

ACCELERATION OF DESENSITIZATION BY AGONIST PRE-TREATMENT IN THE SNAKE

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SUMMARY

1. The kinetics of carbachol-induced desensitization have been studied in snake twitch-muscle fibres maintained in an isotonic potassium propionate solution and voltage clamped to +50 mV.

2. Microperfusion of carbachol (162–756 μM) induces a transient outward current which peaks within a few seconds and then slowly decays towards the base line. The time course of current decay estimates the time course of desensitization onset.

3. Brief exposure (30 s) to a 'conditioning' concentration of agonist (10.8 μM) accelerates the desensitization onset produced by exposure to higher 'test' concentrations of agonist (162–756 μM).

4. The acceleration of desensitization by pre-treatment with 10.8 μM -carbachol was independent of the duration of exposure between 15 and 60 s. This observation indicated that the mechanism responsible for the alteration in desensitization kinetics by treatment with 10.8 μM -carbachol differed from that responsible for the time-dependent development of desensitization produced in the presence of higher carbachol concentrations.

5. Pre-treatment with the muscarinic agonists, methylcholine and bethanechol, did not accelerate 216 μM -carbachol-induced desensitization, suggesting that the alteration of desensitization kinetics by pre-treatment was specific for nicotinic agonists.

6. The conditioning concentrations of carbachol (5.4–10.8 μM) produced no measurable outward current in muscle fibres voltage clamped to +50 mV. Further, in patch-clamp recordings it was observed that, with these concentrations of carbachol, there was no channel activity in many successful patches voltage clamped to +50 mV and, when present, the frequency of channel activity was very low. These results demonstrated that the alteration in desensitization was not the consequence of significant amounts of either receptor activation or desensitization produced by the conditioning concentration.

7. Exposure to 10.8 μM -carbachol for periods of up to 150 s did not change the amplitude of miniature end-plate currents recorded at end-plates voltage clamped to +50 mV. These results also demonstrated that the acceleration of desensitization by pre-treatment with conditioning concentrations of agonist was not due to partial desensitization occurring during the pre-treatment period.

8. Our results are consistent with the view that there are distinct populations of agonist binding sites on the acetylcholine receptor which separately regulate desensitization and channel opening. Further, we suggest that the binding of agonist to the 'desensitization sites' (i.e. the conditioning concentration) promotes the fast phase of desensitization produced during subsequent exposure to 'test' concentrations of carbachol. Consequently, the fast phase of desensitization is accelerated and contributes more to the development of desensitization.

INTRODUCTION

During sustained exposure to agonist a progressive loss of chemosensitivity occurs. This process, known as desensitization, is a characteristic response shared by most receptor-mediated events. Although studied extensively, especially in the case of the nicotinic receptor-channel complex, the molecular events responsible for the development of desensitization remain unknown. Further, the time course of the development of desensitization measured electrophysiologically often exhibits two exponential components: an initial fast phase followed by a second more slowly developing phase (Feltz & Trautmann, 1980, 1982; Chestnut, 1983; Connor, Fiekers, Neel, Parsons & Schnitzler, 1984). Because many pharmacological agents appear to alter the kinetics of desensitization onset without producing demonstrable effects on the kinetics of receptor-channel activation, it has been proposed that there may be distinct sites on the receptor-channel complex which regulate these two processes independently (Conti-Tronconi & Raftery, 1982). Further, the results of recent biochemical studies using isolated *Torpedo* acetylcholine receptor (AChR) indicate that there may also be two distinct classes of agonist binding sites; a high-affinity site regulating desensitization ('desensitization site') and low-affinity site controlling membrane permeability (Dunn & Raftery, 1982; Conti-Tronconi & Raftery, 1982, 1986; Raftery, Conti-Tronconi, Dunn, Crawford & Middlemas, 1984).

The present experiments were undertaken to test whether desensitization in intact muscle preparations also may be regulated by a second class of agonist-binding sites which are separate from the binding sites controlling membrane permeability. In brief, the results of this study demonstrate that prior exposure to low agonist concentrations accelerates the development of desensitization primarily by accentuating the fast phase of desensitization, and are consistent with the view that agonist binding to a separate population of desensitization sites promotes the fast phase of desensitization. A preliminary account of some of these experiments has been presented in abstract form (Parsons, Neel & Fiekers, 1986).

METHODS

General methods

All experiments were done on voltage-clamped twitch-fibre end-plates in the costocutaneous muscle of the garter snake (*Thamnophis*). The method of dissection and the criteria used for identification of twitch fibres were similar to those published previously (Connor *et al.* 1984). The muscle preparations were bathed in a HEPES-buffered isotonic potassium propionate solution of composition (mM): potassium propionate, 161; CaCl₂, 1.0; MgCl₂, 4.2; CsCl, 5.0; HEPES, 1.0, pH = 7.2; 20–23 °C. Potassium-depolarized preparations were used for these experiments to eliminate muscle contraction during exposure to agonist in adjacent muscle fibres which were not

voltage clamped. A few experiments were also done on muscle preparations in which the nerve terminals were removed by enzymatic dissociation following the procedures described previously (Betz & Sakmann, 1973).

The end-plate region of individual muscle fibres was voltage clamped using two-micro-electrode voltage-clamp system identical to that used in previous studies (Fiekers, Spannauer, Scubon-Mulieri & Parsons, 1980; Connor *et al.* 1984). Carbachol and other drugs were applied locally to the end-plate region of individual voltage-clamped fibres by hydrostatically controlled microperfusion (Fiekers *et al.* 1980; Connor *et al.* 1984). Because the neuromuscular junction in snake twitch fibres is very compact, agonist concentration equilibrates quickly and effective voltage control can be maintained over the entire end-plate region during agonist application (Connor *et al.* 1984).

We chose to undertake these experiments on muscle fibres voltage clamped to +50 mV for the following reasons. First, the peak agonist-induced current amplitudes at positive membrane potentials are smaller than at negative membrane potentials with similar agonist concentrations (Connor *et al.* 1984). Therefore, at positive potentials adequate voltage control can be maintained over a wider range of agonist concentrations, and the muscle contractions produced with large agonist-induced currents are minimized. Secondly, since desensitization onset is voltage dependent (Magazanik & Vyskocil, 1970; Fiekers *et al.* 1980; Magleby & Pallotta, 1981; Connor *et al.* 1984), it is possible to determine the fast component of desensitization at positive membrane potentials more accurately. This is especially important with the microperfusion technique which requires 1–2 s for agonist to equilibrate over the end-plate region. Lastly, high concentrations of agonist are known to produce channel blockade (Sine & Steinbach, 1984*a, b*); however, because open-channel blockade is a voltage-dependent process, it does not occur as readily at positive membrane potentials.

All results are expressed as the mean \pm s.e. of mean obtained in a number of different fibres.

Estimation of the extent of receptor-channel activation and time course of desensitization during sustained agonist exposure

The peak value of the carbachol-induced end-plate current (e.p.c._{carb}) estimates the maximal extent of activation (Fiekers *et al.* 1980; Connor *et al.* 1984). The time course of decline of the agonist-induced current from the peak towards the base line, with continuous agonist application, indicates the onset of desensitization (Fiekers *et al.* 1980; Connor *et al.* 1984). In the present study the time course of current decay was fitted using a computerized non-linear, exponential-fitting program as described previously (Connor *et al.* 1984). Because the time course of decay of the current often exhibits more than one component, the decay phase of each individual current was fitted either with a single- or two-exponential expression. If the root mean square of the error to the fit was reduced by more than 50% with two exponentials, then it was assumed that the decay was composed of two components. Under the conditions of the present study, the decay time course of the e.p.c._{carb} in most experiments required two components for adequate fitting. The relative contribution of the two decay components to the decay time course was approximated using the ratio of the amplitude of individual slow and fast current components (I_s/I_f) extrapolated back to the peak e.p.c._{carb} (Connor *et al.* 1984).

A double-perfusion technique was used to test the influence of brief pre-treatment with a conditioning concentration of carbachol (5–10.8 μM) on the desensitization time course occurring during a subsequent exposure to a higher test concentration of carbachol (162–756 μM). Individual voltage-clamped end-plates were initially perfused with the conditioning concentration for 15–60 s prior to the application of a test concentration.

Patch-clamp procedures for single-channel measurements

The patch-clamp recording technique was used on enzymatically treated muscle preparations to estimate the single-channel activity initiated by exposure to low carbachol concentrations. The procedures used to obtain stable single-channel recordings were similar to those described in detail previously (Hamill, Marty, Neher, Sakmann & Sigworth, 1981; Dionne & Leibowitz, 1982). After removal of the nerve terminal, the end-plate region of individual snake muscle fibres can readily be identified for accurate placement of the patch pipette. In all experiments the muscle preparations were maintained in the isotonic potassium solution with 5.4–16.2 μM -carbachol in the patch electrode. All recordings were made in the cell-attached mode (Hamill *et al.* 1981).

RESULTS

Pre-treatment with 10.8 μM -carbachol accelerates the onset of desensitization produced during a subsequent exposure to 216 μM -carbachol

In the majority of fibres voltage clamped to +50 mV and perfused with 216 μM -carbachol, the e.p.c._{carb.} decay exhibited two components: one with a time constant of a few seconds and a second slower component having a time constant of tens of seconds. In control cells most of the current decay time course is represented by the slower component.

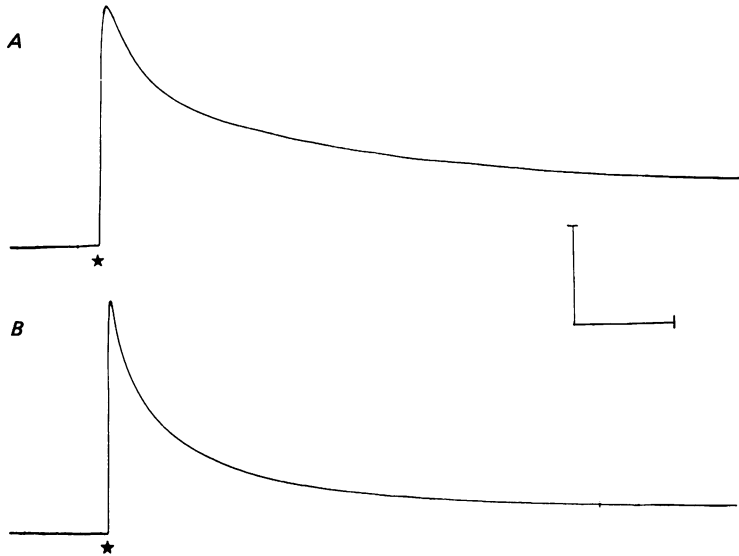


Fig. 1. Examples of the e.p.c._{carb.} measured during continued microperfusion of 216 μM -carbachol to a control twitch-fibre end-plate (*A*) and to another twitch fibre pre-treated with 10.8 μM -carbachol for 30 s (*B*). The stars indicate the initiation of the 216 μM -carbachol application. The peak current value in *A* was 257 nA and the two time constants of decay were 6.1 and 81.6 s. The peak current value in *B* was 329 nA and the two time constants of decay were 4.2 and 23.0 s. The ratio of the two current components, I_s/I_f , was 1.5 in the case of the control fibre and 1.2 for the fibre pre-treated with 10.8 μM -carbachol. The calibration is: Y axis, 100 nA in *A* and 125 nA in *B*; X axis, 20 s.

However, if other fibres maintained under identical conditions are exposed to a conditioning concentration of 10.8 μM -carbachol for 30 s, the development of desensitization during a subsequent exposure to 216 μM -carbachol is markedly accelerated. The acceleration of desensitization was characterized by a decrease in the value of both the fast and slow component time constants as well as by an increased contribution by the fast component to the total current decay time course (i.e. the I_s/I_f ratio was decreased).

Example records which illustrate the time course of the current induced with 216 μM -carbachol in a control fibre (*A*) and in a second fibre pre-treated with 10.8 μM -carbachol for 30 s (*B*) are presented in Fig. 1.

The acceleration of desensitization by the conditioning concentration occurs with brief period of exposure

Additional experiments were undertaken to determine whether the acceleration of 216 μM -carbachol-induced desensitization by the conditioning concentration was dependent upon the duration of pre-treatment. In these experiments the duration of exposure ranged from 15 to 60 s in nine different fibres. At least two experiments

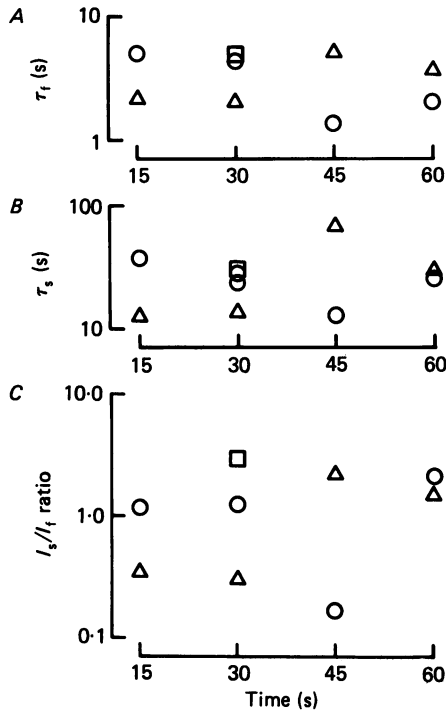


Fig. 2. Results which demonstrate that the acceleration of desensitization by pre-treatment with 10.8 μM -carbachol is similar with exposure times between 15 and 60 s. *A*, the time constant for the fast phase of desensitization (τ_f) obtained in nine different fibres. *B*, the time constant for the slow phase of desensitization (τ_s) in the same nine fibres. *C*, the I_s/I_f ratio calculated for these same nine fibres. The common symbols at each time point represent values obtained in the same fibre.

were done at each time point. The results of these experiments, which are presented in Fig. 2, demonstrate that the alteration in desensitization occurs within 15 s of exposure and remains relatively constant over a 1 min period. Because of this, the results of the initial experiments with different exposure durations were grouped together, and for most of the subsequent experiments the duration of exposure was limited to 30 s.

Pre-treatment with conditioning concentrations accelerates the desensitization onset produced by a range of test concentrations of carbachol

We also determined whether pre-treatment with a fixed conditioning concentration accelerated the desensitization produced by different test concentrations of carba-

chol. In this series of experiments, the conditioning concentration was either 5.4 or 10.8 μM -carbachol, and the test concentration of carbachol ranged between 162 and 756 μM . We were unable to use carbachol concentrations greater than 756 μM as the test concentrations because with higher concentrations muscle contractures occurred which interfered with the estimate of the e.p.c._{carb.} decay time course.

At the lowest test concentration of carbachol, 162 μM , pre-treatment with 10.8 μM -carbachol appeared to accelerate the onset of desensitization. However, only four experiments (two control and two test) were done with 162 μM -carbachol as the test concentration because the results between the two groups could not be compared quantitatively. In the two control fibres the decay time course was adequately described by a single-exponential function; whereas, in the two fibres pre-treated with 10.8 μM -carbachol for 30 s, the e.p.c._{carb.} decay time course consisted of two exponential components. Therefore, to demonstrate the difference in the e.p.c._{carb.} decay time course in the two control and two pre-treated fibres, we used half-time decay values. The half-time values for the control fibres were 88.0 and 60.5 s whereas those for the fibres pre-treated with 10.5 μM -carbachol were 57.5 and 55.0 s.

At the higher test concentrations of carbachol (216–756 μM) the e.p.c._{carb.} decay in most experiments for both control fibres and pre-treated fibres exhibited a two-component decay time course. The only exceptions observed were one control fibre and one pre-treated fibre in which the test concentration of carbachol was 216 μM . In these two cases, the e.p.c._{carb.} decay was adequately fitted as a single-exponential function, with the decay time constant 34.9 s for the control fibre and 16.6 s for the fibre pre-treated with 10.8 μM -carbachol.

In control fibres, as expected from the results of previous studies, desensitization developed more rapidly as the test concentration of carbachol was increased. The acceleration of desensitization resulted from a decrease in the time constants of both the fast and slow components, as well as greater contribution of the fast phase of desensitization to the total current decay (Feltz & Trautmann, 1982; Connor *et al.* 1984). Pre-treatment with 10.8 μM -carbachol consistently accelerated the desensitization produced by all test concentrations of carbachol used. In contrast, pre-treatment with 5.4 μM carbachol produced a significant acceleration of desensitization only at the highest test concentration. In the case of those fibres in which the test concentration of carbachol was 216 μM , the acceleration of desensitization was due to a decrease in both the fast and slow time constants as well as a greater proportion of the current decay being represented by the fast component. In those experiments in which the test concentration was 324 or 756 μM , pre-treatment accelerated the subsequent desensitization by decreasing the fast-component time constant and increasing the contribution of the fast-component current to the total decay time course without affecting the value of the slow-component time constant. A summary of the results obtained in this series of experiments is presented in Table 1.

The e.p.c._{carb.} amplitude varied considerably between end-plates at the same membrane potential and carbachol concentration. However, the slow and fast time constants of desensitization onset were independent of peak current amplitude, as shown in Fig. 3. The peak e.p.c._{carb.} value generally was less in those fibres pre-treated

with $10.8 \mu\text{M}$ -carbachol than in control fibres (Table 1). Although this decrease was present with all concentrations of carbachol used, the change was not significantly different in all experiments.

TABLE 1. Acceleration of carbachol-induced desensitization by pre-treatment with low agonist concentrations

Conditioning conc. (μM)	Test conc. (μM)	Peak e.p.c. (nA)	τ_f (s)	τ_s (s)	I_s/I_f	n
—	216	260 ± 37	7.7 ± 1.0	60.0 ± 8.9	2.7 ± 0.6	13
5.4	216	279 ± 60	7.0 ± 1.9	47.1 ± 10.4	1.0 ± 0.2	4
10.8	216	210 ± 19	3.7 ± 0.5	34.0 ± 6.7	1.4 ± 0.3	11
—	324	534 ± 63	8.5 ± 1.0	44.1 ± 5.8	1.1 ± 0.3	4
5.4	324	376 ± 62	6.8 ± 1.7	49.4 ± 13.1	2.2 ± 1.0	5
10.8	324	378 ± 34	4.0 ± 0.5	40.1 ± 6.4	0.7 ± 0.1	12
—	756	1738 ± 350	4.0 ± 0.4	21.7 ± 1.1	0.8 ± 0.1	5
5.4	756	1100 ± 195	1.8 ± 0.2	21.3 ± 1.2	0.3 ± 0.0	3
10.8	756	1467 ± 455	2.1 ± 0.5	19.0 ± 6.5	0.4 ± 0.1	3

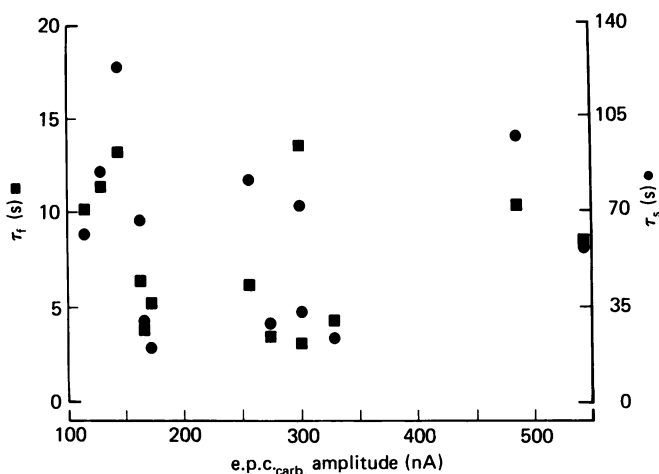


Fig. 3. The e.p.c._{carb.} fast (■) and slow (●) decay time constants plotted as a function of the peak e.p.c._{carb.} amplitude for thirteen control fibres voltage clamped to +50 mV and perfused with $216 \mu\text{M}$ -carbachol.

Pre-treatment with comparable concentrations of the muscarinic agonists methylcholine and bethanechol do not accelerate carbachol-induced desensitization

We investigated whether muscarinic agonists would produce an acceleration of carbachol-induced desensitization which was similar to that seen with pre-treatment with low concentrations of carbachol. A 30 s pre-treatment with either $25 \mu\text{M}$ -methylcholine or $25 \mu\text{M}$ -bethanechol had no significant effect on the peak e.p.c._{carb.} value or the time course of desensitization produced by $216 \mu\text{M}$ -carbachol. These results are summarized in Table 2.

TABLE 2. Muscarinic agonist do not accelerate carbachol-induced desensitization

Condition	Peak size (nA)	τ_r (s)	τ_s (s)	I_s/I_r	n
Control	185 ± 31	8.4 ± 1.6	75.4 ± 12.4	3.0 ± 1.0	6
25 μ M-methylcholine	192 ± 9	7.2 ± 2.2	47.5 ± 6.6	2.8 ± 1.2	4
25 μ M-bethanechol	211 ± 49	6.7 ± 3.4	60.5 ± 16.7	2.0 ± 0.4	4

Pre-treatment accelerates desensitization observed enzymatic removal of the nerve terminals

Experiments were undertaken to demonstrate that brief exposure to a conditioning concentration accelerates the desensitization produced by a test concentration after enzymatically removing the presynaptic nerve terminals (Betz & Sakmann, 1973).

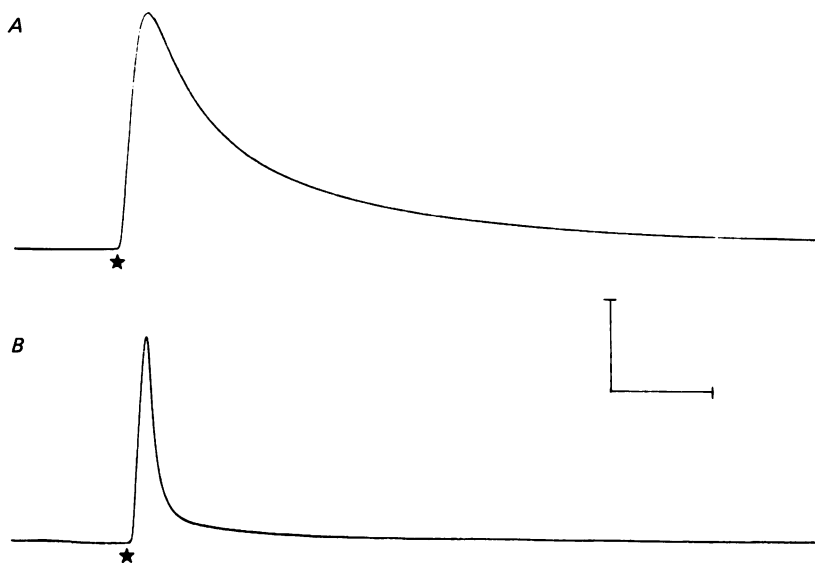


Fig. 4. Example currents which demonstrate that the acceleration of desensitization by pre-treatment occurs after enzymatic dissociation of nerve terminals from muscle fibres. *A*, the e.p.c._{carb.} recorded in a control fibre from an enzyme-treated preparation; *B*, the response obtained in another fibre after pre-treatment with 5.0 μ M-carbachol for 30 s. The stars indicate the initiation of 324 μ M-carbachol application. In example *A* the peak e.p.c._{carb.} value was 325 nA and the fast and slow decay time constants were 2.2 and 8.8 s. In example *B*, the peak e.p.c._{carb.} value was 144 nA and the two decay time constants were 0.5 and 10.8 s. The I_s/I_r ratio was 0.92 in *A* and 0.10 in *B*. The calibration is: Y axis, 125 nA in *A* and 70 nA in *B*; X axis, 5 s.

For these experiments, the conditioning concentration was 5.0 or 10.8 μ M and the test concentration was either 216 or 324 μ M. Only a few experiments were completed in this series because it was extremely difficult to obtain stable voltage-clamp recordings from these enzyme-treated preparations. Although limited in number, the results obtained in preparations with the nerve terminals enzymatically removed were

similar to those obtained in intact preparations. Pre-treatment with low carbachol concentrations markedly accelerated the desensitization time course and decreased the e.p.c._{carb.} amplitude produced by test concentrations of carbachol. This is illustrated in Fig. 4.

The acceleration after pre-treatment with low agonist concentrations is not a consequence of receptor activation

Two types of experimental procedures were used to determine the extent of activation produced by carbachol in the range of concentrations used for pre-treatment. Initially, we measured the peak response obtained during local application

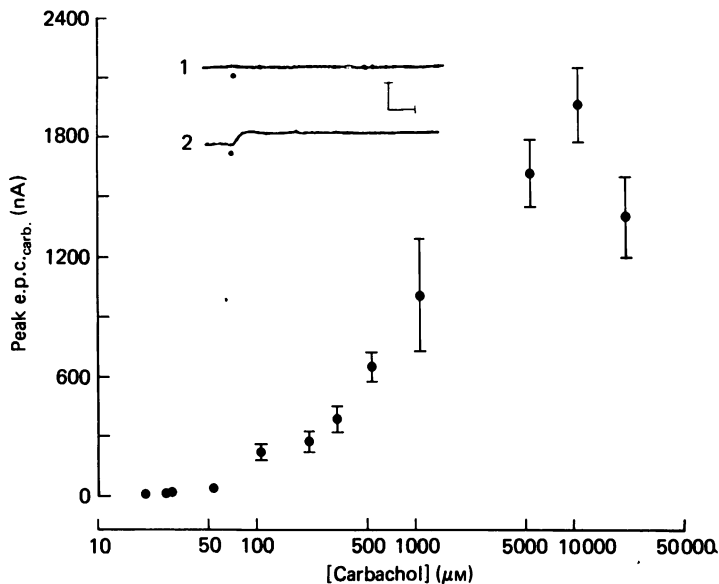


Fig. 5. The concentration dependence of peak e.p.c._{carb.} amplitude at twitch fibre end-plates voltage clamped to +50 mV. The concentration points give the mean \pm s.e. of the mean values of the average current amplitude from at least three fibres, except at 20 μM which is the average response from only two fibres. The inset shows the current record during the application of 10.8 μM -carbachol (1) or 20 μM -carbachol (2); the filled circles indicate the initiation of carbachol application. The calibration is: Y axis, 6nA; X axis, 1 s.

of carbachol (5.4–20 000 μM) to the end-plate region of muscle fibres voltage clamped to +50 mV. No measurable outward current occurred with concentrations of carbachol less than 20 μM . As the concentration of carbachol was raised from 20 μM to higher concentrations the outward current increased. The relationship between e.p.c._{carb.} amplitude and carbachol concentration from a number of fibres is summarized in Fig. 5. The inset in Fig. 5 demonstrates the small outward current produced by local application of 20 μM -carbachol and the lack of response with microperfusion of 10 μM -carbachol.

In other experiments cell-attached patch-clamp recordings were obtained to determine the influence of these low agonist concentrations on receptor-channel activation on enzymatically treated muscle twitch fibres. Muscle fibres were bathed in the isotonic potassium solution with the patch pipettes containing the same solution plus 5.4–16.2 μM -carbachol. In the initial experiments of this series, channel activity was estimated with the patch voltage clamped to +50 mV. With 5.4 or 10.8 μM -carbachol in the patch pipette, channel activity, when present, was very infrequent

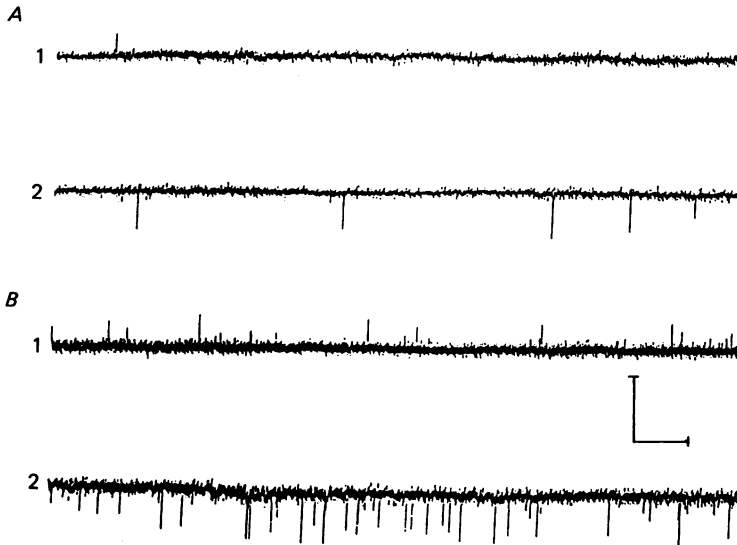


Fig. 6. Patch-clamp recordings which demonstrate the concentration and voltage dependence of the frequency of channel activity. Carbachol concentration in the patch pipette was 5.4 μM in *A* and 10.8 μM in *B*. In traces *A1* and *B1* the membrane potential was +50 mV and in traces *A2* and *B2* the membrane potential was -50 mV. The calibration is: Y axis, 4 pA; X axis, 20 s.

(often less than 1/min). In addition, at this membrane potential individual channel openings were very brief, as expected from the voltage dependence of channel open time (Dionne & Leibowitz, 1982; Connor *et al.* 1984). Also, with both concentrations there were many successful patches from the end-plate region which did not exhibit any channel activity (four of seven cells with 5.4 μM and two of seven cells with 10.8 μM -carbachol). In the presence of 5.4 and 10.8 μM -carbachol no superimposed events were observed. Also, with these concentrations of carbachol channel activity was maintained at the same low frequency throughout the duration of the recording with no evidence of the development of desensitization. In the presence of 16.2 μM -carbachol channel activity was much more frequent and was present in all the successful patches (four of four patches). Even though the channel activity was greater in the presence of 16.2 μM -carbachol, there also was no evidence of desensitization occurring within a time period at least three times that used for pre-treatment. These results demonstrate that 5.4 and 10.8 μM -carbachol represent thresh-

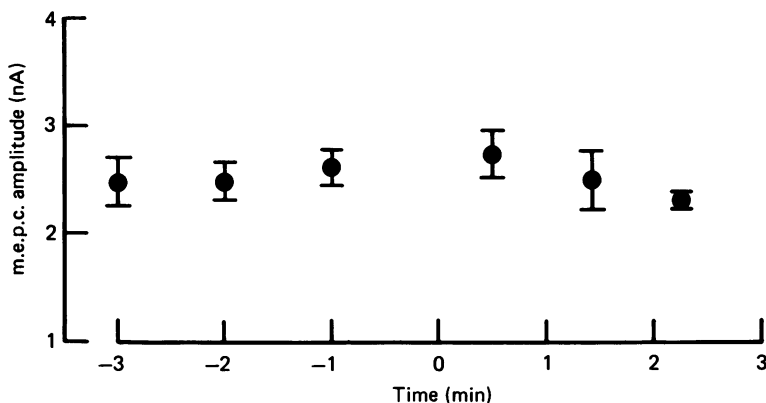


Fig. 7. Results from seven fibres voltage clamped to +50 mV which demonstrate that m.e.p.c. amplitude does not change significantly during exposure to $10.8 \mu\text{M}$ -carbachol for periods up to 150 s. Each point represents the mean \pm s.e. of the mean values for m.e.p.c.s recorded at each time given. At time zero, microperfusion of $10.8 \mu\text{M}$ -carbachol to the end-plate region of each fibre was initiated.

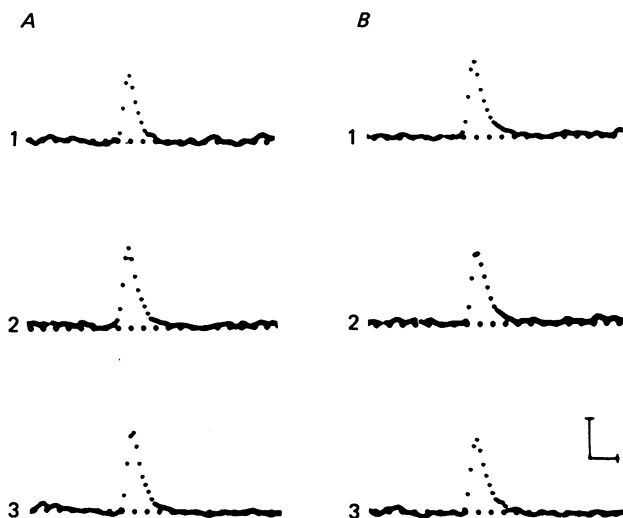


Fig. 8. Examples of averaged m.e.p.c.s recorded at a twitch-fibre end-plate prior to and during perfusion with $10.8 \mu\text{M}$ -carbachol for approximately 145 s. *A* shows average m.e.p.c.s obtained at different times during a perfusion with the isotonic potassium solution. *B* shows averaged m.e.p.c.s obtained during the perfusion of the isotonic potassium solution containing $10.8 \mu\text{M}$ -carbachol for approximately 30 s (1), approximately 83 s (2), and approximately 145 s (3). Each trace is the average of at least sixteen m.e.p.c.s. Calibration is: Y axis, 1.25 nA; X axis, 2.5 ms.

hold concentrations for the initiation of channel activity at positive membrane potentials.

We also observed that the frequency of channel activity at all concentrations of carbachol tested was greater at negative membrane potentials than at positive

membrane potentials. Example results, which demonstrate the single-channel activity recorded with either 5.4 or 10.8 μM -carbachol in patches with the membrane potential set either at +50 mV or -50 mV, are presented in Fig. 6.

The alteration in desensitization kinetics by low carbachol concentrations is not due to residual desensitization

Another series of experiments was undertaken to determine that end-plate sensitivity did not change during the pre-treatment period. For these experiments, miniature end-plate currents (m.e.p.c.s) were recorded from seven fibres prior to and during a sustained application of 10.8 μM -carbachol. If end-plate sensitivity was decreasing during the initial carbachol exposure, it was assumed that the amplitude of m.e.p.c.s would decrease progressively (Magleby & Pallotta, 1981; Pennefather & Quastel, 1982). As demonstrated in Fig. 7, with fibres voltage clamped to +50 mV the sustained application of 10.8 μM -carbachol did not influence the peak size of m.e.p.c.s recorded for periods longer than those used for pre-treatment. Fig. 8 presents example records from one of these experiments which illustrate that m.e.p.c. amplitude does not change during exposure to 10.8 μM -carbachol for 150 s.

DISCUSSION

The results of the present study demonstrate that brief pre-treatment with conditioning concentrations of agonist markedly accelerates the desensitization of end-plate receptor-channel complexes produced by subsequent exposure to higher concentrations of carbachol. Further, we found that the acceleration of desensitization by pre-treatment required only a brief duration of exposure and was similar after either a 15 or 60 s exposure. This observation suggests that the alteration in desensitization kinetics observed with pre-treatment with low conditioning concentrations most probably results from mechanisms different from those responsible for the progressive change in desensitization kinetics observed previously when higher pre-treatment concentrations were used (Connor *et al.* 1984). The results of these experiments also demonstrate that the acceleration of the time course of desensitization onset reflects a direct action of the conditioning concentration of agonist on the ACh nicotinic receptor to modify the kinetic properties of desensitization produced by a test concentration. For instance, this effect is not a secondary non-specific effect related to either receptor-channel activation or desensitization produced by the conditioning concentration during pre-treatment. We eliminated receptor activation by the conditioning concentration as a factor because in patch-clamp recordings the channel openings were very infrequent and often not present when carbachol in the concentration range of 5.4–10.8 μM was used. This very limited channel activity explains the lack of measurable activation noted in macroscopic current determinations with similar concentrations of agonist.

Previously, Akaike, Ikeda, Brookes, Pascuzzo, Rickett & Albuquerque (1984) reported that single-channel activity was voltage dependent, increasing significantly with hyperpolarization. Similar results were obtained in the present study (Fig. 6). Dionne & Leibowitz (1982) observed single-channel activity with concentrations of carbachol lower than those used as the conditioning concentration in the present

study. However, their experiments were done at more hyperpolarized values of membrane potential than those used here.

The possibility that the subsequent change in desensitization kinetics resulted from some receptor-channel complexes undergoing desensitization during the initial exposure to low agonist concentration was also considered unlikely. Under the conditions of our experiments, m.e.p.c. amplitudes did not change during a 30–180 s exposure to $10.8 \mu\text{M}$ -carbachol, indicating that no change in receptor sensitivity was produced during this period of exposure to low concentrations of agonist. Previously, Magleby & Pallotta (1981) and Penefather & Quastel (1982) have demonstrated that a decrease in m.e.p.c. amplitude can be used effectively to estimate the presence and time course of development of desensitization produced either by neurally released acetylcholine or exogenously applied agonist. Consequently, we feel confident that if any significant amount of desensitization occurred during the pre-treatment period, m.e.p.c. amplitudes would have decreased. Actually, because desensitization has a considerable voltage dependence, it is not surprising that in fibres voltage clamped to +50 mV, no desensitization was evident during the brief pre-treatment exposure to $10.8 \mu\text{M}$ -carbachol (Magazanik & Vyskocil, 1970; Fiekers *et al.* 1980; Magleby & Pallotta, 1981; Connor *et al.* 1984).

Following pre-treatment, the peak e.p.c._{carb.} was decreased in many experiments; though this was not usually statistically significant. We suggest that this decrease does not represent a change in sensitivity produced by the initial exposure to low carbachol concentrations, but is most likely to be related to the microperfusion technique. One limitation of the microperfusion technique is that the agonist-induced current does not reach its maximum amplitude instantaneously but rather develops over a few seconds. Therefore, with this method of drug application, some of those receptor-channel complexes activated initially may become desensitized before the peak current value is reached, therefore leading to an underestimate of the peak current value. This truncation of peak size would be expected to be greatest when the initial component of desensitization develops very rapidly.

Brief pre-treatment with a conditioning concentration of carbachol accelerated onset of desensitization by test concentrations after enzymatic removal of nerve terminals. We had considered the possibility that when the the nerve terminal was present the conditioning concentration might enhance spontaneous ACh release. In turn, the acceleration in desensitization might have been an indirect effect of an elevated ACh concentration in the vicinity of the post-junctional receptors. However, the results obtained with enzymatically treated preparations eliminated this possibility.

The conditioning concentration of carbachol which altered the time course of desensitization is considerably less than that which is thought to be required for initiating changes in membrane permeability. Previous studies have demonstrated that the dissociation constant (K_D) for receptor-channel activation by carbachol in vertebrate skeletal muscle is 300–400 μM (Dionne, Steinbach & Stevens, 1978; Dreyer, Peper & Sterz, 1978; Sakmann & Adams, 1978; Trautmann, 1983). Our observations indicate that the concentration of carbachol which alters the desensitization time course produced with a subsequent exposure to higher agonist is at least one order of magnitude less than the K_D for receptor-channel activation. One inter-

pretation of this difference in effective dose is that the agonist binding site modifying desensitization kinetics is distinct from the site controlling the permeability change and has a higher affinity for agonist than the site(s) controlling channel opening. Previously it has been suggested, based on the results of biochemical studies undertaken on isolated *Torpedo* membrane preparations, that there are two agonist binding sites on the ACh receptor: a high-affinity site regulating desensitization (desensitization site) and a low-affinity site controlling membrane permeability (Dunn & Raftery, 1982; Conti-Tronconi & Raftery, 1982; Raftery *et al.* 1984). We conclude that our results are consistent with such a suggestion and propose that there are also two classes of agonist binding sites on the ACh receptor at the motor end-plate of skeletal muscle. We also observed that muscarinic agonists did not produce a similar alteration of desensitization. This suggests that the site regulating desensitization is specific for nicotinic agonists.

The development of desensitization during sustained agonist application has been found to exhibit at least two exponential components: an initial fast phase followed by a more slowly developing component (Feltz & Trautmann, 1980, 1982; Chestnut, 1983; Connor *et al.* 1984). Under the conditions of the present study, desensitization onset also exhibited a two-component time course. Further, pre-treatment accelerated desensitization by accentuating the fast component of desensitization. The results obtained in the present study do not establish a mechanism(s) responsible for the acceleration of desensitization by pre-treatment. However, a very interesting and plausible hypothesis can be postulated by correlating the results of this study with recent observations from other laboratories. In our scheme, we propose that there are two classes of agonist binding sites: a population of high-affinity sites which regulate desensitization ('desensitization sites') and a separate class of lower-affinity sites which control ion permeability. We also assume, in accord with previous suggestions, that there are different states of ACh receptor (Magleby & Pallotta, 1981; Feltz & Trautmann, 1982). Some receptor-channel complexes are in a 'desensitizable state' and these complexes desensitize rapidly when exposed to sustained agonist application (Magleby & Pallotta, 1981). In contrast, those receptor-channel complexes which are not in the desensitizable configuration but are in the 'closed state' desensitize more slowly because they have to undergo a time-dependent transition of closed state to desensitizable state before becoming desensitized.

The results of the present study also appear to correlate well with recent studies which demonstrate that following phosphorylation the rate of desensitization of the AChR is accelerated. Therefore, we propose that the desensitizable state may correspond to the phosphorylated state of the muscle AChR. Phosphorylation of the *Torpedo* and muscle AChR has been demonstrated previously (Huganir & Greengard, 1983; Huganir, Miles & Greengard, 1984; Anthony, Rubin, Miles & Huganir, 1986) and agents which promote phosphorylation have been shown to accelerate desensitization in muscle preparations (Eusebi, Molinaro & Zani, 1985; Albuquerque, Deshpande, Aracava, Alkondon & Daly, 1986; Middleton, Rubin & Schuetze, 1986). Further, in the presence of agents which promote phosphorylation, the acceleration of desensitization is due to a potentiation of the fast phase of desensitization similar to that produced in the present study by pre-treatment. Therefore, one possible mechanism responsible for the acceleration of desensitization by pre-treatment

would be that an interaction of agonist at the high-affinity, desensitization-related binding sites may promote the phosphorylation of the muscle AChR. Consequently, during pre-treatment individual receptor-channel complexes may become phosphorylated so that during a subsequent exposure to a higher test concentration of agonist, the fast phase of desensitization would be potentiated.

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REFERENCES

- AKAIKE, A., IKEDA, S. R., BROOKES, N., PASCUZZO, G. J., RICKETT, D. L. & ALBUQUERQUE, E. X. (1984). The nature of the interactions of pyridostigmine and the nicotinic acetylcholine receptor-ion channel complex. II. Patch clamp studies. *Molecular Pharmacology* **25**, 102-112.
- ALBUQUERQUE, E. X., DESPANDE, S. S., ARACA, Y., ALKONDON, M. & DALY, J. W. (1986). A possible involvement of cyclic AMP in the expression of desensitization of the nicotinic acetylcholine receptor. *Federation of European Biochemical Societies Letters* **199**, 113-120.
- ANTHONY, D. J., RUBIN, L. L., MILES, K. & HUGANIR, R. L. (1986). Forskolin regulates phosphorylation of the nicotinic acetylcholine receptors in rat primary muscle cell cultures. *Society for Neuroscience Abstracts* **12**, 148.
- BETZ, W. & SAKMANN, B. (1973). Effects of proteolytic enzymes on function and structure of frog neuromuscular junction. *Journal of Physiology* **230**, 673-688.
- CHESTNUT, T. J. (1983). Two component desensitization at the neuromuscular junction of the frog. *Journal of Physiology* **336**, 229-241.
- CONNOR, E. A., FIEKERS, J. F., NEEL, D. S., PARSONS, R. L. & SCHNITZLER, R. M. (1984). Comparison of cholinergic activation and desensitization at snake twitch and slow muscle fibre end-plates. *Journal of Physiology* **351**, 657-674.
- CONTI-TRONCONI, B. M. & RAFTERY, M. A. (1982). The nicotinic cholinergic receptor: correlation of molecular structure with functional properties. *Annual Review of Biochemistry* **51**, 491-530.
- CONTI-TRONCONI, B. M. & RAFTERY, M. A. (1986). Nicotinic acetylcholine receptor contains multiple binding sites: evidence from binding of alpha-dendrotoxin. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 6646-6650.
- DIONNE, V. E. & LEIBOWITZ, M. (1982). Acetylcholine receptor kinetics. A description from single-channel currents at snake neuromuscular junction. *Biophysical Journal* **39**, 253-261.
- DIONNE, V. E., STEINBACH, J. H. & STEVENS, C. F. (1978). An analysis of the dose-response relationship at voltage-clamped frog neuromuscular junctions. *Journal of Physiology* **281**, 421-444.
- DREYER, F., PEPPER, K. & STERZ, R. (1978). Determination of dose-response curves by quantitative iontophoresis at the frog neuromuscular junction. *Journal of Physiology* **281**, 395-419.
- DUNN, S. M. J. & RAFTERY, M. A. (1982). Activation and desensitization of *Torpedo* acetylcholine receptor: evidence for separate binding sites. *Proceedings of the National Academy of Sciences of the U.S.A.* **79**, 6756-6761.
- EUSEBI, F., MOLINARO, M. & ZANI, B. M. (1985). Agents that activate protein kinase C reduce acetylcholine sensitivity in cultured myotubes. *Journal of Cell Biology* **100**, 1339-1342.
- FELTZ, A. & TRAUTMANN, A. (1980). Interaction between nerve-released acetylcholine and bath applied agonists at the frog end-plate. *Journal of Physiology* **299**, 533-552.
- FELTZ, A. & TRAUTMANN, A. (1982). Desensitization at the frog neuromuscular junction: a biphasic process. *Journal of Physiology* **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. *Journal of General Physiology* **75**, 511-529.
- HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85-100.
- HUGANIR, R. L. & GREENGARD, P. (1983). cAMP-dependent protein kinase phosphorylates the

- nicotinic acetylcholine receptor. *Proceedings of the National Academy of Sciences of the U.S.A.* **80**, 1130-1134.
- HUGANIR, R. L., MILES, K. & GREENGARD, P. (1984). Phosphorylation of the nicotinic acetylcholine receptor by an endogenous tryosine-specific protein kinase. *Proceedings of the National Academy of Sciences of the U.S.A.* **81**, 6968-6972.
- MAGAZANIK, L. G. & VYSKOCIL, F. (1970). Dependence of acetylcholine desensitization on the membrane potential of frog muscle fibre and on the ionic changes in the medium. *Journal of Physiology* **210**, 507-518.
- MAGLEBY, K. L. & PALLOTTA, B. A. (1981). A study of desensitization of acetylcholine receptors using nerve-released transmitter in the frog. *Journal of Physiology* **316**, 225-250.
- MIDDLETON, P., RUBIN, L. & SCHUETZE, S. (1986). Forskolin increases the rate of acetylcholine receptor desensitization. *Society for Neuroscience Abstracts* **12**, 148.
- PARSONS, R. L., NEEL, D. S. & FIEKERS, J. F. (1986). Kinetics of desensitization onset are modified by pretreatment with low concentrations of agonist. *Society for Neuroscience Abstracts* **12**, 738.
- PENNEFATHER, P. & QUASTEL, D. M. J. (1982). Fast desensitization of the nicotinic receptor at the mouse neuromuscular junction. *British Journal of Pharmacology* **77**, 395-404.
- RAFTERY, M. A., CONTI-TRONCONI, B. M., DUNN, S. M. J., CRAWFORD, R. D. & MIDDLEMAS, D. (1984). The nicotinic acetylcholine receptor: its structure, multiple binding sites, and cation transport properties. *Fundamental and Applied Toxicology* **4**, S34-S51.
- SAKMANN, B. & ADAMS, P. R. (1978). Biophysical aspect of agonist action at frog endplate. In *Advances in Pharmacology and Therapeutics: Receptors*, ed. Jacob, J., vol. 1, pp. 81-90. Oxford: Pergamon Press.
- SINE, S. M. & STEINBACH, J. H. (1984a). Activation of a nicotinic acetylcholine receptor. *Biophysical Journal* **45**, 175-185.
- SINE, S. M. & STEINBACH, J. H. (1984b). Agonist block currents through acetylcholine receptor channels. *Biophysical Journal* **46**, 277-284.
- TRAUTMANN, A. (1983). A comparative study of the activation of the cholinergic receptor by various agonists. *Proceedings of the Royal Society B* **218**, 241-251.