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thesis entitled

THE ROLE OF CALCIUM AND OTHER CATIONS IN SENSORY TRANSDUCTION AND HABITUATION IN THE CILIATED PROTOZOAN SPIROSTOMUM AMBIGUUM

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biophysics

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Date May 22, 1981

O-7639

THE ROLE OF CALCIUM AND OTHER CATIONS IN SENSORY TRANSDUCTION AND HABITUATION IN THE CILIATED PROTOZOAN *SPIROSTOMUM AMBIGUUM*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Biophysics Department

1981

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Repeated mechanical (vibratory) but not electrical stimulation (rate 0.1 Hz) of the ciliated protozoan *Spirostomum ambiguum* leads to a decrement in the probability (habituation) of the contractile response. Habituation is a simpler form of learning/memory. The growth medium for *Spirostomum* contains the chloride salts of Na, K, Mg, and Ca and a pH buffer. Calcium appears to be an important ion in the stimulus transduction and habituation processes. The present experiments were undertaken to investigate further the role of calcium and other cations in these processes. The effects of the following cations were studied: Na^+, K^+, Mg^{2+}, Ca^{2+}, Li^+, Cs^+, Rb^+, Ni^{2+}, Zn^{2+}, Ba^{2+}, Sr^{2+}, Co^{2+}, Mn^{2+}, and La^{3+}; in addition pharmacological agents which alter membrane permeability to calcium or affect calcium binding (A23187, caffeine, and verapamil) were tested for behavioral effects on *Spirostomum*.

Reducing extracellular calcium levels decreased responsiveness to both modes of stimulation. Responsiveness to electrical stimulation was reduced by raising the concentrations of Na^+, K^+, or

Mg^{2+} in the culture medium while increased $[K^{+}]$ and replacement of Ca^{2+} with Ba^{2+} or Mn^{2+} reduced responsiveness to mechanical stimulation.

A fast Ca-45 labeling component was observed in stimulated and unstimulated Spirostomum. Electrical stimulation resulted in increased Ca-45 uptake compared to the control and the amount of uptake was dependent on extracellular $[Ca^{2+}]$. Some indication of Ca-45 uptake in response to mechanical stimulation was observed but this result was not statistically significant. The calcium ionophore A23187 and PO_4^{2-} both increased Ca-45 uptake in unstimulated Spirostomum.

A response decrement to electrical stimulation was observed in low $[Ca^{2+}]$ media; surprisingly no change in decrement level occurred in response to mechanical stimulation in the same media. Replacement of calcium with Ba^{2+}, Sr^{2+}, or Zn^{2+} also resulted in a decrement to electrical stimulation even though Ba^{2+} and Sr^{2+} often substitute for Ca^{2+} in many physiological processes. The presence of Ba^{2+} or Zn^{2+} reduced habituation levels to mechanical stimulation; caffeine also removed habituation. La^{3+} and verapamil, a calcium blocking agent, tended to increase the amount of habituation to mechanical stimulation.

The results show that calcium is necessary for the transduction of mechanical and electrical stimuli and may play a role in the response decrement process. The differing responses to mechanical and electrical stimulation in the presence of the same ions suggest that the mechano- and electro-transduction sites are separate.

With respect to the role of calcium it is not clear whether calcium acts by a binding or an uptake mechanism; the present results give support to both mechanisms. The results are compatible with a model where two separate calcium channels are responsible for sensory transduction and habituation is due to reduced calcium entry through the mechanoreceptor channel. The habituation process may involve regulation of calcium permeability by a metalloenzyme. This work suggests habituation in neural and aneural systems may have similar mechanisms.

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INTRODUCTION

Although extensively studied, the molecular mechanisms of learning have thus far eluded neuroscientists. In complex nervous systems much of the problem has to do with localization of the event and accessibility of the preparation; this has led to the use of model systems. It is also advantageous to eliminate complexity in the behavior to be studied. Thus the study of simpler forms of learning and memory in model systems can be a useful approach to understanding mechanisms of learning and memory.

The work described here aims at obtaining an understanding of habituation, a simpler form of learning and memory, at a cellular level. Habituation is defined as a response decrement to an intermittent but repetitive stimulus. A major problem in studying the molecular basis of any kind of learning and memory is in finding a "simple" enough preparation in which one can locate and isolate the relevant changes. Most nervous systems are too complex. Neural elements involved in a particular learned behavioral act are not easily identified and it is often difficult, if not impossible, to assess the relative contributions to such behavior of intraneuronal vs. interneuronal (i.e., connectivity) factors. If one views the functions carried out by nervous systems as having both intracellular as well as intercellular components then one might view such work on aneural single cells as a strategy to focus on the intracellular

components uncomplicated by intercellular considerations. The protozoa (in particular the ciliates) would appear very promising for such studies. All of the activity to be observed occurs in one cell, which contains all of the sensory, motor, and integrative aspects of the behavior. Thus, in addition to being a single cell it is also an entire organism. The single-celled organisms allow unrestricted access to the cell and its immediate environment and thus allow the use of techniques which require access to the cell, e.g., being able to maintain and alter in a controlled fashion the ionic milieu of the cell. Thus, a single-celled organism allows cellular experiments on habituation to be performed more easily than on nervous systems such as in Aplysia. This study examines habituation in the large ciliated protozoan, Spirostomum ambiguum. This one-celled organism exhibits habituation of its contractile response to repeated mechanical (vibratory) but not to electrical stimuli (Osborn, Blair, Thomas, and Eisenstein 1973a).

One interpretation of the significance of habituation in higher animals is that it represents a mechanism that the nervous system has evolved for filtering out unimportant inputs. Compared to associative and Pavlovian conditioning, habituation is probably the simplest and certainly the most ubiquitous form of learning; it occurs in all animal phyla from single-celled aneural organisms such as the ciliated protozoa (Applewhite and Morowitz 1966; Applewhite 1968a; Applewhite, Gardner, and Lapan 1969; Wood 1970a, 1973; Osborn et al. 1973a) through mammals (Groves and Thompson 1970; Thompson, Groves, Teyler, and Roemer 1973). The characteristics of the

habituation phenomenon are also remarkably similar throughout phylogeny (Thompson and Spencer 1966; Eisenstein and Peretz 1973).

Many of the phenomena studied in neurobiology are not unique to nervous tissue but also occur in aneural systems (e.g., conduction of potentials and sensory transduction in protozoa [see Eisenstein 1975]). There also are striking similarities across phylogeny of many metabolic pathways as well as in membrane structure and function. It is therefore reasonable to expect that there also may be similarity in the mechanisms underlying related behavioral phenomena such as habituation and sensory transduction in neural and aneural systems.

Previous work suggests that habituation is closely tied to the mechanotransduction process in Spirostomum; accordingly this work also attempts to describe molecular mechanisms involved in the transduction of stimuli, especially mechanical stimuli. Studying cellular aspects of mechanotransduction has been difficult in higher systems due to a variety of problems, such as the small size of the receptor cell and the relative inaccessibility of the receptor region. Accessibility may be more easily achieved in Spirostomum. Its large size (1 to 3 mm in length) make it especially attractive for single cell behavioral, molecular, and cellular studies.

In summary then, this study is concerned with the earliest cellular representation of the transduction of mechanostimuli. The goal is to understand how an external stimulus event is converted into its biological representation (transduction) and then coupled to the motor output and further how this coupling changes over time (habituation). The focus is on the nature of both the transduction

and habituation mechanisms, i.e., what they are, where they are located, and what changes occur with time and stimulation.

Background

Many ciliates, including Spirostomum, have two motor systems--contractile and ciliary. The latter is involved in locomotory behavior. Spirostomum ambiguum (Figure 1) contracts to approximately 50% of its resting length in less than 5 milliseconds when stimulated electrically (Jones, Jahn, and Fonseca 1966; Hamilton and Eisenstein 1969; Hamilton and Osborn 1977). Similar rapid contractions occur in response to mechanical (Hamilton and Osborn 1977) as well as photic

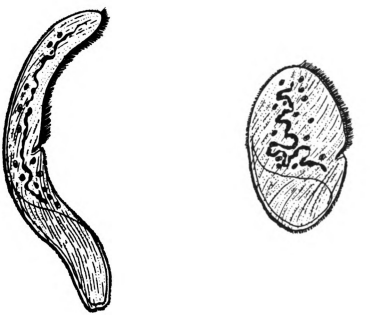

Figure 1.--Drawing (not to scale) of the free-swimming and contracted forms of Spirostomum ambiguum. This ciliated protozoan grows to lengths of 1-3 mm and can contract to less than 50% of this length in a few milliseconds.

stimuli (Borsellino, Cavazza, and Riani 1971). Habituation occurs in response to repeated mechanical stimulation[1] but not to repeated electrical stimulation (Osborn et al. 1973a). This latter result rules out the possibility that habituation occurs on the motor, i.e., contractile, side of the behavior since the same response apparatus is used for both mechanical and electrical stimulation but only the former leads to habituation; thus habituation is not fatigue of the contractile apparatus. The habituation process must occur between the initial mechanotransduction step and the initiation of contraction (Applewhite et al. 1969; Osborn et al. 1973a). The response of Spirostomum to mechanical and electrical stimulation is stimulus specific (Osborn et al. 1973a), i.e., mechanical stimuli do not affect the responsiveness to electrical stimuli while electrical stimulation has only a small effect on responsiveness to mechanical stimulation (Applewhite et al. 1969; Osborn et al. 1973a). Stimulus specific habituation also has been observed in the closely related protozoans, Stentor (Wood 1970a, 1973) and Vorticella (Patterson 1973), as well as in higher organisms (Thompson et al. 1973). In addition, habituation shows generalization from the point at which the ciliate is stimulated to its entire surface (Wood 1972).

Calcium, in contrast to most other ions in a cell's normal environment, is often involved in control processes (Chang and Triggle 1973; Rubin 1974; Carafoli, Clementi, Drabikowski, and Margreth 1975;

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[1]See Kinastowski (1963a,b) for extensive studies on the effects of mechanical stimulus parameters (intensity, frequency, number of trials) on habituation in Spirostomum.

Rasmussen 1975; Doughty and Diehn 1979). In higher systems calcium is important in muscle contraction (Ebashi 1972; Smith 1977; Scarpa and Carafoli 1978), vision (Hagins and Yoshikami 1974), synaptic transmission (Katz and Miledi 1967; Triggle 1972; Miledi 1973; Rahamimoff, Erulkar, Alnaes, Meire, Rotshenker, and Rahamimoff 1976; Weller and Morgan 1977), and secretion (Matthews 1970; Rubin 1974; Scarpa and Carafoli 1978). It also is involved in the regulation of cyclic nucleotide synthesis (Rasmussen 1975). In lower systems calcium has a regulatory role in bacterial chemotaxis (Ordal 1977) and in the ciliary reversal process in Paramecium (Grebecki 1965; Kuznicki 1966, 1970, 1973; Naitoh 1968; Naitoh and Eckert 1969; Eckert 1972).

Using the calcium-sensitive photoprotein aequorin, Ettienne (1970) showed that contraction of Spirostomum is preceded by an increase of cytoplasmic free calcium concentration; contractions also have been elicited by injection of EGTA-buffered calcium solutions at concentrations greater than 10^{-5} M (Hawkes and Holberton 1974) and in calcium activated models (Ettienne 1976). Although it appears that ATP is not required for contraction (Seravin, Skoblo, and Bagnjuck 1965), some aspect of mitochondrial metabolism would appear to be important in the regulation of intracellular calcium in Spirostomum, based on the action of various pharmacological agents which inhibit mitochondrial respiration and affect the contractility of Spirostomum (Ettienne and Selitsky 1974; Holberton and Ogle 1975; Dikstein and Hawkes 1976). The existence of calcium in the mitochondria of Spirostomum has been confirmed by electron microprobe analysis

(Osborn, Hsung, and Eisenstein 1973b; Osborn and Hamilton 1977). In higher forms mitochondria prefer calcium uptake to oxidative phosphorylation; the calcium is apparently stored as a calcium-phosphate compound (Lehninger 1970; Carafoli and Crompton 1976). Based on studies utilizing Ca-45 and P-32, Spirostomum actively accumulates both ions in the cytoplasm (Jones 1966, 1967; Bai and Dilly 1976; Balcerzak 1978) and at least a portion is stored in the form of hydroxyapatite (Pautard 1959; Bien and Preston 1968) which is found in the endoplasmic reticulum of Spirostomum (Osborn et al. 1973a; Osborn and Hamilton 1977). Calcium control of contractility also has been observed in other peritrich ciliates where intracellular calcium is stored in the endoplasmic reticulum and possibly released by a linkage structure between the reticulum and the myonemes (Allen 1973), the tightly packed bundles of microfilaments responsible for contraction (Lehman and Rebhun 1971). Vesicles, possibly containing calcium, have been observed with the electron microscope near the myonemes in Spirostomum (Yagiu and Shigenaka 1963; Finley, Brown, and Daniel 1964; Vivier, Legrand, and Petitpriz 1969; Legrand and Prensier 1976), but no linkage structures between vesicles and myonemes have been reported.

Calcium may be involved in the sensory transduction process as well as the contractile process. For example, previous work shows that the addition of EGTA to the medium in which Spirostomum swims results in a decreased probability of contraction to mechanical but not to electrical stimulation (Osborn et al. 1973b). In Paramecium responses to changes in hydrostatic pressure (which is probably the

direct stimulus Spirostomum receives during mechanical [vibratory] stimulation) are apparently mediated by calcium channels in the plasma membrane (Otter and Salmon 1979). In addition, it appears there may be cholinergic sites involved in sensory transduction in some ciliates (Wood 1975, 1977; Doughty 1978).

Kandel (1976, 1978, 1979) suggests that habituation in the gastropod mollusc Aplysia is due to decreased free calcium concentration in the sensory presynaptic terminals; he postulates that this lowered calcium level is caused either by a decreased calcium influx or increased mitochondrial calcium uptake. The sequence of steps occurring at the site of habituation in a ciliate may be analogous to the presynaptic steps occurring at the site of habituation in nervous systems, e.g., Aplysia.

The normal culture medium of Spirostomum contains the chloride salts of sodium, potassium, magnesium, and calcium (Carter 1957) and a HEPES-PIPES pH buffer. Ions other than calcium also may be involved in the transduction and habituation processes. For example, Applewhite and Davis (1969) found that Mg^{2+} affects habituation as well as responsiveness to mechanical stimulation. Various ions have been found to increase spontaneous contractility (Sleigh 1969, 1970) and increase the threshold to electrical stimulation (Fabczak 1974). Possibly calcium is involved in the regulation of the permeability of Spirostomum to other ions, as it is in a variety of other systems, e.g., nerve and muscle tissue in the mollusc, arthropod, and vertebrates (Frankenhaeuser and Hodgkin 1957; Triggle 1972; Rubin 1974; Meech 1976; Ohki 1978; Schultz and Heil 1979).

The ciliary motor system of protozoa has received much study in recent years. The role of calcium is well defined in the ciliary reversal process of Paramecium (Naitoh 1968, 1974; Naitoh and Eckert 1969; Eckert 1972; Sakai and Hiramoto 1975; Machemer 1976; Machemer and dePeyer 1977; Eckert and Brehm 1979). When the anterior end of Paramecium is mechanically stimulated, calcium enters resulting in ciliary reversal (Ogura and Machemer 1979). A stimulus to the posterior end causes potassium to enter, triggering an increase in the ciliary beat leading to forward movement (Naitoh and Eckert 1969; Eckert 1972). The process seems to be controlled by an ion exchange process on the membrane of Paramecium (Jahn 1962; Grebecki 1965; Kuznicki 1966, 1970; Naitoh and Yasamasu 1967; Naitoh 1968). Reversal is activated when the intraciliary calcium level is greater than 10^{-8} to 10^{-6} M (Naitoh and Kaneko 1972; Sakai and Hiramoto 1975). Since calcium-dependent electrical responses are eliminated by deciliation (Ogura and Takahashi 1976; Dunlap 1977), calcium may enter via channels in the ciliary membrane. Calcium binding sites also have been localized at the base of the cilium in Paramecium (Fisher, Kaneshiro, and Peters 1976; Tsuchiya and Takahashi 1976). Machemer and Ogura (1979) found that mechanoreceptor channels in Paramecium are distributed over the somatic (nonciliary) membrane. Kuznicki (1973) provides evidence arguing against the involvement of extracellular calcium in the ciliary reversal process, thus supporting the possibility the calcium may come from a membrane-bound source.

Electrophysiological studies of Spirostomum have not been particularly successful. Resting potentials of approximately -16 mv

(inside negative) have been reported by Ettienne (1970) and Hawkes and Holberton (1974). Kokina (1972) claims to have measured resting potentials varying from -37 mV to 47 mV. Jahn (1966) reported no potential changes accompanying contraction. Intracellular recording with glass micropipettes is very difficult with this animal; they are far too agile and contractile. Without extensive development intracellular electrophysiological studies are not likely to contribute to the understanding of habituation and mechanotransduction in this organism.

Intracellular recordings have been made from both Paramecium and Stentor (Paramecium shows little or no contraction and Stentor, although contractile like Spirostomum, will attach to a site and remain fixed when impaled). Eckert, Naitoh, and Friedman (1972) and Ogura and Machemer (1979, 1980) found a receptor potential in Paramecium associated with mechanical stimulation; Wood (1970b,c, 1980) recorded a receptor potential in Stentor in response to mechanical stimulation which did not occur to electrical stimulation and which became progressively smaller with repeated mechanostimulation in correlation with habituation of the contractile response (no habituation of the contractile response occurred to electrical stimulation in Stentor just as it does not for Spirostomum). Receptor potentials also have been observed during chemokinesis in Paramecium (Van Houten 1979).

Several approaches have been used to study the basis of habituation of the contractile response to mechanical stimulation in Spirostomum. Applewhite and co-workers, for example, studied RNA and

protein synthesis and failed to find evidence that they were involved in habituation to mechanical stimulation (Applewhite and Gardner 1968; Gardner and Applewhite 1970b; Applewhite 1970). Low temperatures were found to improve retention of habituation but had no effect on the rate or amount of habituation itself (Applewhite 1968b,c; Gardner and Applewhite 1970a).

Specifically, this work involves studying the role(s) of calcium in mechanotransduction and habituation. This is accomplished by: (1) altering calcium levels in the media, including calcium buffering with EGTA; (2) measuring calcium uptake in behaving Spirostomum using a radiolabeled calcium species (Ca-45); (3) replacing calcium with or adding other divalent cations; and (4) applying pharmacological agents which alter membrane permeability to calcium or affect calcium binding, e.g., A23187, caffeine, lanthanum, and verapamil. The effects of altering other ions composing the Carter's medium in which the animals are cultured also is investigated. In addition, deciliation is attempted to explore the role of the cilia in the transduction process. The results of the present study should aid in the characterization of the molecular events occurring during sensory transduction and behavior modification in the ciliated protozoan, Spirostomum ambiguum.

METHODS

Protozoan Cultures

Spirostomum were obtained from Connecticut Valley Biological Supply Company (Southampton, Massachusetts) and cultured in an aqueous medium containing the chloride salts of potassium, sodium, magnesium, and calcium in glass doubly-distilled water; the concentrations used were: KCl - 0.5 mM, NaCl - 2.0 mM, $MgCl_2$ - 0.2 mM, $CaCl_2$ - 0.5 mM, and a 2.5 mM HEPES-PIPES[2] pH buffer (Carter 1957). The pH of the medium was approximately 7.2. Heat-killed wheat seeds were added to support bacteria upon which Spirostomum feed. The protozoans were recultured every 7-21 days. All cultures were grown at room temperature (23-25°C) with continual exposure to room light.

Stimulation and Data Analysis

For behavioral studies Spirostomum were tested in groups of four and contractions observed through a dissecting microscope positioned over the slide in which the protozoans swim (Osborn et al. 1973a). Following transfer to the microscope slide a rest period of at least 5 minutes was given prior to stimulation. Mechanical stimuli were applied by dropping the core of a solenoid on the slide. Electrical stimulation was provided by 2 millisecond biphasic pulses delivered across two 1 cm lengths of .002" silver wire on each side

[2]HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid;
PIPES: Piperazine-N,N'-bis-(2-ethanesulfonic acid).

of the slide well or across platinum plated on opposing sides of the well; the pulses were generated by a Grass S-8 stimulator and SIU-4678 isolation units. Voltages supplied to the slide well ranged from 10 to 75 volts; the stimulus delivered to the animal depends on the size, shape, and contents of the slide well as well as the orientation of the animal to the electrodes. Stimulus intensities were chosen to obtain probabilities of contraction between 0.70 and 0.80 for one minute of electrical or mechanical stimulation in the control Carter's medium. Stimuli were presented at a rate of 0.1 Hz for varying periods of time (usually 2 or 10 minutes). To minimize variability the number of contractions for 6 trials (one minute of stimulation) was combined and expressed as a probability. The probability of contraction for minute 1 of the stimulation period is considered an index of the efficacy of transduction. The amount of habituation was calculated by subtracting the probability of contraction for the final minute of stimulation from that for minute 1; this difference was then divided by the probability for minute 1 to reduce the influence of the starting level of responsiveness and was expressed as a percent. Recovery was measured by testing responsiveness for one minute after a short (4 minute) rest period; it was expressed as the percent of the decrement for which responsiveness has returned. The data were examined by analysis of variance and the least significant difference (LSD) multiple range test was used to locate significant effects. By testing in groups of 4 and using a dichotomous measure (contractions were scored yes-no) the assumptions of analysis of variance can be met (J. L. Gill, personal communication).

Correlation and covariance analysis also were performed to evaluate the relationship of the date and time of the experiment and the age of the culture of Spirostomum to the responsiveness, response decrement, and recovery parameters. All statistical analyses were performed with the CDC Cyber 750 computer at Michigan State University using the Statistical Package for the Social Sciences (SPSS).

Effects of Ions

Calcium

The effects of extracellular calcium on sensory transduction and habituation were investigated by incubating Spirostomum for 15 minutes in media containing several different concentrations of calcium and EGTA[3] (a calcium buffer); following incubation the animals were stimulated for 2 minutes to measure responsiveness and response decrement. The following concentrations of calcium and EGTA were used: (1) 0.5 mM Ca + 1.0 mM EGTA, (2) 0 Ca + 0.1 mM EGTA, (3) 0.5 mM Ca + 0.5 mM EGTA, (4) 0 Ca + 0 EGTA, (5) 0.25 mM Ca + 0 EGTA, (6) 0.5 mM Ca + 0.25 mM EGTA, (7) 0.5 mM Ca + 0 EGTA (control), and (8) 0.75 mM Ca + 0.25 mM EGTA. Free calcium and magnesium concentrations were calculated using the successive approximation method of Portzehl, Caldwell, and Ruegg (1964) and assuming calcium and magnesium contamination levels of 5 μM (Table 1). Atomic absorption spectroscopy indicates calcium-free Carter's media contains about 1 μM Ca; this level does not include calcium contamination due to the

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[3]Ethyleneglycol-bis-(β-amino-ethyl ether)-N,N'-tetra acetic acid.

slide or calcium that is transferred with the animals. The calculated free calcium concentrations can be considered maximal levels.

Other Ions

The effects of several monovalent and divalent cations were investigated by the addition, removal, or replacement of the ion in the Carter's medium. Non-toxic concentrations were chosen on the basis of 24 hour tests. Only Zn^{2+} and Ni^{2+} were toxic at levels equal to that of calcium (0.5 mM). Replacements were as follows: Li^{+} replaced Na^{+}; Rb^{+} and Cs^{+} replaced K^{+}; Sr^{2+}, Ba^{2+}, Mn^{2+}, and Co^{2+} replaced Ca^{2+}; Zn^{2+} and Ni^{2+} replaced a portion of the Ca^{2+} in the Carter's medium. Spirostomum were stimulated at a rate of 0.1 Hz for 11 minutes followed by a 4 minute rest and another minute of stimulation to test recovery.

Ca-45 Uptake

Uptake of calcium by behaving Spirostomum was measured using Ca-45. The animals were first centrifuged, dialyzed, and starved for four days in clean Carter's medium to remove bacteria (Jones 1966). Ca-45 (New England Nuclear, Boston, Massachusetts) was added to a large-welled slide containing either 50 or 500 Spirostomum; the final Ca-45 activity was approximately 1 μC/ml. The organisms were then stimulated or rested (controls) for periods of 2, 5, 10, 30, and 60 minutes. To increase the sensitivity of the technique the 60 minute incubation was in calcium-free Carter's medium. Upon addition of the Ca-45 the calcium concentration was approximately 6 μM. This allowed an increase of the percentage of labeled calcium available for uptake.

Following stimulation the contents of the slide were filtered through 0.8 μ Millipore or Gelman filters (Martonosi and Feretos 1964); the filters were pre-rinsed with 10 ml of Carter's medium to reduce non-specific Ca-45 binding (Browning and Nelson 1976). The filters were post-rinsed with 10 ml of Carter's medium. Each filtration step lasted 30 seconds. The filters were dissolved in 1 ml of 2-methoxy-ethanol (cellosolve) for at least 15 minutes (Gelman filters do not dissolve but rather become clear) and 10 ml of Aquasol (New England Nudlear, Boston, Massachusetts) or tritosol (80 ml Fluoralloy, 320 ml 95% EtOH, 100 ml ethylene glycol, 760 ml triton-X, 1740 ml xylene) was added. The suspension was mixed thoroughly and analyzed by liquid scintillation counting. Quench corrections were not made. However, the counting window was not restricted and self-adsorption is not a problem for the energetic β of Ca-45 (Zarybnicky and Reich 1980). Counting samples as prepared above or after the addition of EGTA or as gels had little effect on the relative Ca-45 uptake measured. The use of EGTA (Van Breemen and Casteels 1974; Aaronson, Van Breemen, Loutzenhiser, and Kelber 1979) or La^{3+} (Mayer, Van Breemen, and Casteels 1972; Weihe, Hartschuh, Metz, and Bruhl 1977; Hellman 1978) to determine the contribution of cell surface binding were not satisfactory (see discussion). Counts per minute (CPM) data were analyzed statistically and the uptake expressed as pmole calcium uptake per animal. This method does not distinguish between uptake and binding of calcium and thus in the results the terms uptake, binding, and incorporation will be used synonymously.

The effects of A23187, La^{3+}, and PO_4^{2-} on Ca-45 uptake in unstimulated Spirostomum also were tested. A23187, a divalent cation ionophore (Reed and Lardy 1972), was a gift from the Eli Lilly Co. (Indianapolis, Indiana). A23187 and La^{3+} were added just prior to the addition of Ca-45. One mM PO_4^{2-} was present in the pH buffer (0.5 mM KH_2PO_4 + 0.5 mM Na_2HPO_4) of the Carter's medium replacing HEPES-PIPES. Ca-45 uptake was measured during a one hour incubation in Carter's medium in the presence of 7.5 mM La^{3+} or 2 μM A23187 (dissolved in EtOH); the uptake of Ca-45 in 2% EtOH-Carter's also was monitored. Since the calcium and phosphate transport systems may be coupled, uptake in phosphate-buffered and HEPES-PIPES-buffered Carter's media were determined after a two week incubation with 50 Spirostomum; wheat seeds and bacteria were added to allow the Spirostomum to feed. The filtration and counting procedures were as above.

Pharmacological Agents

Several agents which affect calcium-dependent processes were examined for behavioral effects on Spirostomum. These included caffeine, lanthanum, verapamil, A23187, serotonin, tubocurarine, and decamethonium.

Caffeine

Caffeine apparently facilitates release of calcium from vesicles (Bianchi 1961; Weber and Herz 1968; Kitazawa and Endo 1976) and increases spontaneous contraction rates in Spirostomum (Sleigh 1970). Spirostomum were incubated in 5 mM caffeine for 10 minutes

and then stimulated mechanically for 10 minutes and after a 4 minute rest stimulated for an additional minute to test recovery.

Lanthanum

La^{3+} displaces Ca^{2+} from Ca^{2+} binding sites and blocks many processes involving calcium (Takata, Pickard, Lettvin, and Moore 1966; Mela 1968; Weiss 1973; Reed and Bygrave 1974; Larsen and Vincenzi 1977). Lanthanum in concentrations of 0, 1, 5, 10, 50, and 100 μM in Carter's medium was used to test the responsiveness of Spirostomum to 2 minutes of mechanical or electrical stimulation following a 15 minute incubation period. The media were prepared such that the sum of the lanthanum and calcium concentrations was always 0.5 mM, the normal calcium concentration of Carter's medium, i.e., differing amounts of Ca^{2+} were replaced by La^{3+}. Incubation times ranging from 6 to 120 minutes in 0, 5, 50, and 100 μM lanthanum-Carter's also were tested.

Verapamil

Spirostomum were incubated for 15 minutes in Carter's medium containing 0, 1, 10, 100, and 1000 μg/ml verapamil (Knoll Industries, Whippany, New Jersey) (Haas and Haerthfelder 1962), a mammalian calcium channel blocking agent. This was followed by a 2 minute mechanical or electrical stimulation period. Also animals were incubated for periods ranging from 6 to 180 minutes in 1, 25, 50 or 100 μg/ml verapamil-Carter's and tested as above.

A23187

A23187, a calcium ionophore, facilitates Ca^{2+} entry across a variety of membranes (Reed and Lardy 1972; Pfeiffer, Reed, and Lardy 1974; Desmedt and Hainaut 1976; Wulf and Pohl 1977). A stock solution of 10 mM A23187 in ethanol was used to make experimental media by adding aliquots of this solution to normal Carter's medium 5-10 minutes before use. Final A23187 concentrations ranged from 10^{-6} to 10^{-4} M; ETOH concentrations were always less than 1% (v/v). Control media containing the same amount of EtOH but without A23187 also were tested. After a 15 minute incubation in one of the above solutions the responsiveness of Spirostomum to mechanical or electrical stimulation was measured.

Serotonin

Serotonin may remove habituation in neural systems by facilitating calcium influx (Kandel 1976, 1977). One to three hour incubations in 0.2 mM to 2.0 mM serotonin were used to investigate the effect of serotonin on responsiveness and habituation in Spirostomum.

Tubocurarine and Decamethonium

These compounds block mechanosensitive calcium channels in the ciliate Stentor coeruleus (Wood 1977). Spirostomum were incubated in 2.0 mM decamethonium Br or 0.1 mM d-tubocurarine Cl for one to three hours followed by 2 minutes of mechanical or electrical stimulation.

Deciliation

Deciliation of Spirostomum was intended to supply information on (1) the role of the cilia in mechanotransduction, and (2) the possible location of voltage-sensitive or mechano-sensitive calcium channels in the ciliary membrane (Ogura and Takahashi 1976; Dunlap 1977; Ogura and Machemer 1979). Several deciliation procedures were tried including chloral hydrate (Kuznicki 1963; Kennedy and Brittingham 1968; Dunlap 1977), dibucaine (Satir, Sale, and Satir 1976; Thompson, Baugh, and Walker 1974), ethanol (Ogura and Machemer 1979), and calcium shock (Watson and Hopkins 1962; Rosenbaum and Carlson 1969; Everhart 1972). None of these techniques was satisfactory. All weakened the membrane and caused lysis of Spirostomum due to loss of membrane integrity. The results with chloral hydrate confirmed those of Kuznicki (1963) who found that this agent causes deciliation in Paramecium but not in Spirostomum.

RESULTS

Analysis of behavioral data focuses on (1) the initial responsiveness, i.e., contractility during the first minute of stimulation, and (2) the response decrement, i.e., the drop in responsiveness between the first and last minutes of stimulation. The initial response level is interpreted as an indicator of the efficacy of sensory transduction (when compared to the control). Recovery from habituation also is studied when possible. Except where indicated all data are analyzed by analysis of variance and tested with the least significant difference (LSD) procedure; all significance levels are for the $p < .05$ level.

Effects of Calcium

The effects of varying free calcium concentration on responsiveness and response decrement to mechanical and electrical stimulation are shown in Table 1. Free calcium and magnesium concentrations were calculated by successive approximation of the equations describing the binding kinetics of EGTA, Ca, and Mg. Free calcium levels ranged from 0.83×10^{-11} mM to 0.505 mM; free magnesium levels were depressed in media with excess EGTA (EGTA very selectively binds Ca^{2+} over Mg^{2+}). The control group (*) contains 0.505 mM Ca^{2+} without the addition of EGTA. The decrements in Table 1 were calculated from the differences in responsiveness between minutes 1 and 2 of the stimulation period and are expressed as percents of the minute 1

TABLE 1.--Effects of Free Calcium Concentration on Responsiveness and Response Decrement to Mechanical and Electrical Stimulation.

Experimental Parameters					Probability of Contraction					
Total		Bound	Free		Mechanical			Electrical		
EGTA (mM)	Ca (mM)	EGTA (mM)	Ca (mM)	Mg (mM)	Min. 1	Min. 2	Decrement (%)	Min. 1	Min. 2	Decrement (%)
1.0	.505	.71	$.83 \times 10^{-11}$	$.30 \times 10^{-7}$	.53	.37	29	.55	.28	52
.1	.005	.10	$.11 \times 10^{-5}$	.110	.67	.41	37	.67	.39	41
.5	.505	.50	.005	.205	.41	.21	51	.55	.27	52
-	.005	-	.005	.205	.68	.47	31	.72	.38	48
-	.255	-	.255	.205	.80	.52	35	.87	.82	5
.25	.505	.25	.255	.205	.79	.51	35	.88	.83	5
-	.505*	-	.505	.205	.79	.54	32	.85	.79	7
.25	.755	.25	.505	.205	.79	.54	32	.80	.75	5

level. The data of Table 1 are from 12 groups of four Spirostomum per group for each concentration.

Mechanical Stimulation

At the assumed calcium contamination level (5 μM) and below responsiveness of Spirostomum to mechanical stimulation was lower than to control calcium levels (0.5 mM). The probability of contraction was reduced for both minutes in these low calcium media. The amount of habituation (percent decrement) was not affected. The high level of habituation for the 0.5 mM EGTA + 0.505 mM Ca medium is probably due to the low initial response level.

Electrical Stimulation.

The initial responsiveness of Spirostomum to electrical stimulation in low calcium levels is reduced in the same manner as for mechanical stimulation (Table 1). The four media with free Ca^{2+} less than 0.005 mM all decreased the probability of contraction during the first minute of stimulation. In addition, these low Ca^{2+} media cause a large decrement; the decrement is as large as that occurring in the mechanically stimulated control group.

In summary, reducing free Ca^{2+} of the Carter's medium to micromolar and below levels results in decreased initial responsiveness to mechanical or electrical stimulation. A large decrement occurs in response to electrical stimulation that is not present in media containing 0.255 mM or more (free) Ca^{2+}.

Effects of Monovalent and Other Divalent Cations

Mechanical Stimulation

Table 2 shows the effects of removal, addition, or replacement of various monovalent and divalent cations on responsiveness, response decrement, and recovery of Spirostomum to mechanical stimulation. Increasing Na levels, replacing Na^+ with Li^+, or replacing K^+ with Cs^+ or Rb^+ had no effect on these parameters for Spirostomum exposed to 11 minutes of mechanical stimulation. Raising (to 10 mM) or lowering (to 0 mM) the Mg^{2+} levels did not alter responsiveness of Spirostomum. Replacing Ca^{2+} with Sr^{2+} or Co^{2+} and partial replacement of Ca^{2+} with Ni^{2+}, Zn^{2+}, or Ba^{2+} also were without effect. Those monovalent and divalent cations with significant effects on responsiveness of Spirostomum to mechanical stimulation are shown in Figure 2. Increasing $[K^+]$ from 0.5 mM (control level) to 10 mM reduced the initial probability of contraction from 0.76 to 0.30. Habituation still occurred as responsiveness dropped to near zero after 11 minutes of mechanical stimulation. Very little recovery occurred in the high $[K^+]$ medium. Replacing Ca^{2+} with Mn^{2+} or Ba^{2+} also reduced initial responsiveness of Spirostomum. Mn^{2+}-treated animals habituated and recovered to the same levels as the control group. Spirostomum in 0.5 mM Ba^{2+} and no Ca^{2+} habituated less than the control groups, as well as exhibiting a depressed initial response level; this did not occur in groups incubated in media containing 0.25 mM Ba^{2+} and 0.25 mM Ca^{2+} (partial replacement) indicating that barium ions exert their effects only in the almost complete absence of calcium. In contrast, replacing one-half of the Ca^{2+} with Zn^{2+}

TABLE 2.--Effects of Monovalent and Divalent Cations on Responsiveness, Response Decrement, and Recovery to Mechanical Stimulation.

Treatment	n	1	2	5	10	15	Percent Decrement	Percent Recovery
Control	5	.76	.58	.21	.10	.53	86.8	65.8
10 mM Na^{+}	5	.73	.49	.12	.08	.48	88.5	62.3
(*)Li^{+}/ Na^{+}	5	.78	.59	.24	.07	.48	91.4	57.7
K^{+}-free	5	.78	.60	.20	.14	.51	81.9	57.1
10 mM K^{+}	5	.30	.09	.03	.02	.08	94.4	20.6
(*)Cs^{+}/ K^{+}	5	.78	.45	.22	.13	.45	83.0	48.7
(*)Rb^{+}/ K^{+}	5	.87	.69	.39	.16	.62	81.7	64.7
Mg^{++}-free	5	.73	.53	.18	.11	.46	85.2	56.0
10 mM Mg^{++}	5	.78	.61	.27	.18	.56	77.6	63.0
(†)Ni^{++}- Ca^{++}	5	.74	.52	.23	.11	.54	85.4	68.4
(‡)Zn^{++}- Ca^{++}	5	.77	.67	.54	.63	.66	18.5	23.5
(‡)Ba^{++}- Ca^{++}	5	.70	.45	.18	.05	.43	92.9	57.7
(*)Ba^{++}/ Ca^{++}	5	.58	.45	.37	.38	.51	35.7	64.0
(*)Sr^{++}/ Ca^{++}	5	.78	.49	.23	.10	.51	87.1	60.5
(*)Co^{++}/ Ca^{++}	5	.83	.56	.33	.16	.57	80.8	61.3
(*)Mn^{++}/ Ca^{++}	5	.49	.31	.18	.10	.43	79.7	83.0

(*)/-first ion replaces second in Carter's medium (†).05 mM Ni^{++} + .45 mM Ca^{++} (‡).25 mM first ion + .25 mM Ca^{++}

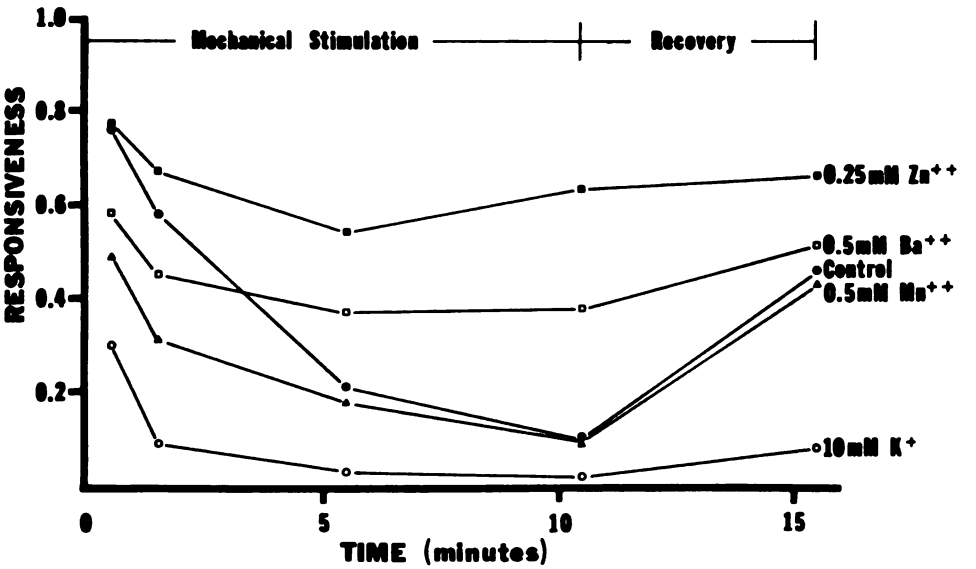

Figure 2.--The effects of monovalent and divalent cations on responsiveness, response decrement, and recovery to mechanical stimulation (0.1 Hz for 11 min.; 4 min. rest; 1 min. stimulation to test recovery). Responsiveness is shown on the ordinate as probability of contraction. Data are plotted from Table 2. Standard errors (SEM) ranged from .01-.12 responsiveness units.

(0.25 mM) almost completely eliminated habituation. Complete replacement of calcium by zinc was not possible due to the toxicity of higher $[Zn^{2+}]$.

Electrical Stimulation

The effects of monovalent and divalent cations on responsiveness, response decrement, and recovery of Spirostomum to electrical stimulation are shown in Table 3. As with mechanical stimulation replacing Na^+ with Li^+ or K^+ with Cs^+ or Rb^+ had no effect on responsiveness. Raising $[Na^+]$ or $[K^+]$ to 10 mM depressed initial responsiveness and this reduced response level was maintained during the 11 minute stimulation period (Figure 3). Initial response

TABLE 3.--Effects of Monovalent and Divalent Cations on Responsiveness, Response Decrement, and Recovery to Electrical Stimulation.

Treatment	n	1	2	5	10	15	Percent Decrement	Percent Recovery
Control	6	.59	.54	.59	.61	.52	- 3.5	-
10 mM Na^{+}	5	.43	.27	.33	.33	.37	21.6	36.4
(*)Li^{+}/ Na^{+}	5	.71	.67	.64	.53	.64	25.9	63.6
K^{+}-free	5	.69	.68	.49	.45	.50	34.9	20.7
10 mM K^{+}	5	.24	.25	.28	.22	.15	10.3	-
(*)Ca^{+}/ K^{+}	5	.69	.67	.59	.43	.38	38.6	-
(*)Rb^{+}/ K^{+}	5	.67	.63	.66	.53	.53	21.3	5.9
Mg^{++}-free	5	.74	.68	.66	.57	.48	23.6	-
10 mM Mg^{++}	5	.28	.21	.27	.27	.20	3.0	-
(†)Ni^{++}- Ca^{++}	5	.67	.51	.71	.49	.45	26.3	-
(‡)Zn^{++}- Ca^{++}	5	.75	.77	.53	.35	.23	53.3	-
(‡)Ba^{++}- Ca^{++}	5	.57	.51	.34	.28	.40	50.0	41.2
(*)Ba^{++}/ Ca^{++}	5	.41	.28	.26	.03	.12	91.8	22.2
(*)Sr^{++}/ Ca^{++}	5	.58	.53	.36	.17	.29	71.0	30.6
(*)Co^{++}/ Ca^{++}	5	.63	.55	.68	.48	.56	23.7	50.0
(*)Mn^{++}/ Ca^{++}	5	.48	.53	.51	.60	.48	-26.3	-

(*)/-first ion replaces second in Carter's medium (†).05 mM Ni^{++} + .45 mM Ca^{++} (‡).25 mM first ion + .25 mM Ca^{++}

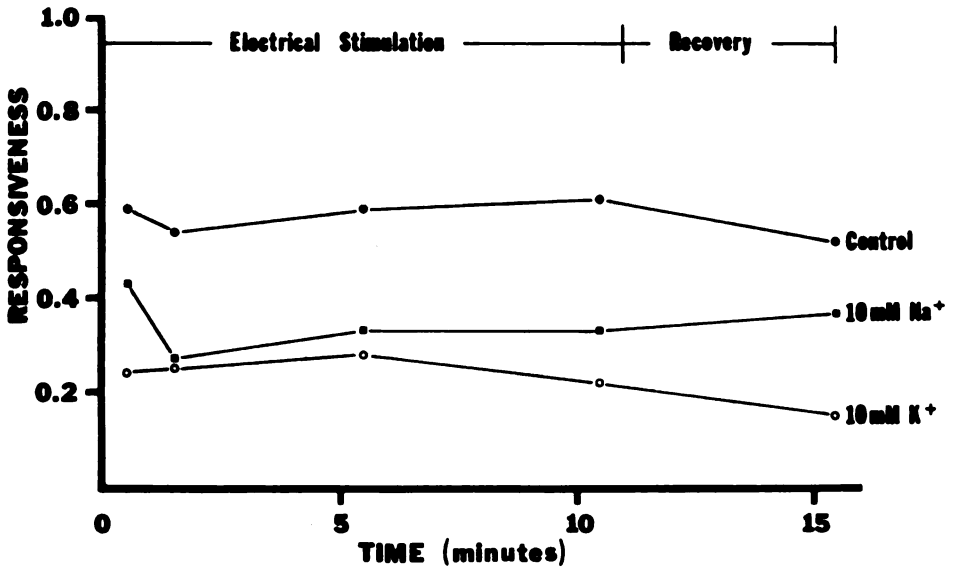

Figure 3.--The effects of monovalent cations on responsiveness, response decrement, and recovery to electrical stimulation. Stimulation and response parameters are the same as in Figure 2. Data are plotted from Table 3. Standard errors (SEM) ranged from .04-.07 units.

levels were not affected by partial or complete replacement of Ca^{2+} by Ni^{2+}, Zn^{2+}, Ba^{2+}, Sr^{2+}, Co^{2+}, or Mn^{2+}; replacement of Ca^{2+} with Ba^{2+} or Mn^{2+} reduced contractility of Spirostomum but not significantly. Raising $[Mg^{2+}]$ to 10 mM depressed responsiveness to electrical stimulation in a manner similar to Na^{+} and K^{+} (Figure 4).

Although they had no significant effect on initial probability of contraction, replacement of Ca^{2+} with Ba^{2+} or Sr^{2+} and partial replacement of Ca^{2+} with Zn^{2+} or Ba^{2+} resulted in a response decrement to electrical stimulation (Figure 4; data for 0.25 mM Ba^{2+} + 0.25 mM Ca^{2+} not shown). This result also was found in low $[Ca^{2+}]$ Carter's media (Table 1). Spirostomum in control Carter's do not show a decrement to repeated electrical stimulation (Table 3, Figures 3 and 4).

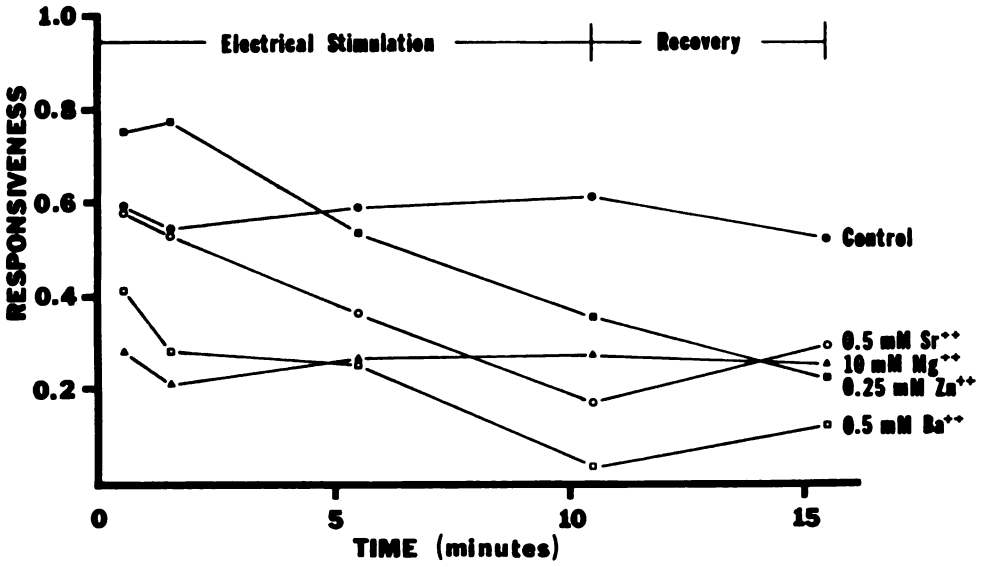

Figure 4.--The effects of divalent cations on responsiveness, response decrement, and recovery to electrical stimulation. Stimulation and response parameters are the same as in Figure 2. Data are plotted from Table 3. Standard errors (SEM) ranged from .03-.11 units.

In summary, initial responsiveness to mechanical stimulation was decreased by high $[K^+]$ and by replacing Ca^{2+} with Ba^{2+} or Mn^{2+}. For electrical stimulation raising $[Na^+]$, $[K^+]$, or $[Mg^{2+}]$ reduced the initial probability of contraction. The amount of habituation was reduced by partial replacement of Ca^{2+} with Zn^{2+} and by complete replacement of Ca^{2+} with Ba^{2+}; partial replacement with Ba^{2+} had no effect. A response decrement occurred during electrical stimulation in media where Ba^{2+} or Sr^{2+} completely replaced Ca^{2+} and in media with partial replacement of Ca^{2+} by Ba^{2+} or Zn^{2+}. This effect is similar to that seen in low $[Ca^{2+}]$ media.

Ca-45 Uptake Studies

Uptake of Ca-45 during 2 to 60 minutes of mechanical, electrical, or no (control) stimulation is shown in Table 4. The uptake is given as picomoles of calcium incorporated per animal. Each figure is based on 2-8 repetitions. Both stimulated and unstimulated Spirostomum show significant uptake (or binding) in the first two minutes of exposure of Ca-45. This very fast binding also can be seen by first filtering Spirostomum and then filtering 0.5 ml Ca-45 Carter's medium through the millipore apparatus; the uptake with this procedure is not as much as the 2 minute uptake but is substantially higher than the non-specific filter binding component. The data for 2-30 minutes of incubation is for 50 Spirostomum per sample and the 60 minute incubation data is from 500 animals per sample. The 60 minute incubation was in low calcium Carter's medium (approximately 6 μM Ca) to increase sensitivity by increasing the labeled to unlabeled calcium ratio. Only electrical stimulation for 30-60

TABLE 4.--Uptake of Ca-45 in Stimulated and Unstimulated Spirostomum.

	Ca-45 UPTAKE (pmole/animal) Incubation Time (minutes)				
	2	5	10	30	60
Control	0.64	0.58	0.58	1.02	0.50
Mechanical	0.54	0.38	0.65	1.42	0.53
Electrical	0.74	0.45	0.78	1.58	1.41

minutes produced Ca-45 uptake that was significantly higher than control levels. Although the amount of Ca-45 uptake during 10-60 minutes of mechanical stimulation is higher than the control, the difference is not statistically significant. Table 4 also shows that lowering the extracellular calcium reduces total calcium uptake in both stimulated and unstimulated Spirostomum.

Table 5 shows Ca-45 uptake by Spirostomum during one hour of incubation in the calcium ionophore A23187 or in $LaCl_3$. Each measurement is the mean of 3-6 repetitions. Filter binding Ca-45 levels are due to non-specific adsorption to the filter. Incubation in 2% EtOH (the ionophore solvent) slightly but not significantly increases uptake compared to control levels. Incubation in 2 μM A23187 significantly increases Ca-45 incorporation during the one hour incubation period. Both 2% EtOH and 2 μM A23187 in 2% EtOH increase spontaneous contraction rates in Spirostomum. Incubation in 7.5 mM LaCl-Carter's for one hour reduces Ca-45 uptake to a level lower than the filter binding. However, this figure is not statistically different from the control. Calcium uptake in unstimulated Spirostomum also depends on phosphate levels; a two week incubation in phosphate-buffered Carter's medium resulted in three times the Ca-45 uptake as a HEPES-PIPES-buffered medium (data not shown).

Thus calcium uptake or binding occurred very quickly in stimulated and unstimulated Spirostomum. Electrical stimulation resulted in increased calcium uptake compared to a control and the amount of uptake was dependent on the extracellular $[Ca^{2+}]$. A23187 and PO_4^{2-} increased Ca-45 uptake in unstimulated Spirostomum while

TABLE 5.--Effects of A23187 and Lanthanum on Ca-45 Uptake in Unstimulated Spirostomum.

Treatment	Ca-45 Uptake (CPM ± SEM)
Filter binding	600 ± 64
Control	715 ± 88
EtOH (2% v/v)	842 ± 82
A23187 (2 μM in 2% EtOH)	1443 ± 363
$LaCl_3$ (7.5 mM)	463 ± 143

La^{3+} may have affected non-specific filter binding as well as incorporation into the animal.

Effects of Pharmacological Agents

Figure 5 gives the effects of incubating Spirostomum in 5 mM caffeine on responsiveness to mechanical stimulation. Initial responsiveness is not affected and the caffeine-treated animals do not habituate during the 10 minute stimulation period. After 10 minutes of stimulation the control response is significantly lower than the caffeine-treated level ($p < .05$, Mann-Whitney, U, two-tailed). Caffeine also increases spontaneous contraction rates in Spirostomum.

Spirostomum were unable to survive in Lanthanum concentrations greater than 10 mM. A 15 minute incubation in concentrations ranging from 10 to 100 μM lanthanum lowered the probability of contraction to mechanical stimulation for both minutes 1 and 2 of the stimulation

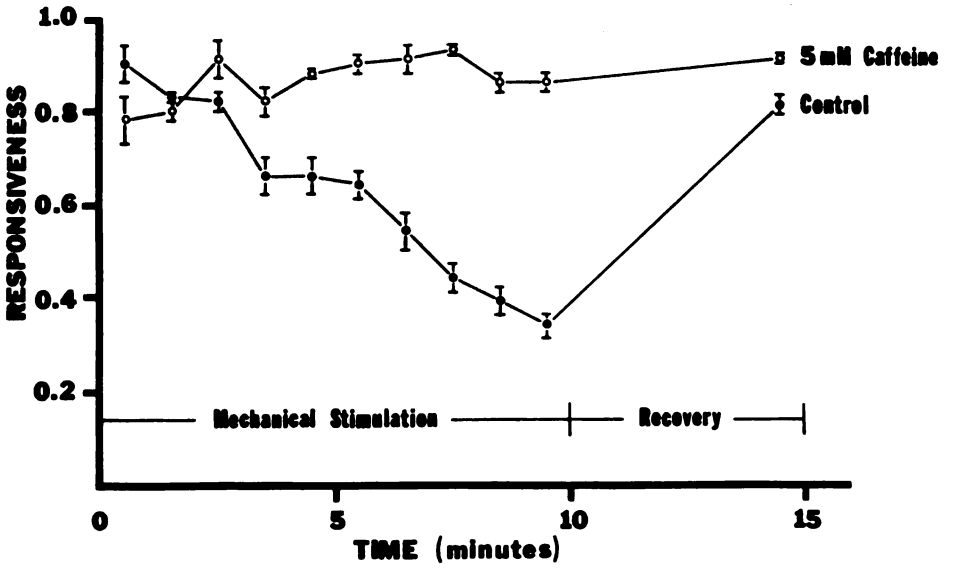

Figure 5.--The effect of caffeine on responsiveness, response decrement, and recovery to mechanical stimulation (0.1 Hz for 10 min.; 4 min. rest; 1 min. stimulation to test recovery). Spirostomum were incubated in 5 mM caffeine for 10 minutes prior to testing. Responsiveness is plotted as probability of contraction. The vertical bars represent the standard errors of the means. Control data are from 9 groups of 4 Spirostomum per group; caffeine data are from 4 groups.

period (Figure 6); the responsiveness at 10 and 100 μM are significantly lower than the control (0 μM La^{3+}) for both minutes. Responses in 1 or 5 μM lanthanum-Carter's were not significantly different from control levels for either minute. Increasing lanthanum concentration reduced initial responsiveness to electrical stimulation only at the 10 μM level (Figure 7). The difference in responsiveness between minutes 1 and 2 is not significant, i.e., no habituation or decrement to electrical stimulation occurs at any La^{3+} concentration. Figure 8 shows that the amount of habituation to 2 minutes of

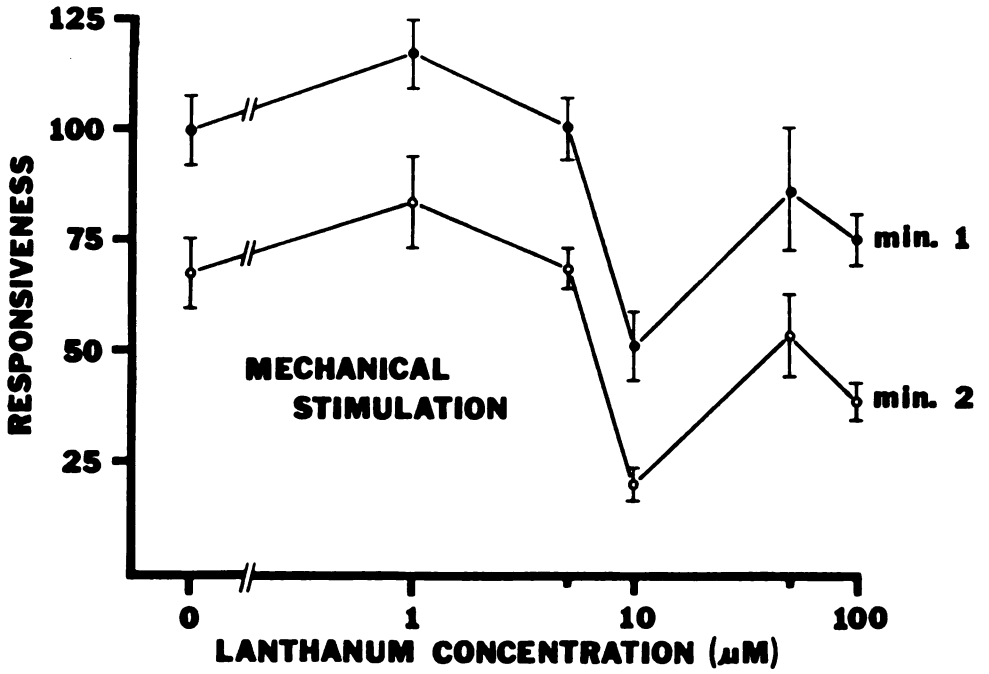

Figure 6.--The responsiveness to 2 minutes of mechanical stimulation as a function of lanthanum concentration (μM). The probability is expressed as a percent of the control group's (0 μM La) response to the first minute of stimulation (percent of control). Note that the abscissa has a logarithmic scale. Data are from 5 groups of Spirostomum per group for each concentration.

mechanical stimulation increased at lanthanum concentrations of 10-100 μM. Increasing verapamil (a calcium channel blocker) concentration also increased habituation levels between minutes 1 and 2 of mechanical stimulation (Figure 9); 100 μM verapamil produced signs of toxicity in Spirostomum.

Only incubation in 100 μM lanthanum significantly affected responsiveness during a 120 minute incubation. Figure 10 shows the responsiveness during the first minute of mechanical or electrical stimulation for Spirostomum in 100 μM La^{3+} at various incubation

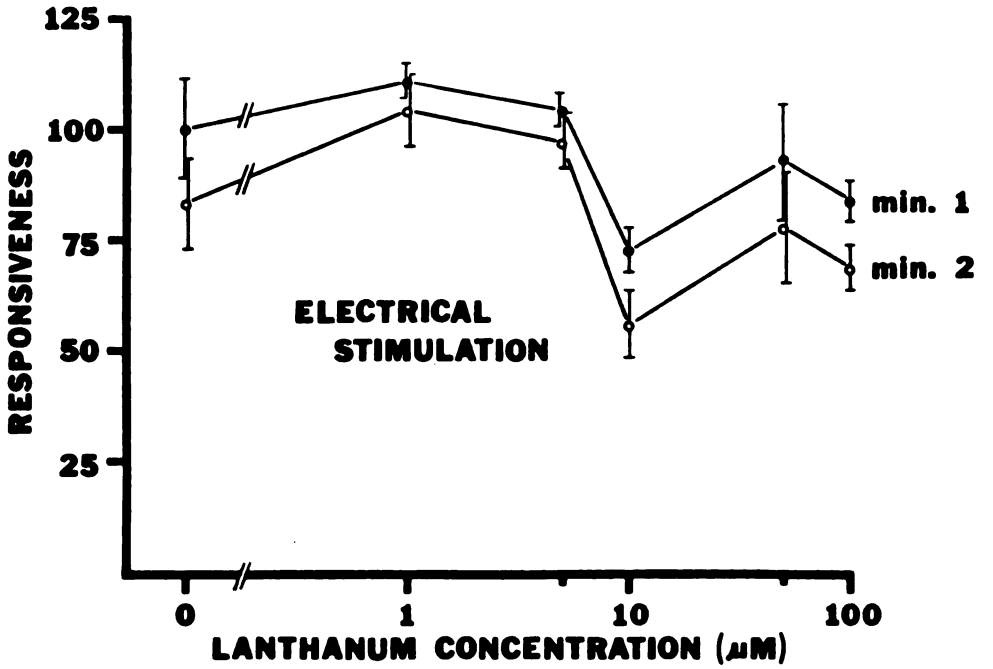

Figure 7.--The responsiveness to 2 minutes of electrical stimulation as a function of lanthanum concentration (μM). The ordinate units are the same as shown in Figure 6. Data is from 5 groups of 4 Spirostomum per group for each concentration.

times. The response to electrical stimulation decreased quickly and recovered faster than the response to mechanical stimulation. The response levels of mechanically and electrically stimulated Spirostomum at 6, 60, and 90 minutes are significantly different.

No significant effects of tubocurarine, decamethonium, or serotonin on responsiveness or response decrement in Spirostomum to mechanical or electrical stimulation were observed after 1-3 hours of incubation in each of these compounds.

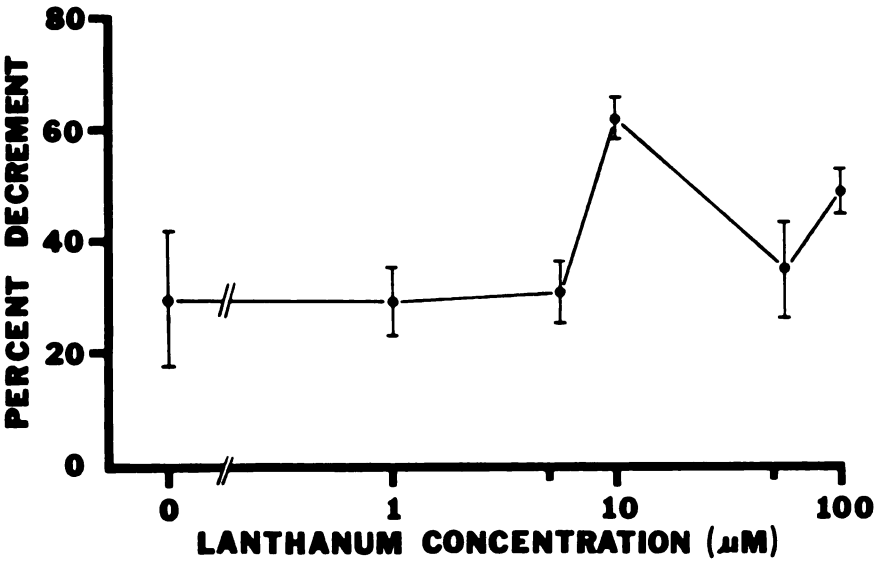

Figure 8.--The percent decrement to mechanical stimulation as a function of lanthanum concentration (μM) for the data of Figure 6. The percent decrement is calculated by subtracting the probability of contraction for minute 2 from that for minute 1 and expressing the result as a percent of the response for minute 1.

In summary, caffeine eliminated habituation of Spirostomum to mechanical stimulation. Lanthanum decreased initial responsiveness to mechanical stimulation and increased habituation levels. Verapamil also increased habituation levels. Incubation in 100 μM La^{3+} showed that response to electrical stimulation drops quickly and recovers; responsiveness to mechanical stimulation dropped and recovered more slowly.

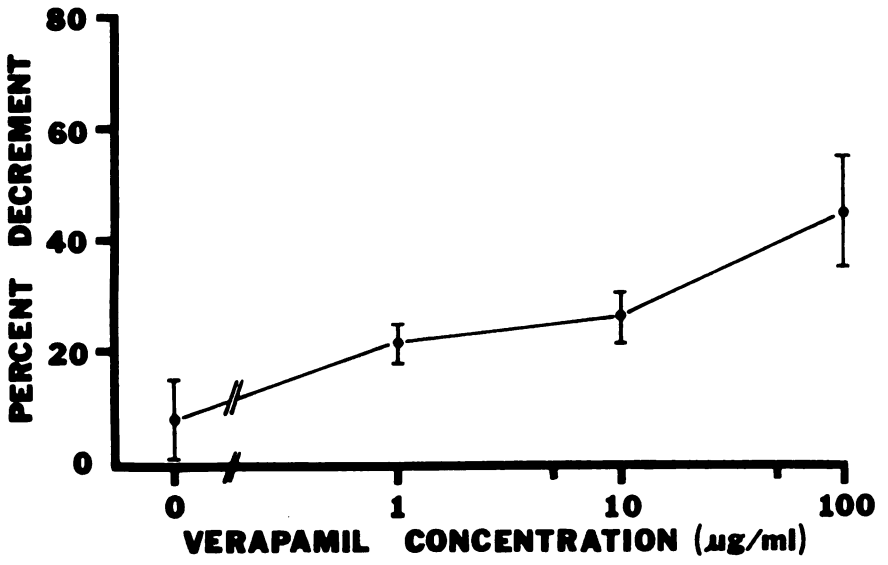

Figure 9.--The percent decrement to mechanical stimulation as a function of verapamil concentration (µg/ml). The percent decrement is calculated as in Figure 8. Data is from 5 groups of 4 Spirostomum per group for each concentration.

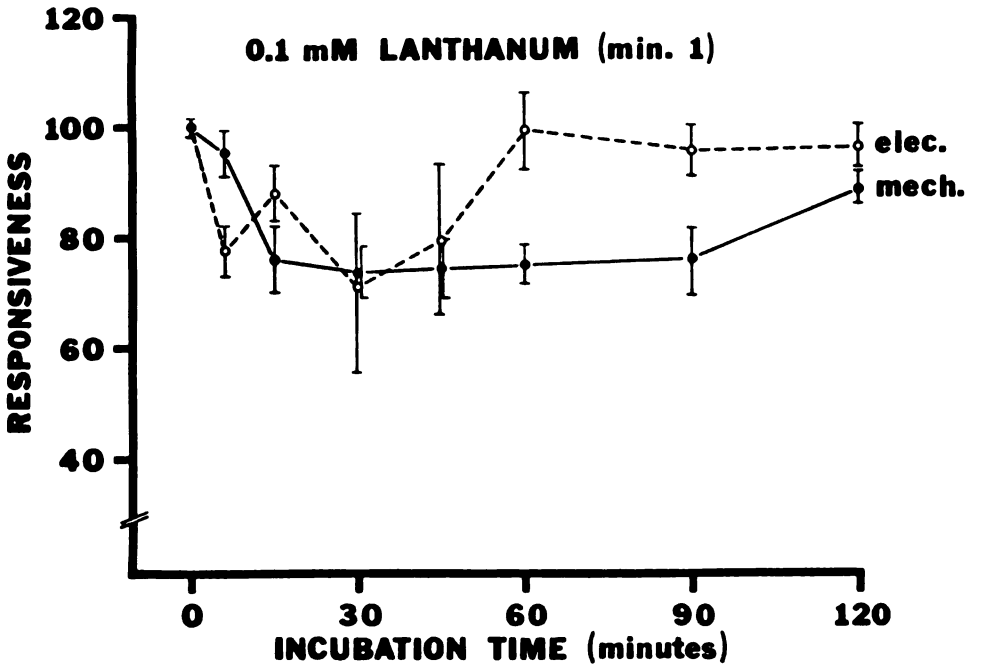

Figure 10.--The responsiveness to one minute of mechanical or electrical stimulation as a function of incubation time in 100 μM lanthanum. The probability of contraction is expressed as a percent of control as in Figure 6. Data is from 9 groups of 4 Spirostomum per group for each incubation time.

DISCUSSION

The results implicate calcium in both transduction and response decrement to mechanical and electrical stimulation. It is not surprising that calcium is involved in the excitability and behavior of Spirostomum since calcium plays a role in the excitability of many organisms, particularly invertebrates (Brink 1954; Hagiwara and Naka 1964; Hagiwara 1975; Hidalgo, Luxoro, and Rojas 1979; Chiarandini, Sanchez, and Stefani 1980; Nelson, Young, and Gardner 1980). A requirement for calcium by excitable tissue was first noticed by Ringer (1883) in studies on heart muscle.

Based on studies in the ciliates Paramecium (Naitoh and Eckert 1969; Machemer and Ogura 1979; Ogura and Machemer 1979, 1980) and Stentor (Wood 1980, personal communication) it was expected that in Spirostomum calcium would carry currents related to both mechano- and electro-reception through separate channel structures in the somatic and ciliary membranes respectively, and further, that habituation to repeated mechanical stimulation would be the result of a decreased calcium entry through the mechanoreceptor channel. A model was developed based on these and other studies (Eisenstein, Lovell, Reep, Barraco, and Brunder 1980) and was subsequently used to design the experiments reported here.

Transduction

The presence of micromolar levels of Ca^{2+} in the extracellular medium is necessary for the transduction of electrical and mechanical stimuli in Spirostomum (Table 1). Responsiveness is reduced to both modes of stimulation when the free calcium concentration is below about 5 μM. The effects are not correlated with total [Ca] or [EGTA] or with the ratio of [Ca] to [Mg]; in some systems the Ca-EGTA complex may act like free Ca^{2+} and thus total calcium is an important variable in such preparations (Sarkadi, Schubert, and Gardos 1979). The presence of a 'threshold' effect suggests that calcium acts by a membrane-binding mechanism rather than by passive channel conductance since an influx mechanism would most likely show a greater dependence on the calcium gradient present across the membrane. In disagreement with the present study Fabczak (1974) reported responsiveness of Spirostomum was unchanged after a short incubation in an EGTA-CaCl solution with free [Ca^{2+}] of 10^{-7} M. However, there were no other cations present in the test solution while the media used in the present experiments contained Na^{+}, K^{+}, and Mg^{2+} in addition to Ca^{2+}; this may account for the observed difference. Removal of excitability by lowering extracellular calcium also is seen in other systems, e.g., barnacle muscle (Hidalgo et al. 1979).

Since most membranes are impermeable to EGTA (Weber, Herz, and Reiss 1966; Van Breemen and Van Breemen 1969; Baker 1972; Wheeler and Weiss 1979), EGTA must exert its effect by buffering extracellular calcium or by altering calcium binding to the membrane (Schlatz and Marinetti 1972; Wheeler and Weiss 1979). Although the use of EGTA

to remove membrane bound calcium was not satisfactory as a quantifiable technique with Spirostomum, the experiments did suggest that EGTA was able to chelate membrane-bound Ca-45. If calcium binding is important one might expect to see media with excess EGTA have a greater effect on responsiveness than media without excess EGTA; this result was not observed. In muscle studies EGTA levels of 80-90 mM are required to reduce excitability (Barrett and Barrett 1978); however, the presence of 'protected' extracellular calcium compartments, e.g., the t-tubule system, must be considered in muscle systems. Thus it appears that EGTA acts primarily by buffering calcium levels and no behavioral effect of EGTA on membrane-bound calcium was indicated.

The present resuls agree with those of Osborn et al. (1973b) for the effects of EGTA on electrical but not mechanical stimulation. Osborn et al. (1973b) found that 0.5 mM Ca + 0.25 mM EGTA reduced initial responsiveness to mechanical but not to electrical stimulation when compared to the control (0.5 mM Ca). The present experiment showed no change to either stimulation mode (compare rows 6 and 7 in Table 1). However, Osborn's experiments were repeated and the results reproduced by this author. The methods used differed slightly for the two experiments in that Osborn stimulated Spirostomum just prior to EGTA addition (as a pre-EGTA control) (see Figure 3 in Osborn et al. 1973b).

Raising the concentrations of Na^+, K^+, or Mg^{2+} of the Carter's medium decreased responsiveness of Spirostomum to electrical stimulation (Table 3). This confirms studies by Fabczak (1974) who

found that increasing concentrations of Ba, Ni, Ca, Mg, K, or Na from 0.5 to 10 mM reduced responsiveness of Spirostomum to electrical stimulation. The present results also agree with Fabczak (1974) in finding no evidence of Donnan equilibrium effects (between binding of Ca^{2+} and monovalent ions) as is seen in Paramecium where ciliary reversal is controlled by the ratio $[Ca^{2+}]^{\frac{1}{2}}/[K^{+}]$ (Jahn 1962; Naitoh and Yasamasu 1967; Naitoh and Eckert 1968; Naitoh 1973). Fabczak finds the effectiveness of cations in reducing responsiveness follows Na < K < Mg while the present experiments order the effects as Na < Mg < K. Fabczak (1974) proposes that the cations exert their effect by acting on the electrical double layer at the cell surface. Preliminary evidence suggests that reducing [Na] to 0.2 mM (from 2.0 mM) increased responsiveness of Spirostomum to electrical stimulation. Oubain also increases contractility in Spirostomum (Sleigh 1970), perhaps through a $[Na^{+}]$ effect on the Na-Ca pump leading to increased availability of intracellular calcium (Baker, Blaustein, Hodgkin, and Steenhardt 1967). Reducing $[K^{+}]$ or $[Mg^{2+}]$ had no effect on responsiveness to electrical stimulation. Extracellular Cs^{+}, which may block K^{+} channels (Gay and Stanfield 1977; Adelman and French 1978), did not alter responsiveness of Spirostomum suggesting no major role for K^{+} in electrotransduction. Perhaps a better indication of the apparent role of each ion would be observed if all ions, especially Cs^{+}, Rb^{+}, and Li^{+}, also were tested at higher levels.

Partial or complete replacement of Ca^{2+} with Ni^{2+}, Zn^{2+}, Ba^{2+}, Sr^{2+}, Co^{2+}, or Mn^{2+} had no effect on responsiveness to electrical

stimulation. In contrast to the present results Fabczak (1974) found incubation of Spirostomum in tris buffer and $BaCl_2$ greatly decreased excitability. Again the lack of additional cations in Fabczak's test media makes comparisons difficult. In the present study Ba reduced response levels but not significantly. Sleigh (1970) found that $NiCl_2$ also reduced responsiveness of Spirostomum to electrical stimulation; although similar toxicity was noted in the two studies no effect of replacing a small amount of Ca^{2+} with Ni^{2+} (0.05 mM) was observed. Reducing calcium concentrations reduced responsiveness to electrical stimulation but no effects of calcium replacement were observed; this is possibly due to a non-specific ion effect on membrane potential, e.g., raising ion concentrations may hyperpolarize the membrane (Machemer 1976).

Responsiveness to mechanical stimulation behaves very differently to changes in the ionic composition of Carter's medium (Table 2). Raising $[K^+]$ to 10 mM still inhibits the contractile response but neither high $[Na^+]$ nor $[Mg^{2+}]$ affect the contractility of Spirostomum to mechanical stimulation. Replacing Ca^{2+} with Ba^{2+} or Mn^{2+} did lower responsiveness. Mn^{2+} (and Co^{2+}) often blocks calcium currents (Baker, Meves, and Ridgeway 1973; Baker and Glitsch 1975; Kryshtal 1976). Ba^{2+}, as well as Sr^{2+}, is usually a good calcium substitute (Meves 1968; Hagiwara, Fukuda and Eaton 1974; Hagiwara 1975; Adams and Gage 1980; Kerrick, Malenick, Hoar, Potter, Coby, Pocinwong, and Fischer 1980; Miledi and Parker 1980). La^{3+} decreased responsiveness to mechanical stimulation and to a lesser extent to electrical stimulation; the La^{3+} results do not discriminate

between uptake and binding since La^{3+} may block both processes (Van Breemen and Deweer 1970; Van Breemen, Farinas, Gerba, and McNaughton 1972; Van Breemen, Farinas, Casteels, Gerba, Wuytack, and Deth 1973; Weiss 1973; Freeman and Daniel 1973; Martin and Richardson 1979; Kurzinger, Stadkus, and Hamprecht 1980). Responsiveness to either mode of stimulation does not correlate with ionic radii or hydrated ion sizes as might be expected for a channel-influx mechanism where channel selectivity is based on size.

The results discussed above suggest that calcium and other cations may affect the excitability of Spirostomum by altering the binding of Ca^{2+} or by some other membrane-linked mechanism; passive channel permeability also may involve ion binding. However, the large uptake of Ca-45 during 60 minutes of electrical stimulation (Table 4) supports a calcium influx mechanism. The uptake is much higher than control levels and is much too large to represent membrane binding. Mechanical stimulation did not result in significant Ca-45 uptake. However, the responsiveness in the Ca-45 test solutions was very low due to limitations of the stimulation apparatus; the probabilty of contraction was about 0.50 during the first minute and habituated to a level of 0.15 by minute 10 of the mechanical stimulation period. Response levels to electrical stimulation were around 0.70 for 10 minutes of testing. If a calcium current flows during transduction then a much higher calcium influx would occur during electrical stimulation in the apparatus used in this study. Electrical stimulation also causes the release of much more Ca^{2+} internally than mechanical stimulation (Osborn et al. 1973b) and

could possibly produce a larger calcium current. Entry of enough calcium during transduction to elicit contraction is not necessary since a calcium-induced calcium release (Endo, Tanaka, and Ogawa 1970; Ford and Podolsky 1970) may occur; such an amplification mechanism in Spirostomum has been suggested by Hawkes and Holberton (1974). Much of the calcium entering during transduction may be expelled following contraction; in Paramecium Ca-45 influx studies must be performed at 0°C to reduce Ca-45 efflux (Browning and Nelson 1976). It is difficult to imagine that the Ca-45 entering would not mix with and label intracellularly sequestered calcium stores in Spirostomum; in Paramecium, where uptake is into the cilia and no calcium stores are present, complete efflux may be possible. In summary, calcium uptake occurs during electrical stimulation and uptake associated with mechanotransduction is not conclusively disproved.

Stimulus specificity and habituation to only one stimulation mode (mechanical) support the separation of the electro- and mechanotransducer sites (Osborn 1971). This separation is sustained by the differential concentration effects (Figures 6 and 7) and differential time effects (Figure 10) of lanthanum on responsiveness to electrical and mechanical stimulation. Separate sites also are suggested by the different effects of altering the ionic composition of the Carter's medium, i.e., ion effects depend on the stimulation mode. The mechano- and electro-transduction pathways may be completely separate except for calcium activation of contraction.

Response Decrement

Before discussing the role of calcium in response decrement in Spirostomum a comparison of the use of the terms 'response decrement' and 'habituation' should be made. Although it is difficult to separate habituation of Spirostomum to mechanical stimulation and receptor fatigue (since no dishabituation occurs in Spirostomum [Eisenstein and Peretz 1973]), the response to electrical stimulation rules out effector or motor fatigue. Thus electrical stimulation acts somewhat as a control to test the state of the effector. When a decrement in responsiveness to electrical stimulation occurs a control situation is not as available. It would be advantageous to see what response level would occur to mechanical stimulation after a decrement-producing electrical stimulation period; this experiment could be tried in the presence of Zn^{2+} where no decrease in initial levels is seen and a response decrement to electrical stimulation occurs. Because of the difficulty in assessing the state of the contractile apparatus of Spirostomum after a decrement to electrical stimulation, the term habituation is being reserved to describe the response decrement to mechanical stimulation only and habituation is hypothesized to occur at the mechanoreceptor site.

Although it was expected that habituation levels would be very susceptible to low calcium concentrations, i.e., assuming that habituation is due to decreased calcium entry (Kandel 1976, 1978; Klein and Kandel 1980; Wood personal communication), reducing $[Ca^{2+}]$ had little effect on habituation during 2 minutes of mechanical stimulation (Table 1). Perhaps after 10 minutes of stimulation a

difference in the decrement levels would have appeared. Replacing Ca^{2+} with Ni^{2+}, Zn^{2+}, Ba^{2+}, Sr^{2+}, Co^{2+}, or Mn^{2+} was expected to increase habituation levels since at least one or two of the ions should block Ca^{2+} currents or interfere with calcium binding. Nearly the opposite was observed--two ions, Ba^{2+} and Zn^{2+}, removed habituation. Applewhite and Davis (1969) report that Mg^{2+} and Mn^{2+} block habituation in the absence of Ca^{2+}; this effect was not tested and cannot be evaluated. Also, those ions that affected transduction did not necessarily alter the presence or amount of the observed response decrement suggesting that responsiveness and decrement levels are not correlated.

The Ba^{2+} and Zn^{2+} results suggest a possible habituation mechanism. Calcium current in Paramecium and in Aplysia inactivates itself through an effect of the intracellar $[Ca^{2+}]$ on the calcium permeability system (Brehm and Eckert 1978, 1979; Eckert and Brehm 1979; Eckert and Tillotson 1979; Tillotson and Eckert 1979; Brehm, Eckert and Tillotson 1980; Marban and Tsien 1981). Ba^{2+} blocks Ca^{2+} inactivation of the Ca^{2+} channel, possibly by binding to the channel macromolecule (Eckert and Brehm 1979; Adams and Gage 1980; Tsuda, Morimoto, and Brown 1981). Thus, if calcium-induced calcium inactivation was the mechanism of habituation, Ba^{2+} would be expected to remove habituation and this was observed. However, Ba^{2+} may block K leakage currents which would result in the same effect; in such experiments K^{+} currents are minimized by the use of K^{+}-channel blockers. Ba^{2+} is known to block K^{+} channels (Bezanilla and Armstrong 1972; Satow and Kung 1976; Valeev, Magura, and Zamekhovskii 1977;

Hagiwara et al. 1974; Standen and Stanfield 1978; Armstrong and Taylor 1980; Eaton and Brodwick 1980; Schwindt and Crill 1980). Zn^{2+} is a powerful enzyme activator (Cotton and Wilkinson 1972) and could very easily regulate permeability controlling proteins; Zn^{2+} is often involved in the regulation of phosphorylating reactions (Cotton and Wilkinson 1972) which may mediate ionic permeability (Hirata and Axelrod 1980). Zn^{2+} also appears to facilitate Ca^{2+} binding to sperm cells based on chlortetracycline fluorescence measurements (Nelson et al. 1980). Naitoh (1969) proposes that Zn^{2+} may interact with ATP and the contractile system of the ciliary apparatus in Paramecium. The action of Zn^{2+} in sperm cells and possibly in Spirostomum may be related to the activation of calcium-dependent metalloenzymes which may control permeability. Applewhite and Davis (1969) also suggest that metalloenzymes are involved in habituation. Zn^{2+} may be a useful tool in the further molecular characterization of habituation since Zn^{2+} (1) is available as an isotope, (2) can be chemically identified and estimated, and (3) can be removed from membranes by EDTA (Brierley, Knight, and Settlemire 1967).

Caffeine, which facilitates calcium release in muscle (Frank 1962; Weber and Herz 1968; Thorpe and Seeman 1971; Langer 1973; Kitazawa and Endo 1976; Fujimoto, Vanamoto, Kuba, Morita, and Kato 1980), removed habituation in Spirostomum suggesting that decreased availability of intracellular calcium may be the basis of habituation. The actions of La^{3+} and verapamil, which increased habituation levels (Figures 8 and 9), also support a decreased calcium entry mechanism

for habituation. If not for the possible toxicity of verapamil at 100 μg/ml (Figure 9) a calcium flux mechanism would be strongly supported since verapamil blocks calcium channels (Crankshaw, Janis, and Daniel 1977; Hoeschen 1977; Bondi 1978) and does not affect calcium binding. Similarly, caffeine probably has little effect on membrane binding of calcium.

Another surprising result was that reducing extracellular calcium levels caused a large decrement to electrical stimulation within the second minute of stimulation (Table 1). Thus, extracellular calcium is necessary to maintain responsiveness to electrical stimulation. A decrement to electrical stimulation in 5 μM Ca^{2+} was observed by Sleigh (1970) by applying approximately 20 stimuli during 200 minutes of incubation. Forty micromolar [Ca^{2+}] and above did not result in a decrement (Sleigh 1970) suggesting that the 'cutoff' for this effect is between 5 and 40 μM free Ca^{2+}.

The partial replacement of Ca^{2+} with Zn^{2+} or Ba^{2+} or the complete replacement of Ca^{2+} with Ba^{2+} or Sr^{2+} also resulted in a response decrement to electrical stimulation. The reasons why these ions cause decrements, and why Ni^{2+}, Co^{2+}, and Mn^{2+} (normally Ca^{2+} blockers) did not, are not clear. These decrements to electrical stimulation may be caused by a reduced availability of extracellular calcium.

The above results are compatible with a model where separate mechanoreceptor and voltage-sensitive calcium channels exist in the membrane of Spirostomum and where habituation is due to a decrease in the mechanoreceptor calcium current. However, the experiments

performed here do not conclusively discriminate between calcium fluxes, i.e., uptake, and calcium binding as the mechanism. The binding mechanism is suggested by (1) the effects of low [Ca^{2+}] on transduction, (2) the inability to observe Ca-45 uptake during mechanical stimulation, and (3) the effects of ions are not based on ionic sizes. Calcium uptake mechanisms are strongly supported by (1) the Ca-45 uptake during electrical stimulation, (2) data from the ciliates Paramecium and Stentor, and (3) the presence of a mechanism (Ca-induced Ca inactivation) which appears to fulfill the requirements of a model for habituation. Mechanoreception is based on calcium influx in the related ciliates Paramecium and Stentor (Ogura and Machemer 1979, 1980; Machemer and Ogura 1979; Wood 1980); however, membrane binding is also extremely important in the regulation of ciliary reversal in Paramecium (Jahn 1962; Naitoh and Yasamasu 1967; Naitoh and Eckert 1968; Naitoh 1973). More work will be necessary to discriminate between these mechanisms. For example, chlortetracycline fluorescence measurements would be an excellent initial experiment to assess the role of calcium binding in the effects observed. Calcium may affect membrane potential by binding to the membrane surface (McLaughin, Szabo, and Eisenman 1971; DiFrancesco and McNaughton 1979); this is also a possible transducer mechanism (Gingell 1971) and could be involved in controlling the membrane permeability to calcium and other ions (Kim and Sanders 1979).

Although Ca^{2+} appears to be the controlling ion it is important to identify the excitation current carrying ion because calcium

may activate the conductance of other ions, i.e., K^+, Na^+, or Mg^{2+}. Ca^{2+}-dependent Na^+ and K^+ currents have been reported in a variety of organisms (Romero and Whittam 1971; Meech 1972; Peach 1975; Lew and Ferreira 1976; Kostyuk, Kryshtal, and Tsyndrenko 1977; Schulz and Heil 1979; Hotson and Prince 1980; Woolum and Gorman 1981) including Paramecium (Satow and Kung 1978; Saimi and Kung 1980). Mechanoreception in Aplysia statocyst cells is apparently mediated by sodium currents (Gallin and Wiederhold 1977). The present study does not rule out the possibility that Na^+ or K^+ ions are directly involved in habituation or transduction.

In evaluation of Ca-45 uptake studies co-transport of PO_4^{2-} also must be considered. Spirostomum actively accumulates both ions (Jones 1967; Balcerzak 1978). The present results show that Ca-45 uptake was three times higher in media containing PO_4^{2-}. Another ciliate Tetrahymena pyriformis also actively accumulates Ca^{2+} in the presence of PO_4^{2-} (Rosenberg and Munk 1969). The calcium and phosphate apparently are stored as hydroxyapatite which is not likely to be readily available for the activation of the contractile filaments. Such non-exciting calcium uptake components must be considered in interpreting the role of calcium in studies involving Ca-45.

The use of La^{3+} and EGTA to estimate calcium binding was not completely successful with Spirostomum. The action of La^{3+} and EGTA on non-specific filter binding (see Table 5) interfere with the ability to observe a decrease in binding. La^{3+} and EGTA did appear to reduce binding of Ca-45 but not in a quantifiable manner with the filtration technique used.

Many differences were noted in comparing the results of the present studies with studies on other ciliates, i.e., Paramecium and Stentor. Differences with Paramecium are not surprising since the calcium currents and mechanoreceptor potentials are related to a different behavior from that observed in Spirostomum--in Paramecium the behavior studied is ciliary reversal while in Spirostomum we study contraction and habituation. However, Stentor exhibits the same behavior pattern as Spirostomum, i.e., habituation of the contractile response to mechanical but not electrical stimulation. One major difference between Spirostomum and Stentor is that responsiveness to mechanical stimulation in Stentor is blocked by tubocurarine and decamethonium (Wood 1975, 1977)--a result that was not observed for Spirostomum. Also, Co^{2+} blocks mechanoreceptor calcium currents in Stentor (Wood 1980) but has no effect on responsiveness in Spirostomum. Thus, the likelihood of identical habituation mechanisms in these two ciliates is doubtful.

Finally, in recent years it has become apparent that many of the regulatory functions carried out by calcium ions may be mediated by calmodulin (Cheung 1980; Roufogalis 1980; Klee, Crouch, and Richman 1980; O'Callaghan, Dunn, and Lovenberg 1980; Blum, Hayes, Jamieson, and Vanaman 1980; Wolff, Cook, Goldhammer, and Berkowitz 1980), a small protein whose structure is highly conserved throughout phylogeny (Vanaman 1980). Calmodulin binds Ca^{2+} and translates Ca^{2+} levels into a large variety of cellular responses. Calmodulin and calcium may control the cyclic nucleotide dependent kinases that have been isolated from protozoans (Kuznicki, Kuznicki, and

Drabikowski 1979; Blum et al. 1980; Lewis and Nelson 1980); these kinases could be involved in regulating membrane permeability. Interestingly, Zn^{2+} may act by inhibiting calmodulin (Brewer, Aster, Knutsen, and Kruckeberg 1979); removal of habituation by Zn^{2+} could be due to inhibition of calmodulin action on a calcium permeability-controlling protein, suggesting that habituation is regulated by calmodulin.

In summary, the results indicate:

1. Extracellular Ca^{2+} is necessary for transduction of mechanical and electrical stimuli.

2. Various effects of ions on transduction were observed; these effects were not related to hydrated or non-hydrated ionic radii. The different ion effects suggest different sites for mechano- and electro-transduction.

3. Removal of habituation to mechanical stimulation by Ba^{2+} and Zn^{2+} suggests a role for calcium in the habituation process. However, reducing extracellular calcium levels had little effect on the amount of habituation during two minutes of stimulation.

4. Maintenance of responsiveness to electrical stimulation requires the presence of extracellular calcium; a response decrement occurs when the availability of extracellular calcium is reduced.

5. The experiments provide support for both binding and uptake as mechanisms for calcium action.

6. The results are compatible with a heuristic model in which two separate calcium channels are responsible for sensory transduction and habituation is due to a reduced calcium current through the mechanoreceptor channel. The habituation process may involve regulation of calcium permeability by a metalloenzyme which regulates mechano-receptor current based on intracellular calcium levels.

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