Journal of Physiology (1989), **415**, *pp.* 159–188
With 8 text-figures
Printed in Great Britain

DESENSITIZATION OF THE ACETYLCHOLINE RECEPTOR OF FROG END-PLATES MEASURED IN A VASELINE-GAP VOLTAGE CLAMP

BY A. B. CACHELIN* AND D. COLQUHOUN

From the MRC Receptor Mechanisms Research Group, Department of Pharmacology, University College London, Gower Street, London WC1E 6BT

(*Received 24 October 1988*)

SUMMARY

1. Desensitization of the nicotinic acetylcholine receptor of the frog end-plate was investigated in dissociated frog muscle fibres using the Vaseline-gap clamp method so that a wide range of well-defined agonist concentrations could be used without having to use α-bungarotoxin to reduce currents, and so that the intracellular medium could be controlled.

2. Acetylcholine (ACh) concentrations between 1 and 1000 μM were used, after inactivation of acetylcholinesterase. The intracellular calcium concentration was usually kept near zero by using 80 mM-K_2EGTA as the intracellular solution.

3. When using the low intracellular calcium solution, desensitization proceeded as a biphasic process with estimates of fast and slow time constants of about 8 and 80 s at 4 °C and 20 μM-ACh (the rates increased with concentration). In contrast, only one (fast) component of desensitization was detected when the intracellular calcium concentration was allowed to increase during ACh application.

4. Despite rapid application of ACh the time to peak response was 0·2 s (with 400 μM-ACh) to 2 s (with 1 μM-ACh); this slow rise was shown to result from diffusion delays. Nevertheless the peak current with 200 μM-ACh corresponded to opening of most of the channels present, so there is probably not much desensitization in the millisecond time range.

5. Both fast and slow time constants for onset of desensitization showed only slight dependence on membrane potential when $[Ca^{2+}]_i$ was buffered with 80 mM-K_2EGTA.

6. Increasing the intracellular cyclic AMP concentration directly, or indirectly with forskolin and IBMX, had no effect on the time course of desensitization.

7. Intracellular application of submicromolar concentrations of phorbol-12,13-dibutyrate (PDBu) and phorbol-12-myristate-13-acetate (PMA) yielded a small but reproducible reduction of the peak response to ACh. The time course of desensitization was, however, not modified by these substances.

8. The implications of these observations for the mechanism of desensitization, and their relationship to single-channel observations, are discussed.

* Present address: Pharmacology Department, University of Berne, Friedbühlstrasse 49, 3010 Berne, Switzerland.

INTRODUCTION

Katz & Thesleff (1957) published the first quantitative study of the desensitizing effect of the physiological transmitter acetylcholine (ACh) on the nicotinic ACh receptors of the frog skeletal muscle end-plate. They found the onset of desensitization showed an exponential time course with a time constant (of the order of seconds) that decreased with agonist concentration. Many subsequent studies also found a simple exponential time course, though the time constant varied considerably from one report to another. Those discrepancies may have resulted from the fact that the time course of desensitization is actually biphasic (at least when intracellular $[Ca^{2+}]$ is allowed to rise during ACh application; see Discussion). This has been shown by several investigations during the last 7 years (for example, Anwyl & Narahashi, 1980; Clark & Adams, 1981; Feltz & Trautmann, 1982; Pennefather & Quastel, 1982; Chesnut, 1983; Connor, Fiekers, Neel, Parsons & Schnitzler, 1984; Adams, 1987, and, in neurones, Slater, Hall & Carpenter, 1984, and Boyd, 1987). The time constants found were of the order of a few seconds for the faster component, and of the order of a minute for the slower component. Much more recently the advent of rapidly perfused outside-out membrane patches, which allow very rapid agonist application, has revealed much faster desensitization processes, with time constants of the order of tens of milliseconds, in the mouse-muscle-like receptor of the BC3H1 cell line (Bekkers, 1986; Brett, Dilger, Adams & Lancaster, 1986). Similar fast rates have been reported in purified *Torpedo* receptor preparations (Heidmann & Changeux, 1984; Cox, Kaldany, DiPaola & Karlin, 1985).

Despite a great deal of work, it is still true that nothing is known about the physical nature of desensitized states of the receptor. It is known that the desensitization process can be influenced by calcium ions (Manthey, 1970; Fiekers, Spannbauer, Scubon-Mulieri & Parsons, 1980; Miledi, 1980; Chemeris, Kazachenko, Kislov & Kurchikov, 1982; Chesnut 1983; Oswald, 1983). Recently evidence has been obtained that phosphorylation of the nicotinic receptor can accelerate desensitization in rat muscle preparations (Albuquerque, Deshpande, Aracava, Alkondon & Daly, 1986; Huganir, Delcour, Greengard & Hess, 1986; Middleton, Jaramillo & Schuetze, 1986) it is, however, unlikely that phosphorylation is the normal mechanism that produces desensitization because the process can occur in reconstituted peripheral receptor (Nelson, Anholt, Lindstrom & Montal, 1980; Schindler & Quast, 1980; Popot, Cartaud & Changeux, 1981) and, as in the present work, when the intracellular medium has been replaced by one that contains no ATP.

Up to now studies of desensitization of muscle receptors under voltage clamp have been limited by the modest current-carrying capacity of the apparatus, or by muscle contracture with high agonist concentrations. As a result previous studies have been restricted to low agonist concentrations, or ionophoretic application of agonist (so the concentration is unknown and non-uniform; e.g. Feltz & Trautmann, 1982; Chesnut, 1983), or to reducing the total current flow with α-bungarotoxin, as in the work cited by Adams (1987), with consequent uncertainty about the behaviour of channels that have only one of their two binding sites blocked. Single-channel studies on isolated membrane patches (e.g. Bekkers, 1986; Brett *et al.* 1986) solve these

problems, but are suitable only for investigation of very rapid desensitization; the very long shut periods that correspond to slow desensitization processes (Sakmann, Patlak & Neher, 1980; Colquhoun & Ogden, 1988) cannot be collected in large enough numbers for quantitative analysis, and cannot be interpreted in the absence of knowledge of the number of channels in the patch. As a result of these problems there is still no published account of, for example, the concentration dependence of the fast and slow rates of desensitization over a wide range. In this paper we report an investigation of desensitization over a wide range of agonist concentrations without the need for toxin block, by taking advantage of the large current-carrying capacity of the Vaseline-gap voltage clamp method (Hille & Campbell, 1976). This method has the additional advantage that the intracellular medium can be changed, by diffusion through the cut ends of the muscle fibre, though it has the disadvantage that investigation of the rate of recovery from desensitization is difficult.

METHODS

Preparation and voltage-clamp apparatus

The left or right semitendinosus muscles of male and female *Rana temporaria* were dissected in chloride-free depolarizing solutions (Adams, Nonner, Dwyer & Hille, 1981). Frogs were killed by decapitation followed by destruction of the brain. Individual muscle fibres were prepared with scissors and forceps and transferred to a Perspex measuring chamber containing intracellular solution (Hille & Campbell, 1976). A membrane cylinder extending about 5 mm on either side of the end-plate was divided into four regions by applying three Vaseline seals (Glisseal, Bohrer Chemie, Switzerland) about 150 μm apart. Diffusional exchange of the intracellular space with the solutions in the end-pools was facilitated by making a longitudinal cut into the muscle fibre that extended as close as possible to the Vaseline seals.

The resting membrane potential was then re-established by perfusing the end-plate region with frog Ringer solution. Potential offsets were adjusted following which the membrane potential was clamped by increasing the gain of the feedback amplifier until it just began to oscillate. The cut-off frequency of the amplifier was adjusted so as to minimize oscillations.

The membrane potential was clamped with an amplifier designed by Hille & Campbell (1976) and built by Dr D. J. Adams. Calomel electrodes were used to connect the recording chamber to the voltage-clamp amplifier. Currents of several microamps could be passed without sign of polarization.

Data acquisition

Current responses were low-pass filtered before acquisition (f_c = 125 Hz, −3 dB for concentration jumps or 1·25 kHz for voltage jumps; EF05-02 Filter, Fern Development, UK). The command pulses were issued, and the responses were digitized, on-line and stored on disc (PDP 11/73, Digital Equipment Corporation; CED502, Cambridge Electronics Design, Cambridge, UK). In many cases a faster sampling rate was used for the first part of the response, during which the current was changing relatively fast, followed by change to a slower sampling rate for the later, slower part of the response. This dual rate sampling method both reduced storage requirements and speeded curve fitting.

Solutions

The usual external frog Ringer solution contained (mM): NaCl, 150; KCl, 2·5; $CaCl_2$, 1·5; Na-HEPES, 5; pH 7·2. The depolarizing solution contained (mM): potassium methylsulphate, 110; $CaSO_4$, 2; K-HEPES, 10; pH 7·2.

Solutions applied to the end-pools diffused readily into the cytoplasm through the cut ends of the fibre. The intracellular solution used in most experiments was 80 mM-K_2EGTA (adjusted to pH 7·2 with KOH). For voltage-jump experiments 40 mM-Cs_2SO_4 + 40 mM-K_2EGTA was used.

The intracellular solution used in phosphorylation experiments contained (mM): potassium glutamate, 75·5; $MgSO_4$, 6·8; Na_2ATP, 5; creatine phosphate, 20; EGTA, 0·2; Na-HEPES, 5; pH

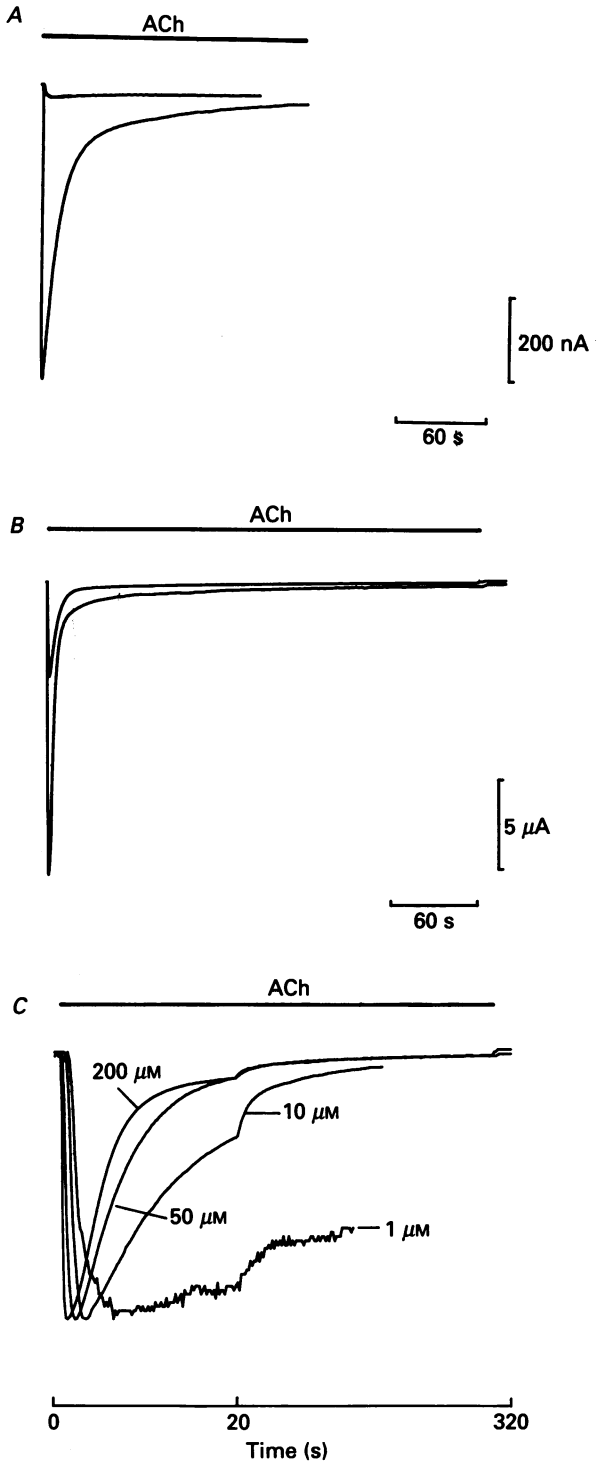

Fig. 1. For legend see facing page.

7·4 (Irving, Maylie, Sizto & Chandler, 1987). The free calcium concentration of this solution was estimated to be below 10^{-7} M, and the free Mg^{2+} concentration about 1 mM.

Freshly made-up ACh (ACh chloride, Sigma) solutions were added to the external medium. External solutions were applied through a thin glass capillary, placed at about 250 μm from the end-plate, at 15 ml/min. This method, combined with the pool volume of about 200 μl, guaranteed very short solution exchange times. The temperature of the perfusing solution was monitored next to the end-plate by a miniature thermistor probe and kept to $4{\cdot}0 \pm 1{\cdot}5$ °C by a cooling system. Experiments in which receptor phosphorylation was investigated were performed near to room temperature (17–18 °C).

In all experiments acetylcholinesterase was inactivated by perfusion with methanesulphonyl fluoride (Eastman Chemicals), 150 μM for 5 min, or 50 μM for 15 min, before the start of the experiment.

Data analysis

The current decay, from shortly after the peak response, was usually fitted with the sum of two exponentials. In general, the equation fitted had the form:

$$I(t) = I(\infty) + \sum_{i=1}^{n} [w_i \exp(-t/\tau_i)],$$

where $I(t)$ is the current at time t, τ_i are the time constants of the components and w_i are the amplitudes (at $t = 0$) of the components, such that $\Sigma w_i = I(0) - I(\infty)$.

At 1 μm-ACh the slower component was too slow to be fully resolved, so it was represented by a linear component with slope b, thus:

$$I(t) = I(\infty) + [I(0) - I(\infty)] \exp(-t/\tau_i) + bt.$$

Estimates of the time constants and the amplitudes for each component, and for the asymptote, $I(\infty)$, were found by equally weighted least-squares fitting of the equations to the digitized points of the decay curve.

The amplitudes w_i are defined at $t = 0$, the moment of drug application, but the development of the response is sufficiently slow (see Results) that this moment cannot be defined unambiguously. The rate of development of the response was assessed by measuring the 20–80 % rise time, and the time, t_0 say, at which 50 % of the peak response was attained was estimated by interpolation. This time was, somewhat arbitrarily, taken as zero time for fitting the decay phase, so values of the relative amplitudes of components given in the Results refer to this time. A rough correction for the attenuation of the observed peak response (I_p) by desensitization was made by extrapolation of the fitted equation back to t_0 to obtain the corrected value, $I(0)$. The extent of this attenuation is illustrated by the observation that $I_p/I(0)$ decreased from 93 % at 1 μM-ACh to 67 % at 200 μM-ACh.

Fig. 1. Current responses induced by the application of various concentrations of ACh to voltage-clamped frog end-plates. *A*, current induced by 1 μM-ACh (top trace) and 10 μM-ACh (bottom trace). The response to 1 μM-ACh was fitted with a single exponential with a time constant $\tau_f = 19{\cdot}7$ s. The response to 10 μM-ACh was fitted with the sum of two exponentials with time constants $\tau_f = 9{\cdot}3$ s and $\tau_s = 121$ s. *B*, responses to 50 μM-ACh (top trace) and 200 μM-ACh (bottom trace). The responses were fitted with the sum of two exponentials with time constants of $\tau_f = 12{\cdot}3$ s and $\tau_s = 110$ s for 50 μM-ACh, and $\tau_f = 3{\cdot}1$ s and $\tau_s = 48{\cdot}4$ s for 200 μM-ACh. *C*, dual time base display of the responses in *A* and *B*. The total length of the record is 320 s but the first 20 s are shown on an expanded scale to display clearly the faster component. The responses have been scaled so their peak currents appear equal to show the increase of the relative amplitude of the fast component of desensitization with increasing ACh concentration. Experimental conditions were: external solution, frog Ringer solution; intracellular solution, 80 mM-K_2EGTA; E_m (membrane potential) = −50 mV; temperature = 4 °C.

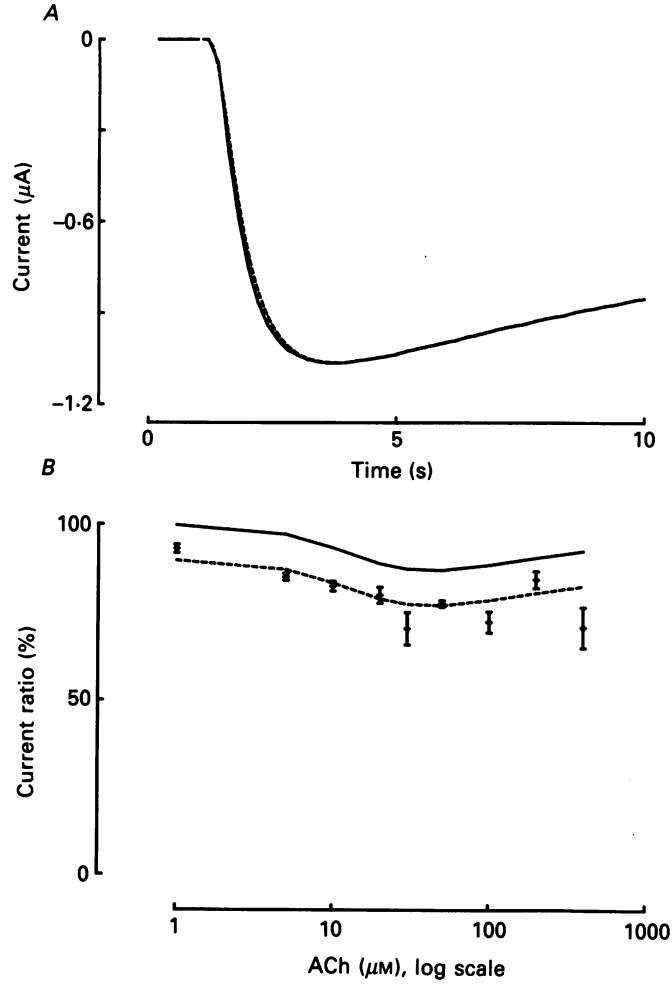

Fig. 2. *A*, comparison between observed (continuous line) and calculated (dashed line) response to 10 μM-ACh. Details of the calculations are given in the Appendix. *B*, effect of desensitization on the peak response to ACh. The continuous line shows the calculated ratio of the response with, and without, desensitization (calculations as in *A*; see Appendix). The experimentally observed ratio of measured to extrapolated peak current, $I_p/I(0)$, is shown at various concentrations of ACh (mean $\pm$ S.E.M.; see Methods). The dashed line through the experimental points was obtained by lowering the continuous line by 10%; the extrapolated responses, $I(0)$, were consistently slightly larger than predicted by the theoretical calculation.

RESULTS

Response as a function of ACh concentration

Figure 1 shows the time course of the responses to 1 and 10 μM-ACh (Fig. 1*A*) and 50 and 200 μM-ACh (Fig. 1*B*) at -50 mV holding potential. After the peak the response declined rapidly at first, and then much more slowly until, after about 5 min, the response had declined to a few per cent of its peak value. Figure 1*C* shows the time course of the responses to a range of concentrations on a dual-rate time scale

(dual rate sampling, see Methods); the curves have been scaled so that they have the same maximum response in order to show more clearly the effect of ACh concentration on the rising and falling phases.

Rising phase of the response

Despite the fact that ACh was applied as rapidly as possible (see Methods) the response took some time to reach its peak. The 20–80 % rise time fell from 2 s with 1 μM-ACh to 0·26 s with 100 μM-ACh. The time course of the rising phase with 10 μM-ACh is shown in Fig. 2*A*, together with the calculated time course based on an assumed unstirred layer with a depth of 35 μm (see Appendix for details). The peak amplitude of the response is clearly likely to have been attenuated by desensitization.

The percentage attenuation, $100\,I_p/I(0)$, predicted by diffusion calculations, is shown in Fig. 2*B* (continuous line), together with the attenuation, implied by extrapolation of the fitted curve back to a point half-way up the rising phase (see Methods). The use of the extrapolated value, $I(0)$, is seen to provide a somewhat larger correction (by about 10 %) than is suggested by the diffusion calculation, but neither correction is very large (as long as there are no faster components of desensitization than those observed here; see below).

The peak response

The peak response, I_p, varied from fibre to fibre, probably as a result of variations from one preparation to another in the proportion of the end-plate which was sitting within the Vaseline gap. Under optimal conditions, up to four different concentrations of ACh could be applied to a single fibre leaving recovery periods of about 10 min between drug applications. The peak current, I_p, invariably decreased as a function of time. This may result partly from residual desensitization, as well as from fibre run-down.

The extrapolated peak response, $I(0)$, is shown in Fig. 3. The response reaches half-maximum (see Fig. 3, and section on models) at about 55 μM-ACh (at -50 mV). This is about what would be expected at -50 mV on the basis of single-channel studies, which allow correction for desensitization, and which suggest a half-maximum response with 15–20 μM-ACh at -120 mV (Sakmann *et al.* 1980; Colquhoun & Ogden, 1988), supposing that the channel shutting rate, α, changes e-fold for about 50 mV at 4 °C. The extrapolation method used here should give an estimate of response corrected for desensitization (see Methods) only as long as there are no components of desensitization with time constants faster than those observed. The response declined at very high ACh concentrations as expected from the known channel-blocking effect of ACh in this preparation (Ogden & Colquhoun, 1985). The maximum response, with 200 μM-ACh (see Figs 1, 3 and 8), was about 16 μA at -50 mV; this current corresponds to the opening of about 10^7 ion channels, which is roughly the number of channels in the end-plate (see Discussion).

The equilibrium response

The asymptote, $I(\infty)$, of the fitted curve was used as an estimate of the equilibrium current. Fitting a linear component in addition to two exponential components produced a larger scatter of values than if the linear component were

omitted so no process *slower* than that with the slower time constant (described below) was detectable. The results are shown in Fig. 3.

The equilibrium responses varied considerably from fibre to fibre, presumably for the same reason as variation in the peak current (see above), but it is clear that the

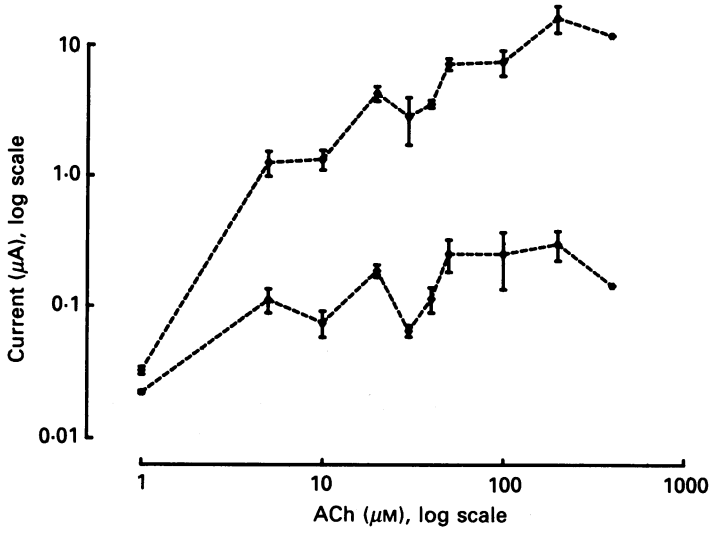

Fig. 3. Peak and equilibrium responses to various concentrations of ACh shown on a log–log scale. The top curve shows the corrected peak responses $I(0)$, and the bottom curve the equilibrium responses $I(\infty)$, as defined in the Methods section. Individual points are means ± S.E.M. of at least three responses on three different cells (except at 400 μM-ACh for which there was only one response). The points have been joined by straight lines here; fitted curves are shown in Fig. 8.

maximum equilibrium response was of the order of 200 nA, i.e. 1–2% of the maximum peak response. The lack of clear concentration dependence above 10 μM-ACh is consistent with the concentration for 50% of the maximum equilibrium response being considerably lower than that for 50% of the peak response, as expected from the models considered below.

The rate constants for onset of desensitization

The decay phase was well fitted by the sum of two exponential components (except at 1 μM-ACh for which the slow component could not be clearly resolved; see Methods). The time constants for these components, and their relative amplitudes, were far more reproducible than the peak and equilibrium currents. Figure 4 shows the fast and slow rate constants for desensitization, $\lambda_f = 1/\tau_f$ (top curve) and $\lambda_s = 1/\tau_s$ (bottom curve), plotted against ACh concentration. The faster time constant, τ_f, clearly got faster as ACh concentration was increased; it was 26 s at 1 μM-ACh and 6 s at 100 μM-ACh (4 °C, $E_m = -50$ mV).

The slower time constant is somewhat less concentration dependent than the faster time constant: an empirical linear fit to the log–log plot suggests a 1·3-fold decrease in τ_s for a 10-fold increase in ACh concentration (compared with a 2·1-fold decrease for τ_f).

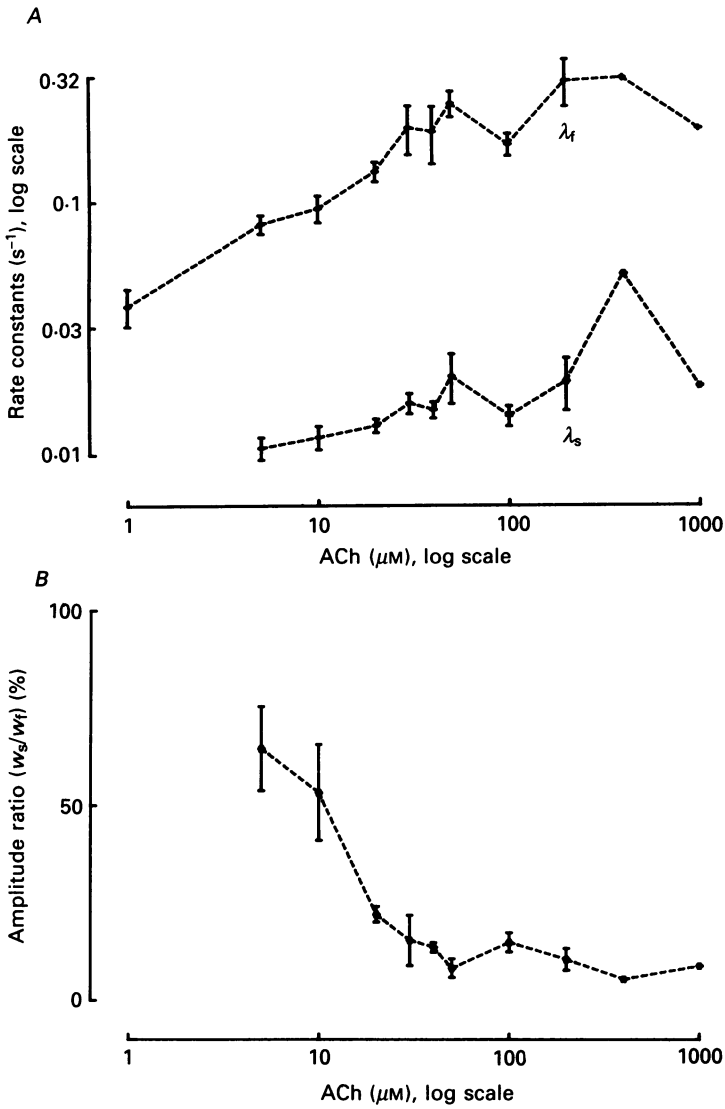

Fig. 4. *A*, concentration dependence of the observed rate constants of desensitization shown as a log–log plot, *B*, relationship between the ratio of the amplitudes (at $t = 0$) of the slow and fast components of desensitization (w_s/w_f) and ACh concentration. The relative amplitude of the fast component of desensitization increases markedly with increasing agonist concentration (see also Fig. 1*C*). There is no value at 1 μM-ACh since the responses were fitted with a single exponential as explained in the Methods section. All points are means $\pm$ S.E.M. of parameters from at least three responses on three different cells (except at 400 and 1000 μM-ACh). The points are joined with straight lines here; fitted curves are shown in Fig. 8.

The concentration dependence of the relative amplitude, w_s/w_f, of the slow and fast components (at $t = t_0$; see Methods) is shown in Fig. 4*B*. The slow component is large at low ACh concentrations, but above roughly 30 μM-ACh the fast component becomes predominant, being 7 to 10 times larger up to 1 mM-ACh.

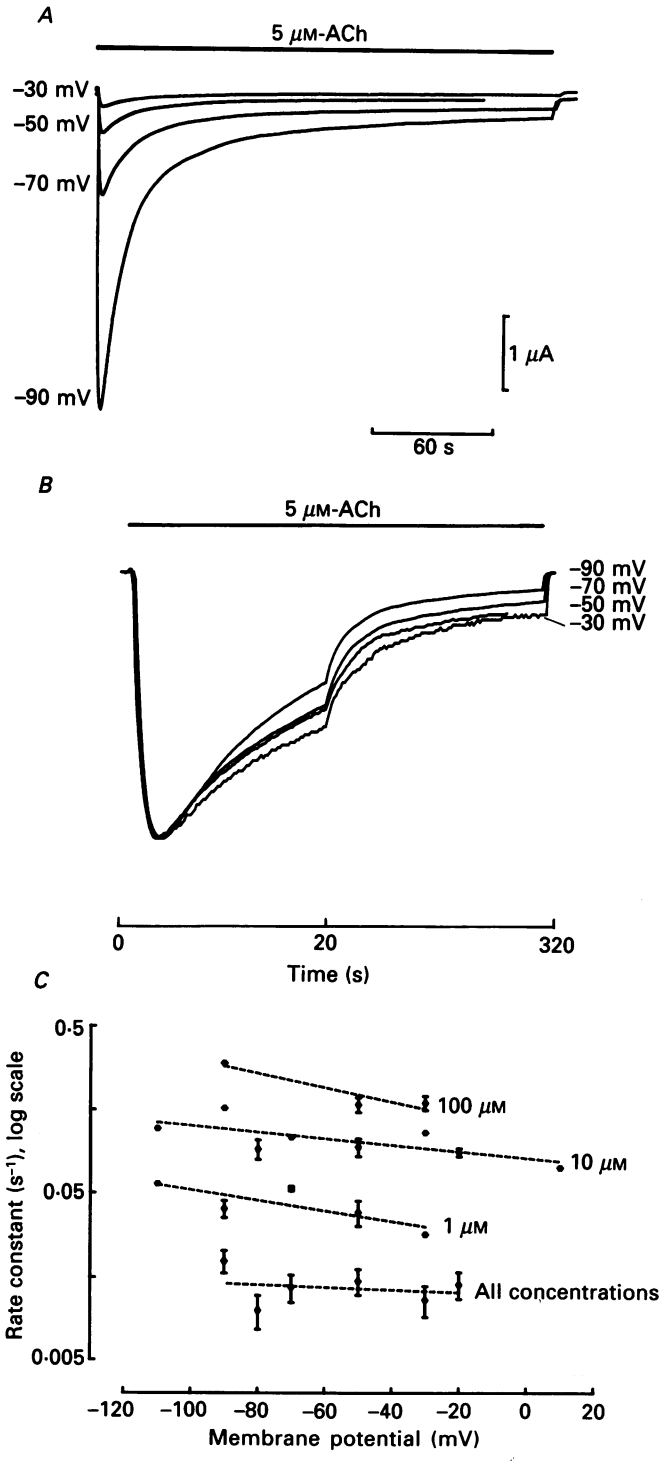

Fig. 5. For legend see facing page.

The effect of membrane potential

Effect of holding potential on the desensitization rate

Responses to 10 μM-ACh at various holding potentials are shown in Fig. 5*A*; in Fig. 5*B* the same responses are shown, this time on a dual-rate time scale, but they have been normalized to have the same maximum response for easy comparison; a modest speeding of the fast component with hyperpolarization is visible. Figure 5*C* shows the effect of varying holding potential of the fibre (from -110 to $+10$ mV) on the rate constants for the two exponential components for the onset of desensitization. In these experiments, in which the internal solution was 80 mM-K_2EGTA, only slight voltage dependence was seen. The faster rate constant, λ_f, became somewhat faster as the end-plate was hyperpolarized; an e-fold increase in rate was produced by a hyperpolarization of $H = 100$–200 mV in different experiments (the value of H bore no consistent relation to ACh concentration). The slower rate constant, $\lambda_s = 1/\tau_s$, was rather more variable; although several individual experiments suggested some increase in λ_s with hyperpolarization, the effect did not usually achieve statistical significance. The slow rate constant was shown above to have only slight dependence on ACh concentration, so values for all concentrations were averaged to obtain the lowest graph in Fig. 5*C*. There is clearly little demonstrable dependence of λ_s on membrane potential.

The relative amplitude, w_s/w_f, of the two components also showed no consistent dependence on membrane potential at any ACh concentration.

Voltage jumps

It is possible that there might exist a component of desensitization faster than those described above (though the arguments above, and in the Discussion, suggest that such a component could not have a very large amplitude). A faster component would not be detectable in concentration-jump experiments because of the diffusion

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Fig. 5. Voltage dependence of desensitization. *A*, currents induced by 5 μM-ACh in one frog muscle cell clamped at four different membrane potentials ($E_m = -30$, -50, -70 and -90 mV). The main effect of hyperpolarization is a marked increase of the peak current from 0·25 μA at -30 mV to 4·24 μA at -90 mV (see also Fig. 6*C*). *B*, responses from *A* are shown normalized to equal peak currents, in order to show more clearly the effect of membrane potential on the time constants of desensitization. Plotted on dual rate time scale (as Fig. 1*B*); the first 20 s of the 320 s record are shown on an expanded scale to emphasize the faster component. The faster time constant, τ_f, was 11·7 s at -30 mV and 10·7 s at -90 mV, whereas τ_s was 78·2 s at -30 mV and 70·8 s at -90 mV. *C*, voltage dependence of the fast rate constant $\lambda_f = 1/\tau_f$ (top three curves) at three ACh concentrations and of the slow rate constant $\lambda_s = 1/\tau_s$ (bottom curve) averaged over all ACh concentrations. Estimates of H, the amount of hyperpolarization for an e-fold increase in the rate constants, were calculated by least-squares fit of a line to the data points. At 100 μM-ACh (top curve), $H = 101$ mV, at 10 μM-ACh (second curve), $H = 222$ mV and at 1 μM-ACh (third curve) $H = 140$ mV. The voltage dependence of the slow rate constant of desensitization, $\lambda_s = 1/t_s$ (bottom curve), was calculated using the average of the rate constants estimated at all concentrations of ACh: $H = 559$ mV when one aberrant point is omitted (inclusion of this point gives $H = 192$ mV).

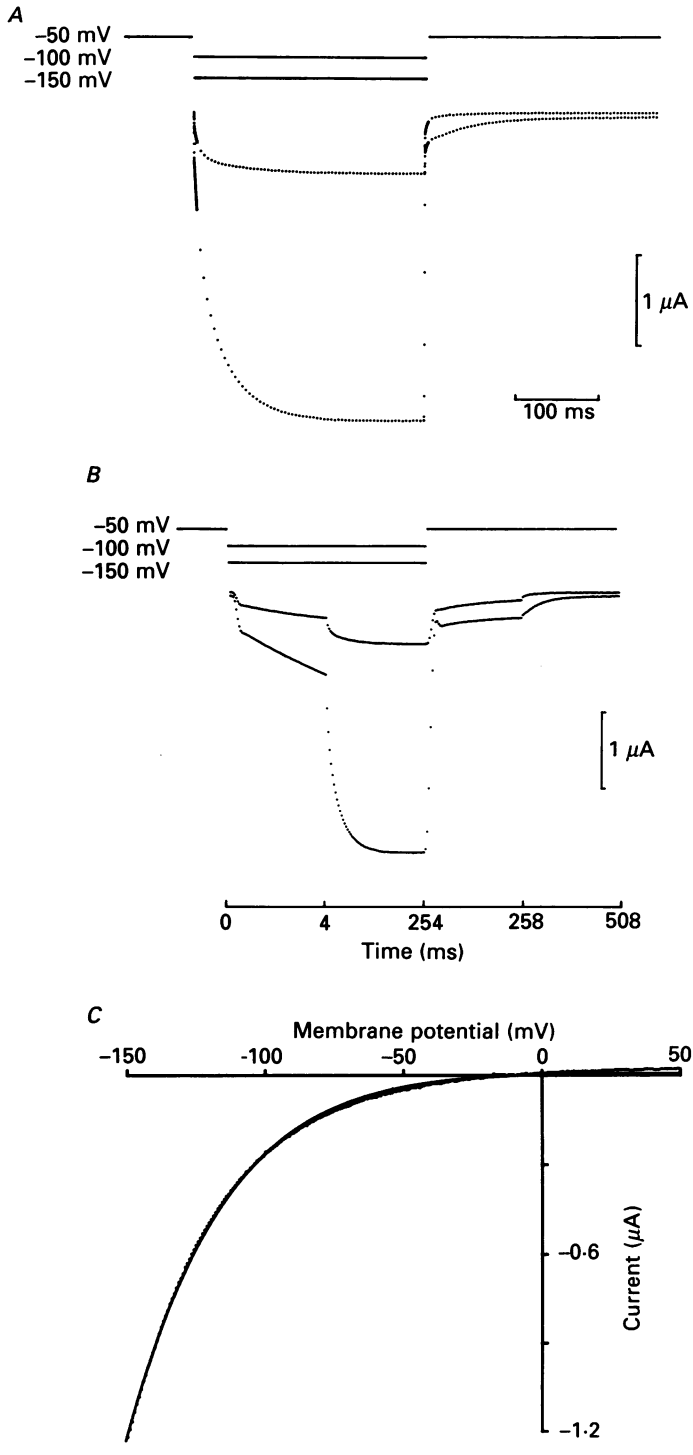

Fig. 6. For legend see facing page.

limitation of the rate of agonist application. However, a faster component might be detectable by voltage jumps, *if* it were voltage dependent.

Following a hyperpolarizing voltage jump from −50 to −100 mV or −150 mV (Fig. 6*A*) there was the normal inward current relaxation that represents opening of more ion channels, mainly as a result of the voltage dependence of the channel shutting rate, α (see for example, Adams, 1974; Lester, Changeux & Sheridan, 1975; Neher & Sakmann, 1975; Colquhoun, Dreyer & Sheridan, 1979). The time constant for this relaxation varied from 14·2 ± 1 ms for 1 μM-ACh at −150 mV to 1·1 ± 0·1 ms for 10 μM-ACh at −50 mV. For 1 μM-ACh, which opens a small fraction of the channels (e.g. Colquhoun & Ogden, 1988), the rate constant for this relaxation decreased e-fold for 56 ± 4 mV hyperpolarization. This voltage dependence was similar to that found for the equilibrium current–voltage curve (Fig. 6*C*) determined by applying a slow voltage ramp; the average value from such experiments was 57 ± 4 mV hyperpolarization for an e-fold increase in equilibrium conductance. This channel opening relaxation was followed by a slower inward current relaxation (Fig. 6*A*); this is likely to be the same (still unexplained) process which, when observed for a shorter time period, is referred to as a sloping baseline (e.g. Neher & Sakmann, 1975; Adams, 1977; Colquhoun *et al.* 1979). In the present experiments the current was sampled for 254 ms for both the 'on' and the 'off' relaxations. The current could usually be fitted well by two exponential components. The slower component paralleled the faster closely; it was about 7·5-fold slower than the fast component at a range of membrane potentials, and at ACh concentrations of 1, 5 and 10 μM. For example, with 1 μM-ACh at −100 mV, we found $\tau_f = 5{\cdot}6 \pm 0{\cdot}5$ ms and $\tau_s = 38 \pm 7$ ms, the slow component having an amplitude about one-third of that of the (normal) fast component. The value for this τ_s is in the range seen by Colquhoun *et al.* (1979). The relative amplitude of the slower component became larger at higher ACh concentrations and at more hyperpolarized membrane potentials.

If there was a rather fast desensitization process, the equilibrium extent of which was *decreased* by hyperpolarization, this could account for the slower inward

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Fig. 6. Voltage-clamp currents recorded during a step change of the membrane potential during ACh perfusion. *A*, the membrane potential was stepped from −50 to −100 mV and from −50 to −150 mV ('on relaxation') at $t = 0$ and back to −50 mV at $t = 254$ ms ('off relaxation'). *B*, responses in *A* shown on a dual time base, with the first 4 ms of the on and off relaxations expanded. On and off relaxations were digitized at 16 kHz for the first 4 ms after the potential change, and then at 256 Hz for 250 ms. *C*, voltage-clamp currents recorded during a ramp-like change of membrane potential during ACh (1 μM) perfusion, after subtraction of a similar record in the absence of ACh. The membrane potential was held at +50 mV for 500 ms before recording and then changed from +50 mV to −150 mV (ramp duration, 256 ms). The coefficient H, the change in membrane potential for an e-fold decrease of conductance, was calculated by least-squares fit to the current response of the equation $I(E) = (E - E_{rev})G(0)\exp(E/H)$ where E is the membrane potential, E_{rev} is the reversal potential and $G(0)$ is the conductance at $E = 0$. An equation of this form is expected from mechanisms of the sort discussed in the text as long as there is little desensitization during the ramp, and the fraction of channels that is opened by ACh is small (which is the case for 1 μM-ACh). In this case (see scheme (3)) the conductance will be proportional to $\beta/(\alpha K_1 K_2)$, with most of the voltage dependence being attributable to α (Neher & Sakmann, 1975; Colquhoun & Sakmann, 1985). In this case the fit gives $H = -45{\cdot}1$ mV and $E_{rev} = -8{\cdot}6$ mV.

relaxation described above. If this were the case it might be expected that the process, at a given ACh concentration, would be simply scaled down by reducing the number of active ion channels. However, this is not what happened when the preparation was perfused with α-bungarotoxin (100 nM) in order to produce

TABLE 1. Effect of α-bungarotoxin on voltage-jump relaxations

$E_m = -150$ mV				$E_m = -50$ mV			
τ_f (ms)	τ_s (ms)	Amplitude (nA)	w_s/w_f	τ_f (ms)	τ_s (ms)	Amplitude (nA)	w_s/w_f
16	64	768	0·86	3·5	58	−212	0·25
15	57	608	0·90	4·0	105	−147	0·19
19	90	479	0·44	4·0	36	−119	0·19
18	85	359	0·38	3·5	71	−88	0·15
17	68	292	0·31	3·5	54	−66	0·12
20	96	222	0·28	3·0	10·2	−55	0·38
17	79	171	0·13	2·7	57	−38	0·02

Two jumps (each from −50 mV to −150 mV, and back) were done as controls. The time constants of the voltage-jump relaxation (τ_f, τ_s), the total amplitude (amplitude = $w_s + w_f$) and the relative amplitude (w_s/w_f) are given above the dashed line. At this point α-bungarotoxin (100 nM) was perfused over the fibre and ACh was applied (to assess the desensitization) at intervals of about 5 min. As expected the amplitude of the relaxation was reduced by α-bungarotoxin, and this results largely from the reduction of the amplitude of the slow component. There is little effect on the time constants.

progressive block of receptors. The results of such an experiment (Table 1) show that although there was no consistent change in the time constants during progressive block, the reduction in amplitude (w_s) of the slow component was much greater than the reduction of the amplitude (w_f) of the fast component; initially w_s/w_f was about 0·88 but by the time w_f had fallen to about 40% of its original value, w_s had fallen to 6% of its original value so w_s/w_f had fallen to 0·13.

This behaviour suggests that the slow component of the relaxation is related to the size of the current that is flowing, rather than being a fast desensitization process (it might, for example, result from transient changes in the ACh concentration in the synaptic cleft; see Appendix and Discussion).

Effect of calcium

With 80 mM-K_2EGTA as the intracellular medium it would be expected that entry of calcium through end-plate channels (Jenkinson & Nicholls, 1961) would have little effect on the intracellular concentration of free calcium ions. It was, therefore, no surprise that reducing the extracellular calcium concentration from 1·5 mM to 20 μM had no clear effect on desensitization under these conditions, as shown in Fig. 7*A* and Table 2A.

The effects of allowing the intracellular free calcium concentration to rise were tested by using as the intracellular medium a potassium glutamate solution containing only 0·2 mM-EGTA (see Methods). With the usual (1·5 mM) extracellular calcium concentration, and an ACh concentration of 40 μM, the results in Table 2B

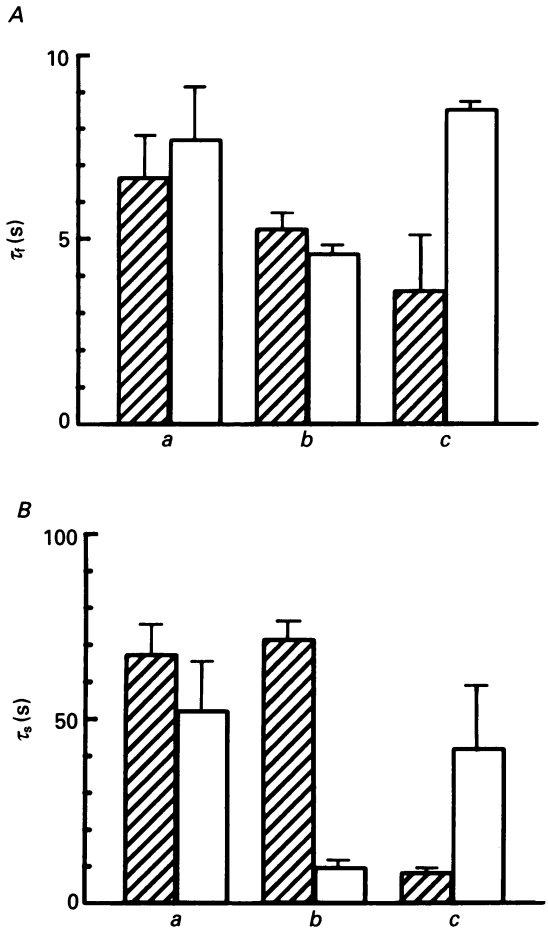


Fig. 7. Effect of calcium on time constants of desensitization. *A*, fast time constant τ_f. *a*, 80 mM-K_2EGTA intracellular solution: hatched bar 1·5 mM $[Ca^{2+}]_o$, unfilled bar 20 μM $[Ca^{2+}]_o$. *b*, 1·5 mM $[Ca^{2+}]_o$: hatched bar 80 mM $[EGTA]_i$, unfilled bar 200 μM $[EGTA]_i$. *c*, 200 μM $[EGTA]_i$: hatched bar 1·5 mM $[Ca^{2+}]_o$, unfilled bar 20 μM $[Ca_{2+}]_o$. *B*, slow time constant τ_s under same experimental conditions as in *A*. Note the marked decrease of τ_s when $[Ca^{2+}]_i$ is allowed to increase. In *c*, τ_s returns to a value similar to that measured with K_2EGTA as intracellular solution when $[Ca^{2+}]_o$ is reduced to 20 μM.

TABLE 2. Effects of internal and external calcium concentration on peak current (I_p), equilibrium current (I_∞), and the time constants (τ_f, τ_s) and relative amplitude (w_s/w_f) of the fast and slow components of desensitization following application of ACh

	A		B		C	
	80 mM-EGTA inside, $[Ca^{2+}]$ outside		1·5 mM-Ca^{2+} outside [EGTA] inside		0·2 mM-EGTA inside, $[Ca^{2+}]$ outside	
	1·5 mM	0·02 mM	80 mM	0·2 mM	1·5 mM	0·02 mM
I_p (μA)	4·2	3·9	3·4	3·9	4·2	3·7
I_∞ (μA)	0·50	0·47	0·11	0·068	0·052	0·17
τ_f (s)	6·7	7·7	5·3	4·6	3·6	8·5
τ_s (s)	67·0	52·0	72·0	9·8	8·4	42·0
w_s/w_f	0·12	0·13	0·14	2·3	3·2	0·21

were observed. There was no detectable effect on the peak current or on the faster time constant; however, the slower component of desensitization virtually disappeared. It was still possible to fit two exponential components but their time constants (4·6 and 9·8 s) are too close together for the separation of the two components, and hence the estimate of their relative amplitudes, to be trustworthy, No clear distinction can be made between, on the one hand, reduction in amplitude of the slow component, and, on the other hand, acceleration of the slow component to a rate similar to that of the fast component.

With glutamate–0·2 mM EGTA as intracellular medium the cell became sensitive to the extracellular calcium concentration: when the latter was reduced to 20 μM the slow component was restored to much the same rate and amplitude as seen when the intracellular calcium was kept low with 80 mM-EGTA, as shown by the results in Table 2C.

The results in Table 2 are consistent with a reduction in the equilibrium current (but not the peak current) by a factor of 2 or 3 under conditions where the intracellular free calcium can rise, but variability from cell to cell in the equilibrium current precludes any more precise conclusions.

Attempts were made to use known buffered intracellular calcium concentrations but as soon as the free calcium concentration approached 1 μM the preparation was destroyed by contracture of the fibre.

Procedures intended to change receptor phosphorylation

It has been suggested that phosphorylation of nicotinic receptors increases the rate of desensitization (Albuquerque *et al.* 1986; Huganir *et al.* 1986; Middleton *et al.* 1986), so experiments were done to test this hypothesis.

The effect of cyclic nucleotides on receptor desensitization was investigated just below room temperature (about 17 °C), with an intracellular potassium glutamate solution that contained an ATP-regenerating system modified from Irving *et al.* (1987), as described in the Methods section. A few experiments were also performed at low temperature using either K_2EGTA or the potassium glutamate solution as the intracellular solution.

Cyclic AMP-dependent phosphorylation

Single muscle fibres were perfused extracellularly with dibutyryl-cyclic AMP in concentrations of 5 ($n = 1$), 10 ($n = 2$) and 50 μM ($n = 1$) with 80 mM-K_2EGTA as the intracellular solution, at 4 °C. The perfusion was interrupted every 5 min (for up to 30 min) for 45 s during which 40 μM-ACh (without dibutyryl-cyclic AMP) was applied. There was no effect of dibutyryl-cyclic AMP on either peak response or on the time constants of desensitization. Forskolin (5 μM) used under the same conditions also had no effect on desensitization ($n = 1$).

Similar experiments were done at 4 °C with the potassium glutamate solution as the intracellular medium. Again neither 10 μM-dibutyryl-cyclic AMP ($n = 1$) nor 10 μM-8-bromo-cyclic AMP ($n = 4$) had any effect on the peak response or the time constants of desensitization.

Further experiments were performed at room temperature with the intention of increasing the phosphorylation rate. Also the cyclic nucleotides were applied

intracellularly through the cut ends of the fibre. Cyclic AMP was applied in concentrations of 1·5 μM ($n = 1$) and 250 μM ($n = 2$) and the response to ACh measured every 15 min for up to 45 min. There was no effect on the parameters of desensitization.

Finally, we applied forskolin intracellularly in a low concentration (10 μM, $n = 1$), with a phosphodiesterase inhibitor (IBMX; 100 μM, $n = 1$), and in a higher concentration (50 μM with 100 μM-IBMX). In neither case was it possible to detect any effect on the parameters of desensitization.

Protein kinase C-dependent phosphorylation

In order to test the hypothesis that phosphorylation by protein kinase C is important, phorbol esters were applied intracellularly in the potassium glutamate solution, and the response to ACh recorded every 15 min at 17 °C. The effect of PMA (phorbol-12-myristate-13-acetate) ($n = 3$) and PDBu (phorbol-12,13-dibutyrate) ($n = 1$) was investigated at concentrations known to fully activate protein kinase C in other systems: 100 nM-PMA and 10 nM-PDBu. Whereas the measured peak response to ACh was reduced in all experiments performed with internal application of phorbol esters, there was no change in the time constants of desensitization even after 45 min exposure. The peak response was reduced to 64 ± 10 % of its initial value ($n = 4$). However, the response to repeated applications of ACh in control experiments also decreases with time. The evidence for a genuine effect of the phorbol esters therefore rests on the observation that 15 min after removing those agents from the end-pools the response had recovered to 80 ± 6 % of control values. This recovery was, however, much quicker than could be expected for an effect on protein kinase C.

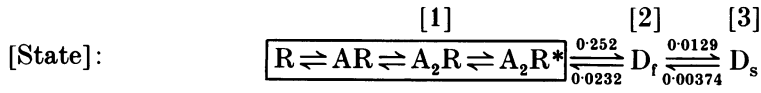
Reaction schemes for desensitization

Three different reaction schemes were fitted to the observations. Five data sets, each with their appropriate weights, were fitted simultaneously according to a weighted least-squares criterion. The parameters so estimated were the rate constants of the mechanisms shown below. The calculations were performed as described by Colquhoun & Hawkes (1977).

The five data sets consisted of values of the following observations at a range of ACh concentrations: (1) the slow rate constant ($1/\tau_s$), (2) the fast rate constant ($1/\tau_f$), (3) the relative amplitudes, w_s/w_f, of the slow and fast components of desensitization, (4) the corrected peak response, $I(0)$, and (5) the equilibrium response, $I(\infty)$.

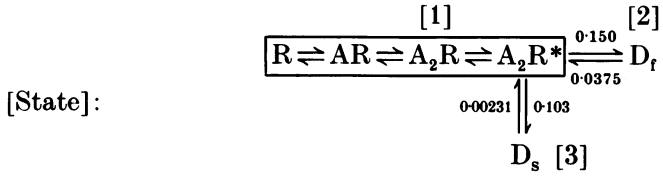
The opening of the channel appears to be very much faster than the desensitization process so the activation steps were treated as though they were permanently in equilibrium. In other words the states enclosed in a box were treated for the purpose of the kinetic calculations as though they constituted a single state (numbered state 1 in each case). The current was calculated from the occupancy of state 1 at time t, multiplied by the (equilibrium) fraction of state 1 in the open state, A_2R^*. The effective rate constants for leaving state 1 were calculated as the 'true' or microscopic, rate constants multiplied by the (equilibrium) fraction of state 1 that was in the species within state 1 that was being left (i.e. the fraction in A_2R^* for the rate constant k_{12} in schemes (1) and (3); the fraction in A_2R^* for the rates k_{12} and k_{13} in

scheme (2), and the fraction in R for k_{15} in scheme (3)). In each case it is necessary to postulate two distinct desensitized conformations of the receptor; they are denoted D_s and D_f. The three reaction schemes are as follows. In scheme (1) these states are arranged sequentially:



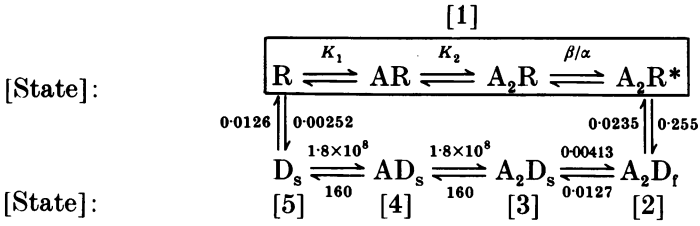
(1)

In scheme (2) the two desensitized states are arranged in parallel:



(2)

Scheme (3) has a cyclic arrangement of states (cf. Katz & Thesleff, 1957; Rang & Ritter, 1970; Sine & Taylor, 1982).



(3)

In all cases the physical nature of the postulated two desensitized states is unknown. In the case of scheme (3) it is tempting to speculate that D_f might correspond in some way to the open state (although it is non-conducting), and state D_s to the shut state. The peak responses were fitted by the calculated response in the absence of desensitization.

In each case the arrows are labelled with the estimated values of the microscopic rate constants (in reciprocal seconds, except for the association rate constant in model 3 which is $1{\cdot}8 \times 10^8$ M^{-1} s^{-1}).

In the fitting of all three schemes the number of channels was fixed at 7×10^6 (see, for example, Colquhoun *et al.* 1979; Matthews-Bellinger & Salpeter, 1983), the equilibrium constant for channel opening (β/α) was fixed at 10 (Colquhoun & Sakmann, 1985; Colquhoun & Ogden, 1988, corrected to -50 mV), and the single-channel conductance was fixed at 30 pS (Gardner, Ogden & Colquhoun, 1984). The equilibrium constants for agonist binding were constrained to be equal ($K_1 = K_2$) and their estimated values were 34 μM for schemes (1) and (2), and 36 μM for scheme (3); these values are not greatly different from the estimates of 77 μM at -120 mV and 32 μM at $+100$ mV made by Colquhoun & Ogden (1988). The values used imply a maximum current of 9·5 μA (at -50 mV) in the absence of desensitization. Scheme (3)

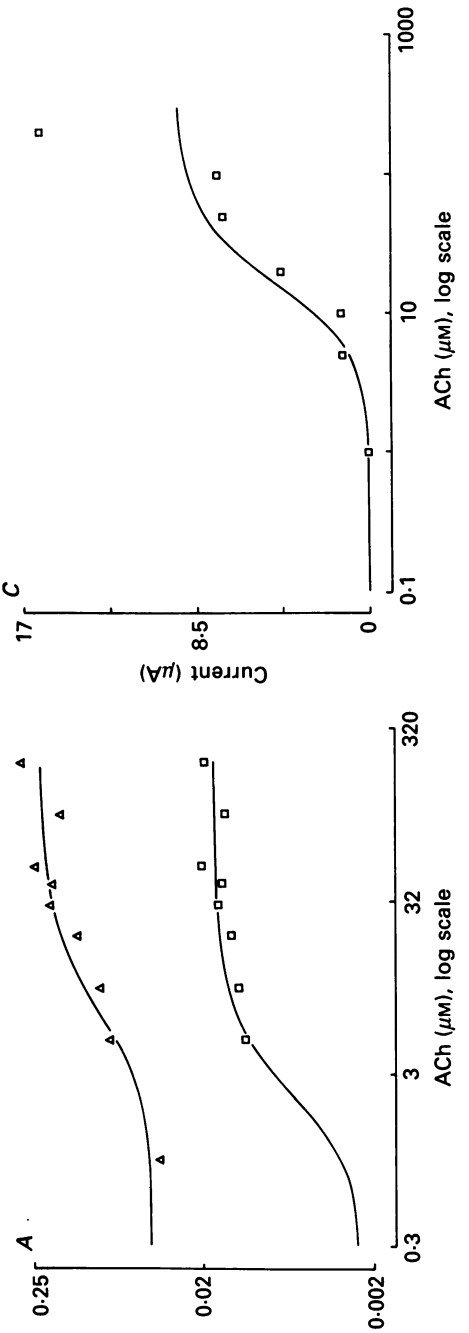


Fig. 8. The fit of the cyclic reaction mechanism (scheme (3)) to the observations. The data are as shown in Figs 3 and 4 except that two points on the peak response curve (at 30 and 40 μM-ACh) and two on the equilibrium response curve (at 10 and 30 μM) were considered too aberrant to use for curve fitting. The parameters are given in the text. *A*, fast and slow rate constants of desensitization. *B*, relative amplitudes of the slow and fast components. *C*, peak response to ACh. *D*, equilibrium response to ACh.

had too many parameters for them all to be uniquely determined: the dissociation rate constant, $k_{45} = 160\ s^{-1}$, was estimated via the microscopic reversibility constraint, and the ratio k_{15}/k_{51} was constrained to be 0·2 (i.e. 17% desensitization in the absence of agonist); a free fit gave a somewhat larger ratio.

Schemes (1) and (2) predict two exponential components as observed and they gave identical fits; they are quite indistinguishable from our data, as might be expected from the known indistinguishability, under certain conditions of C–C–O and C–O–C models (see, for example, Sakmann & Trube, 1984, and Kienker, 1989). Scheme (3) has five states and so predicts four exponential components in the response; two of these turn out to be very fast because, according to the estimates given above, the agonist binding to the desensitized states is rapid compared with the desensitization rates, though not nearly as rapid as agonist binding to the non-desensitized states for which the dissociation rate constant has been estimated as $8000\ s^{-1}$ rather than $160\ s^{-1}$ (Colquhoun & Sakmann, 1985); correspondingly the affinity of ACh is 20 to 40-fold greater than for the non-desensitized state. This means that states 3, 4 and 5 are essentially at equilibrium, except at the earliest times following agonist exposure, and therefore behave kinetically like a single state; thus there are essentially only three kinetically distinguishable states, viz. 1, 2 and (3, 4, 5). Thus two slow exponential components predominate and it is those that were fitted to the observations. The fit was only slightly better than could be obtained with schemes (1) and (2); in order to distinguish these schemes clearly it would be necessary to have data on recovery from desensitization, preferably with more than one agonist, which we do not have in the present work.

The fitted curves (for scheme (3)) are shown in Fig. 8. Good fits are obtained to the fast and slow time constants, and to the relative amplitude of the fast and slow components (Fig. 8*A* and *B*). The results for peak and equilibrium currents (Fig. 8*C* and *D*) are more scattered (for the reasons discussed above) and some points shown (see legend) were omitted from the simultaneous fitting process. The fits are thus not as good as for the rest of the data but they describe adequately the main features of the results. As expected the equilibrium current is predicted to reach a maximum at a substantially lower ACh concentration than the peak current.

DISCUSSION

Dependence of desensitization on ACh concentration

The observation of two exponential components in the onset of desensitization resembles qualitatively recent results in frog end-plates (Feltz & Trautmann, 1982; Chesnut, 1983), in denervated rat muscle (Anwyl & Narahashi, 1980) and in snake muscle (Connor *et al.* 1984); it also resembles results with neuronal nicotinic receptors in PC12 cells (Boyd, 1987) as well as similar receptors in *Aplysia* neurones (Slater *et al.* 1984). In all of these studies of muscle receptors, technical problems such as the avoidance of contracture and the limited current-passing capacity of the two-electrode voltage-clamp method, prevented the determination of the dependence of the time course of desensitization on the agonist concentration over a full range of concentrations that we have attempted. Unpublished work by R. B. Clark and P. R.Adams that shows such results is cited by Adams (1987). In their work

α-bungarotoxin was used to limit the responses to large agonist concentrations, and a nominally Ca^{2+}-free extracellular solution with 10 mM-Mg^{2+} was used to limit contractures (P. R. Adams, personal communication.)

The fast time constant for onset of desensitization clearly became faster as the agonist concentration was increased (Figs 1, 4, and 8); it was 26 s at 1 μM-ACh and 6 s at 100 μM-ACh (4 °C; membrane potential, $E_m = -50$ mV). The slower time constant (60–100 s) was much less dependent on agonist concentration (in contrast with results cited by Adams, 1987). The relative amplitude (see Methods) of the slow and fast components was high (i.e. the slow component predominated at low agonist concentrations) but decreased rapidly at higher agonist concentrations, so the fast component predominates at 200 μM-ACh (see Figs 1, 4 and 8).

The equilibrium current, estimated by fitting the asymptote and two exponential components to the results (see Methods), was no more than 1 or 2% of the peak current so desensitization is intense in this preparation (but it was not complete, as was suggested by Fiekers *et al.* 1980). The equilibrium current appeared to reach saturation at a lower agonist concentration than the peak current as might be expected from the high binding affinity of the desensitized form(s) for agonist (see Figs 3 and 8, and discussion of mechanisms below).

Is there any ultra-fast desensitization?

The diffusion limitation of the response to a 'step' change in agonist concentration prevented the rise time of the response being faster than 0·26 s (with 100 μM-ACh) to 2 s (with 1 μM-ACh). This is much as expected for diffusion through a 35 μm unstirred layer, together with agonist binding (see Fig. 2 and Appendix). It would, therefore, not have been possible to measure accurately any desensitization process that occurred in less than a second or so, as in the other studies cited above. However, much faster desensitization processes have been reported, which result in loss of a large proportion of active channels over a period of tens of milliseconds (Bekkers, 1986; Brett *et al.* 1986). These results were obtained by rapid perfusion of outside-out membrane patches from cultured cells for which the diffusion limitation is far less serious than for intact muscle fibres. A simple argument shows that adult frog receptors are unlikely to behave in this way. A frog end-plate contains, on average, about 10^7 channels (Colquhoun *et al.* 1979; Adams, 1981; Matthews-Bellinger & Salpeter, 1983), and the single-channel conductance is 30 pS (e.g. Gardner *et al.* 1984) so with a membrane potential of -50 mV and a reversal potential close to zero, it would be expected that a current of about 15 μA would flow if all channels were simultaneously open. Such a current is far too big to be measured with a two-electrode clamp but we have observed peak currents of up to 16 μA with 200 μM-ACh (see Figs 1 and 3), and this peak occurs several hundred milliseconds after the start of the response. It is, therefore, improbable that there is any very substantial amount of desensitization that is more rapid than the fastest component measured here, i.e. that with a time constant of a few seconds (see also discussion of mechanisms below). This result is, of course, also consistent with the view that ACh is capable, in the absence of desensitization, of opening a large proportion of channels, the parameters of the peak response *versus* concentration curve being consistent with those found by Colquhoun & Ogden (1988) (see also discussion of mechanisms below).

The voltage dependence of desensitization

Dependence of the rate of nicotinic receptor desensitization on membrane potential has been reported in frog end-plate by Magazanik & Vyskočil (1970), Scubon-Mulieri & Parsons (1978) and by Fiekers *et al.* (1980), and in *Electrophorus* electroplaques by Pallotta & Webb (1980). On the other hand nicotinic-like receptors in neurones of *Lymnea stagnalis* (Chemeris *et al.* 1982) and in *Aplysia* (Slater *et al.* 1984) show no clear voltage sensitivity. There is relatively little quantitative information on the extent of voltage dependence at end-plate receptors; Fiekers *et al.* (1980) found an e-fold increase in the single desensitization rate constant that they measured (roughly comparable to our faster rate) for a hyperpolarization of 83 mV with 250 μM-carbachol (which is roughly equipotent with 15–25 μm-ACh), in potassium-depolarized frog muscle at room temperature with 1·3 mM-external calcium concentration (there was little effect of calcium concentration on the voltage dependence). In *Electrophorus* Pallotta & Webb (1980) found a greater voltage dependence, an e-fold increase in rate for a hyperpolarization of 33 mV in the single rate constant that they observed. We observed, in fibres containing 80 mM-K_2EGTA, a more modest voltage dependence in τ_f, an e-fold increase in rate corresponding to a 100–200 mV hyperpolarization, and we were unable to detect a consistent voltage dependence in the slow component though, if anything, this also became a bit faster on hyperpolarization. Some, or all, of this effect could be secondary to the voltage dependence of channel activation rather than implying only voltage dependence of the desensitization mechanism itself (see discussion of mechanisms below). It is unlikely that the voltage dependence reported here is secondary to the voltage dependence of calcium entry, as has been suggested by Miledi (1980), Chesnut (1983) and Slater *et al.* (1984), because there should be little change in $[Ca^{2+}]_i$ with 80 mM-K_2EGTA inside the fibre.

The effect of calcium

There have been many reports that calcium can increase the rate of desensitization (e.g. Manthey, 1966, 1970; Fiekers *et al.* 1980; Miledi, 1980), though a *decreased* rate has been seen in *Electrophorus* (Pallotta & Webb, 1980). With 80 mM-K_2EGTA as the intracellular solution we observed that the time course of desensitization was unaffected by changing the extracellular calcium concentration from 1·5 mM to 20 μM, so calcium is certainly not necessary for desensitization to occur. The intracellular free calcium ion concentration would be very low in these experiments so the results add weight to the view that calcium ions have their effect at an intracellular site (e.g. Miledi, 1980; Chesnut, 1983). When the intracellular medium was potassium glutamate solution containing only 0·2 mM-EGTA, the intracellular free calcium concentration was much less strongly buffered, and we found, with the usual 1·5 mM-extracellular Ca^{2+}, that the slow component of desensitization virtually disappeared (or accelerated to such an extent that it could no longer be clearly distinguished from the fast component); this was doubtless as a result of entry of calcium from the outside, most probably through the nicotinic receptor channels, because we found that reduction of the extracellular Ca^{2+} concentration with the weakly buffered intracellular solution restored the desensitization rates to values

close to those seen with 80 mM-K_2EGTA inside the cell. We found little trace of a slow component with normal extracellular [Ca^{2+}] and weakly buffered intracellular [Ca^{2+}] and it may be noted that the most convincing and consistent studies of biphasic desensitization at frog end-plates have all used low $[Ca^{2+}]_o$. For example Feltz & Trautmann (1982) used a nominally calcium-free extracellular solution (with 5 mM-magnesium) throughout, and the work cited by Adams (1987) used a similar (but 10 mM-Mg^{2+}) solution. However, a slow component of desensitization in the presence of normal extracellular [Ca^{2+}] has been reported in intact frog muscle by Chesnut (1983), and in snake muscle by Connor *et al.* (1984). The former study employed 52·5 mM $[K^+]_o$ and 1·8 mM $[Ca^{2+}]_o$, but also 10 mM-Tris buffer, which is known to block end-plate channels (Adams *et al.* 1981). The latter was in isotonic potassium propionate with 1·0 mM-Ca^{2+}. Chesnut (1983) found, using ionophoretic agonist application and intracellular injection of EGTA, that the effect of Ca^{2+} was largely on the slow component of desensitization, as in our results.

Procedures intended to alter receptor phosphorylation

We have tested various procedures with an appropriate intracellular solution (see Methods), and working near room temperature, to facilitate receptor phosphorylation. We observed no effect on the time course of desensitization (1) when cyclic AMP was applied intracellularly in concentrations of 1·5 or 250 μM, (2) when forskolin (10 or 50 μM), with the phosphodiesterase inhibitor IBMX (100 μM), was applied intracellularly, and (3) when 8-bromo-cyclic AMP (10 μM) or dibutyryl-cyclic AMP were applied extracellularly (though these last experiments were done at 4 °C rather than room temperature).

These observations contrast strongly with reports that receptor phosphorylation causes substantial acceleration of desensitization (Albuquerque *et al.* 1986; Huganir *et al.* 1986; Middleton *et al.* 1986). It is most unlikely that phosphorylation is the primary cause of desensitization because desensitization can occur with purified and reconstituted nicotinic receptors (Nelson *et al.* 1980; Schindler & Quast, 1980; Popot *et al.* 1981), and intense desensitization is seen in the present experiments in fibres at 4 °C filled with 80 mM-EGTA so phosphorylation could not occur. It is known that receptor phosphorylation can be produced by various agents, including forskolin, on the β- and γ-subunits of *Torpedo* receptor (Huganir & Greengard, 1983; Huganir *et al.* 1986; Yee & Huganir, 1987), on the α- and δ-subunits in primary cultures of embryonic rat muscle (Miles, Anthony, Rubin, Greengard & Huganir, 1987) and on the δ-subunit of the mouse BC3H1 cell line (Smith, Merlie & Lawrence, 1987) (though forskolin *decreased* phosphorylation of the β-subunit in this case). However, the functional consequences of such phosphorylation are still controversial. The only case in which phosphorylation *per se* has been directly demonstrated as a cause of increased desensitization rate is the work of Huganir *et al.* (1986) on *Torpedo* receptor (and even in this case there remains a possibility of error, if, for example, phosphorylation affected the receptor reconstitution process in some way, as discussed by Steinbach & Zempel, 1987). The assessment of the evidence is not made easier by the fact that phosphorylation varies between species, and between fetal and adult forms of the receptor. Middleton *et al.* (1986) showed an increased desensitization rate in adult rat soleus muscle end-plate with forskolin (but not with

dibutyryl-cyclic AMP) in concentrations lower than those that produced an obvious direct effect on the channel; and Albuquerque *et al.* (1986) showed similar effects on both end-plate channels of rat soleus muscle, and on extrajunctional channels of denervated soleus muscle, with less effect being produced by less-active forskolin analogues. Both studies looked for non-specific effects of forskolin (which were observed with concentrations above 20 μM in the former study), but in both cases a different preparation (embryonic rat muscle in culture) was used for this purpose. Several studies have now shown that the effect of forskolin on 'desensitization' rate is unlikely to be a result of cyclic AMP-induced phosphorylation in cultured chicken myotubes (Eusebi, Grassi, Molinaro & Zani, 1987; Häggblad, Erikson, Hedlund & Heilbronn, 1987), or in cultured rat myotubes (Wagoner & Pallotta, 1988), or in (neurone-like) PC12 cells (McHugh & McGee, 1986). In rat myotubes direct intracellular application of cyclic AMP or of catalytic subunit of A-kinase failed to have much effect on desensitization (Wagoner & Pallotta, 1988). These results contrast with the conclusion of Albuquerque *et al.* (1986) that extrajunctional receptors of denervated rat muscle desensitize faster when phosphorylated. However, it remains a possibility that *adult* rat receptors (but not, according to the present work, adult receptors of the frog), as well as *Torpedo* receptors, do indeed show faster desensitization when phosphorylated. Steinbach & Zempel (1987) have discussed some of these questions and have speculated that the proposed phosphorylation site on the γ-subunit of the *Torpedo* receptor may be crucial for this effect, and this site is missing in many other species, though it is found in the adult ϵ-subunit of the bovine receptor (it is not yet known whether it occurs in adult frog or rat receptors).

It is also possible that receptor phosphorylation by protein kinase C which occurs in *Torpedo* receptor (Safran, Sagi-Eisenberg, Neumann & Fuchs, 1987) might be involved in desensitization or other forms of receptor regulation (Eusebi *et al.* 1987). However, in this study we observed that intracellular application of PMA (100 nM) or PDBu (10 nM) had no effect on the desensitization rate, though they produced an easily reversible depression of the peak response to ACh.

In summary, receptor phosphorylation is not the cause of desensitization, and is unlikely to have a universal role in regulating desensitization because it has little or no effect on desensitization in various embryonic tissues or in adult frog, though it probably accelerates desensitization in *Torpedo* receptor, and possibly does so in adult rat receptor too.

Mechanisms of desensitization

Reaction schemes

The foregoing discussion suggests that it is quite likely that desensitization results from the ability of the receptor–channel molecule itself to adopt long-lived shut conformations. It is also likely that these conformations have both agonist binding sites occupied (e.g. Feltz & Trautmann, 1982). The cyclic mechanism proposed by Katz & Thesleff (1957) has received widespread support (e.g. Rang & Ritter, 1970; Feltz & Trautmann, 1982; Sine & Taylor, 1982). Our scheme (3) is merely a development of this mechanism in which the existence of two, rather than one, agonist binding sites is recognized (as in the schemes proposed by Feltz & Trautmann, 1982, and others), and, more unusually, the channel opening step is

envisaged as being separate from the binding step (as is usual in studies of agonist action, e.g. Colquhoun & Sakmann, 1985, but not in discussions of desensitization). If each of the normal states is supposed to have a 'corresponding' desensitized state, as in scheme (3), then biphasic desensitization is predicted and, as shown above, all of our data can be fitted with a mechanism that is rather simpler than some of those that have been proposed to account for biphasic desensitization (e.g. Feltz & Trautmann, 1982; Adams, 1987). In fact our data can be fitted by the even simpler schemes (1) and (2) above, but these fail to predict some of the phenomena observed in other studies. For example all three schemes can account for the observation that the slow component of recovery from desensitization is more prominent after a long pre-incubation with agonist than after a short one (Feltz & Trautmann, 1982), but only the cyclic scheme (3) can account for the observation that rate constants for recovery from desensitization are roughly independent of the nature of the agonist (because recovery is largely via states $3 \rightarrow 2 \rightarrow 5 \rightarrow 1$, the last step being rate-limiting), and only scheme (3) can predict that the onset rate of desensitization may be slower, at low concentrations, than recovery (Katz & Thesleff, 1957).

Indirect voltage dependence

All three schemes described above also predict that desensitization will become faster when the membrane is hyperpolarized, even when the desensitization steps themselves are totally insensitive to membrane potential. This is because desensitization is supposed, in these schemes, to occur via the open state (though this may not be the exclusive route), and at any given agonist concentration there will be more open channels when the membrane is hyperpolarized because, largely, of the voltage dependence of the shutting rate constant, α. Numerical calculations show that the effect is predicted to be mainly on the faster time constant for desensitization (τ_f), which we indeed observed to be more voltage dependent than the slower time constant, and, at moderate agonist concentrations, the voltage dependence predicted for τ_f is similar to that which we observed. However, at higher agonist concentrations this sort of indirect voltage dependence should be reduced because if most channels are already open, hyperpolarization cannot open many more, and so cannot increase much the effective rate of leaving state 1. We did not, however, find that the voltage dependence of τ_f was obviously less with 100 μM-ACh than with lower concentrations (Fig. 5).

Relation of results to single-channel records

Sakmann *et al.* (1980) observed two classes of shut (presumably desensitized) periods in single-channel records obtained with high ACh concentrations on frog junctional-type channels; at 20 μM-ACh they found the mean length of a burst (defined here as a contiguous non-desensitized period) was 0·42 s (and Colquhoun & Ogden, 1988, found it to be 0·84 s). These bursts were grouped into clusters, the gap between bursts being 0·18 s, the mean cluster length 4·8 s and the mean gap between clusters being 34 s. This suggests that desensitization will have two time constants, but the last of these values cannot be interpreted because of lack of knowledge of the number of channels in the patch, so unambiguous predictions of the macroscopic time constants cannot be made from these results. However, it seems reasonable to suppose that, for one channel, the gap between clusters must be much longer than

the cluster length, so a macroscopic time constant comparable to the cluster length, 4·8 s, is expected. This is comparable with the faster time constant, τ_f, observed here (about 5·9 s). The desensitized gaps within a cluster might give rise to a macroscopic time constant of roughly $(1/0{\cdot}42+1/0{\cdot}18)^{-1}$, i.e. about 0·126 s, faster than any of the time constants measured here, and closer to the rates of 'ultra-fast' desensitization discussed above.

The three reaction schemes fitted to our results all make similar predictions for the appearance of single-channel records. Calculations for each were done by the methods of Colquhoun & Hawkes (1982). It is predicted, for 20 μM-ACh, that bursts of mean length 6·8 s will be separated by gaps of 27·7 s with 2·8 such bursts constituting a cluster of mean length 70 s. The mean gap between clusters, for one channel, would be 480 s. The predicted burst length is very close to our slower macroscopic time constant, τ_s, and it is similar to the single-channel *cluster* length observed by Sakmann *et al.* (1980). It seems clear, therefore, that the single-channel record manifests our faster component of desensitization, plus a still faster component; the slow component observed in our results, and those of others, must give rise to shut times that are too long to be detected easily in single-channel records. The existence of an 'ultra-fast' component of desensitization is not necessarily incompatible with our conclusion that our responses are not greatly attenuated by 'ultra-fast' desensitization. The clusters in the records of Sakmann *et al.* (1980) were in short-lived desensitized states for only 30% of the time at 20 μM-ACh, and Colquhoun & Ogden (1988, and work in preparation) found a smaller proportion than this; an attenuation of our responses by 'ultra-fast' desensitization of less than 30% would not have been detectable by our methods, though an attenuation by the much larger factor suggested by the work of Brett *et al.* (1986) or Bekkers (1986) would have been.

APPENDIX

Diffusion and binding in the unstirred layer

Conventional discussions of the time course of relaxations suppose that the agonist concentration remains constant throughout. This assumption is, however, unlikely to be exactly true. An agonist concentration of 1 μM contains $6{\cdot}02\times10^{17}$ molecules/dm^3, i.e. 602 molecules/μm^3. Thus a change in receptor occupancy of only a few per cent can have a large (though transient) effect on the concentration adjacent to the surface. When, as in the present experiments, the nerve ending is intact, the situation is even worse. The width of the synaptic cleft is about 50 nm (= 0·05 μm), so the volume of solution adjacent to 20000 receptors is about 0·05 μm^3 which would contain only thirty agonist molecules. Therefore some calculations have been made in an attempt to predict the time course of diffusion with allowance for the effect of binding on the free concentration. The calculations have two main aims: (1) to estimate the extent to which the peak response to ACh is attenuated by desensitization, and (2) to see whether the slow component in the voltage-jump relaxations can be accounted for by diffusion and binding phenomena.

The end-plate was modelled as an infinite hemicylindrical rod on an impermeable plane. This is geometrically rather crude but has the great computational advantage

of having radial symmetry. It was supposed throughout that the radius of the rod was 1 μm, and that it had on its surface 40000 binding sites per (linear) micrometre. This 'end-plate' was supposed to be surrounded by a hemicylindrical unstirred layer of thickness δ, outside which the concentration of ACh was constant (i.e. a 'perfect' concentraton jump could be achieved at a distance δ from the end-plate surface).

For the purposes of calculating the effect of diffusion on the peak response it was supposed (as in the section on fitting reaction schemes) that the activation steps were sufficiently rapid to be effectively in equilibrium at all times. Furthermore only one desensitized state (the 'fast' one) was included for simplicity (the slow component of desensitization should have little effect on the peak response). The binding mechanism was thus

$$\boxed{R \overset{K_1}{\rightleftharpoons} AR \overset{K_2}{\rightleftharpoons} A_2R \overset{\beta/\alpha}{\rightleftharpoons} A_2R^*} \overset{k_{12}}{\underset{k_{21}}{\rightleftharpoons}} A_2D_f \qquad \text{(A1)}$$

The numerical values used were $k_{12} = 0{\cdot}15\ s^{-1}$, $k_{21} = 0{\cdot}04\ s^{-1}$, $K_1 = K_2 = 80\ \mu M$, $\beta/\alpha = 20$, and the diffusion coefficient was taken as $4 \times 10^6\ cm^2\ s^{-1}$. Changes in occupancy will create an effective flux at the surface of the end-plate, and were allowed for in the boundary condition at this surface. An explicit method was used to integrate numerically the diffusion equation in cylindrical co-ordinates (Cachelin, 1987). Trial and error showed that the rising phase of the response, calculated from the occupancy of the open state in scheme (A1) at time t, could be matched well if the thickness of the unstirred layer was taken as $\delta = 35\ \mu m$, as shown in Fig. 2*A* (this is close to the value of 40 μm estimated by Ogden, 1981, using a different method). The concentration profiles in the unstirred layer were calculated at a series of times starting at $t = 0$, the time when the concentration at distance δ from the end-plate is raised from zero to its final value. The results, when expressed as a percentage of the final value, were not strongly dependent on the ACh concentration. The concentration of ACh at the end-plate surface was very low up to $t = 100$ ms, and then rose steeply to reach 50% of its final value at about 600 ms; the later part of the equilibrium was again rather slow with near-complete equilibration after 3000 ms. The calculated time course of the response of the tissue (as illustrated in Fig. 2*A*) was interpolated to obtain an estimate of the maximum predicted response, which, when expressed as a fraction of the response in the absence of desensitization, gave the predicted attenuation factor shown in Fig. 2*B*.

A similar approach was used in calculations designed to test the effect of diffusion transients on the voltage-jump relaxations. It was assumed that, as a first approximation, only the channel shutting rate constant, α, was voltage dependent. In addition to scheme (A1), calculations were made allowing realistic rates for the activation steps (rather than assuming that they were permanently in equilibrium). A model was also tried in which a thin hemicylindrical well-stirred box (intended to mimic the synaptic cleft) was interposed between the 'end-plate' surface and the unstirred layer; diffusion through the unstirred layer to the surface of this box was followed by equilibration of the concentration at the box surface with the box contents (with a specified time constant). All of these calculations predicted that the observed relaxation rate would be slower than in the absence of diffusion problems, and somewhat non-exponential, but none of them predicted a clear slow exponential

component of the sort observed. It is, therefore, still not clear whether the observed slow component has some cause other than diffusional transients, or whether the models used for these calculations are too simple to predict correctly the effects of diffusional transients.

Note added in proof. Steinbach & Zempel (1987) suggested that the γ-subunit of the *Torpedo* receptor, but not that of many other species, contains phosphorylation sites that are necessary for acceleration of desensitization by phosphorylation. This idea is supported by the recent work of Sumikawa & Miledi (1989) which showed that, when expressed in oocytes, cat receptors desensitized more slowly than *Torpedo* receptors, and hybrid cat–*Torpedo* receptors desensitized at a rate which was dependent on the species from which the γ-subunit mRNA originated.

A. B. C. was supported by the Royal Society, the Schweizerischer National Fonds zur Förderung der wissenschaftlichen Forschung and the Schweizerische Stiftung für medizinisch-biologische Stipendien.

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