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A CALCIUM-DEPENDENT ACETYLCHOLINE DEPOLARIZATION BLOCKED BY METHOXYVERAPAMIL (D600) AND PROCAINE IN SNAIL NEURONES

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1 Repetitive application of acetylcholine (ACh) revealed two types of ACh depolarization in two types of snail neurone, depending on their desensitization properties.

2 Further experiments were carried out on neurones which displayed a rapidly desensitizing response.

3 The amplitude of the response depended on the external sodium and calcium levels.

4 Procaine antagonized ACh effects with the same efficiency as atropine or hexamethonium, half maximal depression being obtained at a concentration of 10^{-4} M. The blocking effect was independent of the dose of ACh.

5 The depression of the ACh-induced depolarization by cobalt ions and D600 suggests that calcium may participate in this response.

Introduction

Procaine and other local anaesthetics are known to exert a depressive action at the neuromuscular junction (Del Castillo & Katz, 1957; Furukawa, 1957). In *Aplysia* neurones, acetylcholine (ACh) currents decrease in the presence of procaine (Marty, 1978). Procaine and related compounds have been extensively studied in the nervous system and it has been suggested that calcium ions are involved in the mechanism of action (Blaustein & Goldman, 1966; Narahashi, Frazier & Takeno, 1976).

D600 (methoxyverapamil) is a selective blocker of calcium flux in squid axon (Baker, Meves & Ridgway, 1973). In *Aplysia* neurones, D600 and cobalt block calcium influx (Klee, Lee & Matsuda, 1973).

Among the snail neurones depolarized by ACh, two categories can be distinguished. The first type (type I) shows no desenzitization of the response and is also depolarized by procaine which increases the membrane conductance (Israel & Meunier, 1979). In this paper, we describe the blocking effects of different pharmacological agents on the second type (type II) of neurone which displays a rapidly desensitizing response to ACh, blocked by procaine.

Methods

The perioesophageal ring of the land snail *Helix aspersa* was removed and pinned down to the bottom of an experimental chamber continuously perfused with snail Ringer (composition mM: Na^+ 120, K^+ 5, Mg^{2+} 3.5, Ca^{2+} 6, Cl^- 144, and Tris HCl 5; pH = 7.3) at room temperature (20 to 22°C). After the connective tissue sheaths were dissected away, naked neuronal somata of the visceral or parietal ganglion were impaled with double barrelled micropipettes filled with a 3 M KCl solution. The resistance of the microelectrode were 5 to 20 Megohms. One barrel was used for recording and the other to pass current in order to measure the membrane resistance or to adjust the membrane potential. A small hyperpolarization was often required to avoid firing.

ACh was iontophoretically applied on to the soma from a single micropipette filled with a 0.5 M ACh chloride solution. Characteristics of the ACh expelling pulse (2 to 30 nA for 100 to 300 ms) were chosen to produce a depolarization of 5 to 15 mV from the resting level (−50 to −60 mV).

For ion substitutions, sodium was replaced by lithium, chloride by propionate and calcium by magnesium. Modified Ringer solution and drugs were added to the bath while repetitive ACh pulses were applied at a frequency of one to two per min.

Cells were selected according to two criteria: first, a depolarizing monophasic response to iontophoretically applied ACh (Tauc & Gerschenfeld, 1961), secondly, a desensitization property for this response which was easily recognized by repetitive application

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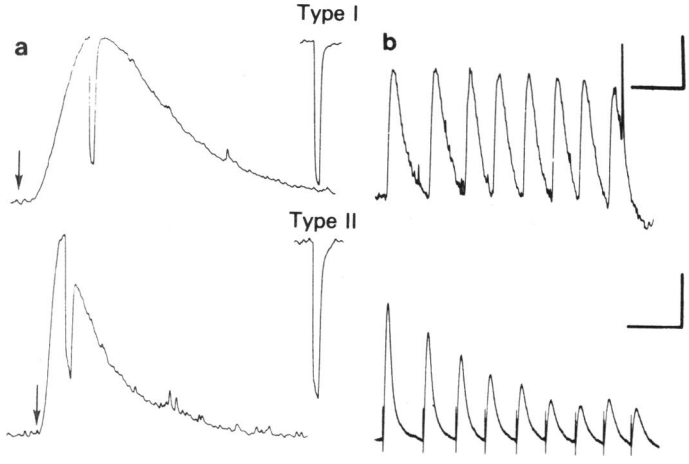

Figure 1 Voltage responses to iontophoretic application of acetylcholine (ACh) on type I and type II *Helix aspersa* neurones. (a) Depolarization obtained by ACh application (at arrow). Downward going traces are resistance measurements at the maximum of the response and after the response at the same potential; the voltage deflection was produced by injecting negative current: 15 nA (type I) and 2 nA (type II). (b) Repetitive application of ACh at the frequency of 0.33/s (type I) and 0.13/s (type II). A decrease in amplitude (desensitization) appears only in type II. Membrane potential: −75 mV (type I); −70 mV (type II); calibration: 4 mV, 4 s (type I); 10 s (type II).

at 0.15/s. A decrease of the amplitude of the response of 50% could then be observed in 30 s. The absence of direct effects of procaine on the membrane potential was also verified.

Results

On neurones of type II at resting potential, ACh induced a rapid depolarization which could trigger spikes. This response was associated with an increase in the conductance of the membrane (Figure 1). When ACh was repetitively applied, the response decreased in amplitude and reached a plateau which depended on the frequency of the ACh pulse. This effect is shown in Figure 1 where the difference between type I and type II neurones is clear.

Changes of the membrane potential do not give information about the ionic dependence of the response; indeed, anomalous rectification resulted in a decrease of the response with hyperpolarization. In order to evaluate the ionic dependence of the response, current-voltage curves have been plotted from experiments performed in normal Ringer and in Ringer containing 10^{-6} M ACh. For comparison, this experiment was also carried out with a type I cell. Figure 2 shows that the curves for a type II cell intersect at a more positive value (+10 mV) than those obtained for a type I cell (−15 mV). Such a shift can be explained by a participation of calcium ions in the ACh response of the type II neurone. The effects of modified Ringer solution were also studied. Substituting half of the chloride ions with propionate had little

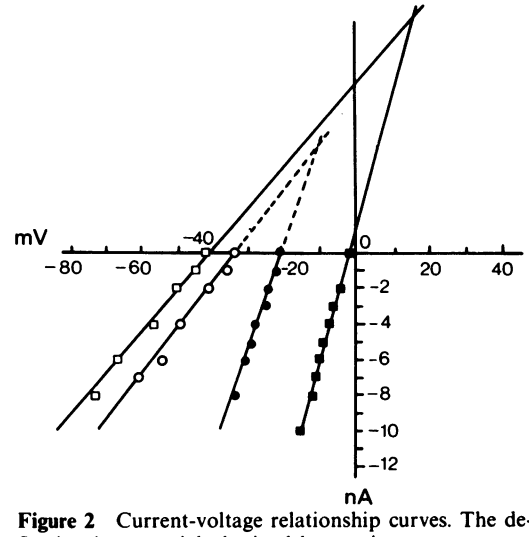

Figure 2 Current-voltage relationship curves. The deflection in potential obtained by passing a square current pulse (1 s duration) through the membrane is plotted versus the value of the current, in normal Ringer solution and in Ringer containing 10^{-6} M acetylcholine (ACh). The two curves for a purely sodium response (type I, ○: normal Ringer; ●: ACh 10^{-6} M) intersect at a value of about −10 mV. With a cell of type II the curves intersect at +15 mV (□: normal Ringer; ■: ACh 10^{-6} M).

or no effect on the amplitude of the response. The response showed very little sensitivity to external sodium changes. When half of the sodium was replaced with lithium, the depression observed was only

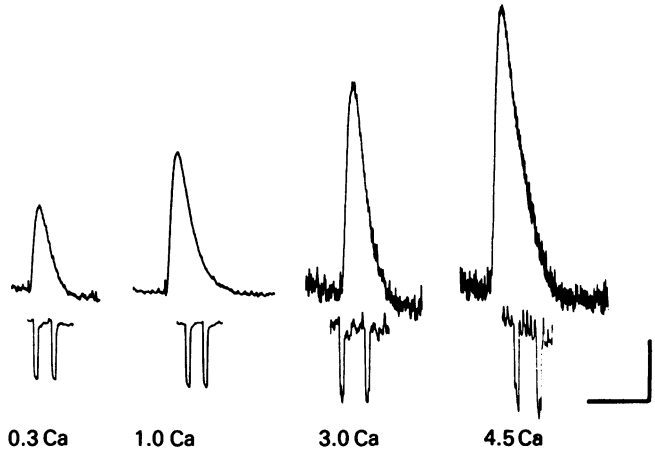

Figure 3 Effects of the variation of external calcium level on the amplitude of the acetylcholine (ACh) depolarization of a type II cell. Calcium concentration was varied between 0.3 and 4.5 in relation to the normal level (6 mM). Membrane resistance changes were small compared with the changes in depolarization. Note the increase of spontaneous synaptic activity by increasing the external calcium; 60% of the sodium was replaced by lithium throughout. Membrane potential: −60 mV. Square pulse 1.5 nA; calibration: 4 mV, 10 s (response); 5 s (square pulse).

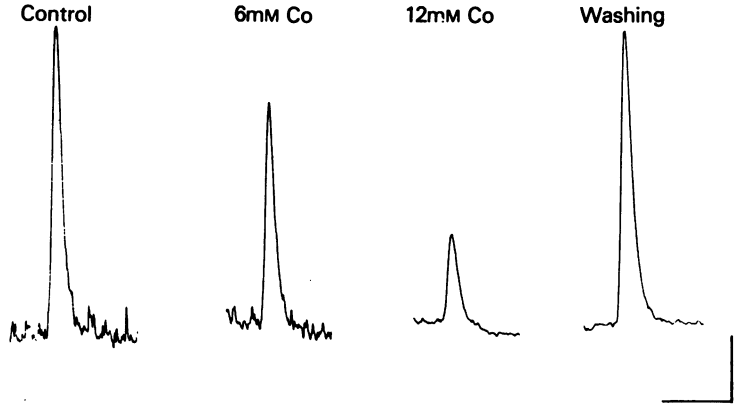

Figure 4 Effect of cobalt ions on the acetylcholine (ACh) depolarization of a type II cell. Responses were obtained by ACh application before and after 12 min of contact with Ringer solution containing 6 mM and 12 mM cobalt ions. The amplitude of the response was reduced by cobalt and recovery was obtained after washing (10 min). Membrane potential: −70 mV; calibration: 4 mV, 2 s.

20%; for the type I neurone, under the same conditions, the depression was 50%. Substitution of half the sodium by magnesium caused a decrease in the response of 40%; under these conditions synaptic activity was blocked.

In addition, the response was dependent on the external calcium concentration. This effect was demonstrated by varying the calcium concentration in a solution where 60% of the sodium ions was replaced by lithium in order to reduce the sodium contribution (Figure 3). The membrane resistance showed only small variations. In type I neurones, the response was not affected by varying the external calcium. Cobalt ions and D600 (methoxyverapamil), an inhibitor of calcium flux were both able to produce a blockade of the response. Figure 4 shows the blocking effect of cobalt (6 mM and 12 mM) on the ACh-induced response of a type II neurone. Figure 5 shows the effect of D600 (10^{-4} M) on both type I and type II neurones. Whereas the ACh-induced response in the type I cell was slightly affected, the depolarization of the type II cell was greatly reduced without any change in the

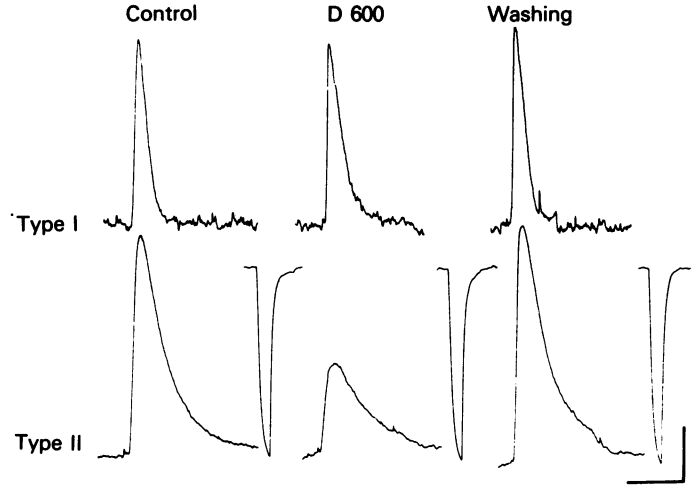

Figure 5 Effects of D600 on acetylcholine (ACh) responses of type I and type II cells. Responses to ACh application were obtained before, during and after exposure to D600, 10^{-4}M (contact time 15 min, type I; 7 min, type II). In the type II cell, the depolarization was reduced by D600 and the amplitude of the square current pulse (1.5 nA) was not modified. Membrane potential: −75 mV (type I), −65 mV (type II); calibration: 5 s, 4 mV (type I); 10 s (response), 2 s (pulse), 4 mV (type II).

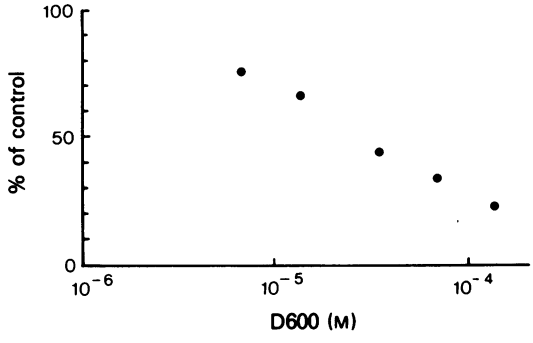

Figure 6 Relation between dose of D600 and amplitude of the acetylcholine (ACh) depolarization in a type II cell. D600 was added in normal Ringer solution at different concentrations and the amplitude of the response is plotted as percentage of the control value. Half maximal depression is 2×10^{-5} M. For each dose, the drug contact time was 5 min. Membrane potential: −65 mV.

membrane conductance, as indicated by the square current pulse. At these concentrations, synaptic activity was reduced slightly by D600 and strongly by cobalt. In Figure 6, the percentage of the maximal response to ACh obtained during perfusion of D600 at different concentrations is plotted versus drug concentration; it indicates that half-maximal depression was reached at 2×10^{-5} M. A total blockade was obtained at 10^{-3} M; thus D600 could inhibit the sodium component of the response. The blocking properties of D600 on the residual ACh response obtained in a calcium-free Ringer are also shown in Figure 7. This effect is totally reversible after washing.

The blocking effects of different drugs on depolarizations induced by ACh on type II neurones were compared. Classical blockers of cholinergic synapses were able to block this response. Half maximal depression was obtained with 2×10^{-5} M tubocurarine and 10^{-4} M atropine and hexamethonium. Procaine also blocked the responses in a dose-dependent manner but was without effect on type I cells up to 10^{-3} M. Figure 8 allows comparison of the effects of these blocking agents; the intensity and time-course of the blocking effect of procaine were similar to those obtained with atropine. Recovery of the initial amplitude of the response occured after washing. None of the above drugs modified the input resistance of the neurone. Varying the membrane potential by passing current did not change the activity of these drugs. Changing the applied dose of ACh (and thus the amplitude of the response) did not change the half-maximal blocking concentration for these different drugs.

Discussion

In view of the calcium dependence of the depolarization response to ACh in type II cells, it could be argued that the response is synaptic. There are two objections to this supposition; firstly, the local application of ACh was carefully controlled such that the

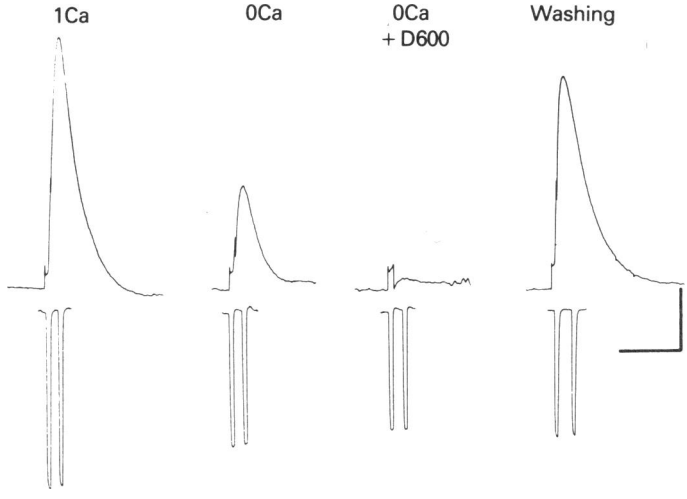

Figure 7 Effect of D600 on the residual acetylcholine (ACh) depolarization of a type II cell in calcium-free Ringer. After the decrease in amplitude of the response induced by calcium privation (10 min), D600 (10^{-4} M) was added to the medium. A blockade of the response was obtained (5 min) which was reversible after washing (10 min). A slight decrease of the membrane resistance was observed. Membrane potential: −65 mV. Square pulse 1.5 nA; calibration: 10 s, 4 mV.

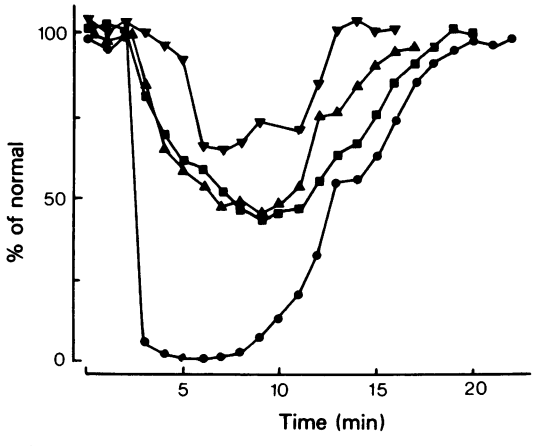

Figure 8 Blocking effect of different drugs on the acetylcholine (ACh) response. Amplitude of the response is expressed as a percentage of the response in normal Ringer solution (10 mV). All drugs were used at a concentration of 10^{-4} M in Ringer and were introduced at time zero: tubocurarine (●), procaine (■), atropine (▲) and hexamethonium (▼). Washing was performed as soon as plateau was reached, 3 min for curare, 6 min for other drugs. Membrane potential: −60 mV.

neurone under study was strongly depolarized with prolonged firing, while neighbouring cells showed only a 1 to 2 mV deflection or no response at all. Secondly, the sensitivities of the ACh response and of the synaptic potentials to different agents were different: in the presence of D600, synaptic activity persisted and the ACh response was blocked whereas in the presence of magnesium, the ACh response persisted in the absence of synaptic activity. From the above results, the participation of calcium ions in the ACh response seems clear. The relative importance of sodium and calcium is difficult to evaluate and seems subject to variations. Reciprocal compensation by a changing ionic composition of the medium is possible. The study of current/voltage relationships gives an indication of the equilibrium potential of the response, but as ACh was being added to the bath in these experiments, the receptors were probably desensitized. Rectification in type II cells, also renders difficult the determination of the intersection point. However, it is clear that the equilibrium potential for ACh is more positive in type II than in type I cells. Calcium influx could be involved in the responses of type II cells to explain this difference.

In normal sodium medium, cobalt and D600 strongly decreased the ACh response and, even in low calcium solutions, D600 blocked the residual response. This fact seems to exclude the possibility of the existence of two separate channels for sodium and calcium. Moreover, low sodium medium increased the sensitivity of the response to external calcium, as if calcium could replace sodium in this response. These facts suggest that both calcium and sodium ions could move through the same non-specific channel which could be blocked by cobalt and D600.

In the type I cell, which seems to be purely sodium-dependent, the response appears insensitive to changes in external calcium but very sensitive to changes in external sodium, is not blocked by D600

10^{-3} M, not affected by addition of cobalt and not blocked by procaine 10^{-3} M. Thus type II cells differ from type I in ionic and pharmacological characteristics.

If a calcium-sodium channel is implicated in type II responses to ACh, it could be blocked by procaine. Indeed, many authors attempt to explain the membrane effects of local anaesthetics as a competition or interaction with calcium ions (Blaustein & Goldman, 1966; Narahashi *et al.*, 1976). The binding of procaine to the ionic channel is proposed by Adams (1977) as an explanation of the procaine endplate current. However, Steinbach (1968) and Katz & Miledi (1975) suggest that xylocaine or procaine bind to the ACh receptor or to an adjacent site. In snail type 1 neurones also, the binding of procaine to the ACh receptor complex is suggested as an explanation for the depolarizing effect of this drug (Israel & Meunier, 1979). Furthermore at the endplate, the effect of procaine resembles that of atropine (Beranek & Vyskocil, 1968; Katz & Miledi, 1973). We show here that, in snail type II neurones, procaine has the same efficiency as atropine or hexamethonium in blocking ACh depolarizations. The present results may also suggest that the blocking effect of procaine could be related to a calcium-dependent mechanism.

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