

EFFECTS OF ETHANOL AND TEMPERATURE ON A CRAB MOTOR AXON ACTION POTENTIAL: A POSSIBLE MECHANISM FOR PERIPHERAL SPIKE GENERATION

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(Received 13 July 1982 — Accepted 10 November 1982)

SUMMARY

1. In autotomized walking limbs of *Pachygrapsus crassipes*, microelectrode recordings of evoked action potentials were made in the meropodite from the E2 excitator axon to the bender muscle.

2. The action potential spike was followed by a depolarizing after-potential. Increases in temperature resulted in a decline in the amplitude and time course of the spike, and an increase in the amplitude of the after-potential. Low levels of ethanol or increased levels of calcium increased the size of the after-potential and decreased the temperature threshold for peripheral spike generation.

3. At high temperatures a single orthodromic E2 axon spike provoked the generation of additional impulses at the periphery, with an inter-spike interval of 2–3.5 ms.

4. The after-potential lasted longer than the refractory period following the spike. The axon membrane, therefore, was depolarized after the refractory period and this resulted in a period of low threshold for spike generation. Increases in temperature shortened the refractory period.

5. We suggest that additional spikes are generated at the periphery where the E2 axon diameter is decreased. The increased membrane resistance at these sites increases the size of the depolarizing after-potential. Therefore, if the depolarization following the refractory period is at or above threshold for firing, additional action potentials will be generated at the periphery.

INTRODUCTION

Cold-blooded animals must remain functional during both short-term and long-term changes in the temperature of their environment. In some crustaceans there is a limited thermal range over which short-term temperature changes have little effect on muscle performance (Harri & Florey, 1977, 1979; Stephens & Atwood, 1982). Moreover, long-term changes in temperature cause the thermal range to shift towards the acclimation temperature. In crayfish (*Astacus leptodactylus*) and in the Pacific

shore crab (*Pachygrapsus crassipes*), increases in temperature above the thermal range result in a decline in the muscle response. The amplitude and time course of the excitatory junctional potentials (EJPs) decline, and the amount of tension produced by a train of EJPs also decreases with temperature. However in the shore crab there is a recovery in muscle performance if the temperature is raised high enough (N. Mindrebo, unpublished observations).

Above a critical temperature threshold, one orthodromic action potential in the single excitor motor axon to the limb stretcher muscle provokes the generation of additional spikes in the peripheral axon branches, and the production of concomitant EJPs in the muscle (Stephens & Atwood, 1981). Facilitation and summation of the EJPs cause an increase in the amount of tension developed by the muscle in response to a short train of motor axon shocks (N. Mindrebo, unpublished observations). The peripheral generation of spikes can be reversibly abolished by cooling and the number of additional spikes increases with temperature. Furthermore, the temperature threshold for this phenomenon is dependent upon the acclimation temperature and can be altered by adding low levels of ethanol to the saline bathing the preparation (Stephens & Lazarus, 1981).

Peripheral generation of additional action potentials has been observed in the single excitor and the specific inhibitor motor axons to the limb stretcher muscle (Stephens & Atwood, 1981), and in one of the excitor motor axons (E2) to the limb bender muscle (Lazarus, Stephens & Mindrebo, 1982). Although the temperature threshold for peripheral spike generation is similar for all three motor axons, this phenomenon does not occur in all efferents – for example in the other excitor to the bender muscle (E1) and the common inhibitor. It is interesting that activity in the specific inhibitor can curtail or abolish the peripherally generated response in the excitor to the stretcher muscle (Stephens & Atwood, 1981). During the curtailed response there is always a matching between the number of excitor axon spikes and EJPs in the muscle, suggesting that the sites of spike generation and modulation in the excitor axon are very close. Since inhibitory presynaptic connections are located at regions where the excitor axon diameter is decreased, at branchpoints and bottlenecks (Jahromi & Atwood, 1974), the generation of additional excitor axon spikes must take place at or near these sites.

In the present study we show how the peripherally generated action potentials in the E2 motor axon to the bender muscle can arise from the depolarizing after-potential that follows the action potential in this axon.

MATERIALS AND METHODS

Pacific shore crabs (*Pachygrapsus crassipes*) were purchased from the Pacific Biomarine Laboratories (Venice, Ca.) and were kept individually at 14°C in the laboratory. Feeding (frozen brine shrimp) and sea water changes were performed every 3–4 days, and under these conditions the crabs lived well.

Preparations were made from autotomized walking limbs (the first three pairs) removed from animals that had been acclimated to the holding temperature for at least 4 weeks. The limb was firmly attached to the bottom of a glass dish with dental wax and bathed in saline of the following composition (mM): 470 NaCl; 8 KCl; 20 CaCl₂; 10 MgCl₂; 5 Hepes buffered to a pH of 7.2. The limb nerve was exposed in

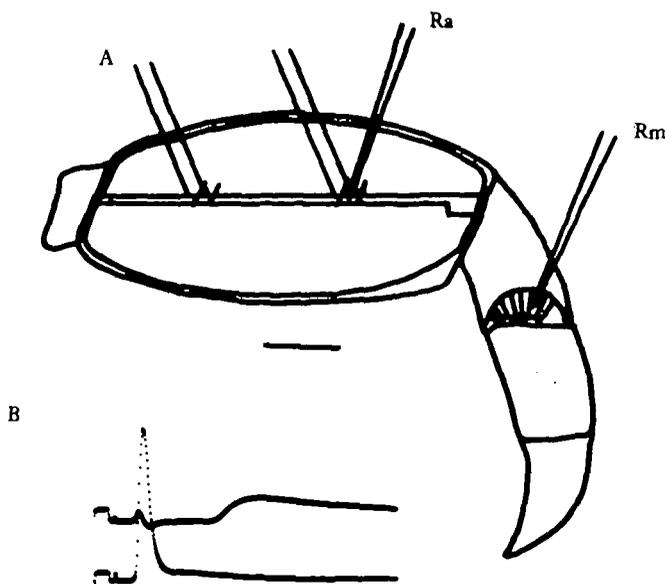


Fig. 1. Recording from the excitor motor axons to the bender muscle. (A) The preparation made from an autotomized walking limb. The limb nerve was exposed and stimulated in the meropodite. Evoked activity was recorded using microelectrodes inserted into single bender muscle fibres (Rm) and motor axons (Ra). (B) Activity in one of the excitor motor axons (E2) to the bender muscle was determined by the simultaneous appearance of an EJP (upper trace) and an axon spike (lower trace) in response to carefully graded limb nerve stimulation. Calibration: 5 mm (A); 5 mV and 1 ms (B).

meropodite and stimulated with brief electrical shocks (0.25 ms duration) applied through proximally located hook electrodes (Fig. 1A). Using glass microelectrodes (filled with 2M-potassium acetate; resistance 10–25 M Ω), recordings were made from fibres of the bender muscle, which was exposed by drilling a small window through the cuticle in the distal portion of the carpodite. Identification of evoked activity in E1 and E2 was achieved by recording evoked EJP activity from fibres in different regions of the bender muscle. The E2 axon supplies all of the muscle fibres accessible through the window, while E1 innervates only a limited number of fibres in the central portion of the exposed bender muscle (Lazarus *et al.* 1982).

Microelectrode recordings were made from the E1 and E2 axons at the distal part of the exposed limb nerve, near the meropodite-carpodite joint, where it was supported by a second pair of hook electrodes (Fig. 1A). In most preparations the limb nerve was split longitudinally and 60–75% of the limb nerve was removed, leaving only a few axon bundles containing the excitor motor supply to the bender muscle. Action potentials were recorded intracellularly from the E1 and E2 axons using glass microelectrodes filled with 2M-potassium acetate (resistance 15–30 M Ω).

Intracellularly recorded responses from the muscle and the axon were conventionally displayed on the screen of an oscilloscope and on a signal averager (Tracor Northern NS-570A). Activity in the E1 and the E2 axon was determined on the basis of two criteria. First, the simultaneous appearance of an action potential and an EJP response to carefully graded electrical stimulation of the limb nerve (Fig. 1B).

Second, the production of EJPs by current injection through the microelectrode in the axon. This latter test was usually performed at the end of an experiment, to ensure that any damage from the large amounts of current required to produce action potentials did not affect the experimental results.

The temperature of the saline in the preparation dish was controlled by heat exchange with a glass coil in the dish. The coil was attached to a thermostatically controlled, constantly circulating bath. The preparation was constantly perfused with fresh saline at a rate of about 10 ml/min. This perfusion, coupled with suction used to maintain constant saline depth, provided sufficient circulation to ensure a uniform temperature throughout the dish. Temperature changes were performed at about 0.2°C/min and were monitored with a thermal probe.

RESULTS

In 30 limb preparations at 14°C, the E2 axon had a mean resting potential of -70.9 mV (s.d. \pm 4.4). The spike had a mean amplitude of 90.3 mV (s.d. \pm 6.8) and was followed by a depolarizing after-potential which lasted about 10 ms (Figs 1B, 2B). In the E1 axon, by contrast, the spike was followed by a hyperpolarizing after-potential (Fig. 2A).

Cross-talk between the two recording electrodes could result in a spike artifact on the muscle EJP trace (Fig. 1B). To eliminate the possibility that the size of the depolarizing after-potential was affected by cross-talk from the muscle EJP response, all observations of the axonal action potentials were made with the muscle recording system grounded.

The effects of temperature

An increase in temperature from 14°C produced a decrease in the amplitude and duration of the E2 axon spike (Fig. 3). By contrast, the amplitude of the depolarizing after-potential increased with temperature (Fig. 4C, E).

An increase in temperature above the critical threshold for peripheral spike generation caused a single E2 axon shock to produce an orthodromic action potential

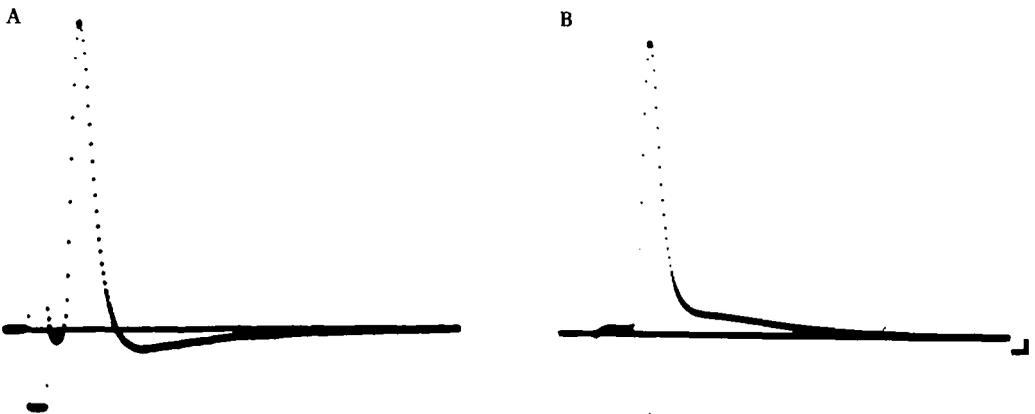


Fig. 2. Microelectrode recordings from E1 (A) and E2 (B) motor axons to the bender muscle. The recordings are the average of 32 responses. Calibration: 5 mV and 1 ms.

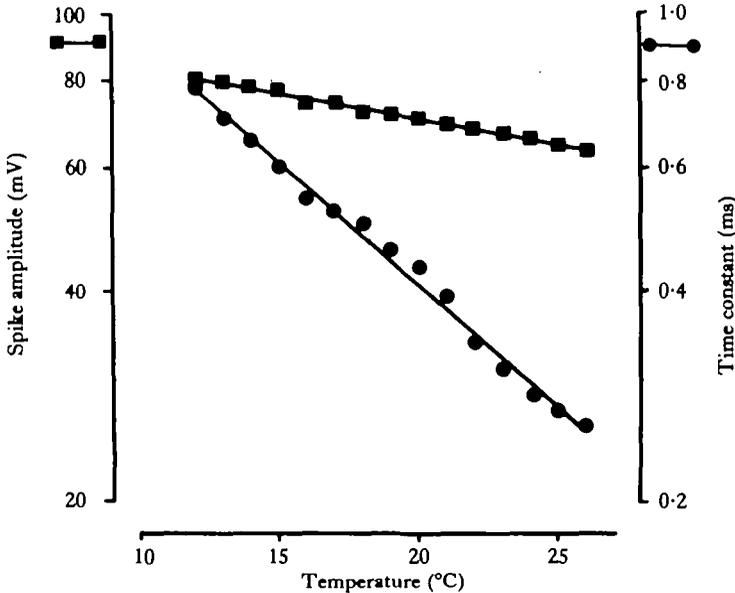


Fig. 3. Graph to show the effects of temperature on the amplitude and time constant of a spike recorded from a single E2 axon.

followed by additional impulses (Fig. 4D). The additional spikes appeared to arise from the depolarizing after-potential of the preceding impulse. The interval between the additional spikes was 2–3.5 ms. However, the interval between the first (orthodromic) spike and the second (antidromic) action potential was longer, presumably due to the time taken for impulse conduction between the recording electrode in the meropodite and the site of spike generation in the peripheral axon branches.

Increases in temperature had no effect on the duration of the increased after-potential (Fig. 4C) and the time interval between the additional spikes (Fig. 4D). These data, coupled with the observation that the E1 axon has a hyperpolarizing after-potential (Fig. 2A) and does not exhibit peripheral spike generation (Lazarus *et al.* 1982), suggest that the depolarizing after-potential is closely associated with the production of additional spikes at high temperatures. Moreover, intracellular microelectrode recordings made from the single excitor and the specific inhibitor motor axons to the stretcher muscle – axons that also exhibit peripheral spike generation (Stephens & Atwood, 1981) – revealed that their spikes were followed by a depolarizing after-potential (P. J. Stephens, unpublished observations). According to this hypothesis, therefore, any agent that increases the depolarizing after-potential should also decrease the temperature threshold for peripheral spike generation.

The effects of ethanol and high calcium

Low levels of ethanol in the saline bathing the preparation cause a decrease in the temperature threshold for peripheral spike generation (Stephens & Lazarus, 1981; Lazarus *et al.* 1982), and also increase the size of the depolarizing after-potential (Fig. 5A).

Increasing the concentration of calcium in the crab saline from 20 mM to 60 mM

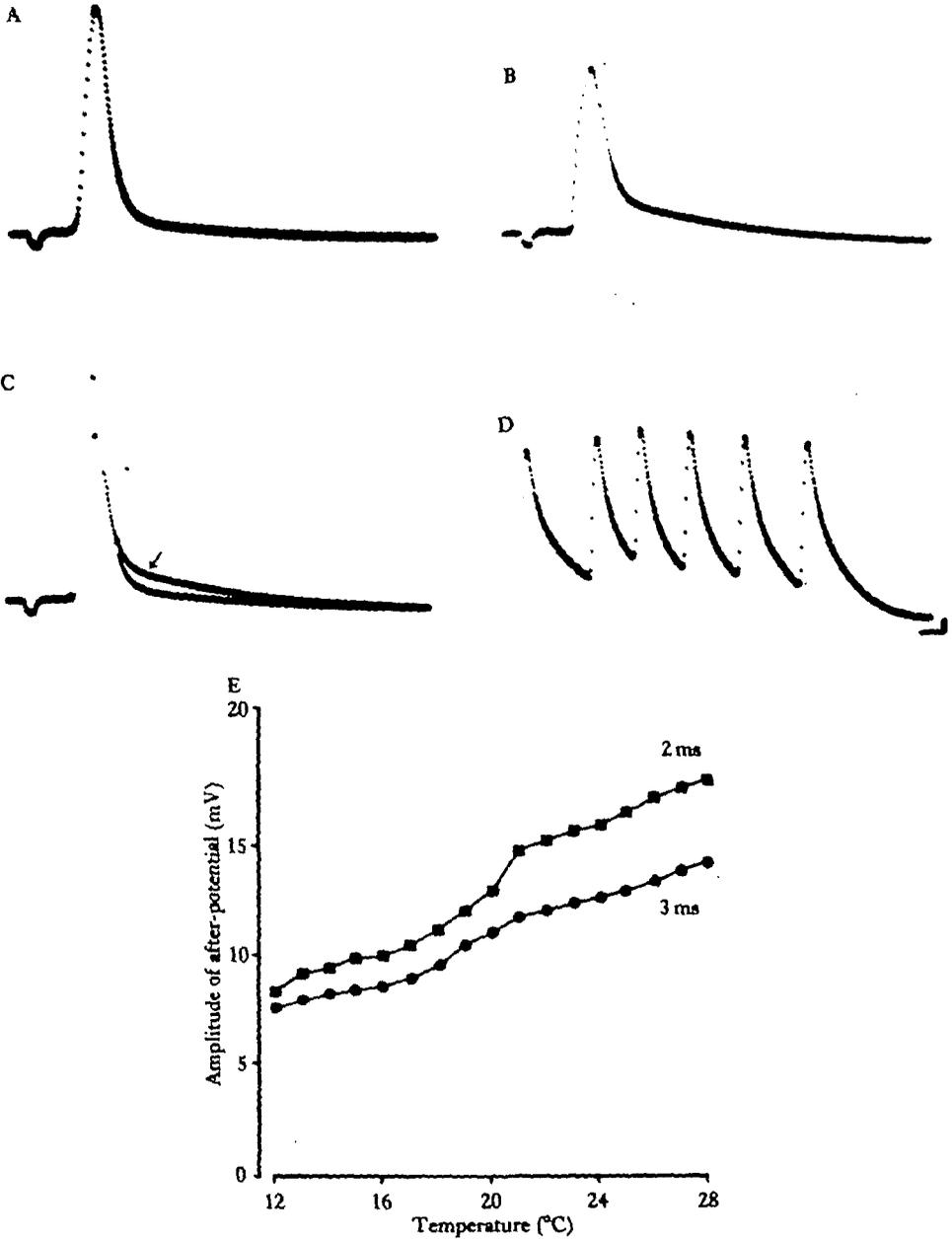


Fig. 4. The effect of temperature on the E2 action potential. (A) At 14°C. (B) At 26°C - average of 32 responses. (C) Records (A) and (B) superimposed to show the larger depolarizing after-potential at the higher temperature (arrow). (D) At 30°C - a single shock evoked six action potentials. (E) Graph showing temperature-dependence of the depolarizing after-potential, as measured 2 ms and 3 ms after the peak of the spike. Calibration: 5 mV and 1 ms.

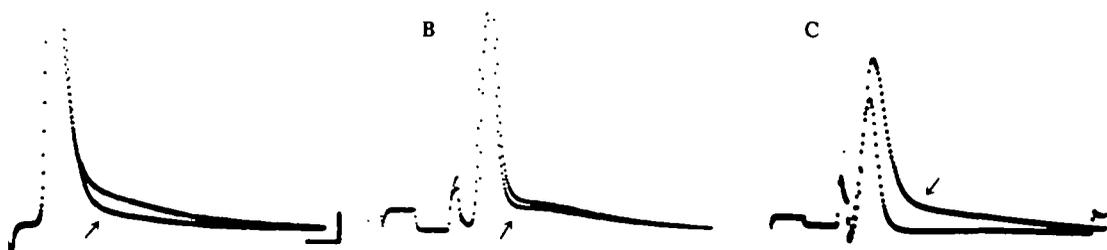


Fig. 5. The effect of ethanol and high calcium on the E2 action potential recorded at 14°C. Pairs of traces recorded from the same E2 axon - average of 32 responses. Action potentials were recorded in normal saline (arrow) and are superimposed upon recordings made in saline made up with 2% ethanol (A), 60 mM-calcium (B), and 0 mM-calcium (C). Any changes in resting potential (± 1 mV) were corrected by current injection through the recording microelectrode. Calibration: 5 mV and 1 ms.

(and decreasing the sodium concentration by 40 mM to maintain osmolarity) decreased the temperature threshold for peripheral spike generation in E2 (Table 1) and increased the size of the depolarizing after-potential (Fig. 5B).

Replacement of external calcium with 2 mM-EGTA (to chelate any calcium ions) and magnesium to maintain the osmolarity, resulted in the appearance of a hyperpolarizing after-potential following the spike (Fig. 5C). This result suggests that calcium ions are associated with the production of the depolarizing after-potential of the E2 action potential.

Refractory and low threshold periods

The duration of the depolarizing after-potential recorded in normal crab saline is longer than the hyperpolarizing after-potential recorded in calcium-free conditions

Table 1. *The effects of extracellular calcium concentration on the temperature threshold for peripheral spike generation*

The threshold for peripheral spike generation was obtained by slowly warming the preparations and determining the temperature at which a single E2 axon shock evoked two action potentials and two EJPs in the bender muscle. Different threshold values recorded in normal (20 mM- Ca^{2+}) saline are due to different animal acclimation temperatures (Lazarus *et al.* 1982)

Animal	Temperature threshold for peripheral spike generation	
	20 mM- Ca^{2+}	60 mM- Ca^{2+}
A	24.0	20.5
B	24.5	21.5
C	30.5	28.0
D	31.5	29.5
E	32.0	29.0
F	34.0	33.0

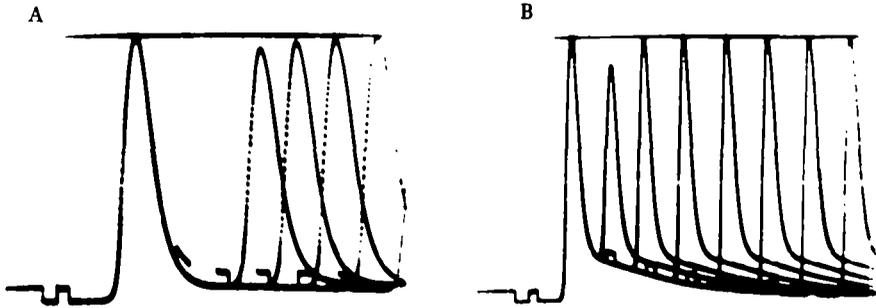


Fig. 6. The effect of temperature on the refractory period following the E2 action potential. The E2 axon was stimulated with pairs of high intensity electrical shocks at short intervals. The shortest interval at which spikes of the same amplitude were recorded was taken to be the limit of the relative refractory period. Recordings were made from the same E2 axon at 14°C (A) and 26°C (B), and relative refractory periods were measured as 4 ms and 2.2 ms, respectively. Calibration: 5 mV and 1 ms.

(Fig. 5C). If the hyperpolarization is indicative of sodium inactivation and an increased potassium conductance, g_K (Hodgkin & Huxley, 1952), it is possible that the refractory period following the E2 spike may have a shorter duration than the depolarizing after-potential. To test this possibility the E2 axon was stimulated with high intensity (3 to 5 times threshold) pairs of pulses at intervals of less than 10 ms. Pairs of action potentials evoked at very short time intervals were not of equal amplitude (Fig. 6). The smaller size of the second spike may be explained by its production during the relative refractory period, when g_K is high. Therefore, the refractory period was simply measured as the minimum time interval required to produce successive spikes of equal amplitude. The refractory period had a shorter duration than the depolarizing after-potential (Fig. 6).

The depolarization of the axon membrane immediately following the refractory period (Fig. 6) may result in a period of increased excitability. To test this, the E2 axon was stimulated at two locations (Fig. 7A). Stimulation at location I evoked an E2 action potential (Fig. 7B), while the stimulus delivered at location II was sub-threshold for spike production (Fig. 7C). However when the sub-threshold stimulus II was critically timed with stimulus I, a second E2 action potential was recorded (Fig. 7D). It may be argued that this result was due to stimulus spread. However, the two pairs of stimulating electrodes were insulated from each other and from the preparation by vaseline walls (Fig. 7A). Furthermore, decreasing the intensity of stimulus I to just below sub-threshold for spike production and increasing stimulus II (but still sub-threshold) did not provoke E2 axon impulses. In fact, the sub-threshold stimulus II only produced an action potential when stimulus I first produced a spike.

Reduction of the intensity of stimulus II resulted in spike production only during a critical time interval after the first action potential (Fig. 7D). The minimum stimulus required was usually about 60% of the threshold value, and at 14°C produced an action potential when applied 4.0 ms after the initial spike (Fig. 7D). However at 26°C the low threshold period was 2.2 ms after the spike (Fig. 7G). This latter value is the same as that measured for the refractory period at 26°C (Fig. 6B) and is similar to the time interval recorded between additional peripherally generated spikes (Fig. 4D).

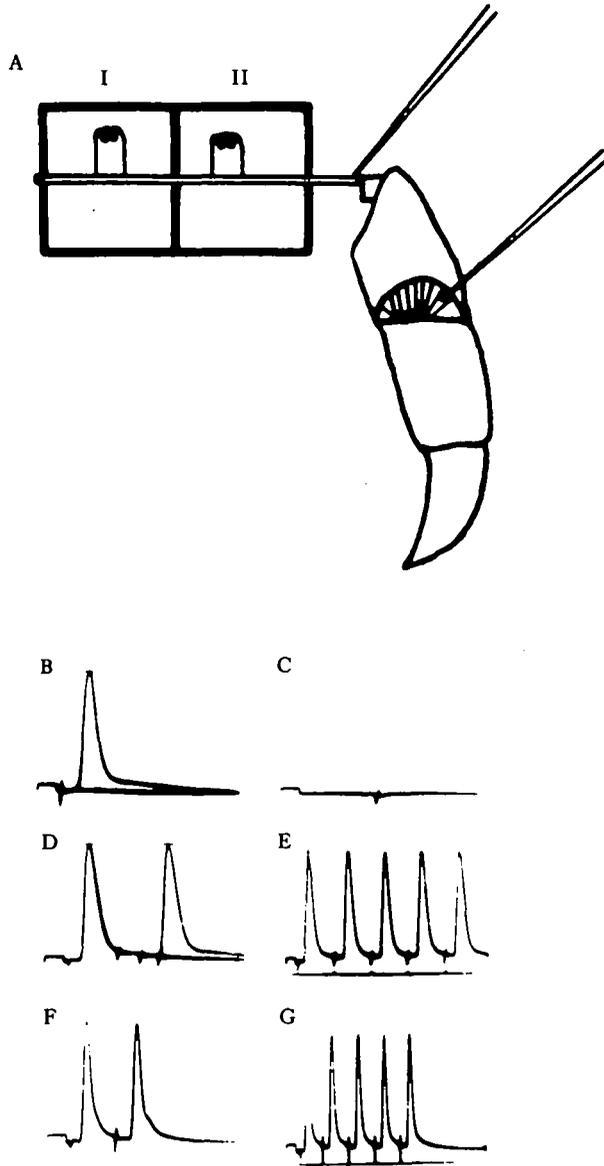


Fig. 7. The period of low threshold. (A) The preparation made from an autotomized walking limb. Vaseline walls were constructed around the stimulating electrodes (I and II) to insulate the shocks and decrease stimulus spread. (B)–(G) Records of E2 action potential made at 14 °C (B–E) and 26 °C (F, G). (B) An action potential evoked by an electrical shock at stimulus site I. (C) A sub-threshold stimulus II shock evoked no E2 action potential. (D) Critical timing between stimulus I and stimulus II shocks evoked two action potentials. Note that stimulus II shocks applied 1 ms before and 1 ms after the critical time did not provoke a second action potential. (E) A stimulus I shock followed by a train of sub-threshold stimulus II shocks evoked successive E2 action potentials. (F) and (G) Records similar to (D) and (E), but at 26 °C. Note the shorter time interval between spikes. Calibration: 5 mV and 1 ms.

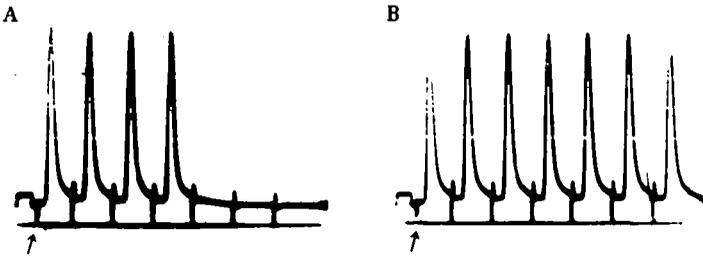


Fig. 8. The effect of repetitive firing on the E2 action potential recorded at 26°C. (A) and (B) Electrical shocks were applied at two locations (I and II) along the E axon – Fig. 7A. (A) A stimulus I shock (arrow) evoked an action potential and was followed by a train of minimum intensity stimulus II shocks which were sub-threshold for E2 spike production when applied alone. Note that four spikes were produced, but were followed by failures. (B) A small increase in stimulus II intensity (but still sub-threshold when applied alone) resulted in a one-for-one following between shock and spike. Calibration: 5 mV and 1 ms.

Repetitive firing

Successive sub-threshold stimulus II shocks evoked short trains of E2 spikes when applied during the low threshold period of the preceding impulse. At 14°C, stimulus II shocks applied every 4 ms evoked successive action potentials (Fig. 7E), while at 26°C a much shorter interval (2.2 ms) between stimulus II shocks was required to elicit a train of E2 spikes (Fig. 7G). As a result, increased temperature caused an increase in the firing frequency.

Successive, minimum intensity, stimulus II shocks evoked only a few additional action potentials; failures were recorded near the end of the stimulus train (Fig. 8A). However a small increase in stimulus intensity resulted in the production of an impulse following each shock (Fig. 8B). Therefore, it seems that there is an increase in the threshold for firing during a short train of spikes.

DISCUSSION

The action potential recorded from the E2 motor axon to the limb bender muscle of *Pachygrapsus crassipes* consists of a spike followed by a depolarizing after-potential (Figs 1B, 2B). Action potentials with similar characteristics have been recorded from other crustacean and molluscan neurones (Watanabe & Grundfest, 1961; Yamagishi & Grundfest, 1971; Fuchs & Getting, 1980; Grossman, Schmidt & Alkon, 1981). The decrease in spike amplitude in zero-calcium saline (Fig. 5C), coupled with the abolition of the spike in the presence of tetrodotoxin (P. J. Stephens, unpublished observations), indicate that the spike may be generated by sodium and calcium ions. Also, calcium ions appear to be implicated in the production of the depolarizing after-potential (Fig. 5C). An increase in temperature causes the spike to decrease in amplitude and time course (Fig. 3) and also results in a decrease in the time course of the refractory period (Fig. 6). These observations are consistent with previous reports that sodium and potassium channels are temperature sensitive (Hodgkin & Katz, 1949; Dierolf & Brink, 1973; Ruiz-Manresa & Grundfest, 1976; Partridge & Connor, 1978).

In many 'encoder' neurones repetitive firing has been explained in terms of a f

potassium channel (Connor, 1981). A fast potassium channel may be present in the E2 axon, as in other crustacean axons (Connor, 1975). However we consider that the characteristics of the E2 axon involved in the production of short trains of additional spikes at high temperatures are an increase in the size of the depolarizing after-potential, a decrease in the time course of the refractory period, and the geometry of the motor axon. However, it may be argued that the decrease in the time course of the refractory period is due to a decline in the slow potassium current and a predominance of the fast potassium current.

A close association between the depolarizing after-potential and peripheral spike generation is drawn from the following evidence. Motor axons with a hyperpolarizing after-potential following the spike (Fig. 2A) do not exhibit peripheral spike generation at high temperatures (Lazarus *et al.* 1982). Increases in temperature provoke peripheral spike generation and also increase the size of the depolarizing after-potential (Fig. 4C, E). Moreover, the time course of the increased response is similar to the time interval between the additional peripherally generated spikes (Fig. 4D). Low levels of ethanol or increased calcium decreased the temperature threshold for peripheral spike generation (Lazarus *et al.* 1982 and Table 1) and increased the size of the depolarizing after-potential (Fig. 5A, B). Recent observations have shown that ethanol increases the calcium current (Oakes & Pozos, 1980).

In the single excitor motor axon to the limb stretcher muscle, the generation of additional spikes takes place at or near the sites of the presynaptic connections made by the specific inhibitor (Stephens & Atwood, 1981). Many of the presynaptic terminals are located where the diameter of the excitor axon is reduced (Jahromi & Atwood, 1974), places where the safety factor for propagation is low and conduction failures take place (Parnas, 1972; Grossman, Spira & Parnas, 1973; Hatt & Smith, 1975; Lang & Govind, 1977; Smith, 1980). Furthermore, at regions where the axon diameter is decreased the membrane resistance must increase (Katz, 1966). Invasion of an action potential with a depolarizing after-potential into a region of increased membrane resistance would result in concentration of the current and an increased depolarizing after-potential. Thus the region of decreased axon diameter functions as an amplifier for sub-threshold potentials. At cool temperatures the long refractory period following the spike (Fig. 6A) would preclude the generation of additional spikes from the after-depolarization. However, increases in temperature increase the size of the depolarizing after-potential (Fig. 4) and shorten the refractory period (Fig. 6). As a result, at high temperatures the period of low threshold occurs about 2 ms after the spike (Fig. 7G). At this time the size of the depolarizing after-potential in the peripheral branches is sufficient to reach threshold for the generation of additional action potentials. Additional increases in temperature presumably provoke further increases in the size of the depolarizing after-potential and decreases in the time course of the refractory period, thus producing short bursts of additional spikes at the periphery.

Increases in temperature above the critical threshold for peripheral spike generation result in a progressive increase in the number of additional spikes evoked by a single orthodromic impulse (Stephens & Atwood, 1981; Lazarus *et al.* 1982). We have shown that the threshold for peripheral spike generation increases during a train (Fig. 8). However the mechanism controlling the length of the discharge is yet to be revealed.

A decrease in EJP amplitude and time course with increased temperature results in a decline in muscle tension (Stephens & Atwood, 1982). However peripheral spike generation causes summation and facilitation of EJPs and increases muscle tension (N. Mindrebo, unpublished observations). Therefore it seems that the peripheral generation of additional motor axon spikes may extend the temperature range in which shore crabs can remain functional. However the sudden and dramatic change in the efferent firing patterns that takes place at the critical temperature threshold for this phenomenon may have a detrimental effect on the animal's ability to perform coordinated movements.

This work was funded by grants from the National Science Foundation (BNS 81 13196), the Whitehall Foundation, and a Faculty Summer Research Grant awarded by Villanova University to P.J.S. We gratefully acknowledge the technical assistance of Ms S. Johnston.

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