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PROLONGED EXPOSURE TO ACETYLCHOLINE: NOISE ANALYSIS AND CHANNEL INACTIVATION IN CAT TENUISSIMUS MUSCLE

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SUMMARY

1. Micro-electrodes were used to record membrane potential and associated noise at the end-plate region of cat tenuissimus muscle (37 °C), during applications of acetylcholine (ACh) in continuously flowing Krebs solution containing eserine and tetrodotoxin.

2. Desensitization was assessed from the frequency of channel opening calculated from the noise variance.

3. At higher concentrations of ACh (10–50 μM), desensitization occurred with an exponential fall to a plateau.

4. At low concentrations of ACh (1–2 μM) only slight desensitization occurred and at a much lower rate. Frequency of channel opening decreased at the rate of $0{\cdot}045 \pm 0{\cdot}024$ min^{-1}. Maximum frequency was $(33 \pm 9) \times 10^7$/sec while maximum depolarization was $20{\cdot}5 \pm 1{\cdot}4$ mV ($n = 11$ cats). Depolarization was well maintained.

5. This slow rate of desensitization at low concentrations of ACh was confirmed in experiments where voltage clamped current, its associated noise, and miniature end-plate current amplitude were measured.

6. At low concentrations of ACh (1–2 μM) in the presence of eserine there was sustained block in neuromuscular transmission when twitch tension was measured.

8. It is concluded that the mechanism of neuromuscular block by ACh at around 1 μM concentration is by depolarization itself, not desensitization.

INTRODUCTION

It is well known (Bacq & Brown, 1937; Burns & Paton, 1951; Rosenblueth, Lindsley & Morison, 1936; Zaimis, 1956) that prolonged exposure to acetylcholine (ACh) produces a block in transmission at the skeletal neuromuscular junction.

Two alternate mechanisms have been suggested: either there is desensitization of the post-synaptic membrane to ACh (Thesleff, 1955*a*, *b*; 1959; Axelsson & Thesleff, 1958; e.g. see also Devore & Nastuk, 1977) or the depolarization itself produces the block (Burns & Paton, 1951; Zaimis, 1956; Zaimis & Head, 1976).

It is known that desensitization increases with the concentration of ACh (Fatt, 1950; Katz & Thesleff, 1957; Axelsson & Thesleff, 1958). However, much previous

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work was carried out using the ionophoretic technique, where the concentration is not known, or by bath application at high ACh concentrations (e.g. Nastuk & Gissen, 1965).

Therefore it is important to study the rate of desensitization at known steady concentrations of ACh, the concentration (and conditions) being chosen as similar to that producing neuromuscular block *in vivo* (around 1 μM). This has been carried out in this paper using the technique of noise analysis (Katz & Miledi, 1972; Anderson & Stevens, 1973; for a recent review see Wray, 1980). From the observed noise, the frequency of opening of post-synaptic channels can be calculated; and hence desensitization rate quantitatively measured. It is worth noting that this rate cannot be obtained quantitatively from (mean) depolarization alone because of large ion shifts (Jenkinson & Terrar, 1973) during long applications of acetylcholine. Cat muscle was used (tenuissimus) for this study because of its similarity (Paton & Zaimis, 1952) to human muscle in its response to depolarizing drugs.

In summary, the object of this work is to decide whether significant desensitization or 'channel inactivation' occurs (under conditions producing neuromuscular block) and hence decide between depolarization or desensitization as mechanisms producing the block.

Some of the results in this paper have already been communicated to the Physiological Society (Wray, 1978*a*).

METHODS

Preparation. The tenuissimus muscle was removed from cats anaesthetized with chloralose (80 mg/kg). It was transferred to a chamber (containing about 2 ml. solution) through which solution flowed at rates 9–26 ml./min (19 ml./min averaged over all experiments). The temperature was maintained in the range 36–39 °C by water jackets (one experiment at 33 °C). The muscle was mounted horizontally on a Sylgard base using fine pins to lightly stretch the muscle.

Solutions. The composition of the bathing Krebs solution was (mM) Na^+ 143·0; K^+ 5·9; Mg^{2+} 1·20; Ca^{2+} 2·52; Cl^- 127·7; HCO_3^- 25·0; $H_2PO_4^-$ 1·2; SO_4^{2-} 1·2; glucose 11·1. Solutions were kept well oxygenated with 95% O_2 + 5% CO_2 mixture. The pH was 7·1–7·4.

To prevent action potentials and subsequent contractions, tetrodotoxin 220–310 nM (Koch-Light) was present in perfusing solutions. The cholinesterase inhibitor eserine 3–6 μM (BDH Chemicals Ltd) was also present.

One reservoir for perfusion contained the above solutions, while a second reservoir contained, in addition, acetylcholine chloride (BDH Chemicals Ltd), added from fresh stock just before each acetylcholine exposure. By the use of an appropriate tap, the solution flow was maintained while changing from one reservoir to the other. The dead time before solution reached the bath from the tap was approximately 0·6 min. This dead time can be allowed for, but limitations on temporal resolution in these experiments arose from solution change in the bath. Drug concentration in the bath changed with a very roughly exponential time constant of 6 sec (range 4·6–13·3 sec), from the above flow rates and volume of solution in the chamber.

Electrical recording. Intracellular voltage recording and voltage clamping was carried out using micro-electrodes filled with 3M-KCl, with resistances in the range 5–15 MΩ.

Intracellular voltage was conventionally recorded by cathode follower (ME 1400 valves) connected to two oscilloscope amplifiers in parallel (Tektronix 5A22N and 5A26). One channel was low gain d.c. coupled for following depolarization, while the other was a.c. coupled at high gain for recording voltage noise (3 kHz low pass filter). The outputs from these amplifiers were to an FM tape recorder (Racal Store 7D) with tape speed 7½ in./sec, Butterworth filter band width 0–2·5 kHz. The low gain voltage was also recorded on a chart recorder (Unicam, SP22).

The voltage clamp circuit utilized the OF720 operational amplifier (Computing Techniques) to provide clamping current via a second micro-electrode and hence give negative feed-back in the conventional way (Takeuchi & Takeuchi, 1959). Gain was approximately 1000. An R-C low

pass filter (time constant 0·1 μsec) was included in the feed-back path to aid stability. Current through a 0·1 or 1 MΩ resistor in the feed-back path (near the micro-electrode) was recorded by a differential voltage follower. The clamp current and current noise were recorded as for unclamped voltage described above (oscilloscope amplifier filter 1 kHz low pass). The behaviour of the clamp was tested in each experiment by hyperpolarizing square pulses of 20 mV at the command input. Voltage was clamped to within 5–10%, 0·2–0·5 msec after the pulse.

Localization of end-plates. End-plates were localized by moving the micro-electrode until miniature end-plate potentials (m.e.p.p.s) were recorded with fast rise time (around 0·5 msec) in the absence of cholinesterase inhibitor. Then tetrodotoxin and eserine were added to the solutions for at least 10 min. The perfusing solution was later switched to one containing ACh + tetrodotoxin + eserine.

Noise analysis. Records were played back from tape at full or half speed and analysed by computer (PDP12 or CED Cambridge/Computer Automation). All records were digitized at 1 kHz sampling rate (unless stated otherwise) after passing through an active Butterworth low pass filter (−3db point 275, 390, 460 or 490 Hz, 48db/octave roll-off). Noise records were edited to remove miniatures and obvious artifacts.

Using 256 data points, firstly the noise variance was calculated, secondly the fast Fourier transform (after cosine tapering (Bendat & Piersol, 1971)) was performed to obtain the power spectrum. Around sixteen to fifty such blocks of data were used to obtain mean values for the variance (and its standard error) and power spectrum. Control values before the application of ACh contained at least 100 blocks, each of 256 points. An average value for the (low gain) membrane potential or current was obtained over the same interval of analysis.

Control values of noise variance, noise power spectra and (low gain) membrane potential or current were subtracted from those during the application of ACh. Log power spectra versus log frequency were plotted. Correction was made for the high pass filter (i.e. a.c. coupling). These log power spectra were fitted by sliding a perspex template in the shape of a Lorentzian curve. The variance was also calculated from the sum of the power spectrum points, after small peaks at 50 Hz (or multiples) were replaced by interpolated values. Changes in membrane potential in these experiments did not occur so rapidly as to provide appreciable errors in the calculation of the noise variance, recorded by a.c. coupling. Numerical estimates showed that errors from this source were small, and only noise records which appeared visually flat were analysed.

Interpretation of noise. As commonly assumed (Katz & Miledi, 1972; Anderson & Stevens, 1973), noise is produced by continual opening of channels, frequency n/sec. On average, each channel produces a voltage depolarization a which decays exponentially with time constant τ. Hence depolarization V and voltage noise variance $\overline{E}^2$ are given by $V = na\tau$ and $\overline{E}^2 = na^2\tau$. A distribution in sizes of a has been assumed (Katz & Miledi, 1972, 1975; Wray, 1980) (i.e. no fraction of $\frac{1}{2}$ in the last equation). The power density spectrum is given by $S(f) = S(0)/(1+(f/f_c)^2)$ where $S(f)$ is the power spectral density at frequency f, and f_c ('corner frequency') is related to the time constant by $\tau = 1/2\pi f_c$. The relation $\overline{E}^2 = S(0)/4\tau$ holds. For the large depolarizations reported here, corrections for non-linear summation (Martin, 1955) were made. This correction can be used when the $I-V$ relation is linear, as is the case in cat tenuissimus muscle (Boyd & Martin, 1959). Corrected values of depolarization ($\tilde{V}$) and noise variance ($\tilde{E}^2$) are given by (Katz & Miledi, 1972) $\tilde{V} = V(V_0/(V_0-V))$ and $\tilde{E}^2 = \overline{E}^2(V_0/(V_0-V))^4$ respectively where V_0 is the potential difference between resting potential and reversal potential (Fatt & Katz, 1951; del Castillo & Katz, 1954). Reversal potential is observed to be in the range 0 to −15 mV (Takeuchi & Takeuchi, 1959, 1960; Katz & Miledi, 1977). The corrected value of elementary depolarization is $\tilde{a} = \tilde{E}^2/\tilde{V}$. The number of channels opened/sec, n is given by $\tilde{E}^2/(\tilde{a}^2\tau)$. Thus a plot of the (corrected) noise variance is proportional to the frequency of opening of channels, with proportionality constant $1/\tilde{a}^2\tau$. For each exposure to ACh, a mean value of $\tilde{a}$ and τ were first obtained (see later), and hence the corrected noise variance could be 'calibrated' in terms of frequency of opening, n.

Effects of chloride ion shifts. Jenkinson & Terrar (1973) have shown that large shifts of chloride ion occur during long depolarizations, which gives large contributions to the membrane potentials. The 'elementary event' for passive diffusion probably consists of the transfer of single molecules through the permeable membrane. Hence the amplitude, a_D, of the elementary event is much smaller than the amplitude a for ACh channels, which pass around 10^4 ions per 'event'. For shot noise from passive diffusion, noise variance is given by $\overline{E}_D^2 = a_D V_D$, where V_D is the associated steady potential change. Since $V_D \sim V$ (V is depolarization reached) and $a_D \ll a$,

then $\bar{E}_D^2 \ll \bar{E}^2_{ACh}$. Thus the contribution to shot noise from passive diffusion is negligible. Another source of noise may perhaps be expected from diffusion: $1/f$ noise. This was neglected in subtracted spectra. Hence depolarization receives contributions from several sources, but noise variance only receives important contributions from the opening of ACh channels. Thus the frequency of opening of ACh channels can be followed throughout from $n = \bar{E}^2/(\bar{a}^2\tau)$, but not from $\hat{V}/(\bar{a}\tau)$ except under certain conditions.

Chloride ion distributes passively such that the chloride ion equilibrium potential E_{Cl} is normally equal to the resting potential, E. After application of ACh, these chloride shifts occur, moving E_{cl} towards the current membrane potential, with time constant of a few minutes. If the ACh is applied fast enough (e.g. ionophoretically) a clear initial depolarization would be seen determined by ACh channels alone. In practice, for bath application there are diffusion delays at the beginning which occur at the same time as chloride shifts, and this considerably complicates the time course of the depolarization. Therefore, at the beginning of the ACh application, the mean potential, V, receives contributions from chloride ion movements *and* from ACh channels.

When do contributions to V from chloride shifts become negligible? In the continued presence of ACh, suppose the frequency of channel opening reaches a constant value; eventually chloride ion will shift passively so that E_{cl} is equal to the membrane potential, i.e. the membrane potential reaches a constant value also. Then there is no further contribution to V from Cl ion shifts and V is given only by ACh channels. It is only after this point on the plateau that a can be calculated with its usual significance.

In summary, the usual a can be calculated in these bath application experiments when (i) n is constant and (ii) V is constant. In practice, in this paper, a was calculated when *both* n and V were only changing slowly, as an approximation to this.

Desensitization rate was obtained from the frequency of channel opening in one of two ways: (i) for higher concentrations, an exponential curve, e^{-t/τ_s}, of decay to a plateau value was fitted by least squares to the values for frequency (t time, $1/\tau_s$ desensitization rate) (ii) for lower concentrations, rate of decay of frequency versus time was very slow and could be fit well by a straight line soon after maximum was reached. The percentage rate of desensitization was calculated from $(n_{max}-n_1)\times 100/n_{max}\Delta t$ where n_{max} is the maximum frequency of channel opening, and n_1 is the value it fell to after a time interval Δt. A linear decay of frequency is approximately equal to an exponential decay if the time constant, τ_s, is large: $e^{-t/\tau_s} \sim 1-t/\tau_s$. Therefore the percentage rate of desensitization calculated from $(n_{max}-n_1)\times 100/n_{max}\Delta t$ is approximately equal to $100/\tau_s$, provided the final plateau value is assumed negligible. If plateau values are non-zero, this formula only provides an approximation (lower limit) to $1/\tau_s$.

Artifacts due to solution flow were sometimes present, but small in magnitude (usually contributing less than 50 μV^2 to the noise variance), and were assumed to be unimportant in subtracted spectra.

Drifts in continuous recording of steady voltages during impalements lasting many minutes were carefully checked at the end of the experiment when the micro-electrode was withdrawn from the muscle fibre. Drifts were usually less than 5 mV.

Twitch tension recording. In some experiments, twitch tension of cat tenuissimus muscle was measured *in vitro* while the nerve was stimulated. The tenuissimus muscle was mounted in the same bath as in the above experiments, and under similar conditions. One end of the muscle was tied and attached by thread to an isometric transducer, while the other end was tied and attached to a firm support. The nerve to the muscle was stimulated by platinum wire electrodes or by a suction electrode. Stimulus strength was adjusted to twice that for maximal twitch tension, while the muscle length was adjusted to that for maximal twitch tension.

Results for measured quantities are quoted where possible as mean ± S.E. of mean in this paper.

RESULTS

(i) *Effect of acetylcholine concentration on desensitization rate*

Before carrying out a detailed study at low concentration (next section), the effect of ACh concentration on desensitization rate was first studied as a preliminary.

Cat tenuissimus muscle was exposed to different, known, concentrations of ACh near 37 °C. Intracellular voltage and noise at the end-plate were observed continuously as described in Methods. Fig. 1 shows a typical record of these variables before and during application of ACh. The corrected noise variance (see Methods) together with the observed membrane potential was plotted versus time, and this can be seen in Fig. 2. The corrected noise variance is proportional to the frequency of channel opening, n (proportionality constant $1/\bar{a}^2\tau$). This fact has been used to calibrate the noise variance directly in terms of frequency of channel opening, and is also shown in Fig. 2.

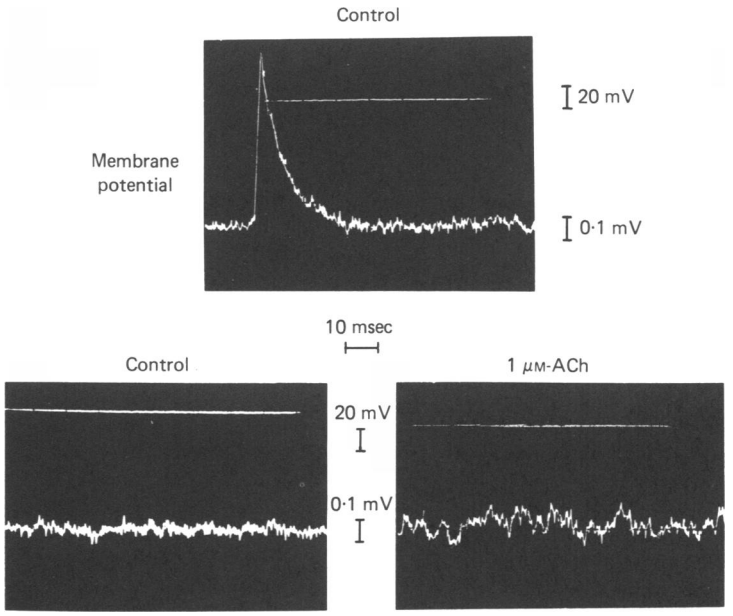

Fig. 1. This Figure shows intracellular recordings of membrane potential on low gain (top trace) and high gain (lower trace), at cat tenuissimus muscle end-plate, at 38 °C. Under control conditions (top and lower left of Figure), noise is contributed by the recording equipment. A miniature end-plate potential is seen. After ACh 1 μM, depolarization and an increase in noise is seen (lower right of Figure). Eserine (3 μM) and tetrodotoxin (250 nM) present throughout. Low pass filter: 3 kHz. Calibrations are marked on the Figure.

It can be seen from Fig. 2 that no marked fall in channel opening frequency can be detected for concentrations of ACh less than 2 μM. In a similar experiment at a different end-plate, no marked fall in channel opening frequency was found during application of ACh at concentrations of 0·1, 0·2, 0·3 and 1 μM. However, at 10 and 50 μM, a clear fall in channel opening frequency can be seen to an eventual plateau (Fig. 2). As shown in Fig. 3*A*, the channel opening frequency falls exponentially with time, t, (e^{-t/τ_s}) with time constant τ_s around 9 sec at an ACh concentration of 50 μM and τ_s around 26 sec at 10 μM. These results are very roughly consistent with desensitization rate ($1/\tau_s$) proportional to concentration.

Defining $1/\tau_e = f[C]$ where f is constant, and [C] is the concentration of ACh, we find an average value $f = 3{\cdot}1 \times 10^3$ M^{-1} sec^{-1}. However, no attempt was made to make detailed tests of this relationship (this was especially difficult at lower concentrations where desensitization was too slow to be measured).

It can also be seen from Fig. 2 that the depolarization does not reflect faithfully the frequency of opening of end-plate channels, presumably because of large shifts in chloride ion concentration (Jenkinson & Terrar, 1973). On the other hand, the noise

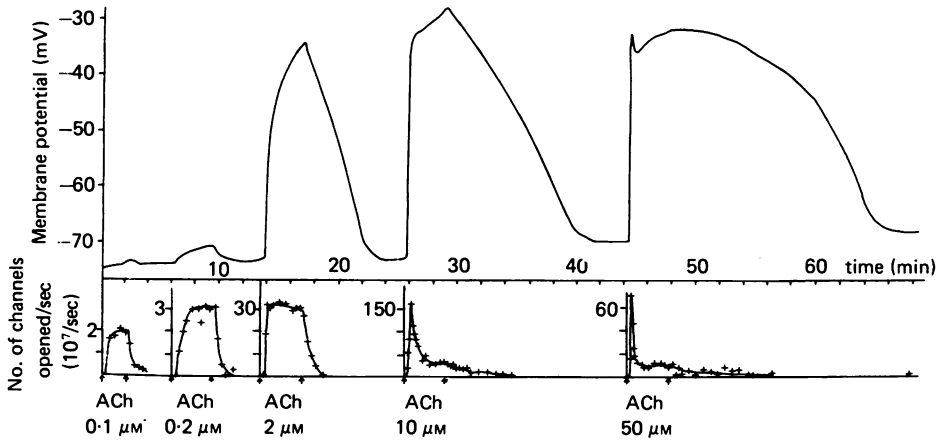

Fig. 2. In this experiment, different concentrations of ACh were applied (between arrows in Figure) to a cat tenuissimus muscle (35–37 °C) while continuous intracellular recording of membrane potential was made at an end-plate (3 μM-eserine present). The tracings show: (upper) membrane potential and (lower) voltage noise variance, after correction to the resting potential (the reversal potential was taken as 0 mV). Most of the values of noise variance were calculated from intercepts of the noise power spectra. The value of a was calculated after 1·5 min exposure to ACh. The average (between 2nd, 3rd and 4th exposures) for $\tilde{a}$ was 0·063 μV (corrected to the resting potential). Mean values of τ were calculated from power spectra for each exposure, yielding τ = 2·4 msec. These values of $\tilde{a}$ and τ were used to convert the noise variance into frequency of channel opening which is marked on the Figure. Continuous curves have been drawn through the noise variance points. Low pass filter: −3 db point 390 Hz. A separate control variance just before each exposure was calculated.

(control subtracted) probably receives negligible contributions from ion shifts (see also Methods), and hence reflects accurately the frequency of opening of channels at the post-synaptic membrane. Dose–response curves for the maximum frequency of opening of channels are shown in Fig. 3*B* for two end-plates. Slopes of the log–log plots for low concentrations ($\leqslant 10$ μM) are 1·0 and 1·4. Detailed quantitative values of the slope were not studied here. For higher concentrations, the values for maximum channel opening frequency are probably higher than those marked in Fig. 3*B* because of desensitization not resolvable by this technique of bath application.

The experiments at two end-plates reported in this section were intended primarily to set the scene for the experiments in the following section.

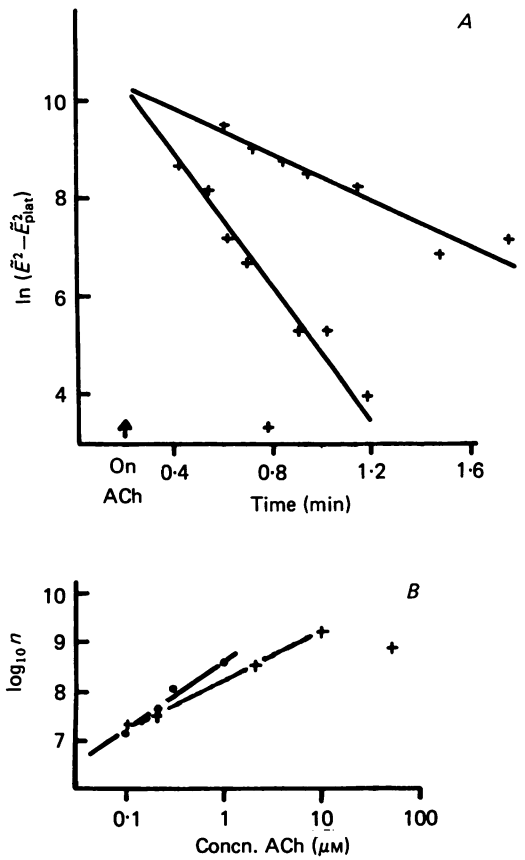

Fig. 3. *A*, upper Figure shows a semilogarithmic plot of the difference between the (corrected) noise variance (in μV^2) and its plateau value versus time. Data are from the end-plate shown in Fig. 2. The upper curve is at 10 μM-ACh (plateau value taken as 2700 μV^2) while the lower curve is for 50 μM-ACh (plateau value 930 μV^2). The lines have been drawn by eye. Because of the plateau value subtraction, errors increase markedly at the lower values, and this would invalidate a simple least-squares fit which ignored this fact. We find τ_s = 25·8 sec at 10 μM and 8·6 sec at 50 μM; assuming an exponential decay of variance e^{-t/τ_s} (t time, $1/\tau_s$ decay constant). *B*, lower Figure shows a plot, on log–log co-ordinates, of the maximum frequency of channel opening, n, (sec^{-1}) *vs.* concentration of ACh. The points marked + are from the data of Fig. 2 while the points marked ● are from similar experiments on a different end-plate. Least-squares fits of the points up to and including 10 μM concentration are shown in the Figure, and have slopes of 1·0 and 1·4.

(ii) *Desensitization rate at low concentrations of ACh*

To measure desensitization rate at concentrations of 1–2 μM ACh, longer applications of ACh (15 min or more) were necessary.

Again intracellular voltage and noise were recorded, and Fig. 4 shows the results of a typical experiment. Depolarization is quite well maintained, while the corrected

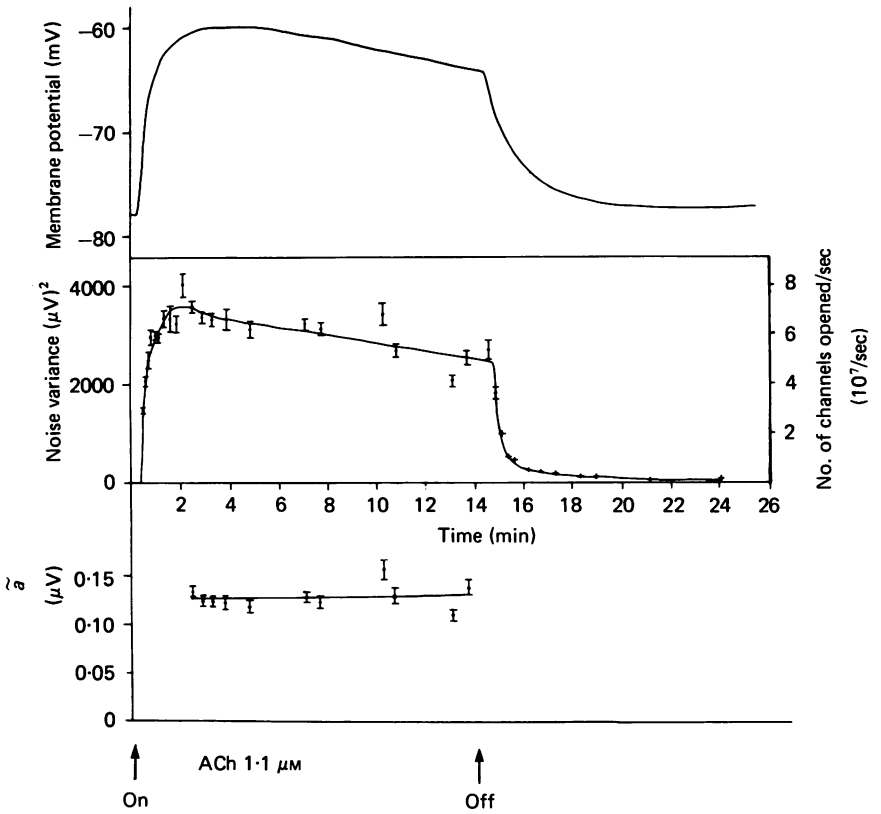

Fig. 4. The Figure shows continuous recording of membrane potential (upper part of Figure) and voltage noise variance (computed directly), corrected to resting membrane potential (middle part of Figure) for cat tenuissimus muscle end-plate (38 °C, resting potential −78 mV). Eserine (3 μM) and tetrodotoxin (250 nM) were present throughout. In the correction to resting potential the reversal potential was taken as −15 mV. Each point on the lower curve shows mean and standard error of sixteen to fifty blocks of data, each 256 msec in length (see Methods). The value of $\tilde{a}$, obtained from the ratio of depolarization to variance (corrected to the resting potential) is plotted in the lower part of the Figure after the first 2 min. It can be seen that $\tilde{a}$ is quite constant with time (a least-squares linear fit is shown) with mean value $\tilde{a} = 0{\cdot}127 \pm 0{\cdot}004$ μV (mean and S.E. of eleven data points). The mean value of τ from the power spectra was $\tau = 3{\cdot}1 \pm 0{\cdot}1$ msec (mean and S.E. of twenty spectra). Using these values of $\tilde{a}$ and τ, the noise variance curve has been calibrated in terms of frequency of opening of channels, n. A least-squares linear fit is shown to these points between time 3·8 and 13·8 min with slope $-0{\cdot}19 \times 10^7$ channels/sec.min, or a decrease of 2·8% of max per minute (i.e. $1/\tau_s = 0{\cdot}028$ min^{-1}). Control variance was 278 μV², while uncorrected subtracted variance during application of ACh ranged from 1120 to 725 μV². Records uncorrected for low pass filter (275 Hz at −3 db point).

noise variance falls off very slowly, at the rate of 2·5–3·0 %/min (i.e. $1/\tau_s$ = 0·025–0·030 min^{-1}, see Methods), an extremely low rate.

Fig. 4 also shows the elementary unit blip $\tilde{a}$ calculated by dividing each variance value by the corresponding depolarization (both corrected to resting potential, see Methods). It can be seen that $\tilde{a}$ is constant with time as expected, with mean value $\tilde{a} = 0{\cdot}127 \pm 0{\cdot}004\ \mu V$ in this experiment. Note that values of $\tilde{a}$ have not been com-

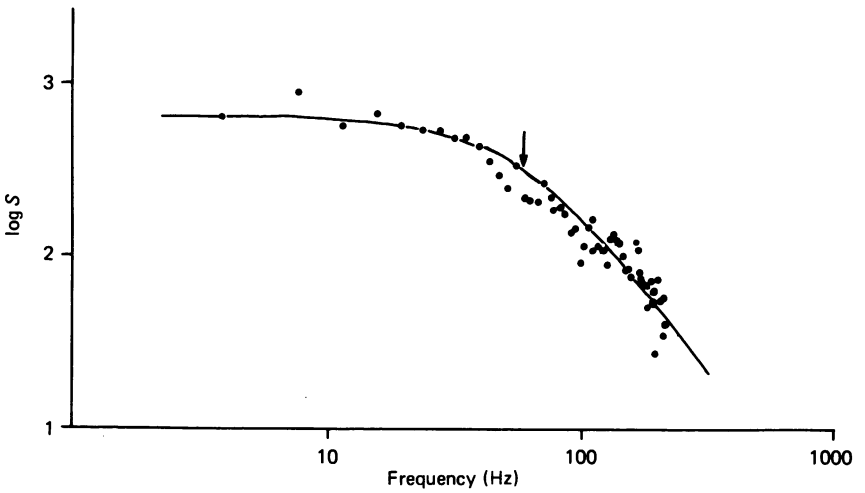

Fig. 5. This Figure shows a power spectrum obtained during the experiment described in the Fig. 4 caption. Noise variance power density (control subtracted) is plotted against frequency on log–log axes. A Lorentzian curve is shown fitted by eye to the experimental points. This spectrum gave a time constant τ = 2·8 msec and was obtained by averaging fifty-four records each 256 msec in duration (1 kHz sampling rate). The average of twenty such spectra during the experiment gave $\tau = 3{\cdot}1 \pm 0{\cdot}1$ msec. The power spectral density S has been plotted in relative units, while the absolute value can be obtained by multiplying by 0·0128 μV^2 sec. (Low pass filter 275 Hz at −3 db point.)

puted when depolarization is changing at the beginning. This is because large chloride ion shifts occur during this initial period (complete in a few minutes), contributing to the depolarization but not the noise, rendering the formula for $\tilde{a}$ invalid (see Methods).

Fig. 5 shows the power spectrum of the noise during the experiment shown in Fig. 4, and this was used to obtain the time constant τ of the elementary voltage blip (mean value in this experiment $\tau = 3{\cdot}1 \pm 0{\cdot}1$ msec, mean of 20 spectra). This time constant, τ, should reflect passive decay of voltage, and hence should be the same as the membrane time constant, τ_m. This in turn should be the same as the time constant of decay of miniature end-plate potentials, m.e.p.p.s, if ACh molecules disappear rapidly from the cleft in the absence of acetycholinesterase inhibition (Anderson & Stevens (1973)). To check this identity of m.e.p.p. and noise time constants, m.e.p.p.s were recorded (before eserine) in the same fibre and the decay phase fit to e^{-t/τ_m}. To the extent that the passive decay of the m.e.p.p. can be

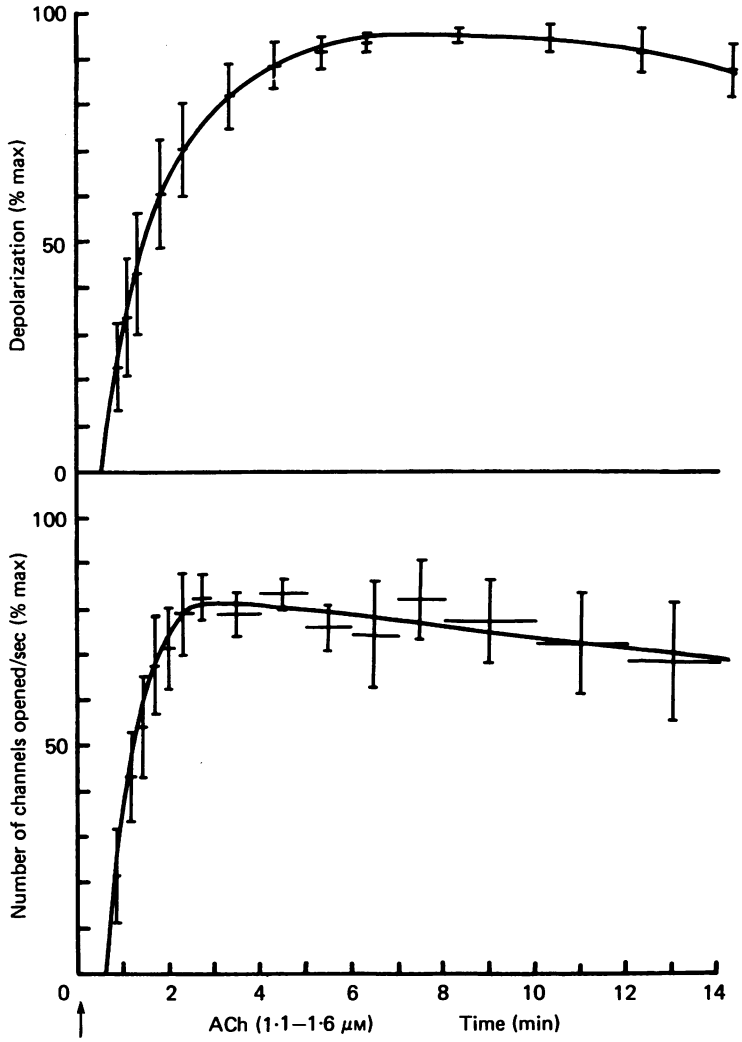

Fig. 6. The Figure shows mean and standard errors, for five cat muscles, of depolarization, V, and frequency of channel opening, n (both variables plotted as % of maximum reached in each experiment), during an application of ACh (1·1–1·6 μM) for 14 min (37–39 °C) in the presence of eserine 3–6 μM and tetrodotoxin 250 nM. These average curves do not pass through 100% because maxima for these quantities are not reached at the same time in different experiments. A continuous curve was drawn by eye through the points for depolarization and the beginning of the curve of channel opening frequency, while the latter curve was fit (by least squares) from time 3 min onwards to a straight line, with slope of 1·1%/min (i.e. $1/\tau_s = 0{\cdot}011\ \text{min}^{-1}$). Other details of these experiments are listed in the first five lines of Table 1.

approximated as a result of instantaneous displacement of charge, the time course of passive decay is proportional to $(1/\sqrt{t}) \exp(-x^2\tau_m/4\lambda^2 t) \exp(-t/\tau_m)$ (Fatt & Katz, 1951). This approximates to e^{-t/τ_m} for larger t and small x. Here t is time, τ_m is the membrane time constant, x is the distance between site of recording and site of action of ACh, and λ is the membrane space constant.

In this experiment, τ_m was $2{\cdot}9 \pm 0{\cdot}1$ msec ($n = 5$ m.e.p.p.s) and thus in agreement with the decay constant τ from noise.

Knowing the constants, $\tilde{a}$, τ, the noise variance can be converted into the frequency of channel opening, n ($n = \bar{E}^2/\tilde{a}^2\tau$) and this is also shown in Fig. 4.

Fig. 6 shows average values of depolarization and frequency of channel opening for tenuissimus muscles (37–39 °C) from five cats during application of ACh 1·1–1·6 μM. Depolarization is well maintained, and the frequency of channel opening falls only slightly during 14 min, at the rate of about 1·1 %/min (i.e. $1/\tau_s = 0{\cdot}011$ min^{-1}).

Even after a longer ACh (1·1 μM) exposure of 26 min for two cat muscles, depolarization fell by 2 mV in one experiment and 5 mV in the other (maximum depolarization 24 mV for both), while frequency of channel opening fell by 5 and 45 % of its maximum value in the respective experiments. In another acetylcholine (1·4 μM) exposure of 21 min (a different cat muscle), depolarization fell by 7·4 mV (max. depolarization 22 mV), while frequency of channel opening fell by 51 % of its maximum value.

Fig. 6 also shows (as does Fig. 4) that, following ACh application, frequency of channel opening changes faster than depolarization, again because of chloride shifts, affecting only the depolarization. This rapid change (in about a minute) in channel opening frequency is similar to the rapid change in clamp current (Jenkinson & Terrar, 1973).

Table 1 is a compilation of results from the eleven cat muscles studied in this section (33–39 °C) at mean initial resting potential of $76{\cdot}0 \pm 2{\cdot}0$ mV. For concentrations in the range 1·1–2·2 μM, the mean rate of desensitization, obtained from frequency of channel opening, was $4{\cdot}5 \pm 2{\cdot}4$%/min or $1/\tau_s = 0{\cdot}045 \pm 0{\cdot}024$ min^{-1} ($n = 11$ muscles). Indeed, after 13·5 min exposure to ACh, the frequency of channel opening only fell by 21 ± 13 % of its maximum value, while depolarization fell by $1{\cdot}9 \pm 1{\cdot}2$ mV ($n = 6$ muscles). For two experiments, there was a slight rise in channel opening frequency, and the change was expressed as a percentage of the initial 'plateau' value near the beginning. The range was a fall of 76 % to a rise of 12 %. For depolarization, the range was a fall of 5·4 mV to a rise of 2·5 mV. Depolarization reached its maximum, or 'plateau' value, after $5{\cdot}3 \pm 0{\cdot}3$ min of exposure to ACh, while for noise variance this was $2{\cdot}9 \pm 0{\cdot}5$ min ($n = 11$ muscles). The mean maximum depolarization recorded was $20{\cdot}5 \pm 1{\cdot}4$ mV corresponding to a mean membrane potential of $55{\cdot}5 \pm 2{\cdot}9$ mV ($n = 11$ muscles), while mean maximum frequency of channel opening was $(39 \pm 11) \times 10^7$/sec ($n = 11$ muscles). Noise spectra could usually be fitted by a Lorentzian curve. As expected (Katz & Miledi, 1972), no statistical difference was found between time constants τ deduced from noise spectra (eserine present) or from m.e.p.p.s before eserine (paired Students' t test, $n = 10$ muscles, 5 % confidence level). The mean values were τ (noise) $= 1{\cdot}9 \pm 0{\cdot}2$ msec and τ_m (m.e.p.p.) $= 2{\cdot}2 \pm 0{\cdot}1$ msec. After eserine, the time constant of exponential decay

TABLE 1. The Table shows the results of measurements on eleven cat muscles, during exposure to ACh in concentration 1·1–2·2 μM at 33–39 °C, in the presence of eserine 3–6 μM and tetrodotoxin 250 nM. Noise variance was computed directly or by adding all power spectrum values (see Methods). The first column, V_{max}, is the maximum depolarization reached. A continuous curve was drawn through the plotted points of frequency of channel opening, n, and hence the maximum frequency, n_{max}, obtained (second column). After an interval of time Δt during exposure to acetylcholine, the frequency usually fell continuously to a new value n_1. The third column shows $(n_{max}-n_1)/n_{max}\,\Delta t$, which represents the desensitization rate, $1/\tau_s$ (see Methods): this rate of desensitization was approximately linear, except in one experiment (a) where $1/\tau_s$ was obtained from a fit to an exponential curve (a plateau value at 18% of max. was reached). The quantity in column 4 is the total time of exposure to acetylcholine. Column 5 shows the value of a corrected (with reversal potential assumed to be −15 mV) to the resting potential of the fibre (65–89 mV), while column 7 shows the time constant τ, from the power spectra. The mean and standard error of n measurements during a single application are shown in columns 5 to 8. Column 9 shows the 'exponential' decay constant of m.e.p.p.s (mean and S.E. of 5 m.e.p.p.s) before eserine. Control noise before ACh was in the range 110–660 μV^2 (one exceptional experiment 1820 μV^2), and the signal/noise ratio during application of ACh was in the range 0·4–4·8 for uncorrected variances. Values are not corrected for the low pass filter (275 or 460 Hz at −3 db). (b) Here τ_{mepp} was used to calculate n

V_{max} (mV)	n_{max} 10^7/sec	Desensitization rate (min^{-1})	Duration of ACh (min)	$\tilde{a}$ (μV)	(n)	τ (msec)	(n)	τ_{mepp} (msec)
18·5	7·15	0·026	14	0·127 ± 0·004	(10)	3·12 ± 0·12	(23)	2·85 ± 0·12
13·5	28·5	−0·0136	20	0·035 ± 0·001	(8)	1·75 ± 0·11	(18)	2·07 ± 0·13
24·2	50·5	0·0216	27	0·048 ± 0·001	(8)	1·68 ± 0·05	(20)	2·1 ± 0·3
24·2	44·8	−0·033	24	0·035 ± 0·001	(17)	1·85 ± 0·07	(35)	2·3 ± 0·1
24·0	128·0	0·263(a)	15·5	0·044 ± 0·002	(10)	1·23 ± 0·03	(18)	1·89 ± 0·34
22·0	21·3	0·0422	20	0·064 ± 0·001	(14)	2·40 ± 0·10	(19)	2·40 ± 0·08
27·3	9·85	0·0156	5	0·226 ± 0·012	(4)	2·47 ± 0·13	(9)	1·9 ± 0·3
18·3	17·1	0·0212	7	0·078 ± 0·003	(6)	1·43 ± 0·03	(17)	2·15 ± 0·19
14·4	25·0	0·106	4	0·070	(2)	1·46	(2)	1·89 ± 0·25
23·3	15·1(b)	0	8·5	0·093 ± 0·004	(8)			2·68 ± 0·18
16·3	80	0·0131	5·5	0·131 ± 0·004	(7)	2·02 ± 0·09	(10)	
Mean and S.E.								
20·5 ± 1·4	39 ± 11	0·045 ± 0·024		0·086 ± 0·017		1·94 ± 0·18		2·22 ± 0·11

of m.e.p.p.s increased to $4{\cdot}1 \pm 0{\cdot}2$ msec ($n = 10$ cat muscles); indicating that ACh molecules now act several times. The mean value of elementary depolarization (corrected to the resting potential of each experiment) was $\tilde{a} = 0{\cdot}086 \pm 0{\cdot}017\ \mu V$ ($n = 11$ muscles).

As in Colquhoun, Large & Rang (1977) the ratio of observed variance/'true' variance $= (2/\pi) \tan^{-1} (f_1/f_c)$ where f_1 is the filter cut off frequency, and f_c is the corner frequency of the power spectrum. Variances were corrected for the low pass filters used, leading to $\tilde{a} = 0{\cdot}099 \pm 0{\cdot}019\ \mu V$ and $n_{max} = (33 \pm 9) \times 10^7$/sec (eleven muscles), while desensitization rate remains as above. As a further check, variances were calculated from the intercepts of the power spectra using $\bar{E}^2 = S(0)/4\tau$, and this gave no significant difference from the latter value of $\tilde{a}$ (paired Students' t test, 5% confidence level).

Finally, in those muscles which were returned to Krebs solution and allowed to recover, resting potential returned eventually to near its initial value (the mean change in resting potential was $1{\cdot}3 \pm 1{\cdot}8$ mV, $n = 6$ cat muscles), and noise variance returned close to its control level.

(iii) *Low concentrations of ACh at voltage clamped fibres*

In these experiments, voltage clamped current and its noise were recorded during long applications of ACh. Fig. 7 shows the results of an exposure to $1{\cdot}1\ \mu$M-ACh (38 °C). Clamp current falls off only slightly with time, at about 1·5% of maximum per minute (upper trace), as does the associated current noise (centre trace). The amplitude of miniature end-plate currents (m.e.p.c.s) were also measured, and these are also plotted in Fig. 7 (lower trace). No desensitization at all was seen in the m.e.p.c. amplitude, at least after the first 2 min of exposure.

In a further experiment on a different cat muscle (clamp potential = resting potential = −73 mV, 36 °C), a 'conditioning' dose of ACh 3 μM was first applied for 5 min, then the muscle was allowed to recover in normal Krebs solution for 8 min, and a further dose of ACh 3 μM then applied for 11 min. It was found that both clamp current and current noise variance reached the same maximum values (within about 5%) in the two exposures. Between the exposures, full recovery took place. During the second exposure, the current noise variance fell off approximately linearly from a maximum of $0{\cdot}0184\ nA^2$ near the beginning to $0{\cdot}0152\ nA^2$ after 11 min, i.e. a desensitization rate of around 1·6%/min (i.e. $1/\tau_s = 0{\cdot}016\ min^{-1}$). The m.e.p.c. amplitude fell off from an initial value of 5·9 nA to 4·2 nA after 11 min, i.e. at around 2·6%/min ($1/\tau_s = 0{\cdot}026\ min^{-1}$).

In these experiments, the power spectra could not be used to obtain channel lifetime because the control current became too noisy above about 300 Hz (the subtracted spectra were flat to this point). From the ratio of current noise variance to mean current, the elementary current blip, a_c, was found to be $a_c = 1{\cdot}3$ and 0·9 pA in the respective experiments, leading to values of single channel conductance, $\gamma = 21$ and 15 pS respectively (assuming a reversal potential of −15 mV). These can only be approximate values however, and no correction for the low pass filters was attempted (−3db point 980 and 390 Hz respectively).

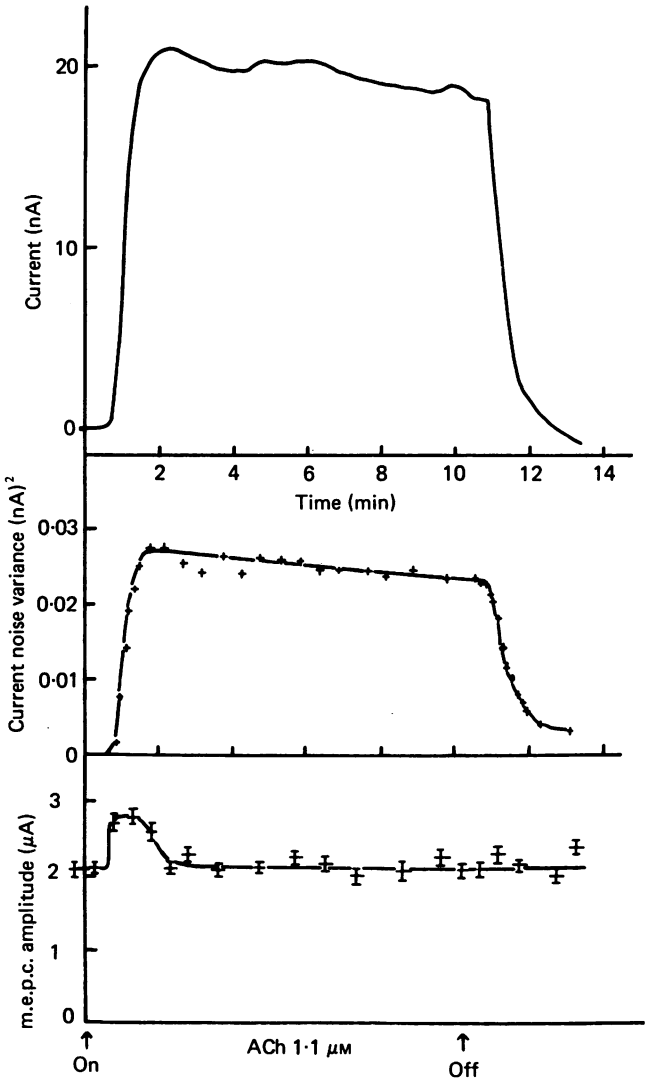

Fig. 7. The Figure shows the results of an exposure to 1·1 μM-ACh (3 μM-eserine and 300 nM-tetrodotoxin present) at 38 °C. The fibre was clamped at its resting potential, 77 mV (input resistance 0·68 MΩ), and current together with its associated noise are plotted *vs.* time in the upper and middle parts of the Figure. Maximum conductance was 0·33 μS. Records were played back from the tape recorder at half speed and digitised at 1 kHz giving an effective 2 kHz sampling rate (the effective low pass filter was up to 980 Hz, −3 db point). Control noise of 0·008 nA^2 was subtracted. The amplitudes of groups of twenty-six to fifty m.e.p.c.s were measured and the means and S.E.s of these plotted in the lower part of the Figure. A straight line was fit by least squares to the current noise variance for time between 2 and 11 min, with slope of $3{\cdot}9 \times 10^{-4}$ nA^2/min or about 1·5% of max per minute ($1/\tau_s$ = 0·015 min^{-1}). A straight line was also fit by least squares to the m.e.p.c. amplitude trace between 2·5 and 11 min and this was found to be essentially flat.

The more rapid rise time of the clamp current (after perfusion of ACh) seen in these experiments occurs because of the absence of chloride ion shifts, confirming the results of Jenkinson & Terrar (1973). The effect occurs even though chloride ion is being injected from the current passing micro-electrode, suggesting that associated build up of chloride ions inside the fibre is small compared with the purely passive chloride shift occurring in unclamped fibres.

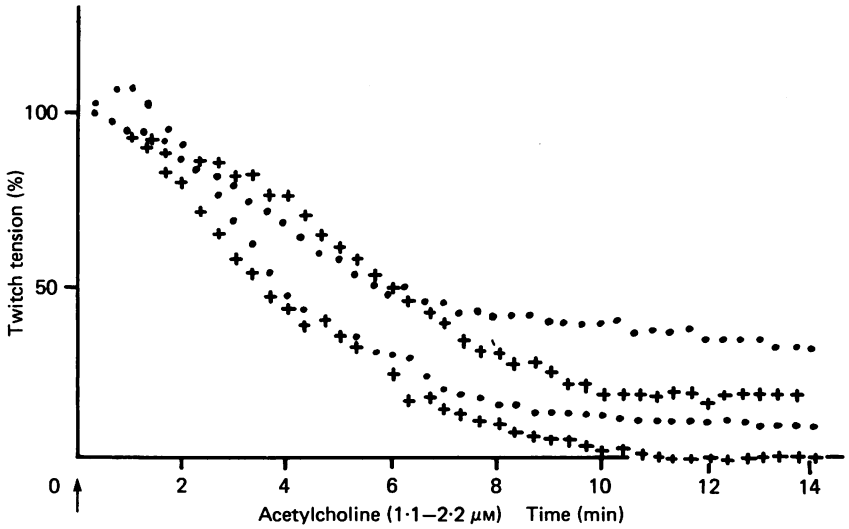

Fig. 8. This Figure shows the results of *in vitro* measurements of twitch tension of cat tenuissimus muscle, while the nerve was stimulated every 10 sec during application of ACh at 37 °C (3 μM-eserine present). The points marked ○ are from experiments with 1·1 μM-ACh, while the points marked + are for 2·2 μM-ACh. Three cat muscles were used (the points on the top and bottom curves are from different applications on the same muscle). The nerve was stimulated (50–200 μsec pulse) at a voltage 2 × that for maximal twitch.

(iv) *Neuromuscular block at low concentrations of ACh*

Block in neuromuscular transmission for cat tenuissimus muscle was studied, during application of ACh at concentrations 1–2 μM. The *in vitro* conditions of these experiments were similar to those of the intracellular recording experiments, except twitch tension was recorded while the nerve was stimulated every 10 sec.

Fig. 8 shows the extent of neuromuscular block produced during 14 min exposures to ACh (eserine 3 μM present). It can be seen that there is 68–100 % reduction in twitch tension due to block in neuromuscular transmission at this concentration range (mean and S.E. 85 ± 7 %, n = 4 experiments). Neuromuscular block is maximum at about 10 min of exposure, after which the level of neuromuscular block is quite constant.

After washout of ACh from the bath, twitch tension recovered to 85 ± 8 % (mean and S.E. of four experiments) of initial twitch tension (with eserine present throughout).

The twitch tension was potentiated by eserine ($1{\cdot}5 \pm 0{\cdot}1$ times, $n = 3$ muscles) and this potentiated value was taken as the initial (100 %) twitch tension in the above. In terms of unpotentiated initial twitch tension, there is 78 % reduction in twitch tension due to neuromuscular block.

Finally, to investigate whether action potentials could be triggered during ACh depolarization, voltage was recorded intracellularly while using a second microelectrode inserted into the fibre to pass square current pulses (duration 5–10 msec). Before the action of ACh, depolarizing current pulses triggered action potentials (at a threshold of very roughly -50 mV membrane poteutial). During the action of ACh (membrane potential -40 to -53 mV, 2 μM, four cat muscles, 37 °C) no action potentials could be triggered at 10 out of 11 end-plates by depolarizing current pulses. The size of the current pulse was adjusted to test sudden depolarizations from a few millivolts to 10–30 mV.

DISCUSSION

The main finding of this work is that desensitization proceeds very slowly in the presence of concentrations of ACh near 1 μM at 33–39 °C, while the depolarization reached is about 20 mV. Detailed studies from voltage noise variance, voltage clamped current and its associated noise, and amplitude of m.e.p.c.s all consistently show desensitization rate of around 4 %/min (i.e. rate constant $1/\tau_s = 0{\cdot}04$ min^{-1}), – a very slow rate.

Desensitization may consist of slow and fast components (e.g. Adams, 1975). The time course of the fast component, if present, cannot be resolved by the technique used here of bath application. Such a fast component may be present, but it cannot be large at low ACh concentrations, since m.e.p.c. amplitudes were not very much reduced.

During the application of ACh (1–2 μM) depolarization is well maintained, even for times longer than 15 min. This concentration of ACh produces sustained neuromuscular block (about 80 % of fibres blocked), and action potentials could not usually be triggered during ACh depolarization. If the situation is similar to squid axon, a steady depolarization from resting potential will inactivate many Na channels responsible for the upstroke of the action potential (Hodgkin & Huxley, 1952), favouring neuromuscular block. In squid axon, a steady depolarization of 10 mV (from around -50 mV) reduces the sodium current associated with a sudden depolarization of 45 mV by about 60 %. For frog muscle, action potentials are completely abolished during sustained membrane potentials more positive than around -55 mV Jenerick & Gerard, 1953), a value similar to that reached in the present experiments.

We therefore conclude from the evidence summarized here that desensitization is not the mechanism producing neuromuscular block at low ACh concentrations, and infer the mechanism is through depolarization itself.

Noise analysis is a useful quantitative tool for these studies on desensitization because the changes found relate directly to changes in frequency of channel opening at the post-synaptic membrane. On the other hand, depolarization is not always a good measure of these changes because of associated chloride ion fluxes (Jenkinson & Terrar, 1973), or because of possible electrogenic components of the Na pump. So,

for instance, at higher concentrations of ACh (10–50 μM), desensitization can be readily measured by studying noise variance, but not from the depolarization.

Desensitization occurs exponentially, as has been found by other authors (Rang & Ritter, 1970; Adams, 1975). At low concentrations, desensitization appeared linear, but this is consistent with an exponential fit of relatively large time constant (see Methods).

In one experiment at 50 μM-ACh, the time constant of desensitization was 9 sec, a magnitude which begins to approach that found by ionophoretic application (Axelsson & Thesleff, 1958). This suggests higher concentrations of ACh were present at the receptors during the latter experiments. The desensitization rate was roughly proportional to the concentration of ACh in two exposures at 10 and 50 μM-ACh.

As is well known (Katz & Thesleff, 1957; Jenkinson, 1960), dose–response curves start with a region of increasing slope. Chloride ion shifts complicate the dose response curve if depolarization is the measured variable (Jenkinson & Terrar, 1973), but this problem does not occur if frequency of channel opening (from the noise variance) is used in these plots. For two end-plates in the present experiments, the log dose–log frequency curves had slopes of 1·0 and 1·4, rather similar to the value (1·5) obtained for carbachol in chloride-free solutions (Jenkinson & Terrar, 1973; see also the review by Rang, 1975). This suggests that some channels may be open when only one agonist molecule is bound to the receptor, but there is a much greater probability of the channel being open when two (or more) molecules are bound.

The time the channel remained open for was short at this temperature, 37 °C, ($< 0{\cdot}5$ msec), in fact too short to measure easily. The channel conductance and size of elementary voltage blip were similar to values found for other species (for a review see Wray, 1980). Thus, for a resting potential of -75 mV, in end-plates from many different preparations, single channel current, i_c, is around 1·5 pA. The size of the corresponding voltage blip is given (Colquhoun, 1975) approximately by $\tilde{a} = \tilde{a}_\infty \tau_c/(\tau_c + \tau_m)$ where $\tilde{a}_\infty$ is the depolarization eventually attained if the channel is held open, τ_c is the channel lifetime and τ_m is the membrane time constant (about 2 msec in these experiments). Taking $\tilde{a}_\infty = i_c R$ where R is the input resistance (around 0·5 MΩ (Boyd & Martin, 1959)), and knowing $\tilde{a}$ (0·099 μV in these experiments), a value of channel lifetime $\tau_c = 0{\cdot}3$ msec is obtained (37 °C).

As a check on methods, membrane time constants, τ_m, were measured. As expected (Katz & Miledi, 1972), similar values were obtained from m.e.p.p. decay rate (before eserine) and from voltage noise power spectra in the presence of eserine. Values of τ_m around 1·9–2·2 msec were found, in agreement with previous measurements from m.e.p.p. decay rate (Boyd & Martin, 1956). After eserine, m.e.p.p.s decayed with a 2× slower time course (cf. Boyd & Martin, 1956), as expected for ACh molecules acting several times (Katz & Miledi, 1973).

The correction of the voltage noise variance to resting potential (Katz & Miledi, 1972) is dependent on the value of reversal potential, which was not measured in these experiments. It has also been assumed that the reversal potential is unchanged during the course of desensitization, and this has been shown to be the case by Katz & Miledi, 1977 and Lambert, Spannbauer & Parsons, 1977. Shifts of Na and K ions would also change the reversal potential, but this difficulty was not investigated.

In this work, the formulae for n, a and τ (see Methods) were assumed to hold throughout. Almost all of the experiments reported here were at low ACh concentrations (1–2 μM), where no deviation from these formulae is expected. In just two exposures at higher concentrations (10 and 50 μM-ACh), frequencies of opening of up to $1{\cdot}6 \times 10^9$/sec were seen. This is an order of magnitude *lower* than the maximum frequency possible and hence no departures from the formulae for n, a and τ are to be expected. Possibly this frequency was rather low because desensitization was occurring while ACh concentration was building up. In any case, results from these latter exposures were not used to assess slopes of dose–response curves.

Channel opening frequency is given by $n = N/(\tau_c + \tau_0)$ where N is the number of channels at the end-plate ($0{\cdot}8 \times 10^7$, Colquhoun, Dreyer & Sheridan, 1979), τ_c is the channel open time (0·3 msec) and τ_0 is the reciprocal of the opening rate constant. Maximum value of frequency, n, is reached for higher ACh concentrations where $\tau_0 \ll \tau_c$ and $n \sim N/\tau_c \sim 3 \times 10^{10}$/sec.

The experiments reported in section (i) (see Results) consisted of several ACh applications during continuous recording for around 1 hr. Only short ACh exposures were technically feasible ($\sim$3 min), but this did not allow the depolarization to fully reach its plateau values. Thus a could only be estimated approximately here (see Methods). This in turn only allows n to be determined approximately. However, the *rate* of desensitization, and the slope of the log dose–log response curves for the same fibre are not affected.

The elementary blip of depolarization is expected to decrease in magnitude as the fibre is depolarized (always after correction to the resting potential) because channel lifetime decreases with depolarization, at least in other species (Anderson & Stevens, 1973; Colquhoun, Large & Rang, 1977). Since channel lifetime was not measured as a function of membrane potential here, no attempt was made to allow for this.

It is well known that desensitization proceeds at a slower rate at depolarized membrane potentials (Magazanik & Vyskocil, 1970), and it may be thought that this is a factor contributing to the low desensitization rates observed here. However, this cannot be an important factor in the present experiments because similar desensitization rates occur in voltage clamped fibres near the usual resting potential.

A limitation of the method is the relatively long time required for concentration of ACh to build up at end-plates following solution change. At the flow rates used in these experiments, the bath solution was changed with a time constant of around 6 sec (see Methods). Therefore the time constant of desensitization τ_s could only be measured accurately when $\tau_s \gg 6$ sec. This was the case in general in these experiments except for one exposure at 50 μM-ACh. For the latter experiment, solutions changed with a time constant of 5 sec, while desensitization time constant τ_s was estimated to be 9 sec. Therefore the latter measurement is only approximate. At lower ACh concentrations, τ_s increases so bath change time constants are no longer important. However, diffusion of ACh to the receptors themselves probably also limits the time for drug change.

It is worth mentioning that this technique of using noise variance to compute channel opening frequency can be used when voltage clamping is not possible (as is usually the case for large concentrations and currents), and yet provides similar information on the time course of desensitization (unaffected by chloride ion shifts).

In some experiments (Adams, 1975), α-bungarotoxin pretreatment was used to reduce the currents which flow. However, Magazanik & Vyskocil (1973) have presented evidence which may indicate that α-bungarotoxin pretreatment itself may accelerate desensitization.

In conclusion, it is found that, at 37 °C, desensitization (or channel inactivation) is extremely slow at concentrations of ACh around 1 μM, while accompanied by large depolarizations and neuromuscular block. Hence the mechanism of neuromuscular block in the cat at this concentration is by depolarization, not desensitization. Higher concentrations produce more rapid desensitization. Studies on different depolarizing drugs (Wray, 1978*b*), and on human muscle are in progress.

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