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THE EFFECT OF CALCIUM AND MAGNESIUM ON THE SPONTANEOUS RELEASE OF TRANSMITTER AT INSECT MOTOR NERVE TERMINALS

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SUMMARY

1. The effect of the extracellular calcium and magnesium concentrations and calcium ionophore, X-537A, on the frequency of miniature excitatory post-synaptic potentials (MEPSPs) was studied in cockroach leg muscle fibres.

2. The frequency of MEPSPs increased as the calcium concentration was increased from 0·1 to 10 mM. In the presence of 10 mM magnesium, however, raising the calcium concentration from 0·1 to 1 mM slightly depressed the frequency. In saline containing elevated potassium (20·8 mM), increasing the calcium concentration produced a much higher frequency than that in the normal potassium saline (10·8 mM) in the absence of magnesium. Raising the extracellular potassium concentration was without effect unless the bathing solution contained calcium.

3. The frequency of the miniature potentials was reduced as the magnesium concentration was raised from 0 to 10 mM, depending on the presence of calcium ions. On the contrary, a slightly increased frequency was observed in the low calcium saline as the magnesium concentration was raised from 1 to 10 mM. The reciprocal relationship between calcium and magnesium and the time course of the effect suggest that both ions act at the same surface sites in the presynaptic membrane.

4. X-537A elicited a transient increase in frequency followed by a fall of the frequency to a very low rate. Further application of the ionophore was without effect. The effect of X-537A on the spontaneous release of transmitter at the insect neuromuscular junction was comparable with that on the spontaneous acetylcholine release in vertebrate neuromuscular junctions.

INTRODUCTION

The spontaneous miniature potentials in insect muscle fibres, which were first recorded intracellularly by Usherwood (1961), have been extensively studied. Usherwood (1963) observed the influence of potassium, calcium and magnesium ions in the external solution on the miniature frequency of the muscle fibres. However, the effect of altering the ionic composition of the external medium of insect muscle fibres has not been comprehensively studied.

On the other hand, it has been well established that calcium ions are essential for

the release of transmitter quanta at the vertebrate neuromuscular junction (Katz, 1969). The relation between spontaneous transmitter release and the extracellular calcium and magnesium concentration was studied in mammalian neuromuscular junctions by Hubbard (1961) and Hubbard, Jones & Landau (1968). They explained the effect of calcium by the combination of calcium molecules with a nerve terminal receptor site. They also showed that calcium and magnesium competed for the same sites.

The investigation reported here represents a systematic study of the effect of altering the concentrations of these ions in the external bathing solution on the frequency of spontaneous miniature excitatory post-synaptic potentials (MEPSPs) in cockroach leg muscle fibres. The effect of divalent cation ionophore, X-537A, which is capable of transferring calcium ions across membranes (Pressman, 1973) was also studied to elucidate how the change in intracellular calcium levels affects the spontaneous release of excitatory transmitter, which is believed to be L-glutamate in crustacean and insect somatic muscles (Takeuchi & Takeuchi, 1964; Usherwood & Machili, 1968).

METHODS AND MATERIALS

All experiments were performed with the coxal depressor muscle number 178 (Carbonell, 1947) isolated from metathoracic legs of the cockroach, *Periplaneta americana*. Muscle 178 is a broad flat muscle lying dorsally in the coxa about 3·6 mm long and innervated by only one excitatory motor axon (Pearson & Iles, 1971). The muscle was mounted in an acrylic bath which had a volume of about 1·5 ml and was illuminated from below.

Spontaneous miniature potentials were recorded with conventional intracellular recording techniques. The microelectrodes were filled with 3 M-KCl and had a resistance of 5–10 MΩ. The occurrence of the potentials just above the position of the noise level was detected by a capacity-coupled discriminator and triggered a pulse generator (Nihon-Kohden Ltd., Tokyo). MEPSPs were recorded on one channel of a magnetic tape recorder at 14·7 cm/sec, and the pulses from the generator on another channel. The generation of pulses was monitored continuously on another beam of the oscilloscope and was identified with the MEPSPs. In other experiments designed for the measurement of membrane constants, two microelectrodes were used, one being a current electrode and the other a recording electrode. The details of the method were similar to those described by Washio (1972).

The standard bathing solution had the following composition (in mM): NaCl 158·0, KCl 10·8, $CaCl_2$, 5·0, $NaHCO_3$ 1·0, NaH_2PO_4 0·1 (Becht, Hoyle & Usherwood, 1960). Additions or withdrawals of $CaCl_2$ and $MgCl_2$ were osmotically compensated for by withdrawal or addition of appropriated amounts of NaCl. Compensation for added KCl was made by removal of equimolar amounts of NaCl. X-537A (Hoffman–La Roche) was dissolved in ethanol, and control solutions were also prepared with the same amount of ethanol, which never exceeded 0·5 %.

In each experiment the preparation was soaked in the original solution for about 2 h before any recordings were made. The effect of different concentrations of calcium and magnesium was measured by comparing the frequency of MEPSPs at the same junction before and after treatment with test solutions. The preparation was perfused

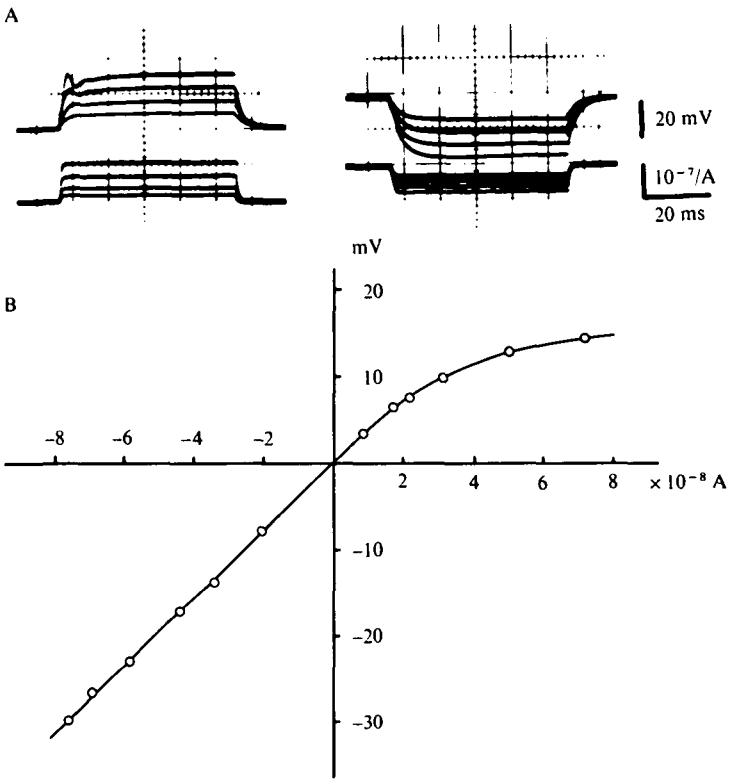

Fig. 1. (A) Membrane potential changes produced by constant current pulses in standard saline (resting potential, −52 mV). (B) Current–voltage relationship in standard saline. The measurements of the membrane potential were made at the steady state of polarization produced by square pulses of 100 ms duration. Negative ordinate represents hyperpolarization.

by constant flow at a rate of 3·0 ml/min, and the rate of flow was raised to 5·0 ml/min during a change in composition of the bathing fluid. The experiments were performed at room temperature (20–24 °C).

RESULTS

Electrical properties of the muscle membrane and spontaneous miniature potentials

The resting potential measured in the standard solution ranged between 45 and 60 mV. When outward current pulses were applied through an intracellular electrode, the muscle fibre responded with graded depolarizations as shown in Fig. 1 A. These responses were accompanied by a hump during their initial parts with large outward current pulses. No all-or-none response was found in standard saline in these fibres. The relation between the steady displacement of potential and the applied current showed delayed rectifications as shown in Fig. 1 B, similar to those seen in other excitable membranes.

The MEPSPs occurred at frequencies ranging from 0·5 to 2·0/s in the standard saline containing 5 mM calcium. The miniature potentials recorded intracellularly

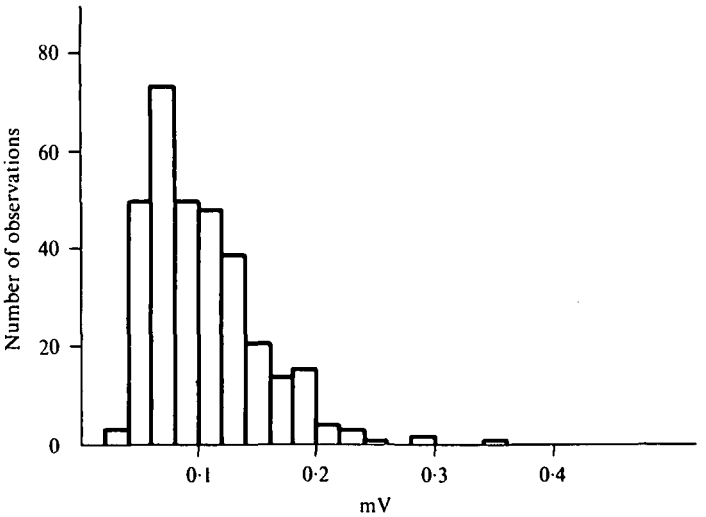

Fig. 2. The distribution of MEPSP amplitudes recorded from the coxal depressor muscle. The potentials recorded from the centre of a fibre (total length, 3·6 mm; λ, 2·6 mm).

occurred basically in random manner at this neuromuscular junction (Washio & Inouye, 1975). Fig. 2 gives a typical amplitude histogram of MEPSPs recorded from a fibre in the coxal depressor muscle. In agreement with the earlier finding (Usherwood, 1963), the histogram shows a positive skew which is to be expected since these muscle fibres are multiterminally innervated. Thus, the miniature potentials originating at various distances from the recording electrode are inevitably attenuated by the cable properties of the fibre before reaching the electrode. However, some of the MEPSPs in Fig. 2 were almost six times the modal amplitude. This cannot be explained on decrement alone as described below. It is conceivable that larger MEPSPs are generated by the synchronous release of several units.

Membrane constants were determined from steady-state changes in membrane potential produced by current passed through an intracellular electrode which was inserted in the middle of the fibre. Since the length constant (λ) is much greater than the fibre length in short insect muscle fibres (Washio, 1972), a short cable model (Weidman, 1952) was used to estimate the length constant.

$$\frac{V_x}{V_0} = \frac{\cos h[(L-X)/\lambda]}{\cos h(L/\lambda)},$$

where V_0 is the electrotonic potential produced by current I at the polarizing electrode, V_x the potential at the distance x from the polarizing electrode and L half the length of the fibre. The calculated values for the electrical characteristics of five fibres from five different coxal depressor muscles of the cockroach are shown in Table 1. From the equation, 16% and 32% attenuation of the electrotonic potential at both ends of the fibre (L: 1·8 mm) correspond to length constants of 2·8 and 2·0 mm, respectively. Since insect somatic muscle fibres are multiterminally innervated (Usherwood, 1974), the recording electrode was usually inserted in the middle of the fibre in order to minimize the attenuation of miniature potentials far from the electrode. Gage & McBurney (1972) found that the decrement of miniature end-plate potentials was

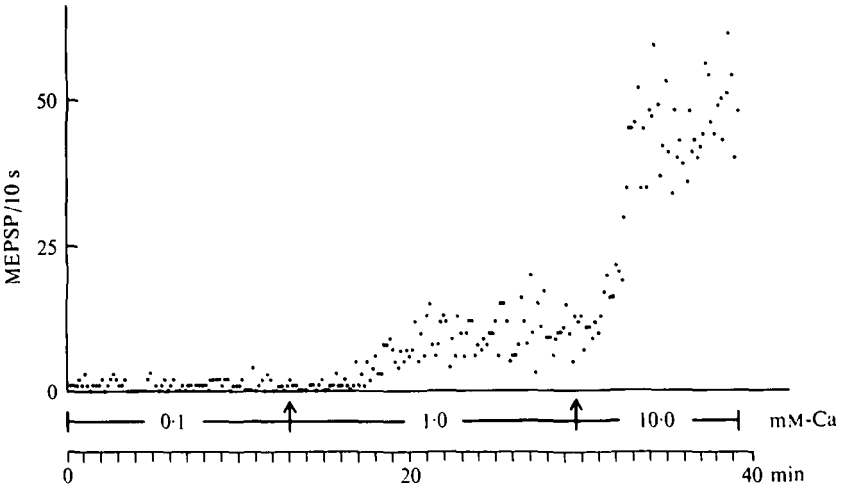

Fig. 3. Time course of the effect of the external calcium concentration (mM) upon the MEPSP frequency in high potassium (20·8 mM) saline. Arrows indicate the changeover points. Data from a single junction.

Table 1. *Input resistance and calculated length constant* (λ) *in cockroach coxal muscle fibres in standard saline*

Input resistance	Length constant (mm)	Fibre length from centre to end (mm)
$3{\cdot}2 \times 10^5\ \Omega$	2·8	1·8
$2{\cdot}3 \times 10^5\ \Omega$	2·5	1·7
$3{\cdot}8 \times 10^5\ \Omega$	2·4	1·8
$1{\cdot}2 \times 10^5\ \Omega$	2·0	1·7
$3{\cdot}5 \times 10^5\ \Omega$	2·6	1·8

larger than that of the steady voltage response. Thus it is conceivable that attenuation of the MEPSPs in the insect muscle fibre is larger than 32% at its maximum. On the other hand, Usherwood (1963) found that extracellular calcium and magnesium ions at concentrations below 10 mM had little effect on the amplitude of the miniature potentials in this insect muscle fibre. Since the amplitude of the miniature potentials should be proportional to the input resistance of the fibre (Katz & Thesleff, 1957), it seems likely that the effect of these ions at concentrations below 10 mM on the cable properties of the muscle fibre is small.

Effect of calcium

To get a reliable relationship between MEPSP frequency and extracellular calcium and magnesium concentrations, measurements of frequency should be made at a time when the miniature potential frequency had reached a steady level after a change in these extracellular ion concentrations. An example may be seen in Fig. 3, which shows the time course of the effect of different concentrations of calcium upon the miniature potential frequency in high-potassium (20·8 mM) saline. Raising the calcium from 0·1 to 1·0 mM and from 1·0 to 10 mM produced a frequency increase which took about 8 min and 5 min for completion in this preparation, respectively. In bathing solutions

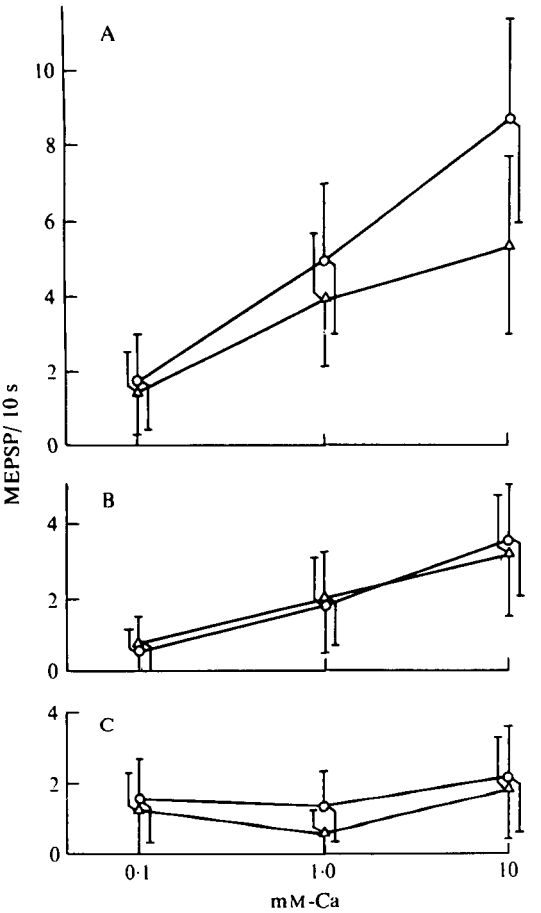

Fig. 4. Effect of external calcium concentration on mean MEPSP frequency in standard potassium (10·8 mM) saline. (A) 0 mM-$MgCl_2$. (B) 2 mM-$MgCl_2$. (C) 10 mM-$MgCl_2$. Each sign in A, B and C indicates mean frequency obtained from an individual junction. Vertical bars indicate S.D. of each mean, the number of observations at each calcium concentration, 120.

containing standard potassium concentration (10·8 mM) it took 2–3 times as long as the time to reach a steady level in the high-potassium saline shown in Fig. 2. The time for completion in insect muscle fibres was compatible with that in frog (Mambrini & Benoit, 1964) and mammalian (Hubbard *et al.* 1968) neuromuscular junctions when the calcium concentration in the the bathing fluid was raised.

The effect of calcium on the miniature potential frequency was studied in the presence of 0, 2 and 10 mM-$MgCl_2$ (Fig. 4). The increase in the frequency was approximately linearly proportional to the logarithm of the extracellular calcium concentrations over the range tested with 0 or 2 mM magnesium (Fig. 4A, B). The rate of increase in the frequency was reduced in the presence of magnesium ions. Finally, in the presence of 10 mM magnesium, raising the calcium concentration from 0·1 to 1 mM slightly depressed the frequency as shown in Fig. 4C (significant in one-tailed Welch test, $P < 0{\cdot}01$ in one series, $P < 0{\cdot}05$ in another). Further increase in the

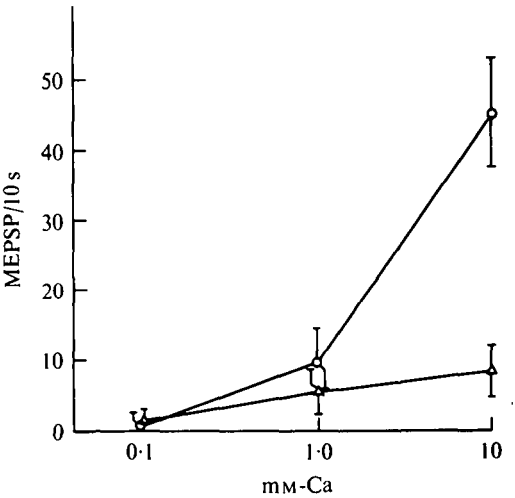

Fig. 5. Effect of external calcium concentrations on mean MEPSP frequency in standard potassium saline (10·8 mM, open triangles) and in high-potassium saline (20·8 mM, open circles) in the absence of magnesium. Each sign was obtained from an individual junction. Vertical bars indicate S.D. of each mean. Number of observations at each calcium concentration, 120.

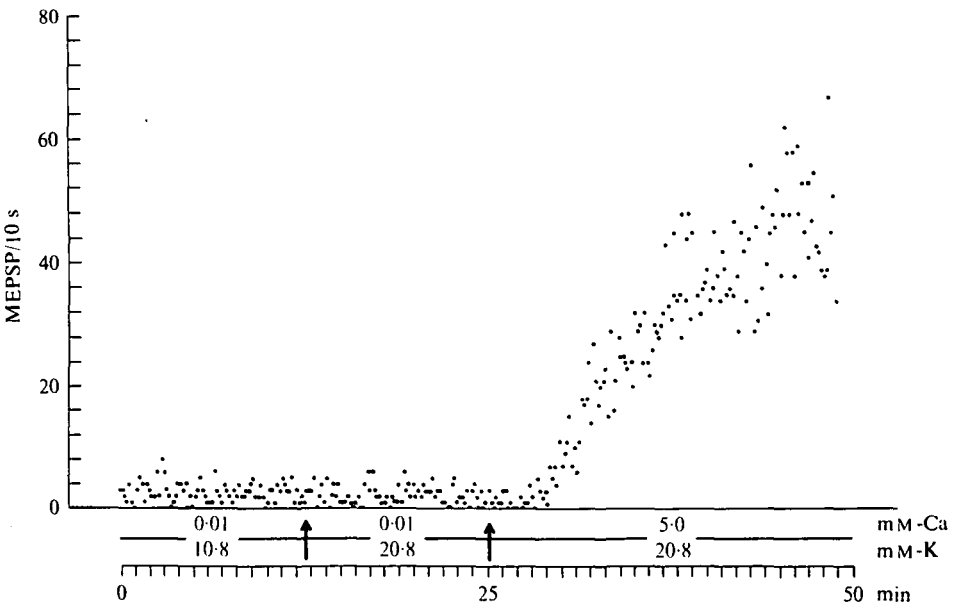

Fig. 6. Effect of external calcium (0·01 and 5·0 mM) and potassium (10·8 and 20·8 mM) concentrations on MEPSP frequency. Arrows indicate the changeover points. Data from a single junction.

calcium concentration to 10 mM increased a fraction of the frequency of the potentials (significant in one-tailed Welch test, $P < 0{\cdot}01$ in both series).

The interaction of calcium and potassium was studied by examining the effect of raising the potassium concentration in the saline on the discharge frequency as calcium concentration was increased. In saline containing elevated potassium ions

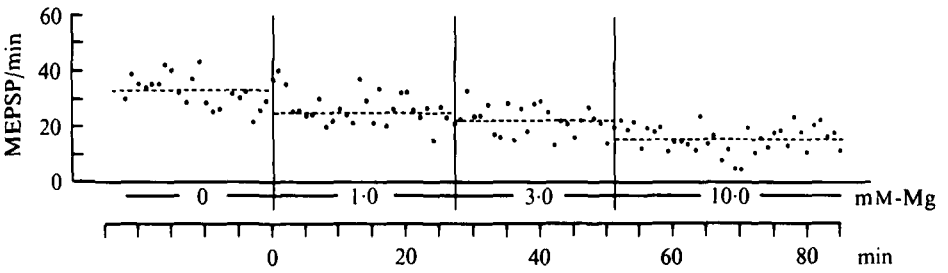

Fig. 7. Effect of external magnesium concentration (mM) on MEPSP frequency in presence of 5 mM calcium. Vertical lines indicate the changeover points. Horizontal dashed lines indicate mean MEPSP frequency in each saline. Data from a single junction.

(20·8 mM) the increase in the frequency was much higher than that in saline containing the standard concentration (10·8 mM) of potassium (Fig. 5). On the other hand, the effect of raising the potassium concentration in the saline containing 0·01 mM-$CaCl_2$ was negligible (Fig. 6). When calcium was added to the saline, the acceleration of the frequency was rapid and substantial. Raising the potassium concentration of the external medium would be expected to produce a stable depolarization of nerve terminals (Liley 1956). Thus, the present results suggest that the increase in the rate of spontaneous transmitter release upon depolarization is highly dependent on the extracellular calcium concentration in insect neuromuscular junctions just as in vertebrate neuromuscular junctions (del Castillo & Katz 1954*a*, *b*, *c*; Hubbard *et al.* 1968; Laudau 1969; Cooke, Okamoto & Quastel, 1973). Recently, reduction in miniature end-plate potential frequency was reported at neuromuscular junctions depolarized by raising the potassium concentration in the saline as the calcium concentration was increased (Cooke & Quastel, 1973; Matthews & Wickelgren, 1977). At insect neuromuscular junctions, however, such a reduction in the frequency was not observed in the saline containing elevated potassium ions up to 20·8 mM.

Effect of magnesium

In agreement with the earlier finding on the mammalian neuromuscular junction (Hubbard *et al.* 1968), the frequency reached a steady level more rapidly following a change in magnesium concentration than in calcium concentration. Fig. 7 shows the time course of the effect of magnesium on the frequency. After increasing magnesium concentrations in the range tested, the new frequency level was reached in about 5 min.

The effect of magnesium was studied in the presence of 0·1, 1 and 5 mM calcium. As shown in Fig. 8A, increasing magnesium concentration from 0 to 10 mM depressed the frequency (significant at 0·05 confidence level). For example, a magnesium concentration of 10 mM depressed the mean frequency by about 48%. It was found that the rate of decrease in the frequency was smaller when the original frequency in the absence of magnesium was low. When the calcium concentration was reduced to 1 mM the effect of magnesium was less effective (Fig. 8B). In this solution the reduction of frequency appeared to be fully developed at 1 mM magnesium and the concentration above 1 mM had little further effect upon the frequency. Finally, magnesium was without effect when the calcium concentration was reduced to 0·1 mM. A slightly

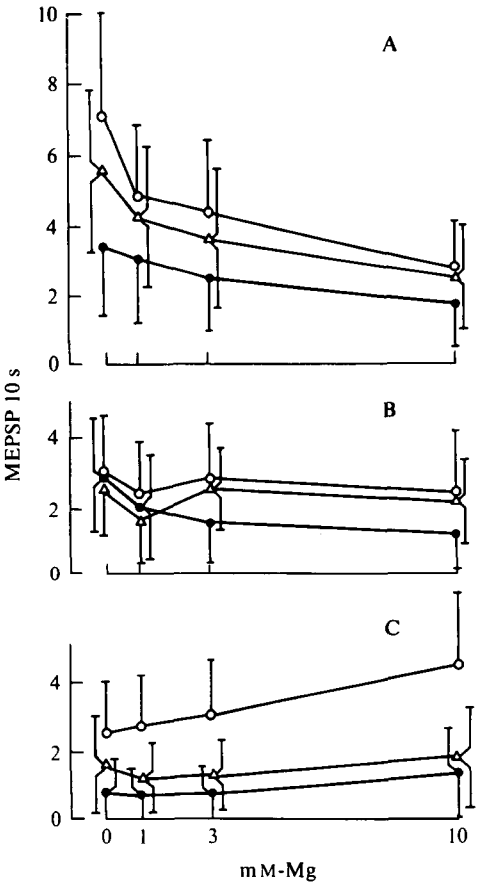

Fig. 8. Effect of external magnesium concentration on mean MEPSP frequency in standard potassium (10·8 mM) saline. (A) With 5 mM-$CaCl_2$. (B) With 1 mM-$CaCl_2$. (C) With 0·1 mM-$CaCl_2$. Each sign in A, B and C indicates mean frequency obtained from an individual junction. Vertical bars indicate S.D. of each mean. Number of observations at each magnesium concentration 120.

increased frequency was recorded in the low calcium saline on raising magnesium concentration from 1 to 10 mM in some junctions, as shown in Fig. 8C ($P < 0.01$).

Effect of X-537A

It has been reported that the ionophoric antibiotic, X-537A, produces a massive and transitory increase in miniature end-plate potentials frequency at the frog neuromuscular junction (Kita & Van der Kloot, 1976). This divalent cation ionophore was also found to produce a substantial increase in the MEPSP frequency in insect muscle fibres. Fig. 9 shows the effect of a series of increasing concentrations of X-537A on the frequency in 5 mM calcium saline. X-537A at 2 and 5 μM had no effect on the frequency, but at 10 μM elicited a transient increase in this preparation. The threshold concentration of X-537A to elicit a massive increase in the frequency was not determined precisely but appeared to be between 5 and 10 μM. In some junctions, X-537A

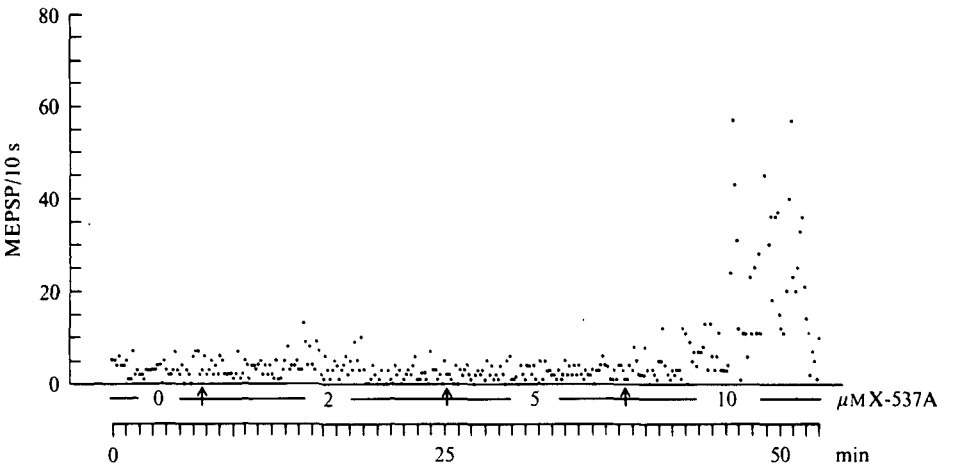

Fig. 9. Effect of a series of increasing concentration of X-537A (μM) on the MEPSP frequency in standard saline. Arrows indicate the changeover points. Data from a single junction.

at 5 μM caused a transient increase approximately 10 min after applying the ionophore, followed by a fall in the frequency to a very low rate. At this stage an application of higher concentration of X-537A was without effect. The higher the concentration of X-537A tested up to 20 μM, the larger and longer was the increase in frequency. However, the frequency increases always began approximately 10 min after the exposure to X-537A, even at a concentration of 20 μM. X-537A at 20 μM depolarized the insect muscle membrane by 8–15 mV, in agreement with earlier findings on frog skeletal muscles (Devore & Nastuk, 1975; Kita & Van der Kloot, 1976). With 10 μM X-537A, the resting potential fell by less than 5 mV about half an hour after introducing the ionophore.

DISCUSSION

It has been shown previously that the process of neuromuscular transmission in insects is affected by the concentration of calcium, magnesium and potassium in the saline, in a similar manner to that in vertebrates (Hoyle, 1955). In this paper we have shown that the spontaneous release of transmitter at excitatory neuromuscular junctions in insect leg muscle is dependent on the concentration of these ions, in a similar way to that in vertebrates. The acceleration of MEPSP frequency by calcium was affected by the presence of magnesium. Also the reduction of the frequency by magnesium was modified by the extracellular calcium concentration. Thus the reciprocal relationship suggests that both ions act at the same site. Furthermore, it seems likely that the rapid actions of magnesium and calcium upon the miniature potential frequency are compatible with surface actions of these ions just as previously pointed out in vertebrate neuromuscular junctions (Hubbard *et al.* 1968). When the magnesium concentration was raised from 0 to 1 mM in the absence of calcium, the depressing effect of magnesium became negligible. On the contrary, a slight increase in the frequency was observed in some neuromuscular junctions as magnesium concentration was raised from 1 to 10 mM in the saline containing 0·1 mM calcium. The result would imply that magnesium has at least some ability to trigger spontaneous release of transmitter in a calcium-free medium at insect neuromuscular junctions as

viously found in frog (Blioch, Glagoleva, Liberman & Nenashev, 1968) and mammalian (Hubbard *et al.* 1968) neuromuscular junctions.

Katz & Miledi (1967, 1969) obtained evidence that depolarization of the presynaptic membrane opens a gate to calcium ions and allows influx of the ions. Thus, an increase in the intracellular calcium concentration in the terminals would increase the spontaneous release of transmitter (Alnaes & Rahamimoff, 1975). Using calcium ionophore, X-537A, Kita & Van der Kloot (1976) proposed that the ionophore increased spontaneous acetylcholine release by acting as an ionophore to raise intracellular divalent cation concentration. Our present results showed that X-537A raised the rate of spontaneous release of transmitter from insect neuromuscular junctions in a similar manner to spontaneous acetylcholine release. The threshold ionophore concentration which caused a massive increase in frequency, and the latent period for the occurrence of this increase after application of the ionophore at insect neuromuscular junctions were very closely comparable with those in vertebrates. Thus, it seems likely that X-537A raises the level of ionized calcium inside the motor nerve terminals by transferring calcium ions across the presynaptic membrane in insect neuromuscular junctions. However X-537A caused a depolarization of the insect muscle fibre as well as the frog muscle fibre. We cannot rule out the possibility that depolarization of the presynaptic nerve terminals by the ionophore-induced intracellular accumulation of sodium ions (Devore & Nastuk, 1975) could partly account for the transient increase in the spontaneous transmitter release at insect neuromuscular junctions.

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