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# Cholinergic interneurons in the feeding system of the pond snail *Lymnaea stagnalis*. I. Cholinergic receptors on feeding neurons

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## SUMMARY

All the identified feeding motoneurons of *Lymnaea* respond to bath or iontophoretically applied acetylcholine (ACh). Three kinds of receptors (one excitatory, one fast inhibitory and one slow inhibitory) were distinguished pharmacologically.

The agonist TMA (tetramethylammonium) activates all three receptors, being weakest at the slow inhibitory receptor. PTMA (phenyltrimethylammonium) is less potent than TMA and is ineffective at the slow inhibitory receptor, which is the only receptor sensitive to arecoline.

At 0.5 mM the antagonists HMT (hexamethonium) and ATR (atropine) selectively block the excitatory response, while PTMA reduces the response to ACh at all three receptors. d-TC (curare) antagonizes only the fast excitatory and the fast inhibitory responses, but MeXCh (methylxylocholine) blocks the fast excitatory and slow inhibitory responses solely.

For each of the feeding motoneurons, the sign of the cholinergic response (excitation or inhibition) is the same as the synaptic input received in the N1 phase of the feeding rhythm.

## 1. INTRODUCTION

Ever since Brown (1911) proposed his model of rhythmic pattern generation, neurobiologists have sought out the interneurons responsible for pattern generation, constructed models to explain the sequence of neural activity and tried to test these models. Progress has been rapid in invertebrate ganglia (for review see Jacklet (1989)), including those involved in gastropod feeding. On the basis of intracellular recordings from pairs of feeding interneurons Elliott & Benjamin (1985*a*) proposed a mechanism for the generation of the rhythmic feeding pattern of the pond snail, *Lymnaea stagnalis*.

One technique available to test this model is pharmacological dissection. This method of blocking identified synapses and so dividing up the neural network has provided new insights into pattern generation in the crustacean stomatogastric ganglion (Bidaut 1980; Eisen & Marder 1984; Marder 1987) and lamprey spinal cord (Grillner *et al.* 1989). Such 'dissection' has been particularly useful in the identification of the functional role of different cells and the modulation of endogenous membrane currents. One important advantage of this technique is that it is not necessary to penetrate all the interneurons involved at the same time, as might be done in direct tests of the pattern generator. This advantage means that the method can be applied to systems more complex than the crustacean stomatogastric ganglion, such as the *Lymnaea* feeding system, where there are many small cells which are difficult to impale simultaneously. With pharmacological dissection, it is thus possible to test a particular hypothesis indirectly and efficiently.

The experiments reported in this series of papers are designed to apply this technique to the model of the *Lymnaea* feeding system, applying cholinergic antagonists to block the output of identified interneurons. The first two papers in this series (this paper, and Elliott & Kemenes (1992)) prepare the ground by examining two questions: (i) which cholinergic antagonists are effective in the *Lymnaea* feeding system, and (ii) which of the feeding interneurons are cholinergic? In the third paper (Elliott 1992), the effective cholinergic antagonists are used to produce precisely known lesions in the feeding system. The results support the predictions of the Elliott & Benjamin (1985*a*) model.

### (*a*) *The Lymnaea* feeding system

In the semi-intact or intact snail, application of sucrose to the lips elicits feeding (Goldschmeding *et al.* 1973; Rose & Benjamin 1979). The neural activity that produces this behaviour has three phases: protraction, rasping and swallowing. The same sequence of activity can be recorded from the isolated central nervous system (CNS), where intracellular recordings show that the motoneurons receive rhythmic synaptic inputs (Benjamin & Rose 1979; Benjamin *et al.* 1979).

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There are ten types of motoneurons, called 1 cell, 2 cell, . . ., 10 cell. The simplest explanation of the three phases of synaptic inputs seen in the motoneurons is that the postsynaptic potentials (PSPs) arise from three classes of multiaction, premotor interneurons called N1, N2 and N3 neurons (Rose & Benjamin 1981*b*). These three types of interneuron have been located and found to be active in turn: only a single type of interneuron is active in each phase of the ingestive behaviour, with the N1 neurons active in protraction, the N2 in rasping and the N3 in swallowing.

Injection of depolarizing current to generate extra bursts of spikes into the N1, N2 or N3 cells reset the phase of the feeding rhythm (Elliott & Benjamin, 1985*a*), demonstrating that these neurons were all part of the central pattern generator. Paired intracellular recordings revealed synaptic interactions between these interneurons, which were often 1:1 with short latency, so that the contacts may be monosynaptic. The synaptic interactions provide a model of the mechanism of rhythm generation which can explain the sequence of neuronal activity. The chief features of this model include recurrent inhibition between the N1 and N2 neurons, reciprocal inhibition between the N1 and N3 neurons, while the timing of the rhythm is controlled by the endogenous properties of the N cells (e.g. endogenous bursting, post-inhibitory rebound (PIR) and plateau potentials).

Three kinds of cell have been shown to modulate the feeding rhythm. One cell, the SO, is located in the buccal ganglia, the others (CGC and CV1 cells) in the cerebral ganglia. Depolarization of the single SO (Elliott & Benjamin 1985*b*; Rose & Benjamin 1981*a*) or paired CV1 cells (McCrohan 1984) is normally sufficient to generate the entire feeding rhythm with a period of 3–5 s, as seen *in vivo*. The SO and CV1 cells activate the feeding system by exciting the N1 cells independently (McCrohan 1984; McCrohan & Kyriakides 1989). In either case, tonic firing in the CGC cells is required for expression of the normal feeding pattern (Benjamin & Elliott 1989).

The feeding system also includes the OM cells, which are oesophageal mechanoreceptors with cell bodies in the buccal ganglia. Dilation of the gut activates these cells which decelerate or terminate the feeding rhythm by inhibitory contacts with the SO and all types of pattern generating N cells (Elliott & Benjamin 1989).

### (*b*) *Pharmacology of the feeding neurons*

In the gastropod molluscs, especially *Helisoma* and *Lymnaea*, a wide range of agonists have been shown to modulate the feeding rhythm. For example, the feeding rate is increased by the peptide $SCP_b$ and by dopamine, but inhibited by FMRFamide (McCrohan & Kyriakides 1989; Murphy *et al.* 1985; Trimble & Barker 1984), but for all of these, the cells which release the agonist are unknown. Until now, the CGC cell is the only neuron in the *Lymnaea* feeding system for which a transmitter (serotonin) has been identified (McCaman *et al.* 1984). The situation is a little better in *Aplysia* where neurons B4 and 5 are known to be cholinergic and to participate in the retraction phase of the rhythm (Gardner 1977; Gardner & Kandel 1972; Jahan-Pawar *et al.* 1983).

Of all the putative transmitters, acetylcholine has the best range of agonists and antagonists. In *Aplysia* their application suggests that three types of cholinergic receptors are present on different cells (Kehoe 1972*a–c*), but there are differences in the receptor sensitivities in *Helix*, *Planorbis* and *Lymnaea* (ter Maat & Lodder 1980; Walker & Kerkut 1977; Witte *et al.* 1985; Zeimal & Vulfius 1968).

In view of differences in sensitivity to antagonists, especially to atropine (ATR) and hexamethonium (HMT), and as the only cholinergic responses in *Lymnaea* have been from non-feeding neurons this paper reports the results of application of ACh to the identified motoneurons of the *Lymnaea* feeding system. The importance of these results is that, in the following papers, the precise pharmacological lesions can be known. Application of agonists and antagonists suggest that each of Kehoe's (1972*b*) three types of receptor are present on the buccal neurons and that the N1 neurons are the most likely interneurons to release ACh as their transmitter.

(Some of these results have been reported previously in abstracts: Elliott (1987); Elliott *et al.* (1988).)

## 2. METHODS

### (*a*) *Snails*

Pond snails, *Lymnaea stagnalis*, were obtained commercially (Blades Biological), kept in standard snail water (SSW2 of Thomas (1986); composition in Elliott & Benjamin (1989)) and fed on lettuce.

### (*b*) *Dissection and recording*

The CNS and a short length of oesophagus was removed and pinned out in a sylgard dish, through which saline could be pumped (0.5–1.5 ml $min^{-1}$).

Table 1. *Composition of the Lymnaea salines*

(The high Mg/low Ca saline blocks chemical synapses in the buccal ganglia, while the Hi–Di saline raises the spike threshold and so reduces polysynaptic pathways and spontaneous synaptic activity. All concentrations in millimoles per litre. Sodium hydroxide was used to adjust the pH to 7.9 in each case; the values for Na concentration take into account the amount of NaOH introduced.)

| | normal | high Mg/low Ca | Hi–Di |
|---|---|---|---|
| Na | 59 | 39 | 35 |
| K | 2 | 2 | 2 |
| Ca | 4 | 0 | 14 |
| Mg | 2 | 18 | 8 |
| Cl | 38 | 38 | 46 |
| $H_2PO_4$ | 0.1 | 0.1 | 0.1 |
| HEPES | 50 | 50 | 50 |



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The volume of the bath was 0.4 ml. The ganglia were bathed in a 0.1% solution of protease (Sigma type XIV) for 1–5 min to soften the ganglionic sheath. For intracellular recordings, glass microelectrodes (resistance 10–50 MΩ) were filled with 1 M lithium sulphate, saturated potassium sulphate or approximately 8 M potassium acetate. The latter allowed the most stable current injection (Elliott & Benjamin 1985*a*, 1989). Signals were amplified using high-impedance amplifiers (which included current clamp circuitry) constructed from LF356 operational amplifiers.

The composition of the salines used in this series of three papers is given in table 1. All the experiments reported in the present paper were made in the high Mg/low Ca saline which blocks chemical synapses. The 'Hi–Di' (high Mg/high Ca) solution raises the threshold of motor and interneurons by 10–15 mV, and so attenuates polysynaptic pathways and spontaneous synaptic activity (Elliott & Benjamin 1989).

### (*c*) *Identification of cells*

The large motoneurons (1, 2, 3 and 4 cluster cells) were identified by size and position (Benjamin & Rose 1979) whereas the other, smaller neurons were identified by their feeding synaptic inputs in normal saline before the pump was switched to the high Mg/low Ca saline. Note that the motoneurons are known as 1, 2, . . ., 10 cells; the pattern generating interneurons as N1–N3 and the modulatory interneurons are SO, CV1 and CGC cells.

### (*d*) *Chemicals*

Drugs were purchased from Sigma or Aldrich, except for methylxylocholine (MeXCh) which was a gift from Smith, Kline and French (McLean *et al.* 1960). In the bath experiments, drugs were dissolved in the high Mg/low Ca saline and used directly or stored frozen in the dark until required. No difference was detected following freezing. Some preparations seemed relatively insensitive to bath-applied ACh and to antagonists. This observation may indicate that the ganglionic sheath provided a restrictive barrier (see Ger & Zeimal 1977; Koike *et al.* 1974; Sigvardt *et al.* 1986); at least in some cases the sensitivity improved with a longer dose of protease. Results from insensitive preparations have been excluded from this series of papers.

In 35 preparations, ACh was applied iontophoretically from pipettes drawn on the same puller settings as the intracellular electrodes and filled with 1 M ACh in distilled water. Current-clamp amplifiers were used to eject the ACh using up to +10 nA current, with no retention current. The electrodes were advanced adjacent to the cell body, close to the site where the axons are known to arise (morphology determined by Benjamin *et al.* (1979)). No cholinesterase inhibitors were applied.

Chemicals are abbreviated as follows: acetylcholine, ACh; arecoline, AREC; atropine, ATR, curare (d-tubocurarine), d-TC; hexamethonium, HMT; methylxylocholine, MeXCh; phenyltrimethylammonium, PTMA; tetramethylammonium, TMA.

## 3. RESULTS

### (*a*) *Motoneuronal responses to ACh*

Ten types of feeding motoneuron have been identified in the feeding system of *Lymnaea* (Rose & Benjamin 1981*a*). These are known as the 1, 2, . . ., 10 cells. All these neurons responded to acetylcholine (ACh), even when the preparation was bathed in a high Mg/low Ca saline to block synaptic transmission. Some cells (1, 2, 7 and 10 cells) were excited, whereas the 3, 4 cluster, 5, 8 and 9 cells were inhibited. Figure 1 shows

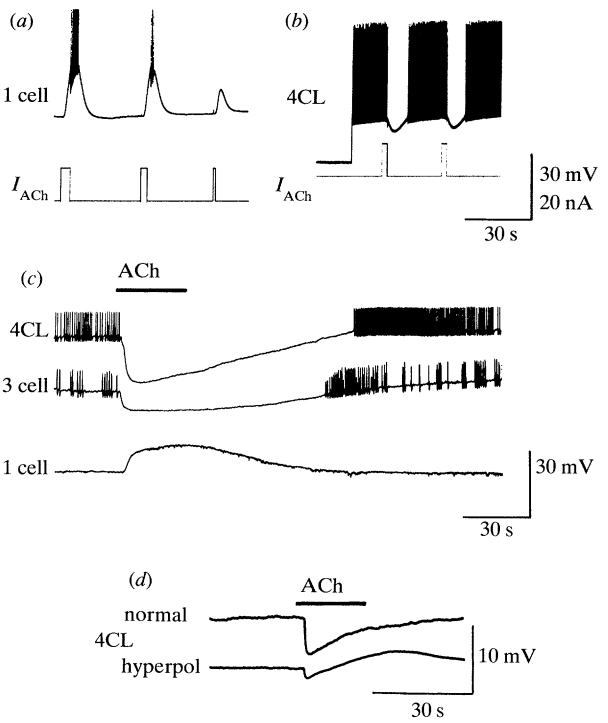


Figure 1. Responses to ACh in identified motoneurons of the buccal ganglia. (*a*) Excitation of a 1 cell. Iontophoretic application from a pipette filled with 1 M ACh, placed close to the axon hillock of a 1 cell. Lower trace: iontophoretic current ($I_{ACh}$); upper trace: intracellular recording from the soma of the 1 cell. The 1 cell is depolarized after each pulse of agonist. (*b*) Inhibition of a 4 cluster cell. Iontophoretic application from a pipette filled with 1 M ACh, placed near to the axon hillock of a 4 cluster cell (4 CL), penetrated with two potassium sulphate electrodes. The upper trace is the intracellular recording from this motoneuron, while the lower trace shows the iontophoretic current ($I_{ACh}$). Shortly after the start of the record, the 4 cluster cell is depolarized by current injection through the second electrode. Both pulses of agonist inhibit the 4 cluster cell. (*a*) and (*b*) are on the same scale. (*c*) Simultaneous intracellular records from a 4 cluster cell, a 3 cell and a 1 cell. The 4 cluster and 3 cells were both depolarized by current injection. Application of 0.5 mM ACh excited the 1 cell and inhibited both the 4 cluster and 3 cells. Note that the 4 cluster cell reaches the peak hyperpolarization immediately and then begins to return, despite the continued presence of ACh. Compare the prolonged hyperpolarization of the 3 cell. (*d*) Superimposed records from a single 4 cluster cell at normal and hyperpolarized membrane potentials, during the application of 0.5 mM ACh. Note the biphasic response to ACh when the cell was hyperpolarized.



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typical responses to ACh application from a pipette (*a* and *b*) and in the bath (*c* and *d*).

The 1 cells are excited by ACh application. The response is dose dependent, so that three successive iontophoretic applications of ACh with shortening current pulses give reducing 1 cell depolarizations (figure 1*a*). Bath application of 0.5 mM ACh also depolarizes the 1 cell (figure 1*c*), but not enough to evoke action potentials. The 2 cell (figure 7), 7 cell (figure 5*b*) and 10 cell (not shown) were also excited by ACh application.

Other motoneurons were inhibited by ACh. Figure 1*b* shows a recording from a 4 cluster cell penetrated by two electrodes. Shortly after the start of the excerpt, depolarizing current is passed through the current electrode, and so the 4 cluster cell starts to fire action potentials. Each ionophoretic application of ACh (from a pipette placed close to the axon hillock) inhibits the 4 cluster cell. Iontophoretically applied ACh also inhibits the 3 cells (Elliott *et al.* 1988), 5 and 8 cells (not shown).

The 3 and 4 cluster cells are also inhibited by bath-applied ACh (figure 1*c*). Note that the 3 cell hyperpolarization is maintained throughout the ACh application and its minimum membrane potential occurs at the same time as the maximum in the 1 cell (figure 1*c*; lower trace), but that, in the simultaneous record from the 4 cluster cell (figure 1*c*; upper trace), the peak hyperpolarization is recorded immediately the ACh arrives. After this, the response gradually declines, despite the continued presence of ACh. This phenomenon is also seen in the response of another 4 cluster cell (figure 1*d*) but when this cell starts from a more hyperpolarized membrane potential the response is biphasic: a rapid hyperpolarization followed by gradual depolarization. The recording can be interpreted as follows: the cholinergic response of the 4 cluster cells is constructed from two ionic components: one acts rapidly and has a reversal potential more negative than the normal resting potential, the second is slower with a reversal potential close to the normal resting potential. The responses of the 5, 8 and 9 cells show similar evidence of a two-component inhibitory response to bath applied ACh (not shown).

### (*b*) *The response to cholinomimetics*

The potencies of four cholinomimetics were compared by simultaneous intracellular recordings from the 1, 3 and 4 cluster cells while the agonists were passed through the bath. No cholinesterase inhibitors were present.

The weak effects of the agonist arecoline (AREC) (0.5 mM in the bath) are displayed in figure 2. The 1 cell (lower trace) shows no change in membrane potential, but the 3 cell (middle trace) is weakly hyperpolarized and its firing rate reduced. The 4 cell stops firing, without any large hyperpolarization. The ineffectiveness of arecoline on the 1 cell's excitatory response and its low potency as an inhibitor of the 3 and 4 cluster cells can be seen by comparing the traces of figure 2 with figure 1*b* as the arecoline traces follow the strong responses to the same concentration

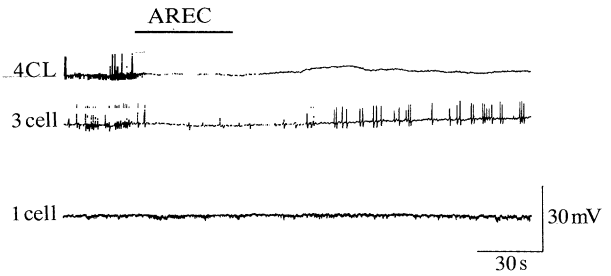


Figure 2. Application of 0.5 mM arecoline to a 4 cluster, a 3 and a 1 cell. The 4 cluster and 3 cells were depolarized by steady current injection. Arecoline weakly inhibits the 4 cluster and 3 cells, but the 1 cell is unaffected. This record follows directly after figure 1*c*, where ACh was applied.

of ACh, without a break. However, in none of the four experiments with arecoline did the inhibitory effect on the 3 or 4 cluster cells wash out quickly, unlike the ACh potentials.

For each agonist, the response increases with concentration and the dose–response curves for TMA and ACh run nearly parallel (figure 3). In eight experiments, the mean threshold for the excitatory response was 12 μM, with a slightly lower threshold for the inhibitory responses (6 μM). In figure 3, note that the potencies of the quaternery ammonium ions differ between the cells. TMA excites the 1 cell four times

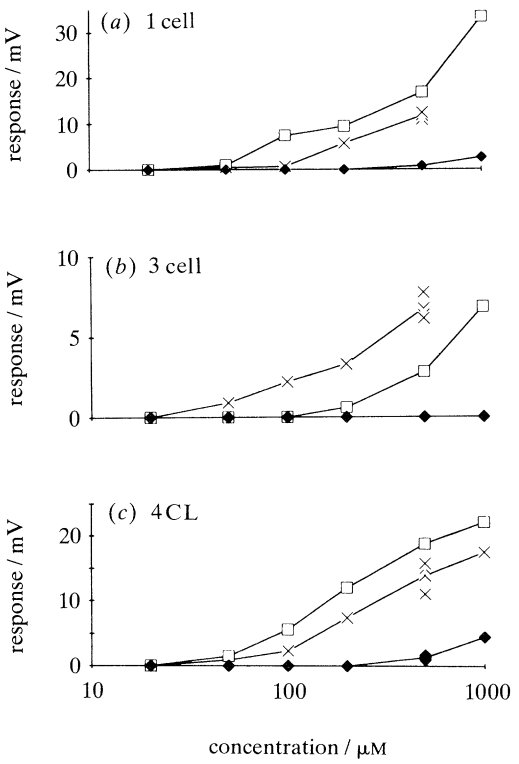


Figure 3. Dose response curves for three agonists (ACh (×), TMA (□) and PTMA (◆)). The responses of the (*a*) 1 cell, (*b*) 3 cell and (*c*) 4 CL cluster cells were recorded simultaneously during bath application of the agonists. Results from one experiment. The points for 500 μM ACh were obtained before, during and at the end of the series of drug applications. Each agonist excited the 1 cell but inhibited the 3 and 4 cluster cells. Note that the 3 cell is much less sensitive to TMA and insensitive to PTMA.



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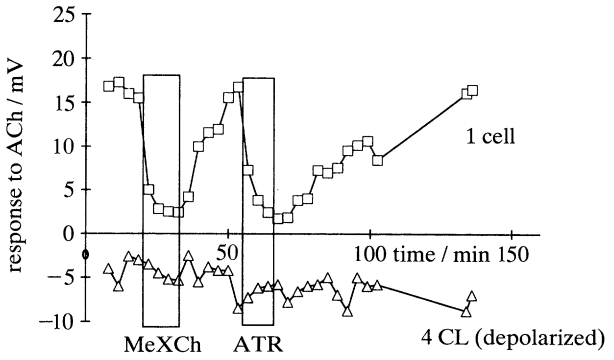


Figure 4. The effects of cholinergic antagonists MeXCh (0.5 mM) and ATR (0.5 mM) on the responses to ACh (0.5 mM, 20 s) of the 1 and 4 cluster cells. The 4 cell was depolarized by injection of 1 nA throughout the experiment. The 1 cell is excited by ACh and the 4 cell inhibited. Both antagonists block the effect of ACh on the 1 cell but do not affect the 4 cluster cell response.

more strongly than ACh (mean of 4 experiments; one shown in figure 3). The 3 cell (inhibited by ACh) is ten times less sensitive to TMA, while the 4 cluster cell (with a two-component inhibition) is more sensitive to TMA. In seven experiments, the mean displacement between the dose–response curves was 1.05 log units.

On all three cell types PTMA is a much less effective agonist than TMA or ACh. Applying PTMA to excite the 1 cell required six times the ACh concentration to produce the same effect (mean of six experiments). In the 3 cell, PTMA produces no discernible inhibition, even at 1 mM. However, even though the 4 cluster cell is clearly inhibited by PTMA, it is 30 times less effective than ACh ($n=6$).

### (*c*) *Antagonists*

Five putative antagonists (HMT, ATR, d-TC, PTMA and MeXCh) were tested with ACh application to the buccal neurons. The effectiveness of the block depends on the cell type. Figure 4 shows one of the 48 experiments in which ACh was applied in the bath, with simultaneous penetrations from a 1 cell and 4 cell. Repeated bath applications of ACh gave a 17 mV excitation of the 1 cell and a 5 mV inhibition of the 4 cell, even though the 4 cell is depolarized throughout the experiment. When either antagonist (MeXCh or ATR) was passed through the bath the 1 cell response was decreased, but the 4 cell response remained unaffected. The strong effect seen with the

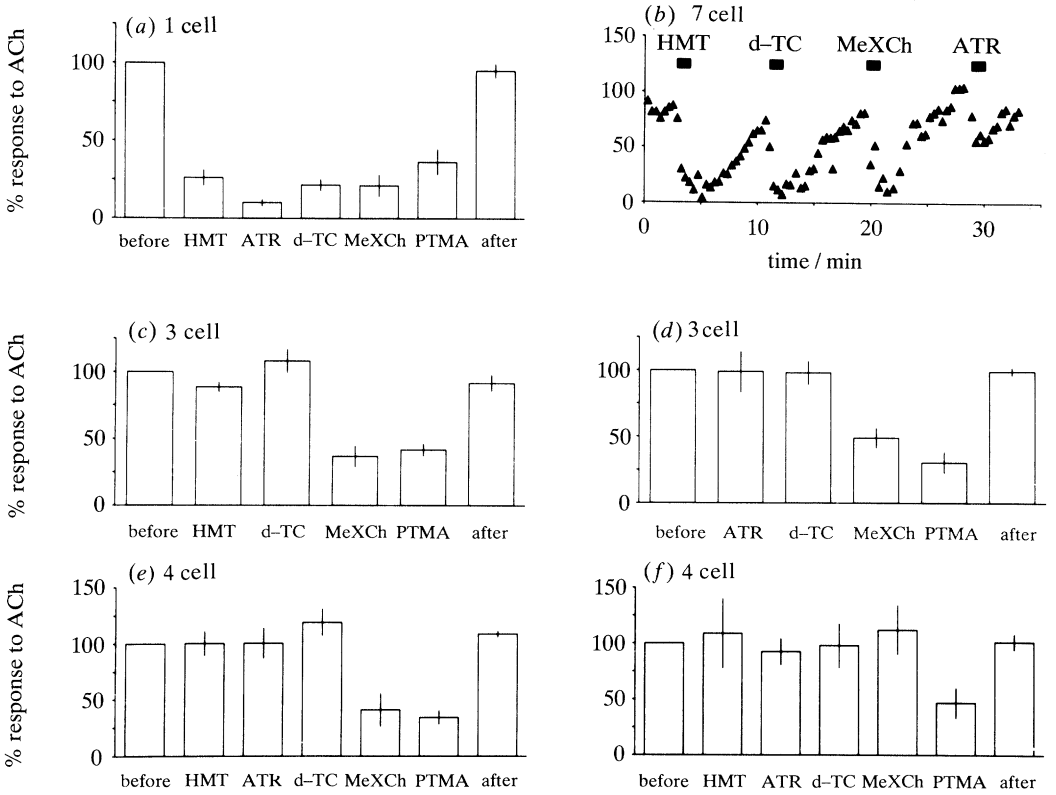


Figure 5. The effects of cholinergic antagonists on the buccal motoneurons' response to ACh. (*a*) Excitation of the 1 cell by bath ACh is blocked by HMT, ATR, d-TC, MeXCh and PTMA (42 applications). (*b*) Antagonism of a 7 cell excitatory response to iontophoretic ACh (10 nC) by HMT, d-TC, MeXCh and ATR. Each antagonist was present in the bath at 0.5 mM for 40 s. (*c*) The inhibitory response of the 3 cell to bath ACh is blocked by MeXCh and PTMA (10 applications). (*d*) The inhibitory response of the 3 cell to iontophoretic ACh is also blocked by MeXCh and PTMA (14 applications). (*e*) The inhibitory response of the 4 cluster cell at its resting potential to bath ACh is blocked by MeXCh and PTMA (47 applications). (*f*) The inhibitory response of the depolarized 4 cluster cell to bath ACh is blocked by PTMA only (14 applications). Results from 56 experiments in which pairs of cells were impaled, with each preparation receiving one application of each of two antagonists only. All antagonists were applied at 0.5 mM for 40 s. Mean ± standard error.



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first test pulse of ACh indicates that most of the block must take place within 1 min of the introduction of the antagonist. Note that the ATR washes out very slowly (over 60 min): recovery from the other antagonists took less than 15 min.

The response of the 1 cell to 0.5 mM bath ACh is normally an excitatory depolarization of 10–20 mV. Figure 5*a* shows that each of the antagonists (HMT, ATR, d-TC, PTMA and MeXCh) applied in the bath at 0.5 mM reduce the response substantially, to 2–3 mV which is just above the noise (1 mV peak-to-peak). The same results are obtained when ACh is applied iontophoretically and only the antagonists are pumped through the bath. Cholinergic excitation of the 1 and 7 cells is blocked by each of the antagonists. For example, the ACh depolarizaton of the small 7 cell is reversibly blocked by successive applications of 0.5 mM HMT, d-TC and MeXCh (figure 5*b*). The same dose of ATR only produced a 20% reduction in the 7 cell response, but a longer pulse of 0.5 mM ATR produces complete inhibition with no recovery within 15 min (not shown).

Only two of the antagonists tested – MeXCh and PTMA – reduce the 3 cell inhibitory response to ACh. This result is obtained with bath and iontophoretic application (figure 5*c*, *d*). The 3 cell cholinergic inhibition persists in the presence of the other antagonists (HMT, d-TC and ATR) although simultaneous recordings with bath application confirmed that the 1 cell response is much reduced.

At first sight, the effects of the antagonists on the 4 cluster cells is the same as that on the 3 cells, with only MeXCh and PTMA blocking the inhibition by bath ACh (figure 5*e*). However, if the 4 cluster cell is depolarized then MeXCh no longer blocks the cholinergic inhibition (figure 5*f*, as in figure 4). The difference from the 3 cell means that there must be two kinds of inhibitory ACh receptor on the 4 cluster cells. The blocking effect of PTMA is seen in every preparation, irrespective of membrane potential. In some preparations, d-TC increased the size of the ACh response by up to 50% (see Elliott *et al.* 1988), but this increase was not consistently seen in all preparations and is not statistically significant overall.

Iontophoretic application of ACh was made to 5 cells in four preparations. Evidence that the smaller 5 cells (like the 4 cluster cells) have a two-component cholinergic inhibition is given in figure 6. At resting potential the 5 cell response to each pulse of ACh is a small depolarization. Introduction of d-TC eliminates this: instead a gradual hyperpolarization is recorded. This shows that the two components of the 5 cell cholinergic potential have different reversal potentials and so are likely to have a different ionic basis. With a depolarized 5 cell the response to ACh is hyperpolariz-

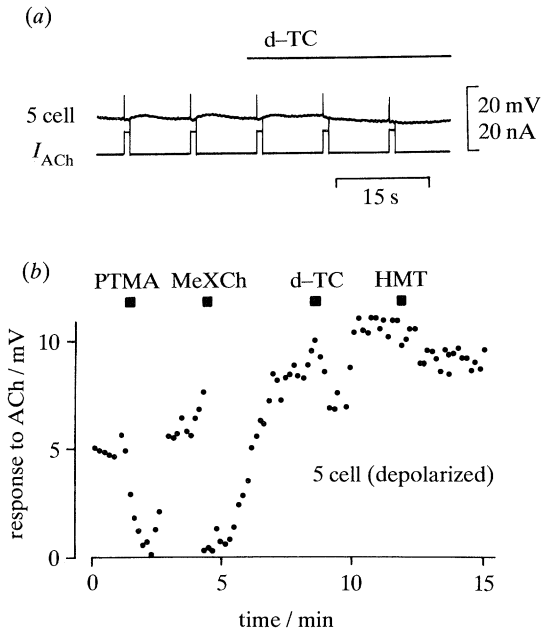


Figure 6. Antagonism of the 5 cell response to iontophoretic ACh. (*a*) Effect of bath applied d-TC. The 5 cell is at resting potential and ACh is repeatedly ejected by positive current ($I_{ACh}$) from a pipette close to the soma. Each time a small depolarizing response is recorded. This is abolished by 0.5 mM d-TC and replaced by a slower hyperpolarizing response to each ACh pulse. (*b*) Bath application of antagonists to a 5 cell, depolarized just over threshold, which was inhibited by iontophoretic application of ACh (10 nC). PTMA and MeXCh both blocked the cholinergic response completely, d-TC had a lesser effect while HMT had little discernible effect. The gradual increase in response during the experiment may be due to movement of the electrode.

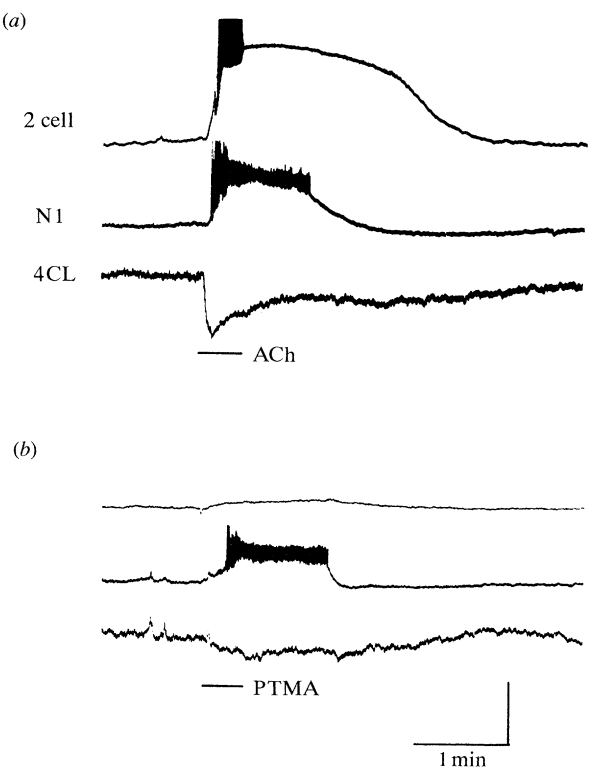


Figure 7. The response of an N1 interneuron, and two motoneurons, the 2 and 4 cluster cells to (*a*) 0.5 mM ACh and (*b*) 0.5 mM PTMA. Note the small spikes on the top of the N1 excitatory response to ACh. The 2 cell spikes are limited by the width of the chart recorder. Scalebar: 2 cell, 20 mV; N1, 20 mV; 4 CL, 10 mV.



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ing. Now perfusion of the antagonists PTMA and MeXCh (0.5 mM) both block the 5 cell inhibition by ACh (figure 6*b*). In each case the block is close to 100% and is rapid and reversible. In this depolarized 5 cell, the application of d-TC has only a small reduction (only 20%), whereas HMT (and ATR, not shown) have no effect on the cholinergic inhibition of the 5 cells.

### (*d*) *Response of N1 interneurons to cholinomimetics*

The N1 interneuron was also tested for its response to bath applied ACh in four experiments. One is shown in figure 7: ACh depolarizes the N1 cell and this leads to a burst of small spikes, probably attenuated action potentials (see Elliott & Kemenes 1992). The agonist PTMA also excites the N1 interneuron (figure 7*b*), but it does not give such a strong depolarization.

## 4. DISCUSSION

### (*a*) *Three kinds of receptors*

The results presented above show that ACh excites some of the motoneurons in the buccal ganglia of *Lymnaea* and inhibits others. Since all the experiments in this paper are performed in a high Mg/low Ca saline, the responses are likely to be endogenous to the cells concerned and not produced as a result of chemical synaptic input, e.g. from sensory- or interneurons. (So far as is known, the motoneurons do not make strong electrical connections with other, unidentified buccal neurons.)

The response of a particular cell is the same irrespective of whether the ACh is applied in the bath, or iontophoretically to either the soma or near to the axon. In this respect, the buccal cells of *Lymnaea* differ from those of *Navanax* and the *Lymnaea* CDC cells where the response varies topographically (Levitan & Tauc 1972; ter Maat & Lodder 1980).

The cells that are excited include the 1, 2, 7 and 10 cells: all identified motoneurons in the feeding system (Benjamin & Rose 1979; Benjamin *et al.* 1979; Rose & Benjamin 1981*a*) and the N1 interneurons. The 1 cell, largest of these motoneurons, is also depolarized by TMA and PTMA, the latter being much less potent than ACh (figure 3). The excitatory response to ACh is much reduced by a 0.5 mM bathing solution of each cholinergic antagonist tested (HMT, ATR, d-TC, MeXCh and PTMA). Note that the antagonism by PTMA cannot be explained by its depolarizing agonist action since 0.5 mM PTMA will only produce a 2 mV depolarization (figure 3).

ACh hyperpolarizes and inhibits the 3, 4 cluster, 5, 8 and 9 cells, which are further identified motoneurons in the *Lymnaea* feeding system (Rose & Benjamin 1981*a*). In the 3 cell a simple inhibitory response is recorded irrespective of membrane potential; hyperpolarization of this neuron by 0.5 mM ACh has the same time course as the 1 cell (excitatory) response (figure 1*c*). The dose–response curves from the cholinomimetics TMA and PTMA show that these are much less effective on the 3 cell than on the 1 cell (cf. figure 3*a*, *b*); indeed no response could be recorded from 1 mM PTMA. However, the 3 cell is sensitive to arecoline which is ineffective at the excitation of the 1 cell. Of the five antagonists tested, only MeXCh and PTMA are effective in blocking the cholinergic potential in the 3 cell (figure 5*c*, *d*). These responses correspond with those expected from the 'slow' inhibitory receptor described in *Aplysia* by Kehoe (1972*b*).

In the 4 cluster, 5, 8 and 9 cells the situation is more complex and a two component response is recorded. The 4 cluster hyperpolarization normally reaches its peak rapidly and then decays, even while the ACh concentration is maintained (figure 1*c*). If the motoneuron is hyperpolarized, then the response may become biphasic, a rapid hyperpolarization followed by depolarization (figure 1*d*). These results suggested that the 4 cluster cells have a two-component response, like the medial cells of the pleural ganglion of *Aplysia* (Kehoe 1972*a*, *c*), with a mixture of fast and slow receptors.

The 4 cluster cells respond to TMA slightly more sensitively than to ACh, and show weak inhibition with PTMA (figure 3*c*). The difference between 3 and 4 cluster dose–response curves cannot simply arise from a higher density of ACh receptors on the 4 cluster cell, as both cells have the same threshold to ACh (figure 3*b*, *c*). Instead, the hyperpolarization of the 4 cluster cells by TMA and PTMA shows that the inhibitory receptors on the 4 cluster cells must include ones not found on the 3 cell. (The possibility that the 4 cluster cells have a mixture of excitatory and slow, TMA-insensitive, inhibitory receptors can be eliminated by the observation that the response to TMA as well as ACh is hyperpolarization.) This is further evidence that the 4 cluster cells possess fast as well as slow inhibitory receptors.

The antagonists HMT and ATR do not affect either the 4 cluster or 5 cells, while PTMA always attenuates the ACh response (figures 4, 5 and 6). However, the results recorded during application of the other antagonists depend on the cell's membrane potential (cf. figures 5*e*, *f* and 6*a*, *b*).

In the resting 4 cluster cell, MeXCh reduces the cholinergic response but d-TC produces a small increase (figure 5*e*). This means the resting inhibitory response to ACh must be dominated by currents flowing through slow receptor-ionophores. However, when the 4 cluster cell is depolarized neither d-TC nor MeXCh reduce the response to ACh, showing that, when the cell is depolarized ions can flow through either the fast or slow receptor-ionophore. Thus the 4 cluster cells have two kinds of receptor, fast and slow. PTMA is effective at any membrane potential (figure 5*f*), as it blocks all three types of ACh receptor.

In the resting 5 cell, a small depolarizing response is seen to ACh. This is blocked by d-TC, leaving a hyperpolarizing response. When the 5 cell is depolarized, d-TC seems relatively ineffective (figure 6). The simplest explanation is that, like the 4 cluster cell, the 5 cell has both types of inhibitory ACh receptors. In both cell types the reversal potential for the fast (d-TC



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Table 2. *The three types of cholinergic receptors in the buccal ganglia of Lymnaea*

(NR, no response; NC, no change.)

| receptor type | excitatory inhibitory | slow | fast |
|---|---|---|---|
| neurons | 1, 2, 7, 10 | 3, 4 CL, 5, 8, 9[a] | 4 CL, 5, 8, 9[a] |
| agonists | | | |
| TMA | excite | inhibit | inhibit |
| PTMA | excite | NR | inhibit |
| arecoline | NR | inhibit | NR |
| antagonists | | | |
| HMT | block | NC | NC |
| ATR | block | NC | NC |
| d-TC | block | NC | block |
| MeXCh | block | block | NC |
| PTMA | block | block | block |

[a] The 4 CL, 5, 8 and 9 cells have fast and slow inhibitory receptors.

Table 3. *A comparison of the synaptic inputs seen in the buccal motoneurons during the feeding rhythm with their response to acetylcholine (ACh)*

(The feeding rhythm has three phases (known as N1, N2, and N3); the tables shows the sign (excitatory or inhibitory) of the synaptic input received by each motoneuron. Note that, at rest, some inhibitory synaptic inputs are reversed and appear to the depolarizing. e, excitation; i, inhibition; —, no input; x, not tested.)

| | motoneuron cell type | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 |
| N1 phase input | e | e | i | i | i | e | i | i | e |
| N2 phase input | — | e | e | i | i | i | i | e | e |
| N3 phase input | — | — | e | i | i | i | i | i | — |
| ACh bath | e | e | i | i | i | e | i | i | e |
| ACh pipette | e | e | i | i | i | e | x | x | x |

sensitive) inhibitory receptor is more positive than that of the slow (MeXCh sensitive) receptor. This evidence would agree with the results of Kehoe (1972*a*), who found the fast current was carried by chloride (rev. pot −60 mV) and the slow by potassium (rev. pot −80 mV).

### (*b*) *Comparative pharmacology of the gastropods*

The results of application of the cholinergic agonists and antagonists to the buccal motoneurons of *Lymnaea* have been explained above in terms of the three kinds of ACh receptors (E, $I_{fast}$ and $I_{slow}$) which Kehoe (1972*a–c*) found in *Aplysia*. The results from the 4 cluster and 5 cells confirm that the reversal potentials of the two inhibitory responses are different, the fast more positive than the slow and so agree with the ionic mechanisms established in *Aplysia*.

The results from the antagonists d-TC and MeXCh closely resemble those of other gastropods. With PTMA there seem to be slight differences in pharmacology: for example in *Lymnaea*, PTMA reduces the response at all three kinds of receptors (figure 5), but in *Aplysia*, it is selective for the slow receptor (Kehoe 1972*b*). Again, arecoline at 0.05 mM produced large effects on the *Aplysia* medial cells, but in *Lymnaea* even when used at ten times this concentration, it produced very weak effects (figure 2). In this respect, neurons in *Lymnaea* resemble the 'D' and 'H' cells' of *Helix*, where arecoline was 64 and 27 times less effective than ACh (Walker 1968).

The main differences concern the antagonists HMT and ATR. In our experiments on *Lymnaea*, the excitatory response of the buccal 1 and 7 cells to ACh is effectively blocked by these antagonists at 0.5 mM (figures 5*a*, *b*), but they were reported to be ineffective at the 'D cells' in the visceral and parietal ganglia of *Lymnaea* and *Planorbis* (Zeimal & Vulfius 1968). Similarly, 10 mM HMT was required to block the cholinergic excitation of neuron B1 in the *Helix* buccal ganglia, but this also blocked some of the inhibitory responses (Witte *et al.* 1985). On the other hand, 0.1 mM ATR was sufficient to block the cholinergic excitation of 'D' and inhibition of 'H' cells in *Helix* (Walker 1968; Witte *et al.* 1985).

One possible cause of the differences is suggested by Witte *et al.* (1985), who found that the completeness of the HMT antagonism at *Helix* neuron B1 depended on the composition of the saline: higher levels of HEPES (5 mM) increased the effectiveness of the HMT. In the standard *Lymnaea* saline, the concentration of HEPES is yet greater (50 mM) and so this may well explain the increased blocking. The higher pH (7.9) used in these *Lymnaea* experiments may also affect receptor affinities.

These differences in experimental results stress the importance of these experiments as controls for the pharmacological dissection experiments described in paper III (Elliott 1992).

### (*c*) *The pattern of cholinergic responses in the motoneurons*

Rose and Benjamin (1981*b*) showed that the feeding rhythm had three phases of synaptic activity and suggested that it was likely that this originated from three sets of premotor multiaction neurons. Table 3 compares the response to ACh with the known feeding inputs to the motoneurons. It is clear that only in the 9 cell do the N2 and N3 inputs differ in polarity. Some of the cells even receive the same sign of synaptic input in the N1, N2 and N3 phases and their ACh response also has the same sign (e.g. 4 cluster cells; all i). These cells do not help decide which premotor neurons might release ACh. However, the 3 and 7 cells receive opposite inputs in the N1 versus N2 (or N3) phases. For both these cells the N1 phase input matches the ACh effect. Indeed all the motoneurons tested have the same input in the N1 phase as ACh response. The simplest hypothesis is that the N1 interneurons are the most likely premotor interneurons to release ACh as



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their transmitter. The alternative hypothesis – that the ACh could be stimulating the N1 neurons (figure 7*a*) which in turn affect the motoneurons – can be rejected as unlikely because of the high Mg/low Ca saline which blocks chemical transmission. Furthermore, in the next paper (Elliott & Kemenes 1992) these hypotheses will be tested by applying antagonists while recording from a N1 neuron and a motoneuron.

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