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Polar innervation of enteric non-cholinergic and non-adrenergic neurons in the isolated guinea-pig ileum.

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Abstract

To study the polarity of the efferent pathway of the myenteric plexus, recordings were made of the mechanical activity of the longitudinal muscle of isolated guinea-pig ileal segments upon stimulation with an electrical field around the myenteric plexus contained within strips of longitudinal muscle (LM-MP) continuous with each end of ileal segment. The amplitude of the contractile response to stimulation of the anal LM-MP was always larger than that to the oral LM-MP. After cholinergic and adrenergic transmission was suppressed by atropine (10 microM) and guanethidine (1 microM), and the tone of the segment was enhanced by histamine (1 microM), the LM-MP stimulation produced non-cholinergic, non-adrenergic (NCNA) ascending contraction and NCNA descending relaxation. The NCNA contraction, but not the NCNA relaxation, was abolished or reduced by desensitization to substance P. The present results suggest that the NCNA innervation of the myenteric plexus participates in the polar effects observed in the guinea-pig ileum, that the NCNA excitatory response may be mediated at least in part by myenteric substance P neurons, and that the NCNA inhibitory response is mediated by non-adrenergic neurons.

KEYWORDS: ileal motility, intrinsic reflex, myenteric plexus, substance P, guinea pig

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To study the polarity of the efferent pathway of the myenteric plexus, recordings were made of the mechanical activity of the longitudinal muscle of isolated guinea-pig ileal segments upon stimulation with an electrical field around the myenteric plexus contained within strips of longitudinal muscle (LM-MP) continuous with each end of ileal segment. The amplitude of the contractile response to stimulation of the anal LM-MP was always larger than that to the oral LM-MP. After cholinergic and adrenergic transmission was suppressed by atropine ($10\,\mu$M) and guanethidine ($1\,\mu$M), and the tone of the segment was enhanced by histamine ($1\,\mu$M), the LM-MP stimulation produced non-cholinergic, non-adrenergic (NCNA) ascending contraction and NCNA descending relaxation. The NCNA contraction, but not the NCNA relaxation, was abolished or reduced by desensitization to substance P. The present results suggest that the NCNA innervation of the myenteric plexus participates in the polar effects observed in the guinea-pig ileum, that the NCNA excitatory response may be mediated at least in part by myenteric substance P neurons, and that the NCNA inhibitory response is mediated by non-adrenergic neurons.

Key words: ileal motility, intrinsic reflex, myenteric plexus, substance P, guinea pig

Bayliss and Starling (1) formulated the law of the intestine, which states that mechanical stimuli in extrinsically denervated small intestine produce ascending excitatory and descending inhibitory reflexes. Hukuhara *el al.* (2) found that receptive fields of such reflexes were located in the mucosa and called them the mucosal intrinsic reflexes, and presumed that the polarity of the efferent pathways of the reflex is formed in the myenteric plexus. The mucosal intrinsic reflex was also demonstrated in the canine colon (3). In isolated segments of guinea-pig large intestine, the ascending excitatory and descending inhibitory reflexes were produced by local distension of the segment (4). Yokoyama and Ozaki (5) showed that stimulation of a node of myenteric plexus attached to a longitudinal muscle strip (LM-MP) of rabbits mainly caused an excitation of electrical activity of longitudinal muscles above and an inhibition below the stimulated node. They also showed that the ascending excitatory pathways were mainly cholinergic and the descending inhibitory pathways were non-adrenergic.

The present study was designed to inves-

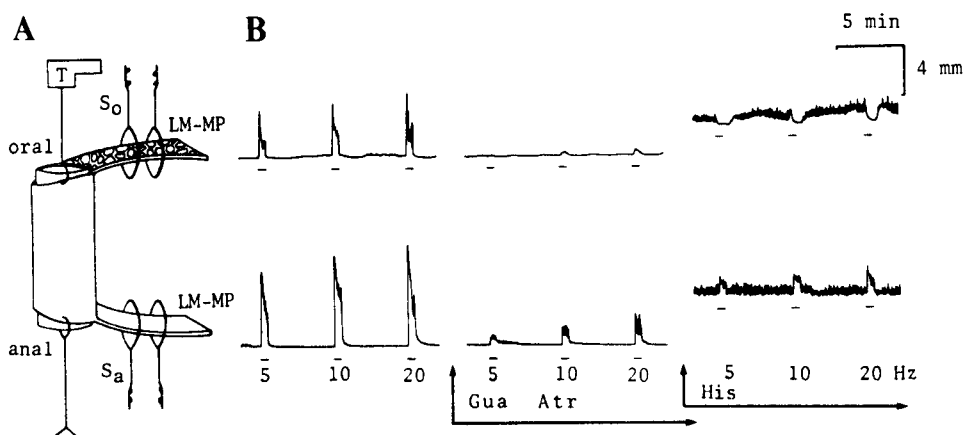* To whom correspondence should be addressed.

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Fig. 1 A: Experimental arrangement. T: isotonic transducer, LM-MP: strips of longitudinal muscle and myenteric plexus, So and Sa: electrodes for stimulating the oral and anal LM-MPs. B: Responses of the intact segment to oral (upper traces) and anal (lower traces) LM-MP stimulation (0.1 msec, supramaximal intensity), Atr: $10 \mu M$ atropine, Gua: $1 \mu M$ guanethidine, His: $1 \mu M$ histamine.

tigate whether polar effects on longitudinal muscle mechanical activity of an intact ileal segment could be produced by stimulation of the myenteric plexus.

Materials and Methods

After adult guinea pigs of either sex weighing 200-450 g were stunned by a blow on the head and bled via the carotid artery, the small intestine was immediately removed, and the lumen was flushed with Tyrode solution of the following composition: 145 mM NaCl, 2.7 mM KCl, 1.5 mM $CaCl_2$, 0.7 mM $MgCl_2$, 4.8 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, and 11.1 mM glucose. A segment 5-7 cm long taken from the mid ileum was gently slipped onto a glass rod. To make the strips of longitudinal muscle with myenteric plexus (LM-MP), a modification of the procedures first described by Ambache (6) was used: The longitudinal muscle was partly stripped from the circular muscle by gently stroking away from the mesenteric attachment with damp cotton. At the oral end, the stroking was continued around to the antimesenteric border, but elsewhere a broad strip of longitudinal muscle along the antimesenteric edge was not touched with cotton. The end of the strip which was completely loosened was then gently pulled so as to lift the remainder of the strip from the circular muscle until the length of the LM-

MP strip was 2-2.5 cm. The ileal segment, from which the LM-MP had been stripped, was then cut away. By the same procedure, an LM-MP strip was dissected at the anal end of the remaining ileum, so that an intact segment about 1-1.5 cm long with LM-MP strips at both oral and anal ends was prepared (Fig. 1, A). The preparation was suspended in a 15 ml organ bath containing Tyrode solution bubbled with a mixture of 95% O_2 and 5% CO_2 at 37 ± 1°C. The intact segment was tied to the supporting glass rod, and the isotonic transducer and its mechanical activity was recorded under a 0.25-0.5 g load. Following 20 min of equilibration, maximal contraction, which was used as a reference, was elicited with acetylcholine (10 nM for 3 min). The experiments started 40 min after the acetylcholine was removed from the organ bath.

The LM-MPs orally and anally attached to the intact segment were pulled through two pairs of ring electrodes placed 0.5 cm apart (Fig. 1, A). The oral or anal LM-MP was stimulated by square pulses (5-50 Hz, 0.1-0.2 msec, supramaximal current) for 30 sec at 5.5-min intervals. The activation of nerve elements in the LM-MP by this stimulation was selective since brief pulses, such as 0.1 or 0.2 msec, have been shown to excite nerve elements but fail to excite directly muscle fibers in the guinea-pig ileum (7), and since, in the present study, muscle responses of the intact segment to LM-MP stimulation were abolished by tetrodotoxin ($0.2 \mu M$) and were not evoked after crushing the

LM-MP between the intact segment and the electrodes. Furthermore, an application of the pulses through the electrode elicited no response on the intact segment after taking the LM-MP out of the electrodes.

The following drugs were used : acetylcholine chloride (Wako Pure Chemical Ind., Ltd., Osaka, Japan), atropine sulfate (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), guanethidine sulfate (Tokyo Kasei Kogyo), hexamethonium bromide (Sigma Chemical Co., St. Louis, Mo, U. S. A.), tetrodotoxin (Sigma Chemical), histamine hydrochloride (Wako Pure Chemical) and substance P (Peptide Institute Inc., Osaka, Japan). Stock solutions of the drugs were prepared in distilled water. Substance P was dissolved with 0.9% saline solution before the experiment. All concentrations are expressed as molarity of the salt of the final solution.

Quantitative data were presented as mean ± S. E. M. Statistical significance was determined with Student's t-test for paired data. A probability of 0.05 or less was considered statistically significant.

Results

Electrical field stimulation of the anal and oral LM-MPs caused longitudinal muscle contractions of the intact segment (Fig. 1, B). The extent of the contractions increased with increasing stimulus frequencies from 5 to 20 Hz, but the responses were slightly reduced at 50 Hz. A 20-Hz stimulation gave the maximum amplitude in most experiments (Fig. 2). The contractile response to the anal LM-MP stimulation was always larger than that to the oral LM-MP stimulation in all experi-
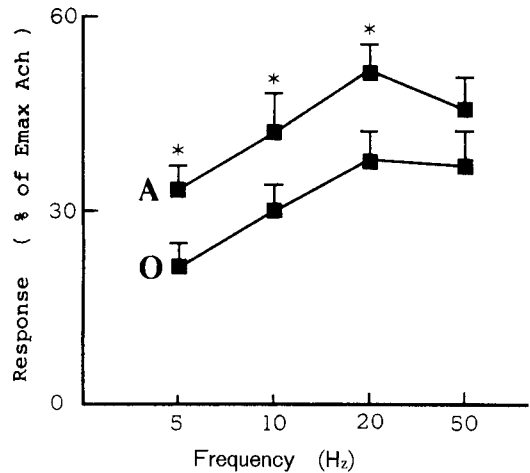

Fig. 2 Frequency-response curves for contractions of the ileal segment elicited by stimulation of oral LM-MP (O) and anal LM-MP (A). The extent of contractions (mean ± S. E. M.) is expressed as a percentage of the maximal response to acetylcholine (10 nM, Emax). ∗ : p < 0.05, A vs O.
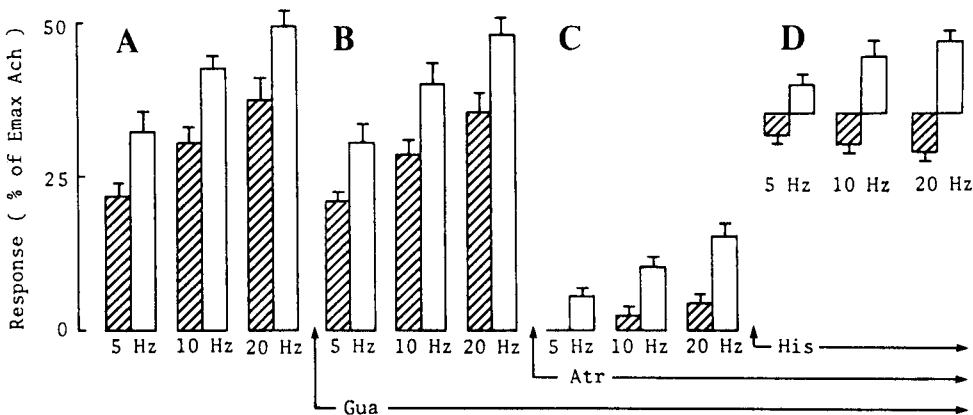

Fig. 3 Effects of guanethidine (Gua, 1 μM; B), atropine (Atr, 10 μM; C) and histamine (His, 1 μM; D) on responses of ileal segments to LM-MP stimulation. Hatched and open columns: responses to oral LM-MP and anal LM-MP stimulation, respectively. The extent of contractions in A-D and relaxations in D are expressed as a percentage of the maximal contraction evoked by 10 nM acetylcholine. Means ± S. E. M. of 19 experiments.

ments. There was a significant difference
(p < 0.05, n = 19) between the responses to
anal and oral LM-MP stimulations at all fre-
quencies of stimulation except for 50 Hz
(Fig. 2).

Guanethidine (1 μM) did not affect the con-
tractions evoked by stimulation of either the
oral or anal LM-MP at 5 to 20 Hz (n = 19)
(Fig. 3,B). Atropine (10 μM) diminished the
contractions evoked by the oral or anal LM-
MP stimulation in guanethidine-treated seg-
ments (Figs. 1,B and 3,C). Atropine inhib-
ited the contractions to the anal LM-MP
stimulation by 81.3 ± 3.1% at 5 Hz, 73.3 ±
3.3% at 10 Hz and 67.5 ± 2.9% of the con-
trol at 20 Hz, while it abolished the contrac-
tions to the oral LM-MP stimulation at 5 Hz
in all cases (n = 19) and strongly inhibited
them by 94.4 ± 1.0% and 91.3 ± 2.9% of the
control at 10 and 20 Hz, respectively. In 6
of 19 preparations, however, the responses
to the oral LM-MP stimulation at 10 and 20
Hz were completely abolished by atropine.
Therefore, the response to the oral LM-MP

stimulation was more sensitive to atropine
than that to the anal one. No relaxation was
produced by the oral and anal LM-MP stimu-
lations in segments pretreated with atropine.

Histamine, an agent which acts directly on
the longitudinal muscle, was used to raise the
basal tone of the intact segment. Histamine
(1 μM) produced an initial phasic contrac-
tion followed by a sustained tonic contraction
in the presence of atropine and guanethidine.
The magnitude of the phasic and tonic con-
tractions corresponded to 64.9 ± 3.8 and
35.2 ± 4.9% (n = 36) of the maximal contrac-
tion produced by acetylcholine (10 nM), re-
spectively. During the histamine-induced ton-
ic contracture, the anal LM-MP stimulation
at 5 to 20 Hz produced a non-cholinergic,
non-adrenergic (NCNA) contraction, but its
amplitude was not significantly different from
that before histamine, while the oral LM-MP
stimulation caused a NCNA relaxation in all
preparations tested (n = 19) (Figs. 1, B and
3, D).

Hexamethonium (10 μM) substantially in-

Fig. 4 Effects of hexamethonium
(C$_6$, 10 μM) on responses of ileal seg-
ments to LM-MP stimulation (A, n
= 11) and on non-cholinergic, non-
adrenergic (NCNA) responses to
LM-MP stimulation during histamine-
enhanced basal tone (B, n = 19). The
symbols are the same as in Fig. 3.
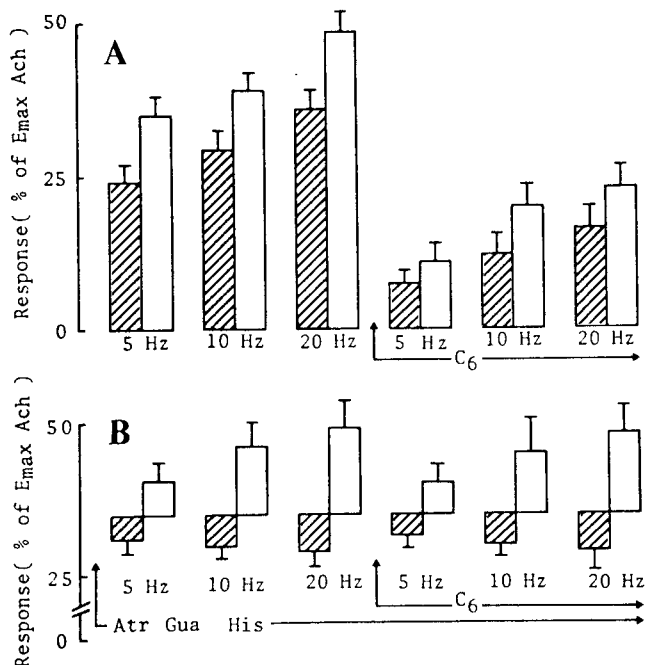
Fig. 5 Effects of desensitization to substance P (SP-D) on responses to LM-MP stimulation (A, n = 11) and NCNA responses to LM-MP stimulation under basal conditions (B, n = 14) and during histamine-enhanced basal tone (C, n = 19). The symbols are the same as in Fig. 3.
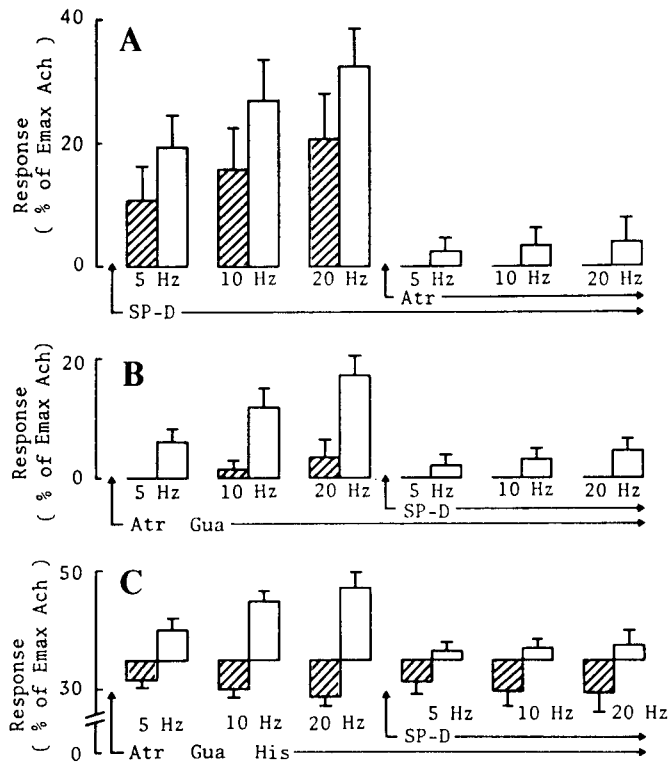
hibited the contraction evoked by either oral or anal LM-MP stimulation. The reductions were statistically significant at all frequencies of stimulation (Fig. 4,A). Reductions in the contractions evoked by anal LM-MP stimulation at 5, 10 and 20 Hz were 69.5 ± 3.1, 48.3 ± 2.8 and $52.6 \pm 3.4\%$ of the control (n = 11) and the reductions in the contractions evoked by oral stimulation at the same frequencies were 70.0 ± 2.7, 58.3 ± 3.0 and $54.6 \pm 3.3\%$ of the control (n = 11), respectively. No significant difference was found between effects of hexamethonium on the response to oral LM-MP stimulation and that to the anal one. The inhibitory effect of hexamethonium was significantly weaker than that of atropine with no exception. However, hexamethonium did not affect the NCNA contraction induced by oral or anal LM-MP stimulation without histamine treatment (data not shown), nor did it affect the NCNA con-

traction induced by the anal LM-MP stimulation, or the NCNA relaxation induced by the oral LM-MP stimulation in the presence of histamine (Fig. 4,B).

A high concentration of substance P can desensitize its own receptors in the guinea-pig small intestine (8). To produce the receptor desensitization to substance P (SP-D), a high concentration of substance P (0.1 μM) was applied. SP-D reduced the amplitudes of the contractions elicited by LM-MP stimulation. The contraction due to the anal LM-MP stimulation was depressed by 44.2 ± 3.1 % at 5 Hz, $30.8 \pm 2.8\%$ at 10 Hz and $33.6 \pm 3.4\%$ of the control at 20 Hz, and the contraction due to the oral one was reduced by $54.3 \pm 2.7\%$ at 5 Hz, $45.1 \pm 3.0\%$ at 10 Hz and $42.3 \pm 3.3\%$ of the control at 20 Hz (n = 11). The remaining contractile response to the oral LM-MP stimulation after SP-D was abolished by atropine (10 μM), and the re-

maining response to the anal LM-MP stimu-
lation was reduced markedly (Fig. 5, A).
The NCNA contraction due to the oral LM-
MP stimulation was abolished, and that to
the anal one was largely reduced to $13.6 \pm$
2.5, 20.2 ± 3.4 and $25.0 \pm 3.7\%$ of the con-
trol at 5, 10 and 20 Hz after SP-D, respec-
tively (Fig. 5, B). In contrast, the SP-D did
not affect the NCNA relaxation due to the oral
LM-MP stimulation on segments treated with
histamine (Fig. 5, C). The degree of inhibi-
tion induced by SP-D on an excitatory re-
sponse to LM-MP stimulation was significantly
smaller than that by atropine at 5 Hz-anal
LM-MP stimulation and at 5 Hz- to 20 Hz-
oral stimulation, although there was no sig-
nificant difference between the effects of
SP-D and atropine on the responses to 10
and 20 Hz-anal LM-MP stimulation.

Discussion

The present results show that an electri-
cal field stimulation of the oral and anal
LM-MPs produces longitudinal muscle con-
tractions of intact ileal segments in drug free
solution. After enhancement of the muscle
tone with histamine and suppression of cho-
linergic and adrenergic transmissions with
atropine and guanethidine, longitudinal muscle
contraction was induced by the anal LM-MP
stimulation, while relaxation of the muscle
was induced by the oral stimulation (an as-
cending NCNA excitation and a descending
NCNA inhibition), with no exception. It is
therefore revealed that there is a polarity,
at least, in the NCNA innervation, although
the ascending excitatory pathway and the de-
scending excitatory and inhibitory pathways
are formed in the myenteric plexus.

Yokoyama and Ozaki (5) showed that local
electrical stimulation of a node of the myen-
teric plexus produced mainly an excitation of
electrical activity of the longitudinal muscle

localized along the longitudinal axis of the
intestine passing through the stimulating point
and oral to the point of stimulation, while
inhibition of electrical activity was produced
anal to the point of stimulation (polarity of
oral excitation and anal inhibition). Howev-
er, local electrical stimulation produced only
an excitation of mechanical activity of the
longitudinal muscle strips both oral and anal
to the point of stimulation. They suggested
that the descending excitation, which may be
evoked by activation of an excitatory side
pathway, masked the descending inhibition,
which may be evoked by activation of an in-
hibitory pathway along the longitudinal axis.
In the present study, therefore, the response
of the ileal segment to the anal LM-MP stim-
ulation may be the sum of the cholinergic and
NCNA excitatory responses, while the re -
sponse to the oral LM-MP stimulation may be
the sum of the cholinergic and NCNA exci-
tatory responses and the NCNA inhibitory
response, because: (a) guanethidine had no
effect on the responses to the oral or anal
LM-MP stimulation; (b) atropine largely
reduced, but did not abolish the responses;
(c) relaxation due to the oral LM-MP stimu-
lation was produced after enhancement of
the muscle tone by histamine in the presence
of atropine and guanethidine. The amplitude
of contractions induced by the anal LM-MP
stimulation was always larger than that by
the oral LM-MP stimulation in drug free
solution. It is likely that the descending in-
hibitory response is masked, *i.e.*, the exci-
tatory components may be dominant to the
inhibitory ones in the response to the oral
LM-MP stimulation.

In the present experiment, the pair of ring
platinum electrodes allowed stimulation of
the entire mesh of the myenteric plexus.
Electrical stimulation of the LM-MP may
cause activation of not only excitatory and
inhibitory motor neurons but also some inter-
neurons and sensory neurons. As a result,

neural pathways other than the pathways eliciting the mucosal intrinsic reflex might be activated simultaneously. Therefore, it may be difficult to demonstrate the oral contraction and the anal relaxation (the law of the intestine or the mucosal intrinsic reflex effects) by electrical field stimulation of the LM-MPs. It seems likely that physiological afferent input from the mucosa and/or submucosa is necessary to activate selectively the efferent ascending excitatory and the efferent descending inhibitory pathways in the myenteric plexus, since mucosal stimulation causes an excitation of the motility in the oral segment to the point of stimulation and an inhibition in the anal segment (2), and since ascending and descending excitatory pathways and descending inhibitory pathways exist in the myenteric plexus as demonstrated in the present experiment and by Yokoyama and Ozaki (5). Therefore, the polarity of the efferent pathways in the myenteric plexus for eliciting mucosal intrinsic reflex may consist of polarized innervations of NCNA motor neurons and/or an ascending innervation of atropine-sensitive cholinergic motor neurons.

Yokoyama and Ozaki (5) suggested the existence of cholinergic interneurons having efferent processes that impinge on cell bodies of both ascending cholinergic excitatory and descending non-adrenergic inhibitory neurons. In the present study, the NCNA contraction induced by anal LM-MP stimulation and the NCNA relaxation induced by oral LM-MP stimulation were not affected by hexamethonium. In contrast, the oral and anal excitatory responses in drug free solution were largely reduced by hexamethonium, although the inhibitory effect of this drug was weaker than that of atropine. From these results, it was suggested that transmission through nicotinic cholinergic synapses was in part involved in atropine-sensitive excitatory pathways, but was not involved in the

NCNA pathways.

Substance P-containing nerves have been demonstrated in the guinea-pig ileum, including the myenteric plexus, by immunohistochemical methods (9). Franco *et al.* (10) demonstrated that non-cholinergic longitudinal muscle contractions of guinea-pig ileum to stimulation of the intramural nerves were abolished by a specific desensitization to substance P. The present experiment reconfirmed their findings. The ascending and descending NCNA contractions elicited by the LM-MP stimulation were abolished or reduced largely by desensitization to substance P, but the descending NCNA relaxation was not changed. These results indicate that there are myenteric nerves which release a substance pharmacologically similar to substance P in the NCNA excitatory pathways. The contractile response sensitive to SP-D was larger than the response resistant to atropine and sensitive to SP-D (44.2 vs 13.6% of the control at 5 Hz-anal LM-MP stimulation), indicating that substance P neurons may also be involved in atropine-sensitive excitatory pathways.

In conclusion, our findings suggest that the NCNA innervation is a polar, *i.e.*, an excitation is produced on the oral side and an inhibition on the anal side of the stimulated point. The NCNA excitatory response may be mediated at least in part by myenteric substance P neurons, and the NCNA inhibitory response may be mediated by non-adrenergic neurons.

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