Control of Contractility in *Spirostomum* by Dissociated Calcium Ions

EARL M. ETTIENNE

From the Department of Biological Sciences, State University of New York at Albany, Albany, New York 12203

ABSTRACT The freshwater protozoan, *Spirostomum ambiguum*, exhibits generalized contraction when electrically stimulated with a DC pulse. Light and electron microscopic studies show a subcortical filamentous network, believed responsible for generating contractile tension, in association with vesicles which were shown to accumulate calcium oxalate precipitates. Organisms microinjected with the calcium-sensitive, bioluminescent protein, aequorin, emit light when stimulated to contract. Analyses of cinefilm records of electrically induced contraction indicate that contraction may occur up to 25 msec after the onset of stimulation at a point when the calcium-aequorin light emission is at a peak. The evidence shows that calcium release from an interval compartment is directly associated with the onset of contraction in *Spirostomum*, and that the removal of calcium, through some internal sequestering mechanism, signals relaxation.

Most of what is known about the mechanism and control of contractile phenomena has come from studies on striated muscle. As early as 1940, L. V. Heilbrunn speculated that calcium ions might play the role of initiator of muscle contraction and many other processes. In 1947 Heilbrunn and Wiercinski showed that calcium alone, among many other substances injected into striated muscle fibers, initiated contraction. Subsequent investigation on vertebrate striated muscle cells has established that an action potential triggers the release of calcium from vesicles of the sarcoplasmic reticulum. The interaction between calcium and muscle proteins initiates myofibrillar shortening (for a review, see (Wilkie, 1968). Relaxation occurs with the sequestering of Ca^{++} ions by the sarcoplasmic reticulum so that they are no longer available to the actomyosin-ATPase system. In this paper we are concerned with the control of contractility in the giant ciliate, *Spirostomum ambiguum*.

The ciliate, *Spirostomum ambiguum*, can contract rapidly to about one-half

of its resting length, either "spontaneously" (i.e. in the absence of known external stimuli) or in response to chemical, mechanical, or electrical stimulation (see Fig. 1).

In this study of contractility in *Spirostomum*, I have taken the approach that was successfully employed in the study of the control mechanisms of striated muscle. The results show that calcium ions are stored in vesicles in

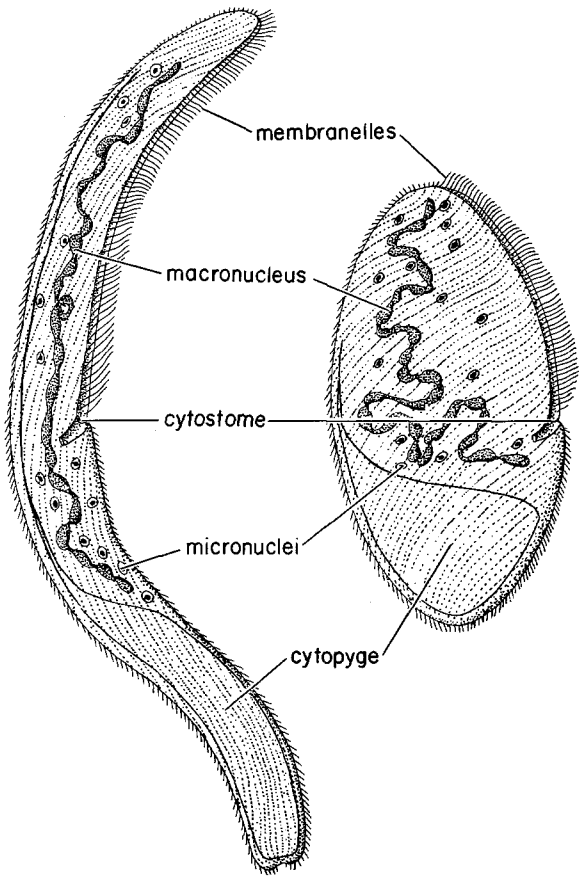

FIGURE 1. Adult *Spirostomum ambiguum*. The organism to the left is representative of the free-swimming form which may grow to 3 mm in length. Contraction to as much as 50% of resting length can be spontaneous or in response to electrical, chemical, or mechanical stimulation.

the inactive organism and that, on stimulation they are released. Apparently calcium plays the same role in the control of contractility in *Spirostomum* that it does in striated muscle.

METHODS

Cell Cultures

Spirostomum ambiguum was cultured on Carter's medium[1] and fed weekly on a mixed bacterial culture. In addition, polyaxenic mass cultures were kept in a medium containing distilled water, sphagnum moss, and barley grains.

Technique of Cell Injection and Stimulation

In order to prepare the organisms for microscopic observation and microinjection, the swimming movements were inhibited by isosmotic calcium-free Carter's medium containing 2.5 % gelatin at room temperature. Brief chilling from 25° to 20°C caused gelation, so that *Spirostomum* were held immobile without detectable injury.

The cells were then injected with aequorin, a bioluminescent protein extracted from the jellyfish, *Aequorea*. Aequorin emits light only in the presence of calcium ions. The light emission is proportional to the aequorin concentration over a wide range of Ca^{++} concentration, and only under special conditions is the rate a measure of Ca^{++} concentration (Shimomura, Johnson, and Saiga, 1962, 1963; Hastings, Mitchell, Mattingly, Blinks, and Van Leeuwen, 1969). After injection the cells were stimulated to contract by exposure to a DC electrical field provided by a Grass stimulator. A current of 1.2×10^{-3} ma was developed between two, parallel, platinum, wire electrodes placed 1 cm apart in a 22 x 50 x 3 mm, glass-bottomed chamber; thus providing a current density during stimulation of 6×10^{-4} ma/cm^2. The duration of stimulation was 20–50 msec determined by presetting the pulse duration on the stimulator and also by reading off as an oscillograph.

Photometric Measurement of Ca-Aequorin Complex

A 1 ml aliquot of an EDTA-buffered solution of aequorin[2] (0.143 mg/ml) was centrifuged, washed, and dissolved in 0.5 ml of 300 mM KCl. Generally, less than 0.2 μl of aequorin dissolved in KCl was microinjected from 3–5 μm tip diameter micropipettes attached to a micromanipulator.

Measurements of photon emission by the free Ca^{++}-aequorin complex were made with an EMI type 9558-B photomultiplier housed in a light-tight box. The tube was operated at −1200 v provided by a Hewlett-Packard 6525-A power supply. The photocurrent was amplified and displayed with gains up to 100 times on a type 565 dual beam oscilloscope. Data from the experiment of Shimomura, Johnson, and Saiga performed with similar methods, indicated that 10^{-6} M Ca^{++} in a small volume could be detected. They also stated that it was possible to make the test 100 times more sensitive (Shimomura et al., 1962).

[1] Carter's medium: 2 mM NaCl, 0.5 mM KCl, 0.5 mM $CaCl_2$, KH_2PO_4, 0.1 mM, and KOH, 0.1 mM to pH 6.3.

[2] Kindly supplied by Dr. O. Shimomura and Professor Frank H. Johnson, Department of Biology, Princeton University.

Electrophysiological Measurements

Resting membrane potentials and membrane resistance measurements were made with glass capillary micropipette electrodes filled with 3 M KCl in contact with a non-polarizable Ag-AgCl electrode. The electrodes were coupled to a high input impedance, negative-capacitance feedback amplifier. The electrical signals were monitored with the same type of oscilloscope that was used to display light emission.

Microscopy

Light microscopic observations and cinefilm recordings of contraction were made with a Zeiss dark-field microscope synchronized with a Milliken high speed camera operated at 400 frames/sec. An additional light flash, synchronized with the stimulating pulse, was used to mark the frame on the film where the electrical stimulus was applied. The accuracy of the time marker was thus ± 2.5 msec. Analysis was made by making drawings from consecutive frames, and the lengths of the animals were plotted against time. Similar studies of contraction-relaxation in *Spirostomum* have been conducted by Jones, Jahn, and Fonseca (1966) and H. Kinosita (1938).

For electron microscope observation, specimens were fixed for 1 hr in 1 % osmium tetroxide in cacodylate buffer at pH 6.3. Dehydration in alcohol was followed by embedding in Epon. Sections were cut on a Reichert ultramicrotome, manufactured by C. Reichert Optische Werke A. G., Vienna, Austria, and observed with an AEI EM-6B electron microscope.

RESULTS

In all organisms, electrically induced contraction resulted in shortening to roughly 50% of resting length. The organisms returned to their original lengths within a period of 500 msec. The threshold stimulus required to induce contraction in organisms in gelatin was a current density about 6×10^{-4} ma/cm^2. The duration of the latent period, the time between the application of an electrical stimulus and the onset of contraction in the calcium-free gelatin medium, ranged from 13 to 25 msec with a mean of 20.5 msec.

Fig. 2 shows sample oscilloscope traces of the photomultiplier output when *Spirostomum* was microinjected with aequorin and subsequently stimulated electrically to contract. The increase in light emission (see Fig. 2 *a*, *b*) indicates an increased formation of the calcium-aequorin complex.

In control experiments with organisms which had not been injected with aequorin, no changes in photocurrent were detected (Fig. 2 *c*) showing that the photocurrent changes in injected cells were not stimulus artifacts. Thus, the observed photomultiplier record is not an electrical artifact due to stimulus interference, but is in fact due to light emission by aequorin. During the latent period between electrical excitation and the initiation of contraction, an increase in photon emission was detected indicating an increase in the concentration of cytoplasmic free calcium (cf. Ridgway and Ashley 1967,

1968). In experiments performed on *Spirostomum* microinjected with aequorin, the rate constants of calcium diffusion into and out of cytoplasm were measured as functions of photon emissions. In *Spirostomum*, the half-time to maximum photon emission is 5 msec, and the time for removal of calcium from the cessation of electrical stimulation is 25 msec. The reaction of calcium

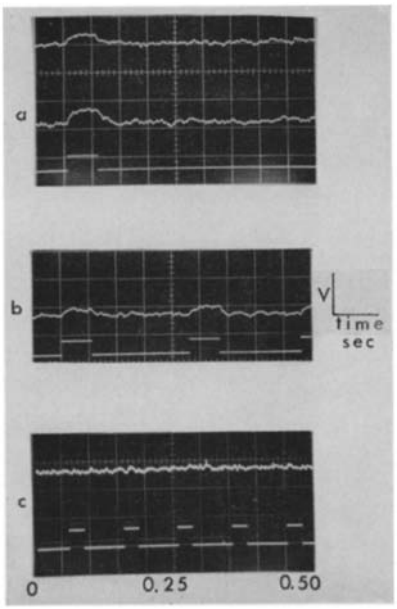

FIGURE 2. Oscillograph recordings of the photomultiplier output induced by luminescence from *Spirostomum* microinjected with aequorin. (*a*) Shows peak output of light 20–30 min after microinjection. A time interval of 3 min exists between the first and second recording. (*b*) Repeated electrical stimulation at a frequency of 175 msec shows no significant diminution between responses. However, response is not as strong as when a single stimulating pulse is applied. (*c*) Control showing that the photomultiplier signals are not some artifact of stimulation since the organisms are not microinjected with aequorin (50 msec/horizontal division and 10 mv/vertical division from each base line).

with aequorin to yield a photon is not precisely stoichiometric; therefore, data on the exact concentration of calcium at peak photon emission cannot be provided. However, since the organism contracts when microinjected with 10^{-5} M calcium chloride, it is assumed that maximum photon emission corresponds to approximately this concentration. The kinetic values for the reaction of calcium and aequorin in *Spirostomum* are in agreement with biochemical kinetic studies of reactions involving calcium and the isolated aequorin molecule (Hastings et al., 1969).

Electrical measurements on eight cells recorded a mean resting potential across the cell membrane of 15 mv negative inside. The mean value of the specific membrane resistance was (25 ± 5.85 SD) $\times 10^4$ ohm.cm^2. In more

than eight separate cells, no measurable depolarization of the cell membrane was detected following the application of electrical stimuli greater than the threshold for mechanical response.

Pretreatment of *Spirostomum* with 10 mM sodium oxalate resulted in a progressive and marked decrease in contractile response to all external stimuli. Electron micrographs of *Spirostomum* taken from fresh cultures, and then treated with 10 mM sodium oxalate before fixation, often revealed multiple precipitating centers of comparatively large, electron-dense, crystalline masses enclosed within membrane-limited vesicles (Fig. 3 *a*, *b*). The situation appears to be similar to that observed in muscle where the calcium concentration in the cytoplasm is too low to form detectable calcium oxalate precipitates; in the vesicles which sequester calcium, deposits of calcium oxalate are plentiful (see review by A. Weber, 1966).

Fig. 3 *b* is a high magnification micrograph of an unstained section showing the crystalline precipitate in *Spirostomum* enclosed within a vesicle membrane. Other calcium precipitates (e.g. hydroxyapatite) are often observed in organisms from cultures that are left unattended for over 2 wk (Bien and Preston, 1968; Pautard, 1959; Jones, 1966, 1967), or in organisms that have been fixed in alkaline, phosphate-buffered, fixatives (Lehman and Rebhun, 1969). These other precipitates consist of smaller crystals and fewer are found within each vesicle. Control organisms from fresh cultures, which had not been pretreated with oxalate, failed to show the characteristic, dense, oxalate, precipitates (Fig. 3 *c*).

DISCUSSION

It is evident from studies on muscle and several other contractile systems that calcium must be present in concentrations approaching 10^{-5} M in order for contraction to occur. Various calcium chelators and precipitators, such as EDTA, EGTA, citrate, and oxalate, have been used to show that the contractile response fails when the concentration of calcium is held constant below 10^{-5} M (A. Weber, 1966).

When *Spirostomum ambiguum* is placed in calcium-free media, spontaneous contractions may occur as they do in organisms in normal media. Furthermore, electrical stimuli still bring about contractions in calcium-free media. In the light of these observations, it is unlikely that a calcium-dependent contraction could be governed solely by the passage of calcium ions across the cell membrane of the organism; in addition, the cell membrane appears to consist of two paired unit membranes (Finley, Brown, and Daniel, 1964) with a large specific membrane resistance. If the high specific membrane resistance represents a permanent state of the cell membrane, then calcium fluxes across the cell membrane would be too slow to account for the rapid changes in Ca^{++} concentration during the contraction-relaxation cycle in

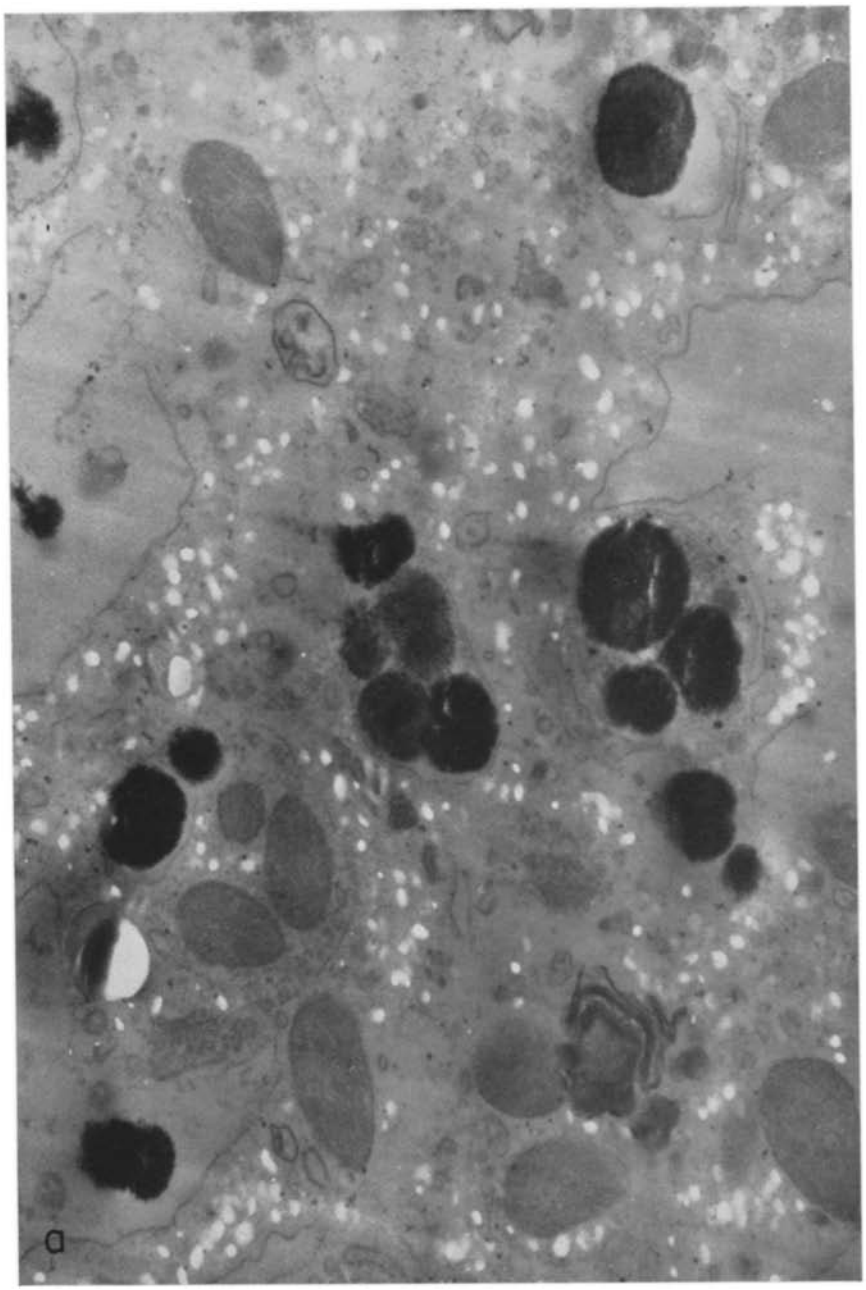

FIGURE 3 *a*. Electron micrograph of an unstained section of *Spirostomum* which has been treated with 10 mM sodium oxlate. The crystalline precipitates are typical of those found within vesicles of oxalate-treated organisms. × 15,000. 3 *b*. High magnification of oxalate precipitates showing radial symmetry characteristic of calcium precipitates and vesicular membrane. × 30,000. 3 *c*. Absence of electron-dense inclusions in an unstained section of *Spirostomum* from a culture which had not accumulated dense inclusions nor been treated with oxalate. × 12,500.

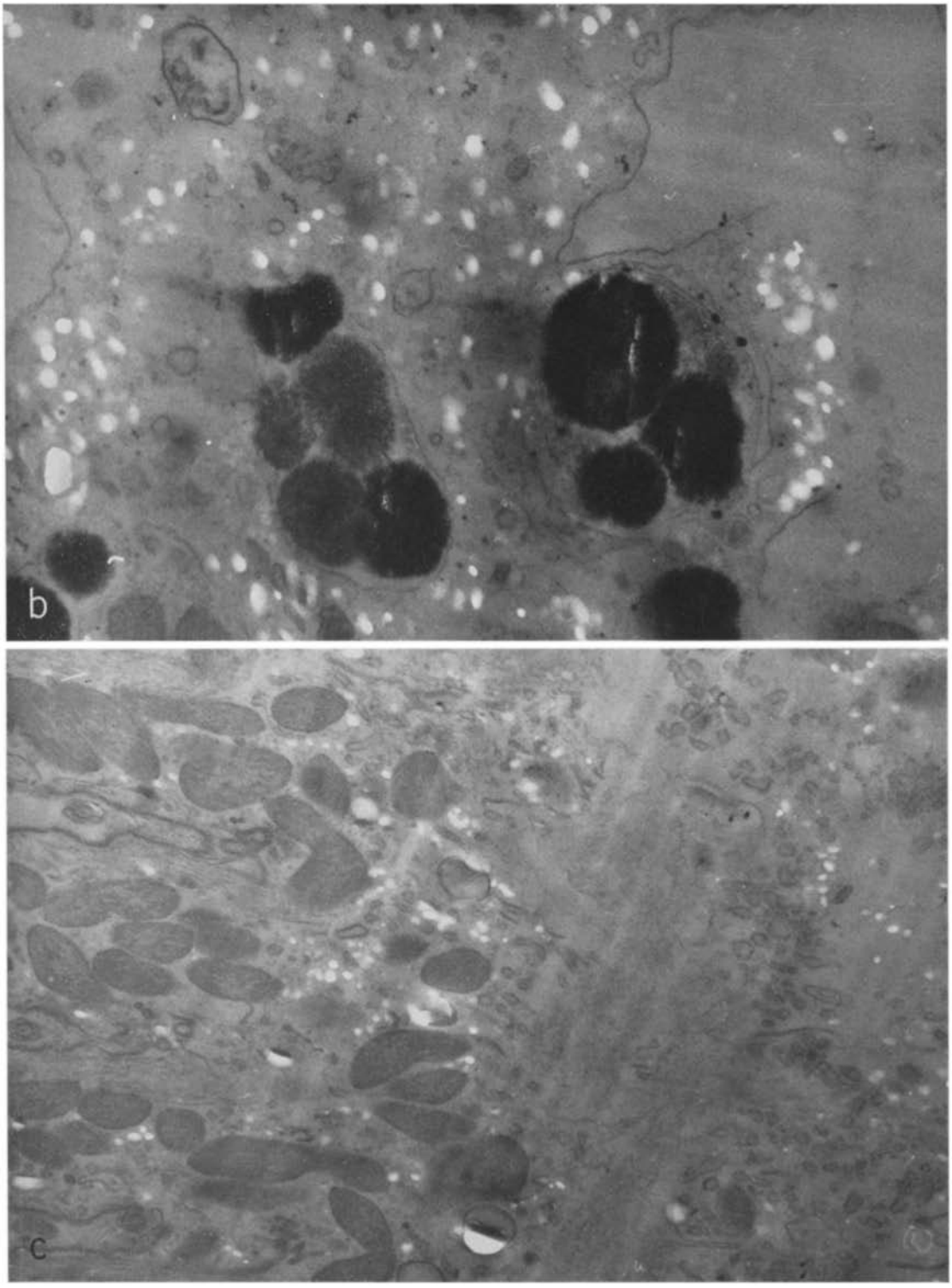

FIGURE 3 *b* and *c*.

Spirostomum. It is therefore likely that the organism has evolved an internal depot from which calcium can be released, in the required amounts over the required time interval, to the contractile elements.

Ultrastructural studies of *Spirostomum* have revealed the presence of many vesicles which were shown in the present study to, presumably, accumulate calcium oxalate precipitate. These are closely associated on the one hand with

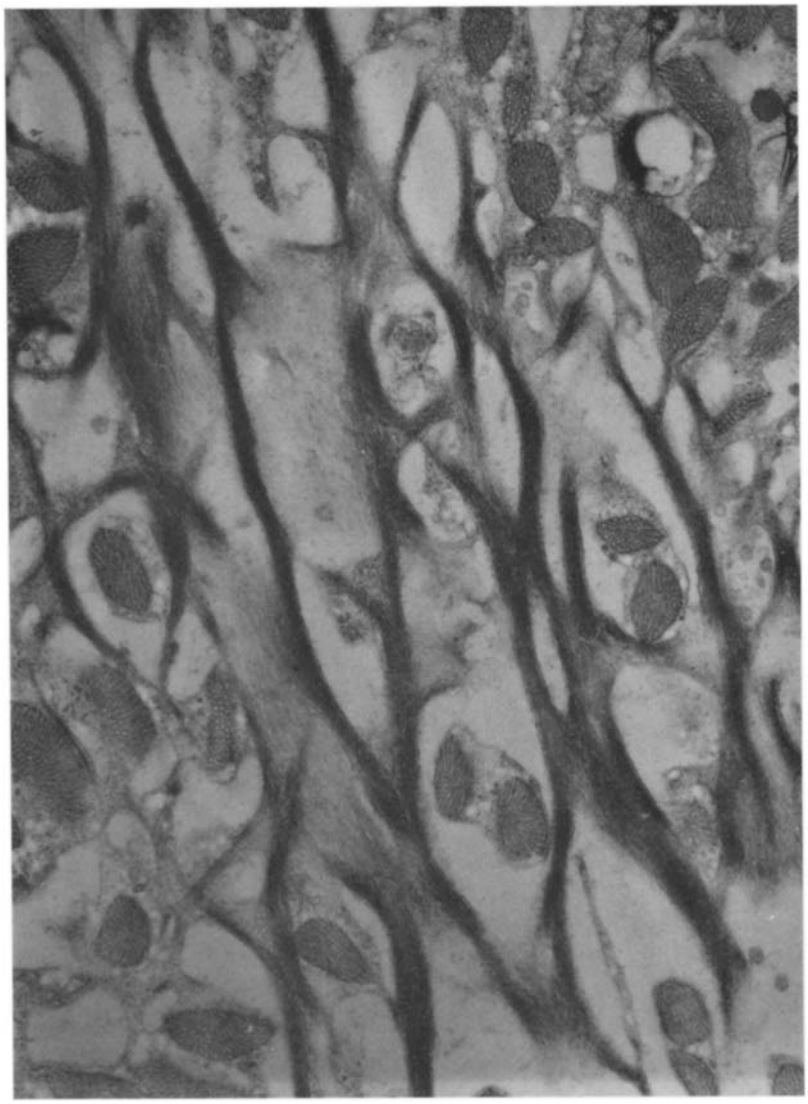

FIGURE 4. Tightly packed 40–50 A filaments form a loose network which appears to extend around the organism. Numerous mitochondria and membrane-limited vesicles exist in close proximity to the filaments. × 14,000.

a subpellicular network of filaments running roughly parallel to the axis of contraction, and on the other with numerous mitochondria (Fig. 4; see Lehman and Rebhun, 1969; Yagiu and Shigenaka, 1963). This situation is similar to that in muscle, where it has been established that the sarcoplasmic reticular vesicles serve to regulate the levels of sarcoplasmic free calcium as a means of controlling contractility (Jobsis and O'Connor, 1966).

If the vesicles store and release free calcium as a means of controlling contractility, then an increase in free calcium should be detectable in the cytoplasm in the interval between excitation and contraction.

Prolonged latent periods in calcium-free media are most likely manifestations of lowered ambient levels of cytoplasmic free calcium, established by the equilibration of calcium ions across the cell membrane (Jones et al., 1966). It is understandable under these conditions that, if the activation of contractility is accomplished by an increase in the concentration of calcium, it would take a comparatively longer time to establish a threshold concentration via an internal calcium-release mechanism. Fig. 5 shows the relationship between maximum latency to contraction and maximum photon emission, demonstrating that contraction is initiated only when the calcium mediated photon emission, and therefore calcium ion concentrations, is maximal.

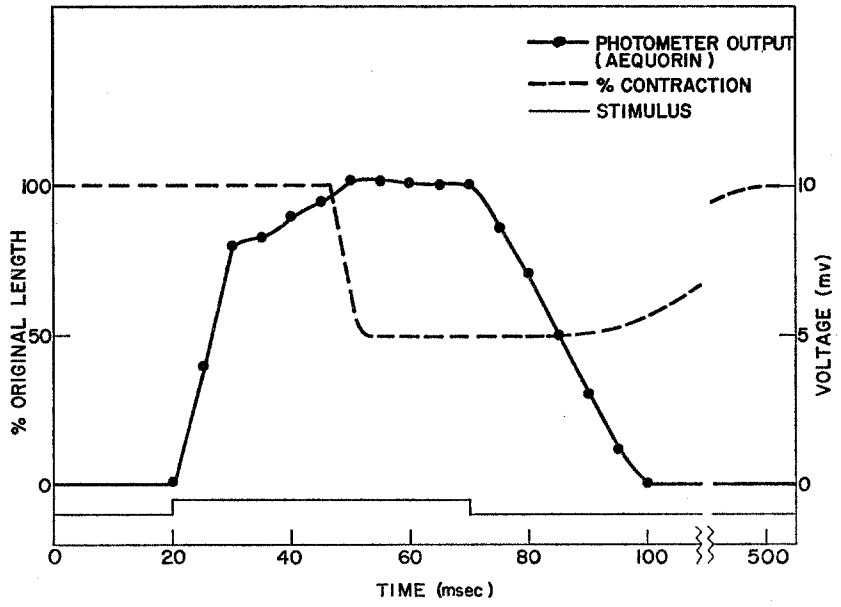

FIGURE 5. Graph shows the relationship among the onset of electrical stimulation, photon emission, and maximum latency to contraction. Mechanical contraction begins when aequorin-calcium induced photon emission is at a peak. Photon emission drops off and relaxation ensues with the cessation of electrical stimulation.

An anterioposterior, morphogenetic, sensitivity gradient has been described for organisms treated with various chemical agents (Seravin, 1962). According to Jones et al. (1966), electrically induced contraction always begins at the anodal end of the organism with the cathodal end lagging 2–3 msec behind. Contraction and ciliary reversals are the most readily detectable effects of disruption of the gradient, and Seravin (1962) speculated that they too are under the control of an "endoplasmic physiological regulating mechanism" sensitive to subtle ionic changes within the gradient.

The spontaneous contractions, which are commonplace in normal organisms, might be regulated by the generation of unstable potentials along the surfaces of internal membranes which are continuous within the gradient. Under these conditions, local depolarizations of internal membranes could occur with accompanying fluxes of calcium from the vesicles. On occasion bursts of internal spontaneous hyperpolarizing and depolarizing spikes were observed.

On the basis of the experiments performed and discussed in this paper, it is proposed that contraction in *Spirostomum ambiguum* is controlled by an internal vesicular system, analogous with the sarcoplasmic reticulum of striated muscle, which regulates the levels of cytoplasmic free calcium. Under the proper ionic conditions, the vesicular network depolarizes and releases calcium which initiates the generation of tension by the contractile machinery of the cell.

Supported by a Program Grant POIGM 14891–03 from the Institute of General Medical Science of the National Institutes of Health.

This investigation was carried out under the supervision of Professor Robert D. Allen with technical advice and assistance from Professors Charles Edwards and Robert Rikmenspoel and Dr. S. M. McGee-Russell.

I am much indebted to Dr. William Lehman for demonstrating his technique of isometric fixation prior to its publication.

Received for publication 19 September 1969.

REFERENCES

BIEN, S. M., and F. B. PRESTON. 1968. Calcification of *Spirostomum ambiguum*. *J. Protozool.* **15**:251.

FINLEY, H. E., C. A. BROWN, and W. H. DANIEL. 1964. Electron microscopy of the ectoplasm and infraciliature of *Spirostomum ambiguum*. *J. Protozool.* **2**:264.

HASTINGS, J. W., G. MITCHELL, P. MATTINGLY, J. BLINKS, and M. VAN LEEUWEN. 1969. Response of aequorin bioluminescence to rapid changes in calcium concentration. *Nature (London).* **222**:1047.

HEILBRUNN, L. V. 1940. The action of calcium on muscle protoplasm. *Physiol. Zool.* **13**:88.

HEILBRUNN, L. V., and F. J. WIERCINSKI. 1947. The action of various cations on muscle protoplasm. *J. Cell Comp. Physiol.* **29**:15.

JOBSIS, F. F., and M. J. O'CONNOR. 1966. Calcium release and absorption in sartorius muscle of the toad. *Biochem. Biophys. Res. Commun.* **25**:246.

JONES, A. 1966. Uptake of Ca^{45} by *Spirostomum ambiguum*. *J. Protozool.* **13**:422.

JONES, A. 1967. Calcium and phosphorus accumulation in *Spirostomum ambiguum*. *J. Protozool.* **14**:220.

JONES, A., T. JAHN, and J. R. FONSECA. 1966. Contraction of protoplasm. I. Cinematographic analysis of the anodally stimulated contraction of *Spirostomum ambiguum*. *J. Cell Physiol.* **68**:127.

KINOSITA, H. 1938. Electrical stimulation of *Spirostomum*. *J. Fac. Sci. Univ. Tokyo. Sect. IV. Zool.* **5**:71.

LEHMAN, W., and L. REBHUN. 1969. The structural elements responsible for contraction in the ciliate *Spirostomum*. *J. Cell Sci.* In press.

PAUTARD, F. 1959. Hydroxyapatite as a developmental feature of *Spirostomum ambiguum*. *Biochim. Biophys. Acta.* **35**:33.

RIDGWAY, E. B., and C. C. ASHLEY. 1967. Calcium transients in single muscle fibers. *Biochem. Biophys. Res. Commun.* **29**:229.

SERAVIN, L. N. 1962. Physiological gradients in the infusarium *Spirostomum ambiguum*. *Tsitologiya.* **4**:545.

SHIMOMURA, O., F. JOHNSON, and Y. SAIGA. 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J. Cell. Comp. Physiol.* **59**:223.

SHIMOMURA, O., F. JOHNSON, and Y. SAIGA. 1962. Microdetermination of calcium by aequorin luminescence. *Science* (*Washington*). **140**:1339.

SHIMOMURA, O., F. JOHNSON, and Y. SAIGA. 1963. Further data on the bioluminescent protein, aequorin. *J. Cell. Comp. Physiol.* **62**:1.

WEBER, A. 1966. Energized calcium transport and relaxing factors. *In* Current Topics in Bioenergetics. D. R. Sanadi, editor. Academic Press, Inc., New York, 203.

WILKIE, D. R. 1968. Muscle. St. Martin's Press, New York.

YAGIU, R., and Y. SHIGENAKA. 1963. Electron microscopy of the longitudinal fibrillar bundles and the contractile fibrillar system in *Spirostomum ambiguum*. *J. Protozool.* **10**:364.