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Original Article

Dietary Glutamate: Interactions With the Enteric Nervous System

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Background/Aims
Digestion of dietary protein elevates intraluminal concentrations of glutamate in the small intestine, some of which gain access to the enteric nervous system (ENS). Glutamate, in the central nervous system (CNS), is an excitatory neurotransmitter. A dogma that glutamatergic neurophysiology in the ENS recapitulates CNS glutamatergic function persists. We reassessed the premise that glutamatergic signaling in the ENS recapitulates its neurotransmitter role in the CNS.

Methods
Pharmacological analysis of actions of receptor agonists and antagonists in concert with immunohistochemical localization of glutamate transporters and receptors was used. Analysis focused on intracellularly-recorded electrical and synaptic behavior of ENS neurons, on stimulation of mucosal secretion by secretomotor neurons in the submucosal plexus and on muscle contractile behavior mediated by musculomotor neurons in the myenteric plexus.

Results
Immunoreactivity for glutamate was expressed in ENS neurons. ENS neurons expressed immunoreactivity for the EAAC-1 glutamate transporter. Neither L-glutamate nor glutamatergic receptor agonists had excitatory actions on ENS neurons. Metabotropic glutamatergic receptor agonists did not directly stimulate neurogenic mucosal chloride secretion. Neither L-glutamate nor the metabotropic glutamatergic receptor agonist, aminocyclopentane-1,3-dicarboxylic acid (ACPD), changed the mean amplitude of spontaneously occurring contractions in circular or longitudinal strips of intestinal wall from either guinea pig or human small intestinal preparations.

Conclusions
Early discoveries, for excitatory glutamatergic neurotransmission in the CNS, inspired enthusiasm that investigation in the ENS would yield discoveries recapitulating the CNS glutamatergic story. We found this not to be the case.
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Key Words
Intestines; Motility; Proteolysis; Receptors, glutamate; Secretion

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Introduction

Neuroscience in the 1980s-1990s experienced an avalanche of ground breaking advancement of concepts related to the pharmacology and classification of receptors for glutamate as an excitatory neurotransmitter in the brain and spinal cord. As interest in glutamate in the central nervous system (CNS) grew and numbers of original papers on glutamatergic neurobiology expanded, enthusiasm for glutamatergic research spread to the enteric nervous system (ENS). Investigators in neurogastroenterology recognized that the findings in the CNS might translate to the ENS (i.e., the brain-in-the-gut) and moved ahead with testing of a premise that glutamatergic signaling in the ENS would be analogous to that in the CNS.[1-3] Published results of this early work generally supported the premise. Nevertheless, closer scrutiny of accumulated literature reveals major inconsistencies in analysis and interpretation and casts doubt on the validity of application of discoveries in the CNS to a glutamatergic hypothesis for functional ENS integration of behavior of the effector systems in the digestive tract.

Work that was done in the ENS was focused on recognition in the CNS that receptors for glutamate are commonly partitioned into ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Ionotropic GluRs are glutamate-gated cation channels that are subdivided into N-methyl-D-aspartate (NMDA) receptors for NMDA and non-NMDA receptors for kainate and a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA).[4] Metabotropic GluR groups comprise a large family of receptors coupled to second messenger systems via GTP binding proteins. The 8 cloned mGluRs (mGluR1-mGluR8) are divided into 3 groups based on their pharmacology, second messenger coupling and sequence homology. Group I mGluRs (mGluR1 and mGluR5) stimulate phosphoinositide hydrolysis in expression systems; whereas, Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7 and mGluR8) mGluRs inhibit adenylylcyclase activity.[5] In view of acceptance of the ENS as an independent integrative nervous system, it was logical to expect that evidence for similar partitioning of glutamatergic receptors into functional roles would emerge for the synaptic microcircuits of the ENS. Nevertheless, in spite of a diverse array of research, evidence of this nature for the ENS is equivocal owing to inconsistency among investigative outcomes at the levels of individual neurons, glia and at the level of the functional digestive tract.

Materials and Methods

Mucosal and muscle preparations from guinea pig ileum and human jejunum were used. Male Hartley-Dawley guinea pigs (0.3-0.6 kg) were killed by rapid stunning and immediate exsanguination from the cervical vessels according to procedures reviewed and approved by the Ohio State University Laboratory Animal Care and Use Committee (Protocol 2010A0023: Exp. 2/11/2013). Fresh human preparations were obtained from segments of jejunum discarded during Roux-En-Y gastric bypass surgeries and transferred immediately to the research laboratory. The human protocols were reviewed and approved by the Institutional Review Board of the Ohio State University Office of Research Risks Protection (Protocol 02H0208).

Electrophysiology

Flat-sheet preparations of myenteric and submucosal plexus (2.0×1.0 cm) were obtained by routine methods of microdissection.[6] The preparations were pinned to Sylgard® resin at the bottom of 2 mL electrophysiological recording chambers. The chambers were perfused at a rate of 10-15 mL/min with Krebs solution warmed to 37°C and gassed with 95% O_2/5% CO_2 to buffer at pH 7.3-7.4. The composition of the Krebs solution was (in mM) NaCl, 120.9; KCl, 5.9; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 14.4; $CaCl_2$, 2.5; and glucose, 11.5. Transmembrane electrical potentials were recorded with conventional intraneuronal "sharp" microelectrodes. The microelectrodes were filled with 2% biocytin in 2 M KCl, buffered with 0.05 M Tris at pH 7.4 as described in detail elsewhere.[6] The preamplifier (M-767; World Precision Instruments, Sarasota, FL, USA) was equipped with bridge circuitry for intraneuronal injection of electrical current. Synaptic potentials were evoked by focal electrical stimulation of interganglionic fiber tracts in the myenteric or submucosal plexus with electrodes made from 20 μm Teflon®-insulated Pt wire connected through stimulus-isolation units (Grass SIN5) to Grass S48 stimulators (Grass Instrument Division, Astro-Med, Inc., Warwick, RI, USA). All data were recorded initially in digital format for later analysis.

Motility

Strips were cut from the longitudinal or circular axes of guinea pig ileum or human jejunum and connected to isometric tension transducers in tissue bath setups in vitro (AD-Instruments, Colorado Springs, CO, USA). Canonical pharmacological meth-

ods were used to analyze actions of L-glutamate and glutamatergic receptor agonists and antagonists on contractile behavior of the segments. Studies were done simultaneously in four tissue baths. Analog changes in muscle contractile tension were digitized and stored on hard disks for analysis.

Mucosal Secretion

This work was designed to address questions related to glutamatergic stimulation of neurogenic mucosal secretion. The objective was to determine if glutamate is able to cross the mucosa and access receptors expressed by secretomotor neurons in the submucosal division of the ENS and to investigate the identity of any glutamatergic receptors involved. This was done by placing L-glutamate on the mucosal or serosal side of full-thickness, flat-sheet preparations in Ussing flux chambers and measuring changes in electrogenic short-circuit current (Isc) as a marker for stimulation of mucosal secretion. Isc is a surrogate marker for movement of chloride across the mucosal epithelium, the direction of which determines H_2O and electrolyte movement into or out of the intestinal lumen.[7] Details of the methods are described in detail elsewhere.[8]

Segments of guinea pig ileum between 10 cm and 20 cm proximal to the ileocecal junction or human jejunal segments were flushed with ice-cold Krebs solution and opened along the mesenteric border. Four to eight of the flat-sheet preparations were obtained from each animal for mounting in Ussing flux chambers. Fresh preparations for Ussing chamber studies were procured from human small intestine. They were obtained from segments of jejunum discarded during Roux-En-Y gastric bypass surgeries and studied immediately in the laboratory. Composition of the Krebs solution was in mM: 120 NaCl, 6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.35 NaH_2PO_4, 14.4 $NaHCO_3$ and 11.5

for glucose. The Krebs solution in the Ussing chambers was bubbled with 95% O_2/5% CO_2 and buffered at pH 7.4.

The Ussing flux chambers were equipped with pairs of Ag/AgCl electrodes via Krebs-agar bridges connected to calomel half-cells for measurement of transmural potential difference (PD). A second pair of electrodes was connected to an automated voltage clamp apparatus, which compensated for the solution resistance between the PD-sensing bridges. The flat-sheet preparations were mounted between halves of Ussing chambers, which had a total cross-sectional area of 0.64 cm^2 for guinea pig preparations and 1.13 cm^2 for human preparations. The tissues were bathed on both sides with 10 mL of Krebs solution and maintained at $37^{\circ}C$ by circulation from a temperature controlled water bath. The current necessary to change the transepithelial PD by 2.5 mV was passed periodically to monitor tissue conductance, calculated according to Ohm's law, and used together with responses to carbachol at the end of the experiment for evaluation of tissue viability. Isc was monitored by electronic voltage-clamp amplifiers (DVC-1000; World Precision Instruments). Concentration-response relations were obtained by adding L-glutamate to the serosal or mucosal compartment of the chamber. Glutamate-evoked changes in Isc were calculated as ΔIsc. Data were normalized to the cross-sectional area of the preparations.

Immunohistochemistry

Whole-mount immunohistochemistry was done in essentially the same manner as we described elsewhere.[9,10] Presentation of methods here summarizes and quotes from these papers. Whole-mount preparations were obtained by microdissection from segments of guinea pig small intestine and transferred to a disposable chamber filled with fixative solution containing 4% formaldehyde and 2% of a saturated picric acid solution in 0.1

Antigen	Host	Code	Dilution	Sources
Anti-EAAC1 transporter	Goat	AB1520	1:200	Millipore-Chemicon
Anti-Glu	Rabbit	AB133	1:200	Millipore-Chemicon
Anti-Glu	Mouse	MAB5304	1:200	Millipore-Chemicon
Anti-Hu	Mouse	A-21271	1:500	Invitrogen
Anti-S-100	Rabbit	S2644	1:200	Sigma-Aldrich
Mouse IgG	Donkey	715-505-150	1:300	Jackson
Mouse IgG	Donkey	715-485-150	1:100	Jackson
Mouse IgG	Horse	CI-2000	1:200	Vector
Rabbit IgG	Donkey	711-505-152	1:300	Jackson
Rabbit IgG	Donkey	715-485-152	1:100	Jackson
Goat IgG	Donkey (Cy3)	705-165-147	1:100	Jackson
Goat IgG	Donkey (FITC)	705-095-147	1:100	Jackson

Figure 1. Codes and sources of primary and secondary antibodies used in immunohistochemical studies. Anti-Hu, anti-human neuronal protein HuC/HuD; anti-S-100, low molecular weight protein.

mol · L^{-1} phosphate buffered saline (PBS). Nonspecific immunological binding was blocked with 10% normal donkey serum in 0.01 M PBS (pH 7.4) for 1 hour at room temperature. The tissues were incubated with the primary antibodies (Fig. 1) diluted in 0.01 mol · L^{-1} PBS containing 10% normal donkey serum, 0.3% Triton X-100 and 0.05% sodium azide for 18 hours at room temperature. The tissues were then washed (3 × 10 minutes) in PBS (pH 7.4) and transferred to an incubation medium that contained a single secondary antibody or a mixture of secondary antibodies (Fig. 1). Combination of primary antibodies for glutamate, glutamate transporter, S-100 protein and HuC/HuD was done at the same time to achieve double immunolabeling. After incubation with the antibodies, the tissues incubated with appropriate secondary antibodies were conjugated with fluorescence isothiocyanate (FITC) or indocarbocyanin (Cy3) diluted in 0.01 mol · L^{-1} PBS. The tissues were then rinsed in PBS and cover slipped with Vectorshield (Vector, Burlingame, CA, USA). Pre-absorption of the primary antibodies with appropriate antigen was done as controls. Specificity of immunostaining was also checked by omitting either the primary or secondary antibody.

All preparations were examined under a Nikon Eclipse E-600 fluorescence microscope (Nikon Inc., Melville, NY, USA) and analyzed with filter combinations that enabled separate visualization of multiple fluorophores. Digital images were obtained with a SPOT RT cooled CCD digital camera (Diagnostic Instruments, Sterling, Heights, MI, USA), stored on disk, and analyzed with SPOT II software. Contrast in the digital images was sometimes enhanced before either converting to JPEG file interchange format (*.jpg) for electronic transfer or printing as photomicrographs with ink jet printers.

The proportions of neurons that expressed glutamate or glutamate transporter immunoreactivity relative to the total neuronal population in the myenteric and submucosal divisions of the ENS were evaluated by double labeling with a mouse monoclonal antibody for human neuronal protein HuC/HuD (anti-Hu) and glutamate. Anti-Hu antibody binds specifically to antigens that are expressed exclusively in neurons and is a useful tool for marking all neurons in the ENS.[11] Distribution of glutamate-immunoreactivity (IR) in relation to glia was evaluated by double labeling with a rabbit monoclonal antibody for S-100 protein and glutamate. Anti-S-100 antibody binds to astrocyte-like glia and is a useful marker for glia in the ENS.[12]

Reagents and Antibodies

L-Glutamate, (RS)-3,5-Dihydroxyphenylglycine (RS)-3,5-DHPG), (1S,3R)-1-Aminocyclopentane-1,3-dicarboxylic acid (1S,3R)-ACPD), (±)-1-Aminocyclopentane-trans-1,3-dicarboxylic acid (ACPD), (S)-4-Carboxyphenylglycine (CPG), (RS)-α-Methyl-4-carboxyphenylglycine disodium salt (MCPG), (RS)-2-Chloro-5-hydroxyphenylglycine sodium salt (CHPG), (3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid (TBOA), L-Quisqualic acid, and N-Methyl-D-aspartic acid (NMDA) were purchased from Tocris (Ellisville, MO, USA). Tetrodotoxin and (±)-AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid) were purchased from Sigma-Aldrich Biochemicals (St. Louis, MO, USA). Antibodies and antisera are listed in (Fig. 1).

Statistical Methods

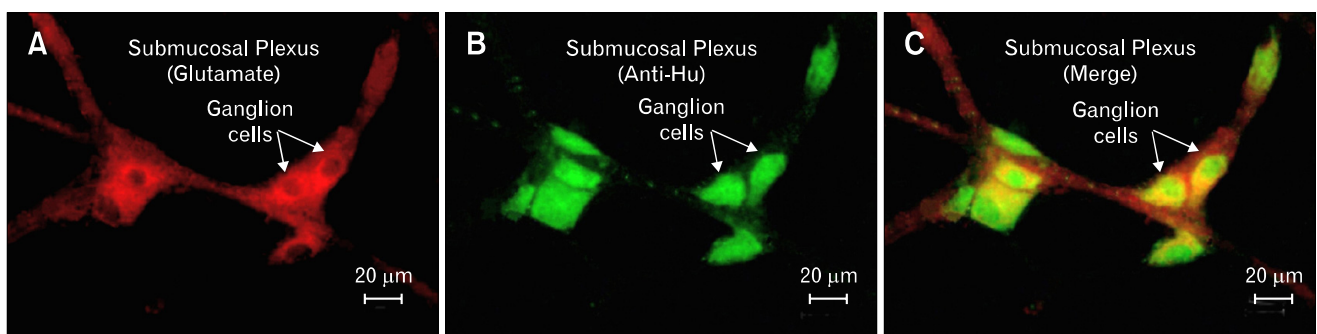Results are expressed as mean ± SEM. Student's t test and

Figure 2. Proportion of neurons expressing glutamate-immunoreactivity (IR) in whole-mount preparations of the submucosal plexus was determined by double-labeling glutamate and anti-human neuronal protein HuC/HuD (anti-Hu) in guinea pig small intestine. IR for the neuron-specific RNA binding protein, Hu, was used to assess the total numbers of neurons in the preparations. (A) Expression of glutamate in a whole-mount preparation of the submucosal plexus. (B) Expression of anti-Hu-IR in the same whole-mount as A. (C) Digital merger of A and B revealed co-expression of glutamate-IR and anti-Hu-IR in 63.9% of the submucosal ganglion cells overall.

paired t test were used for statistical analysis of significance with $P < 0.05$ accepted as significant. Ussing chamber data are presented as means ± SE with n values referring to numbers of patients and preparations. Continuous curves for concentration-response relationships were constructed with the following least-squares fitting routine using Sigmaplot® software (SPSS Inc., Chicago, IL, USA): $V = V_{max} / [1 + (EC_{50}/C)^{nH}]$, where V is the observed increased Isc, V_{max} is the maximal response, C is the corresponding drug concentration, EC_{50} is the concentration that induces the half-maximal response, and n_H is the apparent Hill coefficient.

Results

Immunohistochemistry

Nerve cell bodies, expressing glutamate-IR, were present in the myenteric and submucosal plexuses (Fig. 2 and 3). We examined the distribution of glutamate-IR neurons quantitatively by counting the neuronal cell bodies with glutamate-IR and anti-Hu-IR in ganglia in whole-mount preparations from 4 guinea pigs. In myenteric plexus preparations, 57.6% (157/273) of the neurons expressed glutamate-IR; in the submucosal plexus 63.9% (85/133) of the neurons expressed glutamate-IR.

We used double-labeling immunohistochemistry to compare expression of glutamate-IR and expression of S-100 protein-IR. No significant co-expression of glutamate-IR and S-100 protein-IR was found in whole-mount preparations removed from the myenteric and submucosal plexuses of 4 guinea pigs (Fig. 4).

Immunoreactivity for the EAAC-1 glutamate transporter was co-expressed with the pan neuronal marker, anti-Hu, in 42.7% (88/206) of the nerve cell bodies in the submucosal plexus and 53.5% (244/456) of the nerve cell bodies in the myenteric plexus (Fig. 5 and 6). Immunoreactivity for the EAAC-1 gluta-
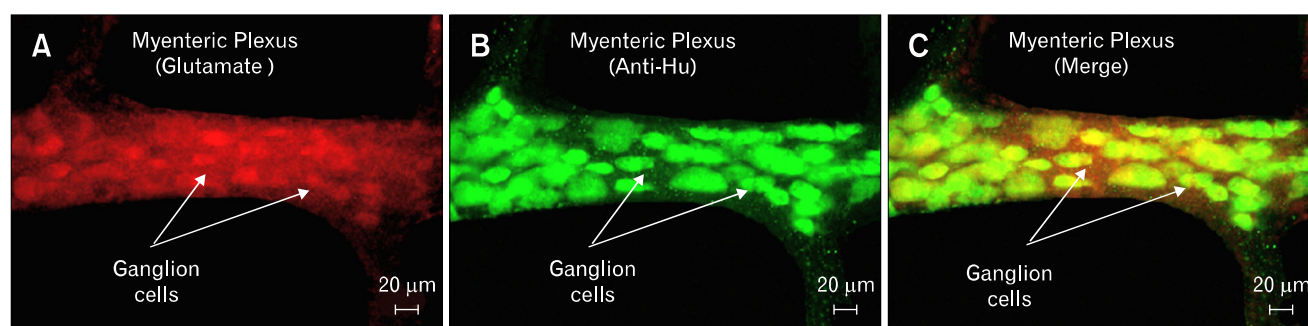

Figure 3. Proportion of neurons expressing glutamate immunoreactivity (IR) in whole-mount preparations of the myenteric plexus was determined by double-labeling glutamate and anti-human neuronal protein HuC/HuD (anti-Hu) in guinea pig small intestine. IR for the neuron-specific RNA binding protein, Hu, was used to assess the total numbers of neurons in the preparations. (A) Expression of glutamate in a whole-mount preparation of the myenteric plexus. (B) Expression of anti-Hu-IR in the same whole-mount as A. (C) Digital merger of A and B revealed co-expression of glutamate-IR and anti-Hu-IR in 57.6% of the myenteric ganglion cells overall.
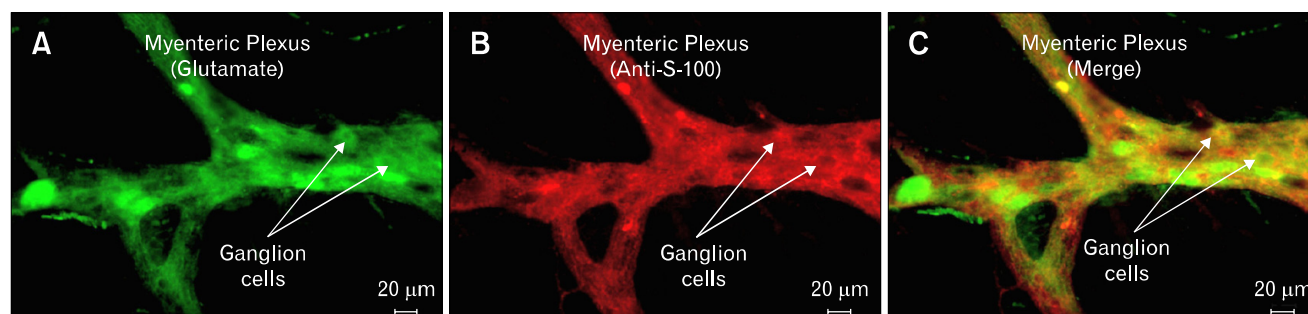

Figure 4. Proportion of glial cells expressing glutamate-immunoreactivity (IR) in whole-mount preparations of the myenteric plexus was determined by double-labeling glutamate and anti-S-100 protein in guinea pig small intestine. IR for S-100 protein was used to assess the population of glial cells in the preparations. (A) Expression of glutamate in a whole-mount preparation of the myenteric plexus. (B) Expression of anti-S-100 protein-IR in the same whole-mount as A. (C) Digital merger of A and B revealed absence of expression of glutamate-IR in enteric glia.
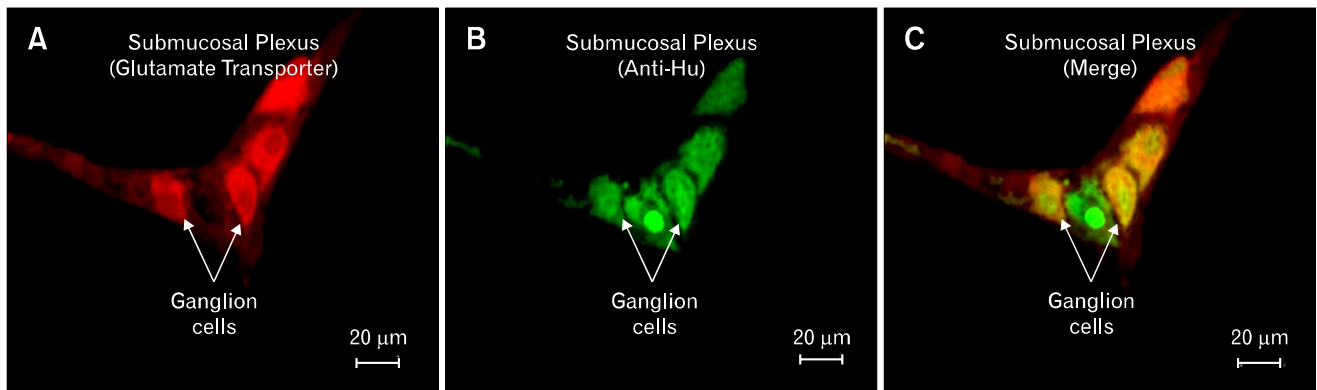
Figure 5. Proportion of neurons expressing immunoreactivity (IR) for the EAAC-1 glutamate transporter in whole-mount preparations of the submucosal plexus was determined by double-labeling EAAC-1 and anti-human neuronal protein HuC/HuD (anti-Hu) in guinea pig small intestine. IR for the neuron-specific RNA binding protein, Hu, was used to assess the total numbers of neurons in the preparations. (A) Expression of EAAC-1-IR in a whole-mount preparation of the submucosal plexus. (B) Expression of anti-Hu-IR in the same whole-mount as A. (C) Digital merger of A and B revealed coexpression of EAAC-1-IR and anti-Hu-IR in 42.7% of the submucosal ganglion cells overall.
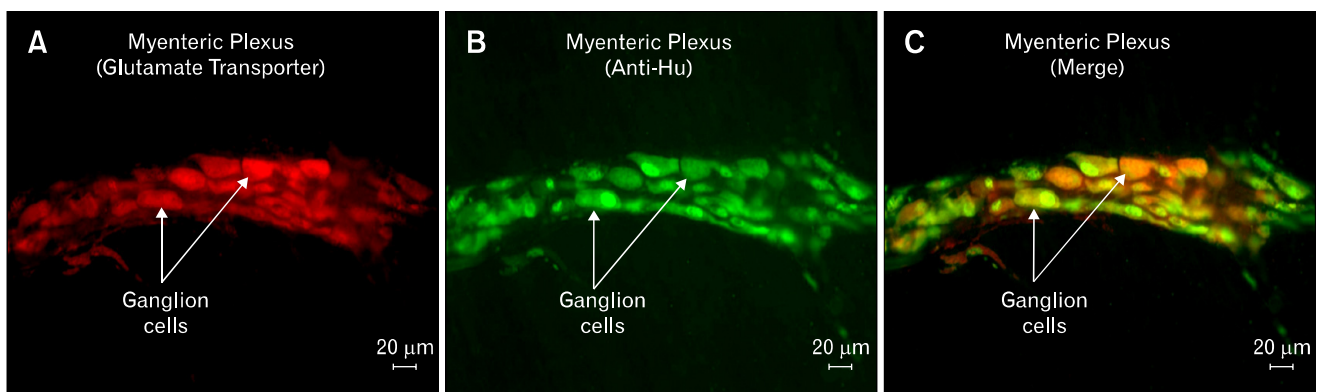

Figure 6. Proportion of neurons expressing immunoreactivity (IR) for the EAAC-1 glutamate transporter in whole-mount preparations of the myenteric plexus was determined by double-labeling EAAC-1 and anti-human neuronal protein HuC/HuD (anti-Hu) in guinea pig small intestine. IR for the neuron-specific RNA binding protein, Hu, was used to assess the total numbers of neurons in the preparations. (A) Expression of EAAC-1-IR in a whole-mount preparation of the myenteric plexus. (B) Expression of anti-Hu-IR in the same whole-mount as A. (C) Digital merger of A and B revealed co-expression of EAAC-1-IR and anti-Hu-IR in 53.4% of the myenteric ganglion cells overall.

mate transporter was found not to be co-expressed with immunoreactivity for S-100 protein in 6 preparations from the submucosal plexus of 4 guinea pigs (Fig. 7) and 6 myenteric plexus preparations from the same 4 guinea pigs.

Electrophysiology

We applied ACPD (±-trans-ACPD), which is a selective agonist for both group I and group II mGlu receptors including subtypes mGluR1-5, to neurons in guinea pig myenteric and submucosal plexus preparations.[13] ACPD had no significant depolarizing action on the membrane potential and did not elevate neuronal excitability in 28 neurons distributed among the 2 kinds

of preparations. We also applied NMDA, which is the prototypic agonist for glutamatergic ionotropic receptors in the brain, to the same 28 neurons in preparations from the 2 divisions of the guinea pig ENS and found no significant excitatory action.[14]

Figure 8 compares lack of effect of L-glutamate, ACPD and AMPA with excitatory actions of MRS2365 and substance P on neuronal excitability and neurotransmission for slow synaptic excitation (excitatory postsynaptic potential [EPSP]) in a secreto-motor neuron in the submucosal plexus of guinea pig small intestine. MRS2365, a P2Y$_1$ purinergic receptor agonist, and substance P are slow EPSP mimetics in the ENS.[15,16] The presence of the glutamatergic agonists in the bathing medium did not
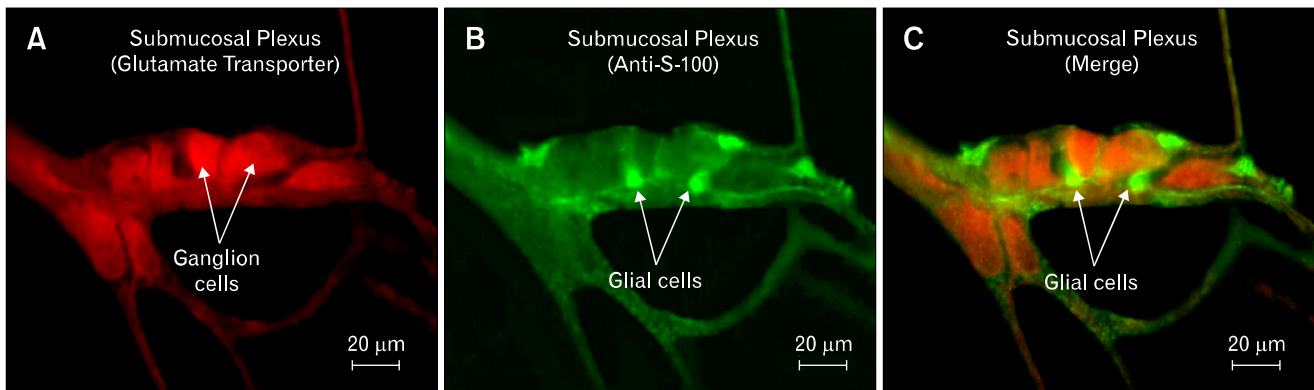
Figure 7. Proportion of glial cells expressing immunoreactivity (IR) for the EAAC-1 glutamate transporter in whole-mount preparations of the submucosal plexus was determined by double-labeling EAAC-1 and S-100 protein-IR in guinea pig small intestine. IR for the glial cell marker, S-100-protein, was used to assess the numbers of glial cells in the preparations. (A) Expression of EAAC-1-IR in a whole-mount preparation of the submucosal plexus. (B) Expression of S-100 protein-IR in the same whole-mount as A. (C) Digital merger of A and B. IR for the EAAC-1 glutamate transporter was co-expressed with S-100 protein-IR in the submucosal or myenteric plexus.
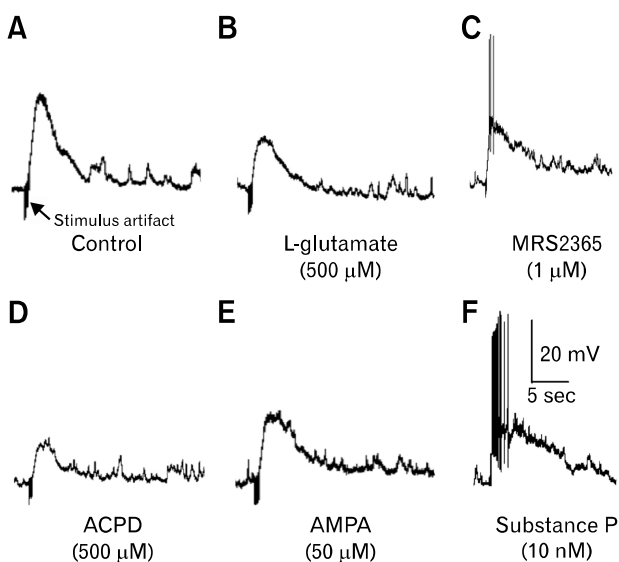

Figure 8. Absence of stimulatory action of glutamatergic receptor agonists on stimulus-evoked slow excitatory postsynaptic potentials (slow EPSP) in neurons of the guinea pig small intestinal submucosal plexus and stimulatory effect of 2 known slow EPSP neurotransmitters, ATP and substance P.[1,2] (A) Control stimulus-evoked slow EPSP. (B) Lack of stimulatory action of L-glutamate on the slow EPSP. (C) Stimulatory action of the purinergic P2Y₁ receptor agonist, MS2365. (D) Lack of stimulatory action of the metabotropic glutamate receptor agonist, aminocyclopentane-1,3-dicarboxylic acid (ACPD). (E) Lack of stimulatory action of the specific glutamatergic receptor agonist, a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). (F) Stimulatory action of substance P. Stimulus parameters were 5 Hz for 3 seconds.

change the latency for the response, the rate of rise of the slowly-activating membrane depolarization or excitability reflected by action potential discharge when compared with the slow EPSP-like actions of MRS2365 or substance P. Figure 9 presents quantitative data for the actions of glutamatergic agonists on slow EPSPs compared with the action of substance P and MRS2365. Figure 10 contains quantitative data for the actions of glutamatergic agonists on resting membrane potentials of S-type and AH-type neurons in the myenteric and submucosal plexuses of the guinea pig small intestine.

Mucosal Secretion

Application of L-glutamate, in the serosal side compartment of the Ussing chambers, evoked increases in Isc with an EC₅₀ of 370 ± 21.6 μM for 12 guinea pig preparations (Fig. 11). Application in the mucosal compartment of the chamber evoked increases in Isc with an EC₅₀ of 291 ± 19.0 μM for 11 preparations (Fig. 11). Maximal concentrations of 500 μM L-glutamate, based on the EC₅₀ values for its stimulation of Isc, were used for pharmacological analysis of the action of L-glutamate (Fig. 12). Application of ACPD, which is an agonist for both group I and group II mGlu receptors, had no effect on Isc when applied on the serosal or mucosal side of the preparations (Fig. 12). Presence of the glutamate transport blocker, TBOA, reversed glutamate-evoked stimulation of Isc. The chloride secretory responses to L-glutamate were converted to chloride absorption in the presence of TBOA (Fig. 12). Blockade of secretomotor neuronal excitability by tetrodotoxin (TTX) reduced L-glutamate-evoked secretory responses by small, but significant incre-
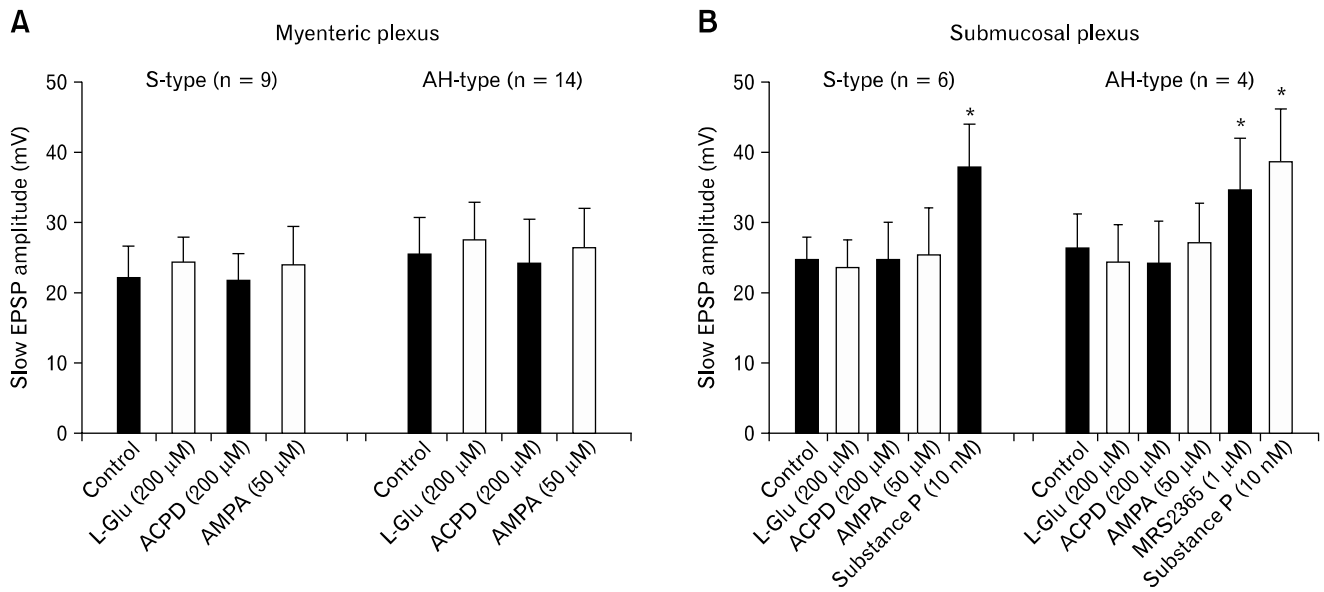
Figure 9. Quantitative data for absence of stimulatory action of glutamatergic receptor agonists on amplitude of stimulus-evoked slow excitatory postsynaptic potentials (slow EPSP) in neurons of the guinea pig small intestinal submucosal and myenteric plexuses as compared with excitatory actions of 2 known slow EPSP neurotransmitters in the enteric nervous system, ATP and substance P. (A) Quantitative data for action of glutamatergic receptor agonists on neurons with S- or AH-type electrophysiological behavior in guinea pig myenteric plexus.[1,2] (B) Quantitative data for action of glutamatergic receptor agonists and slow EPSP mimetics, MRS2365 and substance P on neurons in guinea pig submucosal plexus. *$P < 0.05$ relative to control, n = 28 neurons from a minimum of 3 guinea pigs. L-Glu, L-glutamate.
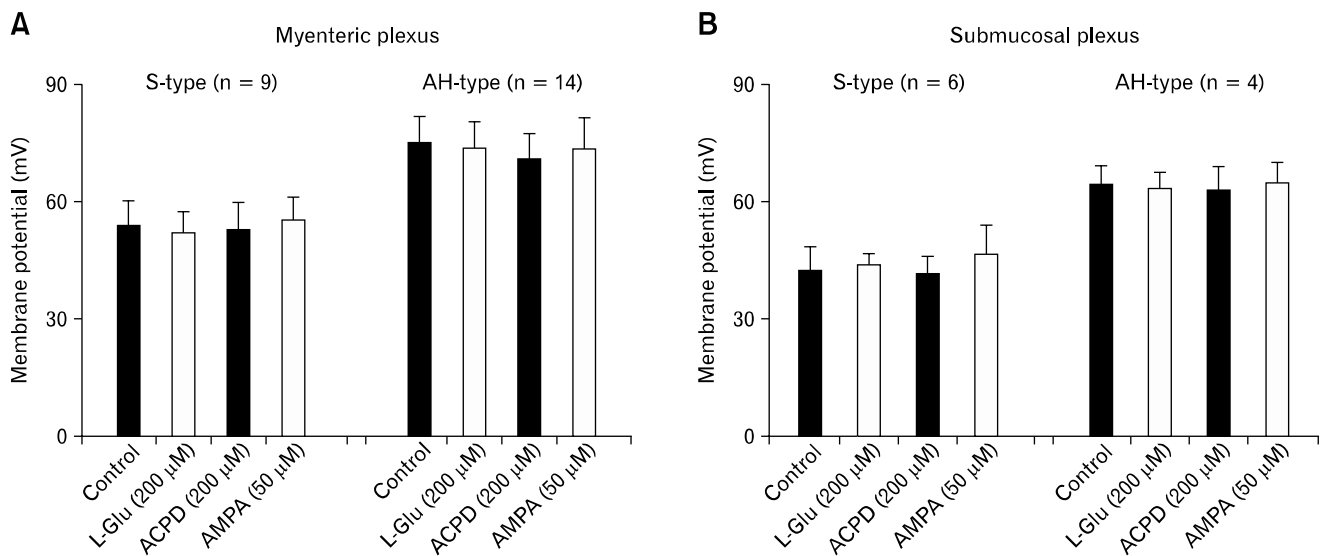

Figure 10. Quantitative data for lack of effect of glutamatergic receptor agonists on the resting membrane potential in neurons of the guinea pig small intestinal submucosal and myenteric plexuses. (A) Quantitative data for action of glutamatergic receptor agonists on membrane potential in neurons with S- or AH-type electrophysiological behavior in guinea pig myenteric plexus.[1,2] (B) Quantitative data for action of glutamatergic receptor agonists on membrane potential in neurons with S- or AH-type electrophysiological behavior in guinea pig submucosal plexus. n = numbers of neurons from a minimum of 3 guinea pigs.

ments (Fig. 12). MCPG is a non-selective antagonist at group I and group II metabotropic glutamatergic receptors.[17] Presence of MCPG, in the bathing solution, did not alter stimulation of Isc

by L-glutamate (Fig. 12). (S)-4-CPG, which is also a competitive antagonist at group I metabotropic glutamatergic receptors, like MCPG, had no effect on stimulation of Isc by

L-glutamate (Fig. 12).[17]

In view of the above results, which suggested absence of expression of excitatory metabotropic glutamatergic receptors by secretomotor neurons in the guinea pig submucosal ENS, we
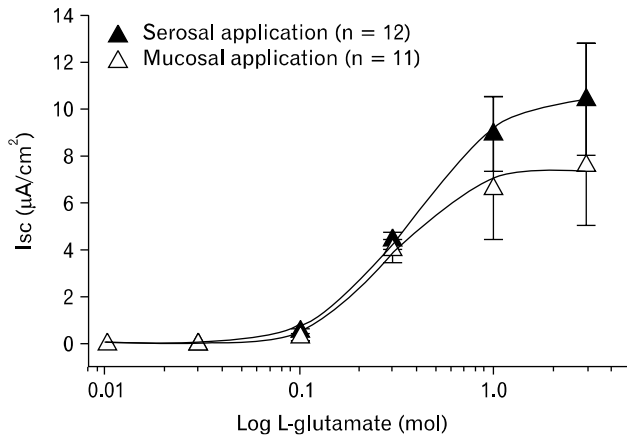
tested multiple selective agonists for glutamatergic action of this nature on mucosal secretion in human and guinea pig small intestine (Fig. 13). Aside from L-glutamate, the following agonists were analyzed: (1) (1S,3R)-ACPD, an agonist at mGluR1, mGluR2, mGluR5 and mGluR6; (2) ACPD; (3) CHPG Sodium Salt, a selective mGluR5 agonist; and (4) carbachol, which is a muscarinic receptor agonist that acts directly on the secretory epithelium to stimulate chloride secretion.[7,18,19] Neither of the mGluR agonists stimulated mucosal chloride secretion relative to vehicle or to the secretory action of carbachol, which served as an indicator of the viability of the preparations.

Musculature Contractility

Strips of the muscularis externa, dissected from the longitudinal and circular axes of guinea pig and human small intestine with the myenteric plexus intact, generated spontaneous-rhythmic contractile activity (Fig. 14). We measured the amplitude of each spontaneously-occurring contraction over a specified time period and used the mean amplitude as a control for any changes evoked by application of L-glutamate or ACPD, which is a se-

Figure 11. Concentration-response relations for stimulation of Isc by L-glutamate. $EC_{50} = 370 \pm 21.6\ \mu M$ for serosal side application; $EC_{50} = 291 \pm 19.0\ \mu M$ for mucosal side application.
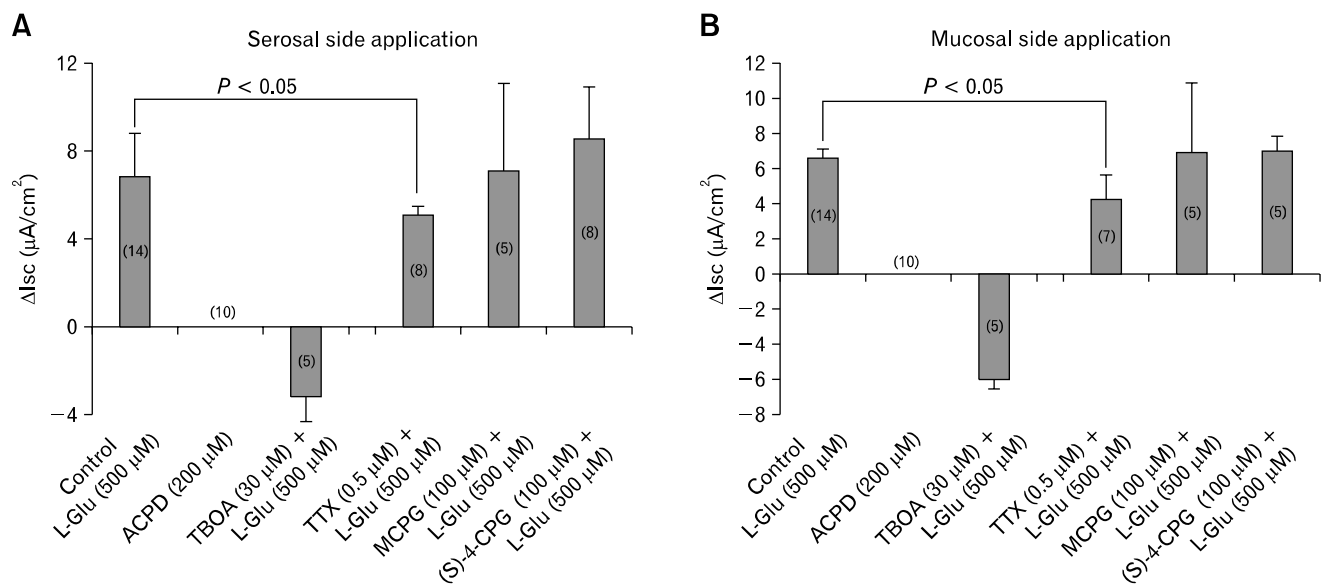

Figure 12. Glutamate secretory pharmacology in guinea pig small intestine. (A) Effects of glutamate agonists, antagonists and inhibitors of mucosal glutamate transport when applied in the Ussing chamber compartment bathing the serosal side of the preparations. (B) Same as for A except with application of the pharmacological agents in the Ussing chamber compartment bathing the mucosal side of the preparations. L-glutamate (L-Glu, 500 μM) stimulated mucosal chloride secretion reflected by increased Isc in the electrically-positive direction when applied to the serosa or mucosa. The selective metabotropic glutamate (mGlu) I receptor agonist aminocyclopentane-1,3-dicarboxylic acid (ACPD, 200 μM) did not stimulate any increase in short-circuit current (Isc). The glutamate transporter inhibitor, 3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid (TBOA), suppressed glutamate-evoked Isc and converted the short-circuit to electrically-negative values indicative of net absorption of chloride. Tetrodotoxin (TTX) suppressed glutamate-evoked increases in Isc. Neither the non-selective mGlu I/II receptor antagonist, (RS)-α-Methyl-4-carboxyphenylglycine disodium salt (MCPG), nor the selective mGlu Iα antagonist, (S)-4-Carboxyphenylglycine (CPG) suppressed stimulation of Isc by glutamate. Numbers of preparations appear in parentheses.
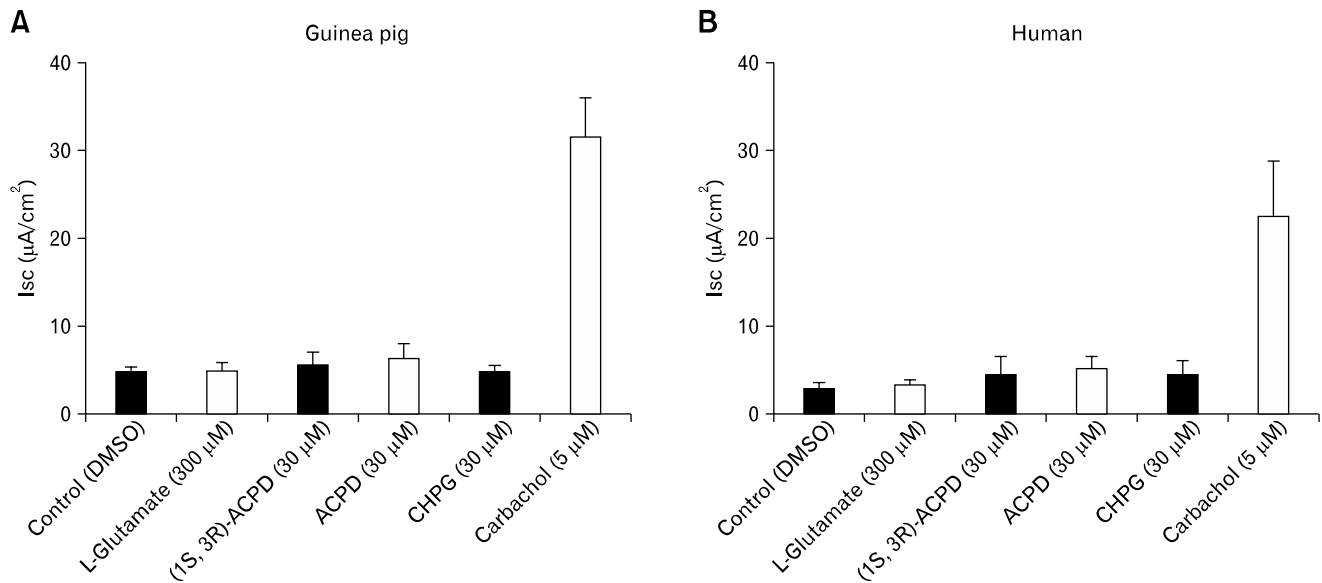
Figure 13. Action of L-glutamate and selective metabotropic glutamatergic receptor agonists on secretion of chloride (short-circuit current [Isc]) in guinea pig and human small intestinal preparations in Ussing flux chambers. Dimethyl sulfoxide (DMSO) was the vehicle for some agents. Carbachol, an agonist at muscarinic receptors, stimulated secretion as an indicator of the viability of the preparations. None of the metabotropic glutamate receptors agonists stimulated mucosal chloride secretion relative to vehicle or to the secretory action of carbachol. Data represent preparations from 8 guinea pigs and 8 human gastric bypass surgeries.

lective agonist for both group I and group II mGlu receptors including subtypes mGluR1-5 mentioned earlier.[13]

Neither L-glutamate nor ACPD changed the mean amplitude of spontaneously occurring contractions in circular or longitudinal strips of intestinal wall from either guinea pig or human small intestinal preparations (Fig. 14). Exposure to L-glutamate or ACPD did not change the frequency at which the spontaneously occurring contractions occurred in either longitudinal or circular strips of intestinal wall from guinea pig or human small intestine (data not shown).

Discussion

When bovine serum albumen, which serves as a model dietary protein, is infused in the small intestine of human subjects, digestion elevates intraluminal concentrations of glutamate to values as high as 7.3 ± 2.17 μM/mL.[20] Concentrations in the blood, leaving the intestine in the portal circulation, are much lower mainly because L-glutamate is oxidized in epithelial cells during its transport from the lumen.[21] Some of the dietarily-supplied glutamate, which escapes metabolic conversions in mucosal epithelial cells, most likely reaches the ENS embedded inside the intestinal wall.

The ENS is an independent integrative nervous system with many properties that mimic the CNS and inspires comparison of glutamatergic signaling in the minibrain-in-the-gut with the CNS brain.[22] In the CNS brain, glutamate is compartmentalized in high concentrations inside neurons and low concentrations in the extraneuronal milieu. This distribution rigidly controls its availability for release as an excitatory neurotransmitter.[23] Blood-borne glutamate does not enter the CNS because the blood-brain barrier is virtually impermeable to glutamate.[24] Glutamate in CNS neurons is synthesized locally from glutamine by the action of phosphate-activated glutaminase.[25]

In view of the fact that the ENS is exposed to glutamate directly, we aimed to address the dogma that the cellular neurophysiology for glutamate in the ENS recapitulates its functional role as an excitatory neurotransmitter in the CNS. This was done by addressing the following: (1) Does application of glutamate excite ENS neurons (i.e., depolarize the membrane potential and evoke action potential discharge)? (2) Does application of glutamate stimulate firing of ENS musculomotor neurons that becomes evident as stimulation of muscle contractile amplitude? (3) Does exposure to glutamate stimulate firing of ENS secretomotor neurons that becomes evident as enhanced mucosal secretion? Moreover, we used immunohistochemical methods to clarify if glutamate is sequestered in ENS neurons and glia in association with expression of glutamate transporters in a comparative manner with the CNS.
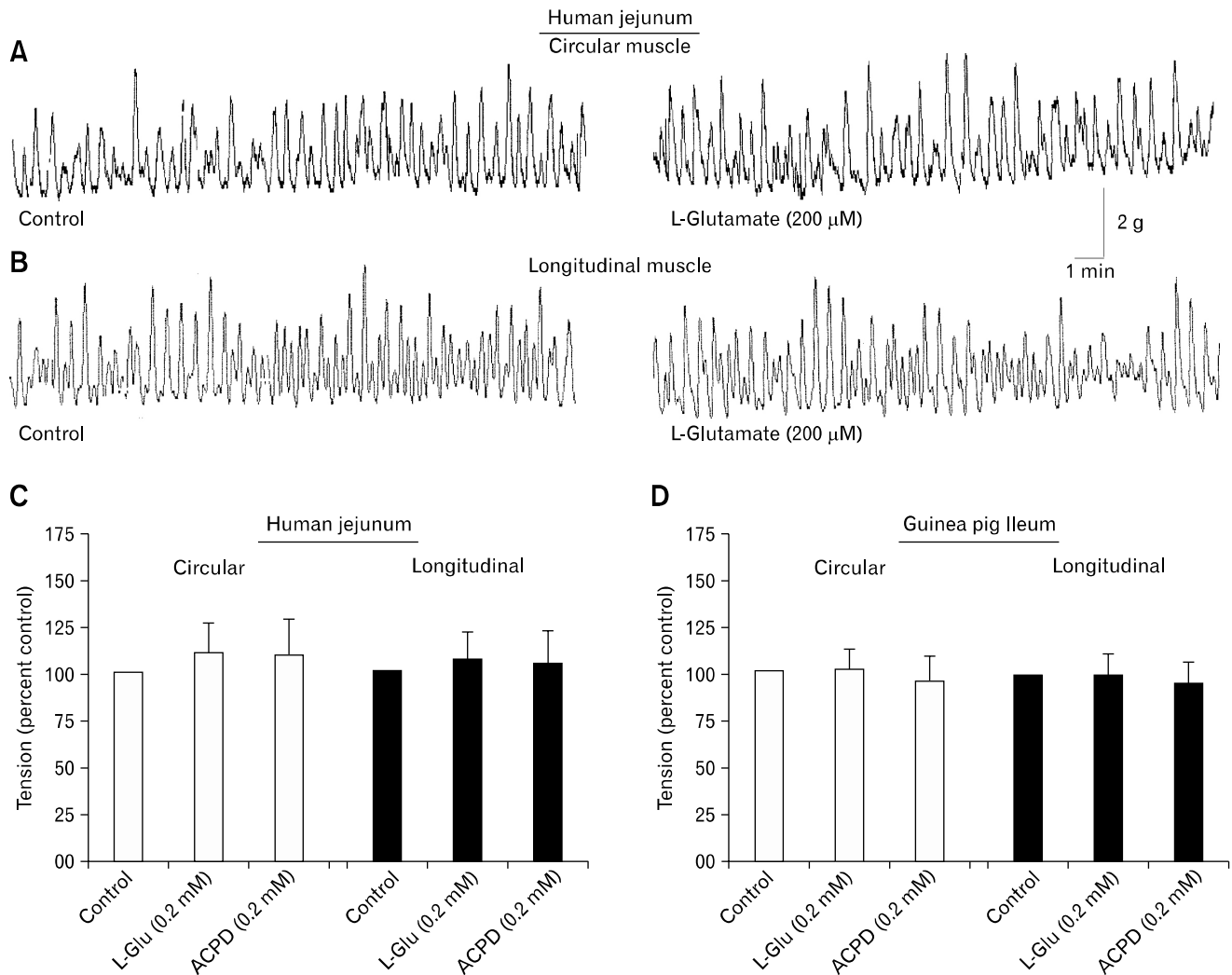
A

Human jejunum
Circular muscle

Control

L-Glutamate (200 µM)

2 g

B

Longitudinal muscle

1 min

Control

L-Glutamate (200 µM)

C

Human jejunum

Circular · Longitudinal

Tension (percent control)

175
150
125
100
75
50
25
00

Control · L-Glu (0.2 mM) · ACPD (0.2 mM) · Control · L-Glu (0.2 mM) · ACPD (0.2 mM)

D

Guinea pig Ileum

Circular · Longitudinal

Tension (percent control)

175
150
125
100
75
50
25
00

Control · L-Glu (0.2 mM) · ACPD (0.2 mM) · Control · L-Glu (0.2 mM) · ACPD (0.2 mM)

Figure 14. Actions of L-glutamate (L-Glu) on contractile activity of strips of intestinal wall removed from the longitudinal or circular axis of human jejunum and guinea pig ileum. (A) Action of L-Glu on circular strips. (B) Action of L-Glu on longitudinal strips. (C) Action of L-Glu and the metabotropic glutamate (mGlu) I receptor agonist, aminocyclopentane-1,3-dicarboxylic acid (ACPD), on contractile tension in circular and longitudinal muscle strips from human jejunum. (D) Action of L-Glu and the mGlu I receptor agonist, ACPD, on contractile tension in circular and longitudinal muscle strips from guinea pig ileum. Data represent preparations from 12 guinea pigs and 8 human gastric bypass surgeries.

As in CNS neurons, a majority, but not all, ENS neurons expressed glutamate-IR indicative of glutamate sequestration. Unlike CNS astrocytes, ENS glial cells did not appear to sequester glutamate. As is the case for CNS neurons, ENS neurons express the EAAC-1 glutamate transporter. On the other hand, glial cells in the ENS appear to differ from CNS astrocytes by not expressing immunoreactivity for the EAAC-1 transporter.

Expression of glutamate-IR in ENS neurons might reflect extraction of glutamate by neuronal transporters as it passes on route to the hepatic portal circulation. On the other hand, the glutamate-IR might be the result of glutamate synthesis in the neurons themselves. Both mechanisms are a possibility. However,

unlike for CNS neurons, little is known about glutamate synthesis in single ENS neurons. Observations, which suggest that the interior of the ENS might be excluded from glutamate entry by a functional barrier, would suggest that synthesis accounts for the glutamate-IR expressed in the ENS neurons. Blood vessels do not enter the ENS ganglia in guinea pigs and a blood ganglion barrier analogous to the blood-brain barrier has been demonstrated for the myenteric plexus.[26,27] Nevertheless, the barrier has been demonstrated only for macromolecules (i.e., labeled albumin or horseradish peroxidase).

In contrast to earlier reports, we found no significant electrophysiological effects of exposure of ENS neurons to L-glutamate or

to ionotropic or metabotropic glutamatergic receptor agonists.[28-31] Our tentative conclusion is that glutamate neuropharmacology for the ENS differs significantly from that for the CNS. Whereas, the brain synthesizes and compartmentalizes glutamate and uses it as a key excitatory neurotransmitter; we found little evidence that it is a neurotransmitter in the ENS. Apparently, neurons in the ENS do not express the receptors necessary for activation by the glutamate that escapes metabolism by the mucosal enterocytes as it passes the neurons on route to the hepatic portal circulation.

Published data for influence of glutamate on intestinal mucosal chloride secretion is sparse. A single paper reported that L-asparagine, an amino acid with similar structure to L-glutamate, stimulated chloride secretion in concentration-dependent manner and neural blockade with TTX suppressed the L-asparagine evoked secretion in piglets.[32] Our results for L-glutamate are reminiscent of the data in this paper. We found that TTX suppressed L-glutamate-evoked increases in Isc, but by only about 33% of the response to L-glutamate alone. A much larger component was non-neurally mediated and related to enterocyte transport. This component most likely reflected electrogenic chloride movement coupled with basolateral sodium-dependent glutamate transport, because blockade of amino acid transporters reversed Isc from glutamate-evoked chloride secretion to chloride absorption.[33]

Aside from the minor suppression of glutamate-evoked chloride secretion by TTX, we found no other evidence that L-glutamate-evoked secretion was mediated by binding to excitatory glutamatergic receptors on ENS secretomotor neurons. This was magnified by failure of selective receptor agonists to mimic stimulation of chloride secretion by L-glutamate and failure of glutamatergic receptor antagonists to suppress stimulation of chloride secretion by L-glutamate.

The TTX effect on L-glutamate-evoked secretion might reflect blockade of conduction in intramural spinal and/or vagal afferents. Collaterals from intramural vagal and spinal afferents are known to innervate ENS neurons and evoke reflexes analogous to axonal reflexes underlying the "triple response" to irritation in the skin. Some of the evidence for this is finding that electrical stimulation of spinal afferents as they enter the intestine in the mesentery evokes excitatory postsynaptic potentials in neurons in the myenteric plexus.[34] Terminals of the same afferents fire during exposure to amino acids, including L-glutamate.[35-37] When the afferents are active, they release calcitonin gene-related peptide and substance P, both of which are known stimulators of neurogenic chloride secretion.[7] Therefore, TTX blockade of glu-

tamate-evoked firing of the afferents might account for the minor reduction in glutamate stimulation of Isc that occurred in the presence TTX (Fig. 11). Glutamate-evoked firing of vagal afferents is mediated by release of 5-hydroxytryptamine and its action at the 5-hydroxytryptamine 3 receptor subtype expressed on the afferent terminals rather than by any action at intramural neuronal glutamatergic receptors.[37]

This work was an alternative test of the hypothesis that glutamate in the small intestinal lumen gains access and activates excitatory glutamatergic receptors expressed by ENS motor neurons. It investigated motility of the longitudinal and circular muscle coat of the guinea pig and human small bowel as an approach for identifying expression of glutamatergic receptors by musculomotor neurons in the ENS. If any stimulatory or inhibitory effects of glutamate on contractile activity were sensitive to neural blockade by TTX, it would suggest glutamatergic activation of excitatory or inhibitory musculomotor neurons in the myenteric plexus. Stimulation of excitatory musculomotor neurons would enhance contractile tension and increased discharge of inhibitory musculomotor neurons would suppress contractile activity.

We found no stimulation of contractile activity or inhibition of contractile activity by L-glutamate itself or commercial glutamate receptor agonists in guinea pig or human small intestine (Fig. 13). Neither the amplitude of ongoing contractions nor the frequency of contractions was altered. These results do not support the hypothesis that dietary glutamate in the small intestinal lumen gains access and activates excitatory glutamatergic receptors expressed by ENS musculomotor neurons.

Conclusion

In view of the ENS as a brain-in-the-gut, exciting discoveries for the neurobiology of excitatory glutamatergic neurotransmission in the CNS brain justified enthusiasm for the likelihood that results of investigation in the ENS would recapitulate the glutamatergic story for the CNS. This has proved not to be the case; although, transport and sequestration of glutamate in ENS neurons might serve to provide a substrate for synthesis of gamma-amino butyric acid, which is a putative neurotransmitter in the ENS.[38]

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