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A STUDY OF DESENSITIZATION OF ACETYLCHOLINE RECEPTORS USING NERVE-RELEASED TRANSMITTER IN THE FROG

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SUMMARY

1. Desensitization of acetylcholine (ACh) receptors was studied at the frog neuromuscular junction under voltage clamp.

2. ACh was applied directly to junctional receptors by stimulating the motor nerve with trains of impulses. End-plate currents (e.p.c.s) were used to estimate the total number of channel openings by the junctional ACh receptors, and miniature end-plate currents (m.e.p.c.s) were used to measure changes in post-synaptic sensitivity. Under the conditions of these experiments the changes in m.e.p.c. amplitudes were shown to be post-synaptic in origin and thus provided a measure of desensitization.

3. When the acetylcholinesterase was inhibited with diisopropylfluorophosphate, neostigmine, or collagenase treatment to prolong the duration of the nerve-released ACh in the synaptic cleft, desensitization developed during repetitive stimulation of 1000 impulses at 5–33 impulses/sec and then recovered after the conditioning trains, with a time constant of about 25 sec.

4. When the acetylcholinesterase was active so that the duration of ACh in the synaptic cleft resulting from each nerve impulse was brief (< 300 μsec), desensitization developed in response to 300–500 pairs of nerve stimuli if the interval between the impulses of each pair was 25 msec or less. When the interval was 30 msec or greater, however, measurable desensitization did not occur, even if the total number of channel openings was many times greater than in the experiments with shorter intervals or inhibited esterase where desensitization readily occurred.

5. The desensitization observed to pairs of impulses was enhanced by chlorpromazine and decreased when the post-synaptic membrane was depolarized, properties similar to those described previously for desensitization to bath and ionophoretic application of ACh.

6. These results indicate that desensitization to nerve-released transmitter is not a simple consequence of receptor activation, is not due to blockade of the open receptor channels by ACh, and does not result from ACh binding directly to desensitized receptors with a resulting shift in the receptor population towards the desensitized state.

7. We suggest that the desensitization observed to nerve-released transmitter is a two-step process with both steps initiated by ACh. In the first step ACh converts some receptors into a desensitizable state which has an apparent lifetime of less than 30 msec; in the second step ACh desensitizes the desensitizable state.

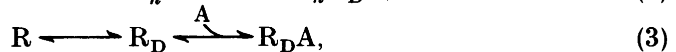
INTRODUCTION

When acetylcholine (ACh) is applied to the motor end-plate it combines with receptors and opens channels in the post-synaptic membrane. Open channels, which have a mean lifetime of a few msec, allow the passage of ions (mainly Na^+, K^+ and Ca^{2+}) which give rise to the end-plate current (del Castillo & Katz, 1957; Takeuchi & Takeuchi, 1960; Takeuchi, 1963; Anderson & Stevens, 1973; Neher & Sakmann, 1976). During prolonged application of ACh the end-plate current will often decrease over a period of seconds to minutes as the post-synaptic receptors enter a desensitized state and no longer respond to ACh (Fatt, 1950; Thesleff, 1955; Katz & Thesleff, 1957). If the agonist is removed the receptors then recover normal ACh sensitivity within seconds to minutes (Katz & Thesleff, 1957; Terrar, 1974; Scubon-Mulieri & Parsons, 1977).

The mechanism underlying desensitization is not well understood. Consequently, several models with quite different molecular mechanism have been considered when describing the onset of desensitization. These models can be summarized by the three following schemes:

$$nA + R \rightleftharpoons A_nR^* \rightleftharpoons A_nR_D, \tag{1}$$

$$nA + R \rightleftharpoons A_nR^* \overset{A}{\rightleftharpoons} A_nR_DA, \tag{2}$$



$$R \rightleftharpoons R_D \overset{A}{\rightleftharpoons} R_DA, \tag{3}$$

where A is the agonist, R is the receptor–channel complex in the non-conducting state, R^* is the conducting state and R_D is the desensitized (non-conducting and inactivated) state. In scheme (1) desensitization is a simple consequence of channel opening such that a small fraction of the receptor–channel complexes with open channels proceed to the desensitized state at some characteristic rate (see del Castillo & Katz, 1957; Katz & Thesleff, 1957; Rang & Ritter, 1970; Weiland, Georgia, Lappa, Chignell & Taylor, 1977; Barrantes, 1978; Heidmann & Changeux, 1979*a*, *b*; Quast, Schimerlick & Raftery, 1979; Pallotta & Webb, 1980). In scheme (2) desensitization occurs as a result of agonist binding to the open-channel state of the receptor–channel complex (see Nastuk & Gissen, 1966; Rang & Ritter, 1970; Adams, 1975). In scheme (3) receptors can interconvert directly between normal and desensitized states. Agonist can bind to the desensitized state and shift the equilibrium towards a larger percentage of desensitized receptors (see Katz & Thesleff, 1957; Rang & Ritter, 1970; Weiland *et al.* 1977; Barrantes, 1978; Heidmann & Changeux, 1979*a*, *b*).

The desensitization models described by schemes (1), (2) and (3) differ in their predicted response to different durations and timing of exposure to ACh. Scheme (1) predicts that desensitization should be proportional to the number of receptor channels which have opened, independent of the duration or timing of agonist application (if appropriate corrections are made for recovery from desensitization). Scheme (2) predicts that desensitization should occur only if free agonist is present when receptor channels are open. Scheme (3) predicts that desensitization should be related primarily to the duration of exposure to agonist rather than the number of receptor channels that have been opened or to the timing of agonist application.

In this paper we use nerve-released transmitter to examine these possible mecha-

nisms for the onset of desensitization. With this technique ACh can be applied and removed from the synaptic cleft on a time scale which is short compared to the mean open time of the activated receptor channels; most of the nerve-released ACh is hydrolysed by acetylcholinesterase within several hundred microseconds while the activated channels typically stay open for several milliseconds (Magleby & Stevens, 1972*b*; Neher & Sakmann, 1976; Rosenberry, 1979; Wathey, Nass & Lester, 1979; Adams, 1980). Thus, when the esterase is active, channels can be open in the absence of free ACh. The precise timing of ACh application to the receptors can then be controlled by changing the interval between pairs of nerve impulses. By decreasing the activity of acetylcholinesterase it is possible to increase the duration of application of ACh resulting from each nerve impulse. The total number of activated receptors resulting from either brief or prolonged pulses of ACh can be controlled by changing the rate and number of times that the nerve is stimulated.

It was found that desensitization to nerve-released transmitter was not a simple consequence of receptor activation as proposed by scheme (1), did not require that free ACh be present when the channels were open as proposed by scheme (2), and was not a simple consequence of exposure of receptors to ACh as proposed by scheme (3). By using pairs of nerve impulses it was found that desensitization did occur if receptors were exposed to ACh a second time within 5–25 msec after being exposed a first time. Even very brief (< 300 μsec) exposure to ACh during this critical period was sufficient.

We suggest that desensitization to nerve-released transmitter is a two-step process with both steps requiring ACh. In the first step ACh converts some receptors to a desensitizable state which has a lifetime of less than 30 msec; in the second step ACh desensitizes the desensitizable state.

METHODS

The sartorius nerve–muscle preparation dissected from the frog *Rana pipiens* was maintained in Ringer solution with the following composition (mM): NaCl, 116; KCl, 2; $CaCl_2$, 3·6; Na_2HPO_4, 2·16; NaH_2PO_4, 0·85; glucose, 5; choline, 0·03; pH 7·3–7·4. Precipitation of calcium phosphate was avoided by adding diluted Ca^{2+} slowly to the buffered solution. Fresh solutions were prepared for each experiment. In some experiments KCl was increased to 5 mM to increase the rate of spontaneous quantal release. Temperature was held at $20 \pm 0·5$ °C by a Peltier-effect thermoelectric cooler. The concentration of Ca^{2+} used in these experiments was 3·6 mM (twice normal) unless otherwise indicated, to increase the amount of transmitter released and the numbers of post-synaptic receptors activated by each nerve impulse. No more than three 1000-impulse trains were applied to any single preparation, and usually only one or two were applied since the amount of transmitter released typically fell with successive trains.

All experiments were performed on glycerol-treated muscles (Gage & Eisenberg, 1967) to avoid muscle contraction upon stimulation. Acetylcholinesterase (esterase) was inhibitied in some preparations after glycerol treatment with the reversible inhibitor neostigmine methylsulphate (Roche Laboratories, New Jersey), or the irreversible inhibitor diisopropylfluorophosphate (DFP; Calbiochem, California). Muscles which were exposed to DFP (1 mM for 30 min) were washed in normal Ringer solution for at least 1 hr prior to use to remove any unbound inhibitor. Esterase was also inactivated through proteolysis by exposing the muscle to 0·02–0·04 % collagenase (Sigma type I; Sigma Chemical Co., Missouri) for 2 hr (Betz & Sakmann, 1973).

Using conventional voltage-clamp techniques (details in Magleby & Stevens, 1972*a*) spontaneously occurring miniature end-plate currents (m.e.p.c.s) and nerve-evoked end-plate currents (e.p.c.s) were recorded on two channels of an FM tape recorder (frequency response 0–10,000 Hz at a tape speed of 30 ips). By recording the current at both high and low gains, a wide range of current

magnitudes (approximately 0·2–3000 nA) could be recorded and analysed at favorable signal-to-noise ratios. The voltage-sensing and current-passing micro-electrodes were filled with 3 M-KCl, with resistances ranging from 2–10 MΩ.

M.e.p.c. amplitudes were determined by two methods. In the first method, currents were actively low-pass filtered at an effective frequency of 1500–2500 Hz and played back at $\frac{1}{16}$ the recorded speed for write-out with a Gould Brush 2200 pen recorder. M.e.p.c. amplitudes were then measured by hand from the chart record. The amplitudes of m.e.p.c.s occurring during the falling phase of e.p.c.s or m.e.p.c.s were measured with respect to the interpolated base line. In the second method, m.e.p.c.s were sampled and stored by a PDP-11 computer directly from the FM tape, examined individually from a display screen and then computer-averaged and analysed (details in Magleby & Weinstock, 1980). Estimates of m.e.p.c. amplitudes obtained before and after the conditioning train by these two methods were in quantitative agreement. Computer analysis was not used to measure m.e.p.c. amplitudes during repetitive stimulation.

The time course of the average ACh-induced current which flowed through the end-plate during a train (example shown in Fig. 1*B*) was determined either from the areas of the e.p.c.s during the train (including any shift in base-line current), or from a highly filtered record (2 Hz, low pass) of the current. From these plots of average current the total amount of net charge which flowed during the train could be obtained by integration. Total net charge was used to calculate total number of channel openings, as explained in the text.

Frog sartorius muscle fibres are innervated at both ends (Katz & Kuffler, 1941). Since the two-micro-electrode voltage-clamp technique only clamps one end-plate region, the unclamped end-plate often generated an action potential when the nerve was stimulated which propagated toward the clamped end-plate and produced an active current on the falling phase of the recorded end-plate current. (Examples of such active currents are shown in Fig. 2*D* and *E* of Kordas, (1969). Active currents from a distant synapse occur after the peak of the end-plate current and can thus be distinguished from active currents from a poorly clamped end-plate which typically occur at the peak of the end-plate current). When active currents due to a propagated action potential were present, total current through the end-plate was determined from the areas of the e.p.c.s excluding the active components. Most experiments have been done in fibres both with and without action potentials from distant synapses, with similar results being obtained.

Values have been expressed as the mean ± S.E. of the mean.

RESULTS

Desensitization to nerve-released transmitter when acetylcholinesterase is inhibited

To determine whether desensitization can occur to nerve-released transmitter we stimulated repetitively under conditions in which the acetylcholinesterase (esterase) had been irreversibly inhibited by prior treatment with DFP. By inhibiting the esterase it is possible to prolong the time course of ACh in the synaptic cleft (Katz & Miledi, 1973, 1975; Magleby & Terrar, 1975) and more closely duplicate the duration of ionophoretic pulses of ACh, which are known to lead to desensitization (Katz & Thesleff, 1957; Magazanik & Vyskocil, 1970).

Fig. 1 presents an experiment of this type. The nerve was stimulated for 30 sec at a rate of ten per sec, and end-plate currents (e.p.c.s) and miniature end-plate currents (m.e.p.c.s) were recorded. The insert in Fig. 1*A* presents examples of an e.p.c. (low gain, left) and a m.e.p.c. falling on the decaying phase of an e.p.c. (high gain, right) recorded during the conditioning stimulation. Both currents are greatly prolonged in this DFP-inhibited preparation when compared to an untreated preparation (insert, Fig. 3*A*) due to the repeated binding and activation of receptors by ACh as it escapes from the synaptic cleft (Katz & Miledi, 1973, 1975; and see Kuba, Albuquerque, Daly & Barnard, 1974). Fig. 1*A*, which plots e.p.c. amplitude against time, shows that e.p.c. amplitudes declined dramatically with the duration of

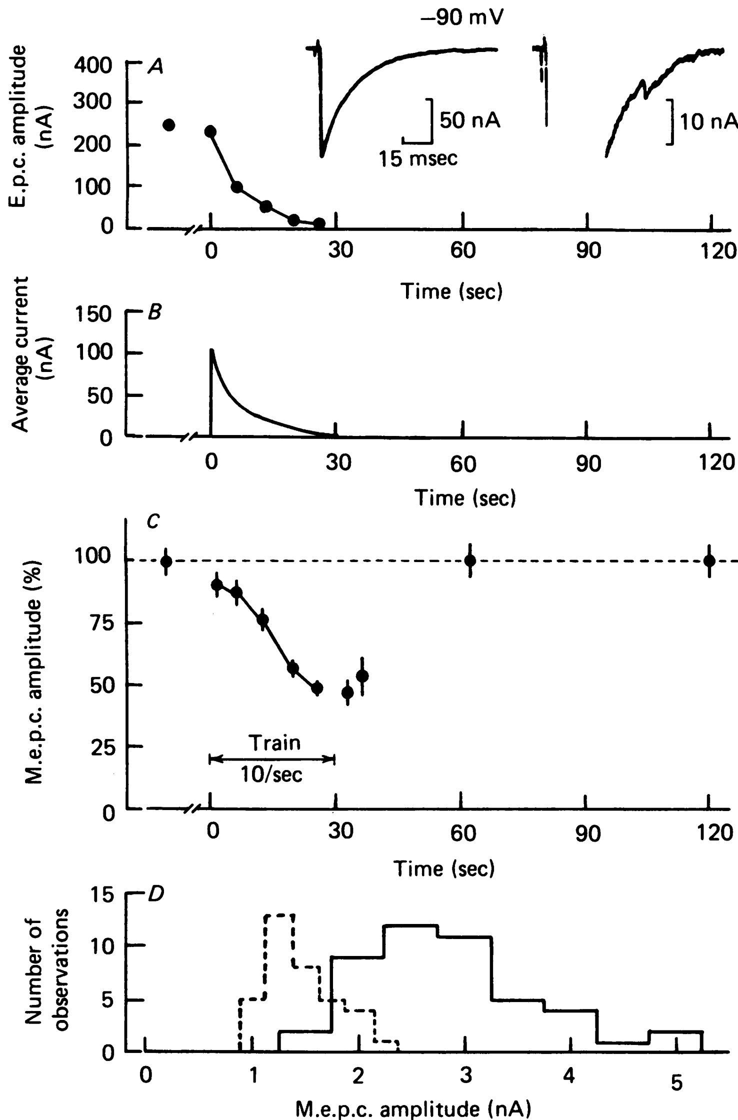


Fig. 1. Effect of repetitive stimulation of the motor nerve at the frog neuromuscular junction on m.e.p.c. amplitudes after irreversible inhibition of the acetylcholinesterase with DFP. Unbound DFP was washed from the muscle before the experiment. Stimulation consisted of 300 impulses at 10/sec. Inserts: nerve-evoked e.p.c.s at low (left) and high (right) gain. A spontaneously occurring m.e.p.c. is present in the high-gain record. Inward current is downward. *A*, e.p.c. amplitudes during the conditioning stimulation. *B*, average stimulation induced current through the end-plate during the conditioning train obtained from the areas of the e.p.c.s including any change in base-line current. *C*, average m.e.p.c. amplitudes before, during (continuous line) and after the conditioning stimulation. Bars indicate the S.E. of mean. *D*, histograms of m.e.p.c. amplitudes obtained during the control period (——) and after stimulation (–––). In this and the following Figures the control period includes those m.e.p.c.s obtained before the train and in some cases also includes those obtained 90 sec after the train, when the response had returned to the control level; the 'after stimulation' histograms include those m.e.p.c.s recorded during the last 5–10 sec of the conditioning train and during the first few sec after the conditioning stimulation. Holding potential: −90 mV.

stimulation. The average current through the end-plate during the conditioning train, determined by measuring the areas of the e.p.c.s and any shift in the base-line current (see Methods), decreased in a similar manner, as shown in Fig. 1 *B*. M.e.p.c. amplitudes also decreased during the conditioning train, to 50 % of their initial value, and then recovered fully after the train as shown in Fig. 1 *C*.

This decrease in the mean amplitudes of the m.e.p.c.s resulted from a shift of the entire distribution of m.e.p.c. amplitudes to lower values. This is shown in Fig. 1 *D* where an histogram of m.e.p.c. amplitudes obtained immediately after and during the last fourth of the conditioning train (when the mean m.e.p.c. amplitudes were decreased) can be compared to an histogram of m.e.p.c. amplitudes obtained during the control period before the train and 90 sec after the train.

In a series of experiments like that shown in Fig. 1, in which the esterase was blocked by prior treatment with DFP, m.e.p.c. amplitudes decreased during repetitive stimulation of the nerve and then recovered following the train. Increasing the stimulation rate led to a greater decrease in m.e.p.c. amplitudes during the trains. If the stimulation rate was greater than about ten to twenty per sec, a long-lasting tail of current often developed during the initial part of the stimulation period (Kuba *et al.* 1974; Katz & Miledi, 1975) and m.e.p.c.s rapidly disappeared into the base-line noise. The decrease in m.e.p.c. amplitudes observed in these additional experiments was also associated with a shift in the distribution of m.e.p.c. amplitudes to lower values. We interpret these results to suggest that post-synaptic sensitivity, as measured by the response to quantal packets of ACh, decreased during and recovered after the conditioning trains. (Support for the use of m.e.p.c. amplitudes to assay post-synaptic sensitivity will be presented throughout the Results section of this paper and will be summarized in the Discussion). The decrease and recovery of post-synaptic sensitivity observed in these esterase-inhibited preparations with nerve-released transmitter was similar (except for some differences in time course to be discussed later) to that observed by Katz & Thesleff (1957) and Magazanik & Vyskocil (1970) with ionophoretic application of agonist.

Desensitization to nerve-released ACh was also seen in experiments in which the esterase was reversibly inhibited with 3 μM-neostigmine methylsulphate. The prolongation of e.p.c.s in these experiments, which gives a measure of the duration of ACh in the synaptic cleft, was less than in experiments in which esterase was blocked with DFP, and the observed desensitization was typically less. A reduced effect when compared with DFP-blocked preparations is consistent with the observation that 3 μM-neostigmine is not sufficient to completely inhibit all the end-plate esterase (Adams & Sakmann, 1978; Katz & Miledi, 1975).

Esterase activity was also decreased by a technique which blocks hydrolysis of ACh in quite a different manner from that of DFP and neostigmine. Pre-treatment of the muscle with collagenase results in the removal of esterase from the synaptic cleft (Hall & Kelly, 1971; Betz & Sakmann, 1971, 1973), and consequently prolongs the duration of ACh in the synaptic cleft (Magleby & Terrar, 1975).

E.p.c.s from a muscle treated for 2 hr with Ringer solution containing 0·04 % collagenase (Sigma type I) were approximately twice as long as those from an untreated muscle (compare the insert in fig. 2 *A* to that in Fig. 3 *A*). During stimulation with a train of 1000 impulses at 33/sec m.e.p.c. amplitudes decreased

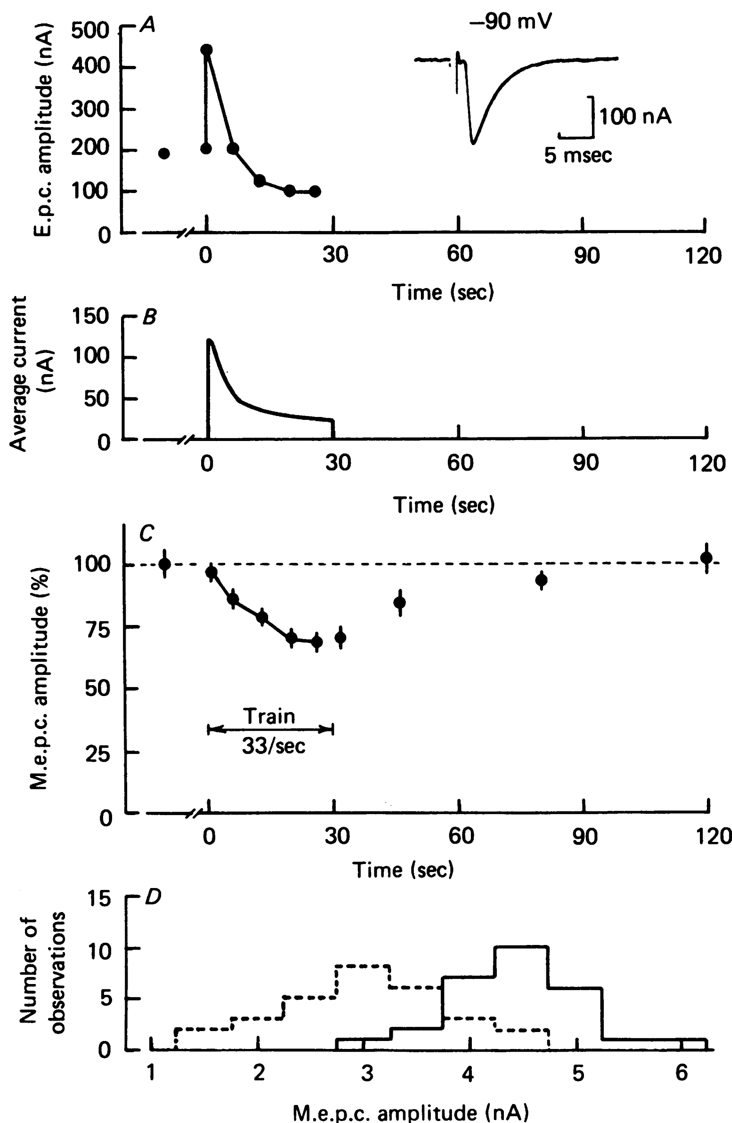


Fig. 2. Effect of repetitive stimulation of the motor nerve on m.e.p.c. amplitudes following inhibition of the esterase by treatment with Ringer containing 0·04 % collagenase (Sigma, type I) for 2 hr. Stimulation consisted of 1000 impulses at 33/sec. Insert: nerve-evoked e.p.c. during the conditioning stimulation. *A*, e.p.c. amplitudes during the conditioning stimulation. E.p.c. amplitudes first increased and then decreased in amplitude in this experiment compared to only a decrease observed in Fig. 1*A*. This difference is mainly due to facilitation of transmitter release that occurred at the higher stimulation rate in this experiment. *B*, average stimulation-induced current through the end-plate during the conditioning train. *C*, average m.e.p.c. amplitudes before, during (——) and after the conditioning stimulation. *D*, histograms of m.e.p.c. amplitudes obtained during the control period (——) and after stimulation (---). Holding potential: −90 mV.

30 %, indicating the onset of desensitization (Fig. 2*C* and *D*). Recovery of m.e.p.c. amplitudes then followed over the next 90 sec. Similar, but less dramatic results were obtained following treatment with 0·02 % collagenase.

The observation that desensitization occurs to nerve-released transmitter when the esterase is inhibited by three different methods (treatment with DFP, neostigmine or collagenase) suggests that the observed desensitization is most likely to be due to the prolonged duration of the nerve released ACh in the synaptic cleft and not due to direct action of these esterase blockers on the receptor–channel complex; it seems unlikely that such different treatments would all have similar direct effects on the receptor–channel complex.

In experiments in which hydrolysis of ACh was blocked by treatment with DFP, neostigmine or collagenase, it was usually possible to anticipate the degree of desensitization from the duration of the e.p.c.s in the absence of repetitive stimulation. If the e.p.c.s were greatly prolonged (more than about three-fold) by the esterase inhibition, indicating a greatly prolonged duration of ACh in the synaptic cleft, then desensitization readily developed during repetitive stimulation, even with low frequency (5–10/sec) stimulation. If the e.p.c.s were prolonged less than about twofold, indicating a shorter duration of ACh action, then it was typically necessary to use high stimulation rates (thirty per sec or greater) for appreciable desensitization to develop. Akasu & Karczmar (1980) have previously reported desensitization during repetitive stimulation when the esterase is blocked.

The observation that the entire distribution of m.e.p.c. amplitudes was typically shifted to lower amplitudes with desensitization (Figs. 1*D* and 2*D*) indicates that the quanta of transmitter that give rise to m.e.p.c.s were released onto the same general population of receptors that was desensitized by the evoked transmitter release. Therefore, the release sites of evoked and spontaneous transmitter release are most likely within less than 1 μm of each other since, when the esterase is active, a quantum of transmitter is thought to spread somewhat less than 1 μm from its point of release (Hartzell, Kuffler & Yoshikami, 1975).

The effect of repetitive stimulation on desensitization when the esterase is active

The results in the previous sections show that desensitization occurred to nerve-released transmitter when the esterase was inhibited. Under these conditions the duration of ACh in the synaptic cleft following each impulse was prolonged to several msec (Katz & Miledi, 1973). To investigate whether desensitization can also occur to very brief pulses of ACh we repeated the experiments when the esterase was active so that the effective duration of ACh in the synaptic cleft following each nerve impulse would be several hundred μsec (Magleby & Stevens, 1972*a*, *b*; Rosenbery, 1979; Wathey *et al.* 1979; Adams, 1980). Fig. 3 presents an experiment of this type. The nerve was stimulated for 30 sec at a rate of 33 impulses/sec when the esterase was active. The e.p.c.s and m.e.p.c.s were brief (inserts, Fig. 3*A*) when compared to those recorded when the esterase was inhibited (Figs. 1 and 2), and m.e.p.c. amplitudes remained relatively constant during and after the conditioning train (Fig. 3*C* and *D*). Similar results were found in a series of experiments in which the stimulation rate was 10–33/sec and the esterase was active: m.e.p.c. amplitudes did not change significantly either during or after stimulation. These results demonstrate that

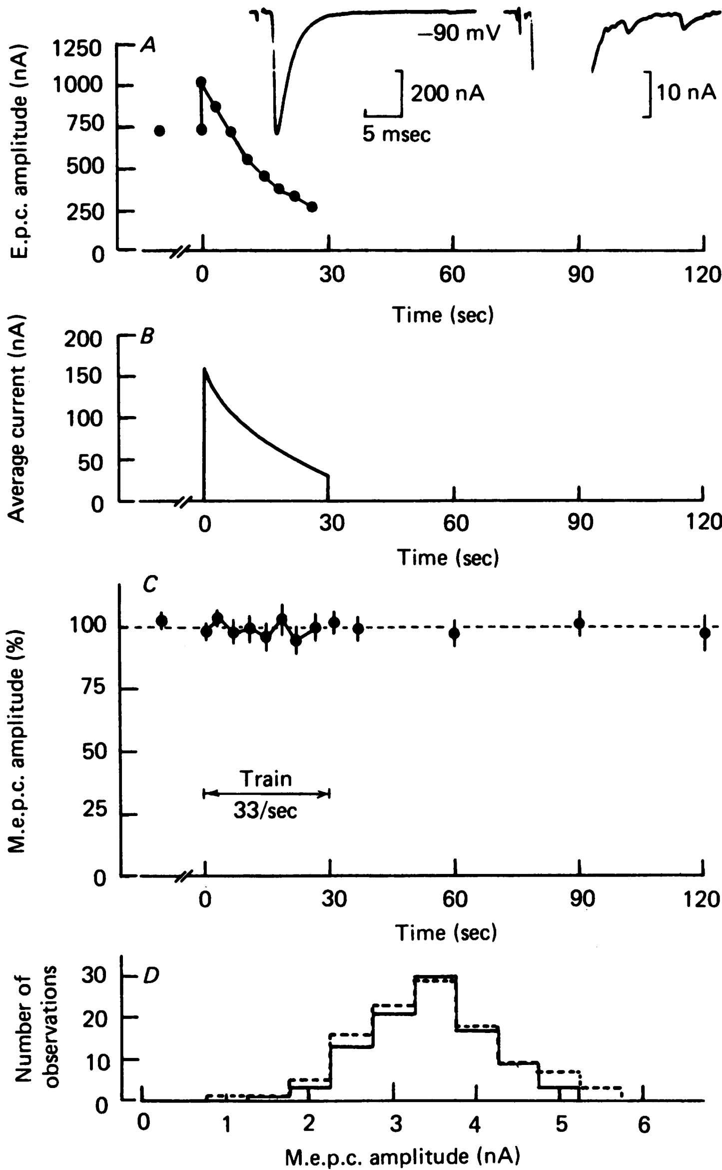


Fig. 3. Effect of repetitive stimulation of the motor nerve on m.e.p.c. amplitudes when the esterase is active. Stimulation consisted of 1000 impulses at 33/sec. Insert: nerve-evoked e.p.c.s and spontaneously occurring m.e.p.c.s recorded during the conditioning stimulation. *A*, e.p.c. amplitudes during the conditioning stimulation. *B*, average stimulation-induced current through the end-plate during the conditioning train. *C*, average m.e.p.c. amplitudes before, during (——) and after the conditioning stimulation. *D*, histograms of m.e.p.c. amplitudes obtained during the control period (——) and after stimulation (---). Holding potential: −90 mV.

measurable desensitization did not occur in these experiments. These findings are consistent with those of Ruzzier & Scuka (1979) who observed little change in m.e.p.c. amplitudes in esterase active preparations after 60 sec of stimulation at 10/sec.

Relationship between channel openings and desensitization

The apparent absence of desensitization during repetitive stimulation when the esterase was active (Fig. 3) can be contrasted to the marked desensitization that occurred when the esterase was inhibited (Figs. 1 and 2). These results suggest that (1) either the very brief pulses of ACh applied to the post-synaptic membrane, as was the case when the esterase was active, were not sufficient to cause measurable desensitizaton, or (2) that too few receptors were activated or exposed to ACh when the esterase was active to allow measurable desensitization to develop.

To explore these possibilities we plotted desensitization against the total number of channel openings for a series of experiments similar to those shown in Figs. 1–3. The results are shown in Fig. 4. Desensitization was calculated from the fractional decrease in m.e.p.c. amplitudes during the conditioning trains, all of which had durations of 30 sec. The number of channel openings during the trains in each experiment was varied by using different stimulation rates or by presenting several trains with a few minutes rest between each train; transmitter release typically decreased with each successive train. Estimates of the total stimulation-induced net charge which flowed through the end-plate were obtained by integrating the average stimulation-induced current that flowed during the trains (examples shown in Figs. 1*B*, 2*B* and 3*B*), or by integrating and summing the areas of all e.p.c.s including any shift in the base-line current during the conditioning trains. Similar results were found by both techniques. Estimates of the total number of channel openings during the conditioning trains were then calculated from the total charge by assuming a single channel current of 2·28 pA (Magleby & Weinstock, 1980) and a mean channel open time of 2·2 msec, estimated from the time constant of decay of e.p.c.s (insert, Fig. 3*A*) in the absence of esterase inhibitors (Magleby & Stevens, 1972*a*, *b*). Dreyer, Walther & Peper (1976) have shown that neostigmine (3 μM) and collagenase treatment do not significantly affect single-channel properties, and we have assumed that inhibiting the esterase with DFP also had little effect on channel properties (see Kuba *et al.* 1974; Katz & Miledi, 1975).

From Fig. 4 it is readily apparent that desensitization occurred in those experiments where esterase was inhibited (open symbols) and was not apparent when the esterase was active (●). Note especially that measurable desentization did not develop in esterase-active preparations at levels of receptor activation considerably greater than those which led to marked desensitization in esterase-inhibited preparations. For example, desensitization was observed at an esterase-inhibited end-plate after thirty million channel openings, yet measurable desensitization was not observed after 180 million, 390 million, or 470 million channel openings in experiments in which the esterase was active.

These results suggest that the desensitization observed to nerve-released transmitter was not a simple consequence of receptor activation, as proposed by scheme (1) in the Introduction. If it were, then desensitization should have been related to the number of channel openings. These results also suggest that exposure to brief pulses

of ACh, as was the case when the esterase was active (Fig. 3), was not sufficient to lead to measurable desensitization by the mechanism proposed by scheme (3).

The results shown in Figs. 1–4 are consistent with the suggestion of Katz & Thesleff (1957) that it is the prolonged duration of ACh in the synaptic cleft that leads to

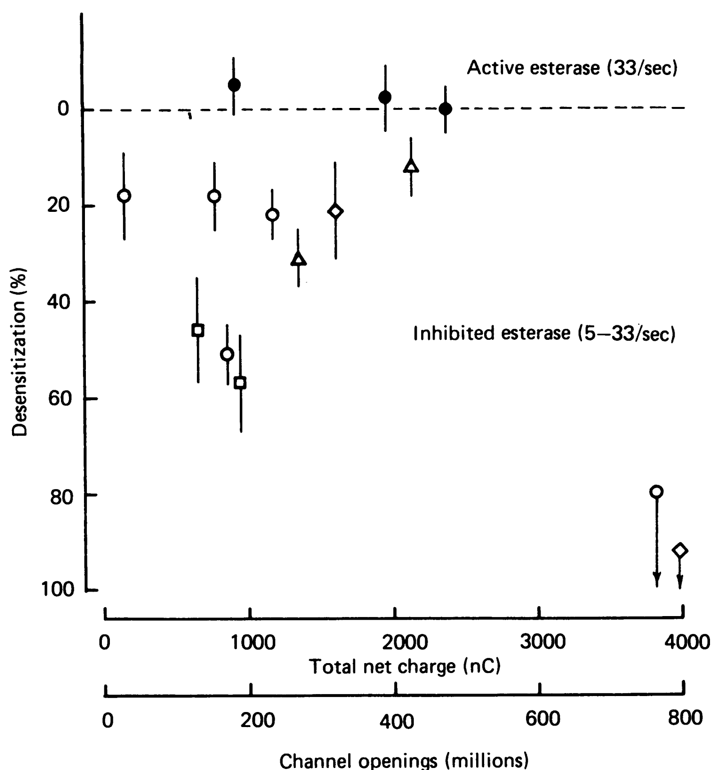


Fig. 4. Relationship between receptor activation and desensitization when the esterase is active (●) or inhibited (○). Desensitization was determined from the percent decrease in m.e.p.c. amplitudes during 30 sec of repetitive stimulation of the motor nerve. Receptor activation, expressed as number of channel openings, was determined from the total stimulation-induced net charge that flowed through the post-synaptic membrane during the conditioning trains. The number of channel openings was then calculated from the total net charge, assuming a mean channel open time of 2·2 msec and single channel current of 2·28 pA. Esterase inhibition by pre-treatment with 1 mM DFP (○, □); unbound DFP was washed from the muscle before the experiment. Esterase inhibited by exposure to 0·04 % (1350 nC point) or 0·02 % (2100 nC point) collagenase for 2 hrs (△). Esterase inhibited with 3 μm neostigmine (◇). Stimulation rate: (●, △, ◇) was at 33/sec; ○, 5/sec for 150 nC point, 20/sec for 760 nC point, 10/sec for 840 and 1150 nC points, and 33/sec for 3750 nC point. □, a pair of impulses separated by a 5 msec interval was delivered once every 200 msec. All data collected in 3·6 mM-Ca^{2+} except for ● at 910 nC, ○ at 760 and 3750 nC, and ◇ at 4000 nC which were collected in 1·8 mM-Ca^{2+}.

desensitization; we found that desensitization readily occurred in esterase-inhibited preparations when the duration of the pulses of nerve-released ACh would be prolonged. Furthermore, by comparing the number of channel openings to desensitization (Fig. 4), we have excluded the possibility that prolonged application of ACh leads to desensitization simply through a greater number of channel openings.

Additional support for the suggestion that it is the prolonged duration of ACh in the synaptic cleft that leads to desensitization was obtained by comparing the results of our experiments using nerve-released ACh to those of Albuquerque, Gage & Oliveira (1979) using bath-applied ACh. The onset of desensitization in our experiments

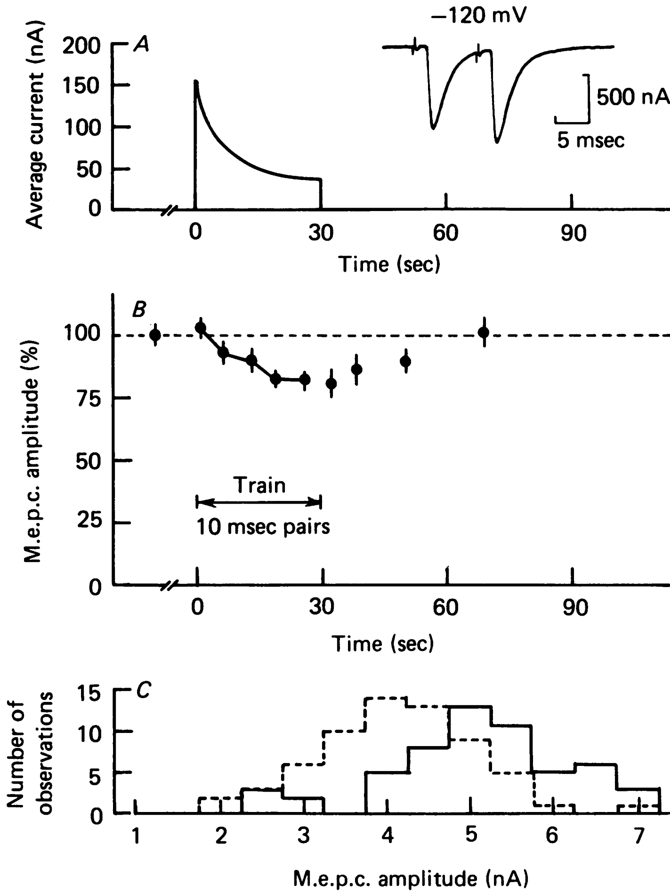


Fig. 5. Effect of paired-pulse repetitive stimulation of the motor nerve on m.e.p.c. amplitudes when the esterase is active. Stimulation consisted of 300 pairs of impulses with one pair delivered every 100 msec; 10 msec separated the impulses in each pair. Insert: a pair of nerve-evoked e.p.c.s recorded during the conditioning stimulation. *A*, average stimulation induced current through the end-plate during the conditioning train. *B*, average m.e.p.c. amplitudes before, during (——) and following the conditioning stimulation. *C*, histograms of m.e.p.c. amplitudes obtained during the control period (——) and after stimulation (---). Holding potential: −120 mV.

when the esterase was inhibited was similar to that observed in the experiments of Albuqerque *et al.*, their Fig. 3*A*, when the esterase was active. A common feature of these two types of experiments was that the duration of ACh in the synaptic cleft was prolonged.

Desensitization to nerve-released ACh when the esterase is active

A simple explanation for the observation that desensitization to nerve-released ACh is related to the duration of exposure to ACh rather than to the number of channel openings (Figs. 1–4) is that desensitization is a two-step process in which sequential exposure of receptors to ACh is required. If this is the case, then it should be possible to obtain desensitization to very brief pulses of ACh if the timing of ACh application is appropriate. To explore this possibility we looked for desensitization to nerve-released ACh when the esterase was active, so that the application of ACh would be brief, but under conditions where free ACh would be present a second time shortly after receptors had been exposed a first time. To accomplish this, receptors were first exposed to ACh by stimulating the nerve, and then another pulse of ACh was applied by stimulating a second time 10 msec after the first; 300 such pairs of impulses were applied by stimulating with a pair of impulses every 100 msec. Fig. 5 presents the results of such an experiment. M.e.p.c. amplitudes decreased 18% during the conditioning train of paired impulses and then recovered, indicating the onset and recovery of desensitization.

Desensitization to nerve-released transmitter when the esterase was active was seen in nine out of eleven additional experiments in which the interval between the impulses of each pair was 5 or 10 msec and the post-synaptic membrane potential was clamped to −90 or −120 mV. In the two experiments in which desensitization was not observed, m.e.p.c. amplitudes did not change in one and increased about 5% during the conditioning train in the other.

The results shown in Fig. 5 in which desensitization occurred to nerve impulses 10 msec apart (average over-all stimulation rate of twenty per sec) can be compared to those shown in Fig. 3 in which measurable desensitization did not develop during stimulation at thirty-three per sec. Since the esterase was active in both these experiments, these results suggests that even very brief pulses of ACh can be sufficient to cause desensitization if the timing of ACh application is favourable.

The timing of receptor desensitization

By changing the interval between impulses of each pair it should be possible to determine the time period during which successive exposure of receptors to ACh can lead to desensitization. An example of such an experiment is shown in Fig. 6. The nerve was presented with three trains of repetitive stimulation, each consisting of 500 pairs of impulses with a pair delivered every 60 msec. The interval between the impulses in each pair was 25 msec for the first train (Fig. 6*A*), 5 msec for the second train (Fig. 6*B*) and 20 msec for the third train (Fig. 6*C*). When the interval separating the impulses in each pair was 5 or 20 msec, appreciable desensitization developed during the conditioning stimulation, as indicated by the the decrease in m.e.p.c. amplitudes; more desensitization was observed when the interval was 5 msec (33%) than when the interval was 20 msec (22%). When the interval between the pulses of ACh in each pair was 25 msec there was still a small amount (8%) of desensitization.

Calculations from the average ACh-induced current during each conditioning train indicated that there were 200 million channel openings during the first train (25 msec pairs), 127 million during the second train (5 msec pairs) and 120 million during the

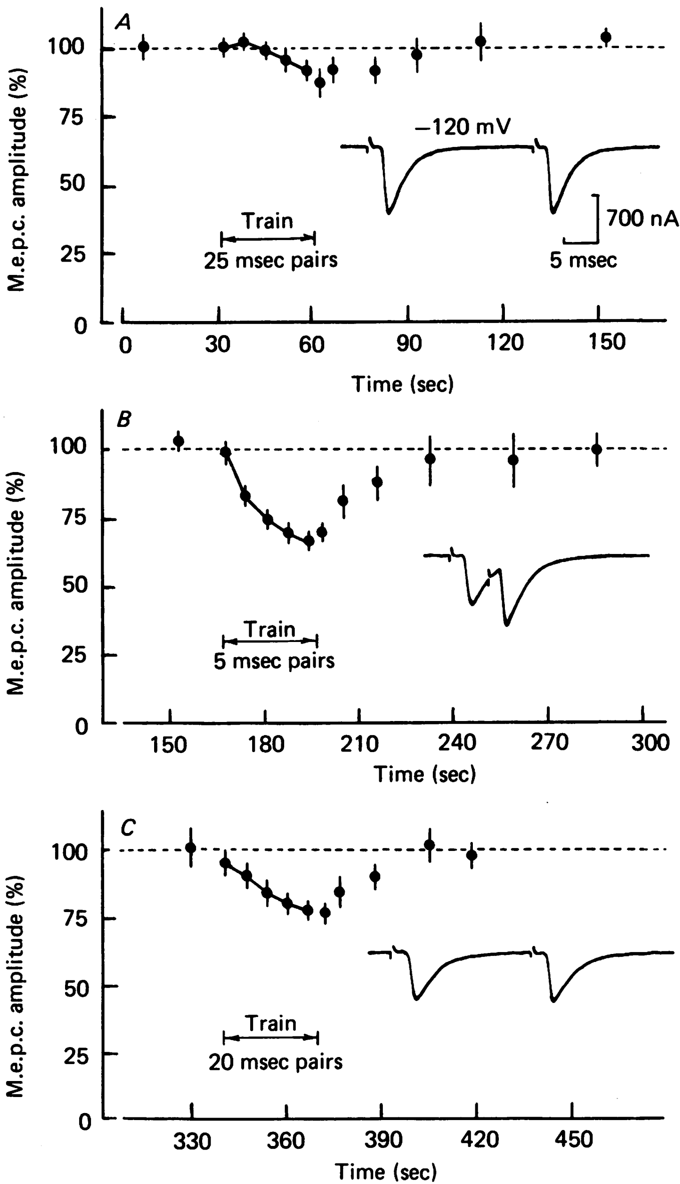


Fig. 6. Effect of the interval of paired-pulse repetitive stimulation of the motor nerve on m.e.p.c. amplitudes when the esterase is active. The nerve was presented with three trains of conditioning stimulation, with each train consisting of 500 pairs of impulses with one pair delivered every 60 msec. The interval between the impulses in the pairs was 25 msec for the data in *A*, 5 msec for the data in *B*, and 20 msec for the data in *C*. Data collected continuously from same fibre. The inserts in each part of the Figure show a pair of nerve-evoked e.p.c.s recorded during the conditioning stimulation for that Figure. *A*, *B*, and *C* plot average m.e.p.c. amplitudes recorded before, during (——), and after the different conditioning trains. Holding potential: −120 mV.

third train (20 msec pairs). Thus, desensitization was not directly related to the number of channel openings or the order of presentation of the conditioning trains, but was related to the interval between the impulses in each pair.

Fig. 7, which plots percent desensitization against the interval between pulses of ACh, summarizes the results obtained in Figs. 3, 5, and 6 on the timing of desensitization. (Ideally the data would all be obtained from the same cell, but this was not possible so we have combined results from several experiments). In these

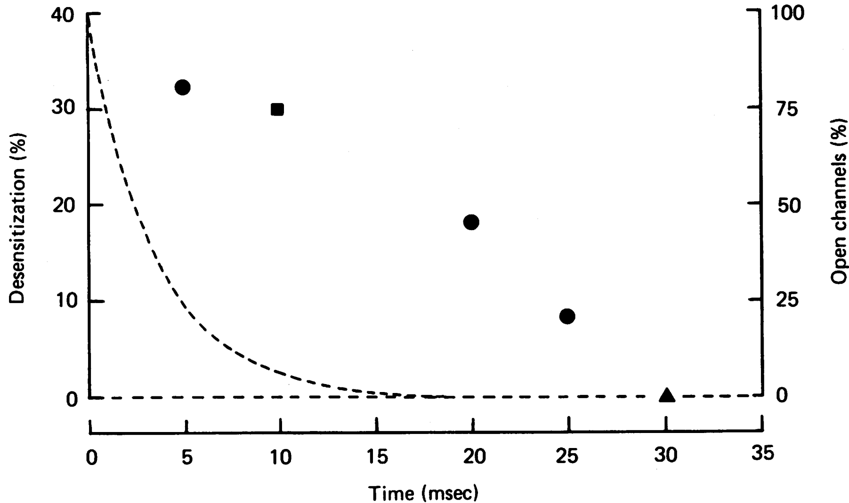


Fig. 7. Effect of varying the interval between nerve impulses on desensitization when the esterase is active. Plotted points, left ordinate: desensitization, determined from the percent decrease in m.e.p.c. amplitudes during 30 sec of repetitive stimulation of the motor nerve with paired impulses is plotted against the interval between the impulses in the pairs. A pair of impulses was presented every 60 msec except for the ■ where a pair was presented once every 100 msce. The plotted data points were obtained from: ▲, Fig. 3; ■, Fig. 5; ●, Fig. 6. The observed desensitization in Fig. 5 was multiplied by (1000/600) before plotting to normalize it to the greater number of conditioning impulses in the other experiments. Right ordinate: time course of channel closing for those channels opened by the first impulse of each pair (----). The line was drawn assuming a mean channel open time of 3·3 msec (−120 mV).

experiments desensitization was greatest when the interval between the impulses in each pair was 5 msec and decreased as the interval increased until there was no measurable desensitization when the interval was 30 msec. The symbols plotted in Fig. 7 may be viewed, then, as approximating the time course of an apparent desensitizable state of receptors. In terms of the two-step model for desensitization, the ACh from the first impulse can initiate formation of this state, and the ACh from the second nerve impulse can desensitize it.

The results shown in Fig. 7 are generally consistent with those obtained in other experiments similar to those presented in Figs. 3, 5, and 6 (3·6 mM-Ca^{2+}, −90 or −120 mV holding potential, active esterase), in which the interval between the impulses in each pair ranged from 5 to 100 msec and a pair of impulses was delivered every 60, 100 or 200 msec. In these experiments desensitization readily developed

during repetitive stimulation if the interval between impulses of each pair was about 25 msec or less. There was no measurable desensitization if the interval was 30 msec or greater. In experiments in which 5 and 10 or 15 msec intervals were given in the same preparation, greater desensitization was often seen with the 10 or 15 msec interval than with the 5 msec interval, as if the proposed desensitizable state developed with a delay.

These findings are consistent with those of Akasu & Karczmar (1980) who have reported desensitization to nerve-released transmitter at the neuromuscular junction with active esterase during 60 sec of repetitive stimulation at fifty per sec (20 msec interval between impulses). They found that the responses to ionophoretic application of ACh used to test for post-synaptic sensitivity were decreased an average of 33 % after the conditioning train. Our findings are also in agreement with those of Otsuka, Endo & Nonomura (1962) who found no desensitization following ten-impulse trains delivered at fifty per sec. Our results suggest that significant desensitization would not be observed after only ten impulses.

The results shown in Figs. 6 and 7 on the timing of receptor desensitization are clearly inconsistent with scheme (1). If the desensitization observed in these Figures were only a simple consequence of receptor activation, as proposed by this scheme, then desensitization should have been related to the number of channel openings and not the timing of ACh application as was observed. These results also appear to be inconsistent with scheme (3) which proposes that desensitization results from the binding of ACh to a population of already desensitized receptors with a resulting shift in the entire distribution of receptors towards the desensitized state. On the basis of this scheme, changing the interval between the impulses in each pair in the range 5–30 msec should not have had any appreciable effect on desensitization; a change in the timing of ACh application of this magnitude would be insignificant when compared to the average lifetime of the desensitized state of the receptors (determined from the recovery from desensitization), which was about 20 sec for the experiments plotted in Fig. 7.

Desensitization can occur after the channels have closed

The results in the previous sections are consistent with two-step models of desensitization of which scheme (2) is a specific case. It should be possible to test scheme (2), which proposes that desensitization occurs if ACh binds to the receptor–channel complex when the channel is open, by applying a second pulse of ACh shortly after all the receptors activated by the first impulse have closed their channels. If desensitization still occurs, then this finding would be inconsistent with scheme (2).

Notice from the insert in Fig. 6*C* that the first e.p.c. of the pair returned to the base line well before the start of the second e.p.c. 20 msec later. This indicates that all the channels opened by the first impulse had closed before the second pulse of ACh was applied. Yet there was still 22 % desensitization; it was not necessary for channels to be open for the applied ACh to cause desensitization. This is more clearly shown in Fig. 7 where a comparison of the time course of channel closing for the first e.p.c. of each pair (dashed line) can be made with the time course of the apparent desensitizable state of the receptors plotted as symbols; desensitization still occurred when ACh was introduced 5–10 msec after all the opened channels had closed. This

finding is inconsistent with scheme (2) which proposes that desensitization occurs when a molecule of ACh binds to (or blocks the channel of) the receptor–channel complex in the open-channel state.

Thus, it appears that the desensitization to nerve-released transmitter observed in our experiments is not easily described by schemes (1), (2) or (3). A two-step model for desensitization which is consistent with our observations will be presented in the Discussion.

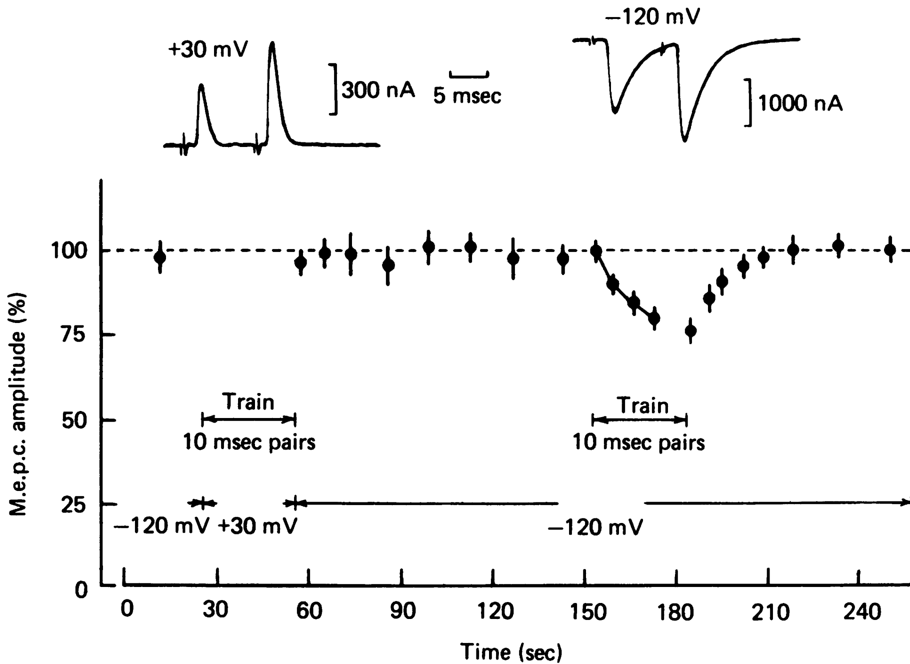


Fig. 8. Effect of post-synaptic membrane potential during repetitive stimulation of the motor nerve on m.e.p.c. amplitudes when the esterase is active. Stimulation for each train consisted of 500 pairs of impulses with one pair delivered every 60 msec; 10 msec separated the impulses in each pair. Inserts: pairs of nerve-evoked e.p.c.s recorded during the conditioning trains delivered at +30 mV (left) and −120 mV (right). Average m.e.p.c. amplitudes are plotted against time. M.e.p.c. amplitudes were too small to measure during the train at +30 mV. M.e.p.c. amplitudes recorded during the train at −120 mV are connected by a continuous line. There were 560 million channel openings during the train at +30 mV and 120 million channel openings during the train recorded at −120 mV.

Voltage sensitivity of desensitization

Fig. 8 presents an experiment in which the voltage sensitivity of desensitization to nerve-released transmitter was examined. There was little or no desensitization when the conditioning stimulation was delivered while the post-synaptic membrane was clamped at +30 mV (left), whereas desensitization developed and then recovered when the conditioning stimulation was delivered while clamped at −120 mV. Similar results were obtained in other experiments in which the order of presentation of the holding potentials was reversed.

These observations, that desensitization to nerve-released transmitter was greatly

reduced at more positive holding potentials, is consistent with the voltage sensitivity of desensitization resulting from bath (Fiekers, Spannbauer, Scubon-Mulieri & Parsons, 1980) and ionophoretic (Magazanik & Vyskocil, 1970) application of agonist.

It should be noted that experiments like those shown in Fig. 8 essentially exclude the possibility that the changes in m.e.p.c. amplitudes observed in our experiments arise from presynaptic mechanisms; since the presynaptic conditions for the two trains were essentially the same in these experiments, the difference in response must result from the difference in the post-synaptic membrane potential and be post-synaptic in origin. These experiments also show that paired stimulation, like that used in Figs. 5 and 6, is not by itself sufficient to cause changes in m.e.p.c. amplitudes through possible presynaptic mechanisms. If it were, then m.e.p.c. amplitudes should have been reduced after the trains at +30 mV. They were not.

Effect of chlorpromazine

Pharmacological evidence that the phenomenon that we are studying is desensitization was obtained in experiments in which 2 μM-chlorpromazine was added to the bathing solution. Desensitization following conditioning stimulation similar to that shown in Fig. 5 was typically greater in the presence of chlorpromazine than in its absence. This increase in desensitization in chlorpromazine is consistent with the effect of chlorpromazine on desensitization induced by ionophoretic application of ACh (Magazanik & Vyskocil, 1975). While chlorpromazine increased desensitization when the post-synaptic membrane potential was clamped to −120 mV in our experiments, it had little effect on m.e.p.c. amplitudes after conditioning trains delivered at +30 mV. This observation demonstrates that the effect of chlorpromazine was not through presynaptic changes in quantal size. If it were, then it should have also reduced m.e.p.c. amplitudes following the trains at +30 mV.

Recovery from desensitization

The recoveries of m.e.p.c. amplitudes after stimulation-induced desensitization shown in the previous figures in this paper are typical of the recoveries we observed in nineteen other experiments. Recovery from desensitization occurred with an apparent exponential time course with a mean time constant of about 25 sec at 20 °C (range: 2·5–65 sec).

The time constants for recovery from desensitization to ionophoretic application of agonist observed by Katz & Thesleff (1957) and Magazanik & Vyskocil (1970) typically fell in the range 3–9 sec. About 20 % of our experiments using nerve-released transmitter had recoveries that fell in this range. The others had somewhat slower recoveries, but the recoveries in these experiments were still faster than the slow recoveries from bath-applied agonist that typically have a time course of minutes (Rang & Ritter, 1970; Scubon-Mulieri & Parsons, 1977). Thus, it appears that the desensitization we observed to nerve-released transmitter has kinetics that are more similar to those of ionophoretically induced desensitization and the fast phase of recovery from bath-applied agonist observed by Adams (1975) than to those of the slow phase of recovery from bath-applied agonist.

To determine if the typically slower rates of recovery from desensitization observed in our experiments using nerve-released ACh when compared to the rates of recovery

in the experiments of Katz & Thesleff (1957) and Magazanik & Vyskocil (1970) using ionophoretically applied agonist arose from possible differences in experimental conditions other than the method of application of ACh, we performed a few experiments using ionophoretic application of agonist under conditions similar to those used for our studies with nerve-released transmitter. Desensitization recovered faster following ionophoretic application of ACh than following nerve application. Thus, the method of application either affects the recovery in some unknown way, or desensitization to nerve-released transmitter may have different properties than that resulting from ionophoretic application of agonist.

DISCUSSION

Nerve-released ACh was used in this paper to study desensitization at the motor end-plate. Using this technique it was possible to study the relationship between receptor activation and desensitization. Central to achieving this goal was the ability to expose the post-synaptic ACh receptors to very brief (< 300 μsec) pulses of ACh; this occurred every time the nerve was stimulated in the absence of esterase inhibitors, since the ACh that was released was rapidly hydrolysed (Magleby & Stevens, 1972*b*; Rosenberry, 1979; Wathey *et al.* 1979; Adams, 1980). To study the effects of longer pulses of ACh it was only necessary to inhibit the esterase; the nerve-released ACh was then no longer hydrolysed so that its duration of action was prolonged to several msec as it diffused from the synaptic cleft (Katz & Miledi, 1973). The timing of ACh application and the total amount applied was changed by altering the pattern, frequency and duration of stimulation. This method allowed the study of desensitization of junctional receptors using precisely timed applications of ACh. It had the disadvantage, in common with focal ionophoretic application of ACh, that the concentration of agonist was unknown.

The spontaneous release of quantal packets of ACh, which give rise to m.e.p.c.s was used to monitor post-synaptic sensitivity. We suggest that the decrease in m.e.p.c. amplitudes observed in our experiments reflects desensitization for the following reasons.

(1) The onset and recovery of 'desensitization' observed in our experiments (Figs. 1, 2 and 5), its voltage sensitivity (Fig. 8) and its enhancement by chlorpromazine are all consistent with the known properties of desensitization reported in previous studies (Katz & Thesleff, 1957; Magazanik & Vyskocil, 1970, 1975; Adams, 1975; Fiekers *et al.* 1980). It would be difficult to explain with presynaptic mechanisms the effects of changes in the post-synaptic membrane potential and micromolar amounts of chlorpromazine, two procedures which would be expected to have little effect on the nerve terminal.

(2) Akasu & Karczmar (1980), using ionophoretic application of ACh to test for post-synaptic sensitivity, have reported desensitization to nerve-released transmitter that is consistent with our findings obtained using m.e.p.c. amplitudes to test for post-synaptic sensitivity.

(3) If the changes in m.e.p.c. amplitudes observed in our experiments were due to presynaptic changes in the size of the quantal packets of transmitter, then it might be expected that the changes in m.e.p.c. amplitudes would be directly related to the

amount of transmitter released during the conditioning trains. This was not the case. Changes in m.e.p.c. amplitudes in our experiments were related to the timing of ACh application rather than the amount of transmitter released (Figs. 4, 6 and 7). In addition, experiments like that shown in Fig. 3, in which there was little or no change in m.e.p.c. amplitude during and after 1000 impulses of stimulation at 33 impulses/sec, and experiments like that shown in Fig. 8, in which stimulation using paired pulses was not by itself sufficient to change m.e.p.c. amplitude, show that the repetitive stimulation used in our experiments by itself had little effect on quantal size.

(4) Experiments like those in Figs. 4 and 6 rule out the possibility that the observed changes in m.e.p.c. amplitudes result from changes in the ionic distributions across the post-synaptic membrane. If the changes in m.e.p.c. amplitudes were due to changes in the ionic distributions, then m.e.p.c. amplitudes should have been directly related to the total current that flowed through the end-plate during the conditioning trains. This was not the case.

In view of the above considerations, then, we feel that the changes in m.e.p.c. amplitudes reported in this paper arise mainly from desensitization. It should be kept in mind, however, that m.e.p.c. amplitudes probably give only an approximate measure of average post-synaptic sensitivity. If the release of the quantal packets of transmitter that give rise to m.e.p.c.s and e.p.c.s is not uniformly distributed over the receptor population, then m.e.p.c. amplitudes could give either an over-estimate or underestimate of the average level of desensitization, depending on the relative distributions of the two types of release. In addition, m.e.p.c. amplitudes may be related in a non-linear manner to the number of functional ACh receptors (Pennefather & Quastel, 1979; Adams, 1980).

In spite of the experimental limitations associated with using m.e.p.c. amplitudes to assay post-synaptic sensitivity some useful results were obtained, since the interpretation of the experiments only required knowing the relative levels of desensitization. The major findings were: when the esterase was inhibited, implying that the pulses of ACh applied to the post-synaptic membrane were several msec in duration, desensitization readily developed during repetitive stimulation (five to thirty-three per sec), even after as few as 30 million channel openings by the receptors in the post-synaptic membrane. When the esterase was active, implying that the pulses of ACh were very brief ($< 300\ \mu$sec), measurable desensitization did not develop during repetitive stimulation if the pulses of ACh were 30 msec or more apart, even after as many as 470 million channel openings. Desensitization readily developed in the esterase-active preparation, however, if the pulses of ACh were applied within 25 msec or less of each other.

These results were shown to be inconsistent with the possibility that the densensitization observed in our experiments was a simple consequence of receptor activation (scheme (1) in the Introduction) resulted from the binding of ACh to the open-channel state of the receptor–channel complex (scheme (2)) or developed from the binding of ACh to receptors already in the desensitized state with a further shift in the receptor distribution towards the desensitized state (scheme (3)). These results are consistent with the suggestion that the desensitization in our experiments is a two-step process requiring sequential exposure of receptors to ACh.

The Katz & Thesleff model

The Katz & Thesleff (1957) cyclic model, originally proposed to describe the kinetics of desensitization resulting from ionophoretic application of ACh to the neuromuscular junction, has also been used to account for desensitization to bath-applied agonist (Rang & Ritter, 1970; Parsons, 1978) and the kinetics of receptor–agonist interactions (Weiland *et al.* 1977; Barrantes, 1978; Heidmann & Changeux, 1979*a*, *b*). It was therefore of interest to determine if this model can account for the kinetics of desensitization we observed using nerve released agonist.

The Katz & Thesleff model can be summarized as follows:

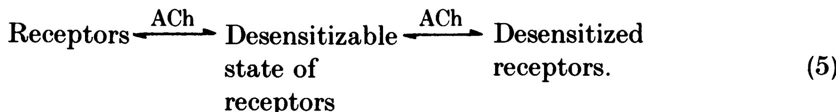
(4)

where A is agonist (ACh in our experiments), R is the receptor–channel complex in the closed state, R* is the open-channel state and R_D is the desensitized (closed-channel) state of the receptor–channel complex.

In this model desensitization can occur in two ways: through the right-hand pathway as a simple consequence of receptor activation, or through the left-hand pathway by binding ACh to already densensitized receptors (R_D) and shifting the receptor equilibrium towards the desensitized state. The Katz & Thesleff model is thus a combination of schemes (1) and (3). In our experiments, desensitization was not a simple consequence of receptor activation (Figs. 4 and 6). In addition, neither of the two proposed mechanisms of desensitization in the Katz & Thesleff model can account for our observations that desensitization readily occurred when brief pulses of ACh were applied to the end-plate at intervals of less than 30 msec, but was not apparent when the pulses were separated by intervals of 30 msec or greater (Figs. 6 and 7). The Katz & Thesleff model predicts approximately equal desensitization for the different intervals. It seems unlikely, therefore, that the desensitization observed in our experiments to nerve-released transmitter arose by the mechanisms described by scheme (4). We cannot exclude, however, that under different experimental conditions, such as during bath or ionophoretic application of agonist, that scheme (4) is a major contributor to desensitization.

A model for desensitization to nerve-released transmitter

A general model for desensitization that is consistent with our observations can be formulated from three non-conducting states of the receptor to give:



(5)

The major feature of this model is that desensitization requires two successive ACh-dependent steps. In the first step receptors are converted into a desensitizable state. In the second the desensitizable state is desensitized. Scheme (5) is the simplest model that accounts for our data. We cannot exclude, however, that desensitized

receptors may also return directly to the receptor state without passing through the desensitizable state.

Our results suggest that the proposed desensitizable state of the receptor can have a lifetime of up to 25 msec (Figs. 6 and 7). On this basis, the two-step sequential model for desensitization shown in scheme (5) would account for our experimental observations as follows. When the esterase was inhibited, ACh from each impulse would be expected to remain in the synaptic cleft sufficiently long to both initiate the conversion of receptors to the desensitizable state and then to desensitize this state. This would account for our observation that desensitization readily occurred with inhibited esterase, even to low frequency stimulation. When the esterase was active so that the nerve-released ACh was rapidly hydrolysed, desensitization would still be expected to occur, in agreement with our observations, if the interval between impulses was less than 30 msec (the apparent lifetime of the desensitizable state); ACh from the first impulse would initiate the formation of the desensitizable state and then ACh from the second impulse would desensitize this state. When the interval between nerve impulses was 30 msec or greater, desensitization would not be expected to occur with active esterase, in agreement with our observations, since the release of ACh by the second nerve impulse would occur after the desensitizable state resulting from the first impulse had decayed to insignificant levels.

Implications of a two-step process for desensitization

If the proposed desensitizable state of the receptor does have a lifetime of up to 25 msec, as suggested by our data, then the desensitizable state in scheme (5) is not likely to be one of the intermediate states ACh_nR (where $n = 1$ or 2) in the generally accepted receptor activation scheme (see del Castillo & Katz, 1957; Anderson & Stevens, 1973; Dreyer, Peper & Sterz, 1978; Dionne, Steinbach & Stevens, 1978; Adams, 1980), as the ACh_nR states appear to have an effective lifetime of less than 1 msec (Magleby & Stevens, 1972*a*, *b*; Rosenberry, 1979; Wathey *et al.* 1979; Adams, 1980). It also appears that the desensitizable state does not represent the open-channel state of the receptor (ACh_2R^* in the generally accepted receptor scheme) since the channel-open times under the conditions of our experiments were too brief (a few msec) to account for the lifetime of the proposed desensitizable state (Fig. 7).

While our results appear to exclude the possibility that the proposed desensitizable state of the receptor is the ACh_nR or the ACh_2R^* states, our results give little additional information about the relationship between receptor activation kinetics and the desensitizable and desensitized state of the receptor in scheme (5). Channel opening may be a necessary factor in one or both steps in the desensitization sequence in scheme (5). Alternatively, desensitization may occur independently of and in parallel with channel opening such that some receptors exposed to ACh desensitize and some open their channels. While our results do not distinguish between these two possibilities, they do suggest that channel opening by itself is not sufficient for measurable desensitization to occur under the conditions of our experiments (Figs. 4 and 6).

We now briefly consider two of many possible mechanisms by which sequential exposure of receptors to ACh could lead first to the proposed desensitizable state and then to the desensitized state. The most straightforward interpretation of scheme (5)

is that both ACh-dependent steps represent direct binding of ACh to receptors with a resulting conformational change in each step. In this model the experimentally observed lifetime of the desensitizable state of the receptor would represent the actual lifetime of a desensitizable conformation of the receptor.

If the proposed desensitizable state of the receptor does represent an additional conformational state, then our results suggest that receptors in this state may stay there for up to 25 msec. If this is the case, then receptors should occasionally stay in a closed-channel state for tens of msec even when exposed to high concentrations of agonist. Sakmann, Patlak & Neher (1980) have observed such a closed-channel state of the receptor that has a lifetime of about 180 msec at 12 °C, and it appears from their data (Fig. 3, 50 μM-ACh) that there may also be a closed-channel state with a lifetime of about 30 msec at 11 °C. Considering that our experiments were performed at a higher temperature (20 °C) and that we may have underestimated the lifetime of the proposed desensitizable state due to the inherent difficulties in detecting low levels of desensitization, it is possible that either the 180 msec or the apparent 30 msec closed-channel states in the data of Sakmann *et al.* may represent the proposed desensitizable state in scheme (5). Further experiments would of course be required to determine if this is the case.

The second interpretation of scheme (5) to be considered in this section is that sequential exposure of receptors to ACh leads to desensitization in a more indirect manner. For example, it is known that the rate of desensitization is highly Ca^{2+}-dependent (Manthey, 1966, 1970; Magazanik & Vyskocil, 1970; Nastuk & Parsons, 1970; Scubon-Mulieri & Parsons, 1977; Fiekers *et al.* 1980) and that Ca^{2+} enters the muscle when the receptors are activated by flowing through the receptor channels (Takeuchi, 1963; Miledi, Parker & Schalow, 1980; Adams, Dwyer & Hille, 1980). It might be that the first step in scheme (5) is indirect such that it is the entry of Ca^{2+} through ACh-activated channels and the subsequent action of Ca^{2+} on the inner side of the post-synaptic membrane that leads to the formation of the desensitizable state of the receptor. Exposure of these altered receptors to ACh in the second step would then desensitize them. In this scheme the desensitizable receptors would not have to be the same receptors that opened their channels in the first step, since the entering Ca^{2+} could also act on neighbouring receptors which were not activated by the first exposure to ACh.

In this indirect model the apparent lifetime of the desensitizable state of receptors may reflect the actual lifetime of an altered conformation of the receptor, as in the previous model, or it may reflect the *effective* time course of the increased Ca^{2+} concentration at the inner surface of the post-synaptic membrane following Ca^{2+} entry. A residual Ca^{2+} hypothesis for the desensitizable state is challenged, however, by the observation that external Ca^{2+} is not required for desensitization to occur at the neuromuscular junction (Fiekers *et al.* 1980) and the findings that external Ca^{2+} has an inhibitory effect on desensitization in the electroplaque (Pallotta & Webb, 1980) or no apparent effect in the mollusc (Bregestovski, Bukharaeva & Iljin, 1979).

Clinical implications

The high frequencies and prolonged durations of stimulation required to produce measurable desensitization in this study when the esterase was active suggest that

desensitization is probably not a significant factor acting to decrease e.p.p. amplitudes under physiological conditions. When the esterase at the end-plate was inhibited, however, desensitization readily developed during repetitive stimulation in the frog, even with low stimulation rates (five to ten per sec) and partial inhibition of the esterase. If a similar phenomenon occurs at the mammalian motor end-plate, then desensitization could contribute to the cholinergic crisis that can occur in patients with myasthenia gravis who receive anticholinesterase drugs for therapy (Engel, 1979).

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REFERENCES

ADAMS, D. J., DWYER, T. M. & HILLE, B. (1980). The permeability of endplate channels to monovalent and divalent metal cations. *J. gen. Physiol.* **75**, 493–510.

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

ADAMS, P. R. (1980). Aspects of synaptic potential generation. *Information Processing in the Nervous System*, ed. PINSKER, H. M. & WILLIS, W. D., JR., pp. 109–124. New York: Raven Press.

ADAMS, P. R. & SAKMANN, B. (1978). A comparison of current–voltage relations for full and partial agonists. *J. Physiol.* **283**, 621–644.

AKASU, T. & KARCZMAR, A. G. (1980). Effects of anticholinesterases and of sodium fluoride on neuromyal desensitization. *Neuropharmacology* **19**, 393–403.

ALBUQUERQUE, E. X., GAGE, P. W. & OLIVEIRA, A. C. (1979). Differential effect of perhydrohistrionicotoxin on 'intrinsic' and 'extrinsic' end-plate responses. *J. Physiol.* **297**, 423–442.

ANDERSON, C. R. & STEVENS, C. F. (1973). Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol.* **235**, 655–691.

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

BETZ, W. J. & SAKMANN, B. (1971). 'Disjunction' of frog neuromuscular synapses by treatment with proteolytic enzymes. *Nature, New Biol.* **232**, 94–95.

BETZ, W. J. & SAKMANN, B. (1973). Effects of proteolytic enzymes on function and structure of frog neuromuscular junction. *J. Physiol.* **230**, 673–688.

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

DEL CASTILLO, J. & KATZ, B. (1957). Interaction at end-plate receptors between different choline derivatives. *Proc. R. Soc. Lond.* B. **146**, 369–381.

DIONNE, V. E., STEINBACH, J. H. & STEVENS, C. F. (1978). An analysis of the dose–response relationship at voltage-clamped frog neuromuscular junctions. *J. Physiol.* **281**, 421–444.

DREYER, F., PEPER, K. & STERZ, R. (1978). Determination of dose–response curves by quantitative ionophoresis at the frog neuromuscular junction. *J. Physiol.* **281**, 395–419.

DREYER, F., WALTHER, C. & PEPER, K. (1976). Junctional and extrajunctional acetylcholine receptors in normal and denervated frog muscle fibres. *Pflügers Arch.* **366**, 1–9.

ENGEL, A. G. (1979). Myasthenia gravis. *Handbook of Clinical Neurology, Vol. 41*, ed. VINKEN, P. J. & BRUYN, G. W., pp. 95–145. Amsterdam: North-Holland.

FATT, P. (1950). The electromotive action of acetylcholine at the motor end-plate. *J. Physiol.* **111**, 408–422.

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

GAGE, P. W. & EISENBERG, R. S. (1967). Action potentials without contraction in frog skeletal muscle fibers with disrupted transverse tubules. *Science, N.Y.* **158**, 1702–1703.

HALL, Z. W. & KELLY, R. B. (1971). Enzymatic detachment of endplate acetylcholinesterase from muscle. *Nature, New Biol.* **232**, 62–63.

HARTZELL, H. C., KUFFLER, S. W. & YOSHIKAMI, D. (1975). Post-synaptic potentiation: interaction between quanta of acetylcholine at the skeletal neuromuscular synapse. *J. Physiol.* **251**, 427–463.

HEIDMANN, T. & CHANGEUX, J.-P. (1979*a*). Fast kinetic studies on the interaction of a fluorescent agonist with the membrane-bound acetylcholine receptor from *Torpedo marmorata*. *Eur. J. Biochem.* **94**, 255–279.

HEIDMANN, T. & CHANGEUX, J.-P. (1979*b*). Fast kinetic studies on the allosteric interactions between acetylcholine receptor and local anaesthetic binding sites. *Eur. J. Biochem.* **94**, 281–296.

KATZ, B. & KUFFLER, S. W. (1941). Multiple motor innervation of the frog's sartorius muscle. *J. Neurophysiol.* **4**, 209–223.

KATZ, B. & MILEDI, R. (1973). The binding of acetylcholine to receptors and its removal from the synaptic cleft. *J. Physiol.* **231**, 549–574.

KATZ, B. & MILEDI, R. (1975). The nature of the prolonged end-plate depolarization in anti-esterase treated muscle. *Proc. R. Soc. Lond.* B, **192**, 27–38.

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

KORDAS, M. (1969). The effect of membrane polarization on the time course of the end-plate current in frog sartorius muscle. *J. Physiol.* **204**, 493–502.

KUBA, K., ALBUQUERQUE, E. X., DALY, J. & BARNARD, E. A. (1974). A study of the irreversible cholinesterase inhibitor, diisopropylfluorophosphate, on time course of end-plate currents in frog sartorius muscle. *J. Pharmac. exp. Ther.* **189**, 499–512.

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

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

MAGLEBY, K. L. & STEVENS, C. F. (1972*a*). The effect of voltage on the time course of end-plate currents. *J. Physiol.* **223**, 151–171.

MAGLEBY, K. L. & STEVENS, C. F. (1972*b*). A quantitative description of end-plate currents. *J. Physiol.* **223**, 173–197.

MAGLEBY, K. L. & TERRAR, D. A. (1975). Factors affecting the time course of decay of end-plate currents: a possible cooperative action of acetylcholine on receptors at the frog neuromuscular junction. *J. Physiol.* **244**, 467–495.

MAGLEBY, K. L. & WEINSTOCK, M. M. (1980). Nickel and calcium ions modify the characteristics of the acetylcholine receptor–channel complex at the frog neuromuscular junction. *J. Physiol.* **299**, 203–218.

MANTHEY, A. A. (1966). The effect of calcium on the desensitization of membrane receptors at the neuromuscular junction. *J. gen. Physiol.* **49**, 963–975.

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

MILEDI, R., PARKER, I. & SCHALOW, G. (1980). Transmitter induced calcium entry across the post-synaptic membrane at frog end-plates measured using arsenazo III. *J. Physiol.* **300**, 197–212.

NASTUK, W. L. & GISSEN, A. J. (1966). Action of certain quaternary ammonium compounds in neuromuscular transmission. In *Nerve as a Tissue*, ed. RODAHL, K. & ISSEKUTZ, B., pp. 305–320. New York: Harper & Row.

NASTUK, W. L. & PARSONS, R. L. (1970). Factors in the inactivation of post-junctional membrane receptors of frog skeletal muscle. *J. gen. Physiol.* **56**, 218–249.

NEHER, E. & SAKMANN, B. (1976). Noise analysis of drug induced voltage clamp currents in denervated frog muscle fibres. *J. Physiol.* **258**, 705–729.

OTSUKA, M., ENDO, M. & NONOMURA, Y. (1962). Presynaptic nature of neuromuscular depression. *Jap. J. Physiol.* **12**, 573–584.

PALLOTTA, B. S. & WEBB, G. D. (1980). The effects of external Ca^{2+} and Mg^{2+} on the voltage sensitivity of desensitization in *Electrophorus electricus* electroplaques. *J. gen. Physiol.* **75**, 693–708.

PARSONS, R. L. (1978). Role of calcium in desensitization at the motor end-plate of skeletal muscle. In *Calcium in Drug Action*, ed. WEISS, G. B., pp. 289–314. New York: Plenum Press.

PENNEFATHER, P. & QUASTEL, D. M. J. (1979). Efficiency of utilizaton of quantal acetylcholine. *Neurosci. Abstr.* **5**, 745.

QUAST, U., SCHIMERLIK, M. I. & RAFTERY, M. A. (1979). Ligand-induced changes in membrane-

bound acetylcholine receptor observed by ethidium fluorescence. 2. Stopped-flow studies with agonists and antagonists. *Biochemistry, N.Y.* **18**, 1891–1901.

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

ROSENBERRY, T. L. (1979). Quantitative simulation of endplate currents at neuromuscular junctions based on the reaction of acetylcholine with acetylcholine receptor and acetylcholinesterase. *Biophys. J.* **26**, 263–290.

RUZZIER, F. & SCUKA, M. (1979). Effect of repetitive stimulation on the frog neuromuscular transmission. *Pflügers Arch.* **382**, 127–132.

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

SCUBON-MULIERI, B. & PARSONS, R. L. (1977). Desensitization and recovery at the frog neuromuscular junction. *J. gen. Physiol.* **69**, 431–447.

TAKEUCHI, A. & TAKEUCHI, N. (1960). On the permeability of end-plate membrane during the action of transmitter, *J. Physiol.* **154**, 52–67.

TAKEUCHI, N. (1963). Effects of calcium on the conductance change of the end-plate membrane during the action of transmitter. *J. Physiol.* **167**, 141–155.

TERRAR, D. A. (1974). Influence of SKF-525A congeners, strophanthidin and tissue-culture media on desensitization in frog skeletal muscle. *Br. J. Pharmac.* **51**, 259–268.

THESLEFF, S. (1955). The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine. *Acta physiol. scand.* **34**, 218–231.

WATHEY, J. C., NASS, M. M. & LESTER, H. A. (1979). Numerical reconstruction of the quantal event at nicotinic synapses. *Biophys. J.* **27**, 145–164.

WEILAND, G., GEORGIA, B., LAPPA, S., CHIGNELL, C. F. & TAYLOR, P. (1977). Kinetics of agonist-mediated transitions in state of the cholinergic receptor. *J. biol. Chem.* **252**, 7648–7656.