Analysis of fast synaptic pathways in myenteric plexus of guinea pig ileum

KATHY J. LEPARD AND JAMES J. GALLIGAN
Department of Pharmacology and Toxicology and Neuroscience Program, Michigan State University, East Lansing, Michigan 48824

LePard, Kathy J., and James J. Galligan. Analysis of fast synaptic pathways in myenteric plexus of guinea pig ileum. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G529–G538, 1999.—Most fast excitatory postsynaptic potentials (fEPSPs) recorded from guinea pig ileum myenteric plexus are mediated by acetylcholine acting at nicotinic receptors and ATP acting at P2X receptors. These studies examine length and polarity of projection of neurons releasing mediators of fEPSPs. Under ketamine-xylazine anesthesia, animals were sham treated or myenteric pathways were interrupted. After severed axons degenerated, fEPSPs were recorded at the operated site using conventional, intracellular electrophysiological methods and were classified as nicotinic or mixed on the basis of sensitivity to hexamethonium. Cholinergic and noncholinergic fEPSPs were recorded from small, operated segments, suggesting that some neurons have projections between adjacent ganglia. The mean amplitudes of nicotinic and mixed fEPSPs were reduced after circumferential and descending pathways degenerated. The proportion of nicotinic vs. mixed fEPSPs recorded from tissues lacking descending projections was greater than that recorded from sham-treated tissues, suggesting that fibers releasing noncholinergic mediators project aborally. Descending projections communicate with neurons in ganglia at least three rows aboral to their origin. The data suggest that fast noncholinergic neurotransmission could contribute to hexamethonium-resistant descending inhibition during the peristaltic reflex.

enteric nervous system; myotomy; electrophysiology; adenosine 5′-triphosphate; noncholinergic fast excitatory postsynaptic potential

ANALYSIS OF PROJECTIONS of identified enteric neurons is essential to understanding the circuitry underlying integrated gastrointestinal reflexes. Specific neuronal markers have been combined with morphology to provide useful information on polarity of projections of identified myenteric neurons (3, 5–10, 28, 33, 34). These studies showed that motoneurons project locally to circular and longitudinal muscle and have Dogiel type I morphology. Excitatory motoneurons contain choline acetyl transferase (ChAT) with or without substance P (SP), and inhibitory motoneurons contain vasoactive intestinal polypeptide (VIP) and nitric oxide synthase (NOS) (7–9, 28). Descending interneurons project long distances aborally, have Dogiel type I or filamentous morphology, and contain ChAT or NOS in combination with serotonin, VIP, or somatostatin (6). Ascending interneurons project short distances orally, have Dogiel type I morphology, and contain ChAT and SP (5, 8, 28). Intrinsic primary afferent neurons (IPANs) send one projection to the mucosa and the other primarily to aborally positioned myenteric ganglia (3, 6, 15, 33, 34). IPANs have Dogiel type II morphology and contain combinations of ChAT, SP, and the calcium-binding protein calbindin. Secretory- and/or vasomotoneurons project to the mucosa or submucosal blood vessels and have Dogiel type III morphology. They contain various peptides with or without CHAT. A detailed summary of the functional class, morphology, and chemical coding of 16 classes of myenteric neurons has been provided by Costa et al. (10).

Immunoactivity in cell bodies and nerve fibers for a neurotransmitter only implies its usage in synaptic transmission. Investigation of polarity of projection of identified myenteric neurons that release specific synaptic neurotransmitters is sparse. Preliminary studies to identify neurotransmitter(s) mediating slow excitatory postsynaptic potentials (sEPSPs) utilized lesion experiments. sEPSPs of normal amplitude persisted in areas where nerve fibers containing serotonin, VIP, somatostatin, enkephalin, or gastrin-releasing peptide had degenerated (4). Because SP-containing fibers have very local projections (13), these sEPSPs were presumed to be mediated by SP (4).

Until recently, fast synaptic neurotransmission was thought to be mediated solely by ACh acting at nicotinic receptors (37). Because CHAT immunoreactivity is found in most classes of myenteric neurons (10), experiments to discern a distinct cholinergic pathway(s) were not possible. In 1994, it was found that not all fast EPSPs (fEPSPs) are mediated exclusively by ACh, because many EPSPs had a hexamethionium (C6)-resistant component (16). Subsequently, two broad groups of fEPSPs have been defined, nicotinic and mixed (24). Nicotinic fEPSPs represent ~25% of all fEPSPs; mixed fEPSPs contain cholinergic and noncholinergic components and represent ~75% of all fEPSPs. Most noncholinergic fEPSPs (90%) are mediated completely or in part by ATP acting at P2X receptors (24). Because two pharmacologically distinct fast neurotransmitters can be released on stimulation of interganglionic fiber tracts, studies were initiated to discern whether distinct cholinergic, mixed, or purinergic pathways exist. The goals of this study were to investigate the length (short or long) and polarity (oral, aboral, or circumferential) of projection(s) that release neurotransmitters contributing to generation of fEPSPs.
MATERIALS AND METHODS

Longitudinal muscle-myenteric plexus preparation. Longitudinal muscle-myenteric plexus (L MMP) preparations were isolated from segments of surgically treated guinea pig ileum using standard methods. Guinea pigs (male, 250–350 g) were anesthetized (halothane inhalation), stunned, and bled through the neck. This protocol was approved by the Michigan State University Animal Care and Use Committee. A segment of ileum was placed in normal Krebs solution of the following composition (in mM): 117 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, and 11 glucose. The Krebs solution contained scopolamine (1 µM) to block muscarinic receptors and nifedipine (1 µM) to block calcium channels on smooth muscle. A segment of gut containing the operated site was cut open and pinned out flat (mucosal surface up) in a dish lined with Silastic elastomer. The mucosal, submucosal, and circular muscle layers were successively stripped away, leaving the myenteric plexus with attached longitudinal muscle. The preparation containing the operated site was transferred to a small recording chamber (2-ml volume), stretched lightly, and pinned to the chamber bottom. The preparations were superfused with warmed (36°C), oxygenated Krebs solution at a flow rate of 7 ml/min. Individual ganglia were visualized at ×200 magnification using an inverted microscope and differential interference contrast optics.

Intracellular electrophysiology. Intracellular recordings were obtained from myenteric neurons using microelectrodes filled with 2 M KCl (tip resistance 60–120 MΩ). Electrode resistance was compensated using an amplifier with an active bridge circuit. Membrane potential and currents were measured using a current- and voltage-clamp amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA). A glass pipette (tip diameter ~60 µm) filled with Krebs solution was used as a focal stimulating electrode. The stimulating electrode was positioned on an interganglionic connective attached to the myenteric ganglion containing the impaled neuron. For some neurons (n = 47), multiple interganglionic connectives were stimulated (2–9) and multiple fEPSPs were recorded (n = 152). For other neurons (n = 131), only one interganglionic connective was stimulated and single fEPSPs were recorded. Single stimuli (0.5-ms duration, 0.02–4 mA) provided by a pulse generator (Master 8, AMPI) and a constant current stimulus isolation unit (A360, WPI) were used to elicit fEPSPs. Cells were included in the study if fEPSPs were recorded on stimulation of interganglionic fiber tract(s); no further attempt was made to classify these cells. The membrane potential of the neuron was hyperpolarized to approximately −100 mV by passing a constant hyperpolarizing current through the microelectrode. Membrane hyperpolarization reduced the chance that the fEPSP would reach spike threshold. Signals were filtered at 1 kHz using a four-pole Bessel filter (Warner Instruments) and digitized at 10 kHz using a DigiData 1200 analog-to-digital converter (Axon Instruments).

Surgical procedures for interruption of intrinsic nerves. All surgical procedures were performed under ketamine (100 mg/ml) and xylazine (10 mg/ml) anesthesia (0.5 ml/kg im). After the animal was placed on a thermal barrier (Vetko), the animal’s fur was carefully removed with a sharp razor blade and the small intestine was exteriorized through a midline abdominal incision. The exposed intestinal segment was kept moist with warmed PBS (0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.2). In sham animals (n = 7), the segment was washed with PBS, manually manipulated, identified with silk suture, and returned to the abdominal cavity. In surgically treated animals, double myotomy, single myotomy, or three longitudinal myotomies were performed on each section. The site of the surgical procedure was indicated by a piece of loosely tied silk suture around the mesenteric vessel closest to the operated site. After surgery, the abdomin al muscles were sewn together with surgical silk (4–0, Deknatel) and the skin was stapled together. All guinea pigs received an injection (0.1 ml im) of broad-spectrum antibiotic (Combiotic, Pfizer Agricultural Division, New York, NY). Animals were allowed to recover for 3–5 days. The operated sections were subsequently identified and excised for utilization in the electrophysiological experiments while the proper oral-to-aboral polarity was preserved.

Double myotomy. To investigate the contribution of both long ascending and long descending pathways to the generation of fEPSPs, in nine guinea pigs, a double myotomy operation was performed (Fig. 1A). In these animals, the long ascending and descending nerve pathways of the myenteric plexus were interrupted by two circumferential incisions through the longitudinal muscle and myenteric plexus (14).
The distance between the two single myotomies was 8 ± 2 mm (range 2–22 mm) or 10 ± 2 rows of ganglia (range 3–23 rows), an average of 0.75 mm/row. fEPSP were recorded from neurons in ganglia located in the island between the two myotomies (shaded area, Fig. 1A).

Single myotomy. To investigate the contribution of either long ascending or long descending pathways to fEPSP generation, in 10 guinea pigs, a single myotomy operation was performed (Fig. 1, B and C). The completeness of each myotomy was confirmed by VIP immunohistochemistry (see Histochemical verification of surgical procedures using VIP immunohistochemistry). fEPSP were recorded from neurons in ganglia within one to three rows oral or aboral of the operated site (shaded area, Fig. 1, B and C).

Longitudinal myotomy. To investigate the contribution of circumferential pathways to the generation of mixed fEPSP, in six guinea pigs we interrupted circumferential nerve pathways by teasing away thick strips of LMMP using blunt forceps (Fig. 1D). Longitudinal myotomy was confirmed at the time of tissue dissection by the presence of longitudinal gaps in the LMMP preparation. The distance between myotomies was 2 ± 0.2 ganglia (range 2–3). fEPSP were recorded from ganglia located at the center of the operated site (shaded area, Fig. 1D).

Histochemical verification of surgical procedures using VIP immunohistochemistry. To confirm that myotomies severed long myenteric neuronal pathways, tissues were treated to visualize VIP nerve terminals aboral to the operated site. After electrophysiological recordings, the experimental portion, flanked by adjacent control sections, was placed in Zamboni’s fixative (2% formaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0) for 24 h at 4°C. The fixative was cleared three times for 10 min with dimethyl sulfoxide. The tissue was rehydrated with three 10-min washes with PBS and then incubated for 24 h in a moist environment at room temperature with 50 µl of VIP-(1–28) primary antibody (code 7913; host, rabbit; dilution, 1:200; gift from CURE Antibody Core, Los Angeles, CA). The unbound antibody was removed by washing three times for 10 min with PBS. The tissue was then incubated for 1–2 h with the secondary antibody, Cy3-conjugated AffiniPure goat anti-rabbit (Jackson ImmunoResearch, 1:200 dilution). The tissues were washed three times more with PBS for 10 min and mounted in buffered glycerol for fluorescence microscopy.

Electrophysiological protocol. In some experiments, only one interganglionic fiber tract entering the ganglia containing the impaled neuron was stimulated. If the fEPSP was <5 mV in amplitude, another interganglionic fiber tract was chosen. For these neurons, the stimulus strength resulting in maximal, control fEPSP amplitude was determined. For other experiments, the stimulating electrode was manually moved to randomly sample many (2–9) interganglionic fiber tracts entering the ganglia containing the impaled neuron. Control fEPSPs, regardless of amplitude, were recorded after stimulation of each fiber tract (1 mA). C6 (100 µM) was superfused, and each fiber tract was stimulated. Care was taken to place the stimulating electrode at approximately the same site on the interganglionic fiber tract. In some experiments, the orientation of the fiber tract to the ganglia (oral, aboral, or circumferential) was noted. In some experiments, the amplitude of the noncholinergic fEPSP after superfusion of C6 (100 µM) plus the P2X antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 10 µM) was recorded.

Statistics. Responses were recorded as a digital average of 12 individual fEPSPs in the absence and presence of drug treatment. Averages were obtained using a personal computer and Axotape software. Data are expressed as means ± SE. Student's t-test for paired data was used to evaluate differences between two groups. For comparisons of more than two groups, the general linear model (GLM) with post hoc least-squares means (LSM) was used. For comparisons of frequency distributions, frequency tables were evaluated by χ-square with post hoc Cochrane-Mantel-Haenszel (CMH, non-zero correlation) statistic. To perform post hoc comparisons of the frequency distribution of nicotinic versus mixed fEPSPs recorded from sham-treated versus surgically operated tissues, we used categorical modeling with maximum likelihood estimates (MLE). An α-level of P < 0.05 was considered significant for any statistical analysis.

Data included in the frequency distribution histograms of fEPSP amplitudes were binned at 2.4 mV because in the presence of C6 the first 0–2.4 mV bin represented nicotinic fEPSPs. Data included in the frequency distribution histograms of percent inhibition (see Fig. 6) were binned at 10% for the minimum needed to produce a significant effect. All histograms were best fit by single or multiple normal Gaussian distributions using the Pstat program of pCLAMP 6 (Axon Instruments).

Drugs. Drugs were applied by superfusion. PPADS was obtained from Research Biochemicals (Natick, MA). All other drugs were obtained from Sigma Chemical (St. Louis, MO).

RESULTS

Verification of effectiveness of surgical treatments. Circumferential myotomy was confirmed by immunohistochemistry as VIP immunoreactivity (VIP-ir) was decreased aboral to the single myotomy (Fig. 1). The first few rows of ganglia were almost devoid of VIP-ir nerve fibers, but cell bodies were seen clearly (Fig. 2, A and B). Animals (n = 22) were allowed to recover from the surgical procedure for 3–5 days.

Pharmacological characterization of fEPSPs. Recordings were obtained from 157 neurons from 32 guinea pigs. One to seven interganglionic fiber tracts entering the ganglion containing the impaled neuron were stimulated to elicit fEPSPs. fEPSPs were categorized as either nicotinic or mixed based on their sensitivity to the nicotinic antagonist C6 (100 µM). If the control fEPSP amplitude (20 ± 1 mV, n = 260) was reduced ≥90% (≤2 mV) by C6, the fEPSP was categorized as nicotinic. If the control fEPSP amplitude was reduced <90% (>2 mV) by C6, the fEPSP was categorized as mixed (Fig. 3).

Effect of surgical procedures on stimulus strength. The effect of surgical treatment on the stimulus strength needed to elicit a control fEPSP of equal, maximal, and stable amplitude was determined for 77 neurons (17 guinea pigs). In these studies, a single fiber tract was stimulated to elicit a fEPSP and the control amplitude (21 ± 1 mV) was independent of surgical treatment (GLM, P = 0.7088) and pharmacological characterization (GLM, P = 0.9859). Overall, the stimulus strength necessary to elicit a fEPSP of equal, maximal, and stable amplitude was 0.67 ± 0.05 mA (range 0.01–2.02 mA) and was independent of surgical procedure (GLM, P = 0.3846) and fEPSP pharmacology (Student's t-test, P = 0.5968). All other fEPSPs were evoked using a stimulus strength of 1 mA.
Effect of surgical procedures on overall fEPSP amplitudes. The control amplitude of all (nicotinic and mixed) fEPSPs recorded from each surgically treated group was not different from the sham-treated group (GLM, \( P = 0.005 \); LSM vs. sham, \( P > 0.05 \); Table 1). However, regardless of surgical treatment, the control amplitude of all nicotinic fEPSPs (14 ± 1 mV, \( n = 47 \)) was smaller than the control amplitude of all mixed fEPSPs (20 ± 1 mV, \( n = 213, P < 0.0002 \)). When the amplitude of the control fEPSP was <10 mV, the fEPSP was about three times more likely to be nicotinic (36% or 22/61) compared with amplitudes >10 mV (13% or 25/199; \( \chi^2, P = 0.001 \)).

The amplitudes of all (nicotinic and mixed) fEPSPs in the presence of C6 were also analyzed. The amplitude of fEPSPs recorded aboral to a single myotomy (4.2 ± 0.1 mV, \( n = 49 \)) was significantly lower than that recorded from sham-treated segments (8.1 ± 0.1 mV, \( n = 53 \); GLM, \( P = 0.0129 \); LSM, \( P = 0.0006 \)).

Effect of surgical procedures on nicotinic fEPSP amplitudes. Surgical treatments altered the control amplitude of nicotinic fEPSPs. Nicotinic fEPSPs recorded both aboral to a single myotomy and between two longitudinal myotomies were smaller than those recorded from sham-treated segments (GLM, \( P = 0.0403 \); LSM vs. sham: aboral single myotomy, \( P = 0.0284 \); longitudinal myotomy, \( P = 0.0326 \); Table 1). The frequency distribution of control amplitudes of nicotinic fEPSPs recorded from sham-treated segments was unimodal with a peak centered at 14 ± 10 mV. The distributions of fEPSPs recorded from surgically operated segments were also unimodal (peaks (mV): double myotomy, 8 (fixed); oral single myotomy, 11 ± 6; aboral single myotomy, 8 ± 1; Fig. 4). Longitudinal myotomy could not be fit by a Gaussian distribution.

Effect of surgical procedures on mixed fEPSP amplitudes. Surgical treatment altered the control amplitude of mixed fEPSPs. Mixed fEPSPs recorded from the island between two circumferential myotomies (double myotomy) were larger than those recorded from sham-treated segments (GLM, \( P = 0.0428 \); LSM vs. sham, \( P = 0.0373 \); Table 1). The frequency distribution of control amplitudes of mixed fEPSPs recorded from sham-treated segments was unimodal with a peak centered at 19 ± 1 mV. When both long ascending and descending pathways were eliminated (double myotomy), the frequency distribution became multimodal with peaks centered at 16 ± 1, 30 ± 1, and 36.2 ± 0.4 mV (Fig. 5). Otherwise, the distributions were unimodal (peaks (mV): longitudinal myotomy, 11 ± 1; oral single myotomy, 19 ± 1; aboral single myotomy, 16 ± 3; Fig. 5).

Effect of surgical procedures on mixed fEPSP amplitudes in presence of C6. Surgical treatment altered the amplitude of fEPSPs in the presence of C6 (100 µM). In the absence of either circumferential or long descending inputs, the amplitude of the mixed fEPSPs in the presence of C6 was significantly lower than those recorded from sham-treated segments (GLM, \( P = 0.0443 \); LSM vs. sham: longitudinal myotomy, \( P = 0.0043 \); aboral single myotomy, \( P = 0.0443 \); double myotomy, \( P = 0.0374 \); Table 1).

Elimination of long myenteric pathways revealed multimodal distributions of fEPSP amplitudes in the presence of C6 (Fig. 6). The distributions of noncholinergic fEPSP amplitudes recorded from sham-treated and longitudinal myotomy segments were unimodal with peaks centered around 5 ± 3 and 3 ± 2 mV, respectively. The frequency distributions of noncholinergic fEPSPs recorded from the island between two myotomies, oral to a single myotomy, and aboral to a single...
myotomy were multimodal [peaks (mV): double myotomy, 1.3 ± 0.3, 8 ± 1, 21 ± 1; oral single myotomy, −9 ± 31, 11 ± 4; aboral single myotomy, −1 ± 2, 9.1 ± 0.4; Fig. 6].

Noncholinergic pathways. To determine the polarity of projection of neurons releasing fast noncholinergic neurotransmitters, the ratio of nicotinic vs. mixed fEPSPs recorded at the operated site was determined for each surgical group. In the sham-treated group, 15% (8/53) of all fEPSPs were nicotinic and 85% (45/53) were mixed (Table 1). The proportion of nicotinic vs. mixed fEPSPs recorded at the operated site was not different from sham (8/45) in oral single myotomy (1/33, P = 0.1612), double myotomy (7/60, P = 0.4469), and longitudinal myotomy (3/43, P = 0.1875) (categorical modeling, MLE). In contrast, the proportion of nicotinic vs. mixed fEPSPs was significantly greater when fEPSPs were recorded aboral to the single myotomy (17/32, categorical modeling, MLE, P = 0.0246).

In a portion of the studies, the distance of the ganglia containing the impaled neuron from the above myotomy was noted. For aboral single myotomy, 92% (34/37) of the fEPSPs were recorded within the first two rows of ganglia (~1.5 mm, see MATERIALS AND METHODS). For double myotomy, only 28% (9/32) of the fEPSPs were recorded within the first two rows of ganglia. The distance of the ganglia containing the impaled neuron from the orally located myotomy significantly influenced the distribution of nicotinic vs. mixed fEPSPs within double myotomy (χ², P = 0.004). The distribution of nicotinic vs. mixed fEPSPs within two rows (1.5 mm) of the double myotomy was similar to that of aboral single myotomy (χ², P = 0.937). In contrast, the distribution of nicotinic vs. mixed fEPSPs recorded from ganglia three to five rows below the myotomy was significantly different from aboral single myotomy (χ², P = 0.001).

Effects of surgical procedures on purinergic fEPSP amplitudes. In a subset of experiments, the sensitivity of the noncholinergic fEPSP to the P2X antagonist PPADS (10 µM; Ref. 24) was determined. If the amplitudes...
tude of the noncholinergic fEPSP was reduced \( \approx 10\% \) by PPADS, the fEPSP was classified as partially purinergic (24). If the amplitude of the noncholinergic fEPSP was reduced \( \approx 10\% \) by PPADS, the fEPSP was classified as “other” (24). Surgical treatment did not affect the ability of PPADS to reduce the amplitude of noncholinergic fEPSPs (ANOVA, \( P = 0.3659 \)). Overall, PPADS reduced the amplitude of noncholinergic fEPSPs by 53 ± 3\% \((n = 89)\).

Purinergic pathways. The previous data suggest a descending, noncholinergic fast synaptic pathway, so data were pooled to compare intact and degenerated descending pathways. Intact descending pathways included sham, oral single myotomy, and longitudinal myotomy. Absent descending pathways included double myotomy and aboral single myotomy. PPADS-sensitive noncholinergic fEPSPs represented 92\% (61/66) and 74\% (17/23) of all noncholinergic fEPSPs recorded at the operated site for intact and degenerated descending pathways, respectively. The proportion of purinergic fEPSPs to other fEPSPs was significantly different for intact (61/66) vs. degenerated (17/23) descending pathways (\( \chi^2, P = 0.020 \)). In addition, PPADS was less effective to inhibit the noncholinergic fEPSPs after descending pathways had degenerated \( [\text{intact} (n = 66), 57 \pm 4\%; \text{absent} (n = 23), 37 \pm 8\%] \) Student’s t-test, \( P = 0.0265 \); Fig. 7).

Orientation of interganglionic fiber tracts. The orientation of the interganglionic fiber tract to the ganglia containing the impaled neuron was noted as oral, aboral, or circumferential for 219 fEPSPs. Orientation of the fiber tract to the ganglia had no significant effect on classification of the fEPSP as nicotinic or mixed (frequency distribution, CMH, \( P = 0.067 \)). In general, mixed fEPSPs were observed more frequently when circumferential fiber tracts (91\% or 58/64), as opposed to oral (79\% or 53/67) or aboral (76\% or 67/88) fiber tracts, were stimulated.

**DISCUSSION**

Processes of enteric neurons that release noncholinergic neurotransmitters involved in fast excitatory synaptic transmission have short, local projections and long, descending projections in the guinea pig ileum. ATP is a neurotransmitter in the local and descending pathways. These conclusions are based in part on electrophysiological results obtained in tissues in which ascending, descending, and circumferential pathways in the intestine had been surgically cut. Surgical treatments severed long myenteric pathways as three distinct parameters were changed: 1) VIP-ir aboral to the single myotomy, 2) the amplitude of fEPSPs in the absence and presence of C6, and 3) the proportion of nicotinic vs. mixed fEPSPs.

Local noncholinergic pathways. Noncholinergic fEPSPs were recorded from experimental segments containing...
only a few ganglia, suggesting that some noncholinergic projections are short (Fig. 8A). In these studies, noncholinergic fEPSPs were recorded from islands that were only two ganglia in length (double myotomy) or width (longitudinal myotomy). Our estimated length of the local projections (<1.5 mm) agrees with a previous study by Bornstein et al. (4) in which fEPSPs were recorded from preparations only four ganglia in length.

Because few submucosal neurons project to the myenteric plexus (21), the short projections must arise from other myenteric neurons. Three types of neuron have local projections in the myenteric plexus: Dogiel type II/AH neurons (3, 28, 35), collaterals of Dogiel type I/S or filamentous/S descending interneurons (25, 29), and Dogiel type I/S longitudinal and circular muscle motoneurons (7−9, 28). Motoneurons do not synapse with other enteric neurons. Therefore, the sources of the local projections are Dogiel type II/AH neurons and/or collaterals of descending interneurons. Our data suggest that some noncholinergic inputs arise from circumferentially directed neurons located farther than two ganglia away, because their elimination by longitudinal myotomy reduced the amplitude of noncholinergic fEPSPs. Dogiel type II/AH neurons have circumferential projections ranging from 1 to 15 ganglia or 7 mm (3, 35), and they can communicate with other neurons via fast excitatory synapses (1, 36). In contrast, Dogiel type I/S neurons do not project in a circumferential direction for more than 600 µm (2, 22, 23, 30). Therefore, some circumferential, noncholinergic inputs must arise from Dogiel type II/AH neurons.

Other local projections may arise from collaterals of descending interneurons (Fig. 8A), because descending but not ascending (5) interneurons can form synapses within the first 100 µm of the cell body (29). There are four neurochemically distinct types of descending interneuron. Three of these have Dogiel type I morphology and contain 5-hydroxytryptamine/ChAT or NOS/VIP/ChAT or bombesin/NOS/VIP. The fourth group contains somatostatin and ChAT, and these neurons have a filamentous-type morphology (25, 29). Descending interneurons communicate with other descending interneurons or motoneurons via fast excitatory synapses (2, 30) and orthodromic or antidromic activation of descending interneurons may release noncholinergic neurotransmitters at collateral synapses.

Descending noncholinergic pathways. Neurons that release noncholinergic neurotransmitters involved in fEPSPs have long, descending projections (Fig. 8B). This is supported by three lines of evidence: 1) the proportion of nicotinic vs. mixed fEPSPs recorded aboral to a single myotomy was greater than in sham-treated tissues; 2) the amplitude of noncholinergic fEPSPs recorded from the island between two myotomies was smaller than those recorded from sham-treated segments; and 3) the amplitude of noncholinergic fEPSPs recorded aboral to a single myotomy was smaller than those recorded from sham-operated tissues.

The proportions of nicotinic vs. mixed fEPSPs recorded from neurons located between two myotomies (7/60) and from sham-treated segments (8/45) were different. However, the distance of ganglia containing the impaled neuron from the oral myotomy influenced the distribution of nicotinic vs. mixed fEPSPs recorded in the isolated island. If recordings were obtained from ganglia one to two rows aboral to the myotomy, the distribution of fEPSP amplitudes was similar to that obtained from recordings aboral to a single myotomy. If the recordings were obtained from ganglia located three or more rows aboral to the myotomy, the distribution of fEPSP amplitudes was similar to sham-treated segments. These data suggest that the shortest length of this noncholinergic pathway is approximately three rows of ganglia (~2.3 mm).

Most neurons have descending projections in the myenteric plexus. Indeed, the majority of all neurons (77%) project in an oral-to-aboral direction (35). Neurons of interest to this study include dendritic Dogiel type II/AH neurons (6, 35) and Dogiel type I/S descending interneurons (29, 36). Dendritic Dogiel type II/AH neurons comprise the largest single source (43%) of...
descending projections longer than 2 mm (6), and they can communicate with other neurons via fast synaptic mechanisms (1, 36). Dendritic Dogiel type II/AH neurons may function as long, descending interneurons to supply noncholinergic synapses within the myenteric plexus.

The short and long noncholinergic projections may arise from the same or different neurons (Fig. 8, A and B). Both dendritic Dogiel type II/AH neurons and Dogiel type I/S descending interneurons have short, collateral projections and long, descending projections. If only one neuron type was responsible for both pathways, the best candidate would be the dendritic Dogiel type II/AH neurons because their circumferential processes are extensive. If more than one neuron is responsible for both pathways, Dogiel type II/AH neurons could account for the local noncholinergic projections and descending interneurons for the long, aborally directed noncholinergic processes. The subclass of descending interneuron could not be determined from this study except to exclude filamentous interneurons because they receive little circumferential input (36).

Cholinergic pathways. Nicotinic fEPSPs were recorded from neurons in every surgical treatment group. Examination of the proportion of nicotinic vs. mixed fEPSPs did not reveal a distinct polarity of nicotinic pathways, but some deductions can be drawn from the control amplitudes of nicotinic fEPSPs. Longitudinal myotomy decreased the control amplitude of nicotinic fEPSPs by 67%, suggesting that circumferential synaptic inputs from Dogiel type II/AH neurons (3, 35) contribute to generation of nicotinic fEPSPs. Similarly, the control amplitude of nicotinic fEPSPs recorded aboral to a single myotomy was smaller than that recorded from sham-treated segments (Table 1), suggesting that some descending Dogiel type I/S, filamentous/S, or dendritic Dogiel type II/AH neurons contribute to generation of nicotinic fEPSPs (Fig. 9).

Fig. 9. Summary of nicotinic pathways. Cartoon represents enteric neurons with their somas (circle) and projections (lines). Oral direction is at top of figure. Acetylcholine is released from terminals of circumferentially projecting neurons with somas at least 2 ganglia away and/or aborally projecting neurons with somas at least 3 rows away. Candidates include Dogiel type II/AH sensory neurons, dendritic Dogiel type II/AH neurons, and/or Dogiel type I/S descending interneurons.

Inhibitory pathways. The control amplitude of mixed but not nicotinic fEPSPs recorded from the island located between two myotomies was larger than that recorded from sham-treated segments. These data suggest that long ascending and descending pathways release a mediator(s) that modulates the amplitude of mixed but not nicotinic fEPSPs. Possible mediators include opioid peptides, neuropeptide Y, somatostatin, VIP, serotonin, and norepinephrine (14).

Frequency distributions of fEPSP amplitudes. Degeneration of fiber tracts after surgical treatment resulted in a loss of synaptic input to the neuron as reflected in smaller control (no C6 present) fEPSP amplitudes. If enough synaptic inputs to the impaled neuron degenerate after surgical treatments, control fEPSP amplitudes would be a measure of neurotransmitter released by the terminals of single presynaptic fibers and gradations in control amplitudes would allow for estimation of the number of intact presynaptic nerve fibers (27).

Gradations in control amplitudes are represented by frequency distribution histograms. The distribution of control fEPSP amplitudes recorded from sham-treated segments was unimodal. Only after elimination of long myenteric pathways did fEPSP amplitudes cluster into multiple peaks. In the absence of C6, elimination of both ascending and descending pathways was necessary to reveal a multimodal distribution. The smallest resolvable peak was 16 ± 1 mV (double myotomy). Double myotomy substantially decreased the number of presynaptic inputs to the impaled neuron. As a result, fEPSP control amplitudes clustered around discrete peaks. In the presence of C6, the amplitude distributions were multimodal after elimination of only one (either ascending or descending) long myenteric pathway. The smallest resolvable peak was 8 ± 1 mV (double myotomy). The number of presynaptic inputs that produced a measurable synaptic response was decreased by both the presence of C6 in the bath and surgical treatment. Because all but one peak were a multiple of 4 mV (30 ± 1 mV, double myotomy), the terminals originating from a single axon may release sufficient neurotransmitters to depolarize neurons by 4 mV. Each additional 4 mV represents release of transmitter from nerve terminals of additional axons. The data suggest that, under control conditions, most neurons are contacted by the terminals of five axons (20 mV). Multimodal distribution of control fEPSP amplitudes is observed within individual myenteric neurons responding to mucosal stimulation, suggesting that synaptic responses arise from multiple sensory nerves (2).

Stimulus strength. The minimum stimulus strength necessary to elicit fEPSPs of equal, maximal, and stable amplitudes was determined for a subset of neurons. Degeneration of nerve terminals may influence the stimulus strength necessary to obtain control amplitudes of magnitude comparable to those recorded from sham-treated segments. The minimum stimulus strength necessary to elicit a fEPSP of maximal and stable amplitude was not altered by surgical treatment. Instead of gradations in stimulus strength resulting in
gradations in control fEPSP amplitude, equal-amplitude fEPSPs were elicited with a wide range of stimulus strengths (0.01–2.02 mA). The variability in control fEPSP amplitudes was caused by degeneration of nerve fibers, not altered sensitivity of fiber tracts to applied stimulus strength. In contrast, stronger focal stimulation was required to elicit eSEPs from surgical segments lacking descending inputs (4).

Conclusion. Noncholinergic fast excitatory neurotransmission has an unknown function in the circuitry of the enteric nervous system. These studies revealed a strong descending projection for neurons releasing ATP. Many studies have described C6-resistant descending relaxation in the peristaltic reflex (20, 30, 31, 32). The resistance of the descending limb of the peristaltic reflex to C6 decreases as the distance from the stimulus increases (30). For example, the descending relaxation was partially reduced at 20 mm (12, 31) but totally blocked at 30–40 mm (17, 18, 31) from the site of stimulation. In one study, the P2 antagonist suramin did not alter C6-resistant descending reflex responses (20). However, conclusions based only on results obtained with suramin are limited because it also interferes with neurotransmission at inhibitory neuromuscular synapses (19, 26). The physiological role of noncholinergic neurotransmission remains unknown, but participation in responses associated with aborally directed ganglionic transmission is most likely.

The authors express gratitude to Dr. David Schneider for assistance with statistical analysis. Address for reprint requests: K. J. LePard, Dept. of Pharmacology and Toxicology, B328 Life Sciences Bldg., Michigan State Univ., East Lansing, MI 48824.

Received 6 July 1998; accepted in final form 27 October 1998.

REFERENCES


