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COMPARISON OF CHOLINERGIC ACTIVATION AND DESENSITIZATION AT SNAKE TWITCH AND SLOW MUSCLE FIBRE END-PLATES

BY ELIZABETH A. CONNOR†, JEROME F. FIEKERS, DARCY S. NEEL, RODNEY L. PARSONS AND RONALD M. SCHNITZLER*

From the Department of Anatomy and Neurobiology, University of Vermont College of Medicine, Burlington, VT 05405, U.S.A.

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SUMMARY

1. Characteristics of receptor-channel activation and desensitization have been compared at voltage-clamped snake slow and twitch fibre end-plates maintained in an isotonic potassium propionate solution.

2. Miniature end-plate current (m.e.p.c.) decay was slower and less voltage dependent at slow fibre end-plates than at twitch fibre end-plates. The peak m.e.p.c. amplitude *versus* voltage relationship and reversal potential were similar at the two end-plate types.

3. Acetylcholine-induced noise and m.e.p.c.s were recorded at slow fibre end-plates. At most slow fibres the spectral density was not adequately fitted by a single Lorentzian function. Rather, the observed spectral density was greater at high frequencies than the values predicted using the m.e.p.c. decay rate. The noise could be well described by the sum of two Lorentzian functions, one of which corresponded to a single Lorentzian function with the corner frequency determined by the m.e.p.c. decay rate.

4. The shape of the carbachol concentration–peak end-plate current relationship was similar at both slow and twitch fibre end-plates. However, for all concentrations tested, the peak carbachol-induced end-plate current ($e.p.c._{carb.}$) value was markedly less at slow fibre end-plates than at twitch fibre end-plates.

5. The onset of desensitization was determined using two methods. The first concerned analysis of the time course of decay of the $e.p.c._{carb.}$ from a peak value during the sustained application of agonist. The second involved a double-perfusion technique in which a 'desensitizing' dose was applied for varying intervals before the application of a second 'test' dose of carbachol. With both methods the development of desensitization at both end-plate types was dependent on carbachol concentration and duration of exposure. At each end-plate type the time course of desensitization onset often exhibited two components; one with a time constant of seconds and a slower component having time constants in the range of tens to hundreds of seconds.

Present address: *Division of Science and Mathematics, Bay Path Junior College, Longmeadow, MA 01106, U.S.A. and †Department of Neurobiology, Stanford University, School of Medicine, Stanford, CA 94305, U.S.A.

6. The slope of the relationship between carbachol concentration and equilibrium desensitization at slow and twitch fibre end-plates was close to two, suggesting that two molecules of agonist are probably bound during the development of desensitization. However, for all concentrations tested, desensitization developed more rapidly and to a greater extent at twitch fibre end-plates than at slow fibre end-plates.

7. The voltage dependence of the 3 min steady-state desensitization produced by 108 μM-carbachol was very similar (~ -0.0250 mV^{-1}) at both fibre types. However, the 3 min steady-state level of desensitization was consistently greater at corresponding voltages for twitch fibre end-plates than at slow fibre end-plates. It was also observed at twitch fibre end-plates exposed to 216 μM-carbachol that the fast component of desensitization and 3 min steady-state level of desensitization could exhibit different voltage dependencies. This is consistent with the view that the fast and slow components of desensitization may represent separate processes.

8. The results indicate that even though the gating kinetics of receptor-channel activation are unique to the fibre type, the characteristics of desensitization appear to have a number of common features at both end-plate types.

INTRODUCTION

Desensitization of the motor end-plate of skeletal muscles during sustained exposure to cholinergic agonists is a well-documented phenomenon (Katz & Thesleff, 1957). In spite of the fact that our understanding of the molecular aspects of receptor-channel gating at the motor end-plate has increased markedly in recent years, the molecular mechanism responsible for the development of desensitization still remains obscure. There are a number of environmental factors such as membrane voltage, extracellular and intracellular ionic concentrations, and agonist concentration which can markedly alter the time course of onset of desensitization (Manthey, 1966; Magazanik & Vyskocil, 1970; Nastuk & Parsons, 1970; Parsons, Schnitzler & Cochrane, 1974; De Bassio, Parsons & Schnitzler, 1976; Parsons, 1978; Chestnut, 1983). The development of desensitization is particularly sensitive to the extent of receptor-channel activation. However, the relationship of the different agonist-induced kinetic states of the receptor-channel complex to the development of desensitization is not known. Recently, Dionne & Parsons (1981) and Dionne (1981) have shown that receptor-channel gating is different at snake slow and twitch end-plates. Slow fibre receptor-channel complexes have a significantly longer intermediate state than those at twitch end-plates (Dionne, 1981). In the present study, we have explored the possibility that this difference in intermediate state lifetime may affect the development of desensitization in these two fibre types. Here, we report the results of a comparative study of the concentration and voltage dependence of carbachol-induced desensitization at snake slow and twitch fibre end-plates. We confirm that the characteristics of receptor-channel gating are different at the two muscle fibre types. Further, we demonstrate that at both end-plate types, desensitization develops during sustained agonist application with a time course which often exhibits two components: an initial fast phase followed by a slower component. The sensitivity of desensitization onset to agonist concentration and voltage are similar at twitch and slow fibres. However, under comparable conditions, desensitization

develops more rapidly and to a greater extent during the initial moments of agonist exposure at twitch fibre end-plates than at slow fibre end-plates.

METHODS

General methods

All experiments have been done on visually identified slow and twitch fibre end-plates in the costocutaneous muscle of the garter snake (*Thamnophis* sp.). Muscle preparations including a portion of rib and scale were stretched out and pinned on to the bottom of a Sylgard-coated glass chamber. The preparations were bathed in a HEPES-buffered isotonic potassium propionate solution (mM: potassium propionate, 161; calcium propionate, 1·0; $MgCl_2$, 4·2; CsCl, 5·0; pH = 7·2, 19–23 °C). The CsCl was added to block the anomalous rectifier potassium channels (Gay & Stanfield, 1977). The advantage of the potassium-depolarized preparation is that (a) muscle contraction is eliminated in adjacent fibres which are not voltage clamped, (b) the fibres can be voltage clamped over a wide range of membrane potentials, and (c) only one cation species (K^+) contributes significantly to the agonist-induced end-plate current at all voltages.

Twitch and slow fibres, which are intermingled in the costocutaneous muscle, were identified by both anatomical and electrical criteria (Hess, 1970; Dionne & Parsons, 1981). Twitch fibres are singularly innervated usually in the central portion of each muscle fibre; slow fibres are multiply innervated at intervals of $\sim 1-2$ mm. Slow fibres have smaller diameters (5–30 μm) than twitch fibres (25–100 μm). Verification of slow fibres was done by recording miniature end-plate currents (m.e.p.c.s) at more than one end-plate on a given fibre.

The end-plate region of individual fibres was voltage clamped using a two-micro-electrode voltage clamp similar to that previously described (Dionne & Parsons, 1981). Because the neuromuscular junctions in both slow and twitch fibres are very compact, the agonist concentration equilibrates quickly during local microperfusion, and effective voltage control can be maintained over the entire end-plate region (Scubon-Mulieri & Parsons, 1978).

Electrophysiological analysis of channel gating

The kinetics of channel gating were determined from analysis of spontaneous m.e.p.c.s and ionophoretically induced acetylcholine (ACh) current fluctuations which were recorded digitally using a laboratory computer (PDP 11/03 or PDP 11/34, Digital Corp., Maynard, MA) and stored on floppy disks for later analysis. For the determination of (average) peak current and decay time constant, 10–20 m.e.p.c.s were recorded and averaged. ACh-induced current fluctuations were recorded digitally, averaged and Fourier transformed to obtain spectral density as a function of frequency (Dionne & Parsons, 1981). Background current fluctuations recorded just before ACh application were subtracted from the ACh-induced current fluctuations.

Mean single-channel conductance, $\bar{\gamma}$, was estimated from the r.m.s. amplitude of the ACh-induced current and its mean value μ. The r.m.s. record was squared, corrected for background to obtain total induced variance σ^2 and $\bar{\gamma}$ was estimated as

$$\bar{\gamma} = \frac{\sigma^2}{\mu(V - V_r)},$$

where V and V_r are membrane potential and ACh reversal potential respectively.

Estimation of the time course of development of desensitization during sustained agonist exposure

In all desensitization experiments, carbachol was the agonist and was applied to the end-plate region of individual muscle fibres by hydrostatically controlled microperfusion (Nastuk & Parsons, 1970). The peak value of the carbachol-induced end-plate current (e.p.c.$_{carb.}$) estimated the maximal extent of activation. The time course of decline of the current from the peak towards the base line in the continued presence of applied agonist indicated the onset of desensitization (Scubon-Mulieri & Parsons, 1978; Fiekers, Spannbauer, Scubon-Mulieri & Parsons, 1980). The onset time course can consist of more than one component depending on the experimental conditions (Feltz & Trautman, 1980, 1982; Clark & Adams, 1981; Magleby & Pallotta, 1981; Chestnut, 1983). In the present study, the development of desensitization during the first 60–70 s of agonist exposure was estimated from the time course of e.p.c.$_{carb.}$ decay. For this, the decline of the current was

quantitated using a computerized non-linear, exponential-fitting program according to the expression:

$$\text{e.p.c.}_{\text{carb.}}(t) = Ae^{-t/\tau_1} + Be^{-t/\tau_2} + C,$$

where A, B and C are constants and τ_1 and τ_2 represent the time constants of two exponential components.

The time course of development of desensitization can extend over many minutes in the continued presence of moderate or low agonist concentrations. This requires that fibres be voltage clamped for prolonged periods. Fluctuations in the background holding current unrelated to the agonist-induced conductances are inevitable, especially in the case of small cells such as the slow muscle fibres. Consequently, we suggest that the time course of e.p.c.$_{\text{carb.}}$ decay can be used to estimate accurately the development of desensitization during only the initial period of agonist application. In addition, with concentrations of carbachol greater than 216 μM and voltages more negative than -50 mV, local contractures often occurred in the end-plate region during the first few seconds of perfusion. These contractures, which are thought to be due to calcium influx through the acetylcholine-gated channels (Parsons & Nastuk, 1969), were strong enough in many instances either to distort the e.p.c.$_{\text{carb.}}$ decay time course or to dislodge the micro-electrodes. For these reasons, we have used a second procedure, a double-perfusion technique, to analyse the time course of development of desensitization over longer periods of agonist exposure. Individual voltage-clamped end-plates were continually perfused with 'desensitizing' doses of carbachol (18–364 μM) and then, after a defined exposure time (15–360 s), a second 'test' carbachol perfusion (5400 μM) was initiated. The peak value of the e.p.c.$_{\text{carb.}}$ produced by the second perfusion (e.p.c.$_2$) was used to assess the populaton of remaining, non-desensitized, activatable receptor-channel complexes. Because there was a range of peak carbachol responses for any given concentration between end-plates of either muscle type, all results were normalized using the ratio e.p.c.$_2$/e.p.c.$_1$ (where e.p.c.$_2$ is the response to the test dose and e.p.c.$_1$ is the peak response produced by the desensitizing dose).

RESULTS

Characteristic differences in receptor-channel gating occur at slow and twitch fibre end-plates maintained in the isotonic potassium solution

Experiments were done to ensure that the kinetic differences in receptor-channel gating observed previously for snake twitch and slow fibre end-plates maintained in a physiological solution also occurred in muscles maintained in the isotonic potassium propionate solution. M.e.p.c. characteristics were compared in both slow and twitch fibre end-plates. Typically the twitch fibre m.e.p.c. rose to its peak value within 300 μs and decayed exponentially. Slow fibre m.e.p.c.s peaked within 500 μs and also decayed exponentially. Only those slow fibre m.e.p.c.s with an abrupt rising phase were taken for analysis. M.e.p.c. decay was markedly slower and less voltage dependent at slow fibre end-plates than at twitch fibre end-plates, thus confirming the previous observations made in physiological solution (Dionne & Parsons, 1981). The m.e.p.c. reversal potential determined by interpolation was similar at slow and twitch fibre end-plates. These observations are summarized in Table 1, and representative results obtained at a slow and twitch fibre end-plate are shown in Fig. 1.

ACh-induced 'noise' and m.e.p.c.s could be recorded in the isotonic potassium solution only at slow fibre end-plates because at twitch fibre end-plates the m.e.p.c. frequency increased almost immediately to levels which interfered with the analysis of noise. In contrast, the m.e.p.c. frequency at slow fibre end-plates increased much more slowly after exposure to the isotonic potassium solution. At most slow fibre

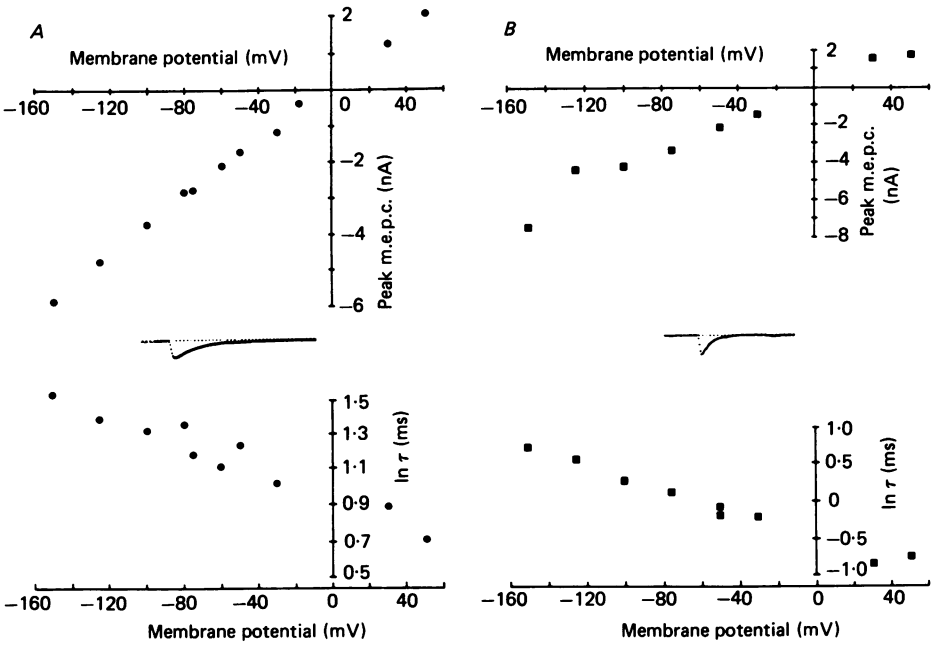

Fig. 1. Characteristics of m.e.p.c.s recorded from a slow fibre (*A*) and twitch fibre (*B*) maintained in the isotonic potassium solution. In each example, the top graph is a plot of the peak m.e.p.c. amplitude–voltage relationship and the bottom graph is a plot of the ln τ–voltage relationship. The insert in each shows an averaged m.e.p.c. recorded at −100 mV. The temperature was 20 °C. The coefficient of voltage dependence for τ was −0·0037 mV^{-1} for the slow fibre and −0·0081 mV^{-1} for the twitch fibre. The peak current value was −3·7 nA and −4·3 nA for the slow and twitch fibre m.e.p.c., respectively. The decay τ was 3·7 ms for the slow fibre m.e.p.c. and 1·3 ms for the twitch fibre m.e.p.c.

TABLE 1. Comparison of m.e.p.c. characteristics at snake twitch and slow muscle end-plates in an isotonic potassium solution

M.e.p.c. characteristics	Snake twitch fibre Exponential decay	Snake slow fibre Exponential decay
Peak amplitude at −100 mV (nA)	−6·05 ± 0·97 (5)	−4·19 ± 0·35 (9)
Decay τ at −100 mV (ms)	1·21 ± 0·08 (5)	3·10 ± 0·25 (9)
Voltage dependence (mV^{-1})	−0·0092 ± 0·0012 (6)	−0·0049 ± 0·0006 (11)
$m.e.p.c._{peak}$ *vs.* voltage	Some non-linearity	Linear
V_r(mV)	−2·2 ± 2·1 (5)	+0·5 ± 1·8 (11)

All values are mean ± S.E. of the mean; number of fibres given in parentheses.

end-plates the spectral density was not adequately fitted by a single Lorentzian function (Dionne & Parsons, 1981). Rather, the observed spectral density was greater at high frequencies than the values predicted by a simple Lorentzian curve whose corner frequency was given by the m.e.p.c. decay rate. This pattern is demonstrated in Fig. 2. The average value of the single-channel conductance, γ, was 22·2 ± 8·8 (mean ± S.D.) in seven slow fibres voltage clamped to −70 mV (21–23 °C).

The results of both the m.e.p.c. and noise experiments indicated that the characteristic differences in the kinetics of receptor-channel gating at slow and twitch

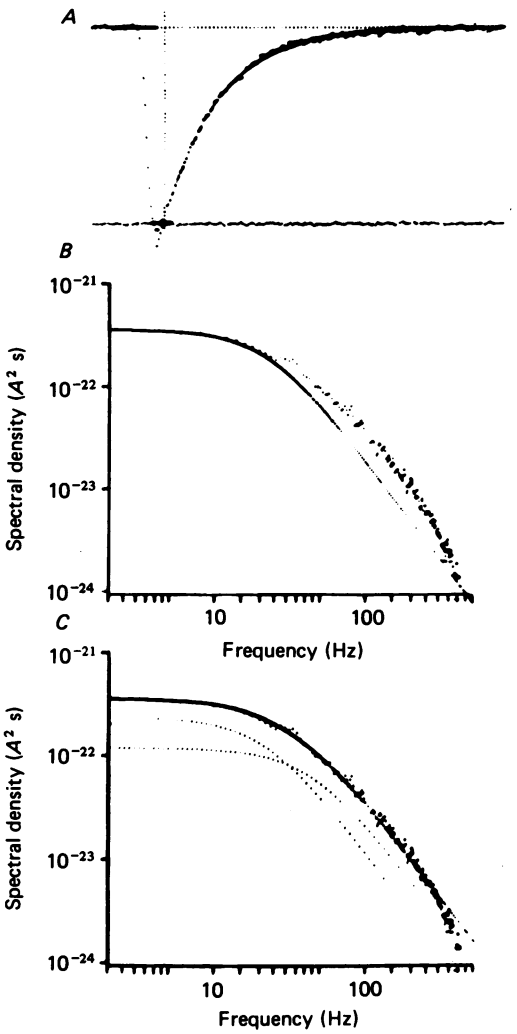

Fig. 2. An example of synaptic responses obtained at the majority of slow fibres studied. M.e.p.c.s and current fluctuations were obtained at −70 mV and 20 °C. *A*, an averaged m.e.p.c. having peak current value of −2·7 nA and an exponential decay with τ equal to 3·3 ms. *B*, a plot of the spectral density recorded in this cell with the dashed line representing a single Lorentzian function whose corner frequency was determined from the m.e.p.c. decay. *C*, a plot of the spectral density with the continuous lines equal to the sum of two Lorentzian components each shown as dashed lines. The characteristic frequencies were 48 and 97 Hz, with the 48 Hz value taken from the m.e.p.c. decay.

fibre end-plates were present in muscles maintained in the isotonic potassium solution.

The carbachol dose–response curve is similar at both slow and twitch fibre end-plates

In order to compare desensitization characteristics at twitch and slow fibre end-plates, it was first necessary to establish whether carbachol produced a comparable

activation level at both end-plate types. Therefore, we compared the carbachol concentration-peak e.p.c.$_{carb.}$ relationship at twitch and slow fibre end-plates. The fibres were voltage clamped to +50 mV for these experiments. At positive voltages desensitization develops more slowly (Fiekers *et al.* 1980), and therefore attenuation of the peak e.p.c.$_{carb.}$ size due to desensitization is minimized.

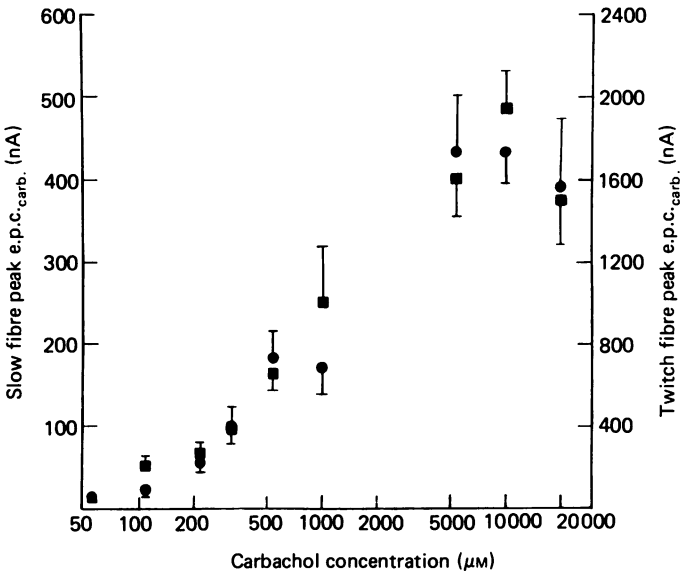

Fig. 3. The concentration dependence of peak e.p.c.$_{carb.}$ amplitude at twitch (■) or slow (●) fibre end-plates voltage clamped to +50 mV. Each concentration point gives the mean ±S.E. of the mean values of the averaged current amplitude from at least three fibres. The scale on the left is for slow fibre data, that on the right for twitch fibre data.

The concentration–e.p.c.$_{carb.}$ relationship for twitch and slow fibres voltage clamped to +50 mV is shown in Fig. 3. We found that with all agonist concentrations the peak e.p.c.$_{carb.}$ values were significantly smaller at slow fibre end-plates than at twitch fibre end-plates. However, the e.p.c.$_{carb.}$ amplitude reached a maximum value between 5·4 and 10·8 mM at both fibre types and the percentage of maximum activation was similar with carbachol concentrations between 50 and 500 μM. Therefore, we felt that similar concentrations of carbachol could be used to compare desensitization in the two fibre types.

Comparison of the initial development of desensitization at twitch and slow fibre end-plates

Microperfusion of carbachol on to the end-plate region of both twitch and slow fibres induced a transient current which developed with a time-to-peak of a few seconds and then slowly decayed towards the base line even though the agonist application was continued. In the present study, we compared the time course and depth of desensitization onset produced during the first 60 s of exposure to 216 μM-carbachol in twitch or slow fibres voltage-clamped to either −60 or −80 mV. At twitch end-plates voltage clamped to −60 mV and perfused with 216 μM-carbachol, the e.p.c.$_{carb.}$ decay phase exhibited two components, one with a time constant of

seconds followed by a much smaller and slower component. At −80 mV, the second component was either absent or so small that in most fibres the entire current decay time course could be adequately described as a single exponential function. The relative contribution of the two decay components to the decay time course was approximated using the ratio of the amplitude of each current component extrapolated back to the time of the peak e.p.c.$_{carb.}$. For all those multicomponent responses from twitch end-plates voltage clamped to either −60 or −80 mV in the presence of 216 μM-carbachol the ratio of the two current components ranged from 0·03 to 0·31.

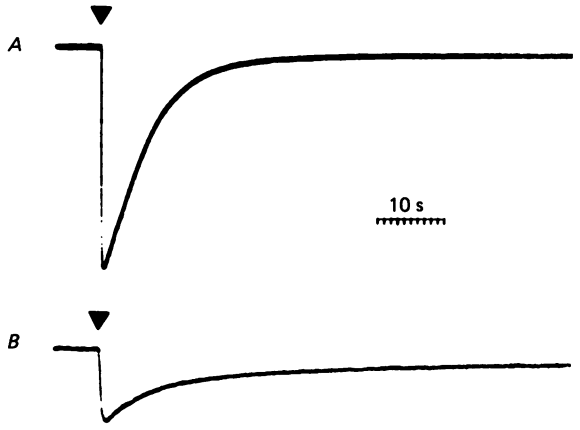

Fig. 4. Examples of the e.p.c.$_{carb.}$ measured in a twitch fibre (*A*) and in a slow fibre (*B*) during continued microperfusion of 216 μM-carbachol. Holding voltage was −60 mV for both fibres. The vertical arrow indicates the initiation of the carbachol application. The initial peak inward current value was 1190 nA in *A* and 125 nA in *B*.

The extent of e.p.c.$_{carb.}$ decay during the initial 60 s of exposure to 216 μM-carbachol at slow fibre end-plates was consistently less than at twitch end-plates. In addition, for all of the slow fibres voltage-clamped to either −60 or −80 mV, the initial decay time course had two components. The first component decayed with a time constant similar to that observed with the twitch end-plates. The second component had a time constant which ranged from tens to hundreds of seconds. The ratio of the two current components ranged from 0·30 to 1·3, which indicated that the second slow current component contributed more to the current decay time course in slow fibres than at twitch fibre end-plates.

Example records which illustrate the carbachol-induced current time course are presented in Fig. 4. Trace *A* was recorded from a twitch fibre voltage clamped to −60 mV and exposed to 216 μM-carbachol. The initial peak current was 1190 nA and the two time constants of decay were 5·9 and 163 s, respectively. Example *B* was recorded in a slow fibre also voltage clamped to −60 mV and perfused with 216 μM-carbachol. The initial peak current value was 125 nA and the two time constants of decay were 6·0 and 57 s, respectively. The ratio of the two current components was 0·03 for the twitch fibre and 0·73 for the slow fibre. The closeness of fit of the computer-generated exponential functions to describe the decay phase

of the two currents shown in Fig. 4 is presented in Fig. 5. A summary of the results obtained in this series of experiments is presented in Table 2.

Desensitization depends on both carbachol concentration and duration of exposure at twitch and slow fibre end-plates

The influence of carbachol concentration on the development of desensitization was compared at slow fibre and twitch fibre end-plates using the double-perfusion technique described in the Methods section. Three different concentrations of

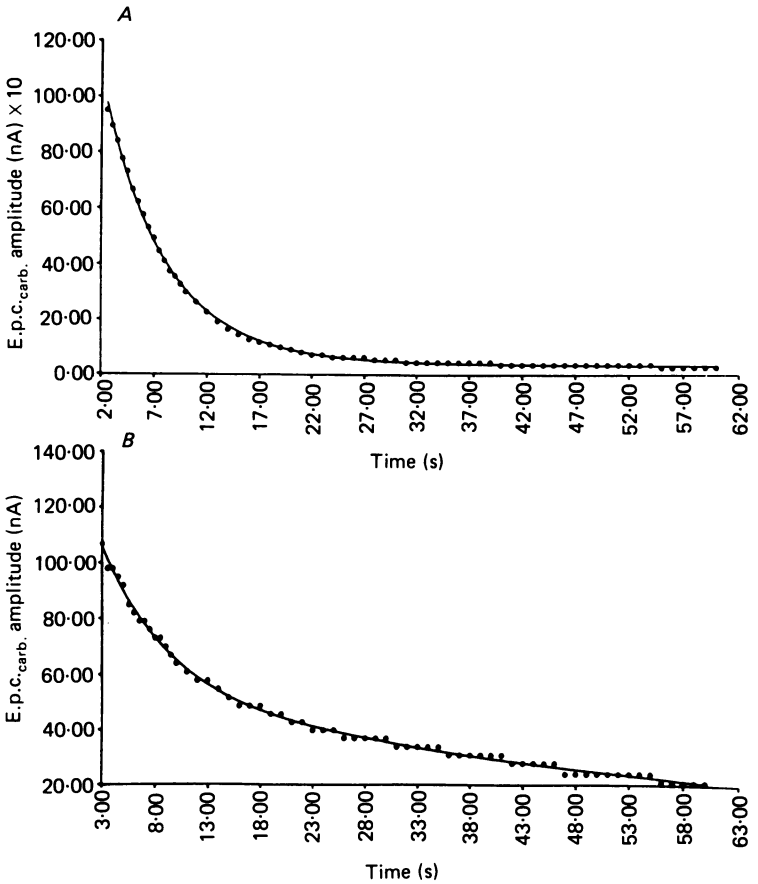

Fig. 5. The decline of the e.p.c.$_{carb.}$ records shown in Fig. 4 is plotted as a function of time. Example *A* shows the data for the twitch fibre and example *B* gives the data for the slow fibre. The filled circles show the data points and the line represents a computer-generated two-exponential function, which illustrates the closeness of fit.

carbachol (54, 108 and 162 μM) were used. The fibres were voltage clamped to -60 mV and the time interval between the desensitizing and test perfusions ranged from 15 s to 6 min. Two experimental records which illustrate the double-perfusion method and the influence of concentration on the development of desensitization are shown in Fig. 6.

With all three desensitizing concentrations, desensitization increased progressively

TABLE 2. E.p.c.$_{carb.}$ decay characteristics for twitch and slow fibre end-plates perfused with 216-μM-carbachol

Fibre type	Cell	Voltage (mV)	Peak e.p.c. (nA)	τ_1 (s)	τ_2 (s)	I_2/I_1†
Twitch	1	−60	1190	5·9	163	0·03
	2	−60	668	6·0	59	0·31
	3	−60	628	6·8	328	0·09
	4	−60	781	6·0	442	0·05
	5	−80	1162	7·7*	—	—
	6	−80	1299	3·3	530	0·04
	7	−80	1068	4·8*	—	—
	8	−80	1281	6·2*	—	—
Slow	1	−60	125	6·0	57	0·73
	2	−60	140	10·7	111	0·34
	3	−60	100	9·9	192	0·80
	4	−80	130	5·3	22	1·33
	5	−80	140	7·4	72	0·46
	6	−80	390	8·4	66	0·58
	7	−80	275	6·2	38	0·48

* Decay phase only had one discernible component when fitted over first minute of agonist application. The current decayed rapidly to a plateau value which remained constant.

† The relative contribution of the two decay components to the decay time course was approximated using the ratio of the amplitude of each current component extrapolated back to the peak e.p.c.$_{carb.}$.

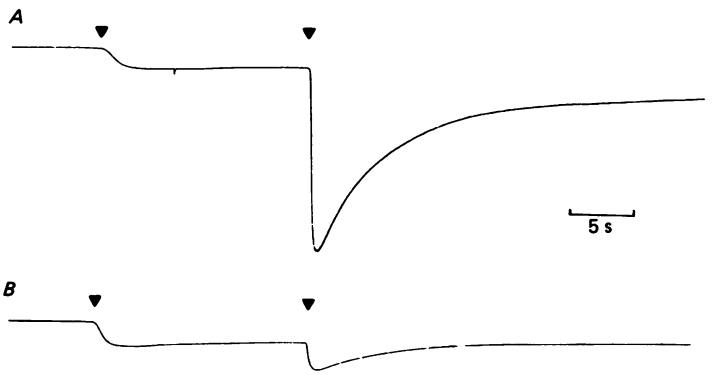

Fig. 6. Example records from two different slow fibres in which *A*, 54 μM- or *B*, 162 μM-carbachol was used as the initial 'desensitizing' dose (onset indicated by the first arrow). After approximately 16 s a second perfusion (indicated by the second arrow) of 5400 μM-carbachol was initiated at the same end-plate. The carbachol-induced current is indicated by a downward deflexion. In example *A*, peak e.p.c.$_1$ was −45 nA and e.p.c.$_2$ was −435 nA. The ratio e.p.c.$_2$/e.p.c.$_1$ was 9·7. In *B*, e.p.c.$_1$ was −115 nA and e.p.c.$_2$ was −125 nA. The ratio e.p.c.$_2$/e.p.c.$_1$ was equal to 1·09. The ratio e.p.c.$_2$/e.p.c.$_1$ demonstrates the influence of concentration on the extent of desensitization. Fibres voltage clamped to −60 mV. The gain was two times greater in *A* than *B*.

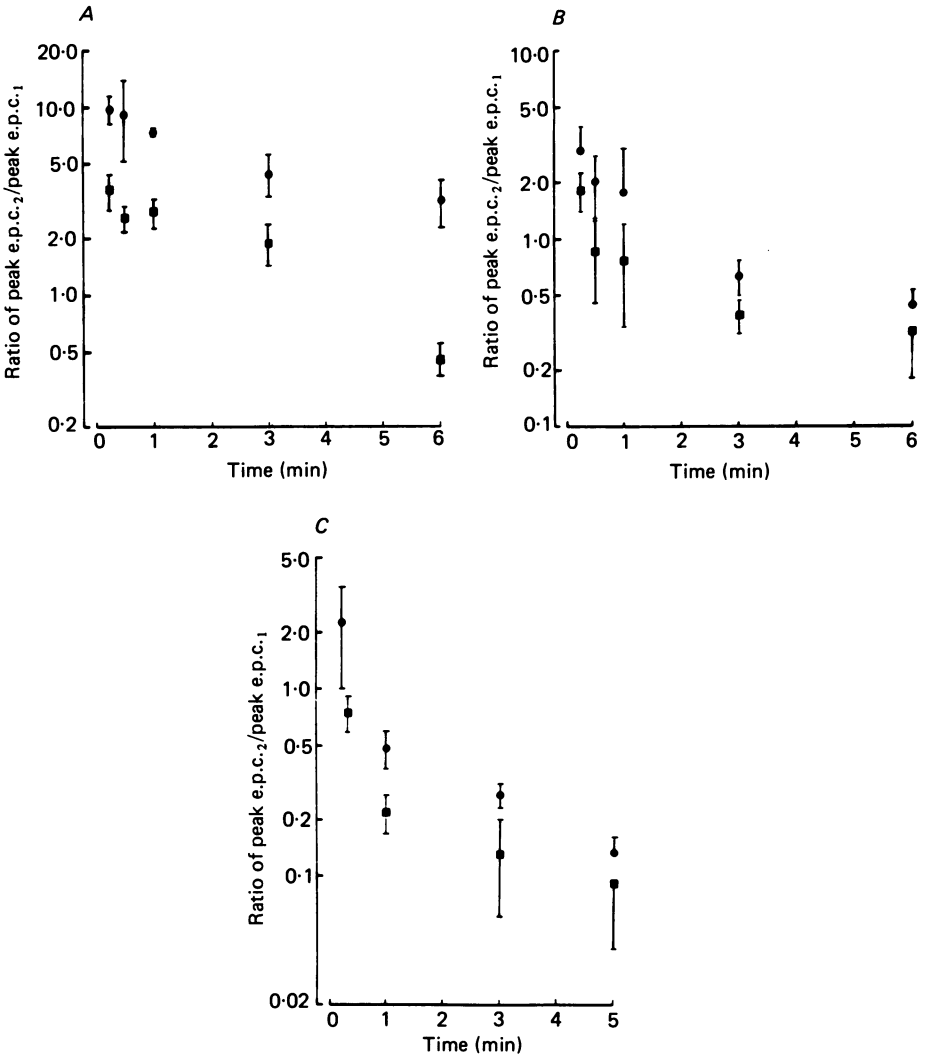

Fig. 7. A comparison of the time course of development of desensitization by *A*, 54 μM-, *B*, 108 μM-, and *C*, 162 μM-carbachol at slow (●) and twitch (■) fibre end-plates. In each instance, the extent of desensitization indicated by the ratio e.p.c.$_2$/e.p.c.$_1$ is plotted semilogarithmically as a function of time. E.p.c.$_1$ is the initial response to the 'desensitizing' dose and e.p.c.$_2$ is the response to 5400 μM-carbachol at different times during a sustained carbachol exposure. Each point represents the mean ± S.E. of the mean from at least three fibres. Fibres were voltage clamped to −60 mV.

as a function of time at both slow and twitch fibre end-plates. In the presence of 54 μM-carbachol, the depth of desensitization increased progressively between 15 s and 6 min at both end-plate types. However, the extent of desensitization was greater for any given time interval at the twitch fibre end-plates than at slow fibre end-plates. These results are summarized in Fig. 7*A*. With either 108 μM- or 162 μM-carbachol as the desensitizing dose, desensitization developed with a more complicated time course, so that the semilogarithmic plot of the e.p.c.$_2$/e.p.c.$_1$ ratio *versus* time

relationship had at least two components: one which occurred within the first 30 s and a second slower phase which extended over many minutes. Also with 108 μM- or 162 μM-carbachol, the extent of desensitization was consistently greater at twitch fibre end-plates than at slow fibre end-plates for the initial period of exposure. However, with longer exposures the difference decreased. The results obtained with 108 μM- and 162 μM-carbachol are summarized in Fig. 7*B* and *C*.

The initial results obtained with the double-perfusion method showed that as the concentration of the desensitizing dose increased, the depth of desensitization at 15 or 30 s increased markedly, and that the amplitude of the second component decreased progressively as a function of concentration. This observation is consistent with the well-established concentration dependence of the fast component of desensitization (Katz & Thesleff, 1957; Nastuk & Parsons, 1970; Scubon-Mulieri & Parsons, 1978).

These results also demonstrate that a slow component of desensitization is present at both end-plate types with prolonged exposure to carbachol concentrations less than 216 μM. We estimated the slope of the decrease in e.p.c.$_2$/e.p.c.$_1$ ratio *versus* time between 60 and 360 s to determine if there was an obvious influence of carbachol concentration on this slow component of desensitization. The values were $6{\cdot}1 \times 10^{-3}$ s^{-1}, $2{\cdot}8 \times 10^{-3}$ s^{-1} and $3{\cdot}7 \times 10^{-3}$ s^{-1} for twitch fibres exposed to 54, 108 or 162 μM-carbachol. The comparable values obtained at slow fibre end-plates were $2{\cdot}7 \times 10^{-3}$ s^{-1}, $4{\cdot}4 \times 10^{-3}$ s^{-1} and $5{\cdot}4 \times 10^{-3}$ s^{-1} with 54, 108 or 162 μM-carbachol. These results suggest that the slow component does not show a consistent agonist concentration dependence when the results from the two fibre types are compared.

The concentration dependence of desensitization is similar at twitch and slow end-plates

We have also used the double-perfusion technique to compare the concentration dependence of steady-state desensitization at snake slow and twitch end-plates. For these experiments, the fibres were voltage clamped to -60 mV. The time interval between the desensitizing dose and test dose perfusions was 3 min, with the desensitizing dose ranging between 18 and 340 μM for twitch fibres and between 27 and 340 μM for slow fibres. The test dose was 5400 μM for each. The results of this series of experiments are summarized in Fig. 8, in which the log of the e.p.c.$_2$/e.p.c.$_1$ ratio (for the 3 min exposure) is plotted as a function of the log of the carbachol concentration. The slope of this log–log relationship, determined by a linear regression using all the individual data values, was 2·1 for twitch and 1·6 for slow fibres. These results indicate that the concentration dependence for the steady-state level of desensitization during a prolonged agonist exposure is similar at both fibre types. The results also demonstrate that over a wide range of concentrations, the depth of desensitization at 3 min remained consistently greater for twitch fibres than slow fibres.

A similar analysis was done using the 15 s points shown in Fig. 7 to determine whether the concentration dependence of the initial extent of desensitization differed at slow or twitch fibre end-plates. The slope of a plot of the log of the e.p.c.$_2$/e.p.c.$_1$ ratio (for the 15 s exposure) *versus* the log of the carbachol concentration (54, 108 or 162 μM) was 1·4 for each end-plate type. This indicates that there is no difference between fibre types in the concentration dependence for both the early phase of desensitization and the later steady-state level of desensitization.

The extent of desensitization during prolonged agonist exposure is voltage dependent at both twitch and slow fibre end plates

We have also compared the voltage dependence of steady-state desensitization produced by 108 μM-carbachol at twitch and slow fibres. For this series of experiments, the e.p.c.$_2$/e.p.c.$_1$ ratio after 3 min exposure to 108 μM-carbachol was compared in slow and twitch fibres voltage clamped to different membrane potentials between

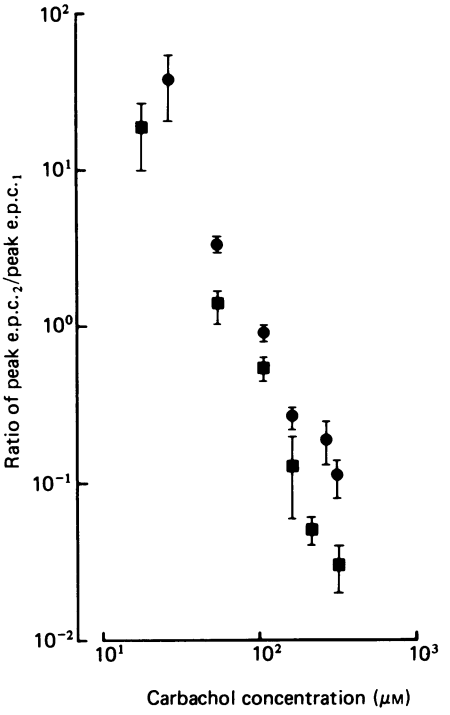

Fig. 8. A log–log plot of the extent of desensitization at 3 min (indicated by the e.p.c.$_2$/e.p.c.$_1$ ratio) *versus* carbachol concentration for slow fibre (●) and twitch fibre (■) end-plates. Each point is the mean ±S.E. of the mean of at least three fibres. Fibres were voltage clamped at −60 mV.

−80 and +50 mV. The voltage dependence of the e.p.c.$_2$/e.p.c.$_1$ ratio was similar at both end-plate types. The results of these experiments are summarized in Fig. 9 in which the log of the e.p.c.$_2$/e.p.c.$_1$ ratio is plotted as a function of membrane voltage. The coefficient of voltage dependence, estimated from the slope of the relationship between log e.p.c.$_2$/e.p.c.$_1$ *versus* voltage determined by linear regression, was 0·0250 mV^{-1} for twitch fibres and 0·0252 mV^{-1} for slow fibres. Although the voltage dependence of the 3 min desensitization level was comparable in the two fibre types, the absolute value of the e.p.c.$_2$/e.p.c.$_1$ ratio was consistently smaller for any given voltage at twitch fibres than at slow fibres.

Given the differences in channel gating properties between twitch and slow fibre end-plates (Dionne, 1981; Dionne & Parsons, 1981), we also expected that the peak carbachol current–voltage relationship would be different at the two end-plate types.

The peak e.p.c.$_{carb.}$–voltage relationship was non-linear at both fibre types and there was a marked difference in the peak e.p.c.$_{carb.}$ value obtained between twitch and slow fibre end-plates at all voltages. This is shown in Fig. 10 in which the average e.p.c.$_{carb.}$ value obtained from at least five different voltage-clamped fibres is plotted as a function of membrane voltage. An estimate of the coefficient of voltage dependence of the peak e.p.c.$_{carb.}$ was determined from the slope of a plot of ln

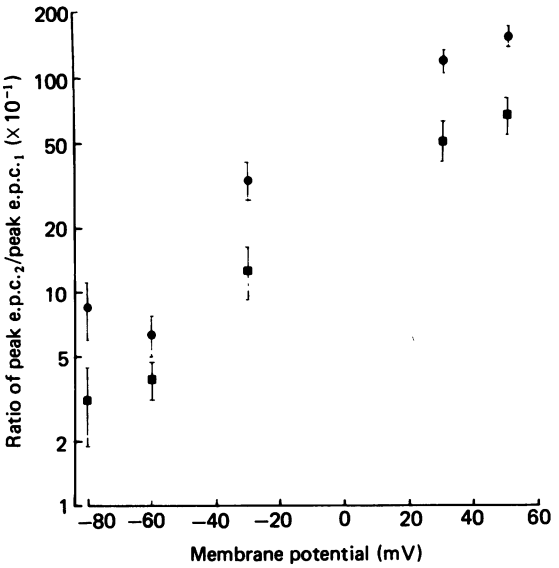

Fig. 9. A semilogarithmic plot of the extent of desensitization at 3 min *versus* membrane voltage for slow fibre (●) and twitch fibre (■) end-plates. Peak e.p.c.$_1$ is the response to 108 μM-carbachol and peak e.p.c.$_2$ is the response to 5400 μM-carbachol. Each point is the mean ±S.E. of the mean from at least three fibres.

(e.p.c.$_{carb.}$/($V - V_r$)) *versus* V, where V is membrane potential and e.p.c.$_{carb.}$ reversal potential V_r is 0 mV (Fiekers *et al.* 1980). The value of the coefficient was $-0{\cdot}0085$ mV^{-1} for twitch fibres and $-0{\cdot}0122$ mV^{-1} for slow fibres which indicates that the peak e.p.c. amplitude was more sensitive to voltage at slow end-plates than at twitch end-plates.

The voltage dependence ($A = -0{\cdot}0250$ mV^{-1}) of the 3 min steady-state level of desensitization obtained in the present study with both twitch and slow fibres is considerably greater than that estimated previously for the fast component of desensitization ($A = -0{\cdot}0100$ mV^{-1}; Fiekers *et al.* 1980). This difference could represent a difference in the voltage dependence of desensitization between frog and snake muscle or perhaps indicates that the initial fast phase of desensitization and longer-term steady-state desensitization exhibit different voltage dependences. To distinguish between these possibilities we determined both the voltage dependence of the initial component of desensitization and the 3 min steady-state level of desensitization. These experiments were done only at twitch end-plates. The desensitizing concentration of carbachol was 216 μM and the fibres were voltage clamped

to −60, +30 and +50 mV. The fast component τ was determined by analysis of the initial phase of $e.p.c._{carb.}$ decay and the 3 min steady-state level estimated by the $e.p.c._2/e.p.c._1$ ratio. Both the decay τ and the $e.p.c._2/e.p.c._1$ ratio could be adequately described as an exponential function of voltage with the coefficient of voltage being $-0{\cdot}0109\ mV^{-1}$ for the decay τ and $0{\cdot}0192\ mV^{-1}$ for the 3 min steady-state level. These results demonstrate that the voltage dependence of the initial component of desensitization and the steady-state level of desensitization can be different.

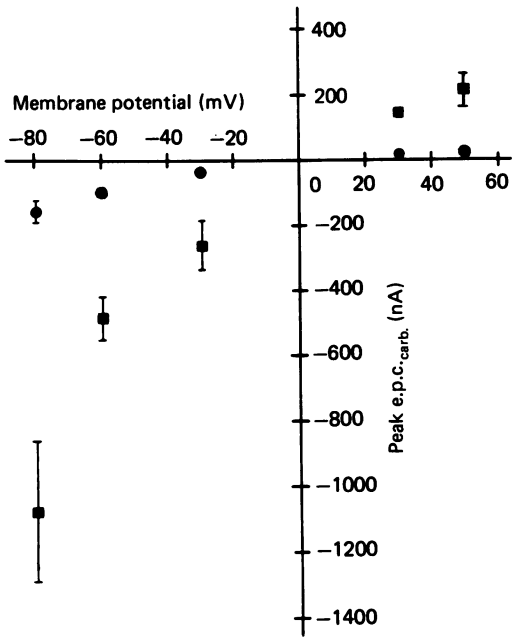

Fig. 10. Peak $e.p.c._1$ plotted as a function of membrane potential for slow fibre (●) and twitch fibre (■) end-plates. Each point represents the mean ±S.E. of the mean from at least three fibres. The carbachol concentration was 108 μM.

DISCUSSION

The results of the present study demonstrate that the characteristic differences in receptor-channel gating between slow and twitch fibre end-plates occur in high-potassium solutions as well as in normal sodium solutions (Dionne & Parsons, 1981). For twitch fibre end-plates the coefficient of the voltage dependence of the m.e.p.c. decay time constant ($-0{\cdot}0092\ mV^{-1}$) was similar to the voltage dependence of the peak $e.p.c._{carb.}$ amplitude ($-0{\cdot}0085\ mV^{-1}$). During sustained agonist application, agonist exposure is much greater than the lifetime of an open channel so that the peak response is primarily a function of the equilibrium population of open channels, which for twitch fibres will depend on the ratio of the opening to the closing rate constants (Dionne & Stevens, 1975). The opening rate constant is not noticeably voltage dependent at snake twitch end-plates (Dionne, 1981). Therefore, the curvature of the carbachol current–voltage curve at twitch end-plates primarily reflects the

voltage dependence of the carbachol-induced open-channel lifetime. The situation at slow fibre end-plates is more complicated since the coefficient of voltage dependence for m.e.p.c. decay ($-0{\cdot}0049\ mV^{-1}$) is much less than the coefficient of voltage dependence for the peak $e.p.c._{carb.}$ ($-0{\cdot}0120\ mV^{-1}$). Dionne (1981) suggested that at slow fibre end-plates the mean channel open time and the duration of the intermediate, non-conducting state increase with hyperpolarization because both the channel closing rate constant and the initial unbinding rate constant appear to be voltage dependent. The observation that the voltage dependence of the peak $e.p.c._{carb.}$ value is slightly greater at slow end-plates than at twitch end-plates is consistent with Dionne's model (1981). Because both the intermediate state and mean channel open time are voltage-dependent at slow fibre end-plates, the probability of channel opening increases with hyperpolarization.

Desensitization developed progressively at both twitch and slow fibre end-plates during the sustained exposure to carbachol. Further, with concentrations of 108–216 μM, the time course of desensitization onset appeared to have at least two separate components at both end-plate types. Other reports have also demonstrated an initial fast phase having a time constant in seconds followed by a second, slower component (Clark & Adams, 1981; Feltz & Trautmann, 1982; Chestnut, 1983). With high agonist concentrations, the decay time course progresses exponentially because the fast component predominates (Fiekers *et al.* 1980). The biphasic decay has been interpreted to indicate that desensitization under certain conditions is a multi-component process (Magleby & Pallotta, 1981; Feltz & Trautmann, 1982; Chestnut, 1983). Our results provide further evidence that the development of desensitization in the presence of moderate agonist concentrations develops with a complex time course: an initial fast component and a second slower component. We have assumed that with the concentrations used in this study no significant amount of recovery occurred in the continued presence of agonist, so that both components represent separate processes leading to the production of desensitized receptor-channel complexes. At all concentrations, twitch fibre end-plates were desensitized to a greater extent during the initial moments of agonist exposure than slow fibres. This was apparent in the first series of experiments using the analysis of the $e.p.c._{carb.}$ decay time course (216 μM-carbachol) and from the $e.p.c._2/e.p.c._1$ ratio at 15 s of exposure with the double-perfusion experiments. Both of these observations suggest that for any particular concentration the extent of desensitization contributed by the initial component of desensitization is greater at twitch end-plates than at slow end-plates. This difference could not be attributed to a difference in the relative extent of activation by carbachol at twitch or slow end-plates. Although peak $e.p.c._{carb.}$ values were significantly less in slow fibres than twitch fibres the shape of the carbacol dose–peak current relationship was not difference between fibre types. At present, we can only speculate as to a possible mechanism responsible for the greater initial desensitization at twitch fibre end-plates. We suggest that this may be related to the differences in the activation gating kinetics between fibre types. The mean channel open time and single-channel conductances are similar at twitch and slow fibre end-plates, but the probability of channel opening is greater at twitch fibre end-plates than at slow fibre end-plates (Dionne, 1981). Therefore, during a sustained exposure to the same concentration of agonist, individual twitch fibre receptor-channel

complexes should be in the open, conducting state a greater percentage of time than receptor-channel complexes at slow end-plates. If the fast component of desensitization develops from the open configuration rather than from an intermediate, non-conducting state, the probability would be greater that twitch fibre receptor-channel complexes will enter the desensitized state than receptor-channel complexes at slow fibre end-plates. Therefore, the initial phase of desensitization would be greater at twitch end-plates than at slow fibres.

The concentration dependence of desensitization was similar at both end-plate types, the slope of the log–log plot being 1·4 for the 15 s time interval and approximately 2 for the 3 min steady-state level. This suggests that two molecules of carbachol are bound to the receptor channel at both end-plate types during the development of desensitization, as well as during receptor activation (Hoffman & Dionne, 1980). Feltz & Trautmann (1982), using different techniques, also concluded that two molecules of agonist are bound to the desensitized receptor-channel complex.

We showed previously at frog end-plates that the fast component of desensitization increases with hyperpolarization (Fiekers *et al.* 1980). A similar value of the voltage dependence of the $e.p.c._{carb.}$ decay τ was also obtained at snake twitch fibre end-plates in this study. In addition, we observed that the 3 min steady-state level with the double-perfusion method can have a voltage dependence different from that of the fast component of desensitization. This is consistent with the recent suggestion of Chestnut (1983) that the fast and slow components of desensitization may represent separate processes. In addition, Chestnut (1983) has reported that the fast component of desensitization is more sensitive than the slow component to agonist concentration. Our results with the double-perfusion technique are quite similar. We observed that the slow component of desensitization did not exhibit a consistent dependency on carbachol concentration whereas the initial extent of desensitization by 15 s of exposure increased markedly with increasing concentration.

In summary, the results of the present study demonstrate that desensitization occurs in a time-dependent fashion at both twitch and slow fibre end-plates during sustained carbachol application and, for both, the time course of desensitization onset can exhibit more than one component. Further, even though the gating kinetics are unique to the fibre types, the characteristics of desensitization onset appear to have a number of common features at both end-plate types.

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