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NEUROMUSCULAR PHYSIOLOGY OF THE LONGITUDINAL MUSCLE OF THE EARTHWORM, *LUMBRICUS TERRESTRIS*

I. EFFECTS OF DIFFERENT PHYSIOLOGICAL SALINES

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INTRODUCTION

The functional properties of the neuromuscular systems of animals are frequently studied by using isolated nerve-muscle preparations and a suitable physiological saline solution. In general suitable salines are obtained by determining the concentrations of major ions in an animal's body fluids and duplicating these concentrations in the form of a saline solution. Any alteration in the concentrations of major ions often leads to malfunctioning or rapid deterioration of the preparation (Lockwood, 1961).

In the case of the earthworm, analyses of the ionic composition of body fluids have been carried out only recently. From these analyses it is obvious that the ionic composition of earthworm body fluids does not coincide with the ionic composition of commonly used earthworm salines. For example, in the saline developed by Pantin (1946) the concentrations of Na^+, Ca^{2+}, and Cl^- are all considerably different from the concentrations of these ions in either blood or coelomic fluid (Kamemoto, Spalding & Keister, 1962; Dietz & Alvarado, 1970).

It is these differences in ionic composition which may account for some of the difficulties encountered in previous investigations of earthworm neuromuscular physiology. For example, the results of Hidaka *et al.* (1969*a–c*) and Chang (1969) indicate the presence of low muscle resting potentials, spontaneous spiking, and erratic junction potentials in earthworm longitudinal muscle. Further difficulties were encountered by Drewes & Pax (1971), who reported prolonged firing in longitudinal muscle following single-pulse stimulation of individual segmental nerves. The nature of such difficulties suggests abnormal excitability of nerve and muscle brought about perhaps by improper ion concentrations in the bathing medium.

For these reasons we have developed a new saline which is much nearer earthworm coelomic fluid and blood with respect to ion composition than the earthworm salines used by previous investigators. In this paper we present a comparison of the activity of earthworm longitudinal muscle when bathed in this new saline and when bathed in the saline most commonly used by previous investigators, the saline recommended by Pantin (1946).

Table 1. *Ionic composition of earthworm salines and body fluids*

	Pantin's saline	Our saline	Coelomic fluid*	Blood*
Na^+ (mM)	135·0	77·0	75·6	85·7
K^+	2·7	4·0	4·0	5·5
Ca^{2+}	1·8	6·0	3·0	8·4
Mg^{2+}	0·4	1·0	—	—
Cl^-	142·0	43·0	42·8	39·0
SO_4^{2-}	0·4	26·0	—	—
PO_4^{3-}	1·0	—	—	—
Tris	—	2·0	—	—
Sucrose	—	55·0	—	—
mOsM	210·0	167·0	167·0†	—
pH	7·4	7·4	—	—

* Values from Kamemoto *et al.* (1962).
† Value from Ramsay (1949).

MATERIALS AND METHODS

Earthworms, *Lumbricus terrestris*, were used in all experiments. Specimens were obtained from the Wholesale Bait Co. (Hamilton, Ohio, U.S.A.) or collected locally. Animals ranged from 0·5 to 1·0 cm in diameter and from 12 to 18 cm in length. All animals were maintained in Buss Bed-ding (Buss Mfg. Co., Lanark, Illinois, U.S.A.) and stored in Styrofoam boxes at 12–14 °C.

Procedures for obtaining the nerve-muscle preparation have been described previously (Drewes & Pax, 1971). Throughout all experiments the temperature was maintained at 12–14 °C.

During the dissection and throughout all experiments the preparation was bathed in either Pantin's (1946) saline or a new saline which we have recently developed (Drewes & Pax, 1972) (Table 1). Our new saline corresponds well with the ionic composition of earthworm coelomic fluid and blood as determined by Kamemoto *et al.* (1962) and Dietz & Alvarado (1970), whereas Pantin's does not. As can be seen from Table 1, in our saline the concentrations of Na^+, K^+, Ca^{2+}, and Cl^- are all nearly identical to those in earthworm coelomic fluid and blood. One major difference between our saline and earthworm coelomic fluid or blood is in the sulphate concentration. The reason for this is the necessity to make up the anion deficit brought about by the relatively low chloride concentration. An appropriate amount of sucrose is also present to obtain an osmotic concentration equivalent to that reported for earthworm body fluid (Ramsay, 1949).

Individual segmental nerves were stimulated by drawing the nerve stump into the tip of a polyethylene suction electrode (inner tip diameter 100–150 μm. Biphasic pulses (0·2 msec duration) were then delivered to the nerve. In all cases each nerve in a preparation was stimulated only once during an experiment.

Electrical activity was recorded using a suction electrode similar to that used for stimulation. Recordings were single-ended against a silver-wire ground electrode placed in the bath. Unless otherwise stated, all records were taken from a position approximately 2 mm lateral to the segmental nerve being stimulated. Electrical activity was amplified using a Grass P15 pre-amplifier and monitored on an oscilloscope.

Intracellular longitudinal muscle activity was recorded using glass microelectrodes

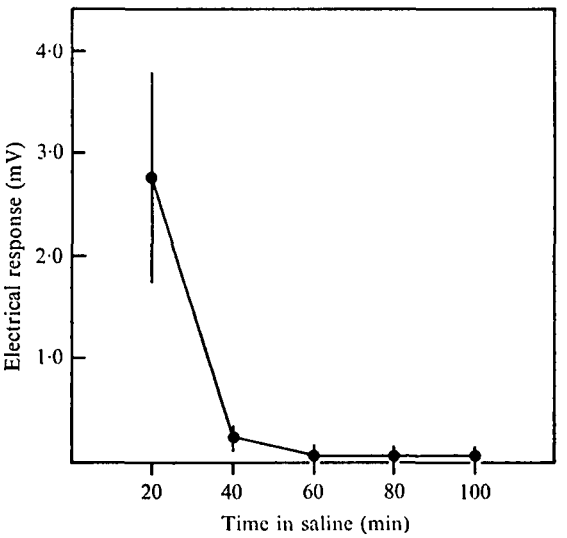

Fig. 1. Amplitude of externally recorded muscle potentials in Pantin's saline following stimulation of SN II–III. Each point represents a mean of six different measurements from one animal. Vertical lines indicate the ranges.

filled with 3 M-KCl. The lead from the microelectrode was connected to a W-P Instruments Model M-4A electrometer. In all experiments microelectrode resistances ranged from 8 to 20 MΩ.

RESULTS

Nerve and muscle activity in Pantin's saline

External electrical activity

In the following experiments the electrical responses of the nerve-muscle preparation were examined in order to determine the stability of the preparation while bathed in Pantin's saline. External electrical potentials were recorded in eight preparations at various times after the initial exposure to the saline during dissection. The suction recording electrode was applied to the surface of the longitudinal muscle and a segmental nerve was stimulated supramaximally with a single stimulus (6·0 V). Since the responses to this stimulation consist of slow smooth waves of electrical activity, such responses are most probably extracellular measurements of postsynaptic activity from many longitudinal muscle fibres. This idea receives more conclusive support in the subsequent paper (Drewes & Pax, 1973).

Results from a typical experiment are shown in Fig. 1. Each point represents the mean amplitude of external muscle potentials recorded following stimulation of the double segmental nerve, SN II–III. Initial recordings, taken 20 min after exposure to the saline, consisted of a large, single negative potential with a mean amplitude of $2{\cdot}75 \pm 1{\cdot}02$ mV S.D. After 40 min of exposure to the saline the mean amplitude had decreased to $0{\cdot}19 \pm 0{\cdot}02$ mV S.D. or less than one-tenth the initial value. The mean amplitude of these potentials continued to decrease until they were almost non-recordable; after 1 h the mean amplitude was only $0{\cdot}02 \pm 0{\cdot}01$ mV S.D. Similar results

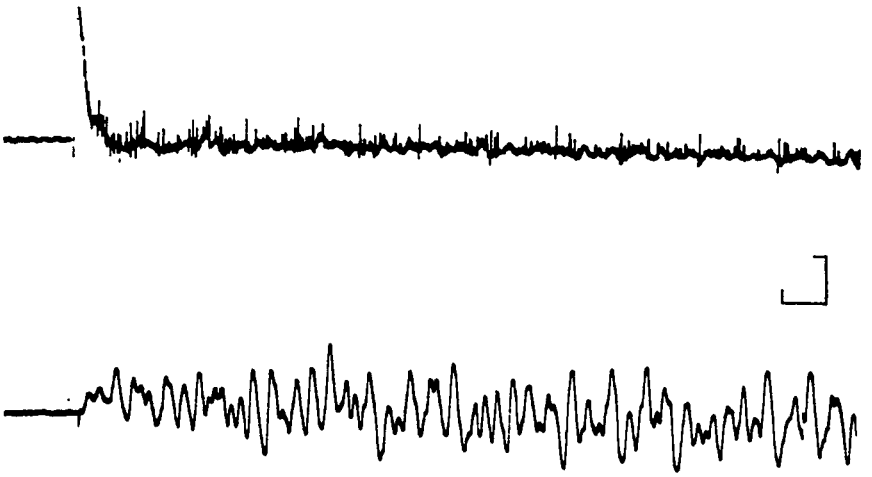

Fig. 2. Activity in nerve and muscle following a single stimulus. In the top record repetitive spiking activity is recorded from SN II–III in response to a single stimulus (8 V). In the bottom record repetitive activity is recorded from the longitudinal muscle following another stimulus to the nerve. Voltage scale: 0·2 mV. Time scale: top record, 100 msec; bottom record, 50 msec.

were obtained in all other preparations and results were indistinguishable for the two different segmental nerves.

Correlated closely with the decrement in external muscle potentials there was a tendency toward repetitive firing in the longitudinal muscle in response to a single stimulus. As shown in Fig. 2, a single stimulus to SN II–III resulted in a prolonged burst of low-amplitude muscle potentials. In general the duration of such activity increased with increasing stimulus intensity. With high stimulus strengths the bursting activity lasted several seconds and resulted in a large and observable mechanical response with the amplitude of this contraction being directly related to the amount of bursting activity recorded from the muscle. Thus there was no clear threshold for the mechanical response and there was no stimulus strength which was clearly supramaximal.

The question arises as to whether such repetitive muscle activity has its origin in the muscle or is due to a hyperexcitability of the nerve such that a single stimulus gives rise to repetitive discharges in the nerve. In order to answer this question a suction-recording electrode was placed along side and slightly distal to the stimulating electrode. Thus both stimulating and recording electrodes were in contact with the nerve. Results obtained from each of the two nerves were similar. Fig. 2 shows the activity in SN II–III in response to a single stimulus. The activity consisted of a series of spikes of various amplitudes, with the spiking activity gradually decreasing in frequency. Such activity lasted up to several seconds and was often recorded even at stimulus strengths only slightly above threshold. The duration of this burst of nerve activity corresponded to the duration of the burst of muscle activity. This correlation between bursting activity in nerve and muscle suggests that the nerve activity is at least partially due to repetitive firing of motor axons innervating the longitudinal muscle.

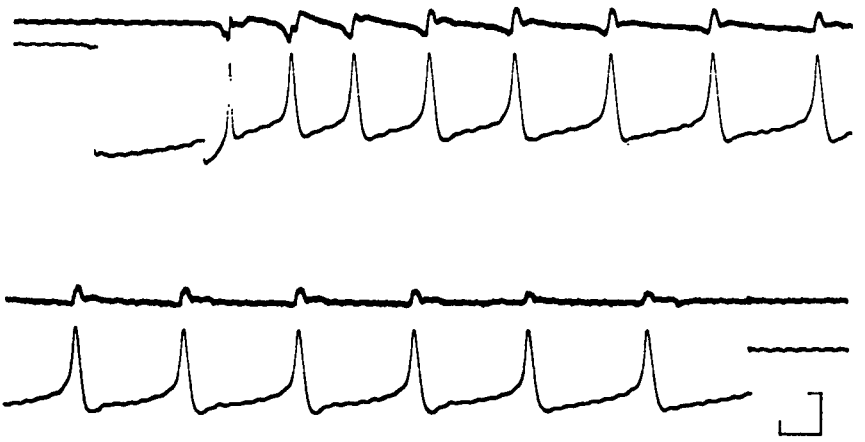

Fig. 3. Spiking activity in a longitudinal muscle fibre following penetration with a microelectrode. Lower traces, intracellular record; upper traces, extracellular record. Record is continuous from upper left to lower right. Penetration with the microelectrode is signalled by the large negative shift in the baseline of the intracellular record. Note spiking activity in external record stops when microelectrode is withdrawn. Voltage scale: upper traces, 0·1 mV; lower traces, 10 mV. Time scale 50 msec.

Intracellular electrical activity

The effect of Pantin's saline on the electrical activity of longitudinal muscle fibres was also studied using intracellular microelectrodes. Initial measurements of resting potentials were made 20 min after exposure to the saline; later records were taken approximately 1 h after exposure to the saline. In all these experiments no stimulation of nerve or muscle was given.

Initial measurements (taken about 20 min after first exposure to Pantin's saline) were made from 37 cells (four animals). The mean resting potential at this time was $-44{\cdot}2 \pm 2{\cdot}9$ mV S.E. and the majority of the cells were quiescent. After 1 h in the saline, the mean resting potential had decreased to $-36{\cdot}1 \pm 2{\cdot}5$ mV S.E. (150 cells, four animals). Of these cells 25% were quiescent, while 75% showed 'spontaneous' spiking activity, such as that shown in Fig. 3.

Generally the spiking activity consisted of a regular series of spikes, ranging in frequency from 0·2 to 15/sec, with the frequency usually decreasing with time. The amplitude of these spikes varied from one cell to another, with spikes in some cells overshooting zero potential by as much as 10–15 mV. In other cells spikes were as small as 7 mV. These results suggest that exposure to Pantin's saline brings about a gradual depolarization in muscle cells which is accompanied by an increased tendency for repetitive spiking.

The origin of such spiking appears to be in the mechanical disruption, or penetration of the muscle fibres. This was clearly demonstrated by monitoring the external electrical activity of muscle fibres prior to, during and after penetration by a microelectrode. To do this the suction-recording electrode and microelectrode were placed in very close proximity (less than 50 μm), so that both electrodes could be used to record activity from the same muscle fibre.

Fig. 3 shows simultaneous intracellular and external electrical activity recorded from a longitudinal muscle fibre. Prior to penetration no external activity was recorded.

Immediately after penetration a series of spikes was recorded intracellularly. Simultaneously there occurred small external potentials with a time course identical to the time course of intracellular spikes. Once the microelectrode was withdrawn no spiking activity was recorded externally. These results indicate that the so-called 'spontaneous' spikes occur only during penetration, and they may therefore be considered injury spikes rather than true spontaneously occurring spikes.

From the preceding results it appears that Pantin's saline has numerous undesirable effects on the nerve-muscle preparation. First, there is a decrease in the amplitude of externally recorded muscle responses to nerve stimulation. There is an apparent hyperexcitability of motor axons when exposed to the saline. Finally there is a decrease in the muscle resting potential, which is accompanied by a tendency for repetitive spiking in muscle fibres following penetration by a microelectrode. These undesirable effects all indicate that any studies made using this saline must be carefully evaluated in order to determine whether results obtained reflect the actual functioning of earthworm muscle or are simply artifacts produced in a deteriorating preparation.

Nerve and muscle activity in the new saline

External electrical activity

In order to compare our saline with that of Pantin a series of experiments parallel to those used for examination of the effects of Pantin's saline was performed using our new saline. External electrical responses of the longitudinal muscle were recorded from 12 animals after exposure to the new saline.

Muscle responses to supramaximal stimuli were large, ranging in amplitude from 1 to 5 mV, and if tested at 20 min intervals the amplitude of these responses, in contrast to the previous experiments, remained stable for more than 1 h of experimentation. For example, in one experiment involving stimulation of SNII–III the mean amplitude of the response 20 min after exposure to the new saline was 3·3 mV ($N = 5$; range 2·5–4·0 mV). After 100 min of exposure to the saline the mean amplitude of the response was still 3·1 mV ($N = 5$; range 2·0–4·2 mV). Responses to stimulation of SNI were essentially the same as those of SNII–III. In all cases responses of the longitudinal muscle consisted of discrete potentials and showed no evidence of repetitive activity. Thus it appears that segmental nerves in preparations bathed in the new saline are not hyperexcitable and do not fire repetitively following a single stimulus.

Intracellular recordings

Intracellular activity of longitudinal muscle bathed in the new saline was also recorded and compared to the intracellular activity previously described in Pantin's saline. Measurements of resting potentials were taken from 200 longitudinal muscle fibres in five preparations. The mean resting potential was $-47{\cdot}9 \pm 2{\cdot}5$ mV S.E., there being no significant changes in the mean potential over a period of 1 h. These values are considerably greater than resting potentials measured in Pantin's saline (mean = $-36{\cdot}1$ mV after 1 h).

Generally cells were quiescent following penetration, this being in contrast to the repetitive injury spiking seen in Pantin's saline. Occasionally one or a few spikes were recorded immediately after penetration but the membrane was stable thereafter.

DISCUSSION

In studying any isolated physiological preparation it is always difficult to determine what constitutes ideal 'normal' conditions for the preparation. One measure which might be used is the stability of the preparation over time after isolation. We have shown that, bathed in Pantin's saline, the earthworm nerve-muscle preparation deteriorates rather quickly; there occurring a decline in externally recorded muscle potentials and resting membrane potentials, as well as the development of hyperexcitability in both nerve and muscle within an hour.

By contrast, in our new saline these measures remain essentially constant. On this basis alone we can conclude that the responses recorded in the new saline probably more closely reflect 'normal' functioning in earthworm muscle than do the responses recorded from preparations bathed in Pantin's saline. The fact that our saline more closely resembles earthworm coelomic fluid and blood corroborates this.

Determination of which ions are most critical in controlling and maintaining normal functioning of earthworm nerve and muscle was not a goal of this study. However, based on other studies, there is reason to think that calcium ions might be particularly important in accounting for the differences in functioning of preparations bathed in the two salines. It is known, for example, that the concentration of external calcium determines the excitability, or polarizability of nervous tissue (Brink, 1954). This effect has been demonstrated in several invertebrate nerve preparations, such as the lobster (Adelman, 1956; Adelman & Adams, 1959) and the spider (Rathmayer, 1969). Such an effect of calcium on nerve excitability may thus explain the hyperexcitability of earthworm motor axons bathed in Pantin's saline.

SUMMARY

1. An earthworm saline commonly used in previous investigations of earthworm neuromuscular physiology has been shown to be inappropriate for such studies because the ionic composition of the saline does not correspond to that of earthworm body fluids.

2. Problems encountered when using this saline include: increased excitability of motor axons innervating the longitudinal muscle, a decrease in the amplitude of external electrical responses of the muscle, an increased susceptibility of longitudinal muscle fibres to injury spiking, and a decrease in resting potentials of longitudinal muscle fibres (to a mean of $-36{\cdot}1$ mV).

3. Such problems have been overcome by the development of a new physiological saline whose composition closely corresponds to the ionic composition of earthworm body fluids.

4. In the new saline responses of the nerve-muscle preparation remain stable for more than 1 h.

5. Resting potentials of longitudinal muscle fibres are also stable with time, the mean resting potential being $-47{\cdot}9$ mV.

This paper is based on part of a thesis presented by Charles Drewes to the Department of Zoology, Michigan State University, in partial fulfilment of the requirements for the Ph.D. degree.

REFERENCES

ADELMAN, W. J. (1956). The effect of external calcium and magnesium depletion on single nerve fibres. *J. gen. Physiol.* **39**, 753–72.

ADELMAN, W. J. & ADAMS, J. (1959). Effects of calcium lack on action potential of motor axons of the lobster limb. *J. gen. Physiol.* **42**, 655–64.

BRINK, F. (1954). The role of calcium ions in neural processes. *Pharmacol. Rev.* **6**, 243–86.

CHANG, Y. C. (1969). Membrane properties of muscle cells from the earthworm *Pheretima hawayana*. *Am. J. Physiol.* **216**, 1258–65.

DIETZ, T. H. & ALVARADO, R. H. (1970). Osmotic and ionic regulation in *Lumbricus terrestris* L. *Biol. Bull. mar. biol. Lab., Woods Hole* **138**, 247–61.

DREWES, C. D. & PAX, R. A. (1971). Mechanical responses of the body wall muscle of the earthworm, *Lumbricus terrestris*, to segmental nerve stimulation. *Can. J. Zool.* **49**, 1527–34.

DREWES, C. D. & PAX, R. A. (1972). Electrophysiological studies of earthworm longitudinal muscle. *Am. Zool.* **12**, xlii.

DREWES, C. D. & PAX, R. A. (1973). Neuromuscular physiology of the longitudinal muscle of the earthworm, *Lumbricus terrestris*. II. Patterns of innervation. *J. exp. Biol.* **60**, 453–67.

HIDAKA, T., ITO, Y. & KURIYAMA, H. (1969*a*). Membrane properties of the somatic muscle (obliquely striated muscle) of the earthworm. *J. exp. Biol.* **50**, 387–403.

HIDAKA, T., ITO, Y., KURIYAMA, H. & TASHIRO, N. (1969*b*). Effects of various ions on the resting and active membranes of the somatic muscle of the earthworm. *J. exp. Biol.* **50**, 405–15.

HIDAKA, T., ITO, Y., KURIYAMA, H. & TASHIRO, N. (1969*c*). Neuromuscular transmission in the longitudinal layer of somatic muscle in the earthworm. *J. exp. Biol.* **50**, 417–30.

KAMEMOTO, F. I., SPALDING, A. E. & KEISTER, S. M. (1962). Ionic balances in blood and coelomic fluid in earthworms. *Biol. Bull. mar. biol. Lab., Woods Hole* **122**, 228–31.

LOCKWOOD, A. P. M. (1961). 'Ringer' solutions and some notes on the physiological basis of their ionic composition. *Comp. Biochem. Physiol.* **2**, 241–89.

PANTIN, C. F. A. (1946). *Notes on Microscopical Techniques for Zoologists*. Cambridge University Press.

RAMSAY, J. A. (1949). The osmotic relations of the earthworm. *J. exp. Biol.* **26**, 46–56.

RATHMAYER, W. (1969). Neuromuscular transmission in a spider and the effect of calcium. *Comp. Biochem. Physiol.* **14**, 673–87.