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CHEMICAL EXCITATION OF THE PROVENTRICULUS OF THE POLYCHAETE WORM *SYLLIS SPONGIPHILA*

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SUMMARY

1. The myoepithelial cells of the proventriculus of *Syllis spongiphila* (which are composed of only one or two sarcomeres that may reach 40 μm in length) are innervated by excitatory and inhibitory nerve fibres. Experiments were performed to examine the chemical excitation of these cells. We found that acetylcholine (ACh) mimics the excitatory transmitter and that the postsynaptic receptors appear to be nicotinic.

2. Bath application of ACh (10^{-5} to 10^{-4} M), carbamylcholine (Carb, 10^{-4} to 10^{-3} M) or nicotine (10^{-4} to 10^{-3} M), but not muscarine (10^{-4} to 10^{-3} M), elicited overshooting depolarizations followed by prolonged depolarized plateaus. Iontophoresis of ACh or Carb (0·1–1·0 M) elicited depolarizing responses which, when elicited in trains, exhibited increased rise times to peak and decreased amplitudes to a plateau level; these results suggest the occurrence of desensitization of the postsynaptic receptors. Depolarizations elicited by indirect stimulation or by iontophoresis of ACh were reduced in amplitude by D-tubocurarine (10^{-5} to 5×10^{-4} M) in a dose-related and reversible manner. Atropine (10^{-6} to 10^{-4} M) had no effect on responses elicted by indirect stimulation.

3. Small potential changes superimposed on responses to cholinergic agents applied in the bath or iontophoretically were abolished in Ca^{2+}-free ASW, Ca^{2+}-free + 1 mM EGTA ASW or in ASW containing 4–6 mM Mn^{2+}. These small potential changes are likely to be from activity in neurones that receive cholinergic synapses.

4. Carb and nicotine applied in the bath consistently induced more overshooting peaks than did ACh, and the plateaus of the responses to ACh were of lower amplitude than those of the responses to Carb. In addition, responses to iontophoretic pulses of ACh were prolonged in the presence of 10^{-4} and 10^{-3} neostigmine. These data suggest the presence of an esterase in the nerve–muscle junctions of the proventriculus.

INTRODUCTION

The proventriculus (i.e. the alimentary canal located between the proboscis and the intestine) of the polychaete worm *S. spongiphila*, is composed of a single layer of myoepithelial cells that are oriented radially around a central lumen (Haswell, 1890). These cells possess only one or two sarcomeres that may reach 40 μm in length

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(Haswell, 1890; del Castillo, Anderson & Smith, 1972; Smith, del Castillo & Anderson, 1973) and appear to be innervated by both excitatory and inhibitory nerve fib (Anderson & del Castillo, 1976). In this paper we discuss experiments performed to determine the identity of the excitatory transmitter and to characterize certain of its effects on the postsynaptic membrane. Some of the observations described here were reported in a brief note (Anderson & Mrose, 1946).

MATERIALS AND METHODS

Animals

Specimens of *S. spongiphila* were collected in the Harbour of San Juan and shipped to Massachusetts. They were kept at 20 °C for periods of up to 2 months in covered 60 mm petri dishes containing a substrate of cheesecloth in sea water about 5 mm in depth.

Bathing solutions

The following chemicals were used. Acetylcholine chloride (ACh), Sigma; atropine sulphate, Calbiochem; carbamylcholine chloride (Carb), Sigma; eserine sulphate, Sigma; ethylenebis (oxymethylenenitrilo) tetra-acetic acid (EGTA), Eastman; DL-muscarine chloride, Sigma; neostigmine bromide, Sigma; D-tubocurarine chloride (DTC), Sigma.

The artificial sea water (ASW) used in all experiments had the following ionic composition (mM): Na, 457; K, 9·7; Ca, 10·1; Mg, 52·5; Cl, 534; HCO_3, 2·5; SO_4, 27·7 (Welsh, Smith & Kammer, 1968). The pH was adjusted to 7·6 with dilute HCl. Calcium-free ASW was made by substituting $MgCl_2$ for $CaCl_2$ Solutions bathing the preparation were changed by drawing off the first solution and then washing at least five times with the new solution.

Preparations

Specimens of *S. spongiphila* were dissected in ASW in a clear, Sylgard-lined Plexiglas dish of 0·35 ml capacity. The portion of the worm posterior to the proventriculus was removed, the cuticular body wall surrounding the region of the proventriculus was dissected away using watchmakers forceps and the preparation was fastened to the bottom of the dish with stainless steel insect pins. For extracellular, indirect stimulation, the head and proboscis were drawn into the tip of a suction electrode. In some experiments the proventriculus was split longitudinally along the dorsal and ventral raphes into left and right halves (see Smith *et al.* 1973, for a detailed description of the anatomy of the proventriculus).

All experiments were performed at 21–23 °C.

Electrical recording and stimulation

For recording from single cells, glass, 3 M-KCl-filled microelectrodes of 5–10 MΩ resistance were connected to the oscilloscope through a high input impedance preamplifier. An agar bridge connected the bath to ground through a calomel cell. For indirect stimulation, trains of pulses were applied to the anterior end of the animal between the internal and external wires of a suction electrode that was connected to a stimulator via a stimulus isolation unit. For the iontophoretic application of dr single or double capillary tubes threaded with glass fibre (obtained from Frede

Haer and Co.) were drawn on a horizontal electrode puller and filled with solutions of 0·1–1·0 M ACh or Carb immediately before use. Braking currents were not applied to the iontophoresis electrodes, since changes in membrane potential of the myoepithlial cell were not observed when outward current was not being applied, nor was any increase in membrane potential observed when the iontophoresis electrode was removed from the vicinity of the recorded cell. Current applied via the iontophoresis pipettes was monitored on a second trace of the oscilloscope by recording the voltage drop across a 10 kΩ resistor between the calomel cell and ground.

Oscilloscope recordings were photographed on film with a kymograph camera.

RESULTS

Addition of antagonists to the bathing solutions

The effects of curare and atropine were tested on intact preparations. Trains of pulses were applied with a suction electrode to elicit indirectly from the myoepithelial cells complexes of hyperpolarizing and depolarizing activity. In the presence of D-tubocurarine (DTC) the depolarizing responses, but apparently not the hyperpolarizing ones were diminished reversibly. The DTC did not affect the resting membrane potential. An example of the effects of 10^{-5} M DTC is shown in Fig. 1. The effects of DTC appeared to be dose-related, since concentrations $\leqslant 10^{-6}$ M did not diminish responses evoked by indirect stimulation up to 15 min after application, while concentrations of 10^{-4} M DTC caused complete abolition of depolarizing activity within 1 min after application.

Atropine sulphate, applied to one preparation in concentrations of 10^{-6}, 10^{-5} and 10^{-4} M, affected neither indirectly evoked activity nor the resting membrane potential.

Addition of agonists to the bathing solutions

ACh was added to the bath of eight intact preparations. Concentrations of 10^{-8} to 10^{-6} M were associated with series of mainly hyperpolarizing postsynaptic potentials (Fig. 2*a*, top trace). More concentrated solutions of ACh caused depolarizations and hyperpolarizations (Fig. 2*b*, *c*, top traces). The initial activity usually subsided within 120 s, but the membrane potential remained depolarized by up to 20 mV more than the control resting level. The resting potential returned to the initial control level within 10 min after return to the control ASW.

The complex nature of the responses elicited by the addition of ACh to the bath suggested that cholinergic synapses within the neural network proximal to the proventriculus may be affected by the drug. Experiments using Ca^{2+}-free ASW, to abolish presynaptic transmitter release, supported this idea (Fig. 2). In the absence of Ca^{2+} only the slow, depolarizing postsynaptic response of the myoepithelial cell was evoked by the addition of ACh to the bath. Indeed, in Ca^{2+}-free ASW, ACh at the concentration of 10^{-6} M had no effect on the myoepithelial cells (Fig. 2*a*, bottom trace).

The complex synaptic activity could be greatly reduced by splitting the proventriculus longitudinally into separate halves. This procedure apparently damaged nerve fibres and broke neural connexions within the raphes, thereby eliminating in large part the synaptic events superimposed on the responses of the myoepithelial cells. Fig. 3 illustrates the responses recorded from a single cell of such a split preparation

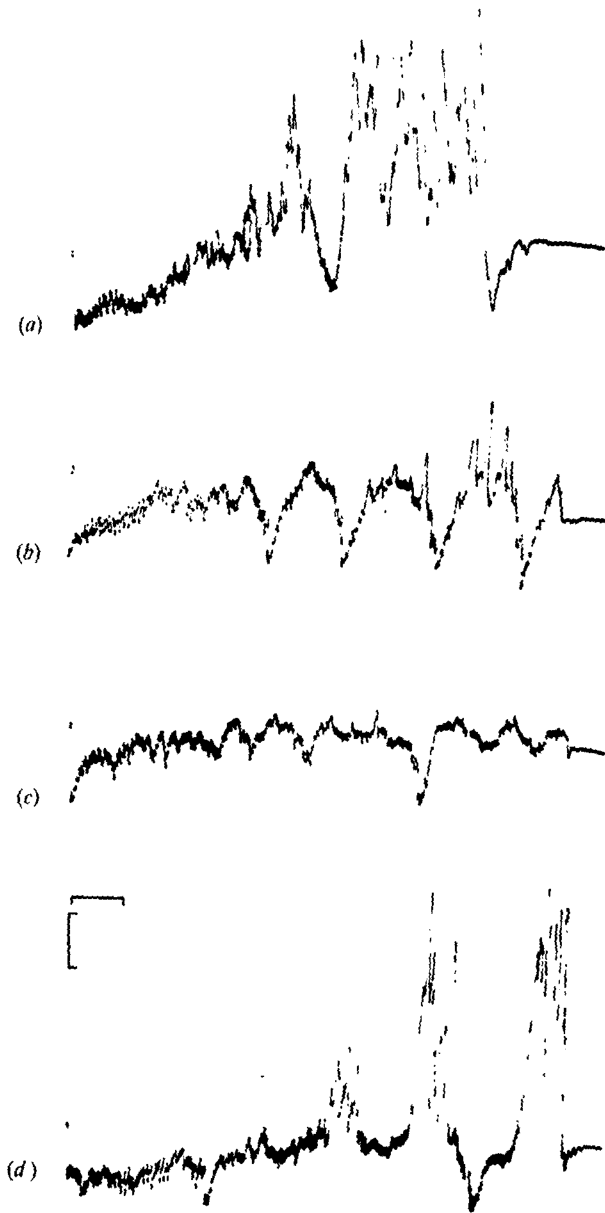

Fig. 1. Application of 10^{-5} M DTC to the bath of an intact preparation. Trains of constant intensity pulses of 0·4 ms duration were applied at a frequency of 35 Hz with a suction electrode. Stimulation was initiated at the beginning of each trace. Responses were recorded in control ASW (*a*), after 3 min in DTC (*b*), after 5 min in DTC (*c*) and approximately 10 min after return to control ASW (*d*). Depolarizing but not hyperpolarizing activity was reversibly abolished in the presence of DTC. Calibrations, 10 mV and 1·0 s.

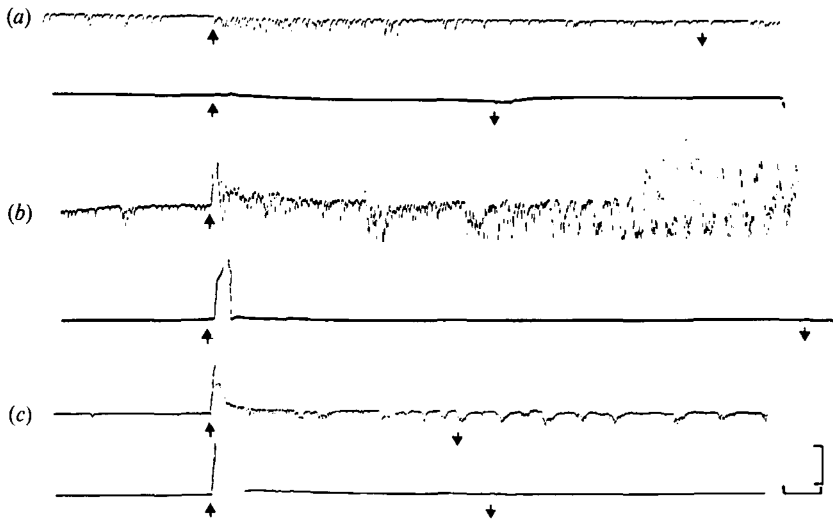

Fig. 2. Effects of the removal of Ca^{2+} from the bathing medium on responses to ACh applied to the bath of an intact preparation. Responses to 10^{-6} M (*a*), 10^{-5} M (*b*) and 10^{-4} M (*c*) ACh were recorded in the presence (top traces) and absence (bottom traces) of calcium. Arrows indicate the application and removal of ACh. Voltage calibration, 20 mV in (*a*) and (*b*) (top trace); 40 mV in (*b*) (bottom trace) and (*c*). Time calibration, 4 s.

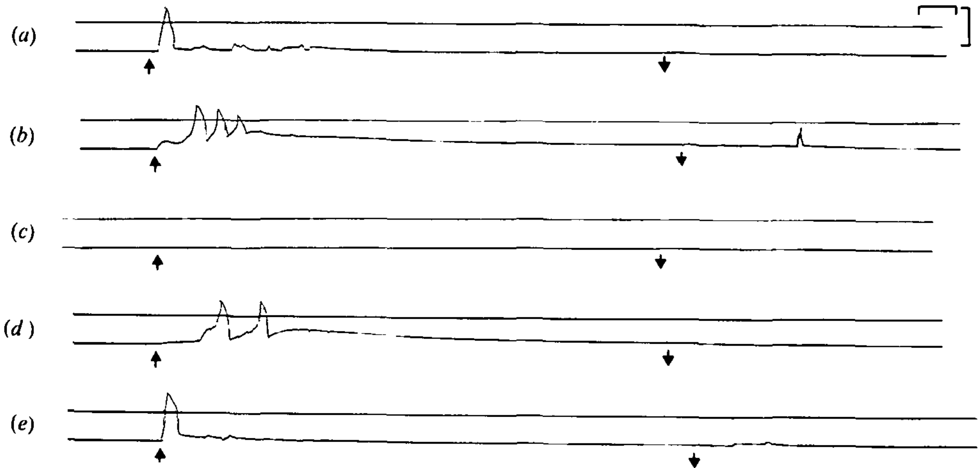

Fig. 3. Responses recorded from a single cell of a split preparation upon the addition to the bath of ACh (*a* and *e*), Carb (*b*), muscarine (*c*) and nicotine sulphate (*d*), all in the concentration of 5×10^{-4} M. The top traces represent the zero reference level. Arrows indicate the application and removal of each agonist. Calibrations, 80 mV, 4·0 s.

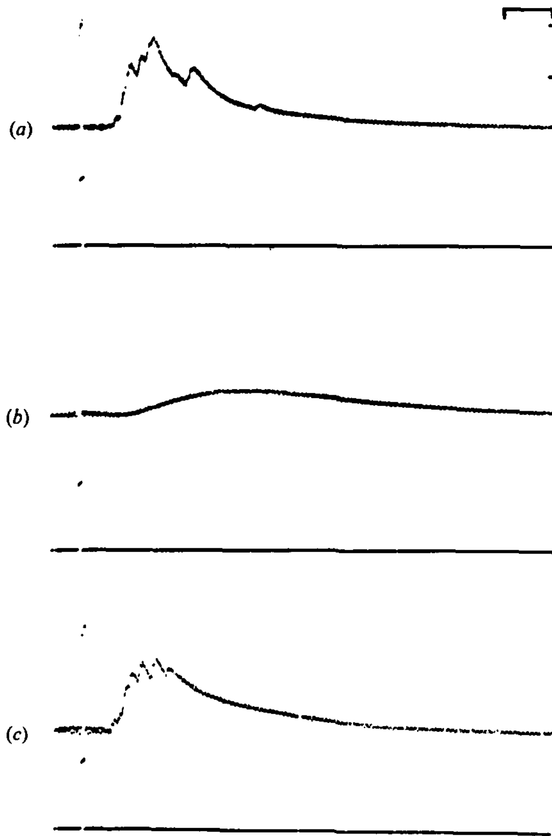

Fig. 4. Effect of the removal of Ca^{2+} on the responses to iontophoretically applied ACh. The superimposed activity, but not the depolarizing response to ACh, was abolished in the absence of calcium. Responses were recorded in control ASW (*a*), after 1 min in Ca^{2+}-free + 1 mM EGTA ASW (*b*) and 2 min after return to control ASW (*c*). All pulses, 15 ms duration, 6.5×10^{-7} A, from pipette containing 10^{-1} M ACh. Calibrations, 5.0 mV, 0.2 s.

upon applications of ACh, Carb, nicotine sulphate and muscarine, all at the concentration of 5×10^{-4} M. Carb, nicotine and ACh caused slow, overshooting depolarizations which were followed by prolonged depolarized plateaus. Upon return to control ASW, the membrane potential in all cases returned to the control resting level within 5 min. Muscarine did not elicit any activity. In all five preparations tested the initial responses to Carb and nicotine typically showed more overshooting peaks than those to ACh. These data suggest the presence of an esterase which limits the effectiveness of ACh.

In addition, the plateaus of the responses to ACh were of lower amplitude than those of the responses to Carb. To test whether the maintained plateaus of depolarizations elicited by ACh were clearly different in amplitude from those elicited by Carb, the ratio of the membrane potential at 20–25 s after the application of ACh or Carb and the membrane potential prior to application was determined for each test. The mean ratios for each of the test conditions in different preparations were found, from analysis of variance, to be not significantly different ($P > 0.1$). With pooled data, the mean ratio of membrane potentials on application of Carb was 0.47 ± 0.031 (S.E.

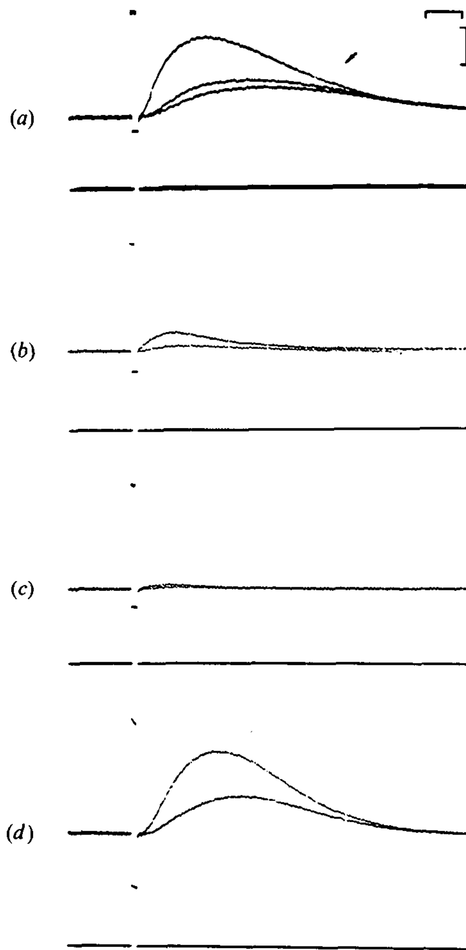

Fig. 5. Reversible abolition by $3 \cdot 5 \times 10^{-4}$ M dTC of responses elicited by the iontophoretic application of ACh. Responses were recorded in control ASW (*a*), after 1 min in dTC (*b*), after 3 min in dTC (*c*) and 5 min after return to control ASW (*d*). In all the records shown, pulses (15 ms duration, $7 \cdot 5 \times 10^{-7}$ A from pipette containing 10^{-1} M ACh) were applied at a frequency of 0·5/s. Responses to successive pulses showed decreased amplitudes and increased times to peak amplitude. Calibrations, 5·0 mV, 0·1 s.

$n = 15$, 5 preparations) and upon application of ACh was $0 \cdot 82 \pm 0 \cdot 024$ (S.E., $n = 19$, 5 preparations) (the 95% confidence intervals did not overlap). These data indicate that the plateaus maintained after the application of ACh and Carb are significantly different, and further suggest the presence of an esterase at the nerve–muscle junction.

Iontophoresis of ACh

Preparations that were split along the raphes were pinned to the base of the dish so that the lumen aspect of each half faced upward. The recording electrode was placed in a cell along the border of the raphe; the iontophoresis pipette was positioned near the lumen end of the recorded cell, its tip between the end of that cell and the tissue that lines the lumen.

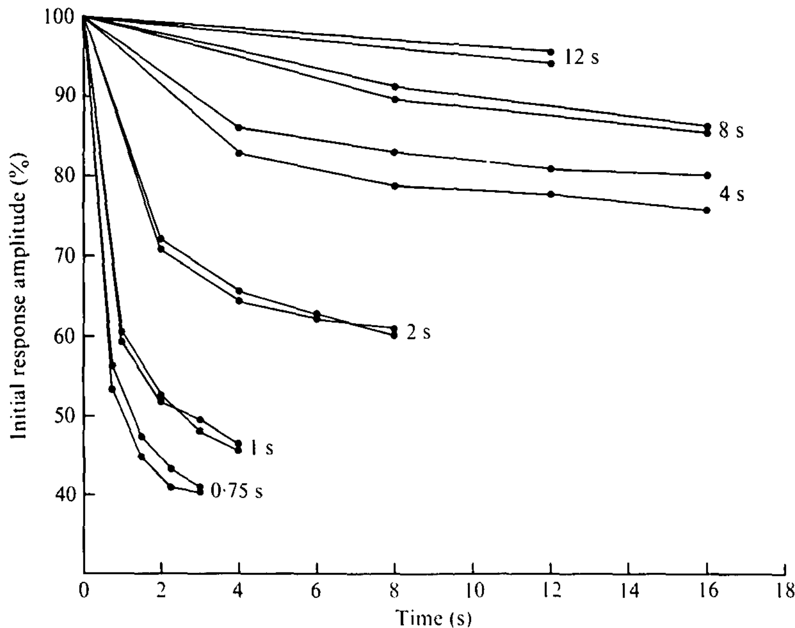

Fig. 6. The degree of desensitization shown as percentage of initial response amplitude plotted against time. Two curves are plotted for each frequency shown. Inter-pulse intervals are indicated on the right. The greatest decrease in amplitude occurred between the first and second responses in a train. The responses of each train reached a plateau level, the amplitude of which depended on the frequency of the train.

The iontophoresis of ACh usually elicited a simple, slow, smoothly rising and falling depolarizing potential change. Occasionally the slow depolarizations exhibited what appeared to be superimposed synaptic activity (Fig. 4*a*). When ACh was iontophoresed in two preparations bathed in Ca^{2+}-free ASW and in two other preparations bathed in 1 mM EGTA in Ca^{2+}-free ASW, the slow depolarizing responses to ACh appeared to be unaltered; however, the superimposed activity was reversibly abolished (Fig. 4). Such activity was also reversibly abolished by the addition of 5 mM Mn^{2+} to the bathing solution. These results again suggest the presence of nerve fibres that are stimulated to fire impulses by the application of ACh.

As expected from experiments using indirect electrical stimulation, DTC added to the bathing medium blocked reversibly the responses to the iontophoretic application of ACh. Fig. 5 illustrates the effect of $3\cdot5 \times 10^{-4}$ M DTC on responses elicited by pulses of ACh. The effects of DTC appeared to be dose-related. In four different preparations it was shown that increases in the concentration of DTC in the bath from 10^{-5} M to 5×10^{-4} M decreased the relative amplitude of the responses to standard pulses of ACh. Below 10^{-5} M, DTC had no detectable effect.

When a series of iontophoretic pulses of ACh or Carb was applied to the proventriculus, the response to each successive pulse decreased in amplitude to a plateau level; in addition, the successive responses in a given series became increasingly prolonged. To examine the decrease in response amplitude with different interpulse intervals, trains of ACh pulses at frequencies from 0·083 to 3 s^{-1} were applied and the amplitudes of successive responses plotted. Fig. 6 shows the results from one prepara-

tion for interpulse intervals from 0·75 to 12 s. At all frequencies the greatest reduction in amplitude, within a given series, occurred between the first and second pulses; the reduction is greatest for the shortest interpulse intervals. In all cases, a plateau level is reached, which is higher for longer interpulse intervals. These results are apparently caused by desensitization of the receptors (Thesleff, 1955; Katz & Thesleff, 1957).

The presence of an esterase, suggested by the addition of agonists to the bath (Fig. 3), was further confirmed by iontophoretically applying ACh and Carb with the anti-esterase neostigmine present in the bath. Whereas the responses to Carb were unaffected, the responses to ACh were prolonged. Thus, it appears that an esterase is present in the nerve-muscle junctions of the proventriculus.

DISCUSSION

The data presented here show that ACh mimics the excitatory transmitter of the proventriculus of *S. spongiphila*. Of the four criteria, suggested by Paton (1958), that should be satisfied before a role as transmitter is accepted for a given substance, two have been met. The application of ACh to the myoepithelial cells, either in the bath or iontophoretically, reproduces the exitatory responses observed during natural activity, and a specific antagonist of ACh, curare, blocks the responses elicited by both indirect stimulation and external application of ACh. Because the nerve fibres that innervate the proventriculus are small and embedded within the raphes, it was not possible to dissect them free to investigate experimentally the remaining two criteria. Thus, it is not known if the neurones which are presynaptic to the proventriculus contain and synthesize ACh or if stimulation of these neurones releases ACh.

The postsynaptic receptors appear to be nicotinic in nature since the application of nicotine, but not muscarine, elicited responses similar to those elicited by ACh; further, curare, but not atropine, antagonized the responses to both the application of ACh and indirect stimulation.

There also may be cholinergic synapses within the neural network of the proventriculus. This conclusion is supported by the demonstration that small potential changes superimposed on responses to ACh applied in the bath or iontophoretically were abolished under conditions of synaptic block.

An esterase which limits the effectiveness of ACh appears to be present in the nerve–muscle junctions of the proventriculus. This is suggested by the observation that responses to nicotine and Carb applied to the bath exhibited more overshooting peaks than did responses to ACh. In addition, it was shown that the plateaus of the responses to ACh were of lower amplitude than those of the responses to Carb. The ratios of the membrane potential 20–25 s after agonist application and immediately prior to application for responses to ACh were shown to be significantly different from those for responses to Carb. These data further support the hypothesis that an esterase is present in the nerve muscle junction.

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