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Jaleel Ahmad Miyan

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Temporal changes in activity during destruction of the thoracic ventral eclosion muscle of the tsetse fly

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SUMMARY

The spontaneous intracellular activity of the thoracic ventral longitudinal eclosion muscle (VLEM) of *Glossina* is described for the period from eclosion up to a short time before the final breakdown of recorded fibres. The VLEM comprises a single motor unit with no inhibitory input. The firing frequency of the motor unit declines over 5 h after eclosion and leg release. Over a period of inactivity lasting between 19–24 h in the sampled fibres, there is no loss of resting membrane potential and occasional miniature potentials. The inactivity is ended by the sudden onset of biphasic potentials very different in form to the motor potentials and having a greatly reduced amplitude. These potentials fired at 6 Hz, lasted 2–4 h and ended with a rise in frequency to 25 Hz. No further activity is recorded and the fibres are observed to lose their striations and rigor.

Experiments to characterize the ionic basis of activity in the VLEM have been done on spontaneous and evoked activity. Like other insect muscles, the VLEM has a major Ca^{2+} potential but unlike insect skeletal muscles, it also appears to have a TTX-sensitive component. This Na^{+} component is revealed by pretreating the system in Na^{+}-free-choline saline, or by treatment with TEA in a Ca^{2+}-free saline. Neither EGTA nor cobalt abolish this potential. Addition of EGTA does not inhibit nerve evoked activity suggesting that the VLEM neuromuscular junction is in some way protected. The similarity of this muscle to insect visceral muscles is discussed.

1. INTRODUCTION

Cell death during metamorphosis and development is a recognized process in both vertebrates and invertebrates (see Finlayson 1975; Bowen & Lockshin 1981, 1985; Truman 1984; Ellis & Horvitz 1986). In most cases it appears to be 'programmed' (genetically) in the sense that cells are lost as part of an orchestrated change in the morphology and organization of the animal concerned. Hormonal triggers have been identified as the prime effectors in the system whatever other factors may influence the fine timing of events (Truman & Schwartz 1980; Weeks & Truman 1985). Recently, Kimura & Truman (1990, personal communication) have found that autolysis of the abdominal intersegmental muscles in *Drosophila melanogaster* is stimulated by exposure to eclosion hormone, whereas their motorneurons respond to a signal originating in the head which is subject to neural triggers that can be manipulated to allow limited survival of the neurons.

In the higher Diptera, I have described a set of eclosion muscles found in the thorax which are insensitive to hormonal signals and which require instead a specific neural signal associated with successful eclosion (Miyan 1989*a*, *b*). One of these muscles, the ventral longitudinal eclosion muscle (VLEM) has been studied in some detail. A single killer immunocyte, activated by a neural trigger, attacks healthy fibres of the muscle (Miyan 1989*b*, 1990). No phagocytosis is observed in the VLEM even at later stages of autolysis. The progressive loss of fibres can be stopped at any

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stage simply by cutting the nerve that innervates the muscle (Miyan 1990). This is clearly different from the situation in all other degenerating muscles in which phagocytes invade the tissue only after the cells are recognizably dead (Lockshin 1985), and after cessation of neural inputs. Direct neural control of immunocyte activity has never been shown in this way before although a vast literature now exists in which neural influences have been inferred in the activation of certain vertebrate immune cells (see, for example, Felten *et al.* (1985); Faith & Murgo (1988); Fiatarone *et al.* (1988); Sibinga & Goldstein (1988); Goetzl & Spector (1989); Jankovic *et al.* (1987)).

In this paper the spontaneous, intracellular activity of the VLEM after eclosion up to a short period before the final breakdown of recorded fibres is described. The ionic requirements for spontaneous and evoked activity in the VLEM are investigated. The VLEM does not show the steady depolarization characteristic of autolysing muscles undergoing 'programmed' cell death. The muscle apparently has protected neuromuscular junctions which, together with a requirement for sodium, makes it unusual when compared with other insect skeletal muscles.

2. MATERIALS AND METHODS

Living pupae of *Glossina m. morsitans* (Westwood) were supplied by the Tsetse Research Laboratory at Bristol. They were kept at 25 °C in a humid atmosphere and closed approximately a month after deposition. Flies were collected as they eclosed and kept in containers labelled with the time of eclosion. Individuals were anaesthetized by cold, CO_2 or N_2; the only noticeable difference between the three techniques was a general increase in excitability following CO_2 treatment (but see Nicolas & Sillans (1989)).

The method of Ikeda for *Drosophila* (1974, described in Miller (1979)), was slightly modified for the physiological studies. Anaesthetized flies were positioned on a wax bed so that at least one pair of thoracic spiracles was exposed to an air chamber accessed through a hole in the wax. The fly was pinned through the base of the mouthparts and posterior abdomen. The legs of the fly were either amputated before pinning out or were manipulated through the hole in the wax to hang free in the air chamber below the preparation. The thorax was opened by a dorsal midline cut and then pinned out over the hole in the wax. Soft wax was then pushed up around the preparation to seal the system. The air supply (from an aquarium pump) was connected to the chamber and adjusted until the thoracic air sacs were clearly inflated and no air escaped from around the preparation. This operation was usually completed within 5–10 min, well before any effects of anoxia are expected (Yamaoka & Ikeda 1988). No further dissection was usually required to access the VLEMs although the section of gut running through the thorax was sometimes removed to reveal the nerve to the muscle. The preparation was covered with physiological saline based upon a modification of Bodenstein's solution (Bodenstein 1946) by Ikeda & Kaplan (1970) and composed of the following (in millimoles per litre): NaCl 128; KCl 4.7; $CaCl_2$ 1.8; Na_2HPO_4 0.74; KH_2PO_4 0.35 at pH 7.2. The osmolarity of this solution is 250 mosm† whereas that of the haemolymph is 350 mosm, therefore the osmolarity was raised to near haemolymph levels by the addition of 3% sucrose. Changes in the concentration of particular ions were accomplished by balancing with changes in Na^+, or substitution with equimolar amounts of glycine. The addition of glycine had no effect upon spontaneous activity and has been used as a sodium substitute in other invertebrate systems (B. Ginsborg, personal communication).

Glass micropipettes with resistance between 10 and 30 MΩ filled with 3 M KCl were used for intracellular recording and stimulation of muscle fibres. Standard electrophysiological equipment was used throughout. Potentials were passed through a Schmidt trigger that provided pulses for a Unilab computer interface. A program supplied by Dr M. Hunter of Edinburgh University, Physiology Department was used to analyse the instantaneous frequency of trains of pulses using a BBC computer. Potentials were displayed through a Thurlby–Thandar DSA524 digital adaptor that allowed pre-triggering and signal averaging to be done on spontaneous activity. Histological preparations were processed according to previously published methods (Miyan 1989*b*). Between five and ten preparations were used in replicates of each experiment. At least five fibres were sampled in each of the two VLEMs of each preparation.

3. RESULTS

(*a*) *Spontaneous activity*

By using the preparation described above it is possible to record intracellular activity from the VLEM for periods up to 50 h. In most cases individual penetrations of fibres were held for up to 4 h. Only a single excitatory potential has been identified in over 200 experiments. For any given preparation, the amplitude of this potential was stable with an oscillation, of variable cycle time, of up to 30% about a mean value. This oscillation in amplitude is probably a reflection of the variations inherent in propagated activity. Between preparations the amplitude varied from 14 to 35 mV. This variability was attributed to the successful aeration of the preparation, the quality of electrodes and penetration, although it may have been due to distance of the electrode from the synaptic site(s) if these have a large separation in the VLEM. No inhibitory potentials were ever recorded. The resting potential was within the range −40 to −55 mV and was constant in any particular preparation for the duration of the experiment. Although the maximum value of the resting potential did not approach that required for electrogenic activity in larval muscles (Yamaoka & Ikeda 1988), the time taken to set up the preparation and the consistency of the maximum suggests that this is indeed the normal potential for this muscle. Cutting the nerve to the muscle results in a loss

† One osmole contains one mole of osmotically active particles.

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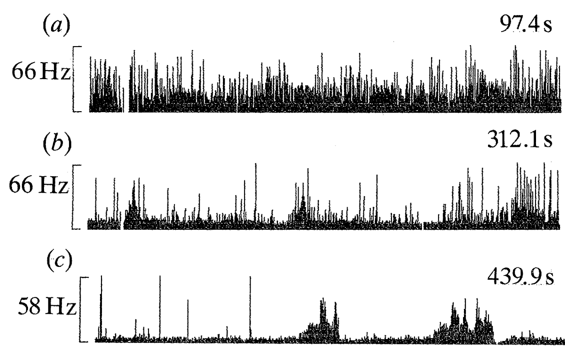


Figure 1. Instantaneous frequency analysis of spontaneous muscle potentials from the VLEM with legs amputated. The analysis program is written so that both axes are variable and autoscaling; the *y*-axis maximum and total record time is noted above each trace (this point is the same for the following two figures). Times since eclosion are (*a*) 0 (*b*) 2 h (*c*) 3 h. Note the persistence of background tonic activity.

of the 14–35 mV potential and only miniature potentials, with amplitudes 0.2–1.5 mV, are recorded.

Initial experiments were done with the legs of the fly either amputated or clamped between the thorax and wax. In this condition the recorded single unit fired tonically at 7–15 Hz with bursts at higher frequencies between 27 and 66 Hz (figure 1*a*). The background tonic activity steadily decreased over the first 3 h to a constant 6–7 Hz with intermittent bursts reaching 50 Hz (figure 1*b*, *c*). With the legs amputated or clamped no degeneration was observed in the muscle fibres, nor did activity stop for the duration of any preparation. Previous experiments (Miyan 1989*b*) have implied that leg joint receptor inputs are probably an important signal triggering degeneration. Experiments were therefore repeated with the legs free beneath the preparation in the air chamber.

With legs free, the activity recorded was much less than with legs clamped or amputated and was different to that described above (figure 2*a*); for as long as potentials were recorded they occurred in discrete

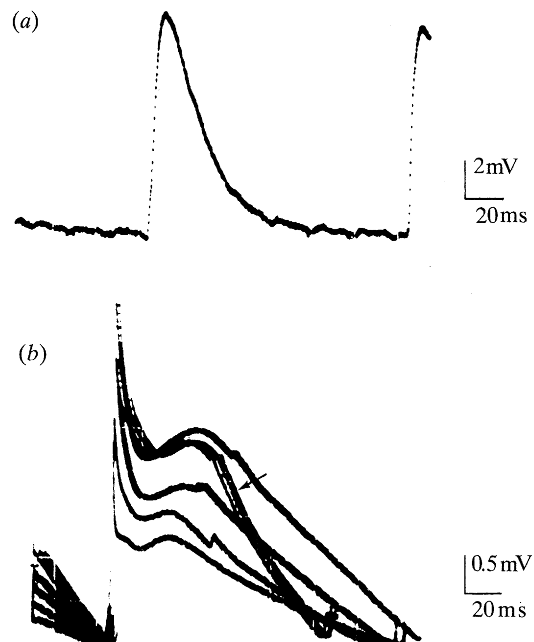
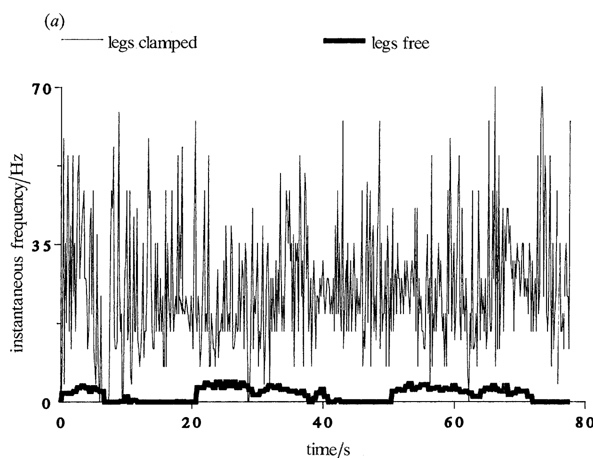


Figure 3. Spontaneous potentials recorded from the VLEM shortly after eclosion (*a*), and after the 19–24 h silent period after cessation of normal potentials (*b*). Notice the difference in the form and amplitude of the potentials. The traces in (*b*) are superimposed to show the rise in amplitude of this potential over the first few minutes of activity. The traces marked by an arrow were taken from the start of the rise in frequency near the end of the train. (From Miyan 1990.)

bursts with no background tonic activity. The bursts continued for 3–5 h during which time burst duration decreased and the frequency of apparent bursts increased (figure 2*b*–*d*). The activity stopped following this slow decline and no potentials were recorded for the next 19–24 h. At the end of this silent period there was a sudden onset of tonic potentials at a constant 6 Hz that were maintained for a period 2–4 h. After a final rise in frequency from 6 to 25 Hz over 10 min, the potentials suddenly stopped. No further activity was



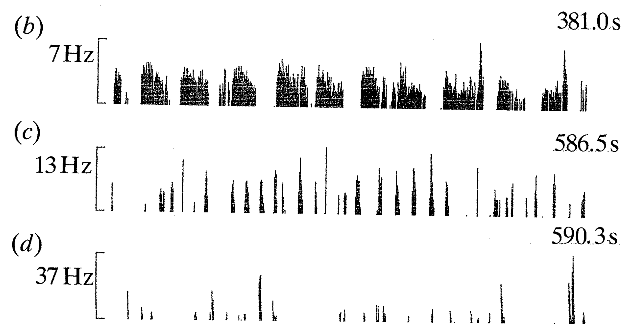


Figure 2. (*a*) Instantaneous frequency of spontaneous VLEM potentials with legs clamped and legs free plotted on the same axes to show the difference in activity. Both recordings were taken immediately after eclosion and it is clear that leg afferents have a significant input to the VLEM. Leg clamps result in maintained tonic activity and inhibition of degeneration. (*b*–*d*) Instantaneous frequency analysis with the legs intact and free to move showing rapid depression in VLEM activity after eclosion and leg release. Times since eclosion are (*b*) 1 h (*c*) 4 h (*d*) 5 h.

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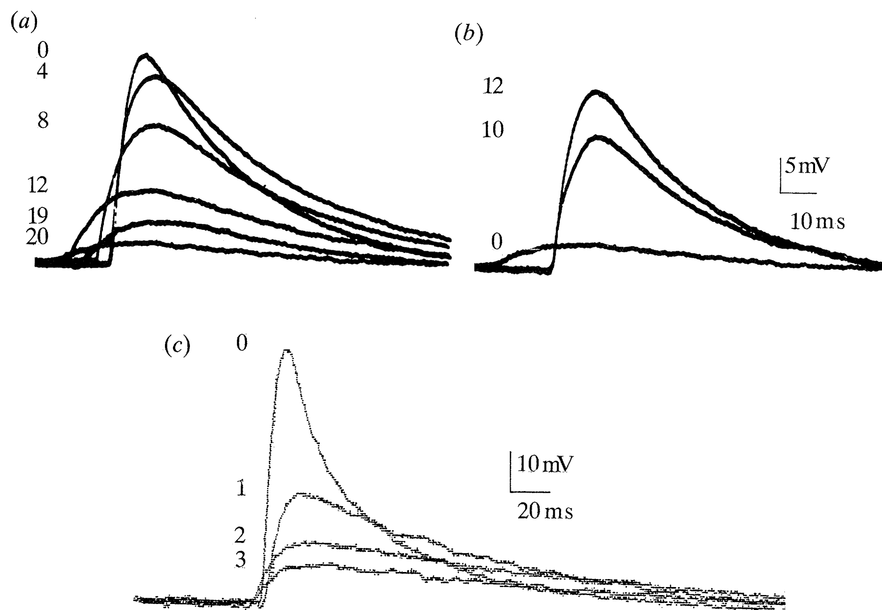

Figure 4. (*a*) Superimposed records showing the effect of removing all extracellular Ca^{2+} upon muscle potentials. Note that although the response collapses to about 5 mV, it is never completely abolished. (*b*) Recovery to a full amplitude is very rapid when the preparation is bathed in normal saline. (*c*) A similar result to (*a*) is obtained by the addition of 2 mM $CoCl_2$ to normal saline in which there is again no complete loss of potential. Numbers beside each trace show minutes since immersion in saline (*a*, *b*) and number of washes in saline (*c*).

recorded although the resting potential was maintained for 2–4 h by which time the fibre had lost its striations and tone. The potentials in this final tonic train were not the same as the muscle potentials recorded immediately after eclosion (figure 3; Miyan 1990); they had an initial depolarization of 3 mV with a rise time of 4 ms; the falling phase was interrupted by a second, slower and longer-lasting depolarization having an initial amplitude of 1 mV and duration (from the start of the fast rise) of 140 ms. The amplitude of the second depolarization increased over the first 10 minutes to a steady value of 2 mV (figure 3*b*). By contrast, the potentials recorded before the silent period, after eclosion, were monophasic depolarizations lasting 85 ms with a rise time of 5 ms and an amplitude of 14 mV (figure 3*a*).

(*b*) *Ionic basis of activity*

Experiments were done on spontaneous activity and upon activity elicited by intracellular current injection. It has already been stated above that cutting the nerve to the VLEM results in the loss of recorded potentials and the presence of only miniature potentials that have a different activity pattern. Treatment with cobalt or EGTA (see below) affects the amplitude of the potentials but not their firing pattern. This, together with other results detailed here, have led me to conclude that the axon and the neuromuscular junction of this system are protected from manipulations of the external bathing medium. Thus the effects are presumed to be operating on the muscle membrane and its response to normal synaptic transmission. This assumption is borne out by experiments done by using intracellular stimulation of muscle fibres during similar manipulations (see below).

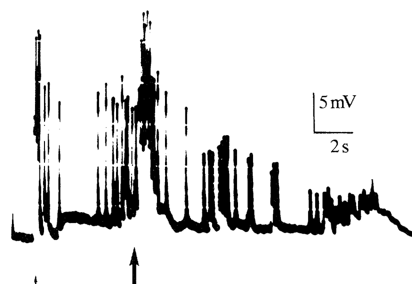

Figure 5. Effect of 0.3 μM TTX upon spontaneous activity of the VLEM. Potentials are completely abolished within 10 s. Small arrow marks the removal of saline from the bath; large arrow, the addition of TTX-saline. A characteristic of this preparation was a burst of impulses to changes in fluid level in the bath (not to fluid movement) probably mediated by cuticular receptors.

Initial experiments were done on spontaneous potentials. Bathing the preparation in a Ca^{2+}-free saline containing 3 mM EGTA did not completely abolish recorded activity (figure 4*a*). A residual potential with an amplitude of 5 mV remained even after repeated washes with fresh Ca^{2+}-free-EGTA saline. These potentials persisted for more than 30 min and rapidly recovered full amplitude when the preparation was returned to normal saline (figure 4*b*). The result was the same if the preparation was exposed to 2 mM Cobalt in normal saline (figure 4*c*). Again the potentials failed to be completely blocked for more than 30 min.

Addition of Tetrodotoxin (TTX), at concentrations down to 0.3 μM, to the bathing solution resulted in rapid (within 10 s) and complete loss of recorded potentials (figure 5). It is unlikely that the axon and neuromuscular junction are accessible to such a rapid

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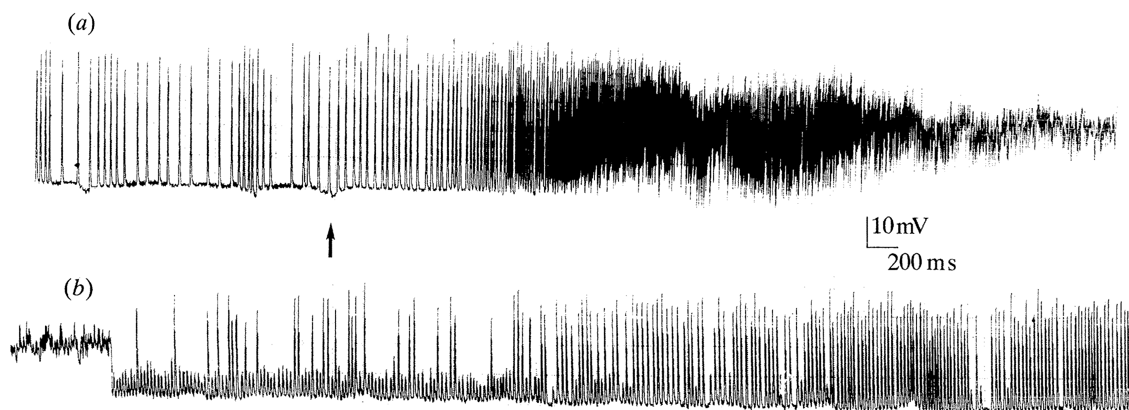

Figure 6. (*a*) Response of the VLEM to a Na^+-free saline containing choline. Notice the rapid rise in frequency and loss of amplitude of potentials together with the depolarization of the baseline after immersion (arrow). This is maintained even after complete abolition of potentials. (*b*) Recovery in normal saline is characterized by the return of a normal baseline and greatly reduced amplitude potentials. As the frequency of these decreases, there is an increase in the frequency of normal amplitude potentials. The overall frequency remains relatively constant.

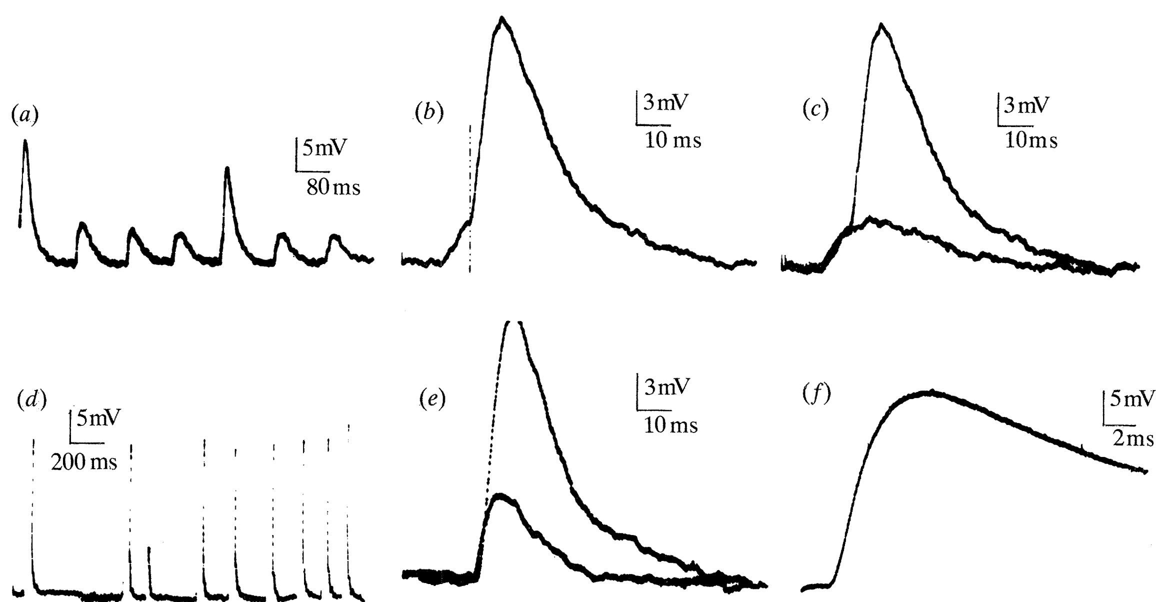

Figure 7. Details of the activity shown in the previous figure. There are clearly two components (the border indicated by the line) to the larger amplitude potential (*b*). The small 'starter' potential has the same form and amplitude as the small potentials as is clear in the two superimposed records (*c*). The coupling that normally exists between the two components appears to have been broken by the choline treatment which has apparently increased the rise time of the small potential, possibly uncovering a propagated response. During spontaneous normal activity, there are very rare occurrences of similar small potentials (arrow in (*d*)) which, however, have a rise time matching that of the larger potential (*e*). There is no indication of a two-component process in the normal muscle potential (*f*).

effect when neither cobalt nor EGTA appear to affect them (see also below).

Bathing the preparation in Na^+-free saline containing choline as the Na^+ substitute (Deitmer & Rathmayer 1976) resulted in a rapid rise in frequency of potentials, and a steady decrease in amplitude as the membrane depolarized (figure 6*a*). The membrane remained held at a depolarized level 10 mV above the normal resting potential. Activity returned after at least two washes in fresh normal saline, initially with small 6.4 mV potentials and occasional normal amplitude potentials. Within a period of 7 min the normal potentials became more frequent and the small potentials decreased and finally stopped occurring (figure 6*b*). A close examination of the potentials in this recovery period reveals a two-phase potential (figure 7*a*, *b*). This is never seen under normal, spontaneous conditions and is rapidly lost from the recording as the rise time of the initial depolarization decreases to match that of the second phase. Under these experimental conditions, it would appear that the two potentials, normally seen as a single unit, have become uncoupled (figure 7*c*). An alternative explanation (suggested by a referee of this paper) is that the treatment has revealed that the VLEM is capable of propagated potentials and that the changes in solution have affected the threshold for the propagated response, hence the appearance of a 'threshold shoulder'.

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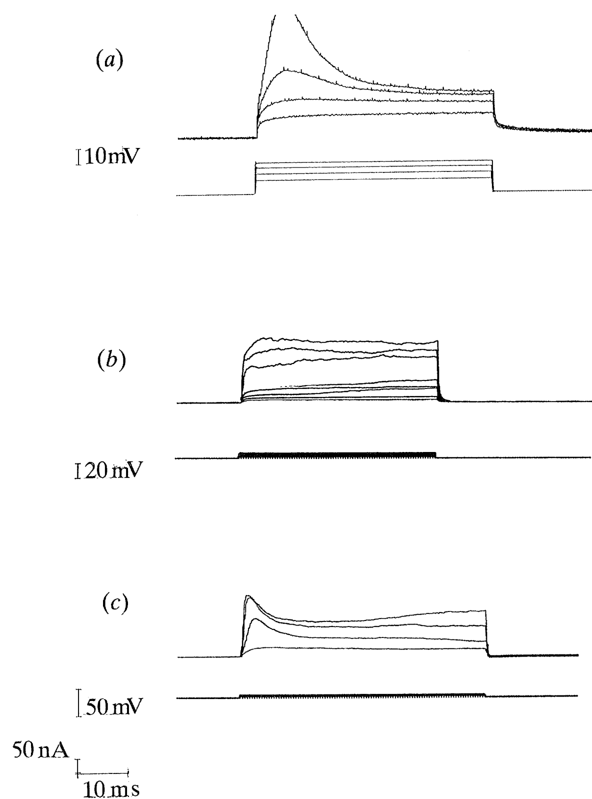

Figure 8. (*a*) Response of the VLEM to intracellular current injection in the presence of 3 mM TEA and 2 mM EGTA in a Ca^{2+}-free saline. A single transient potential is elicited which is completely abolished by the addition of 0.3 μM TTX (*b*). After a wash in normal saline, the response returned with a lower threshold (*c*).

In long-term recordings of spontaneous activity from these muscles, there are infrequent occurrences of potentials that have a similar amplitude to those observed following choline treatment (figure 7*d*, *e*). Under normal conditions there is no indication of this 'starter' potential in the muscle potential recordings (figure 7*e*, *f*).

The sodium component has also been investigated by using intracellular current injection. A single transient response occurs when the preparation is bathed in Ca^{2+}-free saline containing 2 mM EGTA and 3 mM TEA (figure 8*a*). This was abolished by the addition of 3 μM TTX (figure 8*b*) and returned when the preparation was washed out with TTX-free saline (figure 8*c*).

The amplitude of the normal muscle potentials could be doubled by substituting Ba^{2+} (at 1.8 mM) for all external Ca^{2+} (figure 9*b*i). Increasing the Ba^{2+} concentration to 10 mM resulted in long-lasting all-or-none action potentials (figure 9*b*ii, *c*). These potentials were completely abolished when the preparation was bathed in Na^{+}-free saline containing glycine or if 0.3 μM TTX was added to the saline. Further increase in the Ba^{2+} concentration up to 50 mM resulted in greatly lengthened potentials possibly as a result of K^{+} channel blockade (see, for example, Werman & Grundfest (1961); Ashcroft & Stanfield (1982)) (figure 9*d*). These potentials could be elicited in the presence of TTX by intracellular current injection (figure 9*e*).

(*c*) *Effects of transmitter application*

Included in this report are some preliminary observations on the bath application of certain neurotransmitters as these are relevant to discussion of the results already reported above. Specifically, choline has been used previously as a sodium substitute without any observed effects on insect muscle excitability (Deitmer & Rathmayer 1976); the dramatic response of the eclosion muscle to the Na^{+}-free choline saline suggested the presence of cholinergic receptors. Putative transmitter substances were dissolved in standard physiological saline.

Addition of glutamate, the standard insect neuromuscular transmitter, up to 10^{-3} M had no effect upon muscle potential shape or amplitude over several hours of exposure. Similar lack of response was observed to applications of acetylcholine and GABA. Carbachol had an effect that could not be distinguished from that observed in the Na^{+}-free choline saline at 10^{-4} M. Activity could not be blocked by the addition of tubocurarine up to 10^{-3} M but a similar concentration of gallamine produced an inhibition of muscle potentials after a delay of 2–5 min. 5 HT produced the most dramatic response; at bath concentrations between 10^{-5} and 10^{-3} M there was a sudden cessation of potentials and silence. High frequency bursts of potentials characterized recovery in normal saline, the bursts lengthening and finally fusing into sustained tonic activity, which eventually returned to normal. A second exposure after recovery resulted in a slow rise in frequency to fusion followed by a sudden stop. More precise, local release experiments must be done before any definitive conclusions can be made. It is clear, however, that the VLEM has a complicated pharmacology and is an unusual skeletal muscle in an arthropod.

4. DISCUSSION

In this paper I have described the chronology of changes in the electrical activity of the ventral longitudinal eclosion muscle (VLEM) of *Glossina* from eclosion to degeneration. At no stage is there a sustained depolarization and loss of membrane potential that would otherwise characterize a dying, autolysing muscle fibre (Lockshin 1985). There is a slow decline in the number of neurally evoked potentials suggesting a run down of the probable central pattern generator for the eclosion rhythm (Fraenkel 1935; Reid *et al.* 1987*a*, *b*; Miyan 1989*a*). Once the potentials stop, no further activity indicating motorneuron firing is recorded. However, 20–24 h after eclosion, there is a sudden onset of recorded potentials that are different in form and amplitude to the motor potentials and which coincide with the invasion of muscle fibres by processes from a killer immunocyte (Miyan 1990). These potentials, and the resulting degeneration, can be completely abolished by severing the nerve innervating the muscle, implying that there is a direct neural input driving the degeneration process, specifically, driving the killer

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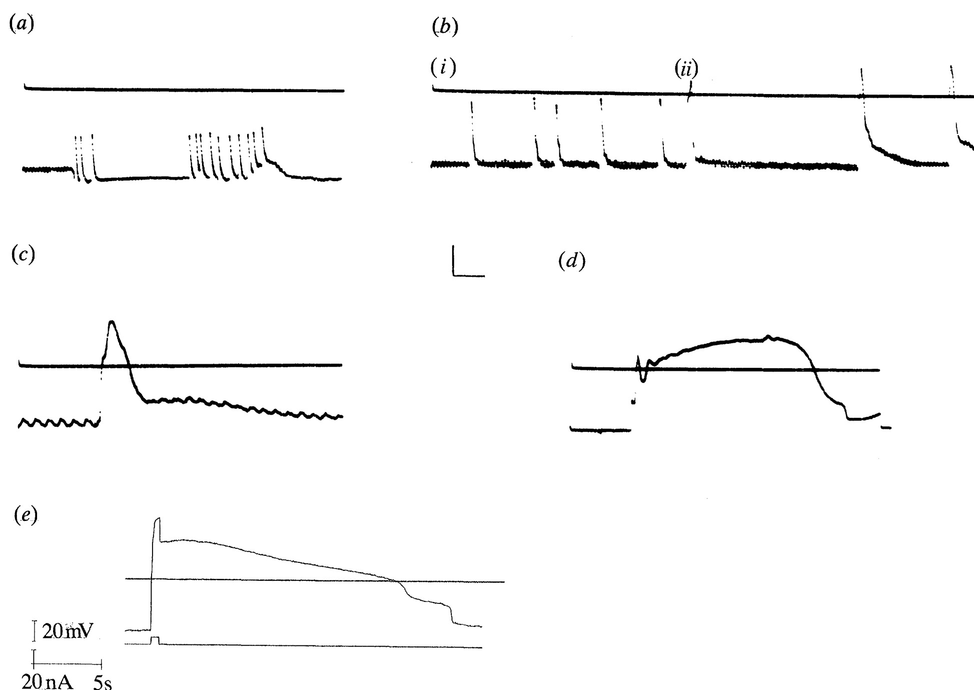

Figure 9. Effects of increasing the external divalent cation ion concentration on VLEM activity. (*a*) Normal potentials with zero potential marked by a line. (*b*i) Doubling of amplitude following substitution of all Ca^{2+} with Ba^{2+} (at 1.8 mM); (*b*ii) conversion to all-or-none action potentials in 10 mM Ba^{2+}. As well as the fast spike there is a small amplitude after potential that lasts for 200–300 ms (shown expanded in (*c*)). Increasing the Ba^{2+} to 50 mM results in long-lasting action potentials (*d*). Only these long lasting potentials could be elicited in the presence of TTX by intracellular current injection (*e*). Scales (*a*, *b*, *d*) 200 ms, 20 mV; (*c*) 50 ms, 20 mV.

immunocyte. It is tempting to conclude that the potentials are the response of the muscle to some substance released by the immunocyte but further experiments are required to show this pathway. Sections of the nerve innervating the VLEM reveal two profiles (Miyan 1990) that can be traced into the muscle. The two can be distinguished on the basis of their ultrastructure and, by using this criterion, I have found that one traverses the length of the muscle, presumably providing the multi-terminal motor innervation, whereas the second appears to be limited to the anterior region of the muscle containing the anchor site of the immunocyte (Miyan 1990). The immunocyte itself is unusual in having a permanent anchor site from whence it sends out processes towards the posterior end of the muscle (Miyan 1989*b*); such an anchor is required for the neural drive as the nerve could not follow a mobile cell. The nature of the 'death' signal as recorded in the muscle fibres awaits further analysis.

The failure of both EGTA and cobalt to block apparent neuromuscular transmission suggests that the synaptic sites are protected on the VLEM. This is very unusual as in most insects there appears to be immediate access to this area from the haemolymph for ions as large as lanthanum (Lane & Treherne 1972) through a leaky glial sheath (Osborne 1970). In ultrastructural studies I have failed to identify similar weaknesses in the sheath surrounding the VLEM neuromuscular junctions (J. A. Miyan, unpublished observations) and this would seem to confirm the physiological observations. In this study there has been a heavy reliance upon spontaneous neural activity to drive VLEM activity. The results must therefore be tested by further intracellular stimulation experiments and by voltage-clamp analysis. The results do show that the VLEM clearly requires the presence of both Ca^{2+} and Na^{+} in the bathing medium for normal excitability. Blocking the Na^{+} component alone results in a collapse of the entire response. The requirement for Na^{+} is similar to that reported for dipteran larval muscles (Yamaoka & Ikeda 1988), although the form of the potential is quite different, and to adult visceral muscles (Huddart 1985). The VLEM is neither larval nor visceral in origin and is thus a very unusual insect adult skeletal muscle.

The effects of choline on the VLEM are not matched by any degree of sensitivity to acetylcholine exposure. Williamson (1989) found a similar lack of sensitivity to ACH and similar response to gallamine in cephalopod statocyst receptors and concluded that a different class of cholinergic receptors from the classical vertebrate ACH receptors must exist in invertebrate systems (see also Kehoe (1972)). The dramatic response of the VLEM to 5 HT, both to exposure and wash out, suggests a significant role for this monoamine. The exact pharmacology of the VLEM system must await a more precise technique for administering drugs directly onto the muscle and for testing other substances, in particular other biogenic amines and peptides. It can be concluded, however, that the VLEM is different from other arthropod skeletal muscles, sharing instead properties with certain insect visceral muscles (Huddart 1985). It is perhaps significant that the VLEM has an ultrastructure similar to visceral muscles and characteristic of supercontracting muscles (Huddart 1985; Miyan 1989*b*).

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