and denervated muscle by direct

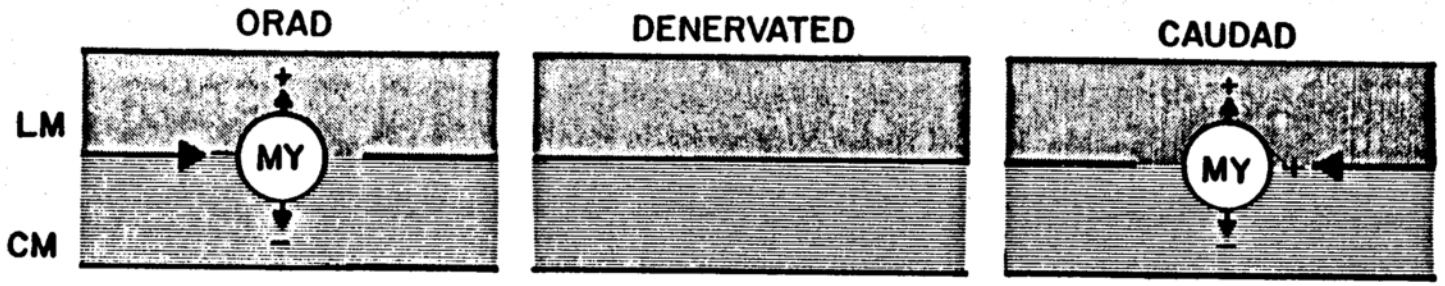
Figure 5. Schematic representation of myenteric innervation in control (A) and myenterically denervated (B) rat jejunum. Myenteric plexus (MY) influence on longitudinal muscle (LM) is predominantly excitatory (+) and predominantly inhibitory (-) on circular muscle (CM). Neuronal influences from orad bowel are predominantly inhibitory (descending inhibition) while such influences from caudad bowel are predominantly excitatory (ascending excitation). These influences are probably mediated by polysynaptic pathways.

Fifteen days after myenteric denervation (B), longitudinal muscle at the site of the lesion exhibited subsensitive responses to carbachol, due to the loss of local excitatory influences. Circular muscle at the site of the lesion was supersensitive to carbachol, due to the loss in local inhibitory influences. Muscle 2 cm orad to the lesion was subsensitive to carbachol, presumably due to alterations in local neurons which mediate ascending excitatory influences. Circular muscle 2 cm caudad to the lesion exhibited supersensitive responses, probably due to alterations in local neurons which mediate descending inhibitory influences.

A. CONTROL



B. MYENTERICALLY DENERVATED



stimulation of muscarinic receptors. Carbachol would also stimulate myenteric neurons (via nicotinic action) innervating control, but not MD circular muscle. Innervation of circular muscle is believed to be predominantly inhibitory (Anuras et. al., 1977; Ozaki, 1979; Wood, 1987; Figure 5A), so the carbachol-induced contraction in control tissue is the composite of direct excitatory and indirect inhibitory actions. The inhibitory component is absent in MD circular muscle (Figure 5B), therefore it appears supersensitive 15 days after myenteric denervation.

Thirty days after myenteric denervation, the response of circular muscle is normal (with a strong trend towards subsensitivity), while at 45 days it appears subsensitive. These changes may be the result of alterations in muscarinic receptors. Alternately, it has been suggested that following denervation, a return of normal sensitivity (and by extension, subsensitivity) may be the result of reinnervation (Cannon and Rosenblueth, 1949). If there is a neuronal influence on circular muscle which accounts for the changes in sensitivity, it is probably a submucosal influence. Although current dogma states that circular muscle receives motor input only from myenteric neurons (Wood, 1987), recent evidence indicates a role for submucosal neurons. Morphologic (Ekblad et. al., 1987) and physiologic (Sanders and Smith, 1986) studies suggest that

submucosal neurons may innervate and influence circular muscle function. Interestingly, increases in neurotransmitter synthesis in MD tissue, probably by submucosal neurons, have been reported (Dahl et. al., 1987) and are consistent with the notion of a submucosal neuronal influence on circular muscle. Myenteric neurons cannot be involved since they were ablated, and we have not observed myenteric neuron cell bodies or fibers immunohistochemically (unpublished observations) nor in toluidine blue stained sections, even at 165 days after myenteric denervation (See et. al., 1988). Regrowth of myenteric neuronal processes from adjacent tissue is considered unlikely in the time frame of these experiments, since such regrowth is reported to be only a few millimeters per month (Furness et. al., 1983). The neuronal influence is not extrinsic, since extrinsic nerve fibers have not been observed in MD tissue (unpublished observations).

Our results also indicate that local lesions of the myenteric plexus can alter the carbachol-induced contractile responses of tissue orad and caudad to the lesion, even though the number of neurons in these regions is normal. These alterations may be due to changes in muscle or myenteric nerves, since carbachol stimulates both nicotinic and muscarinic receptors. However, we believe that the changes in muscle response reflect changes in the nicotinic

(neuronal) component of the action of carbachol rather than alterations in muscle receptors. The direction of the change in response (either super- or subsensitive) is consistent with the polarity of enteric innervation and intrinsic reflexes. When present, influences from neurons orad to a given segment are generally inhibitory (descending inhibition, Bayliss and Starling, 1899; Costa and Furness, 1976; Hirst and McKirdy, 1974; Hirst et. al., 1975; Figure 5A), while influences from neurons caudad to the segment are generally excitatory (ascending excitation, Bayliss and Starling, 1899; Costa and Furness, 1976; Figure 5A). Thus, the subsensitive responses of both longitudinal and circular muscle orad to the MD segment can be explained with alterations in local neurons which mediate ascending excitation Figure 5B). Similarly, the supersensitive responses of circular muscle caudad to the MD segment may be due to alterations in local neurons which mediate descending inhibition (Figure 5B). Modulation of neuronal pathways due to local myenteric plexus ablation may alter neurotransmitter synthesis or release in neurons orad and caudad to the lesion, and thus account for the alterations in the nicotinic component of the carbachol response. Indeed, it is known that neurotransmitter levels in tissue orad to the MD segment vary markedly with time after ablation (Dahl et. al., 1987). In addition, loss of

descending presynaptic inhibition of ACh release by local excitatory neurons (Jule, 1980) may also contribute to the supersensitive responses of circular muscle caudad to the MD segment. The return of normal responses in both longitudinal muscle orad and circular muscle caudad to the MD segment may also involve modulation of neurotransmitter synthesis or release.

Clinically, lesions of the myenteric plexus present as obstruction (Dyer et. al., 1969) or pseudoobstruction (Smith, 1972; Krishnamurthy and Schuffler, 1987; Schuffler et. al., 1978; Schuffler and Zonak, 1982). Obstruction may be due to a lack of relaxation of circular muscle at the site of the lesion, a process normally mediated by myenteric inhibitory neurons (Furness and Costa, 1977; Kendall et. al., 1987). The failure of the circular muscle to relax might also be expected to alter normal postprandial motor activity responsible for segmentatation and net aboral transit (Kendall et. al., 1987; Weisbrodt, 1987). In the present study, the presence of viscous, granular chyme in MD and orad segments suggest abnormal postprandial transit. Interestingly, the interdigestive motor pattern, the migrating myoelectric complex (MMC), is only minimally altered in patients with pseudoobstruction (Sullivan et. al., 1977) and unaffected in MD rats (Fox and Bass, 1984). In these cases, the MMC, in addition to its "housekeeping"

role (Szurzewski, 1969), may be the motor pattern primarily responsible for aboral transit. The MMC may prevent total obstruction, and allow normal growth in the MD rats.

Finally, altered responses of tissue adjacent to a lesion of the myenteric plexus may contribute to the etiology of certain clinical conditions in which denervation is suggested by functional changes but not supported by detectable lesions. Because of altered responses in adjacent regions, the portion of the bowel adversely affected (functionally obstructed for example) may extend well beyond the lesioned area to include regions of the bowel where innervation appears normal. Limited sampling of biopsy or autopsy material may fail to detect localized, primary lesions responsible for tissue alterations which lead to clinical conditions (Spriz et. al., 1961; MacIver and Whitehead, 1972; Martin et. al., 1979).

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Chapter 3

Submucosal Motor Innervation of Rat Jejunal Circular Muscle Following Myenteric Plexus Ablation

Abstract

Neurons located in the myenteric plexus are generally believed responsible for motor control of intestinal circular muscle. We determined the *in vitro* isometric responses of naive and myenterically denervated (MD) rat jejunal circular muscle to bethanachol and carbachol, in the presence and absence of neuronal antagonists (hexamethonium bromide, tetrodotoxin, Botulinum toxin A) 15 and 30 days after myenteric plexus ablation. The responses to bethanachol indicated no differences in muscarinic sensitivity between naive and MD tissue. The apparent affinity to carbachol, which acts at both muscarinic and nicotinic receptors, of MD tissue 15 days after denervation was significantly lower than that of naive tissue. However, 30 days after denervation, the apparent affinities of naive and MD circular muscle were comparable. The presence of neuronal antagonists had no effect on the apparent affinity to carbachol 15 days after myenteric denervation, but significantly altered the responses 30 days after denervation. The effects produced by the neuronal antagonists 30 days after myenteric denervation were qualitatively and quantitatively different than those produced in naive tissue, suggesting that the nature of the innervation in these tissues was different. These results

demonstrate that circular muscle is initially denervated following myenteric plexus ablation but reinnervation occurs within 30 days. The reinnervation observed is likely due to neurons located in the submucosal plexus.

INTRODUCTION

Motor control of intestinal circular muscle is generally attributed to neurons within the myenteric plexus (Wood, 1987; Furness and Costa, 1987). Both inhibitory and excitatory junctional potentials have been recorded in circular muscle after stimulation of myenteric neurons (Hirst, et. al., 1975; Bornstein, et. al., 1986). Surgical removal of longitudinal muscle with attached myenteric plexus in the guinea pig results in the loss of both nerve fibers in the circular muscle (Furness and Costa, 1979; Wilson, et. al., 1987) and the response of circular muscle to nerve selective electrical stimulation (Bornstein, et. al., 1986). Such studies support the concept of motor control of circular muscle function by myenteric neurons.

There is, however, physiologic and morphologic evidence which suggests a direct role for submucosal neurons in the motor control of circular muscle. Electrical field stimulation of submucosal neurons produced inhibitory junctional potentials in canine colonic circular muscle in the presence or absence of the myenteric plexus. Microinjection of acetylcholine onto submucosal neurons mimicked the effect of electrical field stimulation (Sanders and Smith, 1986). Surgical lesioning studies in rat intestine demonstrated that most, but not all nerve fibers

disappeared from circular muscle after removal of longitudinal muscle/myenteric plexus. The authors suggested that the source of the remaining fibers may be neuronal cell bodies located in the submucosal plexus (Ekblad, et. al., 1987). Thus, the possibility that submucosal nerves can directly affect circular muscle has some experimental support.

We recently described the carbachol-induced contractile responses of rat jejunal circular muscle at various times after myenteric plexus ablation (Herman and Bass, 1988). Fifteen days after denervation, circular muscle exhibited a supersensitive response to carbachol, as evidenced by a 3-fold reduction in apparent affinity (- log EC_{50}). However, the responses of denervated circular muscle were comparable to naive responses 30 days after denervation and subsensitive 45 days after myenteric denervation. Since carbachol stimulates both muscarinic and nicotinic receptors, the changes observed in the response of circular muscle with time after myenteric denervation could reflect changes in the muscle (muscarinic effects) or in innervation (nicotinic effects). It is unlikely that changes in muscarinic receptors account for the observed alterations since changes in receptor number or affinity have not been associated with changes in responsiveness of smooth muscle (for review, see Westfall, 1981). If the alterations in

carbachol-induced contractile responses are due to changes in nicotinically-mediated effects, this would suggest initial denervation and subsequent reinnervation may have occurred. Increased neurotransmitter synthesis observed in myenterically denervated tissue (Dahl, et. al., 1987) is also consistent with the idea of reinnervation. If reinnervation of circular muscle occurs after myenteric plexus ablation, it may be from neurons in the submucosal plexus.

The present study was designed to determine if the changes in carbachol-induced contractile responses of circular muscle with time were due to alterations in the muscarinic or nicotinic component of the action of carbachol. We determined the responses of rat jejunal circular muscle to 1) bethanachol, a selective muscarinic agonist, and 2) carbachol, a non-selective cholinergic agonist, in the presence and absence of neuronal antagonists 15 and 30 days after myenteric denervation. Evidence is provided which suggests that after ablation of myenteric neurons, submucosal neurons may innervate circular muscle.

Materials and Methods

Myenteric neuron ablation. Myenteric neurons were ablated as previously described (Fox, et. al., 1983). Male

rats, 200 - 225g (Sprague-Dawley, Madison, WI) were anesthesized with pentobarbital-chloral hydrate anesthetic (0.3 ml/100 g b. wt). A 3 - 4 cm portion of jejunum, 6 - 8 cm caudad to the ligament of Trietz, was exteriorized through a small midline incision and delineated with two serosal sutures placed at the mesenteric border. Benzalkonium chloride (BAC, 0.062 % solution in saline) was applied to the serosal surface of the delineated segment every 5 min for 30 min (6 applications). After treatment, the bowel was rinsed with saline, returned to the peritoneal cavity, and the animals allowed to recover. This treatment protocol has been shown to ablate at least 90% of the myenteric neurons in rat jejunum with no effect on the number of submucosal neurons (Fox, et. al., 1983). The details of the morphologic changes in smooth muscle following BAC treatment have been reported (See, et. al., 1988). Animals were housed in stainless steel wire-mesh-bottomed cages, allowed access to food and water ad libitum and maintained on a 12-h light:dark cycle.

Circular muscle responses. The in vitro isometric contractile responses of circular muscle strips from age-matched naive and myenterically-denervated (MD) rats were determined 15 and 30 days after denervation. Animals were sacrificed by a blow to the head followed by cervical dislocation. The jejunum was opened along the mesenteric

border and the mucosa removed by scraping with a microscope slide. Circular muscle strips (0.5 X 1 - 2 cm) were cut perpendicular to the mesenteric border and suspended in water-jacketed tissue baths which contain Krebs-bicarbonate buffer of the following composition (in millimolar): NaCl, 118; KCl, 4.7; $CaCl_2$ $2H_2O$, 2.5; $MgCl_2$ $6H_2O$, 0.5; NaH_2PO_4, 1.0; $NaHCO_3$, 25; and glucose, 11.0. The solution was maintained at 37 °C, and continuously aerated with 95% O_2, 5% CO_2. One end of the muscle strip was attached to an immobile support, the other to a force transducer (model FT-03, Grass Instruments, Quincy, MA) coupled to a polygraph (model 7D, Grass Instruments). Tissues were allowed to equilibrate 60 min prior to the addition of contractile agonist. During the equilibration period, the tissue was gently stretched to attain the appropriate resting tensions for optimal active tension development. Resting tensions for naive and MD circular muscle strips were 0.5 and 1.5g, respectively (Herman and Bass, 1987). Dose-response data were obtained in a non-cumulative manner. After the maximal steady-state response to an agonist concentration was obtained, the buffer solution was changed 3 times, and 10 min allowed to elapse before the addition of a higher concentration. After the maximal tissue response to an agonist was obtained, the tissue was exposed to 10^{-2} M barium chloride. This concentration of barium interacts

directly with the contractile elements or postreceptor regulatory cascade (Hansen, et. al., 1984) to produce the maximum contraction attainable in a given tissue (Fox and Bass, 1986).

Bethanachol, a selective muscarinic cholinergic agonist, was used to evaluate the muscarinic responses of the circular muscle. Carbachol, a non-selective cholinergic agonist, was used to determine both nicotinic and muscarinic responses of the muscle. Carbachol dose-response data were obtained in the presence and absence of various neuronal antagonists. The neuronal antagonists used were hexamethonium bromide (10^{-4} M), tetrodotoxin (TTX, 3 X 10^{-7} M), botulinum toxin A (BTA, 8 g/ml) and a combination of hexamethonium (10^{-4} M) and BTA (8 g/ml). In experiments with hexamethonium or TTX, the antagonist was present in the buffer throughout the entire experiment, including the equilibration period. In experiments with BTA, the toxin was only present during the equilibration period. This length of exposure to BTA has been shown to be sufficient to produce irreversible blockade of acetylcholine release (Harry, 1963; Bigalke and Habermann, 1980).

Analyses of results. All data are presented as mean ± SEM. Contractile responses of a given tissue to bethanachol or carbachol were normalized to the maximum contraction produced by barium in that tissue. Dose-response curves

were constructed and EC_{50} values (the apparent affinity) were determined from individual dose-response curves. Geometric (- log EC_{50}) means were then calculated and compared statistically (Fleming, et. al., 1972) using a one-way analysis of variance (ANOVA) and least significant difference test (Steele and Torrie, 1980) when the ANOVA was significant ($p < 0.05$). Two separate LSD analyses were performed: 1) bethanachol- and carbachol-induced contractile responses of naive versus 15 and 30 day MD responses, and 2) carbachol-induced contractile responses of a given tissue (naive, 15 or 30 day MD responses) in the presence and absence of neuronal antagonists.

Drugs. BAC (Zephiran chloride) was purchased from a local pharmacy. Bethanachol, carbachol, hexamethonium bromide and TTX were obtained from Sigma Chemical Co. (St. Louis, MO). Barium chloride was purchased from Mallinckrodt Chemical Works (St. Louis, MO). Purified BTA was kindly provided by Dr. E. J. Schantz, Food Research Institute, University of Wisconsin-Madison. Carbachol, bethanachol and barium were dissolved in 0.9% saline and prepared freshly on the day of the experiment. These agonists were added to the tissue baths in a volume equal to 1 % of the bath volume.

Results

Responses to cholinergic agonists. Both bethanachol (Figure 1A) and carbachol (Figure 1B) produced concentration dependent contractions of both naive and MD circular muscle. The apparent affinities and maximal responses of bethanachol and carbachol are presented in Table 1. There were no significant differences between naive and MD circular muscle 15 or 30 days after denervation in apparent affinities or maximal responses to bethachol.

Fifteen days after denervation, the apparent affinity to carbachol of MD circular muscle was significantly decreased (2.8 - fold) relative to naive circular muscle, but was comparable to naive 30 days after denervation. The maximal responses to MD circular muscle to carbachol 15 and 30 days after denervation were significantly greater than that of naive circular muscle.

Effects of neuronal antagonists on carbachol responses. The effects of hexamethonium bromide, TTX, BTA and the combination of BTA and hexamethonium on the carbachol dose-response curves of naive and MD circular muscle 15 and 30 days after denervation are shown in Figures 2 - 5, respectively. The apparent affinities and maximal responses of the tissue in the presence of neuronal antagonists are presented in Table 1. A description of the effects of the

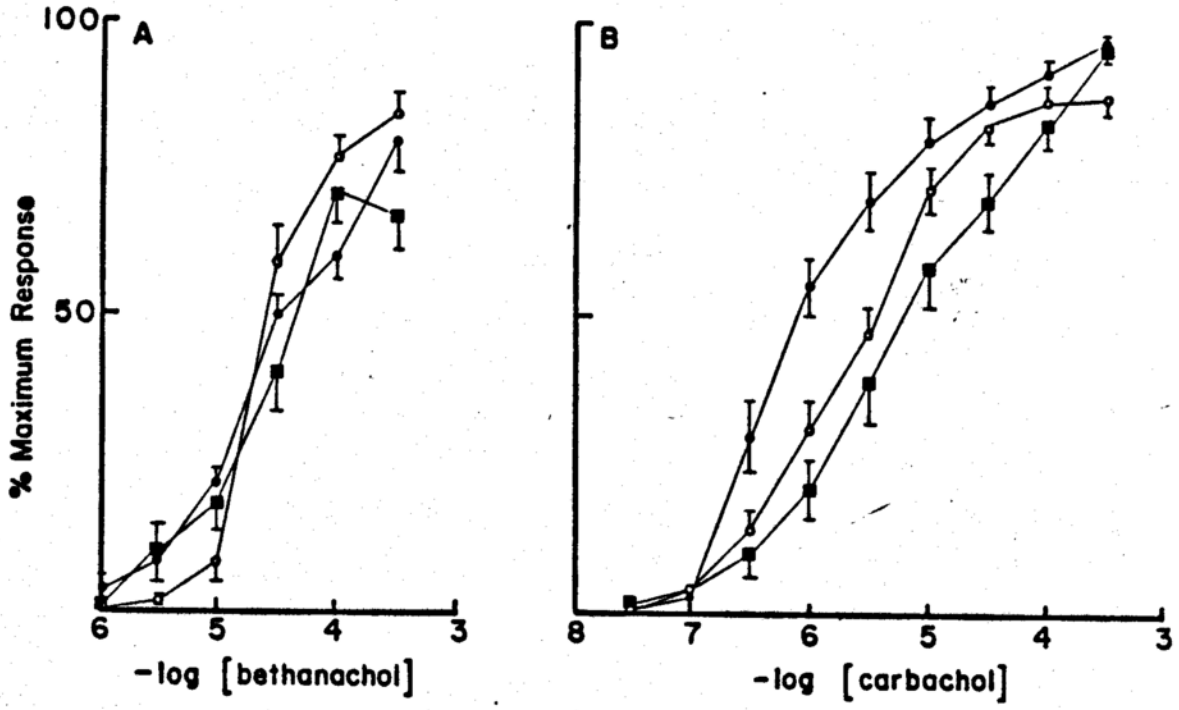

Figure 1. Responses to bethanachol (A) and carbachol (B) of naive (○) and myenterically denervated circular muscle 15 (●) and 30 days (■) after myenteric denervation. Each point represents the mean ± SEM of responses of 6 - 27 rats. Data are expressed as percent of maximum response produced by 10^{-2} M barium chloride.

Table 1. Apparent affinities and maximal responses of jejunal circular muscle from naive and myenterically denervated rats to cholinergic agonists in the presence and absence of neuronal antagonists. Number in parenthesis indicates the number of rats used in a given experiment Hex = 10^{-4} M hexamethonium bromide, TTX = 3 x 10^{-7} M tetrodotoxin, BTA = 8 g/ml Botulinum Toxin A.
* significantly different from naive, $p < 0.05$.
# significantly different from carbachol response in the absence of neuronal antagonist, $p < 0.05$.

Table 1

Apparent Affinities and Maximal Responses to Cholinergic Agonists of Jejunal Circular Muscle from Naive and Myenterically Denervated Rats

	- Log EC50			% Maximum Response		
		Denervated			Denervated	
Agonist	Naive (n)	Day 15 (n)	Day 30 (n)	Naive (n)	Day 15 (n)	Day 30 (n)
Bethanachol	4.67 ± 0.07 (6)	4.69 ± 0.04 (6)	4.56 ± 0.12 (12)	84.2 ± 4.0 (6)	79.7 ± 4.4 (6)	74.3 ± 5.7 (12)
Carbachol	5.61 ± 0.09 (27)	6.05 ± 0.11 (16)*	5.32 ± 0.13 (15)	87.3 ± 3.0 (27)	97.2 ± 1.2 (16)*	96.1 ± 1.8 (15)*
" + Hex	6.50 ± 0.08 (6)#	6.15 ± 0.15 (6)	6.14 ± 0.18 (6)#	98.4 ± 0.8 (6)	96.5 ± 2.2 (6)	98.2 ± 0.9 (6)
" + TTX	6.03 ± 0.16 (5)#	6.23 ± 0.15 (6)	6.51 ± 0.12 (6)#	98.0 ± 2.0 (5)	100.0 (6)	100.0 (6)
" + BTA	5.77 ± 0.11 (6)	6.06 ± 0.16 (6)	5.86 ± 0.15 (6)#	86.4 ± 5.2 (6)	88.7 ± 2.1 (6)#	84.5 ± 5.7 (6)#
" + BTA/ ± Hex	5.11 ± 0.18 (6)#	6.11 ± 0.15 (6)*	5.74 ± 0.09 (6)*,#	70.3 ± 10.7 (6)#	93.1 ± 2.9 (6)*	99.7 ± 0.4 (6)*

* significantly different from native, $p < 0.05$.

significantly different from carbachol in the absence of neuronal antagonist, $p < 0.05$.

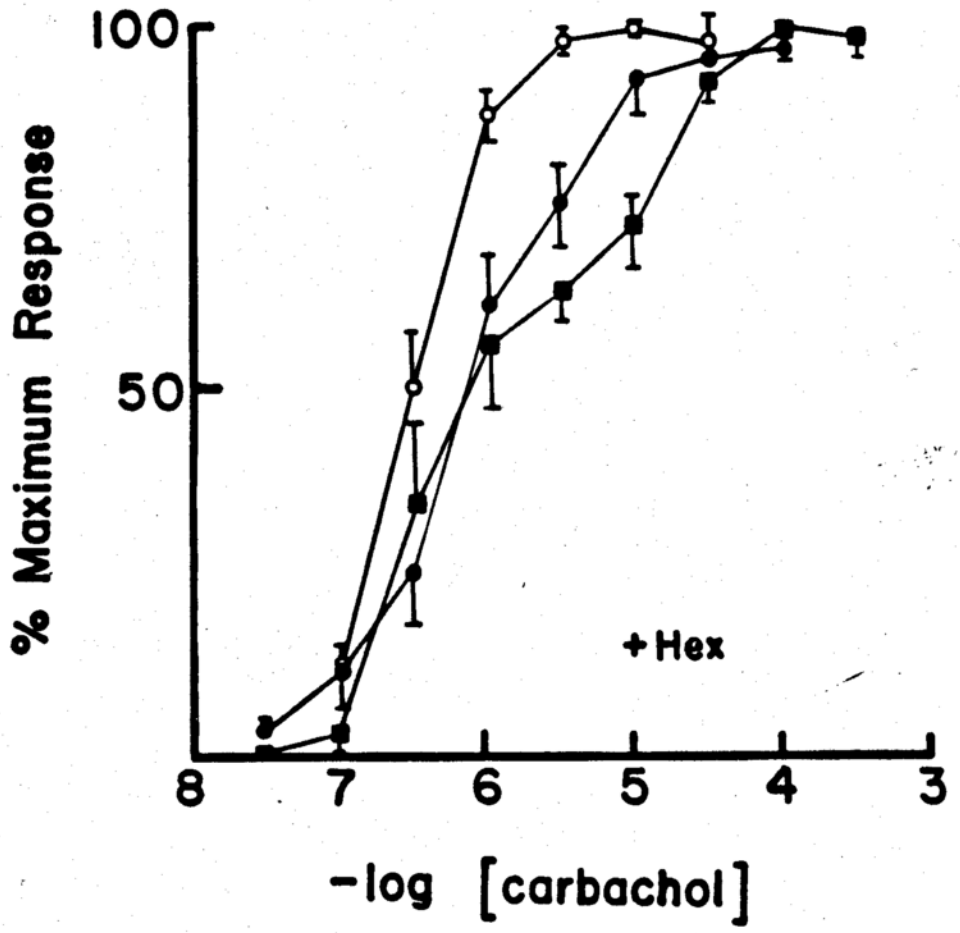

Figure 2. Responses to carbachol of naive (○) and myenterically denervated circular muscle 15 (●) and 30 days (■) after myenteric denervation in the presence of 10^{-4} M hexamethonium bromide. Each data point represents the mean ± SEM of responses of 5 - 6 rats. Data are expressed as percent of the maximum response produced by 10^{-2} M barium chloride.

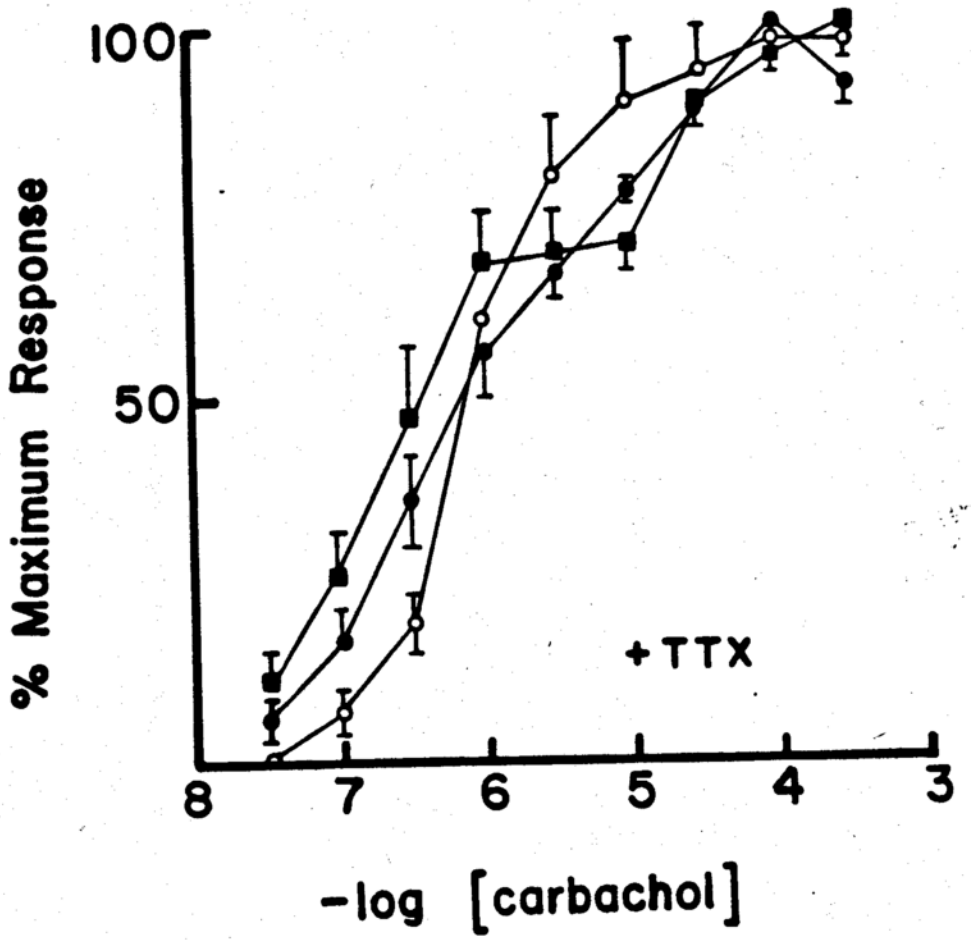

Figure 3. Responses to carbachol of naive (○) and myenterically denervated circular muscle 15 (●) and 30 days (■) after myenteric denervation in the presence of 3×10^{-7} M tetrodotoxin. Each data point represents the mean ± SEM of responses from 5 - 6 rats. Data are presented as percent of the maximum response produced by 10^{-2} M barium chloride.

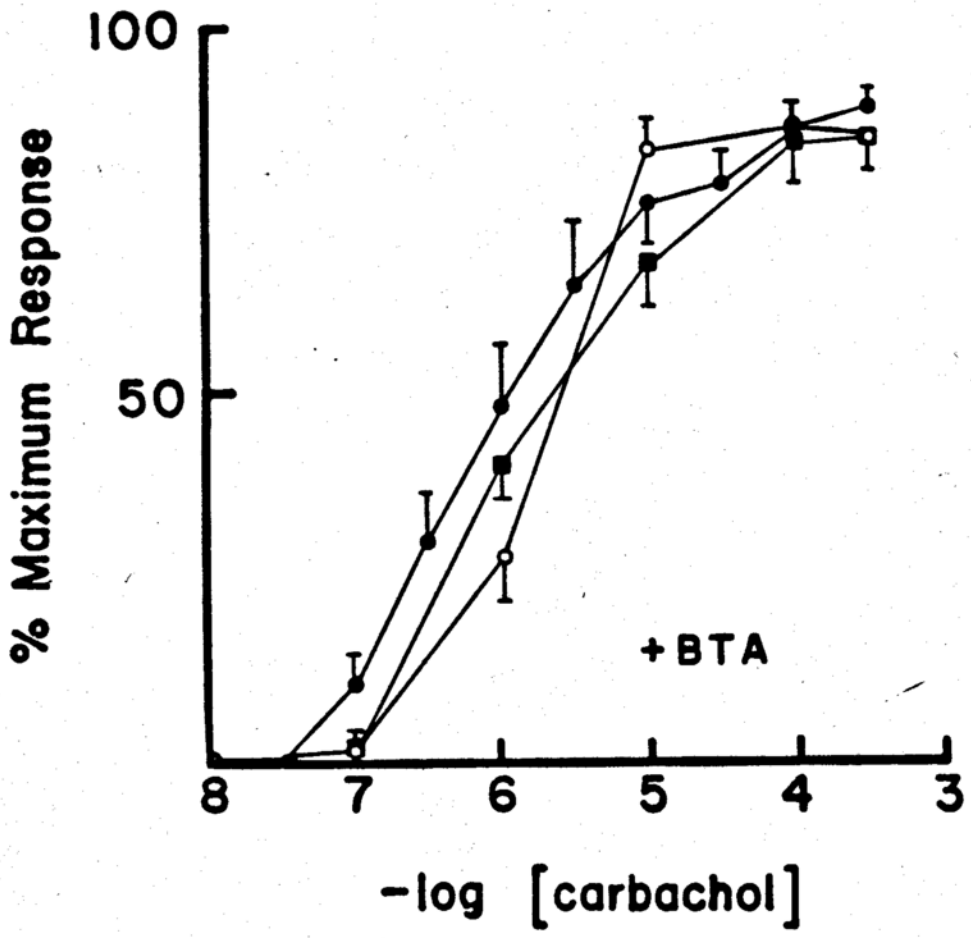

Figure 4. Responses to carbachol of naive (○) and myenterically denervated circular muscle 15 (●) and 30 days (■) after myenteric denervation following pretreatment with 8 g/ml Botulinum Toxin A. Each data point represents the mean ± SEM of responses from 5 - 6 rats. Data are expressed as a percent of the maximum response produced by 10^{-2} M barium chloride.

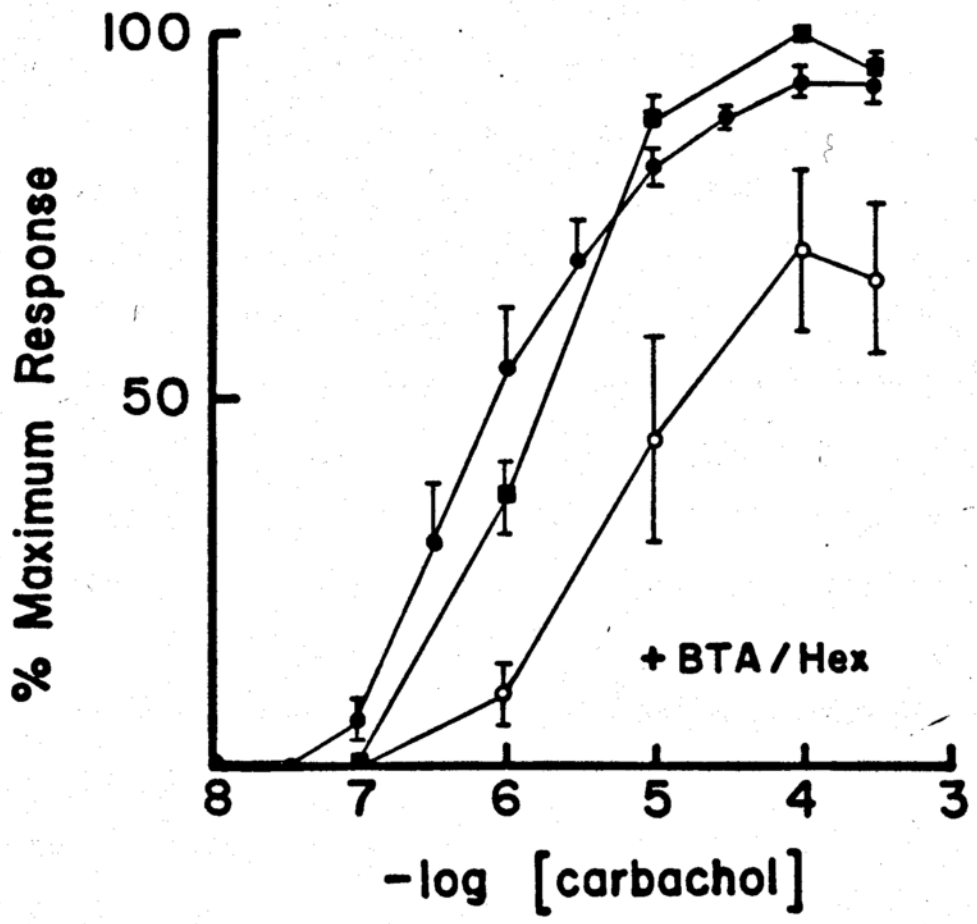

Figure 5. Responses to carbachol of naive (○) and myenterically denervated circular muscle 15 (●) and 30 days (■) after myenteric denervation following pretreatment with 8 g/ml Botulinum Toxin A and in the presence of 10^{-4} M hexamethonium bromide. Each data point represents the mean ± SEM of responses from 5 - 6 rats. Data are expressed as percent of the maximum response produced by 10^{-2} M barium chloride.

antagonists on naive and MD tissue 15 and 30 days after myenteric denervation follows.

Naive circular muscle. The presence of the nicotinic antagonist hexamethonium bromide significantly decreased (7.7-fold) the apparent affinity to carbachol of naive circular muscle. Likewise, the presence of TTX significantly reduced the apparent affinity to carbachol, but the magnitude of the reduction was only 2.6-fold. Pretreatment of naive tissues with Botulinum Toxin A (BTA), which prevents neuronal release of acetylcholine, had no effect on the apparent affinity of the tissue to carbachol. However, pretreatment with BTA and the presence of hexamethonium significantly increased the apparent affinity to carbachol in naive circular muscle. This combination of BTA and hexamethonium also significantly reduced the maximal responses of naive tissue to carbachol.

MD circular muscle 15 days after denervation. The neuronal antagonists had no effect on the apparent affinity to carbachol of circular muscle 15 days after myenteric denervation. The maximal responses of the tissue were slightly, but significantly reduced following pretreatment with BTA. These results suggest the absence of neuronal influences on circular muscle 15 days after myenteric denervation.

MD circular muscle 30 days after denervation. All of

the neuronal antagonists used significantly altered the apparent affinity to carbachol of MD circular muscle 30 days after myenteric denervation. The reduction in apparent affinity (6.6-fold) observed in the presence of hexamethonium in MD circular muscle was comparable to that observed in naive tissue. In contrast, the presence of TTX produced a greater reduction of the apparent affinity to carbachol in MD tissues 30 days after myenteric denervation (15.5-fold) than it did in naive tissue (2.6-fold). BTA pretreatment, which had no effect on the apparent affinity to carbachol in naive tissue, significantly reduced (3.5-fold) the apparent affinity in MD tissue 30 days after denervation. In contrast to the increase in apparent affinity (3.2-fold) in naive circular muscle produced by BTA pretreatment and hexamethonium, the apparent affinity to carbachol of MD circular muscle 30 days after myenteric denervation was significantly decreased (2.6-fold). Thus, 30 days after myenteric denervation, there appears to be a neuronal influence on circular muscle that is both quantitatively and qualitatively different than that in naive tissue.

Discussion

Changes in the apparent affinity to carbachol of

circular muscle following myenteric denervation may be the result of changes in muscarinic receptors on the muscle, or may be due to alterations in the neuronal (primarily nicotinic) component of the carbachol-induced response. The responses of naive and MD circular muscle to bethanachol indicated that muscarinic sensitivity of circular muscle was unaffected following myenteric denervation. Thus, the changes observed in the carbachol-induced contractile responses of MD circular muscle (Herman and Bass, 1988) must reflect alterations in the neuronal (nicotinic) component of the action of carbachol. The apparent decrease in affinity of circular muscle to carbachol 15 days after denervation is due to the loss of myenteric innervation. This conclusion is supported by three lines of evidence. First, the presence of neuronal antagonists (hexamethonium, TTX, BTA) had no effect on the apparent affinity to carbachol in MD circular muscle 15 days after denervation. Second, in the presence of TTX, the apparent affinity to carbachol of naive circular muscle was equal to that of 15 day MD circular muscle. Finally, in the presence of TTX, the maximal responses to carbachol in naive tissue was higher than that obtained in the absence of TTX, and comparable to those of MD tissue. The increases in the maximal responses of naive circular muscle is probably due to blockade of release of inhibitory transmitters(s) from myenteric neurons which

would normally antagonize the maximal contractile responses produced by carbachol.

The responses of MD circular muscle to carbachol were comparable to those of naive muscle 30 days after denervation. This return of normal responsiveness is not due to changes in the muscarinic actions of carbachol, since the bethanachol data indicated that muscarinic responses in naive and MD circular muscle are not different. Thus, the return of normal responsiveness to carbachol must reflect changes in the neuronal (nicotinic) component of the action of carbachol. It is well established that the sensitivity of denervated, supersensitive end organs returns to normal following reinnervation (Cannon and Rosenbleuth, 1949; Simeone, 1937; Vera, et. al., 1957; Luco and Vera, 1964; Ekstrom and Emmelin, 1974). Therefore, it appears that functional reinnervation of circular muscle occurred between 15 and 30 days after myenteric denervation. Further evidence to support the idea of functional reinnervation is provided by the studies in which neuronal antagonists were used. These antagonists (hexamethonium, TTX, BTA) had no effect on the apparent affinity to carbachol of MD circular muscle 15 days after denervation, but significantly altered the apparent affinity to carbachol of MD circular muscle 30 days after denervation. In the absence of neuronal influences, these antagonists would not be expected to alter

tissue responses.

Although the responses of MD circular muscle 30 days after denervation were altered by neuronal antagonists, the alterations were both quantitatively and qualitatively different than those produced by the antagonists in naive tissue. The presence of TTX resulted in a 3-fold leftward shift in the dose response curve of naive circular muscle, while the magnitude of the shift in MD circular muscle 30 days after denervation was 15-fold. Pretreatment of tissue with BTA had no effect on the responses of naive tissue, but significantly lowered the apparent affinity to carbachol in MD circular muscle 30 days after denervation. Pretreatment with BTA and the presence of hexamethonium significantly increased the apparent affinity to carbachol in naive circular muscle (3-fold), but significantly decreased the apparent affinity (3-fold) in MD tissue 30 days after denervation. In addition, BTA pretreatment and hexamethonium lowered the maximal responses obtained in naive tissue relative to those observed in the absence of antagonists, while the maximal response of MD circular muscle 30 days after denervation were unaffected. Thus, the responses of naive and MD circular muscle 30 days after denervation exhibited considerable pharmacologic differences, which suggests that the innervation of the tissues is different.

We believe that the reinnervation observed in MD circular muscle 30 days after denervation is due to neurons located in the submucosal plexus. As noted, there are pharmacologic differences between naive and MD circular muscle 30 days after denervation with respect to the effects of neuronal antagonists. In morphological studies with MD tissue, neuronal cell bodies have never been observed in the myenteric region, even 165 days after treatment (Fox, et. al., 1983; See, et. al., 1988). Immunohistochemical studies likewise do not reveal neuronal cell bodies or intrinsic or extrinsic nerve fibers in the myenteric region or muscle after denervation (Fox, et. al., 1983; unpublished observations). Sprouting of nerve fibers from adjacent, innervated bowel has not been observed, nor is considered likely given the slow growth of myenteric nerve fibers after lesioning (Furness et. al., 1983). Therefore, the submucosal neurons are the only source of the functional innervation of circular muscle observed 30 days after myenteric denervation. Interestingly, increases in neurotransmitter syntheses, presumably by submucosal neurons, have been observed in MD tissue (Dahl, et. al., 1987). Although there is no direct morphologic evidence of reinnervation of circular muscle by neurons in the submucosal plexus after myenteric plexus ablation, the presence of neuronal fibers in the muscle is not a strict

requisite for innervation. It is well established that in the small bowel of most rodents, there are few, if any, nerve fibers in the longitudinal muscle. Presumably, neurotransmitters released from myenteric neurons diffuse to the longitudinal muscle to produce their effects (Gabella, 1987). In an analogous manner, neurotransmitters could diffuse from submucosal neurons to circular muscle following myenteric denervation, and thus account for functional innervation.

In a previous study (Herman and Bass, 1988), MD circular muscle exhibited subsensitive responses to carbachol relative to naive tissue 45 days after denervation. Subsensitive responses of hyperinnervated intestinal muscle have been previously reported (Kuzimicheva, et. al., 1973), and increased or abnormal (submucosal) innervation of circular muscle could account for the subsensitive response of MD circular muscle to carbachol 45 days after denervation.

The motor control of circular muscle is generally believed to be due to neurons located within the myenteric plexus (Wood, 1987; Furness and Costa, 1987). However, recent morphologic (Ekblad, et. al., 1984) and electrophysiologic (Sanders and Smith, 1986) evidence suggest that neurons of the submucosal plexus may innervate and influence circular muscle function. The results of the

present study also suggest a role for submucosal neurons in the control of circular muscle function, in this case, after myenteric denervation. It may prove useful to assess the relationship between circular muscle and submucosal neurons in human conditions, such as pseudoobstruction, in which lesions of the myenteric plexus are observed. (Dyer, et. al., 1969; Tanner, et. al., 1976; Smith, 1982; Krishnamurthy, et. al., 1986; Krishnamurthy and Schuffler, 1987).

Finally, the effects of BTA pretreatment on naive tissue deserves comment. BTA pretreatment alone did not affect the apparent affinity of naive circular muscle for carbachol. However, in BTA pretreated naive tissue, the presence of hexamethonium significantly increased the apparent affinity for carbachol and decreased the maximal responses. Hexamethonium alone decreased the apparent affinity of naive tissue for carbachol. Thus, it appears that in naive tissue, blockade of both ACh release and nicotinic receptors resulted in increased inhibitory neuronal influence on circular muscle, while blockade of nicotinic receptors alone resulted in decreased inhibitory neuronal influence. This suggests that release of ACh within the myenteric ganglia is responsible, either directly or indirectly via interneurons, for inhibition of inhibitory nerves which project to circular muscle.

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Chapter 4

Enteric Neuronal Ablation: Structure-Activity Relationship in a Series of Alkyldimethylbenzylammonium chlorides (ADBAC)

Abstract

Serosal application of a commercial solution of benzalkonium chloride (BAC) has been shown to selectively ablate myenteric neurons in the rat jejunum. This experimental model has proven useful in the study of the role of the myenteric plexus in intestinal function. Commercial BAC is a mixture of at least three homologous N-alkyldimethylbenzylammonium chlorides (ADBAC). The purpose of this study was to determine neuronal ablative activity in a series of ADBAC homologs when applied at equimolar concentrations on the serosa of rat jejunum. Homologs not commercially available were synthesized and purified. Seven ADBAC homologs with alkyl chain lengths ranging from C6 to C18 were tested. All ADBAC homologs, except C18, ablated neurons of the myenteric plexus without affecting the number of submucosal neurons. Intestinal smooth muscle was thickened after treatment. The structure-activity relationship observed in this study paralleled that of the reported antimicrobial activity of the ADBAC homologs, and is related to the aqueous solubility and relative surface activities of the homologs. The C14 homolog was found to be the most effective ablative agent, and reduced the number of myenteric neurons in a concentration-dependent manner. Thus, the C14 homolog can be used to produce a selectively

denervated jejunal model for use in acute or chronic *in vitro* or *in vivo* studies of intestinal function.

INTRODUCTION

The enteric nervous system (ENS) consists of two major ganglionated nerve plexuses located within the walls of the gastrointestinal (GI) tract. The myenteric, or Auerbach's plexus is a ganglionated plexus located between the longitudinal and circular smooth muscle layers. Nerve fibers from myenteric neurons project to the musculature, submucosa and mucosa. It is generally believed that the activity of the musculature is controlled by neurons within the myenteric plexus (Furness and Costa, 1987). The submucosal, or Meissner's plexus, is a ganglionated plexus located within the submucosal region. Nerve fibers from submucosal neurons project to the myenteric plexus, other submucosal ganglia and the mucosa. Neurons located in the submucosal plexus are believed responsible for the regulation of absorptive and secretory activity of the GI tract (Hubel, 1978). The ENS more closely resembles the central nervous system than other peripheral nervous elements with respect to neuronal and glial cell morphology, and the number of neuromodulators present. In addition, the ENS continues to function in the absence of central nervous system input. The interested reader is referred to the excellent monograph on the ENS by Furness and Costa (1987) for further details.

Efforts to study the role of the ENS in GI function have been hampered by the lack of an effective method to selectively ablate enteric neurons. Surgical methods, which have proven useful in studies of the central and peripheral nervous systems, have limited usefulness in the study of the ENS because of the location of the neurons within the wall of the bowel. In addition, studies on surgically denervated intestine (Paton and Zar, 1965; Paton and Zar, 1968) are generally limited to acute, in vitro experiments. Prolonged anoxia (Hukahara, 1961) or close intraarterial profusion of toxicants (McElhannon, 1959; Okamoto et. al., 1963) can alter the function of the ENS, but the effects are often variable (Nagata and Steggerda, 1963) and the methods technically difficult in small experimental animals. Also, it is not possible to selectively ablate either myenteric or submucosal neurons with the anoxia or infusion techniques. Sato, et. al. (1978) demonstrated that serosal exposure of rat anorectum and colon to a solution of benzalkonium chloride (BAC) destroyed enteric ganglia, although quantitative neuronal cell counts were not reported. Fox et. al. (1983) found that serosal application of BAC ablated, in a concentration-dependent manner, the number of neurons in the myenteric plexus without affecting the number of neurons in the submucosal plexus. Intestinal smooth muscle was thickened, but otherwise appeared normal. This

technique has several advantages over previous attempts of enteric denervation. First, because submucosal neurons are intact, the role of the myenteric plexus in intestinal function can be evaluated. Second, both in vivo and in vitro acute or chronic studies can be performed. Finally, the technique is simple, inexpensive, reproducible and applicable to small experimental animals.

The myenterically denervated model has been used to determine the site(s) of action of putative enteric neurotransmitters (Fox et. al., 1986) and the distribution of adrenergic receptor subtypes in the rat jejunum (Fox and Bass, 1986a). Recording of in vivo electrical activity of control and myenterically denervated portions of the rat jejunum have shown that the migrating myoelectric complex propagates through the denervated segment in the absence of a detectable basic electric rhythm (Fox and Bass, 1984). Herman and Bass (1988a) have demonstrated changes in neuronal pathways which modulate intrinsic intestinal reflexes after localized myenteric denervation. Submucosal neurons, which normally do not directly affect circular muscle function, apparently do so after myenteric denervation (Herman and Bass, 1988b). The latter studies may provide insight into human clinical conditions associated with the loss of myenteric innervation (for review, see Krishnamurthy and Schuffler, 1987).

Benzalkonium chloride is a homologous mixture of N-alkyldimethylbenzylammonium chlorides with N-alkyl groups varying from C6 to C18. Commercially, BAC is produced by the reaction of benzyl chloride and coconut oil, and thus the composition may vary from product to product and batch to batch. These compounds, due to their surfactant properties, are used commercially in cosmetics, lubricants, paints, soil stabilizers, and disinfectants. In addition, they are relatively non-toxic (Cutler and Drobeck, 1970). There are, however, considerable differences between the homologs in both antimicrobial (Ross, et. al., 1953; Cutler, et. al., 1967b; Daoud, et.al., 1983) and protein binding (Perrin and Nelson, 1974) activities.

The commercial preparation (Zephiran chloride) used by Fox et. al. (1983) was reported by the manufacturer to consist of the following ratio of homologs: C14 (50%), C12 (40%) and C16 (10%). The purpose of this study was to determine which of these homologs were responsible for the myenteric neuron ablation observed by Fox et. al. (1983). We also examined the myenteric neuron ablative ability of other homologs (C6, C8, C10, C12, C18) which may be minor contaminants in the commercial product. Homologs which were not commercially available were synthesized and purified. The results of serosal application of these homologs indicated that the myenteric neuron ablation structure-

activity relationship paralleled that of the antimicrobial activity of the compounds. The C14 homolog was found to be the most effective ablative agent. The ablative activity of these homologs apparently could be explained on the basis of their physical-chemical properties. Thus, we have identified the most potent ablative agent in the BAC mixture and can use it instead of commercial mixtures in future studies of enteric neuronal ablation.

Materials and Methods

Materials. Benzyldimethyltetradecylammonium (C14) chloride (99 % +) and benzyldimethylstearylammonium (C18) chloride (90 %, remainder C16 homolog) were purchased from Aldrich Chemical Co., Milwaukee, WI. Benzyldimethylhexadecylammonium (C16) chloride (99 % +) was kindly provided by Dr. S. L. Abidi, U.S. Fish and Wildlife Service, National Fishery Research Laboratory, La Crosse, WI. The remainder of the ADBAC homologs were not commercially available, nor available from the Sterling-Winthrop Research Institute(Rensselaer, NY), the source of the homologs for previous investigators (Blois and Swarbuck, 1972a,b; Perrin and Nelson, 1974; Lein and Perrin, 1976; Tomlinson, et. al., 1977). These homologs were synthesized as described below.

Synthesis, analyses of ADBAC homologs. The C6, C8, C10 and C12 ADBAC homologs were synthesized by refluxing the corresponding N-alkyldimethylamines (all obtained from Aldrich Chemical Co., except 1-dimethylaminodecane, which was obtained from Alfa Products, Danvers, MA) and benzyl chloride (Aldrich) in an appropriate solvent (acetonitrile, ether, ether/ethanol, benzene or ethyl acetate) for 4 - 24 hrs (Abidi, 1980; Cutler, et. al., 1967a). The monohydrate salts of the ADBAC homologs, with the exception of the C6 homolog, were formed by the quantitative addition of distilled, deionized water. The resulting product was recrystallized twice. The C6 homolog was isolated as the chloride salt and recrystallized twice from ethyl acetate. Melting points for the ADBAC homologs corresponded to those previously reported (Cutler, et. al., 1967a). Samples of the homologs were also analyzed using a C18 reverse phase HPLC method described by Abidi (1985) or ^{13}C Fourier Transform NMR (JEOL FX 90Q, JEOL Ltd, Tokyo, Japan) to confirm structure and determine purity.

Serosal application of ADBAC homologs. Male rats, 200 - 225g (Sprague-Dawley, Inc., Madison, WI) were anesthetized by an ip injection of a pentobarbital-chloral hydrate anesthetic (0.3ml/100 g body wt). A 3 - 4 cm portion of jejunum, 6 - 8 cm caudad to the ligament of Trietz, was exteriorized through a sall midline incision. The area of

intestine to be treated was delineated with two serosal sutures placed at the mesenteric border. ADBAC homolog (2 mM solution in saline) was applied to the serosal surface of the delineated segment every 5 min for 30 min (6 applications). After treatment, the bowel was thoroughly rinsed with saline and returned to the peritoneal cavity. The midline incision was closed, and the animals allowed to recover. Animals were housed singly in stainless steel wire mesh bottomed cages, and food and water were available ad libitum. A 12-hr light/dark cycle was maintained. Three rats were treated with each homolog.

Additional rats (3 - 9) were treated similarly with 0.5, 1.0, 2.0 or 4.0 mM C14 homolog to determine the concentration-response relationship.

Histologic examinations. Fifteen days after treatment, rats were sacrificed by a blow to the head followed by cervical dislocation. Treated segments were dissected, rinsed with phosphate-buffered saline (PBS) and fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) at 4° C for 12 - 16 hrs. Tissues were then rinsed with PBS and dehydrated through a graded series of ethanols, embedded in a glycol methacrylate embedding media (JB-4, Polysciences, Warrington, PA) and sectioned at 2 m. Sections were then stained with methylene blue-basic fuchsin. The number of myenteric and submucosal neurons per mm jejunum was

determined in three separate sections of intestine from each animal. The thicknesses of the longitudinal and circular muscle layers were measured with an ocular micrometer. Naive, age-matched rats from the same shipment as the treated rats were used as controls.

Analyses of results. All data are presented as mean ± SEM. A one-way analysis of variance (ANOVA) was used to determine significant differences between control and treated rats in neuronal cell counts and muscle thicknesses. When the ANOVA was significant ($p < 0.05$), the data were analyzed further using a two-sided Dunnett's test to identify which groups differed significantly from control (Steele and Torrie, 1980). Again, p values less than 0.05 were considered significant.

Results

ADBAC homolog analyses. The results of the analyses confirmed the structure of the ADBAC homologs, and along with the HPLC data, indicated purities of at least 95 - 99 %. The ^{13}C FT-NMR spectra and HPLC tracing of the dodecyldimethylbenzylammonium chloride (C12) homolog shown in Figures 1 and 2, respectively, are representative of the data obtained with the other ADBAC homologs synthesized. The conditions of synthesis and analyses of the homologs

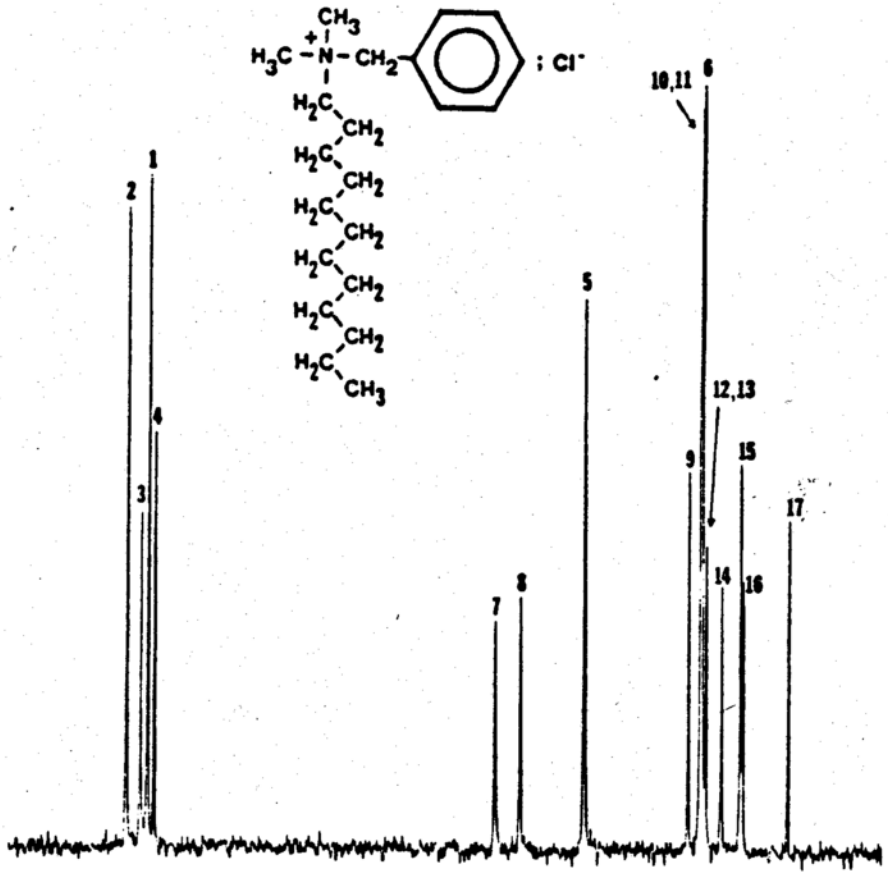

Figure 1. ^{13}C Fourier Transform NMR spectra and structure of dodecyldimethylbenzylammonium chloride (C12). Peaks 1 - 4 represent carbon atoms in the benzene ring. Peak 5 represents the alpha-methylene carbons attached to the nitrogen atom, while peak 6 represents the two methyl carbons attached to the nitrogen atom. Peaks 7 - 17 represent the remaining carbon atoms in the alkyl side chain.

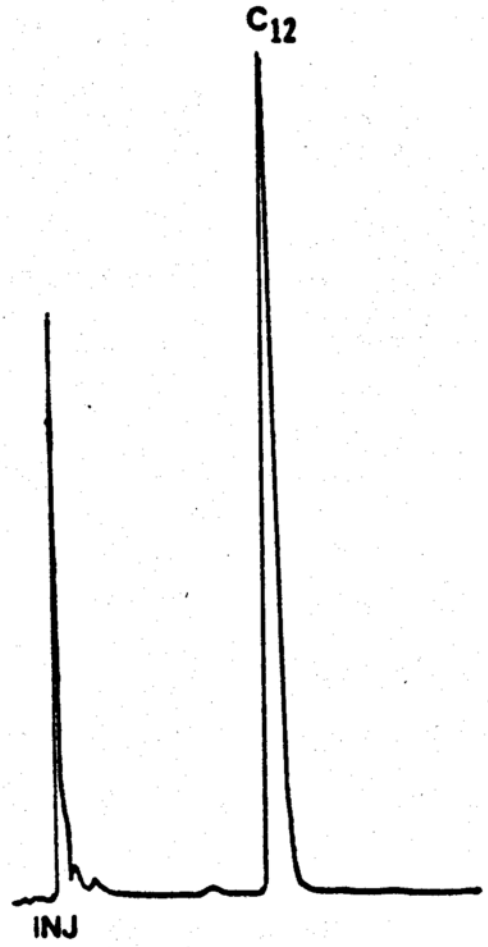

Figure 2. Reverse-phase HPLC chromatogram of dodecyldimethylbenzylammonium chloride (C12). Column: Varian MCH-10 (30 cm x 4.5 mm, 10 m particle size). Mobile phase: acetonitrile-water (7:3), 0.1 M sodium perchlorate, pH 3.0. Detected at 215 nm.

prepared are detailed in Appendix B.

Neuronal cell counts. Neuronal cell counts per mm jejunum were performed on similar sections from all ADBAC homolog treated rats. The number of myenteric neurons per mm jejunum is presented in Figure 3. As the number of carbon atoms in the homologs increased from C6 to C14, the number of myenteric neurons ablated increased to a maximum of 96 % destruction observed with 2 mM C14 homolog. [This is similar to the results obtained with the BAC mixture (Fox, et. al., 1983)]. The C12 homolog was nearly as effective as the C14 homolog in ablating myenteric neurons, but appeared to cause more muscle dyplasia and fibrosis than did the C14 homolog. The C16 homolog ablated about 40% of the myenteric neruons, while the C18 homolog had no effect on the number of myenteric neurons.

In contrast to the ablative effects of the ADBAC homologs on myenteric neurons, the homologs (at 2 mM) had no significant effect on the number of submucosal neurons per mm jejunum (Figure 4).

Smooth muscle thicknesses. Treatment of rat jejunum with 2 mM ADBAC homologs resulted in significant thickening of both longitudinal (Figure 5) and circular muscle (Figure 6) layers. The C18 homolog, which had no effect on the number of myenteric neurons, did not produce muscle

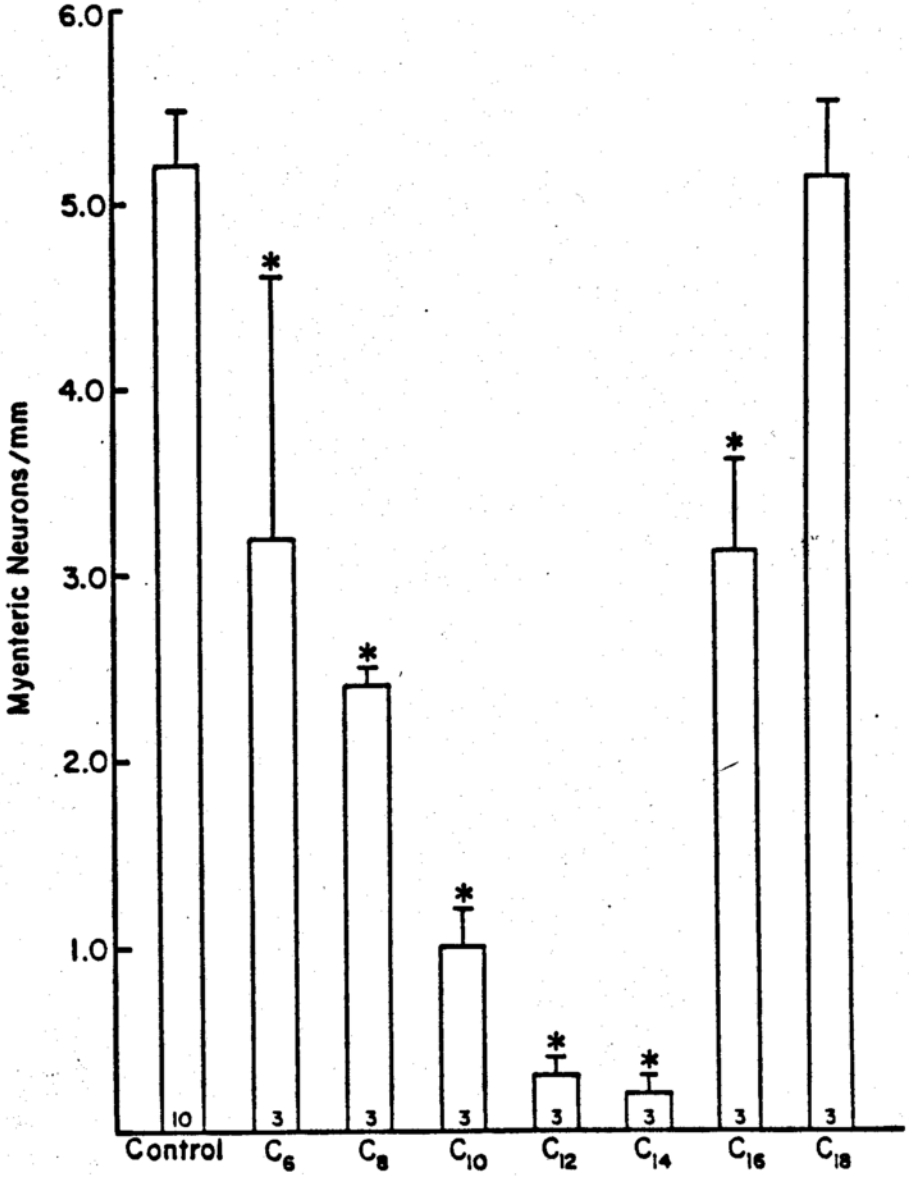

Figure 3. Myenteric neurons/mm jejunum in control and ADBAC homolog treated rats. Rats were treated with 2 mM of each homolog as described in the methods. Data are means ± SEM of the number of rats indicated in the bars. * Significantly different from control mean using Dunnett's test, two-sided, $p < 0.05$.

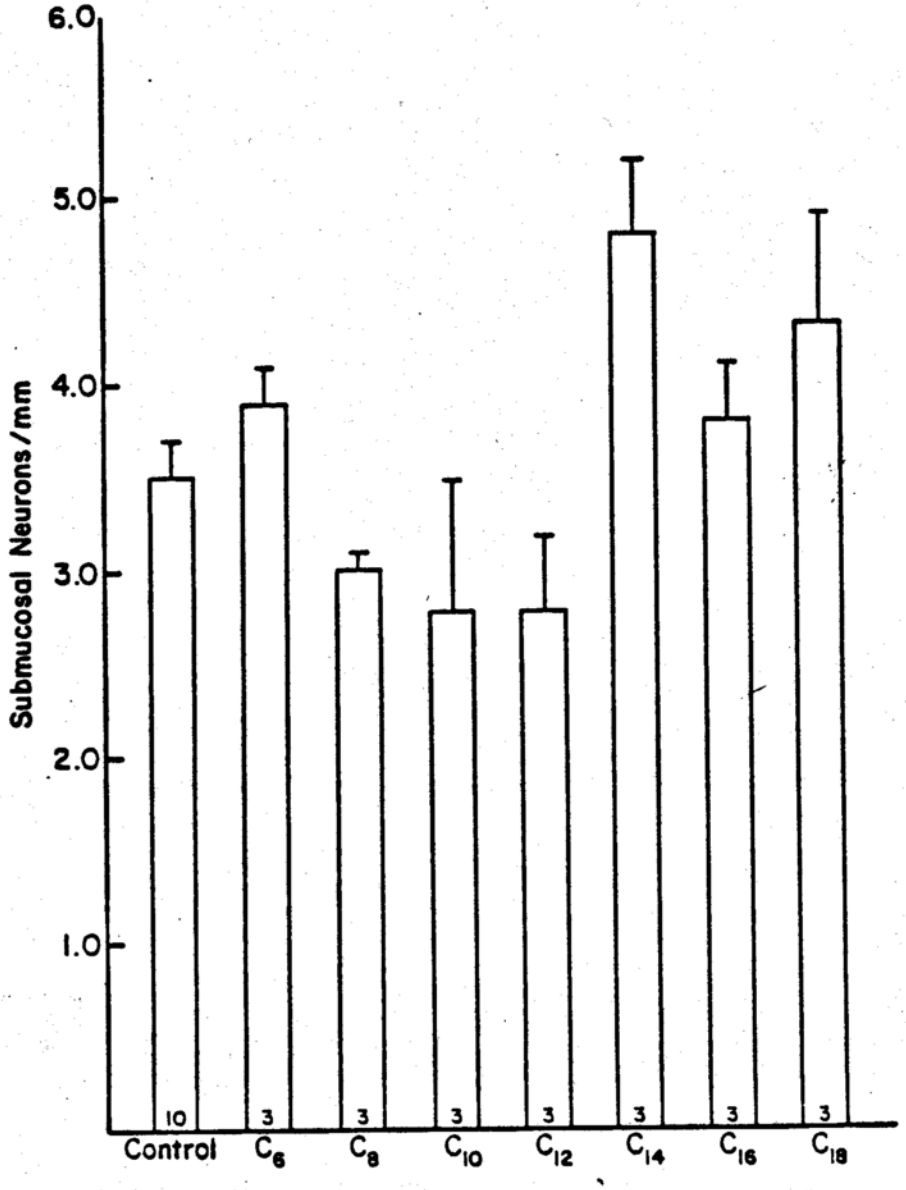

Figure 4. Submucosal neurons/mm jejunum in control and ADBAC treated rats. Rats were treated with 2 mM of homolog as described in the methods. Data are means ± SEM of the number of rats indicated in the bars.

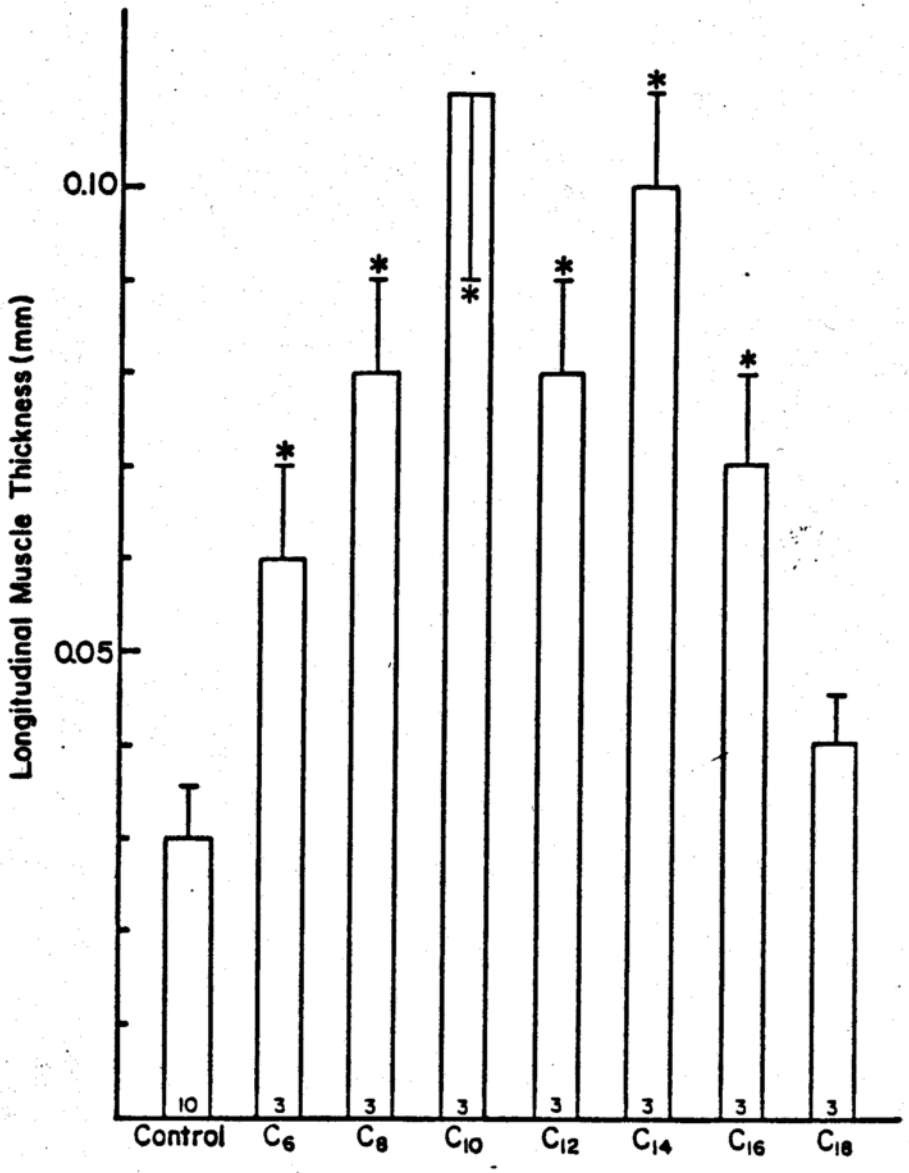

Figure 5. Longitudinal muscle thicknesses of control and ADBAC treated rats. Rats were treated with 2 mM of homolog as described in the methods. Data are means ± SEM of the number of rats indicated in the bars. * Significantly different from control mean using Dunnett's test, two-sided, $p < 0.05$.

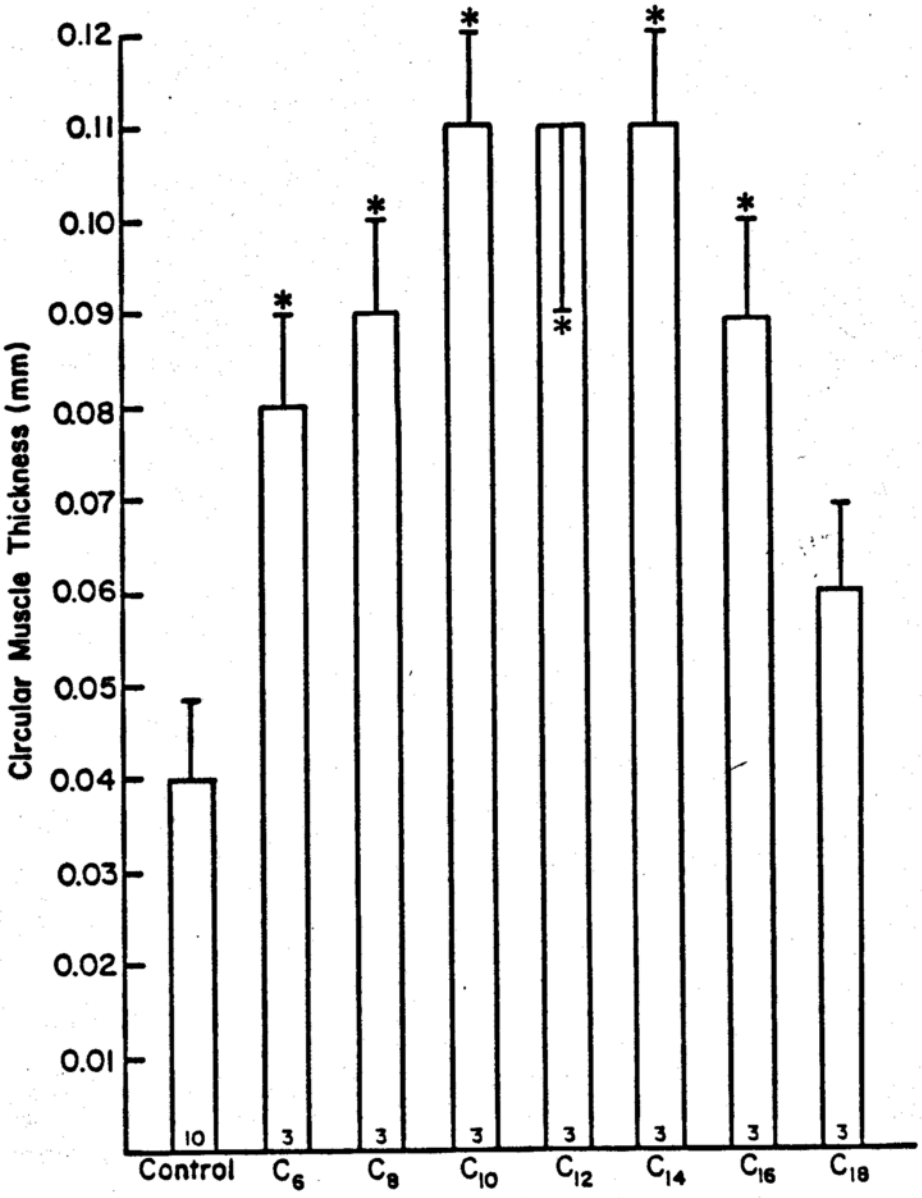

Figure 6. Circular muscle thicknesses of control and ADBAC treated rats. Rats were treated with 2 mM of each homolog as described in the methods. Data are means ± SEM of the number of rats indicated in the bars. * Significantly different from control mean using Dunnett's test, two-sided, $p < 0.05$.

thickening.

C14 homolog concentration response. The effects of 0.5 to 4.0 mM C14 homolog on the number of myenteric and submucosal neurons is shown in Figure 7. A concentration-dependent reduction in the number of myenteric neurons per mm was observed with the C14 homolog. However, the C14 homolog had no effect on the number of submucosal neurons per mm over the concentration range tested. Longitudinal and circular smooth muscle layers were significantly thickened, even at the lowest concentration tested. The degree of thickening was not concentration-dependent.

Discussion

The commercial benzalkonium chloride mixture (Zephiran chloride) used by Fox et. al. (1983) to ablate neurons in the rat jejunum is a mixture of N-alkyldimethylbenzylammonium chlorides. The predominate alkyl species are C14 (50%), C12 (40%) and C16 (10%). The results of the present study indicate that the C12 and the C14 homologs are primarily responsible for the myenteric neuron ablation produced by the mixture. The C16 homolog was found to have a slight, but significant effect on the number of myenteric neurons at an equimolar concentration. Of the two most effective homologs, the use of the C14

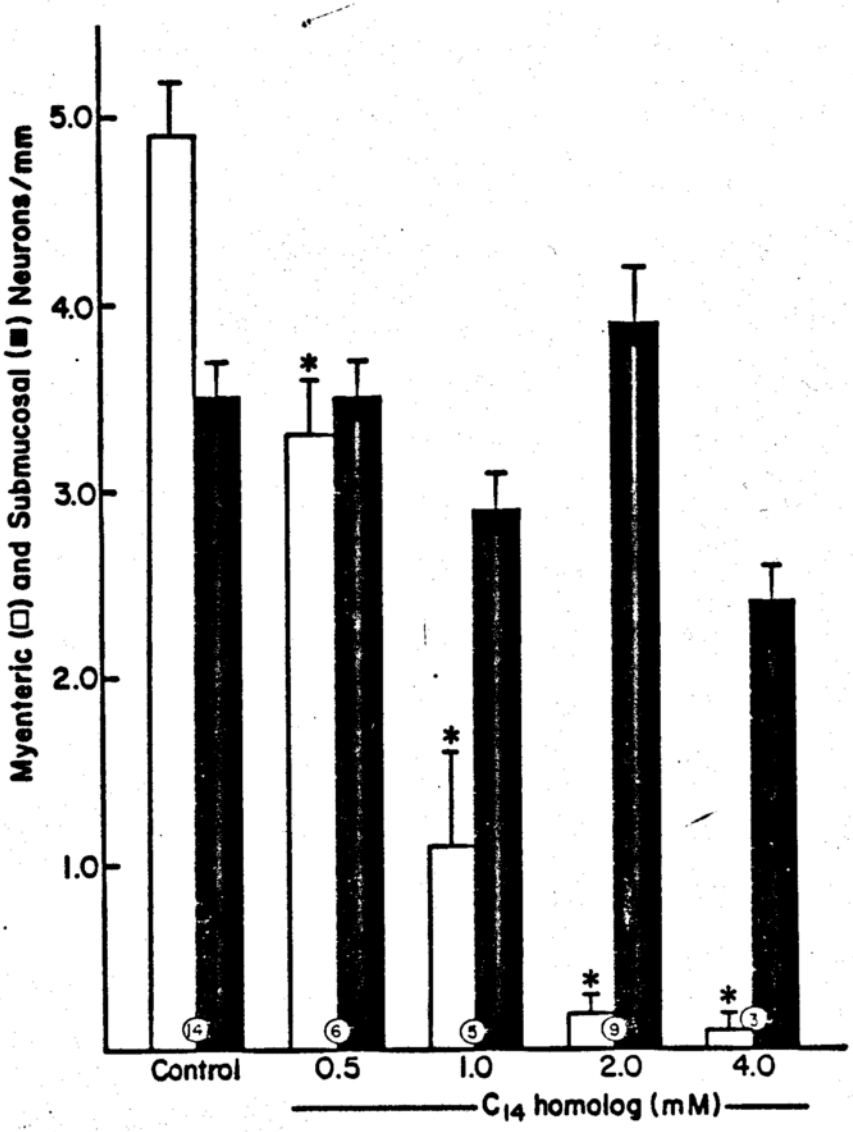

Figure 7. Effects of C14 ADBAC homolog on the number of myenteric (open bars) and submucosal (closed bars) neurons/mm jejunum after serosal application of various concentrations. Data are means ± SEM of the number of rats indicated in the bars. * Significantly different from control mean using Dunnett's test, two-sided, $p < 0.05$.

homolog is recommended to ablate myenteric neurons in rat jejunum since it produced less dyplasia and fibrosis of the intestinal smooth muscle layers than did the C12 homolog. As with the mixture, neither homolog alone affected the number of submucosal neurons. Thus, the C14 homolog can be used to produce a selectively denervated jejunal model for use in acute or chronic in vitro or in vivo studies.

Although the C12 and C14 homologs were the most effective ablative agents, the other ADBAC homologs, except for C18, exhibited significant myenteric neuron ablative activity. The parabolic structure-activity relationship observed in the present study is similar to the structure-antimicrobial activity relationship of the ADBAC homologs observed by others (Ross, et. al., 1953; Cutler, et. al., 1967b; Daoud, et. al., 1983). These investigators found that the C14 homolog was the most potent antimicrobial agent, while the C12 homolog was slightly less potent. Interestingly, the C16 homolog, which was only minimally effective in ablating myenteric neurons, exhibited considerable antimicrobial activity against some species. The antimicrobial potencies of the other ADBAC homologs also paralleled the trend observed in myenteric neuron ablative activities.

The mechanism by which ADBAC homologs exert biologic effects is believed to be non-specific solubilization of

cell membranes due to the surfactant properties of the homologs. In bacteria, this is thought to be a multistep process characterized by adsorption onto and penetration into the cell wall, and subsequently, the cell membrane (Blois and Swarbuck, 1972b). It was suggested that the initial absorption step is the result of electrostatic interactions between negatively charged surface proteins and the positively charged nitrogen group of the homolog. This interaction is believed to facilitate penetration of the hydrocarbon moiety into the the cell wall and membrane. The membrane perturbation caused by penetration results in increased cellular permeability which ultimately leads to bacterial lysis.

There are several lines of evidence in support of a similar mechanism in myenteric neuron ablation. First, Fox et. al. (1983) observed that a variety of surfactants (both ionic and non-ionic) were capable of ablating myenteric neurons. Of those tested, however, the cationic surfactants were the most potent. Second, the demonstration of specific binding of ADBAC homologs to albumin (Perrin and Nelson, 1974) is consistent with the concept of homolog-protein interaction. Interestingly, the C14 homolog appeared to have the highest binding affinity of the homologs tested. Finally, See et. al. (1988) have observed necrosis of virtually all longitudinal and a portion of circular muscle,

in addition to necrosis of myenteric neurons, initially after serosal exposure to BAC. Subsequent cell division of smooth muscle, but not myenteric neurons, is apparently responsible for the generation of the myenterically denervated experimental model.

Given the non-specific mechanism of action of the ADBAC homologs, it is probable that the activity of a given homolog is related to its concentration at and ability to interact with the cell membrane. These factors in turn, are related to aqueous solubility and relative surface activity of the homolog. Within the ADBAC series, it is known that as alkyl chain length increases, aqueous solubility decreases (Daoud, et. al., 1983) and relative surface activity increases (Blois and Swarbrick, 1972b). Also, as aqueous solubility decreases, micelle formation occurs at lower surfactant concentrations. Given these facts, an explanation for the parabolic structure-activity relationship can be proposed. If the aqueous solubility of a homolog (ie, C6) is high, it will exist in the monomeric form capable of membrane interaction (Helenius and Simon, 1975). However, because of low surface activity, the ability to disrupt the membrane will be relatively low. If the aqueous solubility of a homolog (ie, C18) is low, much of the homolog will be present as micelles and little monomer will be available for membrane interaction. Minimal

membrane disruption will occur even though the homolog possesses relatively high surface activity. The C14 homolog apparently possesses the optimum combination of aqueous solubility and surface activity for membrane interaction and is thus the most effective ablative agent.

Additional evidence in support of the explanation of the structure-activity relationship is provided by the C14 homolog concentration-response data. The critical micelle concentration (CMC), the concentration above which micelles form, for the C14 homolog is approximately 1 mM (Cutler, et. al., 1967a). This concentration is, for practical purposes, the maximum concentration of monomer in solution (Helenius and Simon, 1975). Therefore, even though the total homolog concentration in solution was 2 mM, the concentration of monomer available for membrane interaction was probably only 1 mM. Thus, the myenteric ablation produced by 1 and 2 mM C14 homolog should be, and in fact, was comparable (80, 95% respectively; Figure 7). The slightly greater degree of ablation produced by the 2 mM concentration may be due to the presence of homolog in micelles, which could act as a "sink" to provide additional monomer as it was removed from solution by membrane interaction (Helenius and Simon, 1975).

On the basis of the preceding argument, the C16 homolog appears to be the most potent ablative homolog. The free monomer concentration of this homolog, estimated from its

CMC, is about 5-fold lower than that of the C14 homolog. It was not possible, however, to produce a practical myenterically denervated model (greater than 90% ablation) with 30 minutes of application of the C16 homolog. The kinetics of monomer release from micelles may have been too slow for the micelles to provide sufficient monomer for membrane disruption of all myenteric neurons.

Finally, it should be noted that smooth muscle thickening in response to ADBAC homologs occurred only when ablation of myenteric neurons occurred. The magnitude of this thickening, however, did not appear to be related to the degree of myenteric neuron ablation. Thickening of jejunal smooth muscle was also observed following treatment with BAC (Fox, et. al., 1983), but the muscle was responsive to contractile stimuli (Fox and Bass, 1986b). See et. al. (1988) have demonstrated that this thickening is due predominately to smooth muscle hyperplasia. It is likely that the thickening observed in the present study is also due to hyperplasia of smooth muscle following myenteric plexus ablation. It is also likely that the altered mechanical properties of smooth muscle observed after BAC treatment (Herman and Bass, 1987) would be observed following treatment with individual ADBAC homologs.

Acknowledgements

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Summary of Findings

Serosal application of benzalkonium chloride (BAC) destroys neurons located in the myenteric plexus of the rat jejunum. In addition, there is significant thickening of intestinal smooth muscle after treatment. Studies which characterized the ability of both longitudinal and circular muscle to generate active tension at various times after treatment were performed. The results of these studies indicated that initially after treatment the ability of both muscle layers to generate active tension was impaired. Seven days after treatment, the ability of both muscle layers to generate active tension was comparable to that of respective control tissues. However, 15 days after treatment, denervated longitudinal muscle generated significantly greater active tension than did control tissue. These results can be explained on the basis of the morphologic study by See et. al. (1988). These authors demonstrated that initially after treatment, virtually all longitudinal and a portion of the circular muscle was necrotic, and therefore unable to generate tension. As the smooth muscle divided to repair the BAC-induced damage, the ability to generate tension also returned to normal. Normal muscle thickness and cell number on day 7 correlate exactly to normal active tension development at that time. Fifteen

days after treatment, denervated longitudinal muscle was capable of generating almost twice the active tension as the control tissue. This was also the time that See et. al. (1988) observed maximal thickening and hyperplasia of longitudinal muscle.

Changes in the length-stress properties of both smooth muscle layers were also observed after myenteric plexus ablation with BAC. The changes observed in denervated circular muscle were minimal, and consisted of increases in resting tension at L_o (optimum length for active tension development) both 15 and 30 days after treatment. Resting tension at L_o of denervated longitudinal muscle was elevated 15 days after treatment, but was comparable to control on day 30. Active stress (force/cross-sectional area) generation by denervated longitudinal muscle was twice that of control tissue 15 and 30 days after treatment. Since contractile data was normalized to cross-sectional area, the increase in active stress generation is not simply due to the hyperplasia observed. Changes in contractile protein content or calcium metabolism may be responsible for the increases observed.

In addition to the information about smooth muscle structure/function provided by these studies, appropriate resting tensions for denervated tissues were also obtained. These data were required for the proper conduct of the

pharmacologic studies described in Chapters 2 and 3.

Denervation is known to induce changes in the pharmacologic sensitivity of smooth muscle. Local myenteric plexus ablation with BAC induced changes in the sensitivity of both longitudinal and circular jejunal smooth muscle. In contrast to what was expected based on Cannon's law of denervation, denervated longitudinal muscle exhibited supersensitive responses to carbachol at all times examined. The responses of denervated circular muscle depended on the time after denervation that the tissue was examined. Fifteen days after denervation, circular muscle was subsensitive to carbachol, in accord with the law of denervation. Thirty and 45 days after denervation, the responses of denervated circular muscle were comparable and subsensitive, respectively , to those of control tissue. The nature of these changes in both muscle layers probably reflect alterations in the neuronal (nicotinic) action of carbachol since they are consistent with the known patterns of innervation of intestinal muscle.

Denervation of a localized area of jejunum apparently alters the neuronal pathways which mediate enteric reflexes. The responses to carbachol of longitudinal and circular muscle 2 cm orad or caudad to lesion were altered following localized myenteric plexus ablation. Muscle orad to the lesion was subsensitive, suggesting alterations in the

neuronal pathways which mediate ascending excitation. Muscle caudad to the lesion was supersensitive, suggesting alterations in the neuronal pathways which mediate descending inhibition. Alterations in smooth muscle responses, both at and beyond the site of myenteric plexus ablation may account, in part, for altered intestinal motility which could lead to obstruction.

Because carbachol is a non-selective cholinergic agonist, the changes observed in the responses of denervated circular muscle might reflect alterations in nicotinic or muscarinic receptors. Responses of naive and denervated circular muscle to bethanachol were comparable, indicating no difference in muscarinic sensitivity between the naive and denervated tissues. Therefore, the changes observed 15 days after denervation reflect the loss of the nicotinic (neuronal) component of theaction of carbachol, which in the case of circular muscle, is predominantly inhibitory. Thus, the response to carbachol of denervated circular muscle appears supersensitive.

The return of normal responsiveness to carbachol of myenterically denervated circular muscle 30 days after denervation was due to the reemergence of a nicotinic (neuronal) element in the response to carbachol. This was demonstrated by the fact that neuronal antagonists, which had no effect on the responses on day 15 posttreatment,

significantly altered the responses of circular muscle 30 days after myenteric denervation. The effects produced by the antagonists 30 days after denervation were qualitatively and quantitatively different than those produced in naive tissue, suggesting that the nature of the innervation in these tissues is different. The reinnervation of circular muscle observed 30 days after myenteric denervation is probably due to neurons located within the submucosal plexus. This finding may have relevance in clinical conditions where myenteric neurons are absent but submucosal neurons remain.

The BAC mixture used to ablate myenteric neurons is composed of several individual alkyldimethylbenzylammonium chloride homologs. The individual alkyldimethylbenzylammonium chloride homologs , with the exception of the octadecyl homolog, possess the ability to ablate myenteric neurons when applied serosally. The potency of the homologs varies widely, however, and the variations can be explained on the basis of the physical-chemical properties of the homologs. The tetradecyl (C14) homolog was found to be the most effective ablative agent, and reduced the number of myenteric neurons in a concentration-dependent manner. Thus, the C14 homolog, rather than the BAC mixture, can be used in future studies to ablate neurons of the myenteric plexus of the rat jejunum.

Appendix A

Temporal Changes in Mechanical Properties of Rat Jejunal Smooth Muscle After Myenteric Plexus Ablation

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Appendix B

Synthesis and Analysis of Commercially Unavailable Alkyldimethylbenzylammonium Chlorides

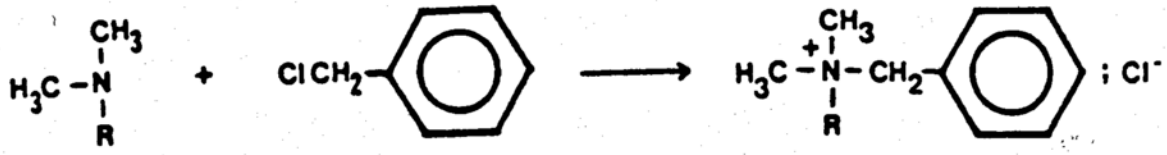
Introduction

Benzalkonium chloride is a mixture of alkyldimethylbenzylammonium chloride homologs, with N-alkyl groups ranging form C8 to C18. The biologic activity of these homologs differs widely. In order to conduct the structure-activity relationship study outlined in Chapter 4, it was necessary to synthesize several of the homologs since they were neither commercially available nor available from Sterling-Winthrop Research Institute, the source of the homologs for previous investigators. This Appendix details the synthesis and analysis of the hexyl-, octyl-, decyl- and dodecyldimethylammonium chloride homologs.

Methods

The general methodology used for homolog synthesis was that outlined by Cutler et. al. (1967). The reaction equation is shown in Figure 1. The homologs were synthesized by refluxing the appropriate N-alkyldimethylamine with benzyl chloride (Aldrich Chemical Co., Milwaukee, WI, 97%) in appropriate solvents. The specific conditions for the individual homologs are presented below.

Hexyldimethylbenzylammonium chloride (C6). This homolog was prepared by refluxing N,N-dimethylhexylamine



dimethyl-alkylamine

benzyl chloride

alkyldimethylbenzyl-ammonium chloride

Figure 1. Generalized reaction equation for the synthesis individual alkyldimethylbenzylammonium chloride homologs.

(Aldrich, 99%) and benzyl chloride in ethyl acetate. Product formation was noted prior to heating, and after 5 hours of refluxing, the ethyl acetate was distilled, and the crystals filtered and allowed to dry. The product was then recrystallized twice from ethyl acetate.

Octyldimethylbenzylammonium chloride (C8). This homolog was prepared by refluxing N,N-dimethyloctylamine (Aldrich, 95%) and benzyl chloride in acetonitrile for 19 hours. The acetonitrile was then distilled, yielding a dark residual oil. The oil was then dissolved in ethyl acetate. The monohydrate salt of this homolog was prepared by quantitative addition of distilled, deionized water. Heating produced a clear solution from which the monohydrate crystallized. The product was recrystallized twice from ethyl acetate.

Decyldimethylbenzylammonium chloride (C10). This homolog was prepared by refluxing 1-dimethylaminodecane (Alfa Products, Danvers, MA, 98%) and benzyl chloride in ethyl acetate for 42 hours. The monohydrate salt of this homolog was prepared by quantitative addition of water. Heating to reflux produced a clear solution from which the product slowly crystallized. The product was recrystallized twice from ethyl acetate.

Dodecyldimethylbenzylammonium chloride (C12). This homolog was prepared by refluxing N,N-dimethyldodecylamine

(Aldrich, 97%) and benzyl chloride in acetonitrile for 18 hours. The acetonitrile was distilled, the residual oil dissolved in ether, and the monohydrate salt of this homolog was prepared by the quantitative addition of water. Heating to reflux temperature produced a clear solution from which the product formed. The product was then recrystallized twice from ether.

Analyses of homologs. Melting points of the homologs were determined, and samples of each homolog in deuterium oxide were analyzed with a ^{13}C Fourier Transform (FT) NMR (JEOL FX 90Q, JEOL Ltd, Tokyo, Japan) to confirm structure and purity. The NMR analyses and interpretation were performed by G. Girdaukas.

Results and Discussion

Melting points of the homologs are presented in Table 1, and indicate close conformity with those reported by Cutler et. al. (1967). The results of the NMR analyses are presented in Figures 2 - 5 for the C6, C8, C10 and C12 homologs, respectively. The spectra are consistent with what is expected from the structures of the homologs, and no additional peaks, which would indicate impurities, were noted. Therefore, on the basis of the melting point and NMR data, the purity of the homologs estimated to be at least 95%. The homologs were then used as described in Chapter 4.

Table 1

Melting Points of Synthesized

Alkyldimethylbenzylammonium Chloride Homologs

N-Alkyl	Observed Melting Point (°C)[1]	Reported Melting Point (°C)[2]	% Yield
6	157.5 - 159.8	-	88.5
8	68.1 - 69.9	71.4 - 72.4	85.9
10	40.8 - 41.6	41.5 - 43.8	83.9
12	48.6 - 48.8	44.9 - 46.8	98.3

[1] uncorrected

[2] Cutler et. al. (1967)

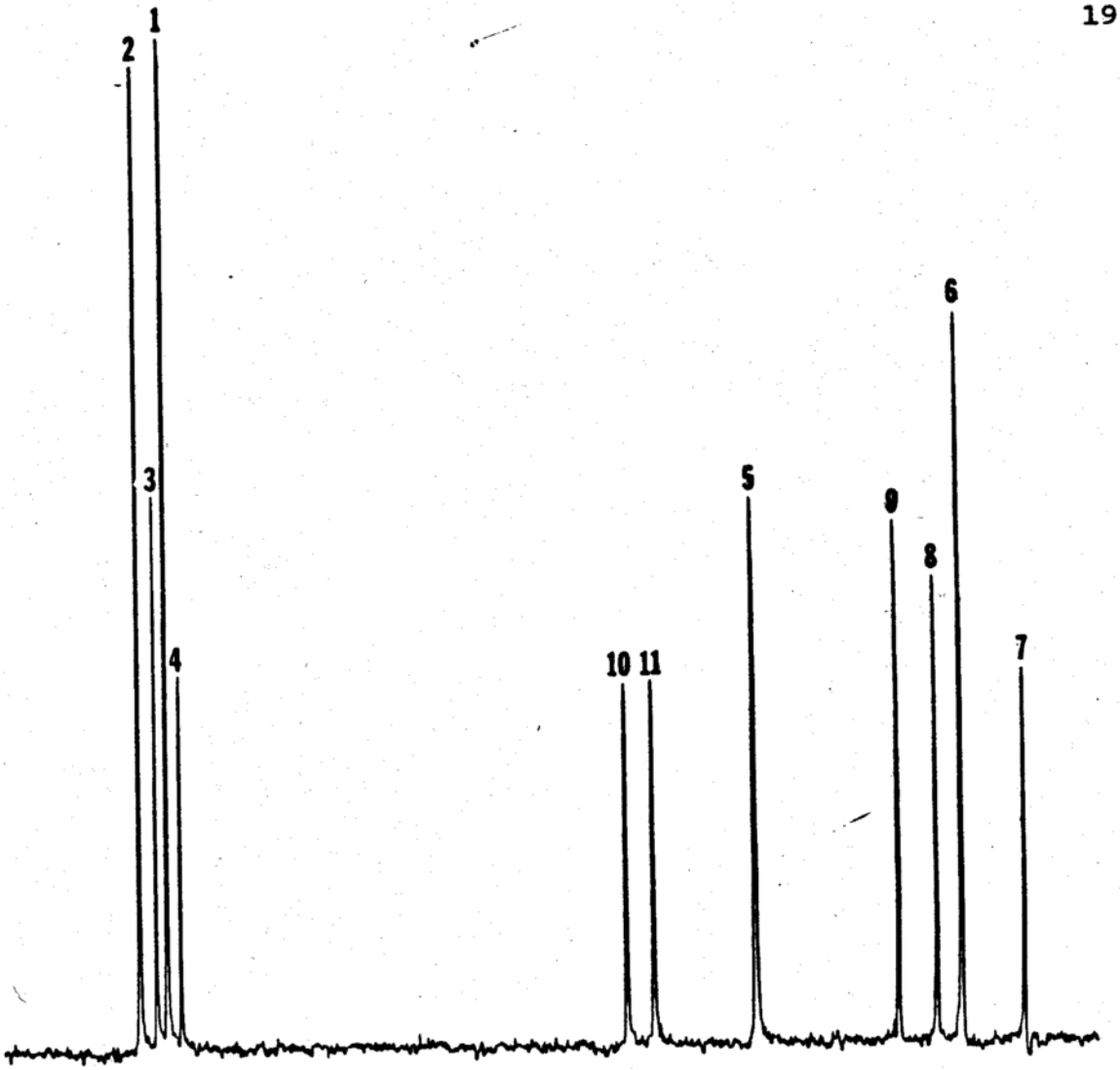


Figure 2. ^{13}C FT-NMR spectrum of hexyldimethylbenzyl-ammonium chloride.

Peaks 1 - 4: benzene carbon atoms

Peak 5: alpha-methylene carbon atoms

Peak 6: methyl carbon atoms

Peaks 7 - 11: N-alkyl carbon atoms

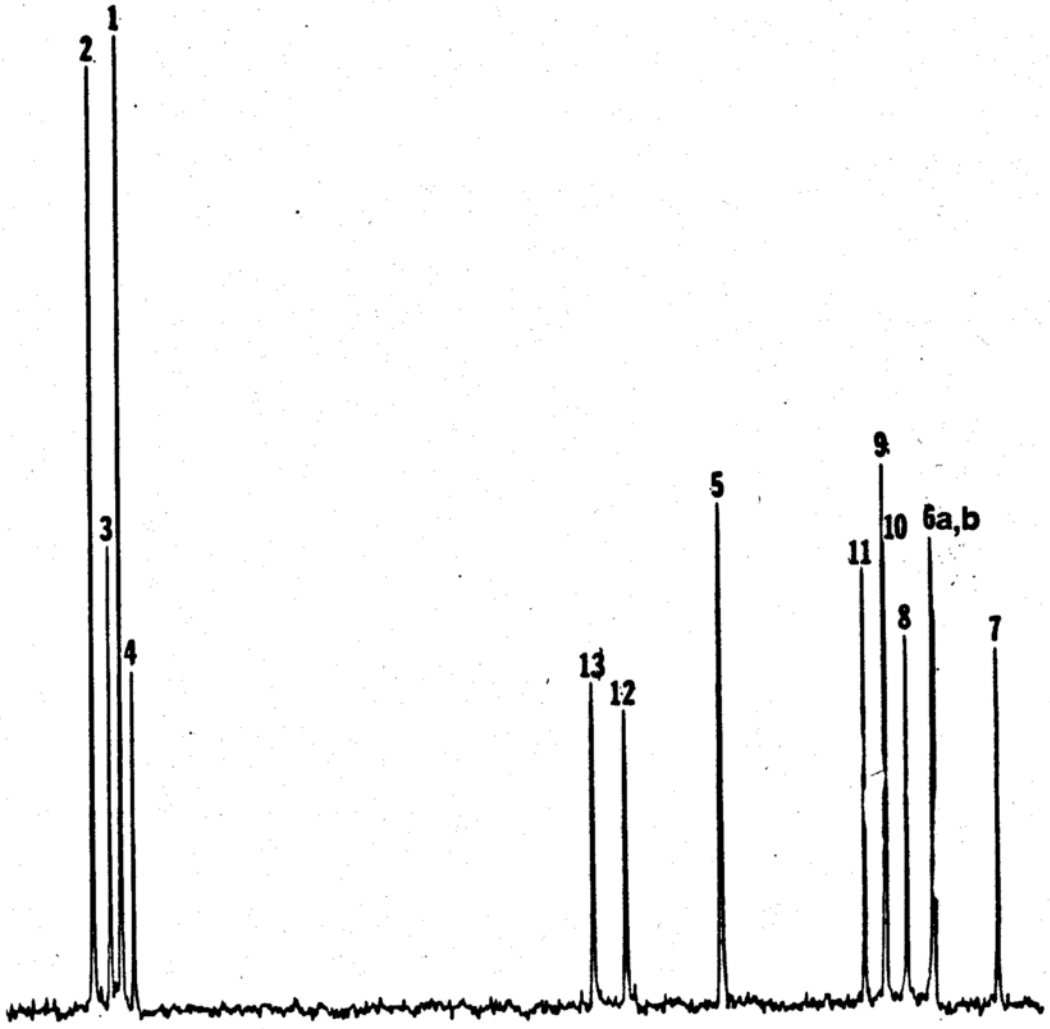


Figure 3. ^{13}C FT-NMR spectrum of octyldimethylbenzylammonium chloride.

Peaks 1 - 4: benzene carbon atoms

Peak 5: alpha-methylene carbon atoms

Peaks 6a,b: methyl carbon atoms

Peaks 7 - 13: N-alkyl carbon atoms

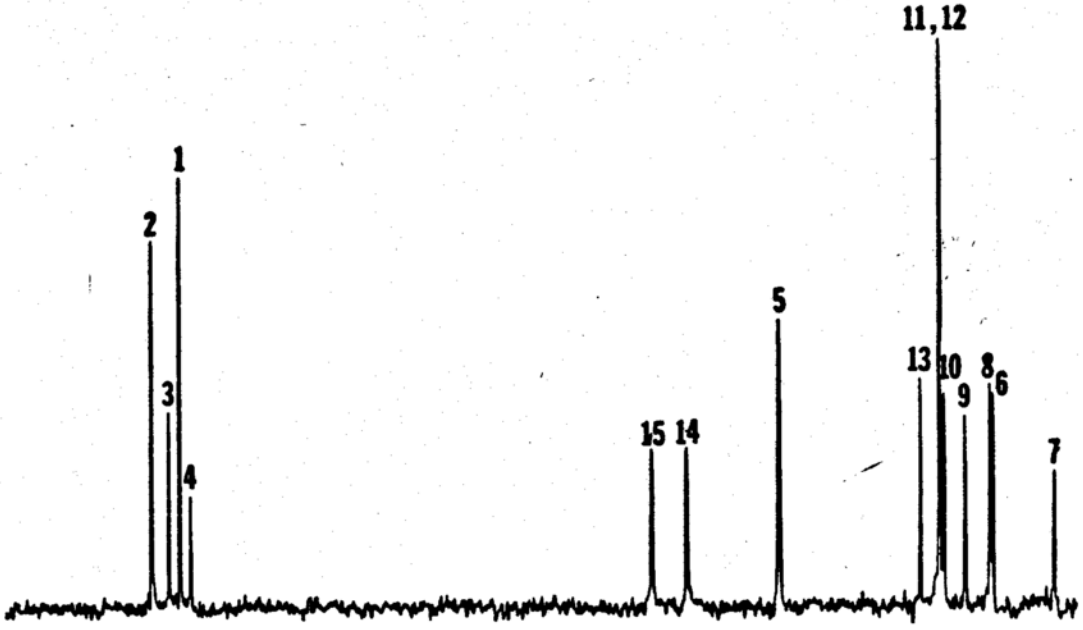


Figure 4. ^{13}C FT-NMR spectrum of decyldimethylbenzyl-ammonium chloride.

Peaks 1 - 4: benzene carbon atoms

Peak 5: alpha-methylene carbon atoms

Peak 6: methyl carbon atoms

Peaks 7 - 15: N-alkyl carbon atoms

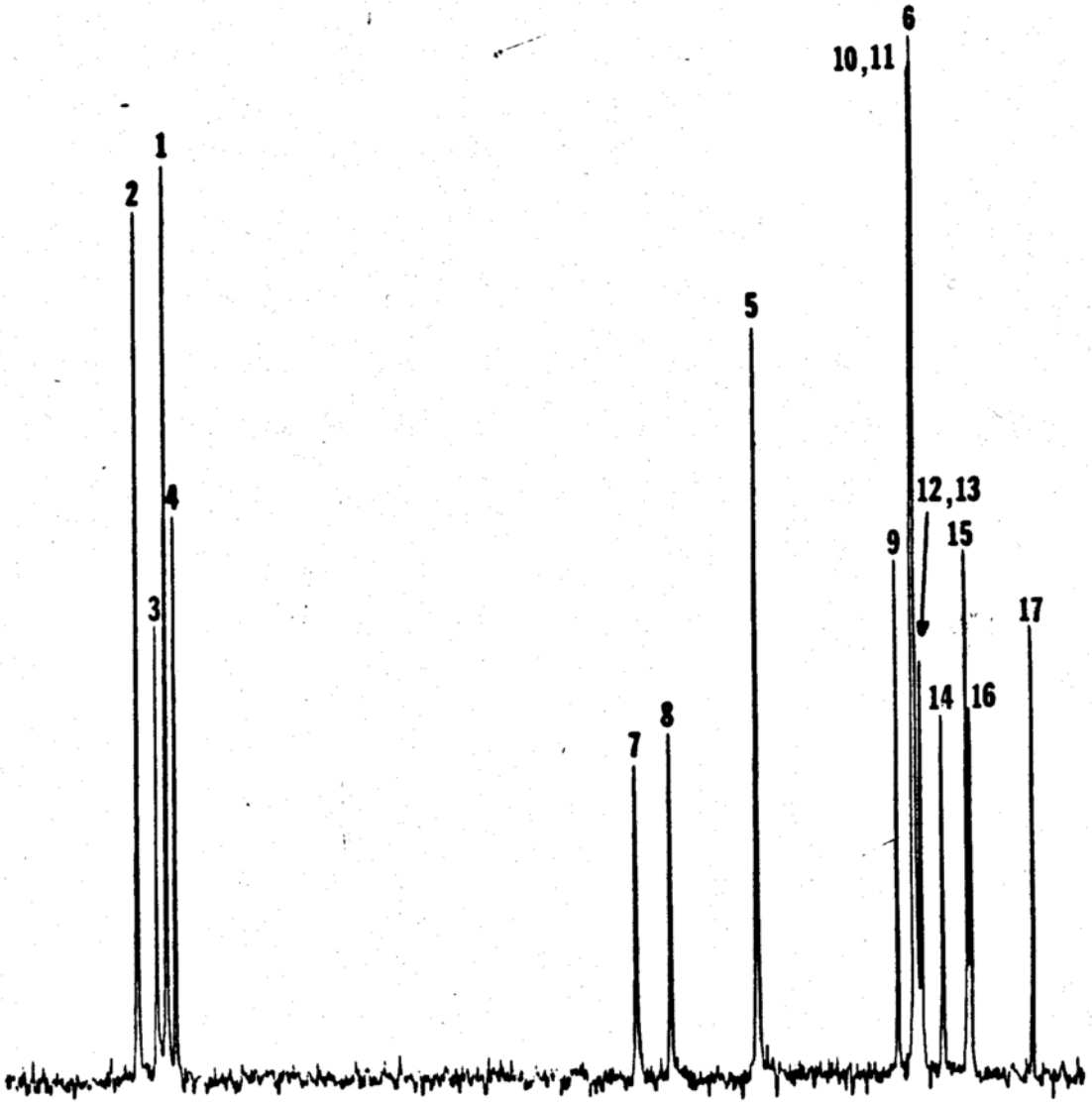


Figure 5. ^{13}C FT-NMR spectrum of dodecyldimethylbenzyl-ammonium chloride.

Peaks 1 - 4: benzene carbon atoms

Peak 5: alpha-methylene carbon atoms

Peak 6: methyl carbon atoms

Peaks 7 - 17: N-alkyl carbon atoms

Reference

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Appendix C

Chemically-Induced Aganglionic Rat Jejunum: Attempts to Produce an Experimental Model

Introduction

A simple, reproducible technique to ablate neurons of both ganglionated plexuses in the small intestine of small laboratory animals is currently not available. Thus, there are currently no experimental models of aganglionosis of the small intestine to complement the myenterically denervated model described by Fox et. al. (1983). If such a model were available, the role of the submucosal plexus in gastrointestinal function could be more accurately assessed. This Appendix will outline a variety of techniques used in attempts to produce an aganglionic rat jejunal model. Unfortunately, these attempts have been largely unsuccessful but have provided clues which may facilitate development of such a model in the future.

Materials and Methods

Initial attempts at producing an aganglionic model were based on the methods of Fox et. al. (1983). These authors found that serosal application of 0.062% benzalkonium chloride (BAC) on the rat jejunum for 30 minutes ablated neurons of the myenteric but not submucosal plexus. It was felt that modification of treatment times or exposure techniques might facilitate destruction of both nerve

plexuses. To this end, rat jejunum was immersed (soaked) in 0.031% or 0.062% BAC for 1 - 2 hrs. It was felt that the soak technique might provide a more uniform treatment and also provide a continuous source of drug for diffusion into the tissue.

Fox et. al. (1983) also demonstrated that 1% ricinoleic acid (RA), when applied serosally, ablated all myenteric and approximately 50% of submucosal neurons. Therefore, rat jejunum was treated with either 1% or 2% RA via paint (Fox technique) or soak for 1 or 2 hrs.

While the studies with BAC and RA were ongoing, it was decided to assess the ability of known or suspected central or peripheral neurotoxins to ablate enteric neurons when applied serosally. To this end, rats were treated using the serosal application protocol of Fox et. al. (1983) for 30 minutes with acrylamide (5%), hexachlorophene (1, 2%), quinacrine (0.1, 0.5, 1, 2.9%), 5-chloro-7-iodo-8-hydroxyquinoline (Vioform, 2, 5%) and chlorpromazine (0.5, 1, 2.5 and 5%).

The blood flow in the submucosal region is considerably greater than that in the myenteric region. It is possible that this higher blood flow may act as a sink for any ablative agent which may penetrate to the submucosal region. If so, it may not be possible to achieve an effective concentration of the ablative agent at the desired site with

blood flow intact. Therefore, a variety of mechanical methods were employed to either reversibly or irreversibly obstruct blood flow to the region of bowel exposed to BAC. Additional rationale for the interruption of blood flow to the bowel was provided by the studies of Hukahara et. al. (1961) who demonstrated that prolonged anoxia resulted in irreversible alterations in enteric nervous system function in the canine intestine. Therefore, three different techniques to interrupt intestinal blood flow were employed in combination with serosal application of 0.062% BAC for 30 or 60 minutes. The first technique involved interruption of the "lateral" or "intrinsic" blood flow to the treated segment. The second technique involved interruption of the mesenteric blood flow to the treated segment. The third technique was a combination of the first two techniques, that is, interruption of both mesenteric and lateral blood flow.

It is believed that massive increases in intracellular free calcium are ultimately responsible for cell death following exposure to a number of different toxicants (Farber, 1981). Therefore, it was felt that a combination of BAC and calcium chloride might be an effective ablative treatment. BAC (0.015, 0.031 or 0.062%) and calcium chloride (0.65, 1.3 or 2.5%) were applied using the painting technique of Fox et. al. (1983) to the serosa of rat

jejunum.

The effects of the various treatments outlined above on the number of enteric neurons were semiquantitatively assessed with immunohistochemical techniques. Frozen sections (10 - 12 microns) of paraformaldehyde-fixed control and treated tissues were prepared and exposed to antisera raised against vasoactive intestinal peptide (VIP), neuron specific enolase (NSE) and/or neurofilament proteins (NF). Antisera binding was visualized with secondary antibody conjugated to the fluorescent dye fluoroscein isothiocynate (FITC) and an epifluorescent microscope.

Treatments which appeared to ablate both myenteric and submucosal neurons (BAC/calcium combinations) were examined further by quantitative cell counts on methylene blue-basic fushcin stained plastic sections. The number of nerve cells in at least three sections were counted from each treated portion of jejunum.

The number of animals in each treatment protocol ranged from 2 to 10, and the effectiveness of each treatment was assessed 15 days after treatment.

Results and Discussion

The results of immersion of rat jejunum in BAC or RA are presented in Table 1. Exposure to BAC for 2 hrs

Table 1

Attempted Chemical Ablation of Enteric Neurons in Rat Jejunum - Serosal "Soaking" Technique[a]

Chemical	Conc. %	Treatment Length (hr)	VIP Myenteric	Staining[b] Submucosal	Muscle Thickening[c]	n
BAC	0.031	2	3	1 - 2	3[d]	6
	0.062	2	3	1 - 2	3[d]	2
RA	1[e]	2	2 - 3	0 - 1	3	4

[a] rats, 6-8 weeks old at the time of treatment, were sacrificed 15 days posttreatment

[b] 0 - staining comparable to control

1 - slight reduction in staining relative to control

2 - moderate reduction in staining relative to control

3 - marked reduction in staining relative to control

[c] 0 - thickness comparable to control

1 - slight thickening relative to control

2 - moderate thickening relative to control

3 - marked thickening relative to control

[d] also diffuse necrosis of mucosa

[e] 1 of 4 rats died prior to sacrifice on day 15

decreased the VIP staining in the myenteric region markedly, but only reduced staining in the submucosal region slightly to moderately. Marked muscle thickening was observed, as well as diffuse necrosis of the mucosa in these animals. Soaking in a 1% solution of RA for 2 hrs resulted in a moderate to marked reduction in VIP staining in the myenteric region, but only a slight reduction in VIP staining in the submucosal region. The musculature was markedly thickened, but no mucosal damage was observed. These results indicated that longer exposure to BAC or RA did not ablate submucosal neurons, although the mucosal damage with BAC suggests penetration of BAC beyond the submucosal region. This suggests that blood flow may play a protective role against BAC-induced neuronal ablation.

The effects of known neurotoxins on enteric neurons are presented in Table 2. Chlorpromazine, hexachlorophene, quinacrine and vioform all exhibited BAC-like effects on VIP staining in the myenteric region, but with the exception of 5% chlorpromazine, had little effect on submucosal staining. This concentration of chlorpromazine markedly reduced submucosal VIP staining, but also produced marked muscle thickening and damage in the rat which survived beyond 7 days. The other compounds mentioned above also produced thickening of intestinal smooth muscle. These compounds were probably effective due to their surfactant

Table 2

Effects of Known Neurotoxins on Enteric Neurons

Following Serosal Application[a]

Chemical	Conc. %	VIP Staining Myenteric	VIP Staining Submucosal	Muscle Thickness	n
acrylamide	5	0	0	0	2
$AlCl_3$	2	0	0	0	2
	5[b]	-	-	-	2
hexachlorophene	2	3	1	3	2
	5[c]	-	-	-	2
quinacrine	0.1	0 - 1	0	1	2
	0.5	3	0 - 1	3	2
	1	3	1	3	2
	2.9	3	2 - 3	3	2
Vioform	2	2	0	1	2
	5	3	1	2	2
Chlorpromazine	0.5	1 - 3	0	1	2
	1	2 - 3	0 - 1	2 - 3	2
	2.5	2 - 3	1 - 2	3	2
	5[d]	3	3	3	2

[a] rats, 6-8 weeks old at the time of treatment, were sacrificed 15 days posttreatment; see Table 1 for staining and thickness legends

[b] 2 of 2 rats died within 6 days of treatment

[c] 3 of 3 rats died prior to 15 days

[d] 3 of 4 rats died within 7 days of treatment

characteristics. Acryamide and aluminum chloride had no effect on enteric VIP staining or muscle thickness, but 5 % aluminum chloride was lethal to 2 of 2 rats within 6 days of treatment. The cause of death appeared to be intestinal perforation.

The effects of serosal painting of RA, and BAC in combination with anoxia, are presented in Table 3. Increasing the concentration of RA or duration of treatment was no more effective than the 30 minute exposure described by Fox et. al. (1983). Painting with BAC in combination with interruption of blood flow markedly reduced the VIP immunoreactivity in the myenteric region. Interruption of lateral blood flow in combination with BAC had little effect on submucosal VIP staining, while interruption of the mesenteric blood flow and BAC exposure resulted in a slight to moderate reduction in VIP immunoreactivity in the submucosal region. The interruption of both mesenteric and lateral blood flow and BAC resulted in a marked loss of VIP immunoreactivity in the submucosal region, but unfortunately, also the loss of a significant portion of treated animals (5 of 10) prior to day 15. It was decided that mortality was too high for this technique to be practical, so no quantitation of neuronal cell ablation was performed. Interruption of the mesenteric and lateral blood flow without BAC treatment had no effect on VIP

Table 3

Attempted Chemical Ablation of Enteric Neurons of Rat Jejunum - Serosal Painting and Interruption of Intestinal Blood Flow[a]

Chemical	Conc. %	Treatment Length (min)	VIP Staining Myenteric	VIP Staining Submucosal	Muscle Thickness	n
RA	1	60	2 - 3	0	0-1	2
	2	60	2-3	0 - 1	2	2
BAC	0.062[b]	30	2 - 3	0 - 1	1 - 2	2
	0.062[c]	30	3	1 - 2	2	2
	0.062[d]	30	3	2 - 3	2 - 3	6
	0[d]	30	0	0	0	4

[a] rats, 6-8 weeks old at the time of treatment, were sacrificed 15 days posttreatment; see Table 1 for staining and thickness legends

[b] interruption of lateral blood flow (30 minutes)

[c] interruption of mesenteric blood flow (30 minutes)

[d] interruption of both lateral and mesenteric blood flow (30 minutes)

immunoreactivity in either myenteric or submucosal regions.

The combination of BAC and calcium significantly reduced the immunoreactivity to VIP, NSE and NF in both myenteric and submucosal regions, and the combination of 0.031% BAC and 1.3% $CaCl_2$ appeared to be the most effective combination. Therefore, quantitative cell counts were performed on rats treated with this combination (Table 4). In experiments with the original source of BAC used by Fox et. al. (1983), 90% of myenteric and 70% of submucosal neurons in mature rats (> 3 months old) were ablated. The remaining cells were swollen, vacuolated and exhibited altered staining characteristics. However, in two separate experiments with a new source of BAC and rats 6 - 8 weeks old, the reduction in submucosal neurons was only 40 - 50%. BAC which was at least as old as the original bottle was obtained from Dr. T. Rudy, but this formulation ablated only 77% of the myenteric neurons and 29% of the submucosal neurons in 6 - 8 week old rats. Thus, the original promising results with the BAC/calcium combination were not reproducible.

It was initially believed that differences in the alkyl homolog composition of the various BAC solutions were responsible for the observed variations in enteric neuron ablative activity. However, chemical analysis (reverse phase HPLC) of the mixtures indicated no significant

Table 4

The Effects on Enteric Neurons of

Serosal Application of BAC and Calcium[a]

BAC Source	Rat Age at Treatment (wk)	MY/mm[b]	SUB/mm[c]	N
Control	6 - 13	5.2 ± 0.3	3.5 ± 0.2	11
Fox[d]	13	0.5 ± 0.2	1.1 ± 0.2	6
new[e]	6 - 8	1.2 ± 0.3	2.0 ± 0.3	6
new[e]	6 - 8	0.7 ± 0.2	1.8 ± 0.2	7
Rudy[f]	6 - 8	1.2 ± 0.6	2.5 ± 0.7	3

[a] rats were treated with a combination 0.031% BAc, 1.3% $CaCl_2$ and sacrificed 15 days after treatment

[b] myenteric neurons/mm jejunum

[c] submucosal meurons/mm jejunum

[d] BAC (Zephiram chloride) originally used by Fox et al. (1983) to ablate myenteric neurons

[e] BAC (Zephiram chloride) purchased from a local pharmacy 10/86

[f] BAC (Zephiram chloride) obtained from Dr. T. Rudy, estimated to be as old as that used by Fox et al. (1983)

differences in homolog ratios, although the original commercial BAC solution was slightly more concentrated than the other solutions used. However, treatment of 6 - 8 week old rats with a combination of higher concentrations of the newer BAC solutions with 1.3% calcium chloride still failed to reproduce the original observations.

It is possible that the age of the rats may affect susceptibility to BAC/calcium treatment. The original observations were made on mature (> 3 mos) rats, while subsequent studies were performed with 6 - 8 week old animals. It is known that in humans the enteric nervous system does not fully mature until about age 2 (Bughaighus and Emery, 1971). Prior to that time, it is possible that undifferentiated neuroblasts could undergo cell division in response to injury. If a similar phenomenon occurs in rats, older animals, which lack neuroblasts capable of cell division, could not replace necrotic nerve cells. Younger animals may be able to replace damaged cells due to the presence of undifferentiated precursor cells. This readily testable hypothesis deserves future consideration.

It is quite likely that the role of calcium in the BAC/calcium combination is that of a prolonged vasoconstrictor since intense, persistent vasoconstriction was noted during and after treatment. Clearly, the extracellular concentration of calcium (at least 4 orders of

magnitude greater than intracellular free calcium) is sufficient to induce cell death without the addition of exogenous calcium.

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