

## BASAL ACETYLCHOLINE RELEASE IN LEECH GANGLIA DEPOLARIZES NEURONS THROUGH RECEPTORS WITH A NICOTINIC BINDING SITE

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*Accepted 6 April; published on WWW 21 May 1998*

### Summary

The response of Retzius neurons, the main neuronal source of serotonin in the leech nervous system, to cholinergic agonists has been extensively investigated. In this study, we analyzed the effects of inhibiting the acetylcholinesterase (AChE) activity in the leech midbody ganglion on the electrophysiological activity of the Retzius neurons. Bath application of neostigmine and physostigmine ( $0.1\text{--}100\ \mu\text{mol l}^{-1}$ ) produced, after a delay, a strong depolarization of the Retzius neurons with a dose-dependent amplitude and latency. The amplitude of this depolarization increased as the extracellular level of  $\text{Ca}^{2+}$  increased and decreased as the extracellular level of  $\text{Ca}^{2+}$  decreased. The response to neostigmine and physostigmine was inhibited by curare ( $100\ \mu\text{mol l}^{-1}$ ), nicotine ( $10\ \mu\text{mol l}^{-1}$ ), atropine ( $100\ \mu\text{mol l}^{-1}$ ) and strychnine ( $100\ \mu\text{mol l}^{-1}$ ), but was not affected by mecamylamine ( $100\ \mu\text{mol l}^{-1}$ ) or hexamethonium ( $100\ \mu\text{mol l}^{-1}$ ). Superfusion with solutions containing  $100\ \mu\text{mol l}^{-1}$  strychnine or atropine produced a progressive

hyperpolarization of the Retzius neurons, while superfusion with  $100\ \mu\text{mol l}^{-1}$  curare did not. The hyperpolarization induced by atropine was inhibited in the presence of curare. Other neurons in the ganglion showed distinctive responses to the AChE inhibitors that were coincident with their responses to cholinergic agonists.

The results suggest the existence of a basal level of acetylcholine (ACh) release in the leech ganglion that is powerfully counteracted by endogenous AChE activity. Under control conditions, this basal release appears to be sufficient to generate an ACh tonus that regulates the membrane potential of Retzius neurons. Since these neurons can support a sustained firing rate, which is dependent on the membrane potential, the results presented in this report suggest that the basal ACh tonus regulates the output of these neuromodulatory serotonergic neurons.

Key words: leech, *Hirudo medicinalis*, acetylcholine, nicotinic binding site, serotonergic neurone.

### Introduction

While the functional role of nicotinic acetylcholine (ACh) receptors in the neuromuscular junction has been well established in vertebrate and invertebrate organisms, their role in the central nervous system has not been clearly defined. Despite extensive characterization of the great diversity of nicotinic receptors, which are present in different areas of the central nervous system of different species, there are few examples where their role in neurotransmission has been properly documented (Nicoll *et al.* 1990; Sargent, 1993; McGehee and Role, 1995).

The nervous system of the leech is one system in which it has been possible to study the effects of activating neuronal nicotinic receptors in identified neurons (Woodruff *et al.* 1971; Pellegrino and Simonneau, 1984; Sargent *et al.* 1977; Bigiani and Pellegrino, 1990; Schmidt and Calabrese, 1992; Kristan *et al.* 1993; Szczupak *et al.* 1993, 1998). The nervous system of this annelid consists of a chain of similar ganglia, innervating each one of the invariant segments that make up its body (Muller *et al.* 1981). Each segment preserves a high

degree of autonomous sensory and motor function and, therefore, research at the single segment level can reveal substantial information on the functioning of the organism as a whole. The regularity of the body organization is partially disrupted in segments 5 and 6, which contain the reproductive organs. Each segmental ganglion contains approximately 200 pair of neurons. Amongst these are the Retzius cells, a pair of large neurons readily identifiable by their soma size and position in the ganglion, which constitute the main source of serotonin in the leech nervous system (Willard, 1981; Lent and Dickinson, 1984; Wittenberg and Kristan, 1992). The Retzius cells are of particular physiological and developmental interest because of their differential anatomical and physiological properties in the normal and reproductive segments (French and Kristan, 1992). The Retzius neurons show a complex electrophysiological response to cholinergic agonists (Kristan *et al.* 1993; Szczupak *et al.* 1993, 1998). Applications of pressure pulses of cholinergic agonists to the soma of Retzius neurons from standard midbody ganglia

produce a three-phase response: a fast depolarization followed by a hyperpolarization and a subsequent delayed depolarization. Similar pulses applied to the soma of Retzius neurons from reproductive segments produce a hyperpolarizing response followed by a relatively small delayed depolarization. In the present study, we have investigated the effects of endogenous cholinergic signals on the Retzius neurons by inhibiting the acetylcholinesterase (AChE) activity in the ganglion while recording the electrophysiological activity of these neurons. The results suggest the existence of a prominent basal level of release of ACh in the leech ganglion, which produces a strong depolarizing effect on Retzius neurons through the activation of receptors with a nicotinic profile. Under control conditions, this basal level of release was sufficient to build an ACh tonus that regulates the resting potential of the Retzius neurons.

## Materials and methods

### Biological preparation

Leeches, *Hirudo medicinalis* L., weighing 2–5 g, were obtained from a commercial supplier (Leeches USA, Westbury, NY, USA) and maintained at 15 °C in artificial pond water. The animals were not fed for at least 1 month prior to dissection. Individual ganglia were dissected out and pinned, ventral side up, to Sylgard (Dow Corning) in a chamber under constant superfusion with saline solution at a flow rate of 50  $\mu\text{l s}^{-1}$ . Unless stated otherwise, the sheath covering the ganglion was left intact, except for those experiments where we explicitly state that we used desheathed ganglia.

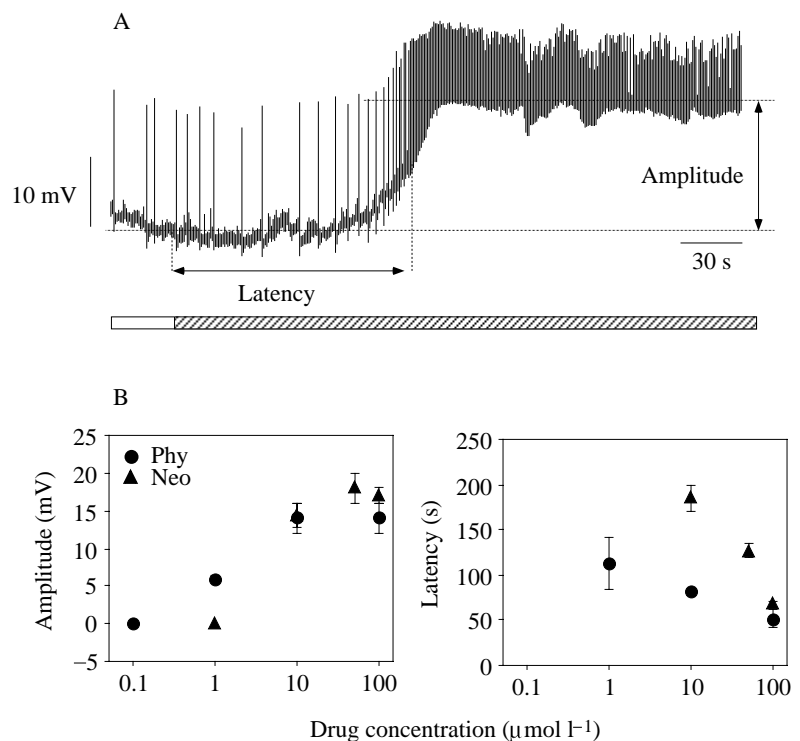
### Solutions and materials

The saline solution had the following composition (in  $\text{mmol l}^{-1}$ ): NaCl, 115; KCl, 4; CaCl<sub>2</sub>, 1.8; MgCl<sub>2</sub>, 1; Tris maleate, 4.6; Tris base, 5.4; and glucose, 10; pH 7.4 (control solution). The solution with a high  $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$  ratio contained 20  $\text{mmol l}^{-1}$  MgCl<sub>2</sub> and 1  $\text{mmol l}^{-1}$  CaCl<sub>2</sub> (the osmolarity was kept constant by reducing the NaCl concentration); and the high- $[\text{Ca}^{2+}]$  solution contained 10  $\text{mmol l}^{-1}$  CaCl<sub>2</sub> and 1  $\text{mmol l}^{-1}$  MgCl<sub>2</sub>. Physostigmine, neostigmine, carbachol, nicotine (hemisulfate salt), *d*-tubocurarine chloride, mecamlamine, atropine, strychnine and hexamethonium (Sigma Co., St Louis, MO, USA) were dissolved in the saline solution and applied *via* the superfusion system.

### Electrophysiological recordings

Retzius neurons of isolated ganglia were impaled with a single intracellular electrode connected to an amplifier (Axoclamp 2B, Axon Instruments, Foster City, CA, USA) operating in the current-clamp configuration using its bridge balance to compensate the voltage drop through the microelectrode. Microelectrodes were pulled from borosilicate capillary tubing (FHC, Brunswick, ME, USA), filled with a 3  $\text{mol l}^{-1}$  potassium acetate solution and had a resistance of 20–40 M $\Omega$ . The recordings were digitized using a TL-1 DMA interface and acquired using Fetchex and Clampex protocols (Axon Instruments) at frequencies of 1000–3200 Hz. Retzius neurons were initially recorded while superfused with control solution, and direct current was injected to set them at a membrane potential of  $-60$  mV. The different drugs were

Fig. 1. Responses of Retzius neurons to acetylcholinesterase inhibitors. (A) A representative response of a Retzius neuron, set at an initial membrane potential of  $-60$  mV, to  $10 \mu\text{mol l}^{-1}$  neostigmine. The horizontal bar underneath the recording, in this and the following figures, describes the time course of the experiment: the ganglion was perfused with control solution (open segment) and then with neostigmine-containing solution (hatched segment). The arrowed lines indicate how the amplitude and latency of the response were measured: the amplitude was measured from the baseline to the level of maximal depolarization (ignoring the spikes), and the latency was measured from the onset of drug perfusion to the time when the neuron reached 50% of its maximal depolarization. (B) Mean amplitudes and latencies of the responses elicited by physostigmine (Phy) and neostigmine (Neo), measured as indicated in A, are plotted against drug concentration. Values are means  $\pm$  S.E.M.,  $N=3$  for physostigmine and  $N=5$  for neostigmine.



applied by switching the perfusion to the test solutions. Responses were characterized in terms of the amplitude and latency of the membrane potential change as indicated in Fig. 1. The analysis of the recordings was performed using commercial software (Axograph 6.0, Axon Instruments). Results are expressed as mean values  $\pm$  the standard error of the mean (S.E.M.), and the number of neurons studied ( $N$ ) is expressed in parentheses. The statistical significance of the results obtained under different experimental conditions was determined using unpaired  $t$ -tests.

## Results

### Responses of Retzius neurons to acetylcholinesterase inhibitors

Superfusion of isolated ganglia from standard midbody segments with solutions containing the AChE inhibitors physostigmine and neostigmine ( $0.1$ – $100 \mu\text{mol l}^{-1}$ ) produced, after a delay, a strong depolarization of the Retzius neurons that was accompanied by high-frequency firing (Fig. 1A). The amplitude and the latency of the depolarization were dose-dependent: increasing concentrations produced an increase in the amplitude and a decrease in the latency (Fig. 1B). At  $10 \mu\text{mol l}^{-1}$ , physostigmine and neostigmine induced a maximal depolarization of Retzius neurons; however, at a concentration of  $100 \mu\text{mol l}^{-1}$ , these AChE inhibitors produced their effect with a significantly shorter latency. These responses were persistent even after a 20 min wash-out in control solution.

The long latencies of the responses to physostigmine or neostigmine were not due to delays inherent in the superfusion system but were intrinsic to the mechanism of action of these drugs. Fig. 2 shows the responses of Retzius neurons to superfusion with solutions containing the cholinergic agonists carbachol ( $10 \mu\text{mol l}^{-1}$ ) and nicotine ( $10 \mu\text{mol l}^{-1}$ ), in comparison with the response to neostigmine ( $100 \mu\text{mol l}^{-1}$ ). The responses to the cholinergic agonists reached their maximal amplitude well before the onset of the responses to the AChE inhibitor and had latencies that were approximately 35 s ( $34.7 \pm 3.1$  s  $N=4$ ) shorter. It is noteworthy that, while the carbachol-induced depolarization reached a plateau that persisted throughout its bath application, the nicotine-induced depolarization had a duration of approximately 35 s ( $35 \pm 7$  s,  $N=3$ ), after which the membrane potential returned spontaneously to a level close to its initial baseline, in spite of the continuous presence of the agonist. This pattern of response was probably due to the stabilization of the receptor in a desensitized state following the activation of the depolarizing current (Schrattenholz *et al.* 1993).

To test whether the glia covering the cell bodies could impose a diffusion barrier on the AChE inhibitors, we performed experiments using ganglia whose sheath was removed. The mean amplitude and latency ( $N=6$ ) of the responses to  $100 \mu\text{mol l}^{-1}$  neostigmine in these ganglia ( $18.9 \pm 5$  mV;  $65.3 \pm 3.1$  s, respectively) were similar to those in intact ganglia ( $16.6 \pm 1$  mV;  $68 \pm 3$  s, respectively).

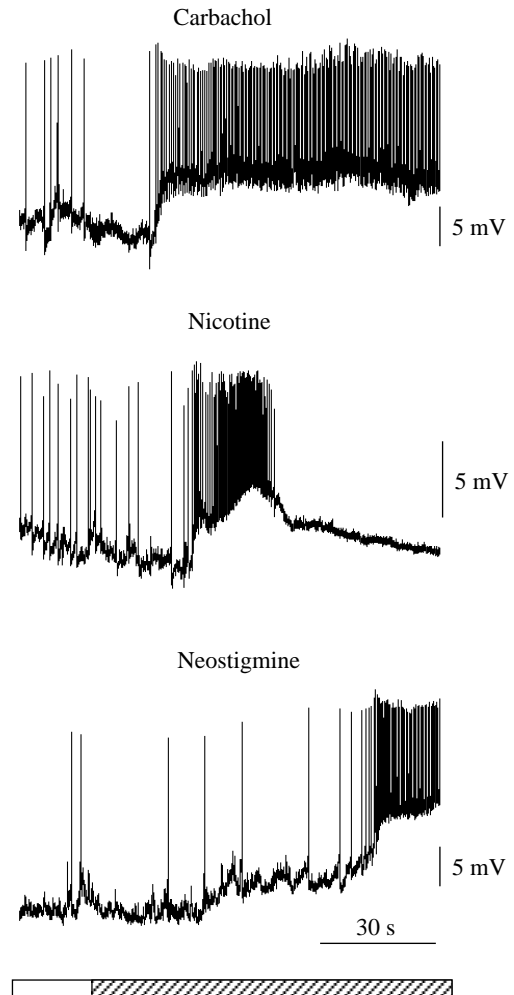


Fig. 2. Comparison of the responses of Retzius neurons to the application of cholinergic agonists and acetylcholinesterase inhibitors. Representative responses of three Retzius neurons, set at an initial membrane potential of  $-60$  mV, to bath applications of solutions containing  $10 \mu\text{mol l}^{-1}$  carbachol,  $10 \mu\text{mol l}^{-1}$  nicotine and  $100 \mu\text{mol l}^{-1}$  neostigmine, at the time indicated by the hatched segment of the horizontal bar underneath the recordings. Similar responses to carbachol and nicotine were obtained in at least three other Retzius neurons examined with each agonist.

### Dependence of neostigmine-induced depolarization on extracellular $[\text{Ca}^{2+}]$

The responses of Retzius neurons to neostigmine were studied in ganglia bathed in saline solutions containing different  $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$  ratios (Fig. 3). In the presence of a  $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$  ratio of 20/1, the mean amplitude of the response induced by  $10 \mu\text{mol l}^{-1}$  neostigmine was approximately 40% the mean amplitude in control solution (with a  $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$  ratio of 1/1.8), and in the presence of a high extracellular  $[\text{Ca}^{2+}]$  ( $10 \text{ mmol l}^{-1}$ ) the mean amplitude of the response was approximately 70% higher. While the mean amplitudes of the responses induced by 10 and  $100 \mu\text{mol l}^{-1}$  neostigmine under standard conditions were similar, the

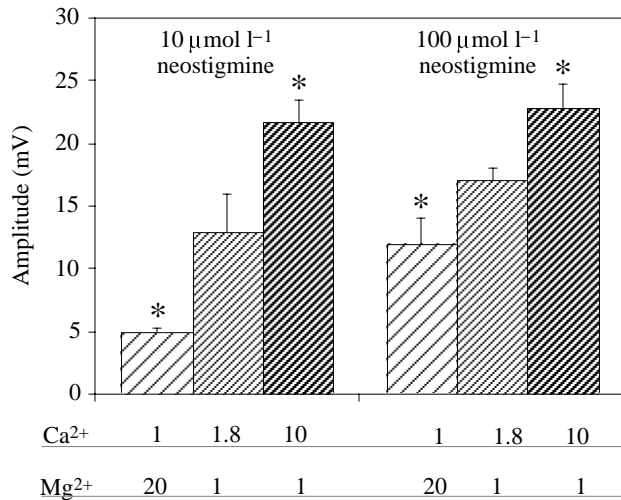


Fig. 3. Responses of neostigmine-induced depolarizations at different extracellular  $\text{Ca}^{2+}$  concentrations. The columns indicate the mean amplitude of the responses of Retzius neurons to  $10 \mu\text{mol l}^{-1}$  ( $N=3$ ) and  $100 \mu\text{mol l}^{-1}$  ( $N=4$ ) neostigmine in ganglia superfused with solutions containing different concentrations of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , as indicated at the bottom of each column (in  $\text{mmol l}^{-1}$ ). The bars indicate the S.E.M. and an asterisk indicates that the mean value is significantly different ( $P<0.05$ ) from the control value ( $1.8 \text{ mmol l}^{-1} \text{ Ca}^{2+}$  and  $1 \text{ mmol l}^{-1} \text{ Mg}^{2+}$ ).

responses produced by the higher neostigmine concentration appeared to be less sensitive to changes in the  $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$  ratio (Fig. 3).

To evaluate whether these different extracellular solutions could affect the responsiveness of Retzius neurons to cholinergic agonists *per se*, we tested the responses of Retzius neurons to pressure pulses of carbachol in the presence of solutions with standard and high  $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$  (20/1) ratios. The amplitudes of the delayed depolarization induced by this cholinergic agonist under the two conditions exhibited a ratio of  $1.1 \pm 0.2$  ( $N=4$ ), showing that the cholinergic response of Retzius neurons was not altered by modification of the concentration ratio of external divalent cations.

#### Pharmacological characteristics of the depolarization induced by acetylcholinesterase inhibitors

To characterize the receptors mediating the effect of the AChE inhibitors, we tested the response induced by  $50 \mu\text{mol l}^{-1}$  neostigmine in ganglia preincubated with the nicotinic antagonists curare, mecamylamine and hexamethonium. Curare inhibited the neostigmine-induced depolarization, in a dose-dependent manner (Fig. 4A,B), without affecting its latency. In contrast, hexamethonium and mecamylamine, at a concentration ( $100 \mu\text{mol l}^{-1}$ ), that strongly inhibited the fast depolarization produced by cholinergic agonists, had no effect on the neostigmine-induced depolarization ( $N=3$ ; data not shown). The response to neostigmine was also tested in ganglia superfused with a solution containing nicotine, after it had produced its transient

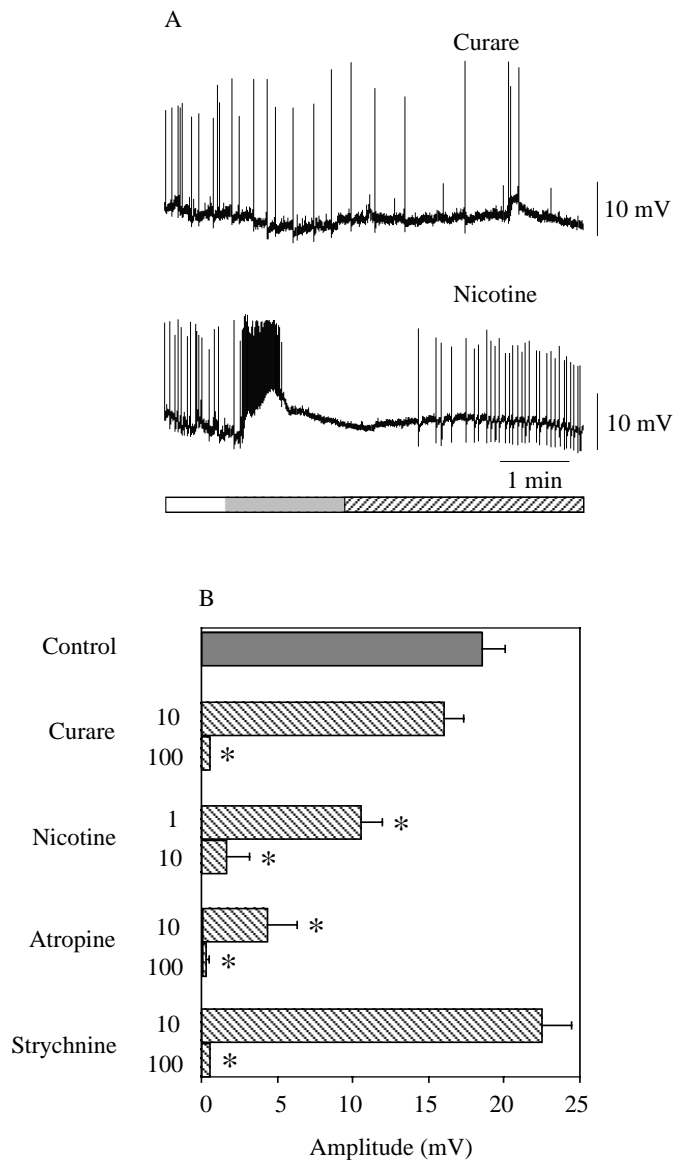


Fig. 4. Effects of different nicotinic antagonists on the depolarization induced by neostigmine. (A) Representative recordings of Retzius neurons, set at a membrane potential of  $-60 \text{ mV}$ , to  $50 \mu\text{mol l}^{-1}$  neostigmine in the presence of  $100 \mu\text{mol l}^{-1}$  curare and  $10 \mu\text{mol l}^{-1}$  nicotine. The dotted segment in the horizontal bar underneath the recordings indicates the timing of antagonist application; the segment with the superimposed patterns indicates the application of a solution containing a mixture of neostigmine and the antagonist. (B) The mean amplitudes ( $N=3-4$ ) of the responses to  $50 \mu\text{mol l}^{-1}$  neostigmine when the ganglia were preincubated in the control solution and in solutions containing curare, atropine, nicotine and strychnine at the concentrations indicated on the left (in  $\mu\text{mol l}^{-1}$ ). The bars indicate S.E.M. and an asterisk indicates that the mean value is significantly different from the control value ( $P<0.05$ ).

agonist effect. Nicotine produced a dose-dependent inhibition of the neostigmine-induced depolarization (Fig. 4A,B). Given that this pharmacological profile resembles that of the delayed depolarization induced by carbachol (Szczupak *et al.* 1998),

we tested the effect of atropine and strychnine, two effective antagonists of this response. These antagonists also inhibited the neostigmine-induced depolarizations in a dose-dependent manner (Fig. 4B).

#### *Segmental specificity of the response of Retzius neurons to acetylcholinesterase inhibitors*

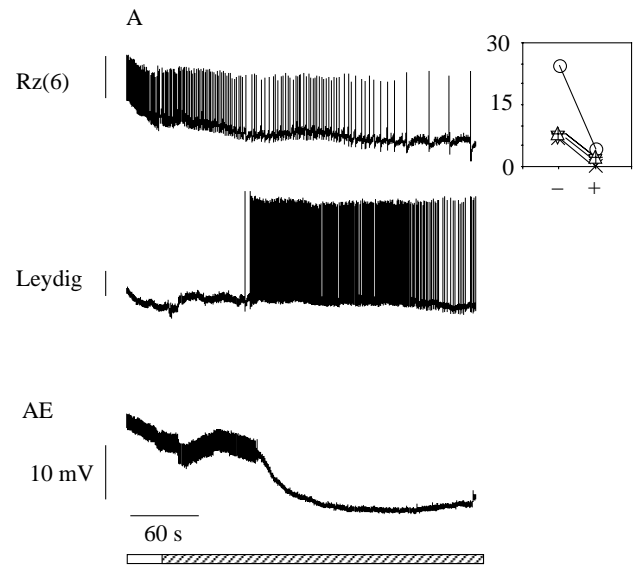
Since Retzius neurons from the reproductive segments 5 [Rz(5)] and 6 [Rz(6)] exhibit a pattern of response to cholinergic agonists that differs from that of Retzius neurons from standard segments [Rz(X)], we investigated the effect of  $100\ \mu\text{mol l}^{-1}$  neostigmine on Rz(5) and Rz(6), set at their resting membrane potential. While Rz(5) neurons ( $N=3$ ) did not exhibit any change in membrane potential or firing frequency, Rz(6) neurons ( $N=4$ ) were slightly hyperpolarized and their firing frequency decreased (Fig. 5A).

#### *Cellular specificity of the response to acetylcholinesterase inhibitors*

To assess whether the observed segmental specificity was due to differential characteristics of the Rz(5) and Rz(6) neurons themselves or of the ganglia in general, we investigated the effect of the AChE inhibitors on other neurons in ganglia from standard and reproductive segments. Leydig neurons from standard ganglia showed a dramatic increase in spike frequency without a significant change in baseline membrane potential (Fig. 5A). Identical responses were observed in Leydig cells from ganglia 5 and 6. The mechanosensory neurons sensitive to touch (T), pressure (P) and nociceptive (N) stimuli and the anterior pagoda (AP) cells showed no response to the AChE inhibitors. In contrast, the cholinergic annulus erector (AE) motor neuron, from standard and reproductive ganglia, hyperpolarized and stopped spontaneous firing (Fig. 5A). The table in Fig. 5B summarizes these results.

#### *Effects of inhibitors of the neostigmine-induced depolarization on the resting electrophysiological properties of Retzius neurons*

We examined the effects of those substances that blocked the neostigmine-induced depolarization on the membrane potential of Retzius neurons at rest. Control experiments showed that superfusion with normal saline for 10 min produced no significant change in the resting membrane potential of Retzius neurons (Fig. 6A). Superfusion with solutions containing  $100\ \mu\text{mol l}^{-1}$  strychnine or  $100\ \mu\text{mol l}^{-1}$  atropine produced a progressive hyperpolarization of the cells, while superfusion with  $100\ \mu\text{mol l}^{-1}$  curare did not (Fig. 6B). The mean membrane potential of Retzius neurons after an 8 min perfusion with strychnine was  $-64\pm 1\ \text{mV}$  ( $N=4$ ). To assess the input resistance of Retzius neurons throughout the perfusion period, we injected small current pulses ( $-0.5\ \text{nA}$ , 5 ms, 0.5 Hz) into the soma and found that, after a 10 min period in control solution, the input resistance was  $150\pm 2\%$  of the value at time zero. The change in input resistance after an equivalent superfusion period with curare, strychnine and



B

| Cell   | Ganglion | N  | Response   |
|--------|----------|----|------------|
| Rz     | X        | 34 | Excitatory |
| Rz     | 5        | 3  | -          |
| Rz     | 6        | 4  | Inhibitory |
| Leydig | X        | 3  | Excitatory |
| Leydig | 5        | 1  | Excitatory |
| Leydig | 6        | 3  | Excitatory |
| AP     | X        | 3  | -          |
| N      | X        | 3  | -          |
| T      | X        | 2  | -          |
| P      | X        | 3  | -          |
| AE     | X        | 4  | Inhibitory |
| AE     | 5        | 1  | Inhibitory |

Fig. 5. Responses of different cells to acetylcholinesterase inhibitors. (A) Representative responses of Rz(6), Leydig and annulus erector (AE) cells to the application of  $100\ \mu\text{mol l}^{-1}$  neostigmine as indicated by the horizontal bar underneath the recordings. The recordings were initiated with neurons at their resting membrane potentials. The thick baseline of the AE motoneuron recording is due to the high-frequency firing of these neurons, which shows little passive reflection of their action potentials in somatic recordings. The inset beside the recording from Rz(6) shows the number of action potentials counted during 10 s in control solution (-) and after 3 min in neostigmine (+) in four different Rz(6) cells (each one represented by a different symbol). (B) The table shows the neurons studied in ganglia of standard (X) and reproductive (5 and 6) segments, the number of cells studied ( $N$ ) and the type of response obtained, classified as excitatory, inhibitory or no response (-). AP, anterior pagoda neuron; N, T, P, mechanosensitive neurons responsive to nociceptive, touch and pressure stimuli.

atropine was indistinguishable from that in control solution ( $150\pm 1\%$  of the value at time zero for curare,  $140\pm 2\%$  for strychnine and  $140\pm 2\%$  for atropine). The method used to evaluate the input resistance can underestimate changes in the membrane resistance in the area of the neuritic arborization since (1) the amplitude of the current pulses reaching that area is smaller than their amplitude in the soma, and (2) the somatic

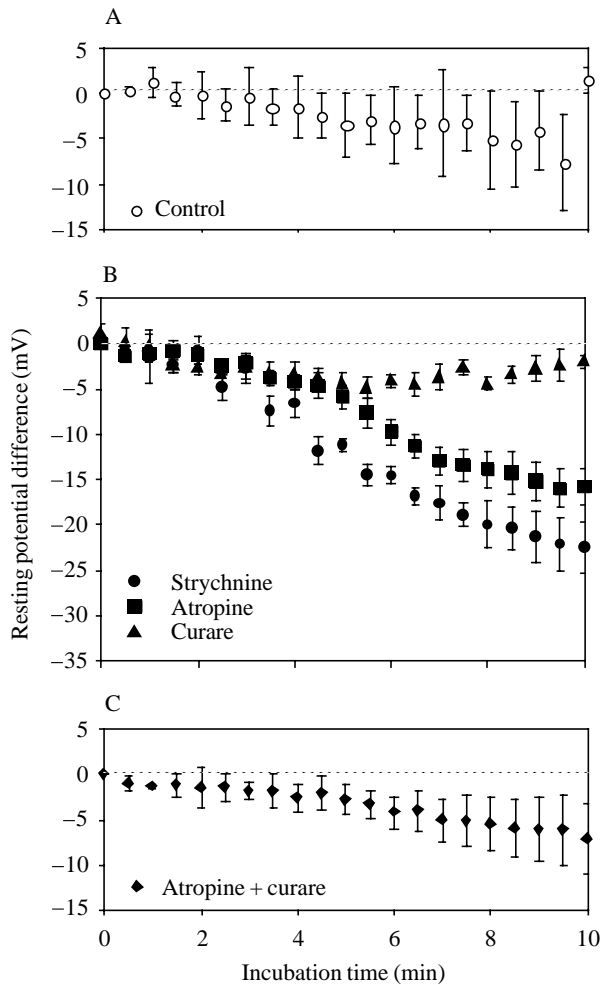


Fig. 6. Effect of inhibitors of the neostigmine-induced depolarization on the electrophysiological properties of Retzius neurons at rest. Differences in the resting potential of Retzius neurons recorded from ganglia that were constantly superfused with (A) normal saline (control,  $N=6$ ), (B) saline containing  $100\ \mu\text{mol l}^{-1}$  strychnine ( $N=6$ ), curare ( $N=4$ ) or atropine ( $N=4$ ) and (C) saline containing both  $100\ \mu\text{mol l}^{-1}$  atropine and  $100\ \mu\text{mol l}^{-1}$  curare ( $N=3$ ). The superfusion was switched to the test solution 30 s after the onset of the recording (time zero). The resting potential recorded at time zero was subtracted from the values recorded at each time. The bars indicate the S.E.M.

recording is a decremented reflection of the changes in membrane potential elicited by the pulses in those distal sites. Thus, the membrane of the soma has a larger contribution than that of the neurites in the input resistance measurement. The method used, therefore, served mainly to ascertain that the drugs applied had no unspecific effects over the neuronal membrane that would have been reflected in the soma as well as in the neurites.

Unlike atropine and strychnine, curare had no effect on the resting potential of Retzius neurons. A possible explanation may reside in the fact that curare also inhibits the ACh-induced hyperpolarization of Retzius neurons, while atropine has no

effect on this phase of the cholinergic response (Szczupak *et al.* 1993). To test this hypothesis, we examined the combined action of atropine and curare. In the presence of curare, atropine did not produce its hyperpolarizing effect (Fig. 6C) and the neurons responded as under control condition (Fig. 6A).

Retzius neurons from reproductive ganglia 6 ( $N=3$ ) incubated in the presence of  $100\ \mu\text{mol l}^{-1}$  strychnine or atropine showed little change in their resting potential. The neurons hyperpolarized by  $-4\pm 3$  mV after a superfusion period of 9 min and this change in membrane potential was similar to that observed in this type of neuron superfused with control saline ( $-4\pm 4$  mV).

## Discussion

### *Basal release of acetylcholine in leech ganglion*

The data presented throughout this paper show that classical inhibitors of AChE activity, such as neostigmine and physostigmine (Taylor, 1996), evoked a strong depolarization of the serotonergic Retzius neurons in the leech, mediated through receptors with nicotinic binding sites. The most plausible explanation of the effects of neostigmine and physostigmine is that, by inhibiting ACh breakdown, these substances generate an increase in extracellular ACh concentration, which is tonically released from endogenous sources and is normally maintained at low levels by the high AChE activity detected in the leech ganglia (Wallace, 1981; Talsala *et al.* 1995).

In agreement with this interpretation, we observed that the amplitude of the neostigmine-induced depolarization was partially dependent on extracellular  $\text{Ca}^{2+}$  levels. The extent of the dependence was as expected for a phenomenon that involves basal neurotransmitter release; namely, the amplitude of the response was decreased but the response was not abolished in a solution containing a high  $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$  ratio, and it was increased by an increase in extracellular  $[\text{Ca}^{2+}]$  (Landau, 1969; Matthews and Wickelgren, 1977).

Since nicotinic receptors bear a specific binding site for AChE inhibitors (Lena and Changeaux, 1993), the effects of these inhibitors could have been due to direct agonistic actions. However, several of our observations preclude this possibility. (i) The neostigmine-induced depolarization of Retzius neurons was inhibited by the desensitizing effect of nicotine, while the direct agonistic action of neostigmine was unaffected by substances that fix the receptor in its desensitized state (Schrattenholz *et al.* 1993; Maelicke *et al.* 1993). (ii) The neostigmine-induced depolarization of Retzius neurons was inhibited by curare, while the direct action of the AChE blockers on ACh receptors was insensitive to it (Lena and Changeaux, 1993). (iii) The latency of the response to both AChE inhibitors was relatively long and was dose-dependent. These observations appear to be incompatible with a direct agonistic effect (Cooper *et al.* 1996; Storch *et al.* 1995).

Taken together, these considerations indicate that the actions of physostigmine and neostigmine were mainly due to their

classic role as AChE blockers. This interpretation also explains the fact that the amplitude of the response reached a plateau at approximately  $10\ \mu\text{mol l}^{-1}$  physostigmine or neostigmine, while its latency was decreased by higher concentrations of the AChE inhibitors (Fig. 1B) since the ACh level that produced the maximal response could be generated at a faster rate.

*Pharmacological profile of the response induced by the acetylcholinesterase inhibitors*

The pharmacological properties of the receptors mediating the neostigmine-induced depolarization, sensitive to nicotine, strychnine and atropine, and insensitive to mecamylamine, fit the pharmacological profile observed for the receptors that mediate the delayed depolarization of Retzius neurons from standard midbody ganglia (Szczupak *et al.* 1998). However, the response evoked by AChE inhibitors was sensitive to curare, while the delayed depolarization was not. A possible explanation for this discrepancy is that the responses were evoked by different agonists, endogenous ACh in one case and carbachol in the other, and it may be that curare is less effective in displacing carbachol than ACh.

It is noteworthy that the prolonged depolarization was mediated by receptors with a nicotinic pharmacological profile. This has not been a classic function ascribed to nicotinic receptors, which have generally been associated with phasic depolarizations (David and Sattelle, 1984; Egan and North, 1986; Trimmer and Weeks, 1989; Lipton *et al.* 1987; McCormick and Prince, 1987; Mulle and Changeaux, 1990).

*Segmental specificity of the responses induced by the acetylcholinesterase inhibitors*

The expression of cholinergic receptors by Retzius neurons is segment-specific (Kristan *et al.* 1993; Szczupak *et al.* 1998), and the pattern of responses of Retzius neurons from standard and reproductive segments to AChE inhibitors was compatible with the segment-specific distribution of cholinergic receptors. The strong excitation of Rz(X) neurons was, as already stated, in agreement with the widespread distribution of the receptors that mediate the delayed depolarization. The expression of these receptors in Rz(5) was very low, and their expression in Rz(6) was even lower (Szczupak *et al.* 1998). However, cholinergic agonists produce a slow hyperpolarizing current in Rz(5) and Rz(6) (Kristan *et al.* 1993). Thus, the lack of response of Rz(5) neurons was compatible with the concomitant activation of moderate hyperpolarizing and depolarizing currents, while the response of Rz(6) neurons agrees with the activation of a moderate hyperpolarizing current and a very weak depolarization, producing a small net hyperpolarizing response. That the different responses were due to a difference in the distribution of cholinergic receptors in the Retzius neurons, rather than to a difference in the pattern of ACh release in ganglia from reproductive and standard segments, was shown by the responses of Leydig and AE neurons, which showed similar responses to neostigmine in both types of ganglia.

*Cellular specificity of the responses*

The responses of different neurons to the AChE inhibitors showed a marked coincidence with the responses obtained when these neurons were stimulated by pressure pulses of carbachol (Szczupak *et al.* 1998). The Leydig neurons are a pair of cells, present in each ganglion, with putative neuromodulatory functions (Lockery and Kristan, 1991), which were, like the Retzius cells, strongly excited by the application of AChE inhibitors. In agreement with this observation, these neurons responded to carbachol applications with a depolarizing response. The AE cells are a pair of motor neurons that are responsible for the erection of the skin of each annulus. These neurons are cholinergic (Sargent, 1977) and they exhibited an inhibitory response to AChE inhibitors, in agreement with their response to carbachol applications. The mechanosensory neurons did not show any response to the application of neostigmine. When subjected to pressure pulses of carbachol applied to their soma, these neurons responded with a transient depolarization, and carbachol pulses at other sites did not evoke any measurable response. The AP neurons are a pair of cholinergic cells of unknown function. These neurons did not exhibit any measurable response to AChE inhibitors. In contrast, they exhibited a hyperpolarizing response to carbachol pulses, when these were applied to the soma, and a relatively small delayed depolarization that could be evoked by carbachol pulses applied at other sites on their neuritic arborization. The inability of the AChE inhibitors to evoke the hyperpolarizing response could be explained on the basis of the location of the receptors that mediate this response: an increase in the concentration of ACh in the neuropil may not affect the somatic region. The inability to generate a slow depolarization could be due to the restricted expression of the corresponding receptors in the membrane of the AP cells, which showed a relatively small response to carbachol pulses.

These results indicate that the AChE inhibitors do not exert a generalized action on the ganglion due to some sort of nonspecific phenomenon. The wide agreement between the responses to AChE inhibition and to carbachol pulses (with the sole exception of the AP neurons) further supports the view that the actions of neostigmine and physostigmine were exerted through an increase in the neuropilar ACh concentration.

*Physiological implications*

The large depolarization induced by exposing leech ganglia to AChE inhibitors is a phenomenon unlikely to occur under physiological conditions. However, the response disclosed the existence of a basal release of ACh in leech ganglia that may build a cholinergic tonus. The hypothesis that the extracellular space in the central nervous system stores low levels of neurotransmitters, which can act tonically on neurons, has been demonstrated for glutamate, which acts through *N*-methyl-D-aspartate (NMDA) receptors to regulate the electrophysiological activity of hippocampal pyramidal neurons (Sah *et al.* 1989).

Retzius neurons from standard segments show three types of response to cholinergic agonists: a fast depolarization, a

slow hyperpolarization and a delayed depolarization. The fast depolarization is mediated by receptors that desensitize very rapidly (Szczupak *et al.* 1993) and are therefore unlikely to respond to basal levels of ACh. The present results indicate that the cholinergic tonus activated the receptors mediating the slow hyperpolarization (Szczupak *et al.* 1993) and the delayed depolarization (Szczupak *et al.* 1998), exerting a concomitant depolarizing and hyperpolarizing action on the Rz(X) neurons. Thus, curare, which inhibits both phases of the ACh response, did not produce any net effect by itself. Selective blockers of the delayed depolarization, atropine and strychnine, which also inhibited the neostigmine-induced depolarization, caused a marked hyperpolarization of Rz(X) neurons, shifting their membrane potential to approximately the reversal potential of the Cl<sup>-</sup>-mediated hyperpolarization (-65 mV) induced by ACh in these neurons (Szczupak *et al.* 1993). This hyperpolarization did not take place in the presence of curare. Taken together, these results suggest that, under control conditions, the counteracting effects of ACh balanced each other out but that, under conditions of high basal levels of ACh, the depolarizing effect predominated. As expected, Retzius neurons from the reproductive segments were not affected by atropine or strychnine, since the expression of the receptors mediating the long-lasting depolarization is very low (Szczupak *et al.* 1998) and they did not therefore depolarize when AChE activity was inhibited.

The release of serotonin from Retzius neurons depends on action potential firing (Bruns and Jahn, 1995). Since these neurons support high constant firing rates that correlate with their membrane potential, the basal ACh tonus could regulate serotonin release in the leech nervous system and, thus, control its neuromodulatory functions.

The authors are grateful to Drs Daniel Calvo, Pierre Drapeau, Diego Golombek, William B. Kristan Jr, Michael Nusbaum and Ruth Rosenstein for their helpful and encouraging discussion of the present manuscript and to Dr Daniel P. Cardinalli for his support and encouragement. This work was supported by grants from Fundación Antorchas to L.S.

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