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SEGMENT-SPECIFIC MODULATION OF THE ELECTROPHYSIOLOGICAL ACTIVITY OF LEECH RETZIUS NEURONS BY ACETYLCHOLINE

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Summary

1. The acetylcholine responses of Retzius neurons were electrophysiologically and pharmacologically characterized *in situ* and in culture. Single-electrode voltage-clamp was used to record currents from leech Retzius neurons from standard segments [Rz(X)] and from reproductive segments [Rz(5,6)].

2. A 1s pressure pulse of acetylcholine (ACh) produced a fast inward current followed by a slower outward current in Rz(X) neurons, whereas it produced only an outward current in Rz(5,6) neurons. These segment-specific responses were maintained when the two types of Retzius neurons were isolated in culture for up to 12 days.

3. The inward current of Rz(X) reversed at around −25mV and was partially carried by Na^+. This cationic current desensitized rapidly. The outward current of Rz(X) and Rz(5,6) neurons reversed at around −65mV and was carried by Cl^-. This anionic current desensitized very slowly upon prolonged applications of ACh.

4. The expression of the ACh-induced outward current in Rz(X) was season-dependent and was recorded in a larger proportion of Rz(X) neurons during the summer than during the winter. The expression of the ACh-induced outward current in Rz(5,6) did not show any seasonal pattern.

5. The fast inward current of Rz(X) was also elicited by nicotine; it was blocked by *d*-tubocurarine, hexamethonium and mecamylamine, but was not affected by α-bungarotoxin. The outward current of Rz(X) and Rz(5,6) was also elicited by nicotine and by 4-[*N*-(3-chlorophenyl)carbamoxyloxy]2-butynyltrimethylammonium chloride (a muscarinic agonist); it was blocked by *d*-tubocurarine and by α-bungarotoxin, but it was not affected by hexamethonium or mecamylamine.

6. The results show that the serotonergic Retzius neurons of the leech could be tonically inhibited by ACh. In addition, the Retzius neurons from standard segments could also be phasically excited by ACh. The receptors responsible for the excitation fit into the classification of neuronal nicotinic receptors, whereas the receptors mediating the inhibition are closer in type to the muscular nicotinic receptor.

Introduction

Leech Retzius neurons are a pair of serotonergic cells easily identifiable in each

Key words: leech Retzius neurons, cholinergic response, nicotinic receptor, cell culture, acetylcholine.

segmental ganglion. Retzius neurons in each midbody segmental ganglion display similar electrophysiological, morphological and connectivity patterns. However, Retzius neurons of the ganglia in the reproductive segments 5 and 6 [referred to as Rz(5) and Rz(6)] show a series of properties different from those of Retzius neurons in standard midbody ganglia [referred to as Rz(X)] (see review by French and Kristan, 1992*a*). Among these differential properties is the fact that the neurotransmitter acetylcholine (ACh) evokes changes in membrane potential of opposite sign in the two groups of Retzius cells: it depolarizes Rz(X) but it hyperpolarizes Rz(5,6) (Kristan *et al.* 1993).

Retzius neurons in *Hirudo medicinalis* contain more than half the total ganglionic serotonin (Glover, 1984; McAdoo and Coggeshall, 1976). Given the involvement of serotonin in the regulation of activity levels in the leech (Williard, 1981; Lent *et al.* 1991), the modulation of the physiological activity of Retzius neurons is of obvious importance to the study of the regulation of behavior in this animal. Moreover, the different responses of Retzius neurons in different segments to the same neurotransmitter represent an interesting developmental phenomenon. Since all Retzius neurons develop in a highly stereotyped manner from a particular embryonic stem cell, the N teloblast (Stuart *et al.* 1983), the differential expression of membrane receptors in Rz(5,6) and in Rz(X) must be regulated during development by their different segmental environments (see review by French and Kristan, 1992*b*). This report presents a characterization of the modulation of electrophysiological activity of Retzius neurons in standard and reproductive segments by exogenously applied cholinergic agents. It shows that both types of Retzius cell display an anionic current that can be activated by nicotinic and muscarinic agents and can sustain a tonic hyperpolarization of the Retzius neurons. In addition, Rz(X) neurons display a fast cationic inward current with a physiological and pharmacological profile similar to that of vertebrate neuronal nicotinic receptors. This current is responsible for a phasic depolarization of the Rz(X) neurons at rest and is absent in the Retzius neurons from reproductive segments.

Materials and methods

Biological preparation

Hirudo medicinalis Linnaeus, weighing 2–5g, were obtained from a commercial supplier 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 in a superfusion chamber lined with Sylgard (Dow Corning). The sheath covering the ganglion was dissected away, leaving the cell bodies exposed to the superfusion solution. The studies were performed on Retzius cells from ganglia 5 and 6 [Rz(5,6)] and on Retzius cells from ganglia 8, 9 and 10 [Rz(X)]. Rz(5,6) could be easily recognized by position and size even though these neurons have smaller somata than Rz(X) (Jellies *et al.* 1987) and ganglia 5 and 6 contain a larger number of cells than standard ganglia (Macagno, 1980).

To isolate Retzius cells in culture we followed the procedures described by Dietzel *et al.* (1986). The neurons were used for electrophysiological recordings after 2–15 days in culture.

Solutions

The ganglia were bathed in a saline solution with the following composition (in $mmol\,l^{-1}$): NaCl, 87.2; KCl, 4; $CaCl_2$, 1; $MgCl_2$, 20; glucose, 10; Tris maleate, 4.6; Tris base, 5.4; adjusted to pH7.2. When higher K^+ concentrations were used, the Na^+ concentration was lowered to maintain the same osmolarity as in normal saline. The Cl^--free solution was obtained by iso-osmolar substitution of chloride salts with sulfate salts. The Na^+-free solution was obtained by substituting sodium salts with Tris salts. A Mg^{2+}/Ca^{2+} ratio of 20 was used in all the experiments in order to isolate the responses of Retzius neurons from inputs through chemical synapses that could be affected by the cholinergic agonists and antagonists (Nicholls and Purves, 1970).

The following drugs were used: acetylcholine chloride, nicotine (hemisulfate salt), α-bungarotoxin, atropine, pirenzepine, hexamethonium chloride, mecamylamine, *d*-tubocurarine chloride, 4-[*N*-(3-chlorophenyl)carbamoxyloxy]2-butynyltrimethylammonium chloride (McN A 343) (purchased from Sigma).

Electrophysiological recordings

Microelectrodes were pulled from borosilicate capillary tubing (FHC, Brunswick, ME) and filled with a $3mol\,l^{-1}$ potassium acetate solution. Electrodes with a resistance of 8–20 MΩ were selected. The tips of the micropipettes were coated with Sylgard to decrease the electrical capacitance of the electrodes.

Retzius neurons were impaled with a single intracellular electrode and voltage-clamped using a sample-and-hold amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA) operating at switching rates of 5–9kHz. When the ionic composition of the extracellular solution was altered during the course of a recording (as in Figs 1 and 6A) a bath reference electrode was used (similar to the intracellular electrode), so that voltage measurements were relative to the bath rather than relative to ground (an Axoclamp 2A device), to overcome possible effects of the different solutions on the junction potential of the bath ground. The current and voltage recordings were digitized using a TL-1 DMA interface (Axon Instruments) and acquired using a Clampex (pClamp, Axon Instruments) protocol at a frequency of 1kHz.

The resting potential was measured at stable recording values. The input resistance of the cells was calculated from the slope of a current–voltage curve, obtained by measuring, at steady state, the current necessary to hold the membrane potential at different levels between −20 and −90mV. The current–voltage relationship was linear for both types of Retzius neurons in this voltage range.

Drug applications

The recordings were performed while the ganglia or isolated cells were under continuous superfusion with saline solution (see *Solutions*). Agonists were applied by pressure pulses. The agonist solution was loaded into a micropipette whose tip dimensions were standardized by measuring their ‘bubble number’ (Corey and Stevens, 1983); pipettes with a bubble number of 9.0 were used. The pressure pulse was applied

by a Picospritzer II (General Valve) using a pressure value of 1 atmosphere (101.3kPa). The micropipettes were positioned 5–10 μm from the cell body of the Retzius neurons and the applications were performed under visual control. Pressure pulses of saline solution applied under similar conditions failed to produce any effect on the holding membrane current. When mechanical artifacts (movement of the cell body) occurred as a result of the pressure pulse, there were some small deflections of the baseline current, but these were of much smaller amplitude than the smallest ACh response observed. In any case, recordings were made only when no mechanical artifacts were seen. When ACh was presented in two different solutions, a micromanipulator with a holder for two micropipettes was used, so that the tips of the two pipettes were placed as close together as possible (usually 1 μm apart). Antagonists were applied through the superfusion solution. The change in saline solution was achieved using manifold valves (General Valve). The effects of the different antagonists tested were measured by applying the agonist before, during and after superfusing the cells with the saline solution containing the antagonist. The degree of inhibition reflected in the amplitude of the current in the presence of the antagonist is expressed as a percentage of the amplitude of the current under control conditions before application of the antagonist. Average values are given ± standard error of the mean (S.E.M.). The reversibility of the drug-induced blockade was checked by subsequently superfusing the ganglia with normal saline.

To quantify the time required in our superfusion chambers to exchange one solution for another, we measured the time required to achieve the maximal change in membrane potential when a 40mmol l^{-1} K^+ saline solution was replaced by a 4mmol l^{-1} K^+ solution (Fig. 1A). It took approximately 45s to obtain the maximal change in membrane potential, with half the maximal change occurring by around 15s.

The application of drugs by pressure ejection has the advantage of the localized application of a small quantity of neurotransmitter with a very sharp onset time. This is particularly important when studying rapidly desensitizing responses. It is difficult, however, to establish the actual concentration reaching the cell membrane. In order to quantify roughly the dilution rate of the volume applied by the pressure pulse, we measured the change in membrane potential in response to a pressure pulse of a 4 mmol l^{-1} K^+ solution onto a Retzius cell under constant superfusion with a 40mmol l^{-1} K^+ solution. This value was superimposed on a curve relating maximal change in membrane potential to extracellular K^+ concentration, which was obtained by complete exchange of the bath solution (Fig. 1B). The curve shows that pressure pulses of a 4 mmol l^{-1} K^+ solution with a duration of 1 and 5s produced the effect of a bath-applied solution of 31 and 29.5mmol l^{-1} K^+, respectively. The change in membrane potential was, then, the equivalent of a change in K^+ concentration of 9 and 10.5mmol l^{-1} K^+ rather than 36mmol l^{-1}. This represents dilutions to 25% and 29%, respectively, of the concentration of the solution delivered by the pipette as it reaches the cell membrane. We measured the hyperpolarization produced by a low K^+ concentration while the ganglion was in a high-K^+ solution, rather than *vice versa*, to avoid the contribution of the voltage-dependent Na^+ channels, whose effect would be more prominent in response to short pressure pulses than to long-lasting bath applications.

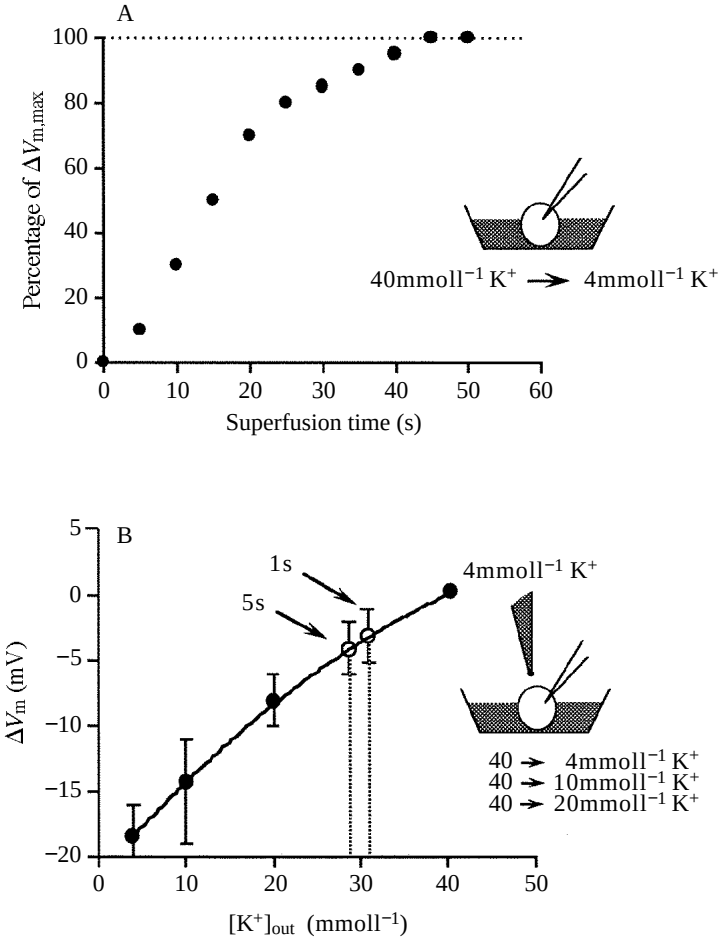


Fig. 1. Responses of Retzius neurons to changes in external K^+ concentration. (A) Time course of the change in membrane potential resulting from a change in the K^+ concentration of the extracellular solution from 40mmoll^{-1} to 4mmoll^{-1} (see inset) through the superfusion system. The maximal change in membrane potential ($\Delta V_{m,max}$) was measured at the steady state. The ordinate indicates the percentage maximal change (maximal is 100%) in membrane potential and the abscissa indicates the time elapsed since the superfusion with 4mmoll^{-1} K^+ solution was initiated. (B) Estimation of the ratio of dilution of solutions applied by pressure pulses. The filled circles indicate the steady-state change in membrane potential (ΔV_m) produced by changing the K^+ concentration of the extracellular solution ($[K^+]_{out}$) from 40 to 4, 10 or 20mmoll^{-1} (see inset). The abscissa indicates the value of the newly exchanged K^+ concentration. The open circles represent the maximal change in membrane potential produced by a pressure pulse of a saline solution containing 4mmoll^{-1} K^+ for 1 and 5s, while the ganglion was bathed in a 40mmoll^{-1} K^+ solution. The results are expressed as the average change in membrane potential in three different experiments ± standard error of the mean (S.E.M.).

Results

Passive properties of Retzius neurons

The passive properties of Retzius neurons were monitored by measuring the resting potential and the input resistance of these cells. These variables were used to test the stability of the tissue in the course of the experiments. *In situ* Rz(5,6) neurons had an input resistance some 77% higher than that of Rz(X) neurons and a membrane potential approximately 8mV more depolarized (Table 1). The input resistance of a neuron is inversely proportional to its membrane surface and, therefore, the lower input resistance of Rz(X) neurons is probably due to their larger somata and more extensive and dense neuropilar arborization (Jellies *et al.* 1987). The lower resting potential of Rz(5,6) neurons may be due to inherent differences in their resting conductances, but could also result from their higher input resistance, since this would amplify the effect of the damage produced by electrode impalement. The current–voltage relationship in both types of neurons was linear in the range −20 to −90mV (data not shown).

Rz(X) and Rz(6) neurons cultured on concanavalin A produced a limited neuropilar arborization, and no significant difference was found in the extent of their neurite growth observed using phase contrast optics. About 50% of the cells studied did not extend any processes, some 40% of the cells produced processes extending to an equivalent of 1 cell diameter (30–80 μm) and the remaining cells produced processes that extended to an equivalent of 2–3 cell diameters. Retzius neurons developed most of their outgrowth after 2 days in culture. Both types of Retzius neurons had similar input resistances and resting potentials in culture (Table 1). This suggests that the difference in input resistance observed *in situ* between the two types of Retzius cells was due to the difference in surface area of their neuropilar arborization and not to differences in their passive electrophysiological properties. Accordingly, the similarity between the resting potentials of both types of neurons in culture supports the view that the differences observed *in situ* may reflect the difference in their input resistance.

Membrane currents elicited by acetylcholine in Rz(X)

The study of the ionic currents induced by ACh in Retzius neurons was performed by applying pressure pulses of the neurotransmitter onto the soma of voltage-clamped neurons, both *in situ* and in culture. As shown in Materials and methods, the application

Table 1. *Passive properties of Retzius cells* in situ *and in culture*

	Rz(X)			Rz(5,6)		
	RP (mV)	R_m (MΩ)	*N*	RP (mV)	R_m (MΩ)	*N*
In situ	−42±1	13±1	21	−34±1	23±1	51
In culture	−32±1	34±3	25	−29±1	35±4	24

Resting potential (RP) and input resistance (R_m) of Retzius cells was studied in individual ganglia *in situ* or in culture. The results are expressed as mean ± S.E.M. and *N* indicates the number of cells studied.

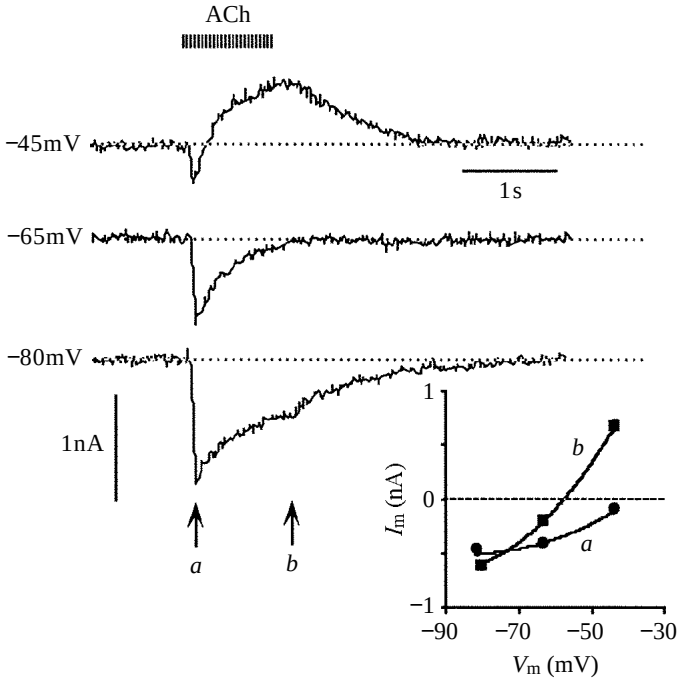


Fig. 2. Response of Rz(X) to acetylcholine (ACh). Membrane currents elicited by the application of a pressure pulse of 1mmol l^{-1} ACh for 1s on an Rz(X) cell clamped at three different membrane potentials (noted on the left of each trace, expressed in mV). The bar above the traces represents the period during which the ACh pulse was applied. The pulses were applied at intervals of 30s. The current amplitude (I_m) was measured at the times indicated by the arrows *a* and *b* in order to plot the current–voltage relationship of these two current components, as shown in the inset.

of a 1s pulse of solution from the pipette was equivalent to exposing the whole membrane surface of that neuron to a solution that is 25% of the concentration in the electrode.

A pressure pulse of a 1mmol l^{-1} ACh solution for 1s on the soma of Rz(X) neurons, *in situ* or in culture, produced a biphasic response: a fast inward current followed by an outward current with slower kinetics (Figs 2, 3). Lower concentrations of ACh in the pipette (10, 100 and 500 μmol l^{-1}) produced qualitatively similar responses of smaller amplitude (see also Kristan *et al.* 1993). The inward current desensitized with repetitive pulses of ACh, revealing that the outward current was initiated at about the same time as the inward current but with a slower rising phase (Fig. 3). Rz(X) neurons preserved this biphasic response to ACh for as many as 12 days in culture (23/25 neurons cultured for up to 7 days and 8/12 neurons cultured for 10–12 days showed both currents). The amplitude of the inward current recorded in culture was around 50% larger than *in situ* (approximately 1.5nA in culture compared with 1nA *in situ*, at −80mV) and the amplitude of the outward current was approximately four times larger in culture than *in situ* (approximately 1.6nA in culture and 0.4nA *in situ*, at −35mV).

We measured the reversal potentials of the ACh-induced ionic currents of Rz(X) in

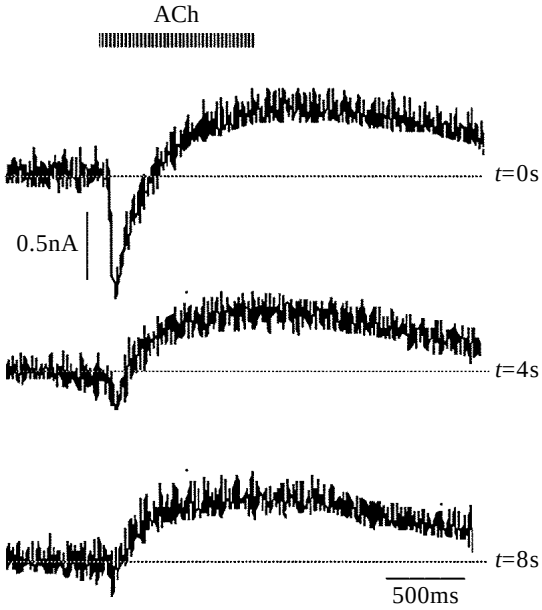


Fig. 3. Desensitization of the fast inward current of Rz(X). Membrane currents elicited by the application of three successive pressure pulses of 1mmol l^{-1} ACh on an Rz(X) cell, clamped at −38mV. The bar above the traces represents the period during which the pulse was on. The pulses were applied at intervals of 4s (first pulse at t=0).

Table 2. *Reversal potential of the cholinergic responses of Retzius cells* in situ *and in culture*

	Rz(X)			Rz(5,6)	
	Inward current E_i (mV)	Outward current E_i (mV)	N	Outward current E_i (mV)	N
In situ	−23±2	−52±1	21	−67±2	40
In culture	−30±2	−61±1	21	−65±1	28

Average values of the reversal potentials (E_i) of the cholinergic inward current and outward current of Rz(X) and outward current of Rz(5,6), measured as indicated in Figs 2 and 4. The results are expressed as mean ± S.E.M. and N indicates the number of cells studied.

order to estimate their ionic composition. The average reversal potential of the inward current measured *in situ* was about 7mV more depolarized than in culture (Table 2). In *in situ* experiments with Rz(X) neurons that did not express the outward current, the average reversal potential of the inward current was −18±2mV (N=13), even more depolarized than the value given in Table 2, which is derived from neurons expressing both currents. These results suggest that the measurement of the inward current amplitude is influenced by the outward current and that this contribution is larger with outward currents of larger amplitudes. Therefore, the actual reversal potential of the inward

current is likely to be closer to the value obtained *in situ*. The value of the reversal potential of the fast inward current suggests that this current is carried by more than one ionic species, since it is not close to the equilibrium potential of any specific ionic species.

The average reversal potential of the outward current *in situ* was about 9mV more depolarized than in culture (Table 2). As will be shown below (*Agonists*), when the outward current was elicited *in situ* by an agonist that did not elicit the inward current, its reversal potential was about −64mV, close to the value obtained in culture. This suggests that the measurement of the amplitudes of the outward current, performed as shown in Fig. 2, also underestimates this current because of the partial temporal overlap with the inward current. Given that the outward current was of larger amplitude in culture than *in situ*, the actual value of the reversal potential of the outward current in Rz(X) is probably closer to the value measured in culture. The reversal potential of the outward current is close to the chloride equilibrium potential (−70 mV) as estimated by Lent (1977).

In a previously reported current-clamp study, we showed that prolonged exposures of Rz(X) to ACh produced a delayed depolarization (Kristan *et al.* 1993) that was not observed in culture. This delayed depolarization had an onset time of around 1s. In the present study, the ACh was applied in pulses of 1s and, therefore, this third component was not seen.

Membrane currents elicited by acetylcholine in Rz(5,6)

The application of 1mmol l^{-1} ACh pulses onto the soma of Rz(5,6) neurons evoked an outward current (Figs 4, 5) that reversed at around −67mV *in situ* and −65mV in culture (Table 2). This outward current desensitized very slowly: the response to a 10s ACh pulse showed hardly any decrease in its amplitude, although some decay could be observed with a 20s pulse (Fig. 5). The reversal potentials of the outward current of Rz(5,6) *in situ* and in culture were statistically indistinguishable from one another. The reversal potential for the outward current of Rz(5,6) was also indistinguishable from the reversal potential of the outward current of Rz(X) measured in culture. Rz(5,6) neurons preserved this response to ACh for as many as 15 days in culture (36/38 neurons cultured for up to 7 days and 8/8 neurons cultured for 10–15 days showed the outward current, but did not show the fast inward current). The amplitude of the outward current of Rz(5,6) in culture was around five times larger than *in situ* (3nA in culture and 0.6nA *in situ*, at −30mV).

Seasonal variation of the response of Retzius neurons to acetylcholine

A seasonal trend was found in the response of Rz(X) to ACh (Table 3). *In situ* studies performed from April to September showed that 60% of the Rz(X) neurons displayed the biphasic response and the remaining 40% did not show the outward current even at very depolarized membrane potentials (up to −25mV). From October to March, the outward current was measurable in only 8% of the cells.

The expression of the outward current was much more stable in Rz(X) cells in culture (Table 3). All cultured Rz(X) neurons showed the biphasic response during the period from April to September and 83% showed the biphasic response from October to May (as opposed to the 8% registered in the same batch of animals *in situ*). The expression of the

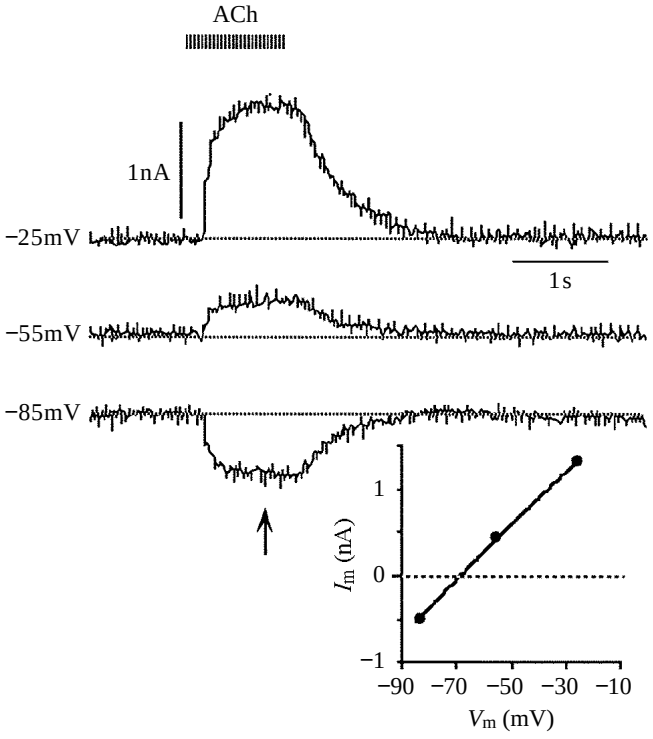


Fig. 4. Acetylcholine response of Rz(6). Membrane currents elicited by the application of a pressure pulse of 1mmol l^{-1} ACh for 1s on an Rz(6) cell clamped at three different membrane potentials (noted on the left of each trace, expressed in mV). The bar above the traces represents the period during which the ACh pulse was applied. The pulses were applied at intervals of 10s. The current amplitude (I_m) was measured at the times indicated by the arrow in order to plot the current–voltage relationship shown in the inset.

Table 3. *Seasonal variation of the expression of the outward current of Rz(X) cells subjected to a 1s pulse of 1mmol l^{-1} acetylcholine and studied at membrane potentials ranging from –85 to –25mV classified as displaying or not displaying the slow outward current following the development of the fast inward current*

	Percentage of Rz(X) cells expressing the outward current	
	April–September	October–March
In situ	60 (10)	8 (12)
In culture	100 (24)	83 (17)

The table indicates the percentage of Rz(X) cells displaying the outward current in studies made *in situ* and in culture. The data were further subdivided according to the months during which the recordings were performed.

The numbers in parentheses indicate the number of cells studied.

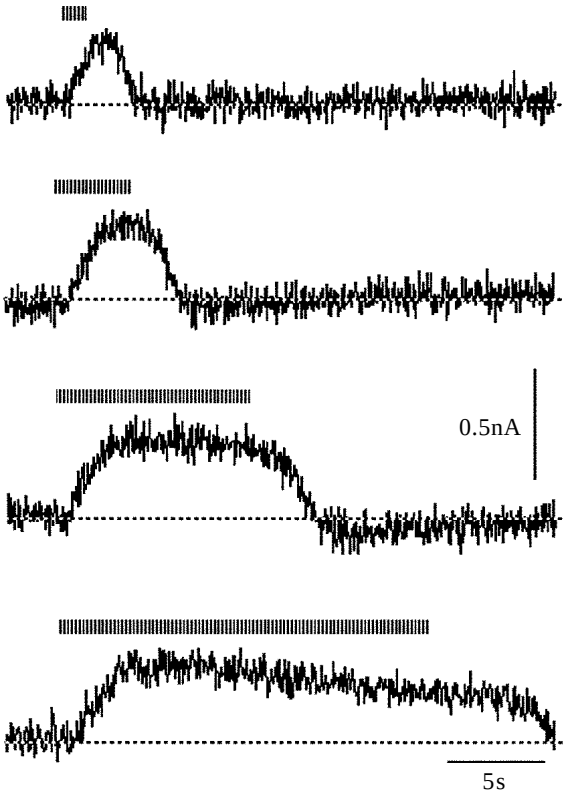


Fig. 5. Responses of Rz(6) to ACh pulses of different durations. Pressure pulses of 1mmol l^{-1} ACh of different duration (1, 3, 10 and 20s) were applied onto the soma of an Rz(6) cell clamped at −35mV. The bar above each trace represents the period during which the ACh pulse was applied. Similar results were obtained in five other preparations.

outward current in Rz(6) did not show any obvious seasonal pattern. In more than 150 cells studied over a 2 year period, the outward current in response to ACh pulses was observed throughout the year.

In summary, the response of Rz(X) to brief pulses of ACh is composed of a fast inward current and a slow outward current, and the expression of the outward current appears to be seasonally regulated. This seasonal difference disappears when Rz(X) neurons are isolated in cell culture. Rz(5,6) neurons appeared to share the outward component of the cholinergic response with the standard Retzius neurons, but did not express the fast inward current. The outward current in these cells does not show any seasonal trend.

Ionic selectivity

Because the outward current in Rz(5,6) and Rz(X) reversed at potentials close to the equilibrium potential calculated for Cl^- (Lent, 1977), the response to ACh was tested in the presence of different extracellular Cl^- concentrations. Total or partial replacement of

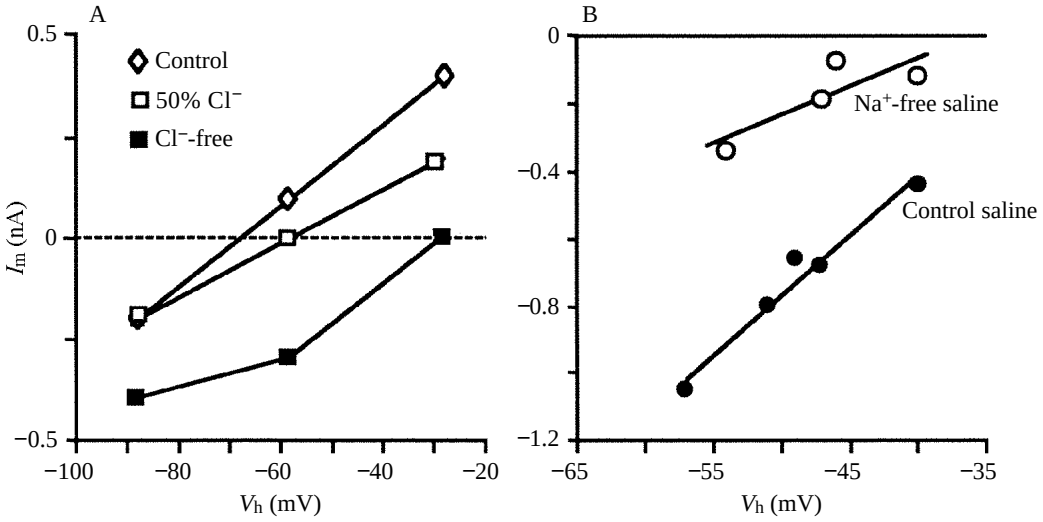


Fig. 6. The ionic nature of the membrane currents elicited by ACh. (A) The contribution of Cl^- to the outward current of Rz(6). The response of an Rz(6) neuron to a pressure pulse of 1 mmol l^{-1} ACh was studied at three different membrane potentials while the ganglia was superfused in normal saline (control), after 3min of superfusion in a solution in which 50% of the Cl^- has been replaced by sulfate (50% Cl^-) and after 3min of superfusion in a Cl^--free solution (Cl^--free). The ordinate indicates the amplitude of the ACh-induced current (I_m) and the abscissa indicates the holding membrane potential (V_h). (B) The contribution of Na^+ to the inward current of Rz(X). The response of an Rz(X) neuron to a pressure pulse of 1mmol l^{-1} ACh for 1s was studied at different membrane potentials. The ACh in the pipette was dissolved in normal saline (filled circles) or in a Na^+-free solution (open circles). The ordinate indicates the amplitude of the ACh-induced current (I_m) and the abscissa indicates the holding membrane potential (V_h).

Cl^- by sulfate in the saline solution bathing Rz(5,6) neurons did not affect their input resistance. Fig. 6A shows the amplitudes of the ACh responses of an Rz(6) neuron at three different membrane potentials: in normal saline, in a saline solution containing 50 % of the standard Cl^- concentration (see *Solutions*) and in a Cl^--free solution. In the presence of half the standard Cl^- concentration, the ACh-induced outward current reversed at a membrane potential approximately 13mV (N=2) more depolarized than in normal saline. In the Cl^--free solution, ACh induced inward currents that zeroed at around −17mV (N=4). The shift in the reversal potential in the presence of half the standard Cl^- concentration is smaller than that calculated from the Nernst equation (17mV), suggesting that the currents we measured may carry some other ionic contribution. However, no outward current was observed in the Cl^--free solution, while the membrane potential was held between −90 and −15mV, strongly suggesting that the main outward current activated by ACh is a Cl^- current.

Replacement of Cl^- by sulfate did not affect the input resistance or the inward current of Rz(X) (N=6, four of these cells did not express the outward current), which was 100±4% of that of the control level. The ionic selectivity of the outward current in Rz(X)

was not tested in detail. Two Rz(X) neurons showing the biphasic response at −40mV (similar to that shown in Fig. 3) were tested at this same membrane potential in a Cl^--free solution and the outward component was reversibly abolished, as in the case of Rz(6) neurons (Fig. 6A).

The reversal potential of the fast inward current expressed by Rz(X) is similar to the reversal potential (between −20 and −5mV in different cells) of the nicotinic receptor, which is known to activate a non-selective cationic channel in mammalian neurons (Egan and North, 1986; McCormick and Prince, 1987). We tried to test the contribution of Na^+ to the fast inward current by replacing this cation with a non-permeable one. The complete or partial replacement of Na^+ salts with Tris salts, however, greatly lowered the input resistance of both Rz(X) and Rz(5,6) neurons. This effect was not specific to the Tris because iso-osmolar replacement of NaCl by sucrose had an identical effect. Because this large decrease in input resistance could modify the expression of all ionic currents in the cell, we tested the contribution of Na^+ to the inward current in a different way: by delivering the ACh from the pipette in a Na^+-free solution. This approach was used based on the fact that the entire ACh-evoked inward current takes place early during the 1s flow of ACh from the pipette (Figs 2, 3). The application of a pulse of Na^+-free solution by itself had no effect on the holding current. Therefore, any change in the response to ACh presented in the Na^+-free solution was due to the decrease in external Na^+ concentration produced by the pressure pulse. The pulse of ACh delivered in the Na^+-free saline solution induced a smaller inward current than the control ACh pulse and made the outward current more apparent. Fig. 6B shows, as an example, the amplitude of the inward current of an Rz(X) neuron at different membrane potentials in response to pulses of ACh delivered in a normal solution and in a Na^+-free solution. The average inward current evoked by a pulse of ACh in Na^+-free was 25±11% (N=4) of the amplitude of the control responses (measured at −55 mV). Five Rz(5,6) cells tested in this way also had their outward currents enhanced by an average of 30±2% of control.

In summary, all Retzius neurons responded to a pulse of ACh with an outward current driven by Cl^-, and Rz(X) neurons responded with an additional fast inward current partially driven by Na^+.

Pharmacological profile of the cholinergic responses

The cholinergic receptors involved in activating the ionic currents described above in Rz(X) and Rz(5,6) neurons were tested using pharmacological reagents that help to discriminate between nicotinic and muscarinic types of cholinergic receptors in vertebrates.

Agonists

The application of a 1s pulse of nicotine onto Rz(X) neurons induced responses qualitatively similar to those evoked by ACh: a fast inward current and a slower outward current (Fig. 7). The muscarinic agent McN A 343, a specific agonist of the muscarinic receptor type I (Nathanson, 1987), elicited only the slow outward current (which reversed at −64±3mV, N=5) and not the fast inward current (Fig. 7).

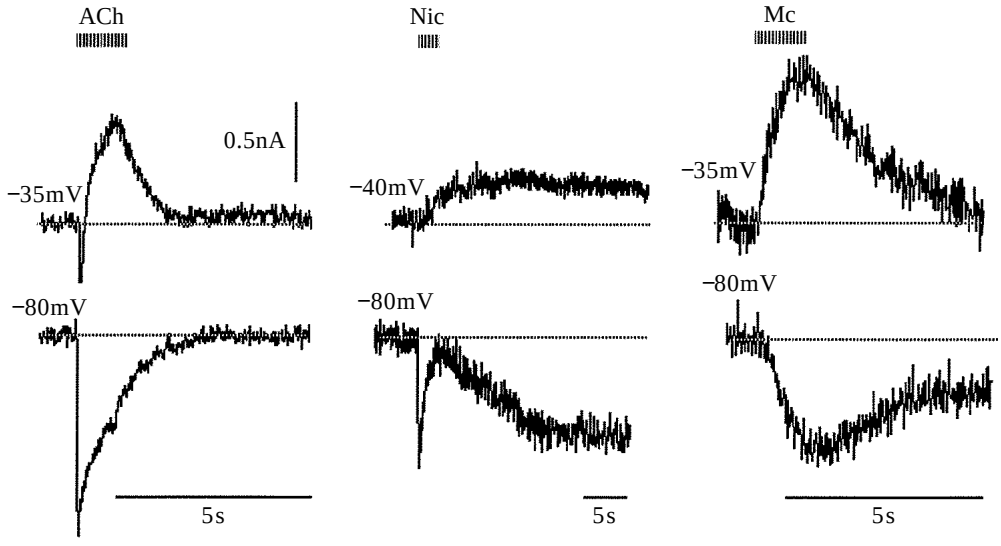


Fig. 7. Responses of Rz(X) to nicotinic and muscarinic agonists. Membrane currents elicited by the application of a pressure pulse of 1mmol l^{-1} solutions of ACh, nicotine (Nic) and the muscarinic agonist McN A 343 (Mc) for 1s on Rz(X) neurons. The neurons were clamped at two different membrane potentials (noted on the left of each trace, expressed in mV). The bar above the traces represents the period during which the agonist pulse was applied.

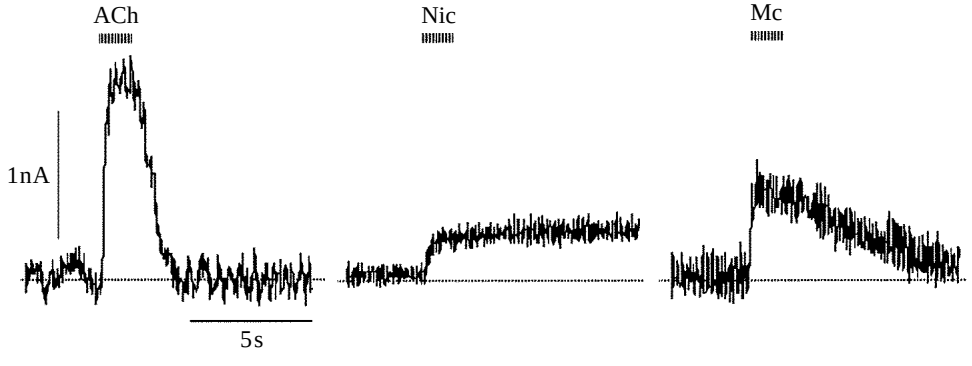


Fig. 8. Responses of Rz(6) to nicotinic and muscarinic agonists. Membrane currents elicited by the application of a pressure pulse of 1mmol l^{-1} solutions of ACh, nicotine (Nic) and the muscarinic agonist McN A 343 (Mc) for 1s on an Rz(6) neuron. The neuron was clamped at around −30mV. The bar above the traces represents the period during which the agonist pulse was applied.

The application of a pulse of nicotine and McN A 343 onto Rz(5,6) mimicked the response elicited by ACh (Fig. 8): both produced an outward current which reversed at approximately −66mV. These currents were strongly decreased when tested in a Cl^{-}-free saline solution at around −40mV. The outward current elicited by nicotine in both types of Retzius neuron had a slower onset and a much longer duration than the current

induced by ACh. McN A 343, however, acted with kinetics similar to these of ACh. These differences in the kinetics of the responses are not surprising since it has been established that different cholinergic agonists interact with the nicotinic receptor in a variety of kinetic patterns (Ogden *et al.* 1987).

Antagonists

The nicotinic antagonists α-bungarotoxin, *d*-tubocurarine, hexamethonium and mecamylamine, and the muscarinic antagonists atropine and pirenzepine, were studied for their action on the ACh-induced inward and outward currents displayed by Retzius neurons. The effects of the antagonists on the inward current of Rz(X) neurons were tested by the application of a 1s pulse of a $1\,mmol\,l^{-1}$ ACh solution while holding the membrane potential at −80mV. In order to test the effect of the antagonists on the outward current of Rz(X) without contamination from the inward current, a 1s pulse of a $1\,mmol\,l^{-1}$ McN A 343 solution was applied while the membrane potential was held at −40mV. The outward current of Rz(5,6) was elicited by the application of a pressure pulse of ACh in similar conditions. Fig. 9 summarizes the results obtained using the nicotinic antagonists. The nicotinic blocker α-bungarotoxin at $100\,nmol\,l^{-1}$ had no effect on the inward current, whereas *d*-tubocurarine, hexamethonium and mecamylamine, all at $100\,\mu mol\,l^{-1}$, partially blocked this current under the conditions detailed in the figure legend. The effect of all these drugs was reversed after the ganglia had been washed with normal saline. The amplitude of the outward current in Rz(X) and in Rz(5,6) was strongly diminished in the presence of α-bungarotoxin and *d*-tubocurarine. Neither hexamethonium nor mecamylamine had any effect under the conditions detailed in the figure legend. α-Bungarotoxin required a more prolonged exposure of the neurons to affect the outward current, probably because this toxin has quite a high relative molecular mass (approximately 8000), and the effect was irreversible: superfusion of the ganglia with normal saline solution for up to 30min did not eliminate the effect of the toxin. The effect of *d*-tubocurarine was reversed within 30s of washing.

A previous report (Kristan *et al.* 1993) concluded that *d*-tubocurarine did not affect the ACh-induced hyperpolarization of Rz(5,6), but in that study *d*-tubocurarine was applied for only 1min instead of the standardized 5min used in this study. In addition, since the previous report measured only voltage changes, a partial blockage by *d*-tubocurarine would be missed if the unblocked current were sufficient to bring the membrane to the ACh reversal potential.

It has been shown that certain nicotinic antagonists act both as competitive blockers of the receptor site and as channel blockers (Ascher *et al.* 1978; Katz and Miledi, 1978; Colquhoun *et al.* 1979). Curare and hexamethonium are classical examples of this type of antagonist. The degree of inhibition that *d*-tubocurarine exerted on the ACh-induced inward and the outward currents was constant at membrane potentials of −30 to −85mV; in contrast, hexamethonium blocked the ACh-induced inward current with a higher potency when the membrane was held at −85mV than it did at −65mV. Hexamethonium had no effect on the ACh-induced outward current with the membrane held in the range −20 to −80mV. These observations suggest that *d*-tubocurarine is a

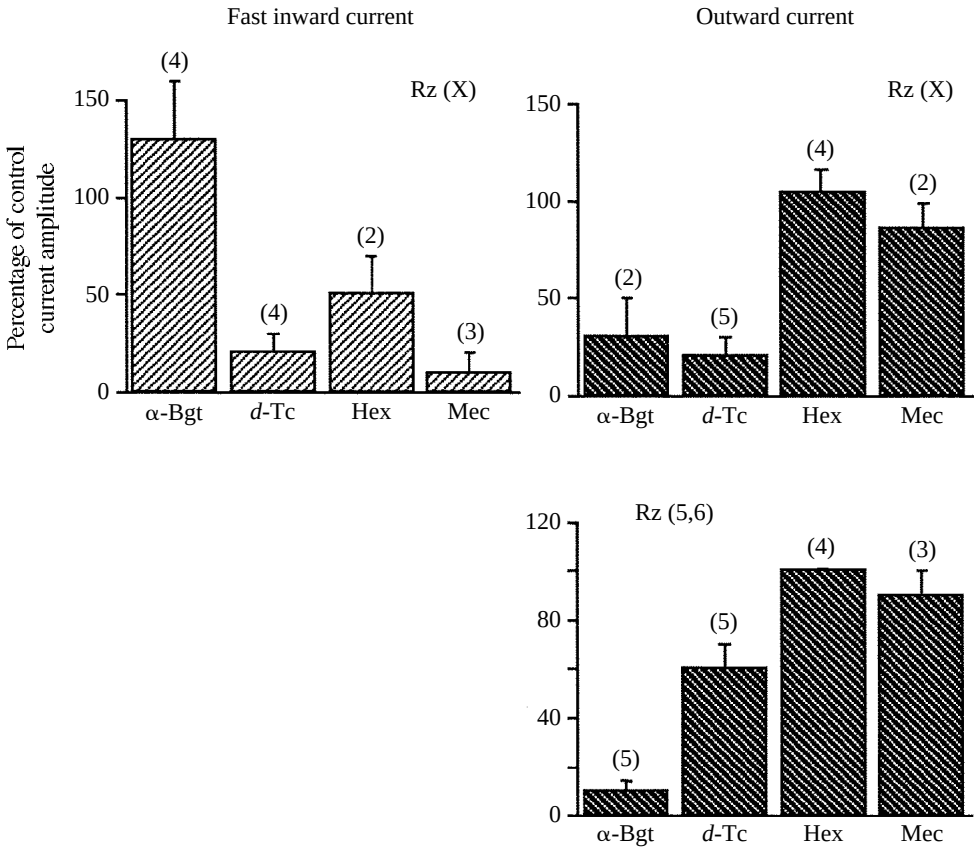


Fig. 9. Antagonist profile of the cholinergic currents of Rz(X) and Rz(5,6). Rz(X) neurons were clamped at −80mV and a pulse of a 1mmol l^{-1} solution of ACh was applied to elicit the fast inward current. Rz(X) and Rz(5,6) were clamped at around −40mV and the outward current was elicited by a pulse of a 1mmol l^{-1} solution of McN A 343 on Rz(X) and of ACh on Rz(5,6). The currents were elicited while ganglia were superfused in normal saline (control) and during the superfusion with normal saline containing the antagonists 100nmol l^{-1} α-bungarotoxin (α-Bgt), 100 μmol l^{-1} *d*-tubocurarine (*d*-Tc), 100 μmol l^{-1} hexamethonium (Hex) and 100 μmol l^{-1} mecamylamine (Mec). The amplitude of each current measured in control conditions was taken to be 100%. The columns represent current amplitude as a percentage of the control amplitude after the treatment with each of the antagonists. The ganglia were superfused for 10min in α-bungarotoxin, 5min in *d*-tubocurarine, 3min in hexamethonium or 3min in mecamylamine. The error bars indicate the S.E.M., and the number in parentheses represents the number of Retzius neurons studied.

true blocker of the receptor site, whereas hexamethonium may act by blocking both the receptor and the channel.

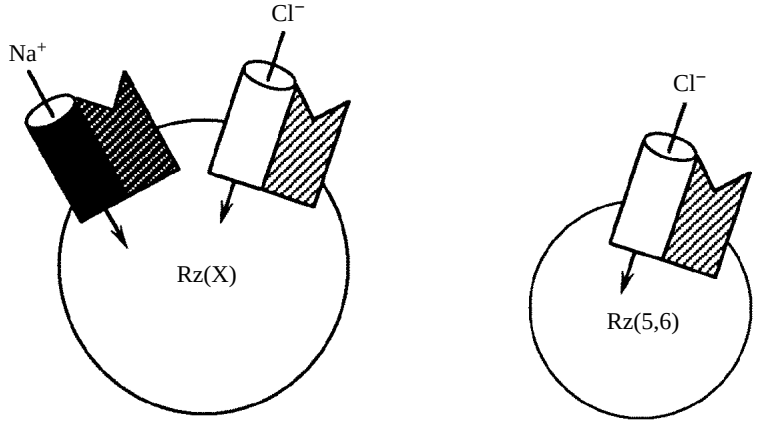
Neither of the responses described was sensitive to atropine (a muscarinic ACh receptor antagonist) or pirenzepine (a blocker of muscarine type I receptors; Nathanson, 1987) when neurons were exposed to these for up to 30min at a concentration of 0.01mmol l^{-1}.

Discussion

Characterization of the cholinergic responses

Our results suggest that Retzius neurons express two types of cholinergic receptors. One receptor is coupled to a cationic conductance with a fast rising phase and a fast desensitization to prolonged exposures to the neurotransmitter. The other receptor is coupled to an anionic conductance with a slower rising phase and is capable of sustained responsiveness to prolonged exposures to the neurotransmitter. Retzius neurons from unspecialized ganglia express both receptors, whereas Retzius neurons from ganglia belonging to reproductive segments express only the cholinergic receptor associated with the anionic current (Fig. 10). These receptors have been shown to be localized in the soma of the neurons since isolated cells with or without dendritic arborizations revealed the same type of responses as the Retzius neurons *in situ.* The distribution of the receptors on the dendritic tree remains to be studied.

Fig. 10 summarizes the physiological and pharmacological information derived from these experiments. The receptor associated with the cationic conductance was activated



Cationic current

Fast desensitization
E_i=−25mV

Agonists
ACh
Nicotine

Antagonists
d-Tubocurarine
Hexamethonium
Mecamylamine

Anionic current

Slow desensitization
E_i=−65mV

Agonists
ACh
Nicotine
McN A 343 (muscarinic)

Antagonists
d-Tubocurarine
α-Bungarotoxin

Fig. 10. Physiological and pharmacological characteristics of the cholinergic responses of Rz(X) and Rz(5,6) neurons. Rz(X) expresses both the cationic and the anionic current, whereas Rz(6) only expresses the anionic current. E_i is the reversal potential.

by nicotine but not by the muscarinic agonist. It was blocked by *d*-tubocurarine, hexamethonium and mecamylamine, but not affected by α-bungarotoxin. The action of hexamethonium does not seem to reflect a blockade of the receptor site but a blockade of the channel. A very similar pharmacological profile has been described for the nicotinic receptor in neurons in the mammalian central nervous system (Egan and North, 1986; Mulle and Changeux, 1990; Rapier *et al.* 1990) and in neurons in other invertebrate ganglia (Kehoe, 1972*a*,*b*, 1976). Therefore, from our data, the fast inward current can be considered to be activated by a 'typical' neuronal nicotinic receptor (see review by Colquhoun *et al.* 1987).

The receptor associated with the chloride current, however, has a less conventional physiological and pharmacological profile (Fig. 10). It was activated by both nicotinic and muscarinic agonists and was blocked by α-bungarotoxin, which is an irreversible blocker of the nicotinic receptor on muscle and also of the neuronal nicotinic receptors composed of only the α7 subunit (Couturier *et al.* 1990). The ACh-induced Cl^- current in Retzius cells was also partially blocked by *d*-tubocurarine, which is known to block both muscular and neuronal nicotinic receptors. The observation that neither mecamylamine nor hexamethonium blocked this receptor, together with its sensitivity to α-bungarotoxin, makes this receptor similar to the muscle type of nicotinic receptor (Colquhoun *et al.* 1987). An ACh-induced Cl^- current of similar characteristics has been reported in *Aplysia californica* ganglionic neurons (Kehoe, 1972*a*,*b*, 1976). The ultimate criterion for distinguishing this receptor from a muscarinic one would be the confirmation that both receptor and channel are part of the same molecular structure (Changeaux and Devillers-Thiery, 1984; Colquhoun *et al.* 1987).

The observation that there is no cationic current in Retzius neurons from the reproductive segments implies that the expression of these nicotinic receptors has been negatively regulated by particular element(s) of the segmental environment during embryonic development (Kristan *et al.* 1993). This negative regulation could not be removed by isolating Rz(5,6) cells for up to 15 days in culture, suggesting that this aspect of the differentiation of Rz(5,6) becomes a stable characteristic of the mature phenotype and is not under the continuous regulation of the segmental environment.

Physiological implications of the cholinergic effect

Our observations indicate that Retzius neurons could be tonically inhibited by a cholinergic input, since the ACh-induced anionic current showed a very slow desensitization upon prolonged exposure to the neurotransmitter. However, Retzius neurons from standard ganglia could be transiently excited by a cholinergic input, whereas Retzius neurons in reproductive segments could not. This implies that cholinergic inputs that activate the outward current can exert their effect in a sustained manner and that the excitatory cholinergic input is temporally regulated by the desensitization pattern of the receptor–channel complex. The expression of cholinergic receptor–channel complexes with a variety of binding and conductance properties has been described in other invertebrate systems (Kehoe, 1972*b*; Gardner and Kandel, 1977).

Stimulation of Retzius neurons and exogenous application of serotonin have been shown to induce a series of behavioral patterns: (1) mucus secretion from skin glands,

implicated both in mechanical protection of the skin and the suckers and in inter-individual communication (Lent, 1977); (2) induction of the swimming motor program (Williard, 1981), concomitant with modulation of the longitudinal muscle tension (Mason and Kristan, 1982) and (3) activation of certain components of feeding behavior (Lent and Dickinson, 1984). Moreover, endogenous serotonin levels were correlated with the state of activity of the animal (Williard, 1981; Lent *et al.* 1991). Therefore, our present study of the modulation of the electrophysiological activity of Retzius neurons by acetylcholine also relates to a broader physiological and behavioral question regarding the nature of the inputs that modulate the activity level of the organism in general, and specific behavioral patterns in particular, and the mechanisms by which they are coded and processed by the nervous system.

Catarsi *et al.* (1990) showed that there is a seasonal variation of serotonin levels in segmental ganglia, with minimal levels during the spring and summer. This coincides with the season when the ACh-induced anionic current was more prominent. Considering that neuronal activity can regulate the rate of neurotransmitter synthesis (Hall, 1992), the former correlation suggests that the activation of the ACh-induced anionic current could be the inhibitory factor maintaining Retzius neurons on a low activity profile which, in turn, could be responsible for low serotonin levels in the ganglia. However, the observation that isolated Rz(X) neurons escape the negative regulation of the anionic current during the autumn and winter implies that the expression of this current is tightly regulated by the segmental environment through an input that is sensitive to season.

To understand the physiological significance of the cholinergic-evoked currents described in this paper it is important to elucidate the cholinergic input pathway(s) that converge(s) on the Retzius cell. It will also be relevant to look at the developmental acquisition of the differential responses of Rz(X) and Rz(5,6) neurons as a model for the mechanisms by which the physiological environment shapes the mature features.

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