Localization and function of metabotropic glutamate receptor 8 in the enteric nervous system

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Tong, Qingchun, and Annette L. Kirchgessner. Localization and function of metabotropic glutamate receptor 8 in the enteric nervous system. Am J Physiol Gastrointest Liver Physiol 285: G992–G1003, 2003. First published June 26, 2003; 10.1152/ajpgi.00118.2003.—The enteric nervous system (ENS) contains glutamatergic neurons, transporters, and functional ionotropic and groups I and II metabotropic glutamate receptors (mGluRs). The aim of this study was to determine whether the ENS contains functional group III mGluRs. RT-PCR demonstrated the expression of mGluR7 and mGluR8 mRNA in rat myenteric ganglia. Western blot analysis confirmed the presence of mGluR8 protein. Immunocytochemistry, in conjunction with confocal microscopy, demonstrated mGluR8 immunoreactivity in the ENS of several species, including humans. mGluR8 immunoreactivity was localized to the membrane of nerve cell bodies that received glutamatergic input. Significant receptor internalization of mGluR8 was observed on activation, and localization to membrane was observed on blocking with the mGluR III antagonist (RS)-cyclopropyl-4-phosphonophenylglycine (CPPG). mGluR8-positive myenteric neurons contained glutamate or nitric oxide synthase (NOS), a marker of inhibitory motorneurons. Enteric group III mGluRs are functional because mGluR8 agonists inhibited forskolin-induced accumulation of cAMP in isolated myenteric ganglia, and CPPG reduced this effect. In addition, an accelerating effect on guinea pig colonic motility was observed after the application of mGluR8 agonists. Increase in motility was specific, because CPPG inhibited it. Moreover, in the presence of hexamethonium or N\textsuperscript{-}nitro-L-arginine methyl ester, an inhibitor of NOS, responses caused by mGluR8 agonists were abolished. mGluR8 agonists also increased longitudinal muscle contractions. These findings suggest that mGluR8 agonists increase motility by inhibiting nitricergic relaxation and possibly by facilitating cholineric contractions.

(RS)-4-phosphonophenylglycine; (S)-3,4-dicarboxyphenylglycine; (RS)-cyclopropyl-4-phosphonophenylglycine; adenosine 3',5'-cyclic monophosphate; colonic motility

METABOTROPIC GLUTAMATE RECEPTORS (mGluRs) are G protein-coupled receptors that are highly expressed throughout the central nervous system (CNS). To date, eight metabotropic glutamate receptors (mGluR1–8) have been cloned and are classified into three major groups on the basis of sequence homology, coupling to second-messenger systems, and selectivities for various agonists (7). Group I mGluRs (mGluR1 and 5) couple to G\textsubscript{q} and activation of phosphoinositide hydrolysis, whereas groups II (mGluR2 and 3) and III (mGluR4, 6, 7, and 8) mGluRs couple to G\textsubscript{i/o} and inhibit adenylate cyclase activity. The mGluRs (with the exception of mGluR6) are widely distributed throughout the CNS and play important roles in regulating cell excitability and synaptic transmission at excitatory and inhibitory synapses (1). Group I mGluRs are mainly located on postsynaptic nerve terminals. In contrast, groups II and III mGluRs are found on both pre- and postsynaptic terminals. Presynaptically localized mGluRs act as autoreceptors mediating the inhibition of glutamate release; however, activation of group III mGluRs also decreases transmission at inhibitory (GABAergic) synapses (34).

Functional mGluRs are also expressed outside the CNS, in nodose ganglia (10, 12), dorsal root ganglia (9, 27), taste buds (4), osteoblasts (11), pancreatic islets (32), murine thymocytes (30), and the retina (18, 19). Groups I and II mGluRs are also found in the enteric nervous system (ENS) (16). In the guinea pig small intestine, activation of group I mGluRs depolarizes submucosal neurons (14, 20), whereas activation of group II mGluRs inhibits Ca\textsuperscript{2+} currents in acutely dissociated myenteric neurons (5). Whether functional group III mGluRs are present in the ENS is not known. Thus the purpose of this study was to determine whether group III mGluRs are expressed in the gut and to examine their role in gastrointestinal physiology.

MATERIALS AND METHODS

Tissue. Adult female rats (150–200 g; Harlan Sprague-Dawley, Indianapolis, IN), male guinea pigs (Harlan Sprague-Dawley), and mice (C57BL/J6) were euthanized by CO\textsubscript{2} inhalation and then decapitated. The Animal Care and Use Committee of State University of New York Downstate Medical Center approved this procedure. Tissue from the human gut (duodenum and ileum) was obtained from surgical specimens. The Karolinska Institutet North Ethics Committee approved the study, and informed consent was obtained from all subjects. The gut was removed and surgical specimens. The Karolinska Institutet North Ethics Committee approved the study, and informed consent was obtained from all subjects. The gut was removed and washed with oxygenated (95% O\textsubscript{2}–5% CO\textsubscript{2}) Krebs solution of the following composition (in mM): 121 NaCl, 5.9 KCl, 2.5 Ca\textsubscript{2+}, 1.2 Mg\textsubscript{2+}, 26.2 NaHCO\textsubscript{3}, 11.1 glucose, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, and 0.50 K\textsubscript{2}HPO\textsubscript{4}.

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2.5 CaCl₂, 14.3 NaHCO₃, 1.3 Na₂HPO₄, 1.2 MgCl₂, and 12.7 glucose.

**RNA isolation and RT-PCR.** Total RNA (5 μg) isolated from rat longitudinal muscle with adherent myenteric plexus (LMM) and brain, prepared by using the TRizol reagent (Life Technologies, Gaithersburg, MD), was reverse transcribed at 42°C (1 h) with the use of random primers and murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). This served as a template for PCR by using Taq DNA polymerase. PCR was performed by using the primer sequences as listed in Table 1. Primer sequences were similar to those published (32). We used GAPDH as a housekeeping gene. After an initial denaturation step at 94°C for 5 min, the PCR conditions were as follows: 95°C, 15 s; 52°C for GAPDH and 50°C for mGlRs, 1 min; 72°C, 2 min for 35 cycles, followed by a final extension step at 72°C for 7 min. In controls, reverse transcriptase was omitted.

Results of this amplification were used to ensure successful mRNA isolation without genomic DNA contamination. The PCR products were resolved in 2% agarose gel with ethidium bromide. The PCR products were subcloned into EcoRI-digested pGEM-T vector (Promega, Madison, WI) for sequencing via dye termination cycle sequencing (ABI Pyramid Automated Sequencer; Perkin-Elmer, Norwalk, CT). The best match of the sequences was determined by using the gapped BLAST and PSI-BLAST programs.

**Immunocytochemistry.** Segments of gut were fixed for 24 h with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After fixation, the tissue was washed in PBS, cryoprotected overnight (at 4°C) in PBS containing 30% sucrose, embedded in OCT (TissueTek, Torrance, CA), frozen with liquid N₂, and sectioned (10 μm) by using a cryostat-microtome (Leica). For whole mount preparation, segments of gut were quickly removed, pinned flat in petri dishes coated with Sylgard (Dow Corning, Midland MI), and fixed as described above. The gut was then dissected in different layers, as previously described (20). To locate mGlur8 protein, preparations were incubated with 4% normal horse serum with Triton X-100 (0.5%) in PBS for 30 min. The preparations were then exposed overnight to guinea pig polyclonal antibodies generated against a peptide corresponding to amino acids 894–908 of rat and human mGlur8 (diluted 1:1,000; Chemicon International, Temecula, CA) (25, 32) or antibodies raised in rabbit against a peptide corresponding to amino acids 894–908 of human mGlur8.

After being washed with PBS, the preparations were incubated with donkey anti-guinea pig or donkey anti-rabbit secondary antibodies coupled to Rhodamine Red-X (RRX) (Jackson ImmunoResearch, West Grove, PA) or FITC (Jackson ImmunoResearch) diluted 1:500, for 3 h. The preparations were washed again with PBS, and then the tissues were coveredslipped with Vectashield (Vector Laboratories, Burlingame, CA).

Double-label immunocytochemistry was used to identify the cells that display mGlur8-like immunoreactivity. Double labeling was made possible by using primary antibodies raised in different species in conjunction with species-specific secondary antibodies (donkey anti-rat, donkey anti-mouse, donkey anti-guinea pig (Kirkegaard and Perry, Gaithersburg, MD), donkey anti-goat, donkey anti-rabbit (diluted 1:500; Jackson ImmunoResearch)) coupled to contrasting fluorophores (FITC or RRX, as described above). Primary antibodies were against the α₁₁-subunit of N-type Ca²⁺ channel (rabbit polyclonal, diluted 1:500; Alomone Laboratories), excitatory amino acid carrier 1 (EAAC1; rabbit polyclonal, diluted 1:100; provided by Dr. Rothstein), glutamate (mouse monoclonal, diluted 1:500; Incastar), vesicular acetylcholine transporter (VACHT; goat polyclonal, diluted 1:1,000; Chemicon International), nitric oxide synthase (NOS; sheep polyclonal, diluted 1:5,000; Chemicon International), vasoactive intestinal peptide (VIP; mouse monoclonal, diluted 1:1,000; provided by Dr. J. Walsh), e-κ-Ι (rat monoclonal, diluted 1:150; Chemicon International), synaptophysin (mouse monoclonal, diluted 1:200; Sigma, St. Louis, MO), or vesicular glutamate transporter 2 (rabbit polyclonal, diluted 1:1,500; Synthetic Systems, Göttingen, Germany) (31).

Control sections used to determine the level of nonspecific staining included incubating sections without primary antibody and/or blocking the primary antibody by preincubation (24 h) with the corresponding peptide (10–20 μg/ml) before incubation of the antibody with the tissue. In both cases, no specific staining was observed.

Preparations were examined by using a Radiance 2000 laser scanning confocal microscope (Bio-Rad, Hercules, CA) attached to an Axioplan 2 microscope (Zeiss). Usually, 5–10 optical sections were taken at 1.0-μm intervals. Images of 512 × 512 pixels were obtained and processed by using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA) and printed with a Tektronix Phaser 440 printer.

**Western blot analysis.** LMM was homogenized on ice in 20 mM Tris-HCl, pH 7.4, containing 1 mM PMSF, 20 μg/ml leupeptin, and 1% aprotinin. Homogenates were then centrifuged to obtain the cell membrane fraction. After protein concentration determination, the boiled samples containing a mixture of protease inhibitors (40 g/ml bacitracin and 4 g/ml leupeptin) at 4°C for 1 h. The tissue was then washed in warm Krebs/nicardipine (10⁻⁶ M) and transferred to Krebs/nicardipine/TTX for 1 h at 37°C. All incubations in Krebs solution (at 37°C) contained TTX (0.3 μM) and nicardipine (10⁻⁶ M).

### Table 1. Group III metabotropic glutamate receptor and control primer sets used for the PCR amplification of cDNAs

<table>
<thead>
<tr>
<th>Primers</th>
<th>PCR Product Size</th>
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<tr>
<td>mGlu4 Forward</td>
<td>5’-TCATTGGCTGTCAGCCAAGG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-GAGATCTGAGCCAGAAGGC-3’</td>
</tr>
<tr>
<td>mGlu6 Forward</td>
<td>5’-CAAGTACAGAAGTTGGATT-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-GGTTACATTTGTTGAGCTAAGC-3’</td>
</tr>
<tr>
<td>mGlu7 Forward</td>
<td>5’-GACCTGGTGGAATGTAACAG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-TTTACAGTGCCAGAATTAGAC-3’</td>
</tr>
<tr>
<td>mGlu8 Forward</td>
<td>5’-CGAAGGTTATTAACCTCAGCT-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-TATGGTCGGTATGGTTATGCT-3’</td>
</tr>
<tr>
<td>GDPDH Forward</td>
<td>5’-CATAGACAGAAGGTGGAAGCTGG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-GCGAAGTGTGCTAGATGACC-3’</td>
</tr>
</tbody>
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mGlu, metabotropic glutamate receptor.
To examine the effects of exogenously applied ligands, (RS)-4-phosphonophenylglycine [(RS)-PPG] (400 nM) and/or the group III mGluR receptor antagonist (RS)-cyclopentyl-4-phosphonophenylglycine [(RS)-CPPG; 200 µM] were added to the 4 and 37°C incubations. At 4°C the ligands are able to diffuse through interstitial spaces into the gut and bind to receptors, but membranes are solidified and receptors are not able to internalize. After tissue is warmed to 37°C, the receptors are able to undergo endocytosis.

For quantitative purposes, single optical sections (0.5-µm thick; 1,024 × 1,024 pixels) of mGluR8-immunoreactive myenteric neurons were taken. The sections were taken through the center of the cell body so that the images included the nucleus and a large area of cytoplasm. mGluR8-immunoreactive fluorescence was quantified by using the NIH Image software program (National Institutes of Health, Bethesda, MD). Because the nucleus does not contain mGluR8 protein, fluorescence was measured in the nucleus and the maximum nuclear intensity value was taken as the threshold intensity value for each cell. A line was drawn around the outside of the cell, and the total cell fluorescence was measured. A second line, ~0.5 µm from the first, was drawn inside the cell membrane, and the intracellular fluorescence was measured. The percentages of surface and of intracellular fluorescence were then calculated. Data were collected from 20 cells from each preparation, and an average value for each preparation was obtained. The mean and SE for each group of values was then calculated.

cAMP assay. This method was modified from Xia et al. (35). Briefly, the entire length of small intestine was removed, flushed with ice-cold Krebs solution, and divided into segments of ~4 cm in length. The LMMP was removed by blunt dissection and placed in an Eppendorf tube containing 500 µl Krebs. After preincubation with 1 mM IBMX (15 min), an inhibitor of phosphodiesterase, forskolin (10 µM), (RS)-PPG, and/or CPPG were added and incubated for another 15 min. After incubation, LMMPs were quickly transferred to ice-cold 50 mM sodium acetate (300 µl) and homogenized. After centrifugation, 50 µl supernatant was taken from each sample for protein concentration determination. The remaining supernatant was used for cAMP determination. The total cAMP content of LMMP was determined as picomoles of cAMP per milliliter by using the cAMP EIA kit from Assay Designs (Ann Arbor, MI) and then normalized to corresponding total protein.

Colonic motility assay. Colonic motility was examined according to established methods (17). Briefly, segments of guinea pig distal colon (~8-cm long) were mounted in Sylgard-coated chambers and incubated with oxygenated Krebs solution (at 37°C). Preparations were allowed to equilibrate and empty themselves of fecal pellets for ~30 min before the experiments were begun. A baseline rate of motility was then determined. To evoke the peristaltic reflex, an artificial fecal pellet made from modeling clay was inserted into the oral end of the isolated segments of colon. The rate at which the pellet was transported distally was measured by determining the time taken by the pellet to traverse a distance of 5 cm in the middle of the segment. The pellet was allowed to complete its passage down the entire segment. The pellet was then retrieved and reinserted at the oral end of the segment of the colon. Experiments were started when the rate of propulsion became constant for three consecutive trials after 1-min intervals. The average rate of propulsion measured for the three consecutive trials counted as the control rate. The mGluR8 agonists (RS)-PPG and (S)-3,4-dicarboxyphenylglycine (DCPG) and the antagonist CPPG or other compounds such as the nicotinic acetylcholine receptor antagonist hexamethonium (C6) or the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) were added to assess their effects on colonic motility. The colon was incubated for 10 min in the presence of compound before resuming the measurement of the rate of propulsion of the pellets. The peristaltic reflex was again quantified by averaging the rate of propulsion for three consecutive trials. The rate of propulsion of the pellet in the presence of compounds was expressed as a percentage of the control rate. Each preparation thus served as its own control.

Recording of responses of longitudinal muscle to electrical field stimulation. The procedure was modified from Cayabyab et al. (3). Briefly, segments (3 cm in length) of guinea pig distal ileum were mounted in Petri dish tissue holders and the tissue and holder were placed in jacketed baths. One end of the segment was attached to a screw at the base of the holder, whereas the other was attached to a force transducer (Harvard Apparatus, South Natick, MA). Tissues were subjected to a load of 1.0 g to obtain the most reproducible responses and were allowed to equilibrate for 30 min before starting the experiment. Responses of the longitudinal muscle to submaximal electrical field stimulation for 10 s with
pulses of 1.0-ms width at 30-V intensity and 10-Hz frequency were recorded isometrically. Every response of the fifth of five consecutive responses in each condition was measured and calculated for the mean and used for comparing the difference among different conditions.

Chemicals and drugs. (RS)PPG, CPPG, and DCPG were obtained from Tocris Cookson (Ballwin, MO). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) or Sigma.

Statistical analysis. Data are expressed as means ± SE, with differences among groups determined by Student’s t-test or ANOVA followed by a Scheffe’s F-test (P < 0.05).

RESULTS

mGluR8 mRNA and protein are found in the rat ENS. Group III mGluR expression in the ENS was analyzed utilizing RT-PCR. The primers used are listed in Table 1 (32). As expected, the brain was positive for mGluR8 (Fig. 1A, lane 2), mGluR4 (Fig. 1B, lane 1), mGluR6 (Fig. 1B, lane 2), and mGluR7 (Fig. 1B, lane 3). The LMMP was also positive for mGluR7 and mGluR8 expression (Fig. 1C) with expected bands at 321 and 440 bp, respectively, whereas no expression was detected in this tissue for mGluR4 or mGluR6 (Fig. 1C). RT-PCR analysis was confirmed after we subcloned and sequenced the PCR products. These results suggest that the LMMP, and perhaps specifically the myenteric ganglia, only express the genes encoding mGluR7 and mGluR8, whereas the brain expresses the genes encoding all four group III mGluR subtypes.

mGluR8 protein in the LMMP was established by Western blot analysis (Fig. 1D). A specific band with an apparent molecular mass of 102 kDa was detected in both brain and LMMP, suggesting that mGluR8 protein is found in the ENS. The density of the band in LMMP suggests that mGluR8 protein is abundant. The
upper bands in both brain and LMMP probably represent dimers of mGluR8.

**Distribution of mGluR8 immunoreactivity in the ENS.** Immunocytochemistry in conjunction with confocal microscopy was used to determine the distribution of mGluR8 in the ENS. mGluR8 immunoreactivity was observed throughout the gastrointestinal tract (Fig. 2, A–F) in both the myenteric (Fig. 2, A–D) and submucosal (Fig. 2, E and F) plexus, as well as in interganglionic connectives and circular muscle (Fig. 2, A and C). Punctate staining, which may indicate terminal labeling, was observed throughout the ENS. In addition, mGluR8 immunoreactivity could also be detected in the cell membrane (Fig. 2E) and cytoplasm (Fig. 2D) of a subset of cells. mGluR8 immunoreactivity was observed in the ENS of all species examined, including mouse (Fig. 3, A and B), humans (Fig. 3, C–E), and guinea pigs (Fig. 3F).

**Fig. 3.** mGluR8 immunoreactivity in the human and guinea pig intestine. mGluR8 immunoreactivity is displayed by both nerve cell bodies (arrows) and processes in myenteric ganglia (A, E, and F) and submucosal ganglia (B–D) in mouse (A and B), human duodenum (C) and ileum (D and E), and guinea pig ileum. A, B, and F: whole mount preparations. C–E: cryostat sections. duo, Duodenum; ile, ileum. Scale bar = 10 μM.

**Identification of mGluR8-immunoreactive cells and structures.** Further studies were done to chemically identify the cells and structures in the rat ENS that display mGluR8 immunoreactivity. The punctate appearance of mGluR8 immunoreactivity in the neuropil suggested that the receptor might be located in synaptic terminals. Double-label immunofluorescence was employed to determine whether or not mGluR8-immunoreactive structures display markers expressed in nerve terminals, such as synaptophysin or the α1B-subunit of N-type Ca2+ channels. In addition, we examined the colocalization of mGluR8 with glutamate transporters, VACHT, NOS, VIP, and c-kit (marker of interstitial cells of Cajal; ICC). mGluR8 immunoreactivity was visualized with FITC, whereas the other markers were visualized with the red fluorescence of TRITC.
Punctate synaptophysin- (Fig. 4A) and α1B-subunit- (Fig. 4B) positive structures were abundant in enteric ganglia and localized on nerve processes that displayed mGluR8 immunoreactivity. mGluR8-positive cell bodies displayed EAAC1 immunoreactivity (Fig. 4C), and mGluR8-immunoreactive nerves were contacted by vesicular glutamate transporter 2 (VGLUT2) nerve terminals (Fig. 4D). These findings suggest that mGluR8-immunoreactive cell bodies are glutamatergic, and nerve processes that receive glutamatergic input express mGluR8. To test this hypothesis, we determined whether enteric glutamatergic neurons express mGluR8.

L-Glutamate is one of the primary neurotransmitters utilized by nodose ganglion neurons (2); therefore, glutamate-immunoreactive neurons were located in the nodose ganglia, as a control (Fig. 5A). As suggested by the abundance of mGluR8 immunoreactivity in the nucleus of the solitary tract (25), nodose ganglion neurons expressed mGluR8 (Fig. 5B). Glutamatergic neurons in the ENS (Fig. 5C) displayed membranous mGluR8 immunoreactivity (Fig. 5D) supporting the idea that activation of mGluR8 modulates the activity of glutamatergic cells.

Dual-label immunofluorescence also established that mGluR8-positive neurons are contacted by VACHT-immunoreactive nerve terminals (Fig. 6A) and contain both NOS (Fig. 6B) and VIP (Fig. 6C). In the myenteric plexus, NOS and VIP are markers of inhibitory motorneurons. VIP is also found in submucosal secretomotor cells. A subset of mGluR8-immunoreactive cells, located in the deep muscular plexus, displayed immuno-reactivity to c-kit (Fig. 6D), a tyrosine kinase receptor, indicating that mGluR8 is also expressed by ICC.

Activation of mGluR8 inhibits forskolin-stimulated cAMP production in myenteric ganglia. Groups II and III mGluRs are negatively coupled to adenylate cyclase activity. To study the possible negative coupling between mGluR8 and adenylate cyclase activity in the ENS, we examined the effects of (RS)PPG on forskolin-induced accumulation of cAMP in isolated guinea pig myenteric ganglia. A significant increase (P < 0.01) of
cAMP content in myenteric ganglia was observed after incubation with forskolin (10 μM; from basal level 0.04 pmol/μg protein to 0.76 pmol/μg protein; Fig. 7). (RS)PPG (400 nM) significantly (P < 0.05) inhibited forskolin-induced cAMP production (to 0.43 pmol/μg protein), and the group III mGluR antagonist CPPG (200 μM) was effective in significantly (P < 0.05) preventing the inhibition of (RS)PPG on forskolin-induced potentiation (Fig. 7). CPPG (200 μM) increased forskolin-induced cAMP production in ganglia, suggesting that group III mGluRs may be tonically activated by glutamate.

**Agonist-induced internalization of mGluR8.** Many G protein-coupled receptors (GPCR), including enteric group I mGluRs (20), internalize on prolonged exposure to agonist. In view of the association of mGluR8 with the plasma membrane, we examined whether the mGluR8 undergoes agonist-induced internalization. Segments of rat ileum were incubated with (RS)PPG at 4°C, washed, and warmed to 37°C. Confocal images were obtained to examine the distribution of mGluR8 immunoreactivity.

(RS)PPG (400 nM), within a 60-min period at 37°C, caused mGluR8 immunoreactivity to be localized in numerous endosomes in the soma (Fig. 8, A and A’). In control preparations, without any ligands added, mGluR8 immunoreactivity was confined to the plasma membrane of the soma (Fig. 8, B and B’). No fluorescence was detected in the nucleus. The internalization induced by (RS)PPG was blocked by coapplication of the group III mGluR antagonist CPPG (200 μM; Fig. 8, C and C’).

To quantitate the change in distribution of mGluR8 on receptor activation, 20 neurons were randomly chosen from each preparation (n = 3) and the percentage of fluorescence in the cytoplasm was calculated. Results are shown in Fig. 8D. (RS)PPG significantly (P < 0.001) increased the percentage of cytoplasmic fluorescence from 50 to 70%, and the effect was prevented by...
CPPG. These findings suggest that like other GPCR, mGluR8 also undergoes agonist-induced internalization.

Activation of mGluR8 increases gut motility. To determine whether mGluR8 plays a role in motility, we examined the effects of mGluR8 ligands on the reflex-initiated rate of propulsion of an artificial fecal pellet in the guinea pig distal colon. As previously observed (17), the rate of propulsion of artificial fecal pellets was constant for segments obtained from the same colon. Incubation of colonic segments with TTX (0.5 μM; n = 4) abolished the reflex, indicating that it was nerve-mediated (not illustrated). Incubation of the segments for 10 min with (RS)PPG (4–400 nM) caused an increase of the rate of propulsion (Fig. 9A). Similar results were obtained when DCPG (50–500 nM) was applied (Fig. 9B). Moreover, the effect of (RS)PPG was blocked by the group III mGluR antagonist CPPG (Fig. 9C). Interestingly, CPPG applied at 20–2,000 μM inhibited colonic motility (Fig. 9D), suggesting that group III mGluRs may be tonically activated by glutamate. The observation that mGluR8 agonists increase reflex-driven propulsion further supports the idea that enteric mGluR8s are functional and suggests that glutamate plays a role in the peristaltic reflex in the guinea pig colon.

We then investigated the pathway by which mGluR8 modulates colonic motility. Hexamethonium (C6; 10 μM) reduced colonic motility to 84% of control and abolished the (RS)PPG-evoked increase in motility (Fig. 10, A and B). This result suggests that mGluR8 agonists increase motility via facilitation of cholinergic excitatory neurotransmission. Treatment of the gut segments with an inhibitor of NOS (L-NAME, 200 μM) significantly increased motility (Fig. 10C). In other words, the excitatory effects of L-NAME and (RS)PPG are not additive, suggesting that both mediators are involved in the same pathway.

mGluR8 increases the contraction of guinea pig longitudinal muscle to electrical field stimulation. To further investigate the role of mGluR8 in gut motility, we examined the effects (RS)PPG (400 nM) on contractions of longitudinal muscle of ileal segments induced by electrical field stimulation. (RS)PPG significantly increased the electrical field stimulation-induced contractions (Fig. 11, A and B), and the effect was reversible on washout of the drug. These data further support a stimulatory role of mGluR8 in gastrointestinal motility.
Fig. 9. Effects of mGluR8 agonists on colonic motility. A and B: (RS)PPG and (S)-3,4-dicarboxyphenylglycine (DCPG) increase the propulsion of an artificial fecal pellet in the isolated guinea pig distal colon. Data are means ± SE of 5–8 experiments. *Significant increase of the velocity of propulsion. C and D: CPPG (200 μM) blocks the (RS)PPG (400 nM)-induced increase in colonic motility and by itself inhibits motility. Data are means ± SE of 5–8 experiments.

Fig. 10. Effects of hexamethonium (C6) and Nω-nitro-L-arginine methyl ester (L-NAME) on (RS)PPG-evoked increase in motility. C6 inhibits colonic motility at 10 μM (A) and, applied at the same concentration, blocks the (RS)-PPG (400 nM)-evoked increase in propulsion of an artificial fecal pellet (B). In B, the inhibition in motility produced by C6 is the control. C: effect of (RS)PPG is also blocked by L-NAME (200 μM), an inhibitor of nitric oxide synthase (NOS). As previously determined, L-NAME significantly increases colonic motility ≥132% of control. *P < 0.05 compared with control; n = 5–8 preparations.
DISCUSSION

The present study demonstrates for the first time that group III mGluRs are present in the ENS. With the use of RT-PCR, myenteric ganglia were shown to express the genes encoding mGluR7 and mGluR8. Expression of mGluR8 was corroborated by Western blot analysis and immunocytochemical data localizing mGluR8 protein in the enteric ganglia. Moreover, functional studies demonstrated that mGluR8 plays a role in modulating gut motility.

mGluR8 immunoreactivity was displayed by enteric neurons throughout the gastrointestinal tract and was found in all species examined, including humans. Punctate mGluR8 structures, suggestive of nerve terminals, were observed in the ganglionated neuropil; however, they did not show synaptophysin or N-type Ca\(^{2+}\) channel immunoreactivity proteins associated with synaptic vesicles. The majority of mGluR8 labeling was associated with the membrane and cytoplasm of nerve cell bodies and processes. Immunogold labeling confirmed that mGluR8 is localized at membranes and not over synaptic vesicles (unpublished observations). Similar observations have been made in mammalian retina (18) and autonomic groups of the medulla oblongata (25) in which analysis at the ultrastructural level revealed localization of this receptor subtype at both the pre- and postsynaptic membranes (18). In view of the functional evidence, which suggests a putative autoreceptor role for group III mGluRs in the CNS, the significance of mGluR8 expression in cell bodies is not clear. It is possible that mGluR8 is involved in the regulation of synaptic transmission in the ENS both pre- and postsynaptically, but currently functional evidence for this is lacking.

Group III mGluRs expressed in the CNS act as autoreceptors mediating the inhibition of glutamate release. We obtained some evidence that mGluR8 is expressed by glutamatergic neurons in the gut. mGluR8-positive neurons in both the submucosal and myenteric plexus displayed glutamate immunoreactivity; however, glutamate is a general metabolite, and some nonglutamatergic cells, including GABAergic neurons, contain the amino acid. On the other hand, VGLUTs transport glutamate into synaptic vesicles, making them selective markers of glutamatergic cells (24). Previously, we showed that most glutamate-immunoreactive enteric neurons express VGLUT2 and are contacted by VGLUT2 nerve terminals (31). Glutamate-immunoreactive enteric neurons also contain the neuronal glutamate transporter, EAAC1 (21), which is responsible for inactivating glutamate to terminate its effects and prevent receptor desensitization (28). In the present study, VGLUT2 nerve terminals contacted mGluR8-positive cell bodies and were expressed in mGluR8-containing nerve fibers. As previously reported (31), a subset of VGLUT2 terminals coexpressed VAChT, indicating that cholinergic neurons costore and release glutamate. Taken together, these data suggest that glutamatergic enteric neurons receive glutamatergic/cholinergic input and express mGluR8.

Studies on segments of rat ileum demonstrated that mGluR8 undergoes agonist-evoked internalization. Neurons in both the myenteric and submucosal plexus displayed immunoreactivity that was primarily associated with the plasma membrane. Exposure to the mGluR8 agonist (RS)PPG induced a significant increase in cytoplasmic fluorescence that was blocked by coapplication of the group III mGluR antagonist CPPG. This finding indicates that agonist binding induces internalization of mGluR8, similar to other GPCRs (6). Whether mGluR8 undergoes reflex-evoked internalization, similar to enteric group I mGluRs (20), is not known and would provide support for the idea that group III mGluRs participate in enteric reflexes.

mGluR8 is also expressed by nonglutamatergic cells. NOS-positive neurons in the myenteric plexus contained mGluR8, as well as VIP neurons in the submucosal plexus. NOS is a marker of descending interneurons and inhibitory motorneurons; therefore, activation of mGluR8 would be expected to modulate motility. VIP is a marker of secretomotor cells (8, 15); therefore, activation of mGluR8 on these cells would be expected to modulate intestinal secretion. We also found that ICC displayed mGluR8 immunoreactivity. ICC are coupled to each other and to smooth muscle cells by gap junctions (13, 33). They act as pacemakers in the gut wall and relay most of the inhibitory as well

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Fig. 11. Effect of mGluR8 agonist on electrical field stimulation-induced contraction of longitudinal muscle. A: representative traces of response of guinea pig ileum to electrical field stimulation at 0.1 Hz. (RS)PPG (400 nM) increased the amplitude of longitudinal muscle contraction to electrical stimulation, and the effect was reversible on washout. B: quantification of the results obtained in A. ACh, acetylcholine; ES, electrical stimulation. *P < 0.05 compared with Krebs or wash; n = 3 preparations.
as part of the excitatory impulses from the ENS to smooth muscle (13). Thus mGluR8 may also modulate gut motility by affecting the activity of ICC.

To investigate the role of mGluR8 in motility, we studied the effects of mGluR8 agonists on the peristaltic reflex in isolated segments of guinea pig colon. The peristaltic reflex, assessed by measuring the propulsion of solid pellets, was enhanced by bath application of (R S)PPG or DCPG. Moreover, the effect was blocked by the mGluR8 antagonist CPPG. Interestingly, CPPG inhibited peristalsis, suggesting that mGluR8 may be tonically activated by glutamate. These data suggest that glutamate and mGluR8 participate in the initiation and/or propagation of the peristaltic reflex; however, this study cannot precisely locate the site of glutamate’s action. As discussed above, mGluR8 immunoreactivity is displayed not only by glutamatergic/cholinergic neurons, but also by nonglutamatergic neurons and ICC; therefore, glutamate could increase peristalsis via mGluR8 located on several types of cell.

We investigated the mechanism whereby mGluR8 activation increases peristalsis by examining the effects of mGluR8 agonists in the presence of C6 or the NOS inhibitor L-NAME. As previously reported, C6 decreased and L-NAME significantly increased peristaltic reflex; however, this study cannot precisely locate the site of glutamate’s action. As discussed above, mGluR8 immunoreactivity is displayed not only by glutamatergic/cholinergic neurons, but also by nonglutamatergic neurons and ICC; therefore, glutamate could increase peristalsis via mGluR8 located on several types of cell.

Acceleration of colonic motility may also involve the inhibition of cAMP formation. Activation of group III mGluRs has been shown to inhibit cAMP formation in expression systems, brain slices, and neuronal cultures (7, 26, 29). Consistent with these results, we found that activation of mGluR8 inhibited forskolin-induced cAMP formation in LMMMP and that the mGluR8 antagonist CPPG prevented this effect. Thus it appears that mGluR8 utilizes the same signaling pathway in the ENS as in the CNS. Inhibitory neurotransmitters, such as VIP, have been suggested as inducing relaxation of the smooth muscle through a cAMP-dependent pathway (22); therefore, mGluR8-mediated control of intestinal contraction may involve inhibition of cAMP levels.

In conclusion, in addition to ionotropic and group I and II mGluRs, the ENS contains functional group III mGluRs. mGluR8 immunoreactivity is abundant in the submucosal and myenteric plexus in which it is localized to both pre- and postsynaptic elements. Activation of mGluR8 results in receptor internalization as well as inhibition of forskolin-induced cAMP formation in enteric ganglia. In addition, mGluR8 agonists increase guinea pig colonic motility, at least in part, via inhibition of nitricergic transmission. The abundance of mGluR8 in the human bowel suggests that group III mGluRs play an important role in gastrointestinal physiology.

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DISCLOSURES

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