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DESENSITIZATION OF THE ACETYLCHOLINE RECEPTOR OF DENERVATED RAT SOLEUS MUSCLE AND THE EFFECT OF CALCIUM

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1 Desensitization of the extrajunctional acetylcholine receptors of denervated rat soleus muscle has been studied by evoking iontophoretic acetylcholine potentials at a high frequency (10 Hz).

2 Desensitization occurred with a slow and at least one fast component.

3 Increasing the dose of acetylcholine decreased the half time of onset of desensitization, but did not alter recovery half time.

4 Alteration of external calcium between 0 and 50 mM, or perfusion with agents which increase intracellular calcium such as 2,4-dinitrophenol and the calcium ionophores, X537A and A23187, did not alter desensitization half time.

5 The cholinoceptor agonists acetylcholine, acetylthiocholine, tetramethylammonium and phenyltrimethylammonium all caused desensitization at the same rate.

Introduction

Application of large amounts of acetylcholine (ACh) to ACh receptors causes an initial activation of the receptors followed by a process of desensitization in which the receptors are converted to an inactive form (Thesleff, 1955; Katz & Thesleff, 1957). The onset of, and recovery from, desensitization have previously been found to be single exponential processes, with the onset half times varying from 0.5 to 15 s when ACh is applied iontophoretically (Katz & Thesleff, 1957; Magazanik & Vyskocil, 1975) and varying from 10 s to several minutes when ACh is perfused (Thesleff, 1955; Adams, 1975; Nastuk & Parsons, 1970; Rang & Ritter, 1970).

The rate of desensitization at the frog endplate has been reported to be increased by raising the external calcium concentration (Manthey, 1966; Nastuk & Parsons, 1970), or by perfusing with the calcium ionophore X537A (DeBassio, Parsons & Schnitzler, 1976) and it has been suggested that there is a calcium binding site on the inner surface of the membrane which is important in regulating the rate of desensitization (Nastuk & Parsons, 1970; DeBassio *et al.*, 1976). In the present study, detailed quantitative measurements have been made on desensitization of the ACh receptor using iontophoretic application of ACh, and the effects of calcium on desensitization have been reinvestigated.

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Methods

Experiments were carried out on the soleus muscles of 100 to 200 g female rats. The muscles were denervated by removal of 0.5 to 1 cm of the sciatic nerve in the mid-thigh region.

Intracellular recordings were made with 5 to 15 MΩ microelectrodes filled with 2 M potassium citrate, and iontophoresis was performed with high resistance (100 to 200 MΩ) electrodes filled with 1 M ACh chloride. A bias current of a few nA was applied to the iontophoretic electrode to prevent diffusion of ACh from the tip of the electrode. The iontophoretic current was monitored with an operational amplifier placed between the bath and ground. The sensitivity to ACh was expressed in mV depolarization per nC of charge (Miledi, 1960).

The muscles were removed 5 to 10 days after denervation, and placed in a bath perfused with saline (Na 135 mM, K 5 mM, Ca 3 mM, HEPES 2 mM, pH 7.2). The saline was oxygenated with 95% O_2 and 5% CO_2.

Results

Acetylcholine receptors are present over the whole surface of denervated mammalian muscle (Axelsson & Thesleff, 1959). The present experiments were carried out on soleus muscles that had been denervated for 5

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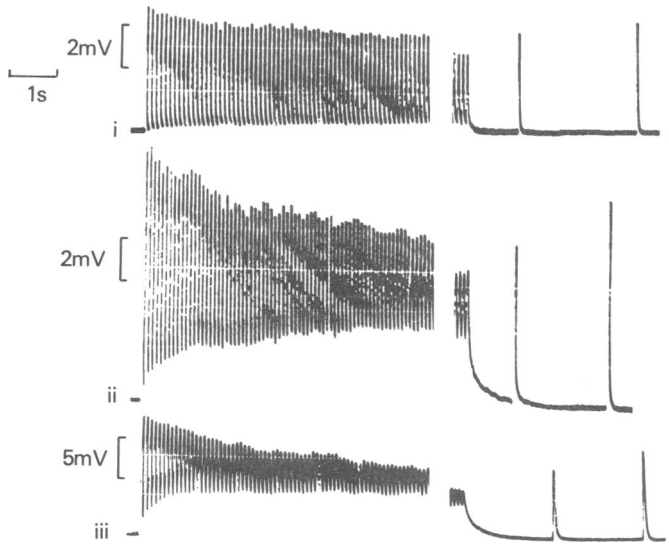

Figure 1 Records of desensitization of trains of acetylcholine (ACh) potentials of different initial amplitudes evoked at 10 Hz. This series of records shows an example of a slow rate of desensitization in normal saline. The amplitude of the initial ACh potentials was (i) 5 mV, (ii) 10 mV and (iii) 12.5 mV. Note the different voltage scale of (iii).

to 10 days in which the extrajunctional sensitivity to iontophoretically applied ACh was 300 to 500 mV/nC. ACh potentials had rise times of 5 to 20 ms.

Onset of desensitization in normal saline

When ACh potentials were evoked at a frequency of 0.5 Hz or less, they always had a constant amplitude. However, stimulation of potentials at higher frequencies caused successive ACh potentials to decline in amplitude, due to desensitization. In the present investigations, desensitization was usually studied by evoking the ACh potentials at 10 Hz, although a few experiments were carried out at 5 Hz. At stimulation frequencies of 5 to 10 Hz, the ACh potentials always declined to zero amplitude if the stimulation was continued for a sufficient period of time.

When evoked at 10 Hz, the ACh potential declined in amplitude very rapidly in the first 2 s, then declined much more slowly (Figures 1, 2a, b). The slow decline in amplitude could always be fitted by an exponential function with a half time varying between 0.5 and 50 s depending on the dose of ACh. Empirically, the very fast desensitization occurring in the initial 2 s of stimulation could be fitted by two additional exponential functions with half times averaging 0.05 and 0.5 s (Figure 2a).

No change in the time course of the ACh potentials occurred during desensitization.

Effect of dose of acetylcholine

Increasing the dose of ACh caused a reduction in the half-time of desensitization, $T_{\frac{1}{2}}$ (Figures 1, 2a-c). The dose was usually increased by raising the iontophoretic current so as to increase the amplitude of the ACh potential. The value of $T_{\frac{1}{2}}$ of large amplitude ACh potentials (15 to 20 mV) was usually 0.5 to 1 s, whereas $T_{\frac{1}{2}}$ of small potentials (2 to 3 mV) was 30 to 50 s. In Figure 2c, $T_{\frac{1}{2}}$ increased from 1.2 s for a 10.5 mV ACh potential to 3.0 s for a 3.5 mV ACh potential, and was exponentially related to the amplitude of the ACh potential. Occasional experiments were also carried out in which the dose of ACh was raised by keeping the iontophoretic current constant and increasing the frequency at which the ACh potentials were evoked. This also resulted in a decrease of $T_{\frac{1}{2}}$ (Figure 2d). Increasing the dose of ACh was found to decrease the half time of the slowest, but not the two fastest exponential functions of desensitization.

Recovery from desensitization

Recovery from desensitization was studied by evoking ACh potentials at intervals of 1.5 to 4.0 s during the 0.1 to 15 s following the termination of the high frequency train of ACh potentials (Figure 2a). Three potentials were usually evoked during each recovery. In Figure 3, the recovery from desensitization is

shown by points averaged from ten recovery experiments. It can be seen that there is a fast recovery component followed by a slower one. The overall half time of recovery averaged 4.3 s (15 expts.). Altering the dose of ACh so as to vary the ACh potential between 3 to 15 mV did not change the recovery half time.

Incomplete recovery from desensitization

When a large continuous 'conditioning' dose of ACh was released from the iontophoretic electrode by reducing the braking current by a large amount, a steady depolarization occurred and the amplitude of the test ACh potential decreased. When the braking current was returned to its original level, the ACh potential only rarely returned completely to its original amplitude, and if large or prolonged conditioning doses of ACh were applied, sometimes only 70 to 90% recovery was attained even 5 to 10 min after the desensitization. A similar incomplete recovery occurred if the conditioning dose of ACh was applied from the second barrel of a double-barrelled electrode.

Ability of different cholinoceptor agonists to cause desensitization

The cholinoceptor agonists acetylcholine, tetramethylammonium (TMA) and phenyltrimethylammonium (phenyl TMA) were compared with ACh in their ability to cause desensitization of the cholinoceptor. The experiments were carried out with double-barrelled electrodes, one barrel being filled with ACh and the other with the particular cholinoceptor agonist being investigated. Identical amplitude and duration agonist potentials were always used. The iontophoretic dose required to evoke such identical potentials was approximately in the ratio 0.5:1:1:10 for TMA, ACh, acetylthiocholine and phenyl TMA, respectively.

The values of $T_{1/2}$ for 8 mV ACh, acetylthiocholine, TMA and phenyl TMA potentials were 9.2, 9.0, 9.4 and 9.5 s, respectively (mean of 3 experiments each agonist) (Figure 4a, b). Similar ratios of half times for

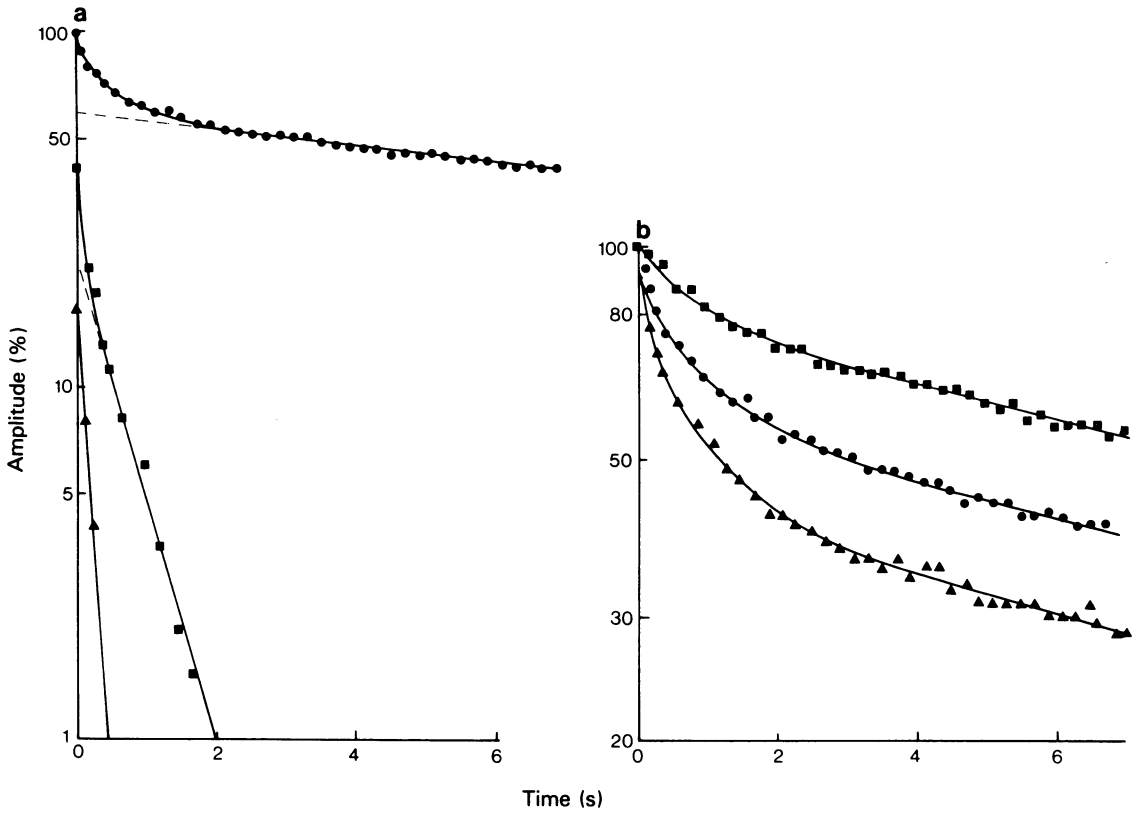

Figure 2 (a) and (b).

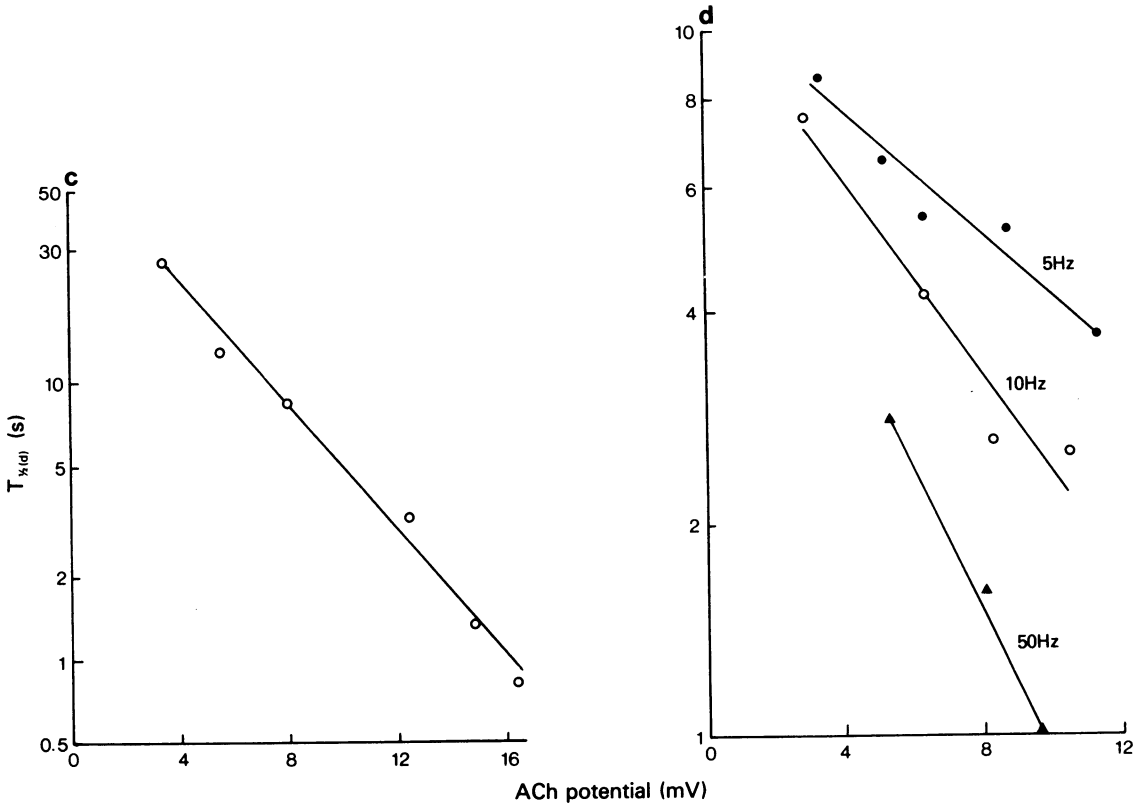

Figure 2 (c) and (d).

Figure 2 (a) Desensitization of a train of acetylcholine (ACh) potentials in normal saline showing the three exponential functions. The amplitude of the initial ACh potential was 6 mV, and the potentials were evoked at 10 Hz. (●): Amplitude of ACh potentials, expressed as a percentage of the initial amplitude and plotted on a logarithmic scale. Only alternate potentials were plotted for clarity. The linear portion of the curve is the slow exponential component. (■): Points obtained by subtraction of the extrapolated slow exponential component from the amplitudes of the ACh potentials. The linear portion of the curve is the intermediate exponential component. (▲): Points obtained by subtraction of the extrapolated intermediate exponential component from the points marked (■). The curve is the fast exponential component. (b) Reduction in amplitudes of successive ACh potentials of different initial amplitudes evoked at 10 Hz in normal saline. The potentials were expressed as a percentage of the initial amplitude, and plotted on a logarithmic scale. The amplitude of the initial ACh potential was 3.5 mV (■), 8 mV (●), and 10.5 mV (▲). The half-time of desensitization was 7.8 s, 3.0 s and 1.2 s for the 3.5 mV, 8 mV and 10.5 mV amplitude of the initial ACh potential, respectively. (c) Changes in the half-time of desensitization ($T_{\frac{1}{2}(d)}$) caused by an increase in the amplitude of the initial ACh potential of a train of potentials evoked at 10 Hz. The half times were plotted on a logarithmic scale. (d) Graph showing the decrease in the half time of desensitization (plotted logarithmically) caused by raising the frequency at which the ACh potentials were evoked and by raising the amplitude of the ACh potential. (●): 5 Hz; (○), 10 Hz; (▲), 50 Hz.

different agonists were found for agonist potentials with different amplitudes.

Effect of calcium

The effect of salines containing high calcium (50 mM) and zero calcium (0 mM Ca plus mM EDTA) were investigated on desensitization. In the experiments on the effects of high Ca, control experiments were carried out in normal saline containing 50 mM Tris in order to keep the osmolarity constant. The muscles were perfused with the altered Ca salines for 1 to 2 h before measurements were made.

Neither the high Ca saline or the zero Ca saline altered the half time desensitization. In normal saline, the half-time of desensitization of 8 mV potentials was 1.7 s (average 8 experiments). In saline containing 50 mM Ca, the half-time of desensitization of 8 mV ACh potentials averaged 1.9 s (7 experiments) (Figure 5).

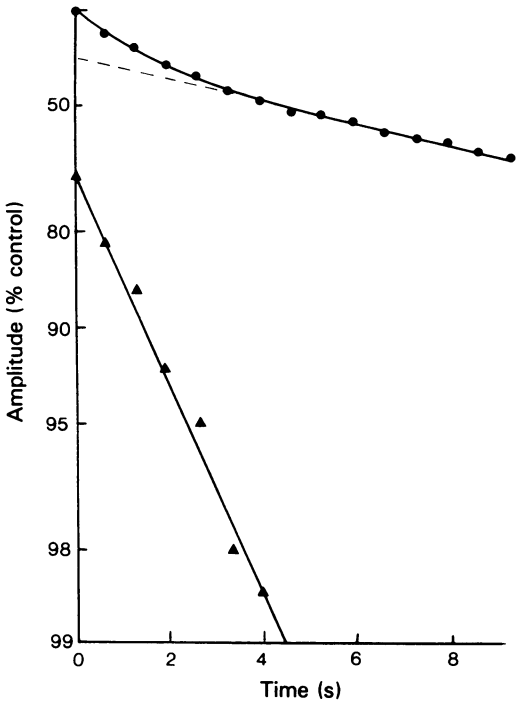

Figure 3 Recovery from desensitization in normal saline. The points were taken from the average of ten recovery curves. (●): Amplitudes of acetylcholine (ACh) potentials during recovery, plotted as a percentage of the initial ACh potential before desensitization was evoked. The amplitude of the potentials was plotted on a logarithmic scale. (▲): Points obtained by subtraction of the extrapolated linear portion of the recovery curve from the amplitude of the ACh potentials.

In saline containing zero Ca and 2 mM EDTA, the resting potential remained at its normal level of about −60 mV for 45 min to 1 h, and then declined to about −30 mV over the subsequent 30 min. The half time of desensitization of 8 mV ACh potentials averaged 2.1 s, 2.4 and 2.2 s in normal saline and after 30 and 90 min in zero Ca respectively.

Effect of 2,4-dinitrophenol (DNP)

Metabolic inhibitors block the ability of mitochondria to take up calcium, and therefore the intracellular concentration of calcium is increased in the presence of these inhibitors (Alnaes & Rahamimoff, 1975). When 1 to 2×10^{-4} M DNP was perfused onto denervated rat soleus muscle fibres, a hyperpolarization of about 10 mV occurred. This hyperpolarization was accompanied by at least a threefold increase in membrane conductance.

During application of DNP, the amplitude of the ACh potentials decreased by 70 to 90%, probably due to the decrease in membrane resistance. The half time of densensitization in the presence of DNP was measured at various times after the application of DNP using the same iontophoretic ejection current as in normal saline. Thus most of the measurements of desensitization in DNP were made on reduced amplitude ACh potentials, although the number of ACh receptors activated will be identical in the presence and absence of DNP.

The half time of desensitization of 12 mV ACh potentials in normal saline was 0.85 s (4 experiments) After 5 min perfusion with 2×10^{-4} M DNP when the membrane was hyperpolarized by about 5 mV but before any reduction in amplitude of the ACh potentials occurred, the half time of desensitization was 0.75 s (4 experiments). After 15 min perfusion with 2×10^{-4} M DNP when the membrane had become hyperpolarized by about 15 mV and the 12 mV ACh potentials were reduced to 3 to 4 mV, the half time of desensitization averaged 2.0 s (4 experiments).

Effect of the calcium ionophores X537A and A23187

X537A caused a gradual reduction in amplitude of the ACh potential. After 10 min and 60 min perfusion with 5 mM X537A, the ACh potential was reduced by 20% and over 75% respectively. The membrane conductance was also increased by a large amount in X537A, which probably accounts for the reduction in amplitude of the ACh potential. The half time of desensitization of 12 mV ACh potentials in normal saline was 3.2 s (3 experiments). After 1 h in 5×10^{-6} M X537A, when the 12 mV ACh potentials were reduced to 3 to 4 mV, the half time of desensitization averaged 3.0 s (3 experiments).

Concentrations of A23187 of 5 to 15×10^{-6} M did not alter the amplitude or half time of desensitization of the ACh potentials even after 1 h of perfusion.

Discussion

The half time of the onset of desensitization measured in the present study varied between 0.5 and 50 s with increasing doses of ACh decreasing the half time. These results agree closely with previous measurements of desensitization onset times obtained by the iontophoretic technique (Katz & Thesleff, 1957; Magazanik & Vyskocil, 1975). Measurements of desensitization using perfusion of the cholinoceptor

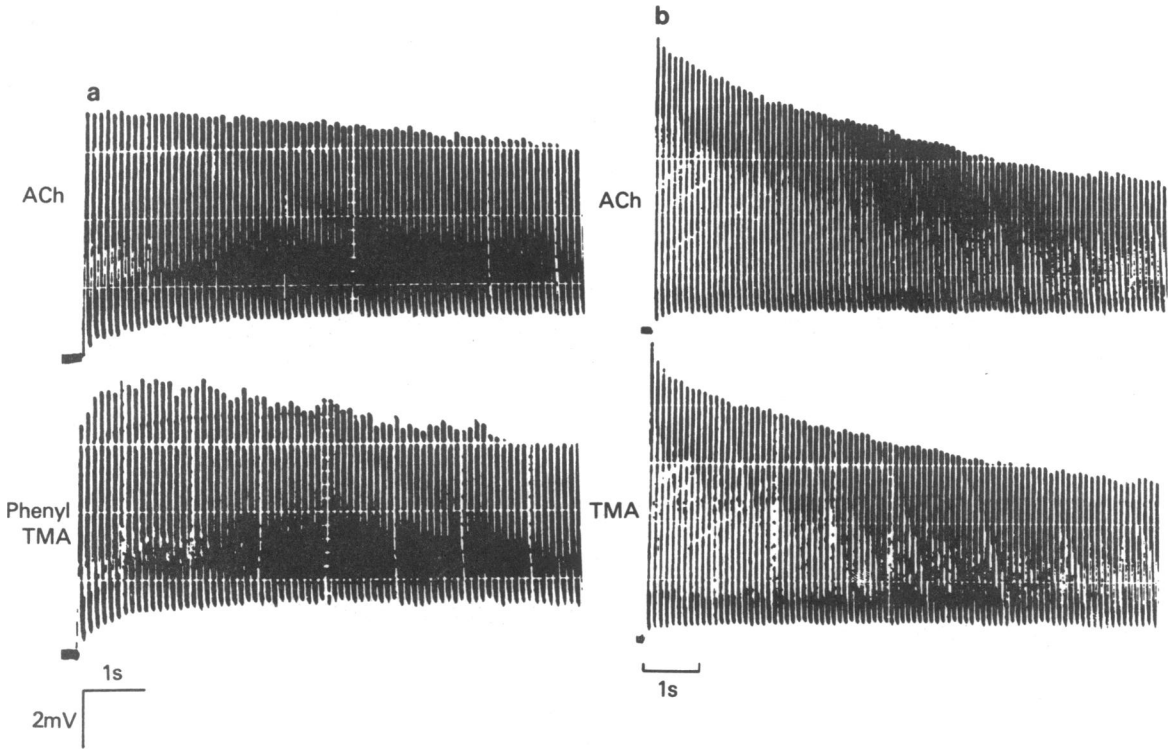

Figure 4 Desensitization produced by iontophoretic application of different agonists onto rat muscle extrajunctional acetylcholine (ACh) receptors. Desensitization was produced by evoking the potentials at 10 Hz. (a) ACh and phenyl tetramethylammonium (Phenyl TMA) potentials. (b) ACh and tetramethylammonium (TMA) potentials.

agonist have given much slower onset times of desensitization, with half times varying from 10 s to several min (Manthey, 1966; Nastuk & Parsons, 1970; Rang & Ritter, 1970; Lester, Changeux & Sheridan, 1975; Adams, 1975). However, the perfusion method suffers from the major disadvantage that the time course of desensitization is determined to a large extent by diffusion of the agonist to the receptors.

Although previously the onset of desensitization has been found to be a single exponential process at the frog neuromuscular junction (Magazanik & Vyskocil, 1975; Adams, 1975), the present study has shown that the onset of desensitization of rat extrajunctional receptors occurs very fast initially, and only after about 2 s becomes exponential. The initial very fast desensitization is much faster than that observed previously, having half times of 50 to 500 ms. This fast desensitization was observed only when ACh potentials were evoked at high frequencies (10 Hz), desensitization then being fast enough to mask potentiation. Previous studies have probably failed to observe such fast desensitization because of the potentiation of the initial response by large doses of the cholinoceptor agonist.

The recovery half time from desensitization averaged 4.3 s, and was not altered by changing the dose of ACh. This agrees closely with previous studies on desensitization evoked iontophoretically (Katz & Thesleff, 1957; Magazanik & Vyskocil, 1975). When desensitization was studied by applying conditioning ACh pulses, recovery of the test potentials was usually incomplete. Such incomplete recovery from desensitization has previously been reported in neurophysiological (Katz & Thesleff, 1957; Adams, 1975) and in biochemical experiments on desensitization (Weber, David-Pfeuty & Changeux, 1975, Sugiyama, Popot & Changeux, 1976). It is therefore likely that there is either an irreversible or very slow recovery component of desensitization.

In the present study, the overall half time of desensitization and the rates of the three phases of desensitization were not changed when the external calcium concentration was altered between 0 and 50 mM. Moreover, treatment with agents designed to

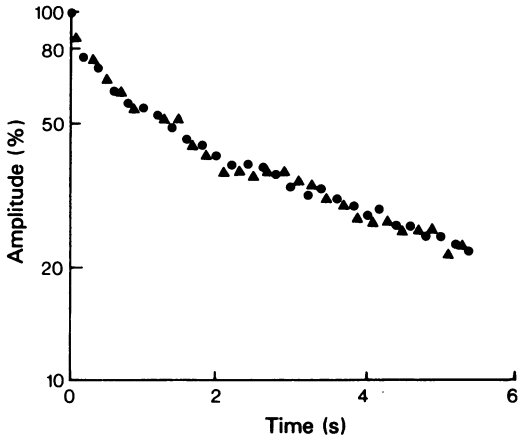

Figure 5 Reduction in amplitude of 8 mV acetylcholine (ACh) potentials due to stimulation at 10 Hz in normal saline (●), and saline containing 50 mM Ca (▲). The half-time of desensitization was 1.6 s in each saline.

increase intracellular concentration of Ca, such as the metabolic inhibitor DNP and the calcium ionophores X537A and A23187 did not increase the rate of desensitization. Although intracellular concentrations of Ca were not measured directly in the present study, the observations that DNP and X537A caused a hyperpolarization and an increase in conductance area strongly indicative that intracellular Ca was increased by these agents. Meech (1974) has previously found that injecting Ca intracellularly causes a hyperpolarization and increased membrane conductance in snail neurones. Although desensitization of the rat extrajunctional receptors is not altered either by changing external calcium or by increasing internal calcium, it is still possible that decreasing internal calcium would decrease the rate of desensitization.

Previous studies on the effects of calcium on desensitization at the frog endplate have given very variable results. The rate of desensitization, measured by local microperfusion of carbachol in concentrations less than 0.27 mM, increased up to seven fold when the external calcium concentration was increased from 0 to 10 mM (Manthey, 1966; Cochrane & Parsons, 1972). Moreover, De Bassio *et al.* (1976) proposed that an internal binding site for calcium regulated the rate of desensitization. However, Manthey (1966) and Cochrane & Parsons (1972) found that altering external calcium concentration did not alter the desensitization rate if carbachol was applied in concentrations greater than 0.27 mM. Moreover, Katz & Thesleff (1957), reported that increasing calcium from 1.8 to 9.0 mM did not obviously alter the iontophoretically measured desensitization rate.

Calcium has been found to have little effect on desensitization at other synapses. Desensitization of ACh receptors of snail neurones is not affected by altering external Ca (Ziskind & Werman, 1975; Bregetowski, Vulflus & Verprintsev, 1975). At the eel electroplaque, desensitization rate determined in various external calcium concentrations between 0.1 and 10 mM, was found to be highest in 0.1 (Pallotta & Webb, 1977). Iontophoretic potentials of identical amplitude and time course evoked by ACh, acetylthiocholine, TMA and phenyl TMA were all found to desensitize at the same rate. This was surprising, since Magazanik & Vyskocil (1977) reported that acetylthiocholine desensitized frog ACh receptors at a lower rate than ACh, whereas Rang & Ritter (1970) have shown that phenyl TMA desensitized frog ACh receptors by a greater amount than ACh. In other previous studies, ACh, carbachol and succinylcholine (Katz & Thesleff, 1957) and ACh, butyrylcholine, suberyldicholine, decamethonium and TMA (Magazanik & Vyskocil, 1977) all induced desensitization at approximately the same rate.

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