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Serotonin induces four pharmacologically separable contractile responses in the pharynx of the leech *Hirudo medicinalis*

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Abstract

Stimulation of the serotoninergic innervation of the leech pharynx or application of serotonin to the isolated pharynx induced four distinct types of contractile activity: an increase in basal tonus, large phasic contractions of 10–15 s in duration, smaller phasic contractions occurring at approximately 1 Hz, and a relaxation after washout of serotonin. Application to the isolated pharynx of the selective serotonin agonists (±)-8-hydroxy-2-(di-n-propylamino)tetralin, *N*-(3-trifluoromethylphenyl)piperazine, 1-(m-chlorophenyl)-piperazine, (±)-2,5-dimethoxy-4-iodoamphetamine, 2-methyl-5-hydroxytrypamine, α-methyl-5-hydroxytryptamine, and 5-methoxytryptamine induced distinct types of pharyngeal contractile activity. The results of this study suggest that the leech pharynx possesses more than one type of serotonin receptor.

Keywords: Biogenic amine; Annelid; Neuromodulation; Feeding; Muscle; Gut; Receptor

Serotonin (5-HT) activates the largest number of different receptors known for any single neurotransmitter. In mammalian systems, there are at least seven different families of serotonin receptors, with many of these families having several receptor subtypes (Hoyer and Martin, 1996). These receptors are coupled to most (if not all) of the major second-messenger systems as well as to ligand-gated ion channels. The multiplicity of serotonin receptor subtypes is believed to be due to the ancient origin (more than 750 million years ago) of the primordial serotonin receptor (Peroutka and Howell, 1994; Walker et al., 1996). On the basis of an analysis of the amino acid sequences of the known G protein-coupled serotonin receptors, Peroutka and Howell (1994) concluded that ancestors to the mammalian 5-HT_1, 5-HT_2, 5-HT_5, 5-HT_6, and 5-HT_7 receptors existed before the divergence of the vertebrates from the invertebrates. However, many of the subtypes of these receptors, known from mammalian systems, first appeared after the divergence of the vertebrates from the invertebrates.

Several invertebrate serotonin receptors have sequence homologies and transduction mechanisms in common with mammalian receptors. For example, the *Drosophila* 5-HT_{dro1} and mammalian 5-HT_7 receptors apparently are homologous (Peroutka and Howell, 1994; Saudou and Hen, 1994; Witz et al., 1990). Similarly, the *Drosophila* 5-HT_{dro2}, the *Lymnaea* 5-HT_{lym} receptor, the *Caenorhabditis* 5-HT_{Ce}, and the mammalian 5-HT_1 family of receptors are apparently homologous (Olde and McCombie, 1997; Peroutka and Howell, 1994; Saudou and Hen, 1994; Sugamori et al., 1993; Witz et al., 1990). However, not all invertebrate serotonin receptors have obvious relationships to vertebrate receptors. On the basis of amino acid sequence homology and transduction mechanism, two serotonin receptors from *Aplysia* (Ap5-HT_{B1} and Ap5-HT_{B2}) are not easily grouped with any of the other described serotonin receptors from either vertebrates or invertebrates (Li et al., 1995).

The combination of a large number of selective serotonin agonists and antagonists along with the large number of workers in the field has lead to an extensive (but by no means complete) understanding of serotonin receptor pharmacology in the vertebrates. In contrast, the field of serotonin receptor pharmacology in the invertebrates is not as thoroughly developed. Attempts to fit invertebrate serotonin receptors into the mammalian scheme by using pharmacological methods have often failed. Locust neuronal somata possess three serotonin-induced currents whose pharmacologies differ from any vertebrate serotonin-induced current (Bermudez et al., 1992). On the basis of the responses of the lobster stomatogastric ganglion to a variety of serotonin agonists

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and antagonists, Zhang and Harris-Warrick (1994) concluded that neurons of the stomatogastric ganglion possess several serotonin receptors. However, the pharmacology of each of these receptors is unlike any known vertebrate receptor. These authors report several occasions in which two drugs that act on the same vertebrate serotonin receptor have different actions on the stomatogastric ganglion. In a similar manner, the pharmacology of molluscan serotonin receptors also does not fit within the vertebrate classification scheme (Fong et al., 1993, 1996; Goldberg et al., 1994; Vehovszky and Walker, 1991).

Serotonin has been known to be an important neurotransmitter in leeches for more than 30 years [reviewed by Leake and Walker (1980) and by Leake (1986)]. More recently, it has become clear that the leech possesses several serotonin receptors; however, their number and relatedness to other described serotonin receptors are largely unknown. The P sensory neurons of *Hirudo medicinalis* may possess three different serotonin receptors. A serotonin-activated Cl^- conductance is mediated by an ionotropic receptor whose pharmacology does not fit within the mammalian classification scheme (Leßmann and Dietzel, 1995; Sanchez-Armass et al., 1991). A second receptor, whose pharmacology resembles that of 5-HT_2 receptors, activates a nonselective cationic conductance by protein kinase C and tyrosine phosphatase (Catarsi and Drapeau, 1997; Sanchez-Armass et al., 1991). A third receptor, whose pharmacology resembles that of 5-HT_{1A} receptors, reduced the open state probability of a potassium channel by protein phosphorylation (Goldermann et al., 1994).

Leake and Koubanakis (1995) examined the effects of a number of selective serotonin agonists on the body wall muscles and Retzius neurons of *Hirudo.* These authors utilized agonists for several of the 5-HT_1 receptor subtypes as well as agonists for 5-HT_2, 5-HT_3, and 5-HT_4 receptors. They concluded that either these two cell types contain a mixed population of serotonin receptors or the individual serotonin receptors activated more than a single second-messenger system. Although native receptors coupled to more than a single second-messenger system have not been unequivocally demonstrated, studies on cloned receptors indicate that individual receptors can be simultaneously coupled to more than one second-messenger system (Gudermann et al., 1996; Reale et al., 1997; Robb et al., 1994).

The muscular pharynx of *Hirudo medicinalis* pumps blood from a wound on the host to the crop, where the blood meal is stored. The pharynx was previously shown to be responsive to serotonin application and to stimulation of the large lateral serotonin-containing neurons (LL cells), whose somata are located in the first neuromere of the subesophageal ganglion (Lent and Dickinson, 1984). The response of the pharynx to either form of stimulation was described as consisting of a series of peristaltic contractions occurring at a frequency of approximately 1 Hz. The work in this report reexamines the response of the pharynx to both serotonin application and LL cell stimulation. The contractile responses of the pharynx elicited by LL cell stimulation or serotonin application were more complex than that described by Lent and Dickinson (1984). With the use of a number of selective serotonin agonists, the complex response of the pharynx to serotonin can be separated into four distinct types of contractions. The differential actions of these agonists indicate that the leech pharynx may possess several different serotonin receptors.

1. Materials and methods

Medicinal leeches, *Hirudo medicinalis*, were obtained from commercial suppliers (Leeches U.S.A., Ltd., Westbury, NY, USA, or ZAUG GmbH, Biebertal, Germany) and maintained in aquaria at about 18°C on a 12 h:12 h L:D photoperiod. Animals maintained in the laboratory for longer than 4 months were fed bovine blood.

1.1. Pharynx: head ganglia preparation

Leeches were anesthetized with ice-cold saline and pinned ventral side down in a frozen wax-bottomed dissection dish. A dorsal midline incision was used to expose the pharynx. The pharynx was freed from its connections to the body wall by first severing the dorsal and lateral extrinsic radial muscles. The pharynx was freed from the animal by first cutting through the crop caudal to the posterior sphincter of the pharynx and then severing the remaining radial muscles on the ventral side of the pharynx as the tissue was lifted out of the animal. The supraesophageal ganglion, the subesophageal ganglion, and the first two segmental ganglia together with the pharynx and mouth were removed from the animal as a unit. The nerve cord was pinned ventral side up in a small Sylgard-lined dish (Dow-Corning, Midland, MI, USA), and the pharynx was reflected anteriorly so that it was not under the nerve cord. Movements of the pharynx were monitored with the use of a force transducer (FORT-10, WPI, Sarasota, FL, USA). The subesophageal ganglion was desheathed by using fine scissors and illuminated during the experiment by a dark-field condenser to allow viewing of the neuronal somata within the ganglion. The preparation was continuously superfused with saline at a rate of 0.5 ml/min. Microelectrodes for electrophysiology had resistances of 20–35 MΩ; and were filled with 4 M potassium acetate and 20 mM KCl. Data from the microelectrode and force transducer were recorded onto magnetic tape and subsequently transferred into computer-based format by using the CODAS data acquisition system (Dataq Instruments, Akron, OH, USA) at a sampling rate of 1000 samples/s/channel.

1.2. Isolated pharynx preparation

The initial dissection was similar to that described in Section 1.1, except that the central nervous system was

not removed from the animal along with the pharynx. To remove the pharynx from the leech, the pharynx was transected just posterior to where it passes through the circumesophageal connectives. A short length of the pharynx, from the mouth to the dorsal surface of the head ganglia, was not removed from the leech. A separate microsurgical needle, bent into a hook shape and attached to 8-0 monofilament nylon suture material (Ethicon 2808G, Sommerville, NJ, USA), was inserted through each end of the pharynx. One suture was anchored to the bottom of a small perfusion tissue bath (volume 0.2 ml), and the suture attached to the other end of the pharynx was attached to an isometric force transducer (FORT-10). This physical arrangement most effectively monitored longitudinal contractions of the pharynx; however, as judged by simultaneous visual observation of the pharynx, circular contractions also were recorded. The pharynx was placed under approximately 20–25 mN of tension and allowed to relax for approximately 1 h. Most pharynxes were initially quiescent but exhibited spontaneous contractions well before the end of the 1-h relaxation period.

The output of the force transducer was fed into a transducer interface (Transbridge, WPI, Sarasota, FL, USA), whose subsequent output was fed into a computer-based data-acquisition system (CODAS or WINDAQ, Dataq Instruments). The signal from the force transducer was digitized at 50 samples/s and recorded to disk. Subsequent data analysis of the records was performed by using the playback software of the data-acquisition system and Advanced CODAS software (Dataq Instruments).

The tissue bath was continuously perfused with saline at a rate of 1 ml/min; saline was removed from the top of the chamber by suction. Control or agonist-containing salines were introduced into an inlet at the bottom of the perfusion chamber through a valve attached to reservoirs containing each of the salines. An air bubble was introduced into the perfusion line to indicate the beginning and end of each treatment. Each application of agonist-containing saline was 1 ml in volume and approximately 1 min in duration. In the determination of concentration–response curves, the lowest agonist concentration was applied first and higher concentrations were added in sequential order.

We quantified the response to each agonist application by measuring the following parameters (Fig. 1): (1) peak tension (i.e., greatest tension produced in response to the agonist); (2) maximal increase in basal tonus (i.e., greatest sustained elevation in muscle tonus); (3) integrated area (i.e., area under the contraction curve during the response to the agonist); and (4) relaxation (i.e., any reduction in tension compared with the baseline tension; e.g., see Fig. 3A). As a practical matter, when phasic contractions were elicited in addition to an increase in basal tone, we defined the increase in basal tonus as the highest point between two phasic

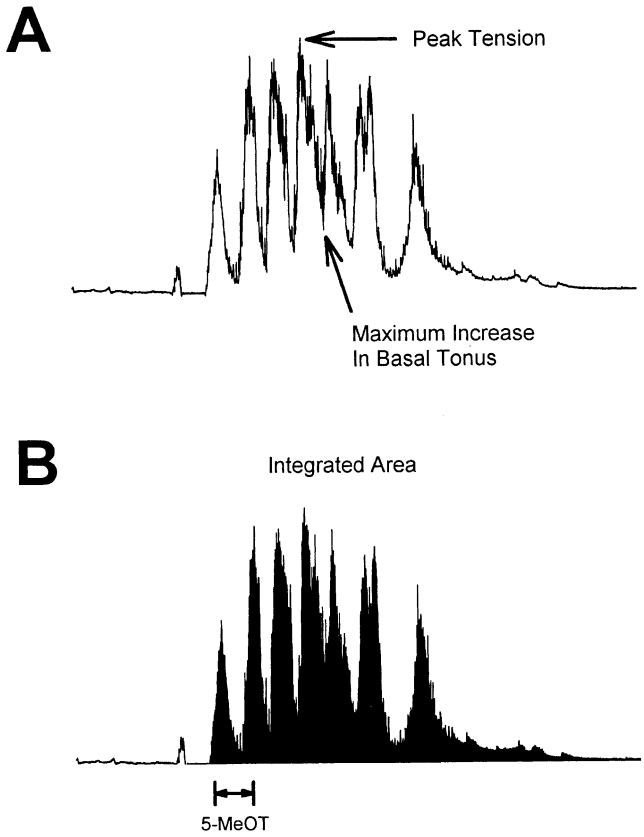

Fig. 1. Illustration of how pharyngeal contractile responses to serotonin agonists were quantified. The contractile response of the pharynx was evoked by an approximately 1 min application of 100 μM 5-MeOT [application time indicated in (B)]. (A) Peak tension was the greatest tension produced after agonist application. The maximal increase in basal tonus was the greatest sustained increase in muscle tonus. (B) Integrated area was the area under the contraction curve from the time of agonist application until the response returned to baseline.

contractions. If the agonist did not induce phasic contractions, peak tension and the maximal increase in basal tonus were identical. Integrated area is a measure that is sensitive to the duration of the induced response as well as to the frequency of phasic contractions.

To estimate the relative efficacy of the agonists examined, the concentration of agonist necessary to produce a half-maximal response (EC_{50}) was estimated from concentration–response curves for each measured parameter (e.g., see Fig. 6). The highest agonist concentration applied to the pharynx was 1 mM. However, for some agonists, the responses had not reached an apparent asymptotic peak by 1 mM (especially true for integrated area). In such a case, the reported EC_{50} (Table 1) is probably an underestimate of the true EC_{50}.

1.3. Drugs and saline

During the dissection and experiment, the preparation was superfused with a physiological saline containing (in mM): NaCl (115), KCl (4), $CaCl_2$ (1.8), $MgCl_2$ (2),

Table 1
EC_{50} values for increases in basal tonus, peak tension, integrated area, and relaxation

Agonist	Basal tonus (μM)	Peak tension (μM)	Integrated area (μM)	Relaxation* (μM)
Serotonin	11	4	190	1.7
8-OH-DPAT	180	200	280	—
TFMPP	170	19	190	—
mCPP	48	48	220	—
DOI	250	122	200	280
α-Me-5-HT	18	1.2	22	30
2-Me-5-HT	1.8	1.4	1.1	—
5-MeOT	31	12	80	<0.01

* The EC_{50} values for agonists that failed to induce relaxation are reported as "—".

HEPES buffer (10), brought to pH 7.4 with NaOH. Experiments were conducted at room temperature (21–25 °C). Drugs were purchased from the following suppliers: 5-hydroxytryptamine creatinine sulfate complex (serotonin), (±)-8-hydroxy-2-(di-n-propylamino)-tetralin HBr (8-OH-DPAT), and 1-(m-chlorophenyl)-piperazine HCl (mCPP) were purchased from Sigma (St. Louis, MO, USA); and (±)-2,5-dimethoxy-4-iodoamphetamine HCl (DOI), *N*-(3-trifluoro-methylphenyl) piperazine HCl (TFMPP), 5-methoxytryptamine HCl (5-MeOT), 2-methyl-5-hydroxytryptamine maleate (2-Me-5-HT), and α-methyl-5-hydroxytryptamine maleate (α-Me-5-HT) were purchased from RBI (Natick, MA, USA).

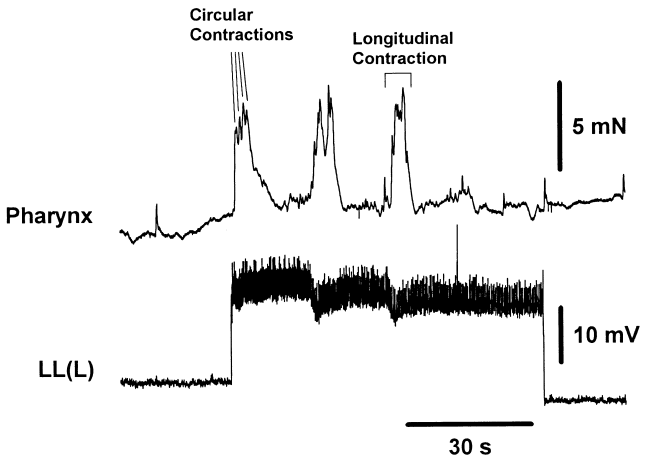

Fig. 2. Pharyngeal contractions elicited by stimulation of the serotonin-containing LL cell. The top trace is a tension recording showing contractions of the pharynx. The bottom trace shows an intracellular recording from the left LL cell during injection of 1.5 nA of current. Shortly after the initiation of current-induced spiking in the LL cell, the contractions of the pharynx changed in pattern and amplitude. The most obvious change was the induction of large phasic deflections, on which were superimposed smaller deflections. Simultaneous visual observation of the pharynx indicated that the large phasic deflections were due to longitudinal contractions, whereas the smaller deflections were produced by circular contractions. The basal tonus was also slightly elevated compared with the tension at the start of LL cell stimulation. The hyperpolarizations in the LL cell membrane potential that are coincident with the longitudinal contractions are movement artifacts.

2. Results

2.1. Response of the pharynx to LL cell stimulation

Stimulation of an individual LL cell in an isolated pharynx–head ganglion preparation ($N > 20$) caused a complex series of contractile responses in the pharynx (Fig. 2). Stimulation of the LL cells caused an elevation in basal tonus and a series of phasic contractions each of which was approximately 10 to 15 s in duration. Superimposed on these phasic contractions were a series of smaller contractions occurring at a frequency of approximately 1 Hz. Subsequent to termination of LL cell stimulation, the basal tonus of most preparations fell to levels below those prior to stimulation (not shown). Simultaneous visual observation of the pharynx during LL cell stimulation indicated that the large phasic deflections were due to longitudinal contractions of the pharynx, whereas the small, rapid deflections were due to circular contractions. The origins of the rise in basal tonus or the relaxation subsequent to LL cell stimulation were not obvious. Tachyphylaxis of the pharyngeal response was noted during repeated LL cell stimulations; however, we did not attempt to quantify the tachyphylaxis. The repetitive pharyngeal peristalsis induced by LL cell stimulation that was described by Lent and Dickinson (1984) occurred at a frequency similar to that of the circular contractions recorded here; thus, we were probably observing the same events described by these workers.

2.2. Response of the isolated pharynx to serotonin and serotonin agonists

2.2.1. Serotonin

The response of the isolated pharynx to serotonin resembled that elicited by LL cell stimulation and consisted of four components (Fig. 3): (1) an elevation of basal tonus, (2) phasic longitudinal contractions 10 to 20 s in duration, (3) circular contractions occurring at a frequency of approximately 1 Hz, and (4) a relaxation of basal tonus after washout of the serotonin. The circular contractions were usually superimposed on the longitudinal contractions.

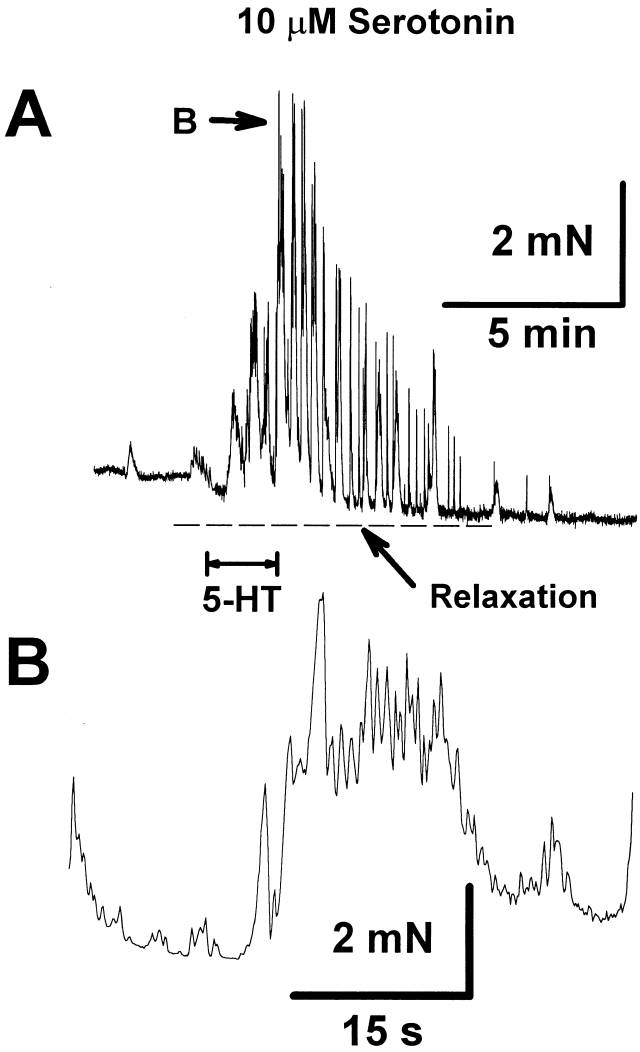

Fig. 3. Typical contractile response of the isolated pharynx elicited by application of 10 μM serotonin. (A) Serotonin induced four distinct types of contractile responses: an increase in basal tonus, large phasic contractions of approximately 10–15 s duration, smaller phasic contractions occurring at approximately 1 Hz superimposed on the large phasic contractions, and a relaxation of tonus after washout of the serotonin. The series of small deflections near the beginning of the serotonin application are artifacts produced by the bubble in the perfusion line used to separate the different solutions. (B) An expanded single large phasic contraction [contraction indicated by arrow in (A)] showing the superimposition of the small contractions occurring at approximately 1 Hz.

Serotonin concentration–response curves for basal tonus, peak tension, integrated area, and relaxation are shown in Fig. 4. The threshold concentration of serotonin for producing effects on each of these four measures was approximately 10 nM. The concentration–response curves for basal tonus, peak tension, and integrated area were each monotonic functions. However, in contrast, the curve for relaxation peaked at 100 μM and exhibited a large reduction at 1 mM serotonin. Examination of Fig. 4 reveals that the development of basal tonus, peak tension, and relaxation (ignoring 1 mM serotonin) occurred with approximately the same sensitivity to different concentrations of serotonin. Compared with the other three curves, the curve for integrated area was shifted to the right. The reason for this shift is that high concentrations of serotonin caused a proportionally larger increase in the response duration compared with the increases induced in peak tension or basal tonus. The rightward shift in the concentration–response curve for the integrated area was also reflected in the higher EC_{50} for integrated area (by approximately an order of magnitude) compared with the other three measures (Table 1).

To determine whether the complexity of the pharyngeal response to serotonin application might be due to the activation of more than one serotonin receptor, we exposed the pharynx to several selective serotonin agonists. If the overall response to serotonin was due to simultaneous activation of several receptors, selective agonists might be able to induce only some components of the overall response to serotonin. The contractile responses of the isolated pharynx to the tested agonists are presented in Figs. 5 and 7A, whereas concentration–response curves for the agonists and serotonin are presented in Fig. 6. EC_{50} values for the induction of the increase in basal tonus, peak tension, integrated area, and relaxation are reported in Table 1. In the text that follows, reference to the receptor specificity of each serotonin agonist in mammalian systems does not imply that a response of the leech pharynx to that agonist is necessarily mediated through a receptor similar to the mammalian receptor. Unless otherwise noted in the descriptions that follow, the actions of each agonist were qualitatively similar at different concentrations. In other words, the magnitude of the response was concentration dependent, but the contractile pattern induced by the agonist was present at all concentrations tested.

2.2.2. 8-OH-DPAT (5-HT_{1A} agonist)

In each of five preparations, the response of the pharynx to 8-OH-DPAT (Fig. 5A) was similar to that of serotonin in that it induced a rise in basal tonus, longitudinal contractions of 10–20 seconds in duration, and circular contractions at approximately 1 Hz. However, 8-OH-DPAT did not induce relaxation after washout (Fig. 6D). As gauged by the estimates of the EC_{50}s, 8-OH-DPAT was equally effective in inducing an increase in basal tonus, peak tension, and integrated area. 8-OH-DPAT was approximately an order of magnitude less effective in inducing increases in basal tonus and peak tension compared with serotonin. However, the EC_{50} for integrated area was similar for both agonists.

2.2.3. TFMPP (5-$HT_{1B/2C}$ agonist)

In each of the 13 preparations examined, the contractile response induced by TFMPP (Fig. 7A) was similar in waveform to that elicited by mCPP (Fig. 5B). Both agonists induced an increase in basal tonus; how-

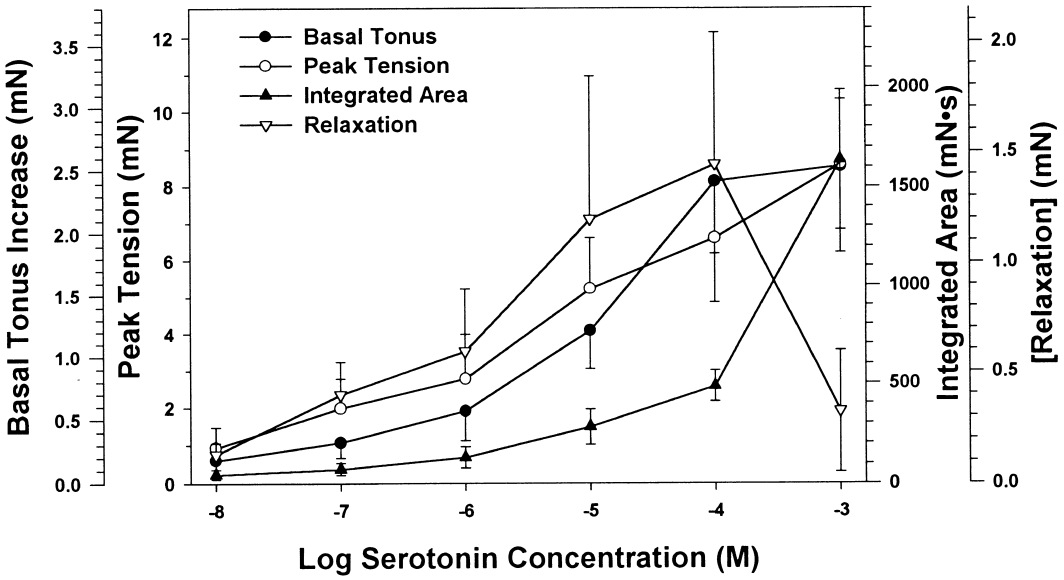

Fig. 4. Concentration–response relations of the effects of serotonin on basal tonus, peak tension, integrated area, and relaxation. Values for relaxation are plotted as absolute magnitude. The ordinate for each set of data is scaled so that the maximum value is approximately the same distance from the abscissa. The threshold concentration for each parameter was approximately 10 nM, with increasing concentrations of serotonin causing increases in each parameter. The data for each of the curves are derived from six preparations.

ever, TFMPP also caused a concentration-dependent increase in phasic contraction frequency. TFMPP was considerably less potent than mCPP in inducing an increase in basal tonus (Fig. 6; Table 1). The EC_{50} of TFMPP for peak tension was approximately an order of magnitude lower than the EC_{50} values for basal tonus or integrated area (Table 1). The lower EC_{50} for peak tension is indicative of the ability of TFMPP to induce phasic contractions at low concentrations (Fig. 7). The frequency of phasic contractions was increased by application of 10 to 100 μM TFMPP, whereas 1 mM TFMPP caused an inhibition of phasic contractions (Fig. 7 A and B). This relation was quantified in Fig. 7B in which the change in phasic contraction frequency during the 5-min period preceding TFMPP application is compared with the 5-min period beginning with TFMPP application.

2.2.4. mCPP (5-$HT_{1B/2C}$ agonist)

TFMPP and mCPP are very similar to each other in structure (mCPP is the chloro counterpart of TFMPP), and both caused an increase in basal tonus. However, mCPP was apparently one of the most selective agonists tested on the leech pharynx in that the response to mCPP in most cases consisted solely of an increase in basal tonus (Fig. 5B). In addition, spontaneously occurring phasic contractions were suppressed by mCPP. We have observed this type of response in several hundred preparations. However, in a few preparations (less than 10%), mCPP also caused an increase in phasic contraction frequency. This increase in phasic contraction frequency was more likely to occur at low concentrations of mCPP than at higher concentrations (even within a single preparation). On rare occasions, phasic contractions persisted through application of high concentrations (1 mM) of mCPP and were superimposed on an increase in basal tonus (not shown). Such occurrences indicate that the increase in basal tonus induced by mCPP is not a summation of phasic contractions into a sustained tension in a manner analogous to tetanus. The increase in phasic-contraction frequency induced by mCPP resembled the increase in phasic-contraction frequency induced by TFMPP (Fig. 7A); however, the propensity of mCPP to induce phasic contractions was much lower than it was for TFMPP. The response to mCPP persisted for a considerable period after drug washout. A 1-min application of 1 mM mCPP commonly produced a response lasting 45 min to 1 h after drug washout. The long duration of the response to high concentrations of mCPP underlies the relatively high potency of mCPP as measured by integrated area (Fig. 6C). This was true even though mCPP was one of the least effective agonists at inducing increases of peak tension compared with other agonists (Fig. 6B). The EC_{50} for producing an increase in basal tonus was slightly greater than that for serotonin, but the EC_{50} values for integrated area were similar for both substances (Table 1). The EC_{50} values reported for basal tonus and peak tension for mCPP are the same because, in the vast majority of preparations, the mCPP-induced increase in basal tonus was also the peak tension.

2.2.5. DOI (5-$HT_{2A/2C}$ agonist)

In seven of ten preparations examined, DOI produced an increase in basal tonus and increased the frequency and amplitude of longitudinal phasic contrac-

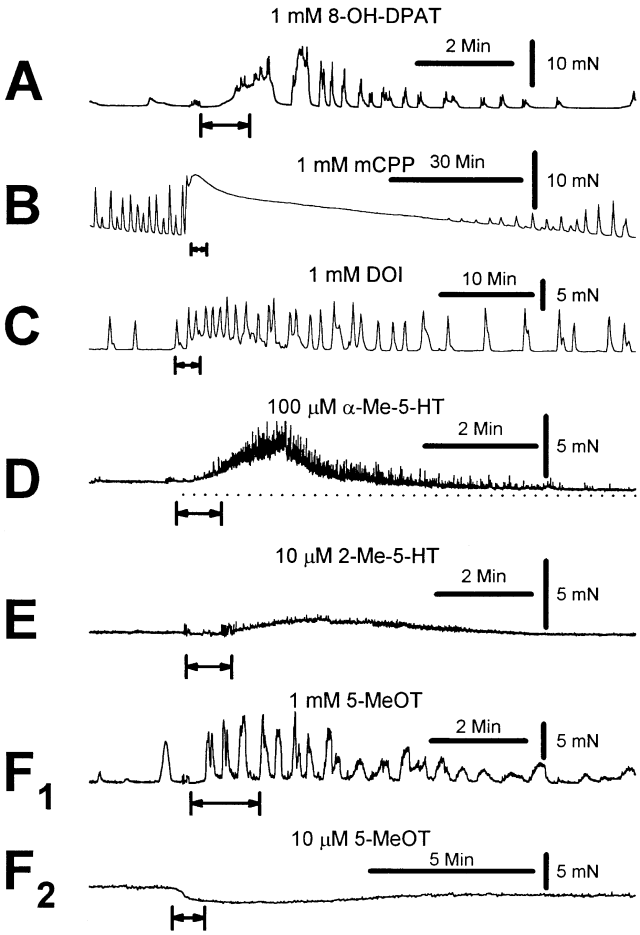

Fig. 5. Contractile responses elicited by different serotonin agonists. Each agonist was applied for approximately 1 min (indicated by arrows). Small deflections occurring near the beginning or end of the agonist application are artifacts due to change of solutions. (A) 1 mM 8-OH-DPAT; (B) 1 mM mCPP; (C) 1 mM DOI; (D) 100 μM α-Me-5-HT (the dotted line below the trace illustrates the relaxation occurring after agonist washout); (E) 10 μM 2-Me-5-HT; (F_1) 1 mM 5-MeOT; (F_2) 10 μM 5-MeOT. Relaxation was induced by 5-MeOT only at concentrations of 10 μM and below. Higher concentrations induced the other components of the overall response to serotonin.

tions (Fig. 5C). The remaining three preparations did not show an obvious response to DOI. DOI did not induce circular contractions or, at most concentrations, a relaxation in basal tonus after drug washout. However, at high concentrations (1 mM), DOI may induce small amounts of relaxation (Fig. 6D). As indicated by its EC_{50} values (Table 1), DOI was one of the least potent agonists tested on all measures.

2.2.6. α-Me-5-HT (5-HT_2 agonist)

In four of five preparations examined, α-Me-5-HT caused an increase in basal tonus, circular contractions of the pharynx, and relaxation after agonist washout (Fig. 5D). The remaining preparation did not show an obvious response to α-Me-5-HT. The α-Me-5-HT-induced relaxation was seen only at concentrations of 100 μM and above. α-Me-5-HT did not induce longitudinal contractions of the pharynx. The EC_{50} values of α-Me-5-HT for the increases in basal tonus and peak tension were similar to those for serotonin, whereas the EC_{50} for relaxation was an order of magnitude higher than that of serotonin (Table 1). In contrast, the EC_{50} of α-Me-5-HT for integrated area was about an order of magnitude lower than that of serotonin.

2.2.7. 2-Me-5-HT (5-HT_3 agonist)

In five of five preparations examined, 2-Me-5-HT caused an increase in basal tonus (Fig. 5E). 2-Me-5-HT did not induce a relaxation after agonist washout or, with rare exceptions (one preparation at 100 μM and 1 mM), longitudinal contractions. Application of 2-Me-5-HT often caused the trace to appear noisier than that prior to agonist application. This may represent the induction of a series of very small contractions of unknown origin. The amplitude of the 2-Me-5-HT response peaked at 10 μM and became smaller at higher concentrations (Fig. 6A–C). Because the response to 2-Me-5-HT peaked at this relatively low concentration, the EC_{50} values for basal tonus and integrated area were the lowest of any of the tested agonists (Table 1). The EC_{50} for peak tension was equal to the lowest produced in this study. Although the primary action of both mCPP and 2-Me-5-HT was to produce an increase in basal tonus, the duration of action of these two agonists was quite different. The mCPP-induced effect persisted for several times the duration of the 2-Me-5-HT-induced response (compare Fig. 5B and 5E). This largely accounts for the much greater magnitude of mCPP-induced integrated area compared with 2-Me-5-HT-induced integrated area (Fig. 6C).

2.2.8. 5-MeOT (5-HT_4 agonist)

At concentrations ranging from 10 nM to 10 μM, 5-MeOT induced only relaxation (five of five preparations; Fig. $5F_2$). At higher concentrations, 5-MeOT induced an increase in basal tonus, longitudinal contractions, and circular contractions (five of five preparations; Fig. $5F_1$). Relaxation was usually not evident at these higher concentrations (Fig. 6D). The EC_{50} of 5-MeOT for inducing relaxation was by far the lowest of any agonist tested for any of the parameters measured in this study (Table 1).

The relative potencies [using the response amplitude at 1 mM (Fig. 6A)] of the tested agonists for the induction of an increase in basal tonus were: 5-MeOT > serotonin ≈ mCPP ≈ α-Me-5-HT > DOI ≈ TFMPP ≈ 2-Me-5-HT ≈ 8-OH-DPAT. However, the order of efficacy (Table 1) was: 2-Me-5-HT > serotonin ≈ α-Me-5-HT ≈ 5-MeOT ≈ mCPP > TFMPP ≈ 8-OH-DPAT ≈ DOI. For peak tension, the order of potency as determined by response amplitude at an agonist concentration of 1 mM (Fig. 6B) was: serotonin ≈ 5-MeOT ≈ 8-OH-DPAT > DOI ≈ α-Me-5-HT > mCPP > TFMPP ≈ 2-Me-5-HT. The order of efficacy (Table 1) for peak

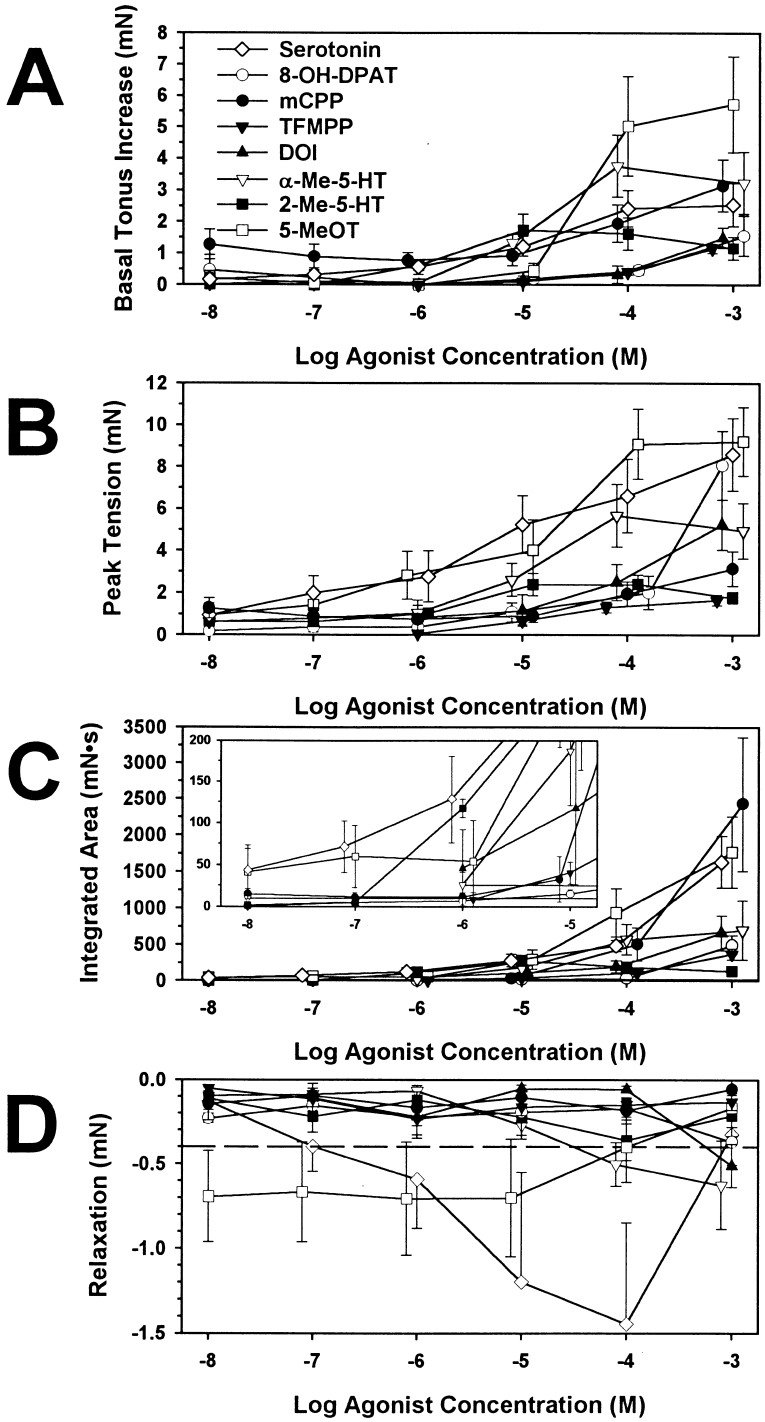

Fig. 6. Concentration–response curves for the serotonin agonists used in this study. Data from Fig. 4 showing responses to serotonin are replotted in this figure for comparative purposes. (A) Basal tonus; (B) peak tension; (C) integrated area (inset shows integrated area data for low agonist concentrations plotted with an expanded ordinate); (D) relaxation. Relaxation is plotted as the reduction in tension induced by the agonist application compared with the preapplication tension. This method of plotting relaxation data contrasts with Fig. 4, where relaxation induced by serotonin is plotted as the absolute magnitude of relaxation. Because pharynxes continuously relax for the duration of the experiment, relaxations of less than −0.4 mN (dashed line) were probably not agonist induced. In each panel, some points have been displaced horizontally for clarity. Points clustered around a particular concentration indicate the response at that concentration. Data in this figure are derived from five preparations for each agonist, except for serotonin ($N = 6$), TFMPP ($N = 13$), and DOI ($N = 10$).

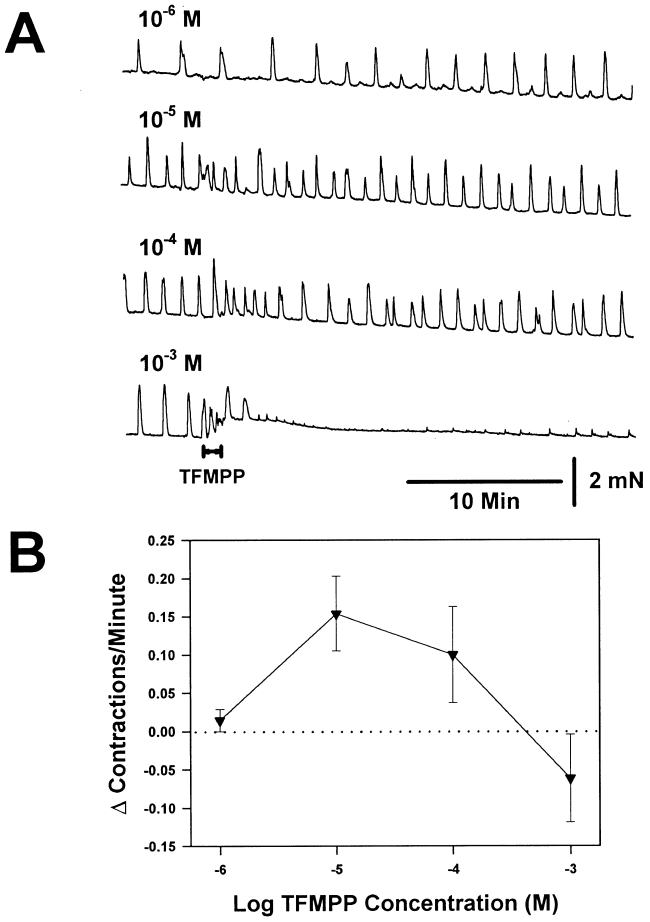

Fig. 7. Effects of TFMPP on phasic-contraction frequency were concentration dependent. (A) Contractile records from a single isolated pharynx when exposed to different concentrations of an approximately 1 min application of TFMPP (application time for all traces is indicated by the bar below bottom trace). Application of 10^{-6} M TFMPP had little if any effect on phasic-contraction frequency, whereas application of 10^{-5} M and 10^{-4} M caused an increase in phasic-contraction frequency. Application of 10^{-3} M TFMPP caused a transient increase in contraction frequency followed by a prolonged suppression of phasic contractions. This response is somewhat atypical in that some very small contractions persisted during the falling phase of the increase in basal tonus. However, even these small contractions were absent during the later parts of the falling phase of the increase in basal tonus. (B) Group data plotting the change in phasic-contraction frequency versus TFMPP concentration. Application of 10^{-5} M and 10^{-4} M TFMPP caused an increase in phasic-contraction frequency, whereas 10^{-3} M TFMPP caused a decrease in phasic-contraction frequency below the preapplication level. Data were derived from seven preparations for 10^{-6} M and 13 preparations for the other concentrations of TFMPP.

tension was: α-Me-5-HT ≈ 2-Me-5-HT ≈ serotonin > 5-MeOT ≈ TFMPP > mCPP > DOI > 8-OH-DPAT. The order of potency for integrated area at 1 mM (Fig. 6C) was: mCPP > serotonin ≈ 5-MeOT > DOI ≈ TFMPP ≈ α-Me-5-HT ≈ 8-OH-DPAT > 2-Me-5-HT. The order of efficacy for integrated area (Table 1) was: 2-Me5-HT > α-Me-5-HT > 5-MeOT > serotonin ≈ TFMPP ≈ DOI ≈ mCPP ≈ 8-OH-DPAT. For relaxation, the order of potency using the response amplitude (Fig. 6D) was: serotonin ⩾ 5-MeOT > α-Me-5-HT > DOI. The order of efficacy for relaxation (Table 1) was: 5-MeOT ⩾ serotonin > α-Me-5-HT > DOI.

The effects of serotonin and the serotonin agonists on each of the four components of the serotonin-induced contractile response are summarized in Table 2. All agonists tested induced increases in basal tonus. The agonists showing the greatest potency for inducing increases in basal tonus were mCPP and 5-MeOT. The high ranking of mCPP is based on the long duration of the mCPP-induced response, which in turn was responsible for its high value for integrated area (Fig. 6C). Both phasic longitudinal and circular contractions were evoked by serotonin, 8-OH-DPAT, and 5-MeOT, whereas mCPP and 2-Me-5-HT failed to evoke either contraction type. Both TFMPP and DOI induced longitudinal contractions but not circular contractions, whereas α-Me-5-HT induced circular contractions but not longitudinal contractions. Relaxation was induced by serotonin, 5-MeOT, α-Me-5-HT, and, at high concentrations, DOI.

3. Discussion

3.1. Response of the pharynx to serotonin and LL cell stimulation

The observations in this study differ from those of Lent and Dickinson (1984) in a number of important aspects. Lent and Dickinson stated that both LL cell stimulation and serotonin application to the isolated pharynx caused only the induction of peristaltic waves. They did not report an increase in basal tonus, phasic longitudinal contractions, or relaxation of basal tonus. There are a number of possible explanations for the discrepancies between their data and ours. In both studies, LL cell stimulation caused a series of circular contractions occurring at an approximate frequency of 1 Hz. We characterized these contractions as merely being circular contractions, whereas Lent and Dickinson characterized these contractions as being peristaltic. In the experiments reported in this paper, the pharynx and central nervous system were removed from the animal, whereas, in the Lent and Dickinson (1984) study, the recordings were made in situ. The most obvious difference between the two preparations is that the extrinsic radial muscles, which connect the pharynx to the body wall, were severed by our dissection but left intact by Lent and Dickinson. The presence of intact radial muscles may be necessary for the production of LL cell-induced peristaltic waves (M.H. Dickinson, personal communication).

The differences in the dissection used in this study and that used by Lent and Dickinson (1984) may account for other differences noted in the complexity of the pharyngeal response to LL cell stimulation. Owing

Table 2
Summary of pharyngeal responses to serotonin and serotonin agonists

Agonist (mammalian receptor)	Basal tonus	Longitudinal contractions	Circular contractions	Relaxation
Serotonin (all)	+	+	+	++
8-OH-DPAT (5-HT_{1A})	+	+	+	−
TFMPP (5-$HT_{1B/2C}$)	+	+	−	−
mCPP (5-$HT_{1B/2C}$)	++	−	−	−
DOI (5-$HT_{2A/2C}$)	+	+	−	+(at 1 mM)
α-Me-5-HT (5-HT_2)	+	−	++	+
2-Me-5-HT (5-HT_3)	+	−	−	−
5-MeOT (5-HT_4)	++	+	+	++

Note: A plus sign indicates that the agonist evoked the component, whereas a minus sign indicates a failure to evoke that component. Two plus signs (++) indicate that the agonist strongly evoked the component (compared with the other agonists). These rankings are based primarily on the amplitude of the response evoked by each agonist.

to the more extensive dissection utilized in this study, the preparations were likely to be more mechanically stable than were those of Lent and Dickinson (1984). In fact, Lent and Dickinson noted that they were not able to drive the LL cell at high-impulse frequencies, because it induced large movements of the pharynx that dislodged the microelectrode from the LL cell. It is possible that these large movements were the longitudinal contractions that we noted (Fig. 2). Owing to the mechanical arrangement of the tissues in the head, longitudinal contractions of the pharynx are more likely to dislodge microelectrodes positioned in neurons of the subesophageal ganglion than are circular contractions.

The differences noted between this study and that of Lent and Dickinson (1984) in the complexity of the response of the isolated pharynx to serotonin application may have simple explanations. Lent and Dickinson exposed their isolated pharynxes to serotonin concentrations ranging from 10 nM to 1 μM, whereas, in this study, the concentration range was extended to 1 mM. In the present study, longitudinal phasic contractions and the increase in basal tonus were more easily observed at serotonin concentrations higher than 1 μM than at the lower concentrations used by Lent and Dickinson. Lent and Dickinson's failure to note the relaxation of basal tonus after serotonin washout also may have a simple explanation. Lent and Dickinson did not use a flow-through perfusion system in their organ chamber, as was used in this study. They terminated the application of serotonin by draining the bathing medium from the chamber. In our hands, draining fluid from an organ chamber in this manner invariably produces artifacts in the tension record that would obscure the relaxation noted here.

3.2. The pharyngeal response to serotonin can be pharmacologically dissected with selective serotonin agonists

As noted previously, serotonin application to the isolated pharynx produced a complex response consisting of four components (Fig. 3). We believe that careful examination of the data in Figs. 5 and 7A and in Table 2 indicates that each of the four components of the overall serotonin-induced response can be pharmacologically dissected from the other components.

An increase in basal tonus was induced by all the agonists tested here. However, two agonists (mCPP and 2-Me-5-HT) evoked only an increase in basal tonus without inducing any of the other components of the serotonin-induced response. It is important to note that the actions of mCPP and 2-Me-5-HT were not identical. The duration of the mCPP-induced response was much longer than the 2-Me-5-HT-induced response (Fig. 5B and 5E). This may indicate that these two agonists activate different receptors, each of which induces a basal tonus increase, or that the interaction of the two agonists with a single receptor is quite different. In mammalian preparations, mCPP activates 5-HT_{1B} and 5-HT_{2C} receptors, whereas 2-Me-5-HT activates 5-HT_3 receptors. Receptors of the 5-HT_1 and 5-HT_2 families are G-protein coupled, whereas 5-HT_3 receptors are ligand-gated ion channels. If mCPP and 2-Me-5-HT differentially act on G-protein coupled and ligand-gated receptors in the leech, the suggestion is that increases in basal tonus can be produced through disparate transduction mechanisms. If mCPP and 2-Me-5-HT do in fact bind to a single receptor, the difference in their actions might be due to differences in the agonist-induced conformational changes of the receptor. These differences in agonist-induced conformational changes might then activate the subsequent transduction mechanisms in distinct manners (Gudermann et al., 1996).

The results of this study with the use of mCPP and TFMPP suggest that a serotonin receptor preferentially activated by phenyl piperazines induced an increase in basal tonus. A similar receptor may exist in trematodes. The body wall muscle of the liver fluke, *Fasciola hepatica*, responds to serotonin application by producing both an increase in basal tonus and a series of phasic contractions (Tembe et al., 1993). Most serotonergic agonists examined by these authors induced both types of

contractions. In contrast, TFMPP induced only an increase in basal tonus without inducing phasic contractions; an action similar to that observed in this study. However, in contrast with the leech pharynx, the fluke body wall muscle was insensitive to mCPP.

The phasic longitudinal and circular contractions of the pharynx could be pharmacologically separated from the other type of phasic contraction. DOI and TFMPP (at low concentrations) induced longitudinal contractions but not circular contractions, whereas α-Me-5-HT induced circular contractions but not longitudinal contractions (Fig. 5).

Circular contractions as well as relaxation were induced by serotonin, α-Me-5-HT, and 5-MeOT (Fig. 5A, 5D, and $5F_2$) but not by 2-Me-5-HT (Fig. 5E). A comparison of the structures of these serotonin analogs suggests that the presence of a methyl group at the 2 position of the indole ring (as is found in 2-Me-5-HT) may lead to the loss of the ability to induce circular contractions or relaxation. The serotonin analogs that induced circular contractions or relaxation all lack a functional group at this position. A serotonin-induced decrease in basal tonus was previously described in leech body wall muscles (Leake and Koubanakis, 1995; Leake et al., 1981; Mason and Kristan, 1982). However, the presence of a methyl group at the 2 position did not seem to be correlated with the ability of an agonist to induce relaxation (Leake and Koubanakis, 1995). A comparison of the actions of serotonin and 5-MeOT with those of α-Me-5-HT showed that α-methylation did not affect the ability of the agonist to induce relaxation or circular contractions. However, that is not to say that the presence of α-methylation is without consequence. This is most easily seen by comparing the integrated area and relaxation amplitude (Fig. 6C and 6D), where α-Me-5-HT is considerably less potent than serotonin or 5-MeOT.

There were considerable differences in the order of potency (Fig. 6) and the order of efficacy (Table 1). The agent showing the greatest discrepancy between the two orders was 2-Me-5-HT. The EC_{50} values of 2-Me-5-HT for basal tonus, peak tension, and integrated area were as low or lower than any of the other agonists tested (Table 1). However, the response amplitude elicited by 2-Me-5-HT at 1 mM was one of the smallest of the tested agonists (Fig. 6A–C). The discrepancies between the orders of potency (Fig. 6) and the orders of efficacy (Table 1) are largely because the response induced by some agonists peaked at lower concentrations than did other agonists. For example, the response induced by 2-Me-5-HT peaked at 10 μM and became smaller at higher concentrations. The lower the concentration at which the response peaks, the lower will be the EC_{50}. Another factor underlying the discrepancies between the orders of potency and efficacy is that the concentration–response curves for some agonists are clearly not parallel to those of the other agonists. Perhaps the most obvious example of this phenomenon is that of the 8-OH-DPAT concentration–response curve for peak tension (Fig. 6B). The unusual shape of this concentration–response curve results in 8-OH-DPAT being one of the most potent agonists (as judged by contraction amplitude) at a concentration of 1 mM, whereas the EC_{50} is the highest of any agonist. The reasons for these nonparallel concentration–response curves cannot be directly deduced from the type of experiments performed in this study. However, one possible explanation is that inflections in the concentration–response curve could be due to interactions of the agonist with different receptor types.

On the basis of the differential effects of the agonists utilized in this study, it is possible that the pharynx possesses as many as four different serotonin receptors, with a separate receptor mediating each of the four components of the serotonin response. However, it is possible that all of the agonists examined interacted with a single receptor but the differential contractile responses were due to agonist specific coupling of the receptor to different transduction mechanisms (Eason et al., 1994: Gudermann et al., 1996; Reale et al., 1997; Robb et al., 1994).

Given the diversity of contractile responses elicited by the serotonin agonists utilized in this study, it is likely that the pharynx possesses more than a single class of receptor. If in fact the pharynx possesses more than one class of serotonin receptor, it is not clear if all pharyngeal muscle cells possess the same complement of receptors or only some subset of receptors. However, the finding that certain agonists preferentially induce circular contractions. whereas other agonists preferentially induce longitudinal contractions, suggests that a differential distribution of these hypothesized receptors may occur on the muscle cells involved in these two types of contractions.

3.3. Comparison of the responses of different leech tissues to serotoninergic agonists

Using the Retzius neurons and the body wall muscles of *Hirudo*, Leake and Koubanakis (1995) examined the effects of several of the same agonists used in this study. However, it is difficult to discern patterns of agonist activity common to the two studies. For example, Leake and Koubanakis noted that many of the agonists induced a relaxation of the body wall muscles. The most effective agonists for inducing body wall relaxation were 5-carboxamidotryptamine (which we did not use) and α-Me-5-HT. Although α-Me-5-HT induced pharyngeal relaxation (Fig. 5D), it did so only at high concentrations (100 μM and above). At lower concentrations, α-Me-5-HT preferentially induced circular contractions and an increase in basal tonus. Although Leake and Koubanakis found that α-Me-5-HT also induced phasic contractions, it did so only at high concentrations. Another agonist showing somewhat different

actions in the two studies is 5-MeOT. Leake and Koubanakis found that 5-MeOT induced contractions of the body wall muscles at low concentrations. When tested at low concentrations in this study, 5-MeOT induced relaxation, and only at higher concentrations were contractions induced (Fig 5F). The agonist showing the most similar actions in the two studies was 2-Me-5-HT. When applied to the pharynx, 2-Me-5-HT induced an increase in basal tonus (Fig. 5E). Leake and Koubanakis (1995) noted that 2-Me-5-HT induced an increase in basal tonus of strips of dorsal body wall; however, when applied to strips of ventral body wall, 2-Me-5-HT induced relaxation and suppression of spontaneous contractions.

Although the pharynx, body wall muscles, and Retzius cells of the leech each respond to a variety of serotonin agonists, it is unclear if particular agonists are acting on the same receptor(s) in each preparation. However, if the different agonist-induced responses in each preparation are in fact mediated by different receptors, the results of this study combined with those of Sanchez-Armass et al. (1991), Goldermann et al. (1994), Leake and Koubanakis (1995), and Leßmann and Dietzel (1995) suggest that the leech possesses a large number of different serotonin receptors. It is not unprecedented to find a large number of different receptors for a particular neurotransmitter in the leech. Leeches possess at least seven different types of nicotinic acetylcholine receptors distributed among neurons, glia, and somatic and visceral muscles (Ballanyi and Schlue, 1989; Calabrese and Maranto, 1986; O'Gara et al., 1997; Szczupak et al., 1993, 1998; Walker et al., 1970). Similarly, it would not be surprising to find different types of serotonin receptors in the nervous system and somatic and visceral muscles of the leech.

We believe that it is premature to draw affinities between the responses elicited by the serotonin agonists used in this study and the receptor subtypes described in other systems. Relatively small changes in the amino acid sequence of critical areas of a receptor could lead to substantial differences in the binding of ligands to the receptor. A widely recognized example of an incorrect classification of a serotonin receptor based on pharmacological data is that of the 5-HT_{2C} receptor. This receptor was initially named the 5-HT_{1C} receptor on the basis of its affinity for a number of ligands compared with those of the previously described 5-HT_1 and 5-HT_2 receptor families (Peroutka, 1988). We believe that the elucidation of the affinities between leech pharyngeal serotonin receptors and other serotonin receptors must await either sequence data on the receptors or, at a minimum, determination of the transduction mechanism(s) activated by a particular receptor.

4. Summary

This study reexamined the effects of serotonin and stimulation of a serotonin-containing neuron on the contractile activity of the leech pharynx. Previous descriptions of the serotonin-induced contractile response of the leech pharynx do not convey the complexity of the induced responses noted in this study. Stimulation of the serotonin-containing large lateral (LL) cells in the subesophageal ganglion induced several types of contractile activity in the pharynx. LL cell stimulation caused the following changes in the contractile activity of the pharynx: an increase in basal tonus, longitudinal contractions of approximately 10 to 15 seconds in duration, circular contractions, occurring at approximately 1 Hz, and a relaxation of basal tonus subsequent to the termination of LL cell stimulation. Exposure of the isolated pharynx to serotonin induced similar types of contractile activity. Application of a number of serotonin agonists (8-OH-DPAT, mCPP, TFMPP, DOI, 2-Me-5-HT, α-Me-5-HT, 5-MeOT), known to preferentially activate different classes of vertebrate serotonin receptors, induced distinct types of pharyngeal contractile activity. On the basis of the different contractile patterns induced by these serotonin agonists, we conclude that it is likely that the leech pharynx possesses several different serotonin receptors.

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