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TEMPERATURE-SENSITIVE ASPECTS OF EVOKED AND SPONTANEOUS TRANSMITTER RELEASE AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. The temperature dependence of presynaptic processes involved in neuromuscular transmission was studied by rapidly increasing the temperature of cooled frog neuromuscular junctions by 4–10 °C using pulses from a neodymium laser. The temperature elevation was complete within 0·5 msec, and decayed back to control levels with a time constant of about 7–8 sec.

2. Temperature jumps completed before nerve stimulation increased the quantal content and decreased the latency of the end-plate potential (e.p.p.). The Q_{10} for e.p.p. quantal content in low [Ca^{2+}] Ringer averaged about 3·9 over the range 1–18 °C.

3. Temperature jumps occurring during the synaptic delay (the interval between the presynaptic action potential and the onset of the e.p.p.) also increased the quantal content and decreased the latency of the e.p.p. These effects diminished as the onset of the temperature jump was moved closer to the expected onset of the e.p.p. Temperature jumps applied after the onset of the e.p.p. immediately accelerated the time course of the e.p.p. but did not significantly alter quantal content. These results demonstrate that the magnitude and timing of evoked release are influenced by temperature-sensitive processes that operate both during and shortly after the presynaptic nerve action potential, but are largely complete before the onset of release.

4. Temperature jumps were applied at various times during the interval between two nerve stimuli. The amplitude of the second e.p.p. decreased as the temperature jump was moved earlier in the interstimulus interval, suggesting that the rise in temperature following the first nerve stimulus accelerates the decay of facilitation. When the temperature jump was moved from 10 msec after to 10 msec before the onset of the first e.p.p., the amplitude of the second e.p.p. either decreased or showed no change. The fact that the second e.p.p. did not increase suggests that the temperature-sensitive processes that increase the quantal content of the conditioning e.p.p. do not greatly increase the facilitation following that e.p.p.

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5. Temperature jumps immediately accelerated the time course of spontaneous miniature end-plate potentials (m.e.p.p.s) and increased their frequency. Experiments using slow temperature changes revealed that the Q_{10} for m.e.p.p. frequency in normal Ringer is about 10 over the range 10–20 °C. M.e.p.p. frequency was much less sensitive to temperature changes below about 10 °C. When the nerve terminal was depolarized by 20 mM-K^+ in the presence of Ca^{2+}, the Q_{10} for the rate of spontaneous release over the range 10–20 °C decreased to about 4, similar to the Q_{10} for e.p.p. quantal content. In the absence of extracellular Ca^{2+} the Q_{10} for m.e.p.p. frequency in 20 mM-K^+ remained near 10.

6. The marked difference in Q_{10}s for spontaneous transmitter release under different experimental conditions suggests that not all transmitter release uses identical mechanisms. A possible explanation for these variations in temperature sensitivity is that the energy of activation and temperature sensitivity of quantal transmitter release is high when intracellular [Ca^{2+}] is low, and that an increase in intracellular [Ca^{2+}] (caused, for example, by nerve stimulation or high [K^+] in the presence of Ca^{2+}, low temperature or injury to the nerve terminal) catalytically reduces this energy of activation, thus increasing the rate and reducing the temperature sensitivity of quantal transmitter release. Calculations show that such a Ca^{2+}-induced reduction in the energy of activation would be more than sufficient to account for the acceleration of transmitter release observed following nerve stimulation.

INTRODUCTION

Temperature changes have a dramatic effect on the rates of many presynaptic processes involved in vertebrate neuromuscular transmission. The rate of spontaneous transmitter release (Fatt & Katz, 1952; Boyd & Martin, 1956; Liley, 1956; Li & Gouras, 1958; Takeuchi, 1958; Hofmann, Parsons & Feigen, 1966; Hubbard, Jones & Landau, 1971; Ward, Crowley & Johns, 1972; Bevan, Grampp & Miledi, 1976), the duration of the action potential in the motor nerve terminal and the minimal synaptic delay (Katz & Miledi, 1965*b*), the time course of transmitter release following nerve stimulation (Katz & Miledi, 1965*b*; Barrett & Stevens, 1972), and the rate of decay of facilitation (Eccles, Katz & Kuffler, 1941; Hubbard *et al.* 1971; Balnave & Gage, 1974) all have reported temperature sensitivities suggesting Q_{10}s of 3 or more at physiological temperatures. These earlier studies used relatively slow temperature changes requiring minutes to complete. We have further investigated the temperature sensitivity of transmitter release using both slow temperature changes and rapid (0·5 msec) temperature jumps induced by discharge of a neodymium laser. These experiments demonstrate that temperature jumps applied before or during the presynaptic action potential or during the synaptic delay increase the average quantal content and decrease the latency of transmitter release, and that the rates of spontaneous and evoked transmitter release can have markedly different temperature sensitivities.

METHODS

Preparation and recording techniques

Laser experiments were performed on small fall and winter frogs (*Rana pipiens*) which were maintained for up to 2 weeks before the experiment in a refrigerator at 10 °C. Cutaneous pectoris nerve-muscle preparations were dissected and placed in a shallow glass chamber filled

with low [Ca^{2+}] Ringer solution (mM: 121 NaCl, 2·5 KCl, 0·5 $CaCl_2$, 2 $MgCl_2$, pH 7·5). Modifications of this Ringer are described in the text. The motor nerve was stimulated at its entrance into the muscle with 1 msec supra-threshold (0·2–5 V) pulses delivered through a Ringer-filled polyethylene suction electrode. The steady-state bath temperature was maintained at 1–12 °C by Peltier-effect thermoelectric cooling modules (Cambion). Metal plates inserted between the thermoelectric devices and the bottom of the glass chamber, and affixed to these surfaces with conducting paste, produced an even steady-state temperature distribution across the bath. The temperature near the neuromuscular junction under study was monitored using a small (0·1 mm diameter) copper-constantin thermocouple. The low temperatures increased the time resolution of the experiments by slowing many of the processes involved in neuromuscular transmission.

Spontaneous miniature end-plate potentials (m.e.p.p.s) and end-plate potentials (e.p.p.s) evoked by nerve stimulation were recorded intracellularly from superficial junctions using glass micropipettes filled with 1 M-KCl (8–20 MΩ tip resistance), displayed on an oscilloscope screen and photographed. The temperature and Ca^{2+} and Mg^{2+} concentrations in the bath were adjusted until m, the average quantal content of the e.p.p., was between 0·5 and 10 at the initial steady-state temperature. Where possible, m was determined by counting quantal potentials or from the ratio of total trials to failures (del Castillo & Katz, 1954*a*). In other cases m was calculated by dividing the average area of the evoked response (the integral of the voltage change over time) by the average area of m.e.p.p.s recorded at the same temperature. Using area ratios rather than amplitude ratios controls for temperature-induced changes in the synchrony of evoked transmitter release and the time course of quantal potentials.

Laser control and specifications

The temperature of a cylindrical region 4–5 mm in diameter enclosing the motor nerve and junctional region under study was rapidly increased 4–10 °C by a 8–20 J pulse from a neodymium laser. The energy emission record in Fig. 1*B* demonstrates that almost all of the light energy was released within 0·5 msec. The laser beam was centred on the synaptic region by aiming a continuous helium laser at the synaptic region through the optical pathway of the neodymium laser, as shown in Fig. 1*A*. The neodymium laser pulse was triggered at various times relative to the motor nerve stimulus. Successive laser pulses were separated by at least 5 min, the minimum time required to cool the neodymium rod.

The physiological effects of the neodymium laser pulse were due mainly to the resulting temperature jump (Moore, Holt & Lindley, 1972; see Discussion). This temperature jump was distributed uniformly across the surface nerve terminal because (1) the $\leqslant$ 0·7 mm length of the nerve terminal is short compared to the 4–5 mm diameter of the laser beam, (2) the 2–5 mm distance between the nerve terminal and the surface of the bathing solution is short compared to the 2·7 cm absorption length of this laser beam in water, and (3) the red (1060 nm) light emitted by this laser appears to be absorbed equally well by protein, lipid, carbohydrate and water molecules (Tanford, 1963).

Measurement of the laser-induced temperature jump

The magnitude of the laser-induced temperature change was measured in three different ways. One method involved measuring the change in conductivity of the bathing solution induced by laser discharge. The temperature-sensitive conductivity change was measured using a linear array of four electrodes. Current passed between the two outer electrodes produced a voltage drop between the two inner electrodes whose magnitude varied with the conductivity of the bathing solution. This voltage drop was plotted as a function of the bath temperature measured by the thermocouple during relatively slow temperature changes. We assumed that this calibration curve extended to times as short as 0·1 msec because on this time scale conductivity is an almost instantaneous function of temperature. Measured in this way, the bath temperature in the path of the laser beam increased 4–10 °C during a 0·5 msec 8–20 J laser pulse, and decayed slowly back to the steady-state bath temperature with an approximate time constant of 7–8 sec. A similar temperature profile was obtained by the second method, which consisted of briefly inserting a small thermocouple into the heated region at various times during the decaying phase of the temperature jump.

The third method for measuring the laser-induced temperature change used the latency of the e.p.p. (the interval between nerve stimulation and the onset of the e.p.p.) as a rapid, sensitive

indicator of junctional temperature. Thus defined, e.p.p. latency includes both nerve conduction time and the synaptic delay, both of which are temperature sensitive (Katz & Miledi, 1965*b*). In order to use e.p.p. latency to estimate bath temperature it was therefore necessary that the full length of the motor nerve (about 3 mm) as well as the nerve terminal be included in the region heated by the laser beam. When the average quantal content exceeded about four, the e.p.p. latency at a given temperature was nearly constant, and decreased monotonically with increasing temperature, showing the same relationship for both fast (laser-induced) and slow temperature changes. Calibration curves of e.p.p. latency *vs.* bath temperature (measured with a small thermocouple near the synapse) made at the onset of the experiment could thus serve to translate e.p.p. latency into junctional temperature during subsequent experiments on that junction. The decay of junctional temperature following the laser pulse measured using this e.p.p. latency method agreed with those measured using the conductivity and thermocouple methods described above.

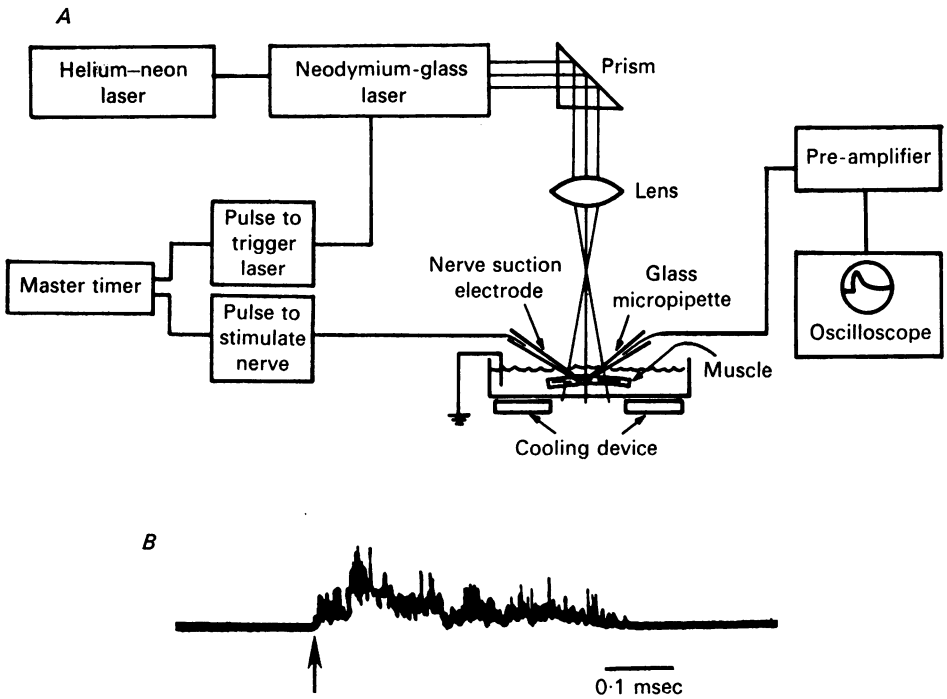

Fig. 1. *A*, block diagram of experimental apparatus. The pulsed neodymium laser beam was centred on the synaptic region under study by directing light from a continuous helium-neon laser through the centre of the optical path of the neodymium laser. A system of pulse generators co-ordinated the timing of nerve stimulation and laser discharge. *B*, time course of light energy emission during a 19 J pulse from the neodymium laser. Arrow indicates onset of laser pulse. Light energy was converted to a voltage signal by a calibrated phototransistor system. Calibrations: arbitrary amplitude units, 0·1 msec.

Voltage artifact associated with laser discharge

The voltage artifact associated with the laser pulse was reduced considerably by electrically shielding the laser, by mechanically decoupling the laser from the recording equipment and by shielding all metal-solution contacts from the laser light. Use of bevelled (Barrett & Graubard, 1970) low resistance micro-electrodes (~ 10 MΩ) with negligible tip potentials further reduced the artifact. Even with these precautions a depolarizing potential was recorded when the laser pulse was applied to an otherwise unstimulated muscle end-plate (Fig. 2, traces 2 and 3). In

some experiments (e.g. Fig. 6) this artifact was small ($< 0{\cdot}5$ mV) and did not interfere with measurements of e.p.p. amplitudes and latencies. In other experiments (e.g. Figs. 5 *B*, 8) artifacts were large, and were subtracted by measuring their amplitude and time course in the absence of nerve stimulation and e.p.p.s. The artifact was probably due to movement of tissue membranes with respect to the electrode tip caused by rapid thermal expansion, rather than to a true change in transmembrane potential, because the artifact persisted in hypertonic KCl and even after prolonged boiling, and could also be recorded when the electrode tip was jammed against connective tissue. Jumping between temperatures associated with approximately the same specific water density would be expected to minimize artifacts associated with thermal expansion. Consistent with this hypothesis, the artifact was smallest when the temperature was jumped from just below to just above 4 °C, the temperature at which water has its minimum specific volume.

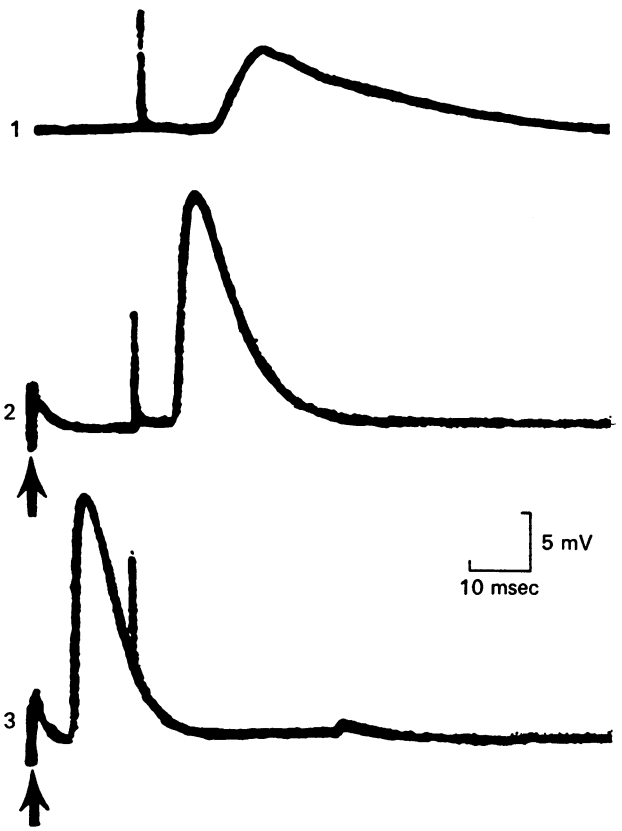

Fig. 2. End-plate potentials produced by motor nerve stimulation and laser discharge. Traces 1 and 2 are e.p.p.s evoked by motor nerve stimulation at 2 °C (trace 1) and after a laser pulse (arrow) elevated the junctional temperature to about 12 °C (trace 2). Trace 3 shows an e.p.p. evoked by a slightly more intense laser pulse (arrow) without prior applied motor nerve stimulation. Subsequent applied nerve stimulation (artifact on falling phase of e.p.p.) failed to evoke any additional transmitter release. Calibrations: 5 mV, 10 msec.

E.p.p.s evoked by very intense laser pulses

Laser pulses which elevated junctional temperature by more than 10 °C occasionally elicited a burst of transmitter release in the absence of applied motor nerve stimulation, as illustrated in trace 3 of Fig. 2. This burst of release was probably mediated by a laser-induced action potential in the nerve terminal because (1) the resultant e.p.p. had a magnitude and time course similar

to those of e.p.p.s evoked by nerve stimulation at the post-jump temperature (trace 2), (2) the interval between the laser pulse and the onset of the e.p.p. (about 6 msec in Fig. 2) was never less than the minimal synaptic delay measured by Katz & Miledi (1965*b*) at the post-jump temperature, and (3) the nerve terminal appeared to be refractory during the laser-evoked e.p.p., since nerve stimuli applied during the falling phase of this e.p.p. released no additional transmitter (trace 3). This presynaptic action potential was probably produced by the rapid thermal expansion associated with the intense laser discharge. In all subsequent figures the laser pulses were less intense than that in Fig. 2, and did not evoke bursts of release in the absence of applied motor nerve stimulation. The effects of these less intense laser pulses on transmitter release were due to the associated temperature change (see Discussion).

Slow temperature changes

Experiments like those plotted in Fig. 4 employed slow (5 min) temperature changes produced by adjusting the current through a thermoelectric device under the experimental chamber. These experiments used small spring and summer *Rana pipiens* maintained at room temperature. The temperature of the bath near the muscle was monitored by a small thermistor probe attached to an ohmmeter, and during any particular determination of m.e.p.p. frequency or e.p.p. quantal content the temperature varied by less than 0·3 °C. M.e.p.p. frequencies were determined by counting from a storage oscilloscope all m.e.p.p.s occurring in a series of 0·1–10 sec time intervals. The interval was adjusted according to m.e.p.p. frequency so that an average of about 0·5–5 m.e.p.p.s occurred in each interval. At least 25 (usually 50–100) m.e.p.p.s were counted at each temperature. To facilitate m.e.p.p. counts in high [K^+], the muscle fibre was frequently hyperpolarized (to increase the amplitudes of the m.e.p.p.s) by passing current through a second electrode inserted near the end-plate region. During measurements of e.p.p. quantal content the nerve was stimulated at very low rates (0·1/sec at 20 °C, 0·025/sec at 10 °C) to avoid possible potentiation of transmitter release.

RESULTS

Temperature jump increases m.e.p.p. frequency

The transient 4–10 °C increase in bath temperature produced by laser discharge had little effect on the resting potential or the passive electrotonic response of the muscle membrane over the tested 1–18 °C range. This result agrees with those obtained using slow temperature changes by del Castillo & Machne (1953), Takeuchi (1958) and Jensen (1972). M.e.p.p.s rose and decayed faster at the higher temperature, and this effect was apparent within 1 msec following laser discharge. Similar effects on m.e.p.p. shape were obtained by slow warming to the post-jump temperature.

Temperature jumps increased the frequency of spontaneous m.e.p.p.s, in agreement with the slow temperature change data of Fatt & Katz (1952), Li & Gouras (1958), Takeuchi (1958), and Bevan *et al.* (1976). However, it was difficult to measure this increase accurately in normal Ringer because of the low rate of spontaneous release, the short duration of the post-laser temperature elevation and the mandatory 5 min interval between laser pulses. The circles in Fig. 3 plot average m.e.p.p. frequency as a function of time following a temperature jump from 16 to 23 °C at a junction whose steady-state m.e.p.p. frequency was 2·4/sec (dashed line). A fivefold increase in m.e.p.p. frequency is evident within the first second following the laser pulse. In this and other similar experiments m.e.p.p. frequency decreases back to control levels with a time course similar to that measured for the decay of local temperature following a laser pulse (Methods). These results suggest that the effect of the neodymium laser pulse on the time course and frequency of m.e.p.p.s can be

accounted for by the change in local temperature produced by non-specific absorption of the laser light energy.

The light-induced elevation of m.e.p.p. frequency reported by Goto & Kuroda (1975) was delayed in time and probably had a different mechanism from that in Fig. 3 because they used ultra-violet light, which is preferentially absorbed by organic molecules, and at the light energies used in their study temperature changes were negligible.

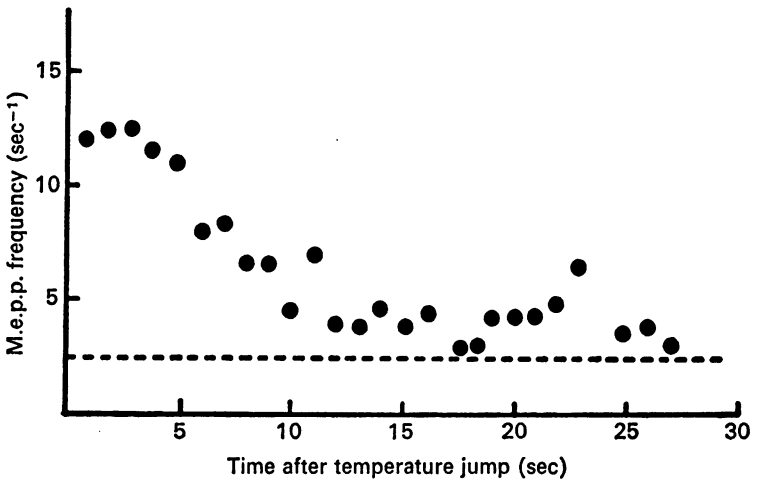

Fig. 3. Increase in spontaneous m.e.p.p. frequency as a function of time (sec) following a laser-induced temperature jump from 16 to 23 °C at a junction bathed in normal Ringer. Frequency measurements were averaged from four successive laser discharges separated by 5 min intervals. Dashed line indicates control m.e.p.p. frequency at 16 °C.

Depolarization reduces the temperature dependence of the rate of transmitter release

Because of the difficulties involved in measuring post-laser m.e.p.p. frequencies in normal Ringer, we re-examined the temperature dependence of the spontaneous release rate by slowly changing bath temperature with a thermo-electric device. Filled circles in Fig. 4*A* plot log m.e.p.p. frequency *vs.* reciprocal absolute temperature for a junction bathed in Ringer containing 0·6 mM Ca^{2+} and 2 mM-K^+. M.e.p.p. frequency increased from 0·037/sec at 10·5 °C to 0·35/sec at 20·5 °C. The slope of this Arrhenius plot over the range 10–20 °C yields a Q_{10} of about 10, in agreement with the value of $9{\cdot}5 \pm 0{\cdot}63$ reported by Bevan *et al.* (1976) over a similar temperature range in normal Ringer. When this nerve terminal was depolarized by increasing bath [K^+] to 20 mM, the m.e.p.p. frequency at 20 °C increased about twentyfold, but the temperature sensitivity decreased (open circles, Fig. 4*A*). The slope of these high [K^+] data suggest a Q_{10} of 3·6 over the range 10–20 °C. Similar experiments at other junctions confirmed this result and showed that the depolarization-induced increase in m.e.p.p. frequency and decrease in temperature sensitivity are Ca^{2+} dependent, since terminals bathed in 20 mM-K^+ and less than 10^{-7} M-Ca^{2+} (0·03 mM-EGTA, no added Ca^{2+}) show low m.e.p.p. rates and high Q_{10}s (crosses, Fig. 4*A*), similar to those seen in normal Ringer.

Nerve stimulation also lowers the temperature dependence of the transmitter release rate. Fig. 4*B* plots data from an experiment in which the temperature dependencies of both spontaneous m.e.p.p. frequency and the quantal content of the e.p.p. were measured at the same neuromuscular junction. Again m.e.p.p. frequency (filled circles, left ordinate) displays a high temperature dependence, with a calculated Q_{10} of about 9·4. The average quantal content of the e.p.p. (open circles, right ordinate) also increases with increasing temperature, from about 1·6 at 12 °C to about 2·7 at 20·5 °C, but the calculated Q_{10} is only about 1·9. At another junction the Q_{10} for e.p.p. quantal content was slightly higher, around 2·8.

M.e.p.p. frequency always showed a reduced temperature sensitivity below 10 °C,

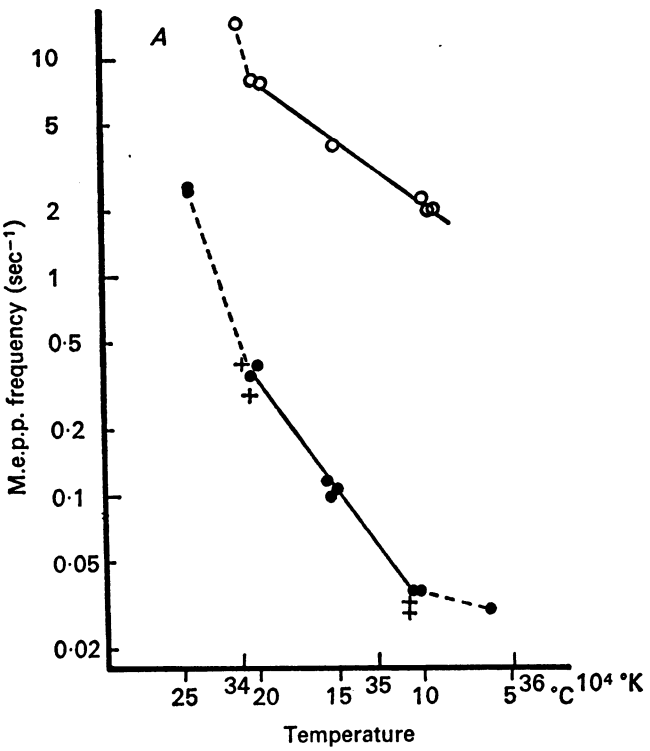

Fig. 4. Arrhenius plots of log transmitter release rate as a function of reciprocal absolute temperature (10^4/°K). Lower scale on abscissa plots corresponding °C. Temperature changes were slow. *A*, filled circles plot spontaneous m.e.p.p. frequencies collected in Ringer containing 0·6 mM-Ca^{2+}, 2 mM-Mg^{2+} and 2 mM-K^+; open circles were obtained at the same junction after increasing [K^+] to 20 mM. Crosses plot m.e.p.p. frequencies from another junction bathed in 20 mM-K^+, 2 mM-Mg^{2+}, 0·03 mM-EGTA and zero added Ca^{2+}. *B*, filled circles plot spontaneous m.e.p.p. frequencies (left ordinate) collected in Ringer containing 0·45 mM-Ca^{2+}, 2 mM-Mg^{2+} and 2 mM-K^+. Open circles plot the average quantal content of the e.p.p. (right ordinate) measured at the same junction. Multiple open circles at a given temperature indicate quantal content estimates calculated using multiple techniques (direct quantal counts, ratio of total trials to failures, e.p.p./m.e.p.p. amplitude ratio). In both *A* and *B* the continuous line segments were drawn by eye to fit points between 10 and 20 °C. In these experiments the transmitter release rate at a given temperature did not vary with the previous temperature history of the terminal.

as reported by Duncan & Statham (1977), but it was difficult to obtain reliable Q_{10} estimates at these low temperatures because the m.e.p.p. frequency at a particular temperature frequently varied with time and with the previous temperature history of the terminal. At temperatures below 3–4 °C m.e.p.p. rates occasionally even increased with decreasing temperature. These variations in m.e.p.p. rate during slow temperature changes below 10 °C may be due in part to redistribution of ions within the nerve terminal.

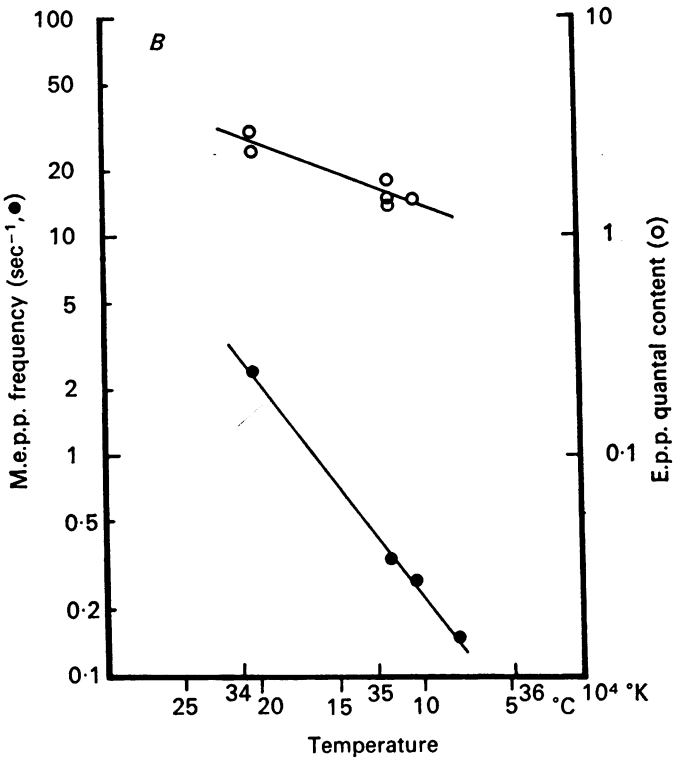

Fig. 4*B*. For legend see facing page.

Temperature jumps preceding nerve stimulation increase e.p.p. quantal content and decrease e.p.p. latency

Previous workers have shown that the amplitude of the post-synaptic potential increases with increasing temperature at the frog neuromuscular junction (Eccles *et al.* 1941; Jensen, 1972) and at the squid giant synapse (Weight & Erulkar, 1976). Takeuchi (1958) and the data of Fig. 4*B* indicate that in the frog at least part of the increase in e.p.p. amplitude is due to an increase in the average number of quantal transmitter packets released during the e.p.p. We attempted to quantify this temperature sensitivity by comparing the quantal contents of e.p.p.s evoked before and after a laser-induced temperature jump. Fig. 5 shows sample records from these experiments, which demonstrate that temperature jumps administered before the action potential invades the motor nerve terminal increase the average quantal content of the e.p.p. and decrease the latency of the e.p.p. The records of Fig. 5*A*

were taken from an experiment in which the steady-state bath temperature and the average quantal content of the e.p.p. were so low that the individual quantal components of the e.p.p. could be distinguished and counted. All but trace 3 were collected at the control temperature of 5·5 °C. The trial-to-trial fluctuations in the

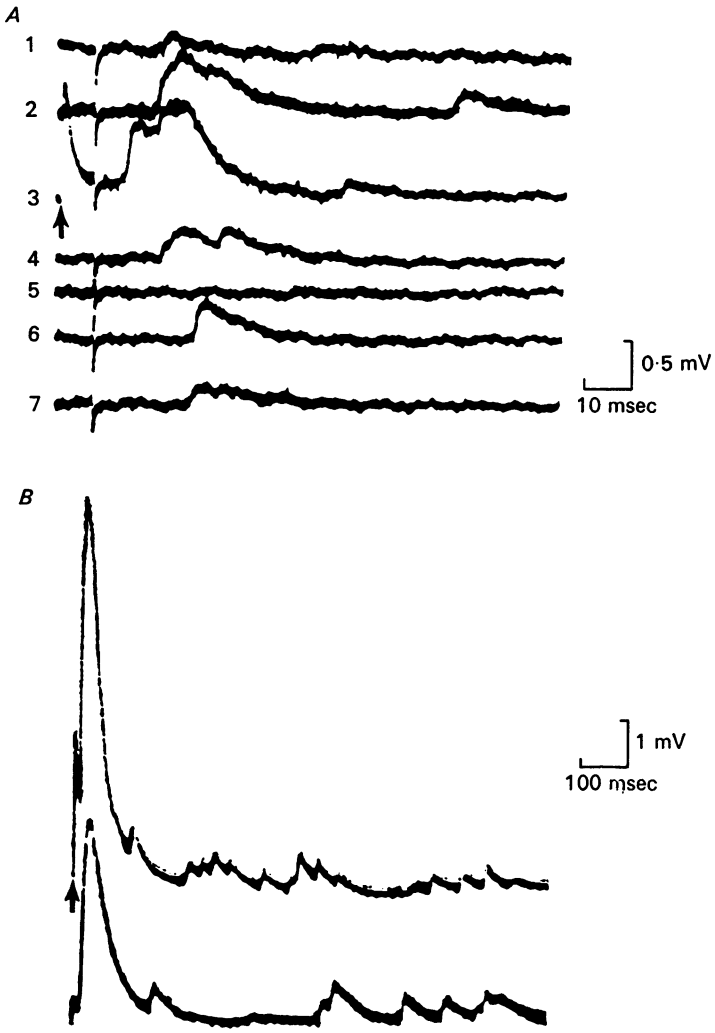

Fig. 5. Effect of laser-induced temperature jump on end-plate potentials at two junctions. *A*, traces 1, 2 and 4–7 were recorded at the control temperature of 5·5 °C. The time of motor nerve stimulation (rate 0·5/sec) is indicated by the negative-going artifact near the beginning of each trace. In trace 3 a laser pulse (arrow) sufficient to raise junctional temperature to 10·5 °C was administered 10 msec before nerve stimulation. The temperature jump decreased the latency and increased the quantal content of the subsequent e.p.p. Records are from junction *E* of Table 1. Calibrations: 0·5 mV, 10 msec. *B*, the lower trace was recorded at the control temperature of 2·5 °C, the upper trace after a laser pulse (arrow) administered before nerve stimulation increased junctional temperature to 9 °C. The temperature jump increased both e.p.p. amplitude and the subsequent delayed m.e.p.p. discharge. Records are from junction *P* of Table 1. Calibrations: 1 mV, 100 msec.

amplitude and latency of the e.p.p. reflect the probabilistic nature of transmitter release, because extracellular recordings indicate that at a given temperature the timing and configuration of the presynaptic action potential are relatively invariant from trial to trial (Katz & Miledi, 1965*a*, *b*, and our observations). In trace 3 a laser pulse administered 10 msec before the nerve stimulus raised the junctional temperature to 10·5 °C. The subsequent e.p.p. contained at least three quanta, more than were seen in any of the control trials, and its latency was shorter than those observed during the control trials. In twenty-five control trials at this junction the average quantal content of the e.p.p. was 1·21; in four trials preceded by a temperature jump the average quantal content rose to 2·25, an increase in transmitter release of about 1·9 times following a temperature jump of 5 °C, corresponding to a Q_{10} of about 3·5.

TABLE 1. Temperature dependence of e.p.p. quantal content. m_c and m_L are the average quantal contents of e.p.p.s evoked before and after the laser-induced temperature jump, respectively

Junction	Initial temp. (°C)	Post-jump temp. (°C)	m_c	m_L	Q_{10}
	I. *m* determined by counting quantal potentials				
A	2	6	0·83	1·6	5·2
B	3	7	0·77	1·25	3·3
C	3	10	0·8	2·0	3·7
D	4	10	0·96	2·67	5·5
E	5·5	10·5	1·21	2·25	3·5
F	5·5	11	0·6	1·3	4·1
G	6	10	1·45	2·3	3·2
H	6	16	1·0	3·6	3·6
	II. *m* determined from e.p.p./m.e.p.p. area ratio				
I	1	11	3·8	13·5	3·6
J	1	10·5	6·1	23	4·0
K	1	8·5	5·5	18·7	5·1
L	1·4	7·2	4·2	9·2	3·9
M	2	8	2	4·7	4·2
N	2	10	9·9	32·7	4·5
O	2·2	10·2	5·6	16·4	3·8
P	2·5	9	8·5	17·4	3·0
Q	5	10·5	4·4	8·7	3·5
R	5·5	13·7	4·1	11	3·3
S	5·5	10·5	7·4	12·6	2·9
T	6·3	13·7	4·3	9·7	3·0
U	6·7	13·7	3·3	7·2	3·1
V	10·7	15·4	10·5	17·4	2·9
W	12·5	18	3·9	8·6	4·2

Mean $Q_{10} = 3{\cdot}8 \pm 0{\cdot}7$ S.D.

Fig. 5*B* shows e.p.p.s and m.e.p.p.s recorded in another preparation with a higher control quantal content. The lower control trace was recorded at 2·5 °C, the upper trace after a laser pulse (arrow) elevated the junctional temperature to 9 °C. Since individual quanta could not be counted, the quantal contents of the e.p.p.s were measured by dividing the area under the e.p.p. by the average area under m.e.p.p.s recorded at the same temperature (see Methods). Measured in this way, the average

quantal content of the control e.p.p. was 8·5, increasing to 17·4 after the 6·5 °C temperature jump, for a Q_{10} of 3·0. Note also the increased m.e.p.p. frequency following the e.p.p. recorded at the higher temperature.

The results of these and other similar experiments are summarized in Table 1. The values for e.p.p. quantal content after the temperature jump are approximate since they were calculated from only three to six e.p.p.s due to the mandatory 5 min interval between laser pulses. Nevertheless in all cases temperature jumps markedly increased the quantal content of the e.p.p., with a mean Q_{10} of $3{\cdot}8 \pm 0{\cdot}7$ (S.D.) over the range 1–18 °C. The average Q_{10} increased to about 3·9 when all e.p.p.s exceeding 5 mV were corrected for non-linear summation according to either Martin (1955) or Stevens (1976). Calculated Q_{10} values were not significantly correlated with the initial steady-state temperature (range 1–12·5 °C), the size of the temperature jump (range 4–10 °C), or the initial quantal content (range 0·6–10·5).

The records of Figs. 5*A*, 6 and 8 show that temperature jumps applied during or slightly before nerve stimulation also reduce the latency of the e.p.p. Extracellular recordings of action potentials in or near the nerve terminals in several preparations indicated that the decrease in e.p.p. latency following the temperature jump was due to both a higher conduction velocity in the nerve and a decreased synaptic delay between depolarization of the nerve terminal and the onset of the e.p.p. The minimal synaptic delays measured in these experiments agreed with those recorded by Katz & Miledi (1965*b*) at similar temperatures. Since the majority of the synaptic delay is of presynaptic origin (Katz & Miledi, 1965*a*, *b*), the onset of the e.p.p. or m.e.p.p. gives a good indication of the onset of transmitter release from the presynaptic terminal.

Temperature jumps during the synaptic delay also increase e.p.p. quantal content and decrease e.p.p. latency

The effect of temperature jumps on the quantal content and timing of transmitter release might be mediated by changing various features of the presynaptic action potential and the associated Ca influx and/or by modifying presynaptic processes occurring during the synaptic delay. Figs. 6 and 7 present data from an experiment in which temperature jumps were applied at various times during, as well as before and after, the synaptic delay. These records demonstrate that temperature-sensitive processes operating after the presynaptic action potential can alter the magnitude and timing of evoked transmitter release.

Each numbered record in Fig. 6 shows superimposed traces of one or two control e.p.p.s (C) recorded at 2 °C, along with a test e.p.p. (T) recorded during a trial in which a laser pulse (arrow) elevated the junctional temperature to 10 °C. In the sequence 1–5 the laser pulse was applied increasingly closer to the expected onset of the e.p.p., while in records 6–9 the laser pulse was applied between the onset and the peak of the e.p.p. Note that the voltage gain was doubled between records 5 and 6. Fig. 7 plots the quantal contents and latencies of the test e.p.p.s recorded at this junction as a function of the interval between the laser pulse and the time the e.p.p. would have begun if the temperature had remained at 2 °C. It is evident that temperature jumps applied before the expected onset of the e.p.p. (records 1–4, Fig. 6) reduce the latency, increase the peak amplitude and quantal content, and accelerate

the time course of the subsequent e.p.p. The increase in e.p.p. amplitude and quantal content becomes smaller as the interval between the temperature jump and the expected onset of the e.p.p. decreases, but temperature jumps occurring just 1 msec before the expected onset of the e.p.p. (record 4, Fig. 6) still have a significant effect. Extracellular records of Katz & Miledi (1965*b*) indicate that at the steady-state (control) temperature of 2 °C the peak inward and peak outward currents of the presynaptic action potential occur about 6 and 4 msec, respectively, before the onset of the e.p.p. (horizontal bar, Fig. 7). Consequently, the temperature jumps in records 2–4 of Fig. 6 were applied during the synaptic delay, after the major presynaptic

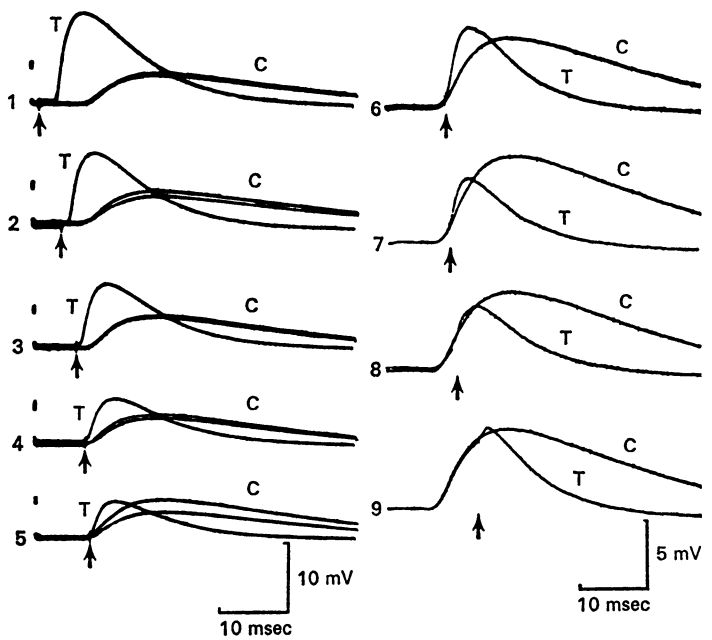

Fig. 6. Effect of temperature jumps applied at various times during neuromuscular transmission. Each record shows superimposed traces of 1 or 2 control e.p.p.s (C) recorded at the steady-state temperature of 2 °C, plus a test e.p.p. (T) recorded during a trial in which a laser pulse (arrow) sufficient to raise junctional temperature to 10 °C was applied. In records 1–5 the laser pulse preceded the e.p.p., while in records 6–9 the laser pulse occurred during the rising phase of the e.p.p. Note change of gain between records 5 and 6. Records are from junction *N* of Table 1. Calibrations: 10 mV, 10 msec in records 1–5; 5 mV, 10 msec in records 6–9.

depolarization. Thus, temperature jumps occurring during the synaptic delay increase and accelerate transmitter release, though to a lesser extent than temperature jumps applied before or during the presynaptic action potential.

Temperature jumps applied between the onset and the peak of an e.p.p. (records 5–9, Fig. 6) do not increase peak e.p.p. amplitude, but almost instantaneously accelerate the remaining time course of the e.p.p. The quantal contents of the test e.p.p.s in records 5 and 6 do not differ from controls. It was difficult to determine quantal content when the temperature jump occurred well after the onset of an e.p.p. (records 7–9), but certainly there is no evidence for a marked increase in release

following the laser pulse. Experiments like that of Figs. 6 and 7 performed at five other junctions also gave similar results. In all cases, temperature jumps applied before and during the synaptic delay increased e.p.p. quantal content, while jumps applied after the onset of the e.p.p. did not. Thus from these experiments it appears that the temperature-sensitive processes that help determine the number of quanta to be released during the e.p.p. operate during the presynaptic action potential and the synaptic delay, before the actual onset of release.

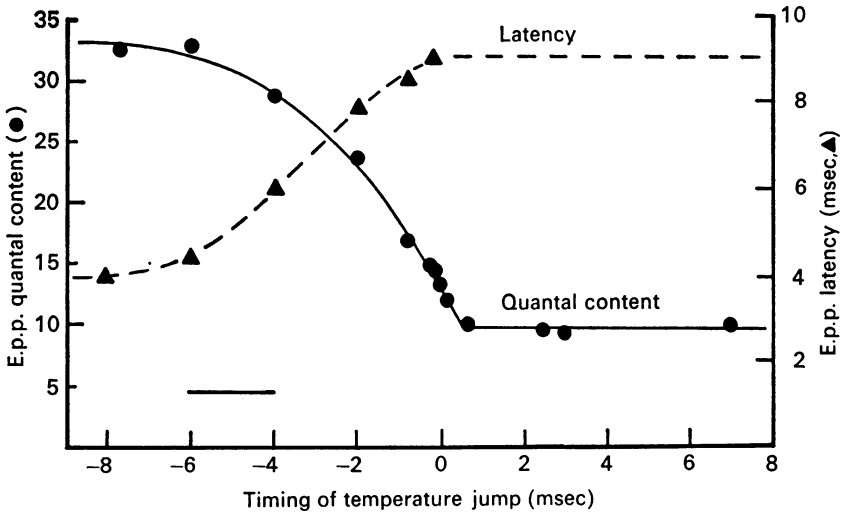

Fig. 7. Variation in e.p.p. quantal content and latency as a function of the timing of a temperature jump from 2 to 10 °C. Quantal contents were calculated from the ratio of the e.p.p. area to the average m.e.p.p. area at the same temperature. E.p.p. latency was measured as the interval between motor nerve stimulation and the onset of the e.p.p. Latency and quantal content values at far right are for a control e.p.p. recorded at 2 °C in the absence of a temperature jump. The timing of the temperature jump is expressed relative to the onset of this control e.p.p. On this time scale the motor nerve stimulus was applied at −9 msec. Same junction as Fig. 6. The bar at the lower left indicates the interval between the peak inward current and the peak outward current of the presynaptic action potential at 2 °C, based on extracellular records of Katz & Miledi (1965*b*).

The faster time course of e.p.p.s following the temperature jump probably reflects temperature-sensitive processes in both pre- and post-synaptic membranes. The timing of evoked transmitter release is highly temperature dependent (Katz & Miledi, 1965*b*; Barrett & Stevens, 1972), as is the rate of closure of ACh-induced end-plate channels (Magleby & Stevens, 1972; Kordaš, 1972; Anderson & Stevens, 1973). We did not collect sufficient data to measure the kinetics of transmitter release following temperature jumps, but limited data like those of Fig. 5*A*, in which individual quantal potentials can be distinguished, do suggest a more synchronous release of transmitter at the higher post-jump temperatures. Data of Barrett & Stevens (1972) indicate that at the control temperature of 2 °C most transmitter release should be complete within about 5 msec of the onset of the e.p.p. Temperature jumps applied after this time (e.g. record 9, Fig. 6) probably accelerate the falling

phase of the e.p.p. mainly by increasing the rate of closure of end-plate channels, since the passive membrane properties of the muscle are only minimally temperature sensitive (del Castillo & Machne, 1953, and our observations).

Temperature jumps increase the rate of decay of facilitation

When two motor nerve stimuli are applied within a few hundred msec of each other in low [Ca^{2+}] Ringer the second e.p.p. is usually facilitated, i.e. is larger and has a higher average quantal content than the first e.p.p. (del Castillo & Katz,

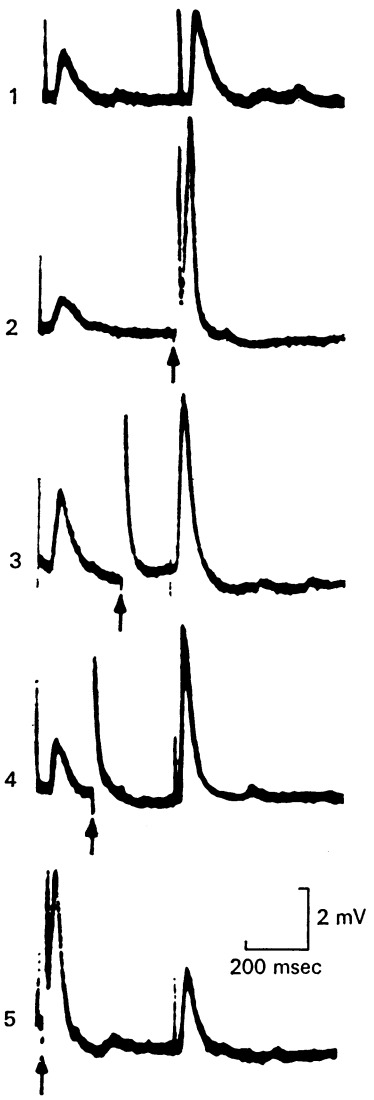

Fig. 8. Effect of temperature jump on facilitation. Upper trace shows two successive e.p.p.s recorded at the steady-state temperature of 2 °C. In traces 2–5, a temperature jump (arrow) sufficient to elevate junctional temperature to 10 °C was applied at progressively earlier times relative to the second e.p.p. The base lines of some records were retouched for clarity. Calibrations: 2 mV, 200 msec.

1954*b*; Mallart & Martin, 1967). Fig. 8 shows records from an experiment investigating the effects of temperature jumps on facilitation. Trace 1, recorded at the control temperature of 2 °C, shows two e.p.p.s, separated by a 350 msec interval. In traces 2–5 junctional temperature was jumped to 10 °C at the times indicated by the arrows. Temperature jumps applied during the interstimulus interval markedly increase the amplitude of the second e.p.p., consistent with the temperature dependence of e.p.p. quantal content demonstrated in Fig. 5 and Table 1. The amplitude (and quantal content) of the second e.p.p. decreases as the interval between the temperature jump and the second e.p.p. increases (compare traces 3 and 4 with trace 2). Very little of this decrease in the amplitude of the second e.p.p. is due to the decay of bath temperature following the laser pulse because single e.p.p.s evoked from 50 to 500 msec following a laser pulse are identical and because the measured time constant of thermal decay (7–8 sec) is long compared to the 350 msec interstimulus interval. The decrease in the amplitude of the second e.p.p. is more likely due to a temperature jump-induced acceleration of the rate of decay of facilitation, such that less facilitation remains as the interval between the laser pulse and the second e.p.p. increases. This result is consistent with demonstrations by Eccles *et al.* (1941), Hubbard *et al.* (1971) and Balnave & Gage (1974) that the time constant of decay of facilitation decreases with increasing temperature.

When the temperature jump is applied a few msec before the first e.p.p. (trace 5) the amplitude of the first e.p.p. increases, but that of the second e.p.p. decreases dramatically. This decrease in the second e.p.p. may be due in part to depression because of the marked increase in release to the first e.p.p., although the quantal content of the first e.p.p. in trace 5 is only about 13. In four similar experiments we compared the amplitudes of the second e.p.p.s when the temperature jump was moved from 10 msec after to 10 msec before the first e.p.p. With this paradigm changes in the amplitude of the second e.p.p. should be mainly due to temperature-induced changes in presynaptic processes surrounding the first e.p.p., since the time constants of decay of temperature (7–8 sec) and facilitation (> 150 msec at these low temperatures, Balnave & Gage, 1974) were large compared to the 20 msec difference in timing of the temperature jumps. In all cases temperature jumps preceding the first e.p.p. yielded second e.p.p.s that were either the same or smaller than those recorded when the temperature jump followed the first e.p.p., even when quantal release rates were only one third of those in Fig. 8. The fact that the second e.p.p. did not increase along with the first e.p.p. suggests that the temperature-sensitive processes which increase the magnitude of the first e.p.p. do not significantly increase the magnitude of facilitation.

DISCUSSION

All of the observed effects of the neodymium laser flash on neuromuscular transmission appeared to be mediated by the associated rapid increase in bath temperature rather than by direct photoactivation. Evidence supporting this conclusion includes the observation that the time courses of the post-laser increase in m.e.p.p. frequency (Fig. 3) and the measured post-laser temperature elevation are similar, and the observation that laser-induced changes in e.p.p. latency and in m.e.p.p. and e.p.p. time course could be duplicated by raising the steady-state bath temperature

to the level measured following laser discharge. Compared with conventional slow temperature changes the laser-induced temperature jump has at least two distinct advantages for studying the direct effects of temperature on neuromuscular transmission. First, the fast temperature jump minimizes the progressive ionic redistributions and metabolic changes that probably occur during slow temperature changes. Secondly, the laser makes it possible to increase junctional temperature during critical intervals such as the synaptic delay, the decaying phase of the end-plate current or a conditioning-testing interval.

The rates of both evoked and spontaneous transmitter release increase with increasing temperature. Temperature jump data of Table 1 give a mean Q_{10} of 3·8 (range 2·9–5·5) for e.p.p. quantal content in low [Ca^{2+}] Ringer over the range 1–18 °C. Two experiments employing slower temperature changes in the range 10–20 °C yielded lower Q_{10} values (1·9 and 2·8) for e.p.p. quantal content. The discrepancy between these Q_{10} values may be due merely to sampling errors, or may reflect slow changes occurring in the nerve terminals during the slow temperature changes. Q_{10} values for spontaneous transmitter release rates in normal Ringer over the range 10–20 °C are much higher, around 10 (Fig. 4*A*, *B*), in agreement with the average value of 9·5 reported by Bevan *et al.* (1976).

The temperature-dependent increase in m.e.p.p. frequency is not due to an increased influx of Ca^{2+} into the motor nerve terminal because a similar temperature-dependent increase in m.e.p.p. frequency is observed when bath [Ca^{2+}] is reduced to very low levels ($\leqslant 5 \times 10^{-7}$ M) with EGTA (Duncan & Statham, 1977, and our Fig. 4*A*). It is also unlikely that the temperature-dependent changes in the e.p.p. are due mainly to a change in Ca^{2+} influx, for several reasons. First, temperature jumps during the synaptic delay decrease the remaining synaptic delay (Figs. 6, 7). The synaptic delay would not be expected to change if the temperature jump merely increased Ca^{2+} influx, because the minimal synaptic delay is not sensitive to extracellular [Ca^{2+}] (Katz & Miledi, 1965*b*). A second reason is based on Katz & Miledi's (1968) demonstration that facilitation is linked to Ca^{2+} influx during the conditioning action potential. If a temperature jump increased the conditioning e.p.p. by increasing presynaptic Ca^{2+} influx, one might expect an increased facilitation of the test e.p.p. as the temperature jump was moved from just after to just before the conditioning e.p.p. Such an increase was never seen, even at low initial quantal contents. A final reason for suspecting that temperature-dependent changes in Ca^{2+} influx do not account for the temperature sensitivity of evoked release is that in squid axons the action potential-dependent Ca^{2+} and Na^{+} influxes actually decrease with increasing temperature (Hodgkin & Keynes, 1957; Landowne, 1977). Thus although the evidence is indirect, it appears that the temperature sensitivity of evoked release is due mainly to changes in rate-limiting steps other than Ca^{2+} influx. Data of Figs. 6 and 7 suggest that these temperature-dependent steps, which influence both the average quantal content and the minimal latency of the e.p.p., operate during both the presynaptic action potential and the synaptic delay, but are largely complete before the actual onset of release.

Temperature-dependent increases in evoked and spontaneous release rates may be due to an increased Ca^{2+} efflux from intracellular stores, as suggested for spontaneous release by Duncan & Statham (1977). However, we favour the idea that the Q_{10}

(10–20 °C) for spontaneous release in normal Ringer and in EGTA-low [Ca^{2+}] Ringer is very high because there is a very high energy barrier for a rate-limiting step in the transmitter release pathway. Reduction of this energy barrier produced by an increase in intracellular [Ca^{2+}] would account for the accelerated release rate and the reduced temperature sensitivity of evoked release and of spontaneous release in K^+-depolarized preparations (Fig. 4). Perhaps the high and low Q_{10}s characterize Ca-independent and Ca-dependent release, respectively. Alternatively, the high Q_{10} could characterize release activated by a single Ca^{2+}, and the lower Q_{10} could characterize release catalysed by 3–4 Ca^{2+}, since numerous workers have presented data suggesting that the rate of depolarization-evoked transmitter release varies linearly (or less than linearly) with extracellular [Ca^{2+}] when [Ca^{2+}] is very low ($< 10^{-5}$ M at the frog neuromuscular junction), but can vary as the third or fourth power of extracellular [Ca^{2+}] over a slightly higher range of [Ca^{2+}] (0·15–0·4 mM at the frog neuromuscular junction) (frog: Dodge & Rahamimoff, 1967; Miledi & Thies, 1971; Crawford, 1974; rat: Hubbard, Jones & Landau, 1968*a*, *b*; mouse: Cooke, Okamoto & Quastel, 1973).

If intracellular [Ca^{2+}] does determine the temperature sensitivity of transmitter release, then any experimental condition that produces an increase in intracellular [Ca^{2+}] should reduce the Q_{10} for m.e.p.p. frequency. We found lower Q_{10}s not only in depolarized terminals, but also in terminals cooled below about 10 °C, where intracellular [Ca^{2+}] might be expected to increase due to a temperature-dependent decrease in active sequestration or extrusion of Ca^{2+}. The relatively low Q_{10} (10–20 °C) values of 3–5 reported by earlier workers (Fatt & Katz, 1952; Li & Gouras, 1958; Takeuchi, 1958) may have been due to an elevated intraterminal [Ca^{2+}] under their experimental conditions. For example, some of Takeuchi's experiments employed 1·5 times hypertonic solutions to increase m.e.p.p. frequency, and hypertonic solutions increase intracellular Ca^{2+} activity in *Chironomus* salivary glands (B. Rose, personal communication) and probably in motor nerve terminals as well (Shimoni, Alnaes & Rahamimoff, 1977). An increase in intracellular [Ca^{2+}] caused by reduced metabolic activity or by electrode-induced mechanical damage to the nerve terminal would also be expected to increase m.e.p.p. frequency and to decrease the temperature sensitivity of m.e.p.p. frequency. Under our experimental conditions nerve terminals with spontaneous m.e.p.p. frequencies of 0·5/sec or less at 20 °C usually had Q_{10}s (10–20 °C) near 10 (see also Fig. 3*B* of Bevan *et al.* 1976), while terminals with resting m.e.p.p. rates exceeding 5/sec at 20 °C usually had lower Q_{10}s. However, there was considerable variability between junctions and frogs, and factors other than intracellular [Ca^{2+}] also influence m.e.p.p. frequency (e.g. nerve terminal area, Kuno, Turkanis & Weakly, 1971).

Assuming that one step in the spontaneous transmitter release pathway is rate-limiting and effectively irreversible, the energy of activation, E_A, of that reaction will influence the temperature dependence of spontaneous release. If the Q_{10} of 9–10 measured for spontaneous release is entirely due to the E_A of this reaction, then the E_A of this rate-limiting step is 35–37 kcal ($E_A = (\ln Q_{10})\ (T^2R/10)$, where T is absolute temperature and R is the gas constant). Similar analysis yields an activation energy of 22 kcal for an irreversible, rate-limiting step in evoked release with a Q_{10} of 4 (Table 1). If Ca^{2+} catalytically reduces an energy barrier involved in the trans-

mitter release process, this lower Q_{10} would apply whenever the rate of Ca^{2+}-activated release greatly exceeds the rate of Ca^{2+}-independent release.

Assuming that spontaneous and evoked release have the same rate-limiting step and that Ca^{2+} reduces the energy barrier associated with this step, the difference between the energies of activation of spontaneous and evoked release, ΔE_A (13–15 kcal), can be used to predict the ratio between the quantal release rate when all release sites are saturated with Ca^{2+} and the release rate when release sites bind little or no Ca^{2+}. Using the Arrhenius equation,

$$\text{Rate} = K\, e^{-E_A/RT},$$

where K represents entropy factors, the predicted ratio of Ca^{2+}-dependent to Ca^{2+}-independent release rates is

$$\frac{\text{Rate}_{\text{Ca}}}{\text{Rate}_{\text{no Ca}}} = e^{\Delta E_A/RT},$$

if the entropy requirements for both release pathways are similar. For ΔE_A of 13–15 kcal, the predicted ratio lies between 10^{10} and 2×10^{11}. A similar ratio is obtained using the Eyring absolute rate equation (Eyring & Eyring, 1963). Observed spontaneous and evoked quantal release rates at 20 °C in normal $[Ca^{2+}]$ are about 0·5/sec and 100/0·5 msec, respectively, yielding a ratio of 4×10^5. Thus the predicted ratio of Ca^{2+}-dependent to Ca^{2+}-independent release rates is considerably higher than the observed ratio of evoked and spontaneous release rates. At least part of this discrepancy probably arises because only a fraction of the release sites are saturated with Ca^{2+} during normal evoked release. Evoked release rates increase dramatically when the action potential is prolonged by tetraethylammonium in high $[Ca^{2+}]$ Ringer (Katz & Miledi, 1969). Other factors, such as a slow turnover of release sites, may also limit the maximal observed release rates. Another possibility is that Ca^{2+} reduces an energy barrier in a separate transmitter releasing pathway with more stringent entropy requirements than the Ca^{2+}-independent pathway.

In summary, the very high Q_{10} of about 10 measured for spontaneous m.e.p.p. frequency in normal or low $[Ca^{2+}]$ Ringer between 10 and 20 °C suggests the existence of a transmitter-releasing pathway containing a very high energy barrier. The accelerated release rates and lower Q_{10}s (3–5) measured after nerve stimulation or during high $[K^+]$ depolarization in the presence of Ca^{2+} suggests that an increase in intracellular $[Ca^{2+}]$ lowers an energy barrier in this or another parallel transmitter releasing pathway. Care should be taken to distinguish between these two types of transmitter release, one completely or relatively Ca^{2+}-independent and very temperature sensitive, the other Ca^{2+} dependent and less temperature sensitive, in future studies directed at characterizing the mechanism of transmitter release.

Note added in proof. Further support for our argument that processes in addition to Ca^{2+} influx contribute to the synaptic delay comes from recent experiments with extrinsically depolarized frog motor nerve terminals (B. Katz & R. Miledi, 1977, *Proc. R. Soc.* B, **196**, 465–469). Terminals depolarized to a level which suppresses transmitter release show a burst of release, the 'off' response, upon termination of the depolarization. Although presynaptic Ca^{2+} influx would be expected to be substantial immediately following release from depolarization, there is a definite delay (6 msec) between the end of the depolarization and the onset of the 'off' response.

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