The Journal of Neuroscience, July 1988, 8(7): 2582–2595

Characterization and Localization of a Peripheral Neural 5-Hydroxytryptamine Receptor Subtype (5-HT_{1P}) with a Selective Agonist, ^{3}H-5-Hydroxyindalpine

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Peripheral neural 5-hydroxytryptamine (5-HT) receptors are different from both classes 5-HT_1 and 5-HT_2, which have been described from studies of 5-HT receptors in the brain. Recently, it has been shown that, as in the CNS, there is more than a single type of neural receptor for 5-HT in the enteric nervous system. One of these, called 5-HT_{1P}, has a high affinity for ^{3}H-5-HT, initiates a long-lasting depolarization of enteric neurons associated with an increase in membrane resistance, and is the physiological receptor through which enteric serotoninergic neurons mediate slow EPSPs. The other receptor, called 5-HT_3 (5-HT_{2P}), does not bind ^{3}H-5-HT with high affinity, and initiates a brief depolarization of enteric neurons with decreased input resistance, but a physiological action of 5-HT mediated by these receptors has not yet been identified. Hydroxylated indalpines have been found to be agonists at 5-HT_{1P} receptors. We have now examined 5-HT_{1P} receptors using 5-hydroxyindalpine (5-OHIP) as a probe. The action of 5-OHIP on enteric neurons was determined electrophysiologically and compared with that of 5-HT; the binding of ^{3}H-5-OHIP to isolated enteric membranes was studied by rapid filtration, and to frozen sections of tissue by radioautography. ^{3}H-5-OHIP binding was compared with that of ^{3}H-5-HT. 5-OHIP, like 5-HT, induced a triphasic response in most enteric neurons: an initial short-lived depolarization, during which input resistance fell, followed by recovery, and then a long-lasting depolarization, during which the input resistance increased. 5-OHIP bound saturably, reversibly, and with high affinity to enteric membranes (K_d = 7.6 ± 0.7 nM; B_{max} = 76 ± 14 fmol/mg protein). Binding of ^{3}H-5-OHIP was not inhibited by agents that bind to α- or β-adrenoceptors, nicotinic or muscarinic receptors, histamine H_1 or H_2 receptors, or 5-$HT_{1(A,B,C, or D)}$, 5-HT_2, or 5-HT_3 receptors, but was displaced by substances, such as hydroxylated indoles and a dipeptide of 5-hydroxytryptophan (5-HTP-DP), that antagonize the binding of ^{3}H-5-HT to enteric membranes or tissue sections. It is concluded that 5-OHIP is an agonist at peripheral neural 5-HT_{1P} receptors and can be used to label these receptors selectively outside the brain. Radioautographs demonstrated enteric 5-HT_{1P} receptors in the lamina propria of the intestinal mucosa and in the submucosal and myenteric plexuses. Extraenteric 5-HT_{1P} receptors were also found in the skin and heart. It is suggested that 5-HT_{1P} receptors may be found on subtypes of primary afferent nerve fibers.

Serotonin (5-hydroxytryptamine, 5-HT) is found in both the wall and the lining epithelium of the gastrointestinal tract. In the epithelium, 5-HT is stored in enteroendocrine cells of the EC type (Erspamer, 1966), while that in the wall of the bowel is found in neurons, the cell bodies of which are located in the myenteric plexus (Gershon, 1982; Costa et al., 1987). These enteric serotoninergic neurons are all interneurons and project extensively within the myenteric plexus and, to a smaller extent, to the submucosal plexus as well (Erde et al., 1985; Costa et al., 1987; Gershon and Sherman, 1987). Although enteric neuronal 5-HT has fulfilled all of the criteria necessary for its identification as a neurotransmitter (Gershon, 1982; Erde et al., 1985; Takaki et al., 1985a; Mawe et al., 1986), the precise role of 5-HT in the physiology of the bowel is not yet clear. The role of enteroendocrine 5-HT is similarly obscure, although it has been proposed that 5-HT released from enteroendocrine cells activates intrinsic enteric primary afferent nerve fibers in the intestinal mucosa to initiate the peristaltic reflex (Bülbring and Crema, 1958; Bülbring and Lin, 1958). To a considerable extent, this lack of clarity is the result of the fact that 5-HT has many different types of action on the bowel (Costa and Furness, 1979a, b; Gershon, 1981). The abundance of these actions, combined with a paucity of specific agonists and antagonists for enteric 5-HT receptors, has made it difficult to determine which of the effects of 5-HT are of physiological significance.

Enteric neuronal 5-HT receptors are known to be different from those that have been studied extensively in the brain, and to be different also from the peripheral 5-HT receptors on smooth muscle (Branchek et al., 1984a; Gershon et al., 1985; Richardson et al., 1985; Bradley et al., 1986). Historically, attempts to characterize enteric 5-HT receptors have relied on analyses of the motility of enteric smooth muscle following the addition of 5-HT or experimental compounds to preparations of gut (Gaddum and Picarelli, 1957; Drakontides and Gershon, 1968; Fozard and Mobarok Ali, 1978; Richardson et al., 1985). Con-

Received Aug. 18, 1987; revised Nov. 18, 1987; accepted Nov. 27, 1987.

We wish to thank Dr. Adam Doble of Rhone-Poulenc Santé Laboratoires, Gennevillers, France for contributing the 5-hydroxyindalpine, 6-hydroxyindalpine, and indalpine used in this study and for arranging the synthesis of ^{3}H-5-hydroxyindalpine by the Commissariat à l'Energie Atomique, Paris, France. We would like to thank Dr. Hadassah Tamir of Columbia University for contributing ^{3}H-5-HTP-DP. We also thank Ms. Valerie Boone for excellent technical assistance. This work was supported by the Council for Tobacco Research and NIH Grants NS 12969, NS 22637, and NS 07062.

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tractions that can be antagonized by muscarinic blockers (Brownlee and Johnson, 1963) or by addition of tetrodotoxin (Gershon, 1967; Drakontides and Gershon, 1968) have been considered to be due to an action on neurons, while those that can be elicited in the presence of these agents have been thought to be due to direct effects of the test compounds on smooth muscle. When applied to the characterization of 5-HT receptors, such experiments have assumed that there is only a single or a dominant type of enteric neural 5-HT receptor (Fozard and Mobarok Ali, 1978; Richardson et al., 1985). When 5-HT is applied to the entire enteric nervous system (ENS), it has excitatory, inhibitory, presynaptic, and postsynaptic effects (Bülbring and Crema, 1958; Bülbring and Lin, 1958; Wood and Mayer, 1979; Johnson et al., 1980a, b; North et al., 1980; Erde et al., 1985; Takaki et al., 1985a, b). These lead finally to a net release of ACh (Vizi and Vizi, 1978), the effect of which is measured when muscle contraction is followed; however, not all the various neural actions of 5-HT need be mediated by the same receptor. The existence of multiple neural 5-HT receptors would thus be masked in this type of experiment.

The recent development of a number of different drugs that are antagonists at peripheral 5-HT receptors has been helpful in clarifying what types of 5-HT receptor are found in the gut and elsewhere in the PNS (Bradley et al., 1986; Richardson and Engel, 1986). Used together with analyses of the binding of ^{3}H-5-HT to enteric membranes and electrophysiological studies of the effects of 5-HT on single neurons, these compounds have made it possible to define 2 classes of 5-HT receptors on enteric neurons, both of which are different from any of the 5-HT receptors that have thus far been found in the CNS (Mawe et al., 1986). One of these classes, for which the name 5-HT_{1P} has been proposed, is characterized by a high affinity for ^{3}H-5-HT. The designation applies the classification of 5-HT receptors in the CNS, in which the 5-HT_1 receptor class is meant to imply a high affinity for ^{3}H-5-HT to the periphery. The "P" stands for peripheral. The 5-HT_{1P} receptor mediates a long-lasting depolarization of myenteric neurons associated with an increase in input resistance. In addition to 5-HT itself, agonists at this receptor include hydroxylated indalpines. 6-Hydroxyindalpine (6-OHIP) has been extensively studied and found to be a relatively pure 5-HT_{1P} agonist; 5-hydroxyindalpine (5-OHIP) also has agonist properties, but has been less well studied than 6-OHIP. 5-HT_{1P}-mediated responses are specifically antagonized by dipeptides of 5-hydroxytryptophan, such as *N*-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide (5-HTP-DP; Takaki et al., 1985a). The other class of 5-HT receptor, for which the name 5-HT_{2P} has been proposed, does not have a high affinity for ^{3}H-5-HT. It appears, from the effects of antagonists, that the 5-HT_{2P} receptor is the same receptor as that which has also been called "5-HT_3" (Bradley et al., 1986; Richardson and Engel, 1986). This receptor mediates a short-lived depolarization of myenteric neurons associated with a decrease in input resistance. 2-Methyl-5-HT, at low concentrations, is a specific agonist at this receptor, and ICS 205-930 (Sandoz) is a specific antagonist. Binding of ^{3}H-5-HT to enteric membranes is inhibited by 5-HTP-DP and hydroxylated indalpines, but not by ICS 205-930. Responses to 5-HT, essentially identical to those found in neurons of the myenteric plexus, have recently been observed in neurons of the submucosal plexus (Surprenant and Crist, 1988). Like their counterparts in the myenteric plexus, these responses can be differentiated on the basis of their different ionic mechanisms and sensitivity to ICS 205-930.

In the current study, enteric neural receptors have been analyzed using 5-OHIP instead of 5-HT itself as a probe. 5-OHIP, rather than 6-OHIP, was selected for this investigation, even though 6-OHIP was previously characterized, because 5-OHIP displays greater chemical stability than does 6-OHIP. Binding sites for ^{3}H-5-OHIP were compared with those for ^{3}H-5-HT, both in terms of their properties and their localization in the gut. In order to help verify that ^{3}H-5-OHIP binding sites are 5-HT receptors, physiological responses of myenteric neurons to the addition of 5-OHIP were examined and compared with those to 5-HT. The abilities of 5-HTP-DP and ICS 205-930 to antagonize these responses were also studied in comparison to the abilities of the same compounds to displace bound ^{3}H-5-OHIP and ^{3}H-5-HT. In addition, the possibility that the effects of 5-OHIP might be due to actions in the gut other than direct activation of 5-HT receptors was also explored. Possible additional effects that were investigated included release of endogenous 5-HT from myenteric neurons and inhibition of the uptake of 5-HT. Finally, ^{3}H-5-OHIP was used to search elsewhere in the body for 5-HT receptors similar to those found on enteric neurons.

Characterization of the properties of 5-OHIP binding sites was done with membranes obtained from dissected strips of longitudinal muscle with attached myenteric plexus obtained from rabbit. Rabbit tissue was chosen for this analysis because it is much easier to obtain a sufficient quantity of membranes for assay from rabbit bowel than from smaller animals. Moreover, 5-HT binding has previously been most extensively characterized in the rabbit longitudinal muscle–myenteric plexus preparation (Branchek et al., 1984a), although subsequent studies have shown that the same 5-HT binding site is also present in the mouse intestine (Branchek and Gershon, 1987). Radioautographic studies of the binding of ^{3}-5-HT have revealed that the same receptors are also present in the intestines of guinea pigs and that the localization of ^{3}H-5-HT binding sites in the gut is the same in all 3 species. Nevertheless, there is a considerable species difference in the density of 5-HT receptors in the bowel wall; therefore, radioautographic studies can most readily be performed in the murine gut, where exposure times are shortest and the small size of the organ facilitates examination of the neural tissue. For this reason, although radioautographic studies of the binding of ^{3}H-5-OHIP were done with intestine from mouse, guinea pig, and rabbit, the murine bowel was examined most extensively. Finally, electrophysiological studies were all done on the guinea pig myenteric plexus because all previous studies of the physiology of responses of individual myenteric neurons to 5-HT have been done on this tissue, the physiology of the guinea pig myenteric plexus has been comprehensively investigated, and there is very little known about the electrophysiology of the ENS of other species (Wood, 1987).

Materials and Methods

Binding of ^{3}H-5-OHIP. Segments of longitudinal muscle with attached myenteric plexus were dissected from the small intestines of adult male New Zealand rabbits (0.25–1 kg) and homogenized in 50 mM Tris-HCl buffer, pH 7.4. A membrane fraction was obtained by differential centrifugation as previously described (Branchek et al., 1984a). Samples containing these membranes were incubated in a shaking water bath with ^{3}H-5-OHIP (82 Ci/mmol; Commissariat a l'Energie Atomique, Paris) for 10 min at 37°C in either 50 mM Tris-HCl or 50 mM sodium phosphate (pH 7.4). Aliquots contained 200 μg protein/ml incubating solution and 0.1–50 nM ^{3}H-5-OHIP. Following incubation, membranes were collected by rapid filtration on Whatman GF/B glass fiber filters (presoaked with 0.3% polyethyleneimine overnight), using a Brandel

Cell Harvester (Gaithersburg, MD). Radioactivity was determined by counting in a Tracor 6895 (Austin, TX) liquid scintillation spectrometer. Specific binding was defined as that displaced by a 1000-fold excess of nonradioactive 5-OHIP or 5-HTP-DP and was ≈55–65% of total binding. Addition of 0.1% ascorbic acid or 1% sodium metabisulfite was found to have variable effects. Total binding of ^{3}H-5-OHIP was reduced; however, this effect was predominantly on nonspecific binding, and specific binding of ^{3}H-5-OHIP was relatively unaffected by the presence of these antioxidants. Under optimal conditions, when specific binding was >50% of total binding, ascorbic acid did not significantly reduce the binding of ^{3}H-5-OHIP (binding was decreased by 1.0 ± 0.4%; n = 5, p, n.s.). On the other hand, when specific binding was <50% of total binding, ascorbic acid significantly reduced specific, as well as nonspecific, binding of ^{3}H-5-OHIP (by 42 ± 12%; n = 5; $p < 0.04$). Because of its variable effects on the specific binding of ^{3}H-5-OHIP, ascorbate was not included in further experiments. Saturation isotherms were analyzed by computer-assisted nonlinear regression using the LUNDON-1 program (Lundon Software, Cleveland, OH) in order to estimate K_d and B_{max}. The ability of a variety of compounds to compete with ^{3}H-5-OHIP for binding sites was studied by adding isolated membranes to solutions containing ^{3}H-5-OHIP and the potential competitor.

Radioautographic localization of ^{3}H-5-OHIP binding sites was done on 15 μm frozen sections of unfixed gut, skin, heart, and brain, according to a modification of the method of Branchek et al. (1984a). Thaw-mounted sections of tissue on slides were incubated for 30 min at 25°C in 300 mM Tris-HCl buffer (pH 7.4) in the presence of 10 nM ^{3}H-5-OHIP or of ^{3}H-5-HT in the presence or absence of nonradioactive 5-HT, 5-OHIP, or 5-HTP-DP. The specificity of radioligand binding was analyzed further by incubating the sections in the presence of a variety of potential displacing agents (10–100 μM). After rapid washing with iced buffer, the slides were air-dried, apposed to a tritium-sensitive film (Ultrofilm, LKB), and exposed for 1–16 weeks prior to development. In order to identify structures in the tissue underlying sites of accumulation of radioautographic silver grains, sections were stained after development of the film with toluidine blue, cresyl violet, Giemsa, or Bodian's silver.

Uptake of ^{3}H-5-HT. The effects of 5-OHIP, 6-OHIP, indalpine, ICS 205-930, and 5-HTP-DP on the specific uptake of ^{3}H-5-HT were determined. Uptake of ^{3}H-5-HT was studied as described previously (Takaki et al., 1985b). Strips of longitudinal muscle with attached myenteric plexus were dissected from guinea pig small intestine, cut into 1 cm segments, equilibrated for 30 min, and incubated for 5 min at 37°C in oxygenated Krebs solution containing 0.5 μM ^{3}H-5-HT. Nonspecific uptake of ^{3}H-5-HT was measured by incubating tissues with the radioactive amine at 4°C. Compounds to be evaluated as inhibitors of ^{3}H-5-HT uptake were preincubated with the tissue for 15 min and were also present in the incubation solution along with ^{3}H-5-HT. Following incubation, tissues were washed with 250 ml of iced Krebs solution for 20 min, weighed, and extracted overnight with 70% ethanol. Radioactivity of the ethanolic extracts was determined by liquid scintillation.

Electrophysiology. Intracellular recordings were made from myenteric neurons exposed by dissection of the longitudinal layer of smooth muscle with attached myenteric plexus from adult guinea pig jejunum. Standard techniques, previously described in detail, were used for dissection of the tissue, recording from neurons, and application of drugs (Erde et al., 1985; Takaki et al., 1985b). Preparations were maintained in a low-volume chamber that was perfused at 37°C with Krebs solution equilibrated with a mixture of 95% O_2 and 5% CO_2. Microelectrodes were filled with 3 M KCl and had resistances of 40–80 MΩ. To measure membrane resistance, constant anodal current pulses were passed into the cells through the recording microelectrode.

Drugs were applied either by superfusion or ejection with pressure from a micropipette. Micropipetes (15–20 μm tip diameter) were filled with drugs at a concentration of 1 mM. Ejection of drugs was accomplished by applying pulses of nitrogen gas (300 kg/cm^2; 10–999 msec in duration). The distance between the tip of the pipette and the recording electrode was 50–100 μm. All drugs were dissolved in Krebs solution except 5-HTP-DP, which was first dissolved in 100% ethanol and then diluted to 10% ethanol in Krebs solution. Solutions containing drugs were never recirculated.

Drugs used. ^{3}H-5-OHIP was graciously synthesized and supplied by the Commissariat a l'Energie Atomique, Paris, France at the request of Dr. Adam Doble of Rhone-Poulenc Santé Laboratoires, Gennevillers, France. The purity and identity of ^{3}H-5-OHIP was evaluated by thin-layer chromatography on silica gel. The solvent system was butanol: acetic acid : water (8:1:1). The radioactive material was found to comigrate with the authentic unlabeled compound. ^{3}H-5-HT was purchased from New England Nuclear. *N*-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide (5-HTP-DP) was graciously supplied by Dr. Hadassah Tamir, Columbia University. ICS 205-930, 2-methyl-5-HT, and methysergide were donated by Sandoz (Basel, Switzerland). 5- and 6-Hydroxyindalpine (5- and 6-hydroxy-[(indolyl-3)-2-ethyl]-4 piperidine) 5-OHIP and 6-OHIP, as well as the nonhydroxylated parent compound, indalpine, were provided by Dr. Adam Doble, Rhone-Poulenc Santé Laboratoires. MDL 72222 (3,5-dichlorobenzoyltropine ester) was donated by Merrell Dow (Strasbourg Center, France). 8-Hydroxy-di-*n*-propylamino tetralin (8-OH-DPAT) was donated by Eli Lilly Laboratories (Indianapolis, IN). Ketanserin and spiroperidol were donated by Janssen Pharmaceutical Co. (New Brunswick, NJ). D-LSD was supplied by the National Institute for Drug Abuse. Zimelidine was donated by Astra Lakemedel Co. (Sweden), trazodone by Mead-Johnson Co. (Evansville, IN); cyproheptadine by Merck, Sharpe, and Dohme Pharmaceutical Co. (West Point, PA). Other compounds included cimetidine (Smith, Kline and French Pharmaceutical Co., Philadelphia, PA), propranalol (Ayerst Pharmaceutical Co., NY), phentolamine (CIBA-Giegy Pharmaceutical Co., Summit, NJ), pargyline (Abbott Pharmaceutical Co., North Chicago, IL), atropine (Elkins-Sinn Pharmaceutical Co., Cherry Hill, NJ), and, from Sigma Chemical Co. (St. Louis, MO), 5-HT, tryptamine, 5-methoxytryptamine, hexamethonium, and ACh.

Results

Saturability of ^{3}H-5-OHIP binding

Saturation of ^{3}H-5-OHIP binding to isolated enteric membranes was examined by evaluating the specific binding of ^{3}H-5-OHIP as a function of its concentration. Specific binding at equilibrium initially increased as the concentration of ^{3}H-5-OHIP was raised; however, saturation was rapidly approached (Fig. 1). The equilibrium dissociation constant (K_d) and the maximal number of binding sites at saturation (B_{max}) were estimated by computer-assisted nonlinear least-squares analysis of the saturation isotherms (LUNDON-1 program). The curves were best fitted by a model that assumed a single, saturable binding site in the range of concentrations tested (0.1–50.0 nM; $p < 0.01$). These experiments (n= 6) yielded a mean K_d of 7.6 ± 0.7 nM and a mean B_{max} of 76 ± 14.2 fmol/mg protein. Analysis of the data for the binding of ^{3}H-5-OHIP according to the Hill equation gave a coefficient (0.96 ± 0.02) that approximated unity, thus indicating a probable lack of positive or negative cooperativity.

Specificity of the enteric ^{2}H-5-OHIP binding sites

In order to evaluate the specificity of the enteric ^{2}H-5-OHIP binding sites, a variety of drugs known to be antagonists at muscarinic or nicotinic receptors, α- or β-adrenoceptors, H_1 or H_2 histamine receptors, dopamine receptors, 5-HT_1, 5-HT_2, 5-HT_{1P}, and 5-HT_3 (5-HT_{2P}; Mawe et al., 1986) receptors were tested for their ability to displace ^{3}H-5-OHIP. In addition, since enteric serotoninergic neurons have a high-affinity membrane uptake system for 5-HT (Gershon and Altman, 1971; Gershon et al., 1976), inhibitors of 5-HT uptake were also examined as potential displacers of the binding of ^{3}H-5-OHIP to enteric membranes. Finally, a number of indolic analogs were studied similarly. The compounds tested are listed in the Appendix. Only compounds that can act as agonists or antagonists at 5-HT_{1P} receptors were found to be capable of inhibiting the binding of ^{3}H-5-OHIP (10 nM) to enteric membranes. All of these substances are indoles with an unsubstituted hydroxyl group on the indole ring. The other compounds had no effect on ^{3}H-5-OHIP binding, even when present at 1000-fold the concentration of ^{3}H-5-OHIP. It can be concluded that the enteric ^{3}H-5-OHIP binding site shows specificity and is not a physiological receptor

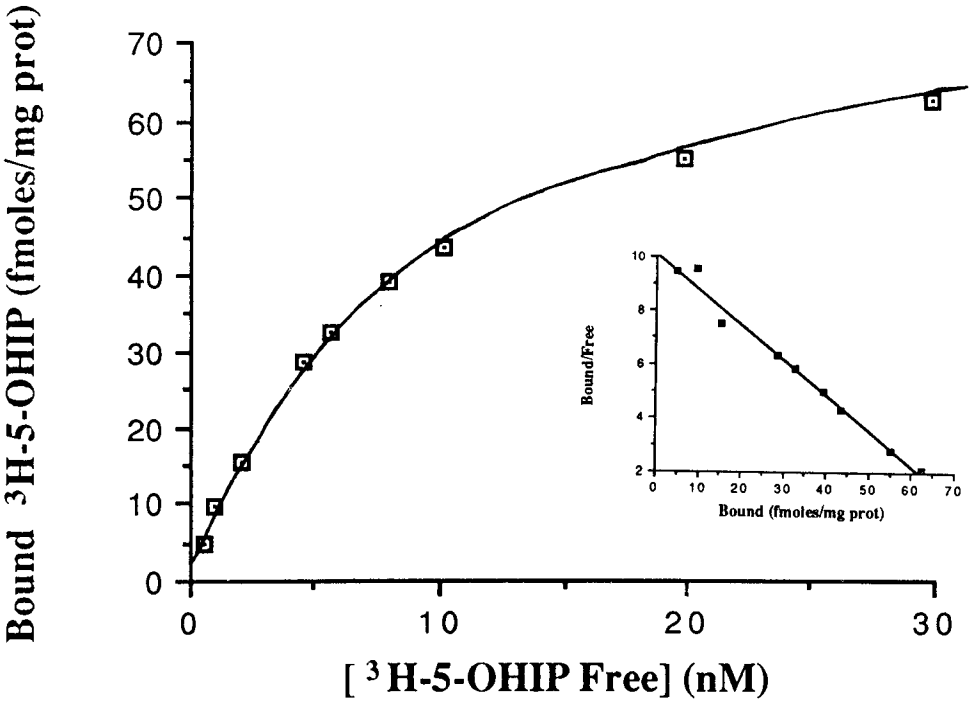

Figure 1. ³H-5-OHIP binding to enteric membranes was plotted as a function of the ³H-5-OHIP concentration. Specific binding was calculated from the total binding minus the binding in the presence of a 1000-fold excess of 5-HTP-DP. The saturation isotherm is a computer-drawn nonlinear least-squares fit of the data (LUNDON-1). *Inset,* Scatchard transformation of the data. K_d = 7.7 nM; B_{max} = 65 fmol/mg protein for the experiment illustrated.

of known type for ACh, a catecholamine, or histamine; moreover, the binding is also probably not to a 5-HT uptake site or to a receptor that resembles either of the previously described classes of receptor for 5-HT in the brain or the enteric neural 5-HT_3 receptor. On the other hand, the data are consistent with the idea that ³H-5-OHIP selectively labels the 5-HT_{1P} receptor in enteric membranes.

Radioautographic localization of enteric ³H-5-OHIP binding sites

In order to locate the sites in the wall of the bowel at which ³H-5-OHIP binds to enteric membranes, radioautography was used. Frozen sections of mouse, guinea pig, and rabbit gut were cut and incubated at room temperature with ³H-5-OHIP (10 nM) in the presence or absence of a 1000-fold excess of either 5-HT or 5-HTP-DP. The washed and rapidly dried sections were exposed to tritium-sensitive film for 1 week. Two areas of concentration of ³H-5-OHIP binding were found, between the layers of smooth muscle of the muscularis externa, corresponding in location to the myenteric plexus, and beneath the epithelium of the mucosa (Fig. 2). This latter site encompassed the lamina propria of both the mucosa and the submucosa. The binding of ³H-5-OHIP could not be distinguished from that of ³H-5-HT in identically prepared sections incubated with that amine (Fig. 3). With neither radioligand could specific binding to smooth muscle, epithelium, or to the dense connective tissue of the submucosa be found. The mucosal–submucosal zone of labeling, however, did overlie ganglia of the submucosal plexus and many mucosal nerve fibers that could be stained with Bodian's silver following the radioautographic processing of the tissue.

The ability of the compounds listed in the Appendix to antagonize the binding of ³H-5-OHIP and ³H-5-HT to enteric membranes was reevaluated radioautographically (Fig. 4). In these experiments, the concentration of ³H-5-OHIP was 10.0 nM and of the potential displacing agents was 10.0 μM. Again, only hydroxylated indoles, which are agonists or antagonists at 5-HT_{1P} receptors, were found to impede the radioautographically detected binding of ³H-5-OHIP or ³H-5-HT at either the myenteric plexus or the mucosal–submucosal sites.

Actions of 5-OHIP on myenteric neurons

Type II/AH neurons were selected for study because this is the only type of myenteric neuron that has definitively been shown to manifest a synaptic response mediated by 5-HT (a slow EPSP; Erde et al., 1985; Takaki et al., 1985a). Type II/AH cells were identified as those with a resting membrane potential of >60 mV, a failure to display anodal break excitation, a pronounced hyperpolarizing afterpotential (the AH for which the cell is named), and a failure to spike repetitively when injected with depolarizing current pulses through the recording micropipette (Wood, 1987). When activated by stimulation of interganglionic fiber tracts, these cells showed fast EPSPs, slow EPSPs, or both. Control responses to 5-OHIP were elicited in 36 cells. The most common response (32/36 cells) was biphasic. There was an initial, brief depolarization associated with a decrease in input resistance (fast response). This response abated and was followed by a prolonged depolarization associated with an increase in input resistance (slow response) (Fig. 5*A*). The long duration of the slow response to 5-OHIP permits it to be detected when fast and slow responses are obtained simultaneously, because the slow response outlasts the obscuring fast response. Often, cells were excited to fire action potentials during the fast response; these spikes were followed by a hyperpolarizing afterpotential that preceded the appearance of the slow response to 5-OHIP. Three of the cells showed only the fast response to 5-OHIP, and one of the cells showed only the slow response to the compound. Control responses to 5-HT were elicited in 27 cells. A biphasic response (Fig. 5*B*), with both fast and slow components, similar to that evoked by microejection of 5-OHIP, was evoked in 22/27 cells, only a fast response in 4/27, and only a slow response in 1/27. In 8 cells, responses were elicited first by 5-HT and then, after recovery, by 5-OHIP. Identical effects,

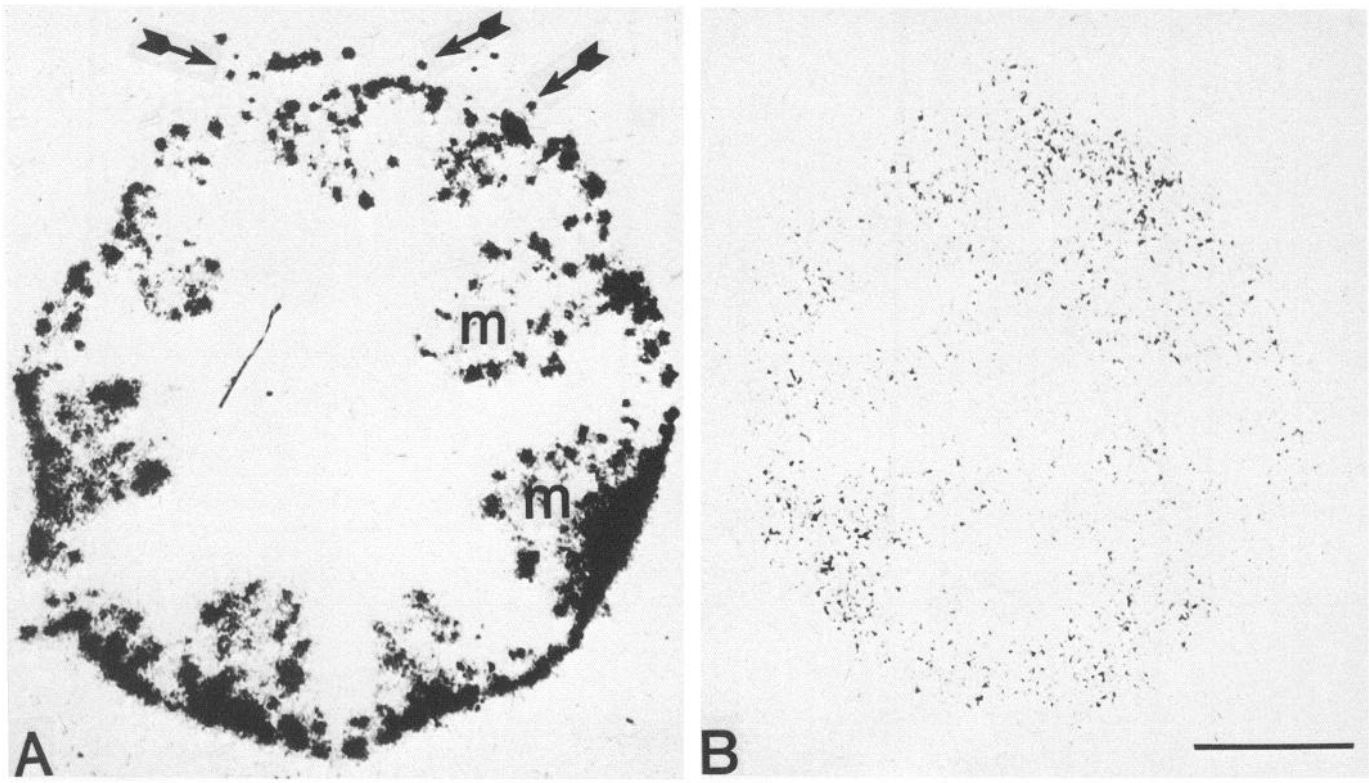

Figure 2. *A*, Radioautograph showing the binding of ^{3}H-5-OHIP (10.0 nM) to sites in the murine colon. Labeling can be seen over ganglia of the myenteric plexus (*arrows*) and over the lamina propria of the mucosa (*m*). *B*, Control. A section serial to that illustrated in *A* was incubated with ^{3}H-5-OHIP (10.0 nM) in the presence of 5-HTP-DP (10.0 μM). Note the complete displacement of ^{3}H-5-OHIP binding from both the myenteric plexus and the lamina propria by 5-HTP-DP. Scale bar, 500 μm.

consisting of an initial fast and a following slow response, were found to be evoked by each agent in all 8 of these cells (Fig. 5, *C*, *D*).

In order to test the possibility that 5-HT receptors of different types are responsible for mediating the fast and slow responses evoked by microejection of 5-OHIP, the abilities of the 5-HT_{1P} antagonist, 5-HTP-DP and of the 5-HT_3 antagonist ICS 205-930 to block responses to 5-OHIP were studied. Microejection

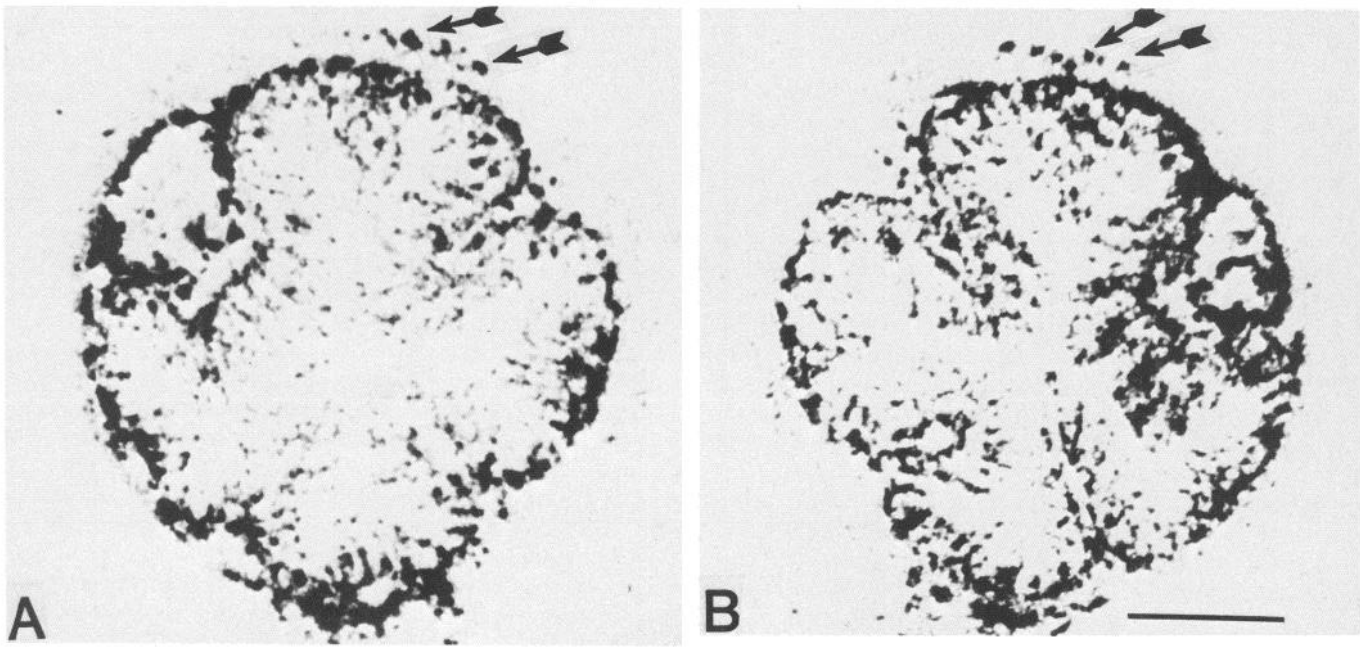

Figure 3. Radioautographs comparing the distribution of ^{3}H-5-OHIP binding sites in the murine colon with those of ^{3}H-5-HT. *A*, ^{3}H-5-OHIP (10.0 nM) binding. *B*, ^{3}H-5-HT (10.0 nM) binding in a section serial to that illustrated in *A*. Note that binding sites for both ligands are found in the lamina propria and myenteric ganglia (*arrows*) and that the patterns of labeling are indistinguishable. Scale bar, 500 μm.

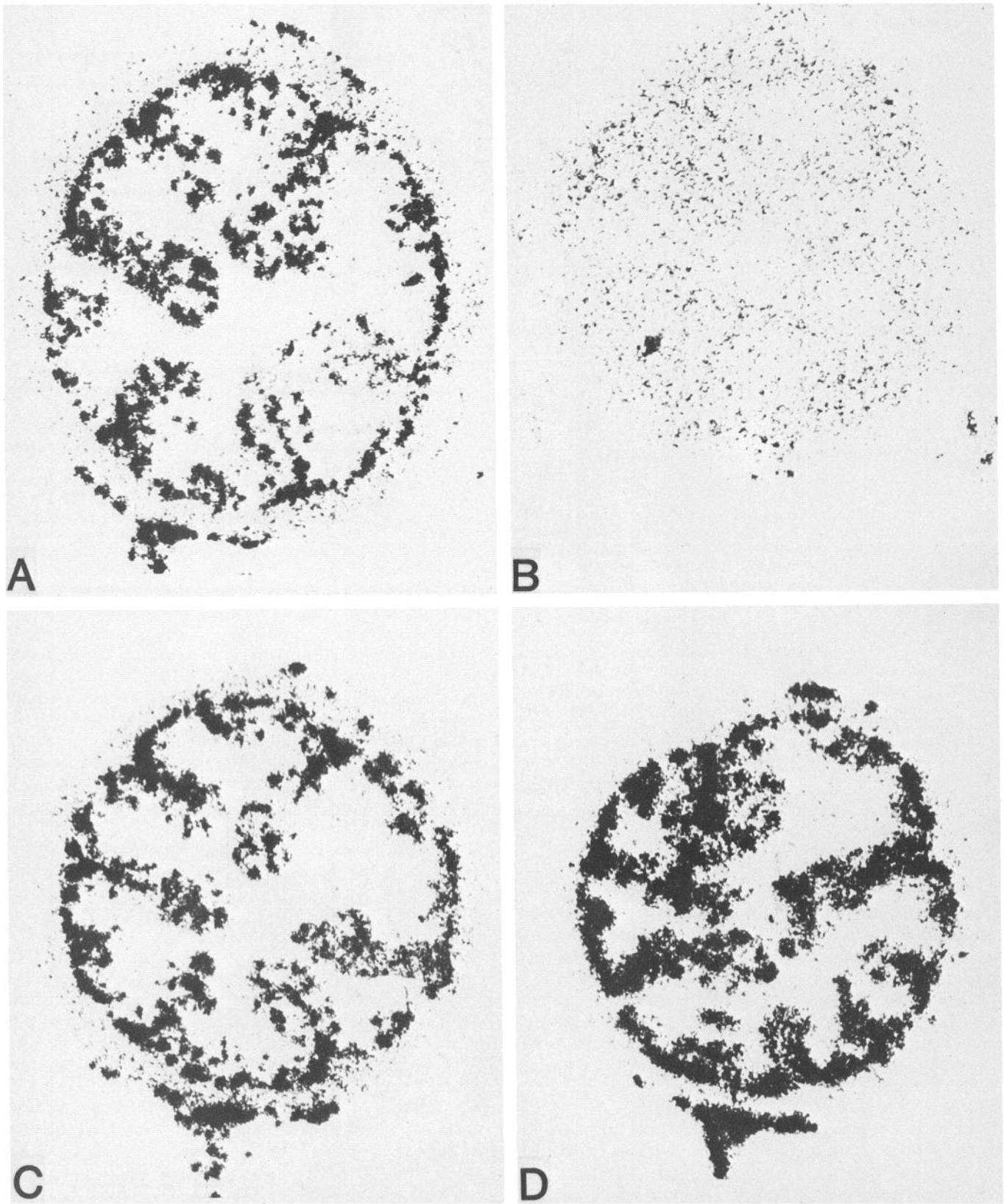

Figure 4. *A,* Radioautograph showing the binding of ^{3}H-5-OHIP (10.0 nM) to sites in the murine colon in the absence of potential competitors. *B,* 6-OHIP (10.0 μM) abolishes the the binding of ^{3}H-5-OHIP in a section serial to that illustrated in *A*. *C,* ICS 205-930 (10.0 μM) fails to inhibit the binding of ^{3}H-5-OHIP in a section serial to that illustrated in *B*. *D,* D-LSD (10.0 μM) fails to inhibit the binding of ^{3}H-5-OHIP in a section serial to that illustrated in *C*.

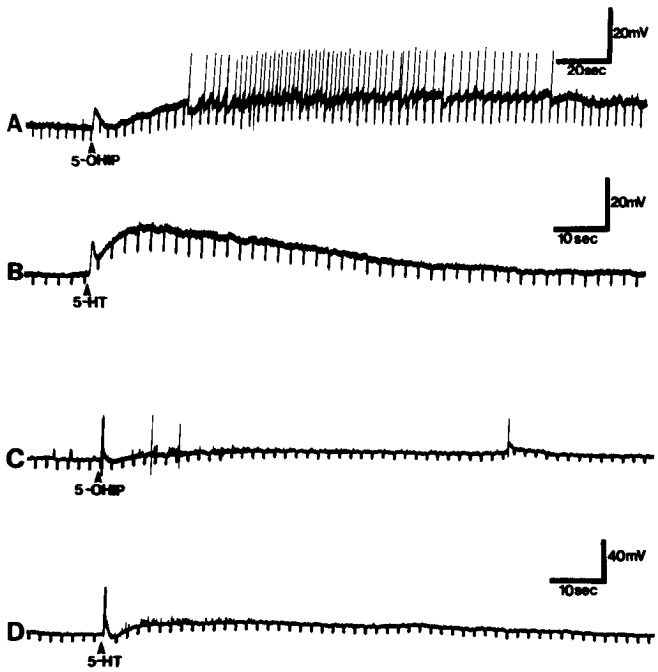

Figure 5. Comparison of the effects of 5-OHIP and 5-HT on type II/AH neurons of the myenteric plexus of the guinea pig small intestine. Membrane potential of 3 type II/AH neurons is illustrated. Constant hyperpolarizing current pulses were injected through the recording microelectrode. *A,* Response to the microejection of 5-OHIP. There is a transient depolarization (the fast response), a partial recovery of the membrane potential, followed by a prolonged depolarization (the slow response). Note the increase in input resistance during the slow response to 5-OHIP. The neuron is hyperexcitable and spikes repetitively during the slow response. The resting membrane potential was −67 mV. *B,* Response of a second myenteric type II/AH neuron to the microejection of 5-HT. As after the microejection of 5-OHIP, there is a transient depolarization, a partial recovery of the membrane potential, followed by a prolonged depolarization. Note that there is also an increase in input resistance during the slow response to 5-HT. The resting membrane potential was −72 mV. *C, D,* Responses of the same myenteric neuron, first to the microejection of 5-OHIP (*C*), then to 5-HT (*D*). Note the similarity of the response of the cell to each agent. The resting membrane potential was −72 mV.

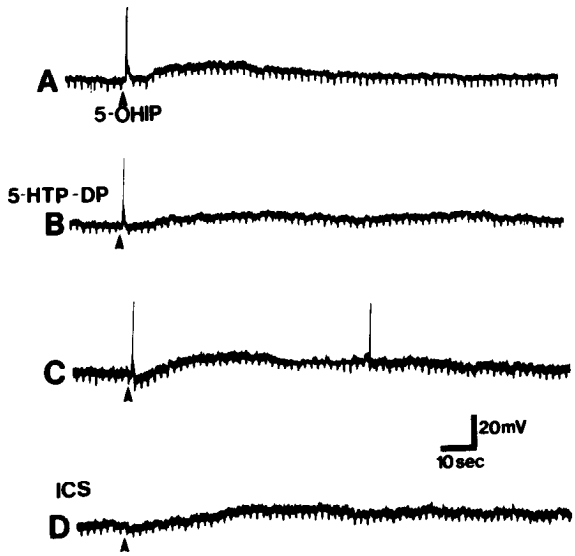

Figure 6. Comparison of the effects of 5-HTP-DP and ICS 205-930 on responses of a type II/AH neuron to 5-OHIP. Constant hyperpolarizing current pulses were ejected through the recording microelectrode. *A,* Control response to 5-OHIP; both a fast and a slow response are evoked. *B,* Response to 5-OHIP in the presence of 5-HTP-DP (10.0 μM). The slow response to 5-OHIP is attenuated, but the fast response is unaffected. *C,* Response to 5-OHIP 20 min following washout of 5-HTP-DP. The effect of 5-HTP-DP is reversible. A fast and a slow response can again be seen. *D,* Response to 5-OHIP in the presence of ICS 205-930 (0.1 μM). The fast response is abolished, but a slow response can still be distinguished. The resting membrane potential was −75 mV.

of 5-HTP-DP (30 pulses, 1 sec each) had no effect on the fast response to 5-OHIP; however, the slow response to 5-OHIP was abolished in the presence of this antagonist (7/7 cells; Fig. 6). In contrast, the fast response to 5-OHIP was blocked by superfusion of the preparations with ICS 205-930 (0.1 μM), while ICS 205-930 (up to 1.0 μM) and had no effect on the slow response to 5-OHIP (13/13 cells; Fig. 7). The effects of these antagonists on responses to 5-OHIP thus are identical to their effects on responses to 5-HT (Mawe et al., 1986); again, the slow response to 5-HT is blocked specifically by 5-HTP-DP and the fast response is blocked specifically by ICS 205-930.

In order to determine whether 5-OHIP acts on receptors directly or indirectly through the release of 5-HT, the effects of 5-OHIP on preparations in which endogenous 5-HT had previously been depleted were studied. Depletion of endogenous 5-HT was accomplished by incubating preparations with tryptamine (20 μM) for 10 min prior to application of 5-OHIP. Tryptamine has been shown to release and deplete endogenous 5-HT from enteric neurons so that, within 4 min, 5-HT-mediated slow EPSPs, evoked by stimulation of interganglionic fiber tracts, are abolished (Takaki et al., 1985b). Receptors close to serotoninergic terminals may be transiently desensitized by the high concentration of released 5-HT in their microenvironment. Responses to microejection of 5-OHIP in preparations previously exposed to tryptamine, like those to 5-HT, were initially blocked; however, after about 30 min, responses to 5-OHIP recovered (6/6 cells; Fig. 8). Slow EPSPs do not recover in preparations that have been superfused with tryptamine because the tissue no longer contains a significant quantity of endogenous 5-HT (Takaki et al., 1985b). These observations suggest that the responses of myenteric type II/AH neurons to the microejection of 5-OHIP are direct effects and not secondary to the release of 5-HT.

Release of ^{3}H-5-HT by 5-OHIP

Although physiological responses to the application of 5-OHIP cannot be accounted for by a release of endogenous 5-HT, an additional effect of 5-OHIP of releasing 5-HT from endogenous stores cannot be excluded by physiological experiments alone. In order to evaluate directly the ability of 5-OHIP to release 5-HT, strips of longitudinal muscle with adherent myenteric plexus were preloaded by incubation *in vitro* with ^{3}H-5-HT (0.5 μM). Washout of tritium was then followed; when a relatively stable rate of efflux was achieved, the strips were challenged twice, once with 20 μM 5-OHIP and once with 20 μM tryptamine, with an interval of 20 min between challenges. Control tissue was manipulated identically, but was not exposed to 5-OHIP or tryptamine. Monoamine oxidase was inhibited. Under these conditions, radioactive metabolites of 5-HT (mainly 5-HT-O-glucuronide) wash out of tissue more rapidly than does ^{3}H-5-HT itself (Gershon and Ross, 1966). As a result, when ^{3}H-5-HT-loaded strips are challenged after 20 min of washout, most of the radioactive material remaining in the tissue is ^{3}H-5-HT.

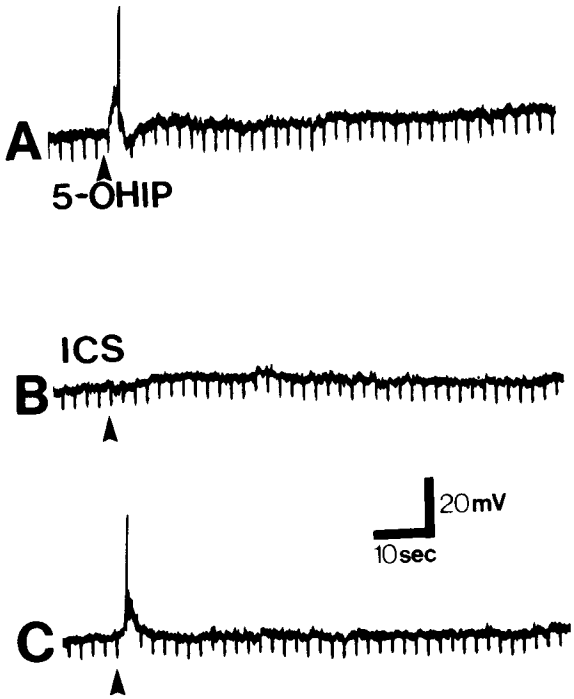

Figure 7. Reversibility of the effect of ICS 205-930 on the response of a type II/AH neuron to 5-OHIP. Constant hyperpolarizing current pulses were injected through the recording microelectrode. *A*, Control response to 5-OHIP; both a fast and a slow response are evoked. *B*, Response to 5-OHIP in the presence of ICS 205-930 (0.1 μM). The fast response to 5-OHIP is attenuated, but the slow response remains. *C*, Response to 5-OHIP 20 min following washout of ICS 205-930. The effect of ICS 205-930 is reversible. A fast response can again be seen. The resting membrane potential was −70 mV.

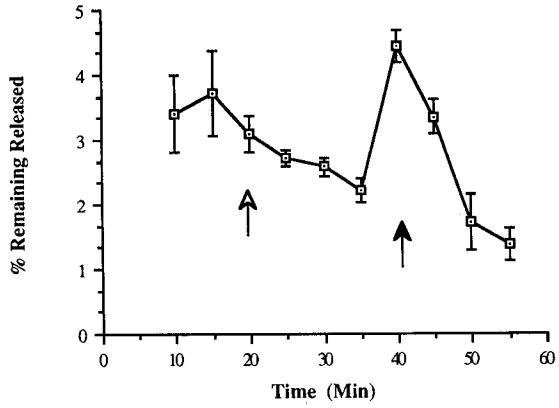

Figure 9. Effects of 5-OHIP and tryptamine on the release of ^{3}H-5-HT from strips of myenteric plexus adherent to longitudinal muscle from the guinea pig ileum. Tissue was preloaded with ^{3}H-5-HT (0.5 μM) and subjected to serial transfer through successive vials of oxygenated Krebs solution at 5 min intervals at 37°C. The percentage of radioactive material remaining in the tissue at the beginning of each interval that was released during that interval (% remaining released) was plotted as a function of time. 5-OHIP (20 μM) was present during the interval marked by the *open arrow*, and tryptamine (20 μM) during the interval marked by the *solid arrow*. Each value is the mean ± SE of 5 tissues.

In contrast to tryptamine, 5-OHIP did not significantly alter the rate of release of ^{3}H-5-HT (Fig. 9).

Uptake of ^{3}H-5-HT

The structure–activity requirements for compounds to be active at 5-HT$_{1P}$ receptors (Branchek et al., 1984a; Gershon et al., 1985; Mawe et al., 1986) are similar to those that have previously been reported in order to inhibit the specific high-affinity uptake of ^{3}H-5-HT (Gershon et al., 1976). For this reason, the effect of 5-OHIP on the specific uptake of ^{3}H-5-HT was studied and compared with the effects on the uptake of ^{3}H-5-HT of 6-OHIP and indalpine (the nonhydroxylated parent compound). In addition, the abilities of the 5-HT$_{1P}$ antagonist 5-HTP-DP and the 5-HT$_3$ antagonist ICS 205-930 were also evaluated. Inhibition of ^{3}H-5-HT uptake by the known competitive inhibitor of 5-HT uptake, zimelidine (Takaki et al., 1985b), served as a standard of reference. All of the tested compounds (10 μM) inhibited the specific uptake of ^{3}H-5-HT ($p < 0.001$; Fig. 10). Indalpine was more potent (at this concentration) than its hydroxylated derivatives ($p < 0.005$). Both receptor antagonists inhibited ^{3}H-5-HT uptake, although ICS 205-930 was more potent than 5-HTP-DP ($p < 0.05$) and equal in potency to indalpine. Moreover, ICS 205-930 also inhibited the uptake of ^{3}H-5-HT at 1 μM (to 50% of control), while 5-HTP-DP did not. Zimelidine was significantly more effective in antagonizing the uptake of ^{3}H-5-HT than were any of the other compounds tested.

Localization of sites of ^{3}H-5-OHIP in tissues other than gut

Radioautography was used to look for the presence of specific ^{3}H-5-OHIP and ^{3}H-5-HT binding sites in heart, skin, and brain. Specific binding, both of ^{3}H-5-HT and ^{3}H-5-OHIP, was found in the heart (Fig. 11). The anatomical localization of the binding sites of each radioligand was similar. Sites of specific binding were limited to the endocardial–myocardial and pericardial–myocardial junctions. The sites appeared as punctate accumu-

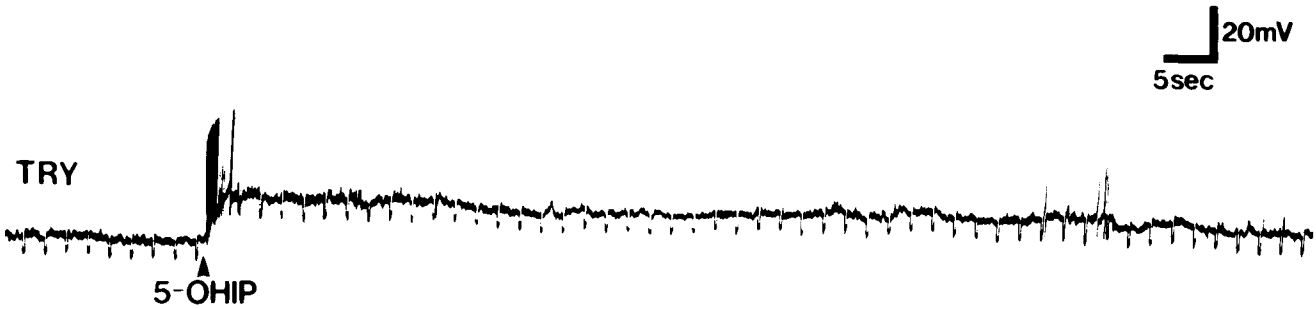

Figure 8. Effect of incubation with tryptamine (20 μM) to deplete endogenous 5-HT on the responses of type II/AH neurons to 5-OHIP. Constant hyperpolarizing current pulses were injected through the recording microelectrode. The preparation was incubated with tryptamine for 45 min. Microejection of 5-OHIP evoked a fast and a slow response. The resting membrane potential was −70 mV.

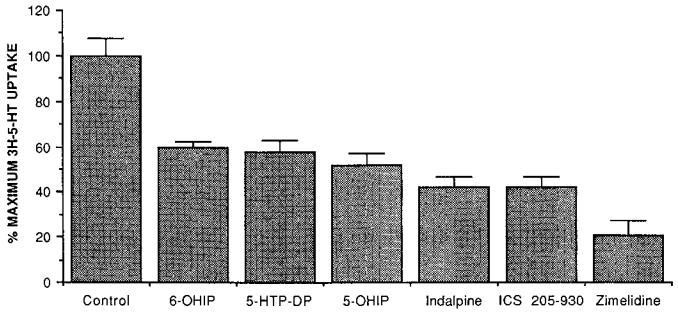

Figure 10. Effect of inhibitors on the myenteric uptake of ^{3}H-5-HT. Uptake of ^{3}H-5-HT by strips of myenteric plexus adherent to longitudinal muscle from the guinea pig ileum was measured. Uptake was measured as nmol/gm per minute and expressed for each condition as a percentage of the control uptake of ^{3}H-5-HT in the absence of potential inhibitors. The accumulation of ^{3}H-5-HT at 4°C was used to estimate the nonspecific uptake of the amine and was subtracted from the amount of ^{3}H-5-HT accumulated at 37°C to give the specific uptake of ^{3}H-5-HT. Means ± SE of 20 tissues for each condition. All drugs were evaluated at 10.0 μM.

lations ("hot spots") of radioautographic silver grains. Many of the "hot spots" in the pericardial region were perivascular in location. When the tissue sections (from which the radioautographs were prepared) were stained with Bodian's silver, nerve fibers were found to have been located under each of the sites of ^{3}H-5-HT or ^{3}H-5-OHIP labeling; however, the resolution of the radioautographic technique was inadequate to establish whether or not these nerve fibers were the labeled structures. Binding of both ^{3}H-5-HT and ^{3}H-5-OHIP (10 nM) in the heart was antagonized by 5-HT, 5-OHIP, 5-HTP-DP, 6-OHIP, and 2-methyl-5-HT (10 μM). Binding of neither ^{3}H-5-HT nor 5-OHIP was affected by 8-OH-DPAT, ketanserin, methysergide, ICS 205-930, phentolamine, propranalol, spiroperidol, 5-methoxytryptamine, or indalpine. Cardiac ^{3}H-5-HT and ^{3}H-5-OHIP binding sites thus resemble enteric binding sites for these radioligands.

Radioautographic localization of ^{3}H-5-HT and ^{3}H-5-OHIP binding sites in the skin (perianal and flank) showed a similar distribution for each radioligand (Fig. 12). Punctate labeling was seen in the dermis, and was especially heavy near hair follicles and sebaceous glands. Subsequent staining of the slides from which the radioautographs had been prepared, using Bodian's silver, again revealed many nerve fibers in the most heavily labeled regions; nevertheless, the radioautographic resolution was not adequate to establish whether or not these nerve fibers were labeled. As is true of gut and heart, binding of ^{3}H-5-HT and ^{3}H-5-OHIP was antagonized by excess (10 μM) nonradioactive 5-HT and 5-HTP-DP, but not by ICS 205-930 or D-lysergic acid diethylamide (D-LSD). The binding sites thus are not 5-HT$_3$, 5-HT$_1$, or 5-HT$_2$ receptors, but instead had characteristics of 5-HT$_{1P}$ receptors.

In the brain, ^{3}H-5-OHIP binding sites were found radioautographically in a number of different locations. The highest relative densities of binding sites were found in hippocampus, entorhinal cortex, lateral amygdaloid nucleus, ventromedial and mammillary nuclei of the hypothalamus, anterior and central thalamic nuclei, nucleus accumbens, caudate nucleus, medial and lateral septal nuclei, substantia nigra, interpeduncular nucleus, periaqueductal gray matter, and the superficial layer of the frontal cortex. ^{3}H-5-HT binding sites were found in all of the same locations; however, in contrast to binding of ^{3}H-5-OHIP in peripheral tissues, binding of ^{3}H-5-OHIP to sites in the brain could be displaced, not only by excess nonradioactive 5-HT and 5-HTP-DP, but also by ICS 205-930 and D-LSD. These results indicate that ^{3}H-5-OHIP is not a selective label for the 5-HT$_{1P}$ receptor in the brain.

Discussion

Two different types of neural 5-HT receptor, 5-HT$_{1P}$ and 5-HT$_3$ (5-HT$_{2P}$), have been found in the enteric nervous system (Mawe et al., 1986). These receptors mediate different effects on myenteria type II/AH neurons and differ in their affinity for 5-HT. Until recently, the only probe available for labeling 5-HT$_{1P}$ receptors was ^{3}H-5-HT itself. This restriction has led to some uncertainty in the interpretation of results, because it has been reported that oxidation of ^{3}H-5-HT can lead, under nonideal conditions of assay, to the formation of an unknown radioactive product that saturably binds to tissue and is displaced by excess nonradioactive 5-HT (Peroutka et al., 1986). Binding of this product can give rise to artifactual results that might be misinterpreted as binding to 5-HT receptors. In order to identify 5-HT$_{1P}$ receptors with certainty, therefore, it would be useful to be able to do so with another radioligand, the binding of which could be compared with that of ^{3}H-5-HT. Neither ^{3}H-D-LSD nor ^{3}H-DPAT is a suitable radioligand for 5-HT$_{1P}$ receptors because neither compound has an affinity for this type of 5-HT receptor (Branchek et al., 1984a; Branchek and Gershon, 1987). The 5-HT$_{1P}$ receptor antagonist 5-HTP-DP has been tritiated and an attempt was made to use ^{3}H-5-HTP-DP as a radioligand for the study of the distribution of 5-HT$_{1P}$ receptors; however, this attempt was confounded because ^{3}H-5-HTP-DP has a relatively low affinity for the 5-HT$_{1P}$ receptor and binds nonspecifically to membranes because ^{3}H-5-HTP-DP is hydrophobic (unpublished observations). Hydroxylated indalpines have been found to be agonists at 5-HT$_{1P}$ receptors and are potent competitors of the binding of ^{3}H-5-HT to enteric membranes (Mawe et al., 1986). We thus reasoned that ^{3}H-5-OHIP might be a good radioligand with which to study the distribution and properties of 5-HT$_{1P}$ receptors in the bowel and elsewhere in the body. Although 6-OHIP was found to be a specific agonist at 5-HT$_{1P}$ receptors, 6-OHIP is less stable chemically than is 5-OHIP. Moreover, unlike ^{3}H-5-HTP-DP, ^{3}-5-OHIP is hydrophilic; therefore, assays of its binding are less confounded by nonspecific adsorption by membranes.

^{3}H-5-OHIP was found to bind saturably, reversibly, and with high affinity to enteric membranes. Its K_d for binding, 7.6 nM, is significantly greater than that of ^{3}H-5-HT (2.7 nM; Branchek et al., 1984a; $p < 0.001$); nevertheless, the B_{max} is not significantly different when ^{3}H-5-OHIP (76 fmol/mg protein) is used as a radioligand instead of ^{3}H-5-HT (92 fmol/mg protein). Thus, although ^{3}H-5-HT has a slightly higher affinity for enteric binding sites than does ^{3}H-5-OHIP, both ligands appear to recognize the same number of sites in preparations of enteric neural membranes. Furthermore, since 5-HT competes with ^{3}H-5-OHIP and 5-OHIP competes with ^{3}H-5-HT, it is probable that these radioligands label the same binding sites.

Examination of the ability of agents known to have affinities for a variety of receptors to displace bound ^{3}H-5-OHIP indicates that the sites in enteric membranes to which this radioligand binds have the same specificity as do 5-HT$_{1P}$ receptors. For example, binding of ^{3}H-5-OHIP, like that of ^{3}H-5-HT to 5-HT$_{1P}$ receptors, can be displaced by the 5-HT$_{1P}$ receptor antagonist 5-HTP-DP, but not by the 5-HT$_3$ receptor antagonist ICS 205-

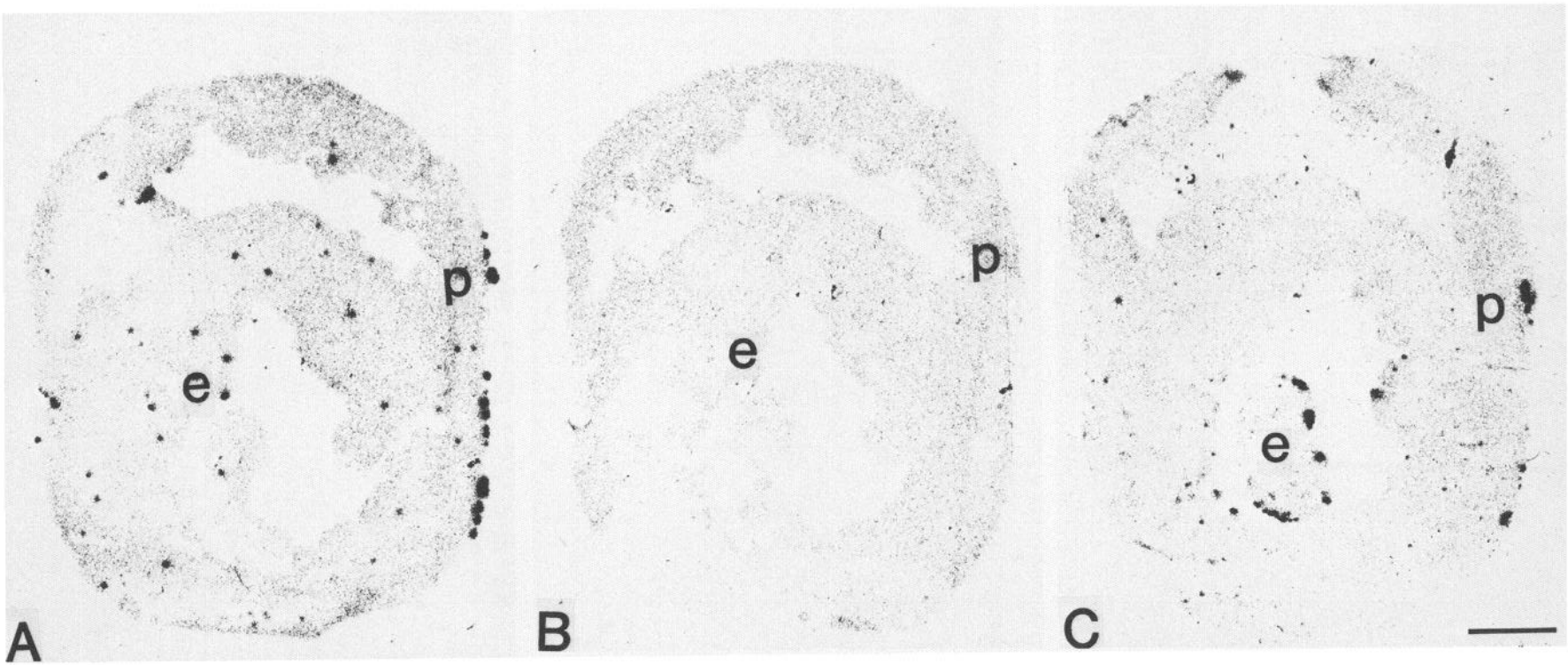

Figure 11. Radioautographs comparing the distribution of ^{3}H-5-OHIP binding sites in the guinea pig heart with those of ^{3}H-5-HT. *A*, ^{3}H-5-OHIP (10.0 nM) binding. *B*, Control. A section serial to that illustrated in *A* was incubated with ^{3}H-5-OHIP (10.0 nM) in the presence of 5-HTP-DP (10.0 μM). Note the complete displacement of ^{3}H-5-OHIP binding from both pericardial (*p*) and endocardial (*e*) accumulations of binding sites ("hot spots") by 5-HTP-DP. *C*, ^{3}H-5-HT (10.0 nM) binding in a another section of guinea pig heart. Note that binding sites for both ligands are found as "hot spots" in pericardial (*p*) and endocardial (*e*) regions, and that the patterns of labeling are indistinguishable. Bar, 2.0 mm.

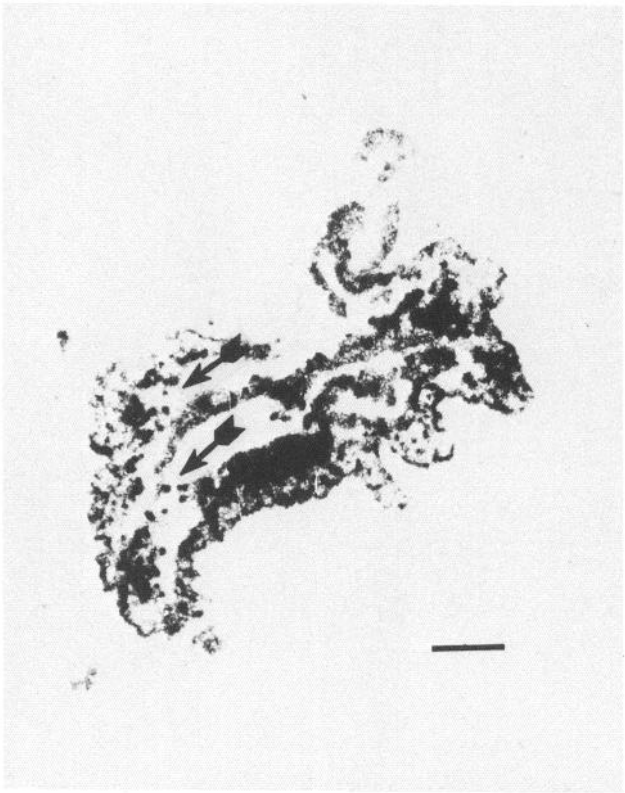

Figure 12. A radioautograph showing the binding of ^{3}H-5-OHIP (10.0 nM) to sites in the perianal skin of a mouse. Labeling can be seen in local accumulations. These accumulations occur in dermal and hypodermal connective tissue, especially near hair follicles (*arrows*). Bar, 1.0 mm.

930. Furthermore, again like the binding of ^{3}H-5-HT, ^{3}H-5-OHIP binding is inhibited by indolic compounds that have an unsubstituted hydroxyl group on the indole ring, including 5-HT, 5-HTP-DP, 6-OHIP, and 5-OHIP itself, but not by indoles that lack such a moiety, including tryptamine, 5-methoxytryptamine, ICS 205-930, and the parent compound, indalpine. Moreover, substances (see list in Table 1) that bind to α- or β-adrenoceptors, dopamine, muscarinic, nicotinic, histamine (H1 and H2), 5-HT$_1$, or 5-HT$_2$ receptors, as well as to the transporter responsible for high-affinity transmembrane uptake of 5-HT, all fail to inhibit the binding of ^{3}H-5-OHIP to enteric membranes. Further evidence that ^{3}H-5-OHIP binds to 5-HT$_{1P}$ receptors comes from the radioautographic localization of ^{3}H-5-OHIP binding sites in the gut. These sites have the same distribution as do 5-HT$_{1P}$ receptors localized with ^{3}H-5-HT, and are found in the enteric ganglia and in regions of the lamina propria of the mucosa that overlie nerve fibers. Not only are the anatomical locations of ^{3}H-5-OHIP binding sites the same as those previously determined to be 5-HT$_{1P}$ receptors, but displacement of ^{3}H-5-OHIP from radioautographically detectable binding sites, like that of ^{3}H-5-HT, can be accomplished only by compounds that act as agonists or antagonists at 5-HT$_{1P}$ receptors. In fact, the same types of compounds that inhibit the binding of ^{3}H-5-OHIP to isolated enteric membranes are able to antagonize radioautographic labeling of enteric sites in frozen sections of gut, and no compound that was unable to block the binding of ^{3}H-5-OHIP to isolated membranes was able to do so in a radioautographic assay. It can be concluded, therefore, that the radioautographic and rapid filtration assays of ^{3}H-5-OHIP binding measure binding to the same sites, that these sites have pharmacological properties and an anatomical distribution that is quite similar to those of ^{3}H-5-HT binding sites, and that these sites have characteristics of 5-HT$_{1P}$ receptors.

The properties of 5-OHIP were further investigated physiologically in order to test the hypothesis that high-affinity ^{3}H-5-OHIP binding sites are 5-HT$_{1P}$ receptors. 5-OHIP was found to mimic the actions on myenteric type II/AH neurons of 5-HT. The compound, like 5-HT, was able to induce both a long-lasting depolarization associated with an increase (slow response) and a short-lived depolarization associated with a decrease (fast response) in input resistance. Not every cell displayed both types of response; however, in those cells in which both compounds could be tested, the effects of the application of 5-OHIP and 5-HT were indistinguishable. Moreover cross-desensitization was found in responses to the 2 compounds; that is, once cells became desensitized to 5-HT, they no longer responded to 5-OHIP, and cells desensitized to 5-OHIP did not respond to 5-HT. Slow responses desensitized more quickly than did fast responses for both compounds. Finally, the slow response to each agonist was specifically inhibited by 5-HTP-DP, and the fast response to each was antagonized by ICS 205-930. These observations are compatible with the conclusion that 5-OHIP and 5-HT are both agonists at 5-HT$_{1P}$ (slow response antagonized by 5-HTP-DP) and 5-HT$_3$ (fast response antagonized by ICS 205-930) receptors. An alternative possibility, however, is that 5-OHIP might release endogenous 5-HT from enteric nerve terminals. Such an effect, similar to that of tryptamine (Takaki et al., 1985b), would mimic responses to exogenous 5-HT and would also exhibit apparent cross-desensitization.

In order to investigate the possibility that the action of 5-OHIP is indirect, mediated by released 5-HT, its action was studied in preparations in which endogenous 5-HT had been depleted prior to the application of 5-OHIP. This was accomplished by preincubating tissue with tryptamine at a concentration and for a time previously found to empty enteric neural stores of 5-HT. Elimination of slow EPSPs, which are mediated by 5-HT, served to monitor the degree of 5-HT depletion. Initially, incubation with tryptamine was found to inhibit responses to 5-OHIP, an effect also observed when responses to 5-HT were assayed. This effect, which was transient, has been attributed to the desensitization of 5-HT receptors secondary to the outpouring of 5-HT from nerve terminal induced by tryptamine (Takaki et al., 1985b); thus, although slow EPSPs are permanently abolished following incubation with tryptamine, responses to 5-HT and 5-OHIP return in parallel. Moreover, direct experiments in which the release of preloaded ^{3}H-5-HT from enteric nerve terminals was followed showed that 5-OHIP was unlike tryptamine, and was unable to release ^{3}H-5-HT. These experiments demonstrate that responses to 5-OHIP could not have been mediated indirectly by endogenous 5-HT. It is concluded that 5-OHIP is an agonist that acts directly on 5-HT receptors; however, it has a relatively broad spectrum, like that of 5-HT itself, and can act both at 5-HT$_{1P}$ and 5-HT$_3$ receptors.

It is of interest that neither the binding of ^{3}H-5-HT nor of ^{3}H-5-OHIP is antagonized by ICS 205-930; thus, although both compounds act at 5-HT$_3$ receptors, neither labels these sites in enteric membranes. It can be concluded, therefore, that 5-HT$_3$ receptors, like 5-HT$_2$ receptors (Pazos et al., 1985), have a low binding affinity for agonists and do not interfere with assays of 5-HT$_{1P}$ receptors that employ agonists as radioligands. The low

affinity of 5-HT_2 receptors for ^{3}H agonists probably also explains why neither ^{3}H-5-HT nor ^{3}H-5-OHIP radioautographically labels intestinal smooth muscle cells.

5-OHIP was found to have one additional action. The compound was a moderately potent inhibitor of the uptake of ^{3}H-5-HT by enteric nerve terminals. Nevertheless, it is doubtful that this action explains the effects of 5-OHIP on myenteric neurons or its binding to enteric membranes or frozen sections. The potency of compounds as inhibitors of 5-HT uptake does not correlate either with their ability to mimic the actions of 5-HT at enteric 5-HT_{1P} or 5-HT_3 receptors or with their ability to interfere with the binding of ^{3}H-5-HT or ^{3}H-5-OHIP. Indalpine, for example, is more potent than 5-OHIP as a ^{3}H-5-HT uptake inhibitor, yet it has no effect on the binding of ^{3}H-5-HT or ^{3}H-5-OHIP. Moreover, the most potent and specific inhibitor of the uptake of ^{3}-5-HT known, zimelidine, also fails to affect the binding of either radioligand. It is of interest that both of the antagonists, 5-HTP-DP and ICS 205-930, that act at enteric neural 5-HT receptors (of either type) also inhibit uptake of ^{3}H-5-HT. These compounds are quite different in structure from one another and from 5-OHIP. Since both agonists (5-OHIP, 6-OHIP, and 2-methyl-5-HT) and antagonists at 2 types of 5-HT receptor also affect transmembrane uptake of ^{3}H-5-HT, it is possible that the transporter for 5-HT in enteric neural membranes is similar, but not identical, in structure to 5-HT_{1P} and 5-HT_3 receptors.

Although 5-OHIP is an agonist at both types of enteric neural 5-HT receptor, ^{3}H-5-OHIP can still be used as a probe with which to label selectively 5-HT_{1P} receptors outside the CNS. It binds with high affinity only to 5-HT_{1P} sites, and thus the simultaneous presence of 5-HT_3 or 5-HT_2 (previously called "D"; Gaddum and Picarelli, 1957) receptors does not interfere with assays of 5-HT_{1P} receptors that use ^{3}H-5-OHIP as a radioligand. Moreover, its binding is also resistant to displacement by drugs that have activity at the CNS type of 5-HT_1 receptors. By using ^{3}H-5-OHIP as a probe, we were able to detect apparent 5-HT_{1P} receptors in the skin and the heart, as well as in the gut. The heart was selected for study because peripheral neural 5-HT receptors have been reported to be highly concentrated in cardiac tissue (Humphrey, 1983; Humphrey et al., 1983; Fozard, 1984; Richardson et al., 1985). The skin was selected because it has previously been found to have specific ^{3}H-5-HT binding sites (Branchek et al., 1984b; Branchek and Gershon, 1987). Since the distribution of ^{3}H-5-OHIP binding sites in these organs was identical to that of ^{3}H-5-HT and had the same properties as 5-HT_{1P} receptors in the gut, it is likely that the ^{3}H-5-OHIP binding sites in the heart and skin are 5-HT_{1P} receptors. There is evidence that there are 5-HT receptors on primary afferent nerves in the skin. 5-HT is a potent activator of pain fibers when applied to the base of blisters in humans (Armstrong et al., 1953, 1957). Since pain can be alleviated by administration of ICS 205-930, some of these receptors may be the 5-HT_3 subtype; however, the distribution of ^{3}H-5-OHIP binding sites in proximity to hair follicles and sebaceous glands, where afferent nerves are concentrated, suggests that there are 5-HT_{1P} receptors on nerves as well. This hypothesis receives some support from the high concentration of 5-HT_{1P} receptors in the lamina propria of the gut. This is the location of the primary afferent nerve terminals in the bowel that are responsible for the mediation of the peristaltic and other enteric reflexes, and is the site where application of 5-HT initiates the peristaltic reflex (Bülbring and Crema, 1958; Bülbring and Lin, 1958). The distribution of ^{3}H-5-OHIP binding sites in skin and gut is consistent with the idea that several types of primary afferent nerve fibers have 5-HT_{1P} receptors on their surface. Such a location of 5-HT_{1P} receptors would also explain the distribution of ^{3}H-5-OHIP binding sites in the heart. Many nerve fibers are found underlying the endocardium and epicardium. Clearly, ^{3}H-5-OHIP does not bind to connective tissue structures, which are also located in the zones of concentration of ^{3}H-5-OHIP binding sites; nevertheless, a neural location of cardiac and skin 5-HT_{1P} receptors remains to be confirmed.

The brain was examined to determine whether ^{3}H-5-OHIP binding sites are limited in their distribution to the PNS or whether they also occur in the CNS. Binding of ^{3}H-5-OHIP to sites in the CNS was observed. This binding has been found to be saturable, reversible, and of high affinity ($K_d \sim 5$ nM; A. Doble, personal communication). Moreover, the brain regions that were found to display the highest levels of ^{3}H-5-OHIP binding are sites also known to be rich in 5-HT_{1A} and especially 5-HT_{1B} receptors (Meibach, 1984; Pazos and Palacios, 1985; Pazos et al., 1985). On the other hand, since ^{3}H-5-OHIP binding was antagonized by D-LSD and ICS 205-930, which do not do so in the PNS, the ^{3}H-5-OHIP binding sites in the brain are not simply 5-HT_{1P} receptors. Since no regions of the brain were found that bound ^{3}H-5-OHIP but not ^{3}H-5-HT, it seems likely that all of the CNS ^{3}H-5-OHIP binding sites are one or more of the subtypes of the 5-HT_1 receptor category. ^{3}H-5-OHIP thus appears to label central 5-HT_1 receptors of many, if not all, subtypes, and cannot be used, by itself, to locate 5-HT_{1P} receptors (if they are present) in the CNS. It is still possible that receptors similar to the 2 types of peripheral neural 5-HT receptor exist in the brain, along with central 5-HT receptors to which ^{3}H-5-OHIP binds, but which are not present in the periphery. For example, 5-HTP-DP (a 5-HT_{1P} antagonist), but not methysergide, ketanserin, or naloxone, can block 5-HT-induced pain volleys recorded from thalamic nuclei in rats (Emmers et al., 1987). ICS 205-930 (a 5-HT_3 antagonist) also has central actions. For example, intracerebral injections of ICS 205-930, but not of methysergide or ritanserin, into the hypothalamus enhance gastric emptying in the guinea pig (Costall et al., 1986a). In addition, intravenous administration of ICS 205-930 has been shown to block cisplatin-induced emesis in the ferret (Costall et al., 1986b). Finally, a new class of 5-HT binding site in the brain has been found which is neither a 5-HT_1 nor a 5-HT_2 receptor, and which has many properties, such as displacement by bufotenin, but not by D-LSD, that are similar to those of 5-HT_{1P} receptors (Robaut et al., 1985). The hypothesis that 5-HT_{1P} and 5-HT_3 receptors are present in the CNS needs further study.

Appendix

Ability of compounds to compete with ^{3}H-5-OHIP for binding

Serotinin receptors	
5-HT_{1P}	
5-HT	(K_i = 44.0 nM)
5-OHIP	(K_i = 7.6 nM)
6-OHIP	(K_i = 4.4 nM)
5-HTP-DP	(K_i = 44.0 nM)
2-Methyl-5-HT	(K_i = 44.0 nM)
5-HT_{2P} or "5-HT_3"	
ICS 205-930[a]	
MDL 72222[a]	

5-HT_1
- 8-OH-DPAT[a]
- D-LSD[a]
- Spiroperidol[a]

5-HT_2
- Ketanserin[a]
- D-LSD[a]
- Methysergide[a]
- Spiroperidol[a]

5-HT uptake inhibitors
- Fluoxetene[a]
- Zimelidine[a]
- Trazodone[a]
- Indalpine[a]

Catecholamine receptors
- Phentolamine (α)[a]
- Propranalol (β)[a]
- Spiroperidol (DA)[a]

Cholinergic receptors
- Atropine (muscarinic)[a]
- Hexamethonium (nicotinic)[a]

Histamine receptors
- Cyproheptadine[a]
- Cimetidine[a]

Inactive indoles
- 5-Methoxytryptamine[a]
- Tryptamine[a]
- Indalpine[a]
- ICS 205-930[a]

The compounds listed above were tested for their ability to compete with ^{3}H-5-OHIP (10 nM) for binding in both rapid filtration assays and in radioautographic studies.

[a] K_i > 500 nM.

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