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SPONTANEOUS MINIATURE POTENTIALS FROM INSECT MUSCLE FIBRES

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The most widely applicable hypothesis for transmission across the synapse between the motor nerve terminals and the muscle membrane envisages the release of quantal packets of chemical transmitter, each packet containing many molecules, from the presynaptic terminals, and the attachment of these packets to receptor sites on the post-synaptic membrane to produce a transient depolarization; the magnitude of the depolarization is held to be partly dependent on the number of quanta released. The arrival of the nerve impulse at the presynaptic terminals is thought to produce the discharge of a great number of these quantal packets of transmitter within a millisecond or so to produce a depolarization of the post-synaptic membrane called the post-synaptic potential. In the absence of nerve stimulation randomly occurring spontaneous potentials, usually less than 1 mV in magnitude, are recorded from the junctional regions of both vertebrate (e.g. Fatt & Katz, 1952) and crustacean muscle (Dudel & Orkand, 1960). It has been suggested that these miniature potentials result from a sporadic release of transmitter from the nerve terminals.

Spontaneous miniature post-synaptic potentials have now been observed in insect muscle fibres. A preliminary note on these has already appeared (Usherwood, 1961). This note also described some of the effects which changes in the osmotic pressure of the bathing solution produced in the discharge frequency of the miniature potentials. In the present paper these observations are described in greater detail, together with additional observations, made subsequently, on the action of cations and drugs on the spontaneous discharge.

METHODS

Recordings were obtained from the metathoracic flexor and extensor tibiae muscles of the locust, *Schistocerca gregaria*, and the cockroaches, *Blaberus giganteus* and *Periplaneta americana*. Most experiments were performed with the metathoracic legs attached to the animal but with the central connexions of the nerves to the femoral muscles cut. The femoral muscles of a few isolated legs were also examined. The properties of these preparations have been described in previous publications (Hoyle, 1954; Usherwood, 1962*a*). For

Schistocerca, Hoyle's (1954) locust saline was used, containing (mM): NaCl 140, KCl 10, $CaCl_2$ 2, $NaHCO_3$ 4, NaH_2PO_4 6. For *Periplaneta*, Becht's cockroach saline was used, containing (mM): NaCl 158, KCl 10·8, $CaCl_2$ 9, $NaHCO_3$ 2, NaH_2PO_4 0·1 (Becht, Hoyle & Usherwood, 1960). For *Blaberus*, the following saline was used (mM): NaCl 161·4, KCl 18·5, $CaCl_2$ 9, $NaHCO_3$ 4, NaH_2PO_4 6 (Usherwood, 1962*a*). Changes in the concentrations of magnesium ions, calcium ions and potassium ions in the salines were made by substitution for, or addition of, osmotically equivalent quantities of sodium ions. Changes in saline tonicity were made either by dilution with de-ionized water or by addition of sucrose. Drugs were applied in solution, in the appropriate saline.

The bathing fluid was exchanged by circulating fresh saline over the preparation with a fine syringe. Since it was considered impossible to exchange completely the saline around the deeper bundles of muscle fibres by this technique, recording was restricted to surface fibres. The spontaneous activity of a single fibre was usually recorded before, during and after exposure to the test solution, but on occasions this procedure was modified by starting the series of recordings in the test solution.

On each occasion the preparation was soaked for 5 min in the new saline before any recordings were made. The effects of changes in the cation concentrations were examined by recording, in both test saline and normal saline, the miniature potentials arising over a period of 3 min and calculating the average amplitude and frequency during this period. The results for a number of different fibres were collated.

Micro-electrodes, filled with 3M-KCl and of 5–20MΩ resistance, were used, together with conventional recording apparatus. Spontaneous movements of the preparations, which often persisted after the femur had been isolated from the rest of the leg, frequently shifted the position of the electrode inside the muscle fibre and sometimes dislodged it altogether. If these movements persisted for long the preparation was discarded.

The miniature potentials were recorded throughout by photographing successive oscillograph traces, and the records were subsequently magnified and measured by projection on to a calibrated screen. Del Castillo & Katz (1954*a*) pointed out that errors might be incurred by recording small rapid signals on a slowly moving 'noisy' base line. The usual procedure of measuring a transient from the upper edge of the base line to peak of excursion was adopted, and any errors that may have resulted from this procedure have not been compensated for.

RESULTS

As might be expected from the multi-terminal innervation that is known to be usual in insect muscle (e.g. Marcu, 1929) it is possible to record spontaneous miniature potentials by impaling a muscle fibre at any point along its length with a micro-electrode. The amplitudes of the spontaneous potentials vary to some extent, with the membrane resistance of the muscle fibre. In the present experiments the amplitudes of potentials

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Legend to Fig. 1

Fig. 1. Histograms of potentials recorded from three muscle fibres with markedly different frequency distributions of amplitudes. *A*, series of 824 potentials (arithmetic mean amplitude, 180 μV) from a fibre (length about 8 mm, λ about 3 mm) of the metathoracic flexor tibiae muscle of *B. giganteus*. *B*, series of 434 potentials (arithmetic mean amplitude, 229 μV) from a proximal fibre (length about 3 mm, λ about 2·8 mm) of the locust metathoracic extensor tibiae muscle. *C*, series of 756 potentials (arithmetic mean amplitude, 311 μV) from a distal fibre (length about 1·0 mm, λ about 2·8 mm) of the locust metathoracic extensor tibiae muscle. Note reduction of positive skew as length of fibre decreases relative to length constant.

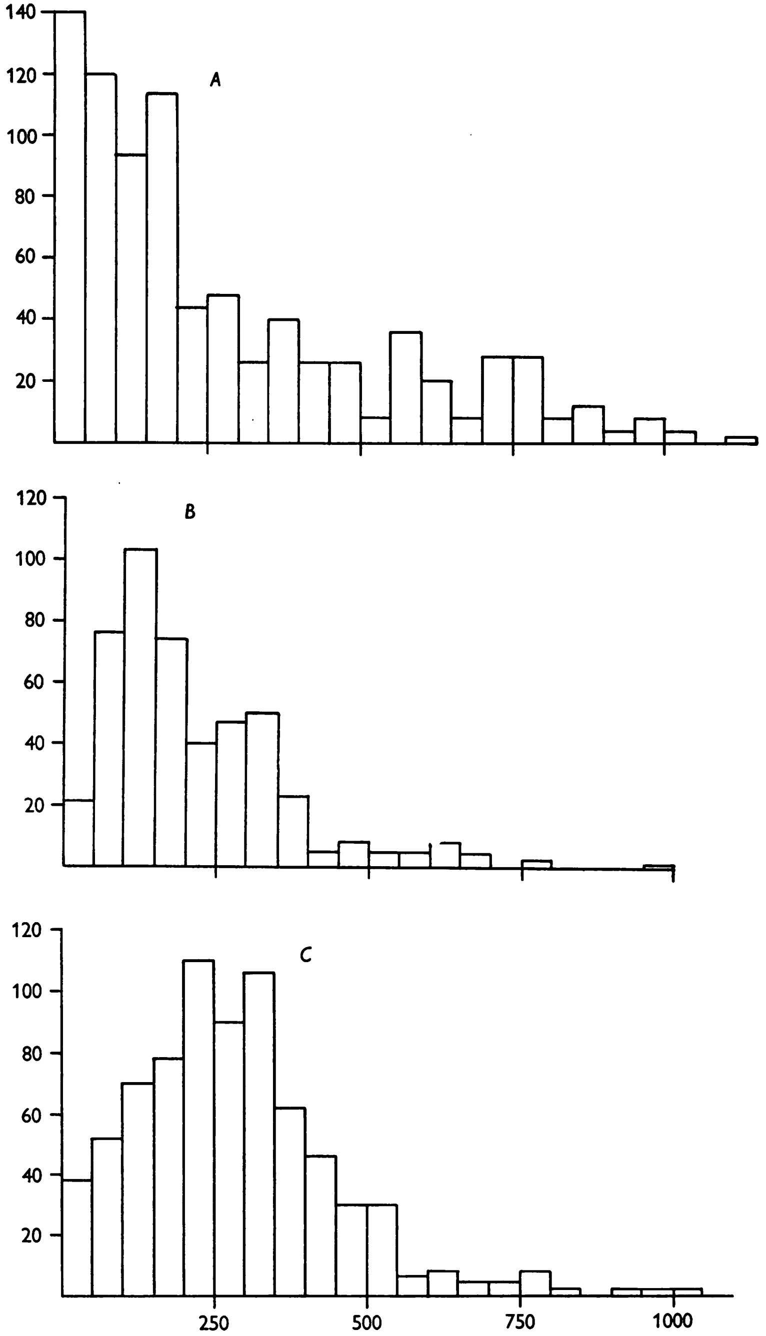

Fig. 1. For legend see opposite page.

recorded from fibres with effective membrane resistances greater than 200 kΩ were, on the average, about 250 μV.

The temporal relations of the miniature potentials and also the frequency distributions of amplitudes vary according to the length of the muscle fibre. Potentials recorded from fibres of the flexor tibiae muscles of both locust and cockroach (about 15 mm long in *Schistocerca*) gave markedly skewed frequency distributions of amplitudes (Fig. 1*A*) with a high proportion of smaller potentials, and considerable variations were observed in both rise times and half-decay times. The spontaneous discharges of these insect flexor tibiae muscles are clearly comparable to the discharges seen in the 'slow' muscle fibres of the frog (Burke, 1957) and the chick (Ginsborg, 1960).

At the distal end of the locust extensor tibiae muscle there are about four bundles of fibres each less than 1·2 mm long. The length constants, as determined by 'square pulse analysis', of both flexor and extensor tibiae muscle fibres are about 2·8 mm (Usherwood, 1962*b*); therefore, in the distal fibres of the extensor muscle a miniature potential originating at one end of a fibre is only slightly attenuated when it is recorded at the other end. The frequency distributions of amplitudes for potentials recorded from these fibres (Fig. 1*C*) approach the approximately normal distributions characteristic of the miniature potentials recorded from frog 'fast' muscle fibres (Fatt & Katz, 1952). Figure 1*B* illustrates the frequency distribution of amplitudes for a proximal fibre of the locust extensor muscle. Since the typical proximal fibre is between 2 and 3·5 mm long some attenuation of potentials originating at sites distant to the recording electrode inevitably takes place, and the histogram shows a positive skew which is less than that for the longer flexor muscle fibre but greater than that for the shorter distal fibre of the extensor muscle.

Miniature potentials with rise-times less than 1 msec were recorded from muscle fibres of the two cockroach species. In the locust, however, the minimal rise-time recorded was 3·5 msec. It is of interest to note that the differences between the rise-times of the post-synaptic potentials of locust and cockroach muscle are of the same order of magnitude (Usherwood, 1962*a*). Values for discharge frequencies of the miniature potentials ranged from 0·2/sec to 15/sec. Higher frequencies were often recorded, especially from muscle fibres (which were often less than 25 μ in diam.) of the two cockroach species, but these were considered to result from localized damage at the electrode tip leading to an abnormal increase in the discharge frequency. Apparently the cockroach muscle fibres were damaged more easily by the electrodes. In many fibres the mean frequency varied only slightly over a period of 1 hr, but in others progressive increases or decreases in the mean frequency were noted.

The miniature potentials recorded from insect muscle fibres appear at irregular intervals in a manner characteristic of vertebrate miniature postsynaptic potentials. The distribution of intervals between 312 successive potentials recorded from a distal fibre of the locust extensor tibiae muscle is shown in Fig. 2. The curve is drawn according to the equation

$$n = \frac{N\Delta t}{T}\exp(-t/T) \quad \text{(see Fatt \& Katz, 1952).}$$

The fit of the observations with the curve means that there is no obvious interaction between successive potentials.

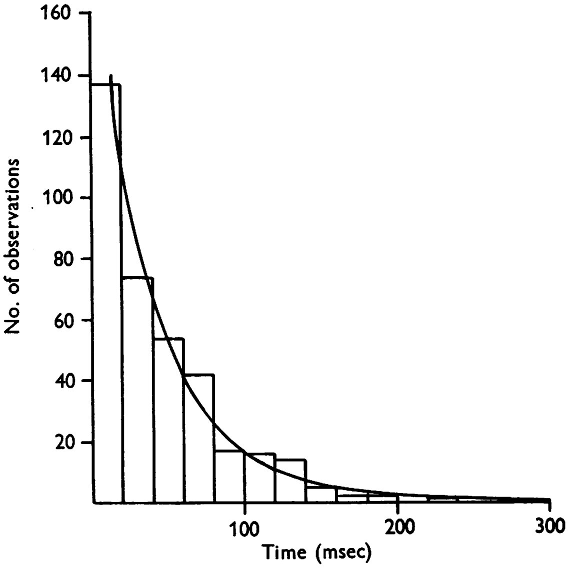

Fig. 2. Distribution of time intervals between discharges in a series of 364 miniature potentials recorded from a distal fibre of the locust metathoracic extensor tibiae muscle. Observations were grouped in classes of 20 msec. Mean interval was 42 msec. Curve is theoretical distribution for random events.

Extracellular recordings. Attempts to record the spontaneous potentials of insect muscle fibres with extracellular electrodes filled with saturated NaCl were not very successful. Two main factors may have contributed to the failure of this recording technique. First, incessant small spontaneous movements in the muscles constantly altered the position of the electrode-tip relative to the muscle fibre under examination. Secondly, the mode of innervation of the insect muscle fibre, with the closely packed bundles of fibres apparently innervated on their adjoining faces and not on their more easily accessible outer faces, made it very difficult to find the synapses. Furthermore, the sheath-like membrane which invests the insect synapse (Edwards, Ruska & de Harven, 1958) would make it almost impossible to find 'active' spots (Fatt & Katz, 1952) on the post-synaptic membrane without causing considerable damage to the nerve terminals.

The action of cations

Magnesium. An increase in the magnesium concentration depresses the post-synaptic potentials arising from nerve stimulation in both cockroach and locust muscle fibres (Hoyle, 1954). In the present experiments treatment with 20–40 mM-Mg rapidly reduced the post-synaptic potential to zero. When the potentials reached a level of about 2·5 mV they fluctuated in amplitude in an approximately step-wise manner. The resting potential also declined in high Mg (by about 5 mV in 20 mM-Mg and by about 15 mV in 40 mM-Mg) and this must be taken into consideration when interpreting the effects of Mg on the miniature discharge, since the amplitude of the post-synaptic potential is known to vary in approximate proportion to the resting potential (Cerf, Grundfest, Hoyle & McCann, 1959). Figure 3 shows the effects of Mg on the frequency and amplitude of the

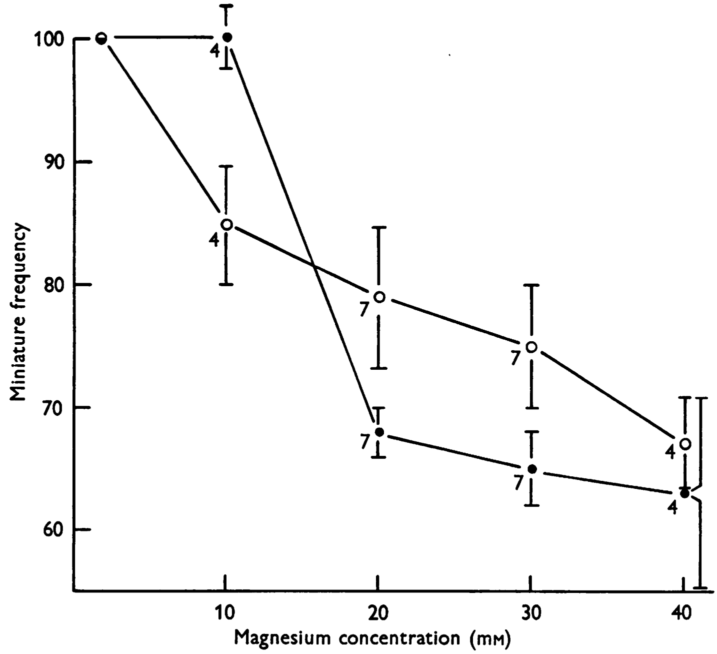

Fig. 3. Influence of magnesium concentration on miniature potential frequency (○) and amplitude (●). Ordinate: miniature frequency (amplitude) as a percentage of frequency (amplitude) in normal locust saline (2 mM-Mg). Vertical bars indicate ± S.E. of mean percentage of frequency (amplitude). Figures under points indicate number of fibres examined.

miniature potential of the locust muscle fibre. Mg appears to have little effect on the amplitude at concentrations below 10 mM, but with higher concentrations than this the amplitude is markedly reduced. The frequency of the spontaneous discharge appears to be sensitive to Mg at all concentrations, being markedly depressed with high concentrations of this ion, e.g. a Mg concentration of 40 mM depressed the mean frequency by about 30 %.

TABLE 1. Effect of calcium on the frequency of spontaneous miniature potentials of the locust extensor tibiae muscle

Recordings were made in normal locust saline containing 2 mM-Ca and 2 mM-Mg (*N*) and also in locust saline containing 10 mM-Ca and 0 mM-Mg (*E*). The number of miniature post-synaptic potentials counted in any one experiment varied between 200 and 400. Frequency ratio is calculated from the arithmetic mean of either the two *N* or the two *E* values

	Average no. of potentials/sec			
Expt.	*N*	*E*	*N*	Frequency ratio, *E*/*N*
1	1·16	1·74	1·40	1·391
2	1·70	1·96	2·16	1·016
3	2·38	2·30	1·14	1·307
4	2·06	3·04	2·98	1·206
	E	*N*	*E*	
5	0·92	0·56	1·42	2·089
6	1·46	1·36	1·90	1·235
7	1·50	1·14	1·72	1·412
8	2·88	2·38	2·84	1·215
Mean ± S.E. of mean ratio of frequencies				1·361 ± 0·033
Frequency as % of control				136·1 + 3·3

TABLE 2. Mean amplitudes of potentials recorded in experiments summarized in Table 1

	Amplitudes (μV)			
Expt.	*N*	*E*	*N*	Amplitude ratio, *E*/*N*
1	230	180	200	0·837
2	205	175	190	0·886
3	130	145	140	1·074
4	160	175	155	1·104
	E	*N*	*E*	
5	170	150	160	1·100
6	140	110	135	1·250
7	210	215	185	0·919
8	270	165	205	1·215
Mean ± S.E. of mean ratio of amplitude				1·077 ± 0·020
Amplitude as % of control				107·7 ± 2·0

Calcium. An increase in the Ca content of the saline from its normal value of 2 to 10 mM increased the discharge frequency by about 40 % (Table 1). At the same time only a slight increase in amplitude was recorded (Table 2). Unfortunately it was not possible to examine the effects of Ca in concentrations greater than 10 mM, since with higher concentrations fibrillation ensued and the preparation quickly deteriorated.

Potassium. The locust muscle fibre is depolarized by from 10 to 15 mV by doubling the K content of locust saline. The depolarization is accompanied by an increase of discharge frequency of 31·9 ± 9·8 (S.E. of mean of 6 observations) % and a reduction in amplitude of 11·1 ± 6·7 % (6). Treatment with zero K saline reduced the frequency by 15·3 ± 3·5 % (9) and increased the amplitude by 8·2 ± 2·7 % (9). On every occasion the resting potential at first increased in zero K saline, but in a few fibres it then fell rapidly to about 10 mV below normal.

The action of drugs

Curare, prostigmine and acetylcholine. Although the action of these drugs on vertebrate neuromuscular transmission is well established, they had no effect on the spontaneous miniature potentials of the locust or cockroach muscle fibre, even in concentrations as high as 10^{-2} M. It is concluded, therefore, that either the acetylcholine system is absent from the insect nerve-muscle junction, or alternatively these drugs, when applied externally, fail to penetrate the sheath which invests the synaptic regions.

5-*hydroxytryptamine.* Hill & Usherwood (1961) recently showed that high concentrations of 5-HT block transmission at the insect nerve-muscle synapse. Blockage of the spontaneous miniature discharge which results from treatment of insect muscle with this drug is, therefore, not entirely unexpected if, as appears possible from analogy with vertebrate miniature potentials, the insect miniature potentials arise from a spontaneous release of chemical transmitter from the nerve endings. Preliminary experiments have shown that 5×10^{-2} M 5-HT apparently almost completely blocks the miniature potentials in the distal fibres of the locust extensor tibiae muscle within 3 min of application (Fig. 4*B*). An immediate reduction of the resting potential also occurs, to a value between 15 and 40 mV below normal. On removal of the drug the miniature potentials reappear and the resting potential quickly returns to its former value. 5×10^{-3} M 5-HT is less effective on the more closely packed proximal fibres of this muscle, and a concentration of 10^{-2} M is required before the spontaneous discharge is almost completely blocked (Fig. 4*C*). A concentration of 10^{-3} M 5-HT reduced the amplitude of the spontaneous potentials in the distal fibres but failed to block the discharge completely (Fig. 4*A*). The effect of 5-HT on the amplitude of the spontaneous discharge is much greater than that expected solely as a result of the muscle depolarization which occurs in the presence of this drug.

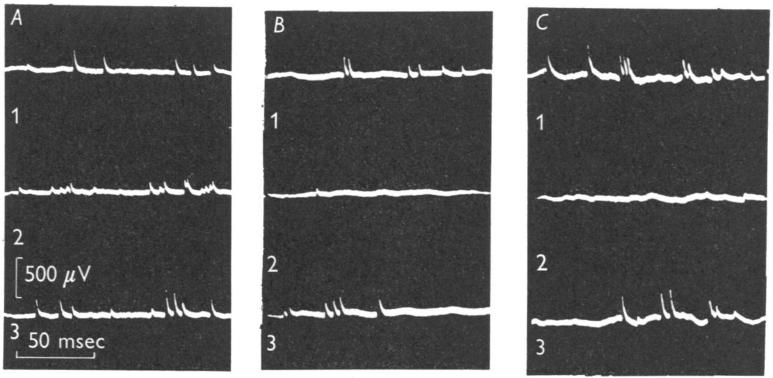

Fig. 4. Effect of 5-hydroxytryptamine on the spontaneous miniature potentials recorded from two distal fibres (*A*, *B*) and one proximal fibre (*C*) of the locust metathoracic extensor tibiae muscle. Fibre *A*: (1) normal locust saline; (2) 10^{-3} M 5-HT; (3) muscle returned to normal saline. Fibre *B*: (1) normal locust saline; (2) 5×10^{-2} M 5-HT; (3) normal locust saline. Fibre *C*: (1) normal locust saline; (2) 10^{-2} M 5-HT; (3) normal locust saline. Time and voltage calibrations apply to all three records.

DISCUSSION

An attempt has been made in the present investigations to compare the spontaneous potentials recorded from insect muscle fibres with the spontaneous subthreshold activity seen at vertebrate neuromuscular junctions. From the results it appears that a quantal discharge process similar to that characteristic of the vertebrate nerve ending is to be found in insects, although a complete analogy between the two systems has not yet been established. The presence of synaptic vesicles in insect nerve terminals (Edwards *et al.* 1958) suggests that they might be involved in transmission, either in the formation of the transmitter or its transmission across the synapse (de Robertis & Bennett, 1954).

The multiterminal innervation of the insect muscle fibre, together with the spatial decrement of the miniature post-synaptic potential, obscures the general picture of amplitude distribution of the intracellularly recorded spontaneous discharge. As a result it has not been possible to test for the possibility of quantal liberation of a transmitter agent from the pre-synaptic terminals. The technique of recording extracellularly from 'active spots' on the post-synaptic membrane would have shown whether or not transmission is quantal, but unfortunately attempts to use this technique have not proved successful. There is, therefore, no conclusive evidence for quantal transmission across the insect synapse. It is of interest to note, however, that in the presence of excess Mg the intracellularly recorded post-synaptic potentials arising from nerve stimulation are reduced in magnitude to the level of the spontaneous miniature potentials and fluctuate in an approximately step-wise fashion. A step-wise fluctuation in the amplitude of the post-synaptic potential (Fig. 5) is seen also during the final stages of the break-down of transmission following denervation of insect muscle (Usherwood, 1963). Step-wise fluctuations of the post-synaptic potentials have been seen in the denervated and magnesium-treated vertebrate muscle fibres, and are thought to result from random changes in the number of quantal units released by the presynaptic nerve terminals (del Castillo & Katz, 1954*b*; Birks, Katz & Miledi, 1960). Further evidence for an analogy between the vertebrate and insect miniature potential is afforded by the results of experiments with Ca and Mg. In both insect and vertebrate muscle Ca increases and Mg decreases the spontaneous discharge frequency. The Mg concentration required to produce a significant change in frequency is much higher for insect muscle than for vertebrate muscle (Hubbard, 1961). This is to be expected, however, since in nature insects appear to be very tolerant to concentrations of Mg in their haemolymph that would paralyse any vertebrate skeletal muscle (e.g. Duchateau, Florkin & Leclercq, 1953).

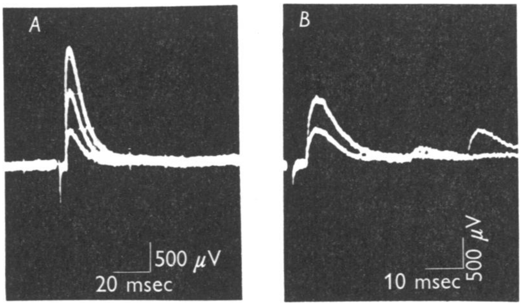

Fig. 5. Effect of motor nerve section on the post-synaptic potential of the locust metathoracic extensor tibiae muscle fibre. Response of muscle 18 days after section of central ends of nerves 5 and 3. The records were obtained by superposition of sweeps (successive sweeps at 2 sec intervals) during each of which nerve (5) was stimulated. Record *A* shows post-synaptic responses to nerve stimulation varying in a step-like manner between about 0·5 mV and 2 mV. On other occasions, not shown in above records, there were no post-synaptic responses. Record *B* shows two post-synaptic responses to nerve stimulation each followed by a spontaneous potential.

The reduction in amplitude of the insect miniature potentials seen in Mg concentrations greater than 10 mM cannot be entirely related to the concomitant depolarization of the muscle fibre, since an apparently equivalent depolarization, produced by addition of potassium to the saline, produced a much smaller reduction in the magnitude of the miniature potential. The slight increase in amplitude seen in high Ca could be related, however, to the accompanying small hyperpolarization (about 3 mV). Perhaps high concentrations of Mg reduce the sensitivity of the chemically sensitive post-synaptic membrane to the chemical transmitter. Del Castillo & Katz (1954*b*) have invoked a similar explanation to account for the reduction in amplitude of the vertebrate miniature post-synaptic potential in the presence of this ion.

In vertebrates, changes in the membrane potential of the peripheral nerve endings produce changes in the spontaneous discharge frequency (del Castillo & Katz, 1954*c*). The changes of spontaneous discharge frequency which result from alteration of the external potassium concentration at the insect nerve-muscle synapse are due possibly to the effects of potassium on the level of membrane potential of the presynaptic terminals.

The effects of 5-HT on the spontaneous discharge give further support to the hypothesis that this drug prevents transmission at the insect neuromuscular synapse by post-synaptic rather than by presynaptic action. 5-HT had apparently no effect on the frequency of the spontaneous miniature potentials, but merely caused their amplitude to decrease.

SUMMARY

1. Spontaneous miniature potentials have been recorded with intracellular micro-electrodes from a variety of insect muscle preparations. The potentials are, on the average, 0·25 mV in magnitude and occur at frequencies ranging from 0·2 to 0·5/sec.

2. Potassium increases the frequency and decreases the amplitude of the spontaneous discharge. Calcium has little effect on the amplitude but significantly increases the frequency. Concentrations of magnesium above 10 mM decrease the amplitude and the frequency.

3. Curare, prostigmine and acetylcholine have no effect on the spontaneous discharge, even at concentrations as high as 10^{-2}M 5-hydroxytryptamine at a concentration of 10^{-3}M reduces the amplitude but has little effect on frequency. With higher concentrations of this drug the discharge is blocked completely.

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