LONG-LASTING DEPOLARIZATION OF LEECH NEURONS MEDIATED BY RECEPTORS WITH A NICOTINIC BINDING SITE

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Summary

The serotonergic Retzius neurons of the leech midbody ganglia respond in a complex manner to pressure pulses of acetylcholine (ACh) applied onto their soma with a fast depolarization followed by a slower hyperpolarization and an additional delayed long-lasting depolarization. The delayed depolarization is the subject of the present study. The delayed depolarization could be elicited by long (>1 s) ACh pressure pulses or by short pulses (10–100 ms) of carbachol, nicotine and DMPP, but not by muscarinic agonists. It was inhibited by bath application of nicotine (10–100 μmolL⁻¹), strychnine (100 μmolL⁻¹) and atropine (10–100 μmolL⁻¹). Nicotinic antagonists that blocked the fast depolarization and the slow hyperpolarization (100 μmolL⁻¹ mecamylamine and d-tubocurarine) did not affect the delayed depolarization induced by carbachol. Partial replacement of the extracellular Na⁺ by glucamine caused a decrease in the amplitude of the response and a shift of its reversal potential to more negative values. Carbachol pulses applied to Retzius neurons of the ganglia innervating the reproductive segments elicited delayed depolarizations of much smaller amplitude than the ones recorded in Retzius neurons from standard segments. The delayed depolarization could be elicited by the application of short agonist pulses onto different loci over the surface of the ganglion, at a distance from the soma. Isolated cultured Retzius neurons did not exhibit the delayed depolarization although they readily expressed the earlier phases of the complex cholinergic response. Carbachol pulses applied to the soma of other neurons in the leech ganglion produced a variety of specific responses.

The results suggest that the delayed depolarization was produced by the activation of a cationic conductance mediated by receptors with a pharmacological profile similar to that of the α9 nicotinic receptors and was not a byproduct of the early phases of the cholinergic response. The response seemed to be initiated in the extensive neuropilar processes of the Retzius cell, enabling a persistent excitatory signal.

Key words: serotonergic neurone, cholinergic response, delayed depolarization, leech, Hirudo medicinalis, Retzius neurone, nicotinic receptor, α9 nicotinic receptor.

Introduction

The neuronal nicotinic receptors are a highly diverse family of ligand-gated ion channels present in neurons of the central nervous system of vertebrates and invertebrates (Gerschenfeld, 1973; McGehee and Role, 1995; Sargent, 1993). Detailed molecular research on the diversity of nicotinic subunits, carried out in artificial expression systems, has produced extensive information on their pharmacological and physiological properties. However, the functional role(s) of the nicotinic receptors in the central nervous system is poorly understood (Sargent, 1993; McGehee and Role, 1995). Single neurons may co-express more than one type of nicotinic receptor (Ulian and Sargent, 1995), and in situ physiological experiments have shown that nicotinic agonists can elicit complex responses mediated by different nicotinic receptor subtypes (Kehoe, 1972; Moss and Role, 1993; Kristan et al. 1993). Investigation of the responses mediated by nicotinic receptors in well-characterized neurons in situ is likely to provide more direct information on their physiological role.

The Retzius cells in the leech are a pair of well-characterized serotonergic neurons with neuromodulatory functions (Willard, 1981). They display a complex response to acetylcholine (ACh) that is mediated by receptors bearing different nicotinic pharmacological profiles. Application of short ACh pulses (<1 s) to the soma of the Retzius neurons from standard body segments produces a fast depolarization, followed by a slow hyperpolarization driven by a Cl⁻ current (Szczupak et al. 1993). In addition, a more prolonged (>1 s) application of ACh to Retzius neurons produces a third phase in the response, a delayed and long-lasting depolarization (Kristan et al. 1993). The present study seeks to characterize this late phase of the response to ACh and its relationship to the earlier phases, particularly as it exhibits unusual properties related to both the electrophysiological and pharmacological
characteristics of the receptors themselves and to their localization on the neuron.

**Materials and methods**

**Biological preparation**

Leeches, *Hirudo medicinalis* L., weighing 2–5 g, were obtained from a commercial supplier (Leeches USA, Westbury, NY) 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 of the animal and pinned ventral side up to Sylgard resin (Dow Corning) in a superfusion chamber. The sheath covering the ganglion was dissected away, leaving the cell bodies exposed to the superfusate. For the experiments on cultured neurons, Retzius cells were isolated and maintained in culture following procedures described previously (Dietzel et al. 1986). The neurons were plated on Concavalin A and were studied after periods ranging from 2 to 15 days in culture.

**Solutions and materials**

Ganglia were bathed in a saline solution with the following composition (in mmol L⁻¹): NaCl, 87; KCl, 4; CaCl₂, 1; MgCl₂, 20; glucose, 10; Tris maleate, 4.6; Tris base, 5.4; adjusted to pH 7.2. When higher K⁺ concentrations were used, the Na⁺ concentration was lowered to maintain the normal osmolarity. A 50% [Na⁺] solution was obtained by substituting glucamine chloride for 50% of the sodium chloride. A [Mg²⁺]/[Ca²⁺] ratio of 20 was used in all experiments to prevent chemical synaptic transmission (Nicholls and Purves, 1970), thereby eliminating the effects of the cholinergic agonists and antagonists on neurons presynaptic to the Retzius cells. Acetylcholine chloride, carbachol, nicotine (hemisulfate salt), 1,1-dimethyl-piperazinium iodide (DMPP), muscarine, 4-[N-(3-chlorophenyl)carbamoyloxy]-2-butynyltrimethylammonium chloride (McN A-343), atripine, mecamylamine, d-tubocurarine chloride and strychnine were obtained from Sigma (St Louis, MO, USA).

**Electrophysiological recordings**

Microelectrodes were pulled from borosilicate capillary tubing (FHC, Brunswick, ME, USA) and filled with a 3 mol L⁻¹ potassium acetate solution. Electrodes with a resistance of 10–20 MΩ were selected. Retzius neurons were impaled with a single intracellular electrode, and the membrane potential (V_m) was measured in the discontinuous current-clamp configuration using a sample-and-hold amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA, USA) operating at switching rates of 5–9 kHz. The recordings were digitized using a TL-1 DMA interface (Axon Instruments) and acquired using a Clampex (pClamp, Axon Instruments) protocol at frequencies of 33–100 Hz (with the exception of data for Fig. 5 where a sampling frequency of 400 Hz was used). The low sampling frequencies were sufficient to capture the long-lasting delayed depolarization, but did not resolve the action potentials well, so that many recordings show action potentials with less than maximal amplitudes.

**Drug applications**

The characteristics of our perfusion chamber and of the method of application of the agonists have been described previously (Szczupak et al. 1993). In brief, the recordings were obtained while the ganglia or isolated cells were continuously superfused with saline solution. Agonists were dissolved in the extracellular saline solution at a concentration of 1 mmol L⁻¹ (unless otherwise stated) and loaded into calibrated micropipettes. They were applied by pressure pulses (6.9 kPa) using a Picospritzer II (General Valve, Fairfield, NJ, USA). The dilution of the solution ejected through a 1 s pulse as it reached the cell surface was estimated to be 25% of the concentration in the pipette (Szczupak et al. 1993). In order to diminish the contribution of the ACh-induced hyperpolarization to the late phase of the cholinergic response, the somata of the Retzius neurons were set at a membrane potential of approximately −75 mV, close to the reversal potential of the hyperpolarization (Szczupak et al. 1993), by the injection of direct current (−0.5 to −1 nA). In some studies, pressure pulses of different duration were delivered. To evaluate the delay before the ejected solution was washed out by the superfusion system, we recorded the responses to pressure pulses of a solution containing a high K⁺ concentration. Fig. 1A shows the responses to pulses with a duration of 10, 1000 and 3000 ms from a pipette filled with saline solution containing 100 mmol L⁻¹ K⁺. The duration of the K⁺-evoked depolarization was coincident with the length of the pulse. When the pulse was turned off, the membrane potential rapidly returned to its initial value, showing that the perfusion system effectively removed the pressure-applied solutions. Antagonists were applied through the superfusion solution. Manifold valves (General Valve, Fairfield, NJ, USA) were used to change the perfused solution. To analyze the antagonist profile of the responses, we used carbachol pulses with a duration of 500–1000 ms. These pulses produced robust signals but had a relatively short time course to avoid saturation of the receptor sites by the agonist. The agonist pulses were applied before, during (allowing 3–5 min of diffusion) and after superfusion of the cells with the antagonist solution. Each agonist application was preceded by the injection of a −0.2 nA square pulse of 1 s duration to evaluate whether the antagonists had any effect on the input resistance of the neurons. The pressure pulses of agonist solutions were typically applied at 2 min intervals to allow for full recovery of the response. The effectiveness of each antagonist was expressed as the amplitude of the carbachol response in the presence of the antagonist divided by the amplitude recorded in its absence.

Values are presented as means ± S.E.M.

**Results**

**Responses of Retzius neurons to cholinergic pulses of different duration**

The application of pressure pulses of ACh to the soma of
Retzius neurons, set at a membrane potential of −75 mV, produced two types of depolarizing responses, depending on the duration of the pulse (Fig. 1B): a 10 ms pulse produced only a small, fast and transient depolarizing response; a 1000 ms pulse produced an increase in the amplitude of the fast depolarization followed after a delay by a depolarization of small amplitude. A 3000 ms pulse produced no further increase in the amplitude of the fast depolarization but produced an increase in the amplitude and duration of the delayed depolarization. A 1000 ms pulse produced an increase in the amplitude of the fast depolarization followed after a delay by a depolarization of small amplitude. A 3000 ms pulse produced no further increase in the amplitude of the fast depolarization but produced an increase in the amplitude and duration of the delayed depolarization. A 3000 ms pulse produced no further increase in the amplitude of the fast depolarization but produced an increase in the amplitude and duration of the delayed depolarization. A 1000 ms pulse produced a delayed long-lasting depolarization that was larger than that produced by a 3000 ms pulse of ACh.

Pharmacological characterization of the delayed depolarization

Pressure pulses of nicotine and the nicotinic agonist DMPP produced delayed depolarizations similar to those produced by carbachol (Fig. 2A–C). However, after the first application, nicotine caused the Retzius neurons to become unresponsive to further applications of nicotine or any other agonist. Such
an effect was never observed when ACh, carbachol or DMPP was used as an agonist.

To compare the relative potencies of carbachol and DMPP, we used pulses of different duration to generate a dose–response curve. This method (Waldrop and Hildebrand, 1989) was considered suitable since (i) the volume of agonist solution ejected by the pressure pulses is proportional to the duration of the pulse, and (ii) the rise time of the delayed depolarization was longer than the duration of the pulse needed to induce it. The pulses lying on the linear section of the curve (10–500 ms) ended well before the delayed response began, whereas the longest pulse used in these experiments (3000 ms, marked as the interval between the two vertical lines in Fig. 2A–C) ended just as the delayed depolarization was beginning. Fig. 2D shows that the amplitude of the delayed depolarization increased with increasing doses of carbachol and DMPP. Both agonists produced maximal responses of similar amplitude, but the durations of the pressure pulses of DMPP and carbachol needed to produce half the maximal response were significantly different ($P<0.05$, t-test).

Pressure pulses of muscarine (1 mmol l$^{-1}$ solution in the pipette) produced weak hyperpolarizations of the Retzius neurons ($N=18$), whereas the muscarinic agonist McN A-343 produced pronounced hyperpolarizations ($N=7$) caused by a strong outward current measurable in the soma of the neurons (Szczupak et al. 1993).

These results indicate that the delayed depolarization was mediated by receptors with a nicotinic binding site. To substantiate this pharmacological characterization, we analyzed the effect of several nicotinic antagonists on the carbachol-induced delayed depolarization. Nicotine, the agonist that induced a persistent desensitization when bath-applied, inhibited the carbachol-induced depolarization in a dose-dependent manner (Fig. 3A,D). The effect of nicotine was reversed after washing the ganglia with control solution for 8 min. Mecamylamine and $d$-tubocurarine, two nicotinic antagonists that were effective blockers of the fast transient depolarization (Szczupak et al. 1993), did not inhibit the delayed depolarization significantly. After 5 min of incubation in the presence of 100 $\mu$mol l$^{-1}$ $d$-tubocurarine ($N=10$), the amplitude of the delayed depolarization was 85±11 % of the control value; after 5 min of incubation in the presence of 100 $\mu$mol l$^{-1}$ mecamylamine ($N=3$), the depolarization was 126±3 % of the control value. Atropine, a muscarinic antagonist (Brown and Taylor, 1996), caused a decrease in the amplitude of the response that was also dose-dependent (Fig. 3B,D). This inhibitory effect was reversed by a 10 min wash with control solution. The sensitivity of the delayed depolarization to nicotine and atropine hinted at a similarity between the receptor mediating this response and the $\alpha_9$ nicotinic receptor. As the $\alpha_9$ receptor is inhibited by strychnine, a typical glycine receptor-blocker (Elgoyhen et al. 1994), we tested its effect on the delayed depolarization and
found that, at 100 μmol L⁻¹, strychnine caused a decrease in the amplitude of the response (Fig. 3C,D). A prolonged wash-out (more than 20 min) was required to reverse this effect. Superfusion with the solutions containing the antagonists did not cause, by themselves, any significant change in the input resistance of the cells (data not shown), indicating that the effectiveness of some of the inhibitors was not due to cell damage.

*Changes in the ionic conductance during the delayed depolarization*

To estimate the reversal potential of the delayed depolarization, carbachol pulses were applied as the Retzius neurons were preset at different membrane potentials. The amplitude of the delayed depolarization decreased as the membrane was depolarized (Fig. 4). The extrapolation of the amplitude versus $V_m$ curves suggests a reversal potential close to 0 mV ($-1\pm5$ mV, $N=12$). Replacement of 50% of the extracellular Na⁺ ([Na⁺]₀) by glucamine abolished the firing of action potentials by Retzius neurons, allowing us to preset the neurons at more depolarized values than under control conditions. The reversal potential of the delayed depolarization in the 50% [Na⁺] solution was approximately $-15$ mV ($-15\pm5$ mV, $N=4$). This represents a shift in the extrapolated reversal potential close to the $-17$ mV predicted by the Nernst equation for a 50% change in [Na⁺]₀. The amplitude versus $V_m$ curves were nonlinear: a marked rectification was observed as the neurons were hyperpolarized beyond $-60$ mV and this rectification was more pronounced in the presence of low [Na⁺]₀.

It is noteworthy that in a different series of experiments in which we replaced [Na⁺]₀ by choline, rather than by glucamine, this cation completely abolished the delayed depolarization at a concentration of 10 mmol L⁻¹ ($N=3$) and diminished its amplitude to 50% ($50\pm10\%$, $N=3$) at 1 mmol L⁻¹. Since choline is a synthetic precursor of ACh and
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has been shown to bind to the *Torpedo californica* nicotinic receptor with low affinity (Damle and Karlin, 1980), its effect on Retzius neurons may be that of a weak competitive antagonist.

**Firing threshold of the Retzius neurons during the delayed depolarization**

During the prolonged depolarization in response to pressure pulses of carbachol, Retzius neurons began to fire action potentials (Fig. 5Ai) at a membrane potential of approximately −60 mV (−61±2 mV, N=17). When compared with the firing frequency of Retzius neurons depolarized by injection of direct currents into their soma, we observed that this potential was subthreshold for ganglia bathed in a solution with a high divalent cation content (Fig. 5Aii). Moreover, the neurons attained a firing frequency during the delayed depolarization that was only observed when the somata were electrically driven to a potential approximately 35 mV more depolarized (Fig. 5B).

During delayed depolarizations, action potentials of two different sizes were often recorded in the somata of the Retzius neurons (Fig. 5A; see also Figs 1, 2). Large action potentials with a pronounced after-hyperpolarization phase were seen together with smaller action potentials with distinctly smaller after-hyperpolarizations. These differences were not due to the low data acquisition rate, because they were apparent both at faster sampling rates and on the oscilloscope during the experiments.

**Segment specificity**

We studied the responses of Retzius (Rz) neurons from the reproductive segments 5 and 6, Rz(5) and Rz(6) respectively, to pressure pulses of carbachol applied to their soma as the neurons were set at their resting potential and compared them with the responses of Retzius neurons from standard segments, Rz(X), studied under similar conditions. After an initial hyperpolarizing phase, Rz(5) and R(6) developed a delayed depolarization phase whose amplitude was much smaller than that of Rz(X) (Fig. 6); the responses of Rz(6) were significantly smaller than those of Rz(5).

**Responses of Retzius neurons to carbachol applications to different sites on the ganglion**

The disparity between the firing thresholds during carbachol application and during current injection could be explained by assuming a difference in the site of initiation of the depolarizing responses. Whereas the current pulses were injected into the soma and spread from there to the spike-initiation zone, the carbachol-induced depolarization could have been initiated at more distant sites, closer to the spike initiation zone in the neuropilar processes. To test whether the delayed depolarization could be induced at the neurites, carbachol pulses were applied at sites distant from the soma but close to the processes of the neuron.

We recorded the responses of Retzius neurons while applying 100 ms pressure pulses of carbachol in distant anterior, posterior, right and left sites to the surface of the...
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The Retzius neurons responded with depolarizing responses of similar amplitude, wherever the carbachol was applied (Fig. 7, left-hand column). To test whether the carbachol acted at the site of application and did not reach the soma, we applied equivalent pulses of 100 mmol l\(^{-1}\) K\(^+\) to the distant sites. Such K\(^+\) pulses produced small and transient depolarizations when applied to the soma (Fig. 1A), but had no effect on the somatic recordings when applied at the distant sites, probably because the small electrophysiological responses in the processes decayed electrotonically and were not detectable in the soma. This result supports the idea that the short carbachol pulses applied to distant sites acted mainly at restricted local regions of the neuropil and that the responses in the somata were electrotonically decayed versions of the neuropilar responses.

Responses of Retzius neurons in culture

Retzius neurons (N=9) were isolated and cultured for 3–11 days, a period during which they developed a neuritic tree 0.5–2 cell diameters in length. Pulses of carbachol applied to the soma of these neurons, while they were held at \(-70\) mV, produced only a fast depolarization that was not followed by any other phase. None of these neurons responded with the delayed depolarization regardless of the extent of the arborization developed in culture (data not shown).

Fig. 6. Segment-specificity of the delayed depolarization. Mean amplitudes of the delayed depolarizations recorded in Retzius neurons from standard and reproductive ganglia stimulated by pressure pulses (1 s) of carbachol (0.5 mmol l\(^{-1}\) in the pipette) as the neurons were initially set near their resting potential of approximately \(-40\) mV. The bars indicate the S.E.M. The mean amplitudes recorded in Rz(5) and in Rz(6) were both significantly different from that in Rz(X) (P<0.05); the asterisk indicates that the mean amplitude recorded in Rz(6) was significantly different from that in Rz(5) (P<0.05).

Fig. 5. Comparison of the firing frequency of Retzius neurons depolarized during the delayed depolarization and by current injection. (A) Recording of a Retzius neuron (i) stimulated with a 1000 ms pressure pulse of 1 mmol l\(^{-1}\) carbachol at the time indicated by the arrow and (ii) driven to different membrane potentials, in random order, by passing a direct current. Data acquisition of these recordings was performed at 400 Hz. The axis on the left indicates the membrane potential of the neurons. (B) Relationship between the firing frequency and the membrane potential (V\(_m\)) from experiments such as that described in Aii. Each point represents the mean frequency and the bars indicate the s.e.m. (N=9). The open circle shows the mean frequency displayed by carbachol-stimulated Retzius neurons (N=17) at the somatic membrane potential at which the cell started firing. This frequency was measured during the 5 s following the first action potential (as indicated by the thick bar below the recording in Ai). Within this period, the soma of the Retzius neurons depolarized further, on average by 5.9±0.9 mV.
different sites of the ganglion. Application of carbachol to the soma of an anterior pagoda (AP) neuron, held at its resting potential, produced a hyperpolarization followed by a delayed depolarization (Fig. 7, middle column). The application of similar pulses of carbachol at other sites of the ganglion evoked only the delayed depolarization and not the phasic hyperpolarization. The annulus erector (AE) motor neurons hyperpolarized in response to the application of carbachol to their soma or at the lateral site, contralateral to the location of the recorded neuron, but they did not respond to carbachol application at the anterior and the lateral-ipsilateral site of the ganglion (Fig. 7, right-hand column). Qualitatively similar recordings were obtained in four additional Retzius neurons, six AP neurons and three AE neurons. Each trace had a total duration of 600 ms.

**Discussion**

*The cholinergic receptors of Retzius neurons*

The present results extend a description of the responses of Retzius neurons to pressure pulses of cholinergic agonists applied to their soma and lead to the conclusion that these neurons bear at least three types of cholinergic responses: a fast depolarization, a slow hyperpolarization and a delayed depolarization. These responses, elicited by nicotinic agonists in conditions that precluded neurotransmitter release by other neurons, were mediated by receptors with different physiological and pharmacological characteristics (Fig. 8).

The pharmacological properties of the delayed depolarization, presented in this study, show that this response was not a byproduct of any of the two earlier responses: it was not affected by mecamylamine, an effective blocker of the fast depolarization, and it was not induced by McN A-343, a selective agonist of the slow hyperpolarization (Szczupak et al. 1993). The delayed depolarization was mediated, in part, by an increase in the Na⁺ permeability of the neurons, elicited by the nicotinic agonists carbachol, DMPP and nicotine. DMPP was more potent than carbachol, whereas nicotine desensitized the response after a first application, blocking the responsiveness to any of the other agonists. In addition, the response was also...
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inhibited by bath applications of atropine and strychnine. This pharmacological profile is similar to that of ACh-induced currents measured in oocytes expressing the nicotinic α9 subunits (Elgoyhen et al. 1994), except that the latter were sensitive to curare. Curare did not affect the delayed depolarization induced by carbachol, but it did inhibit the prolonged depolarization induced by endogenous ACh (Marín Burgin and Szczupak, 1998), which exhibited a pharmacological profile similar to that of the delayed depolarization. We therefore suggest that the lack of effect of curare in the present study was due to a lower effectiveness in displacing carbachol than ACh.

Activation of nicotinic receptors in neurons of invertebrate and vertebrate organisms has been associated with fast depolarizations (David and Sattelle, 1984; Trimmer and Weeks, 1989; Egan and North, 1986; Lipton et al. 1987; McCormick and Prince, 1987a; Mulle and Changeaux, 1990), while slow depolarizations have been associated with muscarinic receptors (Curro Dossi and Steriade, 1991; Trimmer and Weeks, 1993; Trimmer, 1994; Trimmer and Weeks, 1989; McCormick and Prince, 1987b). However, in a few cases where activations of slowly developing currents were elicited by nicotinic agonists, these currents were shown to result from the activation of an earlier phasic current (Evans, 1996; McCormick and Prince, 1987a). Thus, the long-lasting response described here, which has a nicotinic pharmacological profile and is not secondary to the earlier transient phases, represents an unusual observation. The delayed depolarization was elicited after a relatively long latency, it had a slow rise time and, when elicited by acetylcholinesterase (AChE)-insensitive agonists, the response continued far beyond the end of the agonist pulse. The time course of the responses to pulses of high [K+] (Fig. 1A) showed that the pressure-ejected solution reached the soma membrane with no measurable latency and was efficiently washed out, indicating that the prolonged rise time and duration of the response were not likely to be due to the diffusion time of the agonist towards or away from receptors located on the soma membrane. Two possible explanations can account for the time course of the delayed depolarization: (i) the response was mediated by metabotropic receptors involving a cascade of second messenger mechanisms that determine its slow time course; (ii) the response was mediated by nicotinic receptors located on the neurites, away from the soma, that have a high affinity for the agonist and a slow desensitization rate (see Edmond et al. 1995). A more definitive classification of this receptor requires a more thorough examination of its molecular properties. However, two considerations lead us to favor the second explanation. First, no metabotropic cholinergic receptor so far characterized

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<th>Fast depolarization</th>
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<td>Neurites</td>
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Fig. 8. Summary of the pharmacological and physiological properties of the three cholinergic responses of standard Retzius neurons. The three thick horizontal lines at the top schematically represent the three types of voltage changes produced by pressure pulses of cholinergic agonists measured in standard Retzius neurons. The thin dotted vertical lines mark the peaks of the three kinds of responses: the initial fast depolarization, the slow hyperpolarization and the prolonged delayed depolarization. The first two responses have been described previously (Szczupak et al. 1993; Kristan et al. 1993); the third response is the subject of the present paper. The table compares the responses in terms of the agonists that elicit them and the antagonists that inhibit them, the apparent locations of the receptor sites and the times to peak from the onset of the pressure pulses. Blank spaces indicate an absence of data. ACh, acetylcholine.
presents the pharmacological profile described for the delayed depolarization, while the profile matches that of ionotropic nicotinic receptors. Second, the electrophysiological properties of nicotinic receptors have been characterized largely in artificial expression systems, and it has been suggested that native neurotransmitter receptors may have larger conductances and reside in the open state longer than oocyte-expressed varieties (McGehee and Role, 1995). In addition, several lines of evidence suggest that the receptors responsible for the delayed depolarization are located in the neuropil, as discussed below.

Location of the receptors that mediate the delayed depolarization

The receptors that mediate the delayed depolarization appear to be distributed throughout the neuropil, probably along the neurites of the Retzius neurons, but are absent from their soma. The main support for this hypothesis comes from two observations. (1) The response could be elicited by short agonist pulses applied, distant from the soma, to areas reached by the extended arborization of the Retzius neurons. (2) The response could not be elicited in isolated cultured somata devoid of their extended arborization, in spite of the fact that these isolated neurons readily express the fast depolarization and the slow hyperpolarization (Szczupak et al. 1993).

In addition, this conclusion gives a coherent explanation for other observations. (3) During the rising phase of the carbachol-induced depolarization, the Retzius neurons fired action potentials in a range of $V_m$ that is usually subthreshold for the delayed depolarization rules out the possibility that it was mediated by an increase in extracellular [K+] (Schlué and Walz, 1984). However, the large amplitude of the delayed depolarization rules out the possibility that it was mediated by an increase in extracellular [K+], because the concentration needed to produce the large-amplitude response would be close to 100 mmol l$^{-1}$.

The responses in other neurons

To test the cellular specificity of the delayed depolarization, we studied the responses of other neurons in the ganglion to carbachol pulses, confirming and extending the observations made in other studies (Sargent et al. 1977; Pellegrino and Simonneau, 1984). The variety of responses that has been observed shows that the delayed long-lasting depolarization, although not peculiar to the Retzius neurons, was not a generalized response to carbachol application. However, when present in cells such as Retzius, Leydig and AP neurons, it could be elicited by applications made at any site on the surface of the cells. In contrast, responses such as the fast depolarization of the Retzius, P and N neurons and the hyperpolarization of the AP neurons were only observable when the carbachol pulses were applied to their respective soma, but not when the applications were made at a distance from them. The spatial pattern of response of the AE neuron was coincident with the branching pattern of this neuron (Stuart, 1970), which sends neurites to the periphery through the contralateral roots, but not through the ipsilateral roots. In our view, the differential response displayed by the neurons, depending on the site of application of the agonist pulse, further confirms the local effect of the pressure pulses, suggesting that this experimental approach can be used to construct a map of the neurotransmitter-mediated responses of neurons in the leech ganglia.

While the fast depolarization is confined to Retzius neurons from standard segments, but is absent in Retzius neurons from the contralateral root.
the reproductive segments, the segmental specificity of the delayed depolarization is of a quantitative nature, being poorly expressed in Rz(5) and Rz(6) neurons. In a previously reported investigation (Kristan et al. 1993), we failed to observe the delayed depolarization in the Retzius neurons from the reproductive segments when they were stimulated with prolonged pressure pulses of ACh. This was probably due to the relative inefficiency of ACh as an agonist of this response and to the low responsiveness of Rz(5) and Rz(6) neurons.

Biological relevance of the delayed depolarization

The data presented suggest that the long-lasting delayed depolarization was due to the activation of nicotinic receptors widely distributed along the neurites of the Retzius neuron. Neuronal nicotinic receptors were shown to be concentrated at synaptic sites but, unlike those in muscle fibers, extrasynaptic patches of nicotinic receptors were also identified (Sargent and Pang, 1989; Wilson Horch and Sargent, 1995). A similar situation has been described for GABA_A receptors (Somogyi et al. 1989), leading to the interpretation that extrasynaptic receptors may be activated when the GABAergic neurons are excited above the level at which the mechanisms of GABA removal could not prevent the ‘spill-over’ of the neurotransmitter to extrasynaptic areas. Our results do not allow us to ascertain whether the cholinergic receptors mediating the delayed depolarization are synaptic or extrasynaptic. The broad spatial responsiveness of Retzius neurons to exogenously applied ACh agonists could indicate either that cholinergic synapses are widely distributed over the surface of the Retzius neurons or that the cholinergic receptors are not confined to synaptic sites and that endogenously released ACh acts in a tonic manner. In the leech ganglion, an effective AChE barrier limits the access of ACh to the ganglion neuropil (Wallace, 1981; Talesa et al. 1995). In the accompanying paper (Marin Burgin and Szczupak, 1998), we present evidence that inhibitors of AChE activity in the ganglion produce a significant excitation of the Retzius neurons, indicating the existence of a basal ACh tonus, which appears to be effective under control conditions. The receptors mediating the excitatory effect of the basal ACh release showed physiological and pharmacological properties similar to those of the delayed depolarization. These results indicate the possibility that basal release of endogenous ACh activates the receptors described in the present study. The physiological characteristics and spatial distribution of these receptors confer on them the properties necessary to support a strong excitatory input onto the Retzius neurons. Because these neurons show very little accommodation, this sustained depolarization can support a persistent firing pattern which, in turn, could generate significant serotonin release. Since Retzius neurons exert modulatory actions in the nervous system of the leech through the release of serotonin (Willard, 1981), an excitatory signal with properties such as those of the delayed depolarization could convey an important drive to serotonin neuromodulatory phenomena. Further work is required to establish the cellular origin of the cholinergic signals and the type of physiological information they convey.

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