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EFFECTS OF MEMBRANE POTENTIAL, TEMPERATURE AND NEOSTIGMINE ON THE CONDUCTANCE CHANGE CAUSED BY A QUANTUM OF ACETYLCHOLINE AT THE TOAD NEUROMUSCULAR JUNCTION

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SUMMARY

1. Miniature end-plate currents were recorded at neuromuscular junctions of toads, either in voltage-clamped fibres or with extracellular electrodes. The two methods gave similar results.

2. Two types of m.e.p.c.s, differing in their growth times (50–300 μsec and 0·5–5 msec) were found. The more frequent had the shorter growth times.

3. The decay of m.e.p.c.s was exponential with a single time constant. The time constant was an exponential function of membrane potential, becoming less as the membrane was depolarized. In contrast, there was little change, or in some cases an increase, in the growth times of m.e.p.c.s when the membrane was depolarized.

4. The decay time constant had a Q_{10} of $3{\cdot}13 \pm 0{\cdot}22$ (mean $\pm$ S.E. of mean) whereas the growth time had a significantly lower Q_{10} of about 1·2. The change of decay time constant with temperature followed the Arrhenius equation giving an activation energy of $18 \pm 1{\cdot}0$ kcal (mean $\pm$ S.E. of mean). The amplitude of m.e.p.c.s increased with temperature and had a Q_{10} of $1{\cdot}5 \pm 0{\cdot}14$ (mean $\pm$ S.E. of mean) in voltage-clamped fibres.

5. Neostigmine prolonged the decay phase and increased the amplitude of m.e.p.c.s but had little effect on the growth phase. The changes in m.e.p.c.s caused by membrane potential and temperature were not affected by neostigmine.

6. The results show that the growth phase and decay phase of m.e.p.c.s are governed by processes with quite different characteristics. The reaction which limits the decay phase appears first-order, is voltage sensitive and

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has a Q_{10} of about 3, whereas the reaction underlying the growth phase does not appear first-order, is less voltage-sensitive and has a Q_{10} of about 1·2. It is suggested that diffusion of transmitter across the synaptic cleft may be the rate-limiting step during the growth phase.

INTRODUCTION

The interaction of acetylcholine with post-synaptic receptors at the neuromuscular junction causes an increase in the sodium and potassium conductance of the end-plate membrane and a net, inward current flow. When the membrane potential is constant, the time course of this end-plate current reflects the time course of the underlying conductance change.

It has been found that the time course of the later part of the decay phase of end-plate currents (e.p.c.s) is exponential (Takeuchi & Takeuchi, 1959; Magleby & Stevens, 1972*a*; Kordas, 1972*a*), is affected by membrane potential (Takeuchi & Takeuchi, 1959; Kordas, 1969; Magleby & Stevens, 1972*a*) and has a Q_{10} of 2–3 (Takeuchi & Takeuchi, 1959; Magleby & Stevens, 1972*a*; Kordas, 1972*b*). These results have formed the basis of some interesting theories about the mechanisms responsible for end-plate conductance changes (Magleby & Stevens, 1972*a*, *b*; Kordas, 1972*a*, *b*). At the same time as some of these results were being obtained, we were studying factors which affected miniature end-plate currents (m.e.p.c.s). Although the signal-to-noise ratio is much less favourable for m.e.p.c.s than for e.p.c.s, it was thought that the advantages outweighed the disadvantages for the following reasons.

If the time course of e.p.c.s in voltage-clamped fibres is to be used to analyse the kinetics of post-synaptic events leading to a conductance change, the membrane potential over the whole of the end-plate region must be well controlled, and the time course of transmitter release (the release function) must be known and taken into account. From the results of Katz & Miledi (1965*a*, *b*) and Barrett & Stevens (1973) it is clear that the release function must contribute considerably to the growth phase and the early part of the decay of e.p.c.s. Also, it has been found that it is often impossible to voltage-clamp the whole of an end-plate region with two micro-electrodes (Gage & McBurney, 1972). Therefore, we report here the effects of membrane potential, temperature and neostigmine on miniature end-plate currents generated by a single quantum of transmitter. This avoids the problems of the release function and the necessity to space-clamp the whole of the end-plate region. Unclamped m.e.p.c.s can be recognized (Gage & McBurney, 1972) and rejected. Another advantage of recording m.e.p.c.s rather than e.p.c.s is that we often recorded two rather

different types of m.e.p.c.s with different growth times, as will be described below. Presumably, both would contribute to e.p.c.s.

Notwithstanding the above reservations, many of the observations on the decay phase of m.e.p.c.s confirm previous observations on the later part of the decay of e.p.c.s. However, measurements of the growth phase of m.e.p.c.s, which is not affected by asynchronous release of quanta, show that it has different properties from the decay phase. The time course of the growth phase has a low Q_{10} (less than 1·5) and is not very sensitive to membrane potential. Thus the processes which determine the time course of the onset and decay of the end-plate conductance change caused by acetylcholine have different characteristics. Some of the results have been described briefly elsewhere (Gage & McBurney, 1971, 1972; McBurney, 1973; McBurney & Gage, 1973; Caldwell, Gage & McBurney, 1974).

METHODS

The preparation used in these experiments was the isolated sartorius muscle from the cane toad (*Bufo marinus*). Some of the techniques, and the standard Ringer solution, have been described previously (Gage & McBurney, 1972). Modifications and additional techniques were as follows.

Membrane potential was recorded differentially between an intracellular microelectrode (3 M-KCl, 2–10 MΩ) and an extracellular macroelectrode (3 M-KCl in agar). This was done to eliminate the possibility of any errors in the recording of membrane potential caused by DC 'holding' currents polarizing the standard ground electrode (3 M-KCl in agar).

Extracellular m.e.p.c.s were recorded using macroelectrodes filled with 1 M-NaCl in agar. These electrodes generally had resistances of 0·2–1 MΩ and tip diameters of 10–100 μm. Care was taken when placing these electrodes over junctional regions not to press too hard against the muscle fibre. In this way it was hoped that possible alterations in the time course of extracellularly recorded m.e.p.c.s (Katz & Miledi, 1973) would be avoided.

The temperature of preparations was controlled (within 0·2° C) by circulating an alcohol–water mixture from a refrigerated unit (Lauda) through a jacket which surrounded the muscle bath. The temperature in the muscle bath (usually set at 20° C) was monitored by a small thermistor which was placed as close as possible to recording electrodes.

Two new methods were used to 'capture' low frequency, spontaneous m.e.p.c.s. In some experiments, m.e.p.c.s were captured by use of an on-line laboratory computer (LAB 8/I, Digital Equipment) which was programmed to store and display the m.e.p.c.s in a stationary position on the oscilloscope screen. These m.e.p.c.s could be photographed and, if desired, 'hard copies' of them could be obtained in digital form on paper tape for further analysis. In later experiments a hard-wired device which performed the same function (Neurograph N3, Transidyne General Corp.) was used to capture m.e.p.c.s.

Usually, the time course of m.e.p.c.s was measured from film of oscilloscope traces obtained during an experiment. On some occasions, however, the digital form of the m.e.p.c.s was analysed by computer. Measurements were made from the mid-point of each section of a trace magnified with a film projector. An m.e.p.c. has normally

two main phases consisting of a relatively rapid increase to a maximum (peak) current and a slower decrease in current. Because inward currents are conventionally shown as downward deflexions, we have called these two phases the 'growth phase' and 'decay phase' respectively, rather than 'falling' and 'rising' phases. Because the growth phase of m.e.p.c.s tended to be S-shaped, and the base-line noise obscured the early part of this phase, the 'growth time' was measured as the time for an m.e.p.c. to increase from 20 to 80 % of its maximum amplitude. Using the Neurograph, it was possible to 'sweep out' individual m.e.p.c.s after they had been captured and hence accurate measurements could be made of the growth time. The decay phase of m.e.p.c.s was analysed by a log-linear (least squares) regression analysis (logarithm of the amplitude of the current against time).

Neostigmine ('Prostigmine', Roche) was added to standard Ringer solution to give the stated concentrations, and new neostigmine solutions were made every 2–3 days. In experiments in which fibres were to be voltage-clamped at positive potentials, muscles were first glycerol-treated (see e.g. Gage & McBurney, 1972).

RESULTS

Two types of miniature end-plate currents

In many fibres two types of m.e.p.c.s were recorded. The usual difference was in their growth phase though occasionally their decay phases were also different. The 'normal' m.e.p.c.s which were more frequent had growth times (see Methods) of 50–300 μsec. In contrast 'slow' m.e.p.c.s had growth times of 0·5–5 msec. Slow m.e.p.c.s were not found in all fibres. Rarely, some fibres had only slow m.e.p.c.s.

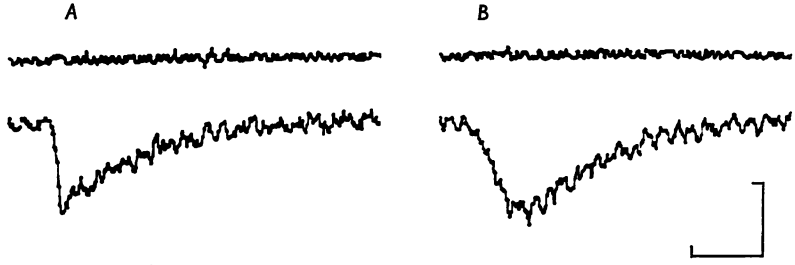


Fig. 1. Miniature end-plate currents recorded in a voltage-clamped fibre. The upper traces monitor membrane potential and the lower traces show the m.e.p.c.s. A normal m.e.p.c. with a relatively fast growth phase is shown in *A*, and a 'slow' m.e.p.c. with slower growth phase is shown in *B*. Calibrations: horizontal, 4 msec; vertical, 1 mV for voltage, 4 nA for current.

As described previously (Gage & McBurney, 1972), normal m.e.p.c.s generally decayed exponentially from the peak with a single time constant. Occasionally, rounded peaks lasting up to 200 μsec were seen. In most cases the later part of the decay phase of slow m.e.p.c.s also conformed to a single exponential with a time constant in the range found for the decay

phase of normal m.e.p.c.s in the same fibre at the same membrane potential. However the decay phase of some slow m.e.p.c.s was considerably slower than that of normal m.e.p.c.s. A normal and a slow m.e.p.c. recorded at the same membrane potential (-70 mV) in a voltage-clamped fibre are shown in Fig. 1. The normal m.e.p.c. (*A*) had a growth time of 170 μsec and the slow m.e.p.c. (*B*) had a growth time of 1·5 msec. It can be seen that the later part of the decay phase of the slow m.e.p.c. has a similar time course to the decay phase of the normal current.

Extracellular and intracellular miniature end-plate currents

The results described below were obtained from records of normal m.e.p.c.s, recorded either extracellularly, or intracellularly in voltage-clamped fibres. The time courses of m.e.p.c.s obtained with either method were very similar. The decay phases of m.e.p.c.s, whether intracellular or extracellular, were exponential from the peak and gave correlation coefficients (log-linear least squares fits) greater than 0·9. For example, in one fibre in which 71 m.e.p.c.s were recorded extracellularly at temperatures which ranged from 10 to 22° C, the mean correlation coefficient was 0·9985 (S.E. of mean = 0·0003). Peaked m.e.p.c.s, which were recorded intermittently in some voltage-clamped fibres when the voltage trace betrayed limitations in the spatial control of potential (Gage & McBurney, 1972), were never seen in extracellularly recorded m.e.p.c.s. The growth phase of m.e.p.c.s recorded in voltage-clamped fibres was generally more linear (less S-shaped) than in extracellular m.e.p.c.s. Therefore, because extracellular recording does not have the high-frequency limitations of the voltage-clamp technique, measurements of the growth phase were generally made with extracellular m.e.p.c.s, except when testing the effects of membrane potential when it was necessary to measure the growth phase of m.e.p.c.s in voltage-clamped fibres.

The similar time courses of well-clamped intracellular m.e.p.c.s and extracellularly recorded m.e.p.c.s confirm the previously described differentiation between 'clamped' and 'unclamped' m.e.p.c.s in voltage-clamped fibres (Gage & McBurney, 1972). The results also indicate that the exponential decay of m.e.p.c.s from the peak is real, and not an artifact introduced in some way by the voltage-clamp technique.

Effects of membrane potential on m.e.p.c.s

Decay phase

The effects of membrane potential on m.e.p.c.s were examined in voltage-clamped fibres only. These were generally glycerol-treated (see e.g. Gage & McBurney, 1972) to prevent activation of contraction during depolarization. At all clamp potentials ($+50$ to -150 mV) miniature

end-plate currents had an exponential decay over the whole of the measurable decay phase. Because of the variability in the time constants of miniature end-plate currents recorded at a single junction at any potential (Gage & McBurney, 1972) the decay time constants of five to twenty m.e.p.c.s were averaged to give a mean decay time constant for any one membrane potential. The results of one of the experiments in which the clamp potential was varied are shown in Fig. 2. Inset are shown representative m.e.p.c.s at four different clamp potentials (+56, −40, −70 and

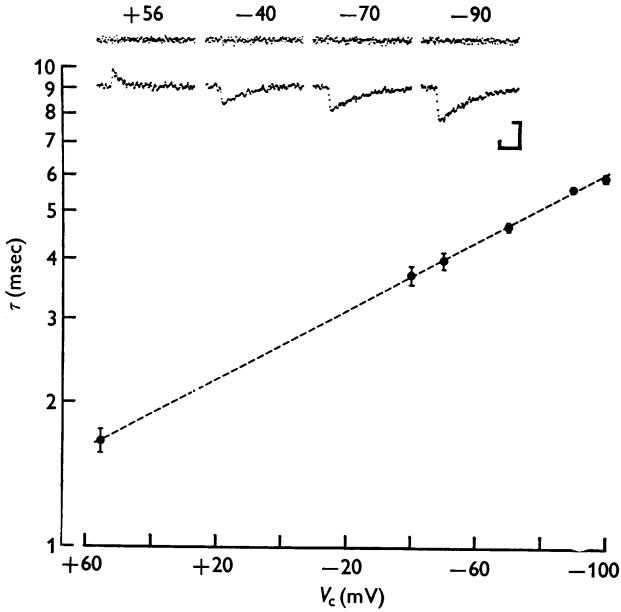


Fig. 2. The relationship between clamp potential and the time constant of decay of m.e.p.c.s is exponential. Inset are shown records obtained in one fibre at the clamp potentials indicated. Horizontal calibration, 4 msec. Vertical calibration, 1 mV or 4 nA. The graph shows the mean ± S.E. of mean of the decay time constant of m.e.p.c.s at various clamp potentials in this fibre. Note the logarithmic ordinate (15 °C).

−90 mV). The top traces (inset) show the membrane potential during the m.e.p.c.s and the lower traces show the m.e.p.c.s. The exponential relationship reported by Magleby & Stevens (1972*a*) for the tail of end-plate currents was found with miniature end-plate currents (see also, Anderson & Stevens, 1973) as can be seen from the graph (Fig. 2) in which mean decay time constant (τ) is plotted (semilog plot) against clamp potential. This relationship between the rate of decay of m.e.p.c.s and membrane potential can be described by the equation (cf. Magleby & Stevens, 1972*a*).

$$\tau(V) = \tau(0).\exp(V/H),$$

where $\tau(V)$ is the time constant of the decay phase at clamp potential, V, $\tau(0)$ is the time constant at clamp potential of zero, and H is a constant which indicates the voltage sensitivity of the decay phase. Values for $\tau(0)$ and H obtained in thirteen preparations in this way are shown in Table 1. The mean values with S.E. of means were $\tau(0) = 1{\cdot}38 \pm 0{\cdot}09$ msec, $H = -102{\cdot}5 \pm 3{\cdot}1$ mV (20° C). These values are in the range of those reported for e.p.c.s ($\tau(0) = 0{\cdot}36$ to $1{\cdot}88$ msec, $H = -84$ to -185 mV at 25° C; Magleby & Stevens, 1972*a*).

TABLE 1. The effect of membrane potential on the decay phase of m.e.p.c.s

Fibre	Temp. (° C)	$\tau(0)$ (msec)	H (mV)
*41–1	26	0·62	−94
45–4	20	1·06	−103
*93–2	15	2·63	−122
117–2	20	0·88	−91
125–1	20	1·40	−105
125–2	20	1·35	−104
126–2	20	1·08	−83
131–1	20	1·78	−99
132–1	20	1·25	−90
152–2	20	1·70	−105
182–1	20	1·30	−108
192–1	20	1·60	−111
192–3	20	1·80	−118
	Mean	1·38	−102·5
	S.E. of mean	0·09	3·1

* Not used in calculations of mean or S.E. of mean of $\tau(0)$ because of temperature differences.

Growth phase

There was not a comparable effect of clamp potential on the growth time (20–80 % of peak amplitude) of miniature end-plate currents. Results obtained in ten preparations are shown in Table 2. In four of the ten experiments, there was a statistically significant ($P < 0{\cdot}01$, Student's t) *increase* in growth time when the membrane potential was depolarized 100 mV. In the other six, there was no significant change. With the same change in clamp potential in the same experiments, the time constant of decay of the m.e.p.c.s *decreased* by a factor of 2–3 and this change in all ten preparations was clearly significant ($P < 0{\cdot}001$, Student's t). It is possible that a significant increase in growth times of m.e.p.c.s with depolarization would have been found in all cases if more m.e.p.c.s had been recorded (ten to twenty m.e.p.c.s were measured at each membrane potential in these experiments). The variance in growth times at any one potential would tend to lessen the significance of real differences. However,

it can be concluded that membrane depolarization had little effect or, if anything, increased the growth time of m.e.p.c.s in contrast to the significant decrease in the time constant of the decay phase.

TABLE 2. The effect of membrane potential on the growth phase of m.e.p.c.s

Fibre	Clamp potential (mV)	Growth time (μsec) Mean	S.E. of mean	P (Student's t)
5–3	−80	159	8	
	−60	130	10	
	−40	136	13	
5–6	−80	132	9	
	−40	134	16	
21–3	−50	86	7	
	+50	146	10	< 0.001
21–7	−50	95	8	$0.05 < P < 0.1$
	+50	142	14	
21–9	−50	120	25	
	+50	150	27	
22–4	−50	156	16	
	+50	182	23	
22–6	−50	118	15	$0.01 < P < 0.02$
	+50	176	16	
22–7	−50	125	21	$0.001 < P < 0.005$
	+50	207	10	
22–8	−50	120	13	$0.001 < P < 0.005$
	+50	181	18	
	−50	133	13	
23–2	−50	98	10	
	+50	116	16	

Effects of temperature on m.e.p.c.s

Decay phase

The decay phase of miniature end-plate currents was very sensitive to changes in temperature. Graphs of the variation of decay time constants with temperature are shown in Fig. 3 at two clamp potentials, −90 mV (above) and −65 mV (below), at the one junction. The Q_{10}'s measured from these two graphs were 3·5 at −90 mV and 3·0 at −65 mV (10–20° C). At 12 voltage-clamped junctions Q_{10}'s ranged from 1·88 to 4·6 (mean, 3·13; S.E. of mean, 0·22). The results are shown individually in Table 3. In three experiments in which m.e.p.c.s were recorded extracellularly, the Q_{10}'s of the decay time constants were 2·4 (Fig. 6), 2·4 and 1·9, which are within the range obtained in voltage-clamp experiments.

As the Q_{10} of a process gives only an indication of its temperature sensitivity and depends on the range over which it is obtained (Q_{10}'s

measured at high temperatures are less than Q_{10}'s measured at low temperatures), we decided to determine whether the temperature sensitivity of the decay was consistent with the Arrhenius equation which describes the relationship between temperature and the rate of a single reaction.

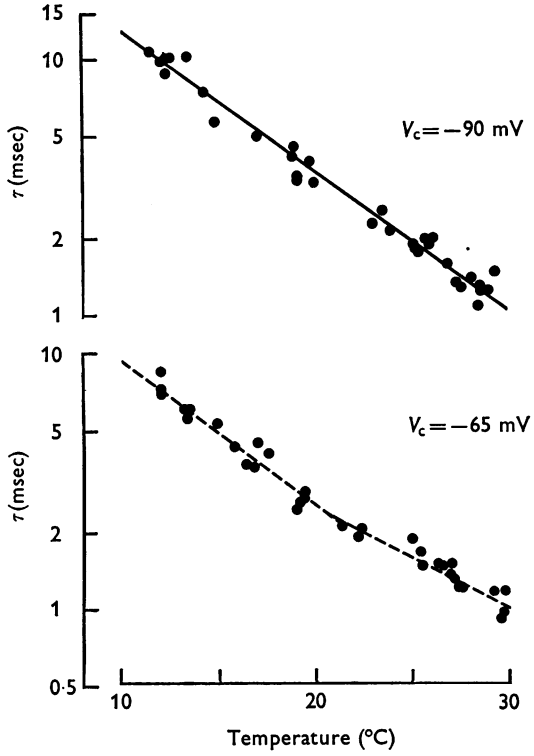


Fig. 3. The effect of temperature on the time constant of decay of m.e.p.c.s (τ, msec). The results for this graph were obtained in one fibre at the two clamp potentials indicated. Note the logarithmic ordinates.

The Arrhenius equation may be written (for our purposes) in the form

$$-\ln \tau = \ln A - E_a/RT,$$

where $1/\tau$ is the rate constant of a hypothetical first-order reaction controlling the decay phase, A is a constant, E_a is the activation energy for the reaction, T is absolute temperature in degrees Kelvin, and R is the universal gas constant. Graphs were made of $-\ln \tau$ against $1/T$ ($°K^{-1}$) and a least-squares regression line fitted to the points. In some fibres, in which there was not too much variation in τ, good correlation coefficients ($> 0{\cdot}95$) were obtained. The slope of a regression line gave E_a, and the intercept at $1/T = 0$ gave $\ln A$. Results obtained in one of these fibres are shown

in Fig. 4. The least squares regression line through the points had a correlation coefficient of 0·965. The conformity of the experimental results to the Arrhenius equation suggests that the process controlling the decay of

TABLE 3. Effect of temperature on the decay phase of m.e.p.c.s

Fibre	V_C (mV)	Range °C)	Q_{10} of τ	E_a (kcal)	ln A
92–2	−75	15–25	2·30	12·23	26·96
93–1	−90	15–25	3·04	19·05	38·65
93–2	−70	11–35	2·20	13·22	28·43
94–1	−100	17–30	2·10	14·38	30·16
95–1	−70	15–25	1·88	10·81	24·52
	−50	15–25	2·15	13·55	29·39
98–1	−90	14–22	3·25	20·00	40·56
99–1	−70	11–23	4·20	23·35	46·20
100–1	−90	10–20	4·58	23·00	45·50
	−65	10–20	4·29	22·24	44·48
100–3	−90	17–28	2·82	17·60	36·13
	−65	17–28	2·80	17·29	35·82
100–4	−90	12–19	4·60	24·57	48·10
	−65	12–19	3·86	22·28	44·42
102–1	−90	11–30	3·50	20·96	41·62
	−65	11–30	3·00	18·12	36·99
125–5	−70	10–25	2·78	15·79	33·02
		Mean	3·13	18·14	37·11
		S.E. of mean	0·22	1·04	1·80

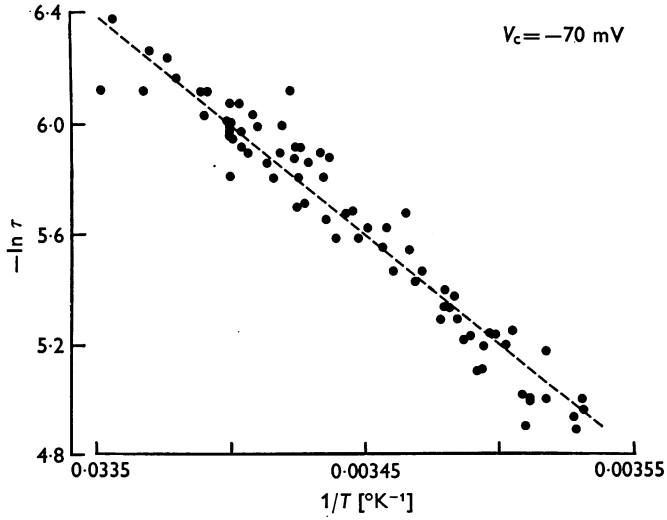


Fig. 4. An Arrhenius plot of the effect of temperature on the time constant of decay of m.e.p.c.s in a voltage-clamped fibre (V_C = −70 mV). The activation energy calculated from the regression line was 15·8 kcal in this case.

m.e.p.c.s. is indeed a single first-order reaction. The activation energy calculated from the slope in this experiment was 15·8 kcal. The values of E_a and ln A obtained in twelve voltage-clamp experiments are shown in Table 3. The mean activation energy was 18 kcal (S.E. of mean = 1·0 kcal).

Growth time

The growth phase of m.e.p.c.s recorded in voltage-clamped fibres was much less sensitive to temperature than the decay phase. In fact the growth time changed only slightly over a 20° C range of temperature. In extracellularly recorded m.e.p.c.s, a small, but statistically significant, slowing of the growth phase could be detected as the temperature was lowered. Fig. 5 illustrates the relative effects of temperature on the growth time and time constant of decay of extracellularly recorded m.e.p.c.s. The m.e.p.c. shown on two different time scales in *A* and *C* was recorded at

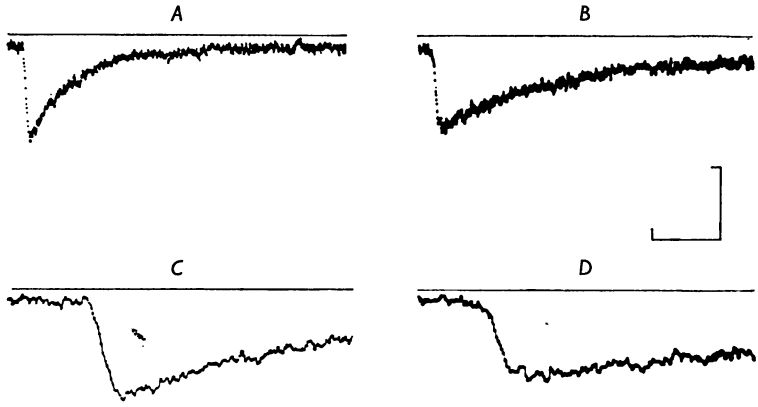


Fig. 5. M.e.p.c.s recorded extracellularly at one junction at 22·1° C (*A* and *C*) and 11° C (*B* and *D*). Calibrations: vertical, 0·5 mV; horizontal, 5 msec for the top traces (*A* and *B*) and 1 msec for the lower traces (*C* and *D*). The Figure illustrates the greater effect of temperature on the decay phase than on the growth phase.

22·1° C and has a growth time of 180 μsec and decay time constant of 3 msec. The m.e.p.c. shown in *B* and *D* was recorded shortly afterwards at the same spot when the temperature had been lowered to 11° C. The growth time had not changed (Fig. 5*D*) whereas the decay time constant (Fig. 5*B*) had increased, by a factor of 3, to 9 msec. Clearly the growth phase was less affected by temperature changes than the decay phase. The temperature sensitivity of the growth phase and decay phase of extracellularly recorded m.e.p.c.s in another experiment can be seen over a range of temperatures in Fig. 6. The least squares regression line (r = 0·92) through the points in Fig. 6*A* gave a Q_{10} of 2·4 for the decay time constant.

In Fig. 6*B* the growth times of the same m.e.p.c.s are plotted against temperature and the regression line ($r = 0{\cdot}29$) gave a Q_{10} of 1·23. In spite of the wide scatter in growth times, it is clear that temperature had much less effect on this phase of m.e.p.c.s than on the decay phase. This suggests that the onset and decay of the current may be governed by different processes: the initial phase by a process with a low activation energy, possibly diffusion, and the decay phase by a process with high activation energy, possibly a conformation change in a membrane protein (Magleby & Stevens, 1972*a*).

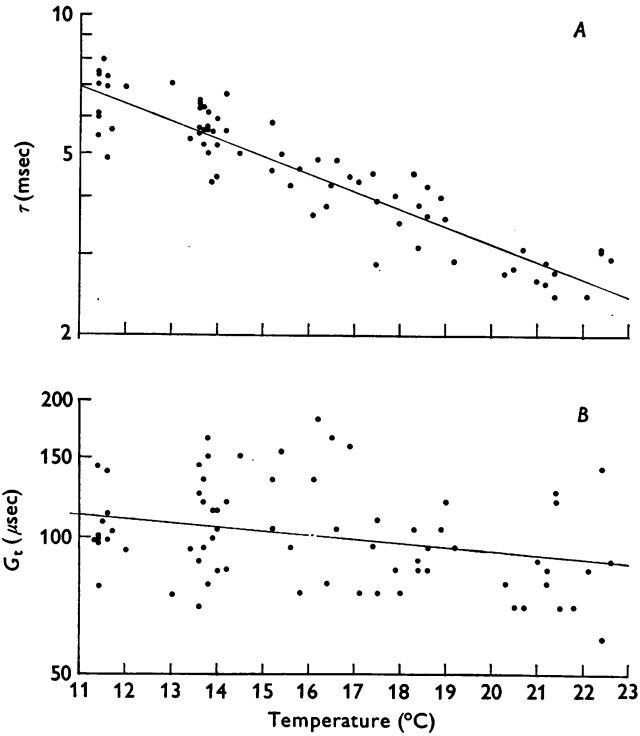


Fig. 6. Graphs of the regression with temperature, of the time constant of decay (*A*) and growth time (*B*) of extracellularly recorded m.e.p.c.s. (note the logarithmic ordinates). The regression lines gave a Q_{10} of 2·4 for the decay time constant and 1·23 for the growth time.

Amplitude

It has been reported that the magnitude of the elementary conductance change is independent of temperature (Anderson & Stevens 1973). However, the amplitude of m.e.p.c.s was clearly affected by temperature. This is shown in Fig. 7*A* and *B*. The effect of temperature on the peak heights

of m.e.p.c.s recorded extracellularly from one fibre is shown in Fig. 7*A*, and the change in the peak heights of m.e.p.c.s with temperature in a voltage-clamped fibre is shown in Fig. 7*B*. Despite the large scatter of m.e.p.c. amplitudes, the mean m.e.p.c. amplitude is clearly less at lower temperatures. The least-squares regression lines gave a Q_{10} of 1·9 in Fig. 7*A*, and 1·3 in Fig. 7*B*. In twelve voltage-clamped fibres, the Q_{10} of m.e.p.c. amplitude was 1·52 ± 0·14 (mean ± S.E. of mean). Little emphasis can be placed on the larger change in amplitude obtained with extracellular recording because of the possibility of differential movements of the electrode and tissue during temperature changes.

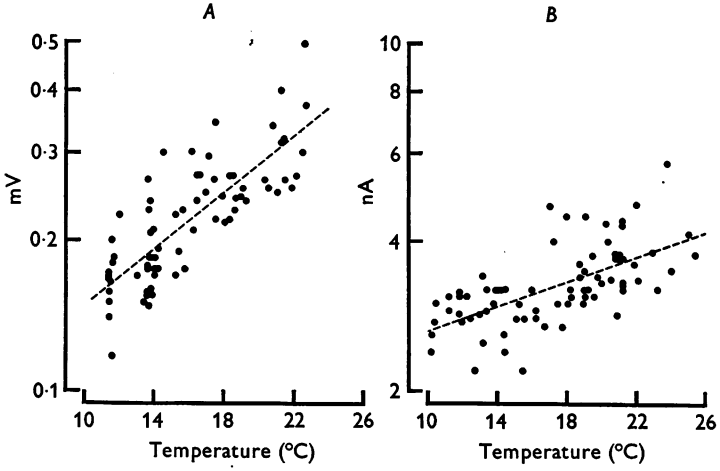


Fig. 7. The effect of temperature on the peak amplitude of m.e.p.c.s which were recorded extracellularly for *A* and in a voltage-clamped fibre for *B* (note logarithmic ordinates). The regression lines gave a Q_{10} of 1·9 in *A*, and 1·3 in *B*.

The effect of neostigmine on m.e.p.c.s

Neostigmine prolongs the decay phase of end-plate currents (Magleby & Stevens, 1972*a*; Kordas, 1972*b*; Katz & Miledi, 1973). We have examined the effects of neostigmine on m.e.p.c.s and have found that there is a considerable influence of concentration on the nature of the effect produced. With concentrations from 0·5 to 2·0 mg/l. there was, in general, an increase in the peak height of m.e.p.c.s and a prolongation of their decay phase. However the time constant of the decay phase was seldom increased by more than a factor of two in this preparation. At neostigmine concentrations greater than 2·0 mg/l., the frequency and amplitude of m.e.p.c.s was markedly reduced. The effect of the higher concentrations of neostigmine on the frequency of m.e.p.c.s appeared to be confined to normal m.e.p.c.s. The relative frequency of slow m.e.p.c.s was increased. Only results obtained

with neostigmine concentrations between 0·5 and 2·0 mg/l. are reported in detail here.

The effect of neostigmine at a concentration of 1 mg/l. on m.e.p.c.s is illustrated in Fig. 8. The m.e.p.c. shown in Fig. 8*A* was recorded in a voltage-clamped fibre in standard Ringer solution and the m.e.p.c. in Fig. 8*B* was recorded in the same fibre 30 min after commencement of an infusion of Ringer solution containing 1 mg/l. neostigmine. An increase in amplitude and prolongation of the decay phase of the m.e.p.c. in neostigmine is clear in Fig. 8*B*. However, there was no clear increase in the growth time of m.e.p.c.s recorded in voltage-clamped fibres in neostigmine.

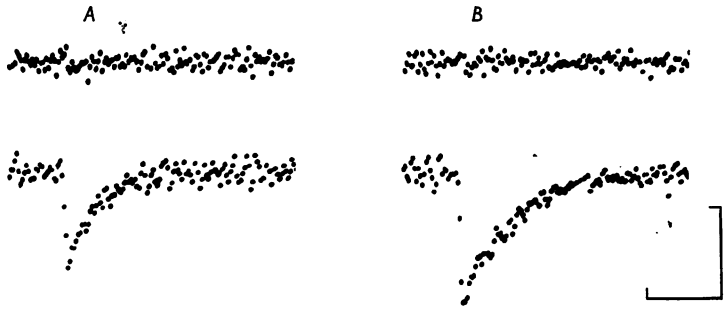


Fig. 8. Records illustrating the increase in decay time constant and amplitude of m.e.p.c.s caused by neostigmine. The m.e.p.c. in *A* was recorded in standard solution, and the m.e.p.c. in *B* was recorded after 30 min in a solution containing 1 mg/l. neostigmine. Calibrations: horizontal, 4 msec; vertical, 1 mV and 4 nA.

With extracellularly recorded m.e.p.c.s, there was sometimes an increase in growth times of m.e.p.c.s. However, the effect is minor compared to the effect of neostigmine on the decay phase. It was disappointing that there was not a greater prolongation of m.e.p.c.s. in neostigmine especially as Katz & Miledi (1973) have obtained much larger effects with the same concentration and type of neostigmine. There seems no reason to believe that the acetylcholinesterase would not have been significantly inhibited (see also, Magleby & Stevens, 1972*a*) with neostigmine concentrations of 1–2 mg/l.

The following experiments were done in an attempt to exclude acetylcholinesterase as a cause for the voltage sensitivity or temperature sensitivity of the decay phase of m.e.p.c.s. Acetylcholinesterase activity has a high Q_{10} of 2–3 *in vitro* (Butterworth, Ely & Stone, 1953), and could conceivably be voltage-sensitive. The recent observation that the voltage sensitivity of end-plate currents is abolished by the irreversible acetylcholinesterase inhibitor, diisopropylfluorophosphate (Kuba, Albuquerque & Barnard, 1973) adds weight to such a possibility. On the other hand,

changes in temperature and membrane potential affect the frequency of spectrum acetylcholine noise (Katz & Miledi, 1972; Anderson & Stevens, 1973) whereas neostigmine does not (Katz & Miledi, 1973).

Effect of neostigmine on the voltage sensitivity of the decay phase of m.e.p.c.s

To test whether the voltage sensitivity of the decay phase of m.e.p.c.s (Fig. 2) was due to, or influenced by, an effect of membrane potential on cholinesterase activity (Magleby & Stevens, 1972*a*), m.e.p.c.s were recorded in voltage-clamped fibres in standard Ringer and then in Ringer solutions containing 1 or 2 mg/l. neostigmine, over a range of membrane potentials. Results obtained at four junctions are shown in Table 4 which gives the parameters obtained from fibres in neostigmine together with 'control' values (in brackets) in the same fibres. It can be seen that the parameter influenced by neostigmine is $\tau(0)$ which is greater in the presence of neostigmine than in control solution whereas H, the voltage sensitivity parameter, appears unaffected. Therefore, 1–2 mg/l. neostigmine does not affect the voltage sensitivity of the decay phase of m.e.p.c.s.

TABLE 4. Effect of neostigmine on the voltage sensitivity of the decay of m.e.p.c.s

Fibre	Temp. (°C)	Neostigmine (mg/l)	$\tau(0)$ (msec)	H (mV)
117–2	20	1	1·43 (0·88)	−90 (91)
131–2	20	1	2·47	−125
132–1	20	1	1·63 (1·25)	−90 (90)
192–3	20	2	3·00 (1·80)	−120 (118)
			Mean	−106·3
			S.E. of mean	9·4

Effect of neostigmine on the temperature sensitivity of the decay phase of m.e.p.c.s

M.e.p.c.s were recorded both extracellularly and in voltage-clamped fibres, in standard Ringer and in solutions containing neostigmine, and over a range of temperatures. Neostigmine produced no significant change in the temperature sensitivity of τ. Results obtained in four experiments are shown in Table 5. The Q_{10} of τ in standard Ringer was $3{\cdot}13 \pm 0{\cdot}22$ (mean $\pm$ S.E. of mean) and in neostigmine (1 mg/l.), a value of $3{\cdot}4 \pm 0{\cdot}14$ (mean $\pm$ S.E. of mean) was obtained. The difference is clearly not statistically significant. The result indicates that the temperature sensitivity of the decay phase does not reflect the temperature sensitivity of acetylcholinesterase activity.

TABLE 5. The effect of neostigmine (1 mg/l.) on the temperature sensitivity of the decay phase of m.e.p.c.s

Fibre	V_C (mV)	Range (°C)	Q_{10} of τ	E (kcal)	ln A
105–3	−78	10–20	2·98	17·56	35·16
106–3	−70	10–18	3·42	18·94	38·10
116–1	−70	12–23	3·48	20·54	40·91
116–1	−100	12–23	3·86	21·00	41·47
132–1	−60	8–18	3·26	18·90	38·11
		Mean	3·40	19·39	38·75
		S.E. of mean	0·14	0·62	1·14

DISCUSSION

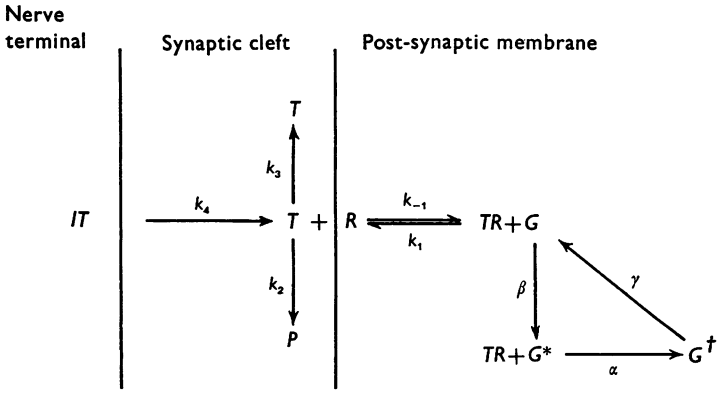
Some of our results concerning the decay phase of m.e.p.c.s are in general agreement with observations made previously on the exponential part of the decay end-plate currents. We have shown that the whole of the decay of m.e.p.c.s generally conforms to a single exponential. It has been reported before that the decay of end-plate currents is exponential after a rounded peak (Takeuchi & Takeuchi, 1959; Magleby & Stevens, 1972*a*; Kordas, 1972*a*). The absence of rounded peaks in m.e.p.c.s suggests that the rounding of the peak end-plate currents is related to the time course of transmitter release. The time constant of the decay of m.e.p.c.s has a high Q_{10}, as has been found for end-plate currents (Takeuchi & Takeuchi, 1959; Magleby & Stevens, 1972*b*; Kordas, 1972*b*) and 'elementary' currents (Anderson & Stevens, 1973), and is affected by membrane potential (Takeuchi & Takeuchi, 1959; Gage & Armstrong, 1968; Kordas, 1969; Gage & McBurney, 1972; Anderson & Stevens, 1973) exponentially (Magleby & Stevens, 1972*a*). The agreement between the results obtained with end-plate currents and m.e.p.c.s indicates that the whole of the end-plate membrane must have been reasonably well clamped during the exponential part of the decay phase of end-plate currents.

We have shown, further, that the change in the rate of decay of m.e.p.c.s with changes in temperature conforms to the Arrhenius equation. This observation, taken in conjunction with the exponential decay of m.e.p.c.s, suggests that a single first-order reaction controls the rate of decay of the acetylcholine-induced conductance increase. The high Q_{10} of the decay of end-plate currents has been used as an argument against diffusional dilution of acetylcholine being rate-limiting. However, if the diffusion coefficient for acetylcholine in the synaptic cleft changed with temperature, the process underlying the decay of the end-plate conductance could still be diffusion, and have a high Q_{10}, but it would be difficult to explain how

diffusional loss of acetylcholine should be affected by the post-synaptic membrane potential. On the other hand, acetylcholinesterase activity could conceivably be affected by membrane potential but the unchanged voltage-sensitivity of the decay of m.e.p.c.s in preparations exposed to neostigmine make this explanation unlikely.

The sensitivity of the rate-limiting reaction to membrane potential indicates that the reaction occurs within the post-synaptic membrane and probably involves molecules with dipole moments. These conclusions about the nature of the process controlling the decay of end-plate currents are in general agreement with those of Magleby & Stevens (1972*a*, *b*) and Kordas (1972*a*, *b*) though their more explicit explanations are different. Kordas and others have suggested that the rate-limiting step is dissociation of acetylcholine from the receptors, whereas Magleby & Stevens (1972*a*, *b*) have presented the hypothesis that the rate-limiting reaction involves a conformation change in the transmitter–receptor complex which has a dipole moment. If the rate-limiting step is dissociation of acetylcholine from receptors, the reaction would have to 'see' and be affected by the membrane potential. Without further evidence, it is difficult to decide which, if either, is the rate-limiting reaction. However, it is probably worth while discussing our other results in terms of a model which includes provision for either type of process (dissociation of acetylcholine from receptors or a later reaction, possibly a conformational change in a macromolecule) being rate-limiting.

The events that are thought to occur during the activation and decay of the end-plate conductance may be represented as follows,



Intracellular transmitter (*IT*) in the nerve terminal is released giving free transmitter, *T*, some of which is lost by diffusion (rate k_3), and some by hydrolysis (rate k_2) to reaction product, *P*. The transmitter which reaches the receptors (*R*) forms the complex *TR*. Magleby & Stevens (1972*b*)

suggest that TR then undergoes a conformational change to TR^*: end-plate conductance is proportional to TR^*. Because there is no evidence for such a change in the transmitter-receptor complex, we prefer to describe a more general reaction activated by the formation of the transmitter-receptor complex. A molecule G is changed to G^* (rate β) which controls the end-plate conductance. G^* decays to an inactive form $G^\dagger$ (rate α) and $G^\dagger$ then goes back to G (rate γ). (The reaction

$$G \overset{\beta}{\rightarrow} G^* \overset{\alpha}{\rightarrow} G^\dagger$$

may eventually prove to be the reaction $TR \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} TR^*$ as proposed by Magleby & Stevens, 1972*b*.)

With diffusion and hydrolysis of acetylcholine excluded as limiting the rate of decay of end-plate currents under normal circumstances, in terms of the above model the decay must be determined by k_1 (the rate of dissociation of acetylcholine from receptors) or α (the rate of conversion of the activated conductance molecule, G^*, to an inactive (desensitized) form, $G^\dagger$. The information available does not yet allow a distinction but it can be stated that the reaction must have a high activation energy and be sensitive to membrane potential, presumably because molecules having dipole moments are involved (see Gage, McBurney & Schneider, 1974).

That membrane potential influences the decay of e.p.c.s and m.e.p.c.s has been reported previously (Takeuchi & Takeuchi, 1959; Gage & Armstrong, 1968; Kordas, 1969; Magleby & Stevens, 1972*a*; Gage & McBurney, 1972). Because of the signal-to-noise ratio, Gage & Armstrong (1968) recorded m.e.p.c.s at clamp potentials of -50 and $+50$ mV only, not at potentials between. They tentatively suggested, on the basis of the effects of membrane potential and procaine, that the different time courses at the two membrane potentials might reflect different time courses of sodium and potassium conductance changes. However the graded effects of membrane potential over a wide range suggest that the voltage sensitivity of τ must have another explanation. The voltage sensitivity of the decay of synaptic currents does not seem to be a general property of synapses. For example, the time course of the decay of synaptic current at the squid giant synapse is not detectably affected by clamp potential (P. W. Gage and J. W. Moore, unpublished observations; Joyner, 1973).

Although the time course of m.e.p.c.s in voltage-clamped fibres varied over a wide range (see also, Gage & McBurney, 1972) it was noticeable that the scatter of decay time constants was less with extracellular recording which monitors m.e.p.c.s from only a small fraction of an end-plate. The greater scatter seen in voltage-clamped fibres may reflect heterogeneity of

the post-synaptic membrane along the length of an end-plate. It would not be surprising if such factors as local field strength and the environment of the receptors (e.g. the dielectric, Gage & McBurney, 1972) varied from spot to spot along an end-plate. The same sort of explanation may apply to the variation in the Q_{10} of the decay phase of m.e.p.c.s recorded in different fibres (Table 3).

The amplitude of m.e.p.c.s was clearly affected by temperature and had a Q_{10} of 1·5–2. As the amplitude of elementary currents is not affected by temperature (Anderson & Stevens, 1973) the reduction in amplitude of m.e.p.c.s at lower temperatures must reflect a reduction in the number of activated receptors, perhaps because fewer acetylcholine molecules reach them (because of reduced secretion or increased loss in the synaptic cleft) or perhaps because fewer complexes can overcome a critical energy barrier at the lower temperatures.

The prolongation of m.e.p.c.s caused by neostigmine was never as great as reported by Katz & Miledi (1973) but was of the same order as the effects reported by Magleby & Stevens (1972*a*) and Kordas (1972*a*). A more striking effect in some fibres was the increased proportion of slow m.e.p.c.s. We do not know how effectively acetylcholinesterase was inhibited at the toad end-plate but have no reason to believe that neostigmine would have been less effective in toads than in frogs. Katz & Miledi (1973) have suggested that the prolongation of m.e.p.c.s produced by neostigmine is related to the number of receptors available to acetylcholine as it diffuses out of the synaptic cleft. The degree of prolongation of m.e.p.c.s produced by neostigmine may reflect the number or density of receptors and the geometry of the synaptic cleft rather than the degree of inhibition of acetylcholinesterase. Assuming that acetylcholinesterase was significantly inhibited by the neostigmine in our experiments, it can be concluded that a reduction in the activity of acetylcholinesterase does not change the voltage or temperature sensitivity of the decay phase of m.e.p.c.s. Magleby & Stevens (1972*a*) also found that the sensitivity of the decay constant of e.p.c.s to membrane potential was unchanged by neostigmine. On the other hand the unchanged Q_{10} in neostigmine differs from the observation of Kordas (1972*b*) who found that the effect of neostigmine varied with temperature, and we have no explanation for this. Other evidence makes it very unlikely that the effect of temperature on the decay of m.e.p.c.s is due to a change in acetylcholinesterase activity. The duration of elementary events, which is related to the time constant of decay of m.e.p.c.s (see e.g. Katz & Miledi, 1973; Anderson & Stevens, 1973), is affected by temperature but not by neostigmine (Katz & Miledi, 1972, 1973; Anderson & Stevens, 1973).

It was proposed (Magleby & Stevens, 1972*b*) that the time course of

end-plate currents is determined by the two rate constants (α and β) associated with a conformational change in the transmitter–receptor complex. We have found that the growth and decay phases of m.e.p.c.s have different characteristics. The growth phase is not exponential, is little influenced by membrane potential, and has a low Q_{10}. Takeuchi & Takeuchi (1959) obtained a higher Q_{10} of about 2 for the growth phase of end-plate currents. This could be due to the contribution of the asynchronous release of transmitter, which has a high Q_{10} (Katz & Miledi, 1965*a*, *b*; Barrett & Stevens, 1973), to the growth phase of end-plate currents. On the whole, it seems unlikely, therefore, that the same reaction (whatever its nature) determines the time course of the growth phase and the decay phase. Rather, it would seem that a process with low temperature coefficient, such as simple diffusion, controls the growth phase. In the above model, k_4 is the term which would be determined, at least in part, by diffusion of acetylcholine across the synaptic cleft. It should be noted that with e.p.c.s, k_4 would include the rate of release of transmitter. This may explain why the time course of the growth phase, and the early part of the decay phase, are different for e.p.c.s and m.e.p.c.s. As the release of a single quantum of transmitter is presumably essentially instantaneous, it would be predicted from the above hypothesis that the growth phase of m.e.p.c.s would be faster than that of e.p.c.s, and this is, in fact, what is found (Takeuchi & Takeuchi, 1959; Gage & Armstrong, 1968; Magleby & Stevens, 1972*a*, *b*; Gage & McBurney, 1972).

If free diffusion does determine the growth phase of m.e.p.c.s, it should be possible to use the time course of the growth phase to place limits on the diffusion coefficient for acetylcholine in the synaptic cleft, or, alternatively, on the approximate distance between the release site and postsynaptic receptors.

Using a model with simple geometry, such as the diffusion of a substance from an instantaneous point source into an infinite medium (Carslaw & Jaeger, 1959) it is possible to calculate that if the diffusion pathway across the synaptic cleft at toad neuromuscular junctions is taken as 500 Å (P. W. Gage and N. Lane, unpublished observations), the diffusion coefficient for acetylcholine would have to be about 10^{-8} $cm^2.sec^{-1}$ to give a time course of increase in acetylcholine concentration comparable to the growth phase of m.e.p.c.s. This is several orders of magnitude less than in free solution. The free solution diffusion coefficient for acetylcholine, of about 10^{-5} $cm^2.sec^{-1}$ (Eccles & Jaeger, 1958; Krnjević & Mitchell, 1960), would require a diffusion distance of 15,000 Å to give a concentration time course superimposable on the growth phase.

These calculations indicate that either the diffusion coefficient for acetylcholine in the synaptic cleft is considerably less than in free solution

or that the diffusion distances involved are much longer than the width of the synaptic cleft. With regard to the first possibility, an electron opaque substance can be seen in the synaptic cleft under the electron microscope (P. W. Gage and N. Lane, unpublished observations). It is possible that this substance presents a diffusion barrier to acetylcholine and reduces the diffusion coefficient of acetylcholine to well below its free solution value. On the other hand it is possible that there is considerable lateral diffusion of acetylcholine and the growth phase of m.e.p.c.s represents the activation of receptor sites both directly opposite the release site and up to 15,000 Å away. If this were so one might expect that the acetylcholine molecules which diffused along the length of the nerve terminal would have a better chance of activating a receptor than those which diffused across the breadth of the terminal. This might help to explain the discrepancy which is observed between the number of acetylcholine molecules released from the nerve terminal per quantum (Straughan, 1960; Bowman & Hemsworth, 1965; Potter, 1970) and the number which are effective in causing an m.e.p.c. (Katz & Miledi, 1972).

A simple diffusion process in which the rise time of the concentration profile at some point could be superimposed on the growth phase of an m.e.p.c. would be expected to have a rather slow decay phase, with a half-time of 1–2 msec and this would be expected to contribute to the decay of m.e.p.c.s. The main evidence against the decay of end-plate currents being controlled by the rate of diffusional loss of acetylcholine is the high Q_{10} of the decay phase but not the growth phase. However, the concentration of acetylcholine at the receptors may not decay slowly following the time course predicted from diffusional dilution. For example, if cholinesterase is 'activated' by the arrival of acetylcholine at the receptor region, the later-arriving molecules may be rapidly hydrolysed and never be able to activate receptors. Thus the decay of acetylcholine concentration would have a half-time much less than 1 msec. Results obtained with octanol (Gage, McBurney & Van Helden, 1974) which show that the time constant of decay of m.e.p.c.s can be as low as 0·25 msec (following a normal growth time), suggest that the decay of acetylcholine concentration normally does have a half-time of less than 1 msec. Although we have discussed only diffusion as the rate-limiting process for the growth phase, the rate of the transmitter–receptor interaction (k_{-1}) or the rate of the change from G to G^* (β), have not been excluded. The evidence available is not strong enough to support a firm choice of k_4, k_{-1} or β, though on balance we would favour k_4.

In summary, the results show that the rate-limiting reactions controlling the time course of the growth and decay phases of m.e.p.c.s have different characteristics: the growth phase has a low Q_{10}, is comparatively insensitive

to membrane potential and does not follow an exponential time course, whereas the decay phase has a higher Q_{10}, is clearly affected by membrane potential, and is exponential. We suggest that the growth phase is determined by the rate of arrival of acetylcholine at the post-synaptic receptors. The decay phase could be related to the rate of dissociation of acetylcholine from receptors or to the rate of a conformational (or other) change in a macromolecule with dipole moment in the post-synaptic membrane.

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