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Coexistence of Contractile and Relaxant 5-Hydroxytryptamine Receptors Coupled to Distinct Signaling Pathways in Intestinal Muscle Cells: Convergence of the Pathways on Ca^{2+} Mobilization

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SUMMARY

Muscle cells were dispersed separately from circular and longitudinal muscle layers of guinea pig intestine, and 5-hydroxytryptamine (5-HT) receptors were characterized in naive cells and in cells in which one receptor type was preserved by selective receptor protection. In naive cells from both regions, 5-HT caused contraction and stimulated increases in cytosolic free calcium concentration ($[Ca^{2+}]_i$) (3-fold; $p < 0.01$) and cAMP levels (40–60%; $p < 0.01$) that were inhibited, respectively, by the 5-HT_2 antagonist ketanserin and the 5-HT_{1p} antagonist *N*-acetyl-5-hydroxytryptophyl 5-hydroxytryptophan amide (5-HTP-DP). In circular muscle cells, where agonist-induced increase in $[Ca^{2+}]_i$ is mediated by Ca^{2+} release from inositol (1,4,5)trisphosphate-sensitive stores, 5-HT caused an increase in inositol (1,4,5)trisphosphate levels that was inhibited by ketanserin. In cells maximally contracted with a non-5-HT agonist (cholecystokinin octapeptide), 5-HT caused relaxation when the contractile effect mediated by 5-HT_2 receptors was blocked with ketanserin; relaxation and the concomitant increase in cAMP were inhibited by 5-HTP-DP. The singular contributions of the Ca^{2+} and cAMP signaling pathways were identified in cells where only one receptor type was preserved. In cells with only 5-HT_2 receptors, 5-HT caused contraction and an increase in $[Ca^{2+}]_i$ but not in cAMP levels; contraction and the increase in $[Ca^{2+}]_i$ were inhibited by ketanserin. Conversely, in cells with only 5-HT_{1p} receptors, 5-HT caused relaxation and an increase in cAMP levels but not in $[Ca^{2+}]_i$; relaxation and the increase in cAMP levels were inhibited by 5-HTP-DP. The two signaling pathways were functionally linked, converging to regulate the level of $[Ca^{2+}]_i$. Thus, the increase in $[Ca^{2+}]_i$ was augmented 1) when cAMP production was inhibited by 5-HTP-DP in naive cells or 2) when cAMP production was suppressed in cells where 5-HT_{1p} receptors were inactivated and only 5-HT_2 receptors were preserved. The results imply that the increase in cAMP levels mediated by 5-HT_{1p} receptors acted to attenuate the increase in $[Ca^{2+}]_i$ mediated by 5-HT_2 receptors. We conclude that the response to 5-HT in muscle cells is a compound effect involving activation of two receptor types coupled to distinct signaling pathways that converge on $[Ca^{2+}]_i$ as the determinant of mechanical activity.

Several serotonin (5-HT) receptors belonging to the family of guanine nucleotide-binding protein-coupled receptors have been cloned, including homologous 5-HT_2 and 5-HT_{1c} receptors coupled to phospholipase C and Ins(1,4,5)P_3-dependent mobilization of Ca^{2+} (1–4) and homologous 5-HT_{1a} and 5-HT_{1d} receptors coupled negatively to adenylate cyclase via a pertussis toxin-sensitive guanine nucleotide-binding protein (5–7). A 5-HT_4 receptor coupled positively to adenylate cyclase is present in collicular neurons and smooth muscle of rat esophagus but has not been cloned (8–11). A distinct neural receptor, 5-HT_3, belongs to the family of ligand-gated ion channels and is sensitive to low concentrations of the antagonist ICS205–930 (12–14).

Several of these receptors have been identified in the enteric nervous system by pharmacological, radioligand binding, and electrophysiological techniques. Among these are 5-HT_3, 5-HT_{1a}, and 5-HT_4 receptors (15–17) and a distinct receptor type, labeled 5-HT_{1p}, that is characteristically activated by hydroxylated indalpines and antagonized by 5-HTP-DP (15, 18, 19). Studies on intestinal muscle strips in the presence of the axonal conductance blocker tetrodotoxin suggest the presence of two

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 5-HTP-DP, *N*-acetyl-5-hydroxytryptophyl 5-hydroxytryptophan amide; ICS205–930, (3α-tropanyl)-1*H*-indole-3-carboxylic acid ester; NEM, *N*-ethylmaleimide; Rp-cAMPS, Rp isomer of adenosine-3′,5′-cyclic phosphorothioate; Ins(1,4,5)P_3, inositol (1,4,5)trisphosphate; CCK-8, cholecystokinin octapeptide; $[Ca^{2+}]_i$, cytosolic free calcium concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCK, cholecystokinin; $InsP_3$, inositol trisphosphate.

5-HT receptor types on muscle cells, a ketanserin-sensitive 5-HT_2 receptor that mediates contraction (20–22) and a distinct receptor type not yet fully characterized that mediates relaxation (23, 24). In rat esophageal muscle, the receptor type mediating relaxation and increase in cAMP appears to be 5-HT_4 (9, 10).

In the present study we have attempted to characterize the types of 5-HT receptors on intestinal smooth muscle cells and the signal transduction pathways to which they are coupled. Muscle cells were isolated separately from circular and longitudinal muscle layers of guinea pig intestine, and 5-HT receptors were characterized in naive muscle cells as well as in muscle cells in which only one receptor type was preserved by a technique of selective receptor protection (25, 26). The results indicate that two 5-HT receptor types coexist on intestinal muscle cells, a dominant 5-HT_2 receptor antagonized by ketanserin that mediates Ca^{2+}-dependent contraction and a 5-HT_{1p}-like receptor antagonized by 5-HTP-DP and by high concentrations of ICS205–930 that mediates cAMP-dependent relaxation. The 5-HT_{1p}-like receptor on intestinal muscle cells appears to be similar and may be identical to the 5-HT_4 receptor. The signal transduction pathways activated by the two receptors are linked to each other, converging to regulate the level of cytosolic Ca^{2+}.

Materials and Methods

Dispersion of intestinal muscle cells. Muscle cells were isolated separately from the longitudinal and circular muscle layers of guinea pig intestine as described previously (26, 27). Briefly, muscle strips were incubated at 31° for two successive 45-min periods in 15 ml of HEPES-buffered medium containing 0.1% collagenase (type II) and 0.01% soybean trypsin inhibitor. The partially digested muscle strips were washed free of enzyme, and spontaneously dispersed muscle cells were harvested by filtration through 500-μm Nitex mesh.

Measurement of contraction and relaxation in isolated muscle cells. The contractile response of isolated muscle cells was measured by scanning micrometry as described previously (26, 27). Aliquots of cell suspension (0.5 ml) containing 10^4 cells were incubated for 30 sec with test agents and the reaction was terminated after 30 sec with 1% acrolein. The length of 50 muscle cells was measured and contraction was expressed as percent decrease in cell length from control. Relaxation was measured in muscle cells maximally contracted with CCK-8 (1 nM) and the response was expressed as the decrease in maximal contraction.

Enrichment of muscle cells with specific 5-HT receptor types. The antagonists ketanserin (28) and 5-HTP-DP (19) were used as protective agents to enrich the muscle cells with one receptor type. As described previously (25, 26), muscle cells were treated with 0.1 μM ketanserin or 5-HTP-DP for 2 min at 31° and incubated for another 20 min with 5 μM NEM to inactivate all residual unprotected receptors. In some experiments, CCK receptors were also preserved by addition of 1 nM CCK-8 as protective agent. The treated muscle cells were centrifuged twice at 150 × *g* for 10 min to remove NEM and protective agents and were resuspended in fresh medium.

Measurement of Ins(1,4,5)P_3 by radioreceptor assay. Ins(1,4,5)P_3 levels were measured separately in suspensions of circular and longitudinal muscle cells as described previously (29), using Amersham's assay system, which utilizes ^{3}H-labeled *myo*-D-Ins(1,4,5)P_3 and bovine brain microsomes. One milliliter of cell suspension (10^6 cells/ml) was incubated with 10 mM LiCl for 10 min at 31°; agonists with or without antagonists were added for 30 sec, after which the reaction was stopped with 10% perchloric acid (v/v). After centrifugation for 10 min at 750 × *g*, the supernatant was extracted and Ins(1,4,5)P_3 content in the aqueous phase was measured. The results were expressed as pmol of Ins(1,4,5)P_3/10^6 cells or as percent increase above basal levels.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured separately in circular and longitudinal muscle cells as described previously, using the fluorescent dye fura-2/acetoxymethyl ester (30–32). The cells (10^6/ml) were incubated with fura-2/acetoxymethyl ester (2 μM) at 31° for 30 min, centrifuged, and resuspended in the same medium. Fluorescence was measured at 510 nm, with excitation wavelengths alternating between 340 and 380 nm. Autofluorescence of unloaded muscle cells was determined in each suspension and subtracted from fluorescence values of fura-2-loaded muscle cells. Ca^{2+} levels were calculated in the resting state and upon addition of agonists or antagonists from the ratio of observed, minimal, and maximal fluorescence, as described by Grynkiewicz *et al.* (30).

Measurement of cAMP. cAMP levels were measured separately in circular and longitudinal muscle cells as described previously (33). The cells were incubated for 10 min at 31° with 1 μM isobutylmethylxanthine and treated with test agents for 30 sec, and the reaction was terminated by addition of 6% (v/v) trichloracetic acid. The samples were centrifuged at 2000 × *g* for 10 min at 4° and the supernatant was extracted with saturated diethyl ether. The lyophilized samples were reconstituted for radioimmunoassay in 500 μl of 50 mM sodium acetate and were acetylated with tetraethylammonium/acetic anhydride (3:1 v/v) for 30 min.

Materials. CCK-8 was purchased from Bachem (Torrance, CA); 5-HTP-DP from Dr. Hadassah Tamir, Columbia College of Physicians and Surgeons (New York, NY); collagenase (type II) and soybean trypsin inhibitor from Worthington Biochemicals (Freehold, NJ); Ins(1,4,5)P_3 assay system from Amersham (Arlington Heights, IL); ^{125}I-cAMP from New England Nuclear (Cambridge, MA); ketanserin and ICS205–920 from Research Biochemicals Inc. (Natick, MA); Rp-cAMPS from Biolog (Bremen, Germany); and other chemicals from Sigma Chemical Co. (St. Louis, MO).

Statistics. Results were expressed as means ± standard errors of *n* cell samples derived from different animals. Statistical significance was evaluated using Student's *t* test for paired or unpaired values.

Results

Mechanical response of isolated muscle cells to 5-HT. 5-HT elicited concentration-dependent contraction of circular (EC_{50}, 50 ± 5 pM) and longitudinal (EC_{50}, 25 ± 5 pM) muscle cells (Fig. 1). Maximal contractile responses in the two cell

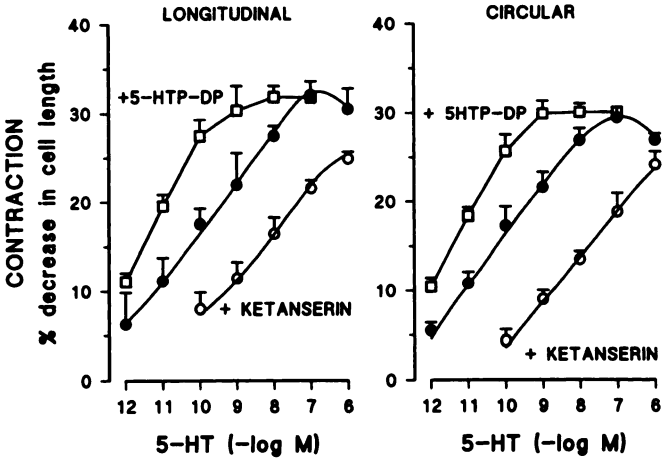

Fig. 1. Contraction induced by 5-HT in muscle cells isolated separately from the circular and longitudinal muscle layers of guinea pig intestine. Concentration-response curves were constructed for 5-HT alone (●) and in the presence of 1 μM ketanserin (○) or 1 μM 5-HTP-DP (□). Contraction is expressed as the percent decrease from resting cell length. Values are means ± standard errors of six to eight experiments.

types (29.2 ± 0.7% and 29.7 ± 1.0% decrease in cell length, respectively) were similar to those elicited by 1 nM CCK-8 (30.2 ± 0.7% and 31.9 ± 1.6%). The $5HT_2$ receptor antagonists ketanserin (1 μM) and spiperone (1 μM) inhibited the response to 5-HT, shifting the concentration-response curve to the right without altering maximal response. K_i values estimated from the lateral shifts of the curves were 3 and 4 nM for spiperone and 1 to 6 nM for ketanserin in the two cell types.

In contrast, 5-HTP-DP (1 μM), a specific antagonist of 5-HT_{1p} receptors (14), augmented contraction induced by 5-HT in both circular and longitudinal muscle cells, shifting the concentration-response curves to the left (Fig. 1). Augmentation of contraction by 5-HTP-DP implied that 5-HT activated additionally a 5-HT_{1p}-like receptor that mediates relaxation. ICS205-930, an antagonist of 5-HT_3 receptors at low concentrations (10 nM), had no effect on contraction induced by 5-HT (12).

Coexistence of two 5-HT receptor types on isolated muscle cells. Two approaches were used to determine the effects mediated by each receptor type. In the first approach, muscle cells were maximally contracted with CCK-8 and the effects mediated by each receptor type were determined by adding 5-HT in the presence of one or the other antagonist. In both muscle cell types, 5-HT alone did not augment maximal contraction induced by CCK-8; upon addition of ketanserin to block effects mediated by 5-HT_2 receptors, 5-HT inhibited the response to CCK-8, i.e., induced relaxation (Fig. 2). The relaxant effect of 5-HT was concentration dependent and was reversed by 5-HTP-DP (Fig. 3) but was not affected by pretreatment of the cells for 1 hr with 200 ng/ml pertussis toxin.

In the second approach, muscle cells were first enriched in one 5-HT receptor type by selective receptor protection. CCK and 5-HT_2 receptors were protected by incubating the cells with CCK-8 and ketanserin followed by inactivation of all unprotected receptors with NEM; the procedure yielded cells in which only CCK and 5-HT_2 receptors were preserved. A similar procedure using CCK-8 and 5-HTP-DP yielded cells in which only CCK and 5-HT_{1p} receptors were preserved.

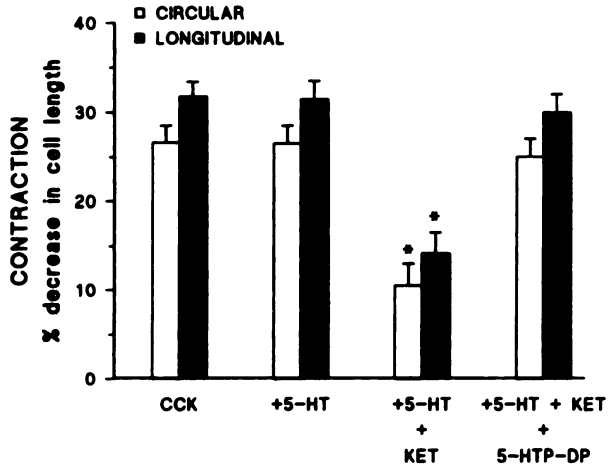

Fig. 2. Contractile and relaxant effects of 5-HT in circular and longitudinal muscle cells. From *left* to *right:* contraction in response to a maximal concentration of CCK-8 (1 nM) alone, CCK-8 with 5-HT (0.1 μM), CCK-8 with 5-HT and ketanserin (*KET*) (1 μM), and CCK-8 with 5-HT, ketanserin, and 5-HTP-DP (1 μM). Inhibition of CCK-induced contraction (i.e., relaxation) occurred upon addition of 5-HT with ketanserin and was reversed upon addition of 5-HTP-DP. Values are means ± standard errors of five to seven experiments. *, $p < 0.001$.

Circular or longitudinal muscle cells in which CCK and 5-HT_2 receptors were preserved retained their ability to contract in response to CCK-8 or 5-HT (Fig. 4). The magnitudes of the maximal responses to CCK-8, 5-HT, or a combination of 5-HT and CCK-8 were identical to those observed in untreated cells (Figs. 2 and 4). In treated cells, however, 5-HT in the presence of ketanserin did not inhibit the contractile response to CCK-8, consistent with the absence of 5-HT_{1p} receptors mediating relaxation (Fig. 4).

In contrast, circular or longitudinal muscle cells in which CCK and 5-HT_{1p} receptors were preserved retained their ability to contract in response to CCK-8 but not to 5-HT (Fig. 5). In these cells, 5-HT without ketanserin inhibited the contractile response to CCK-8 by 46 ± 6% ($p < 0.001$) in circular muscle cells and by 42 ± 5% ($p < 0.001$) in longitudinal muscle cells; the inhibition was completely reversed by 5-HTP-DP (Fig. 5). The pattern of response in these cells was consistent with the absence of 5-HT_2 receptors that mediate contraction and preservation of 5-HT_{1p} receptors that mediate relaxation. Cells treated with NEM without prior protection of receptors lost their response to all agonists but not to contractile agents, such as ionomycin and KCl, or relaxant agents, such as dibutyryl-cAMP and forskolin, that act by bypassing receptors (25, 26).

Signal transduction pathway mediating 5-HT-induced contraction. 5-HT increased $[Ca^{2+}]_i$ to the same extent in both cell types (Table 1). Ketanserin inhibited the increase in $[Ca^{2+}]_i$ by 90 ± 4% ($p < 0.001$), whereas 5-HTP-DP augmented the increase in $[Ca^{2+}]_i$ by 147 ± 5% ($p < 0.001$).

The increase in $[Ca^{2+}]_i$ induced by 5-HT was abolished by methoxyverapamil (1 μM) in longitudinal but not circular muscle cells (Fig. 6). This pattern of response, previously observed with other contractile agonists (31, 32), implied that the initial increase in $[Ca^{2+}]_i$ and contraction was mediated by Ca^{2+} release in circular muscle cells and Ca^{2+} influx in longitudinal muscle cells. Consistent with this notion, and as with other contractile agonists (29, 31, 32), 5-HT elicited an increase in $Ins(1,4,5)P_3$ levels in circular (6.4 ± 0.9 pmol/10^6 cells; $p < 0.01$) but not longitudinal (0.1 ± 0.2 pmol/10^6 cells) muscle cells. Ketanserin inhibited the increase in $Ins(1,4,5)P_3$ in circular muscle cells by 90% (0.6 ± 0.2 pmol/10^6 cells).

Signal transduction pathway mediating 5-HT-induced relaxation. 5-HT increased cAMP levels significantly in both cell types (Table 1). Ketanserin had no effect on the increase in cAMP, whereas 5-HTP-DP inhibited the increase in circular muscle cells by 72 ± 10% ($p < 0.01$) and abolished the increase in longitudinal muscle cells.

A pattern whereby 5-HTP-DP augmented contraction and $[Ca^{2+}]_i$ but inhibited relaxation and cAMP levels suggested that cAMP acting via cAMP-dependent protein kinase induced relaxation by causing a decrease in $[Ca^{2+}]_i$. This notion was tested directly with Rp-cAMPS, which suppresses protein kinase activity by inhibiting the dissociation of the catalytic and regulatory subunits of cAMP (34). In muscle cells that had been pretreated for 10 min with Rp-cAMPS (10 μM), relaxation of CCK-contracted cells induced by a combination of 5-HT and ketanserin was inhibited by 84 ± 1% in longitudinal and by 84 ± 2% in circular muscle cells ($p < 0.001$).

Signal transduction pathways in cells with one 5-HT receptor type. In muscle cells where only 5-HT_2 receptors were preserved, 5-HT retained the ability to increase $[Ca^{2+}]_i$ but not cAMP levels in both cell types (Table 2). Ketanserin

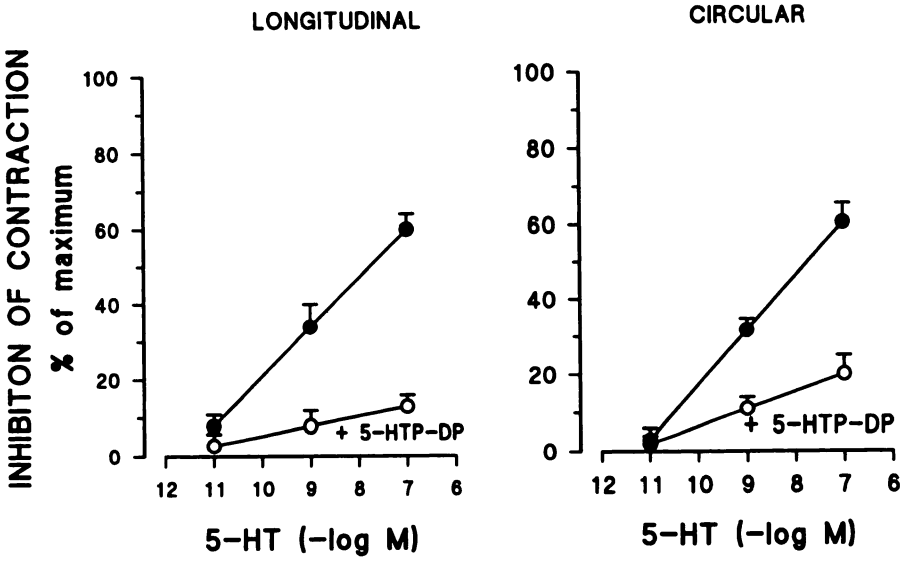

Fig. 3. Concentration-response curves for the relaxant effect of 5-HT in circular and longitudinal muscle cells. Relaxation induced by 5-HT in the presence of ketanserin (1 μM) was measured in cells maximally contracted with CCK-8 (1 nM) and is expressed as percent inhibition of contraction. Relaxation (●) was inhibited by 5-HTP-DP (1 μM) (○). Values are means ± standard errors of six or seven experiments.

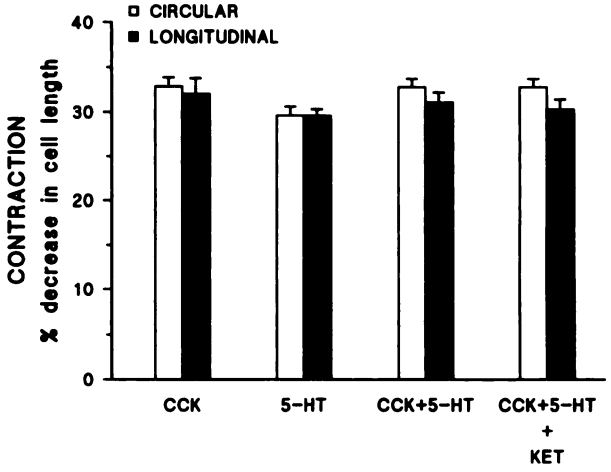

Fig. 4. Contraction of circular and longitudinal muscle cells in which 5-HT_2 and CCK receptors were preserved. From *left* to *right:* contraction in response to CCK-8 (1 nM), 5-HT (0.1 μM), CCK-8 and 5-HT, and CCK-8, 5-HT, and ketanserin (*KET*) (1 μM). Addition of ketanserin did not cause relaxation (i.e., inhibition of CCK-induced contraction). For comparison, see Fig. 2. Values are means ± standard errors of five or six experiments.

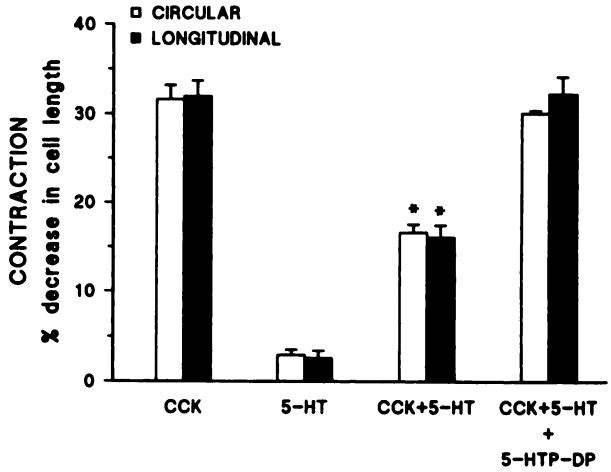

Fig. 5. Contraction and relaxation of circular and longitudinal muscle cells in which CCK- and 5-HTP-DP-sensitive receptors were preserved. From *left* to *right:* contraction in response to CCK-8 (1 nM), 5-HT (0.1 μM), CCK-8 and 5-HT, and CCK-8, 5-HT, and 5-HTP-DP (1 μM). Note virtual absence of contraction with 5-HT, inhibition of CCK-induced contraction by 5-HT without concomitant addition of ketanserin, and reversal of the inhibition by 5-HTP-DP. Values are means ± standard errors of four or five experiments. *, $p < 0.001$.

inhibited the increase in $[Ca^{2+}]_i$ by 84–95% ($p < 0.001$), whereas 5-HTP-DP had no effect. 5-HT also retained the ability to increase $InsP_3$ levels in circular muscle cells (4.0 ± 0.5 pmol/10^6 cells; $p < 0.01$); the $InsP_3$ response was abolished by ketanserin. The increase of $[Ca^{2+}]_i$ in longitudinal but not circular muscle cells was abolished by methoxyverapamil.

The magnitude of the increase in $[Ca^{2+}]_i$ was significantly greater than that in untreated muscle cells exposed to 5-HT alone but similar to that in untreated muscle cells exposed to a combination of 5-HT and 5-HTP-DP (see Tables 1 and 2). Thus, eliminating the influence of 5-HT_{1p} receptors by inactivation with NEM or by blockade with a specific antagonist (5-HTP-DP) unmasked the potent singular influence of the 5-HT_2 receptor on $[Ca^{2+}]_i$.

In muscle cells where only 5-HT_{1p} receptors were preserved, 5-HT retained the ability to increase cAMP levels but not $[Ca^{2+}]_i$ in both cell types (Table 3). The ability of 5-HT to increase $InsP_3$ levels in circular muscle cells was also lost (0.2 ± 0.5 pmol/10^6 cells; not significant). 5-HTP-DP abolished the increase in cAMP, whereas ketanserin had no effect.

In muscle cells where both CCK and 5-HT_{1p} receptors were preserved, CCK-8 caused an increase in $[Ca^{2+}]_i$ (longitudinal, 370 ± 37 nM, $p < 0.01$; circular, 338 ± 28 nM, $p < 0.01$). This was significantly inhibited by 5-HT acting via 5-HT_{1p} receptors (51 ± 11%, $p < 0.01$, in longitudinal and 44 ± 3%, $p < 0.001$, in circular muscle cells). In these cells, 5-HT inhibited CCK-induced contraction (Fig. 5). The fact that CCK-8 retained the ability to increase $[Ca^{2+}]_i$ implied that elimination of 5-HT_2 receptors did not interfere with the operation of the signal transduction cascade responsible for Ca^{2+} mobilization.

Identity of the 5-HT receptor mediating relaxation. The similarity between the pattern of response mediated by 5-HT_{1p} receptors in intestinal muscle cells and 5-HT_4 receptors in rat esophageal muscle strips raised the possibility that the receptors might be analogous. Because selective 5-HT_4 antagonists are not currently available commercially (11), the notion

TABLE 1

$[Ca^{2+}]_i$ and cAMP levels in response to 5-HT in isolated smooth muscle cells of guinea pig intestine

$[Ca^{2+}]_i$ represents the increase above basal levels (basal $[Ca^{2+}]_i$ in parentheses). cAMP levels represent the percent increase above basal levels (4.0 ± 0.6 in circular and 3.9 ± 0.6 pmol/10^6 cells in longitudinal muscle cells). *n*, number of experiments.

	Circular		Longitudinal	
	$[Ca^{2+}]_i$ (*n* = 8)	cAMP (*n* = 7)	$[Ca^{2+}]_i$ (*n* = 8)	cAMP (*n* = 7)
	nM	%	nM	%
5-HT (0.1 μM)	198 ± 8 (68)	40 ± 8	176 ± 29 (45)	67 ± 7
5-HT + Ketanserin (1 μM)	18 ± 3[a] (68)	40 ± 7	23 ± 4[a] (48)	68 ± 8
5-HT + 5-HTP-DP (1 μM)	287 ± 22[b] (62)	12 ± 7[a]	353 ± 21[b] (57)	−13 ± 5

[a] Significant inhibition of response to 5-HT, $p < 0.01$ to $p < 0.001$.
[b] Significant increase above response to 5-HT alone, $p < 0.001$.

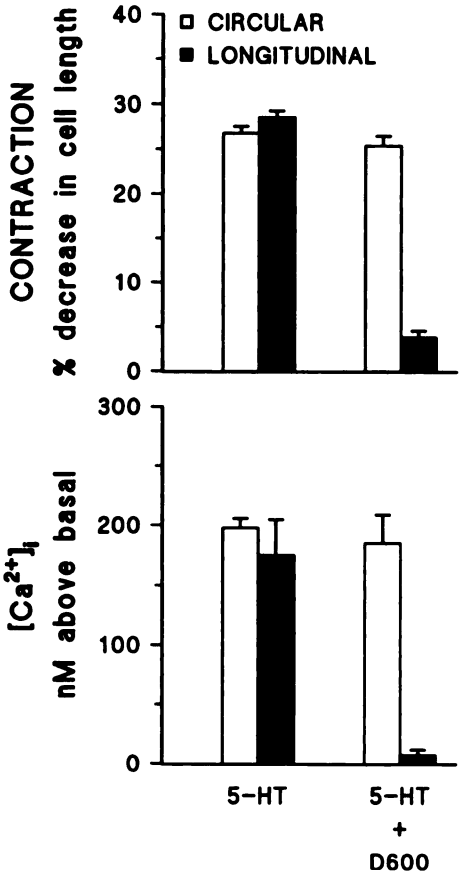

Fig. 6. Contraction and increase in $[Ca^{2+}]_i$ induced by 5-HT in circular and longitudinal muscle cells in the presence and absence of the Ca^{2+} channel blocker methoxyverapamil (D600). Contraction and the increase in $[Ca^{2+}]_i$ were abolished by D600 in longitudinal muscle cells only. Values are means ± standard errors of five to eight experiments.

was tested with high concentrations of ICS205–930. At 10 μM but not at 10 nM ICS205–930 (i) augmented contraction induced by 10 nM 5-HT by 27 ± 7% in circular and by 24 ± 4% in longitudinal muscle cells and (ii) inhibited the relaxation of CCK-contracted cells induced by a combination of 5-HT and ketanserin by 50 ± 3%. Furthermore, the receptors mediating relaxation could be preserved by treatment with 10 μM ICS205–930 as protective agent. In cells treated with ICS205–930 as protective agent, relaxation of CCK-contracted cells by 5-HT was inhibited to the same extent by 5-HTP-DP and 10 μM ICS205–930 (77–82% of control).

Discussion

The present study shows that two 5-HT receptor types coexist in freshly dispersed intestinal smooth muscle cells devoid of neural elements. One receptor type is equally sensitive to ketanserin and spiperone and mediates Ca^{2+}-dependent contraction; the other receptor type is sensitive to 5-HTP-DP and mediates cAMP-dependent relaxation. A receptor sensitive to 5-HTP-DP ($5\text{-}HT_{1p}$ receptor) had previously been shown to exist on neurons of the enteric nervous system, but its intracellular mechanism of action had not been identified (15, 18, 19, 35). The results of the present study demonstrate the existence of this receptor type on smooth muscle cells and its direct coupling to activation of adenylate cyclase. Preliminary evidence based on the use of high concentrations of ICS205–930 that are effective against $5\text{-}HT_4$ receptors suggests that the $5\text{-}HT_{1p}$ receptors on intestinal muscle cells may be analogous to $5\text{-}HT_4$ receptors. However, this notion should be tested further when potent $5\text{-}HT_4$ antagonists become commercially available (36, 37).

No evidence was obtained for the presence of other 5-HT receptor types. Neither contraction nor relaxation induced by 5-HT was sensitive to low concentrations of the $5\text{-}HT_3$ receptor antagonist ICS205–930 (10). $5\text{-}HT_{1c}$ receptors, like $5\text{-}HT_2$ receptors, are coupled to phospholipase C and mobilization of Ca^{2+} but they are insensitive to spiperone and less sensitive than $5\text{-}HT_2$ receptors to ketanserin (1–4, 38). $5\text{-}HT_{1a}$ and $5\text{-}HT_{1d}$ receptors are coupled negatively to adenylate cyclase and induce a pertussis toxin-sensitive decrease in intracellular levels of cAMP (6, 7); relaxation induced by 5-HT in either cell type was insensitive to pertussis toxin.

The use of freshly dispersed muscle cells made it possible to characterize the signal transduction pathways to which $5\text{-}HT_2$ and $5\text{-}HT_{1p}$ receptors were coupled. Consistent with simultaneous activation of both receptor types, 5-HT stimulated increases in both $[Ca^{2+}]_i$ and cAMP levels that were inhibited by ketanserin and 5-HTP-DP, respectively. In circular muscle cells, where agonist-induced increase in $[Ca^{2+}]_i$ is mediated by $InsP_3$, 5-HT stimulated an increase in $InsP_3$ levels that was inhibited by ketanserin.

The singular contribution of each signal transduction pathway was identified separately in muscle cells where only one receptor type was preserved. In cells enriched with $5\text{-}HT_2$ receptors, 5-HT retained the ability to increase $[Ca^{2+}]_i$ but not cAMP levels; the increase in $[Ca^{2+}]_i$ was inhibited by ketanserin but not by 5-HTP-DP. Conversely, in cells enriched with $5\text{-}HT_{1p}$ receptors, 5-HT retained the ability to increase cAMP levels but not $[Ca^{2+}]_i$; the increase in cAMP levels in these cells was inhibited by 5-HTP-DP but not by ketanserin. The pat-

TABLE 2

$[Ca^{2+}]_i$ and cAMP levels in response to 5-HT in muscle cells where only 5-HT_2 receptors were preserved

As in Table 1, $[Ca^{2+}]_i$ and cAMP levels represent the increases above basal levels (basal $[Ca^{2+}]_i$ in parentheses). *n*, number of experiments.

	Circular		Longitudinal	
	$[Ca^{2+}]_i$ ($n = 5$)	cAMP ($n = 4$)	$[Ca^{2+}]_i$ ($n = 5$)	cAMP ($n = 4$)
	nM	%	nM	%
5-HT (0.1 μM)	312 ± 73 (62)	4 ± 8	347 ± 75 (46)	7 ± 13
5-HT + Ketanserin (1 μM)	49 ± 18[a] (58)	3 ± 5	17 ± 5[a] (48)	9 ± 6
5-HT + 5-HTP-DP (1 μM)	338 ± 28 (58)	NT[b]	370 ± 27 (43)	NT

[a] Significant inhibition of response to 5-HT, $p < 0.001$.
[b] NT, not tested.

TABLE 3

$[Ca^{2+}]_i$ and cAMP levels in response to 5-HT in muscle cells in which only 5-HT_{1p} receptors were preserved

As in Table 1, $[Ca^{2+}]_i$ and cAMP levels represent the increases above basal levels (basal $[Ca^{2+}]_i$ in parentheses). *n*, number of experiments.

	Circular		Longitudinal	
	$[Ca^{2+}]_i$ ($n = 5$)	cAMP ($n = 4$)	$[Ca^{2+}]_i$ ($n = 5$)	cAMP ($n = 4$)
	nM	%	nM	%
5-HT (0.1 μM)	13 ± 2 (53)	31 ± 8	19 ± 2 (47)	40 ± 8
5-HT + Ketanserin (1 μM)	NT[a]	30 ± 7	NT	39 ± 7
5-HT + 5-HTP-DP (1 μM)	NT	−9 ± 7[b]	NT	−10 ± 6[b]

[a] NT, not tested.
[b] Significant inhibition of response to 5-HT, $p < 0.01$.

terns imply that 5-HT can activate distinct signal transduction pathways coupled to each receptor type.

In cells where 5-HT_2 receptors were eliminated and both CCK and 5-HT_{1p} receptors were preserved, CCK-8 retained the ability to increase $[Ca^{2+}]_i$, implying that elimination of 5-HT_2 receptors did not interfere with the operation of the signal transduction cascade responsible for Ca^{2+} mobilization. This is further corroborated by the fact that cells in which all receptors were inactivated retained their ability to contract or relax in response to agents that bypass receptors (25, 26).

Several results, however, implied that the two signaling systems activated by 5-HT were linked, converging to regulate $[Ca^{2+}]_i$. Firstly, contraction and the increase in $[Ca^{2+}]_i$ induced by 5-HT were significantly augmented when cAMP production was inhibited by 5-HTP-DP. Secondly, in muscle cells where only 5-HT_2 receptors were preserved, cAMP production was suppressed and the increase in $[Ca^{2+}]_i$ was significantly greater than in untreated cells and could not be augmented further by 5-HTP-DP. Thirdly, in muscle cells where both CCK and 5-HT_{1p} receptors were preserved, the relaxation of CCK-contracted cells induced by 5-HT was accompanied by a significant decrease in $[Ca^{2+}]_i$ and could be blocked by 5-HTP-DP as well as by Rp-cAMPS, an inhibitor of protein kinase A activity (34). Taken together, these observations imply that the increase in cAMP levels resulting from interaction of 5-HT with 5-HT_{1p} receptors acts to attenuate the increase in $[Ca^{2+}]_i$ resulting from interaction of 5-HT with 5-HT_2 receptors. This occurred in circular muscle cells, where the increase in $[Ca^{2+}]_i$ is mediated by Ins(1,4,5)P_3-dependent Ca^{2+} release, and in longitudinal muscle cells, where the increase in $[Ca^{2+}]_i$ is triggered by influx of Ca^{2+} from extracellular sources. The contractile effect of 5-HT is a compound response resulting from activation of two pathways that converge to regulate the cytosolic level of Ca^{2+}, the main determinant of mechanical activity in muscle cells.

It is important to note that the interaction between cAMP and Ca^{2+} in muscle cells was initiated by the same agonist acting via distinct receptor types. The interaction differs from, yet confirms, observations that the increase in cAMP induced by one agent modulates the increase in $[Ca^{2+}]_i$ induced by another agent. The interaction between the cAMP and Ca^{2+} pathways varies with the cell type, with increases in $[Ca^{2+}]_i$ in some cells (39, 40) and decreases in other cells (41, 42).

Pharmacological studies in innervated muscle strips and radioligand binding studies in heterogeneous cells or membranes must contend with the presence of 5-HT receptors on different cell types. The receptors can mediate opposite actions, as shown in the present study, that would further obscure the interpretation of pharmacological responses. Previous studies using intact vascular and visceral (e.g., tracheal, intestinal) smooth muscle tissue showed that 5-HT causes contraction by releasing contractile neurotransmitters as well as by interacting with 5-HT_2 receptors on smooth muscle cells (12, 20–22). Interaction with 5-HT_2 receptors is evident in homogeneous cell systems, such as cultures of aortic smooth muscle cells, where 5-HT causes a ketanserin-sensitive increase in inositol phosphate production and Ca^{2+} release (38). Evidence for the existence of 5-HT receptors mediating relaxation was based on the ability of 5-HT to cause relaxation of smooth muscle precontracted with various agents (23, 24, 43, 44). However, neither the receptor type nor the signal transduction pathway mediating relaxation was identified, except for recent studies on rat esophageal muscle where 5-HT_4 receptors positively coupled to adenylate cyclase were found (9, 10).

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