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VOLTAGE CLAMP ANALYSIS OF ACETYLCHOLINE RECEPTOR DESENSITIZATION IN ISOLATED MOLLUSC NEURONES

BY P. D. BREGESTOVKSI*†, E. A. BUKHARAEVA AND V. I. ILJIN

*From the * Department of Biophysics, University College London, London WC1E 6BT and the*
† Institute of Biological Physics, Pushchino, Moscow Region, U.S.S.R.

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SUMMARY

1. Desensitization produced by acetylcholine (ACh) in completely isolated *Limnaea stagnalis* neurones with chloride-selective membrane channels was studied using a voltage-clamp technique.

2. A difference in the time course of the neurone responses to ACh, depending on whether the measured parameter was voltage or current, was observed and explained on the basis of an equivalent electric scheme of the neurone soma membrane.

3. Desensitization onset was shown not to depend on membrane potential in the range of -30 to -120 mV.

4. Variation of external Ca^{2+}, Na^+ and Cl^- concentrations over a wide range had little influence on the onset of desensitization and recovery from it.

5. An obvious difference is shown to exist between features of desensitization in mollusc neurone and frog muscle end-plate ACh receptors.

INTRODUCTION

It is well known that the response of a cell to acetylcholine (ACh), and to some of its analogues, decreases when the drug is applied continuously or repeatedly. This phenomenon, termed 'desensitization', was found at frog neuromuscular junction and denervated muscles (Thesleff, 1955; Katz & Thesleff, 1957; Manthey, 1966; Magazanik, 1968), mammalian skeletal muscles (Axelsson & Thesleff, 1958), electric eel electroplaques (Lester, Changeux & Sheridan, 1975) and mollusc neurones (Tauc & Bruner, 1963).

Detailed investigation of this phenomenon has been performed at the frog muscle end-plate. It was shown that Ca^{2+} and other polyvalent cations accelerate the decay of the response to ACh (Manthey, 1966, 1970; Magazanik, 1968; Nastuk & Parsons, 1970; Scubon-Mulieri & Parsons, 1977).

Moreover, the time course of the end-plate desensitization strongly depends on membrane potential: depolarization slows down this process, hyperpolarization accelerates it (Magazanik & Vyskočil, 1970, 1975; Scubon-Mulieri & Parsons, 1978).

Preliminary studies on mollusc neurones (Bregestovski, Vulfius & Veprintsev, 1975) did not reveal any dependence of desensitization on external Ca^{2+} or membrane potential. We suggested that the effects of both Ca^{2+} and membrane potential on the

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rate of the end-plate response decay was, possibly, associated with the properties of cation-selective ionic channels of the muscle fibre membrane. In several identified neurones of the fresh-water mollusc *Limnaea stagnalis* ACh induces an increase in membrane permeability to Cl^- only (Kislov & Kazachenko, 1974). We have re-examined the effects of membrane potential and external ionic concentrations on desensitization onset and recovery of neurone ACh receptors and compared our data with the results obtained at muscle fibre receptors. We present here data indicating that neurone desensitization does not depend on membrane potential or on Ca^{2+}, Na^+ and Cl^- concentrations in the medium. We also confirm the results of Manthey (1966) and Lester *et al.* (1975) on different time courses of voltage and current changes following ACh application. A model describing this difference is proposed.

METHODS

Experiments were carried out at identified giant neurones from the left (LP1, LP2, LP3) and right (RP1, RP2) parietal ganglia of *Limnaea stagnalis* (for neuronal map see Jurchenko, Vulfius & Zeimal, 1973) in summer and autumn at room temperature (18–23 °C). Neurones were isolated according to the method described by Kostenko, Geletyuk & Veprintsev (1974). Dissected brain was treated with pronase (3·5 mg/ml., 45 min) or with trypsin (3·5 mg/ml., 15 min) and then with pronase (2·5 mg/ml., 20 min). Enzymes were dissolved in Ringer solution (NaCl 100 mM, KCl 1·6 mM, $CaCl_2$ 4·0 mM, $MgCl_2$ 8·0 mM, Tris 0·12 g/l., pH adjusted to 7·5 with 1N-HCl). After enzyme treatment ganglia were rinsed in fresh Ringer solution. Neurones were isolated with tungsten microneedles and transferred into a lucid chamber of about 0·3 ml. in volume which permitted continuous flow of perfusing fluid at a rate of 15–20 ml./min. The neurones were pressed to the glass bottom of the chamber with two micro-electrodes (so no additional fixation was needed) and impaled by a gentle tapping on the steel platform of the manipulator.

Glass micro-electrodes filled with 2·5 M-KCl or 2·0 M K propionate were used. The latter permitted clamping of the neurone membrane at the level of reversal potential (E_r) and so estimation of the possible involvement of diffusion polarization in the process of desensitization (see Discussion). With KCl micro-electrodes it was impossible to record ACh responses at the membrane potentials close to the level of E_r (-20 to -30 mV) because of the inactivation of cholinoceptive neurone membrane in this voltage range (Kazachenko & Kislov, 1974).

The two-micro-electrode voltage-clamp circuit described earlier was used (Iljin & Bregestovski, 1977). One micro-electrode served for monitoring the membrane potential and the other for passing the current. Membrane currents were registered by means of the virtual ground circuit. The perfusing medium was connected with the virtual ground of an operational amplifier through an agar bridge. The time constant of the voltage-clamp system was about 1 msec. For the current-monitoring circuit the time constant was about 100 msec. Such a slow resolution was quite sufficient for our measurements since the quickest process studied had a time scale of seconds.

An oscillograph with U.V.-sensitive paper was used for recording the membrane currents and potentials.

The protocol for a typical experiment was as follows: the membrane was voltage-clamped near the resting potential level (-50 to -60 mV) unless otherwise mentioned, and rectangular depolarizing and hyperpolarizing pulses (± 10 mV, each step 500–700 msec in duration) were applied from the holding potential. ACh was added to the bath and currents corresponding to each potential level were measured (Fig. 1).

The membrane was always unclamped during the interval between ACh applications.

The quantitative parameters of desensitization measured were the half-time ($T_{\frac{1}{2}}$) and the time constant (τ) of the response decay and the ratio I_{pl}/I_{max} or G_{pl}/G_{max}, where I_{max}, I_{pl}, G_{max} and G_{pl} were respectively the values of current and conductance at the peak of the response and at the plateau after desensitization had been developed (see Fig. 1).

To determine the extent of recovery from desensitization ACh 2×10^{-6} M was applied to achieve a plateau. It was then washed out for 1 min and the same ACh concentration was tested again (Fig. 1). From the ratio of the peak responses I'_{max}/I_{max} (or G'_{max}/G_{max}) a coefficient

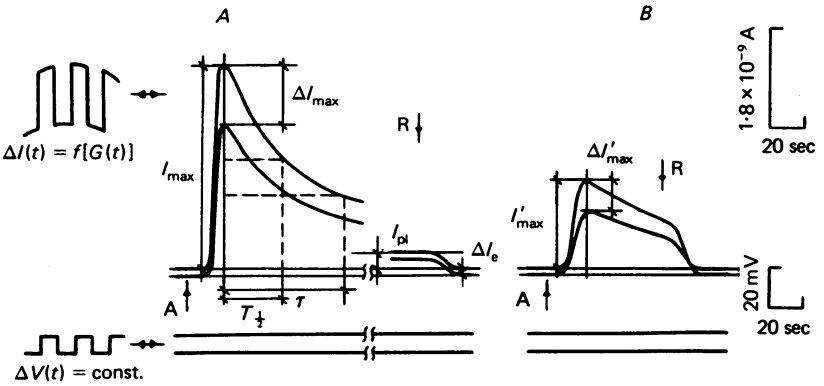

Fig. 1. The parameters of neurone soma responses at excitation by an agonist. Time course of transmembrane current (the upper traces in *A* and *B*) and potential (lower traces) are presented. Here and elsewhere inward currents are upward. Holding potential is equal to resting potential (-60 mV); from this level the sequence of square hyperpolarizing pulses is applied to the membrane (the shape of varying membrane potential $\Delta V(t)$ is given in the bottom left hand corner). Two current levels correspond to those of voltage; the ratio $\Delta I_e/\Delta V$ is the value of leakage conductance G_e of the electrically excitable membrane. *A*, adding ACh (2×10^{-6} M) to the bath (marked by an arrow A) induces rapid rising of transmembrane current up to a maximum value I_{max} (in the top left corner a faster sweep of current changes $\Delta I(t) = f[G(t)]$ at the peak of response is given). Conductance at the peak response is determined as $G_{max} = \Delta I_{max}/\Delta V$. When the maximum is reached, current declines over 2–3 min to a steady level I_{pl}. This decline, or desensitization, is not strictly exponential. To characterize it we use $T_{\frac{1}{2}}$ ($I(T_{\frac{1}{2}}) = I_{max}/2$) or τ ($I(\tau) = I_{pl} + (I_{max} - I_{pl})/e$). Both parameters do not adequately describe the response decline, but are quite acceptable for comparative analysis. When washing ACh out (marked by an arrow R) membrane conductance returns quickly to the initial value G_e. *B*, second testing with ACh (2×10^{-6} M) to estimate the extent of recovery from desensitization after a wash period of 1 min. Here the maximum response, I'_{max} (or G'_{max}), is smaller than that in *A* because of the desensitization developed at the first ACh testing. Calibrations: current, $1{\cdot}8 \times 10^{-9}$ A; potential, 20 mV; time, 20 sec. Temperature 22 °C.

characterizing the extent of recovery was calculated. This method does not allow measurement of the time course of recovery and determination of its rate constant, but it is useful for comparative analysis.

In several experiments responses to ACh were measured on the same neurone, (*a*) as the current under voltage-clamp conditions and (*b*) as membrane potential changes.

When ACh was applied repeatedly, the intervals between applications were of no less than 8 min. This time was found to be sufficient for complete recovery of membrane sensitivity to ACh from desensitization at room temperature.

The effect on the action of ACh, of varying the ionic composition of the bathing solution, was studied in a number of experiments. In each experiment the concentration of ACh, when applied, was kept constant. Each application of ACh was preceded by a washing time of 4 min in the solution of ionic composition under investigation, and followed by at least 4 min washing in the same solution before proceeding to the next ionic composition. The rate of perfusion during ACh testings was maintained throughout the experiment within $\pm 1{\cdot}3$ ml./min of its nominal value. It was necessary since all the parameters measured were strongly dependent on the rate of ACh concentration changes at the cell membrane (Fig. 2).

The composition of the solutions with varying Ca^{2+}, Na^+ and Cl^- concentrations is presented in Table 1. Number 1 is the basic solution. Acetylcholine iodide (Chemapol, Czechoslovakia) was

dissolved in distilled water and kept in a refrigerator. Stock ACh solution was diluted to the desired concentration with the appropriate ionic solution.

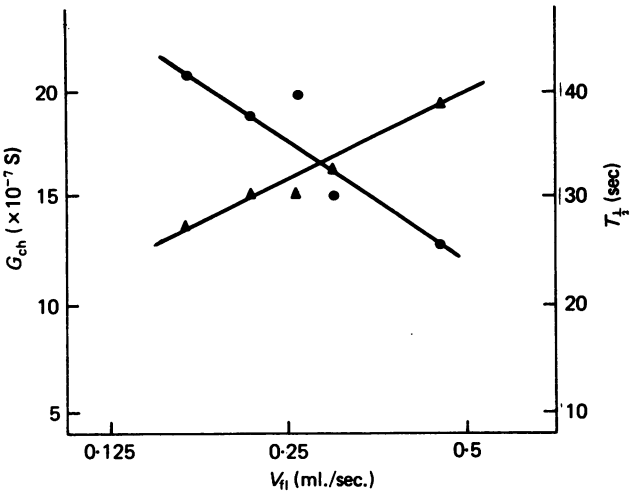

Fig. 2. Changes in G_{max} (▲) and $T_{\frac{1}{2}}$ (●) of neurone response to ACh (2×10^{-6} M) induced by variation of rate V_{fl} of bathing solution flow. G_{max} increases and $T_{\frac{1}{2}}$ decreases approximately 1·5 times for a three-fold increase in V_{fl}. The lines are drawn by eye.

TABLE 1. Composition of physiological solutions (mM)

Solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	Na propionate	Sucrose	Tris-HCl
Normal	100	1·6	4·0	1·5	—	—	—
$\frac{1}{8}$ of normal Ca^{2+} concn.	106	1·6	0·5	1·5	—	—	—
10 × normal Ca^{2+} concn.	28	1·6	40	1·5	—	36	—
$\frac{1}{10}$ of normal Na^+ concn.	10	1·6	4·0	1·5	—	—	90
$\frac{1}{10}$ of normal Cl^- concn.	—	1·6	4·0	1·5	100	—	—

RESULTS

Comparison of the time course of desensitization when ACh action was estimated from current and from potential changes

Addition of ACh to the bathing solution induced depolarization of the neurone membrane and an increase in transmembrane current (Fig. 3*A*). Both these changes were transient when ACh concentration was maintained at a constant level. Depolarization decayed much more slowly than current. Similar results were obtained earlier at the frog motor end-plate (Manthey, 1966) and the electroplaque of *Electrophorus electricus* (Lester *et al.*, 1975).

The difference in the time courses of desensitization onset can be described based

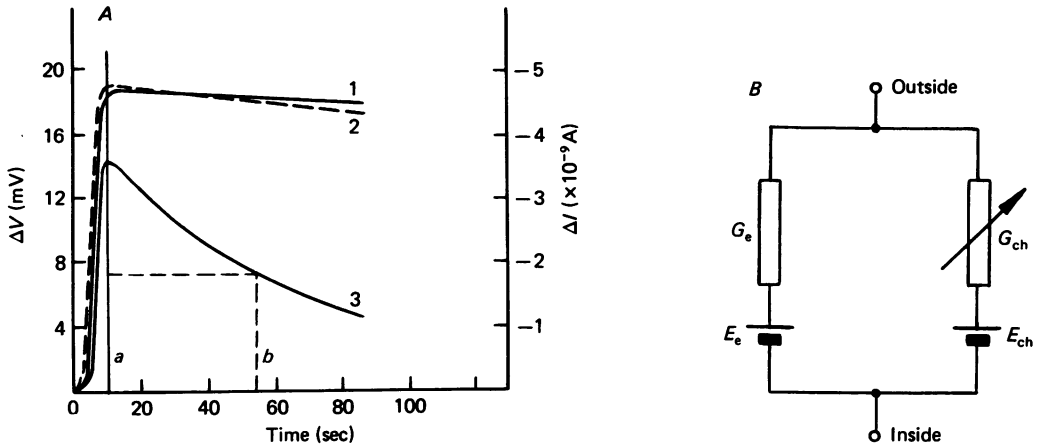

Fig. 3. *A*, time course of neurone response to ACh when recording the membrane potential ΔV (curve 1) or transmembrane current ΔI (curve 3). Membrane potential course calculated from an equivalent electric scheme of neurone soma membrane (see *B*) is given by thick dashed line 2. Segment *ab* of the abscissa is numerically equal to half-time of the current decay. On the time scale chosen, the half-decay of membrane potential is not reached.

$$\text{Curves 1 and 2: } \Delta V = \frac{G_{ch}}{G_{ch}+G_e} \times (E_m - E_r)$$

$$\text{Curve 3: } \Delta I = G_{ch} \times (E_m - E_r).$$

B, a simplified equivalent electric scheme of neurone soma membrane (see Katz & Miledi, 1972). G_e, equivalent leakage conductance of electrically excitable membrane. E_e, equivalent e.m.f. for ions taking part in generation of resting membrane potential. G_{ch}, conductance of cholinoceptive membrane (in the absence of an agonist it is assumed to be equal to zero). E_{ch}, e.m.f. for Cl^- ions penetrating through the membrane during agonist-induced excitation.

on a simple electrical scheme of the neurone membrane (Fig. 3*B*). Increments of membrane potential and transmembrane current at excitation of ACh receptors are respectively:

$$\Delta V = \frac{\Delta G_{ch}}{\Delta G_{ch} + G_e} \times (E_m - E_r) \quad (1)$$

and

$$\Delta I = \Delta G_{ch} \times (E_m - E_r), \quad (2)$$

where ΔG_{ch} is the increment of the conductance of the cholinoceptive membrane, G_e is the shunting conductance of electrically excitable membrane assumed to be constant at excitation of ACh receptors, E_m is the resting (eqn. (1)) or holding (eqn. (2)) potential, and E_r is the reversal potential of the neurone responses to ACh.

If ΔG_{ch} is assumed to be the only time dependent parameter, the time derivatives of V and I are:

$$\frac{\partial(\Delta V)}{\partial t} = \frac{G_e \times \dfrac{\partial(\Delta G_{ch})}{\partial t}}{(\Delta G_{ch} + G_e)^2} \times (E_m - E_r) \quad (3)$$

and $$\frac{\partial(\Delta I)}{\partial t} = \frac{\partial(\Delta G_{ch})}{dt} \times (E_m - E_r). \quad (4)$$

Then the ratio of (3) to (4) is

$$\frac{\frac{\partial(\Delta V)}{\partial t}}{\frac{\partial(\Delta I)}{\partial t}} = \frac{G_e}{(\Delta G_{ch} + G_e)^2}. \quad (5)$$

When ACh is applied, ΔG_{ch} is usually much larger than G_e. Consequently,

$$\frac{\partial(\Delta V)}{\partial t} \Big/ \frac{\partial(\Delta I)}{\partial t} \simeq \frac{G_e}{\Delta G_{ch}{}^2} \ll 1.$$

This means that the current declines in the course of drug action much more quickly than does potential. Curve 2 in Fig. 3*A* is the time course of potential changes calculated according to eqn. (1) and the experimental values G_{ch} and G_e. It coincides almost exactly with the experimental curve 1.

Thus, current recording under voltage-clamp conditions permits more accurate estimation of desensitization time course than does potential recording. All the subsequent studies were carried out using voltage-clamp techniques.

The time course of desensitization at different membrane potential levels

Voltage dependence of desensitization was explored in two sets of experiments, using micro-electrodes filled with K propionate or KCl. With K propionate microelectrodes, the current responses of a neurone to successive application of ACh at constant concentration decreased in amplitude. After four to five applications, I_{max} reduced irreversibly by a factor of 2–3. The value of E_r was in the range of -50 to -75 mV, but it did not remain at the same level; it could shift during a 1 min application of ACh by as much as 5–10 mV.

The time course of ACh-induced currents varied at different membrane potential levels. An example is given in Fig. 4. The neurone was voltage-clamped at -75 mV and short voltage pulses of ± 25 mV were applied. It can be seen that the half-times ($T_{\frac{1}{2}}$) of the current decay are substantially different at the three potential levels; $T_{\frac{1}{2}}$ at membrane potential -50 mV is nearly 3 times as large as $T_{\frac{1}{2}}$ at -100 mV (Fig. 4*A*). From these data it would appear that desensitization is a voltage-dependent process. However, the difference in the time courses of the neurone responses can be accounted for by the progressive shift in E_r during 1 min perfusion with ACh (Fig. 4*B*). Indeed, when the membrane potential was clamped at E_r and a succession of hyperpolarizing and depolarizing voltage pulses of equal amplitude and duration were applied, the time course of ACh-induced currents was similar at the two different potential levels (Fig. 4*C*). Under these conditions E_r was constant during the ACh application (Fig. 4*D*).

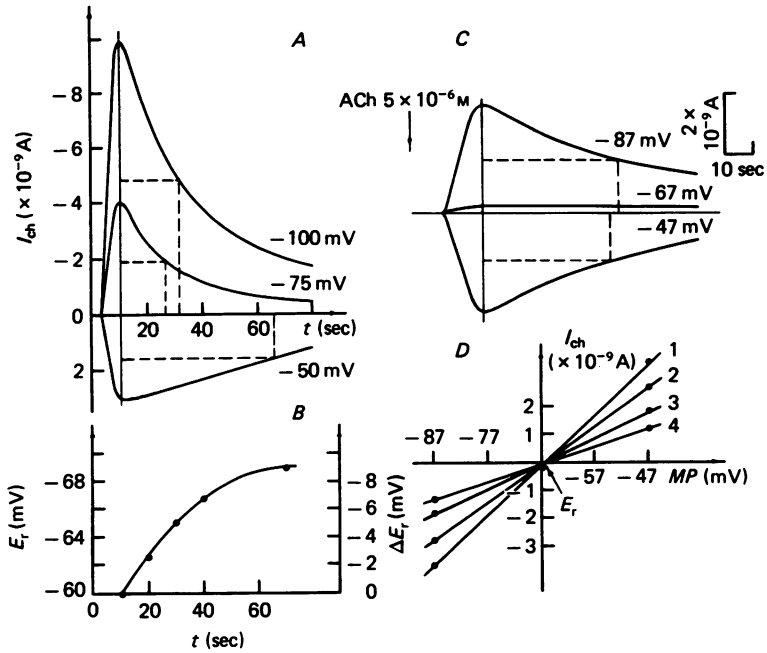

Fig. 4. Excitation of a neurone by bath-applied ACh (5×10^{-6} M) when using K propionate micro-electrodes. *A*, time course of transmembrane currents at different voltage levels. Membrane potential is held at resting potential level (−75 mV). From this level depolarizing and hyperpolarizing square voltage pulses (each 25 mV in in amplitude) are applied. Graphical determination of $T_{\frac{1}{2}}$ for each current curve is shown by dashed lines here and throughout. The half-time appears to depend on membrane potential. *B*, the change of absolute E_r value and the increment ΔE_r in the course of the same ACh testing. The continuous curve is drawn by eye. *C*, transmembrane currents when membrane potential is held at the level of E_r (−67 mV), with superimposed depolarizing and hyperpolarizing pulses of equal dimension. The time courses of currents at the two different voltage levels are practically identical. Calibrations: current, 2×10^{-9} A; time, 10 sec. *D*, voltage–current curves of excited cholinoceptive membrane drawn by eye according to the data obtained in *C*. The curves are taken 10 sec (1), 30 sec (2), 50 sec (3) and 70 sec (4) after starting the ACh testing. E_r does not change in the course of ACh application. *MP*, membrane potential; I_{ch}, current through the excited cholinoceptive membrane.

All these facts can be explained by redistribution of ions through the membrane. At potentials more negative than E_r, ACh evokes a flux of Cl^- ions out of the neurone. Hyperpolarizing current pulses are formed by a non-penetrating anion (propionate) flow through the current electrode, and depolarizing pulses by penetrating K^+ cations. Thus, ACh application leads to a gradual replacement of intracellular Cl^- by non-penetrating propionate. This results in shifting E_r in the hyperpolarizing direction, slowing down the current decay of depolarizing potentials and accelerating the decay at hyperpolarizing ones. Redistribution of anions can be additionally enhanced by passive diffusion of propionate from both electrodes inducing an outward passive flux of Cl^- through the membrane. A gradual shifting of E_r in the hyperpolarizing direction accounts for the decrease in neurone responses to ACh.

If the membrane potential is clamped at E_r, Cl^- flux outward during hyperpolarizing current pulses is equal to that inward during equal amplitude depolarizing ones. Thus, intracellular chloride concentration and E_r change very little.

With KCl-electrodes, E_r was practically constant and varied from −35 to −20 mV

in different cells. So the same neurone could be of D- or H-type (i.e. responding to ACh with depolarization of hyperpolarization) depending on the type of anion in the electrode.

Good reproducibility of responses to successive application of ACh at constant concentration was observed over several hours. The time courses of ACh-induced currents were practically the same at different membrane potential levels, with only a slight tendency to decrease the half-time of desensitization onset with depolarization (Fig. 5, top). E_r was nearly constant during each ACh application (Fig. 5, bottom).

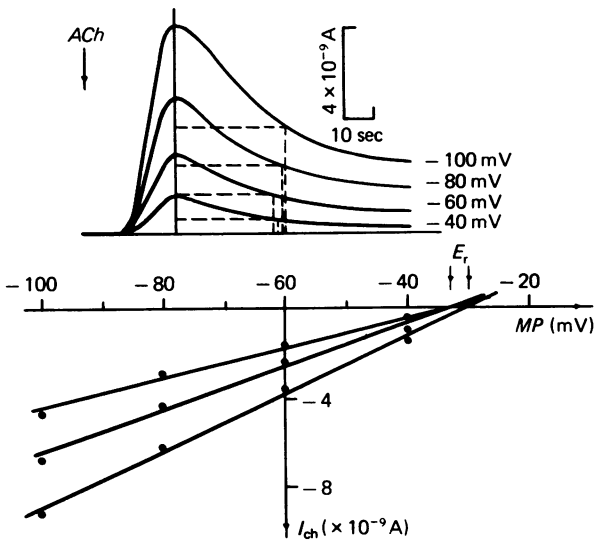

Fig. 5. Excitation of a neurone by ACh (5×10^{-6} M) when micro-electrodes filled with KCl were used. Top: time course of transmembrane currents which correspond to four voltage levels. No significant variation of $T_{\frac{1}{2}}$ from curve to curve is seen. Bottom: voltage–current curves of excited cholinoceptive membrane taken (starting from the lower one) 10, 30 and 50 sec after ACh was added into the bathing solution. The curves are drawn by eye from experimental data in the upper trace. E_r value determined by linear extrapolation varies in the time course of neurone response by about 5 mV (variation range is marked by arrows).

Symbols MP and I_{ch} are as in Fig. 4*D*.

We suggest the following explanation for the constancy of E_r and responses to ACh. At membrane potential levels more negative than the resting potential one, current through the membrane during ACh application is equal to current through the electrode, and both are carried by Cl^- ions. Thus, there can be no significant changes in inside Cl^- concentration.

We studied the time course of desensitization while testing ACh in different concentrations and at potential levels over a wide range (−30 to −120 mV). For each ACh concentration only slight variation of $T_{\frac{1}{2}}$ (by no more than 15 %) with membrane polarization was observed.

The time course of desensitization onset was also independent of membrane potential when ACh was tested at different temperatures, pH or when various ACh analogues (tetramethylammonium, carbamylcholine, suberyldicholine) were applied.

Most of the measurements of parameters of desensitization onset were made using

the pulse polarization technique (see Methods). One may suggest that 0·5–0·7 sec steps are too short to induce some potential-dependent changes in the rate of desensitization. To check this possibility we performed several experiments with constant potential level during one ACh testing. A standard ACh concentration was tested at a number of different levels of membrane potential in the range -50 to -120 mV. This set of experiments also showed that the half-time of desensitization varied only slightly (by not more than 10–15 %).

Desensitization onset and the extent of recovery in solutions with various concentrations of calcium, sodium and chloride ions

Calcium ions. External Ca^{2+} concentration was changed from 0·5 to 40·0 mM (see Table 1). Several minutes after substitution of normal physiological solution for that containing 0·5 mM-$CaCl_2$, leakage conductance of the membrane had increased from $2–3 \times 10^{-8}$ S to $1–2 \times 10^{-7}$ S. This effect was often irreversible. Low external Ca^{2+} concentration affected only slightly the responses to ACh (Table 2). Some acceleration of desensitization was observed but τ was reduced by less than 25 %. The recovery was a little slower (18 %).

TABLE 2. The influence of external calcium $[Ca^{2+}]_o$ on the parameters of neurone responses to bath-applied ACh (2×10^{-6} M). The values are the means ± S.E. of means. For each value the total number of measurements performed on six neurones is given in parentheses, a.u. = arbitrary units.

$[Ca^{2+}]_o$ (mM)	I_{max} (a.u.)	I_{pl} (a.u.)	τ (sec)	I'_{max}/I_{max} (× 100 %)
0·5	50 ± 2·4 (6)	7 ± 1·0 (6)	14 ± 1·2 (6)	59 ± 2·0 (3)
4·0 (normal)	58 ± 5·7 (15)	10 ± 1·6 (15)	18 ± 0·8 (15)	72 ± 1·6 (9)
40	62 ± 7·3 (14)	16 ± 2·0 (14)	23 ± 1·6 (14)	78 ± 1·2 (10)

Elevation of external Ca^{2+} concentration up to 40·0 mM evoked a three-to five-fold decrease in leakage conductance. I_{max} of responses to ACh did not essentially change and onset of desensitization became a little slower (Table 2). τ of desensitization changed by not more than 2 times for an eighty-fold alteration of external Ca^{2+}. Increase of I_{pl} and some acceleration of recovery of neurone sensitivity to ACh after desensitization (column I'_{max}/I_{max} in Table 2) were observed in this set of experiments.

Sodium ions. A tenfold lowering of the external Na^+ concentration led to a small decrease in I_{max} and slowed down desensitization (Table 3), but the difference in the values of τ was negligible.

The reason for the small decrease of I_{max} was not studied. It could not have been due to participation of Na^+ ions in ACh-induced transmembrane currents since E_r values in normal and low sodium solutions were not significantly different (-27 ± 4 and $-26 \pm 2·6$ mV respectively; mean + S.E., $n = 5$). The degree of recovery from desensitization was also independent of Na^+ concentration (Table 3).

TABLE 3. The influence of external sodium concentration $[Na^+]_o$ on the parameters of neurone responses to bath-applied ACh (2×10^{-6} M). The values are the means ± S.E. of means from five observations. a.u. = arbitrary units

$[Na^+]_o$ (mM)	I_{max} (a.u.)	$T_{\frac{1}{2}}$ (sec)	I'_{max}/I_{max} (× 100 %)
100 (normal)	69 ± 2·2	22 ± 1·3	68 ± 5·4
10	59 ± 1·3	28 ± 2·2	69 ± 4·5

Thus, the data provide an evidence that Na^+ ions only slightly affect the properties of the cholinoceptive membrane of the neurones studied.

Chloride ions. Variation of external Cl^- concentration affected E_r and G_{max}, as would be expected from Cl^- selectivity of the cholinoceptive membrane of *Limnaea* neurones.

With K propionate micro-electrodes, maximal responses (G_{max}) to the standard ACh concentration were on average 3 times smaller in a solution with ten-fold lower Cl^- concentration than in the normal one. Lowering of the outside Cl^- concentration led to a shift of E_r (determined by the method of linear extrapolation at the peak of ACh-induced current) by 29 mV (Table 4). This value was half that predicted by the Nernst equation ($\Delta E_r = 58$ mV). It is possible that the discrepancy between observed and theoretical E_r values was due to the loss of internal Cl^- when the solutions were exchanged and ACh was applied.

Using ionophoretic micro-application, Kislov & Kazachenko (1974) showed that E_r shifts during perfusion of a cell with a solution containing low Cl^- concentration. Approximately 1 min after solution exchange E_r reached its maximum value, corresponding to theoretical predictions, and then declined progressively, apparently due to ion redistribution. We tested ACh always 4 min after substitution of the solutions to avoid the involvement of redistribution in the time course of neurone responses. Within this time the internal Cl^- concentration and E_r could change considerably.

In solutions with low Cl^- concentration and with the membrane potential held at E_r, ACh-induced currents were too small to allow accurate estimation of their time courses. If the membrane potential was held at a more hyperpolarized level, E_r shifted during the response to ACh, and thus precise estimation of τ of desensitization was impossible under these conditions. We determined the extent of desensitization and the degree of recovery comparing the responses within a short period of time. Chloride concentration changes had very little effect on these parameters (Table 4).

TABLE 4. The influence of external chloride concentration $[Cl^-]_o$ on the parameters of neurone responses to bath-applied ACh (2×10^{-6} M) when using micro-electrodes filled with K propionate. The values are the means ± S.E. obtained on six neurones. The total number of measurements for each value is given in parentheses

$[Cl^-]_o$ (mM)	G_{pl}/G_{max} (× 100 %)	G'_{max}/G_{max} (× 100 %)	E_r (mV)
112·6 (normal)	22 ± 1·6 (20)	49 ± 4·0 (20)	66 ± 3·6 (15)
12·6	19 ± 1·6 (8)	42 ± 4·5 (9)	37 ± 4·9 (10)

With KCl-micro-electrodes, a tenfold lowering of the external Cl^- concentration evoked a slight acceleration of desensitization; approximately 25 % decrease in the value of τ was found (Table 5). The influence on the ratio G_{pl}/G_{max} and the extent of recovery was even less (10–15 %). Thus the external concentration of Cl^- ions does not significantly affect the parameters of desensitization.

TABLE 5. The influence of external chloride concentration $[Cl^-]_o$ on the characteristics of neurone responses to bath-applied ACh (2×10^{-6} M) with micro-electrodes filled with KCl. The data obtained on six neurones are presented. The values given are means ± S.E. For each value sixteen measurements were performed

$[Cl^-]_o$ (mM)	G_{pl}/G_{max} (× 100 %)	G'_{max}/G_{max} (× 100 %)	τ (sec)
112·6 (normal)	25 ± 4·5	51 ± 2·4	31 ± 3·3
12·6	22 ± 2·8	45 ± 4·1	24 ± 2·4

DISCUSSION

Desensitization appears to be an essential feature of both nicotinic and muscarinic ACh receptors in nerve and muscle cells of different species (Katz & Thesleff, 1957; Axelsson & Thesleff, 1958; Paton, 1961; Tauc & Bruner, 1963; Rang & Ritter, 1969, 1970 *b*). The question arises whether desensitization of various kinds of receptors is due to a common mechanism, or whether different way of decreasing ACh-induced excitation have been developed in different tissues in the course of evolution. To answer this question one should consider the effect of some factors on desensitization. The following discussion is confined to nicotinic receptors.

The effect of 'diffusional polarization'

A decay of the response to ACh, looking like desensitization, can be due to local exhaustion of ions at one side of the membrane and their accumulation at the other side, when the density of ACh-induced current through the membrane is high. This phenomenon, termed 'diffusional polarization' (Vetter, 1961), can evoke a marked decrease in membrane conductance for some seconds in model systems; recovery requires some minutes (Neumcke, 1971; Sidorova, Fridrikhsberg & Mel'nikov, 1972). If diffusional polarization were taking part in desensitization onset of *Limnaea* neurones the following might be expected:

1. Desensitization would have to accelerate with hyperpolarization because of the increase in transmembrane current.

2. The time course of ACh-induced outward current would have to differ very much from that of the inward current, as the internal Cl^- concentration is nearly an order lower than the external one.

3. E_r would always shift in the course of one response to ACh.

4. When membrane potential is held at E_r and depolarizing and hyperpolarizing pulses equal in amplitude are applied, there would be no desensitization at all, since ion redistribution would not proceed.

None of these predictions were confirmed in *Limnaea* neurones (Fig. 4*C* and *D*; Fig. 5).

It is difficult to evaluate the possible contribution of diffusional polarization to desensitization of muscle end-plate. To check this possibility a thorough analysis is needed. Lambert, Spannbauer & Parsons (1977) and Katz & Miledi (1977) showed the coincidence of E_r in normal and desensitized muscle fibres. These data allow the suggestion that any effect of diffusional polarization during ACh action is negligible.

The influence of ion concentrations

Manthey (1966, 1970), Magazanik (1968), Magazanik & Vyskočil (1970), Nastuk & Parsons (1970) and Scubon-Mulieri & Parsons (1977) observed that elevation of Ca^{2+} concentration in the medium accelerates desensitization of frog muscle end-plate. These observations were the basis for a hypothesis of the desensitization mechanism, explaining the decay of the response by the binding of Ca^{2+} ions to negatively charged sites in cation-selective channels inside the membrane (Magazanik & Vyskočil, 1970; Manthey, 1970, 1972) or on the inner surface of the membrane near the channel mouth (Nastuk & Parsons, 1970).

Unlike the situation at the end-plate, variation of external Ca^{2+} concentration from 1 to 10 mM in *Limnaea stagnalis* neurones did not affect the time course of the response to ACh (Bregestovski *et al.* 1975).

In the present work we studied in more detail the dependence of neurone desensitization on outside Ca^{2+} concentration ranging between 0·5 and 40·0 mM. Only very small changes in desensitization parameters were found (see Table 2). Eightfold reduction of external Ca^{2+} evoked a small increase in the rate of desensitization (τ decreased by 20–25 %), while a tenfold elevation of Ca^{2+} led to some retardation of desensitization. Thus, comparing the present results with data obtained at the frog neuromuscular junction, the dependence of *Limnaea* neurone desensitization on Ca^{2+} concentration is small and of opposite direction. Increase in the plateau amplitude and in the ratio I_{max}/I_{pl}, I'_{max}/I_{max} suggest that Ca^{2+} has some effect on the recovery of neurones' sensitivity to ACh, but specificity of this effect needs investigations.

As Manthey (1966) and Magazanik (1968) showed, Na^+ ions, in contrast to Ca^{2+}, slow down the decay of end-plate responses to ACh. Studying desensitization of *Levantina heirosolima* neurones, Ziskind & Werman (1975) observed acceleration, not retardation, of this process with rising external Na^+ concentration. The authors suggest that Na^+ is necessary for desensitization onset in mollusc neurones. In *Limnaea stagnalis* neurones we did not detect any reliable changes of τ when Na^+ concentration was reduced tenfold.

Tenfold lowering of the Cl^- concentration evoked a decrease in the time constant of desensitization by only 25 %, while I_{max} was reduced 2–3 times. Thus, neither cation concentration in the external solution nor concentration of the main anion participating in generation of the response to ACh affect desensitization onset in *Limnaea* neurones.

ACh receptor desensitization at identified *Limnaea stagnalis* neurones, as opposed to the receptors of frog muscle fibres, depends very little on the level of membrane potential. It is interesting to note that at *Electrophorus electricus* electroplaques no dependence of desensitization on membrane potential was found (Lester *et al.* 1975), though the ionic mechanism of responses to ACh is the same as at frog muscle fibres.

Two explanations of these facts can be put forward: either the mechanism of desensitization of mollusc neurone and electric eel electroplaque ACh receptors is quite different from that at the muscle, or there exist some additional factors which affect the time course of the muscle fibre response. The second suggestion seems to be more probable. Dependence of desensitization on agonist concentration (Katz & Thesleff, 1957; Paton, 1961; Magazanik, 1968; Adams, 1975; Lester *et al.* 1975; Rang & Ritter, 1970 *a*), on chemical structure of the agonist (Paton, 1961; Rang & Ritter, 1970 *a*; Bregestovski *et al.* 1975), on temperature (Magazanik & Vyskočil, 1975; Bregestovski, 1975), and the increase of the receptor affinity for some antagonists (Rang & Ritter, 1969, 1970 *b*) and the decrease of affinity for cobra toxin or bungarotoxin (Lester, 1972; Weber, David-Pfeuty & Changeux, 1975; Colquhoun & Rang, 1976) during desensitization, are difficult to explain by the calcium hypothesis, but agree well with the cyclic model advanced by Katz & Thesleff (1957). Studies on ACh receptors in membrane fragments of electric fish electric organs under pre-treatment with agonists in high concentration revealed: (1) quenching of intrinsic flourescence (Bonner, Barrantes & Jovin, 1976), (2) changes in fluourescence of membrane-bound anaesthetic (Grünhagen & Changeux, 1976) and (3) decrease in the receptor SH groups' reactivity (Suárez-Isla & Hucho, 1977). These results support the idea that the conformational changes occur just in the receptor macromolecule during desensitization.

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