ELECTROPHYSIOLOGY OF THE SOMATIC MUSCLES IN THE NEMATODE *ASCARIS LUMBRICOIDES*

Thesis by

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Marathi saying

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ABSTRACT

The electrical activity of the somatic muscles of the parasitic nematode Ascaris lumbricoides has been investigated. The functional muscle syncytium overlying the nerve cord is preferentially excited by anodal stimulation with an extracellular electrode. Cathodal stimulation preferentially excites the nerve cord, allowing determination of separate conduction velocities for the nerve cord and syncytium. The propagation velocity of the nerve cord is 16.2 ± 1.2 cm/sec; that of the syncytium varies with the calcium concentration, being 21.6 ± 1.3 cm/sec for unitary slow waves under normal conditions. Both values are too high to account for the propagation of contractile waves in the intact animal.

Ascaris muscle gives rise to complex spontaneous depolarizations consisting of slow waves and graded spike potentials. Often, the spikes and slow waves are modulated into periodic bursts of electrical activity, which gives rise to rhythmic contractions on a behaviorally significant time scale. Spikes appear to be mediated exclusively by calcium ions; the spike active potential varies with calcium concentration as expected for a calcium electrode and spikes persist in sodium-free media. Slow waves can be mediated by either sodium or calcium ions; they persist when calcium or sodium are removed separately, but not when both are removed together.

In rhythmically active preparations, a burst of slow waves and spikes accompanies each contraction. The modulation shows dorsal-ventral coordination if the right lateral line is intact, in accord with known nervous system asymmetry. Anterior-posterior coordination is also observed .

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PART ONE

PROPAGATION OF ELECTRICAL ACTIVITY IN THE NERVE CORD AND MUSCLE SYNCYTIUM OF *ASCARIS*

INTRODUCTION

Because of their relative simplicity, nematodes may be useful for correlating nervous system structure and function with behavior. Genetic and anatomical approaches to these problems are being made with the small, genetically tractable species, Caenorhabditis elegans (Brenner, 1974; Ward et al., 1975; Ware et al., 1975; Ward, 1973; Dusenbery et al., 1975), and it is hoped that such studies will be complementary with anatomical and physiological studies of the larger nematode, Ascaris lumbricoides (Goldschmidt, 1908,1909; De Bell et al., 1963; Del Castillo et al., 1967). Available evidence suggests considerable structural conservatism between these two species, despite the marked size discrepancy, as within the nematode class as a whole (Bird, 1971). Thus, a combined approach, using physiological studies of Ascaris, behavioral genetics of Caenorhabditis, and biochemical, pharmacological, and anatomical techniques with both species should shed light on the functional organization of the attractively simple nematode nervous system.

Previous studies of the neuromuscular system of Ascaris somatic muscle have been reviewed by De Bell (1965). As shown schematically in Figure 1, the locomotor musculature consists solely of dorsal and ventral longitudinal muscles, which contract in opposition to produce motion. In Ascaris

and other nematodes, the neurons are essentially unbranched. Instead, the dorsal and ventral muscle cells send noncontractile "arms" to their respective nerve cords. These arms divide and interdigitate over the nerve cord, from which they receive innervation by en passant synapses (Rosenbluth, 1965). Muscle cell nuclei are contained within swellings on the muscle arms. These muscle "bellies" are convenient sites for impaling cells with microelectrodes, although the unusual structure of the muscle cells suggests that electrical properties of the bellies may differ from those of other anatomically distinct regions within the muscle cell.

Although the first electrical recording from Ascaris was made by Jarman in 1959, the bulk of the physiological studies on Ascaris emerged from Del Castillo's laboratory in the 1960s. Recording from muscle bellies, spontaneous electrical activity was observed, comprised of slow depolarizations of several hundred milliseconds duration with superimposed, graded spike potentials (Jarman, 1959; De Bell et al., 1963). The depolarizations of nearby cells within one field (dorsal or ventral) were shown to be correlated, suggesting electrical coupling between cells (Jarman, 1959); direct evidence for coupling was obtained when hyperpolarizing currents injected into a given muscle belly were shown to produce inhibition of activity in neighboring cells (De Bell et al., 1963). From the fact that cells on opposite sides of the midline were shown to be coupled, the site of coupling was assumed to be at the

point above the nerve cord where processes from muscle cells of opposite sides interdigitate.

This "syncytial" area was also implicated as the site of origin of the observed spontaneous activity, primarily by two observations. First, current injected through microelectrodes into muscle processes very close to the syncytial region was shown to regulate the level of spontaneous electrical activity (Del Castillo et al., 1967). Second, depolarizations recorded from muscle bellies over the nerve cord surpassed in amplitude and preceded in time those recorded from bellies located more laterally (Jarman, 1959). From these early studies arose a relatively simple view of the Ascaris somatic muscle system, that arms from the muscle cells within each field interdigitate over the nerve cord to form a functional syncytium of electrically coupled processes within which spontaneous electrical activity originates and from which the activity propagates passively to the contractile portion of the cell.

A major problem remaining after this early work was the mechanism(s) by which the spontaneous myogenic activity might be regulated to give the organized contractions necessary for motion. It seems inevitable that the nervous system must be involved in this regulation, but relatively little progress was made in separating any nervous effects from those due to the muscle cells themselves. Much of the difficulty arose

from the inseparability of the nerve cord and the overlying muscle syncytium. For instance, De Bell and coworkers (1963), stimulating the region of the nerve cord and muscle syncytium with a pair of closely spaced wire electrodes, were able to elicit slow waves and spikes, but found that the response was variable and sensitive to the position of the electrodes and the polarity of the current. Although they were able to measure a propagation velocity of about 6 cm/sec, they were unable to tell whether the excitation was propagated by the nerve cord or the muscle syncytium. Similarly, Jarman (1959) erroneously concluded that the nerve cord was responsible for the correlation between the depolarizations in neighboring muscle bellies.

In the first part of this thesis, three methods are reported for circumventing the inseparability of nerve cord and muscle syncytium. The first involves selective stimulation of either the nerve cord or the syncytium by varying the polarity of the stimulus. The selective sensitivities of the two excitable elements to different polarities of stimulus allows measurement of their separate conduction velocities, and also rationalizes earlier observations on the variability of effects of bipolar stimulation (De Bell et al., 1963). The second method involves local interruption of syncytial conduction by mechanical disruption of the muscle arms and bellies over a limited area. Propagation of nervous activity past

such a block allows its effects to be selectively observed. The third method involves blockage of neuromuscular transmission by the use of low-calcium bathing solutions. Taken together, these methods indicate that the passage of behaviorally relevant contractile waves down the length of Ascaris cannot be solely governed by conduction rates of either the nerve cord or the muscle syncytium.

In the second part of this thesis, the ionic mechanisms of the myogenic activity of Ascaris muscle are investigated. Some evidence is provided that the muscle cells are endogenously capable of modulating their activity to generate contractile waves.

MATERIALS AND METHODS

Ascaris were obtained from Farmer John Packing House, Vernon, California. They were maintained for up to five days at 38-39° in artificial perienteric fluid (APF), with daily transfers to fresh solution. The composition of APF and that of a high-magnesium, low-calcium solution (HMLC) are given in Table I.

The plexiglas recording chamber had a Sylgard bottom and a glass heating coil connected to a recirculating, heating water bath. The volume of the chamber was 35 ml, and the temperature was maintained at 37-38°. Two yoked 50 ml syringes were connected to the chamber by plastic tubing and could be used to perfuse the chamber with preheated solutions. The composition of the bathing fluid was changed either using this apparatus or by direct replacement of a portion of the bathing fluid.

To record from the muscle bellies, a large female Ascaris was bisected in front of the gonad. The anterior fragment was pinned down with the lateral lines in a vertical plane. After the lips were examined to determine the position of the dorsal and ventral muscle fields, the anterior centimeter was removed. The remaining portion of the body was then opened along the upper lateral line, the walls were pinned out, and the gut was removed. Fig. 2 shows a typical preparation.

Electrical recordings were made using standard techniques. 3 N KCl-filled microelectrodes were mounted to a platinum wire soldered to the end of a spring attached to a micromanipulator. This allowed the microelectrode to move during muscular contractions, without disimpaling. Intracellular recordings were made from muscle bellies close to the ventral or dorsal nerve cord. The nerve cord/syncytium was stimulated in early experiments with a pair of platinum wires about 1 mm apart placed directly over the nerve cord. In later experiments, only one wire was placed over the nerve cord, the other resting in the bath distant from the preparation. In both cases, the wire(s) over the nerve cord/syncytium were insulated up to the tip with a sheath of glass capillary tubing. Stimuli were 3-10 V in amplitude and 1-4 msec in duration.

A computer of averaged transients (CAT) was used to separate a stimulus evoked response from the spontaneous activity of the preparation. The signal from the electrometer connected to the recording electrode was amplified 100X using an AC preamplifier (.1 Hz to 10 KHz), and fed into the CAT. The CAT was triggered with an output from the stimulus generator. Depending on the strength of the response and the level of the spontaneous activity, variable numbers of sweeps were accumulated. Even if the response appeared clear after a single sweep, several trials were stored in order to obtain an average latency. Usually, 5-30 trials were summed, then fed

out to a chart recorder for later measurement. The amplitude of the CAT traces was not calibrated.

To determine the degree of correlation between the activity of two cells, and to measure the propagation velocities of spontaneous depolarizations, the signal from one cell was amplified and used to trigger the CAT. The electrometer outputs from both this trigger cell and the follower cell were amplified 100X and fed into the CAT.

Latencies were measured from the stimulus artifact to the maximum of the response appearing in the CAT output, or from the peak of the trigger cell response to the peak of the follower cell response, depending on the activity being investigated. This method is prone to error, since changes in the spontaneous activity or excitability of the preparation with time or position can affect the shape of the response measured on the CAT. This is especially true for inhibitory response. For experiments which gave latency vs. separation plots that were nonlinear, no propagation velocities were measured.

Distances were measured using a calibrated, graduated reticle in the eyepiece of the dissecting microscope. To deal with errors in both the time and distance measurements, straight lines were fitted to the data by the method of Acton (1959).

RESULTS

Spontaneous Activity in APF

In Ascaris, decremental conduction between the electrically active region (syncytium), and the recording site (muscle bellies), distorts the time course and amplitude of the recorded electrical events. However, it is usually possible to distinguish three different types of electrical activity, clear examples of which are shown in Fig. 3. These are (a) long lasting potential changes modulating the level of spontaneous activity, (b) slow wave depolarizations, and (c) superimposed, graded spike potentials. Resting potentials range from 30 to 40 mV; spontaneous activity may occur throughout the range. Spike potentials vary in duration from 10 to 50 msec and in amplitude from barely perceptible bumps to spikes of 16 mV positive overshoot. The larger amplitude spikes are usually observed in muscle bellies or muscle processes directly over the nerve cord, but even within a single cell large variations in amplitude are observed. Slow wave depolarizations vary in duration from 100 to 400 msec and in amplitude from 2 to 20 mV. The persistence of slow waves under conditions where spike potentials and contractions have been abolished (to be described below) indicates that they are not movement artifacts. The long lasting potential changes give rise to periods of activity and inactivity lasting up to 20 sec. Inactivity is often accompanied by a visible hyper-

polarization of several mV. The modulation varies with the geometry of the preparation as will be discussed later.

This spontaneous activity differs somewhat from that reported in previous studies of Ascaris (De Bell et al., 1963; Del Castillo et al., 1967; Jarman, 1959). In particular multiple spike potentials are frequently observed on a single slow wave, as in Fig. 3b and 3c, and visible muscle contractions are usually correlated with modulated electrical activity as in Fig. 3a, and even with unitary electrical events as in Fig. 4. Previous workers found only a single spike on each slow wave, did not observe modulation of spontaneous activity, and apparently observed active muscular contractions only rarely. These observations indicate that the preparations used in the present investigation may be in a more natural physiological state. This may result from the temperature and ionic conditions under which the animals are maintained.

Response to Cathodal Stimulation

With cathodal stimulation the response is usually inhibitory. Occasionally the inhibitory response occurs as a true hyperpolarization below the resting potential of the cell. More frequently, especially in spontaneously active preparations, it is manifested as a consistent absence of slow wave depolarizations. An average of the time course of such events is obtained using the CAT, as illustrated in Fig. 5c.

Measurements of the propagation velocity of this response give an average value of 16.2 ± 1.2 cm/sec. A histogram presenting the velocity distribution from 16 experiments is shown in Fig. 6b. The cathodally evoked inhibitory response is reversibly blocked by high-magnesium, low-calcium solution (HMLC), propagates past a region in which the syncytium has been mechanically disrupted (see below), and can be observed several centimeters from the stimulating electrode. Thus, it appears to result from the stimulation of nerves with inhibitory synapses on the muscles from which the recordings are made.

It can be seen in Fig. 5c that the cell depolarizes immediately following the inhibitory response. At times, as in Fig. 5a, this after-depolarization is to a higher level than before stimulation. This depolarization might be *a priori* attributed to stimulation of excitatory nerves. However, since the cell undergoes several oscillatory cycles of depolarization after the initial hyperpolarization (Fig. 5c), it seems more likely to result from rebound upon release from inhibition.

Response to Anodal Stimulation

In response to anodal stimulation in APF, there is an immediate depolarization of the muscle bellies near the stimulating electrode, without a preceding hyperpolarization. At greater distances, especially with larger stimuli, inhibitory

responses are observed, indicating some incidental stimulation of inhibitory nerves; the distinction between anodal and cathodal stimulation is, however, quite clear as can be seen in Fig. 5b and 5c. As shown in Fig. 5b, this depolarizing response is quite similar in time course and amplitude to the spontaneous slow wave depolarizations, and is often accompanied by spike potentials. This response to anodal stimulation does not propagate as well in APF as does the inhibitory response, and often dies out within several millimeters of the stimulating electrode.

Conduction velocities for the anodally evoked depolarizations average 21.6 ± 1.3 cm/sec; a velocity distribution from 19 experiments is presented in Fig. 6a. Unlike the cathodally evoked inhibitory response, the anodally evoked and spontaneous depolarizations persist in HMLC, supporting the earlier suggestion that they are myogenic (De Bell et al., 1963). In HMLC these depolarizations do change markedly in time course and amplitude, however. Spike potentials are absent or much reduced in amplitude, duration of the slow waves increases to up to 1 sec, and the propagation velocity for the anodally evoked depolarization drops to 4.4 ± 0.3 cm/sec. Figure 6c contains a velocity distribution for anodally evoked depolarizations in HMLC, from 9 experiments.

In HMLC the response to anodal stimulation is often larger in amplitude and propagates over greater distances

than in APF. This may be due to the lack of interference from the nervous inputs to the syncytium. Although repeated washings tend to hasten the degeneration of the preparation, HMLC effects are almost always reversible.

At calcium concentrations between those of HMLC and APF, intermediate propagation velocities are observed for the depolarizing response. The 30% seawater used by De Bell and coworkers is intermediate in calcium concentration (3 mM) to HMLC and APF, and the propagation velocity obtained by them in that solution is consistent with the present results, as is shown in Fig. 7.

Further evidence for the syncytial nature of the anodally evoked depolarizations is obtained by selective disruption of the syncytium. This is achieved by carefully plucking out the muscle bellies across the width of the muscle field using a pair of fine forceps, under the dissecting microscope. As demonstrated in Fig. 8, the anodally evoked response does not propagate past the disrupted region in HMLC. Washing with APF restores the inhibitory response to cathodal stimulation, which can still be seen to propagate past the destroyed region of syncytium. This indicates that the nerve cord is intact, but is not sufficient to propagate the depolarizing response.

Response to Mixed Stimulation

In early experiments, the nerve cord/syncytial region was stimulated using a pair of closely spaced platinum wires. As reported by De Bell and coworkers (1963), the nature of the response in such circumstances varies unpredictably with

the stimulus parameters. This is presumably due to the competing effects of simultaneous anodal and cathodal stimulation. Often the CAT-averaged response to mixed stimulation contains an early depolarization follwed by a hyperpolarization. When either response was sufficiently predominant to permit measurement of propagation velocities, the results obtained were similar to those obtained later with the relatively selective "unipolar" stimulation. Mixed stimulation in HMLC gives results similar to those obtained with anodal stimulation, because the inhibitory response is blocked.

Spontaneous Activity in HMLC

The electrical coupling between muscle cells in Ascaris can be clearly seen in HMLC, as well as in APF, by the synchronized activity of adjacent cells. In HMLC contractions are blocked, but the spontaneous depolarizations can be seen to propagate over distances of several centimeters (see Fig. 9). At times these measurements are complicated by an apparent shift in the site of origin of the spontaneous depolarizations, or by the interference of depolarizations arising at different sites. As illustrated in Fig. 9c, the depolarizations propagate both anteriorly and posteriorly, and sometimes undergo propagation failure, as does the anodally evoked response. The synchrony between cells is eliminated if the syncytium between them is disrupted, or if the cells are on opposite sides of the lateral line, as shown in Fig. 9a and 9b.

Using the CAT and the self-triggering circuit, it is possible to measure propagation velocities for the spontaneous depolarizations, as shown in Fig. 10. Usually, the spontaneous depolarizations propagate at the same velocity as the anodally evoked slow waves in the same preparation, and their velocity increases with increasing calcium concentration in a similar manner, as shown in Fig. 11. However, at calcium concentrations approaching that of APF, depolarizations sometimes occur simultaneously over a wide region of the preparation. This happens when the preparation is exhibiting highly regular depolarizations, such as are commonly observed in the isolated dorsal field in APF (see below). It seems that the population of homogeneous, tightly coupled oscillators tends to synchronize when left unperturbed. When the system is continually perturbed by nervous inputs as in the fully innervated ventral field, when the oscillators are less tightly coupled as may be true in HMLC, or when irregular depolarizations occur as in response to electrical stimulation, finite conduction velocities are observed.

Modulation of Spontaneous Activity

The spontaneous activity of these preparations appears to be modulated to an extent not observed by earlier workers. In addition to the quiet, hyperpolarized, and continually active, depolarized states reported previously (De Bell et al., 1963) many preparations alternate with periods on the order of seconds between electrically active and inactive states. Usu-

ally the muscle bellies hyperpolarize during inactive periods; periods of activity coincide with muscular contractions. The times involved are comparable with those involved in motions of intact worms. Examples of such activity are shown in Fig. 12.

If the activity of the ventral muscle field is taken as an indication of whether a given preparation can exhibit modulated activity, then the presence of such activity in the dorsal field depends on which lateral line is severed to expose the muscles. When the right lateral line is cut, thus severing the bulk of the ventral-to-dorsal traveling commissures (Stretton, personal communication), the dorsal side is uncoupled from the ventral side and usually reaches a state in which it exhibits highly regular slow wave depolarizations with superimposed spike potentials. These continue for long periods of time with only gradual changes in frequency and amplitude (see Fig. 12a). When the left lateral line is cut in generating the Ascaris preparation, most of the nerve commissures are left intact. In this case, modulated activity, if present, is recorded from both the dorsal and ventral muscles, and is coordinated so as to produce either simultaneous, or antagonistic activity between the two muscle fields, as in Fig. 12b.

In some such preparations even the individual slow waves are correlated between the dorsal and ventral fields.

This correlation can be seen whether the dorsal and ventral activity periods are in phase, as in Fig. 12b1, or out of phase as in Fig. 12b2. This suggests the possibility of direct electrical connections between muscle cells in the dorsal and ventral fields. The existence of such direct connections is further indicated by experiments in which dorsally placed recording electrodes monitor the response to stimulation of the ventral nerve cord/syncytial region. As expected, cathodal stimulation usually gives clear inhibition on the dorsal side when the right lateral line is left intact, and much less often when only the left lateral line remains intact. Suprisingly, the depolarizing response to anodal stimulation also crosses the lateral line in a number of cases. This response, when seen, is usually weaker and does not propagate well compared with the response to direct anodal stimulation of the dorsal nerve cord/syncytial region, but shows up clearly with the CAT. This response, like the cathodally evoked hyperpolarization is more frequently and clearly seen when the left, rather than the right lateral line is cut in making the preparation.

In addition to the correlation between modulated activity in the dorsal and ventral muscle fields, similar correlations between anterior and posterior regions within the same field are occasionally observed, as shown in Fig. 13. Along with the dorsal-ventral coordination discussed above,

this anterior-posterior coordination must be necessary to allow waves of contraction to pass down the length of the intact worm. Although such coordination must be subject to some degree of neural control, observations have been made that indicate the need for some form of proprioceptive feedback as well. This question is discussed further in the second section of this thesis.

CONCLUSIONS

1. Del Castillo and coworkers suggested that the slow depolarizations in Ascaris muscle cells are myogenic in origin, but the distinction between neural and myogenic propagation along the anatomically inseparable nerve cord and muscle syncytium could not be made (De Bell et al., 1963; Del Castillo et al., 1967). Three sets of experiments reported here allow a separation of myogenic from neurogenic effects, and affirm the myogenic nature of the slow depolarizations.

Firstly, the qualitatively different responses to anodal and cathodal stimulation suggest that the structures involved in slow wave generation are different from those giving rise to inhibition. Secondly, the persistence of the slow waves where the (neurogenic) inhibition is blocked suggests that the muscle syncytium alone is sufficient for the generation and propagation of slow waves. Finally, the interruption of slow wave propagation by mechanical disruption of the syncytium, with the nerve cord still intact, indicates that the syncytium is necessary for slow wave propagation.

2. Observations on the effect of stimulus polarity have been made in three nematode species. Bradley reported that Ascaris and Phocanema contract in response to extracellular anodal stimulation and relax in response to cathodal stimulation (Bradley, 1961). She suggested that anodal stimulation affects muscle cells directly. Similar observations

have been made in Caenorhabditis during iontophoretic microinjections (unpublished results from this laboratory). The results reported here demonstrate the electrophysiological correlates of these earlier observations, and support Bradley's interpretation.

A possible explanation for the direct anodal excitation is that the excitable channels in the muscle processes are localized on the lower face of the muscle fingers, in juxtaposition to the nerves. If the close apposition of the muscle processes with one another over the nerve cord requires that most of the current flowing in response to stimulation goes through, rather than around the muscle fingers, then the lower membrane surfaces, which are postulated to contain the excitable channels, will be depolarized by anodal stimulation. The nerves, presumably having a uniform distribution of excitable channels, would be better excited by cathodal stimulation, as commonly observed in other systems.

3. The dependence of the propagation velocity of the anodally evoked depolarizations on calcium concentration could be explained in several ways. If steady state transmitter release determines the propagation velocity of the syncytium by altering membrane potential and resistance, calcium concentration might control propagation velocity by affecting the amount of transmitter being released. However, since bath applications of gamma-aminobutyric acid (GABA) block activity

without measurably affecting the propagation velocity (unpublished results from this laboratory), this possibility seems unlikely. Another possibility is that calcium affects the strength of the electrical coupling between adjacent cells, thus controlling the propagation velocity. Such effects have been reported (Rose and Lowenstein, 1975). The present results could be similarly explained. A final possibility is calcium is directly involved in the generation of the myogenic activity. The observations that HMLC blocks spike potentials, and alters the time course of the slow depolarizations tend to support this hypothesis, which is examined further in the second part of this thesis.

4. Physiological correlates of the bilateral asymmetry in the Ascaris nervous system have been observed, both in the spontaneous activity of the preparations and in response to stimulation. The results suggest that, in addition to the nerve commissures crossing from the ventral to the dorsal muscle fields, some sort of syncytial connections may also exist. Del Castillo and coworkers (1967) reported cells near the lateral lines that appear to have their electrically active regions near the lateral lines rather than at the nerve cord; in both Ascaris and Caenorhabditis, head muscle cells appear to send processes to both the nerve ring and the nerve cord (Stretton, personal communication; Ware, personal communication). If analogous connections exist in somatic muscle cells between the nerve cord and the lateral lines, this could explain the

observations described above.

5. Modulation occurs in Ascaris preparations (via inhibition of myogenic activity) that is of much longer duration than the response to a single stimulus. This suggests either that the muscles are capable of simultaneous long and short time course oscillations, or that the nerve cord, in isolation from the nerve ring, is capable of bursting spontaneous activity patterns. The observation that isolated ventral preparations, but only rarely isolated dorsal ones, tend to exhibit modulated activity suggests involvement of the nervous system, since nerve cell somata for both cords are exclusively ventral (Stretton, personal communication). The possibility of feedback effects of contraction is suggested by observations of coordinated modulation within an isolated dorsal field.

6. Propagation velocities for both the nerve cord and the muscle syncytium under physiological conditions are greater than 15 cm/sec, while waves of contraction in the intact worm travel as slowly as 1 cm/sec. This finding, coupled with the observations of modulated activity, leads to the conclusion that in Ascaris, the function of the muscle syncytium may be to coordinate burst of myogenic activity, and to smooth the boundaries between relaxed and actively contracting regions of muscle, rather than to propagate individual waves of contraction. The latter function seems most likely to be accom-

plished instead by neural modulation with some sort of proprioceptive feedback, as in more complicated organisms. How the nematode achieves this, with its relatively simple nervous system, is a question of interest.

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Table I

Composition of APF and HMLC

	Concentration (mM)	
	HMLC	APF
$MgCl_2$	10.0	5.0
$CaCl_2$	0.5	6.0
KCl	24.0	24.0
NaCl	23.0	23.0
NaOAc	110.0	110.0
Dextrose	11.0	11.0
Tris	10.0	10.0

Tris stock solution consists of 0.1 M Tris base adjusted to pH 7.6 at 25°. Final pH at 37° is about 7.5. APF is a modified version of that used by Ellison (1959),

Fig. 1. Schematic cross section of Ascaris neuromuscular system. C, cuticle; H, hypodermis; LL, lateral line; VNC, ventral nerve cord; DNC, dorsal nerve cord; MS, muscle spindle; MA, muscle arm; MB, muscle belly. Inset shows the functional syncycytium formed by electrical coupling of muscle processes interdigitating over the nerve cord. N, neuron in nerve cord; MF, muscle finger.

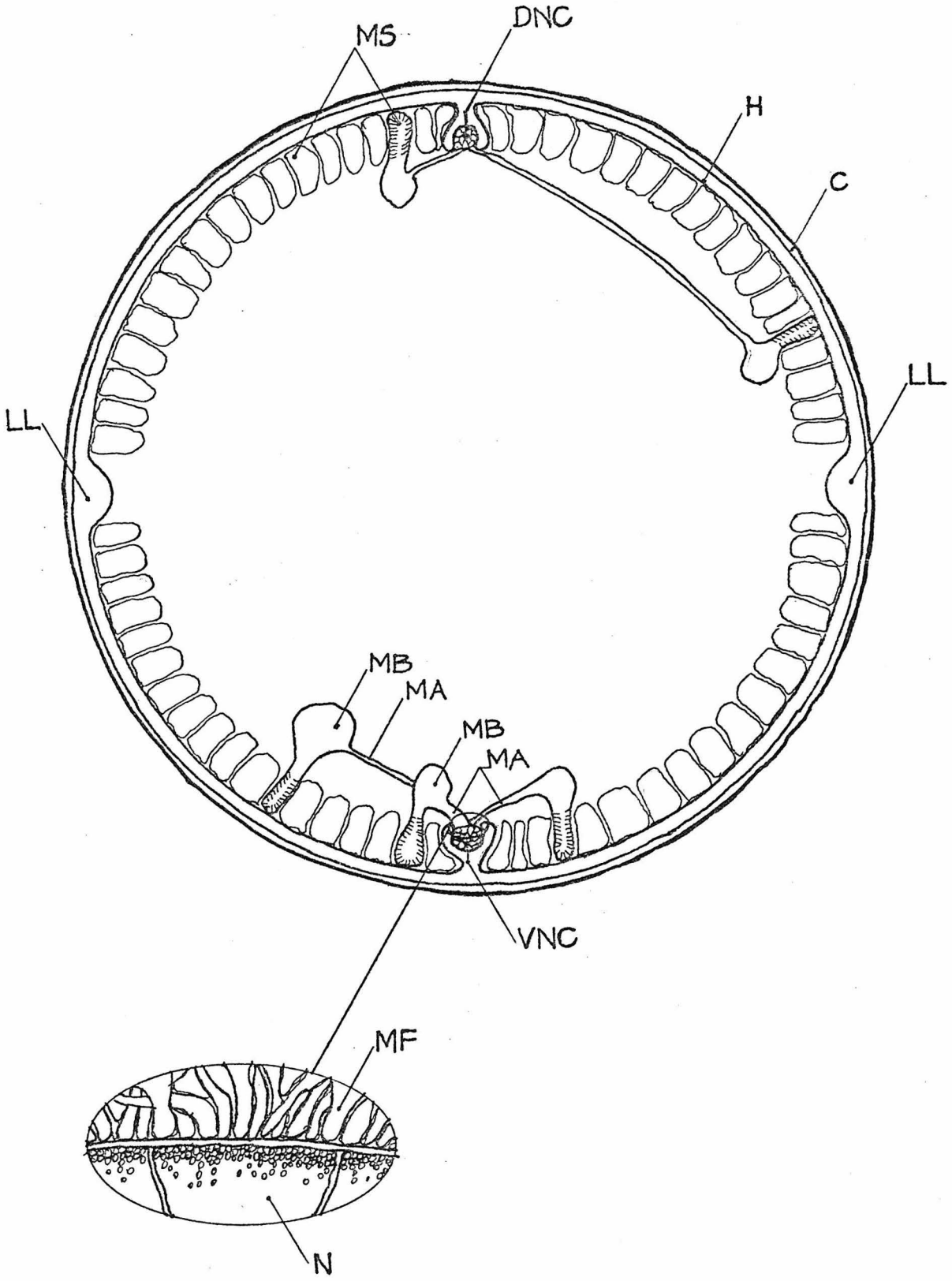

MS
DNC
H
C
LL
LL
MB
MA
MB
MA
VNC
MF
N

Fig. 2. Photomicrograph of a typical preparation. SE, glass sheathed, platinum stimulating electrode; RE1, RE2, micropipet recording electrodes; VNC, ventral nerve cord; LL, lateral line; VMB, ventral muscle bellies; DMB, dorsal muscle bellies. Calibration, 1 mm.

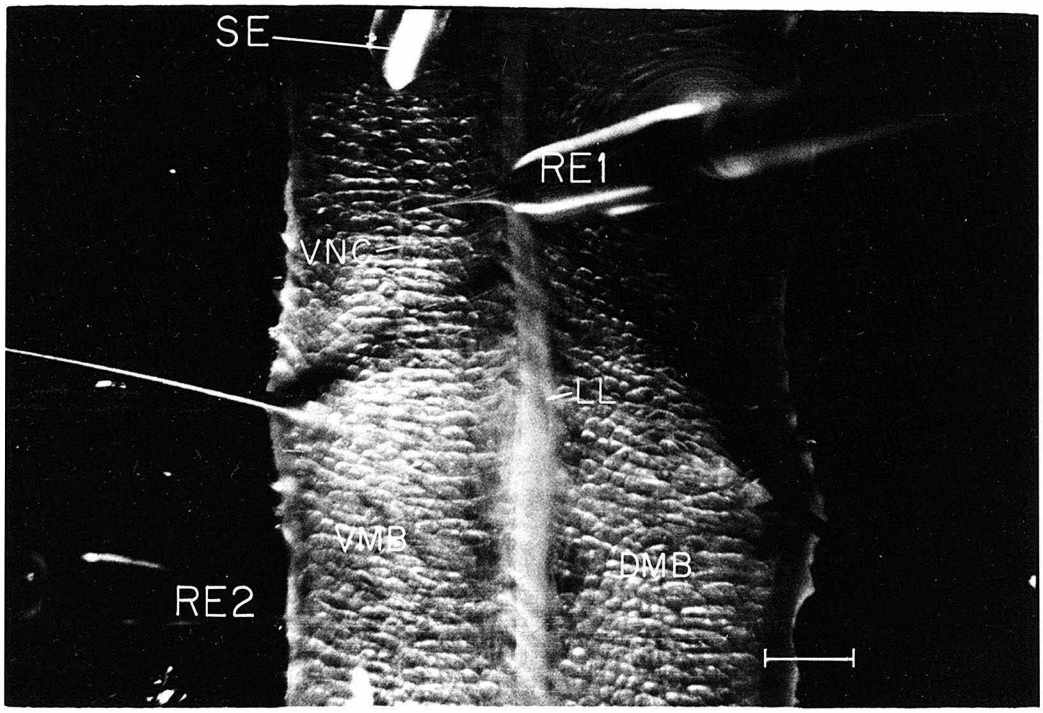

SE
RE1
VNC
LL
VMB
DMB
RE2

Fig. 3. Spontaneous electrical activity in Ascaris muscle bellies. (a) modulated spontaneous activity with a period of about 20 seconds; (b) expanded time scale recording from the same cell, showing how each period of activity consists of slow waves with superimposed, graded spike potentials; (c) slow waves and spikes from a different preparation, showing smaller slow waves with larger spike potentials. Calibration, 10 mV x 12 sec in (a), 10 mV x 1 sec in (b) and (c).

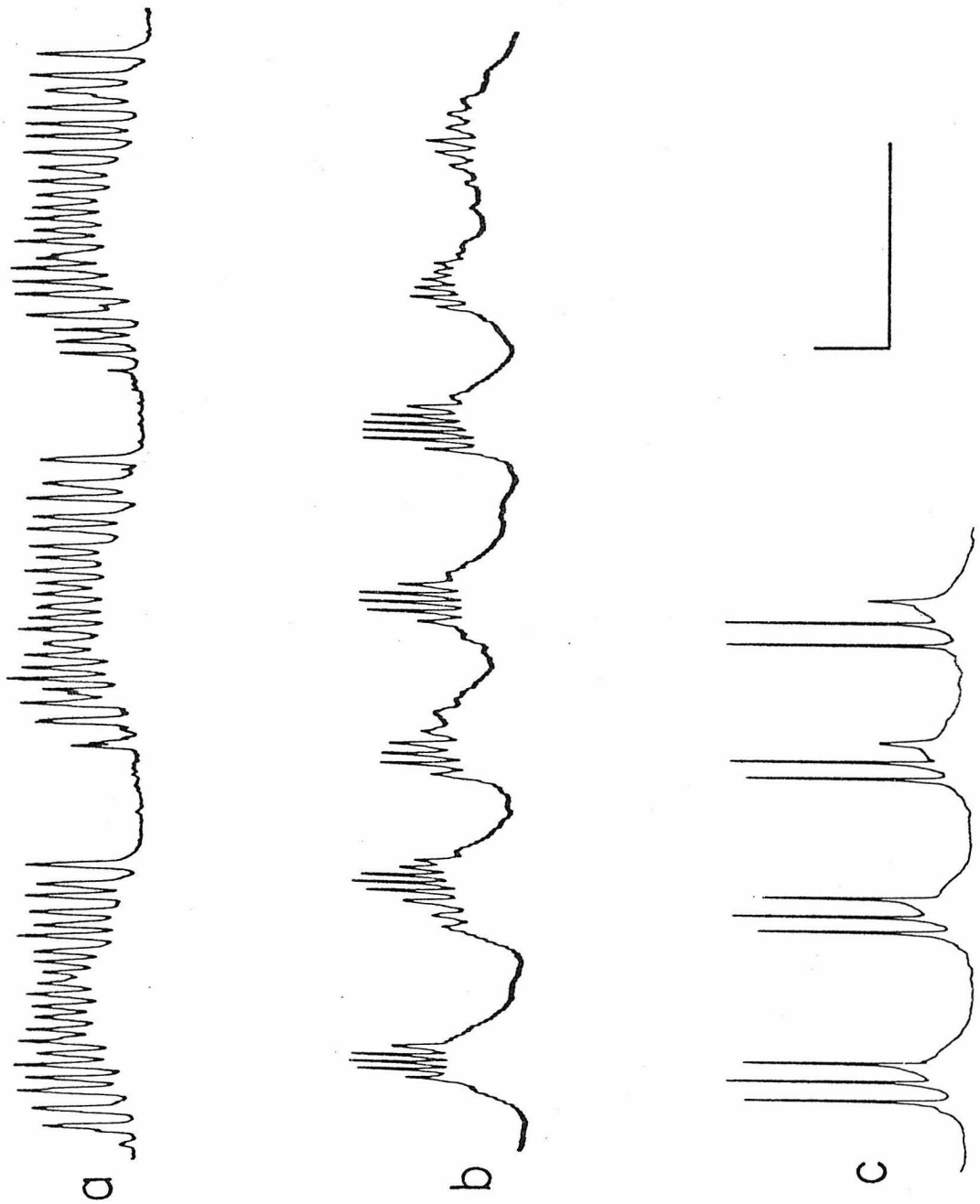

a
b
c

Fig. 4. Correlation of visible contractions with electrical activity. (a) electrical activity, consisting of occasional slow waves with superimposed spike potentials; (b) trace of event marker, blindly activated when a visible contraction was observed through the dissecting microscope. Calibration, 10 mV x 12 sec.

a
b

Fig. 5. Individual and averaged responses to stimulation. Several trials are superimposed in the oscilloscope photographs a-c. (a) inhibitory response to mixed stimulation at a distance of 1.7 cm from the stimulating electrode. (b) depolarizing response to anodal stimulation at a distance of 0.5 cm. (c) inhibitory response to cathodal stimulation at a distance of 0.5 cm (same cell as in (b)). (a1-c1) CAT averages of a-c. Calibration, 5 mV x .1 sec in (a), 10 mV x .1 sec in (b) and (c), .1 sec in (a1-c1).

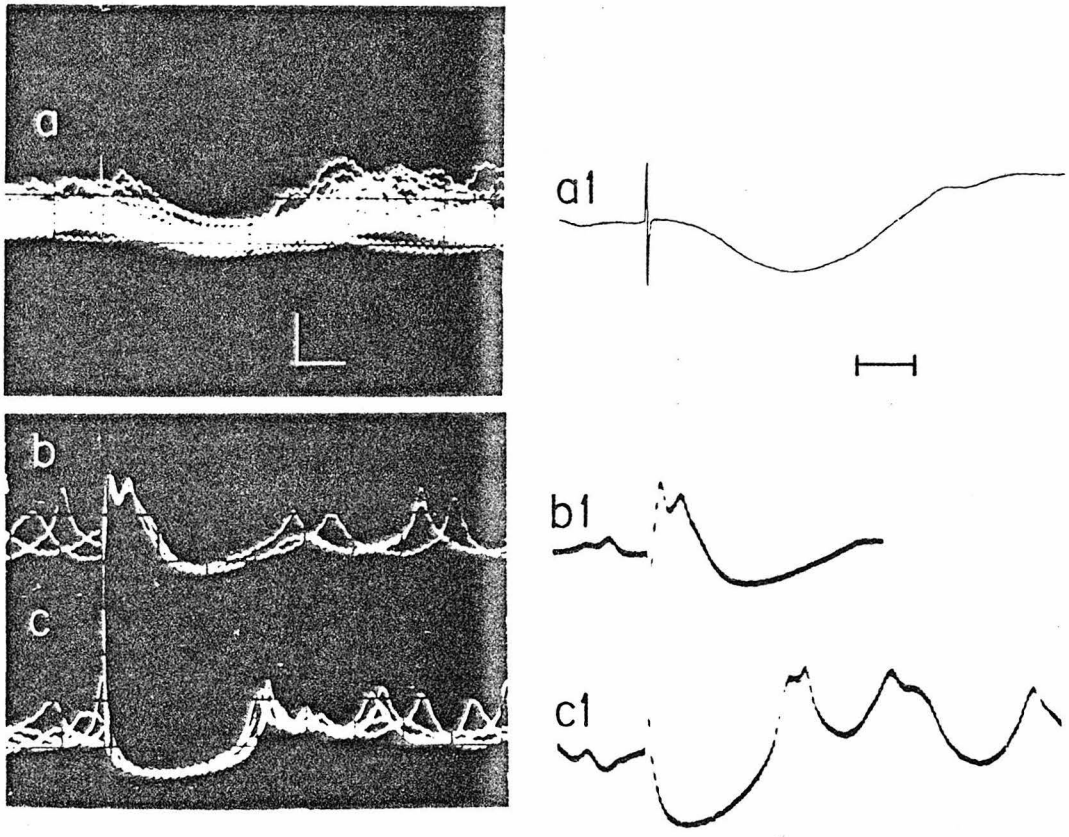

a
a1
b
b1
c
c1

Fig. 6. Velocity distributions for (a) depolarizing response to anodal stimulation in APF; (b) inhibitory response to cathodal stimulation in APF; (c) depolarizing response to anodal stimulation in HMLC. The values given are the average velocity $\pm$ the standard deviation of the mean.

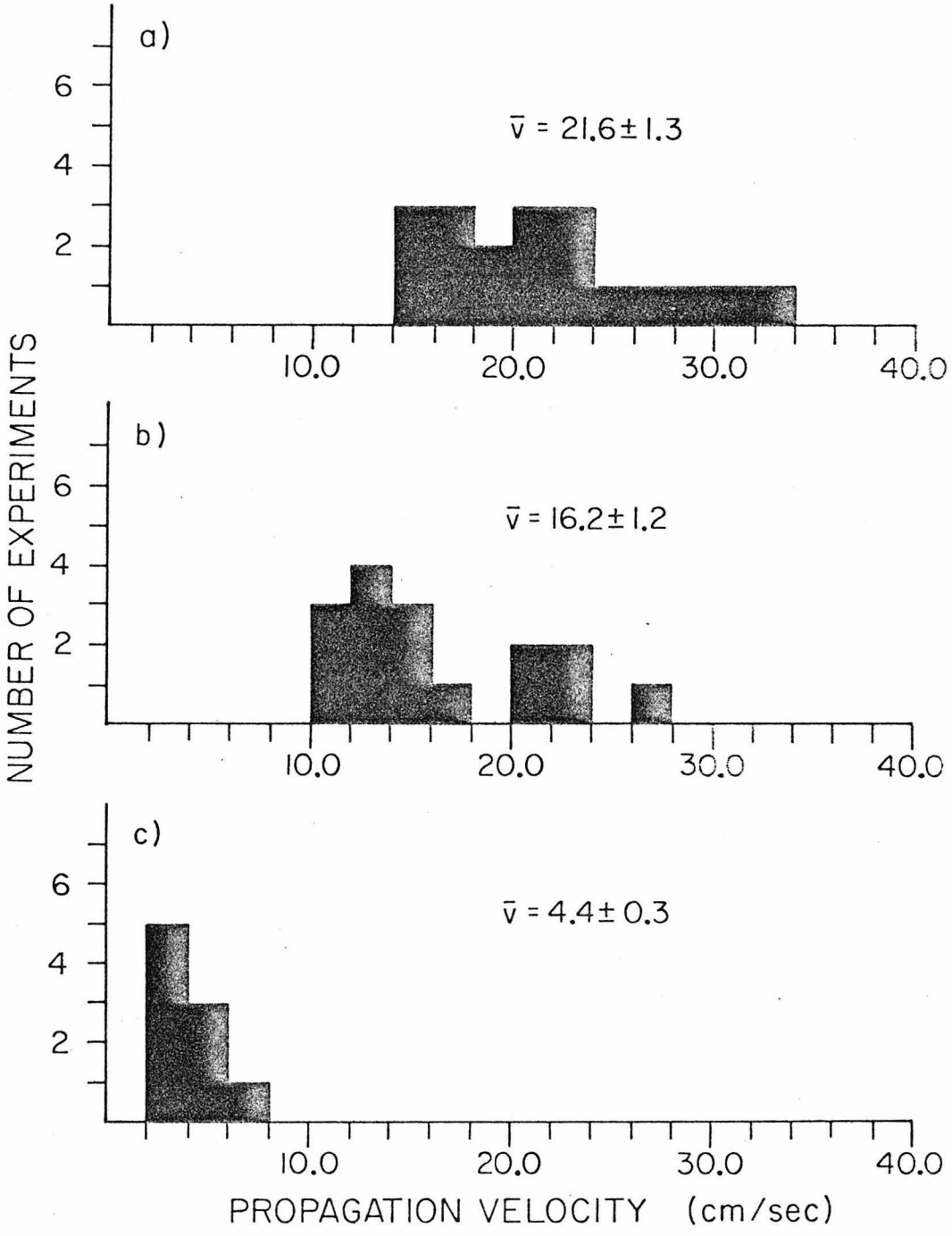

a)
$\bar{v}$ = 21.6 ± 1.3
b)
$\bar{v}$ = 16.2 ± 1.2
c)
$\bar{v}$ = 4.4 ± 0.3
NUMBER OF EXPERIMENTS
2
4
6
10.0
20.0
30.0
40.0
PROPAGATION VELOCITY (cm/sec)

Fig. 7. Propagation velocity of anodally evoked depolarizations as a function of calcium concentration. After measuring the propagation velocity in HMLC, a known volume of APF was substituted for an equal volume of bath solution, or the preparation was washed with APF. After equilibration, the velocity was remeasured. The value obtained by earlier workers in 30% artificial seawater (DeBell et al. 1963) is shown as an open circle.

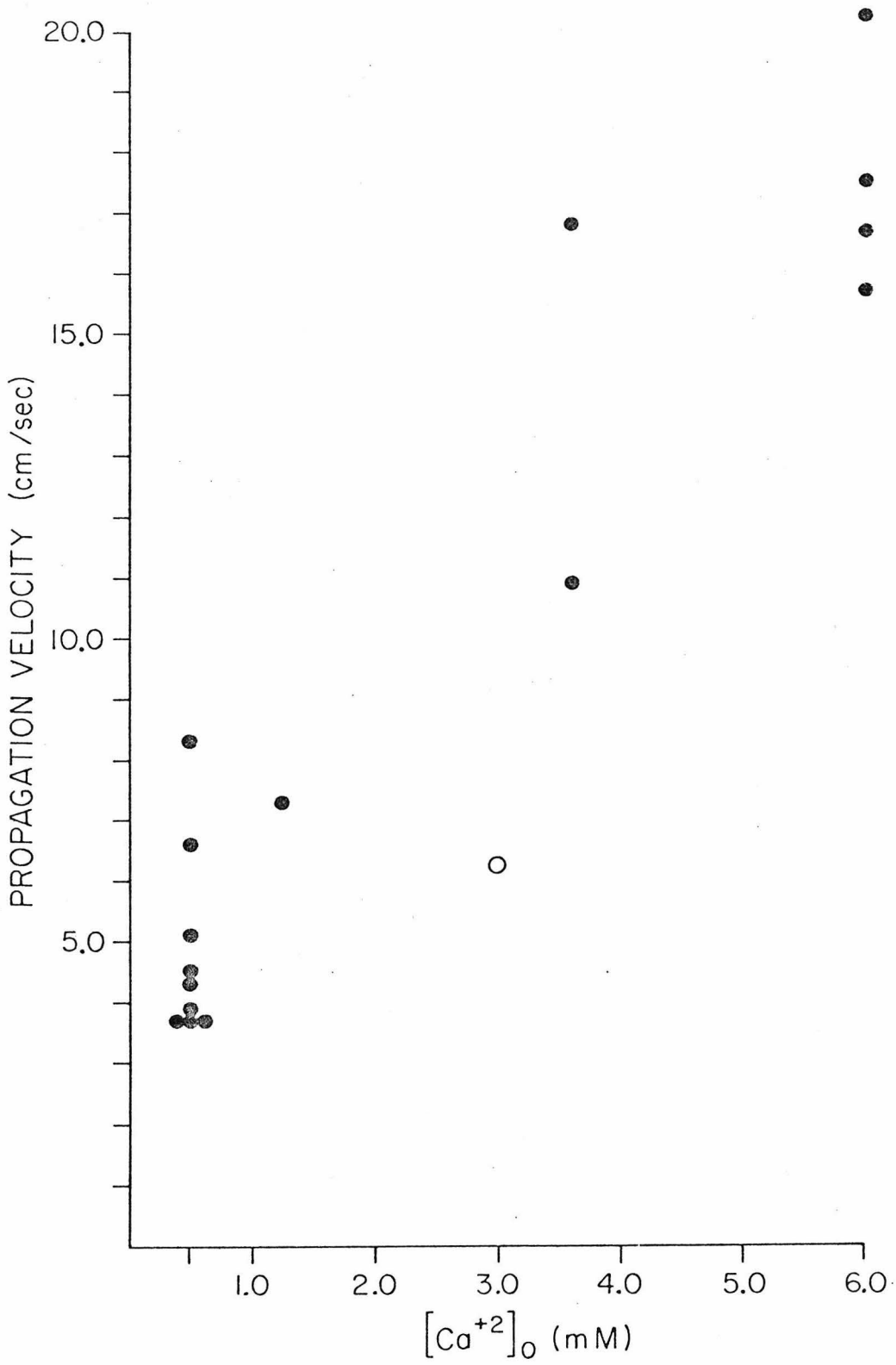

20.0
15.0
10.0
5.0
PROPAGATION VELOCITY (cm/sec)
1.0
2.0
3.0
4.0
5.0
6.0
$[Ca^{+2}]_o$ (mM)

Fig. 8. Selective effects of mechanical disruption of the syncytium. Recordings on the left were made from cells about 0.5 cm from the stimulating electrode; those on the right were made simultaneously from cells about 2.0 cm from the stimulating electrode. (a) Response to anodal stimulation in HMLC, before disruption of the syncytium. (b) Same conditions as (a), but after mechanical disruption of the syncytium at a distance of 1.3 cm from the stimulating electrode. The anodally evoked depolarization no longer propagates to the distal electrode. (c) After washing with APF, response latency to anodal stimulation is reduced in the proximal electrode; still no response in the distal electrode. (d) Evidence that the nerve cord is still intact is provided by the strong inhibitory response observed in both electrodes in response to cathodal stimulation. Calibration, .2 sec.

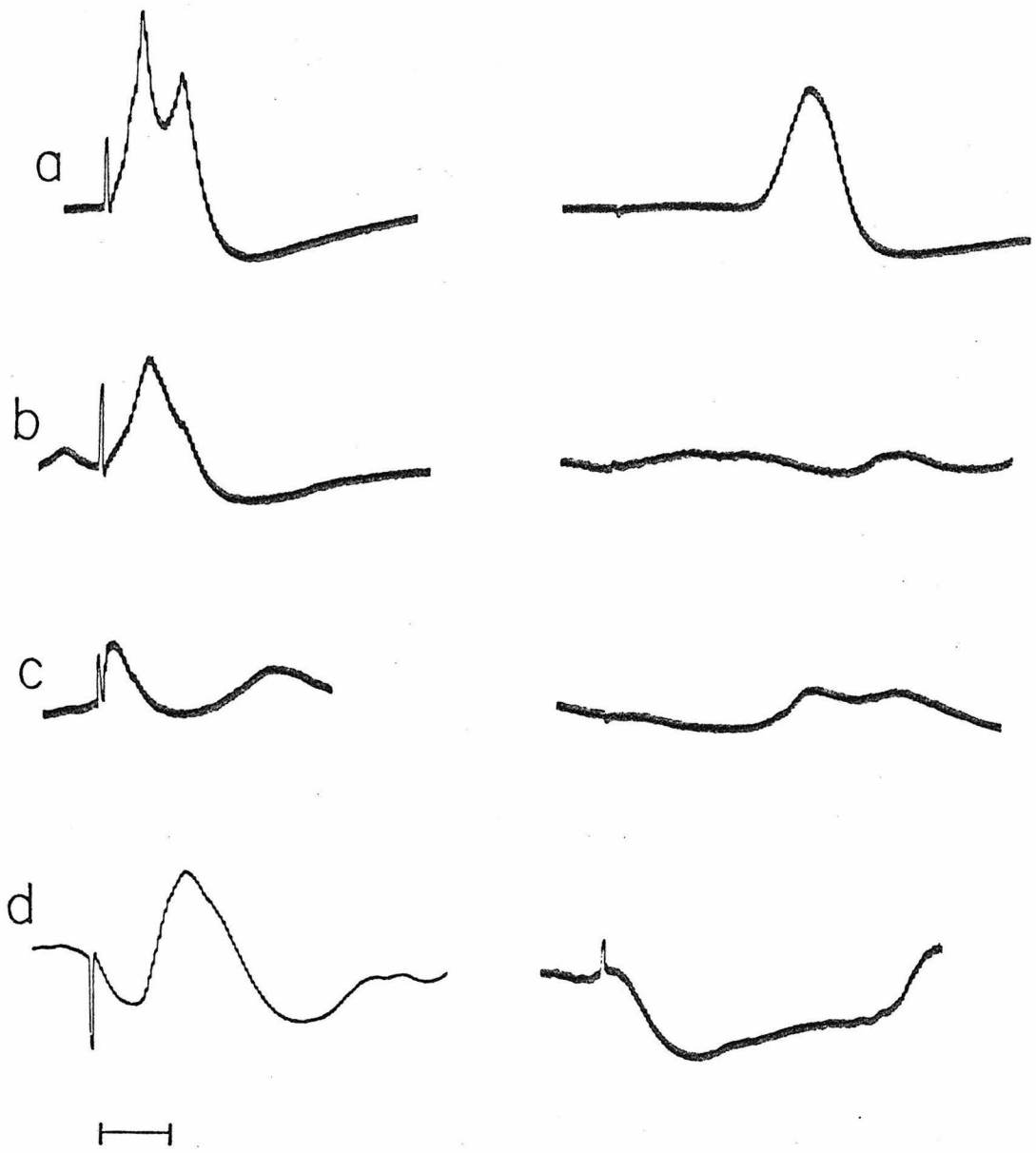

a
b
c
d

Fig. 9. Spontaneous activity in HMLC. (A) Simultaneous recordings from cells separated by 1.2 cm, both on the dorsal side. (b) Same preparation as (a), with one electrode now moved to the ventral side. (c) Simultaneous recordings from three cells in the dorsal muscle field, over a total distance of 3.8 cm. Top to bottom is anterior to posterior. Note that the slow waves propagate from posterior to anterior, except for the first and eighth, which travel in the opposite direction. Calibration, 10 mV x 10 sec in (a) and (b), 20 mV x 5 sec in (c).

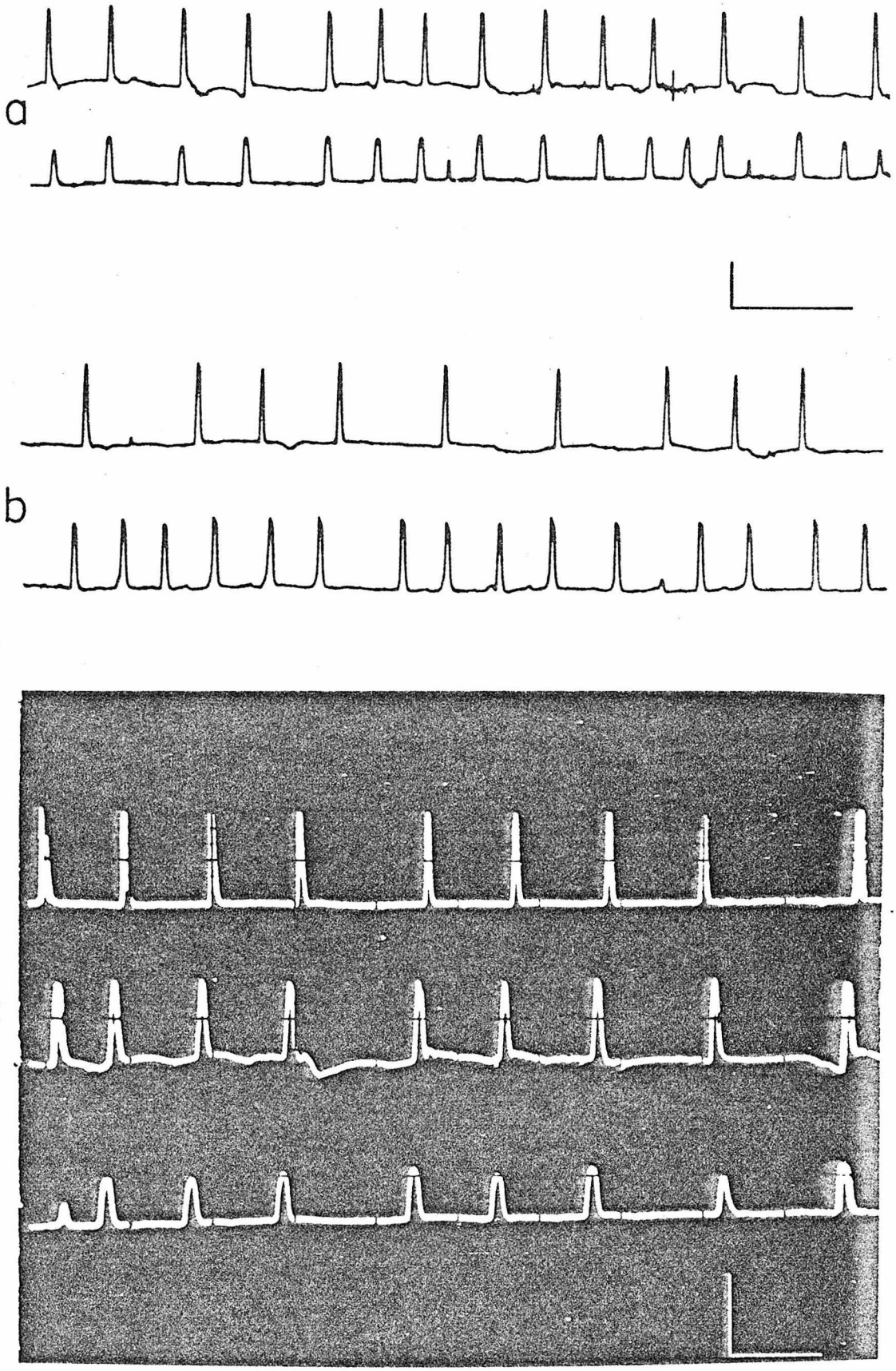

a
b
c

Fig. 10. Increasing latencies of spontaneous depolarizations with increasing separation in HMLC. CAT traces from the trigger cells are on the left, those from the follower cells are on the right. Distances between the trigger cell and follower cells are (a) 1.0 cm; (b) 1.4 cm; (c) 1.8 cm; (d) 2.3 cm. Calibration, .1 sec.

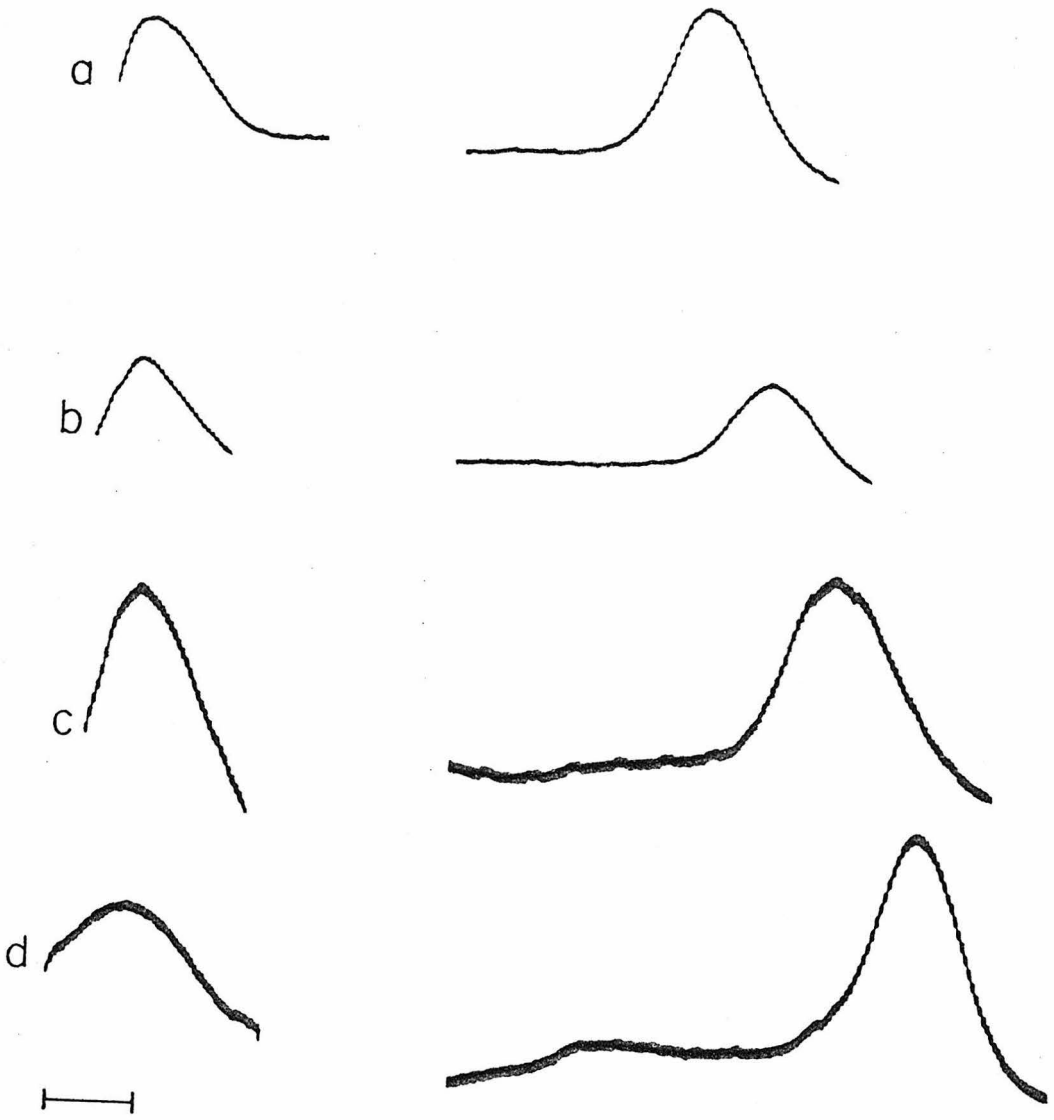

a
b
c
d

Fig. 11. Propagation velocity of spontaneous depolarizations with changes in calcium concentration. Closed circles, HMLC, .5 mM calcium, 3.5 cm/sec; open circles, 1.2 mM calcium, 6.2 cm/sec; crosses, APF, 6 mM calcium, 30.4 cm/sec.

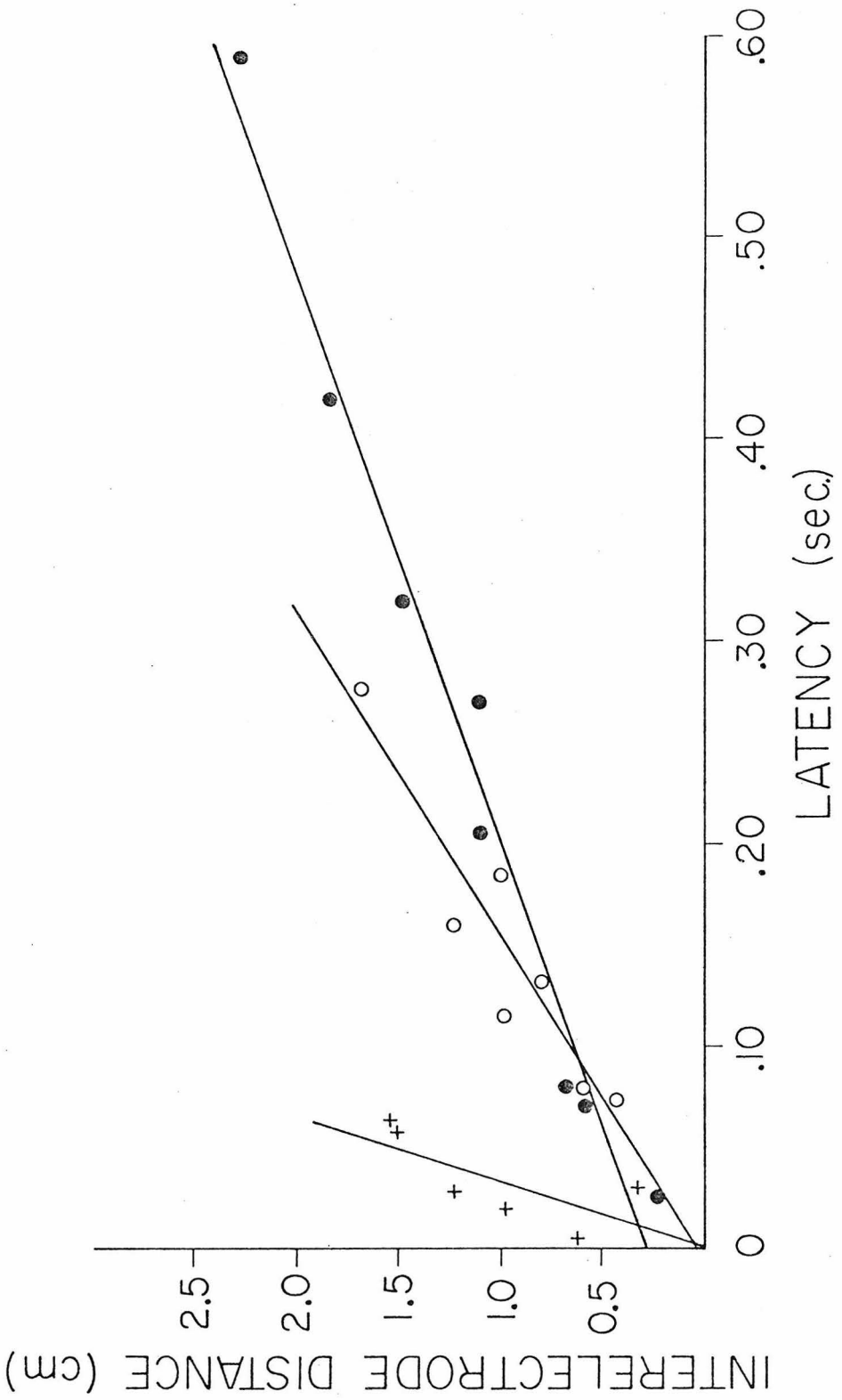

INTERELECTRODE DISTANCE (cm)
2.5
2.0
1.5
1.0
0.5
0
.10
.20
.30
.40
.50
.60
LATENCY (sec.)

Fig. 12. Effects of cutting right or left lateral line on the coordination between dorsal and ventral muscles. Top trace in each pair is from ventral muscles, bottom trace is from dorsal muscles on the opposite side of the lateral line. (a) Absence of modulated dorsal field activity when the right lateral line has been cut. (b) Coordinated dorsal and ventral activity when only the left lateral line has been cut. (b1) synchronized long lasting potential changes between dorsal and ventral fields. Note that the hyperpolarization is not sufficient to block slow wave activity in the dorsal field. (b2 and b3). Antagonistic long lasting depolarizations in dorsal and ventral muscle fields. Lines between dorsal and ventral traces in b1 and b2 indicate examples of correlated slow wave activity. Calibration, 5 mV x 5 sec in (a1); 10 mV x 25 sec in (a2); 5 mV x 12 sec in (b1) and (b3); 5 mV x 5 sec in (b2).

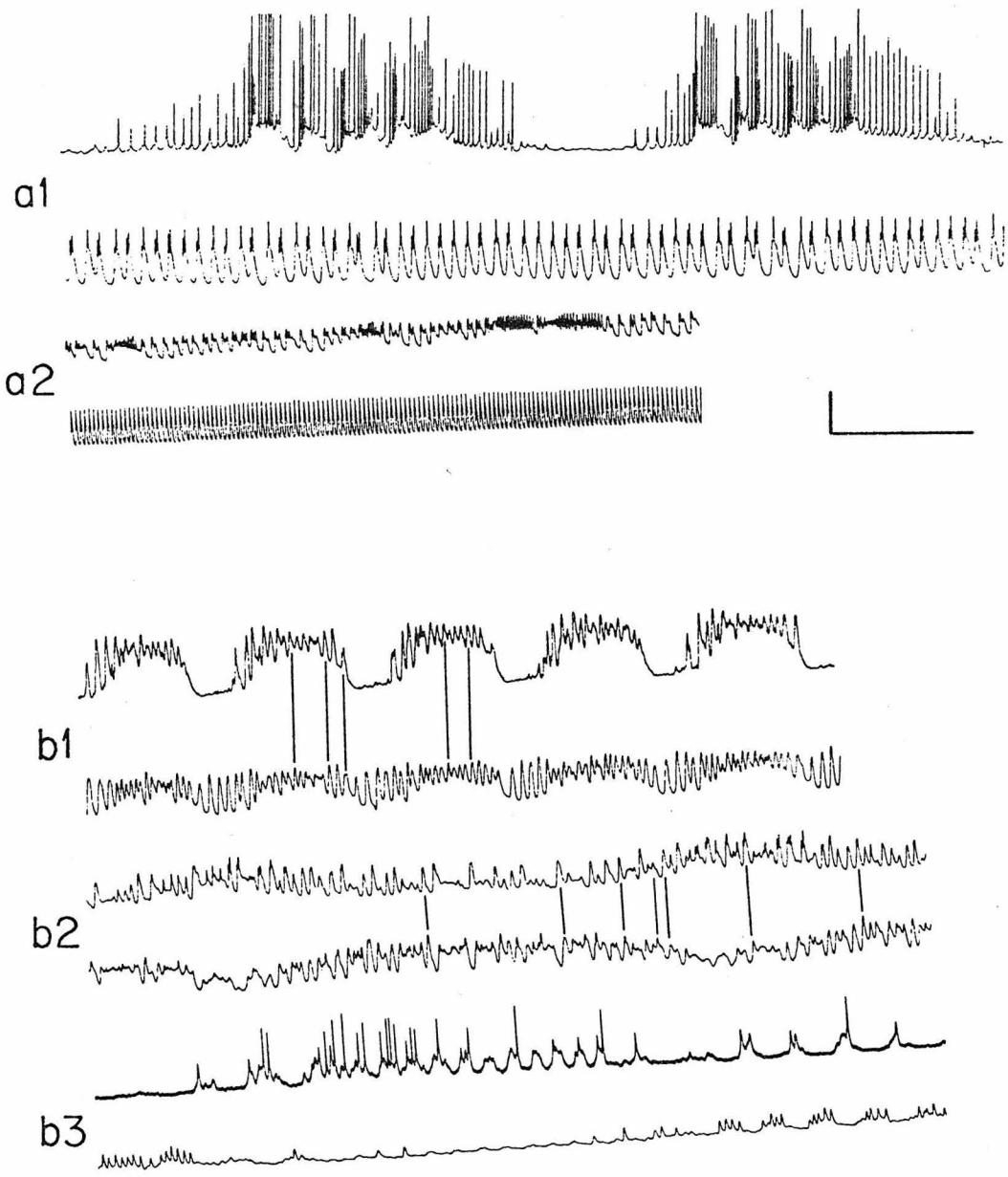
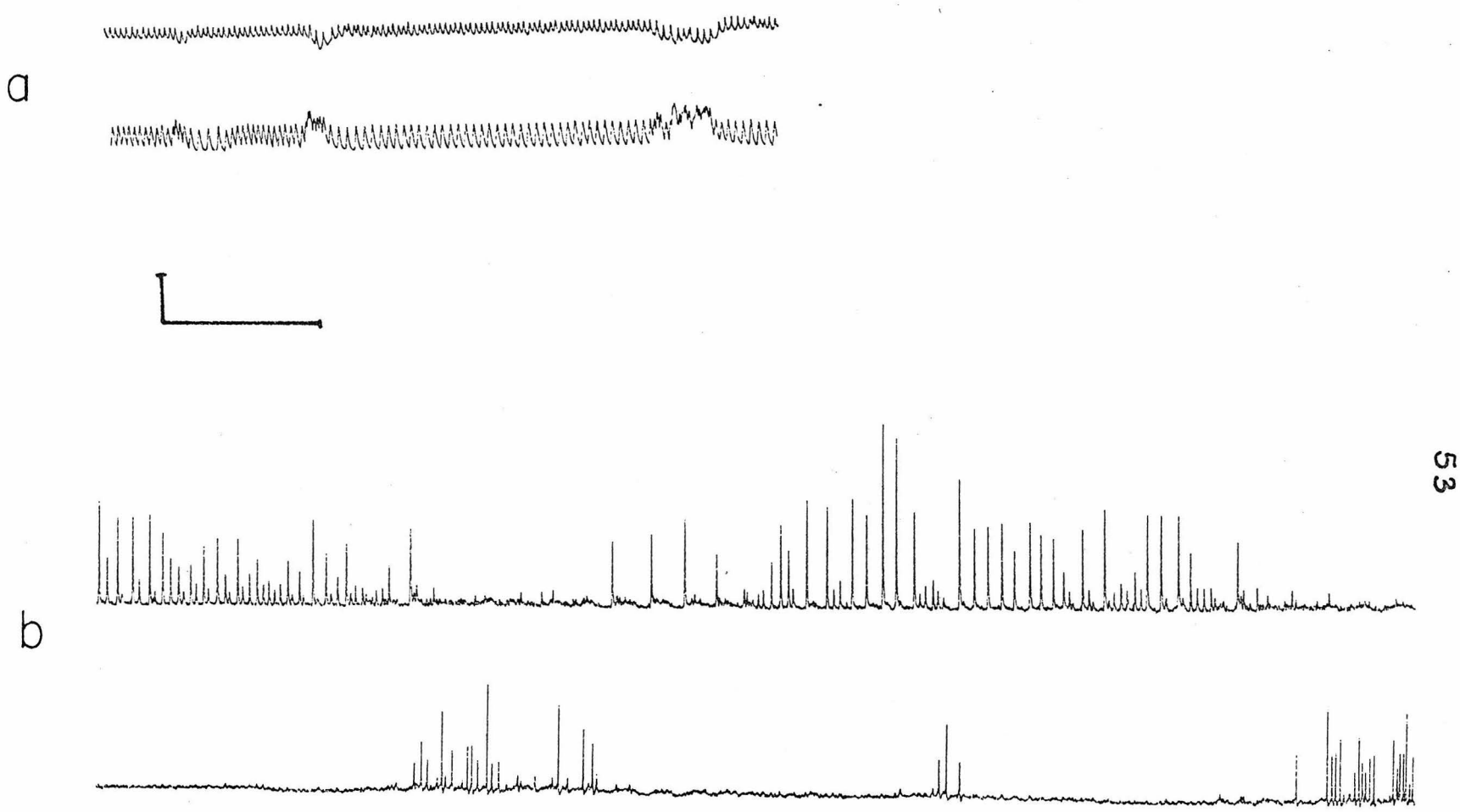

a1
a2
b1
b2
b3

Fig. 13. Anterior-posterior coordination of spontaneous activity. (a) Both electrodes in an isolated dorsal fiels, more than 2 cm apart. (b) Both electrodes in the ventral field, 1.5 cm apart. Calibration, 10 mV x 5 sec in (a); 10 mV x 12 sec in (b).


a
b

PART TWO

THE IONIC BASIS OF ELECTRICAL ACTIVITY IN THE SOMATIC MUSCLES OF *ASCARIS*

INTRODUCTION

In the first part of this thesis, evidence has been presented to show that the passage of contractile waves in Ascaris is not governed by simple conduction rates of the nerve cord or muscle syncytium. Instead, more complicated explanations must be sought. Amongst factors potentially involved in these explanations, several lines of evidence, discussed in detail at the end of this section, indicate that modulatory properties of the muscles themselves may be important.

As a first step in examining these modulatory properties, the second part of this thesis describes the ionic basis of the spontaneous depolarizations observed in Ascaris muscle, and demonstrates that the muscles are capable of relatively long term activity modulations, consistent in time course with the passage of contractile waves.

MATERIALS AND METHODS

Ascaris were obtained from Farmer John Packing House, Vernon, California. The details of the recording chamber, and the procedures for maintaining and preparing the animals have been described in the previous section. Recordings were made from 4 to 5 cm long sections of large, female *Ascaris*, taken from between the head and the vulva, and opened longitudinally along one lateral line.

Electrical recordings were made using standard techniques. Currents passed intracellularly were measured using a virtual ground. All recordings and intracellular current injections were made in muscle bellies near the nerve cord (see Del Castillo *et al.*, 1967, for anatomical details). Membrane potentials were measured by disimpalement. For brevity, the term "resting potential" is used throughout this section to refer to the transmembrane potential observed between spontaneous depolarizations. "Active potential" refers to the most positive transmembrane potential achieved during a depolarization. Values given for membrane potentials are the average of about 10 cells in each experiment; the error given is the expected standard deviation of the mean.

The compositions of the solutions used in these experiments are given in Table I. For two reasons, experiments dealing with spike potentials were carried out in media in

which chloride was the only anion. First, slow waves in such media are smaller and therefore less likely to obscure the spike potentials. Second, sodium-substitution experiments in acetate-based media are complicated by a depolarization and loss of electrical activity not encountered in all-chloride media. When sodium is replaced by cesium or protonated tris(hydroxymethyl)aminomethane (Tris) in artificial perienteric fluid (APF), cells show a transient hyperpolarization and then depolarize by 10 to 15 mV within 30 min. The depolarization is accompanied by an eventual loss of spontaneous activity and excitability, and is partially reversible if sodium is returned within 60 min. In contrast, cells bathed in Cl^-APF show a lasting hyperpolarization when sodium is replaced by cesium or protonated Tris, in agreement with previous findings (Del Castillo et al., 1964), and spontaneous activity and excitability are retained. Resting potentials observed in APF and Cl^-APF, with and without sodium, are reported in Table II. The depolarizations observed when APF is replaced by Cl^-APF are reversible, and suggest that an electrogenic pump contributes to the resting potential in Ascaris muscle. Further evidence of such a pump will be presented later in this section.

The various pharmacological agents used in these experiments were made up as concentrated stock solution in either distilled water (cobalt chloride, lanthanum nitrate, gamma-aminobutyric acid (GABA), tetraethylammonium chloride (TEA)),

or in bathing media of the appropriate composition (tetrodotoxin, ouabain, ethyleneglycol-bis-(beta-aminoethyl ether) N, N'-tetraacetic acid (EGTA)); small volumes of the stock solutions were added to the bath to obtain the desired concentrations.

RESULTS

Three types of spontaneous activity can be seen in *Ascaris* somatic muscle bathed in APF. As shown in Fig. 1, these are graded spike potentials (10-50 msec), slow waves 100-1000 msec) and long-lasting modulation (3-20 sec). The ionic mechanisms of these types of activity have been investigated as described below.

Spike Potentials

Figure 2 shows the relationship between calcium ion concentration in the bath and the active potential of spontaneous and evoked depolarizations. Because *Ascaris* muscle gives rise to graded spike potentials, and because the muscle bellies vary in effective distance from the active region (De Bell *et al.*, 1963), there is a distribution of active potentials up to the maximum in a given solution; only the most positive 2 to 5 active potentials observed in each of 26 experiments are shown. At calcium concentrations above 1 mM, the active potential increases about 30 mV per decade increase in calcium concentration, as expected for a calcium electrode. (At calcium concentrations below 1 mM, spike potentials are abolished, but slow waves persist. Concomitantly, the calcium dependence of the active potential is greatly reduced.) These results suggest that spikes are mediated by calcium ions; however, since the ability of calcium to stabilize the membrane better than magnesium could also contribute to an increase in

active potential with increasing calcium concentration (Frankenhaeuser and Hodgkin, 1957), the spikes might still be mediated by other ions, such as sodium. Direct evidence that sodium is not important in mediating spike potentials in Ascaris muscle is provided by the inability of sodium to mediate spikes in low-calcium media and by the persistence of spikes in sodium-free media. Total replacement of sodium by choline, cesium, or protonated Tris fails to block spike activity, as illustrated in the inset to Fig. 2. A quantitative study of spike active potential in sodium-free media has not been made.

Experiments with agents known to block excitable channels in other systems support the idea that spike potentials in Ascaris muscle are mediated by calcium and not sodium. Tetrodotoxin at 10^{-5} M has no effect on the electrical activity of these preparations, but 1 mM lanthanum or 5 mM cobalt abolish spontaneous and evoked depolarizations. The loss of electrical activity obtained with cobalt or lanthanum is partially reversible if the blocking ions are washed out within 20 min; activity reappears but active potentials fail to regain initial values. Both cobalt and lanthanum induce hyperpolarizations in the muscle cells that could account for the loss of spontaneous activity, but application of ouabain can bring the resting potential to normal values, without restoring spontaneous activity.

Externally applied TEA, at concentrations of up to 30

mM does not prolong spike potentials (see Fig. 4b). In addition, variations of the potassium ion concentration do not affect the duration of the spike potentials or the magnitude of the spike after-potential. These observations suggest that potassium is not important in terminating spike potentials in Ascaris somatic muscle.

Slow Waves

Given the electrical coupling of muscle cells in Ascaris, it is a priori possible that the slow waves recorded in any one muscle belly are just the sum of the spike potentials in neighboring cells, filtered through the resistance and capacitance of the functional syncytium. However, the persistence of slow waves under conditions where no spike potentials are observed (low calcium), leads to the conclusion that slow waves result from ionic mechanisms distinct from those responsible for generation of spike potentials.

In media containing calcium as the only divalent cation, reduction of calcium from 11 mM to 0.5 mM abolishes spike potentials; slow waves persist as very regular, almost sinusoidal oscillations in membrane potential. Further reduction of the calcium concentration to 0.15 mM results in a depolarization of 10 to 15 mV; slow waves increase in duration, but their amplitudes are unaffected. (Reduction of calcium below 0.15 mM, or addition of EGTA, results in a further depolarization and loss of coherent electrical activity.) The

persistence of slow waves at low calcium concentrations does not exclude a current-carrying role for calcium in slow waves, but does suggest that some other ion, such as sodium, might be able to carry the inward current. Slow waves can be recorded from muscle bellies in sodium-free media, indicating that slow waves are not absolutely dependent on sodium ions, either; so the effects of combined sodium-free, low-calcium conditions were examined, as shown in Fig. 3a. When sodium is replaced by protonated Tris in a solution containing 11 mM magnesium and 0.15 mM calcium, a transient hyperpolarization of about 5 mV is observed, after which the cells slowly depolarize. Spontaneous slow waves are lost at the onset of the hyperpolarization and often do not return even when the cells have depolarized beyond the initial resting potential. If slow waves do return, they are of much reduced amplitude and are EGTA-sensitive (see Fig. 3a), indicating that they are mediated by the residual calcium. (Effects of EGTA on preparations bathed in 11 mM magnesium, 0.15 mM calcium, at normal sodium concentrations will be discussed later.) Replacement of all but 50 mM sodium with protonated Tris causes a similar transient hyperpolarization and loss of slow waves, but when the cells have regained the initial resting potential, slow waves invariably return, at slightly reduced amplitude. (Washing back to 133 mM sodium may give some increase in slow wave amplitude; however, as noted previously, the effects of sodium

removal are not fully reversible.) These results suggest that both calcium and sodium ions can carry current during slow waves.

The possibility that slow waves might be mediated by an oscillating electrogenic pump (Connor et al., 1974) is tested by applying 1 to 2 mM ouabain to a preparation exhibiting regular slow waves. Although the cells depolarize 8 to 12 mV within 5 min, indicating an electrogenic component to the resting potential in Ascaris, slow waves persist, at decreased amplitude. The decrease in amplitude is no greater than that observed when cells are depolarized by other means, such as intracellular current injection (see Fig. 5c).

Direct evidence that slow waves are myogenic and voltage-sensitive is obtained by evoking slow waves by injection of large depolarizing currents into muscle bellies, as shown in Fig. 5c. These responses to stimulation are obtained both in APF and in high-magnesium, low-calcium media; the activity evoked in either case is similar to that arising spontaneously. Similar results have been obtained previously (Del Castillo et al., 1967). This suggests that slow waves observed in APF and in high-magnesium, low-calcium media arise from the same membrane processes.

Externally applied TEA, 1 to 30 mM, does not affect slow waves (see Fig. 4b). It is possible that delayed rectification is important in shutting off slow waves, but little is known about the termination process.

Divalent Cation Substitutions

In view of the importance of calcium ions in both spikes and slow waves, we compared the effects of calcium, magnesium, strontium, and barium ions on spontaneous activity, and the effects of TEA in the presence of each of those ions. When ions other than calcium were tested, calcium was maintained at 0.15 mM to stabilize the membrane and to swamp out the effects of calcium contamination in commercially available salts.

Calcium: The effects of low calcium concentrations in the absence of other divalent cations have been described above. At high calcium concentrations, between 10 and 20 mM, the spontaneous activity consists of isolated spike potentials (see Fig. 4a). Slow waves, if present, are of small amplitude and duration; spike potentials appear to rise from irregular, small amplitude oscillations in the membrane potential. As shown earlier, the active potential of the spikes increases with the log of the calcium concentration. At calcium concentrations above 20 mM, resting potential and threshold for stimulation increase, and all spontaneous activity is lost.

The effect of TEA on a preparation in calcium bathing medium is to induce a modulation (Fig. 4b) of the previously sporadic activity. The modulation consists of slow, rhythmic 1 to 5 mV depolarizations with superimposed slow waves and spike potentials of normal amplitude and duration. The overall

activity is strikingly similar to that which sometimes occurs spontaneously in APF (cf. Fig. 1). The same modulation of spontaneous activity can be induced in continuously active preparations in APF by the addition of TEA (1 to 30 mM). Even the highest concentrations of TEA used do not prolong spike potentials, but merely induce modulation. A possible explanation for the bursts of spontaneous activity is that TEA is affecting transmitter release from the nerves. This is probably not the case, since passage of 1 to 2 sec-long depolarizing current pulses into muscle bellies in TEA-treated preparations often induce bursts of slow waves and spikes that continue for seconds after the stimulus is turned off (see Fig. 4c). Thus, TEA-induced modulations, which appear identical to the spontaneously occurring ones, seem to be myogenic in nature.

Magnesium: When magnesium is the predominant divalent cation, the spontaneous activity consists of isolated slow waves of 400 to 1000 msec in duration and 5 to 20 mV in amplitude, as shown in Fig. 5a. The activity differs from that observed in .15 mM calcium without magnesium in that the slow waves are irregular in occurrence. (This may result from the membrane stabilizing effects of the magnesium.) Spike potentials are absent under these conditions; the amplitude of the slow waves does not vary with the magnesium concentration. As the magnesium concentration is increased, resting potential

and threshold for stimulation increase. At magnesium concentrations above 25 mM, spontaneous activity is not observed.

Addition of TEA does not affect the amplitude or duration of individual slow waves in magnesium bathing media, but often induces modulation, as shown in Fig. 5b. The underlying depolarizations are weak and sometimes occur only as a shoulder on the trailing edge of occasional individual slow waves. Similar modulation of slow waves into bursts is frequently observed during the transition from APF to the low-calcuim solution, which suggests a role for calcium in the modulation process.

Strontium: When Ascaris muscle preparations are bathed in media containing strontium as the major divalent cation, the spontaneous electrical activity is non-uniform, consisting of both large slow waves and spikes, occasionally interrupted by large depolarizations of up to 12 sec duration, as shown in Fig. 6a. When the preparation is first washed with strontium solution, the slow waves and spikes increase in amplitude, suggesting that strontium passes through the channels responsible for the generation of slow waves and spikes. However, as strontium concentration is varied between 2 and 26 mM, the active potential varies only 5 to 6 mV per decade, as shown in Fig. 8a. At strontium ion concentrations above 15 mM, the resting potential is increased, but spontaneous activity persists.

Addition of TEA to strontium bathing media increases the probability of the large, slow depolarizations, and increases their duration and amplitude, as shown in Fig. 6b. In several experiments, addition of 10 mM TEA resulted in regular, uniformly large depolarizations of up to 2 min duration, separated by repolarizations lasting 1 to 2 sec. When slow waves and spikes are present in preparations bathed in strontium media with TEA, their duration appears normal.

Barium: When barium is the predominant divalent cation, the spontaneous activity consists of large, long depolarizations at regular intervals. As shown in Fig. 7a, these depolarizations are similar to those observed in strontium solutions. For brevity, these depolarizations are referred to as "square waves". Both active potential and duration of barium square waves vary nonlinearly with the log of the barium concentration, as shown in Fig. 8a and 8b, so that, in 26 mM barium, square waves of up to 60 mV in amplitude (+20 mV active potential) and greater than 2 min in duration are observed. The nonlinear dependence of square wave active potential and duration on the log of barium concentration suggests that barium has a second effect, such as reducing the conductance of an opposing ion, in addition to its presumptive role of carrying current during square waves (Hagiwara and Naka, 1964; Hagiwara et al., 1974). At barium concentrations above 15 mM, resting potentials increase, but spontane-

ous square waves continue.

When a preparation is first washed with barium medium, spike potentials increase in amplitude and slow waves increase in amplitude and duration. Spike potentials soon fail to be visible above the slow waves, but show no increase in duration while they are present. Square waves may either appear gradually, as underlying depolarizations inducing merger of consecutive slow waves, or suddenly, with large amplitude and duration, while still clearly distinguishable. Small slow waves may be observed after long periods in barium solutions, but usually disappear within 30 min. Barium square waves persist for periods comparable to the useful lifetime of Ascaris preparations in APF (more than 2 hours), indicating that barium can be pumped out of the cells or sequestered internally so as to prevent the decline of its electrochemical potential.

In both calcium and magnesium solutions, application of TEA induces modulation of the spontaneous activity by underlying depolarizations similar in time course to the barium square waves, and does not prolong spikes or slow waves. In contrast, application of TEA to barium solutions does not induce modulation of the square waves, but instead increases resting potential, makes the active potential more positive, and increases square wave duration, as if the barium concentration had been raised (see Fig. 7b). This raises the possibility that square waves and myogenic modulations are

mediated by common mechanisms. Additional experiments were carried out to characterize the square waves.

Square Waves

The strong amplitude dependence of square waves on barium concentration suggests that barium carries most of the depolarizing currents during the square waves. This conclusion is supported by experiments in which sodium is removed from barium medium. As shown in Fig. 7c, this results in a hyperpolarization of the muscle bellies; square waves increase in active potential and decrease in duration. Hyperpolarizations induced by application of GABA (0.1 mM) to the bath can have similar effects on square waves. Thus, it appears that sodium is not essential for mediating square waves. After 15 to 30 min in sodium-free solution the cells depolarize, as is observed in almost all experiments in which sodium is removed from acetate-based media.

Although sodium ions are not necessary for square waves when barium is present in the bathing medium, sodium-mediated square waves are observed under certain conditions. As illustrated in Fig. 3b, when 1 mM EGTA is applied to a preparation bathed in 11 mM magnesium, 0.15 mM calcium, a complex transition ensues, finally giving way to regular square waves of long duration. When sodium is replaced by protonated Tris under these circumstances, the square waves are abolished, indicating that they are mediated by sodium and not by magnesium ions.

Results in other barium- and TEA-sensitive systems have been interpreted as showing that barium and TEA block potassium currents which normally counteract the inward currents (Kleinhaus and Prichard, 1975; Hagiwara and Naka, 1964; Hagiwara et al., 1964, 1974; Eckert and Lux, 1976). To test for such effects in Ascaris, the potassium content or the barium medium was varied between 2 and 47 mM. When the external potassium concentration is lowered (see Fig. 7d), the resting potential rapidly becomes more positive; the average membrane potential in 2 mM potassium for three concentrations of barium are shown in Table III. Note that, in 26 mM barium and 2 mM potassium, muscle bellies exhibit stable transmembrane potentials as high as 10 mV positive to ground. When potassium is restored to its normal level of 24 mM, the cells repolarize and resume square wave activity. Conversely, when the external potassium concentration is raised to 47 mM (see Fig. 7e), the muscle bellies hyperpolarize and square waves become shorter in duration. Some small depolarizations are observed that are of the same time course as the square waves. These may result from the passive spread of square waves that have undergone propagation failure in nearby cells.

In APF and high-magnesium, low-calcium media, resting potentials and spontaneous activity are not consistently affected by variations in potassium concentration between 2 and 47 mM. No large depolarizations are seen in these solutions

in 2 mM potassium, but cells in several preparations hyperpolarized slightly when washed with 47 mM potassium solutions. These observations, and those made when potassium is varied in barium media, support the findings of earlier workers (Del Castillo et al., 1964; Brading and Caldwell, 1971) that the electrochemical potential for potassium does not determine the resting potential directly in Ascaris muscle, and suggest that potassium is affecting an electrogenic pump Kerkut and York, 1971). The hypothesis that an electrogenic pump contributes to the resting potential but is not itself the source of the square waves is supported by the effects of ouabain on square wave activity (see Fig. 7f); 1 mM ouabain induces marked depolarizations within minutes after its addition to the bath, but small depolarizations of about the same time course as the square waves persist.

The effects of the residual calcium in the barium solutions were observed by varying the calcium concentration in 11 mM barium media from 0.05 to 0.55 mM. The results, shown in Table IV, indicate that increasing the calcium concentration decreases the duration of the square waves, as if the barium level were being decreased. Square wave amplitudes may decrease slightly at the higher calcium concentrations, but the effect is not pronounced. A similar antagonism between barium and calcium has been observed in Paramecium (Naitoh and Eckert, 1968).

The slow time course and high degree of uniformity of

barium square waves permits the measurement of membrane resistance at various stages of the square waves. Figure 9a shows a typical barium square wave with superimposed hyperpolarizations produced by 400 msec, constant current pulses from a second electrode within the same muscle belly. It can be seen by the decreased response to the current pulses that there is a decrease in the input resistance of the cell during the depolarization; this decrease is more apparent in Fig. 10a where the transmembrane potential during a square wave and the corresponding membrane resistance are shown as separate curves. The decrease in membrane resistance agrees with that predicted from the current-voltage relationship obtained from cells in APF. As shown in Fig. 11, the membrane resistance drops sharply at depolarizations greater than 20 mV above resting potential.

An unexpected result is obtained when the input resistance is measured using depolarizing current pulses during square waves (see Fig. 9b). The membrane resistance drops as expected at the beginning of the depolarization, but then rises almost to the resting level, as shown in Fig. 10b. Similar results are obtained with sodium and strontium square waves (see Fig. 9c and 9d).

By varying the amplitude and polarity of the current pulses during successive square waves, current-voltage relationships can be constructed for various parts of the square

wave, as shown in Fig. 10c. Consistent results are obtained, indicating an initial drop in the membrane resistance to both depolarizing and hyperpolarizing pulses; the decreased resistance to hyperpolarizing pulses is maintained throughout the square wave, but the resistance to depolarizing pulses returns to normal values while the cell is still depolarized.

Figure 10d illustrates the presence of an active component in the response to large hyperpolarizing current pulses during square waves. During the early part of the square wave, small spikes followed by a slow rise in membrane potential occur after the rapid depolarization at the end of the current pulse. Similar spikes can be seen at the front edge of depolarizing pulses (see Fig. 9). As can be seen in Fig. 9c, the transients coincide with the temporary decrease in membrane resistance to depolarizing pulses during the early part of the square wave; their amplitudes are roughly equivalent to the difference between the height of the underlying voltage pulse and the height of the transient-free pulses observed at the end of the square wave. Apparently, the transients result from delayed rectification, and the increase in membrane resistance to depolarizing pulses during the square wave results from a time- and voltage-dependent inactivation of the delayed rectification.

CONCLUSIONS

In the somatic muscle of Ascaris lumbricoides, spontaneous electrical activity consists of spike potentials, slow waves, and modulations, which can be distinguished from one another by their characteristic time courses and amplitudes. The ionic mechanisms underlying these various types of activity have been studied; although the experiments are complicated by the recording geometry, by the inseparability of the nerve cord from the functional muscle syncytium, and by the interdependence of the various types of activity, the results allow definite conclusions to be drawn concerning the ionic mechanisms of spike potentials and slow waves. In addition, observations on the square waves obtained in media of altered divalent ion composition suggest possible mechanisms for the modulations.

1. The spike potentials in Ascaris muscle are mediated by calcium ions and not to any significant extent by sodium ions. The active potential of the spikes varies linearly with the log of the calcium ion concentration above 1 mM, and although the slope of the line cannot be determined exactly (due to the recording geometry and the graded nature of the spike potentials) it is consistent with that of a calcium electrode. Replacement of sodium ions with choline, cesium, or protonated Tris ions does not block spikes. Tetrodotoxin does not affect electrical activity in Ascaris muscle, but low

concentrations of lanthanum or cobalt ions block spontaneous and evoked depolarizations, as expected for calcium-mediated activity. Since externally applied TEA and variation in the external potassium ion concentration do not measurably affect spike potentials, spike termination may not be mediated by potassium currents.

2. Slow waves in Ascaris vary in duration from 80 msec to 2 sec, depending on the ionic composition of the bath. Although the possibility exists that slow waves in different media arise from different mechanisms, three observations suggest otherwise. First, the propagation velocity of slow waves changes gradually with calcium concentration rather than abruptly as might be the case if new ionic mechanisms were becoming operative (Weisblat and Russell, 1976). Second, the similarity between spontaneous and evoked slow waves under all conditions indicates a common voltage dependence and myogenic nature for slow waves in the various media used. Third, at least part of the observed variation in slow wave duration can probably be ascribed to the network properties of the electrically coupled muscle cells, since a fairly wide variation in slow wave durations is observed in single solutions. Changes in network properties might also result from changes in the ionic composition of the bathing media; such effects are an uncontrolled variable in these experiments.

The ionic dependence of the slow waves is more compli-

cated than that of the spikes. Slow waves are observed in sodium-free media, and at calcium concentrations as low as 0.05 mM, but are abolished in combined sodium-free, calcium-free conditions, indicating that both calcium and sodium can carry inward current during slow waves. The increase in slow wave amplitude following addition of barium or strontium ions suggests that these ions may substitute for calcium during slow waves. Depolarizations mediated by both sodium and calcium have been seen in other organisms (Geduldig and Junge, 1968; Meves, 1968; Krishtal and Magura, 1970; Iwasaki and Satow, 1971). Whether calcium and sodium flow through the same or separate channels in Ascaris cannot be determined from these experiments; however, if separate channels exist, both are blocked by cobalt and lanthanum. Slow waves persist in ouabain, suggesting that they do not result from voltage-sensitive fluctuations of an electrogenic pump. Externally applied TEA does not affect amplitude or duration of individual slow waves. The mechanism(s) of slow wave termination remains unknown.

3. Reliable conditions have not been found for the selective study of modulation, which is of particular interest because it corresponds to the contractile waves seen in semi-intact animals. Thus, although observations with TEA-induced modulation suggest that it may be voltage-sensitive and myogenic (see below), the ionic mechanisms of modulation are not known. However, the square waves seen in strontium and

barium solutions share some properties with normal modulations, suggesting the possibility of common mechanisms for these phenomena. In strontium solutions, TEA induces square waves, just as it induces modulations in APF; these square waves are comparable in duration to modulations. In barium solutions, square waves persist even at the lowest practical barium concentrations precluding a test of their TEA-inducibility, but the large, regular barium square waves otherwise resemble the strontium square waves in that their amplitude and duration are enhanced by TEA. These results suggest that both barium and strontium square waves may be mediated by the channels responsible for modulation.

Square waves seem to be mediated by divalent alkali cations (other than magnesium) and by sodium ions, as indicated by their persistence in sodium-free barium solutions and by their abolition in sodium-free magnesium solutions. In addition, several other factors affect square wave amplitude and duration. At low barium concentrations, or with strontium, the active potential of square waves varies only 5 to 10 mV per decade change of concentration, suggesting that conductance to some opposing ion is also important in determining the active potential. A large increase in active potential occurs at higher barium concentrations, or after TEA addition, suggesting that this opposing conductance is barium- and TEA-sensitive. In other systems where similar barium and

TEA effects are observed, they have been shown to result from blockage of a potassium conductance; however, attempts to demonstrate such a potassium conductance in Ascaris directly (by varying external potassium concentrations) are complicated by overriding effects on an apparent electrogenic pump. (Ouabain depolarizes the cells, but square wave-like activity persists, indicating that variations in the pump are unlikely to be involved in square wave generation.)

Resistance measurements during the early part of square waves show a decrease in membrane resistance to depolarizing current pulses, as expected from the delayed rectification observed in Ascaris muscle in APF. This resistance decrease is transient, indicating that the delayed rectification inactivates. It is likely that this inactivation is a function of time and transmembrane potential, and does not represent the blockage of potassium channels by barium or TEA, since the same transient decrease is observed in sodium square waves, in the absence of barium and TEA, and since TEA does not affect the delayed rectification measured in APF (unpublished results from this laboratory).

The rapid rise and fall of square waves suggests the presence of voltage-sensitive "calcium" channels (which can also pass strontium, barium and sodium) but it remains to be explained how the turning on and off of such channels might be triggered at the beginning and end of square waves. Square wave duration decreases with increasing calcium (at constant

barium concentrations), and increases with increasing barium (at constant calcium concentrations), indicating that calcium and barium compete to determine square wave duration. In magnesium solutions, however, no such competition appears, and square waves are obtained only if calcium concentrations are drastically reduced by addition of a molar excess of EGTA. Since magnesium probably does not permeate the membrane, this leads to the conclusion that the site of the barium-calcium competition is intracellular, and concomitantly, to the notion that the accumulation of internal calcium somehow terminates square waves (cf. Eckert and Lux, 1976).

Calcium accumulation might turn on a potassium conductance (Meech, 1972), bringing the membrane potential to the point at which the calcium channels voltage-inactivate, or it might decrease the calcium conductance directly. Three observations argue in favor of the latter hypothesis. First, variations in external potassium have effects opposite to those predicted by the Meech effect. Second, during strontium square waves, no gradual drop in membrane potential precedes the rapid repolarization. Third, no resistance decrease is observed toward the end of the square wave, as would be expected if potassium conductance were increasing.

If internal calcium does inactivate calcium conductance directly, the calcium channels must be sensitive both to voltage and to internal calcium. Furthermore, in order for the calcium channels to be reactivated for subsequent square waves,

it would be necessary to reduce internal calcium levels, presumably by pumping. It is tempting to think that the interval between square waves might be the time required for this pumping. While this view of square waves is tentative, it does serve to indicate how relatively simple ionic mechanisms might generate endogenous electrical activity of long duration in Ascaris muscle.

4. Throughout this section, spike potentials, slow waves, and modulation have been treated as arising from distinct channels, and the argument has been made that square waves are probably mediated by the modulation channels. For the spike potentials, this separation is justified by the observation that spikes, in contrast to slow waves and square waves, cannot be mediated by sodium ions. However, the distinction between slow waves and square waves is less clear, and although it is simplest to interpret the overall results as indicating separate channels, no evidence unequivocally distinguishes these two phenomena.

Phenomena similar to the barium- and TEA-sensitive square waves have been reported in other systems. In barnacle muscle, a calcium spike can be obtained which is prolonged by barium or TEA; the prolongation is shown to result from potassium channel blockage (Hagiwara and Naka, 1964; Hagiwara et al., 1964, 1974). In the leech Retzius cell, TEA or barium cause the appearance of a slowly activating calcium conductance (Kleinhaus and Prichard, 1975). In snail,

pacemaker neurons are known which show prolonged slow waves in barium solutions; these are believed to result from barium flowing in through calcium channels and blocking a late potassium current (Eckert and Lux, 1976). The results for Ascaris differ from these cases in that, although the barium and TEA effects are phenomenologically similar, involvement of a potassium current has not been demonstrated. In addition, the insensitivity of Ascaris spike potentials and slow waves to TEA distinguishes these calcium-mediated phenomena from those described in the other systems.

5. Application of TEA to preparations in APF or calcium media induces modulations of spontaneous activity very similar to that frequently seen to occur spontaneously in APF. In occasional TEA-treated preparations where the modulating depolarizations occur infrequently, a relatively brief depolarizing current pulse can trigger a long burst of spontaneous activity, indicating that modulation may be myogenic in nature.

Several lines of evidence suggest that myogenic modulation probably plays a central role in coordinating contractions in nematodes. First, the simplicity of the patterns of neuromuscular innervation in Caenorhabditis elegans appears to rule out the possibility that the state of activity of each different region of muscles is solely controlled by nervous signals in generating contractile waves (White et al., 1976). Second, the discrepancy, in Ascaris lumbricoides, between the

velocity of contractile waves in the intact animal and the propagation velocity inferred for the nerve cord argues against the hypothesis that contractile waves result from slowly propagating impulses in nerves having uniform synapses along the length of the animal (Weisblat and Russell, 1976). Finally, the variation of wavelength and wave frequency shown by nematodes in media of varying viscosity (Gray and Lissman, 1964) strongly suggest that proprioceptive mechanisms must exist for regulating nematode motion.

Additional evidence indicates that myogenic modulation, while important, must interact with other controlling factors in Ascaris. In particular, neural involvement in the control of physiologically significant modulation of spontaneous activity is indicated by comparing activity in dorsal and ventral muscle fields, as shown in the first part of this thesis. In addition, the level of spontaneous activity is also affected by the degree of stretch in the preparation (work in progress in this laboratory). The relationships among these controlling factors remains to be investigated, but it appears likely that neural control and proprioceptive feedback may influence the generation of contractile waves by regulating the expression of myogenic modulation endogenous to Ascaris somatic muscle.

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Table I

Compositions of Solutions

Solutions	Ion Concentrations (mM) Ca^{+2}	Other M^{+2}	K^+	Na^+	Tris	OAc^-	Cl^-	Dextrose	HCO_3^-
APF[1,2]	6.0	5.0 Mg^{+2}	24.0	133.0	10.0	110.0	78	11.1	-
M^{+2}APF[2]	0.15[3]	11.0	24.0	133.0	10.0	110.0	78	11.1	-
(.05-.50 mM) Ca^{+2}APF	X	-	24.0	148.6	10.0	110.0	78	11.1	-
(2-26 mM) M^{+2}APF[2]	0.15	X	24.0	$149.6-\frac{3X}{2}$	10.0	110.0	78	11.1	-
(2-47 mM) K^+, M^{+2}APF	0.15	11.0	X	157.0-X	10.0	110.0	78	11.1	-
Cl^-APF[2]	6.0	5.0 Mg^{+2}	24.0	133.0	10.0	-	188	11.1	-
(.05-15 mM) Ca^{+2}, Cl^-APF	X	15.0-X Mg^{+2}	24.0	127.0	10.0	-	190	11.1	-
30% sea water[4]	3.0	15.7 Mg^{+2}	3.0	135.0	-	-	175.4	-	0.8

The pH of all solutions is about 7.5 at 37°.

[1]APF=Artifical perienteric fluid.

[2]Sodium-free or reduced-sodium solutions have sodium replaced with equal concentrations of choline or cesium, or with tris(hydroxymethyl)aminomethane (Tris) at a concentration equal to 1.16 times that of the sodium replaced. Thus, the chloride and acetate concentrations are held constant.

[3]In the experiments reported in Table IV, calcium was varied between .05 and .55 mM.

[4]The composition of the bathing media used in most earlier work is shown for reference (Del Castillo et al., 1964).

Table II

Resting Potentials in Media of Various Anion and Cation Compositions

Solution	Resting Potential (mV)	Transition	Change in Resting Potential (mV)
APF	-33.8 ± 0.8 (11 expts)	APF to Cl^-APF	+7.6 ± 2.4 (7 expts)
Cl^-APF	-26.9 ± 1.6 (9 expts)	APF to 0 Na^+APF	+11.0 ± 1.8 (4 expts)
0 Na^+APF	-23.2 ± 1.1 (4 expts)	Cl^-APF to 0 Na^+, Cl^-APF	-11.6 ± 3.9 (4 expts)
0 Na^+, Cl^-APF	-37.1 ± 0.6 (4expts)		

In the sodium-free solutions, sodium has been replaced by protonated Tris.

Table III

Final Membrane Potential in 2 mM Potassium

Barium concentration (mM)	2	11	26
Membrane potential (mV)	-15.8 ± 0.6 (2 expts)	-6.1 ± 0.4 (3 expts)	+4.8 ± 0.7 (2 expts)

Table IV

Duration of Square Waves in 11 mM Barium as a Function of Residual Calcium

Calcium concentration (mM)	0.55	0.15	0.05
Square wave duration:			
mean (sec)	15.2 ± 1.0	21.4 ± 0.5	42.3 ± 1.8
range (sec)	9.0 to 21.6	18.0 to 22.8	34.8 to 63.0
	(2 expts)	(1 expt)	(2 expt)

Fig. 1. Spontaneous electrical activity in artificial perienteric fluid (APF). (a) Modulated spontaneous activity. (b) Expanded time scale recording from the same cell to show details of slow waves and superimposed spikes. (c) Slow waves and spikes from a different preparation. Calibration, 10 mV x 12 sec in (a), 10 mV x 1 sec in (b) and (c). This figure is identical to the third figure in the first part of this thesis.

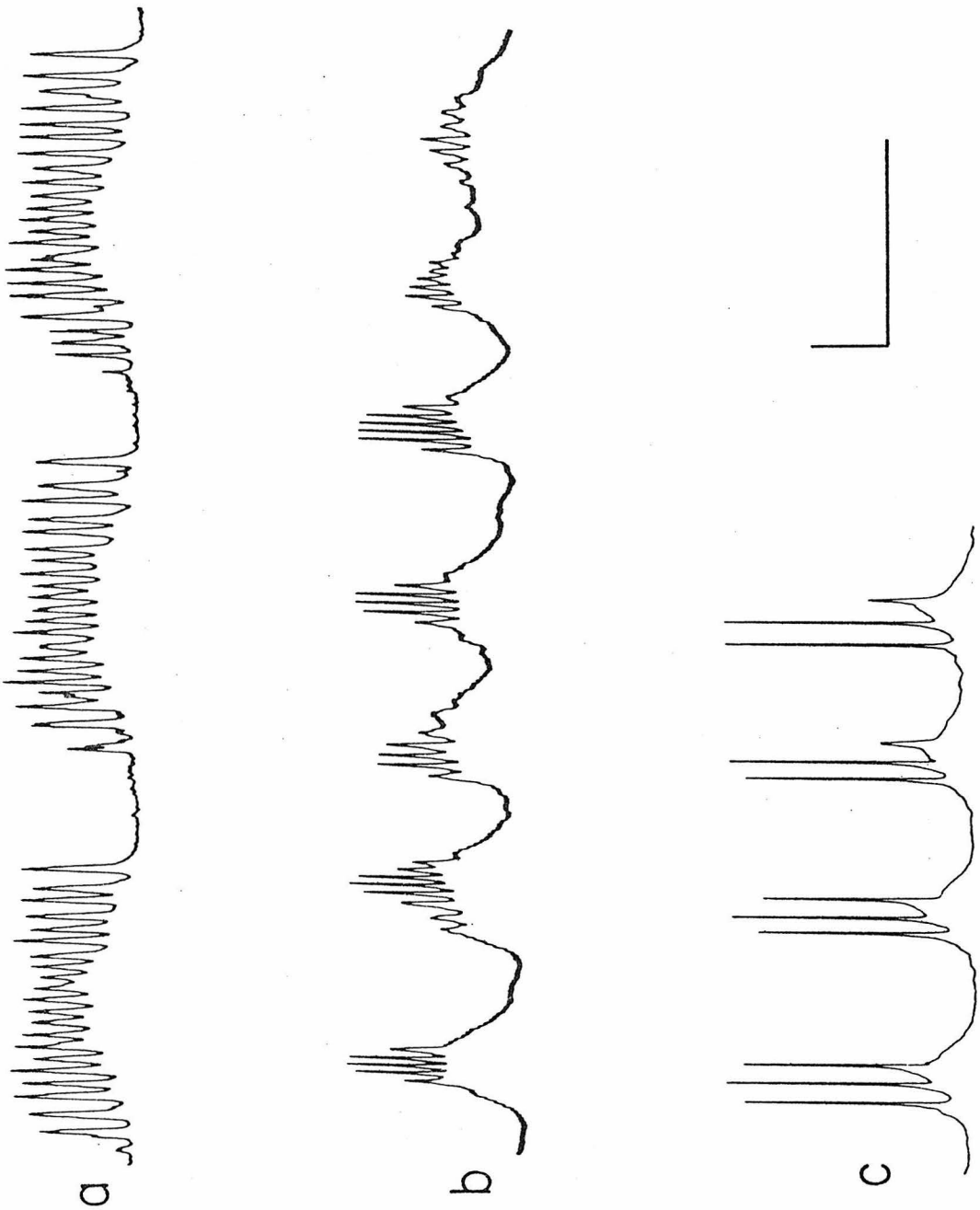

a
b
c

Fig. 2. Calcium dependence of active potential. The active potentials for the 2 to 5 largest depolarizations observed in each of 26 experiments are plotted (filled circles) against the log of the calcium concentration. (.05-15 mM) Ca^{+2}, Cl^{-} APF solutions were used. Calcium concentrations for solutions A through E are 15, 4.8, 1.5, 0.5, and 0.05 mM. Some points have been displaced horizontally for clarity. Data have been included for spikes in APF (solution X, open circles) and for spikes measured in 30% seawater (solution Y, arrow) (De Bell et al., 1963). The straight line drawn through the data has the slope of 31 mV per decade change in concentration (the expected value for a calcium electrode at 38°C). The inset shows spikes evoked in APF (a) and sodium-free medium (b). The upper trace in each case is zero potential. Calibration, 10 mV x 20 msec.

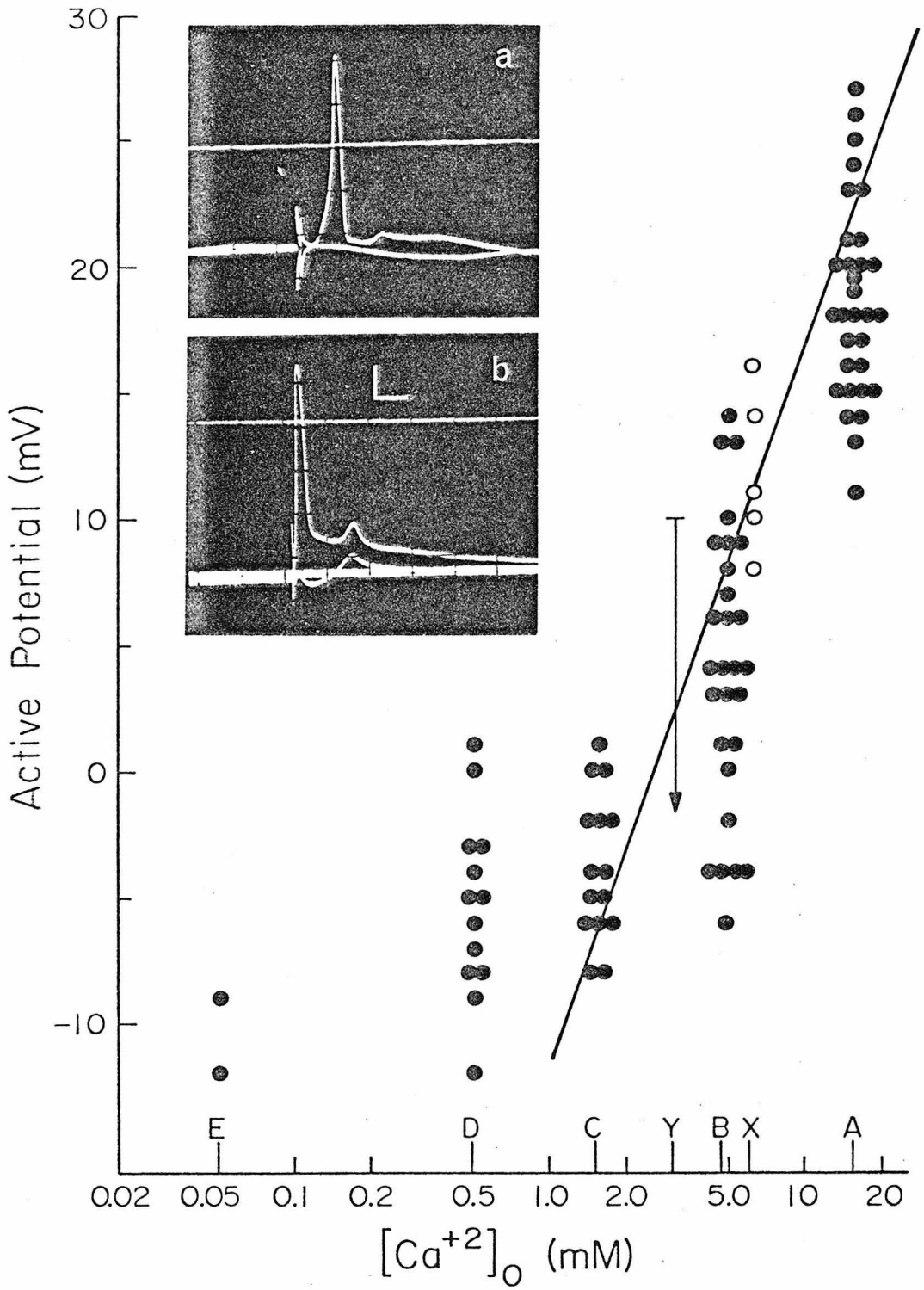

30
20
10
0
-10
Active Potential (mV)
a
b
L
E
D
C
Y
B
X
A
0.02
0.05
0.1
0.2
0.5
1.0
2.0
5.0
10
20
[Ca+2]o (mM)

Fig. 3. Dependence of electrical activity on sodium and calcium media. (a) Variation in activity when first sodium and then calcium is removed. (b) Variation in activity when first calcium and then sodium is removed. Both records begin with cells bathed in 11 mM magnesium, 0.15 mM calcium. Solid bars indicate transitions as follows: (1) Washing the preparation with medium in which the sodium has been replaced by protanated Tris. (2) Addition of a molar excess of EGTA. Arrow indicates change of chart speed. Calibration, 10 mV x 1 min in (a) and before arrow in (b), 10 mV x 5 min after arrow in (b).

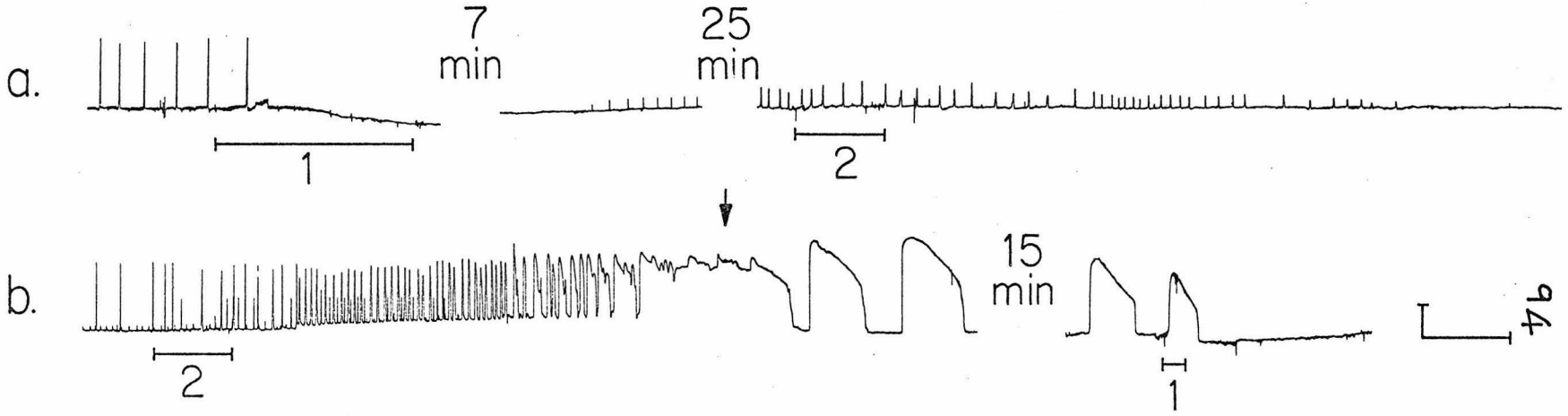

a.
7
min
25
min
1
2
b.
15
min
2
1
94

Fig. 4. Electrical activity in calcium media. (a) Sporadic spike potentials in 11 mM calcium. (b) Modulations induced by 10 mM TEA in 11 mM calcium. (c) Response of cell in APF with 1 mM TEA to 2 sec pulse of depolarizing current (indicated by bar) injected into a neighboring cell. Calibration, 10 mV x 5 sec in first part of (a) and in (c), 10 mV x 12 sec in first part of (b), 10 mV x 1 sec in second parts of (a) and (b).

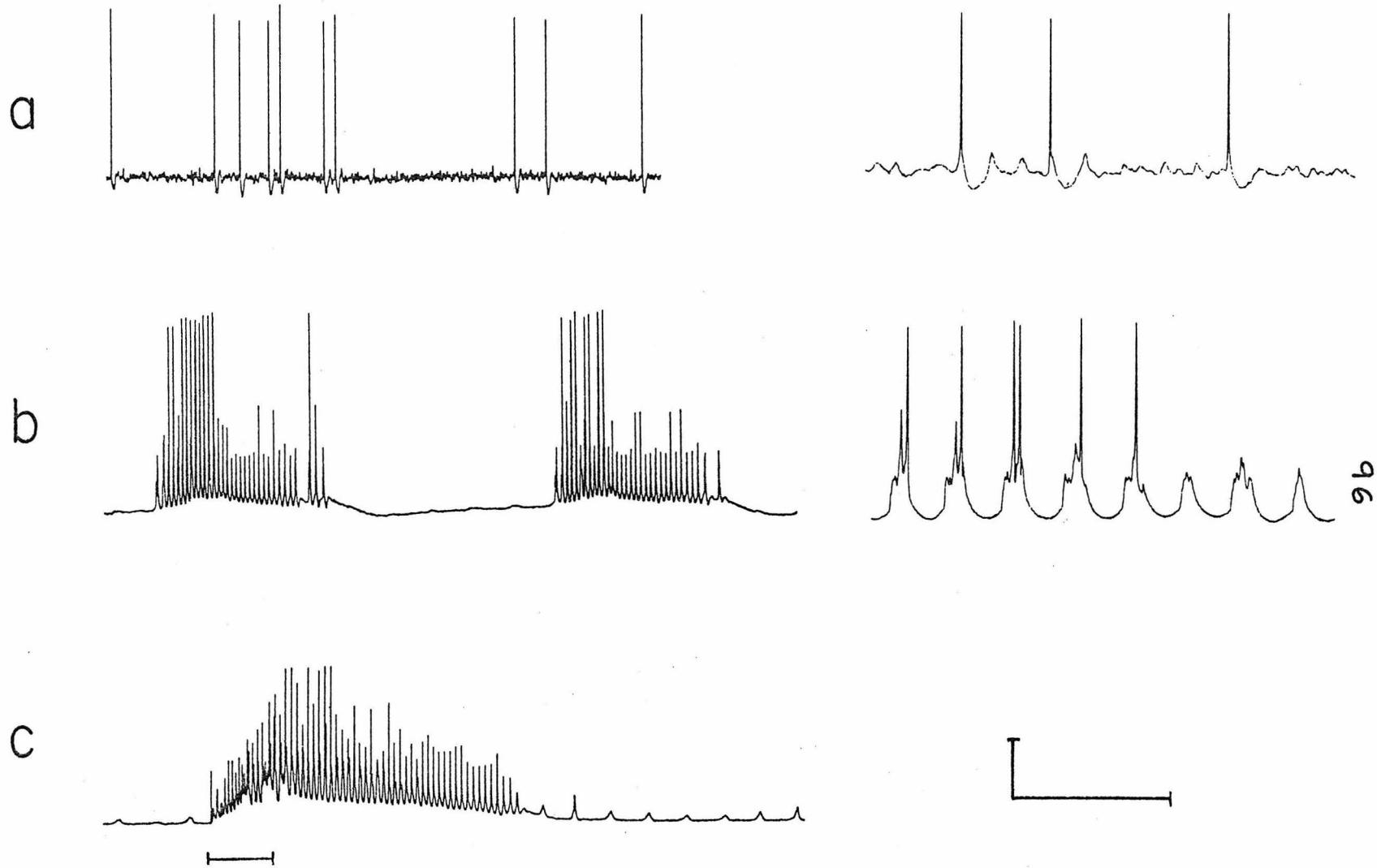

a
b
c
96

Fig. 5. Electrical activity in magnesium media. All records were made in media containing 11 mM magnesium, 0.15 mM calcium. (a) Spontaneous slow waves. (b) Modulations induced by 10 mM TEA. (c) Increase in slow wave frequency induced by injection of depolarizing current into neighboring muscle cell. Three 4 sec pulses (identified by ON and OFF artifacts) are shown. Calibration, 10 mV x 12 sec.

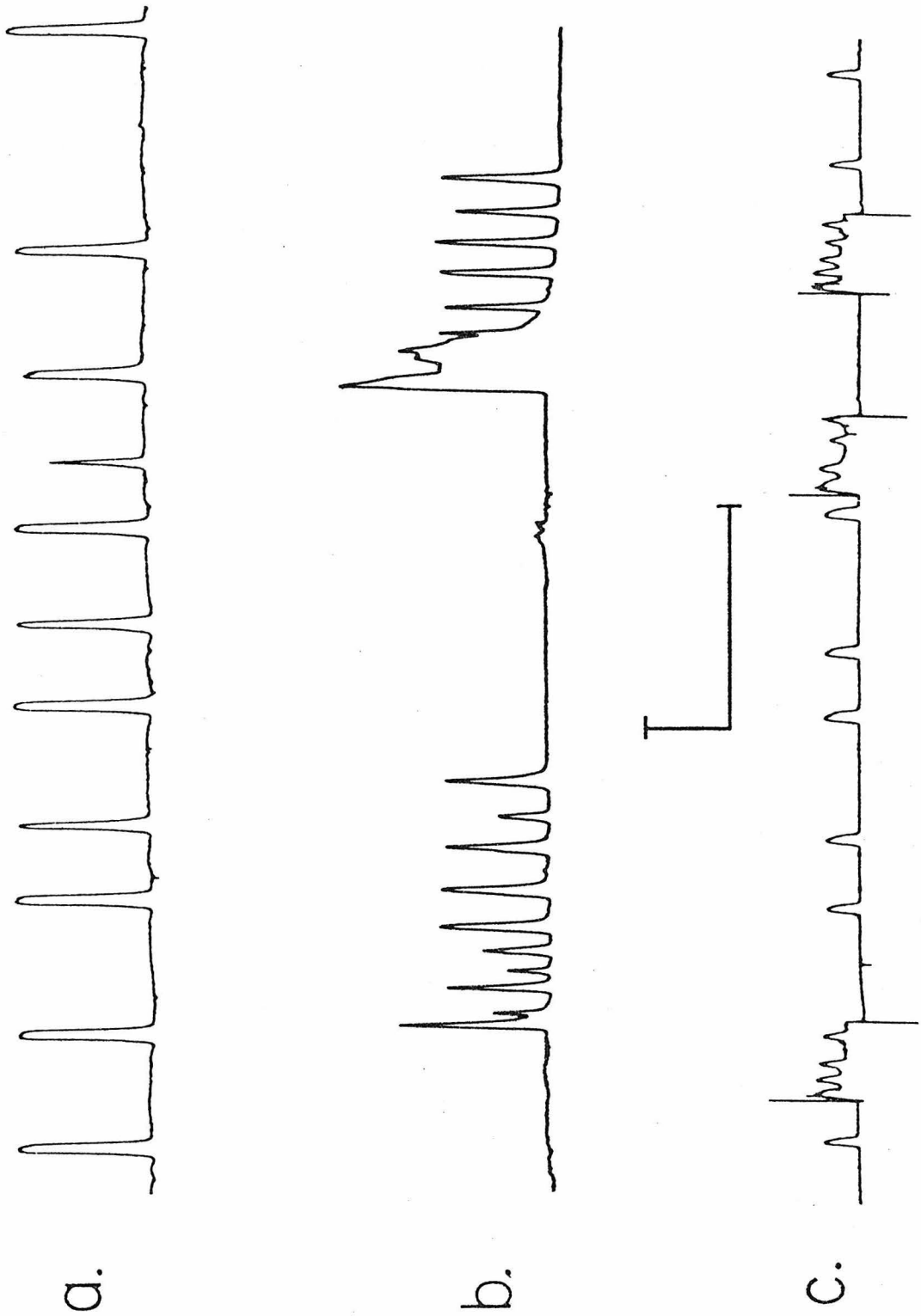

a.
b.
c.

Fig. 6. Spontaneous activity in strontium media. (a) Spike potentials, slow waves and occasional square waves in 11 mM strontium, 0.15 mM calcium. (b) Enhanced square waves induced by 10 mM TEA. Calibration, 10 mV x 12 sec in upper trace in (a) and lower trace in (b), 10 mV x 1 sec in lower trace in (a), 20 mV x 1 min in upper trace in (b).

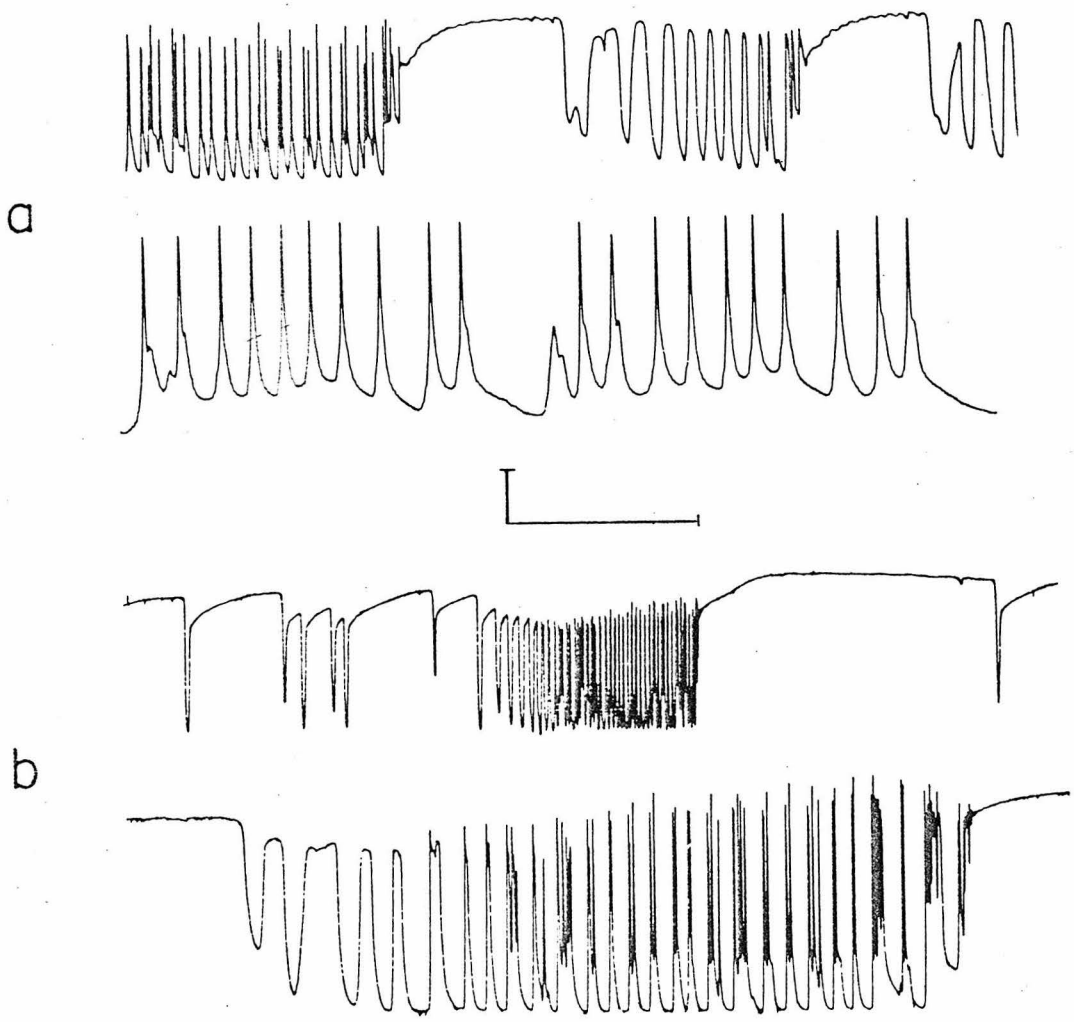

a
b

Fig. 7. Spontaneous activity in barium media. All records except (b) were made in media containing 11 mM barium, 0.15 mM calcium. (b) was made in medium containing 11 mMbarium, 0.05 mM calcium. Solid bars indicate transitions as follows: (b) Addition of TEA for a final concentration of 3 mM. (c) Washing the preparation with medium in which sodium was replaced with protonated Tris. (d) Washing with medium in which the potassium was reduced from 24 to 2 mM. (e) Washing with medium in which the potassium was increased from 24 to 47 mM. (f) Addition of ouabain for a final concentration of 1.2 mM. Calibration, 10 mV x 1 min.

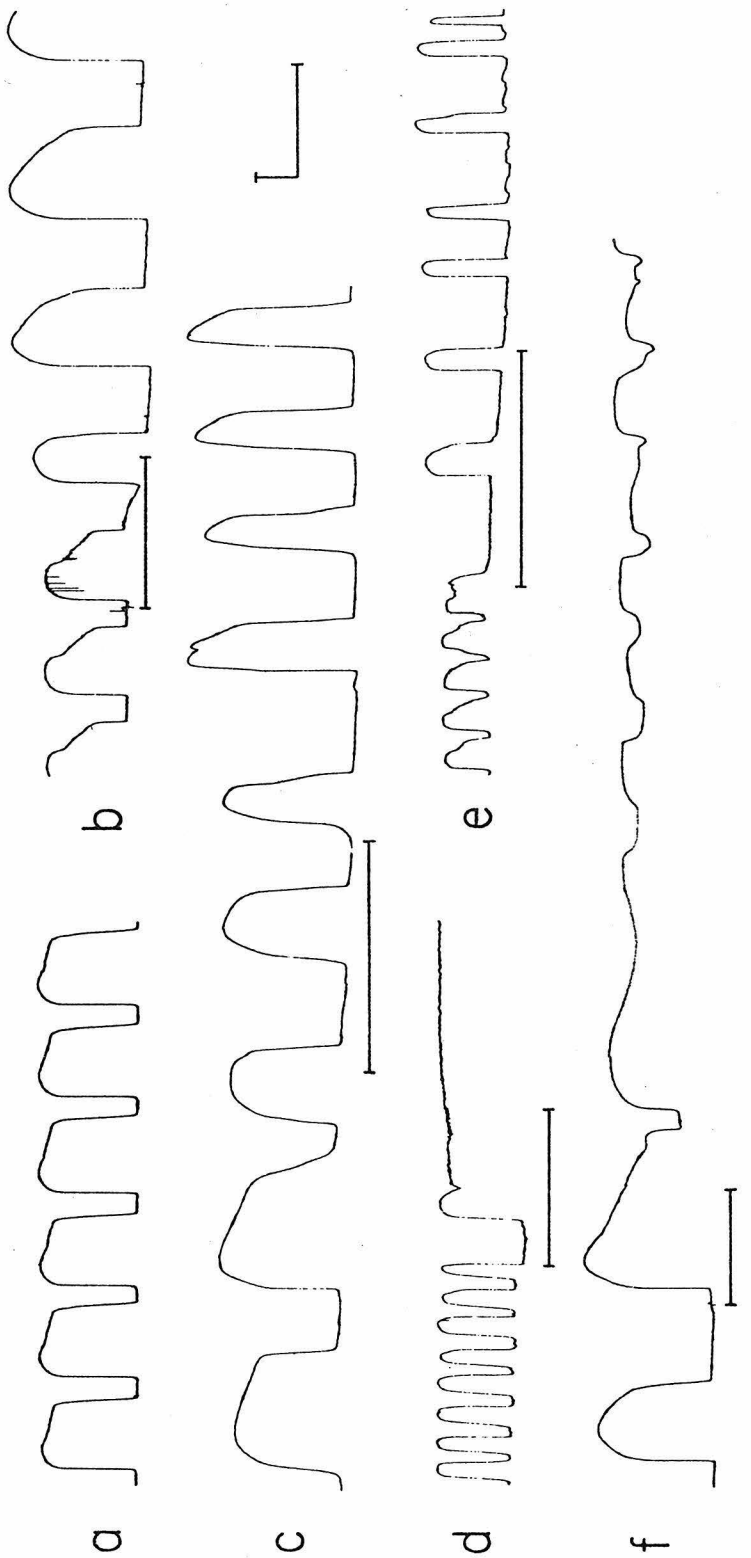

a
b
c
d
e
f

Fig. 8. Square wave dependence on barium and strontium concentration. (a) Variation in active potential with the log of barium or strontium concentration. The straight lines represent the least-squares fit to all the data points for strontium and to all the data points for barium except those obtained at 26 mM. Error bars represent the expected standard deviation. (b) Variation of square wave duration with barium concentration. Error bars represent the range of observed durations. The curve was drawn by eye. Some points in both (a) and (b) have been displaced horizontally for clarity.

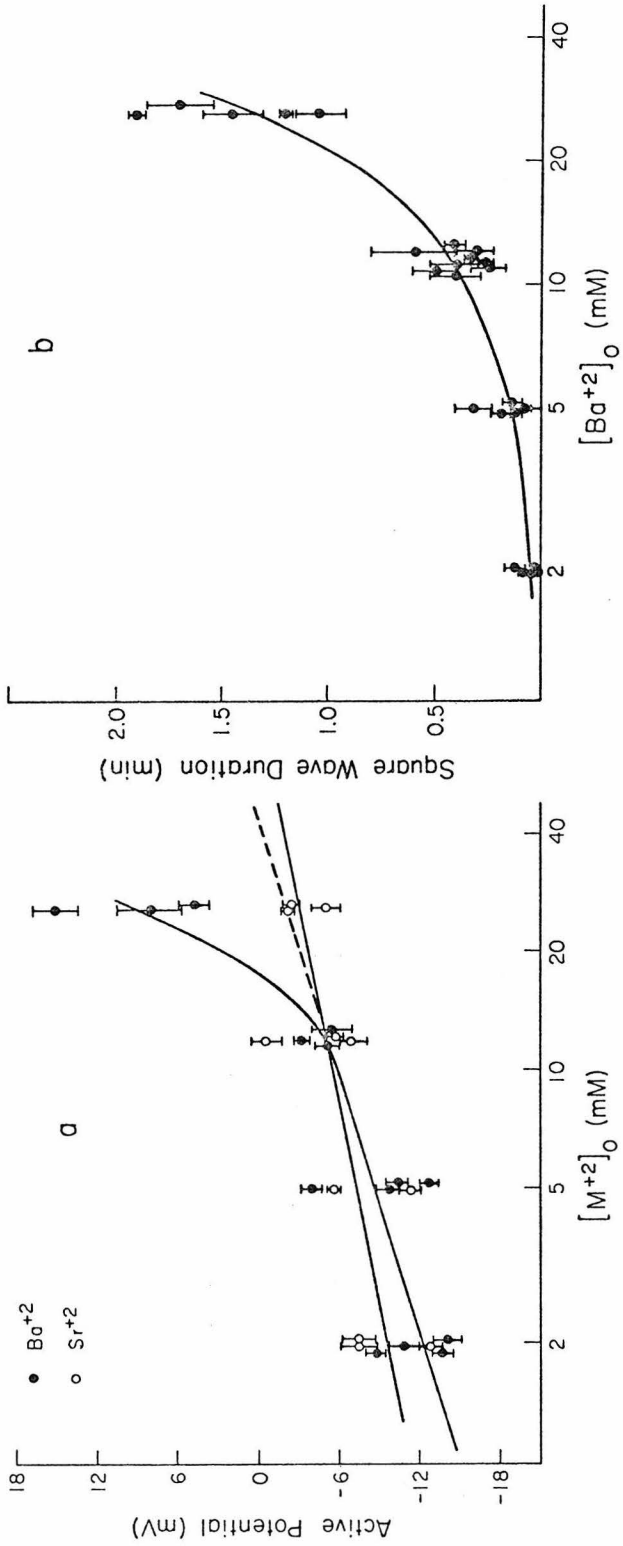

a
b
Ba+2
Sr+2
Active Potential (mV)
Square Wave Duration (min)
[M+2]o (mM)
[Ba+2]o (mM)
18
12
9
0
-6
-12
-18
2.0
1.5
1.0
0.5
2
5
10
20
40

Fig. 9. Resistance changes during square waves I. 400 msec, constant current pulses were passed from one electrode while recording from a second electrode in the same muscle belly. (a) 26 mM barium, 0.15 mM calcium; hyperpolarizing 200 nAmp pulses. (b) 26 mM barium, 0.15 mM calcium; depolarizing 150 nAmp pulses. (c) 11 mM magnesium, 0.15 mM calcium, 1 mM EGTA (sodium square waves); depolarizing 70 nAmp pulses. (d) 26 mM strontium, 0.15 mM calcium, 10 mM TEA; Depolarizing 40 nAmp pulses. Calibration, 20 mV x 10 sec in (a), (b), and (d), 10 mV x 10 sec in (c).

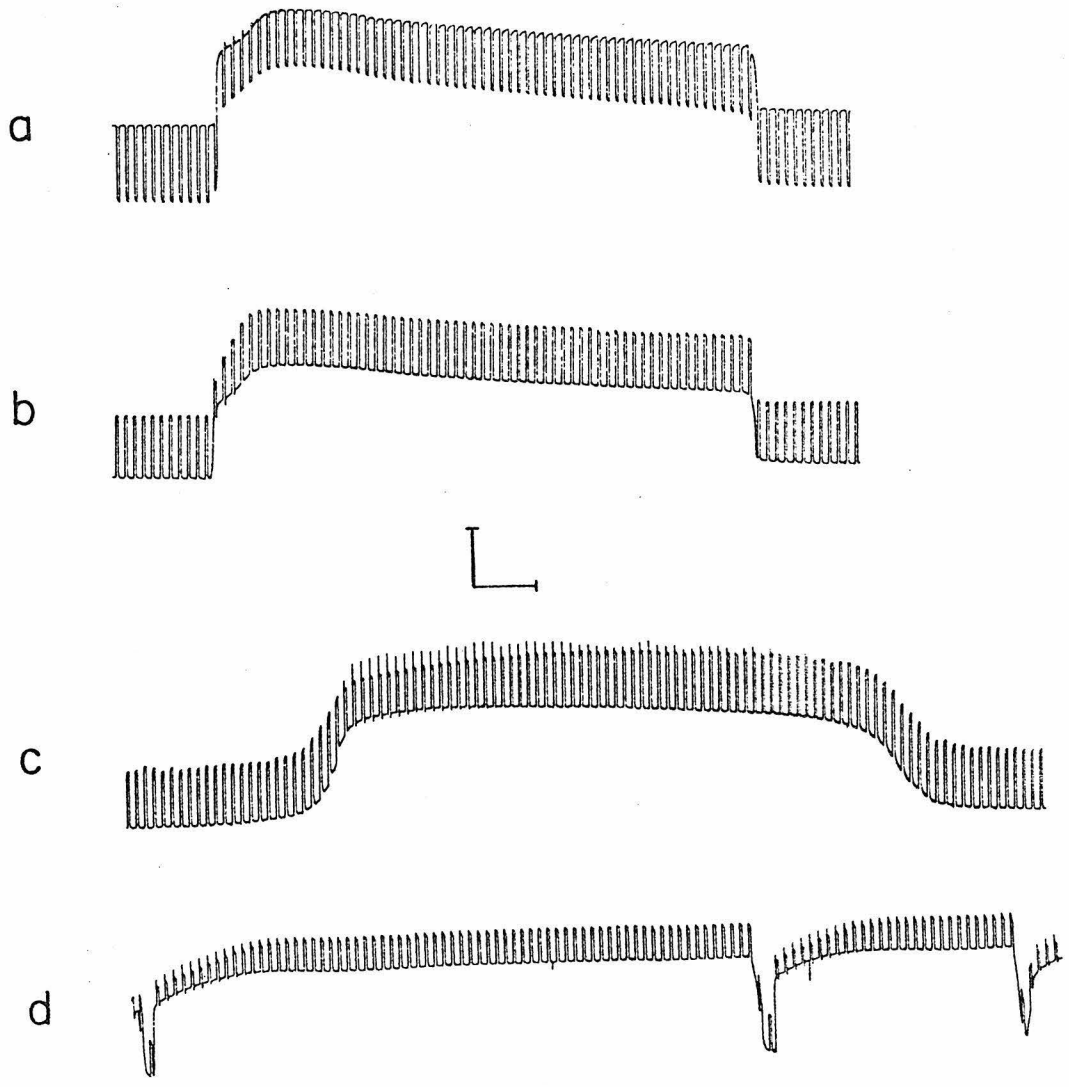

a
b
c
d

Fig. 10a, b. Resistance changes during square waves II. (a) and (b) Input resistance (R_m) and transmembrane potential (V_m) during square waves in 26 mM barium, 0.15 mM calcium. R_m is obtained from records such as those shown in the preceding figure, using the voltage pulse amplitude at the end of the 400 msec current pulse. V_m shows the transmembrane potential without the superimposed voltage pulses. (a) 50 nAmp hyperpolarizing pulses. (b) 100 nAmp depolarizing pulses.

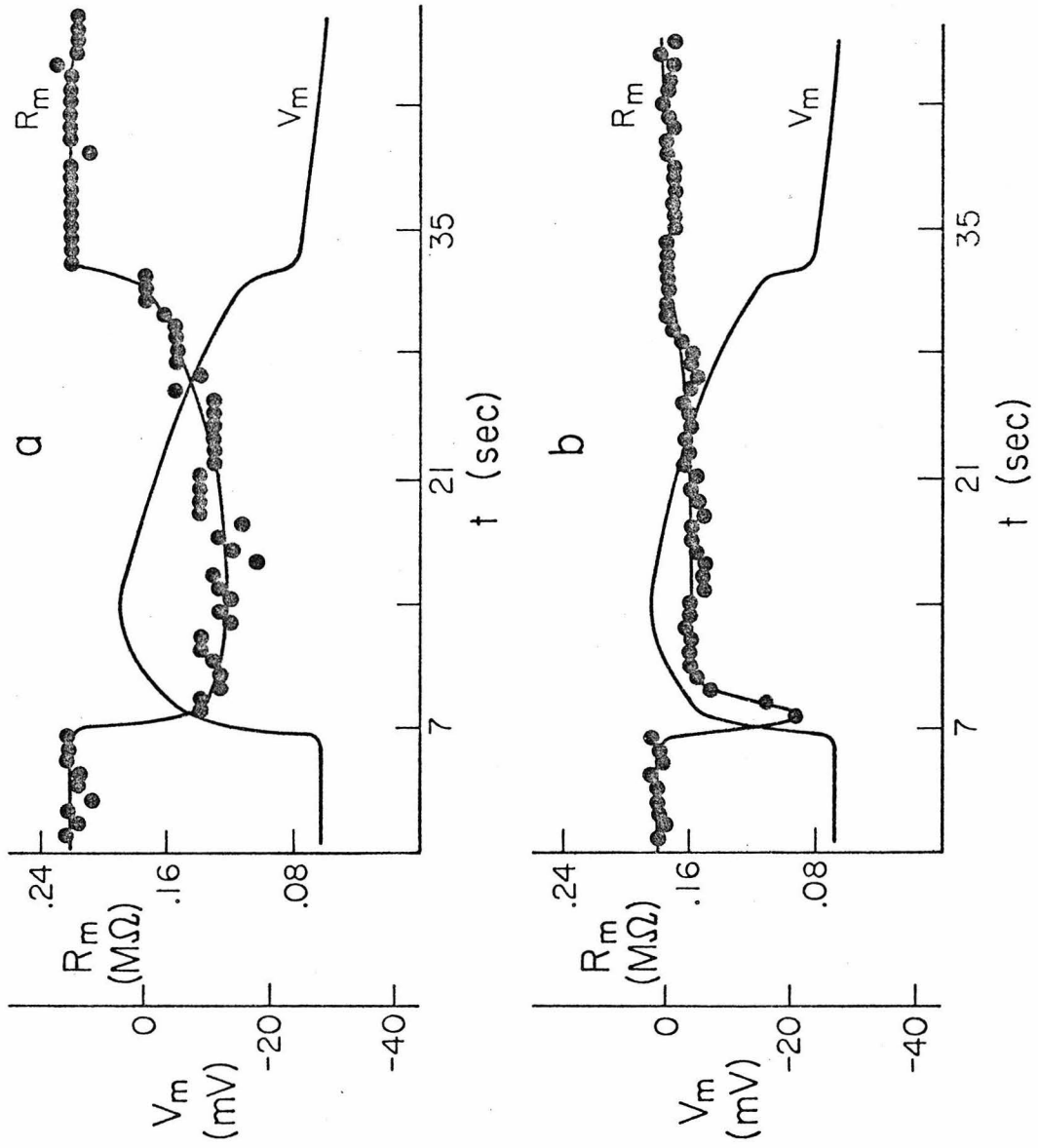
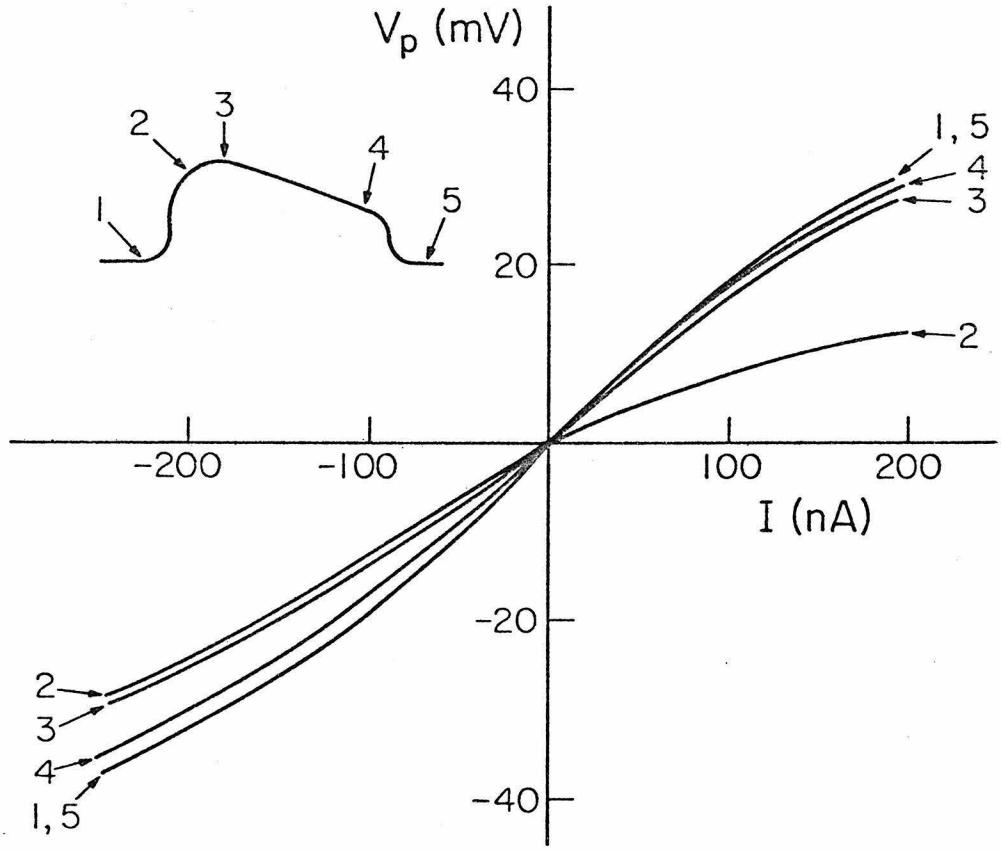

a
b
R_m
V_m
R_m (MΩ)
V_m (mV)
.24
.16
.08
0
-20
-40
7
21
35
t (sec)

Fig. 10c, d. Resistance changes during square waves II. (c) Current-voltage relationships at various points (see inset) during square waves. Voltage pulse amplitude (V_p) is measured at the end of the 400 msec current pulse. For clarity, data points have been omitted from the curves; the fit between the data points and the drawn curves is comparable to that shown in Fig. 11. (d) Transient responses following 300 nAmp hyperpolarizing current pulses, believed to result from delayed rectification (see text for details). Calibration, 20 mV x 5 sec.

c



d

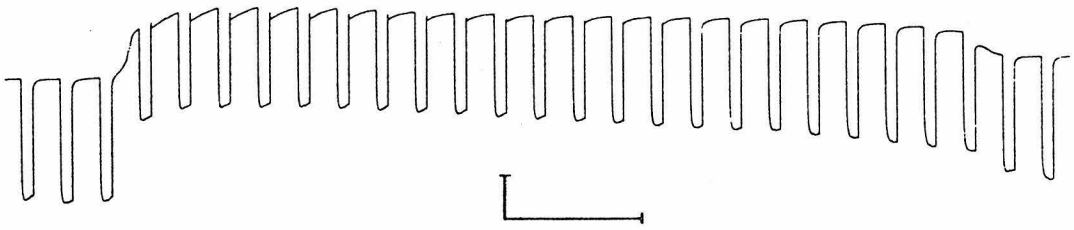
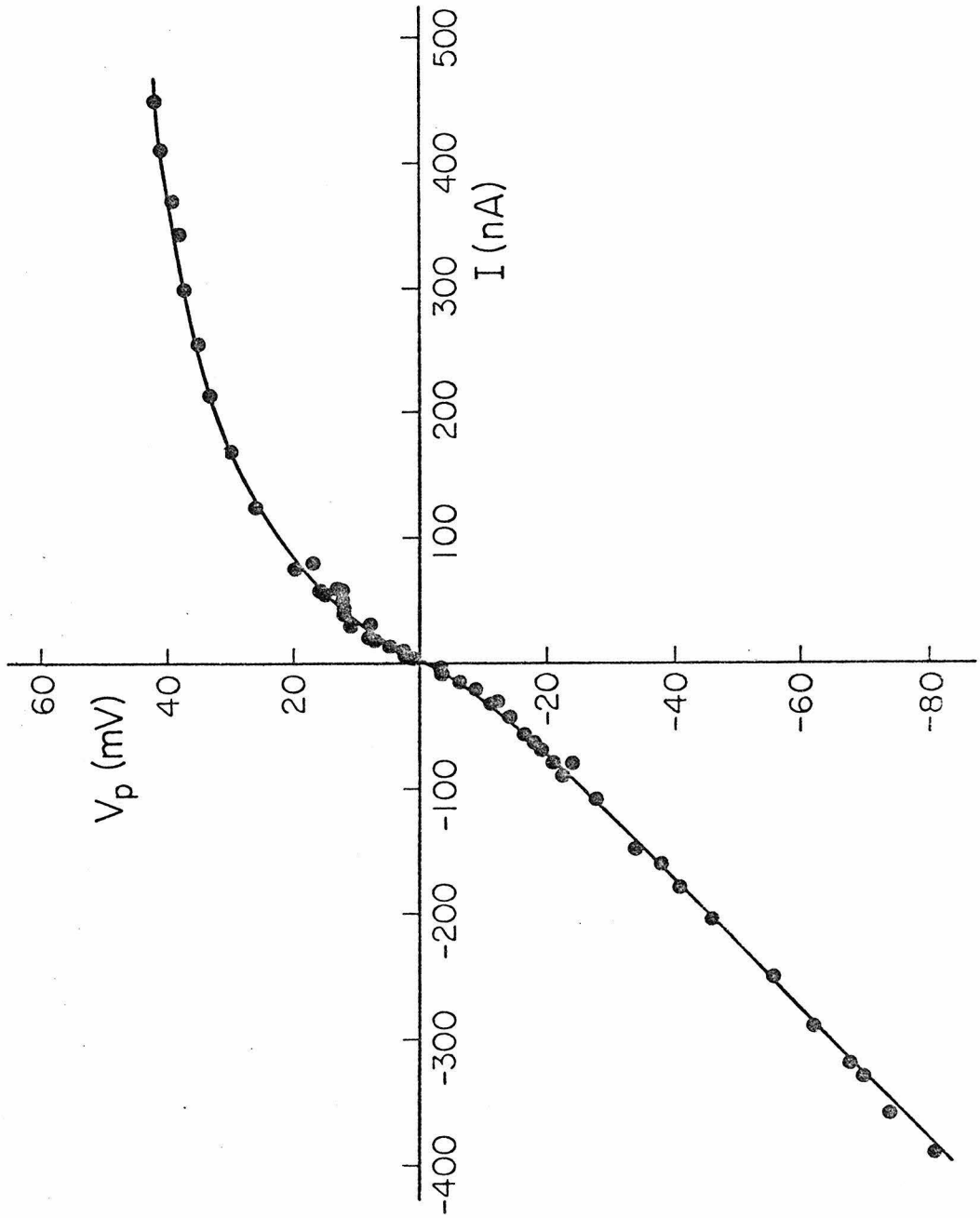


Fig. 11. Current-voltage relationship in APF. Voltage pulse amplitudes (V_p) measured at the end of 400 msec current pulses. Cell input resistance at origin (resting potential) is 0.34 MΩ; cell input resistance when cell is hyperpolarized drops to 0.20 MΩ.


60
40
20
-20
-40
-60
-80
V_p (mV)
500
400
300
200
100
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PROPOSITIONS

Abstracts of the Propositions

Proposition One: It is proposed to continue the investigation of the visual system of the leech, by determining the neuronal basis of a simple photo-motor reflex, and looking for lateral inhibition in the output of the ocular photosensory system. The possibility of plasticity in the connections of the photosensory system can be examined by testing for competition between sensilla in the innervation of second order neurons.

Proposition Two: Contradictory results have been obtained with regard to the effects of barium ions on the potassium conductance in pacemaker neurons in snails. It is proposed to resolve the conflict by measuring effects of injected barium on potassium currents arising during depolarizing voltage steps.

Proposition Three: It is proposed to use identified neural elements in the segmental ganglion of the medicinal leech as a simple model for classical conditioning in invertebrates. Light touch would be used as the conditioned stimulus (CS), pressure or noxious force as the unconditioned stimulus (UCS), and longitudinal shortening as the response (R).

Proposition Four: The synthesis of macrocycles of stacked phenyl groups is proposed. It is suggested that such compounds might exhibit aromaticity arising from a transannular ring current. Such aromaticity, if observed, might be sensitive to the number of phenyl groups comprising the macrocycle.

Proposition Five: Covalent labeling of membrane components associated with potassium conductance is proposed. A route for the synthesis of triethyl-5-diazohexylammonium iodide, and possibilities for its use as a photoaffinity reagent for potassium channels are suggested.

Proposition One: Connectivity and Plasticity in the Photosensory System of the Leech, Hirudo medicinalis

Previous investigators have elucidated the anatomical details and electrical responses of leech photoreceptors, and have identified some neurons receiving inputs from the photoreceptors (Kretz et al., 1976). It is proposed here to identify additional higher oreder neuron in the photosensory pathways, and to investigate the behavioral significance of the leech photosensory system. This will allow one to answer questions about the development and plasticity of nervous systems. The leech is well suited for such investigations, since its nervous system, while sophisticated enough to permit a wide behavioral repertoire, is simplified by its repeating, bilaterally symmetric segmental ganglia, and the large, identifiable neurons.

The photosensory organs in the leech, Hirudo medicinalis, are of two kinds, ocular and sensillar. The ocular system consists of five bilateral pairs of eyes located on the dorsal surface of the head. Each eye consists of 30 to 50 photoreceptors in the bottom of a cylindrical cup, the long axis of which is normal to the body surface. The photosensitivity of these organs was first demonstrated by Walther (1963). Kretz and coworkers (1976) used intracellular and suction electrodes, with electronic signal processing, to

study the output of the ocular system. They showed that the response to a light pulse, recorded by suction electrodes on the optic nerve, consists of an early transient response which decays to a steady state plateau. This electronically processed signal is basically a measure of spike frequency in the optic nerve, and agrees with the response measured intracellularly from the photoreceptors, a transient peak of depolarization followed by a steady state plateau.

The response of the ocular system adapts almost completely to variations in background illumination. Both the transient and plateau responses to light pulses are proportional to the log of the stimulus intensity; they differ in the proportionality constant and in the range of intensities over which the log-linear relation is obeyed. The location of the ocular photoreceptors in the bottom of the eyecups gives rise to a directional selectivity for the response, which may serve to localize visual stimuli, if taken in combination with the spatial arrangement of the five eye pairs along the head of the leech.

The second photosensory system in Hirudo is made up of the sensilla, of which there are seven bilateral pairs in each body segment. The first evidence that these presumptive photoreceptors do give an appropriate response to illumination was obtained by Kretz and coworkers (1976). The sensillar response to that of the ocular system consisting of an early transient peak of membrane depolarization and

spike frequency, followed by a steady state plateau. The background signal from the sensilla differs from that of the ocular system in that it decreases with the intensity of the backgound illumination; in addition, the sensilla are not located within restricting eyecups, and show very little directional selectivity. These differences between the sensillar and ocular photosensory sytems may be indicative of different behavioral functions for the two systems.

There are several promising lines of investigation in this system, falling into two main areas:

I. Photomotor reflexes.

A simple photomotor reflex in Hirudo is the longitudinal contraction observed in response to a sudden increase in intensity of the illumination. The shortening is known to be controlled by the L motor neurons, of which there is one bilateral pair in each segmental ganglion.

Limiting the stimulus by selective masking of photoreceptors should allow one to determine whether the shortening reflex is controlled by the ocular or the sensillar system, or both. The identification of the interneurons involved in this reflex could then proceed with relative ease, since the target motor neuron, the L cell, is already identified.

II. Plasticity and signal processing.

A. The suggestion has been made, on the basis of response

characteristics, directional selcetivity, and spatial organization of the ocular photosensory system, that the leech's eyes may serve to crudely localize visual stimuli in the field above its head (Kretz et al., 1976). With this in mind, it would be interesting to discover whether the higher order neurons in the leech's ocular system exhibit lateral inhibition. Such connections serve to enhance the ability of more highly evolved nervous systems to discriminate between light and dark areas of the visual field. The discovery of lateral inhibition in the leech's ocular system would further support the suggestion that this sytem does serve to identify the position of localized visual stimuli.

B. Kretz and coworkers (1976) have already identified some interneurons in the sensillar system, along with the sensilla from which they receive input. They have found that a given second order neuron is driven only by certain ones of the sensilla from that segment. In light of the known regerative properties of the leech nervous system, it is of interest to determine whether extirpation of the sensilla innervating a given second order neuron would result in the senstization of that neuron to the input of a different sensillum, either by neuronal growth or by the activation of previously nonfunctional synapses. If such a result were obtained, the leech will provide a valuable system to study this type of plasticity, analogs of which have been observed

in higher organisms.

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Proposition Two: Barium Effects on Potassium Conductance in Pacemaker Neurons of Snails

This proposition involves an investigation of potassium conductance regulation by divalent cations. Such effects may be important in processes of membrane excitation that operate by different mechanisms than those proposed by Hodgkin and Huxley, as is described below.

According to the model proposed by Hodgkin and Huxley (1952) to explain the properties of action potentials in the giant axon of the squid, spikes are mediated by selective sodium and potassium channels, each with distinct voltage sensitivities and kinetic properties. The depolarizing phase of the action potential is mediated by regenerative activation of the sodium channels. Spike termination is accomplished by a time-dependent inactivation of the sodium conductance, and a slightly delayed activation of the potassium channels.

Potassium channel activation in the squid giant axon appears to be primarily the direct result of membrane depolarization, but Baker and coworkers (1971) have demonstrated a component of the potassium conductance that appears to be activated by internal calcium. This effect may be ignored in systems such as the squid giant axon, where the spike potentials are essentially sodium-mediated, but given a system in which depolarizations are mediated by inward calcium cur-

rents, (such as the bursting pacemaker neurons in the snail) it is attractive to visualize the repolarization of the membrane as being achieved by a calcium-sensitive potassium conductance.

Meech (1974) monitored membrane resistance in the pacemaker neuron of the snail, Helix aspersa, with .5 sec hyperpolarizing current pulses from an electrode which was also used to inject salts into the cell. He found that injection of calcium, strontium, or barium ions induces a transient hyperpolarization and decrease in membrane resistance which result from an increase in the potassium conductance. (The reversal potential for the hyperpolarized membrane potential was equal to the Nernst potential for potassium, and varied as expected with variations in the external potassium concentration.)

This result appears to contradict results obtained by Eckert and Lux (1976), who used voltage clamp techniques to measure transmembrane currents in analogous cells in a different species of snail. They measured a net inward current in response to small depolarizations of the pacemaker neuron which appears to be normally carried by calcium ions, and a larger net outward current for larger depolarizing voltage steps which is presumably carried by potassium ions. Replacement of calcium with barium in the bathing medium gave a large increase in the inward current and reduction of the outward current. These results were interpreted as indicat-

ing that barium ions flowed readily through the calcium channels and also blocked the potassium conductance.

Thus, two groups of workers, studying essentially the same system, have reached contradictory conclusions regarding the effect of barium on the potassium conductance. The discrepancy could be explained by the difference between the species of snails used in the experiments, but, given the evolutionary conservatism that has been found for many phenomena in neurobiology, this is unlikely. Other possible explanations are differences between the effects of internal and external barium ions, or to differences in the experimental techniques used. (Meech measured membrane resistance with hyperpolarizing current pulses; Eckert and Lux measured currents flowing in response to depolarizing voltage steps.) This problem is of sufficient importance that the discrepancy should be resolved. The following experiments are proposed to accomplish this:

1) Repeat the experiments using a single species of Helix.

2) Repeat the voltage clamp experiments of Eckert and Lux, but with barium injected rather than externally applied. Voltage clamp measurements made before and after barium injection should show whether the effect of external barium is to flow in through the calcium channels and act from within on the potassium conductance. One would expect to see a re-

duction in the late outward current if barium is blocking the potassium conductance, without a strong effect on the inward current. If barium is increasing potassium conductance, the outward current should be increased after barium injection.

Subsequent experiments are dependent on the outcome of these initial ones; if barium increases the potassium conductance, experiments might be done to see if the observations made by Eckert and Lux result from a large inward barium current obscuring the outward current, since the voltage clamp technique measures only the net transmembrane current. If barium blocks the outward potassium current as measured by the voltage clamp, it would then be necessary to explain Meech's results. More complete resistance measurements, before and after barium injection, should reveal any possible effects of barium on the membrane rectification, but such experiments would be difficult due to the active response of the cells to depolarization.

References

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Proposition Three: A Proposal to Use the Leech as a System to Study the Cellular Basis of Classical Conditioning

The study of how learning occurs is often limited by the biological system used in the investigation. Each organism offers inherent advantages and disadvantages that place limits on the experimental approaches that can be applied to the problem.

With vertebrates, it is easy to induce widely varied learned behavior, but the large numbers and small size of the neurons make it very difficult to identify and study individual synapses whose properties might change during learning. Some of the lower invertebrates give one the advantage of simple nervous systems and identifiable nerve cells, but their limited behavioral repetoire makes it difficult to convincingly demonstrate that learning occurs.

It has been suggested that what is needed is a rapidly reproducing, haploid organsim with three large nerve cells and the ability to play piano. In lieu of that, it is proposed here that the leech may be a useful organism in which to study the cellular and synaptic basis of changes occurring during classical conditioning.

The leach exhibits a touch-contraction reflex, the neuronal elements of which have been delineated (Stuart,1970;

Nicholls and Baylor, 1968; Nicholls and Purves, 1970). It is proposed that this reflex system might be modified using a paradigm of classical conditioning, with light touch as the conditioned stimulus (CS), pressure or noxious mechanical stimulus as the unconditioned stimulus (UCS), and contraction of the longitudinal muscles as the response (R). Single, identified neurons would be used to mediate the stimuli and the response.

There are three pairs of T cells, two pairs of P cells, and two pairs of N cells in each ganglion, but for these experiments, only the most laterally located of each cell type would be used to facilitate multiple impalements (Nicholls and Purves, 1970). The key observation in the design of these experiments is the difference in the strength of the synaptic inputs of the different mechanosensory cells to the LMN. In particular, the input of the T cell to the LMN is much weaker than that of the P and N cells. A single impulse in the T cell is usually insufficient to bring the LMN to threshold, whereas single inpulses in either the P or N cell produce an excitatory post synaptic potential sufficient to excite the LMN (Nicholls and Purves, 1970).

It is proposed to train the leech to contract in response to a light touch to the skin, by repetitive pairing of the light touch with a heavy pressure that is certain to produce the contraction. At the cellular level, this is equiv-

alent to facilitating the ability of the T cell to induce action potentials in the LMN by repeatedly pairing impulses in the T cell with input from the P or N cell that is always sufficient to excite LMN.

This experiment can be done better in the isolated ganglion, stimulating the cells directly, than in the intact animal, for the following reasons. First, in the whole animal, the sensitivity of the T cell to such slight stimuli as eddy currents in the bathing medium must necessarily give rise to frequent "spontaneous" acitvity in the T cell. Since this activity is not under the experimenter's control, it results in large numbers of "conditioning trials" in which T cell activity is not paired with P or N cell activity, (heavy pressure or noxious mechanical stimulation). This amounts to extinction of any learned response and makes it all the more difficult to demonstrate learning. In the isolated ganglion there need be no such random input from the sensory neurons.

A second difficulty in doing learning experiments with the intact leech arises in controlling the intensity of the CS. Even gentle touch applied to the leech skin can cause contraction to occur. Presumably this is because the normal response of the T cell to natural stimuli is to fire a rapid burst of nerve impulses which is often enough to bring the LMN to threshold. In the intact organism one would argue that this response would quickly extinguish, permitting condition-

ing to be carried out. However, it would be easier to control this variable in the isolated ganglion, where, with intracellular electrodes, one can regulate the number of spikes put out by the T cell during each trial.

If one met with success at facilitating the T cell input to LMN by pairing it repeatedly with input from the P or N cell, it would be necessary to show that the effect did not result from repeated stimulation of the T cell or P cell alone (sensitization). A second control would be to achieve cellular facilitation in the ganglion of an operated animal and then show learning in the intact animal.

If long term learning could be demonstrated in this proparation, involvement of macromolecular processes could be looked for by incubating the ganglion in inhibitors of RNA and/or protein synthesis. If such a gross treatment prevented conditioning, subtler experiments could be perfromed by injecting inhibitors into the identified neurons.

If inhibitor experiments proved fruitful, it would be desirable to look for the macromolecules synthesized during conditioning. Techniques are available for dissecting out single cells after labeling an entire ganglion with radioactive precursor, and analyzing their proteins on very small gels (Wilson, 1971). This technique suffers from the shortcoming of leaving behind all the synaptic processes when the cell body is dissected out of the ganglion. Although protein synthesis may occur in the nucleus of the cell of

interest, it is possible that the important proteins are rapidly transported out of the cell body to the dendrites or nerve terminals, where they might escape detection. An alternate technique would be to label single cells by microinjection with labeled precursor. One could then work up the entire ganglion, including the neuropil, and yet measure the proteins of a single cell, including its pre- and post-synaptic processes. This technique would give the added advantage of allowing one to work with slightly larger volumes of tissue.

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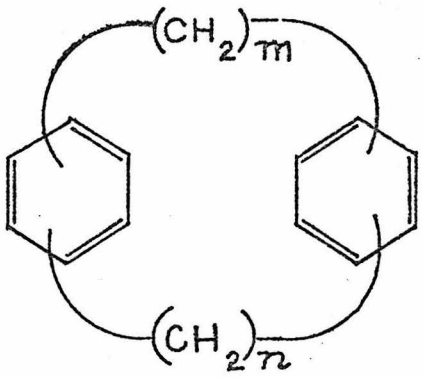
Proposition Four: Transannular Pi Electron Delocalization

This proposal inyolves a study of transannular pi electron delocalization, by looking at the properties of large rings composed of stacked cyclophanes. It is hoped that such compounds would exhibit a transannular ring current analogous to that observed in classically aromatic systems. It is possible that the aromaticity in such large rings would vary markedly, subject to the number of stacked phenyl groups in the molecule.

Cyclophanes are molecules of the general form:



When the aliphatic bridges are meta or para to one another, and m and n are less than four, the aromatic rings are forced much closer together than is found in unconstrained systems (Cram and Cram, 1971). For example, the interring separation in 2,2 paracyclophane is as low as 2.80 Å (Brown, 1953), whereas in graphite the separation between sheets is 3.35 Å (Ubbelohde and Lewis, 1960). Apparently, at the smaller separations the increase in energy due to pi-pi repulsion is greater than any energy gained by delocalization of electrons between the rings. Crystallographic studies have shown that in 2,2 paracyclophane, partial rehy-

bridization occurs, allowing greater pi electron density on the backside of the rings (Cram and Cram, 1971; Ingraham, 1950).

The overriding contribution of strain and repulsive energies do not mean that favorable transannular interactions do not occur, however. Especially in extended cyclophane analogs, where the pi electrons cannot escape to the backside of the molecule, one can imagine that transannular pi electron delocalization is the only means left to minimize the energy of the system. Evidence for this delocalization is as follows:

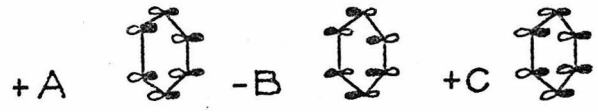
1. A valence bond calculation for an idealized cyclophane sytem with 3 Å separation gave an extra 4 kcal/mole of resonance energy (Ingraham, 1950).

2. When one ring in 2,2 cycloparaphane is substituted with an electron withdrawing substituent, electrophilic substitution into both rings is retarded (Reich and Cram, 1969).

3. The rate of electron transfer between rings in the anion radial of 2,2 paracyclophane is much larger than that observed for the open chain analog, and compares favorably with the rate measured for biphenyl (Williams et al., 1971).

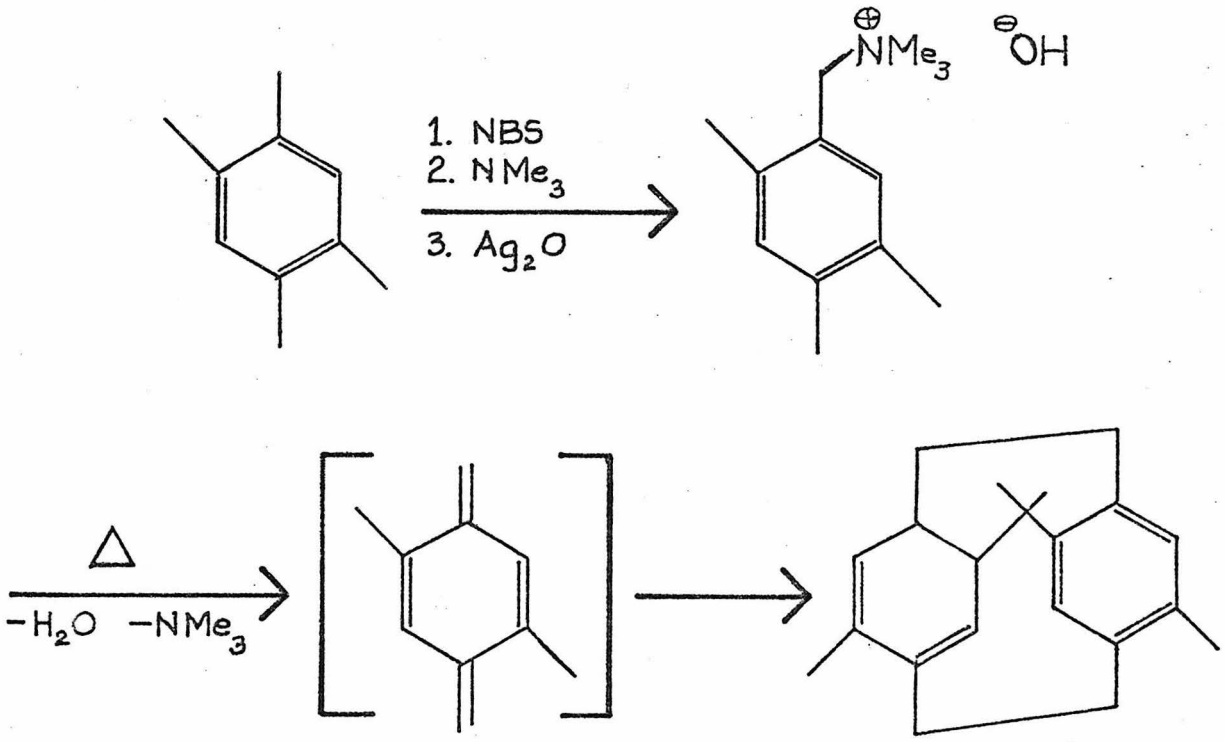
4. The photoelectron spectrum of 2,2 paracyclophane shares features with biphenyl (rather than diphenylmethane or diphenylethane) that are associated with pi electron delocalization (Pignataro et al., 1971).

In constructing molecular orbitals for extended cyclophane systems containing additional stacked aromatic rings, it is necessary to alternate the signs of the wave functions of the component rings in order to maintain positive overlap within the chain, e.g.:



Resonance stabilization for normal conjugated systems is best observed in cyclic analogs. Thus, one is lead to ask whether larger transannular delocalization would be observed in rings formed by joining the ends of a sufficiently long chain of stacked cyclophanes. It can be seen from the consideration mentioned above that there will be no new nodes introduced into the molecular orbitals of the system by the delocalization if there is an even number of stacked rings forming the macrocycle. However, if an odd number of subunits is used, there must be one extra negative overlap in the molecular orbital, where the "+" lobes from one end of the chain are forced into the "-" lobes of the subunit at the other end of the chain. This gives rise to a Mobius-type system; one might predict that aromaticity would not be observed in that case. (It is interesting to note that the postulated nonaromatic system here contains 4n+2 pi electrons, while the one for which greater transannular aromaticity is anticipated would contain 4n pi electrons.)

Stacked cyclophanes are available by the following route, which could be modified to attain large rings of the desired size (Longone and Chow, 1970):



The reaction leading to tetramethylparacyclophane gives a 20% yield and the process has been repeated, starting with the cyclophane and giving the analog containing four stacked rings in 10% yield. The yields would probably be smaller still when making yet larger chains, but only three more steps are required to produce a 32 ring chain, which should be long enough to cyclize. This could be accomplished using the dibrominated product of the first step so that the compound could, at high dilution, close intramolecularly.

Odd size rings could be obtained by reacting intermediate sized chains with single tetramethylbenzene units, and then crossreacting these subunits with other of even length (Otsubo et al., 1971)

Predictions of superaromaticity could be tested in the NMR, comparing rings of odd and even size. If significant transannular ring current is developed, a magnetic field should be induced in the molecule that would shift the protons on the outside of the ring downfield with respect to those on the inside.

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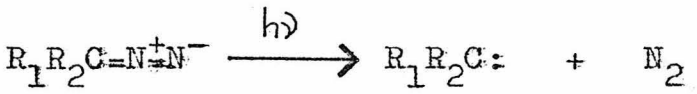
Proposition Five: Photoaffinity Labeling of Potassium Channels in the Giant Axon of the Squid with Triethyl-5-diazohexylammonium Iodide (TEDHA)

A barrier to the characterization of the potassium conductance channels of excitable membranes is the impossibility of assaying the relevant biological activity once the membrane has been disrupted. Although quaternary ammonium compounds (QA) of the form $CH_3(CH_2)_nN^+(CH_2CH_3)_3$ (n is typically from 0 to 8) are relatively specific blockers of the potassium conductance when applied to the inside of the membrane, the ready reversibility of the binding precludes their use in ways analogous to the use of bungarotoxin in the purification of acetylcholine receptors. Furthermore, the relatively high effective concentrations for the QAs (.01 to 1.0 mM) (Armstrong, 1975) and the likelihood of a multiplicity of nonspecific cation binding sites on the membrane makes it seem unlikely that equilibrium dialysis or derivatives of that technique could be effectively used to follow the purification of potassium channels. This proposal suggests that photoaffinity labeling might be used to identify the molecules associated with the selective potassium conductance in the squid giant axon.

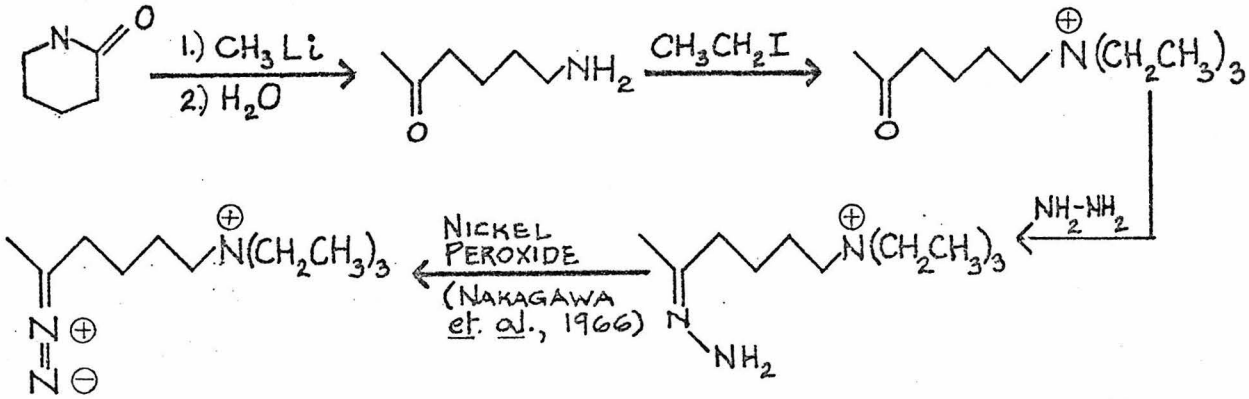
In phtoaffinity labeling (Knowles, 1972), a photosensitive functionality, such as a diazo group, is incorpo-

rated into a compound known to bind to the molecule of interest. Upon illumination, the diazo analog, which is presumably in close proximity to the active site of the target molecule, decomposes as follows:



The highly reactive carbene intermediate can react by insertion into C–H bonds to form a covalent link between the affinity reagent and the target molecule. The short lifetime of the carbene insures that it will not diffuse from the binding site before reacting and the nonselective nature of its reactivity insures that the molecule of interest will be labeled, regardless of the structure of the binding site.

To label potassium channels using this technique, a pharmacologically active diazo QA might be synthesized by the following route:



Radioisotopic label could be introduced in either or both of the alkylation steps, using carbon-14 and/or tritium; thus,

double label experiments are possible. Although one cannot be certain that the proposed reagent, triethyl-5-diazohexyl-ammonium iodide (TEDHA), will block potassium channels, the degree of acceptable variability in the fourth alkyl substituent argues that it should be active.

If this photoaffinity reagent proves active at blocking potassium channels, the problem of nonspecific binding of the reagent to anionic sites on the membrane remains. The problem of nonspecificity might be overcome by pretreating the squid axon with nonradioactive TEDHA; it has been demonstrated that blockage of potassium channels by QAs occurs only after the channels have opened in response to a depolarization of the membrane. Apparently, the probability of the blocking agent being in place when the channel is closed is low (Armstrong, 1975). Hyperpolarizing the membrane causes an inward potassium flow through the channels which is also effective at removing QAs from the channels (Armstrong, 1975). Thus, if photolysis of unlabeled TEDHA were performed during a hyperpolarization of the axon membrane, cation binding sites not associated with the potassium channels should be blocked. Subsequent perfusion of the axon with radiosiotopically labeled reagent and photolysis during a depolarization of the membrane should selectively label only the potassium channels. The proposed photoaffinity technique has the built-in drawback of irreversibly blocking the potassium channels, but,

if successful, it should allow the characterization of the channels to proceed to the extent that active channels might then be isolated by conventional means.

Although QAs appear to be effective only when applied internally in the squid giant axon, QAs have been shown to affect potassium conductance when applied when applied either internally or externally to the Retizius cell of leech, apparently acting at different sites (Kleinhaus and Prichard, 1975). It should be possible in principle to use double label techniques to determine whether the potassium channel sites sensitive to internal and external QAs are really two different sites, and, if so, whether they are contained within the same molecule.

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