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Pharmacological Characterization of the Response of the Leech Pharynx to Acetylcholine

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ABSTRACT In this study we document the sensitivity of the leech pharynx to acetylcholine and begin to characterize the acetylcholine receptor mediating this response by examining the effects of selective cholinergic agonists and antagonists on the contractile behavior of the pharynx. The order of potency derived from the EC_{50} of each agonist was (±)epibatidine > acetylcholine (in the presence of physostigmine) » McN A-343 » carbachol > nicotine. However, when response amplitude was considered, the order of potency to the tested agonists was (±)epibatidine » nicotine » McN A-343 » carbachol > acetylcholine. Acetylcholine-induced contractions of the pharynx were antagonized by d-tubocurarine, but not by α-bungarotoxin, α-conotoxin M1, or mecamylamine. Application of high concentrations of hexamethonium (1 mM) augmented the acetylcholine-induced contractions. However, this augmentation was apparently due to inhibition of acetylcholinesterase by hexamethonium. The muscarinic antagonist atropine produced complex actions and apparently acted as a mixed agonist/antagonist. Atropine by itself produced an increase in basal tonus and increased the frequency and amplitude of phasic contractions. Atropine increased the peak tension of the acetylcholine-induced response; however, it reduced the amplitude of both the acetylcholine-induced increase in basal tonus and integrated area. Based on the pharmacological profile of the pharyngeal acetylcholine response, we conclude that the acetylcholine receptor mediating the response is a nicotinic receptor. However, the responsiveness of the pharynx to muscarinic agents diverges from that of a classical nicotinic receptor. *J. Exp. Zool. 284:729–741, 1999.*

Six different acetylcholine (ACh) receptors have been pharmacologically characterized in the leech (Flacke and Yeoh, '68b; Walker et al., '70; Ross and Triggle, '72; Calabrese and Maranto, '86; Ballanyi and Schlue, '89; Szczupak et al., '93, '98). These receptors are located in four different cell types: body wall muscle, heart muscle, glial cells, and neurons. As in vertebrates, the central nervous system ACh receptors of the leech are pharmacologically distinct from muscle ACh receptors. The pharmacology of each of these receptors suggests that they all belong to the nicotinic family of ACh receptors. No muscarinic receptors have been described in the leech.

The two other groups of annelids, the polychaetes and oligochaetes, also use ACh as a neuromuscular transmitter (Wu, '39b; Nicol, '52; Alvarez et al., '69). The ACh receptors of earthworm and polychaete body wall muscle are not easily classified as nicotinic or muscarinic receptors using pharmacological data (Hassoni et al., '85; Alvarez et al., '69). However, the ACh receptors of earthworm and polychaete guts are apparently muscarinic receptors (Wu, '39a; Ambache et al., '45; Anctil et al., '84; Ukena et al., '95).

The pharynx of the leech *Hirudo medicinalis* is the muscular first region of the gut. Contractions of the pharynx draw blood from a wound on the prey and transport it to the crop, where the blood is stored (Dickinson and Lent, '84). The pharynx is responsive to a number of neurotransmitters, including serotonin, FMRFamide, and SCP_B (Lent and Dickinson, '84; O'Gara, '90; O'Gara et al., '99). In this report, we show that the leech pharynx is responsive to ACh and we characterize the actions of a number of cholinergic agonists and antagonists on the pharynx. Based upon the actions of these agents, we suggest that the receptor mediating this response is a nicotinic receptor, and that

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the pharmacological profile of this receptor is unique. Some of these results have previously appeared in abstract form (O'Gara et al., '97).

MATERIALS AND METHODS

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

Isolated pharynx 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 extrinsic radial muscles on the dorsal and lateral sides. The ventral radial muscles were severed 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. 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), was inserted through each end of the pharynx. One nylon suture was anchored to the bottom of a small perfusion tissue bath (volume 0.2 ml), while the suture attached to the other end of the pharynx was attached to an isometric force transducer (FORT-10, WPI, Sarasota, FL). This physical arrangement most effectively monitored longitudinal contractions of the pharynx; however, as judged by simultaneous visual observation of the pharynx, circular contractions were also recorded. The pharynx was placed under approximately 20–25 mN of tension and allowed to relax for approximately 1 hr. Most pharynxes were initially quiescent, but exhibited spontaneous contractions well before the end of the 1 hr relaxation period.

The output of the force transducer was fed into a transducer interface (Transbridge, WPI, Sarasota, FL, or ETH-200, CB Sciences, Dover, NH), whose subsequent output was fed into a computer-based data acquisition system (WINDAQ, Dataq Instruments, Akron, OH). The signal from the force transducer was digitized at 50 samples/s and recorded to disk. Subsequent data analysis of the records was performed 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 (rate set via a drip chamber); saline was removed from the top of the chamber by suction. Control or drug-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. At any one time, the drug reservoir contained a measured volume of drug-containing saline appropriate for the next experimental manipulation. The application of drug was initiated by the switching of a valve connected to the drug and control saline reservoirs. The entire volume contained within the drug reservoir was applied to the preparation at a rate of 1 ml/min, and was followed by a saline wash. An air bubble was introduced into the perfusion line to indicate the beginning and end of each treatment. The beginning and end of each treatment was noted on the data acquisition file by manually entered event markers. Each application of agonist-containing saline was 1 ml in volume and approximately 1 min in duration. This volume of agonist-containing saline was approximately five times the volume of the organ chamber. When determining 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 peak tension and the integrated area under the contraction curve during the response to the agonist. Integrated area is sensitive not only to peak tension, but also to response duration. The concentration of agonist that produced a half-maximal response (EC_{50}) was estimated from a logistic curve fit to the concentration-response data for peak tension. Responses to agonist concentrations that apparently induced desensitization were not included in the curve fitting procedure.

Protocol for examining the effects of cholinergic antagonists

An isolated pharynx was exposed to three 1 ml applications of 100 μM ACh. The first and third applications were control applications of ACh alone, while the second application was preceded by a 10-min pretreatment of the antagonist im-

mediately followed by a 1-ml application of 100 μM ACh plus the antagonist. Each application of ACh (and antagonist) was followed by a saline wash. The responses of the pharynx to each ACh application were quantified as noted above. However, the response to ACh in the presence of atropine (Fig. 7) was more complex than the response to ACh alone, in that it consisted of a series of phasic contractions superimposed on a rise in basal tonus. Aside from peak tension and integrated area, we also quantified the amplitude of this increase in basal tonus. 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 contractions.

Statistics were performed using SigmaStat 2.0 (SPSS Inc., Chicago, IL). Values are presented as mean ± SE if the data were normally distributed (Kolmogorov-Smirnov test with Lilliefors' correction). Data that were not normally distributed are presented as the median (25th percentile, 75th percentile). Normally distributed data were subjected to a repeated-measures ANOVA, while data that were not normally distributed were subjected to a Friedman repeated measures ANOVA on ranks. If the results of either type of ANOVA were significant, we performed multiple comparisons between the treatments using Dunnett's method. For these multiple comparisons, the response to the first application of ACh was considered the control against which the data for the other two treatments were compared.

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), HEPES buffer (10), brought to pH 7.4 with NaOH. Experiments were conducted at room temperature (21–25°C). Acetylcholine chloride, atropine sulfate, α-bungarotoxin, carbachol (carbamylcholine chloride), (±)epibatidine dihydrochloride, hexamethonium chloride, McN A-343 {4-(N-[3-chlorophenyl]-carbamoyloxy)-2-butynyl-trimethylammonium chloride}, mecamylamine hydrochloride, (-)-nicotine hydrogen tartrate were purchased from Sigma (St. Louis, MO). Physostigmine (eserine) was purchased from Aldrich (Milwaukee, WI) and α-conotoxin M1 was purchased from RBI (Natick, MA). All drugs except α-bungarotoxin and α-conotoxin M1 were dissolved in saline just prior to use. α-Bungarotoxin and α-conotoxin M1 were dissolved in saline, divided into experiment-sized aliquots, and frozen. The frozen toxin was diluted in saline just prior to use.

RESULTS

Response of the isolated pharynx to application of acetylcholine and cholinergic agonists

The isolated pharynx responded to a 1-min application of ACh by producing a smooth contraction (Fig. 1), whose amplitude was dependent upon the ACh concentration (Fig. 2). Spontaneous contractions, when present, were suppressed by ACh application and they remained suppressed for several minutes following ACh washout. The threshold concentration for inducing a change in both peak tension and integrated area was approximately 1–10 μM ACh.

To further characterize the acetylcholine receptor, the pharynx was exposed to the nicotinic agonists epibatidine and nicotine, to the muscarinic M_1 agonist McN A-343, and the nonselective cholinergic agonist carbachol (Fig. 2). The responses of the pharynx to high concentrations of epibatidine, carbachol, and nicotine all show apparent receptor desensitization. Responses of the pharynx to high concentrations of these agonists were smaller than the responses elicited by lower concentrations (although for nicotine, this pattern did not hold for integrated area). The concentration

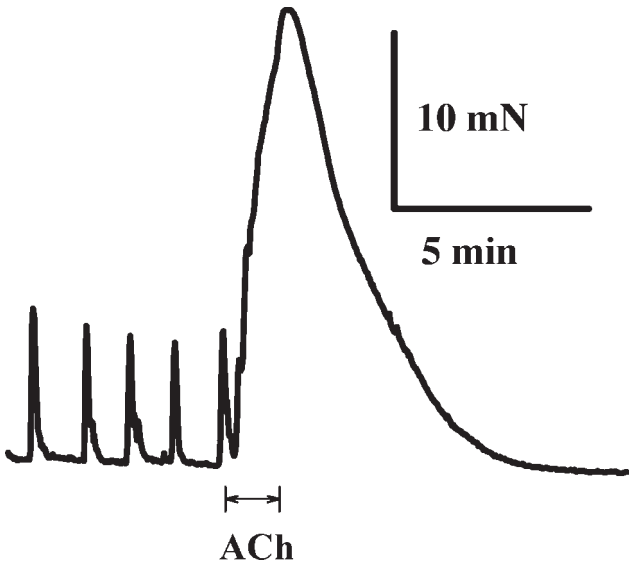

Fig. 1. Contractile response of the leech pharynx to acetylcholine. An approximately 1-min application (indicated by arrowed line) of 1 mM ACh induced a smooth contraction and a suppression of phasic contractions during and after the ACh exposure.

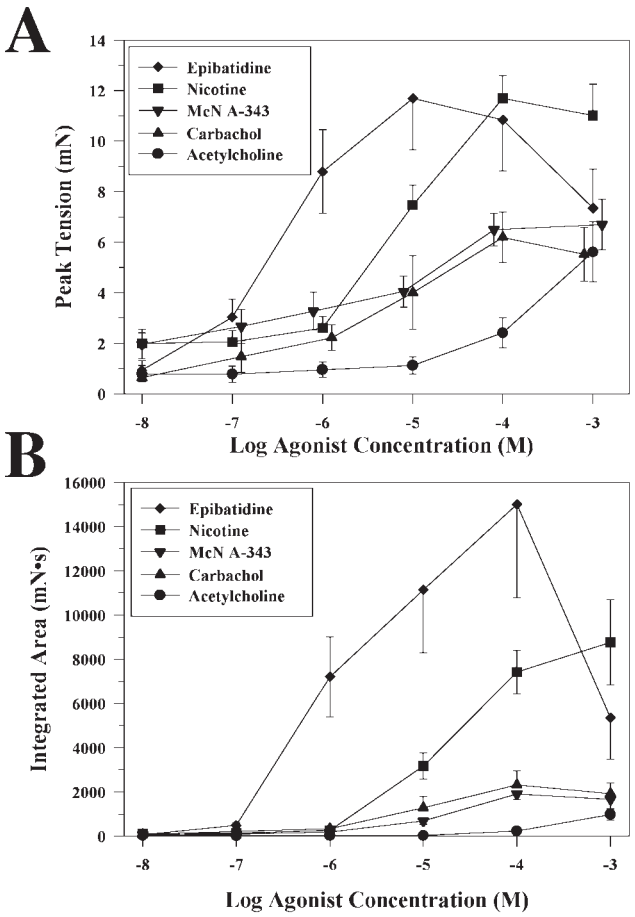

Fig. 2. Concentration-response curves for cholinergic agonists. (**A**) Peak tension. Some data points have been displaced horizontally for clarity. All points clustered around a tick mark for a particular concentration reflect the data at that concentration. The curves for each agonist probably overestimate the sensitivity of the pharynx at low agonist concentrations. The values for peak tension at low agonist concentrations often indicate the presence of a "spontaneous" phasic contraction occurring shortly after the agonist application. The amplitudes of such contractions are reported here since there was no way of determining if the phasic contraction was induced by the agonist application or if the contraction was truly spontaneous. (**B**) Integrated area. The data in both panels was derived from the following number of preparations: epibatidine (9), nicotine (21), McN A-343 (8), carbachol (10), ACh (9). When error bars are not visible, they are smaller than the symbol.

of each agonist that produced a half-maximal response (EC_{50}) for peak tension was estimated from a logistic curve fit to the peak tension data in Fig. 2A. The EC_{50} for each agonist is reported in Table 1. Since the response induced by ACh at high concentrations did not reach an asymptotic peak as shown in Fig. 2A, the EC_{50} reported in Table 1 is an underestimate of the true EC_{50}. Based on the

TABLE 1. EC_{50}s of cholinergic agonists for peak tension

Agonist	EC_{50}
Acetylcholine	>100 μM
Acetylcholine (0.1 μM physostigmine)	0.97 μM
Carbachol	32 μM
Epibatidine	0.32 μM
Nicotine	60 μM
McN A-343	1.2 μM

EC_{50} of each agonist, the order of efficacy was: epibatidine > McN A-343 > carbachol > nicotine » ACh. When maximal response amplitude was considered, the order of potency for both peak tension and integrated area was: epibatidine > nicotine » McN A-343 ≈ carbachol > ACh. The largest difference between using the EC_{50}s vs. the response magnitude to access the relative potency of the tested agonists occurred when considering nicotine. Nicotine produced contractile responses that were nearly equal in amplitude (albeit at a higher concentration) to the most effective agonist, epibatidine. However, the only agonist with a higher EC_{50} than nicotine was ACh (Table 1).

The agonist-induced contraction induced by either epibatidine or nicotine persisted for a considerably longer period (following washout) than did responses to other agonists. For example, a 1-min application of epibatidine at concentrations of 0.1 μM or above, necessitated a wash period of 2–3 hr before the response returned to baseline levels of tension. Nicotine was less potent in this respect, but often the contractions induced by high concentrations of nicotine did not return to baseline levels in less than 30 min. The prolonged decay of the epibatidine- and nicotine-induced responses accounts for the relatively greater potency of these agonists as measured by integrated area as compared to their potency as measured by peak tension (Fig. 2). The initiation of the response following application of epibatidine also had a longer latency than the response to the other tested agonists. Once the drug-containing saline entered the organ chamber, the response to other agonists was usually visible within 5 sec; however, epibatidine-induced contractions frequently were evident only after a delay of 20–60 sec following drug addition.

The pharynx possesses an endogenous cholinesterase

Application of carbachol, a cholinesterase resistant analog of acetylcholine, induced larger responses than those induced by ACh (Fig. 2). This

suggested that the pharynx possesses an endogenous cholinesterase. To examine this possibility directly, we recorded the response of the pharynx to ACh application during continuous exposure to the cholinesterase inhibitor, physostigmine (Fig. 3). The effects of physostigmine upon ACh-induced contractions were concentration-dependent. Application of 0.1 μM physostigmine augmented the amplitude of the subsequent ACh-induced response; this augmentation was more prevalent for peak tension (Fig. 4A) than integrated area (Fig. 4B). The EC_{50} for peak tension was reduced by approximately two orders of magnitude by 0.1 μM physostigmine (Table 1). In contrast, a higher concentration of physostigmine (1 μM), did not augment the ACh-induced response (Figs. 3B, 4).

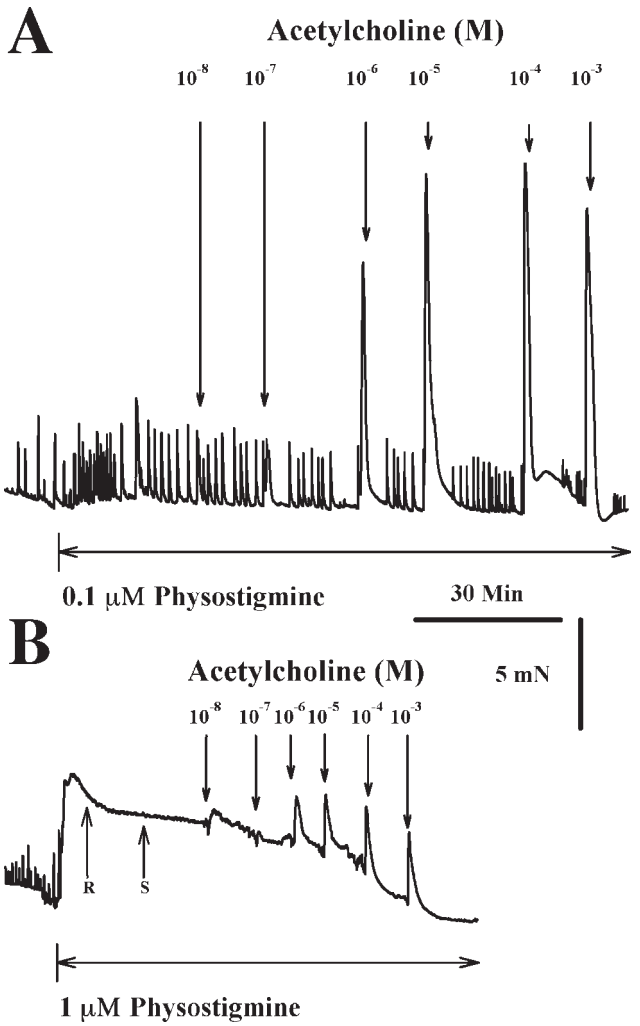

Fig. 3. Effects of the cholinesterase inhibitor physostigmine were concentration-dependent. (**A**) Application of 0.1 μM physostigmine caused a small increase in basal tonus as well as a transient increase in phasic contraction frequency. Numbers above arrows indicate the concentration (in M) of ACh applied. (**B**) Application of 1 μM physostigmine caused a large increase in basal tonus and a suppression of phasic contractions. Note that the increase in basal tonus was not maintained. This decrement in basal tonus occurred in two stages, an initial rapid stage (R) occurring over the first few minutes after physostigmine application, followed by a slower rate of relaxation (S).

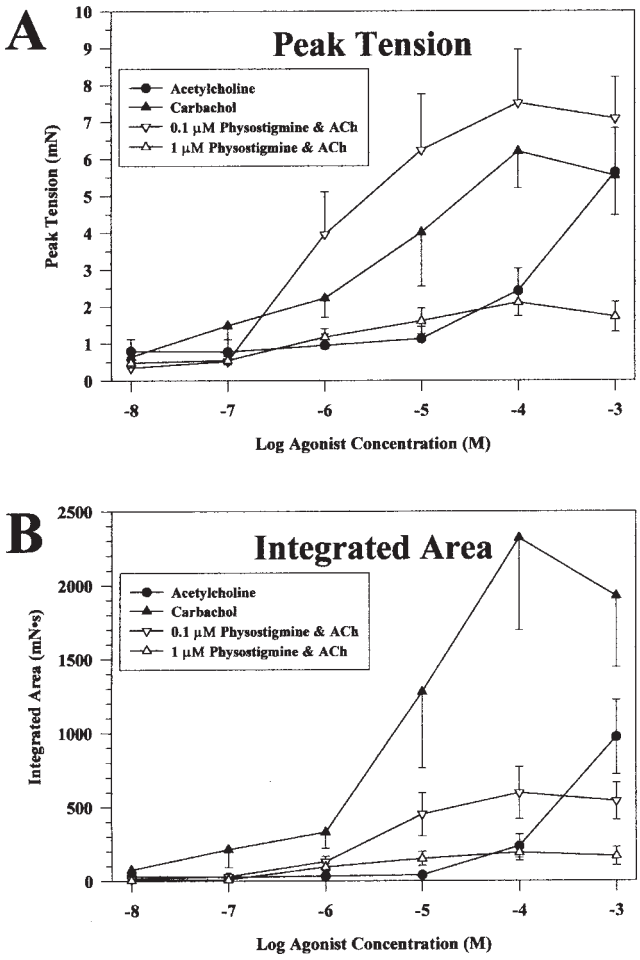

Fig. 4. Concentration-response curves showing the potentiation of ACh-induced responses due to low, but not high, concentrations of physostigmine. (**A**) Physostigmine (0.1 μM) caused a substantial increase in ACh-induced peak tension. Values for ACh-induced peak tension in the presence of 1 μM physostigmine were similar to those of ACh alone, except at 1 mM (10^{-3} M) where the value was substantially lower than that of ACh alone. (**B**) For integrated area, 0.1 μM physostigmine augmented the ACh-induced response at 10 μM (10^{-5} M) and 100 μM (10^{-4} M), but not at 1 mM (10^{-3} M). Values for integrated area in the presence of 1 μM physostigmine were similar to those of ACh alone, except at 1 mM (10^{-3} M) where the value was lower than that of ACh alone. Data in each panel for ACh and carbachol are replotted from Fig. 2 for comparative purposes. Data for the two concentrations of physostigmine were derived from 10 preparations each.

Application of 1 μM physostigmine alone caused a substantial rise in basal tonus. The physostigmine-induced increase in basal tonus decayed rapidly at first, and than at a slower rate. Subsequent application of ACh induced smaller responses than were produced by ACh application in the presence of the lower concentration of physostigmine. In addition, baseline tension decayed more rapidly during the ACh exposure series than it had prior to the first application of ACh. The responses to ACh application in the presence of 1 μM physostigmine were no larger than the responses induced by ACh alone (Fig. 4). And in fact, the contractile response of the pharynx to 1 mM ACh was considerably smaller in the presence of 1 μM physostigmine than in its absence.

Effects of cholinergic antagonists upon the ACh-induced response

To further characterize the pharyngeal ACh-induced response, the ability of a number of cholinergic antagonists to inhibit the response of the pharynx to 100 μM ACh were examined. Each experiment consisted of three ACh applications: the first and third applications were control runs, while the second ACh exposure occurred following a 10-min pretreatment of the antagonist. To determine if the response amplitudes to repeated applications of ACh were consistent, we exposed six preparations to three 1-min applications of 100 μM ACh at 1-hr intervals. Statistical analysis of these data indicated that for both peak tension and integrated area, there were no significant differences between the three responses (for peak tension: $F = 1.47$; $P = 0.276$; df = 2, 10; for integrated area: $F = 0.543$; $P = 0.597$; df = 2, 10). These data indicate that the pharyngeal response to ACh remained consistent when applied repeatedly at 1-hr intervals.

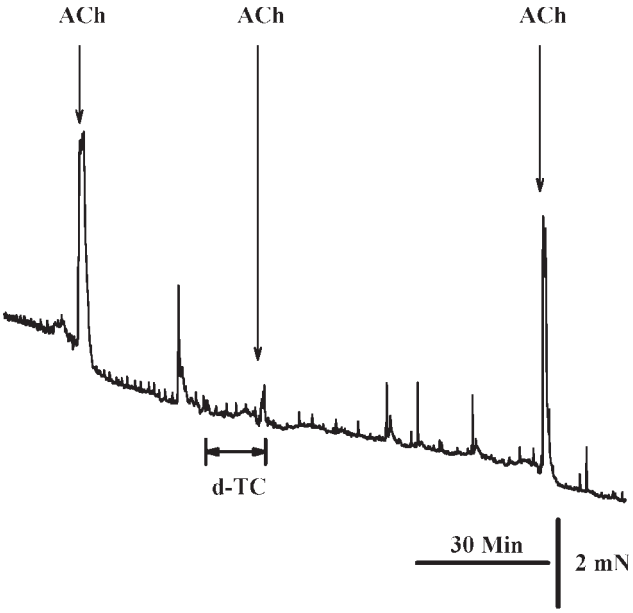

Fig. 5. Antagonism of the ACh-induced response by d-tubocurarine. Continuous tension record from a pharynx during three approximately 1-min exposures to ACh. The first and third applications (arrows) were control applications of 100 μM ACh alone. The second ACh application was preceded by an approximately 10-min pretreatment of 100 μM d-tubocurarine (duration indicated by arrows). The pharynx responded to the first and third applications by producing a vigorous contraction; however, the response to ACh in the presence of d-tubocurarine was markedly reduced. The d-tubocurarine-induced antagonism of the ACh-induced response in this preparation was among the largest we observed. Also note that the frequency of small phasic contractions is lower during d-tubocurarine exposure than prior to the drug treatment (compare the frequency of small deflections prior to the large contraction which just precedes the start of the d-tubocurarine with the contraction frequency during d-tubocurarine application). The general downward drift (relaxation) of the record is a nearly universal characteristic of tension recordings from isolated pharynxes.

d-Tubocurarine

The most effective cholinergic antagonist tested on the pharynx was d-tubocurarine (Fig. 5). Pretreatment of the pharynx with 100 μM d-tubocurarine caused a significant reduction of the ACh-induced response as measured by peak tension and integrated area (Fig. 6). The effects of d-tubocurarine were reversible, in that both peak tension and integrated area in response to the third ACh application were not significantly different from the first ACh application. In six of seven preparations producing spontaneous contractions prior to d-tubocurarine application, d-tubocurarine application by itself caused a reduction in the frequency of spontaneous contractions (Fig. 5).

Atropine

Atropine is normally considered a muscarinic antagonist in vertebrates. However, the actions of atropine on the pharynx were complex and not consistent with a solely antagonistic action (Fig. 7). Application of 1 mM atropine by itself induced an increase in the frequency and amplitude of phasic contractions, as well as causing an increase in basal tonus. Normally, when ACh was applied alone there was a suppression of spontaneous phasic contractions (Fig. 1). However, in the presence of atropine, these phasic contractions were not

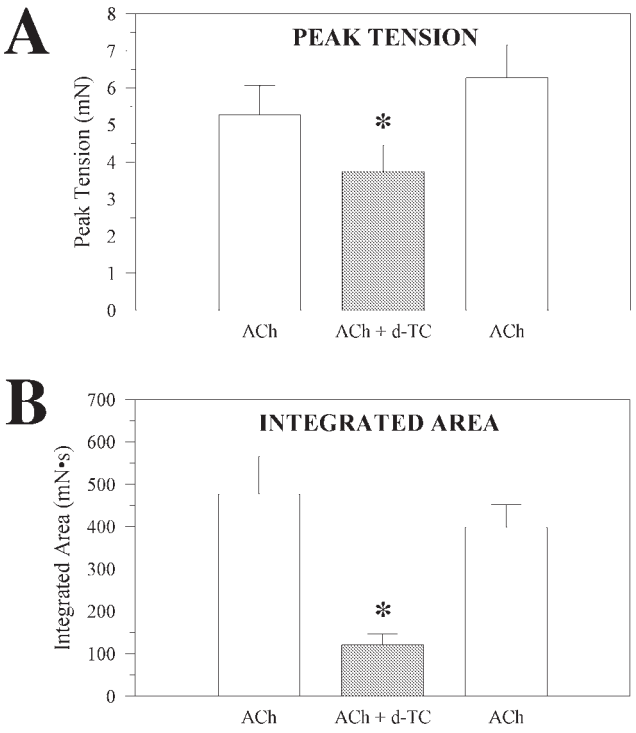

Fig. 6. Quantification of antagonism produced by 100 μM d-tubocurarine on the ACh-induced response (N = 10). (**A**) Peak tension was significantly reduced compared to the first ACh application (F = 6.82; $P = 0.006$; df = 2, 18). In this and subsequent figures, an asterisk in the figure indicates that the treatment was significantly different from the first control ACh application (Dunnett's test, $P < 0.05$). (**B**) Integrated area was also significantly reduced compared to the first ACh application (F = 12.74; $P < 0.001$; df = 2, 18).

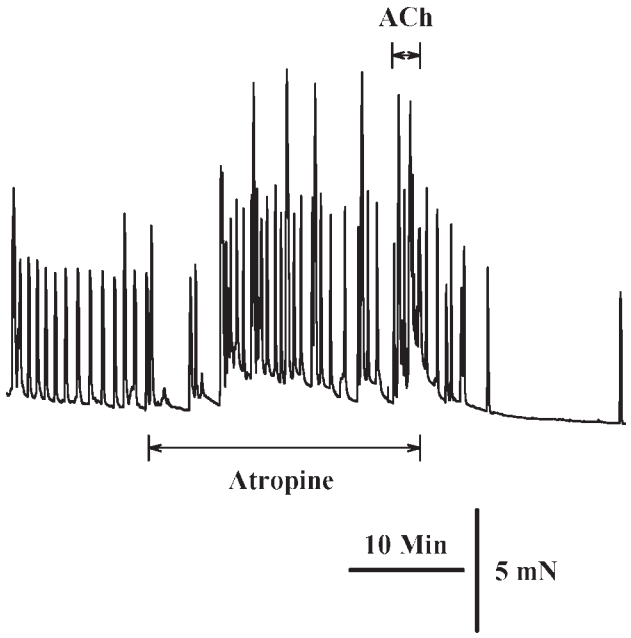

Fig. 7. Effects of atropine upon the contractile behavior of the pharynx. Application of 1 mM atropine elicited an increase in the frequency and amplitude of phasic contractions superimposed upon an increase in basal tonus. Note that the phasic contractions were not suppressed during the exposure to ACh (compare with Fig. 1).

suppressed (Fig. 7). The ACh-induced response in the presence of atropine consisted of a series of phasic contractions superimposed upon an increase in basal tonus. To better assess the actions of ACh in the presence of atropine, we also measured the maximal increase in basal tonus as well as peak tension and integrated area. Atropine caused a significant increase in the ACh-induced peak tension that was not reversible over the 1-hr wash time between the termination of atropine treatment and the last exposure to ACh alone (Fig. 8A).In contrast to its effects on peak tension, atropine reversibly reduced the ACh-induced response as measured by integrated area and basal tonus (Fig. 8B, C). These results suggest that atropine had both agonistic and antagonistic actions on the pharyngeal ACh receptor.

Hexamethonium

The effects of the ganglionic antagonist hexamethonium upon the ACh-induced response were concentration-dependent. Pretreatment with 100 μM hexamethonium had no effect on the subsequent ACh-induced response (Fig. $9A_1$, A_2). However, pretreatment with 1 mM hexamethonium caused a reversible augmentation of peak tension and integrated area (Fig. $9B_1$, B_2). Application of 1 mM hexamethonium by itself, induced a small increase of basal tonus and an increase in the frequency of "spontaneous" contractions in seven of nine preparations (not shown). In five of the seven preparations that exhibited this increase in basal tonus and contraction frequency, the phasic contractions ceased approximately 4 min into the hexamethonium application. In the other two of these seven preparations, the phasic contractions became smaller in amplitude and decreased in frequency. The augmentation of the ACh-induced response in the presence of 1 mM hexamethonium could be due to the known ability of hexamethonium to inhibit acetylcholinesterase (Rang and Rylett, '84; Seto and Shinohara, '88). However, augmentation of the ACh-induced response could also occur if hexamethonium was a weak cholinergic agonist. To distinguish between these two possibilities, we repeated the previous experiment, except that it was conducted in the continuous

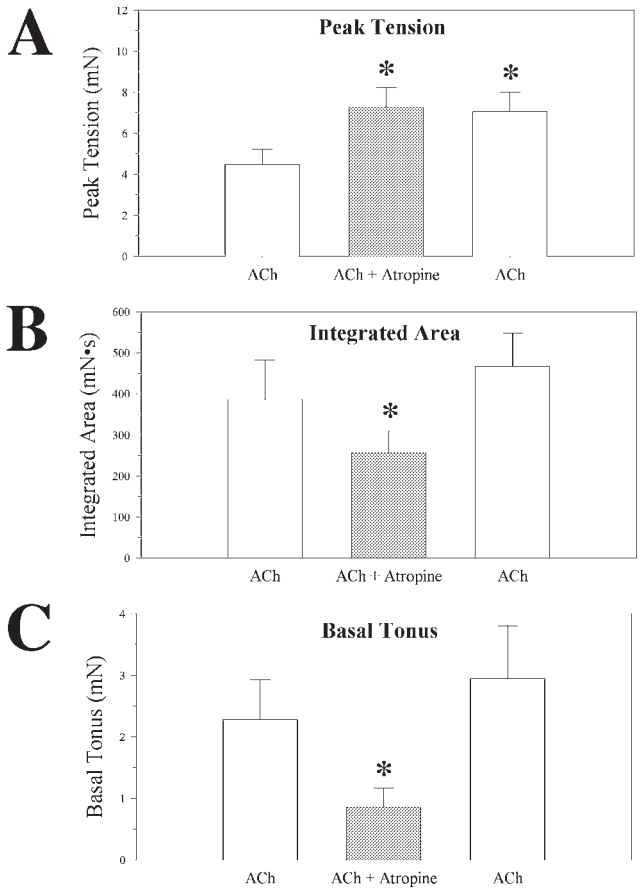

Fig. 8. Quantification of the effects of 1 mM atropine on the ACh-induced response. (**A**) Peak tension was significantly elevated by exposure to atropine (N = 11) (F = 13.95; $P < 0.001$; df = 2, 20). Peak tension in response to ACh application remained significantly elevated following a prolonged (1 hr) washout of atropine (3rd bar). (**B**) Integrated area was significantly reduced by atropine exposure (F = 9.75; $P = 0.001$; df = 2, 20). (**C**) Basal tonus was also significantly reduced by atropine exposure (F = 8.91, $P = 0.002$, df = 2, 20).

presence of 0.1 μM physostigmine to inhibit the endogenous cholinesterase. Under these conditions, the responses to ACh were not augmented by 1 mM hexamethonium (Fig. 9C_1, C_2). These results indicate that the previously observed augmentation in the presence of 1 mM hexamethonium was due to cholinesterase inhibition by hexamethonium. In the continuous presence of 0.1 μM physostigmine, peak tension in response to the third ACh application was significantly reduced compared to the initial ACh application (Fig. 9C_1). This reduction could be the result of receptor desensitization.

Ineffective cholinergic antagonists

The following antagonists failed to affect the ACh-induced response: 1 μM α-bungarotoxin (a vertebrate muscular antagonist), 1 μM α-conotoxin M1 (a vertebrate muscular antagonist), and 100 μM mecamylamine (a vertebrate ganglionic antagonist). The statistical analysis is presented in Table 2.

DISCUSSION

This study demonstrates that the leech pharynx responds to acetylcholine application by producing a smooth contraction (Fig. 1). This response pattern contrasts with the pharyngeal responses induced by FMRFamide and serotonin (O'Gara, '90; O'Gara et al., '99). Each of these neurotransmitters induces a series of phasic contractions superimposed upon an increase in basal tonus (although these response patterns are distinct from one another). The pharmacology of the ACh-induced response suggests that it is mediated by a nicotinic receptor. When response amplitude is considered, the pharynx was much more responsive to the nicotinic agonists epibatidine and nicotine than it was to the muscarinic M_1 agonist McN A-343 (Fig. 2). Epibatidine was the most potent cholinergic agonist tested on the pharynx (Table 1, Fig. 2). On vertebrate neuronal nicotinic receptors, epibatidine is one of the most potent nicotinic agonists known, although it has considerably lower affinity for muscle-type receptors (Gerzanich et al., '95). The high potency of epibatidine on the leech pharynx strongly suggests that the pharyngeal receptor is a nicotinic receptor.

The ability of atropine to reduce some components of the ACh-induced response (Fig. 8) is unusual for a classical nicotinic receptor. However, atropine is known to block some types of nicotinic receptors in insects, nematodes, leeches, and vertebrates (David and Sattelle, '84; Ballanyi and Schlue, '89; Colquhoun et al., '91; Elgoyhen et al., '94; Olmez et al., '94). We have interpreted the atropine-induced increase in peak tension (Figs. 7, 8) as being due to an agonistic effect of atropine. It is possible that atropine could produce this effect via inhibition of cholinesterase. However, we are unaware of any reports of cholinesterase inhibition by atropine.

Comparison of the cholinergic pharmacology of the pharynx with the pharmacology of other leech ACh receptors

The effectiveness of the antagonists used in this study is compared with their effectiveness on the

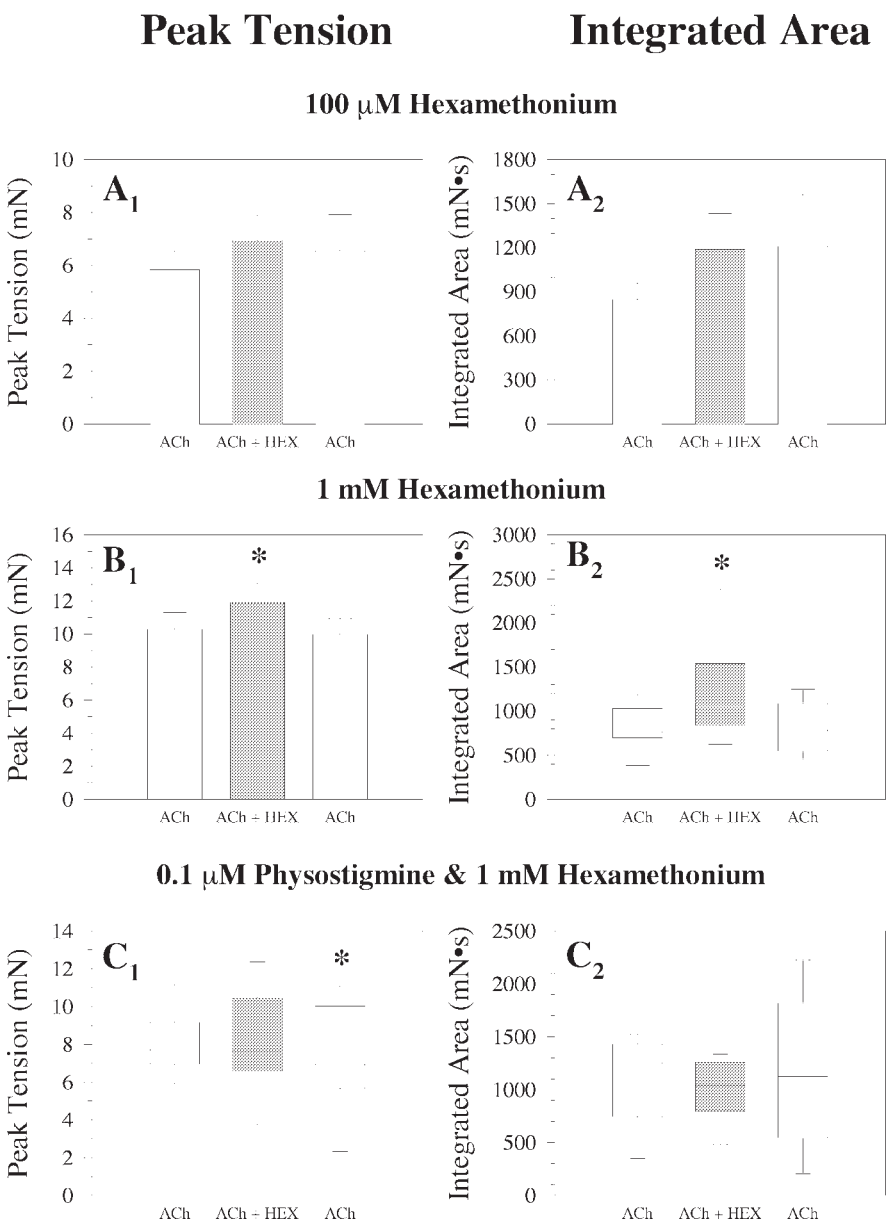

Fig. 9. Augmentation of the ACh-induced response by hexamethonium was due to inhibition of cholinesterase. Graphs in the left column show data for peak tension, while graphs in the right column show data for integrated area. (**A**) Pretreatment of the pharynx with 100 μM hexamethonium (N = 10) had no effect on peak tension (A_1)(F = 1.231; P = 0.320; df = 2, 15) or integrated area (A_2) (F = 1.033; P = 0.380; df = 2, 15). (**B**) Pretreatment of the pharynx with 1 mM hexamethonium (N = 9) caused an increase in peak tension (B_1) (F = 5.72; P = 0.013; df = 2, 16) and integrated area (B_2) (χ^2_r = 11.56; P = 0.001; df = 2 [Friedman ANOVA on ranks]). In this figure, data that were not normally distributed are displayed using a box plot. The line in the center of the box is the median, the lower and upper limits of the box are the 25th and 75th percentile respectively, and the whisker bars are the 10th and 90th percentiles. (**C**) Continuous exposure to 0.1 μM physostigmine (N = 18) eliminated the hexamethonium-induced augmentation of peak tension (C_1). However, peak tension in response to the third application of ACh was significantly smaller than that to the first application (χ^2_r = 13.78; P = 0.001; df = 2). The hexamethonium-induced augmentation of integrated area was also eliminated by continuous exposure to 0.1 μM physostigmine (C_2) (χ^2_r = 2.11; P = 0.348; df = 2).

TABLE 2. Statistical analysis of ineffective cholinergic antagonists[1]

		Drug					
		1 μM α-Bungarotoxin (N = 13)		1 μM α-Conotoxin M1 (N = 10)		100 μM Mecamylamine (N = 9)	
Peak tension (mN)	1st Control (100 μM ACh)	5.66 ± 0.93	F = 2.64	1.53 (0.84, 1.91)	χ^2_r = 2.60	1.46 ± 0.21	F = 0.224
	Drug (ACh & drug)	6.24 ± 0.91	*P* = 0.09 df = 2, 24	1.61 (1.11, 1.94)	*P* = 0.27 df = 2	1.60 ± 0.25	*P* = 0.80 df = 2, 16
	2nd Control (100 μM ACh)	6.58 ± 0.92		1.47 (1.02, 2.23)		1.64 ± 0.18	
Integrated area (mN · s)	1st Control (100 μM ACh)	533.55 (295.58, 693.90)	χ^2_r = 0.62	76.32 (18.86, 147.82)	χ^2_r = 3.80	49.23 (31.45, 83.56)	χ^2_r = 0.667
	Drug (ACh & drug)	415.92 (304.67, 690.23)	*P* = 0.74 df = 2	79.69 (55.65, 92.63)	*P* = 0.15 df = 2	53.63 (46.38, 127.57)	*P* = 0.81 df = 2
	2nd Control (100 μM ACh)	561.57 (424.58, 684.34)		88.39 (79.12, 156.56)		61.15 (34.91, 79.00)	

[1]Normally distributed data are presented as the mean ± the SE, while data that were not normally distributed are presented as the median (25th percentile, 75th percentile).

six pharmacologically described leech ACh receptors, as well as vertebrate muscular and neuronal/ganglionic receptors in Table 3. Two leech ACh receptors have not been included in the table because they have not undergone extensive pharmacological characterization (Pellegrino and Simonneau, '84; Schmidt and Calabrese, '92). These two studies describe ACh-induced chloride currents in anterior pagoda neurons (AP cells) and heart interneurons (HN cells) respectively. The receptors mediating these responses may be similar to the more thoroughly characterized ACh receptor mediating an anionic current in the Retzius cells (Szczupak et al., '93). The presentation of the vertebrate receptors is generic and fails to reflect some of the diversity present within these groups (especially the neuronal/ganglionic receptors).

A comparison of the pharmacological profile of the pharyngeal response with the other receptors presented in Table 3, indicates that the pharyngeal receptor is markedly different from the two vertebrate nicotinic receptors and the other leech ACh receptors. Of the receptors listed in Table 3, the pharyngeal ACh receptor is most similar to the receptor of leech heart muscle (Calabrese and Maranto, '86). The only difference noted in Table

TABLE 3. Comparison of the effects of cholinergic antagonists on vertebrate nicotinic and leech acetylcholine receptors[1]

	Vertebrate receptors		Leech receptors						
Antagonist	Vertebrate muscular receptor[2]	Vertebrate neuronal/ganglionic receptor[2]	Leech giant glial cell[3]	Retzius cell fast cationic current[4]	Retzius cell slow cationic current[5,6]	Retzius cell anionic current[4]	Leech body wall muscle[7,8,9]	Leech heart muscle[10]	Leech pharynx (this study)
d-Tubocurarine	+	+	+	+	–	+	+	+	+
α-Bungarotoxin	+	–	+	–	?	+	+	–	–
α-Conotoxin M1	+	–	?	?	?	?	?	?	–
Hexamethonium	–	+	+	+	+/?	–	–	+	–
Mecamylamine	+ (weak)	+	?	+	–	–	?	?	–
Atropine	–	–	+	–	+	–	–	Mixed	Mixed

[1]In each cell of the table, a "+" indicates that the drug antagonizes the ACh-induced response, while a "–" indicates the drug does not antagonize the response. A "?" in the cell indicates that the effect of the drug has not been examined on that ACh receptor. "Mixed" indicates that the agent has both agonistic and antagonistic actions.
[2]Feldman et al. ('97).
[3]Ballanvi and Schlue ('89).
[4]Szczupak et al. ('93).
[5]Szczupak et al. ('98).
[6]Marín Burgin and Szczupak ('98).
[7]Flacke and Yeoh ('68b).
[8]Walker et al. ('70).
[9]Ross and Triggle ('72).
[10]Calabrese and Maranto ('86).

3 is that hexamethonium antagonizes the ACh-induced response on heart muscle, but not the pharynx. However, there are a number of more subtle differences in the responses of the heart and pharyngeal ACh responses. The pharyngeal response was substantially more sensitive than the heart receptor to the antagonistic actions of d-tubocurarine and to the agonistic actions of carbachol and nicotine. Calabrese and Maranto ('86) reported that the threshold concentrations for carbachol and nicotine were 5 μM and 1000 μM respectively, while in this study the threshold concentrations for both substances were below 0.1 μM. The differences in the responses to cholinergic agents indicate that although the pharyngeal and heart ACh receptors are similar, they are in fact distinct.

The responses of the leech body wall muscles and the pharynx to cholinergic antagonists and agonists indicate that these receptors are also distinct. α-Bungarotoxin blocks the body wall receptor but not the pharyngeal receptor (Table 3; Ross and Triggle, '72); while atropine blocks components of the pharyngeal response (Figs. 7, 8), but does not block ACh-induced body wall responses (Walker et al., '70). In addition, there are also differences in the relative potency of a number of cholinergic agonists between the two preparations. In the body wall, the maximal contraction amplitude induced by ACh, carbachol, or nicotine was approximately the same magnitude (Flacke and Yeoh, '68a). However, in the pharynx, nicotine produced a much larger maximal contraction than carbachol or ACh (in the presence of 0.1 μM physostigmine) (Figs. 2, 4).

The actions of physostigmine on the body wall and pharynx also suggest differences between the ACh receptors on these tissues. In the body wall, application of physostigmine in concentrations ranging from 10 nM to 10 μM potentiates the ACh-induced response in a monotonic relationship (Flacke and Yeoh, '68a). However, in the pharynx 0.1 μM physostigmine augmented the ACh-induced response, but 1 μM physostigmine did not (Figs. 3, 4). The response pattern of the pharyngeal receptor suggests that it desensitized during exposure to the higher physostigmine concentration. This may indicate that the pharyngeal receptor is more prone to desensitization than is the body wall receptor. However, there are other possible explanations. For example, higher rates of desensitization in the pharynx could be induced if the endogenous ACh release rate was high. Upon physostigmine application, this could expose the pharyngeal receptor to a higher ACh concentration than would be experienced by the body wall receptor. The substantial response of the pharynx to physostigmine application (Fig. 3) suggests that neural elements associated with the pharynx were spontaneously releasing significant quantities of ACh. In addition, the observation that application of d-tubocurarine alone decreased pharyngeal activity (Fig. 5) also suggests that tonic release of ACh may regulate pharyngeal activity. It is also prudent to note that the actions of physostigmine are not always due to its ability to inhibit cholinesterase. In both vertebrate and invertebrate preparations, physostigmine can act as a noncompetitive agonist (Maelicke et al., '95; van den Beukel et al., '98).

Comparison of the cholinergic pharyngeal response with other annelid ACh receptors

The pharmacological profile of the pharyngeal ACh response is substantially different from the earthworm and polychaete body wall muscle receptors. Each of the antagonists listed in Table 3 (except for α-conotoxin M1, which has not been tested on the earthworm) successfully antagonizes ACh-induced responses of earthworm body wall muscle (Hassoni et al., '85). Although the cholinergic pharmacology of the polychaete body wall muscles has not been extensively characterized, it appears to differ substantially from that described in leeches or earthworms (Wu, '39b; Nicol, '52; Alvarez et al., '69).

The earthworm gut responds to a number of cholinergic agents in a similar qualitative manner to that which occurs in the leech pharynx; however, there are also a number of major differences. ACh application to several regions of the earthworm gut causes a rapid increase in tension, accompanied by an abolition of rhythmic movements (Wu, '39a). Atropine, when applied alone, has an excitatory effect on *Lumbricus* and *Allolobophora* crop-gizzard (Wu, '39a), but not on the crop-gizzard of *Eisenia* (Ukena et al., '95). Perhaps the most noticeable distinction between the two tissues is that the response of the earthworm crop-gizzard to nicotine was quite different from that produced in the leech pharynx. In the leech pharynx, both ACh and nicotine induced a smooth contraction accompanied by a suppression of spontaneous activity (Fig. 1). In the earthworm crop-gizzard, the actions of ACh and nicotine are distinct (Ambache et al., '45; Ukena et al., '95). Although ACh induced a qualitatively similar contraction in the earthworm gut to that produced by ACh (and nicotine) in the leech pharynx,

nicotine application in *Lumbricus* and *Allolobophora* caused a cessation of peristaltic activity without a simultaneous loss of ACh-induced excitability (Ambache et al., '45). While in contrast, nicotine application had no effect on *Eisenia* crop-gizzard motility (Ukena et al., '95). In addition, curare did not antagonize the actions of ACh in *Eisenia*; however, muscarine produced an ACh-like excitatory response. Thus, the pharmacology of the earthworm gut resembles that of a classical muscarinic receptor, while that of the leech pharynx resembles a nicotinic receptor. The ACh receptor of the anterior intestine of the polychaete *Chaetopterus variopedatus*, although not extensively characterized, is more similar to the earthworm gut receptor than the leech pharyngeal receptor. The ACh-induced response of the *Chaetopterus* anterior intestine is blocked by atropine, but not by curare (Anctil et al., '84).

The pharmacological profile of the pharyngeal response is also substantially different from described insect, nematode, or molluscan ACh receptors (Colquhoun et al., '91; Benson, '92; Walker et al., '92; Darlison et al., '93; Hannan and Hall, '93; Leech and Sattelle, '93; Tornøe et al., '95). The findings of this study combined with the data summarized in Table 3 indicate that the leech is likely to possess at least seven different nicotinic ACh receptors. Insects also appear to possess a large family of different nicotinic receptors (Gundelfinger, '92). This recent research suggests that organization of invertebrate nicotinic receptors may rival the complexity of that found in vertebrates.

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