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AN ANALYSIS OF THE ACTION OF A FALSE TRANSMITTER AT THE NEUROMUSCULAR JUNCTION

BY D. COLQUHOUN, W. A. LARGE AND H. P. RANG

From the Department of Pharmacology, St George's Hospital Medical School, Blackshaw Road, London SW17 0QT

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SUMMARY

1. The action of monoethylcholine (MECh) on neuromuscular transmission has been studied by electrophysiological methods.

2. End-plate potentials (e.p.p.s) in curarized rat muscle were unaffected or slightly increased in amplitude by MECh (0·1–1 mM). Stimulation at 3 Hz for about 30 min in the presence of MECh caused a progressive decline in e.p.p. amplitude, and a shortening of the e.p.p. time course. These changes were reversed by addition of choline to the medium. Similar changes in amplitude, but no change in time course, occurred when the preparation was stimulated in the presence of hemicholinium or triethylcholine.

3. Extracellular recordings of miniature end-plate potentials in frog muscle showed that stimulation in the presence of MECh caused the time constant of the exponential decay of the m.e.p.p.s. to decrease by 42 %. The amplitude of intracellular m.e.p.p.s was reduced by 45 %. These changes were maximal by the time about 3×10^5 quanta had been released.

4. Voltage clamp experiments in rat muscle in which miniature end-plate currents (m.e.p.c.s) were recorded showed that stimulation in the presence of MECh reduced the amplitude (by 33 %) and the decay time constant (by 42 %).

5. Analysis of end-plate current fluctuations produced by local application of acetylcholine (ACh) and acetylmonoethylcholine (AMECh) to voltage clamped rat end-plates showed that the amplitude of the elementary current events was the same for both compounds, whereas the average channel lifetime was 44 % shorter for AMECh than for ACh.

6. The voltage-sensitivity of the channel lifetime (measured from end-plate current fluctuations) was the same for ACh and AMECh. The voltage-sensitivity of the m.e.p.c. decay time constant was the same as that found from noise measurements. The shortened m.e.p.c.s (false

m.e.p.c.s) occurring after stimulation in the presence of MECh also showed the same voltage-sensitivity.

7. Both normal and false m.e.p.c.s were prolonged by neostigmine by almost the same factor; false m.e.p.c.s were thus shorter than normal m.e.p.c.s even when cholinesterase was inactivated. Experiments with progressive curarization of neostigmine-treated end-plates suggested that the fraction of transmitter molecules bound is smaller for false than for normal m.e.p.c.s. The difference implies that the false transmitter has one quarter of the affinity of ACh for the receptors.

8. It is concluded that stimulation in the presence of MECh gives rise to a false transmitter, presumably AMECh, which has a lower affinity for receptors than ACh, and gives rise to ionic channels with a shorter average lifetime than those activated by ACh.

INTRODUCTION

It has recently been found that brain tissue (Frankenberg, Heimbürger, Nilsson & Sörbo, 1973; Barker & Mittag, 1975) and the sympathetic ganglion (Ilson & Collier, 1975; Collier, Barker & Mittag, 1976) can accumulate and acetylate analogues of choline, and that increasing the K concentration or electrical stimulation will then lead to the release of these acetylated analogues in place of the normal transmitter, acetylcholine (ACh). Choline analogues that have been studied by biochemical methods in brain and sympathetic ganglion include monoethylcholine (MECh), triethylcholine, pyrrolcholine and also analogues in which the N-atom of choline is replaced by P or S. Since acetylmonoethylcholine (AMECh) has appreciable depolarizing activity at the neuromuscular junction (Holton & Ing, 1949) MECh was a suitable analogue with which to carry out an electrophysiological study at the neuromuscular junction.

There is evidence from studies on end-plate noise (fluctuations in end-plate conductance which are superimposed on the over-all conductance increase that occurs when depolarizing compounds are applied to the end-plate membrane) that the depolarizing effect of ACh and related compounds is produced by the summation of many minute but discrete increases in membrane conductance (Katz & Miledi, 1972), each representing the opening of a single ionic channel (Neher & Sakmann, 1976). Studies of end-plate noise have revealed that depolarizing substances differ in respect of the mean duration for which these channels remain open (Katz & Miledi, 1973*a*; Colquhoun, Dionne, Steinbach & Stevens, 1975).

There is also evidence (Anderson & Stevens, 1973) that the rate of decay of the conductance change evoked by neurally released ACh is closely related to the mean channel lifetime. Anderson & Stevens (1973) found in

studies of frog muscle that the time constant, τ, for the closing of channels estimated from noise studies was identical with the time constant for the exponential decay of end-plate currents (e.p.c.s) and miniature end-plate currents (m.e.p.c.s) recorded under voltage clamp conditions. Both time constants showed exactly the same dependence on temperature and membrane potential, suggesting that the rate at which e.p.c.s. and m.e.p.c.s decline reflects the rate of channel closure rather than the rate of removal of ACh from the synaptic cleft. Other work (Katz & Miledi, 1973*b*) suggests that the rate of decline of m.e.p.c.s is slower than can be accounted for simply in this way, and that ACh hydrolysis is slow enough to limit the rate at which the conductance change declines. If a false transmitter is released in place of ACh, the time course of the 'false' m.e.p.c. might thus differ from that of a normal m.e.p.c. (*d*) because kinetic parameters (e.g. channel lifetime or rate of dissociation) for the false transmitter may differ from those for ACh, and because the false transmitter may be hydrolysed at a different rate.

Since our preliminary results suggested that nerve stimulation in the presence of MECh led to the release of a false transmitter, which was assumed to be AMECh, we have studied the effect of this change on the time course of m.e.p.c.s, and compared this with estimates of channel lifetime obtained by measuring end-plate noise produced by application of AMECh. We have also investigated the effect of neostigmine on the time course of normal and false m.e.p.c.s in order to test whether both transmitters were equally susceptible to enzymic hydrolysis. It is likely that AMECh would differ from ACh in its affinity for post-synaptic receptors, so that a larger or smaller fraction of the released transmitter molecules would be bound to receptors. Katz & Miledi (1973*b*) suggested that, when acetylcholinesterase is inhibited, this binding has a considerable retarding effect on diffusion of ACh molecules out of the synaptic cleft (see also Magleby & Terrar, 1975). Thus, blocking the receptors with tubocurarine or a similar agent accelerates the loss of transmitter by diffusion, and this principle can be used to estimate the fraction of transmitter molecules that are normally bound, giving an indirect measure of the affinity of the transmitter for the receptors. We have used this approach to compare the false transmitter with ACh.

From our experiments it appears that AMECh has a briefer post-synaptic effect than ACh, which is related to a shorter channel lifetime. AMECh is rapidly hydrolysed by acetylcholinesterase. Its affinity for receptors appear to be lower than that of ACh. Consequently, when acetylcholinesterase is inhibited it diffuses away more quickly than ACh.

Preliminary accounts of some of this work have already appeared (Colquhoun, 1976; Large & Rang, 1976).

METHODS

Experiments were carried out on either the rat phrenic nerve–diaphragm preparation or the frog *cutaneus pectoris* muscle with its motor nerve; the preparations were pinned down to a bed of Sylgard resin (Dow–Corning) and were superfused continuously with normal Krebs solution or frog Ringer respectively. Normal Krebs solution contained (mM) NaCl 119, KCl 4·7, $CaCl_2$ 2·5, $MgSO_4$ 1·2, KH_2PO_4 1·2, $NaHCO_3$ 25, glucose 11, and was bubbled with 5% CO_2–95% O_2. The composition of frog Ringer was (mM) NaCl 135, KCl 2·5, $CaCl_2$ 1·8 and it was buffered at pH 7 with 2 mM Tris-HCl; the high NaCl concentration (135 mM as opposed to 115 M in normal frog Ringer solution) was used to increase the frequency of miniature end-plate potentials (m.e.p.p.s) which made easier the localization of end-plates with a focal extracellular electrode. Experiments were done at room temperatures of 19–23° C and in most cases the temperature of the bath fluid was controlled at 20° C, passing the Krebs solution through a water-jacket circulated with water from a refrigerated unit (Churchill); the temperature was checked routinely with a thermistor probe placed close to the preparation.

A stock solution of 100 mM-MECh was kept at −15° C and made up to the stated concentrations in the Krebs or Ringer solution immediately before use. Fresh solutions of neostigmine sulphate were prepared for each experiment.

Recording of m.e.p.p.s and m.e.p.c.s. In the experiments on frog muscle end-plates were localized by recording extracellular m.e.p.p.s with a micro-electrode of about 1 MΩ resistance filled with 2 M-NaCl, and then the KCl-filled intracellular electrode was placed within about 100 μm of the extracellular electrode in the same cell; this was confirmed by checking that extracellular m.e.p.p.s were always accompanied by intracellular potentials.

The rat diaphragm was pinned on a 2 mm deep layer of Sylgard resin placed on a microscope slide which formed the bottom of the muscle chamber, which was placed on the modified stage of a Zeiss-Nomarski microscope.

The diaphragm is too thick to permit the best use of the Nomarski differential interference contrast optics, but, even so, it was usually possible to see quite clearly the fine nerve terminals. It was assumed that the electrodes were sufficiently close to the end-plate when the intracellular m.e.p.p.s had a fast rise time (0·8–1·5 msec). In experiments where end-plate potentials (e.p.p.s) were studied in a curarized preparation, impalement was considered focal if the e.p.p.s had a rise-time of less than 2 msec.

M.e.p.c.s and fluctuations about mean end-plate current produced by agonists were measured with a voltage clamp arrangement similar to that of Dionne & Stevens (1975), and the currents were measured as the voltage drop across a 1 MΩ resistor placed in series with the current-passing electrode. To diminish high frequency noise the signal was fed into a low-pass active filter (V-F Instruments) where the cut-off frequency was usually 2 kHz with 80 dB/decade roll-off (low Q mode). Both current-passing and recording electrodes were usually filled with 3 M-KCl and had resistances of 5–12 MΩ.

In experiments in which tubocurarine was applied locally to end-plates a coarse micropipette filled with 20 mM (+)-tubocurarine chloride was brought close to the recording site. Diffusional leakage of tubocurarine was usually enough to inhibit the m.e.p.c.s completely. The pipette was then removed and records taken at intervals during the subsequent 5–10 min recovery period.

All data were temporarily stored on an FM tape recorder (Racal Store 4) and subsequently photographed on moving film or on Polaroid film from a storage

oscilloscope, from which measurements of amplitudes and time constants of decay were made. In later experiments m.e.p.c.s or m.e.p.p.s recorded on tape were captured by means of a transient recorder (DataLab DL 901) and transferred to paper by an X–Y plotter. The time constants of decay of m.e.p.c.s were calculated either by comparison with a series of exponential curves of varying decay constants printed on a template, or by plotting the time course with a Hewlett Packard desk calculator (HP9820A) and estimating the time constant by fitting a least-squares line to the semilogarithmic plot.

Measurement of current fluctuations. Rat diaphragm strips were perfused with Krebs solution containing 200–400 nM tetrodotoxin (Sigma). In these experiments the membrane potential was clamped for long periods, so the current-passing electrode was usually filled with 3 M-K citrate neutralized to pH 6·8. The electrode resistances were 3–12 MΩ.

The electrodes were placed within about 100 μm of each other under a dissecting microscope. The criteria for proximity to the end-plate were (*a*) m.e.p.p. greater than about 0·5 mV in amplitude, and a fast rise time with the voltage electrode alone, (*b*) m.e.p.p.s looked similar when both electrodes were used for voltage recording, (*c*) after clamping, m.e.p.c.s had fast rise time and were not less than about 3 nA in amplitude at -80 mV (mean $\pm$ S.E. for ten experiments, $3{\cdot}33 \pm 0{\cdot}12$ nA).

Agonists were applied by perfusion from a wide-tipped pipette (Cooke & Quastel, 1973) placed 0·2–2 mm from the end-plate, in concentrations sufficient to produce currents of 10–100 nA. The current signal was filtered to produce a bandwidth of (*a*) zero to 2–5 kHz for m.e.p.c. measurement as above, and for drug-induced current (Barr & Stroud EF3-02; 160 dB/decade roll-off; damped mode) or (*b*) 0·02–1·0 to 500 Hz for current fluctuation measurement (Krohn-Hite 3700; 80 dB/decade; maximally flat mode). After recording m.e.p.c.s the agonist was applied for about 20 sec; desensitization occurred fairly rapidly, but it was usually possible to obtain a 10 sec sample of noise during which the current was constant, or fell by, at most, a few nA. This sample, recorded on tape, was digitized at 1000 or 1024 Hz and edited to remove m.e.p.c.s and electrical artifacts. The edited data were used to calculate the one-sided spectral density function, $G(f)$, from which was subtracted the spectrum computed from a control period of noise recorded immediately before or after the application of agonist.

Spectra were calculated, usually with 4 Hz resolution, using a Hanning window and ensemble averaging according to the method of Welch (1967). A single Lorentzian component, $G(f) = G(0)/[1 + (f/f_c)^2]$, where f = frequency, f_c = half power frequency, was fitted to the computed log $G(f)$ values by the method of least squares using the patternsearch procedure (see Colquhoun, 1971). The log $G(f)$ values at each frequency were equally weighted.

These analyses were carried out on a PDP 11/45 computer, kindly made available by the Institute of Sound and Vibration Research, University of Southampton.

RESULTS

Effects of MECh in the absence of prolonged nerve stimulation

When MECh (0·5–1 mM) was applied to curarized rat phrenic nerve-diaphragm preparations, the amplitude of end-plate potentials recorded at low stimulation frequency (0·1 Hz) was usually increased appreciably (Fig. 1). With 0·1 mM-MECh the effect was negligible. A similar and more pronounced effect was seen with triethylcholine. This was previously

reported by Bowman, Hemsworth & Rand (1962) and was interpreted by them as an enhancement of transmitter output similar to that produced by tetraethylammonium, which is thought to be due to prolongation of the action potential in nerve terminals. Most of our experiments were done with 0·1 or 0·2 mM-MECh, and this effect was small or absent. These concentrations of MECh had no effect on the amplitude or time course of m.e.p.p.s or m.e.p.c.s in rat or frog muscle, and caused no change in membrane potential. Concentrations exceeding 1 mM caused the membrane to depolarize by a few millivolts.

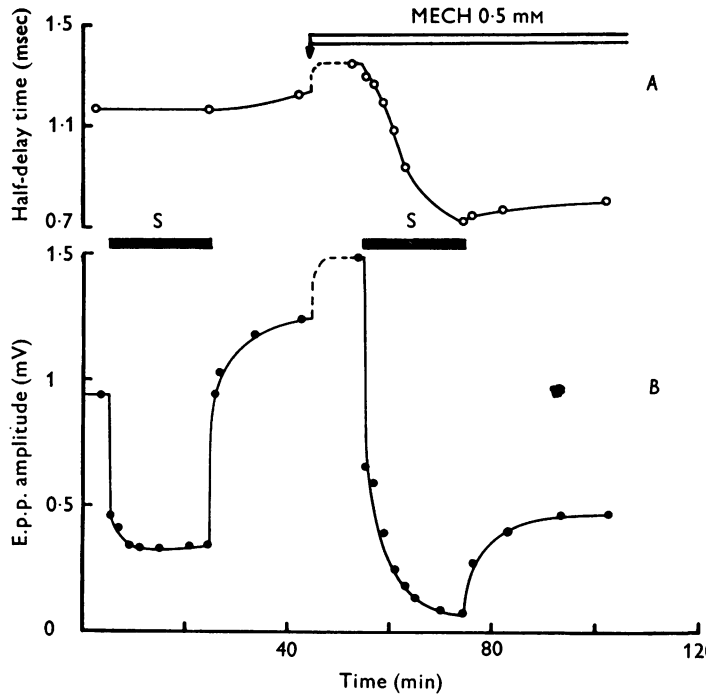

Fig. 1. Effect of MECh on the half-decay time (*A*) and the amplitude (*B*) of e.p.p.s in a curarized rat phrenic nerve-diaphragm preparation. At the horizontal solid bars marked S the frequency of nerve stimulation was increased from 0·1 to 3 Hz. Note that a maintained reduction in both the half-decay time and the amplitude of e.p.p.s was achieved only when the frequency of nerve stimulation was increased in the presence of MECh.

Effects of stimulation in the presence of MECh

Fig. 1 shows the effect of MECh on the amplitude and half-decay time of e.p.p.s recorded from a rat diaphragm fibre. In the absence of MECh, stimulation at 3 Hz caused an immediate decline in the e.p.p. amplitude, which was thereafter well sustained for 20 min. When the frequency was

returned to 0·1 Hz, the amplitude quickly returned, and remained above the control level for some time owing to post-tetanic potentiation.

These changes in e.p.p. amplitude were not associated with any change in the half-decay time of the e.p.p. In contrast, when the nerve was stimulated at 3 Hz in the presence of 0·5 mM-MECh, the immediate decline in e.p.p. amplitude was followed by a further slow decline until after 10–15 min and the e.p.p. became almost undetectable. When the stimulation frequency was reduced to 0·1 Hz the e.p.p. amplitude recovered partly, but only to about 30 % of the control level. The half-decay time of the e.p.p.s was also affected by stimulation in the presence of MECh, showing a gradual decline by about 35 % during the period of stimulation at 3 Hz. No appreciable recovery of the half-decay time occurred when the frequency was reduced to 0·1 Hz. In other experiments it was found that washing out the MECh and continuing to stimulate at 0·1 Hz led to a gradual recovery of the e.p.p. amplitude and half-decay time over the course of 2–3 hr. Addition of choline to the bathing medium (Fig. 2) restored the e.p.p. to normal more quickly.

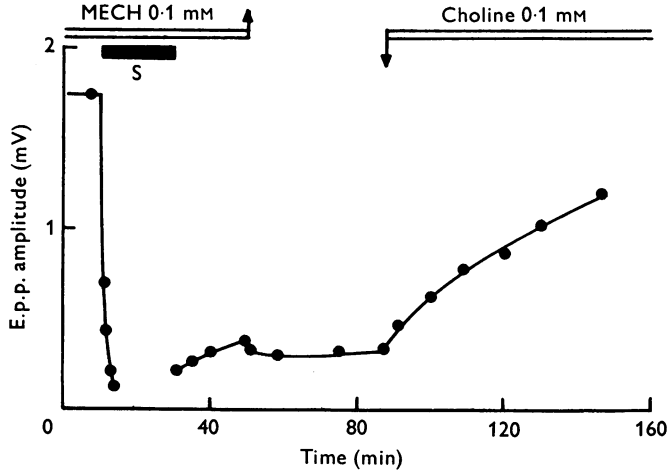

Fig. 2. Reversal by choline of the reduction in e.p.p. amplitude produced by stimulation of the preparation in the presence of MECh. At the solid bar marked S the frequency of nerve stimulation was increased from 0·1 to 3 Hz.

These results are consistent with the false transmitter hypothesis in that the changes in the e.p.p. occurred only when the nerve was stimulated at a moderately high frequency for a period of time, suggesting that the changes depend on the release of pre-formed ACh and its replacement by the false transmitter. It was necessary to check, though, whether mere depletion of ACh without synthesis of an active false transmitter could

produce the same effects. Similar experiments were therefore done with triethylcholine and hemicholinium (Table 1), which are known to inhibit ACh synthesis by motor nerve terminals. These experiments showed that in spite of a greater reduction in e.p.p. amplitude by hemicholinium or triethylcholine (TECh), only MECh caused any change in the half-decay

TABLE 1. Effect of inhibitors of ACh synthesis on the characteristics of end-plate potentials recorded in curarized rat diaphragm muscles. End-plate potentials were recorded during stimulation at 0·1 Hz. The period of stimulation used to deplete the tissue of ACh was usually 30 min at a frequency of 3 Hz

	E.p.p. amplitude (mV)			Half-decay time (msec)		
Inhibitor	Before stimulation *a*	After stimulation *b*	*b/a*	Before stimulation *c*	After stimulation *d*	*d/c*
MECh 0·1 mM	1·90	0·75	0·39	1·62	1·15	0·71
MECh 0·1 mM	0·84	0·32	0·38	1·25	0·98	0·78
MECh 0·5 mM	1·48	0·46	0·31	1·35	0·81	0·60
MECh 1·0 mM	1·65	0·39	0·24	2·08	1·52	0·73
Triethylcholine 0·1 mM	1·36	0·20	0·15	1·64	1·70	1·04
Hemicholinium 0·03 mM	1·22	0·44	0·36	1·68	1·59	0·95

time. The decrease in half-decay time cannot therefore be the result only of depletion of the ACh stores of the terminal, and is consistent with the release of a false transmitter which produces a somewhat briefer synaptic current than ACh. Consistent with this interpretation is the fact that stimulation in the presence of MECh decreased the e.p.p. amplitude to about 40% of the control value. Prolonging the period of stimulation (Fig. 3) or increasing the MECh concentration did not increase the magnitude of the effect. Similar periods of stimulation in the presence of triethylcholine (0·1–1 mM) or hemicholinium (3×10^{-5} M) decreased the e.p.p. amplitude by 90% or more. It therefore seemed likely that the reduced e.p.p.s seen after 20–30 min stimulation in the presence of MECh result from the substitution of ACh by AMECh (acetylmonoethylcholine) in the nerve terminals, which would be consistent with the biochemical findings of Collier *et al.* (1976) on the sympathetic ganglion.

Analysis of m.e.p.p.s in frog muscle

Since the time course of intracellularly recorded e.p.p.s and m.e.p.p.s reflects the passive properties of the membrane as well as the time course

of the underlying end-plate current, it was desirable to measure the end-plate current more directly. In one series of experiments this was done by recording with extracellular NaCl-filled pipettes from end-plates of frog *cutaneus pectoris* muscles. The result of an experiment in which intracellular recordings were made simultaneously is shown in Fig. 4.

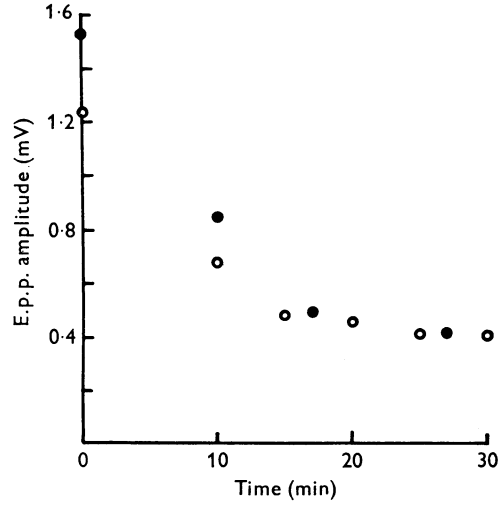

Fig. 3. Time course of the reduction of e.p.p. amplitude produced by MECh in two curarized rat phrenic nerve–diaphragm preparations. The points on the graph represent the amplitudes of e.p.p.s when the nerve was stimulated at 0·1 Hz. The abscissa is the total time for which the nerve was stimulated at 3 Hz and the e.p.p. amplitudes were measured 4 min after returning to 0·1 Hz. Concentration of MECh was 0·1 mM. Note that the effect of MECh on e.p.p. amplitude was fully developed after 30 min stimulation at 3 Hz.

As noted before, MECh had no direct effect on the time course of the m.e.p.p. but after the nerve had been stimulated in the presence of MECh the time constant for the externally recorded m.e.p.p. decay was reduced by nearly 50 %.

In Table 2 are given the results of a number of experiments in which the effect of stimulation in the presence of MECh on (*a*) the amplitude of m.e.p.p.s recorded intracellularly and on (*b*) the half-decay time of m.e.p.p.s recorded extracellularly, was measured at single end-plates. On average, the m.e.p.p. amplitude was reduced to 55 %, and the half-decay time of the end-plate current was reduced to 58 % of control values.

In the frog nerve–muscle preparation (unlike the rat diaphragm) transmission rapidly failed when the nerve was stimulated at 2 or 3 Hz. However, although stimulation failed to produce e.p.p.s it caused a great increase in the frequency of m.e.p.p.s (cf. Brooks, 1956; Miledi & Thies, 1971) which was effective in bringing about the incorporation of the false

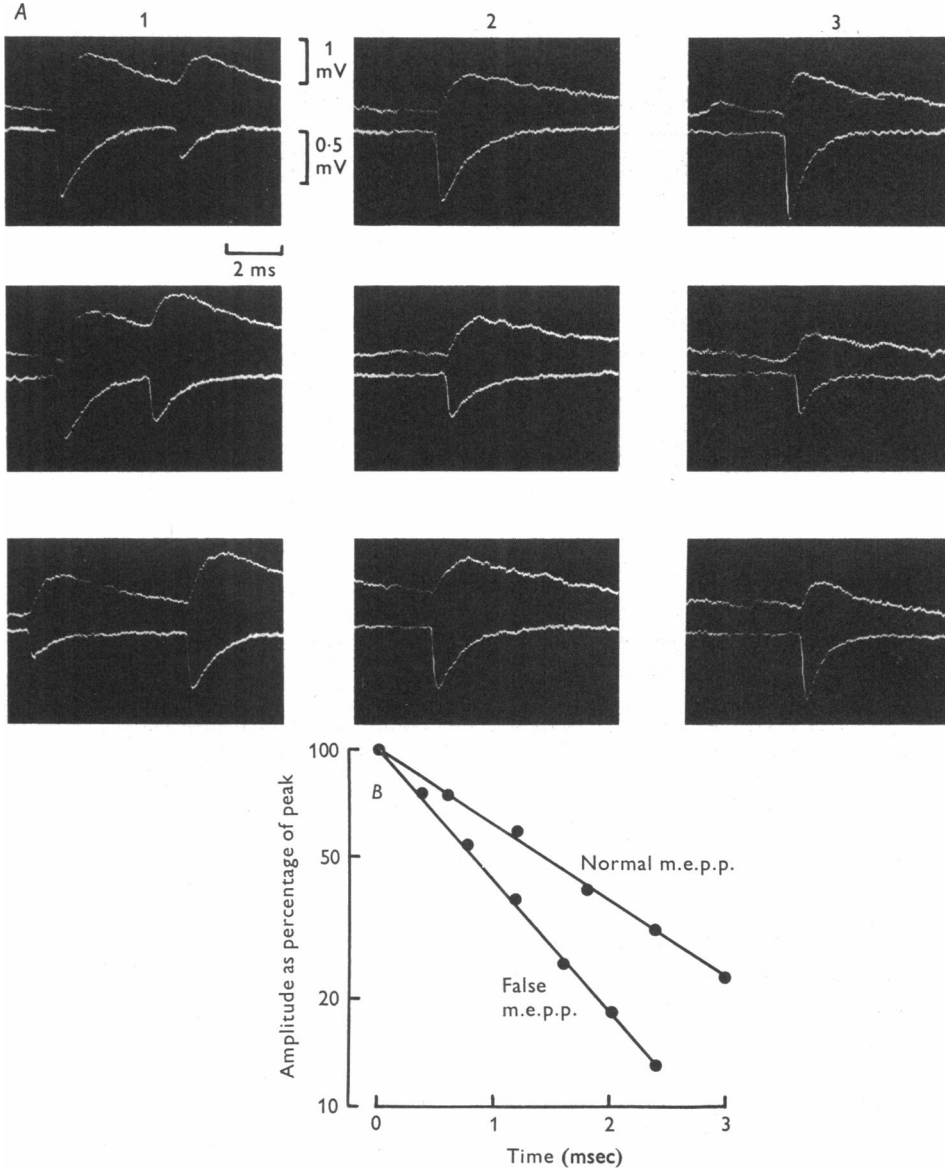

Fig. 4. Effect of MECh on m.e.p.p.s recorded intracellularly (upper traces) and extracellularly (lower traces) simultaneously from the same end-plate in a frog *cutaneus pectoris* preparation. *A*1: m.e.p.p.s in normal frog Ringer solution. *A*2: 35 min after the addition of 0·1 mM-MECh to the bathing fluid (no nerve stimulation). *A*3: after a 30 min period of nerve stimulation (4 Hz) in the presence of MECh. *B*, decay of extracellular m.e.p.p.s shown in top photographs of *A*1 and *A*3 plotted on semilogarithmic co-ordinates. The time constant of decay (τ) of the normal m.e.p.p. was 2·10 msec, and the τ of the false m.e.p.p. was 1·25 msec (retouched).

transmitter. This was convenient in that it allowed the electrodes to be kept in place during the period of rapid turnover of transmitter. Fig. 5 shows the results of an experiment in which choline was found to restore the normal time constant of externally recorded m.e.p.p.s after they had been shortened by incorporation of MECh. In this experiment the high rate of m.e.p.p. discharge (200–300/sec) continued for long enough for reintroduction of MECh to cause a further reduction in the time constant.

TABLE 2. Effect of MECh on m.e.p.p.s recorded with intracellular and extracellular micro-electrodes in frog muscle. After the controls had been recorded the nerve was stimulated for about 30 min at 4 Hz in the presence of 0·1–0·2 mM-MECh

	Amplitude of intracellular m.e.p.p.s (mV)			Half-decay time of extracellular m.e.p.p.s. (msec)		
Fibre	Before stimulation *a*	After stimulation *b*	*b/a*	Before stimulation *c*	After stimulation *d*	*d/c*
1	—	—	—	1·09 ± 0·04	0·65 ± 0·05	0·60
2	—	—	—	1·15 ± 0·05	0·63 ± 0·04	0·55
3	0·51 ± 0·02	0·29 ± 0·01	0·57	1·61 ± 0·07	0·83 ± 0·02	0·52
4	0·59 ± 0·03	0·26 ± 0·02	0·44	0·96 ± 0·03	0·49 ± 0·03	0·51
5	0·69 ± 0·03	0·41 ± 0·02	0·59	1·59 ± 0·10	0·94 ± 0·03	0·59
6	—	—	—	1·21 ± 0·07	0·62 ± 0·04	0·51
7	1·03 ± 0·12	0·44 ± 0·03	0·43	1·61 ± 0·04	0·99 ± 0·05	0·61
8	1·04 ± 0·15	0·65 ± 0·04	0·63	1·34 ± 0·04	0·77 ± 0·03	0·57
9	0·65 ± 0·34	0·34 ± 0·04	0·52	1·29 ± 0·05	0·90 ± 0·05	0·70
10	1·00 ± 0·04	0·44 ± 0·08	0·44	1·58 ± 0·09	0·76 ± 0·05	0·48
11	—	—	—	2·11 ± 0·15	1·07 ± 0·07	0·51
12	0·89 ± 0·05	0·52 ± 0·04	0·58	1·21 ± 0·05	0·78 ± 0·03	0·64
13	—	—	—	1·24 ± 0·06	0·80 ± 0·07	0·65
14	—	—	—	1·20 ± 0·05	0·80 ± 0·05	0·67
15	—	—	—	2·04 ± 0·06	1·02 ± 0·06	0·50
16	0·53 ± 0·05	0·39 ± 0·04	0·74	1·23 ± 0·06	0·76 ± 0·05	0·62
Mean ± S.E. of mean	0·77 ± 0·07	0·42 ± 0·04	0·55 ± 0·03	1·40 ± 0·08	0·80 ± 0·04	0·58 ± 0·02

It was of interest to measure how many quanta of transmitter had to be released in order to incorporate AMECh into the stores. Fig. 6 shows the results of six experiments on frog muscle in which the fractional change in the m.e.p.c. half-decay time was measured as a function of the total number of m.e.p.p.s (recorded with an intracellular electrode) occurring since the MECh was added. In these experiments muscle contraction was prevented by the use of a bathing solution containing 0·5 mM-Ca^{2+} and 8·0 mM-Mg^{2+}. The total number of m.e.p.p.s was calculated from measurements of m.e.p.p. frequency made every few minutes during the experiment.

Though there is a good deal of scatter in the measurements, it appears that the maximal effect was produced by the time about 3×10^5 quanta had been released. This may be compared with the estimate of $4{\cdot}7 \times 10^5$ (range $2{\cdot}9$–$7{\cdot}4 \times 10^5$) for the total number of vesicles released by black widow spider venom from single junctions in this muscle (Ceccarelli, Hurlburt & Mauro, 1973). In a few experiments, the MECh concentration was varied. It appeared that varying the concentration between 50 and 500 μM did not effect the results; in a single experiment with 20 μM-MECh, the effect was smaller and slower to develop. Subsequent experiments were done with 0·1 or 0·2 mM-MECh.

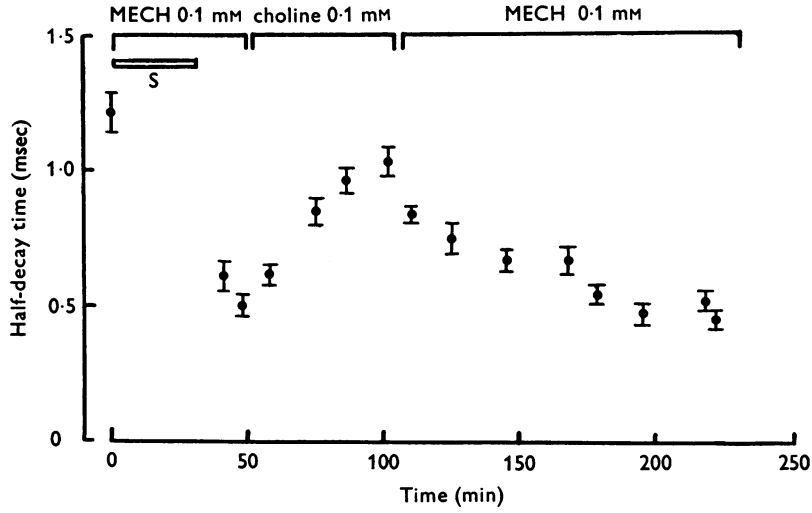

Fig. 5. Reversal by choline of the reduced half-decay time of extracellular m.e.p.p.s produced by stimulating a frog nerve–muscle preparation in the presence of MECh. At the horizontal bar marked S, the nerve was stimulated at 4 Hz for 30 min. After cessation of stimulation, the m.e.p.p. frequency remained high (200–300 sec) throughout the rest of the experiment, and both intracellular and extracellular electrodes were left in position at the end-plate.

Analysis of m.e.p.p.s and m.e.p.c.s in rat muscle

Rat muscle was preferable to frog muscle in that transmission was well maintained during prolonged stimulation, so it was much easier to regulate the turnover of transmitter. The voltage clamp technique was used to record m.e.p.c.s in this tissue partly because it enabled the absolute amplitude of the m.e.p.c.s to be measured, and partly because extracellular recording is considerably more difficult in rat than in frog muscle. In order to produce complete exchange of the transmitter the phrenic

nerve was stimulated in the presence of 0·1 or 0·2 mM-MECh for 30–40 min (see Fig. 3).

The effect of this treatment on the m.e.p.p. is shown in Fig. 7. The amplitude is reduced by about half, the rise time is slightly shorter and the half-decay time is also shorter. These changes are similar to those which can be seen in the records obtained from frog muscle (Fig. 4). In three diaphragms the mean m.e.p.p. amplitude was compared before and after stimulation. In two experiments twenty fibres were impaled at random before and after stimulation; in the third experiment, five identified

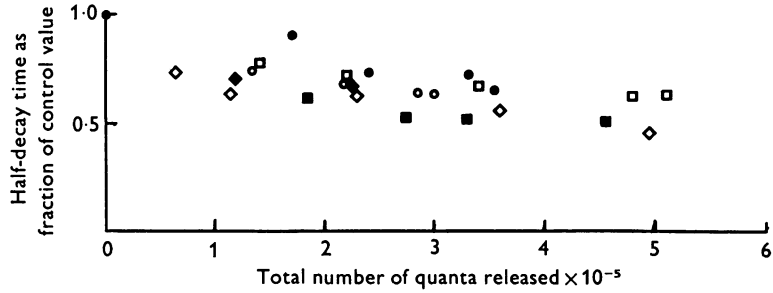

Fig. 6. The half-decay time of extracellular m.e.p.p.s plotted as a function of the total number of quanta released after the addition of MECh to the bathing fluid. In these experiments the frog Ringer solution contained 0·5 mM-Ca^{2+} and 8 mM-Mg^{2+} which greatly reduced or abolished the e.p.p. but there was still an increase in m.e.p.p. frequency when the nerve was stimulated at 4 Hz; this enabled the electrodes to be left in position throughout the experiment. The number of quanta released was estimated by counting m.e.p.p.s on several single sweeps on a storage oscilloscope every few minutes. The results were taken from six separate experiments as indicated by the different symbols.

fibres were impaled before and after stimulation. After stimulation the mean m.e.p.p. amplitude in these three experiments was 0·51 ± 0·04 times the control amplitude, a value similar to that found in frog muscle (Table 2). The reduction in the e.p.p. amplitude found in experiments like those of Figs. 1–3 was generally greater (60–70 %) than the decrease in m.e.p.p. amplitude that we measured (49 %). This discrepancy has not yet been investigated further.

Voltage clamp experiments were carried out to see whether the shortening of the e.p.p.s and m.e.p.p.s was due to a shortening of the membrane current as in frog muscle. It was not practicable to re-impale identified fibres after stimulation in the presence of MECh, so recordings of m.e.p.c.s were made from six to eight fibres at random before and after stimulation. The results are given in Fig. 8 and Table 3. Compared with normal

m.e.p.c.s the false m.e.p.c.s had a lower amplitude (2·18 nA compared with 3·30 nA), and also a shorter decay time constant (1·06 msec compared with 1·84 msec). The degree of shortening of the decay phase was identical with that found for externally recorded m.e.p.p.s in frog muscle.

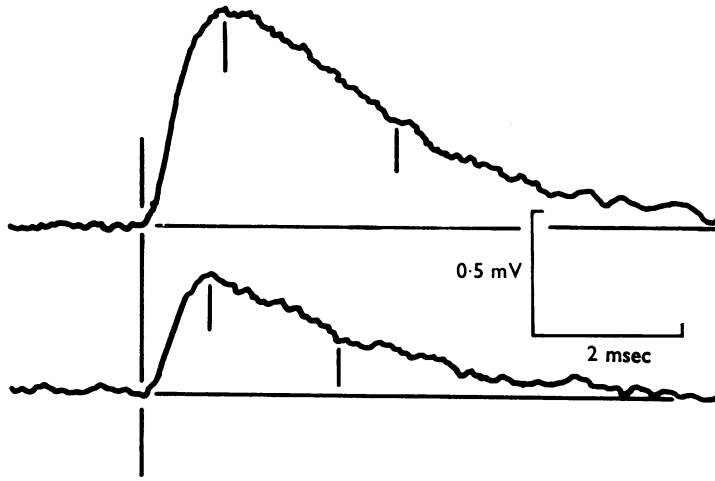

Fig. 7. Effect of MECh on m.e.p.p.s in rat muscle. The top record is a normal m.e.p.p. recorded from an end-plate in Krebs solution and the bottom trace is a false m.e.p.p. from another cell after the preparation had been stimulated at 3 Hz for 30 min in the presence of 0·1 mM-MECh. Note the reduction in not only the amplitude but also the rise-time and half-decay time (indicated by bars) of the false m.e.p.p. (m.e.p.p.s were captured on a transient recorder and subsequently printed out by means of an X–Y plotter; the records shown were traced from these hard copies).

Analysis of end-plate current fluctuations in rat muscle

The concentration of ACh in the wide-mouthed perfusion pipette needed to produce an adequate response varied from 20 to 200 μM in different experiments. The concentration of AMECh needed for a similar response was usually about five times larger. Although no formal assay was done, this result is close to the relative potency for producing contracture of the frog *rectus abdominis* muscle (Holton & Ing, 1949).

Samples of the end-plate current fluctuations are shown in Fig. 9. The noise in control records was usually 40–150 pA (r.m.s.), and two- to five-fold larger in the presence of drug. It can be seen by eye that the fluctuations extend to higher frequencies in the AMECh record than in the ACh record. This impression can be made more quantitative by calculating the spectral density function, examples of which are shown in Fig. 10. In the example in Fig. 10*A*, the control spectral density is ten- to 45-fold less

than that in the presence of ACh, at low frequencies (except at the lowest, 4 Hz); and only above about 350 Hz does the difference between control and ACh spectra fall below fourfold. The effect of subtracting the control spectrum was thus small in this case, except at the highest frequencies. In experiments in which the drug-induced current was smaller, subtraction of the control spectrum had a correspondingly larger effect. As other workers have found (e.g. Anderson & Stevens, 1973), the control spectrum falls linearly at first and then rises again at higher frequencies. Fig. 10*B* shows the net spectrum, G_{drug}–$G_{control}$, plotted on double logarithmic scales.

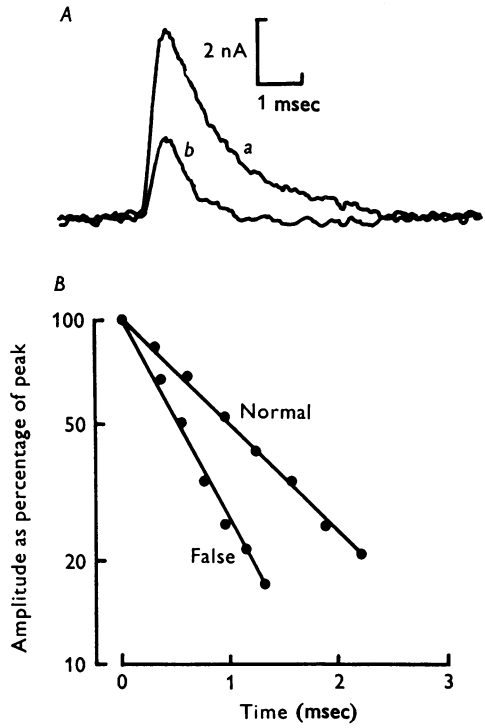

Fig. 8. Normal and false m.e.p.c.s in rat muscle. *A*: superimposed (by tracing) records of a normal (*a*) and a false (*b*) m.e.p.c. *B*: decay phases of (*a*) and (*b*) plotted semilogarithmically against time. Control m.e.p.c. (*a*) τ = 1·45 msec; false m.e.p.c. (*b*) τ = 0·75 msec. In this and subsequent figures inward currents are shown as upward deflexions. Holding potential = −80 mV.

A single Lorentzian component, i.e.

$$G(f) = \frac{G(0)}{1+(f/f_c)^2}, \qquad (1)$$

where f = frequency and f_c = half-power frequency, has been fitted to the

log $G(f)$ values using equally weighted least squares (see pp. 380, 392 for discussion of the propriety of this method, and of the deviations from the fitted line). If it is assumed that (*a*) drug and control noise are independent so their spectra can be subtracted, (*b*) that ion channels are independent of each other, (*c*) that each ion channel fluctuates between a shut state, and an open state with conductance. γ, producing rectangular current pulses,

TABLE 3. Comparison of normal and false m.e.p.c.s in rat muscle. The muscle was stimulated in the presence of 0·1–0·5 mM MECh to obtain false m.e.p.c.s

	Amplitude (nA)			$\tau_{m.e.p.c.}$ (msec)		
Muscle	Normal m.e.p.c.s *a*	False m.e.p.c.s *b*	*b*/*a*	Normal m.e.p.c.s *c*	False m.e.p.c.s *d*	*d*/*c*
1	3·30 ± 0·03	2·11 ± 0·20	0·64	2·16 ± 0·16	1·16 ± 0·06	0·54
2	3·09 ± 0·35	2·79 ± 0·26	0·90	2·04 ± 0·19	1·10 ± 0·09	0·54
3	3·16 ± 0·36	2·14 ± 0·26	0·68	2·01 ± 0·14	1·12 ± 0·11	0·56
4	3·87 ± 0·34	2·44 ± 0·16	0·63	1·88 ± 0·11	1·01 ± 0·08	0·54
5	3·91 ± 0·31	2·33 ± 0·16	0·60	1·99 ± 0·19	1·00 ± 0·09	0·50
6	3·14 ± 0·22	2·16 ± 0·20	0·69	1·97 ± 0·08	1·29 ± 0·11	0·65
7	2·92 ± 0·11	2·63 ± 0·13	0·90	1·49 ± 0·13	0·90 ± 0·06	0·60
8	3·05 ± 0·18	2·51 ± 0·13	0·82	1·81 ± 0·10	1·19 ± 0·06	0·66
9	3·54 ± 0·14	1·55 ± 0·06	0·44	1·73 ± 0·13	1·07 ± 0·13	0·62
10	3·04 ± 0·03	1·67 ± 0·22	0·55	1·49 ± 0·07	0·82 ± 0·05	0·55
11	3·26 ± 0·21	1·63 ± 0·14	0·50	1·66 ± 0·12	0·97 ± 0·05	0·58
Mean ± S.E. of mean	3·30 ± 0·10	2·18 ± 0·13	0·67 ± 0·05	1·84 ± 0·07	1·06 ± 0·04	0·58 ± 0·02

(*d*) the rate of drug binding is rapid compared with the rate of conformation change, (*e*) the open lifetime of each channel is an exponentially distributed random variable with mean τ and (*f*) that each channel is open, on average, for only a small fraction of the time, then a single component spectrum (eqn. 1) should be observed at equilibrium, from which the mean channel lifetime can be calculated as

$$\tau = 1/(2\pi f_c). \qquad (2)$$

The above assumptions are plausible, at least as approximations, although apart from (*f*) the evidence for them is indirect, and the evidence for (*d*) is tenuous (see, for example Anderson & Stevens, 1973; Neher & Sakmann, 1976). In Fig. 10B f_c = 156 Hz, so τ_{ACh} = 1·02 msec. In Fig. 10C, a spectrum produced by AMECh is shown. It is very similar to that produced by ACh, except that it extends to somewhat higher frequencies, so τ_{AMECh} is rather smaller than τ_{ACh}.

Relative time constants from fluctuations. The mean ratio of τ_{AMECh}/τ_{ACh}, from experiments in which both were tested on the same cell was

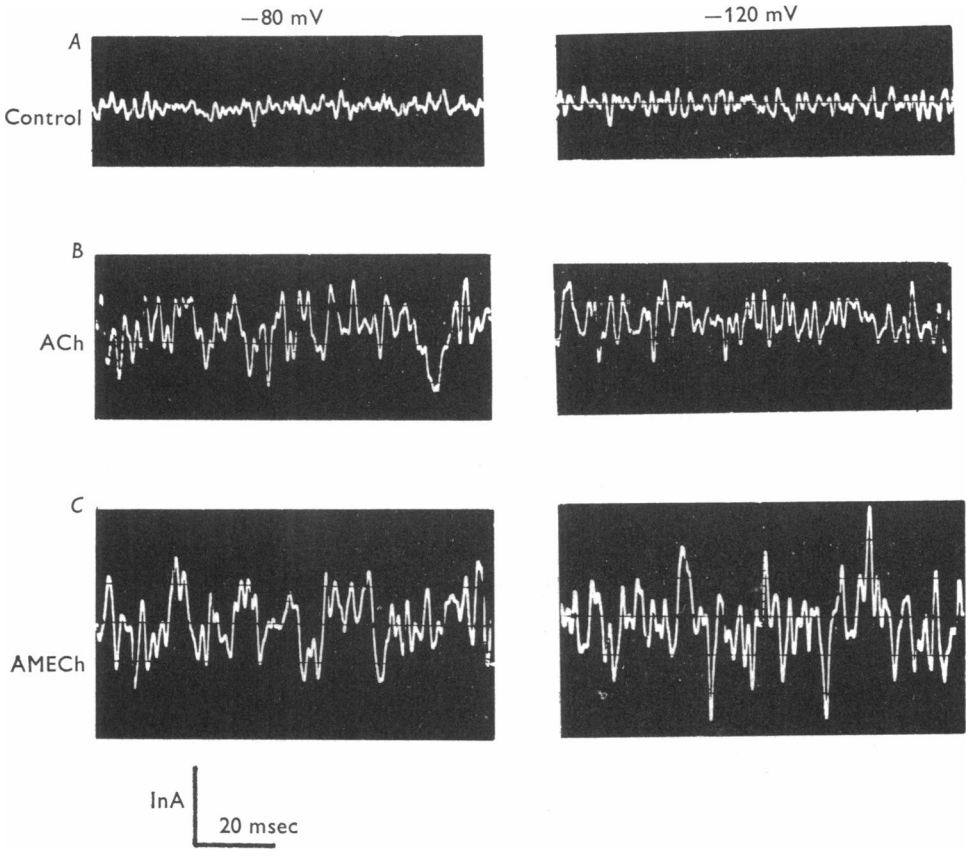

Fig. 9. End-plate current fluctuations produced by agonists (high gain, bandwidth 0·5–500 Hz) at a clamp potential (V_c) of −80 mV (left hand) and −120 mV (right hand, different cell). T = 18° C. *A*, control noise 100 pA (r.m.s.) at −80 mV, 109 pA (r.m.s.) at −120 mV. *B*, ACh. At −80 mV, 80 μM-ACh was applied producing an end-plate current of 59 nA and 279 pA (r.m.s.) fluctuations. At −120 mV 20 μM-ACh produced a 64 nA current and 358 pA (r.m.s.) fluctuations. *C*, AMECh. At −80 mV, 320 μM-AMECh produced a 27 nA current and 205 pA (r.m.s.) fluctuations. At −120 mV, 320 μM-AMECh produced 75 nA current and 393 pA fluctuations.

0·56 ± 0·05 (n = 15). This can be interpreted, on the above assumptions, as meaning that the average lifetime of an open channel, when the channel is opened by AMECh, is about 56 % of its mean lifetime when opened by ACh. This ratio is very similar to the ratio (0·58 ± 0·02) of decay time constants for normal (ACh) and false (AMECh) m.e.p.c.s found above (see Table 3). In one experiment with carbachol, the time constant was 36 %

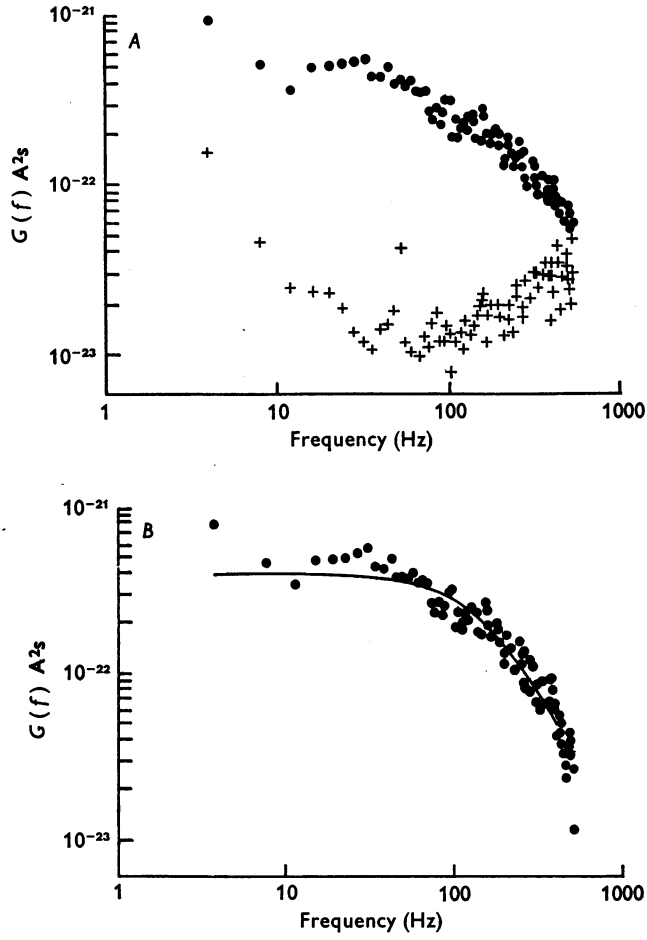

Fig. 10. Spectral density plotted against frequency (both scales logarithmic). Bandwidth 0·5–500 Hz, resolution 4 Hz. Points are too close together for all of them to be plotted at higher frequencies. *A*, +, spectrum of noise recorded in absence of drug; ●, spectrum during subsequent application of ACh (40 μM in pipette) which produced an end-plate current of 59 nA. *B*, net ACh spectrum. Holding potential −80 mV, T = 18·5° C. The points are the differences between the drug and control spectra in (*A*). The line is a single Lorentzian component fitted by equally weighted least squares to points between 4 and 400 Hz; $G(0) = 3{\cdot}86 \times 10^{-22}$ A²s, f_c = 156 Hz, τ = 1·02 msec, γ = 21·4 pS. *C*, net AMECh spectrum from another cell. Holding potential −80 mV, T = 17·5° C. The points and fitted line are as in *B*; end-plate current 25 nA, $G(0) = 1{\cdot}33 \times 10^{-22}$ A²s, f_c = 191 Hz, τ = 0·83 msec, γ = 21·4 pS.

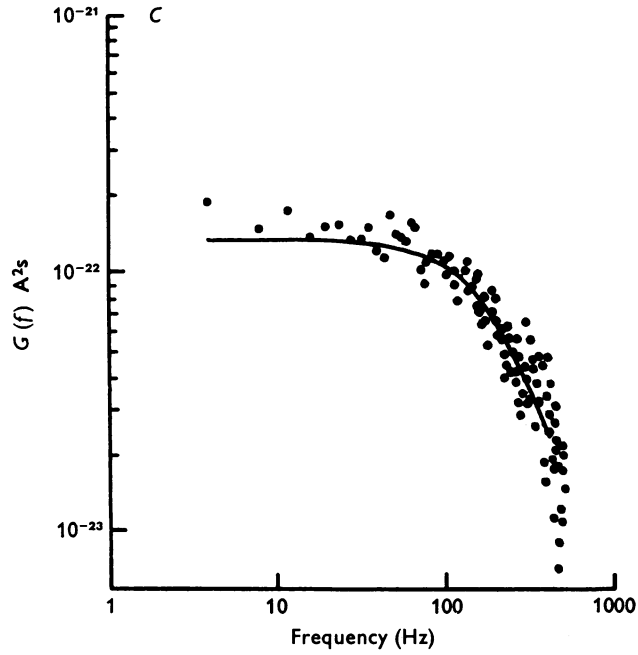

Fig. 10*C*. For legend see opposite.

of that inferred from ACh fluctuations, which is in close agreement with the result on frog muscle (Katz & Miledi, 1972, 1973*a*).

Single channel conductance. The mean conductance, γ, of an open channel can be found, if the above assumptions are correct, as

$$\gamma = \frac{G(0)}{4\, m_I \tau (V - V_{eq})},$$

where $G(0)$ is the spectral density at zero frequency found by fitting eqn. (1). m_I is the mean drug-induced current, τ is the mean channel lifetime from eqn. (2), V is the (clamped) membrane potential, and V_{eq} is the reversal potential (assumed to be -5 mV for both ACh and AMECh). This equation gave $\gamma = 24{\cdot}9 \pm 1{\cdot}2$ pS ($n = 36$) for ACh, which is very close to the value for frog muscle ($25{\cdot}0 \pm 0{\cdot}9$ pS) reported by Colquhoun *et al.* (1975). The conductance for channels opened by AMECh was $26{\cdot}7 \pm 1{\cdot}4$ pS ($n = 16$) which is not significantly different. Rather smaller conductance values were obtained when they were estimated directly from the variances of the observed (edited) noise current using $\gamma = \mathrm{var}(I)/m_I(V - V_{eq})$, but this appeared to result mainly from the variance being too small because of loss of fluctuations above 500 Hz. When corrected for this loss (assuming

a single Lorentzian component outside the observed frequency range) by dividing by $(2/\pi)\tan^{-1}(500/f_c)$, the results were $\gamma_{ACh} = 24{\cdot}8 \pm 1{\cdot}2$ pS (36) and $\gamma_{AMECh} = 26{\cdot}9 \pm 1{\cdot}6$ pS (16), very close to the estimates above.

Absolute values of time constants. If it is necessary to compare absolute values of time constants, particular care must be taken (*a*) with the methods used for curve fitting, (*b*) with correction for any temperature differences between experiments because of the high temperature sensitivity of the time constant. M.e.p.c. decay was found not to deviate consistently from a single exponential. The time constant, $\tau_{m.e.p.c.}$, for this decay (mean of 5–25 m.e.p.c.) was estimated on each cell in which ACh current fluctuations were measured. The mean ratio $\tau_{m.e.p.c.}/\tau_{ACh}$ was $1{\cdot}47 \pm 0{\cdot}06$ (25) when equally weighted least-squares estimates of τ_{ACh} were used (see Methods). However, it can be seen in Fig. 10 that the points at the lowest frequencies tend to lie above the least-squares curve. This was frequently observed with both ACh and AMECh, so an attempt was made to fit a single Lorentzian curve by eye with the aid of a plastic template. This method weighted the relatively small number of low frequency points much more heavily than those at higher frequencies, and produced consistently larger estimates of τ_{ACh} (by a factor of $1{\cdot}17 \pm 0{\cdot}01$) and of τ_{AMECh} (by a factor of $1{\cdot}19 \pm 0{\cdot}02$). This barely affected the ratio $\tau_{AMECh}/\tau_{ACh} = 0{\cdot}55 \pm 0{\cdot}02$(15), but resulted in a lowered ratio of $\tau_{m.e.p.c.}/\tau_{ACh} = 1{\cdot}26 \pm 0{\cdot}05$ (25). The method of curve fitting may make such a consistent difference (*a*) because of different weighting of points by different methods, or (*b*) because the spectrum has more than one component.

Improper weighting must certainly contribute to the uncertainty, but it seems likely that there is a small but consistent deviation from the Lorentzian form. The data are not precise enough to allow fitting of a second minor component, or to decide whether any minor component is of low or high frequency compared with the major component. Such a second component could arise (*a*) as an artifact, e.g. caused by local drug-induced muscle contracture, or by contributions from extrajunctional receptors (Dreyer, Walther & Peper, 1976), or (*b*) because the spectra genuinely have more than one component as is, in fact, predicted by even the simplest theories of channel opening (A. G. Hawkes & D. Colquhoun, in preparation). In the latter case, even if the major component could be fitted separately, the mean channel lifetime could not be estimated simply from it.

The quality of voltage control could also affect the values of τ_{ACh} and $\tau_{m.e.p.c.}$. If both electrodes were distant from the end-plate, both time constants would be too large, but their ratio should be relatively little affected unless the drug-induced conductance change affects voltage control. This seems implausible in a species with such a compact end-plate

as the rat, and, in any case, it might be expected to increase τ_{ACh}, for which the conductance increase is larger, more than $\tau_{m.e.p.c.}$

Subject to these uncertainties, it seems likely that $\tau_{m.e.p.c.}$, which was 1·6 msec at − 80 mV and 20° C (see below and Fig. 11*B*)), was slightly longer than τ_{ACh}, as found by Katz & Miledi (1973*b*), but not by Anderson & Stevens (1973) in frog muscle. The most obvious, but not the only, explanation of this would be that the transmitter persists in the cleft for long enough to lengthen slightly the m.e.p.c. decay. Fig. 11*A* shows τ_{ACh}, τ_{AMECh} (least-squares estimates), and $\tau_{m.e.p.c.}$, as a function of temperature. The temperature dependence was not detectably different for all three, so a pooled slope has been fitted to all the data. This slope corresponds to $Q_{10} = 3{\cdot}26 \pm 0{\cdot}63$ which is similar to the value reported for frog and toad muscle (Takeuchi & Takeuchi, 1959; Kordaš, 1972; Magleby & Stevens, 1972*b*; Gage & McBurney, 1975).

Voltage-dependence of time constants in rat muscle

The time constants for (*a*) normal m.e.p.c. decay, (*b*) false m.e.p.c. decay, (*c*) ACh-induced current fluctuations and (*d*) AMECh-induced current fluctuations were all found to have similar dependence on the potential at which the membrane was clamped (Figs. 11*B* and 12).

The τ values from noise experiments, corrected to 20° C, were taken from the lines in Fig. 11*A*, and the same Q_{10} was used also to make the (small) correction to 20° C of the τ values at clamp potentials (V_c) of − 60 and − 120 mV. The resulting relation between τ and V_c is shown in Fig. 11*B*. The relationship was found to be exponential, $\tau(V) = \tau(0) \exp(-V/H)$, as in frog muscle. The pooled slope from the data in Fig. 11*B* corresponds with an e-fold change in τ for a change of $H = 109 \pm 7$ mV in membrane potential.

In parallel experiments, normal and false m.e.p.c.s were directly compared and again an exponential dependence of τ on potential was observed, as shown in Fig. 12. The potential dependence for normal m.e.p.c.s in Fig. 12 is given by $H = 117 \pm 6$ mV ($n = 12$), which is not significantly different from the value in Fig. 11*B*. The potential dependence of τ for false m.e.p.c.s, $H = 118 \pm 11$ mV ($n = 13$), was very close to that for normal m.e.p.c.s.

The observed potential dependence of time constants is very similar to the values reported for frog and toad muscle (Takeuchi & Takeuchi, 1959; Magleby & Stevens, 1972*a*; Anderson & Stevens, 1973; Gage & McBurney, 1975).

Effect of anticholinesterases on normal and false m.e.p.c.s

AMECh is hydrolysed by acetylcholinesterase as fast as ACh (Holton & Ing, 1949; Collier *et al.* 1976), so it was of interest to measure the effect of

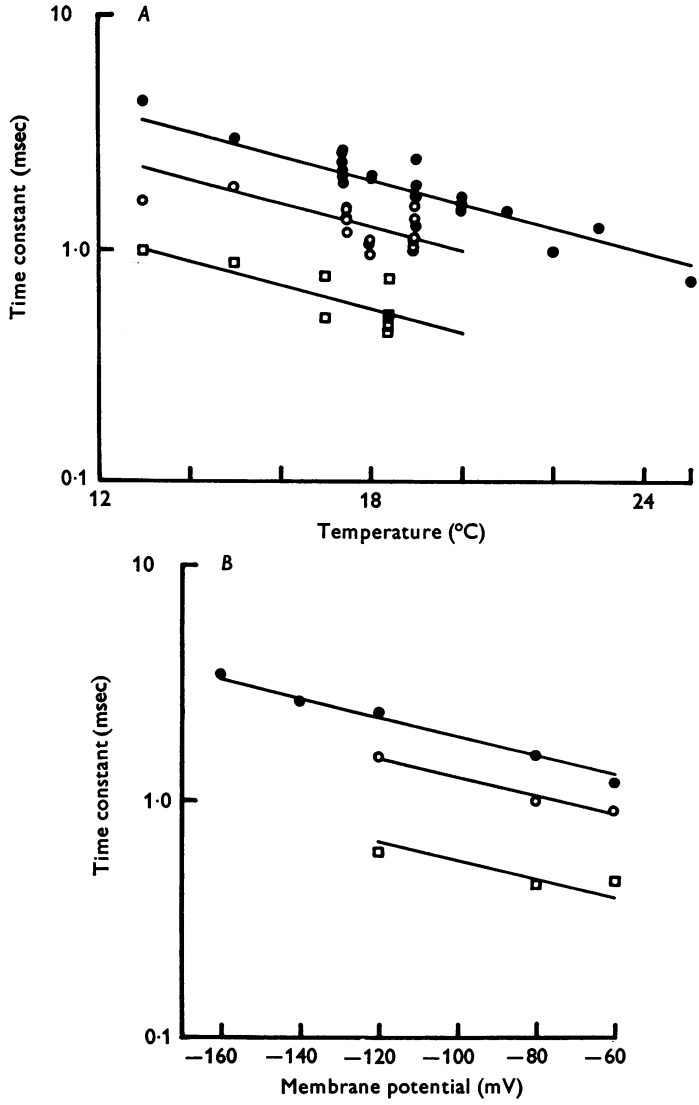

Fig. 11. Dependence of time constants on temperature and membrane potential. Time constants from fluctuations are equally weighted least squares estimates (higher values; see text). *A*, time constant, τ (msec), at a holding potential of −80 mV, plotted on a logarithmic scale against the temperature of the bath solution. ●, $\tau_{m.e.p.c.}$; ○, τ_{ACh} from current fluctuations; □, τ_{AMECh} from current fluctuations. Lines are plotted with the pooled slope for all data, which corresponds with $Q_{10} = 3{\cdot}26$. *B*, time constant, τ (msec), interpolated value at 20° C, plotted on a logarithmic scale against (clamped) membrane potential, V_c. ●, $\tau_{m.e.p.c.s.}$; ○, τ_{ACh} from current fluctuations; □, τ_{AMECh} from current fluctuations. Lines are plotted with the pooled slope for all data according to the equation $\tau(V) = \tau(0) \exp - V/H$, using the value $H = 109$ mV.

cholinesterase inhibitors on false m.e.p.c.s. Tests were done on rat muscle with neostigmine (3 μM) and edrophonium (20 μM) which gave identical results. If spontaneous twitching became excessive, tetrodotoxin (100 nM) was added to the bathing solution. In studies on false m.e.p.c.s, the preparation was stimulated in the presence of MECh, which was present throughout the experiment. M.e.p.c.s were then recorded from six to eight fibres before and after adding the anticholinesterase. It was found that

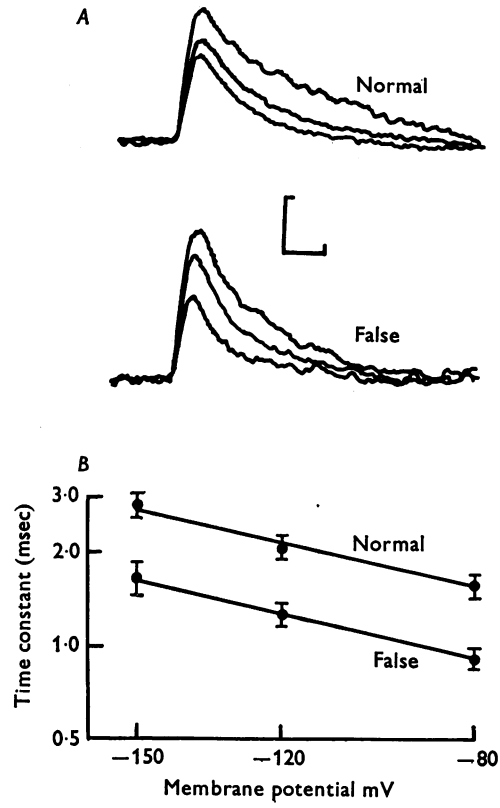

Fig. 12. The effect of (clamped) membrane potential on the time course of the decay of normal and false m.e.p.c.s. *A*: records of normal (top) and false (bottom) m.e.p.c.s. at three different membrane potentials, −80, −120, −150 mV (reading upwards in each set of records). *B*: the relationship between the time constant of decay (τ) and membrane potential (V_c) of normal and false m.e.p.c.s. The bars represent standard errors of the means of five to ten observations in different muscle fibres. The straight lines were drawn according to the equation $\tau\,(V) = \tau\,(0)\,\exp - V/H$ using the values of 171 mV and 118 mV for H and the values of 0·78 msec and 0·45 msec for $\tau(0)$ for normal and false m.e.p.c.s respectively. Calibrations: vertical bar, 4 nA for top records, 2 nA for bottom records, horizontal bar, 1 msec.

both normal and false m.e.p.c.s were prolonged (Fig. 13, Table 4) in the presence of anticholinesterases, normal m.e.p.c.s by a factor $2{\cdot}21 \pm 0{\cdot}15$, false m.e.p.c.s by a factor $2{\cdot}01 \pm 0{\cdot}11$. Thus the false m.e.p.c.s remained shorter than normal m.e.p.c.s ($\tau = 2{\cdot}18 \pm 0{\cdot}13$ msec compared with $\tau = 3{\cdot}60 \pm 0{\cdot}23$ msec) in the presence of the anticholinesterase. The amplitude of normal m.e.p.c.s was somewhat increased (by a factor $1{\cdot}23 \pm 0{\cdot}04$). False m.e.p.c.s were probably also increased (by a factor $1{\cdot}11 \pm 0{\cdot}13$) but the difference was not statistically significant.

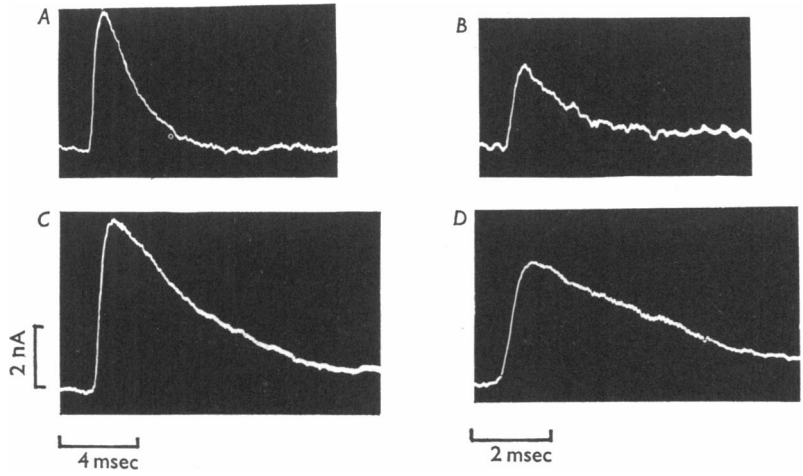

Fig. 13. Effect of neostigmine on normal and false m.e.p.c.s in rat muscle. Normal m.e.p.c.s recorded before (*A*) and after (*C*) the preparation had been exposed to 3 μM neostigmine for at least 30 min; false m.e.p.c.s recorded before (*B*) and after (*D*) neostigmine. Note that the horizontal calibration is 4 msec for (*A*) and (*C*) and 2 msec for (*B*) and (*D*). Holding potential = -80 mV.

At first sight it seemed surprising that false m.e.p.c.s should remain shorter than normal m.e.p.c.s even in the presence of a cholinesterase inhibitor. Since AMECh would be expected to have virtually the same diffusion coefficient as ACh, it might have been expected that AMECh would diffuse out of the synaptic cleft at about the same rate as ACh, and hence that normal and false m.e.p.c.s would have the same time course in the presence of neostigmine. One factor that could account for the observed difference in time course is the degree of binding of transmitter molecules in the synaptic cleft (Katz & Miledi, 1973*b*; Magleby & Terrar, 1975). Thus if the affinity of AMECh for the receptors is lower than that of ACh a smaller fraction of the molecules released into the synaptic cleft will be bound, and a correspondingly greater fraction will be free to diffuse. Even though the binding may be rapidly reversible, the over-all effect is to

TABLE 4. The effect of cholinesterase inhibitors on normal and false m.e.p.c.s

A Normal m.e.p.c.s

Muscle	Cholinesterase inhibitor	Amplitude (nA) Control *a*	Amplitude (nA) Anti-ChE Present *b*	*b/a*	τ (msec) Control *c*	τ (msec) Anti-ChE present *d*	*d/c*
1	Edrophonium 20 μM	3·08 ± 0·32	3·72 ± 0·39	1·21	1·61 ± 0·21	3·28 ± 0·23	2·04
2	Edrophonium 20 μM	3·48 ± 0·35	4·00 ± 0·27	1·15	1·44 ± 0·10	3·23 ± 0·24	2·24
3	Edrophonium 20 μM	3·25 ± 0·26	3·81 ± 0·20	1·17	1·86 ± 0·15	3·40 ± 0·13	1·83
4	Neostigmine 3 μM	3·17 ± 0·11	3·94 ± 0·33	1·24	1·68 ± 0·10	4·42 ± 0·33	2·63
5	Neostigmine 3 μM	2·82 ± 0·16	3·95 ± 0·30	1·40	1·63 ± 0·08	3·03 ± 0·34	1·86
6	Neostigmine 3 μM	2·79 ± 0·10	3·40 ± 0·26	1·22	1·59 ± 0·08	4·21 ± 0·18	2·65
Mean ± S.E. of mean		3·10 ± 0·11	3·80 ± 0·09	1·23 ± 0·04	1·64 ± 0·06	3·60 ± 0·23	2·21 ± 0·15

B False m.e.p.c.s

1	Edrophonium 20 μM	2·33 ± 0·16	2·04 ± 0·24	0·88	1·00 ± 0·09	2·21 ± 0·27	2·21
2	Edrophonium 20 μM	2·16 ± 0·20	2·20 ± 0·20	1·02	1·29 ± 0·11	2·07 ± 0·24	1·60
3	Neostigmine 3 μM	2·63 ± 0·13	2·09 ± 0·12	0·79	0·90 ± 0·06	1·78 ± 0·15	1·98
4	Neostigmine 3 μM	2·51 ± 0·13	3·70 ± 0·37	1·47	1·19 ± 0·06	2·55 ± 0·35	2·14
5	Neostigmine 3 μM	1·55 ± 0·06	2·12 ± 0·12	1·37	1·07 ± 0·13	2·27 ± 0·15	2·12
Mean ± S.E. of mean		2·24 ± 0·19	2·43 ± 0·32	1·11 ± 0·13	1·09 ± 0·07	2·18 ± 0·13	2·01 ± 0·11

reduce the effective rate of diffusion, and the higher the affinity of the transmitter for the receptors the slower will diffusion become. This type of interaction between binding and diffusion has been discussed in detail in relation to the kinetics of action of various agents in intact tissues (e.g. atropine, Rang, 1966; tetrodotoxin, Colquhoun, Henderson & Ritchie, 1972). The magnitude of the effect can be estimated by measuring the effect on the time course of the m.e.p.c. of inactivating a fraction of the receptors with agents such as tubocararine or α-bungarotoxin. This would be expected to reduce the number of transmitter molecules bound, and hence to increase the rate of diffusion of transmitter out of the synaptic cleft (Katz & Miledi, 1973*b*; Magleby & Terrar, 1975).

Effect of tubocurarine on m.e.p.c. decay rate

The binding of AMECh compared with that of ACh was studied in a series of experiments in which normal or false m.e.p.c.s were recorded in the presence of neostigmine, and the effect on their time course of bringing close to the end-plate a fairly coarse micropipette containing 20 mM tubocurarine was observed. The m.e.p.c.s were usually reduced to unrecordable levels but recovered gradually in 5–10 min when the pipette was removed. Records were taken at intervals during this recovery phase and the mean amplitude and time constant of batches of 8–15 m.e.p.c.s were calculated. The relative amplitude (α) and the relative time constant (β) were calculated with respect to the m.e.p.c.s recorded before the tubocurarine pipette was applied. Most of these experiments were done with the membrane clamped at -100 mV, which usefully increased the m.e.p.c. amplitude compared with measurements at -80 mV. Some experimental records are shown in Fig. 14*A*, and it can be seen that tubocurarine had a greater effect on the time course of normal m.e.p.c.s than of false m.e.p.c.s, a result that suggests that the binding of AMECh is normally less than that of ACh.

With certain assumptions these results can be used to calculate b, the fraction of transmitter molecules bound when the transmitter packet is released into the synaptic cleft. These assumptions are: (*a*) that the transmitter molecules equilibrate rapidly (with respect to the rate of diffusion) with the available receptors (i.e. those receptors not occupied by tubocurarine); (*b*) that the transmitter escapes from the cleft at a rate proportional to the free concentration of transmitter molecules; (*c*) that the cleft can be regarded as a single well-stirred compartment; (*d*) that the fraction of receptors occupied by transmitter molecules is directly proportional to the free transmitter concentration, i.e. that the occupancy of receptors by transmitter molecules does not approach saturation and is not cooperative; (*e*) that the amplitude of the end-plate current at any

moment is directly proportional to the number of transmitter-receptor complexes: (*f*) that during the time taken for the m.e.p.c. to decay no appreciable dissociation of tubocurarine can take place; and (*g*) the peak end-plate current occurs sufficiently early that a negligible amount of the released transmitter has diffused out of the cleft.

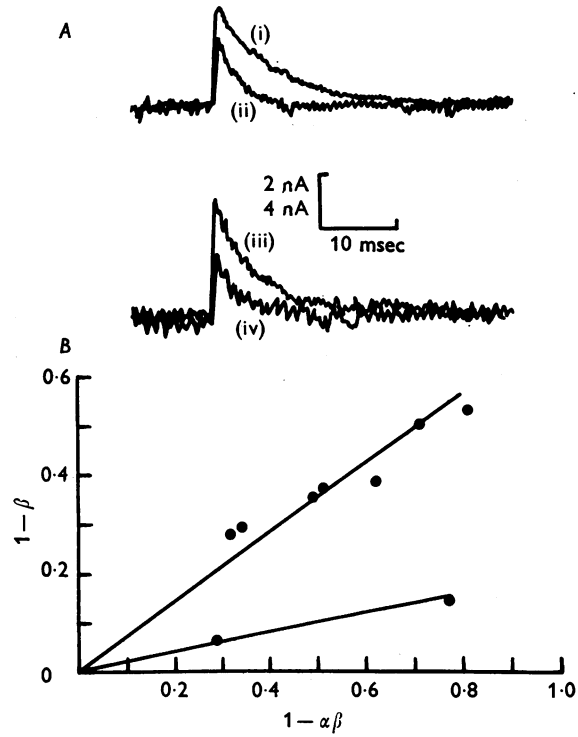

Fig. 14. Effect of (+)-tubocurarine on m.e.p.c.s in rat muscle previously exposed to 3 μM neostigmine. *A*: records of normal m.e.p.c.s (i) and (ii) and false m.e.p.c.s (iii) and (iv) before (i) and (iii) and after (ii) and (iv) the application of (+)-tubocurarine. Vertical calibration is 4 nA for (i) and 2 nA for (ii)–(iv). Note that the reduction in amplitude produced by (+)-tubocurarine was accompanied by a greater shortening of the time course of the normal m.e.p.c.s than of the false m.e.p.c.s. *B*: from the experiment illustrated in 14*A*. The slope of the relation $(1-\beta)/(1-\alpha\beta)$ is 0·70 for the normal m.e.p.c.s and 0·20 for false m.e.p.c.s. (see text for full explanation of the analysis).

From assumptions (*a*), (*d*) and (*f*), the fraction of receptors occupied by transmitter (A) molecules at time t, $p_A(t)$, is given by

$$p_A(t) = x_A(t)\,(1-p_c)/K, \qquad (3)$$

where $x_A(t)$ is the free concentration of transmitter, p_c is the fraction of

receptors occupied by tubocurarine and K is the equilibrium constant for the binding of the transmitter. If $y(t)$ moles of transmitter (bound and free) are present in a compartment of volume V, which contains M moles of receptor sites then

$$x_A(t) = [y(t) - Mp_A(t)]/V. \tag{4}$$

Combining eqns. (3) and (4) gives

$$p_A(t) = y(t)\,(1-p_c)/[KV + (1-p_c)M]. \tag{5}$$

The relative amplitude, α, of the end-plate current in the presence and absence of tubocurarine should, from assumptions (e) and (g), be given by the ratio of the value of $p_A(0)$ with tubocurarine to that in its absence. Thus, from eqn. (5)

$$\alpha = \frac{[(M/KV)+1](1-p_c)}{M(1-p_c)/KV+1}. \tag{6}$$

The time constant, τ, for escape of transmitter from the compartments in the absence of tubocurarine can be found from eqn. (6) of Rang (1966), or more directly from its solution, eqn. (3) of Colquhoun & Ritchie (1972), by supposing that $x_A(t) \ll K$, as implied by assumption (d).

The result is

$$\tau = \tau_0(M/KV+1), \tag{7}$$

where τ_0 is the time constant that would be found if there were no binding. The ratio (β) of the τ value in the presence of tubocurarine to that in its absence, from eqn. (7) and assumption (f), is thus

$$\beta = \frac{[M(1-p_c)/KV]+1}{M/KV+1}. \tag{8}$$

The fraction, b, of the released transmitter molecules that are bound, is $Mp_A(t)/y(t)$. In the absence of tubocurarine this is, from eqn. (5), $(M/KV)/(M/KV+1)$; combining eqns. (6) and (8) then gives the following expression for b in terms of the experimentally obtained estimates of α and β.

$$b = \frac{1-\beta}{1-\alpha\beta}.$$

This is the result given by Katz & Miledi (1973b).

Plotting $1-\beta$ and $1-\alpha\beta$ for a series of records made at different levels of curarization should thus yield a straight line with slope b. Plots of this kind for experiments with normal and false m.e.p.c.s are shown in Fig. 14B, from which it can be seen that the estimated value of b for false m.e.p.c.s was lower than that for normal m.e.p.c.s. Successful experiments were done on seven fibres with normal m.e.p.c.s. and seven fibres with false m.e.p.c.s. On each fibre estimates of α and β were obtained by averaging six to

fifteen individual m.e.p.c.s recorded at two or more levels of curarization, and a mean value of b for that fibre was obtained by pooling the results. The estimates obtained were: Normal m.e.p.c.s, $b = 0{\cdot}52 \pm 0{\cdot}04$ ($n = 7$); false m.e.p.c.s, $b = 0{\cdot}21 \pm 0{\cdot}04$ ($n = 7$).

The question to which these measurements were directed was whether the faster decay of false m.e.p.c.s compared with normal m.e.p.c.s in the presence of neostigmine could be accounted for by a difference in the binding of the two transmitters. The difference in b found for normal and false m.e.p.c.s appears to be of the right magnitude to explain the difference in time course. Thus, for ACh, the fraction of transmitter molecules $(1-b)$ that are not bound and are therefore free to diffuse is 0·48, while for AMECh the unbound fraction is 0·79. Crudely, the rates of diffusion of the two transmitters should therefore be in the ratio $0{\cdot}79/0{\cdot}48 = 1{\cdot}65$, which corresponds with the relative rates of decay of false and normal m.e.p.c.s in the presence of neostigmine ($3{\cdot}60/2{\cdot}18 = 1{\cdot}65$; Table 4).

Although the results strongly suggest that AMECh binds less strongly to the receptors than ACh, it should be realized that the quantitative analysis rests on assumptions that are probably much too simple. It is, for example, known that the increase in conductance is approximately proportional to the square or cube of the ACh concentration (for references see Rang, 1975), so that assumptions (*d*) and/or (*e*) must be incorrect. Furthermore, the time course of m.e.p.c.s becomes highly variable as well as longer, in the presence of neostigmine, presumably because the diffusion distance depends on the site of release of the transmitter quantum within the synaptic gutter (Katz & Miledi, 1973*b*). This would imply that assumption (*c*) is an oversimplification, as are (*a*) and (*g*). There is at present no realistic way of allowing for these complicating factors.

Since M and V are the same for the two transmitters, and

$$M/KV = b/(1-b),$$

the ratio of the equilibrium constants for the two transmitters, $K_{\text{false}}/K_{\text{normal}}$ can be calculated from

$$K_{\text{false}}/K_{\text{normal}} = \frac{b_{\text{normal}}(1-b_{\text{false}})}{b_{\text{false}}(1-b_{\text{normal}})}$$

For the values given above $K_{\text{false}}/K_{\text{normal}} = 4{\cdot}08$, i.e. the affinity of AMECh for the receptors is 0·25 times that of ACh. The relative numbers of AMECh molecules and ACh molecules that are bound ($b_{\text{normal}}/b_{\text{false}} = 2{\cdot}48$) is not a direct measure of the relative binding affinities because of the 'buffering' effect of the receptors within the synaptic cleft.

DISCUSSION

The results described in this paper show that stimulation of rat or frog nerve-muscle preparations in the presence of MECh leads to: (*a*) a decrease in amplitude, and (*b*) an increase in decay rate (but little change in growth rate) of the conductance change produced by one quantum of transmitter. Because it was necessary to increase transmitter turnover in the pre-synaptic nerve terminals in order to see the effect, it seems unlikely that the briefer conductance change results from a direct effect of MECh on the post-synaptic membrane. The fact that hemicholinium did not affect the time course of e.p.p.s in rat muscle shows that the shortening seen with MECh cannot be attributed simply to inhibition of ACh synthesis.

Reitzel & Long (1953) studied the ability of various choline analogues to restore neuromuscular transmission in cats injected with hemicholinium. They found that MECh had some restorative effect, though less than choline, and suggested that it might give rise to an active false transmitter. Recently Collier *et al.* (1976) have demonstrated, by biochemical methods, that cat sympathetic ganglia, following exposure to MECh, release AMECh in response to nerve stimulation, and that this release is Ca dependent. Therefore the most plausible explanation for our results is that nerve terminals at the neuromuscular junction can take up, and acetylate, MECh to form AMECh, which is subsequently released either spontaneously, in quantal fashion, or in response to nerve stimulation; i.e. AMECh is a false transmitter.

The reduced amplitude of false m.e.p.c.s could be explained in several ways. (1) The number of molecules of AMECh in a quantum of false transmitter could be lower than the number of ACh molecules in a normal quantum; (2) the affinity of AMECh for the receptors could be lower than that of ACh; (3) AMECh could have lower efficacy than ACh, i.e. the probability that occupation of a receptor results in opening of an ionic channel could be lower, and (4) the channel opened by AMECh could have a lower conductance than that opened by ACh. The current fluctuation measurements imply very similar single channel conductances (about 25 pS, very close to that observed in frog muscle) for both ACh and AMECh, so the fourth possibility cannot be the explanation. It is difficult to test the first hypothesis by electrophysiological methods, but in biochemical studies Collier *et al.* (1976) have shown sympathetic ganglia can synthesize radioactivity labelled ACh and AMECh at similar rates, and that stimulation (2 min at 20 Hz) causes the same fractional release of the two transmitters; synaptosomes from brain tissue also take up and acetylate choline and MECh at the same rate (Barker & Mittag, 1975).

These results suggest that the first possibility is also unlikely. The following evidence suggests that the second possibility (low affinity) rather than the third (low efficacy) possibility is the major reason for the reduced size of false m.e.p.c.s. Our experiments with tubocurarine, in the presence of a cholinesterase inhibitor, suggest that about 21 % of a quantum of false transmitter is bound to the post-synaptic receptors, compared with about 52 % of a normal quantum. If the buffering effect is taken into account (see Results) this means that the affinity of AMECh for the receptors is about one quarter of the affinity of ACh. This factor of four is close to the ratio (roughly five) of concentrations of ACh and AMECh that has to be applied to the end-plate to produce similar currents in the noise experiments, and is also similar to the relative potency of 5 found for these two agonists in causing contracture of the frog *rectus abdominis* muscle (Holton & Ing, 1949). If the efficacies of the two transmitters were equal, it might be expected that the relative amplitudes of normal and false m.e.p.c.s would directly reflect the difference in binding, e.g. that false m.e.p.c.s would be 40 % (0·21/0·52) of the amplitude of control m.e.p.c.s. Actually, the amplitude of false m.e.p.c.s was 66 % of controls. It should be realized, though, that the peak current represents a transient rather than an equilibrium state (see Dionne & Stevens, 1975) and its magnitude depends *inter alia* on the rate constant for channel opening, which is not known for AMECh.

It is unlikely that differences between the rate of hydrolysis of ACh and AMECh by acetylcholinesterase are an important factor determining the relative amplitudes of normal and false m.e.p.c.s. Biochemical measurements have generally found that both esters are equally rapidly hydrolysed (Holton & Ing, 1949; Collier *et al.* 1976). Moreover, neostigmine had only a small effect on the amplitude of normal and false m.e.p.c.s.

The observation that false m.e.p.c.s are briefer than normal could also have several explanations. The decay phases of both normal and false m.e.p.c.s showed no consistent deviation from a single exponential, and the time constant for decay of false m.e.p.c.s was about 58 % of that for normal m.e.p.c.s.

It is thought that the decay rate of normal m.e.p.c.s is controlled entirely (Anderson & Stevens, 1973) or primarily (Katz & Miledi, 1973*b*) by the lifetime of open channels (rather than by the time course of transmitter concentration in the synaptic cleft) on the grounds that the mean channel lifetime inferred from noise analysis is equal to, or not much greater than, the time constant for m.e.p.c. decay. Our analysis of current fluctuations from voltage-clamped rat diaphragm end-plates suggests that the lifetime of the channel, when it is opened by AMECh, is about 56 % of its lifetime when opened by ACh. This is very close to the relative decay rates (58 %) of

the normal and false m.e.p.c.s. Moreover, the potential dependence (about 107 mV for an e-fold change) was found to be similar for $\tau_{m.e.p.c.}$ (false), $\tau_{m.e.p.c.}$(normal), τ_{ACh} and τ_{AMECh} (noise); the last three also had a similar dependence on temperature (Q_{10} about 3). This reinforces the idea that the same phenomenon, channel closure, is the main factor controlling all of these rates. These values are close to those observed in frog and toad muscle (Takeuchi & Takeuchi, 1959; Kordaš, 1972; Magleby & Stevens, 1972*a*, *b*; Anderson & Stevens, 1973; Gage & McBurney, 1975). There thus seems to be little doubt that the relative brevity of false m.e.p.c.s results primarily from the briefer lifetime of the channel opened by AMECh compared with that opened by ACh.

In the light of these results, it was perhaps surprising to find that the absolute value of the channel lifetime for ACh was apparently slightly shorter than the decay time constant for m.e.p.c. The discrepancy was about 20–50 % and depended rather consistently on the method used for fitting spectral density curves (see Results). The reason for this appeared to be that the curves showed a slight deviation from the single Lorentzian form. Whether this deviation is genuine or artifactual (see Results), it is not possible at present to assess the minor errors that may occur in the estimation of mean channel lifetime from such spectra, or to interpret in terms of mechanism the rather small apparent discrepancy between $\tau_{m.e.p.c.}$ and τ_{ACh}. Katz & Miledi (1973*b*) observed a rather larger discrepancy of this sort, and attributed it to the persistence of transmitter in the synaptic cleft for long enough to lengthen the m.e.p.c. slightly.

The growth times for normal and false m.e.p.c.s were similar, but the factors controlling the growth rate are not sufficiently well understood for this to be interpreted with any certainty. In so far as the rate is diffusion controlled, this result is expected because ACh and AMECh are not likely to have appreciably different diffusion coefficients.

Measurements of potentials in unclamped end-plates showed that the amplitude of false m.e.p.p.s was 50–55 % of normal m.e.p.p.s, and that of false e.p.p.s, in curarized muscle, was 30–40 % of normal. The latter figure may be smaller than the former because of reduced quantal contents after MECh treatment, or because m.e.p.p. and e.p.p. arise from different pools of transmitter. The observed reduction of m.e.p.p. amplitude (to 50–55 % of normal) is actually rather less than expected from the product of the reductions by 0·66 in m.e.p.c. amplitude, and by 0·58 in m.e.p.c. decay rate. It may be relevant, however, that unless the membrane time constant is long relative to the open lifetime of the channel, reduction of channel lifetime by a given factor will result in an elementary voltage pulse that is reduced by a smaller factor (Colquhoun, 1975, eqn. (3)).

These experiments suggest an approach to various questions about the

mechanism of transmitter synthesis in the nerve terminals. During the incorporation of AMECh into the stores, it might be expected that empty vesicles would be refilled with AMECh, so that the store would consist of a mixture of ACh and AMECh quanta giving rise to a bimodal distribution m.e.p.c.s. In experiments where ACh synthesis was completely blocked by hemicholinium, Elmqvist & Quastel (1965) found that the m.e.p.p amplitude decreased uniformly, suggesting that under these conditions the available transmitter remains evenly distributed throughout the population of vesicles. If the same thing happens when ACh is being replaced by AMECh, there should be no stage at which heterogeneity of the released quanta is evident. On the simplest model, when the total vesicle population has been turned over once the replacement of transmitter should be 63 % $(1 - 1/e)$ complete. From the studies on frog muscle (Fig. 6) it appears that the transition is 63 % complete when only about 10^5 quanta have been released, whereas Ceccarelli *et al.* (1973) working with the same muscle found that about 5×10^5 quanta could be released by black widow spider venom. Thus it appears that the transmitter pool into which AMECh is initially distributed is a good deal smaller than the total available pool, and it should be possible to devise kinetic experiments to study the kinetics of the exchanges between newly synthesized transmitter and the various pools of stored quanta.

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