J. Physiol. (1984), **353**, *pp.* 375–391
With 8 *text-figures*
Printed in Great Britain

TWO-COMPONENT DESENSITIZATION OF NICOTINIC RECEPTORS INDUCED BY ACETYLCHOLINE AGONISTS IN *LYMNAEA STAGNALIS* NEURONES

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(*Received 1 November 1983*)

SUMMARY

1. The kinetics of desensitization induced by different agonists of acetylcholine (ACh) as well as the kinetics of recovery from desensitization, have been studied using the voltage-clamp technique in isolated, identified *Lymnaea stagnalis* neurones.

2. Desensitization follows the sum of two exponentials: one fast and one slow.

3. The time constant of the fast desensitization component (τ_{ds}^{I}) under ACh application is in the range of seconds at room temperature (18–23 °C). It increases upon cooling ($Q_{10} = 2{\cdot}8 \pm 0{\cdot}9$), decreases with increasing ACh concentration and is independent of membrane voltage.

4. The time constant of the slow component of desensitization (τ_{ds}^{II}) is in the range of tens of seconds. It decreases with increasing drug concentration and is weakly dependent upon temperature ($Q_{10} = 1{\cdot}3 \pm 0{\cdot}4$).

5. The relative amplitude of the fast component, estimated by back extrapolation to the position of the peak current, increases with agonist concentration and decreases upon cooling.

6. Recovery from desensitization follows the sum of two exponentials with time constants (τ_{r}^{I} and τ_{r}^{II}) of the order of seconds and minutes, respectively. Cooling prolongs the slow component (Q_{10} of τ_{r}^{II} is approx. 3) and reduces its contribution during recovery.

7. A comparison of the desensitization induced by various agonists indicates that for the small monoquaternary agonists the onset and recovery of desensitization resemble the onset and recovery observed with ACh. For more bulky agonists, like ethoxysebacylcholine, sebacylcholine and suberylcholine, the decay of the response during prolonged application of the agonist may involve an additional blocking process.

INTRODUCTION

Continuous or repetitive application of acetylcholine (ACh) on frog muscle end-plates, electric eel electroplaques, or molluscan neurones leads to a decline of the cell response, termed 'desensitization' (Katz & Thesleff, 1957; Lester, Changeux & Sheridan, 1975; Tauc & Bruner, 1963). Katz & Thesleff (1957) put forward the

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hypothesis that this phenomenon results from the transition of the receptor molecule into a refractory state. Conformational changes of nicotinic receptors under prolonged exposure to ACh agonists were subsequently demonstrated by both indirect (Rang & Ritter, 1970*b*; Weber, David-Pfeuty & Changeux, 1975; Suárez-Isla & Hucho, 1977) and direct evidence (Barrantes, 1978; Heidmann & Changeux, 1980). The fact that cooling slows both desensitization onset and desensitization recovery (Bregestovski, 1975; Magazanik & Vyskočil, 1975) has shown that these conversions are highly temperature dependent.

Recent studies of the desensitization on the frog muscle and electric organs have indicated that both desensitization onset and desensitization recovery are better described by two exponentials than by one (see, e.g. Feltz & Trautmann, 1980, 1982; Sakmann, Patlak & Neher, 1980; Neubig & Cohen, 1980; Heidmann & Changeux, 1980). We now report similar observations in a system in which the ACh-induced conductance increase is selective for anions rather than cations. Some preliminary results of this study have been published previously (Andreev & Vulfius, 1980; Andreev, Vulfius & Veprintsev, 1980).

METHODS

Isolation of neurones

Experiments were carried out with identified giant neurones (about 150 μm in diameter) isolated from the left (LP1, LP2, LP3) and right (RP1, RP2) parietal ganglia of *Lymnaea stagnalis*.

These neurones bear a homogeneous population of nicotinic ACh receptors controlling Cl^- permeability (Kislov & Kazachenko, 1974; Chemeris, Kazachenko, Kislov & Kurchikov, 1982). The rates of rise and decay of the neurone response to ACh are an order of magnitude slower and the mean open time of the channels associated with ACh receptors an order of magnitude longer than those in the frog muscle end-plate (Bregestovski & Iljin, 1980; Chemeris *et al.* 1982; for comparison see Rang, 1975). Because of these characteristics and the lack of satellite cells it was possible to achieve a rate of drug application and removal that is much faster than the rates of the processes studied.

Neurones were isolated according to the method described by Kostenko, Geletyuk & Veprintsev (1974). Pronase (3·5 mg/ml for 40 min at room temperature) or papain (10 mg/ml for 40–45 min at 37 °C) were used for treatment of the dissected molluscan brain. Isolated neurones were transferred into a chamber (*ca.* 0·06 ml in volume) continuously perfused with physiological solution.

Solutions; application of drugs

The basic physiological solution had the following composition (in mM): NaCl, 100; KCl, 1·6; $CaCl_2$, 4·0; $MgCl_2$, 1·5; Tris(hydroxymethylaminomethane), 2·0; the pH was adjusted to 7·6 with HCl. In several experiments, a possible effect of Tris was tested by comparing the responses of a neurone to ACh dissolved in Tris or $NaHCO_3$-buffered solutions: no differences in the peak current amplitude or desensitization kinetics were observed. Physiological solution with an elevated $MgCl_2$ concentration (8·0 mM) was used for enzyme dissolution and neurone isolation. Mg^{2+} concentrations higher than that in *Lymnaea* haemolymph (de Witt, 1977) facilitated disaggregation of neurones.

ACh and its analogues were applied by superfusion from a plastic tube (0·2 × 1 mm tip orifice) placed at a distance of 1–1·5 mm from the neurone. Direction and rate (15 ml/min or four volumes of the bath per second) of the flow from the tube were adjusted to be the same as those of macroperfusion through the chamber, to reduce the mechanical disturbance induced by flow change from macroperfusion to superfusion and vice versa. The superfusion system provided complete substitution of the bathing solution near the neurone in 100–200 ms judging from the rate of current rise in response to high ACh concentration. Successive drug applications were separated by intervals of no less than 12 min. No treatment with inhibitors of acetylcholinesterase was made since isolated *Lymnaea* neurones possess no cholinesterase activity (Kostenko *et al.* 1974).

The temperature in the chamber was maintained at a given level by thermostatic control of the perfusion solutions, and was measured near the cell surface with a miniature thermoresistor.

To study the recovery from desensitization, brief repetitive ACh test pulses were delivered by ionophoresis from a micropipette (0·5 μm tip diameter) filled with 1 M-ACh. The pulses were applied before, during and after prolonged exposure of a neurone to ACh or some agonist at high concentration. Ionophoretic currents and the frequency of pulses (0·1–0·5 Hz) were adjusted to provide stable responses in the control.

Electrical arrangement

All the experiments were carried out using the two-micro-electrode voltage-clamp system. Both voltage and current electrodes (tip diameter less than 0·5 μm with resistance ranging from 5 to 40 MΩ) were filled with 2·5 M-KCl. Drug-induced membrane currents were measured by an operational amplifier supplying a virtual ground to the bath via a feed-back resistor and were recorded on a loop oscillograph. The current-monitoring circuit had a time constant of about 1 ms. The voltage-clamp system generated a clamping current of up to 1 μA, so that no disturbance in membrane voltage was generally observed at the largest currents induced by ACh (otherwise the data were rejected). The time constant of the voltage clamp was approximately 20 ms. Slow resolution of the voltage clamp did not markedly affect either the amplitude of the peak current induced by ACh or its agonists (rising time was usually 200 ms or more), or the time course of desensitization proceeding on a time scale of seconds. The influence of desensitization on the peak currents evoked by drugs was estimated in several experiments by back extrapolation of current decay (see below).

The membrane was clamped throughout each drug testing near the resting potential level (−60 to −70 mV) unless otherwise indicated.

Drugs

Acetylcholine iodide or bromide and butyrylcholine iodide were obtained from Chemapol (Czechoslovakia); tetramethylammonium bromide from Reaktivkhim (U.S.S.R.); caprylylcholine chloride was synthesized by Drs I. P. Andrianova and E. A. Gracheva at the Novosibirsk State University; ethyl, propyl, butyl, pentyl, and heptyl trimethylammonium (TMA), bromide or iodide salts, were the gifts of Professor I. J. Kvitko (Leningrad Technological Institute); suberylcholine diiodide, sebacylcholine diiodide and ethoxysebacylcholine iodide were synthesized at the Laboratory of Academician O. L. Mndjoyan (Institute of Fine Organic Synthesis, Academy of Sciences of the Armenian S.S.R., Yerevan), and succinylcholine diiodide was from VEB Chemische Werke Radebeul (G.D.R.).

RESULTS

Desensitization onset is described by the sum of two exponentials

L. stagnalis neurones respond to ACh superfusion with an inward current rising rapidly to its peak (I_p) and then decaying slowly to some steady-state level (I_{ss}), which, as a rule, is about 1·3–4·5 % of I_p (Fig. 1*A*). The times of rise to I_p and decline to I_{ss} depend on the ACh concentration (being about 200 ms and 2–3 min, respectively at 10–50 μM-ACh).

Current amplitudes were measured at regular intervals during decay (I_{ss} being subtracted) and plotted against t in semilogarithmic coordinates. Fig. 1*B* shows that the ACh-induced current declines following the sum of two well-distinguished exponentials with time constants (τ_{ds}^{I} and τ_{ds}^{II}) of 1·2 and 9 s, respectively.

The time constant of the fast component of desensitization decreases with increasing ACh concentration (Fig. 2*C*, see also Andreev & Vulfius, 1980) in agreement with the data obtained by Feltz & Trautmann (1980) at the frog neuromuscular junction.

The curve is very steep in the low concentration range up to a value twice the

apparent dissociation constant, K_d. The limiting value of τ^{I}_{ds} is reached at the ACh concentration inducing maximal response (Fig. 2*A* and *C*). τ^{I}_{ds} does not change significantly with further increase of ACh concentration.

The time constant of the slow component of desensitization also decreases with increasing ACh concentration (Fig. 2*D*), the limiting value being about 12 s. However, this effect was weak in about half the neurones (Fig. 2*D*). This may be related to uncertainty in plotting the slow exponential.

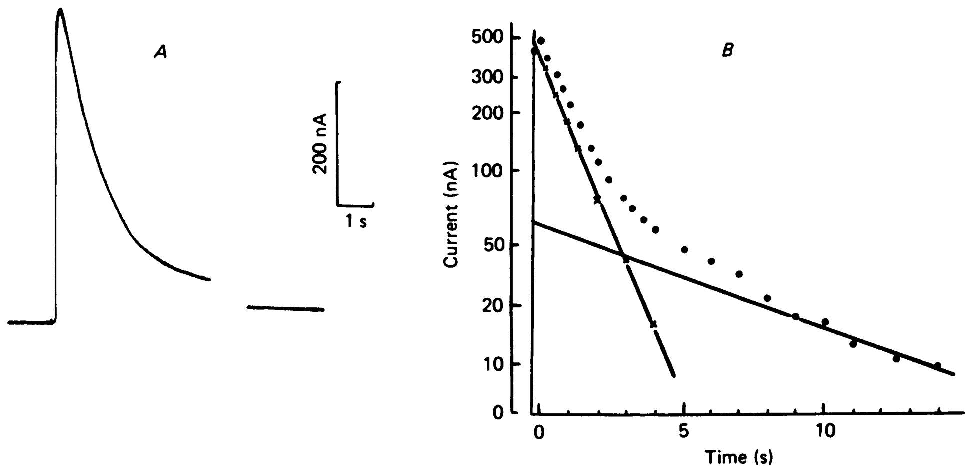

Fig. 1. Desensitization produced in an isolated *Lymnaea* neurone by 50 μM-ACh. *A*, response to a prolonged ACh application. The record is interrupted for 2·5 min to show I_{ss}. The time of ACh wash-out is outside the panel. Here and elsewhere inward current is upward. *B*, the current decay (after subtraction of I_{ss}) is plotted in semilogarithmic coordinates. The rising time of the response is equal to 300 ms; current rising is shown by a line on the left of the plot. The abscissa 0 is the time of I_p. The fast exponential is obtained by subtraction of the slow component from the total current amplitudes. The time constants, τ^{I}_{ds} and τ^{II}_{ds}, are 1·2 and 9 s, respectively. The fraction of the fast component amplitude at the peak current (I^{I}_{p}/I^{tot}_{p}) is 0·87. To correct for a possible underestimation of I_p due to desensitization both exponentials are extrapolated to the point where they cross the line of rising current. The resulting maximal value (I^{tot}_{max}) is 111 % of the measured I_p.

Extrapolation of the two exponentials to the time of I_p gives I^{I}_{p} and I^{II}_{p} (Fig. 1*B*). The sum ($I^{I}_{p}+I^{II}_{p}$) is termed I^{tot}_{p}. The fraction of the fast component in the current decay (I^{I}_{p}/I^{tot}_{p}) is about 0·1 at 0·2 μM-ACh and increases sharply with ACh concentration (Fig. 2*B*). The mean ratio, I^{I}_{p}/I^{tot}_{p}, is 0·84 when ACh is applied at the concentration inducing maximal response; it barely changes upon further elevation of ACh concentration (Fig. 2*B*). Thus, the current induced by a high ACh concentration decays mainly along the fast exponential at room temperature.

Occasionally, high ACh concentrations induced a fast monophasic desensitization. In these neurones I_{ss} was equal to 0 and the time constant of desensitization (1·1 ± 0·1, mean ± S.E. of the mean, n = five neurones, 100 μM-ACh) was close to the τ^{I}_{ds} of the cells with two-exponential desensitization under the action of the same ACh concentrations. Low concentrations of ACh were not tested on these cells.

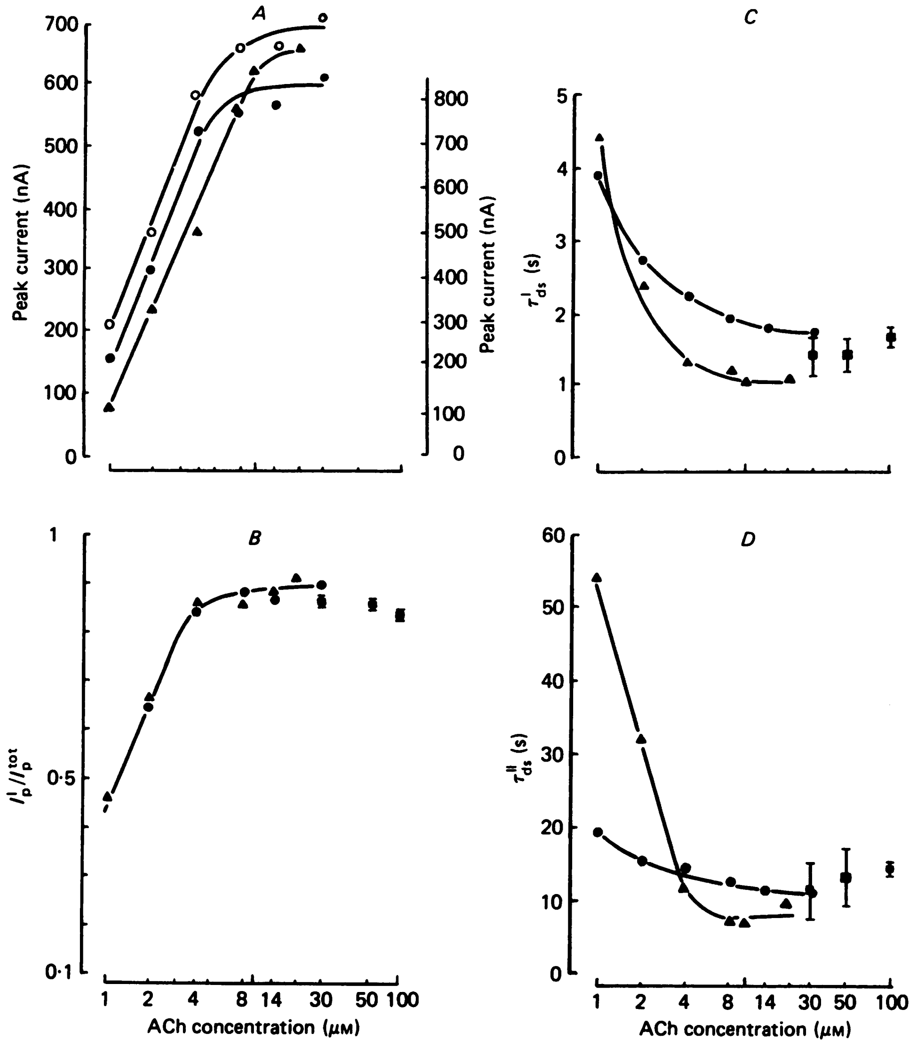

Fig. 2. Peak current and parameters of desensitization as functions of ACh concentration. *A*, peak currents induced in two neurones by ACh (closed symbols); open circles are corrected values for the measured peak currents which are marked by closed circles (corrections were performed as described in the legend to Fig. 1*B*). The left and right ordinates refer to triangles and circles, respectively. The ratio of the rapidly desensitizing component over the total current (*B*), τ^{I}_{ds} (*C*) and τ^{II}_{ds} (*D*) are plotted against ACh concentration for the same two neurones. The data for 30, 50 and 100 µM-ACh, marked by squares with vertical bars, are means ±S.E. of the means taken from three, three and sixteen neurones, respectively. All the curves are drawn by eye.

Voltage dependence of the ACh-induced current

The reversal potential (E_r) of peak currents induced by 1, 10 or 100 µM-ACh is equal to -13 ± 6 mV (mean ±S.D., n = six neurones). E_r was determined by extrapolation except for one neurone (1 µM-ACh) where true reversal of I_p was achieved at -18 mV. The value obtained is in agreement with E_r reported for the same identified *Lymnaea*

neurones when KCl or CsCl electrodes were used (Chemeris *et al.* 1982). An example of the I_p–V relation when 10 μM-ACh was tested at four different holding potentials is illustrated in Fig. 3 (open circles). The reversal potential of I_{ss} was estimated for the same neurone with successive voltage pulses of varying amplitude applied from the holding potential (−128 mV) after I_{ss} had been established (Fig. 3, closed circles). It can be seen that both current–voltage relations are linear and that the E_rs coincide at about −9 mV.

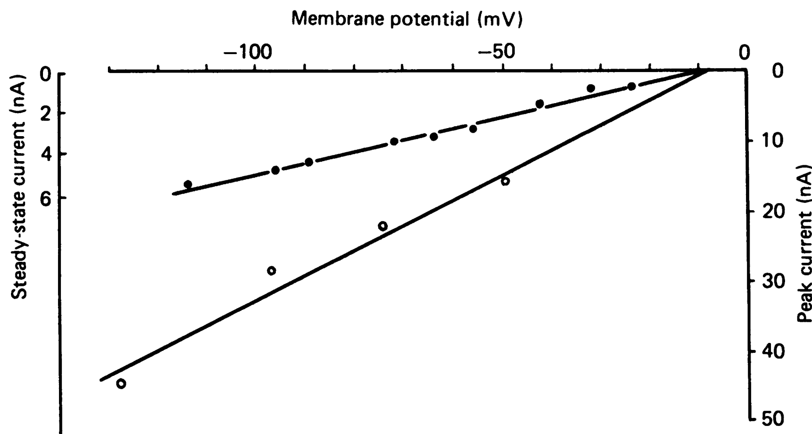

Fig. 3. Current–voltage relations for peak and steady-state values of the response to 10 μM-ACh. Open circles are I_p^{tot}s registered when ACh was tested with a neurone at four holding potentials; the line is calculated by the linear regression analysis; the points deviate from the line insignificantly at $P < 0{\cdot}025$. Closed circles correspond to currents measured during successive voltage steps (6 s or more in duration) applied from the holding potential (−128 mV) after the ACh-induced current has reached its steady-state level, I_{ss}. The same series of voltage pulses was applied in the absence of ACh and the corresponding currents were subtracted; the line is drawn by eye. The extrapolated reversal potentials for both I_p and I_{ss} are about −9 mV.

The mean E_r of I_{ss} is equal to -16 ± 5 mV ($\pm$S.D., n = eight neurones). Thus, E_r hardly shifts during desensitization onset, i.e. no Cl^- redistribution occurs in spite of very large (up to 500 nA) currents. The same conclusion was reached by Bregestovski, Bukharaeva & Iljin (1979) from their data on desensitization at *Lymnaea* neurones studied with the use of KCl electrodes. These authors suggested that the intracellular Cl^- concentration remains approximately constant during the response to ACh due to equivalent Cl^- flow from the current electrode into the cell. The rather short duration of the membrane current under prolonged application of high ACh concentrations (at 100 μM-ACh current declines to approx. 1/10 of its peak amplitude in about 5–8 s) may be another reason why internal Cl^- does not decrease.

Fig. 4 illustrates a semilogarithmic plot of the responses of a neurone to 100 μM-ACh at two holding potentials. The time constants of the fast and slow desensitization components are slightly shorter at −55 mV (2·0 and 10·7 s, respectively) than at −135 mV (2·4 and 11·5 s), but the differences are even less than the S.D. of the means for τ_{ds}^{I} and τ_{ds}^{II} (0·5 and 4·7 s, n = seventeen neurones) when 100 μM-ACh is applied at the resting potential level. No significant changes in τ_{ds}^{I} were found at other

neurones when ACh was tested at different membrane potentials. The same is true for the τ_{ds} obtained for neurones with monophasic current decay. Thus the voltage dependence of the fast desensitization rate, if any, is much weaker than that at the frog neuromuscular junction, where an e-fold change of τ_{ds}/70–80 mV was found (Magazanik & Vyskočil, 1970; Fiekers, Spannbauer, Scubon-Mulieri & Parsons, 1980) and where the τ_{ds} shift is in the opposite direction. Our data were not sufficient to allow any conclusion on the voltage dependence of τ_{ds}^{II} and I_{ss}.

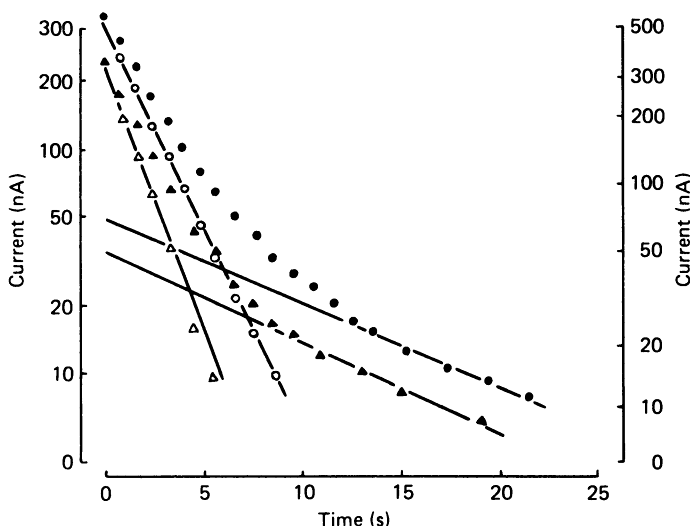

Fig. 4. Voltage dependence of desensitization. Two responses of a neurone to 100 μM-ACh at −55 mV (triangles, left ordinate) or −135 mV (circles, right ordinate) are presented in semilogarithmic coordinates. Closed symbols are experimental values of the currents measured at different intervals. Open symbols (fast exponentials) are obtained by subtraction of the slow components from corresponding total currents. The time constants of the fast desensitization component are 2·0 s at −55 mV and 2·4 s at −135 mV, and the τ_{ds}^{II}s are 10·7 and 11·5 s, respectively. The fraction I_p^I/I_p^{tot} is equal to 0·87 at −55 mV and 0·88 at −135 mV.

Effect of temperature on desensitization

Fig. 5*A* shows two responses of a neurone to 20 μM-ACh at 18 and 9 °C. At 9 °C, I_p is slightly less than at room temperature and desensitization is retarded due to the increase of τ_{ds}^{I} from 1·6 to 2·9 s and the attenuation of the I_p^I fraction from 0·78 to 0·59. The time constant of the slow desensitization component changes slightly with cooling (10·5 and 11·2 s, respectively). The mean changes of τ_{ds}^{I} and τ_{ds}^{II} per 10 °C in the range from 22 to 2 °C are equal to 2·8±0·9 and 1·3±0·4 (±S.E. of the mean, n = four neurones), respectively.

At low temperature I_{ss} is reached much more slowly and the amplitude of I_{ss} increases approximately 1·5-fold/10 °C. Increase of the steady-state response at low temperature was revealed earlier at the frog muscle end-plate (Magazanik & Vyskočil, 1975).

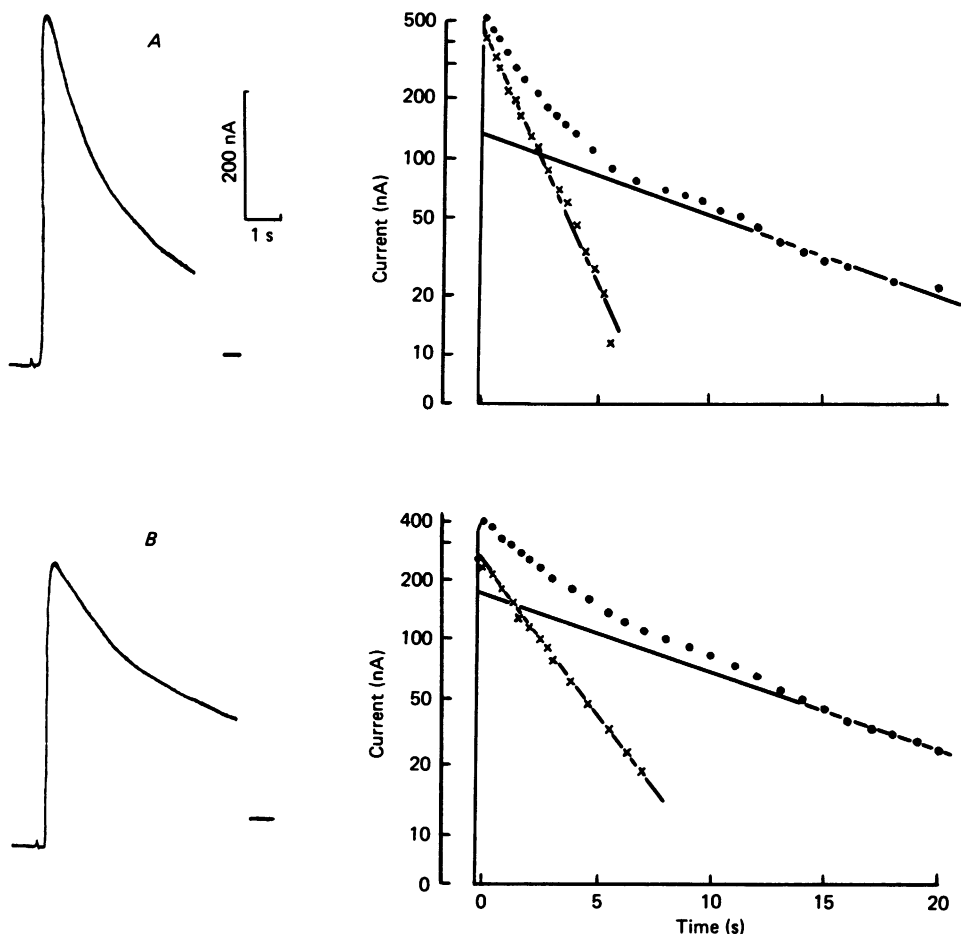

Fig. 5. Effect of temperature on desensitization. Two responses of a neurone to 20 μM-ACh at 18 °C (*A*) and 9 °C (*B*). The records are interrupted for 80 s in *A* and 98 s in *B*. Calibrations are the same for both records. The current decays are plotted in semilogarithmic coordinates on the right. Rising times of the responses are about 200 ms at 18 °C and 300 ms at 9 °C. The time constants of the fast desensitization component are 1·6 s at 18 °C and 2·9 s at 9 °C; those of the slow components are 10·5 and 11·2 s, respectively. The fraction of the fast component in desensitization is equal to 0·78 at room temperature and drops to 0·59 at 9 °C. Underestimation of I_ps experimentally measured was checked as in Fig. 1*B*; corrections are 7 and 5% at room and low temperature, respectively. The holding potential is the same (about −70 mV) for both records.

Desensitization onset under the action of ACh agonists

Fig. 6 shows records of currents induced in a neurone by 100 μM-ACh (*A*), 1 mM-methyl TMA (*B*) and 1 mM-caprylylcholine (*C*) (1 mM is just the concentration at which methyl TMA and caprylylcholine evoke maximal responses). The responses are very similar, except for the fact that in the case of caprylylcholine, I_p is approximately two times smaller than I_p for ACh or methyl TMA. Desensitization in all cases follows the sum of two exponentials as is evident from semilogarithmic

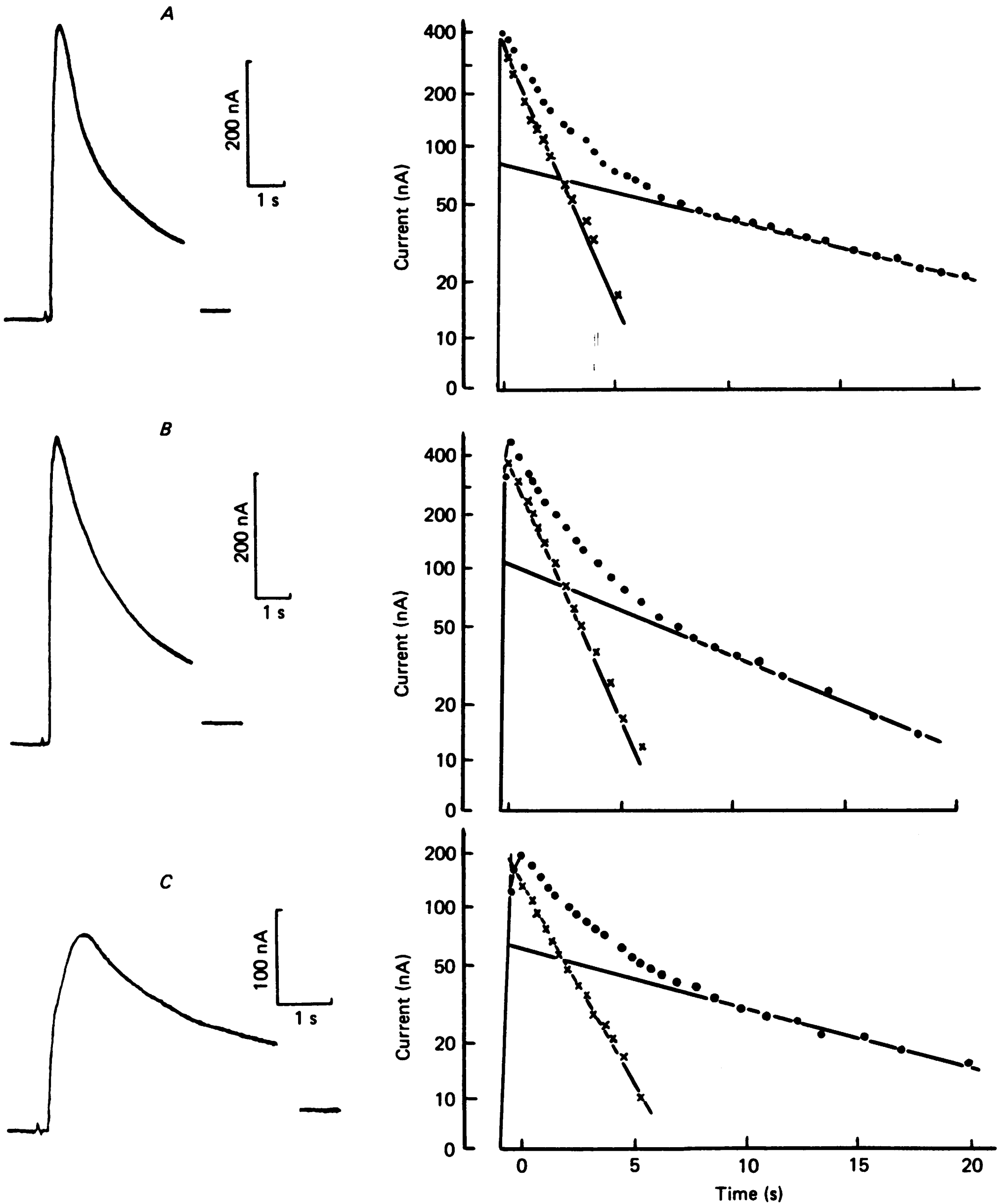

Fig. 6. Responses of a neurone to 100 μM-ACh (*A*), 1 mM-methyl TMA (*B*) and 1 mM-caprylylcholine (*C*). For methyl TMA and caprylylcholine 1 mM is the concentration at which the drugs induce maximal responses. The records are interrupted for 72, 30 and 40 s, respectively. Rising times of the responses are about 300 ms for ACh, 400 ms for methyl TMA, and 700 ms for caprylylcholine. Note that the gain and the sweep speed are different in *C*. The corresponding current decays are plotted on the right in semilogarithmic coordinates. The time constants of the fast and slow components are equal to 1·4 and 15 s for ACh, 1·6 and 9 s for methyl TMA and 1·9 and 14 s for caprylylcholine. The fraction of the fast component is practically the same under the action of ACh and methyl TMA (0·80 and 0·79) and notably less for caprylylcholine (0·70). Back extrapolation of both desensitization components to determine corrections for I_ps gives 4, 18 and 28% for the three drugs, respectively. The holding potential is the same (−54 mV) for all records.

plots of Fig. 6. The time constant of the fast desensitization component is 1·4 s for ACh, 1·6 s for methyl TMA and 2·0 s for caprylylcholine. The corresponding values of τ^{II}_{ds} are 15, 9 and 14 s, respectively.

When other ACh agonists were applied to *Lymnaea* neurones the same two-exponential desensitization was observed. As in the case of ACh, elevation of agonist concentration led to a decrease of τ^{I}_{ds} and an increase of the fast component fraction in desensitization.

TABLE 1. Parameters of desensitization onset produced by different agonists

Agonist	Concentration inducing G_{max} (mM)	Concentration used (mM)	$\tau^{I}_{ds}/\tau^{I}_{ds, ACh}$	τ^{II}_{ds} (s)	I^{I}_{p}/I^{tot}_{p}
ACh	0·01	0·01	1	10·0 ± 1·8 (11)	0·84 ± 0·02 (7)
Butyrylcholine	0·1	0·1–1	0·96 ± 0·04 (4)	11·1 ± 6·3 (5)	0·85 ± 0·02 (5)
Caprylylcholine	0·5	1–2	1·13 ± 0·08 (4)	12·9 ± 1·8 (5)	0·75 ± 0·02 (4)
Methyl TMA	1	1–2	0·99 ± 0·12 (6)	8·1 ± 2·5 (8)	0·75 ± 0·04 (7)
Ethyl TMA	—	1	0·99 ± 0·01 (4)	18·4 ± 4·7 (4)	0·81 ± 0·04 (4)
Butyl TMA	—	1–2	0·98 ± 0·03 (5)	15·1 ± 4·1 (5)	0·84 ± 0·03 (5)

$\tau^{I}_{ds}/\tau^{I}_{ds, ACh}$ is the ratio of τ^{I}_{ds} for an agonist over that for ACh obtained on the same neurone. Results are expressed as mean ± S.E. of the mean. The number of experiments is given in parentheses. G_{max} is the maximal conductance increase induced by a given agonist.

The value of τ^{I}_{ds} for each agonist was compared with the value for τ^{I}_{ds} obtained when ACh was applied on the same neurone. Both the agonist and ACh were applied at a concentration close to or larger than that eliciting a maximal response. As shown by Table 1, the ratio of the values of τ^{I}_{ds} was very close to 1 for six agonists: ACh, butyrylcholine, caprylylcholine, methyl TMA, ethyl TMA and butyl TMA. Similarly, the values of τ^{II}_{ds} were similar for these six agonists, although they showed a wider dispersion.

With other compounds, however, and in particular ethoxysebacylcholine, sebacylcholine, suberylcholine, heptyl TMA, the pattern of desensitization deviated from that observed with ACh. The main deviation was the fact that the dose–response curve was bell-shaped, as illustrated in Fig. 7*A* for ethoxysebacylcholine. The agonist-induced current, after reaching a maximum for concentrations of agonist between 10 and 100 μM, declined at higher agonist concentration. The maximum current was only 28 % of the maximal current induced by ACh on the same neurone.

This anomalous dose–response curve was correlated with an atypical concentration dependence of the ratio I^{I}_{p}/I^{tot}_{p} as well as of τ^{I}_{ds} and τ^{II}_{ds}. The ratio I^{I}_{p}/I^{tot}_{p} increased much less steeply than with ACh (compare Fig. 7*B* with Fig. 2*B*). It eventually reached a maximum value comparable to that obtained with ACh (about 0·85) before decreasing as the agonist concentration was further increased.

As with ACh desensitization, onset was well described by the sum of two exponentials. The time constants of the two exponentials decreased with increasing

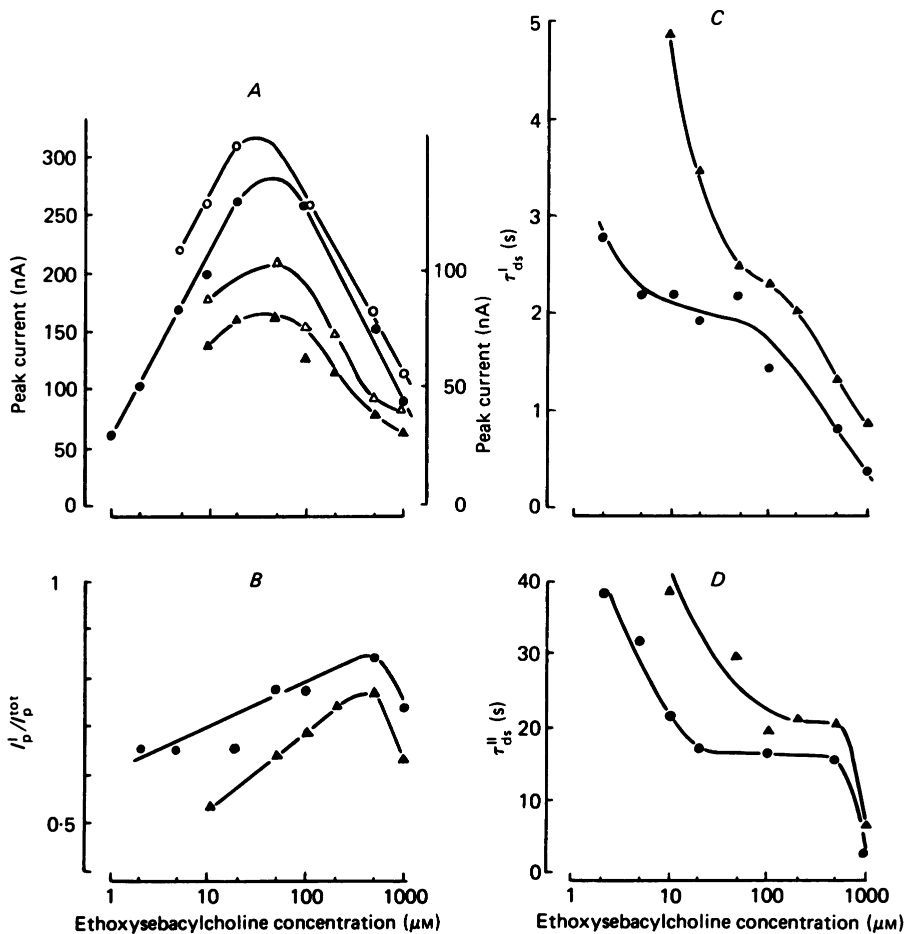

Fig. 7. Peak current and parameters of desensitization as functions of ethoxysebacylcholine concentration. *A*, peak currents induced in two neurones by ethoxysebacylcholine (closed symbols); open symbols are corrected values for measured I_ps found by back extrapolation of both desensitization components to the rising phases. Note that the shapes of corrected dose–response curves are not distinct from those obtained experimentally and that the most effective concentrations are the same. The left ordinate is for circles, and the right ordinate for triangles. *B*, *C*, *D*, the fraction of the fast component in desensitization (*B*), τ^{I}_{ds} (*C*) and τ^{II}_{ds} (*D*) are plotted against ethoxysebacylcholine concentration for the same two neurones. The holding potentials were −50 mV (circles) and −40 mV (triangles).

agonist concentrations until they reached plateau values comparable to those obtained with ACh for concentrations of 10–100 μM. However, when the agonist concentration was raised above 100 μM, both time constants decreased again (Fig. 7*C* and *D*).

Recovery from desensitization

Fig. 8 illustrates an experiment on recovery from the desensitization induced by prolonged application of 50 μM-ACh (Fig. 8*A*, left). The ionophoretic current and the frequency of repetitive ACh test pulses were adjusted to induce responses of constant

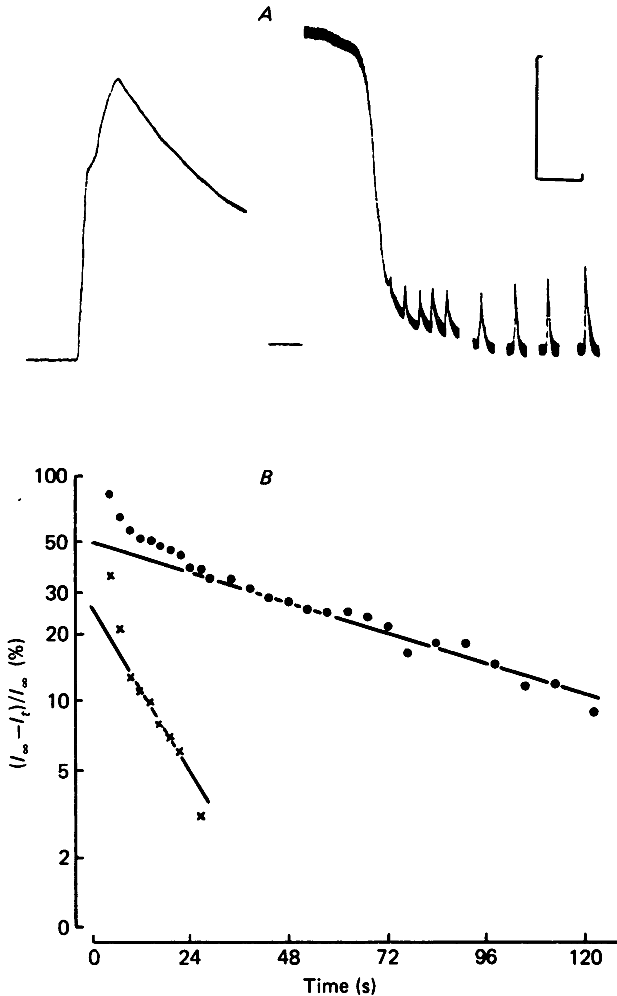

Fig. 8. Recovery from desensitization. *A*, neurone response to bath application of 50 μM-ACh (left), the record is interrupted for 1 min. Before wash-out of ACh, the gain was augmented by 10 and the sweep was slowed (calibrations are 200 nA and 1 s for the left part and 20 nA and 8 s for the right part). During the wash-out of ACh the current induced by ACh declined from I_{ss} to 0 in about 8 s. Ionophoretic test pulses of ACh were applied under continuous flow of physiological solution; the frequency of pulses (1/2·4 s) was adjusted to provide responses of constant amplitude in the control (not shown). Responses to the 1, 2, 3, 4, 5, 11, 31, 51, and 81 test pulses after wash-out of desensitizing ACh are presented (right). Test responses reach constant amplitude (I_∞) at approximately the 80th pulse (after about 3 min). *B*, the progressive decrease of the extent of desensitization, $(I_\infty - I_t)/I_\infty$, is plotted in semilogarithmic coordinates against *t*. Time 0 corresponds to the beginning of ACh wash-out. Two exponentials are plotted as in Fig. 1. In drawing the line of the fast component the first two points were not taken into account because they are presumed to be over-estimated (I_ts underestimated) due to the slow decline of the current induced by the conditioning dose of ACh. The time constants of the fast and slow components of recovery are 14 and 79 s, respectively. The contributions of the fast and slow components are 33 and 67 %, respectively.

amplitude in the control. After the steady-state level of the current induced by bath-applied ACh had been reached, wash-out of ACh was initiated through the superfusion tube. Responses to several test pulses after removal of 50 μM-ACh are shown on the right of Fig. 8*A*. The first two responses appear to be attenuated due to non-instantaneous disappearance of the current induced by the conditioning dose of ACh. The test responses reach constant amplitude (denoted I_{∞}) in about 3 min (the last test response in Fig. 8*A*). The extent of desensitization at time *t* after removal of bath-applied ACh is assumed to be proportional to $(I_{\infty}-I_t)/I_{\infty}$, i.e. to the relative reduction of the test response amplitude. A semilogarithmic plot of $(I_{\infty}-I_t)/I_{\infty}$ against time (Fig. 8*B*) shows that the recovery follows the sum of two exponentials with time constants (τ_r^{I} and τ_r^{II}) of 14 and 79 s, respectively. Approximately 67 % of the recovery occurs during the slow phase.

The estimation of τ_r^{I} is not very precise since this time constant is of the same order as the interval between ionophoretic current pulses (higher frequency of ACh applications is impossible because it would induce desensitization) and also because the amplitude of the fast component is small (it usually does not exceed 25 % at room temperature, in our conditions, i.e. after a prolonged exposure to the desensitizing drug). It was therefore impossible to study the dependence of τ_r^{I} on agonist structure and concentration as well as on membrane potential. The mean value of τ_r^{I} was 11 ± 1 s (mean $\pm$S.E. of the mean, n = thirteen neurones).

A difference in τ_r^{II} was observed between neurones from the right and left parietal ganglia: τ_r^{II} is equal to 84 ± 13 s (mean $\pm$S.E. of the mean, n = eight neurones) and 162 ± 13 s (mean $\pm$S.E. of the mean, n = fourteen neurones), respectively.

Cooling delays the slow recovery component and increases the fast component fraction from $0{\cdot}27 \pm 0{\cdot}05$ to $0{\cdot}64 \pm 0{\cdot}06$. The change of τ_r^{II}/10 °C in the range from 22 to 2 °C is equal to $3{\cdot}0 \pm 0{\cdot}5$ (mean $\pm$S.E. of the mean, n = six neurones); τ_r^{I} is practically independent of temperature ($Q_{10} = 1{\cdot}1 \pm 0{\cdot}1$, n = five neurones).

To study the effect of agonist structure on the slow recovery component two or three drugs (ACh, methyl TMA, and pentyl TMA; ACh and succinylcholine; methyl TMA and pentyl TMA; ACh and suberylcholine) were tested as desensitizing agents on the same neurone and the recovery was measured by the gradual increase of the response to ACh ionophoretic micro-application after wash-out of the drug. At room temperature and at a membrane voltage near the resting potential level, the ratio of τ_r^{II}s for each pair of drugs is close to 1. Thus, recovery proceeds with the same kinetics independently of the desensitizing agonist as was shown earlier at the frog neuromuscular junction (Katz & Thesleff, 1957; Feltz & Trautmann, 1982).

DISCUSSION

Kinetics of desensitization and recovery

Desensitization of acetylcholine receptors in *L. stagnalis* neurones proceeds according to the sum of two distinct exponentials (Fig. 1*B*) just as in the frog muscle end-plate (Feltz & Trautmann, 1980, 1982) and frog denervated muscle (Sakmann *et al.* 1980). This observation differs from those of other authors who described mono-exponential desensitization at neuromuscular junctions and electric eel electroplaques. Analysis of the data on monophasic response decay shows that the

desensitization rate constants were grouped in two ranges: from tenths of a reciprocal second to 2/s (Katz & Thesleff, 1957; Adams, 1975; Magazanik & Vyskočil, 1975; Scubon-Mulieri & Parsons, 1978; Fiekers *et al.* 1980) and from tenths of a reciprocal minute to 3·9/min (Manthey, 1970; Scubon-Mulieri & Parsons, 1977; Rang & Ritter, 1970*a*; Lester *et al.* 1975; Pallotta & Webb, 1980). The first range is close to k_{ds}^{I} (this paper; Feltz & Trautmann, 1980; Sakmann *et al.* 1980) and to the rate constant of intrinsic fluorescence quenching of membrane-bound ACh receptors in the presence of agonists (Barrantes, 1978); the second range is of the same order as k_{ds}^{II} (k_{ds}^{I} and k_{ds}^{II} are reciprocals of the extreme values of τ_{ds}^{I} and τ_{ds}^{II}, respectively).

The discrepancy between the data concerning both the number of components in desensitization and the values of k_{ds} are probably due to differences in experimental procedures. (i) The fast exponential can be revealed only when drug application is sufficiently rapid (Andreev & Vulfius, 1980; Feltz & Trautmann, 1980, 1982); perfusion or superfusion at a rate comparable to that of fast desensitization or slower (Adams, 1975; Lester *et al.* 1975; Fiekers *et al.* 1980; Pallotta & Webb, 1980) slows down the rising of the current and increases τ_{ds}^{I}, bringing it close to τ_{ds}^{II}. (ii) Measurements of the response by muscle contraction, depolarization (Rang & Ritter, 1970*a*), or input resistance changes (Manthey, 1970; Scubon-Mulieri & Parsons, 1977) result in an underestimation of the rate of desensitization as compared with the voltage-clamp technique (cf. Adams, 1975; Scubon-Mulieri & Parsons, 1978; Fiekers *et al.* 1980) and may obscure the fast component. (iii) To distinguish reliably between the two components of desensitization, I_{ss} must be known, i.e. prolonged exposure to the agonist (up to 2–5 min) is needed. (iv) Under repetitive ionophoretic drug application, the slow desensitization component does not develop sufficiently to allow its analysis (see discussion by Feltz & Trautmann, 1982).

The rate of recovery is also influenced by the methods of drug wash-out and response recording (Adams, 1975; Feltz & Trautmann, 1982). As is evident from the data at the frog muscle end-plate (Feltz & Trautmann, 1982), frog denervated muscle (Sakmann *et al.* 1980) and *Lymnaea* neurones (Andreev *et al.* 1980; this paper) recovery is a two-exponential process with τ_{r}^{I} and τ_{r}^{II} varying in the ranges of seconds and minutes, respectively. Thus, complete removal of the desensitizing drug should take less than a second and ACh test pulses should be delivered at a frequency of one per second or higher and for a sufficiently long time (several minutes). The reason for which the fast recovery component was not detected in earlier work (Rang & Ritter, 1970*a*; Scubon-Mulieri & Parsons, 1977, 1978; Bregestovski *et al.* 1979) is probably the slow wash-out of the agonist and the low frequency of the test pulses.

Temperature dependence of the two components of desensitization onset and of desensitization recovery

Both for onset and for recovery, the two components of desensitization differ in their dependence on temperature.

The time constant of the fast component of desensitization onset increases considerably upon cooling ($Q_{10} = 2·8$). This is consonant with the data on the strong temperature dependence of desensitization at the frog neuromuscular junction (Magazanik & Vyskočil, 1975) as well as with earlier results from *Lymnaea* neurones (Bregestovski, 1975). In contrast, τ_{ds}^{II} is almost independent of temperature. The

opposite is evident for recovery: τ_r^{II} lengthens remarkably upon cooling ($Q_{10} = 3$) in agreement with the data of Magazanik & Vyskočil (1975), and τ_r^{I} does not change. The fractions of the fast component in desensitization and of the slow component in recovery are considerably reduced at low temperature.

It is tempting to conclude from these observations that the components having a similar temperature dependence correspond to a common process. Thus, the fast component of desensitization onset would be correlated with the slow component of recovery, while the slow component of the onset would be correlated with fast recovery.

The exact description of the two processes remains to be done. It is worth noting that the cyclic model first proposed by Katz & Thesleff (1957) has been strongly supported by both electrophysiological (Feltz & Trautmann, 1980, 1982; Sakmann *et al.* 1980) and biochemical observations (Neubig & Cohen, 1980; Heidmann & Changeux, 1980) and that a 'double cyclic scheme' as proposed by Feltz & Trautmann (1982) and Heidmann & Changeux (1980) is likely to account also for our observations.

Dependence of desensitization on agonist structure

For six of the compounds tested (three monocholinic esters: ACh, butyrylcholine, caprylylcholine; three alkyl trimethylammonium salts: methyl TMA, ethyl TMA, butyl TMA) the two desensitization processes appeared essentially similar. (i) τ_{ds}^{I} decreased steeply when the agonist concentration was increased, and reached similar plateau values at agonist concentrations corresponding to the maximal responses. (ii) τ_{ds}^{II} also decreased when the agonist concentration was increased, although less steeply than τ_{ds}^{I}. The plateau value was, here again, in the same range for the six agonists, although a greater dispersion of the data does not allow as firm a conclusion as with τ_{ds}^{I}. (iii) The time constant of the fast recovery component was not determined with sufficient accuracy to allow conclusions to be drawn on the possible effects of agonist structure. On the other hand, the time constant of the slow component of the recovery (the main component in our conditions) was the same for all drugs tested.

In general our observations agree with those of previous authors suggesting that a number of small monoquaternary agonists induce similar desensitization onset (Katz & Thesleff, 1957; Magazanik, 1968; Feltz & Trautmann, 1982) and desensitization recovery (Katz & Thesleff, 1957; Rang & Ritter, 1970*a*; Feltz & Trautmann, 1982).

With some of the agonists tested, however, desensitization did not follow the pattern described for ACh. At high agonist concentrations, the agonist-induced current decreased and desensitization was speeded beyond its usual maximal rate. It is not possible at present to decide whether these effects indicate that the rate constants of desensitization are in fact different according to the agonist used, or whether, when one uses bulky agonists, one introduces an additional process. It has been shown in recent years, that some agonists have antagonist actions on the 'activated' receptor channel complex, leading to a decline of the response mimicking desensitization (e.g. Adams & Sakmann, 1978). That such a process could account for desensitization has been suggested by Adams (1975) for ACh. This hypothesis has not been further supported by more detailed studies of ACh-induced desensitization

in frog muscle (see in particular, Sakmann *et al.* 1980). However, it is not impossible that such a 'sequential' scheme may be applicable to the effects of the bulky agonists observed at high concentrations.

We thank Academician O. L. Mndjoyan, Professor I. J. Kvitko and Drs I. P. Andrianova and E. A. Gracheva for kindly providing us with some ACh analogues, Drs P. D. Bregestovski and V. I. Iljin for valuable comments and Mrs Olga N. Shvirst for the help in preparing the manuscript.

REFERENCES

ADAMS, P. R. (1975). A study of desensitization using voltage clamp. *Pflügers Arch.* **360**, 135–144.

ADAMS, P. R. & SAKMANN, B. (1978). Decamethonium both opens and blocks endplate channels. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2994–2998.

ANDREEV, A. A. & VULFIUS, C. A. (1980). Two components of desensitization of acetylcholine receptors in mollusc neurones. *Stud. Biophys.* **78**, 87–93.

ANDREEV, A. A., VULFIUS, C. A. & VEPRINTSEV, B. N. (1980). Two mechanisms of desensitization of acetylcholine receptors at *Lymnaea stagnalis* neurones. *Dokl. Akad. Nauk SSSR* **253**, 1490–1492.

BARRANTES, F. J. (1978). Agonist-mediated changes of the acetylcholine receptor in its membrane environment. *J. molec. Biol.* **124**, 1–26.

BREGESTOVSKI, P. D. (1975). On the influence of the life-time of acetylcholine-receptor complex on the end-plate equilibrium potential. In *Nature of Acetylcholine Receptor and Structure of its Active Site*, ed. VEPRINTSEV, B. N. & VULFIUS, C. A., pp. 140–148. Pushchino: U.S.S.R. Acad. Sci. (in Russian).

BREGESTOVSKI, P. D., BUKHARAEVA, E. A. & ILJIN, V. I. (1979). Voltage clamp analysis of acetylcholine receptor desensitization in isolated mollusc neurones. *J. Physiol.* **297**, 581–595.

BREGESTOVSKI, P. D. & ILJIN, V. I. (1980). Effect of 'calcium antagonist' D-600 on the postsynaptic membrane. *J. Physiol., Paris* **76**, 515–522.

CHEMERIS, N. K., KAZACHENKO, V. N., KISLOV, A. N. & KURCHIKOV, A. L. (1982). Inhibition of acetylcholine responses by intracellular calcium in *Lymnaea stagnalis* neurones. *J. Physiol.* **323**, 1–19.

DE WITT, N. D. (1977). Evidence for the independent regulation of specific ions in the haemolymph of *Lymnaea stagnalis* (L.). *Proc. K. ned. Akad. Wet. Series C. Biological and Medical Sciences* **80**, 144–157.

FELTZ, A. & TRAUTMANN, A. (1980). Interaction between nerve-released acetylcholine and bath applied agonists at the frog end-plate. *J. Physiol.* **299**, 533–552.

FELTZ, A. & TRAUTMANN, A. (1982). Desensitization at the frog neuromuscular junction: a biphasic process. *J. Physiol.* **322**, 257–272.

FIEKERS, J. F., SPANNBAUER, P. M., SCUBON-MULIERI, B. & PARSONS, R. L. (1980). Voltage dependence of desensitization. Influence of calcium and activation kinetics. *J. gen. Physiol.* **75**, 511–529.

HEIDMANN, T. & CHANGEUX, J.-P. (1980). Interaction of a fluorescent agonist with the membrane-bound acetylcholine receptor from *Torpedo marmorata* in the millisecond time range: resolution of an 'intermediate' conformational transition and evidence for positive cooperative effects. *Biochem. biophys. Res. Commun.* **97**, 889–896.

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol.* **138**, 63–80.

KISLOV, A. N. & KAZACHENKO, V. N. (1974). Ion current of excited cholinergic membrane of the giant isolated snail neurones. In *Biophysics of a Living Cell*, vol. 4, ed. FRANK, G. M., pp. 39–44. Pushchino: U.S.S.R. Acad. Sci. (in Russian).

KOSTENKO, M. A., GELETYUK, V. I. & VEPRINTSEV, B. N. (1974). Completely isolated neurones in the mollusc *Lymnaea stagnalis*. A new objective for nerve cell biology investigation. *Comp. Biochem. Physiol.* **49**A, 89–100.

LESTER, H. A., CHANGEUX, J.-P. & SHERIDAN, R. E. (1975). Conductance increases produced by bath application of cholinergic agonists to *Electrophorus* electroplaques. *J. gen. Physiol.* **65**, 797–816.

MAGAZANIK, L. G. (1968). Mechanism of desensitization of the post-synaptic membrane of the muscle fibre. *Biofizika* **13**, 199–202 (in Russian).

MAGAZANIK, L. G. & VYSKOČIL, F. (1970). Dependence of acetylcholine desensitization on the membrane potential of frog muscle fibre and on the ionic changes in the medium. *J. Physiol.* **210**, 507–518.

MAGAZANIK, L. G. & VYSKOČIL, F. (1975). The effect of temperature on desensitization kinetics at the post-synaptic membrane of the frog muscle fibre. *J. Physiol.* **249**, 285–300.

MANTHEY, A. A. (1970). Further studies of the effect of calcium on the time course of action of carbamylcholine at the neuromuscular junction. *J. gen. Physiol.* **56**, 407–419.

NEUBIG, R. R. & COHEN, J. B. (1980). Permeability control by cholinergic receptors in *Torpedo* postsynaptic membranes: Agonist dose–response relations measured at second and millisecond times. *Biochemistry* **19**, 2770–2779.

PALLOTTA, B. S. & WEBB, G. D. (1980). The effects of external Ca^{++} and Mg^{++} on the voltage sensitivity of desensitization in *Electrophorus* electroplaques. *J. gen. Physiol.* **75**, 693–708.

RANG, H. P. (1975). Acetylcholine receptors. *Q. Rev. Biophys.* **7**, 283–399.

RANG, H. P. & RITTER, J. M. (1970*a*). On the mechanism of desensitization at cholinergic receptors. *Molec. Pharmacol.* **6**, 357–382.

RANG, H. P. & RITTER, J. M. (1970*b*). The relationship between desensitization and the metaphilic effect at cholinergic receptors. *Molec. Pharmacol.* **6**, 383–390.

SAKMANN, B., PATLAK, J. & NEHER, E. (1980). Single acetylcholine-activated channels show burst-kinetics in presence of desensitizing concentrations of agonist. *Nature, Lond.* **286**, 71–73.

SCUBON-MULIERI, B. & PARSONS, R. L. (1977). Desensitization and recovery at the frog neuromuscular junction. *J. gen. Physiol.* **69**, 431–477.

SCUBON-MULIERI, B. & PARSONS, R. L. (1978). Desensitization onset and recovery at the potassium-depolarized frog neuromuscular junction are voltage sensitive. *J. gen. Physiol.* **71**, 285–299.

SUÁREZ-ISLA, B. & HUCHO, F. (1977). Acetylcholine receptor: -SH group reactivity as indicator of conformational changes and functional states. *FEBS Lett.* **76**, 65–69.

TAUC, L. & BRUNER, J. (1963). 'Desensitization' of cholinergic receptors by acetylcholine in molluscan central neurones. *Nature, Lond.* **198**, 33–34.

WEBER, M., DAVID-PFEUTY, T. & CHANGEUX, J.-P. (1975). Regulation of binding properties of the nicotinic receptor protein by cholinergic ligands in membrane fragments from *Torpedo marmorata*. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3443–3447.