Mediation by Protein Kinases C and A of G_o -Linked Slow Responses of Enteric Neurons to 5-HT

Hui Pan,¹ Hoau-Yan Wang,² Eitan Friedman,² and Michael D. Gershon¹

¹Department of Anatomy and Cell Biology, Columbia University, College of Physicians and Surgeons, New York, New York 10032, and ²Department of Pharmacology, Allegheny University of the Health Sciences, MCP-Hahnemann School of Medicine, Philadelphia, Pennsylvania 19129

5-HT activates the peristaltic reflex and is the neurotransmitter of a subset of myenteric interneurons. Hyperpolarizing afterpotential (AH)/type 2 neurons respond to 5-HT with a long-lived depolarization that is caused by the inhibition of a Ca^{2+} -activated K^+ conductance (gK_{Ca}). This effect is mediated by a G-protein-coupled receptor, 5-HT_{1P}. 5-HT_{1P} agonists specifically activate G_{α_o} , the immunoreactivity of which was found to be highly abundant and membrane-associated in almost all enteric neurons. Responses of hyperpolarizing AH/type 2 neurons to 5-HT were inhibited by intracellular injection of GDP β S or anti- G_{α_o} Fab fragments but were potentiated and prolonged by intracellular GTP γ S. Responses to 5-HT were antagonized by pertussis toxin, downregulation of protein kinase C (PKC) and inhibitors of phosphatidylcholine phospholipase C (PC-PLC), PKC (including pseudosubstrate peptides, chelerythrine, and the α/β isoform-specific inhibitor G_6 6976), protein kinase

A (PKA), and adenylate cyclase. Responses to 5-HT were mimicked by activators of PKC, and 5-HT induced a concentration-dependent increase in the membrane-associated PKC activity in isolated myenteric ganglia. Immunocytochemical studies suggested that the most abundant isoforms of PKC in enteric neurons are α and δ . These data suggest that signal transduction of the 5-HT_{1P}-mediated slow response to 5-HT involves activation of PC-PLC by G_{α_o} to liberate diacylglycerol, which stimulates PKC (most likely α). PKC probably activates adenylate cyclase, which through cAMP, activates PKA. Activation of both PKA and PKC lead to closure of gK_{Ca} .

Key words: enteric nervous system; serotonin; 5-hydroxytryptamine; 5-HT_{1P} receptor; protein kinase C; protein kinase A; phosphatidylcholine phospholipase C; G_o ; G-proteins; signal transduction

Responses of the gut to exogenous 5-HT may be nerve- or muscle-mediated, excitatory, or inhibitory (Mawe and Gershon, 1993; Gershon et al., 1994; Wade et al., 1994). The complexity of these responses is attributable to the multiplicity of 5-HT receptor subtypes in the enteric nervous system (ENS) (Gershon, 1995) and musculature (Engel et al., 1984). At least five subtypes are present on nerve, 5-HT_{1A} (Galligan et al., 1988; Galligan and North, 1991), 5-HT_{1P} (Branchek et al., 1984; Mawe et al., 1986; Mawe and Gershon, 1993), 5-HT_{2B} (unpublished data), 5-HT₃ (Mawe et al., 1986; Derkach et al., 1989), and 5-HT₄ (Clarke et al., 1989; Craig and Clarke, 1990; Pan and Galligan, 1994) and two subtypes on muscle, 5-HT_{2A} and 5-HT_{2B} (Engel et al., 1984; Cohen et al., 1985; Foguet et al., 1992a,b; Kursar et al., 1992). Of these receptors, only 5-HT_{1P} has been shown to play roles in specific physiological responses. These include the mediation of slow EPSPs in myenteric neurons (Takaki et al., 1985a,b; Mawe et al., 1989; Wade et al., 1991, 1994; Galligan, 1995) and the initiation of the peristaltic reflex (Kirchgessner et al., 1992, 1997; Wade et al., 1996).

The response of myenteric neurons that is 5-HT_{1P}-mediated is known as the slow response (Wood, 1989; Gershon et al., 1994; Galligan, 1995). These responses are most common in hyperpolarizing afterpotential (AH)/type 2 neurons, a cell that is defined

by a characteristic Ca^{2+} -activated K^+ conductance (gK_{Ca}). This gK_{Ca} gives rise to a pronounced hyperpolarizing afterpotential (AH) and also contributes to the resting membrane potential (Galligan, 1995). 5-HT inhibits gK_{Ca} , resulting in a slowly developing but long-lived depolarization, associated with an increase in input resistance and inhibition of the AH (Wood and Mayer, 1979; Grafe et al., 1980; Johnson et al., 1980; Morita et al., 1982; Hirst et al., 1985; North and Tokimasa, 1987; Galligan, 1995). Slow responses to 5-HT are identical to slow EPSPs evoked in the same neurons (Wood and Mayer, 1979), and both are blocked specifically by *N*-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide (5-HTP-DP) (Takaki et al., 1985a; Mawe et al., 1986), renzapride (Mawe et al., 1989), and anti-idiotypic antibodies that recognize 5-HT receptors (Wade et al., 1994). The 5-HT_{1P} receptor binds 5-HT with high affinity ($K_D \approx 2$ –3 nM) (Branchek et al., 1984; Gershon et al., 1985; Mawe et al., 1986) and requires that potential ligands be hydroxylated indoles (Branchek et al., 1988; Mawe and Gershon, 1993).

5-HT receptor subtypes are defined by their primary structure, transductional properties, and operational characteristics (Humphrey et al., 1993). Although the transductional properties of the 5-HT_{1P} receptor have not previously been fully characterized, following three observations have led to the suggestion that this receptor is a member of the heptahelical G-protein-coupled receptor family: (1) the binding of ³H-5-HT by the receptor is antagonized by GTP γ S (Gershon et al., 1991; Fiorica-Howells et al., 1993); (2) intracellular injection of GTP γ S potentiates and prolongs slow responses to 5-HT; and (3) similar injections of GDP β S inhibit slow responses (Wang et al., 1996). The G-protein

Received July 3, 1996; revised Oct. 21, 1996; accepted Nov. 22, 1996.

This work was supported by National Institutes of Health Grant NS12969. H.P. is a fellow of the Pharmaceutical Manufacturers Association Foundation.

Correspondence should be addressed to Dr. Hui Pan, Department of Anatomy and Cell Biology, Columbia University (P&S 12-513), 630 West 168th Street, New York, NY 10032.

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to which the 5-HT_{1P} receptor is coupled, moreover, is likely to be G_o, because G_o is activated specifically when enteric neuronal membranes are incubated with 5-HT or 5-hydroxyindalpine (Wang et al., 1996), a 5-HT_{1P} agonist (Branchek et al., 1988). Activation of G_o by either 5-HT or 5-hydroxyindalpine is antagonized by 5-HTP-DP (Wang et al., 1996). The current experiments were carried out to identify the signal transduction pathway responsible for 5-HT_{1P}-mediated slow responses.

MATERIALS AND METHODS

Tissue preparation. Male guinea pigs weighing 250–350 gm were stunned and exsanguinated. A segment of ileum was excised 10–20 cm proximal to the ileocecal junction and placed in oxygenated (95% O₂/5% CO₂) Krebs' solution of the following composition (in mM): NaCl 121.3, KCl 5.95, CaCl₂ 2.5, NaHCO₃ 14.3, NaH₂PO₄ 1.34, MgCl₂ 1.2, and glucose 11.5. The Krebs' solution contained nifedipine and scopolamine (1 μM each) to block longitudinal muscle contractions while intracellular recordings were obtained. A 1.5 cm segment of ileum was cut open along the mesenteric border and pinned out flat (mucosal surface up) in a Petri dish lined with a silicone elastomer. Preparations of longitudinal muscle with adherent myenteric plexus (LMMP) were dissected by removing the mucosa, submucosa, and circular muscle with a fine forceps and scissors under microscopic control. A 5 mm² LMMP segment was transferred to a small recording chamber (volume, 0.5 ml) that was coated with a silicone elastomer. The LMMP was stretched lightly and pinned with small stainless steel pins to the silicone coating of the bottom of the chamber. Preparations were superfused (3.5 ml/min; 36°C) with Krebs' solution oxygenated with a mixture of 95% O₂/5% CO₂.

Intracellular recording. Individual myenteric ganglia were visualized at a magnification of 6.3×. Intracellular recordings were obtained from neurons using glass microelectrodes filled with 2 M KCl (tip resistance, 90–160 MΩ). An amplifier with an active bridge circuit (Axoclamp 2A, Axon Instruments, Foster City, CA) was used to record the transmembrane potential difference and to inject current via the recording electrode.

Drugs, chemicals, and antibodies. 5-HT (Sigma Chemical, St. Louis, MO), forskolin (Research Biochemicals International, Natick, MA), histamine (Research Biochemical International), and the PKC activators phorbol 12,13-dibutyrate (PDBu, Research Biochemical International), (–)-7-octylindolactam V (LC Laboratories, Woburn, MA), and 1-oleoyl-2-acetyl-rac-glycerol (OAG, Sigma) were applied to neurons by ejection with pressure from a micropipette (filled with a 1.0 mM solution) or by addition to the fluid superfusing the preparations. In six cells used to evaluate the reproducibility of responses, the ratio of the amplitude of a second response to 5-HT to that of its predecessor was 0.97 ± 0.05 ($p = 0.5669$). The interval between trials of 5-HT was at least 5 min to avoid desensitization of 5-HT receptors. In all experiments, the effect of drugs on responses to 5-HT, histamine, or forskolin was not investigated until after reproducible responses to the agonist were obtained. Membrane-permeable potential antagonists of steps in signal transduction were added to the superfusing solutions. These included the phosphatidylcholine-phospholipase C (PC-PLC) inhibitor D609 (Kamiya Biomedical, Thousand Oaks, CA); the adenylate cyclase inhibitor 2',5'-dideoxyadenosine (DDA, Biomol Research Laboratory, Plymouth Meeting, PA); and the PKC inhibitors chelerythrine (LC Laboratories), staurosporine (Research Biochemical International), K252a (Kamiya), Gö 6976, an indolcabazole that selectively inhibits the PKC isozymes, α, β1, β2, and γ, but not δ, ε, or ζ (Martiny-Baron et al., 1993) (Calbiochem, La Jolla, CA); and a myristoylated pseudosubstrate sequence from PKC α and β, myr-FARKGALRQ (Biomol). Compounds that do not readily cross cell membranes were microinjected into neurons via the recording microelectrode. These included GTPγS (Sigma), GDPβS (Sigma), Fab fragments of antibodies to Gα_s (preparation described below), the PKC α, β pseudosubstrate, RFARKGALRQKNV (PKC_(19–31)) (Kemp et al., 1994) (LC Labs, 1.0 mM), and the protein kinase A (PKA) inhibitor Rp-adenosine 3',5'-cyclic monophosphothiolate triethylamine cAMPS (Rp-cAMPS, 70 mM, Research Biochemical International). Because pertussis toxin (PTx, Research Biochemical International) penetrates membranes slowly, preparations were incubated for 6 hr ± PTx before determining the response to 5-HT.

Antibodies to Gα_s were obtained from Upstate Biotechnology, Lake Placid, NY. Fab fragments (Mage and Lamoyi, 1987) were prepared by digestion with papain (in an enzyme-to-protein ratio of 1:100) in 500 μl

of 20 mM sodium phosphate buffer, pH 7.4, that contained 20 μg of antibody to Gα_s, dithiothreitol (1.0 mM), and EDTA (2.0 mM). Digestion was allowed to proceed at 37°C for 4–5 hr. After incubation, the reaction mixture was cooled to room temperature and transferred to dialysis tubing and dialyzed against PBS overnight. Protein A-Sepharose 4B beads were then added to the dialyzed mixture to bind Fc fragments of the antibody. The beads were then removed by centrifugation to provide a final clear solution containing Fab fragments that could be loaded into a micropipette.

Immunocytochemistry. Laminar preparations of LMMP or the dissected submucosa (containing the submucosal plexus) were fixed for 3 hr with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer, pH 7.4, at room temperature, and washed three times with PBS. To locate proteins in the tissue by immunocytochemistry, free-floating LMMP or submucosal preparations were exposed to PBS containing 1.0% Triton X-100 and 10% horse serum for 30 min to permeabilize the tissue and reduce background staining. Gα_s immunoreactivity was demonstrated with rabbit polyclonal antibodies (Upstate Biotechnology) at a concentration of 1.0 μg/ml. Preparations were incubated overnight in a humidified chamber at room temperature. Bound antibody was visualized by incubating tissues for 2 hr with biotinylated affinity-purified goat anti-rabbit IgG secondary antibodies (diluted 1:400; Kirkegaard & Perry, Gaithersburg, MD) at room temperature and then for 2 hr with avidin-FITC (diluted 1:200; Vector Laboratories, Burlingame, CA). Calbindin immunoreactivity, which marks 70–80% of neurons classified anatomically as Dogiel type II and physiologically as AH/type 2 (Pompolo and Furness, 1988; Furness et al., 1990), was located simultaneously in the same sections used to visualize Gα_s immunoreactivity by double-label immunocytochemistry. To demonstrate calbindin immunoreactivity, preparations were incubated overnight at room temperature with mouse monoclonal antibodies to calbindin (diluted 1:100; Sigma), and bound primary antibodies were located with affinity-purified goat anti-mouse secondary antibodies labeled with tetramethylrhodamine isothiocyanate (TRITC, diluted 1:100; Kirkegaard and Perry). Double-label immunocytochemistry was made possible, because primary antibodies were raised in different species and immunoreactivity was visualized with species-specific secondary antibodies. Specific antibodies to the following isozymes of PKC, α, β1, β2, γ, δ, ε, ζ, η, and θ (Jiang et al., 1994), were donated by Dr. Todd Sacktor (State University of New York, Downstate Medical Center). These were all polyclonal rabbit antibodies and thus were visualized, as described above, with biotinylated goat anti-rabbit secondary antibodies, and avidin-FITC. Immunostained tissues were examined with a Leica DMRB Microscope equipped for vertical fluorescence microscopy. FITC fluorescence was detected using a Leica "L-4" filter cube (exciting filter band pass, 470–490 nm; dichroic mirror reflection short pass, 510 nm; suppression filter bandwidth, 520 nm). TRITC fluorescence was detected using a Leica "N-2.1" filter cube (exciting filter band pass, 546/14 nm; dichroic mirror reflection short pass, 580 nm; edge wavelength, 580 nm). There was no cross-detection between the FITC- and TRITC-selective dichroic mirror-filter cubes.

Isolation of myenteric ganglia. Ganglia were isolated from dissected LMMP preparations by a modification of the method described previously (Fiorica-Howells et al., 1993). This procedure takes advantage of the absence of collagen from the interior of the myenteric plexus and the role of glia in providing support for neurons (Gershon et al., 1994). When the LMMP is exposed to collagenase, therefore, the ganglia of the myenteric plexus remain largely intact, whereas the non-neuronal components of the preparation dissociate and form a suspension of single cells (Yau et al., 1989). When the resulting suspension of dissociated cells and intact ganglia is filtered through a wide-pore (8.0 μm) filter (Nucleopore), the individual cells pass through, whereas the ganglia are trapped on the filter (Fiorica-Howells et al., 1993). Studies using desmin immunoreactivity as a muscle marker and neuron-specific enolase as a neuronal marker have verified that the filtered preparations are highly enriched with ganglia and not contaminated by muscle.

For immunoblotting, the ganglia were scraped from the filters and homogenized in iced buffer [50 mM Tris, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 25 μg/ml each of the peptidase inhibitors leupeptin and aprotinin (Sigma), 2 mM EDTA, and 1.0 mM EGTA]. The homogenate was spun for 10 min in a microcentrifuge, and the proteins in the resulting supernatant were separated by electrophoresis through 10% PAGE. The proteins were then blotted onto nitrocellulose membranes and probed with G-protein-purified rabbit polyclonal antibodies selective for the α, β, γ, δ, ε, and ζ isozymes of PKC. Immunoreactive bands were demonstrated with goat anti-rabbit secondary antibodies conjugated to horseradish peroxi-

dase. Horseradish peroxidase activity was visualized with TMB peroxidase substrate (Kirkegaard & Perry).

Protein kinase C-mediated protein phosphorylation and translocation. Isolated myenteric ganglia were incubated for 10 min at 37°C in Krebs' solution with 1.0 μ M 5-HT, 10.0 μ M 5-HT, or 1.0 μ M PDBu. Reactions were terminated by addition of 0.5 mM EGTA in Ca^{2+} -free Krebs' solution. Preparation of tissue fractions and assessment of PKC activity was performed as described previously (Friedman and Wang, 1989). All subsequent procedures were carried out at 4°C, unless otherwise indicated. Tissues were harvested and homogenized in 10 vol of buffer A (20.0 mM Tris-HCl, pH 7.5, 0.32 M sucrose, 2.0 mM EDTA, 0.5 mM EGTA, 50.0 μ g/ml leupeptin, 0.1% 2-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride), and the homogenate was centrifuged at $800 \times g$ for 10 min. The supernatant obtained was sonicated (Kontes Micro Cell Disrupter) and centrifuged at $25,000 \times g$ for 15 min. The resultant supernatant was removed, diluted to 1.0 ml with buffer A, chromatographed on diethylaminoethyl cellulose (DE52, Whatman, Maidstone, UK) anion exchange columns and used as the cytosolic fraction. The pellet was resuspended in 1.0 ml buffer A with 0.2% Nonidet P-40 and solubilized on ice for 1 hr. The sample was centrifuged at $25,000 \times g$ for 15 min. The resultant supernatant was chromatographed on DE52 columns, and the eluate was used as the membrane extract. Samples were applied to 1.0 ml DE52 columns equilibrated in buffer A, and the columns were washed with 5.0 ml of buffer A followed by 0.5 ml of 20 mM NaCl in buffer A. Enzyme was eluted with 0.75 ml of 200 mM NaCl in buffer A, and the eluate was used immediately for assessing PKC activity.

The standard assay mixture (250 μ l) containing 24.0 mM Tris-HCl, pH 7.5, 20.0 mM NaCl, 0.1 mM EGTA, 0.4 mM EDTA, 0.03% 2-mercaptoethanol, 60 μ g/ml leupeptin, 0.04 mM phenylmethylsulfonyl fluoride, 0.25 mg/ml histone type III-s (Sigma), 1.2 mM CaCl_2 , 20 μ g/ml phosphatidyl-L-serine, 8.0 mM phorbol-12-myristate,13-acetate, 10.0 mM $\text{Mg}(\text{CH}_3\text{CO}_2)_2$, and 0.03 mM [^{32}P]ATP (500,000 cpm, DuPont, Boston, MA) was preincubated at 30°C for 5 min, and the reaction was initiated by the addition of eluted protein. After 1 min, the reaction was terminated by transferring 125 μ l onto a 2×4 cm phosphocellulose (Whatman P81) strip that was subsequently immersed in 75.0 mM phosphoric acid (10.0 ml per strip). The strips were washed three times (2 min per wash) in fresh phosphoric acid and air dried. The strips were then placed in scintillation fluid, and radioactivity was determined by liquid scintillation spectrometry (LKB RACKBETA). PKC activity was defined as the phosphorylation that occurred in the presence of phosphatidyl-L-serine and phorbol-12-myristate,13-acetate and expressed as pmol ^{32}P -incorporated per unit protein of column eluate. Protein was determined by the method of Lowry et al. (Lowry et al., 1951).

RESULTS

5-HT evokes a uniphasic slow depolarization in AH/type 2 neurons when receptors for 5-HT receptor subtypes other than 5-HT_{1P} are antagonized

Recordings were made from cells that were classified as AH/type 2 neurons. Criteria used in classification included (1) the presence of an AH, and (2) a Ca^{2+} shoulder on the falling phase of the action potential (Gershon et al., 1994; Wood, 1994). A total of 235 AH/type 2 neurons were studied with a mean resting membrane potential of 73 ± 1 mV and an input resistance of 108 ± 5 M Ω . Except where otherwise stated, the 5-HT_{1A} antagonist NAN-190 (0.3 μ M) and the 5-HT_{3/4} dual antagonist tropisetron (1.0 μ M) were added to the superfusing medium so that the other subtypes of 5-HT receptor to which these cells are known to respond, would not interfere with 5-HT_{1P}-mediated responses. Tetrodotoxin (1.0 μ M) was also present to confine recordings to postsynaptic events in the impaled neurons. In a series of 81 AH/type 2 cells studied under these conditions, 71 responded to the microejection of 5-HT (Fig. 1). The response was uniphasic under these conditions and consisted of a prolonged (104 ± 6 sec) membrane depolarization (16 ± 1 mV) associated with an increase in input resistance (mean increase = $102 \pm 8\%$). No response to 5-HT was observed in 10 of the 81 neurons; such cells were not studied further. Superfused 5-HT (1.0 μ M) reduced the amplitude of the AH from 14 ± 1 mV ($n = 14$) in control

Figure 1. 5-HT induces a slow depolarization in AH/type 2 cells during which membrane conductance decreases and the AH is inhibited. *A*, In the presence of the 5-HT_{1A} antagonist NAN-190 and the 5-HT_{3/4} antagonist tropisetron, the response to 5-HT is a uniphasic depolarization associated with an increase in input resistance. The top trace depicts the membrane potential, and the bottom trace indicates current injected through the recording pipette. The downward deflections in the top trace represent the electrotonic responses to the injections of hyperpolarizing current pulses. *B*, At a faster sweep speed, the pronounced AH (top trace) can be seen after the action potential in the control record. The addition of 5-HT (1.0 μ M) to the superfusing medium blocks the AH. The bottom trace depicts depolarizing current injected through the recording pipette to evoke action potentials.

preparations to 7 ± 3 mV ($n = 5$; $p < 0.02$). The duration of the AH was also reduced by 5-HT from 13 ± 2 sec in control preparations to 6 ± 2 sec ($p < 0.05$).

The slow response to 5-HT is G_o protein-dependent

The hypothesis that the slow response of AH/type 2 neurons to 5-HT is mediated by a G_o protein was tested. Initial studies were carried out to determine whether the previous report (Wang et al., 1996) that slow responses to 5-HT are potentiated by the intracellular injection of GTP γ S and inhibited by GDP β S could be confirmed in preparations in which responses to subtypes of 5-HT receptor other than 5-HT_{1P} were blocked. Subsequent experiments evaluated the sensitivity of the slow response to inhibition by PTx, which would be expected to antagonize a G_o-mediated response (Casey and Gilman, 1988). Finally, the effects of the intracellular injection of Fab fragments of antibodies to G α_o into AH/type 2 neurons were ascertained. To study the effects of the guanine nucleotides on responses to 5-HT, recordings were made with beveled electrodes loaded with GTP γ S or GDP β S. Control recordings were obtained first and, after stable responses to 5-HT were observed, the cells were injected with the guanine nucleotide by passing a negative DC current and simultaneously applying pressure to the recording electrode. The amplitude of control responses to 5-HT and that of responses to 5-HT recorded 10–15 min after the injection of GTP γ S or GDP β S were measured and their ratios determined (Fig. 2). Intracellular injection of GTP γ S more than doubled the amplitude of the response to 5-HT ($p <$

Figure 2. The slow response to 5-HT is inhibited by the intracellular injection of GDPβS and potentiated by the intracellular injection of GTPγS.

0.05; $n = 6$); furthermore, after the intracellular injection of GTPγS, the depolarization induced by the microejection of 5-HT was greatly prolonged and the membrane potential did not fully return to the resting level. In contrast, intracellular injection of GDPβS reduced the amplitude of the response to 5-HT to ~35% of that of the control (Fig. 2) ($p < 0.001$; $n = 6$).

To study the effects of PTx, we initially tried to obtain control responses to 5-HT and then to maintain impalements long enough, after the addition of PTx (1.0 μg/ml) to the medium, to determine whether the slow response to 5-HT was affected by the toxin. Unfortunately, PTx is very slow to take effect when it is applied extracellularly. As a result, very long impalements (4–6 hr) were necessary to investigate the action of PTx. The membrane properties of AH/type 2 cells, even in control experiments, were found to be unstable during such long impalements; consequently, although responses to 5-HT were found eventually be inhibited in cells exposed to PTx (Wang et al., 1996), it was difficult to be certain that the inhibition was a specific effect of PTx. To circumvent this problem and to maximize the effectiveness of exposure to PTx, preparations were incubated with PTx for 6 hr before recordings were obtained. Controls were incubated similarly, but PTx was not present in the medium. As an additional control, to evaluate possible nonspecific effects of incubation with PTx, the responses of AH/type 2 neurons to forskolin were studied in the same cells used to investigate responses to 5-HT. Forskolin, which activates adenylate cyclase, evokes a slow depolarization similar to that elicited by 5-HT, but the response to forskolin (Nemeth et al., 1986; Bertrand and Galligan, 1995) would not be expected to be PTx-sensitive. The ratios of the amplitude of the 5-HT response to that of forskolin were computed for control cells and cells exposed to PTx. After incubation ± PTx, therefore, AH/type 2 cells were identified and their responses to 5-HT and forskolin determined (Fig. 3). The amplitude of the response to 5-HT was significantly lower ($p < 0.0001$) in cells incubated with PTx (2 ± 1 mV; $n = 8$) than in controls (14 ± 1 mV; $n = 35$). The amplitude of the response to forskolin was also somewhat lower in cells incubated with PTx (7 ± 1 mV; $n = 8$) than in controls (15 ± 1 mV; $n = 35$). The ratio of the amplitudes of responses to 5-HT to those of forskolin (Fig. 3), however, was 1.05 ± 0.09 in control cells and 0.34 ± 0.07 after

Figure 3. Slow responses to 5-HT are inhibited by pertussis toxin (PTx). Preparations were superfused with PTx (1.0 μg/ml) for 6 hr. Control preparations were superfused for an equivalent period of time but in the absence of PTx. To control for nonspecific effects of prolonged superfusion, responses of AH/type 2 cells to 5-HT were compared with the similar responses of the same cells to forskolin in the presence or absence of PTx. The graph shows the ratio of the 5-HT to the forskolin response for control and PTx-treated preparations.

exposure to PTx ($p < 0.0001$). The response of AH/type 2 neurons to 5-HT thus was much more strongly affected by exposure to PTx than were the responses of the same cells to forskolin. These observations indicate that the slow response to 5-HT is PTx-sensitive.

Preliminary studies revealed that AH/type 2 neurons could not consistently be injected through the recording micropipette with whole antibodies to G_{α_o} . Preparations in which injections were attempted were fixed and later immunostained with biotinylated secondary antibodies and avidin-FITC to determine whether antibodies to G_{α_o} had actually entered cells. In practice, the pipettes filled with whole antibodies tended to clog, leading to increases in electrode tip resistance, and antibodies only rarely entered impaled cells in quantities adequate for immunocytochemical detection. Fab fragments were therefore prepared and used for intracellular injection. In contrast to whole antibodies, the Fab fragments consistently entered injected cells. An initial control was to inject cells with Fab fragments of anti-Gas. Although these injections did not inhibit slow responses to 5-HT, the effects of anti- G_{α_o} and anti-Gas could not be compared in the same cells. To determine whether injected anti- G_{α_o} exerts nonspecific effects, therefore, responses to 5-HT in anti- G_{α_o} -injected neurons were studied in the absence of tropisetron, so that the 5-HT₃-mediated fast response to 5-HT (Mawe et al., 1986; Derkach et al., 1989) could be measured simultaneously with the 5-HT_{1P}-mediated slow response and used as a control. Before the injection of anti- G_{α_o} Fab fragments, therefore, the response to 5-HT was biphasic instead of monophasic. A rapidly developing transient depolarization (the fast response, which is known to be 5-HT₃-mediated) (Mawe et al., 1986; Wade et al., 1991) preceded the much more slowly developing and longer-lived 5-HT_{1P}-mediated slow response. A second control was to determine the effect of injection of anti- G_{α_o} Fab fragments on responses of AH/type 2 neurons to histamine. Histamine evokes a slow depolarization similar to that elicited by 5-HT; however, the response to histamine is thought to be attributable to the activation of adenylate cyclase (Wood, 1994) and, thus, is probably coupled to a G_s protein. Intracellular injection of anti- G_{α_o} Fab fragments

was found to substantially inhibit the slow response to 5-HT, but these injections had little or no effect on fast responses to 5-HT of the same neurons (Fig. 4*A,B*) and did not significantly affect slow responses of these cells to histamine (Fig. 4*A, ii*). The amplitude of the slow response to 5-HT was reduced by injection of anti- G_{α_o} Fab fragments from 13 ± 1 mV to 7 ± 1 mV ($n = 23$; $p < 0.001$). In contrast, the mean amplitude of the fast response in the same cells was 14.0 ± 1.8 mV before injection and 14.5 ± 2.1 mV ($n = 16$; p is not significant). These data support the hypothesis that the 5-HT_{1P} receptor is coupled to a G_o protein.

Enteric neurons express G_{α_o} immunoreactivity

The presence of G_{α_o} in enteric neurons and their processes was investigated by immunocytochemistry. G_{α_o} immunoreactivity was observed to be widely distributed in neurons of both the submucosal (Fig. 5*A,B*) and myenteric (Fig. 5*C*) plexuses. The immunoreactivity was strikingly marginal in these cells and appeared to be concentrated in their plasma membranes (Fig. 5*B,C*). The cell bodies of neurons thus appeared as negative images outlined by G_{α_o} -immunoreactive membranes. G_{α_o} immunoreactivity was also abundant in the processes of these neurons and thus was seen in interganglionic connectives, as well as in ganglia. Calbindin immunoreactivity, which has been used as a marker for AH/type 2 neurons and is found in the majority of these cells (Pompolo and Furness, 1988; Furness et al., 1990) was demonstrated in the same preparations to determine whether there was a coincident cellular distribution of the two markers (Fig. 5*C,D*). All calbindin-immunoreactive neurons (Fig. 5*D*) were ringed by G_{α_o} -immunoreactive membranes (Fig. 5*C*); however, G_{α_o} immunoreactivity was more widely distributed than that of calbindin and also ringed cells that did not contain calbindin. G_{α_o} immunoreactivity was not observed in the intestinal musculature, but it was abundant in the ganglia and interganglionic connectives of the submucosal plexus (Fig. 5*A*). In the submucosa, G_{α_o} immunoreactivity was not confined to neural tissue but was also found in the smooth muscle of arteries (Fig. 5*A*). Within submucosal ganglia, the localization of G_{α_o} immunoreactivity was similar to that seen in the myenteric plexus. Again, G_{α_o} immunoreactivity was concentrated around neurons, which appeared as negative images (Fig. 5*B*). Because submucosal neurons were as G_{α_o} immunoreactive as myenteric neurons, we evaluated the response of submucosal AH neurons to 5-HT. In the presence of tetrodotoxin ($1.0 \mu\text{M}$), tropisetron ($1.0 \mu\text{M}$), and NAN 190 ($0.3 \mu\text{M}$), 5-HT evoked a slow response that was very similar to that observed in AH/type 2 neurons of the myenteric plexus (Fig. 5*E*).

Activators of PKC mimic the slow response to 5-HT

Because the slow response to 5-HT was found to be associated with the activation of a G_o protein, it seems unlikely that the response is directly linked to adenylate cyclase, which is normally activated by G_s . The hypothesis that the slow response to 5-HT is PKC-dependent was therefore tested. Each of three different activators of PKC mimicked the slow response to 5-HT. When applied by microejection onto the surface of AH/type 2 neurons, PDBu induced a slow depolarization (Fig. 6*A, record 1*) with a mean amplitude of 16 ± 2 mV ($n = 8$) associated with an increase in input resistance of $87 \pm 15\%$. The duration of the slow depolarization was extremely long (>10 min). A similar, but even longer-lasting, response was evoked by adding PDBu ($1.0 \mu\text{M}$) to the superfusing medium (Fig. 6*A, record 2*). Almost identical responses were elicited by OAG or (–)-7-octylindolactam V (data not shown). In addition to the slow depolarization evoked by

Figure 4. Intracellular injection of anti- G_{α_o} Fab fragments inhibits slow responses to 5-HT. *A, i*, In the absence of tropisetron, 5-HT evokes a biphasic response (control), consisting of a fast 5-HT₃-mediated depolarization, followed by the 5-HT_{1P}-mediated slow depolarization. The injection of anti- G_{α_o} Fab fragments antagonizes the slow but not the fast response to 5-HT. *ii*, Control responses of the same neuron to 5-HT and histamine are shown. The control response to 5-HT is again biphasic; the fast response is very small. An action potential is seen during the depolarization. Injection of anti- G_{α_o} Fab fragments inhibits the slow response to 5-HT far more than that to histamine. The mean ratio of the amplitude of slow responses to histamine after injection of anti- G_{α_o} Fab fragments to control responses obtained in the same cells (1.3 ± 0.1) was not significantly different from 1 ($n = 6$; $p = 0.0804$). *B*, The effects of intracellular injection of anti- G_{α_o} Fab fragments on fast and slow responses of AH/type 2 neurons to 5-HT are compared.

Figure 5. $G\alpha_o$ immunoreactivity is present in most submucosal and myenteric neurons. *A*, Both ganglia and interganglionic connectives of the submucosal plexus contain $G\alpha_o$ immunoreactivity. Connective tissue cells of the submucosa do not exhibit $G\alpha_o$ immunoreactivity; however, the smooth muscle of submucosal arteries (arrow) and associated paravascular nerve fibers are also $G\alpha_o$ immunoreactive. *B*, At higher magnification, the $G\alpha_o$ immunoreactivity within a submucosal ganglion can be seen to outline the membranes of component neurons. *C*, The $G\alpha_o$ immunoreactivity of the neurons of a myenteric ganglion (visualized with FITC) is also marginal and outlines neuronal plasma membranes. *D*, The same section as shown in *C*, but the filters are set to reveal calbindin immunoreactivity, which is visualized with TRITC. Calbindin-immunoreactive neurons are ringed by $G\alpha_o$ immunoreactivity; however, so too are most of the neurons of the ganglion. Scale bars, 30 μ m. *E*, An intracellular record obtained from a submucosal AH neuron showing a typical 5-HT-evoked slow response associated with an increase in input resistance.

microejection of PDBu, the AH, which is inhibited by superfused 5-HT (Figs. 1*B*, 6*B*) was also inhibited by superfusion of PDBu (1.0 μ M) or OAG (100 μ M) (Fig. 6*A*, record 3, *B*).

PKC antagonists inhibit slow responses of AH/type 2 neurons to 5-HT

The observation that activators of PKC mimic the slow response of AH/type 2 neurons to 5-HT suggests that PKC might be involved in the transduction of responses mediated by the 5-HT_{1P} receptor. To determine whether PKC actually does play such a role, the ability of PKC inhibitors to antagonize slow responses to 5-HT was determined. Superfusion of stau-

rosporine (1.0 μ M) (data not illustrated) and K-252a (3.0–10 μ M) (Fig. 7*B*, *C*), both of which inhibit PKC, antagonized slow responses of AH/type 2 neurons to 5-HT. These compounds, however, do not discriminate sufficiently well between PKC and other kinases to enable their antagonism of slow responses to 5-HT to be attributed specifically to inhibition of PKC. Studies were thus carried out with more selective PKC antagonists. These included the pseudosubstrate peptide PKC_[19–31] and the membrane permeable PKC inhibitors chelerythrine (3.0 μ M), G₆ 6976 (10.0 μ M), and myr-FARKGALRQ (40 μ M) (Martiny-Baron et al., 1993). Because PKC_[19–31] does not cross cell

Figure 6. Activators of PKC mimic slow responses to 5-HT. *A*, The phorbol ester PDBu evokes a slow depolarization in AH/type 2 neurons associated with an increase in input resistance, no matter whether it is applied by microejection (1) from a pipette positioned close to the impaled neuron or by superfusion (2) in the ambient medium. Superfusion of PDBu also inhibits the AH (3) that follows the action potential in these cells. Note that although the effects of PDBu are qualitatively similar to those of 5-HT, the response to PDBu is much longer lasting. *B*, The effects of 5-HT (1.0 μ M), OAG (100 μ M), and PDBu (1.0 μ M) on the amplitude of the AH in AH/type 2 neurons are compared. All three compounds inhibit the AH; PDBu is the most potent.

membranes, it was injected into AH/type 2 neurons through the recording micropipette. Superfusion with chelerythrine (Fig. 7*A–C*), G_{α} 6976 (Fig. 7*A*), or myr-FARKGALRQ (Fig. 7*A*) and injection of PKC_[19–31] (Fig. 7*A–C*) all inhibited slow responses to 5-HT. For these experiments, control responses to 5-HT were obtained and, when these were stable in amplitude, PKC_[19–31] was injected into cells or the membrane permeable antagonists were added to the superfusing medium. The ratio of the amplitude of the response to 5-HT after the injection of

PKC_[19–31] or superfusion of chelerythrine to that of the immediate preceding control response was determined (Fig. 7*C*). Each cell thus served as its own control. A ratio of 1.0 would have been obtained if PKC_[19–31] or chelerythrine had failed to antagonize slow responses to 5-HT. In fact, the ratios obtained for both of the specific PKC inhibitors were significantly less than 1.0 [$p < 0.001$ for PKC_[19–31] ($n = 12$); $p < 0.01$ for chelerythrine ($n = 8$)]. Similarly, a ratio of less than 1.0 was also obtained for the less PKC-specific K252a [$p < 0.05$ ($n = 3$)] (Fig. 7*C*). The membrane-permeable myristolated peptide myr-FARKGALRQ also reduced the amplitude of the slow response to 5-HT from 22.0 ± 2.0 mV to 7.0 ± 2.0 mV; in contrast to the amplitude of slow responses, the amplitude of fast responses to 5-HT before addition of myr-FARKGALRQ, 12.5 ± 6.5 mV, was not significantly different from that obtained after exposure to the peptide, 14.5 ± 7.5 mV ($n = 3$).

Downregulation of PKC antagonizes slow responses to 5-HT

The observations that six different inhibitors of PKC each antagonized the slow response of AH/type 2 neurons to 5-HT supports the idea that PKC plays a critical role in the transduction of responses mediated by the 5-HT_{1P} receptor. If so, then the downregulation of PKC would be expected to interfere with slow responses to 5-HT. To downregulate PKC, dissected preparations of LMMP were cultured overnight in the presence of PDBu (10 μ M). Control preparations were cultured similarly, but in the absence of PDBu. The amplitude of slow responses of AH/type 2 neurons to 5-HT in the preparations exposed overnight to PDBu was compared with that of slow responses to 5-HT in the controls. To test the possibility that a decrease in the amplitude of slow responses to 5-HT in cells chronically exposed to PDBu might have been attributable to nonspecific effects, responses to forskolin were also obtained from each of the cells used for testing responses to 5-HT. Responses to forskolin, which stimulates adenylate cyclase, would not be expected to be dependent on PKC; moreover, the slow depolarizing response of AH/type 2 neurons to forskolin (amplitude, 16 ± 1.0 mV; duration, 165 ± 9.0 sec, increase in input resistance of $107 \pm 11.8\%$; $n = 46$) is very similar to the slow response of these cells to 5-HT. The ratio of the amplitudes of the slow responses of each neuron to 5-HT to that of the same cells to forskolin was determined. In preparations that were not exposed to PDBu, the amplitudes of slow responses to 5-HT and forskolin were about equal (Fig. 8*A*) ($n = 32$); however, in preparations that were treated with PDBu, the amplitudes of the responses of AH/type 2 neurons to 5-HT were quite small and considerably less than those to forskolin (Fig. 8*B*) ($n = 11$). As a result, the 5-HT to forskolin ratio was significantly reduced by the chronic exposure of preparations to PDBu (Fig. 8*C*) ($p < 0.001$). Chronic exposure to PDBu also reduced the duration of slow responses to 5-HT and the associated increase in input resistance. The ratio of the duration of the 5-HT response to that of forskolin fell from 0.7 ± 0.1 in untreated preparations to 0.4 ± 0.1 in those exposed to PDBu ($p < 0.05$). The ratio of the maximal change in input resistance during the 5-HT response to that during the response to forskolin decreased from 1.3 ± 0.2 in untreated preparations to 0.3 ± 0.2 in preparations treated chronically with PDBu ($p < 0.001$). These observations indicate that the slow response of AH/type 2 neurons is inhibited specifically by downregulation of PKC.

Figure 7. Inhibitors of PKC antagonize slow responses to 5-HT. *A*, Slow responses to 5-HT of an AH/type 2 neuron are antagonized by the intracellular injection of the PKC pseudosubstrate PKC_[19-31] (top trace, left) and by the superfusion of the membrane permeable PKC pseudosubstrate myr-PKC_[19-27] (myr-FARKGALRQ; bottom trace, left). Slow responses are also inhibited by the superfusion of chelerythrine (3.0 μ M; top trace, right) and by the selective PKC α and β antagonist Gö 6976 (10 μ M; bottom trace, right). *B*, *C*, The abilities of PKC_[19-31], chelerythrine and K252a to reduce the amplitude of the 5-HT-induced slow depolarization of AH/type 2 cells are compared.

5-HT activates PKC in isolated myenteric ganglia

To test directly whether 5-HT activates PKC *in situ*, we measured the membrane association of PKC activity $\pm$ the exposure of isolated myenteric ganglia to 5-HT. As a positive control, the membrane association of PKC activity was also followed in preparations exposed to PDBu. The action of 5-HT was investigated in isolated ganglia to avoid interference from effects 5-HT might exert on smooth muscle. The isolated ganglia, trapped on filters, were equilibrated for 5 min at 37°C in oxygenated Krebs' solution, to which was added the 5-HT_{1A} antagonist NAN-190 (0.3 μ M), the 5-HT₂ antagonist ketanserin (10 μ M), and the 5-HT_{3/4} dual antagonist tropisetron (1.0 μ M) to block responses to subtypes of 5-HT receptor other than 5-HT_{1P}. 5-HT (1.0 or 10.0 μ M) or PDBu (1.0 μ M) were then applied for 10 min in the continued presence of the cocktail of antagonists. Control preparations were incubated similarly in the absence of 5-HT. After incubation, all preparations were washed for 5 min with iced Krebs' solution and transferred to iced buffer for homogenization. PKC activity was measured in both the membrane and supernatant fractions. Exposure to 5-HT induced a concentration-dependent increase in

the membrane associated PKC activity (Table 1). At 10 μ M, the response to 5-HT was $\sim$ 75% that to PDBu.

Inhibition of PC-PLC antagonizes slow responses to 5-HT

The observations that the slow response of AH/type 2 neurons to 5-HT are dependent on a G_o protein and PKC raise the question

Table 1. PKC translocation in myenteric ganglia

	PKC activity		M/C ratio
	Membrane bound	Cytosol	
	(pmol/min/ μ g protein)		
Control	2.09 $\pm$ 0.08	2.90 $\pm$ 0.11	0.72 $\pm$ 0.03
5-HT (1.0 μ M)	2.72 $\pm$ 0.16*	2.23 $\pm$ 0.06*	1.22 $\pm$ 0.07*
5-HT (10.0 μ M)	3.17 $\pm$ 0.19*	1.96 $\pm$ 0.03*	1.61 $\pm$ 0.10*
PDBu (1.0 μ M)	3.86 $\pm$ 0.04*	1.78 $\pm$ 0.04*	2.17 $\pm$ 0.05*

The ability of the PKC α/β pseudosubstrates myr-FARKGALRQ and PKC_[19-31] as well as the α/β isozyme-selective Gö 6976 to antagonize slow responses to 5-HT suggests that one of the classical isozymes of PKC is likely to be responsible for the effect. * P < 0.01, compared with control. Data are mean $\pm$ SEM. n = 3–6.

Figure 8. Downregulation of PKC inhibits the slow response of AH/type 2 neurons to 5-HT. *A*, In control preparations, the amplitudes of the slow depolarizations evoked by 5-HT and forskolin are approximately equal. *B*, After overnight exposure of preparations to PDBu to downregulate PKC, the 5-HT-induced slow depolarization is reduced in amplitude far more than is the response to forskolin. *C*, In control preparations, the ratio of the amplitude of the 5-HT to the forskolin response is about 1; however, this ratio is greatly reduced as a result of the inhibition of the response to 5-HT after long-term exposure of preparations to PDBu.

of how G_o , after its activation by the 5-HT_{1P} receptor, is coupled to PKC. This coupling could be dependent on the activity of a PLC; however, because the slow response to 5-HT is attributable to the inhibition of gK_{Ca} , it would seem unlikely that the activation of PKC would be linked to a mechanism that would increase intracellular Ca^{2+} ($[Ca^{2+}]_i$). The hypothesis was therefore tested that the slow response depends not on a phosphatidylinositol-specific isozyme of PLC but on PC-PLC. The liberation of inositol-1,4,5-trisphosphate (IP_3) by a phosphatidylinositol-specific PLC would be expected to cause the release of Ca^{2+} from internal stores and thus to increase $[Ca^{2+}]_i$ (Exton, 1994). In contrast, the action of PC-PLC would liberate the PKC activator, DAG, without simultaneously liberating IP_3 (Schütze et al., 1992). The effect of D609 (100 μM), a specific inhibitor of PC-PLC (Schütze et al., 1992), on the slow response of AH/type 2 neurons to 5-HT was therefore tested. Although D609 did not affect the membrane potential of AH/type 2 cells, the amplitude of the 5-HT-induced slow depolarization was significantly reduced by D609 (Fig. 9*A,B*) ($p < 0.001$; $n = 9$).

PKA antagonism inhibits slow responses of AH/type 2 neurons to 5-HT

The data outlined above, which suggest that PKC participates in mediating slow responses of AH/type 2 neurons to 5-HT, do not imply that PKC is the only protein kinase that does so. Because the downregulation or inhibition of PKC antagonized, but did not abolish, slow responses to 5-HT, it is possible that another protein kinase contributes to the response. To test the hypothesis that PKA contributes to the slow response of AH/type 2 cells to 5-HT, the selective PKA inhibitor Rp-cAMPS was injected into AH/type 2 neurons through the recording electrode. Responses to 5-HT, applied by microejection to the surfaces of AH/type 2 neurons, were then determined. The ability of injected Rp-cAMPS to effect the slow depolarization evoked in the same cells by pressure applications of the PKC activator PDBu was assessed as an expected negative control. Forskolin and histamine were also studied as expected positive controls, because they increase cAMP and thus probably act via PKA. The resemblance of the response of AH/type 2 neurons to forskolin has been described above. When applied by microejection to the surfaces of AH/type 2 neurons, histamine evoked a similar slow depolarization (amplitude, 20 ± 1.2 mV; duration, 93 ± 14.5 sec; increase in input resistance, $100 \pm 0\%$; $n = 3$). As expected, Rp-cAMPS blocked

Figure 9. An inhibitor of PC-PLC antagonizes slow responses to 5-HT. *A*, Slow responses to 5-HT are inhibited by the PC-PLC inhibitor, D609. *B*, The amplitudes of 5-HT-evoked slow depolarizations in the presence or absence (control) of D609 are compared.

both the responses of AH/type 2 neurons to histamine and those to forskolin (data not shown). Because Rp-cAMPS rapidly diffused into impaled cells from the recording electrode, even in the absence of pressure or application of current, it was impossible to be certain that responses to 5-HT or PDBu obtained before the injection of Rp-cAMPS were not affected by the compound. The mean amplitudes of the pooled responses to 5-HT ($n = 8$) or PDBu ($n = 4$) obtained from neurons after the intracellular

Figure 10. Inhibition of PKA antagonizes slow responses to 5-HT. *A*, Slow responses to 5-HT of an AH/type 2 neuron are antagonized by the intracellular injection of the PKA antagonist Rp-cAMPS. *B*, Under control conditions (*top* tracings), 5-HT ($1.0 \mu\text{M}$) inhibits the AH that follows the action potential in an AH/type 2 cell. Superfusion of Rp-cAMPS ($200 \mu\text{M}$; *bottom* tracings) does not itself affect either the action potential or the AH; however, superfusion of Rp-cAMPS prevents the inhibition of the AH by 5-HT. *C*, Although the slow depolarization of AH/type 2 neurons by 5-HT is inhibited by the intracellular injection of Rp-cAMPS, the slow depolarization evoked by PDBu is not affected by Rp-cAMPS.

injection of Rp-cAMPS were thus compared with the mean amplitudes of the pooled responses to 5-HT ($n = 81$) or PDBu ($n = 8$) obtained from control neurons. Intracellular injection of Rp-cAMPS significantly ($p < 0.001$) reduced the amplitude of the

slow response of AH/type 2 neurons to 5-HT (Fig. 10*A,C*) but did not affect the amplitude of the slow response of these cells to PDBu (Fig. 10*C*). In addition to the reduction in the amplitude of slow responses to 5-HT caused by the intracellular injection of Rp-cAMPS, superfusion of Rp-cAMPS antagonized the ability of 5-HT to inhibit the AH of AH/type 2 neurons (Fig. 10*B*).

Antagonism of adenylate cyclase inhibits slow responses of AH/type 2 neurons to 5-HT

The data obtained with Rp-cAMPS are consistent with the idea that PKA, as well as PKC, contributes to the transduction of responses mediated by the 5-HT_{1P} receptor. The participation of PKA suggests that adenylate cyclase is also likely to play a role in the response. This hypothesis was tested by determining the effects of the specific adenylate cyclase inhibitor DDA (0.5 mM) on slow responses of AH/type 2 neurons to 5-HT. The addition of DDA to the superfusing medium did not affect the membrane potential but reduced the amplitude of the 5-HT-induced slow depolarization from $16.0 \pm 3.7 \text{ mV}$ before the addition of DDA to $4.0 \pm 1.5 \text{ mV}$ in the presence of the compound ($p < 0.05$; $n = 5$). These observations are consistent with the idea that adenylate cyclase participates in the transduction of 5-HT_{1P}-mediated responses.

A variety of isozymes of PKC can be identified in enteric neurons

Antibodies to various isozymes of PKC were used to investigate the expression of PKC in the ENS by immunocytochemistry. Western blots were carried out with proteins extracted from isolated myenteric ganglia to confirm results obtained by immunocytochemistry. Calbindin immunoreactivity was located simultaneously with the PKC isozymes to identify probable AH/type 2 neurons in the same preparations. Of the classical (group A) isozymes, only PKC α immunoreactivity was extensively expressed in the enteric plexuses. The distribution of PKC α immunoreactivity was similar to that of G α_o . As with G α_o , the immunoreactivity of PKC α was strikingly marginal, forming rings around most myenteric neurons (Fig. 11*A*). Virtually all of the calbindin-immunoreactive neurons (Fig. 11*B*) were surrounded by such rings of PKC α immunoreactivity (compare Fig. 11*A* and *B*). In addition, PKC α immunoreactivity was abundant in the ganglionic neuropil and was found in processes coursing through interganglionic connectives. The immunoreactivity of PKC α was equally abundant in the submucosal plexus, where it again outlined most neurons and extended into interganglionic connectives (Fig. 11*A*, *inset*). In contrast to PKC α , almost no PKC β_1 or β_2 immunoreactivity could be detected in enteric neurons, although smooth muscle contained PKC β_2 immunoreactivity (data not shown). Of the novel (group B) isozymes of PKC, only PKC δ immunoreactivity was present in abundance in the ENS. The pattern obtained with antibodies to PKC δ (Fig. 11*C*) was quite similar to that obtained with antibodies to PKC α . Again, the immunostaining of myenteric neurons was strikingly marginal and PKC δ immunoreactivity outlined myenteric nerve cell bodies, leaving the bulk of the neuronal cytoplasm unstained. All calbindin-immunoreactive neurons (Fig. 11*D*) were surrounded by PKC δ immunoreactivity; however, PKC δ immunoreactivity was not specifically associated with calbindin-immunoreactive cells and surrounded almost all of the neurons of the myenteric plexus. PKC δ immunoreactivity was also found in the ganglionic neuropil and in axonal varicosities (Fig. 11*C*). The abundance of PKC δ immunoreactivity was only slightly less than that of PKC α but was more prominent than that

Figure 11. The immunoreactivities of PKC α and PKC δ are prominent in enteric neurons. *A*, PKC α immunoreactivity (visualized with FITC) is concentrated at the margins of neurons in both a myenteric and a submucosal ganglion (*inset*) and thus outline enteric neurons. Axons entering interganglionic connectives (*arrows*) are also PKC α -immunoreactive. *B*, Calbindin immunoreactivity (visualized with TRITC) is demonstrated in the same section as shown in *A*. Although calbindin-immunoreactive neurons are outlined by PKC α immunoreactivity, so too are virtually all of the neurons of the myenteric plexus. *C*, PKC δ immunoreactivity (visualized with FITC) is concentrated at the margins of neurons in a myenteric ganglion but does not outline cells quite as sharply as does the immunoreactivity of PKC α . Axons entering interganglionic connectives are also PKC δ -immunoreactive. *D*, Calbindin immunoreactivity (visualized with TRITC) is demonstrated in the same section as shown in *C*. Calbindin-immunoreactive neurons are outlined by PKC δ immunoreactivity, but again virtually all of the neurons of the myenteric plexus are ringed by PKC δ immunoreactivity. *E*, PKC γ immunoreactivity is seen in some highly granular cells (*arrows*) located outside of myenteric ganglia (above or next to the ganglia). In addition, smooth muscle cells contain PKC γ immunoreactivity, which is concentrated in their plasma membranes. The bright streaks running parallel to the nonimmunoreactive myenteric ganglion are the immunofluorescence of the membranes of circular muscle cells. Although only the appearance of PKC γ immunoreactivity in neurons is extremely rare, some very large axonal varicosities are PKC γ -immunoreactive (*arrowhead*). *F*, Calbindin immunoreactivity (visualized with TRITC) is demonstrated in the same section as shown in *E*. Note that neither the calbindin-immunoreactive neuronal cell bodies within the ganglion nor the calbindin-immunoreactive axons in the interganglionic connectives are PKC γ -immunoreactive. The very large PKC γ -immunoreactive varicosities are not calbindin-immunoreactive (compare with *E*). Scale bars, 30 μ m.

of α in axonal varicosities. No specific immunostaining of the enteric plexuses were obtained with antibodies to another novel isozyme, PKC ϵ , although ϵ immunoreactivity was seen in the

smooth muscle (data not shown). Of the atypical isoforms of PKC that were investigated, ζ , ι/λ , and η , none immunostained enteric neurons. Cells with an appearance and distribution consistent

with that of peritoneal macrophages, however, were immunostained with antibodies to PKC η , which was also observed in circular muscle. The immunoreactivity of PKC γ , a classical enzyme, was observed in small numbers of myenteric axons, which were distinguished by very large varicosities (Fig. 11*E*). Weak PKC γ immunoreactivity was also observed in rare neurons (data not shown), which did not contain calbindin. In addition, small extraganglionic cells exhibited strong PKC γ immunoreactivity, which appeared to be located in intracellular granules (Fig. 11*E*); these cells did not contain calbindin (compared *F* and *E*). These observations indicate that only two isozymes of PKC, α and δ , are abundant in enteric neurons. Data obtained from Western blots of proteins obtained from preparations of isolated myenteric ganglia were consistent with the observations made by immunocytochemistry; thus, only PKC α , γ , and δ immunoreactivities were observed in isolated ganglia (data not shown).

DISCUSSION

The current studies confirm that the slow response of AH/type 2 neurons [inhibition of gK_{Ca} (Galligan, 1995)] to 5-HT is linked to G_o and cooperatively mediated by PKA and PKC. Evidence that this response is G_o -linked includes: (1) inhibition by intracellular injection of GDP β S and potentiation/prolongation by GTP γ S; (2) sensitivity to PTx; (3) activation, by 5-HT $_{1P}$ agonists, of G_o , but not of other G-proteins (Wang et al., 1996); (4) antagonism by intracellular injection of anti- G_{α_o} Fab fragments of slow responses to 5-HT but not to histamine, or antagonism of fast, 5-HT $_3$ -mediated responses to 5-HT; and (5) the presence of G_{α_o} immunoreactivity on the plasma membranes of many neurons in both plexuses, including all of those that express the AH/type 2 cell marker calbindin. Evidence that PKC plays a role in mediating slow responses to 5-HT includes: (1) mimicry by the PKC activators, PDBu, (–)-7-octylindolactam V, and OAG; (2) antagonism by PKC inhibitors that have little in common besides their ability to inhibit PKC, including staurosporine, K-252a, G_6 6976, chelerythrine, and the pseudosubstrate peptides myr-FARKGALRQ and PKC $_{[19-31]}$ (by intracellular injection), as well as by (3) overnight exposure to PDBu to downregulate PKC; and (4) the concentration-dependent induction by 5-HT of PKC translocation into membranes in isolated myenteric ganglia. Evidence that PKA participates in mediating slow responses to 5-HT includes: (1) antagonism by the selective PKA inhibitor Rp-cAMPS of slow responses to 5-HT, histamine, and forskolin, but not those to PDBu; and (2) interference by Rp-cAMPS with inhibition of the AH by 5-HT.

G_o is not commonly linked to the activation of PKC and PKA. Because the selective PC-PLC inhibitor D609 antagonizes slow responses to 5-HT, it seems likely that the coupling of G_o to PKC is mediated by PC-PLC. The activity of this enzyme generates DAG, which activates PKC without simultaneously releasing IP $_3$ (Schütze et al., 1992), as would a phosphatidylinositol-specific isozyme. Because the slow response to 5-HT involves the inhibition of gK_{Ca} (Galligan, 1995), the liberation of IP $_3$ would be counterproductive, because IP $_3$ would be expected to increase intracellular Ca^{2+} (Exton, 1994). In fact, the P_2 purinoceptor-induced elevation of intracellular Ca^{2+} is associated with effects, a membrane hyperpolarization and decreased input resistance, that are the converse of the slow response to 5-HT (Palmer et al., 1987a; Christofi et al., 1995). The 5-HT $_{1P}$ -related activation of PKA is probably stimulated by an adenylate cyclase-mediated elevation of cAMP, because slow responses are antagonized by the adenylate cyclase inhibitor DDA and 5-HT increases cAMP in

isolated myenteric ganglia (Xia et al., 1991, 1994; Fiorica-Howells et al., 1993). Slow responses to 5-HT are also mimicked by forskolin (Nemeth et al., 1986) and by analogs of cAMP (Zafirov et al., 1985a; Palmer et al., 1986). Despite these observations, available evidence suggests that G_s is not responsible for the 5-HT-induced activation of adenylate cyclase. When applied to isolated myenteric ganglia, 5-HT activates G_{α_o} , but not G_{α_s} , (Wang et al., 1996). The adenosine-induced stimulation of G_{α_i} does not inhibit slow responses to 5-HT (or slow EPSPs) (Palmer et al., 1987b), but it does block slow responses to forskolin, histamine, vasoactive intestinal peptide, or pituitary adenylate cyclase activating peptide (Zafirov et al., 1985b; Palmer et al., 1987a,b; Christofi and Wood, 1993). Because adenylate cyclase thus appears to be involved in mediating both the G_{α_i} -resistant slow responses to 5-HT and the G_{α_i} -sensitive slow responses to forskolin, histamine, vasoactive intestinal peptide, or pituitary adenylate cyclase activating peptide, the activation of adenylate cyclase associated with the response to 5-HT must differ from that associated with forskolin or compounds that probably act via receptors linked to G_s . PKC is known to be able to activate type II adenylate cyclase (Jacobowitz et al., 1993; Yoshimura and Cooper, 1993); moreover, when adenylate cyclase is activated by PKC, instead of by G_{α_s} or forskolin, it is not inhibited by G_{α_i} (Pieroni et al., 1993). These considerations suggest that the following signal transduction pathway may be responsible for the slow response to 5-HT: 5-HT $\rightarrow$ 5-HT $_{1P}$ receptor $\rightarrow$ activates G_o $\rightarrow$ activates PC-PLC $\rightarrow$ liberates DAG $\rightarrow$ stimulates PKC $\rightarrow$ activates adenylate cyclase $\rightarrow$ elevates cAMP $\rightarrow$ stimulates PKA. Because the slow response to 5-HT is not abolished by inhibitors of PKA or PKC alone but by their combination, PKA and PKC probably each inhibit gK_{Ca} . Why PKC inhibitors or downregulation do not eliminate the slow response to 5-HT is not clear. Conceivably, a Ca^{2+} -independent novel or atypical isozyme of PKC, in addition to one of the classical (group A) Ca^{2+} -dependent PKC isozymes, becomes activated by 5-HT and is not fully inactivated by PKC inhibitors or downregulation.

Immunocytochemical studies indicated that PKC α is present in most of the neurons of both submucosal and myenteric plexuses. In fact, the distribution of the immunoreactivities of G_{α_o} and PKC α was similar and each was found in virtually every cell that displayed calbindin immunoreactivity, investigated as an AH/type 2 cell marker (Furness et al., 1990). In contrast, enteric neurons contained no PKC β_1 or β_2 immunoreactivity and PKC γ immunoreactivity was observed only in rare myenteric neurons, none of which were calbindin immunoreactive. Because many neurons in each plexus and most AH/type 2 neurons exhibit the slow response to 5-HT, PKC α is the only classical (group A) isozyme of PKC that is distributed in a manner consistent with a role in transduction of 5-HT $_{1P}$ -mediated slow responses. Subject to the limitation that the proteins might have been present in forms or quantities that could not be detected by immunocytochemistry, the β_1 , β_2 , and γ isozymes thus are unlikely to be involved in transducing the slow response. The distribution of the immunoreactivity of the novel (group B) isozyme PKC δ , however, was found to be very similar to that of PKC α . Again, many neurons in each plexus and all calbindin-immunoreactive neurons were PKC δ immunoreactive. PKC δ is thus also a candidate to play a role in the slow response to 5-HT. In contrast, the distributions of neither PKC ϵ , nor any of the atypical isozymes of PKC, ζ , ι/λ , and η , were compatible with roles in 5-HT slow response signal transduction. These observations suggest that only the α and δ PKC isozyme are potential participants in transduction of slow

responses to 5-HT. At least some of the PKC inhibitors that were found to antagonize slow responses to 5-HT, however, including G₆ 6976 and the pseudosubstrate peptides FARKGALRQ and PKC_[19–31] are specific for the α and β isozymes of PKC and have little or no activity against PKC δ . The β PKC isozymes, moreover, are evidently not present in enteric neurons; thus, these data suggest that PKC α is probably primarily responsible for transducing the 5-HT_{1P}-mediated slow response. Although PKC α is Ca²⁺-sensitive and intracellular Ca²⁺ is unlikely to increase during slow responses to 5-HT, the constitutive level of cytosolic Ca²⁺ may be sufficient to support the activation of PKC α by DAG, which increases the affinity of PKC for Ca²⁺ (Nishizuka, 1992; Tanaka and Nishizuka, 1994). Conceivably, local elevations of intracellular Ca²⁺ could occur, which could enhance stimulation of PKC α without contributing to gK_{Ca}.

Many characteristics of slow responses to 5-HT are similar to those that have been reported previously for slow EPSPs. For example, slow EPSPs are potentiated by GTP γ S, mimicked by forskolin and PDBu, and inhibited by D609 and staurosporine (Bertrand and Galligan, 1995). No studies have yet been carried out to determine whether slow EPSPs are, like slow responses to 5-HT, affected by GDP β S, antibodies to G α_o , or inhibitors of adenylate cyclase or PKA. The one difference between slow responses to 5-HT and slow EPSPs is that only slow EPSPs have been reported to be PTx-resistant (Bertrand and Galligan, 1995). When slow EPSPs were studied, however, PTx was applied extracellularly for only 2 hr, whereas in the current investigation, at least 4 hr of exposure was found to be required before PTx inhibited slow responses to 5-HT. It is possible, therefore, that slow EPSPs might actually be PTx-sensitive but not observed to be so, because insufficient time was allowed for PTx to become effective. Both the slow response to 5-HT and slow EPSPs are resistant to inhibition by adenosine (Palmer et al., 1987b). The resistance of slow EPSPs to inhibition by adenosine implies that they are, like slow responses to 5-HT, G α_i -resistant. Thus it seems unlikely that any receptor that induces a slow EPSP could be coupled by a G_s protein to adenylate cyclase; nevertheless, because 5-HT is only one of the transmitters that mediate slow EPSPs (Bornstein et al., 1984), the observation that PTx did not inhibit slow EPSPs (Bertrand and Galligan, 1995) could also be explained by the existence of subsets of slow EPSPs transduced by different transmitters using receptors coupled to different G-proteins. Given the many other identities between slow EPSPs and slow responses to 5-HT, however, it seems premature to postulate subsets of slow EPSPs triggered by different G-proteins.

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