

## CHOLINERGIC ACTION ON THE HEART OF THE LEECH, *HIRUDO MEDICINALIS*

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*Accepted 15 April 1986*

### SUMMARY

Experiments were performed to determine the role of acetylcholine (ACh) in neuromuscular transmission in the heart of the leech *Hirudo medicinalis*.

1. Superfused or iontophoretically applied ACh rapidly depolarized both isolated heart muscle cells and muscle cells in isolated hearts in a dose-dependent manner. The depolarization was associated with a conductance increase of the muscle membrane that had a reversal potential of  $-9$  mV.

2. Eserine potentiated the response to superfused ACh, reducing the threshold from  $10^{-6}$  to  $10^{-8}$  mol l $^{-1}$ . Acetylcholinesterase was localized histochemically to be in the immediate area of neuromuscular terminals.

3. Superfused nicotinic agonists mimicked the effects of ACh, while superfused nicotinic antagonists reversibly blocked the iontophoretic response of heart muscle fibres to ACh.  $5 \times 10^{-7}$  mol l $^{-1}$  curare,  $5 \times 10^{-5}$  mol l $^{-1}$  nicotine and  $1 \times 10^{-4}$  mol l $^{-1}$  atropine reduced the iontophoretic response to half its original amplitude.

4. Alpha-bungarotoxin did not block the response of heart muscle cells to iontophoretically applied ACh.

5. Curare was used to determine whether the neurones that innervate the heart – HE motor neurones and HA modulatory neurones – use ACh as a neuromuscular transmitter. The fast depolarizing component of the HE cell's neuromuscular transmission was reversibly blocked by  $10^{-4}$  mol l $^{-1}$  curare, while the HA cell's modulatory effects on the heart were apparently unaffected by  $10^{-4}$  mol l $^{-1}$  curare.

Our results indicate that heart muscle cells have nicotinic acetylcholine receptors that open in the presence of ACh, thereby increasing membrane conductance. The HE motor neurone is probably cholinergic and engages these receptors in its neuromuscular transmission, while the HA modulatory neurone is probably not cholinergic.

### INTRODUCTION

The longitudinal muscle of the leech body wall is very sensitive to superfused acetylcholine (ACh) in the presence of an anticholinesterase such as eserine (Bacq &

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Key words: acetylcholine, heart, leech.

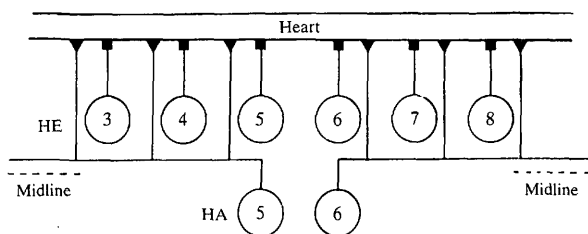


Fig. 1. Schematic drawing showing the innervation of one of the leech's bilaterally paired hearts in the third to eighth body segments. Circles indicate cell bodies, lines are processes and the numbers are body segments. Filled bars and triangles indicate the neuromuscular contacts of the HE motor neurones and HA modulatory neurones, respectively. The dashed line delineates the midline of the nerve cord. HA cell pairs occur only in segmental ganglia five and six and project intersegmentally and contralaterally to the heart, while HE cell pairs occur in segmental ganglia three to eighteen and project segmentally and ipsilaterally to the heart. HE and HA cells are indexed according to segment of origin and body side, thus HA(L,6) indicates the HA cell on the left side of the sixth segmental ganglion.

Coppee, 1937; Flacke & Yeoh, 1968a). Superfused (Walker, Woodruff & Kerkut, 1970) and iontophoretically applied (Kuffler, 1978) ACh depolarizes the muscle, and these responses are reversibly blocked by curare (Walker *et al.* 1970; Kuffler, 1978). More recently, significant amounts of choline acetyltransferase (CAT) and acetylcholinesterase (AChE) have been detected in the cell bodies of several motor neurones, including those that innervate the longitudinal muscles (Wallace, 1981a,b; Wallace & Gillon, 1982). These observations, taken together, strongly implicate ACh as an excitatory neuromuscular transmitter in the leech. Nevertheless, the pharmacological properties of the muscular acetylcholine receptors (Flacke & Yeoh, 1968a,b) and the nature of their ionic channels remain relatively unexplored.

The leech's paired hearts are tube-like vessels that extend the length of this segmented worm (Mann, 1962). The walls of these vessels are made up of spiral muscle cells that receive direct innervation from two identified classes of neurones, the HE motor neurones and the HA modulatory neurones (Maranto & Calabrese, 1984a) (Fig. 1). Pairs of HE motor neurones occur in nearly every segmental ganglion of the nerve cord (Thompson & Stent, 1976); each HE cell innervates the ipsilateral heart in its own segment. Pairs of HA modulatory neurones occur only in the fifth and sixth segmental ganglia (Calabrese & Maranto, 1984); each HA cell innervates the contralateral heart in several segments. The neuromuscular contacts of both types of neurones have been described at the ultrastructural level (Hammersen & Staudte, 1969; Maranto & Calabrese, 1984a) and are similar to those described for the longitudinal muscles (Rosenbluth, 1973; Yaksta-Sauerland & Coggeshall, 1973) and the muscles in the central nervous system (Tulsi & Coggeshall, 1971).

The cell bodies of the HE motor neurones contain both CAT and AChE (Wallace, 1981a,b; Wallace & Gillon, 1982) and action potentials in these motor neurones elicit

excitatory junctional potentials in heart muscle cells (Thompson & Stent, 1976; Maranto & Calabrese, 1984a). These observations suggest that there are cholinergic receptors on leech heart muscle cells associated with the HE cell's neuromuscular contacts. HE motor neurones also contain an FMRFamide-like peptide (Kuhlman, Li & Calabrese, 1985a). Thus, the HE motor neurones may use both ACh and an FMRFamide-like peptide as transmitters.

The HA modulatory neurones also contain an FMRFamide-like peptide (Kuhlman *et al.* 1985a), and their activity modulates the myogenic properties and beat tension of the heart without apparent junctional potentials (Maranto & Calabrese, 1984a; Calabrese & Maranto, 1984). Superfused FMRFamide mimics these modulatory actions on the heart (Kuhlman *et al.* 1985b). Thus, like the HE motor neurones, the HA modulatory neurones may use an FMRFamide-like peptide as a transmitter. Since HA cells have not been assayed for CAT or AChE, it is not clear whether they use ACh as a transmitter.

In this study we localize acetylcholinesterase in the heart, characterize cholinergic receptors on the heart muscle cells and test neuromuscular transmission by the HE motor neurones and HA modulatory neurones in the presence of curare, a potent antagonist of those receptors.

#### MATERIALS AND METHODS

##### *Animals*

Leeches, *Hirudo medicinalis*, were purchased from commercial suppliers and maintained in artificial pond water at 15°C for up to 4 months before use.

##### *Preparations and intracellular recordings*

Enzymatically isolated heart muscle cells, isolated hearts and innervated hearts were prepared and maintained as described previously (Maranto & Calabrese, 1984a,b; Calabrese & Maranto, 1984). Intracellular recordings from these preparations were also made as previously described (Maranto & Calabrese, 1984b; Calabrese & Maranto, 1984).

##### *Acetylcholinesterase staining*

The original method of Karnovsky & Roots (1964) or a modification of this method for the leech (Wallace & Gillon, 1982) was used to stain for acetylcholinesterase activity. Only tissues stained by the first method were suitable for ultrastructural examination, because the other method uses Triton X-100 in the reaction medium. Pseudocholinesterases were blocked with 0.03% ethopropazine in the first method and with 0.1 mmol l<sup>-1</sup> phospholine iodide in the second. Controls for either staining method consisted of tissue incubated in a reaction mixture supplemented with 1 mmol l<sup>-1</sup> eserine sulphate to block acetylcholinesterase, or tissue incubated in a reaction mixture lacking the substrate (acetylthiocholine iodide). Fixation and staining procedures were those described by the authors for

each method. After treatment the tissue was prepared for light or electron microscopy as previously described (Maranto & Calabrese, 1984a). Criteria for the identification of neuromuscular junctions were those of Hammersen & Staudte (1969) and Maranto & Calabrese (1984a). At neuromuscular junctions axon terminals contained clear synaptic vesicles approximately 50 nm in diameter and large dense-core vesicles 80–120 nm in diameter. The junctional cleft was occupied by the basement membrane and was 50–60 nm thick. The postjunctional membrane often had filamentous material associated with it.

#### *Application of pharmacologically active agents*

Pharmacological agents to be applied to heart muscle preparations were dissolved in cold leech saline prior to each experiment and allowed to equilibrate to room temperature. The pH of each drug solution was then readjusted to the original pH of the saline. Drug solutions and normal saline were then alternately superfused over the preparation at flow rates of 2–6 ml min<sup>-1</sup>.

Acetylcholine was microiontophoretically applied according to the method of Curtis & Eccles (1958). Short-shanked (10 mm) micropipettes were back-filled with a 3 mol l<sup>-1</sup> solution of acetylcholine chloride in distilled water. The micropipette was positioned as close as possible to the cell without penetrating it, and acetylcholine was ejected with positive current supplied by a WPI microiontophoresis unit. The dose (measured in nanoCoulombs, nC) was controlled by altering the duration or amplitude of the current pulse. A negative backing current of up to 40 nA was applied to prevent leakage of acetylcholine from the tip of the pipette; the backing current was adjusted to the minimum current that permitted the electrode to approach the recorded muscle cell without depolarizing it.

In a few experiments drugs were applied from the tip of a micropipette with pressure. Drugs dissolved in saline were ejected with 1-s (timed manually) pulses of pressure applied to the back of the pipette.

Alpha-bungarotoxin was purchased from Biotoxins, Inc., St Cloud, Florida, USA.

### RESULTS

#### *Acetylcholinesterase staining*

The histochemical methods of either Karnovsky & Roots (1964) or Wallace & Gillon (1982) indicated the presence of AChE in nerves and endplates of the heart (Fig. 2A). An interconnecting network of stained nerves was present over the entire length of the stained tissue. The stained endplates were small (approximately 5 µm in diameter), circular and more densely stained than the nerves. All staining was blocked by adding eserine to the reaction medium, or removing acetylthiocholine.

In preparations stained for AChE, axon terminals contained clusters of clear synaptic vesicles approximately 50 µm in diameter and large, dense-core vesicles 80–120 nm in diameter (Fig. 2B). Glycogen particles and mitochondria were also present. The axon terminal was surrounded by a glial covering except at the region of the synaptic cleft. The junctional cleft was occupied by a basement membrane and

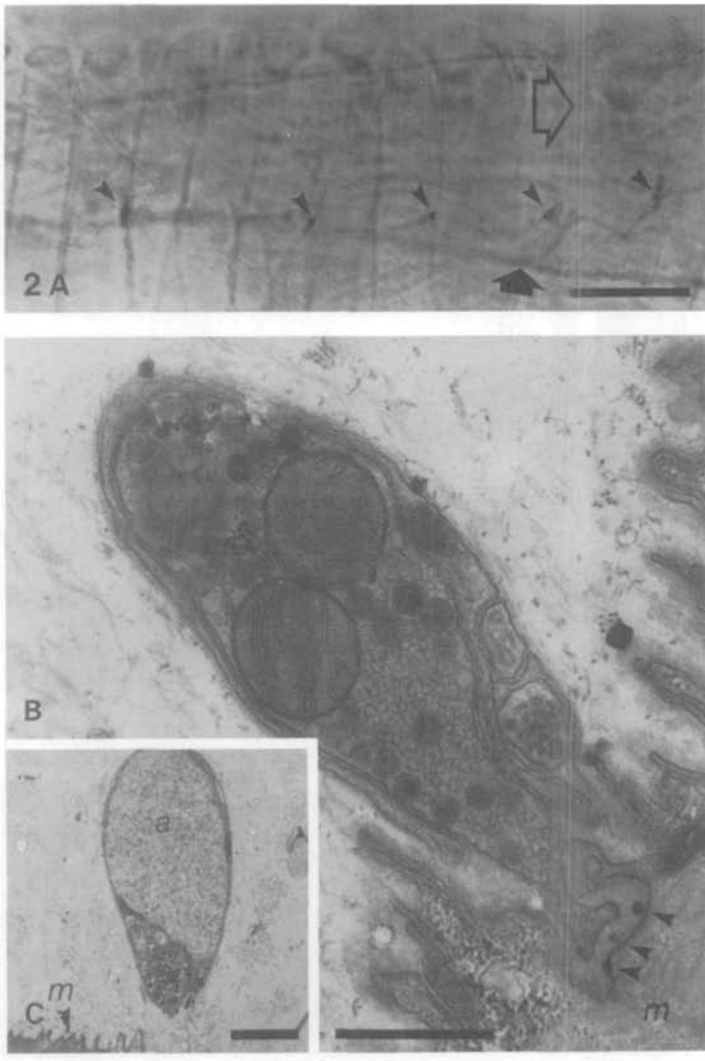
was approximately 60 nm thick. In all respects such terminals were *indistinguishable* from the terminals of the HE motor neurones identified by intracellular labelling with horseradish peroxidase (HRP) (Maranto & Calabrese, 1984a). The granular quality of the AChE reaction product observed here was similar in appearance, though less densely packed, than that observed in vertebrates (Karnovsky & Roots, 1964; McMahan, Sanes & Marshall, 1978). Particles of AChE stain were often found in the cleft of the neuromuscular junction in these preparations, just as they are at vertebrate neuromuscular junctions (Karnovsky & Roots, 1964; McMahan *et al.* 1978). Control preparations contained no such particles and were indistinguishable from those previously reported by Hammersen & Staudte (1969) and ourselves (Maranto & Calabrese, 1984a). Intracellular AChE was not observed in nerve terminals, although a considerable amount of stain was observed in larger axons running along the heart (Fig. 2C).

#### *Cholinergic pharmacology of isolated heart muscle cells*

Although many isolated heart muscle cells could express a polarization rhythm (Maranto & Calabrese, 1984b), many cells were initially quiescent or became quiescent during intracellular recording. Quiescent cells had resting potentials of approximately  $-40$  mV and input resistances of  $50$ – $150$  M $\Omega$ . These cells responded to iontophoretic application of acetylcholine (ACh) with a dose-dependent depolarization (Fig. 3). Maximum responses were  $20$ – $30$  mV in amplitude and these large depolarizations often triggered action potentials (Fig. 3A). The rise time to the peak response was  $300$ – $350$  ms for iontophoretic pulses varying in duration from  $10$  to  $30$  ms. To eliminate conductance changes associated with action potentials, iontophoretic pulses of ACh were adjusted to produce subthreshold depolarizations in succeeding experiments. Most of the thick central portion of the cells seemed sensitive to ACh without well-defined hot spots.

To investigate whether ACh opened ion channels, positive and negative current pulses ( $5$  s in duration) of equally spaced amplitudes were injected into a cell to alter membrane potential. One second after the start of each current pulse, a pulse of ACh was applied from an iontophoretic pipette. The arrangement of voltage, current and ACh pipettes is illustrated in Fig. 4A. Fig. 4B shows superimposed records from a typical experiment in which ACh was applied during seven hyperpolarizing and seven depolarizing current pulses. At more negative membrane potentials the iontophoretic responses became larger, while at more positive membrane potentials they became smaller.

We tested the effect of membrane potential on the amplitudes of the iontophoretic responses to ACh in eight cells. In each case, although the amplitude of the iontophoretic response varied with membrane potential as shown (Fig. 4B), we were unable to reverse the iontophoretic responses with the currents injected. We estimated the reversal potential by extrapolating the data to the abscissa. The mean reversal potential thus determined was  $-9$  mV (range  $-12$  mV to  $-5$  mV), approximately  $30$  mV more positive than resting potential. Graphic analysis of the data from one cell is shown in Fig. 4D. Although the cell's input resistance changed



over this range of membrane potential (Fig. 4C), the resistance change can only account for a fraction of the amplitude change in the iontophoretic response. At the largest hyperpolarization the cell's input resistance was approximately three times the input resistance at the largest depolarization, yet the amplitude of the iontophoretic response was more than ten times the amplitude of the response at the largest depolarization. Changes in the cell's input resistance would, of course, reduce the accuracy of the extrapolation, but the data clearly indicate that ACh acts by causing a conductance increase with an equilibrium potential near 0 mV. Assuming that the ionic equilibrium potentials for these cells resemble those of other muscle cells, a reversal potential near 0 mV suggests that ACh increases conductance to both sodium and potassium ions.

We tested the effect of the cholinergic antagonist curare (*d*-tubocurarine) on the iontophoretic responses of isolated heart muscle cells to ACh (Fig. 5). Superfusion of the cell culture with  $10^{-4}$  mol l<sup>-1</sup> curare reversibly reduced the amplitude of the iontophoretic response at all doses tested. Higher concentrations of curare completely blocked the iontophoretic response.

#### *Cholinergic pharmacology of the heart*

To examine the responses of heart muscle cells to a variety of cholinergic agents including agonists, antagonists and cholinesterase inhibitors, intracellular recordings were made from muscle cells in an isolated heart while these agents were superfused over the preparation. Polarization rhythms observed in these preparations usually had interburst intervals in excess of 30 s, and thus did not interfere with our measurements of membrane potential. Membrane potential was constant during the interburst intervals and was approximately -40 mV. Input resistance of the cells was approximately 4 M $\Omega$ . This low input resistance probably reflects the extensive electrical coupling between cells in the intact heart (Maranto & Calabrese, 1984a).

Muscle cells were rapidly depolarized when ACh was applied at concentrations as low as 1  $\mu$ mol l<sup>-1</sup> in normal saline. Maximum responses at high ACh concentrations were between 20 and 30 mV in amplitude. Such large depolarizations normally did not elicit action potentials in the intact heart, as they did in isolated muscle cells. When the superfused saline contained 50  $\mu$ mol l<sup>-1</sup> eserine sulphate, muscle cells depolarized in response to ACh concentrations as low as 10 nmol l<sup>-1</sup>. This enhanced sensitivity was probably caused by inhibition of the above described acetylcholinesterase by eserine. Dose-response data for four separate experiments are plotted in Fig. 6. The sensitivity of the heart muscle to ACh is similar to the sensitivity of leech

Fig. 2. Acetylcholinesterase staining of the heart. (A) Outer surface of the heart showing a nerve (solid arrow) and several endings (arrowheads), which were stained for acetylcholinesterase (AChE) by the method of Karnovsky & Roots (1964). The hollow arrow indicates the longitudinal axis of the heart. Scale bar, 20  $\mu$ m. (B) Electron micrograph of a nerve terminal forming a synapse with a muscle cell (*m*) in the heart. Arrowheads point to clumps of AChE reaction product in the synaptic cleft. Scale bar, 0.5  $\mu$ m. (C) Electron micrograph of a nerve running over the heart (*m*). A large axon (*a*) in the nerve contains numerous particles of AChE reaction product scattered throughout its cytoplasm. Scale bar, 2  $\mu$ m.

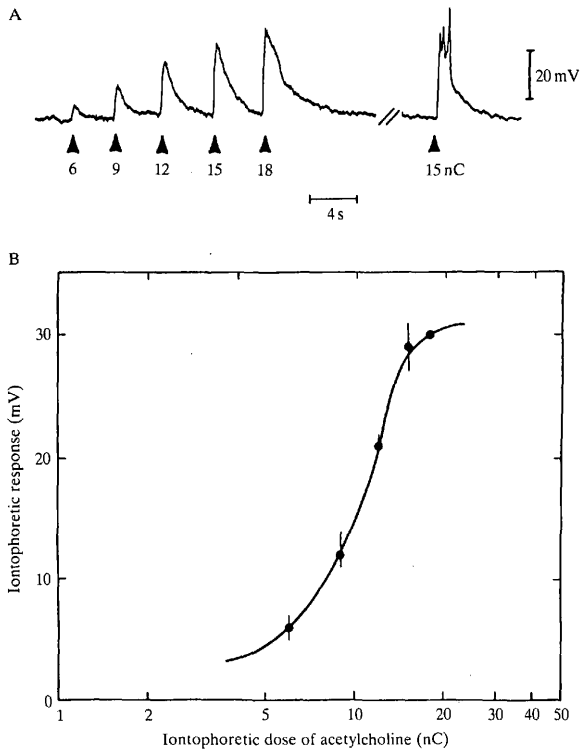


Fig. 3. Isolated heart muscle cells respond to iontophoretically applied acetylcholine (ACh) in a dose-dependent manner. (A) Depolarizing responses of an isolated heart muscle cell to a graded series of iontophoretic pulses of ACh. In this experiment the dose of ACh applied (expressed as charge in nC ejected from the iontophoretic pipette) was varied by varying the duration of the iontophoretic pulses from 10 to 30 ms. Depolarizations large enough to trigger action potentials were often elicited by doses of 15 nC or greater. (B) Dose-response curve for the same cell recorded in A. Each point is the average of at least three trials at the specified dose, except for the point at a dose of 18 nC which represents the only trial at this dose that did not elicit action potentials in the muscle cell. Vertical lines associated with the points indicate the range of values observed. Similar dose-response curves were observed in all cells tested ( $N > 20$ ).

longitudinal muscle reported by others (Bacq & Coppee, 1937; Walker *et al.* 1968; Flacke & Yeoh, 1968a; Kuffler, 1978). Although the heart became more sensitive to ACh in the presence of eserine, the slope of the dose-response curve declined; receptor desensitization at high ACh concentrations in the absence of active AChE could account for this decrease in slope. (See the discussion of the action of other anticholinesterases below.)



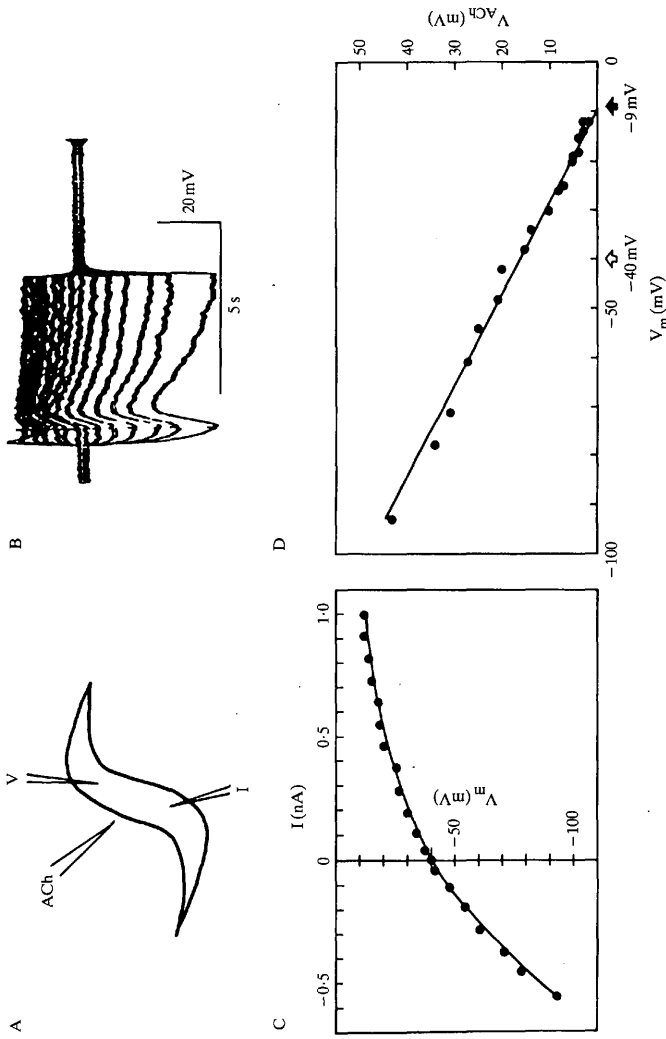


Fig. 4. Reversal potential of the iontophoretic response to acetylcholine (ACh) in isolated heart muscle cells. (A) Schematic drawing of an isolated muscle cell illustrating the intracellular placement of voltage recording (V) and current injecting (I) electrodes and the extracellular placement of an iontophoretic pipette (ACh) for making reversal potential measurements. (B) Superimposed oscilloscope traces from a typical reversal potential experiment in which iontophoretic pulses (fixed charge of 30 nC with a duration of 50 ms) of ACh were applied while membrane potential varied with injected current. (C) Voltage ( $V_m$ ) vs current (I) relationship for another typical cell indicating how the cell's input resistance varies over the range of membrane potentials tested. (D) The amplitude of the iontophoretic response to ACh ( $V_{ACh}$ ) vs membrane potential ( $V_m$ ) is plotted for the cell illustrated in C.

Iontophoretic application of ACh to muscle cells in isolated hearts gave dose-dependent responses (Fig. 7A) similar to those observed in isolated muscle cells, except that no action potentials were elicited even at large doses. The data of Fig. 7A yield a dose-response curve that is linear over a broad dosage range (Fig. 7B), but begins to saturate with large iontophoretic pulses. The rise time to the peak response was 1.2–1.3 s for iontophoretic pulses varying in duration from 40 to 300 ms.

The ability to record in heart muscle cells stable, dose-dependent responses to ACh applied either by superfusion (Fig. 6) or by iontophoresis (Fig. 7) permitted us to use the isolated heart preparation to study in more detail the pharmacology of the ACh receptor. Cholinergic agents were superfused over the heart and tentatively classified as: (1) agonists, if they depolarized muscle cells; (2) antagonists, if they reduced the size of iontophoretic responses to ACh without affecting resting potential or resting input resistance; (3) AChE inhibitors, if they increased the size and duration of iontophoretic responses to ACh (Table 1). In each case the reported responses were reversed by superfusion with normal saline.

The agonistic effects of carbachol (carbamylcholine), DMPP (1,1-dimethyl-4-phenylpiperazinium), nicotine and TMA (tetramethylammonium) suggest the presence of a nicotinic receptor similar to the type found in vertebrate autonomic ganglia. The ability of curare (*d*-tubocurarine) (Fig. 8), decamethonium and hexamethonium to block iontophoretic responses supports this notion (Chen, Portman & Wickel, 1951; Ginsborg & Guerrero, 1964), although the inhibitory effect of nicotine at lower concentrations (Fig. 8) was inconsistent. Muscarinic agonists, methacholine and pilocarpine, and the muscarinic antagonist, scopolamine, had no effects even at millimolar concentrations. Atropine (another muscarinic antagonist) at reasonably low concentrations ( $50\text{--}100\ \mu\text{mol l}^{-1}$ ) did block iontophoretic responses to ACh (Fig. 8), while  $1\ \text{mmol l}^{-1}$  atropine depolarized the muscle.

As expected, neostigmine blocked AChE at the heart but also appeared to antagonize the iontophoretic response to ACh. Decamethonium and hexamethonium

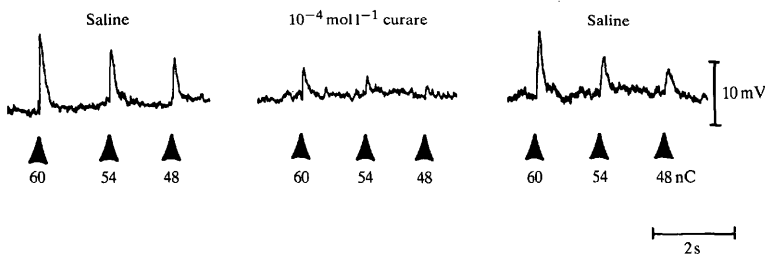


Fig. 5. Blockade of the iontophoretic response to acetylcholine (ACh) in an isolated heart muscle cell by curare. Three different doses (60, 54 and 48 nC) of ACh from the linear portion of the cell's dose-response curve were applied in the presence and absence of curare. (Dose was varied by varying the duration of the iontophoretic pulse from 100 to 80 ms.) The iontophoretic response at each dose was reversibly reduced when curare ( $10^{-4}\ \text{mol l}^{-1}$ ) was superfused through the cell culture.

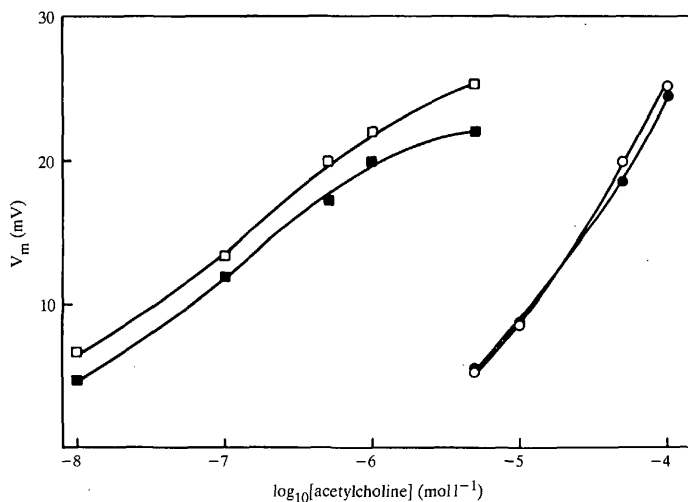


Fig. 6. Dose-response relationships for acetylcholine (ACh) superfused over the heart. Each point represents a single measurement of membrane depolarization ( $V_m$ ) in response to superfusion of ACh in normal saline (solid and filled circles) or in the presence of eserine (solid and filled squares). Each curve represents data collected from a different cell.

produced similar effects. The dual effects were observed as an initial augmentation, followed by a decrement, of the iontophoretic response to ACh. The apparent antagonistic action of these compounds on the heart ACh receptor might result from desensitization of the receptor in the absence of active AChE.

Three antagonists, atropine, nicotine and curare, were singled out for further study because they were free from other effects at low concentrations. We measured the relative potency of these antagonists by determining the concentration of the antagonist which inhibited 50% of iontophoretic responses from the middle of the linear portion of the ACh dose-response curve ascertained for each muscle cell recorded (Fig. 8). Curare was found to be the most potent antagonist, inhibiting 50% of the iontophoretic responses at approximately  $0.5 \mu\text{mol l}^{-1}$ . Nicotine and atropine blocked 50% of the response at 50 and  $100 \mu\text{mol l}^{-1}$ , respectively.

Alpha-bungarotoxin, even at a concentration of  $1 \mu\text{mol l}^{-1}$ , did not antagonize the iontophoretic response of heart muscle cells to ACh. At higher concentrations the toxin reversibly depolarized heart muscle cells. To determine if this effect was mediated through the ACh receptors, the ability of curare to block the response to toxin was tested. Alpha-bungarotoxin was applied to a muscle cell in the heart by ejecting it from a micropipette with pressure. The concentration of toxin in the pipette was  $1 \text{ mmol l}^{-1}$ . Iontophoretic responses to ACh were elicited as usual. Pulses of ACh and alpha-bungarotoxin depolarized the heart rapidly and reversibly

(Fig. 9). When the heart was superfused with  $5 \mu\text{mol l}^{-1}$  curare, the responses to both compounds were attenuated. The antagonistic effects of curare on both the ACh- and toxin-induced depolarizations were reversed by superfusion with normal saline.

*Functional significance of acetylcholine receptors on heart muscle*

Since curare was a potent antagonist of the acetylcholine receptor described above, we used curare to determine if either the HE motor neurones or the HA modulatory

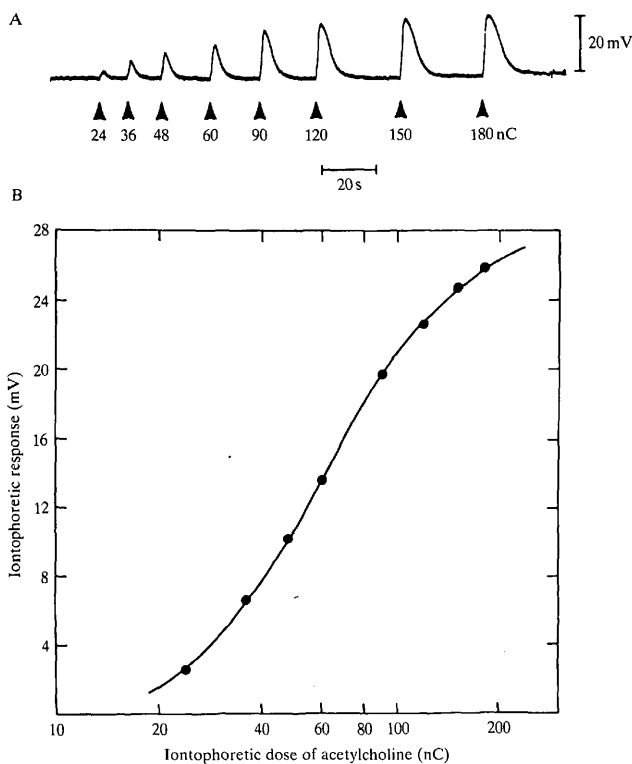


Fig. 7. Muscle cells in isolated hearts respond to iontophoretically applied acetylcholine (ACh) in a dose-dependent manner. (A) Depolarizing responses of a muscle cell in an isolated heart to a graded series of iontophoretic pulses of ACh. Dose is indicated as total charge in nC ejected from the iontophoretic pipette, and was varied by varying the duration of the iontophoretic pulse from 40 to 300 ms. (B) Data of A plotted as a dose-response ( $V_{\text{ACh}}$ ) curve. Similar dose-response curves were observed in all preparations tested ( $N > 15$ ).

Table 1. *Effects of superfused cholinergic compounds on the heart*

Compound	Concentration*
<b>Agonistic</b>	
Atropine sulphate	1 mmol l <sup>-1</sup>
Carbamylcholine chloride	5 µmol l <sup>-1</sup>
DMPP iodide†	100 µmol l <sup>-1</sup>
Nicotine bitartrate	1 mmol l <sup>-1</sup>
Tetramethylammonium bromide	100 µmol l <sup>-1</sup>
<b>Antagonistic</b>	
Atropine sulphate	50 µmol l <sup>-1</sup>
Decamethonium bromide	1 mmol l <sup>-1</sup>
Hexamethonium bromide	1 mmol l <sup>-1</sup>
Neostigmine bromide	100 µmol l <sup>-1</sup>
Nicotine bitartrate	50 µmol l <sup>-1</sup>
<i>d</i> -Tubocurarine chloride	1 mmol l <sup>-1</sup>
<b>Anti-acetylcholinesterase</b>	
Decamethonium bromide	1 mmol l <sup>-1</sup>
Hexamethonium bromide	1 mmol l <sup>-1</sup>
Neostigmine bromide	100 µmol l <sup>-1</sup>
<b>No effect</b>	
Alpha-bungarotoxin	1 µmol l <sup>-1</sup>
Methacholine chloride	1 mmol l <sup>-1</sup>
Pilocarpine hydrochloride	1 mmol l <sup>-1</sup>
Scopolamine	1 mmol l <sup>-1</sup>

\* Concentrations indicated are threshold concentrations below which no effects were observed. Compounds continued to have the same effect at higher concentrations except as indicated for atropine and nicotine, which were antagonists at lower concentrations but became agonists above 1 mmol l<sup>-1</sup>. The special case of high concentrations of alpha-bungarotoxin is treated in the text.

† DMPP iodide is 1,1-dimethyl-4-phenylpiperazinium.

neurones engaged such receptors in neuromuscular transmission. We used a preparation in which a single segment of a heart was left innervated by its segmental ganglion. Previous studies have shown that in such preparations the heart often expresses a myogenic rhythm, and that impulses in HE motor neurones produce excitatory junctional potentials in heart muscle cells (Maranto & Calabrese, 1984a). The individual junctional potentials are small, however, and are not usually resolvable, but bursts of impulses in HE motor neurones do cause rapid summated junctional potentials that clearly reset the heart's myogenic rhythm (Maranto & Calabrese, 1984b) (Fig. 10A). Impulses in HA modulatory neurones do not cause measurable junctional potentials in heart muscle cells (Maranto & Calabrese, 1984a), but their activity can accelerate the myogenic rhythm of the heart or induce it in quiescent preparations (Calabrese & Maranto, 1984). In the present study, curare (10<sup>-4</sup> mol l<sup>-1</sup>) reversibly blocked the ability of the HE motor neurone to depolarize the heart muscle rapidly and reset its myogenic rhythm (Fig. 10B,C), but did not substantially interfere with the ability of the HA modulatory neurone to accelerate the heart's myogenic rhythm (Fig. 11).

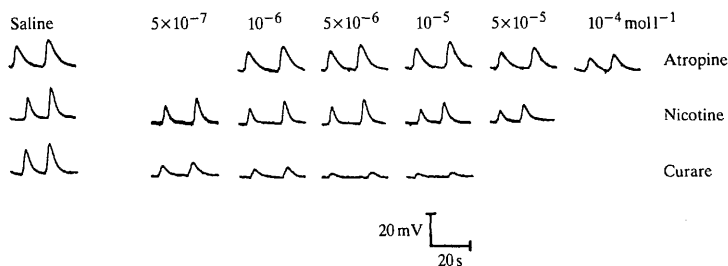


Fig. 8. Relative antagonistic effects of atropine, nicotine and curare (*d*-tubocurarine) on the iontophoretic responses to acetylcholine (ACh) of muscle cells in the isolated heart. For each antagonist a dose-response curve to ACh was first determined in the absence of antagonist, and two doses in the middle of the linear portion of the curve were chosen as criterion responses. Several concentrations of the antagonist were then superfused over the preparation, and the concentration of antagonist that inhibited the criterion responses by approximately 50% was determined. Records are from three different preparations. Doses were 36 and 48 nC for nicotine and 48 and 60 nC for atropine and curare (600 nA pulses).

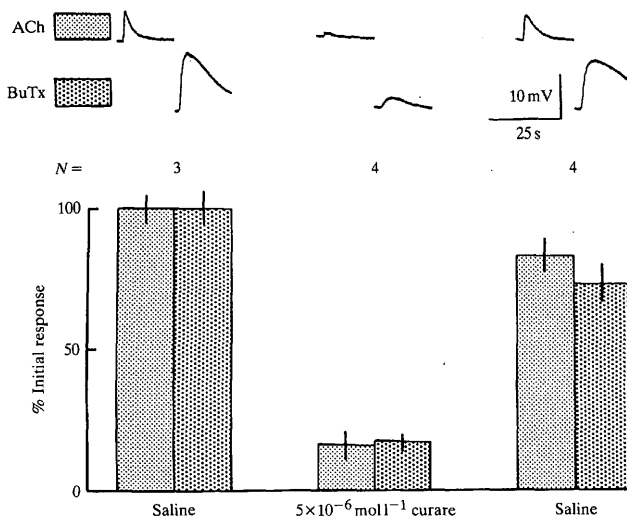


Fig. 9. The action of acetylcholine (ACh) and alpha-bungarotoxin (BuTx) on the heart. Depolarizing responses to iontophoretically applied ACh and pressure applied BuTx are shown before, during, and after blocking with  $5 \mu\text{mol l}^{-1}$  *d*-tubocurarine. Data from the indicated number of trials (*N*) were averaged and displayed as percentage of the initial response. Vertical lines indicate the standard error of the mean.

*Responses of the heart muscle cells to other putative transmitters*

To examine the sensitivity of the heart muscle cells to other putative neurotransmitters, intracellular recordings were made from cells in the isolated heart while the compounds were superfused over the preparation. At concentrations up to  $1 \text{ mmol l}^{-1}$ , the following compounds had no effect on membrane potential: adenosine triphosphate, aspartate, carnosine, dopamine, epinephrine, gamma-aminobutyric acid, glutamate, glycine, histamine, norepinephrine and octopamine.

Serotonin, applied at  $1 \text{ mmol l}^{-1}$ , depolarized muscle cells by about 5 mV. No membrane potential changes were observed when  $0.1 \text{ mmol l}^{-1}$  serotonin was applied. Walker *et al.* (1968) have reported that  $50 \mu\text{mol l}^{-1}$  serotonin reduces the size of excitatory junctional potentials and iontophoretic responses to ACh in leech longitudinal muscle. When we tested the effect of  $0.1 \text{ mmol l}^{-1}$  serotonin on the iontophoretic response to ACh, we observed no changes in amplitude or duration.

## DISCUSSION

*Acetylcholine receptors on heart muscle*

Superfusion and iontophoretic application of ACh to isolated heart muscle cells or muscle cells in isolated hearts demonstrated the presence of acetylcholine receptors. These receptors are linked to channels which open in the presence of ACh; the opening of these channels increases the membrane's conductance apparently to both sodium and potassium ions, since the reversal potential of the depolarizing response is near 0 mV. Because these receptors were not localizable to distinct hot spots in iontophoretic experiments, it is likely that they represent both junctional and extrajunctional receptors.

Pharmacologically, these receptors are similar to those described in the molluscan central nervous system (Kehoe, 1972), the crustacean central nervous system and muscle (Marder & Paupardin-Tritsch, 1978, 1980) and in vertebrate autonomic ganglia (Chen *et al.* 1951; Ginsborg & Guerrero, 1964). The features common to these acetylcholine receptors are: (1) they mediate depolarizing responses to ACh; (2) carbamylcholine, nicotine, TMA and DMPP mimic the effects of ACh; (3) curare, decamethonium and hexamethonium are antagonists of ACh; (4) muscarinic agonists and antagonists such as methacholine and pilocarpine have either weak or no effects on these receptors.

The failure of alpha-bungarotoxin to block ACh-mediated depolarizations in leech heart muscle is also characteristic of this class of nicotinic receptors in sympathetic ganglia (Brown & Fumagalli, 1977), crustacean muscle (Marder & Paupardin-Tritsch, 1980) and the molluscan central nervous system (Kehoe, 1972).

Hassoni, Kerkut & Walker (1985) have characterized an ACh receptor on the body wall muscle of the earthworm, *Lumbricus*, that is sensitive to alpha-bungarotoxin. This receptor is similar pharmacologically both to the receptor on leech body wall muscle (Flacke & Yeoh, 1968*a,b*) and to the receptor described here. The sensitivity of the leech body wall receptor to alpha-bungarotoxin has not been determined: either leech and earthworm muscular ACh receptors differ in their

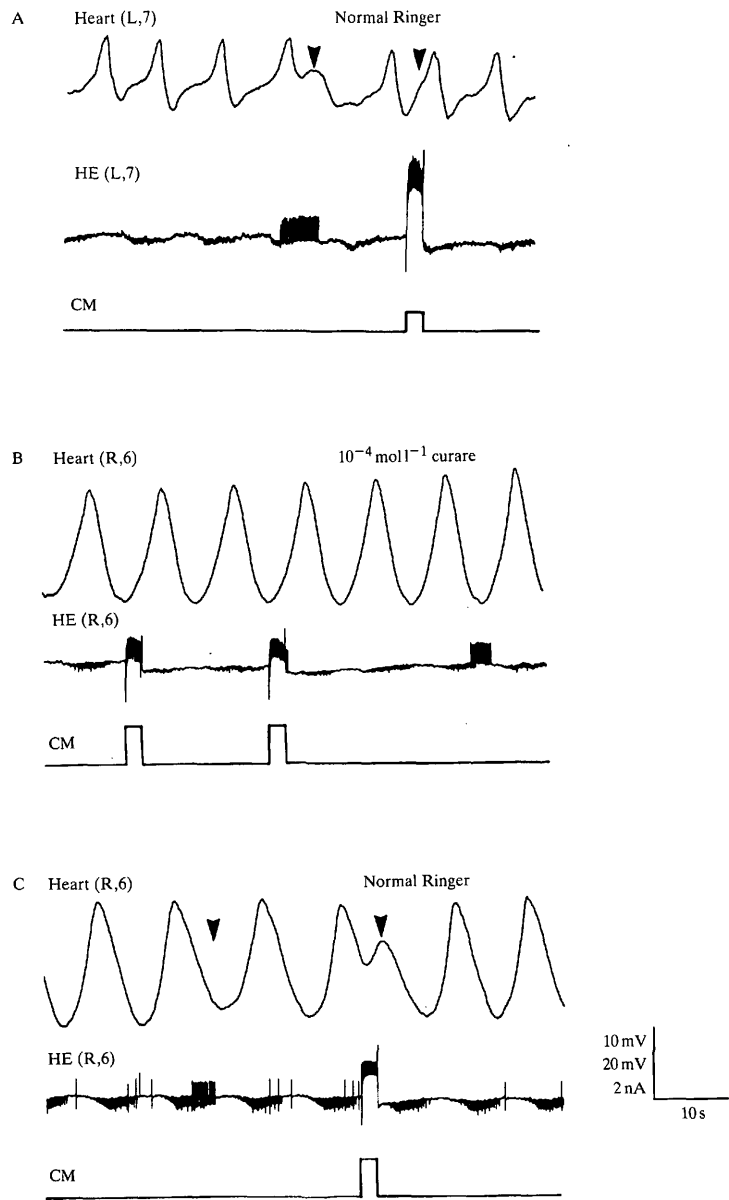


Fig. 10



sensitivity to alpha-bungarotoxin, or leech cardiac muscle has an ACh receptor distinct from that on leech body wall muscle. Neural nicotinic acetylcholine receptors in insects both bind alpha-bungarotoxin and are irreversibly blocked by it (Harrow & Sattelle, 1983; Sattelle *et al.* 1983).

A depolarizing response to alpha-bungarotoxin, such as the one observed here with high toxin concentrations ( $1 \text{ mmol l}^{-1}$ ), has not been previously reported. This response appears to be mediated through acetylcholine receptors, because it is sensitive to curare, but the possibility that it is caused by a contaminant in the toxin preparation cannot be eliminated.

#### *Acetylcholinesterase*

We have demonstrated the presence of acetylcholinesterase associated with nerves and nerve terminals on leech heart muscle using histochemical stains (Karnovsky & Roots, 1964; Wallace & Gillon, 1982). Ultrastructurally, this staining was localized inside axon branches coursing near the heart and to the vicinity of neuromuscular junctions. The sensitivity of the heart muscle to superfused ACh is greatly enhanced by anticholinesterases, such as eserine sulphate. This observation indicates that the cholinesterase may rid heart neuromuscular junctions of released ACh.

#### *Functional significance of acetylcholine receptors on the heart*

Previous studies have demonstrated that the leech's hearts are innervated by two classes of efferent neurones, heart motor neurones (HE cells) and heart modulatory neurones (HA cells) (Maranto & Calabrese, 1984a). Since the HE motor neurones contain both CAT and AChE activity, they are probably cholinergic (Wallace, 1981a,b; Wallace & Gillon, 1982).

The results presented here demonstrate that there are cholinergic receptors on heart muscles cells which could subserve neuromuscular transmission from HE motor neurones. These receptors were sensitive to curare and the HE cell's transmission to heart muscle was blocked by curare. These results provide further

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Fig. 10. Curare reversibly blocks the ability of the HE motor neurone to depolarize rapidly the heart muscle and reset its myogenic rhythm. Intracellular recordings were made from muscle cells in a section of heart innervated by only one segmental ganglion and from the innervating HE motor neurone. The motor neurone was hyperpolarized to suppress its central activity with a steady injected current, and the heart exhibited a rapid myogenic polarization rhythm. The motor neurone was then depolarized with current pulses to induce activity and to test its effects on the recorded muscle cell. As seen in the traces presented, spontaneous bursts of impulses are often recorded in HE cells when they are hyperpolarized; these bursts originate from a peripheral initiation site and are termed 'peripheral neurogenic bursts' (Maranto & Calabrese, 1984b). (A) Both induced bursts and peripheral neurogenic bursts in the motor neurone caused rapid depolarization of the heart muscle cell and reset its myogenic rhythm. (B) Saline containing  $10^{-4} \text{ mol l}^{-1}$  curare blocked the ability of both induced and peripheral neurogenic bursts in the motor neurone to depolarize rapidly the heart muscle cell and reset its myogenic rhythm. (C) The blockade of  $10^{-4} \text{ mol l}^{-1}$  curare was reversed by superfusion of the preparation with normal saline. The traces in B and C are segments from a continuous recording of the same muscle cell and heart motor neurone, while the traces in A are from a different preparation. Arrowheads indicate depolarizations induced by bursts in the heart motor neurone. CM, HE cell's current monitor.

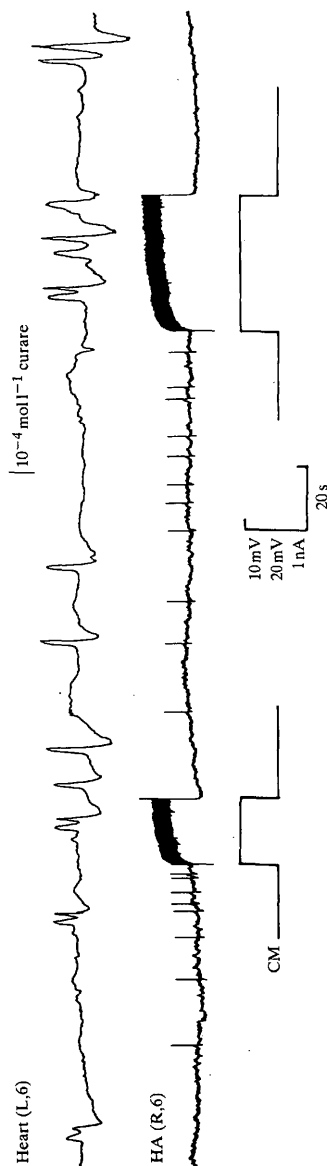


Fig. 11. Curare does not interfere with the ability of the HA modulatory neurone to accelerate the heart's myogenic rhythm. A preparation similar to that described in Fig. 10 was bathed in saline containing  $10^{-4} \text{ mol l}^{-1}$  curare, a muscle cell and its innervating HA neurone were recorded with intracellular electrodes. The HA neurone was hyperpolarized with a steady injected current to suppress its central activity, and the heart exhibited a slow myogenic polarization rhythm. When activity was then induced in the HA neurone with injected depolarizing current, the heart muscle cell's myogenic rhythm accelerated. CM, HA cell's current monitor.

evidence that HE motor neurones, like other leech motor neurones (Walker *et al.* 1970; Kuffler, 1978), use ACh as a neuromuscular transmitter. HE motor neurones also contain an FMRFamide-like peptide (Kuhlman *et al.* 1985a); therefore, they possibly use more than one transmitter. Since FMRFamide is active on the heart (Kuhlman *et al.* 1985b), a slower peptidergic component of the HE cell's transmission might remain when cholinergic transmission is blocked by curare. We intend to use curare to explore this possibility.

The HA neurones have not been assayed for cholinergic enzymes, but they too contain an FMRFamide-like peptide (Kuhlman *et al.* 1985a). We have demonstrated here that the HA cell's action on the heart is insensitive to curare, while previous work has shown that the HA cell's effects on the heart muscle are mimicked by superfusion of micromolar amounts of FMRFamide (Kuhlman *et al.* 1985b). It is, therefore, likely that HA cells are peptidergic but not cholinergic.

We thank Dr Eve Marder for much helpful advice and Christine S. Cozzens for valuable editorial assistance. This research was supported by NIH grant 1 R01 NS21232 to RLC.

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