Inward Currents in Neurons from Newborn Guinea Pig Intestine: Mediation by 5-Hydroxytryptamine Type 3 Receptors

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Accepted for publication June 7, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

The whole-cell patch-clamp technique was used to analyze the effects of 5-hydroxytryptamine (5-HT) and alosetron on cultured myenteric neurons from newborn guinea pigs. All neurons responded to 5-HT (EC50 \( \approx 38.7 \mu M \)) with a concentration-dependent inward current (reversal potential \( \approx 7.1 \pm 1.7 \) mV) with a short latency and rapid decay. Because the 5-HT-induced inward current was mimicked by 2-methyl-5-hydroxytryptamine (50 \( \mu M \)) and blocked by ondansetron (5.0 \( \mu M \)) and MDL 72222 (0.05 \( \mu M \)), it was 5-HT3-mediated. Alosetron (0.2 \( \mu M \)) blocked (IC50 \( \approx 0.05 \mu M \); Hill coefficient \( \approx 1.24 \)) the 5-HT- and 2-methyl-5-hydroxytryptamine-induced inward currents. This effect was independent of membrane potential and was not seen when alosetron was delivered to the inside of cells. Alosetron-sensitive sites are, thus, accessible only on the ectodomain of the plasmalemma. The effect of alosetron was reversible, but not surmountable. Although nicotine (100 \( \mu M \)) mimicked the 5-HT-induced inward current, the response was antagonized by hexamethonium (100 \( \mu M \)), but not by alosetron, implying its potential to be a selective 5-HT3 antagonist. Hexamethonium did not affect responses to 5-HT. Most neurons in the cultures were 5-HT-immunoreactive and immunostained with an antibody raised against 5-HT3 receptors. The 5-HT-selective uptake inhibitor, fluoxetine (30 \( \mu M \)), gradually reduced the amplitude of the current induced by 5-HT; the residual response was abolished by alosetron (0.2 \( \mu M \)). The effect of fluoxetine could have been caused by either the desensitization of 5-HT3 receptors or by a nonspecific 5-HT3 antagonistic effect of fluoxetine. It is concluded that alosetron is a potent and noncompetitive 5-HT3 antagonist on myenteric neurons.

Enteric ganglia differ from those of the sympathetic and parasympathetic nervous systems because they can control the behavior of an organ in the absence of input from the central nervous system (CNS). The independence of the enteric nervous system (ENS) is made possible by the presence within the intestinal wall of primary afferent neurons and interneurons. Although the bowel can function independently of the CNS, it does not normally do so, and there is a considerable exchange of signals between the ENS and the CNS. Serotonin (5-hydroxytryptamine; 5-HT) has been postulated to play roles, both in initiating intrinsic enteric reflexes (Bühbring and Crema, 1959; Foxx-Orenstein et al., 1995; Grider et al., 1996; Kirchgessner et al., 1992, 1996; Wade et al., 1996; Chen et al., 1998) and in activating extrinsic sensory nerves (Hillsley and Grundy, 1998). In both cases, enterochromaffin (EC) cells of the gastrointestinal mucosal have been proposed to be sensory transducers that respond to luminal stimuli by secreting 5-HT, which stimulates the intrinsic or extrinsic nerves.

Several subtypes of 5-HT receptor have been identified on enteric neurons. These include the 5-HT1A (Pan and Galligan, 1994; Kirchgessner et al., 1995), 5-HT1B (Mawe et al., 1986; Wang et al., 1996; Cooke et al., 1997; Pan et al., 1997), 5-HT2A, 5-HT2B (E. Fiorica-Howells and M.D.G., unpublished observations), 5-HT3 (Mawe et al., 1986; Derkach et al., 1989; Galligan, 1995; Johnson and Heinemann, 1995), and 5-HT4 (Pan and Galligan, 1994; Grider et al., 1996; Kadowaki et al., 1996) receptors. The 5-HT4 (Foxx-Orenstein et al., 1995; Grider et al., 1996) and 5-HT3 (Kirchgessner et al., 1992; Kirchgessner et al., 1996; Wade et al., 1996; Cooke et al., 1997; Chen et al., 1998) receptors have been associated with the excitation of the intrinsic sensory nerves that initiate peristaltic and secretory reflexes, whereas 5-HT3 receptors seem to be involved in extrinsic signaling (Hillsley and...
Grundy, 1998). In contrast to its function in the activation of extrinsic nerves, the role played by 5-HT₃ receptors in ganglionic transmission has been unclear. This receptor is expressed by myenteric (Mawe et al., 1986; Wade et al., 1991) and submucosal neurons (Derkach et al., 1989), but 5-HT₃ antagonists have neither been observed to block synaptic transmission (Galligan, 1995), nor to interrupt the peristaltic reflex (Kadowaki et al., 1996).

5-HT₃ antagonists are widely used to treat the nausea and vomiting associated with cancer chemotherapy (Gregory and Ettinger, 1998) and one, alosetron, has been proposed to be of particular value in the treatment of irritable bowel syndrome (IBS; Delvaux et al., 1998). Alosetron and other 5-HT₃ antagonists appear to reduce the abdominal discomfort associated with IBS, an effect that is consistent with the role played by 5-HT₃ receptors in the activation of extrinsic sensory nerves; however, relatively few effects of 5-HT₃ antagonists on motility have been reported. Alosetron slows transit in the left colon and does not affect that in small bowel or distal colon, even in patients with the hyper moteric syndrome (Saslow et al., 1998) or IBS (Delvaux et al., 1998). Alosetron also fails to affect cholera toxin-induced secretion (Bearcroft et al., 1997), which involves the participation of 5-HT (Beubler et al., 1989). In contrast, alosetron is highly effective in preventing cisplatin-induced vomiting (Rudd and Naylor, 1994) and has been estimated to be 5 to 10 times more potent than ondansetron as a 5-HT₃ antagonist in vitro (Saslow et al., 1998). Because alosetron is now undergoing clinical testing, it is important to determine the nature of its effects on enteric neurons.

In the current study, myenteric ganglia were isolated from newborn guinea pigs to characterize the responses of enteric neurons to 5-HT and alosetron. Dissociated ganglion cells were cultured for 5 to 10 days, and responses were analyzed by using the whole-cell patch-clamp recording technique. The predominant response to 5-HT was a fast inward current that was blocked by alosetron and the 5-HT₃-selective antagonist ondansetron, and mimicked by the 5-HT₃-selective agonist, 2-methyl-5-HT. Almost all of the cultured neurons were 5-HT₃-immunoreactive, and many also displayed 5-HT immunoreactivity. These observations confirm that alosetron is a potent and selective antagonist of 5-HT₃ receptors on enteric neurons. The exceptional abundance of serotonergic neurons and 5-HT₃ receptors in preparations from newborn animals suggests that both may play more of a role in the physiology of the developing than the mature ENS.

### Materials and Methods

**Culture of Myenteric Neurons.** A single-cell suspension was prepared from myenteric ganglia of the newborn guinea pig small intestine (Zhou and Galligan, 1996). Briefly, male guinea pig pups were stunned and exsanguinated. The Animal Care and Use Committee of Columbia University approved this procedure. The small intestine was removed, cleaned, and placed in ice-cold Krebs’ solution of the following composition: 121.3 mM NaCl, 5.95 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.34 mM NaH₂PO₄, 14.3 mM NaHCO₃, and 12.7 mM glucose. The bowel was mounted on a pipette placed in the intestinal lumen. The longitudinal muscle and adherent myenteric plexus were then rapidly stripped from the gut with a fine cotton swab. The resulting preparations were minced and the tissue suspension was divided into two aliquots. Each aliquot was incubated with trypsin (Sigma Chemical Co., St. Louis, MO; 1600 U/ml, dissolved in sterile Krebs’ solution) for 30 min at 37°C. The digested tissue was gently trituated 30 times with a fire-polished Pasteur pipette and centrifuged at medium speed for 7 to 10 min. The resulting pellet was resuspended and incubated with crab hepatopancreas collagenase (Calbiochem; La Jolla, CA; 4000 U/ml, dissolved in sterile Krebs’ solution) for 30 min at 37°C. The suspension was again tritiated and centrifuged as above. The final pellet was resuspended in 1 ml of F-12K Nutrient Mixture, Kaighn’s Modification (Gibco; Grand Island, NY) containing 10% fetal bovine serum, gentamicin (10 μg/ml), penicillin (100 U/ml), and streptomycin (50 μg/ml; all additives from Sigma). Cells were plated in 35-mm plastic Petri dishes (Corning Glass Co., Corning, NY). Cultures were incubated at 37°C in an atmosphere of 5% CO₂. Cultures were incubated for 5 to 10 days, and responses were analyzed by using phase-contrast optics. Whole-cell patch-clamp recording (Hamill et al., 1991) was carried out at room temperature using an Axopatch 200B amplifier connected to a Digidata 1200 interface (Axon Instruments, Inc., Foster City, CA) to a computer (OptiPlex GS®; Dell, Round Rock, TX). Gigaohm seals were made using borosilicate glass microelectrodes (World Precision Instruments, Sarasota, FL) with tip resistances of 2 to 5 MΩ and series resistances of 3 to 10 MΩ were compensated by 40 to 80%. Unless otherwise stated, membrane potential was held at −60 mV. Data were filtered at 5 kHz, and displayed and recorded on the computer and a chart recorder (Dash IV XL; Astro-Med, Inc., Quincy, MA). Cells were superfused at 1 to 2 ml/min in an external solution containing: 150.0 mM NaCl, 5.0 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM d-glucose. The osmolality was adjusted with sucrose to 340 mOsm/kg, and the pH was buffered to 7.4 using NaOH. The patch-pipettes were filled with an internal solution containing: 140 mM potassium gluconate, 1 mM CaCl₂, 2 mM MgCl₂, 11 mM EGTA, 10 mM HEPES, and 2 mM ATP (magnesium salt); the pH was buffered to 7.4 with KOH and the osmolality was brought to 310 mOsm/kg with sucrose. Drugs were dissolved in the external solution and applied through a fast perfusion system. This system consisted of a series of fused silica tubes (i.d. of each pipette −200 μm) glued together via a short common outlet (length of ~5 mm and i.d. of ~200 μm) placed within 100 μm of patched neurons. These tubes were held by a micromanipulator (Narishige Inst. Co., Greenvale, NY) and connected to reservoirs containing control or experimental solutions. Neurons were continuously superfused (3–5 μl/s) with control external solution flowing from one barrel of the fast perfusion system and switched to experimental solutions by opening the appropriate valve. The valves were electrically controlled enabling solutions to be rapidly exchanged. The time for the junction potential at an open pipette tip to rise from 10 to 90% was ~50 ms. Agonists were applied at intervals of 3 min so that 5-HT₃ receptors would not become desensitized.

**Whole-Cell Patch-Clamp Recording.** Neurons were viewed with an inverted microscope (Axiovert S100; Zeiss, Oberkochen, Germany) using phase-contrast optics. Whole-cell patch-clamp recording (Hamill et al., 1991) was carried out at room temperature using an Axopatch 200B amplifier connected to a Digidata 1200 interface (Axon Instruments, Inc., Foster City, CA) to a computer (OptiPlex GS®; Dell, Round Rock, TX). Gigaohm seals were made using borosilicate glass microelectrodes (World Precision Instruments, Sarasota, FL) with tip resistances of 2 to 5 MΩ and series resistances of 3 to 10 MΩ were compensated by 40 to 80%. Unless otherwise stated, membrane potential was held at −60 mV. Data were filtered at 5 kHz, and displayed and recorded on the computer and a chart recorder (Dash IV XL; Astro-Med, Inc., Quincy, MA). Cells were superfused at 1 to 2 ml/min in an external solution containing: 150.0 mM NaCl, 5.0 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM d-glucose. The osmolality was adjusted with sucrose to 340 mOsm/kg, and the pH was buffered to 7.4 using NaOH. The patch-pipettes were filled with an internal solution containing: 140 mM potassium gluconate, 1 mM CaCl₂, 2 mM MgCl₂, 11 mM EGTA, 10 mM HEPES, and 2 mM ATP (magnesium salt); the pH was buffered to 7.4 with KOH and the osmolality was brought to 310 mOsm/kg with sucrose. Drugs were dissolved in the external solution and applied through a fast perfusion system. This system consisted of a series of fused silica tubes (i.d. of each pipette −200 μm) glued together via a short common outlet (length of ~5 mm and i.d. of ~200 μm) placed within 100 μm of patched neurons. These tubes were held by a micromanipulator (Narishige Inst. Co., Greenvale, NY) and connected to reservoirs containing control or experimental solutions. Neurons were continuously superfused (3–5 μl/s) with control external solution flowing from one barrel of the fast perfusion system and switched to experimental solutions by opening the appropriate valve. The valves were electrically controlled enabling solutions to be rapidly exchanged. The time for the junction potential at an open pipette tip to rise from 10 to 90% was ~50 ms. Agonists were applied at intervals of 3 min so that 5-HT₃ receptors would not become desensitized.

**Western Blotting.** A synthetic peptide representing the carboxyl terminal octadecapeptide of the rat 5-HT₃ receptor (IRHFLE-KRDMEREVARDW) with a d-tyrosine residue added at the amino terminus was conjugated to keyhole limpet hemocyanin by use of bisdiazotized benzidine. New England white rabbits (8 weeks of age) were immunized by multiple intradermal injections at intervals of 8 weeks as described previously (Sternini et al., 1997). Antibody screening was done by enzyme-linked immunosorbent assay on plates coated with the immunizing peptide. Further characterization was carried out by Western blotting using lysates of human embryonic kidney 293 cells stably transfected with 5-HT₃ receptors using a rat 5-HT₃ receptor cDNA plasmid inserted into the vector LNCX (Marniq et al., 1991). Antibody no. 95247 was selected for future use, based on its high titer (1:100,000) by enzyme-linked immunosorbent assay, its strongly positive and specific identification of an 80-kD
band on Western blotting, and its positive specific immunocytochemical staining of cells transfected with full-length cDNA encoding the 5-HT₃ receptor and of enteric neurons. The antibody was immuno-purified by affinity chromatography as described previously (Ster-nini et al., 1997). Ammonium sulfate-precipitated γ globulin was incubated with EAH Sepharose 4B (Amersham Pharmacia Biotech, Newark, NJ) beads to which immunization peptide was covalently linked by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (Pierce, Rockford, IL), eluted in 0.1 M citrate buffer, pH 3.2, then dialyzed against 0.05 M PBS, pH 7.4.

Immunocytochemistry. Antibodies to 5-HT were purchased from Inestar (Minneapolis, MN). Cultured neurons were fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer (pH 7.4) for 1 h at room temperature and washed 3 times with PBS. When 5-HT immunoreactivity was to be demonstrated, cultures were exposed before fixation, first to 6-hydroxydopamine (2 h) and then to 5,7-dihydroxytryptamine (30 min; Costa et al., 1982). After fixation, preparations were exposed to PBS containing 0.5–1.0% Triton X-100 and 4% horse serum for 30 min to permeabilize the tissue and reduce background staining. Immunoreactivity was then demonstrated by incubating the fixed and permeabilized cultures with primary antibodies (24 h at 4°C) to 5-HT₃ receptors or 5-HT. Bound antibodies were located by incubating preparations for 3 h with biotinylated secondary antibodies to rabbit IgG and alkaline phosphatase-labeled avidin (diluted 1:400, incubated for 24 h at 4°C; Vector Labs, Burlingame, CA). The alkaline phosphatase-labeled avidin was visualized by using an alkaline phosphatase substrate kit (Vector Labs; Kit III) that produces a blue reaction product. The staining was performed in the presence of 1 mM levamisole (Sigma), which inhibits the endogenous alkaline phosphatase activity of the neural tissue. In addition, 10 mM MgCl₂, which enhances the enzyme activity of alkaline phosphatase, was added to the solution containing the substrate. Preparations were incubated with the substrate in the dark for 30 to 60 min or until the blue reaction product was seen. Preparations were then washed in distilled water, dehydrated, cleared in Histoclear (National Diagnostics) and coverslipped with Permount (Fisher Scientific, Springfield, NJ).

Data Analysis. Averaged values in the text and figures are expressed as means ± S.E. Means were compared statistically by the use of ANOVA or paired Student’s t tests. Concentration–response data were analyzed by using a computer-assisted nonlinear curve-fitting program (SigmaPlot 2.01; Jandel Scientific, San Raphael, CA). Values reported for maximal inhibition (Eₘₐₓ) and the concentration of an antagonist producing half-maximal inhibition (IC₅₀) were obtained by fitting the data to the following logistical equation: 

\[
y = \frac{E_{\text{max}} - E_{\text{min}}}{1 + \left(\frac{[x]}{EC_{50}}\right)^n} + E_{\text{min}},
\]

where \(x\) and \(y\) are concentration and response (i.e., percent inhibition), respectively, \(E_{\text{max}}\) is the maximum response, \(E_{\text{min}}\) is the minimal response, \(EC_{50}\) is the half-maximal concentration, and \(n\) is the slope factor (apparent Hill coefficient).

Drugs. 5-HT hydrochloride, 2-methyl-5-HT, fluoxetine, nicotine, and hexamethonium were purchased from Research Biochemicals Incorporated (Natick, MA). Ondansetron and alosetron were supplied by Dr. Allen Mangel of Glaxo Wellcome, Inc. (Research Triangle Park, NC).

Results

5-HT Evokes a Fast Inward Current in Newborn Guinea Pig Myenteric Neurons. All cultured myenteric neurons responded to 5-HT (0.5–140 μM) with a fast inward current (Fig. 1A). This current rapidly decayed to a steady-state level within 15 s when the concentration of 5-HT was ≥ 20 μM. The mean reversal potential of the 5-HT-induced inward current was 7.1 ± 1.7 mV (n = 4). Both the amplitude (Fig. 1, A and B) of the current evoked by 5-HT and its rate of decay (Fig. 2A) were concentration-dependent. The EC₅₀ of the response to 5-HT was 38.7 μM and the Hill coefficient was 1.49. The peak and steady-state currents activated by 5-HT (40 μM) were each antagonized by ondansetron (5.0 μM) and alosetron (0.2 μM; Fig. 1C). The response to 5-HT was also blocked by MDL 72222 (0.05 μM; data not shown).

The fast inward current evoked by 5-HT was mimicked by 2-methyl-5-HT (50 μM), although the potency of 2-methyl-5-HT was lower than that of 5-HT (Fig. 1D). The response of myenteric neurons evoked by 2-methyl-5-HT, like that elicited by 5-HT, was antagonized by alosetron (0.2 μM). These observations are consistent with the conclusion that both the peak and steady-state currents elicited in cultured myenteric neurons by 5-HT were 5-HT₃ receptor-mediated.

We investigated the effects of applying alosetron before neurons were exposed to 5-HT (40 μM – 80 μM). Three concentrations of alosetron were studied: 0.06 μM, 0.2 μM, and 0.6 μM (Fig. 2, A–C). The alosetron-induced reduction in the amplitude of the peak response to 5-HT increased as a function of the time between the exposure of cells to alosetron and that to 5-HT for up to 20 s. Preapplication of alosetron for >20 s did not further increase its effect. The maximum amplitude
of the inward current elicited by 5-HT was more sensitive to inhibition by alosetron than was the steady-state current. At concentrations of 0.06 to 0.2 μM alosetron strongly inhibited the fast inward component of the 5-HT-evoked response, whereas the slower steady-state component of the response was only moderately reduced or scarcely inhibited at all (Fig. 2, A and B). In contrast, at 0.6 μM, preapplication of alosetron strongly inhibited both components of the response to 5-HT. Because both the peak and steady-state components of the response to 5-HT were sensitive to alosetron (Figs. 1C and 2C), ondansetron (Fig. 1C), and MDL 72222 (data not illustrated), it is likely that activation of 5-HT₃ receptors accounts for the entirety of the inward current evoked by 5-HT. This idea is supported by the ability of 2-methyl-5-HT to mimic fully the 5-HT response. The enhanced efficacy of alosetron when it was preapplied suggest that this compound binds relatively slowly to the 5-HT₃ receptor, but that its binding is fully functional (in antagonizing responses to 5-HT) within 20 s. When applied at moderate concentrations (~0.6 μM), the inhibitory effects of alosetron were almost completely reversible.

The effect of alosetron on the peak amplitude of the response to 5-HT was independent of membrane potential. The presence of alosetron did not significantly affect the reversal potential (7.12 ± 1.75 mV; n = 4) of the 5-HT-induced current (Fig. 3A). Moreover, the percent inhibition by alosetron (0.06 to 0.6 μM) was preapplied before challenge with 40 or 80 μM 5-HT. The effect of alosetron, at each concentration, is reversible.

**Fig. 3.** The antagonism by alosetron of responses to 5-HT is independent of membrane potential. A, the current-voltage relationship is plotted for the response to 5-HT (40 μM) in the presence or absence of alosetron (0.06 μM). The reversal potential under both conditions is ~7.12 ± 1.75 mV. B, varying the membrane potential (from −80 to +20 mV) does not change the peak amplitude of the current induced by 5-HT in the presence of alosetron (0.06 μM).

**The Ability of Alosetron to Inhibit Currents Evoked by 5-HT is Concentration-Dependent.** Alosetron in varying concentrations (0.005 μM to 1.0 μM) was preapplied to myenteric neurons for 20 s before the cells were exposed to 5-HT (40 μM; 15 s) to allow adequate time for alosetron to bind to receptors (see Fig. 2). The threshold effect for inhibition of the peak amplitude of inward current induced by 5-HT was ~0.005 μM (Fig. 4, A and B). The steady state of the response was not affected by alosetron until the concentration was increased to >0.2 μM (Fig. 4A). The IC₅₀ for the inhibition by alosetron of the peak amplitude of the response to 5-HT was 0.05 μM and the Hill coefficient was 1.24.

**Antagonism by Alosetron of 5-HT-Induced Currents in Myenteric Neurons is Not Surmountable.** Concentration-response curves were obtained for the peak current evoked by 5-HT (0.5–140 μM) in the absence or presence of alosetron (0.06 μM and 0.2 μM; Fig. 5). In all experiments, alosetron was applied for 20 s before the exposure of neurons to 5-HT. All of the responses were normalized to the peak amplitude of the inward current induced, in the absence of alosetron, by 140 μM 5-HT. Alosetron at both concentrations was found to significantly decrease the maximal response to 5-HT without at the same time producing any apparent shift in the IC₅₀. In the absence of alosetron, the IC₅₀ and Hill coefficient values were 38.08 and 1.21. In the presence of 0.06 μM alosetron, the IC₅₀ was 36.22 μM and the Hill coefficient was 1.22, whereas in the presence of 0.2 μM alosetron, the IC₅₀ was 35.34 μM and the Hill coefficient was 1.21. This observation suggests that alosetron noncompetitively inhibits the 5-HT-induced inward current in myenteric neurons.

**5-HT and ACh Evoke Similar Currents But Act on Different Receptors in the Same Myenteric Neurons.** The nicotinic agonist, nicotine (100 μM), was found to induce a fast, rapidly decaying inward current in about half of the
myenteric neurons that were sampled (Fig. 6A). This current qualitatively resembled that evoked by 5-HT; however, the current evoked by nicotine was probably entirely caused by the activation of neuronal ACh receptors (nAChR), because it was abolished by hexamethonium (100 μM). Alosetron (0.06 μM), moreover, did not affect the response of myenteric neurons to nicotine (101 ± 4% of control, n = 4). In contrast, neither the peak amplitude, nor the steady state of the inward current evoked by 5-HT (40 μM) were affected by hexamethonium (100 μM; P > .05; n = 4; Fig. 6B); nevertheless, in the same cells in which hexamethonium failed to influence the response to 5-HT, the 5-HT-evoked peak current was significantly inhibited by alosetron (0.06 μM; 54 ± 3% of control; n = 4).

Fig. 6. The response of myenteric neurons to nicotine is similar to that of 5-HT but is inhibited by hexamethonium and not by alosetron. A, nicotine (100 μM) evokes a fast inward current with a short latency and rapid decay. Hexamethonium (100 μM) reversibly blocks the fast response to nicotine, which is then resistant to inhibition by alosetron (0.06 μM). B, in contrast to the response to nicotine, the fast response to 5-HT (40 μM) is inhibited by alosetron (0.06 μM), but not by hexamethonium (100 μM).

Receptors Inhibited By Alosetron Are Accessible Only from the External Medium. Alosetron (0.06 μM) was added to the solution contained within the patch pipette to

Fig. 4. Alosetron inhibition of the inward current evoked by 5-HT is concentration-dependent. A, increasing concentrations of alosetron reversibly inhibit the response to 5-HT (40 μM); the magnitude of alosetron inhibition is greater at higher concentrations. B, the concentration-effect relationship for the inhibition of the response to 5-HT by alosetron. The percent inhibition of the response to 5-HT is plotted as a function of the log of the alosetron concentration.

Fig. 5. Antagonism by alosetron of the 5-HT-induced inward current is not surmountable. The concentration-effect relationship is illustrated for the 5-HT-induced inward current under control conditions and in the presence of 0.06 or 0.2 μM alosetron. Alosetron reduces the maximal effect of 5-HT, but does not significantly change the IC₅₀ (see text).

Fig. 6. The 5-HT receptors that are antagonized by alosetron are accessible only on the surface of the cell membrane. A, the inward current evoked by 5-HT (40 μM) is reversibly inhibited by alosetron (0.06 μM). B, the inclusion of alosetron (0.06 μM) in the patch pipette does not interfere with the inward current evoked by 5-HT (40 μM). In addition, the presence of alosetron in the patch pipette fails to inhibit the ability of alosetron (0.06 μM) in the external medium to antagonize the 5-HT-induced inward current. C, pooled data. The percent inhibition of the peak amplitude of the inward current evoked by 5-HT (40 μM) is not significantly different, no matter whether alosetron is or is not present within the patch pipette.
deliver the drug to the inside of the patched cells. Cells responded normally to 5-HT (40 μM), despite the inclusion of alosetron in the patch pipette (Fig. 7, compare A and B). In contrast, the external application of alosetron (0.06 μM) for 20 s before challenge with 5-HT (40 μM) inhibited the resulting inward current to the same degree, no matter whether the patch pipette did (Fig. 7A; mean inhibition = 54.8 ± 2.2%), or did not (Fig. 7B; mean inhibition = 52.0 ± 4.7%), contain alosetron (Fig. 7C). Alosetron, therefore, acts only on externally accessible sites to antagonize 5-HT3 receptors.

5-HT3- and 5-HT-Immunoreactive Neurons Are Present in Cultures of Cells from Newborn Guinea Pigs. The observation that 5-HT evoked an alosetron-sensitive inward current in virtually every neuron examined in cultures of cells from newborn guinea pig suggested that all of these cells expressed 5-HT3 receptors. Electrophysiological studies, however, examine only a small proportion of the neurons in each culture and, thus, it is possible that the cells that were patched represent a biased sample of the total neuronal population. Immunocytochemistry therefore was employed to investigate the distribution of neurons expressing 5-HT3 receptors in the cultures. Antibodies (95247) to a synthetic octadecapeptide were raised in rabbits. The ability of these antibodies to recognize 5-HT3 receptors was assessed by Western blotting of lysates prepared from cells transfected with cDNA encoding the rat 5-HT3 receptor. A dense band corresponding to a molecular weight of 80,000 and a smaller secondary band were seen on the Western blots (Fig. 8). Both bands were almost completely eliminated by preabsorption with the peptide used as the immunogen. Antibody 95247 pretreated with excess immunization peptide. Molecular weight markers are included in the center of the blot. Samples were subjected to electrophoresis on a Bio-Rad 4 to 15% ready gel followed by analysis with a Pierce super-signal detection system. The figure reveals a dense band of MW 80,000 and a small secondary band of slightly lower molecular weight. Both bands were completely eliminated by peptide immunosorption. No signal was detected in cells transfected with vector alone (not illustrated).

Fig. 8. Western blots of extracts of HEK 293 cells transfected with rat 5-HT3 receptors. Approximately 107 cells were lysed in 300 μl Laemmli buffer, and 2.5-, 5-, and 10-μl samples were applied to lanes 1, 2, and 3 from left to right; the left side of the plate was treated with antibody 95247 alone, whereas the right hand side was treated with antibody 95247 pretreated with excess immunization peptide. Molecular weight markers are included in the center of the blot. Samples were subjected to electrophoresis on a Bio-Rad 4 to 15% ready gel followed by analysis with a Pierce super-signal detection system. The figure reveals a dense band of MW 80,000 and a small secondary band of slightly lower molecular weight. Both bands were completely eliminated by peptide immunosorption. No signal was detected in cells transfected with vector alone (not illustrated).

The proportion of neurons in the adult guinea pig that are 5-HT-immunoreactive is quite low (2–3%; Costa et al., 1982); therefore, serotonergic neurons are far more common in cultures of newborn intestine than they are in the adult bowel in situ. The morphology of the cultured serotonergic neurons, moreover, appeared to be that of Dogiel type II neurons (Gershon et al., 1994), with smooth cell somata and multiple long thin processes. Mature serotonergic neurons have been reported to be Dogiel type II in shape (Costa et al., 1982). In the adult myenteric plexus, most Dogiel type II neurons are classified physiologically as AH/type 2, and contain calbindin immunoreactivity, which is considered a marker for these cells (Furness et al., 1990). Many of the cultured neurons were found to be calbindin-immunoreactive (Fig. 9D). The morphology of these cells seemed to be Dogiel type II, suggesting that at least this type of mature neuron has developed in newborn guinea pigs and is present in the cultures.

Fluoxetine Inhibits Responses to 5-HT. Because the immunocytochemical studies suggested that many of the cultured neurons were serotonergic and expressed 5-HT3 receptors, the cultured neurons might well be affected by the endogenous release of 5-HT. 5-HT is inactivated in the ENS by reuptake mediated by the 5-HT transporter (SERT; Gershon and Altman, 1971; Wade et al., 1996; Chen et al., 1998). The inhibition of enteric neuronal SERT has been found to potentiate serotonergic effects initially, but ultimately to inhibit them because of 5-HT accumulation and desensitization of receptors. The effects of fluoxetine on the inward current evoked by 5-HT were investigated to determine whether the 5-HT3 receptors expressed by the cultured neurons could be affected by the endogenous release of 5-HT. Fluoxetine (30 μM) inhibited the amplitude of the current induced by exposure to 40 μM 5-HT (Fig. 10). This effect of fluoxetine was time-dependent and reached a maximum within 4 min after the introduction of fluoxetine. Even at this high concentra-
ward current evoked by 5-HT is 5-HT3-mediated. Mature neurons in situ have not been studied with the patch clamp technique; however, studies with sharp intracellular microelectrodes have defined three types of responses to 5-HT. One is a 5-HT3-mediated fast depolarizing response (Mawe et al., 1986; Derkach et al., 1989; Galligan, 1995). This response is mediated by 5-HT3 receptors and is undoubtedly the voltage equivalent of the fast inward current observed in the present study. In addition to this fast response, however, a slow depolarizing response to 5-HT, mediated by 5-HT1P receptors (Mawe et al., 1986), and a hyperpolarizing response mediated by 5-HT1A receptors (Pan and Galligan, 1994), have also been observed. No membrane currents were evoked in the cultured newborn neurons that might correspond to the slow depolarizing or hyperpolarizing responses to 5-HT.

It is of interest that two components of the fast inward current evoked by 5-HT could be distinguished. The initial fast current decayed to a steady state within 15 s. Alosetron was most effective in antagonizing the initial component and did not inhibit the steady state current except in the higher range of concentrations at which it was tested (see, for example, Figs. 2, 4, and 7; compare with Fig. 10). Ondansetron was effective in blocking both the fast initial component and the steady-state current. Because more than one 5-HT3 receptor unit is now known to exist (Davies et al., 1999), it is possible that myenteric neurons express a variety of 5-HT3 receptors, composed of different subunits. Alosetron might selectively inhibit only one of these, which, if so, would constitute a therapeutic advantage for the compound.

Discussion

Myenteric neurons from the newborn guinea pig intestine were grown in dissociated cell culture, so that the patch clamp technique could be used to analyze their response to 5-HT and the effects of alosetron. A single type of response to 5-HT was encountered. All of the neurons responded with a fast inward current, which was antagonized by the 5-HT3 antagonists alosetron and ondansetron, but not by the nicotinic antagonist, hexamethonium. The response to 5-HT also was mimicked by the 5-HT3 agonist, 2-methyl-5-HT. This pharmacology, as well as properties of the response such as its short latency, brief peak, and rapid decay (Galligan, 1995), are consistent with the conclusion that the fast, inward current evoked by 5-HT is 5-HT3-mediated. Mature myenteric neurons in situ have not been studied with the patch clamp technique; however, studies with sharp intracellular microelectrodes have defined three types of responses to 5-HT. One is a 5-HT3-mediated fast depolarizing response (Mawe et al., 1986; Derkach et al., 1989; Galligan, 1995). This response is mediated by 5-HT3 receptors and is undoubtedly the voltage equivalent of the fast inward current observed in the present study. In addition to this fast response, however, a slow depolarizing response to 5-HT, mediated by 5-HT1P receptors (Mawe et al., 1986), and a hyperpolarizing response mediated by 5-HT1A receptors (Pan and Galligan, 1994), have also been observed. No membrane currents were evoked in the cultured newborn neurons that might correspond to the slow depolarizing or hyperpolarizing responses to 5-HT.

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It is not entirely clear why 5-HT responses other than those mediated by 5-HT3 receptors were not encountered in the present study. It is likely that the 5-HT1P receptor is present in the newborn guinea pig myenteric plexus, because these receptors are known to arise very early in development in the murine ENS (Branchek and Gershon, 1987) and the guinea pig is even more mature than the mouse at birth. Alternatively, the 5-HT1P (Wang et al., 1996; Pan et al., 1997) and 5-HT1A (Gale and Bunce, 1995) receptors are G-protein coupled, and, thus, require the participation of cytosolic proteins to change the conductance of ion channels in the plasma membrane. It is conceivable that critical elements of the cytosol are diluted out or buffered by the solution in the patch-clamp pipette and, thus, cannot be activated by G proteins. In contrast, the 5-HT3 receptor is a ligand-gated ion channel (Derakhch et al., 1989) and, thus, is not as readily influenced by changes in the cytosol of patched cells. In fact, the 5-HT3 receptor was found in the current study to be accessible to alosetron only on the outside of the plasma membrane. The failure of newborn guinea pig myenteric neurons to exhibit responses mediated by subtypes of 5-HT receptor other than 5-HT3 should not, therefore, be taken as evidence that these neurons do not express additional 5-HT receptor subtypes. It is clear, however, that these neurons do express the 5-HT3 receptor, that this receptor is expressed by virtually all of the neurons in culture, and that the myenteric neuronal 5-HT3 receptor is quite sensitive to alosetron.

Although the effects of alosetron were concentration-dependent and reversible, the antagonist behaved as if its action at the 5-HT3 receptor was noncompetitive. Thus, the effects of alosetron were nonsurmountable and administration of alosetron did not significantly change the IC50 for the action of 5-HT. Experiments that showed that alosetron was more effective when it was preapplied >20 s before 5-HT suggested that alosetron might bind more slowly to the 5-HT3 receptor than 5-HT. It is possible that the inability of 5-HT to displace alosetron bound to 5-HT3 receptors will be an advantage in the use of this compound in therapy. Alosetron also appeared to be 5-HT3-selective in that it failed to affect responses of myenteric neurons to nicotine. No attempt was made to determine whether alosetron interacts with other ligand-gated ion channels; nevertheless, alosetron is not a nicotinic antagonist and thus should not interfere with ganglionic transmission in the bowel. On the other hand, 5-HT3 receptors have been found to be expressed by the enteric processes of extrinsic sensory nerves and are important in mediating the transmission of afferent information from the bowel to the CNS (Hillsley and Grundy, 1998). The secretion of 5-HT from EC cells induced by luminal stimuli is thought to be the source of the 5-HT that stimulates both the extrinsic sensory nerves and the intrinsic primary afferent nerves that initiate peristaltic and secretory reflexes (Wade et al., 1996; Cooke et al., 1997; Chen et al., 1998). Alosetron should therefore be helpful in interrupting the transmission of 5-HT-induced noxious signals to the brain, as illustrated by the utility of 5-HT3 antagonists in treating the nausea associated with cancer chemotherapy (Gregory and Ettinger, 1998). Because the receptors on intrinsic primary afferent neurons are 5-HT1P (Kirschgessner et al., 1992; Kirschgessner et al., 1996; Cooke et al., 1997) and/or 5-HT4 (Foxx-Orenstein et al., 1995; Grider et al., 1996), 5-HT3 antagonists can be administered without interfering with the initiation of in-
5-HT$_3$ receptor immunoreactivity was abundant on cell bodies, distributed differently on neurons. In the cultured cells, 5-HT$_3$ receptors mediate responses occurring on processes far from cell bodies. 5-HT$_3$-mediated responses (Mawe et al., 1986) and relatively few are serotonergic (Costa et al., 1982). It is possible that the high concentration of serotonergic neurons in the newborn bowel reflects its immaturity. 5-HT-containing neurons are among the first to be born during ontogeny and all of the serotonergic neurons that will ever be generated in an animal are already present in the newborn gut (Pham et al., 1991). In contrast, other types of neuron continue to be born for several weeks of postnatal life. In the process, serotonergic neurons are diluted by the generation of other types of cell. It is possible that the difference in frequency of 5-HT$_3$-mediated responses between the cultured newborn neurons and mature myenteric neurons in situ can be explained similarly; however, it is also conceivable that the receptors are distributed differently on neurons. In the cultured cells, 5-HT$_3$ receptor immunoreactivity was abundant on cell bodies, as well as on varicose neurites. It is likely that 5-HT$_3$-mediated responses occurring on processes far from cell bodies would not be detected by intracellular recording obtained from cell bodies. 5-HT$_3$ receptors have been found on myenteric neurons in situ (Kirchgessner et al., 1998), but the fine structure of their distribution on cell surfaces remains to be established. In the current study, the abundance of serotonergic neurons might explain the ability of fluoxetine to inhibit 5-HT$_3$-mediated responses. Interference by fluoxetine with the uptake of 5-HT could have caused 5-HT to accumulate and/or linger at receptors to cause them to desensitize. The relatively long delay between the application of fluoxetine and the maximum inhibition is consistent with this explanation. Alternatively, fluoxetine has been found to inhibit 5-HT$_3$-mediated responses in other systems and might have been acting nonspecifically as a 5-HT$_3$ antagonist.

References


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