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THE ANTAGONISTIC EFFECTS OF ANTIMITOTIC AGENTS ON CONTRACTION AND RE-EXTENSION IN THE CILIATE *SPIROSTOMUM AMBIGUUM*

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SUMMARY

Contraction in *Spirostomum* is apparently directed by 2 antagonistic systems which respond to changes in free calcium concentration. The system responsible for imparting contractile tension consists of bundles of 4–5 nm microfilaments which can be calcium-activated. The second system consists of pellicular microtubules which may have the capacity to impart tensile force and react with each other through cross-bridging. Cells subjected to electrical stimulation to induce contraction in the presence of 0·1 mM 2,4-dinitrophenol and KCN failed to recover their original resting lengths. Similar treatment with colchicine produced the same effect, implying an interruption in the activity or structural integrity of the microtubular system. Treatment with 0·5 μg/ml cytochalasin B inhibited all contractile responses, presumably by blocking the activity of microfilaments.

INTRODUCTION

Recent observations suggest that contractile protozoa of the order Peritrichida possess rubber-like contractile proteins which are activated by calcium in the absence of direct metabolic energy (Weis-Fogh & Amos, 1972; Huang & Pitelka, 1973). In *Spirostomum ambiguum*, the contractile proteins are in the form of filamentous aggregates which join to form a cylindrical web around the cell approximately 10 μm below the cell membrane (Lehman & Rebhun, 1971; Ettienne, 1970). The filamentous bundles are often found in close association with endoplasmic vesicles and mitochondria. It has been shown that with excitation there is an increase in the concentration of cytoplasmic, free calcium (Ettienne, 1970) and that the filamentous aggregates undergo a change in orientation and diameter in *Stentor* and in *Spirostomum* (Newman, 1972; Lehman & Rebhun, 1971; Huang & Pitelka, 1973). However, Huang provides evidence for a contractile system which depends on the integrity of 2 elements. This first is based on the calcium-induced shortening of the filamentous proteins; the second is based on the passive interdigitating of microtubules which constitute the longitudinal bundles adjacent to the pellicle in *Stentor*. She further suggests that relaxation (elongation) is accompanied by a 'variation in the axial position' of adjacent microtubules mediated by 'cross-bridge' formation.

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There is very little information on the interactions and energy requirements of tubules and filaments in the systems currently under study. Wessels *et al.* (1971) have shown that the filament-dependent contractile machinery of various motile cell types can be reversibly inhibited by cytochalasin B. In all cases, the microtubular components of these systems are unaffected except when treated with colchicine or inhibitors of protein synthesis. This study shows that the contraction in *Spirostomum ambiguum* can be separated into 2 independent phases. The shortening phase can be inhibited reversibly by treatment with cytochalasin B and becomes insensitive to any stimulation to contract, including that of fixatives; the re-extension phase appears to be dependent on the integrity of the cortical microtubules and can be inhibited by treatment with colchicine, 2,4-dinitrophenol, and KCN.

MATERIALS AND METHODS

Cell culture

Spirostomum was cultured in 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) supplemented with barley grains. The cells were strain 7W1350 obtained from Wards Biological Suppliers, Rochester, New York, U.S.

Electrical stimulation and inhibition

Cells were stimulated to contract by supplying a threshold level d.c. pulse of 25 ms duration from a Grass stimulator at 15-s intervals across a rectangular field defined by platinum electrodes. A minimum of 6 cells were kept in the chamber for each of 5 separate runs in all experiments. Cell response to stimulation was monitored through an Ampex 500 videorecorder attached to the microscope. Measurements of the maximum recovery lengths of the cells during pulse intervals following stimulation were made directly from the monitor screen when the tape was played back. Cell length during excitation was also recorded by holding the image on the monitor screen as the cells exhibited maximum contraction. Specific quantities of the test chemical were added to the stimulation chamber via a μL Hamilton syringe and allowed to equilibrate for 5 min before electrical stimulation. The final concentration in the chamber of each of the test chemicals was as follows: cytochalasin, 1 μg/ml; 2,4-DNP, 0·1 mM; KCN, 0·1 mM, and colchicine, 0·1 mM. Values for standard deviations at each trial and for the correlation coefficients between the initial (L_1), contracted (L_2), and recovery (L_3) lengths of the cells were determined through a comparison statistic programme in an IBM 360 computer.

Cytochalasin-treated specimens were prepared for transmission electron-microscopic observation of microfilaments by fixing in cacodylate-buffered (0·05 M), glutaraldehyde-acrolein (1 and 5 %) at pH 6·3 for 1 h at 4 °C; 2·5 % osmium tetroxide in the same buffer was applied as a post-fixative for 1 h. Epon-embedded sections were cut on a Porter-Blum ultramicrotome, stained with lead citrate and viewed with a Philips EM 300 electron microscope.

Specimens treated with colchicine, 2,4-DNP, KCN, and cytochalasin were quick-frozen in liquid nitrogen to preserve cell shape. Following dehydration and substitution with amyl acetate, the cells were desiccated through critical-point drying and observed with a Jeolco scanning electron microscope.

RESULTS

Inhibition of recovery during electrical stimulation

The organisms were induced to contract by means of a threshold d.c. pulse, duration 25 ms, which was repeated at intervals of 15 s. During 5 min of stimulation the mean recovery length L_3 declined progressively to a value 11 % less than the initial

recovery length (Fig. 1). Normal recovery time to full length following electrical stimulation by a single d.c. pulse has been reported to have a maximum duration of 5 s (Ettienne, 1970). However, repeated stimulation extends the recovery time, thus

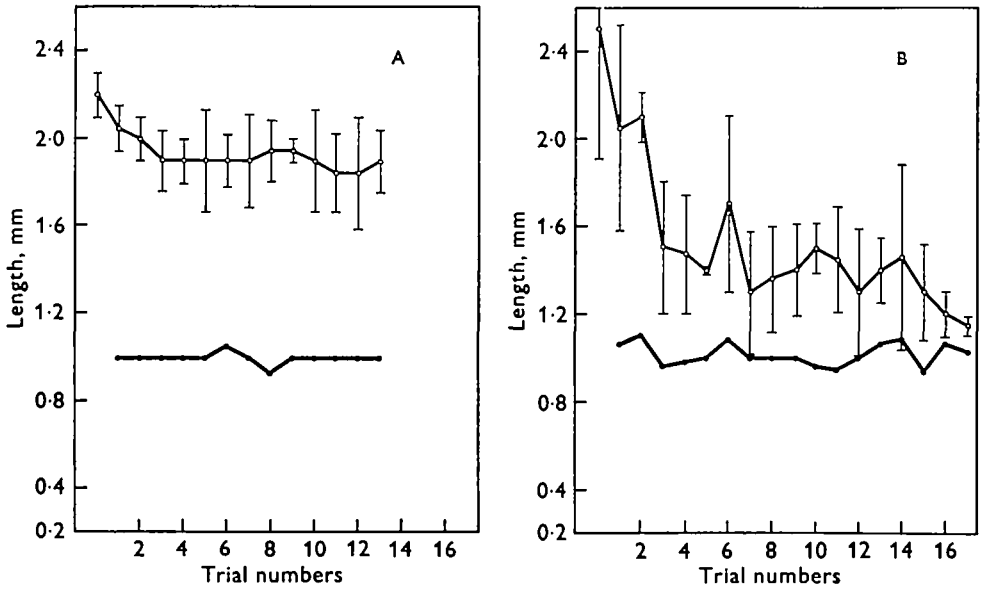

Fig. 1. Following repetitive electrical stimulation above threshold, in the absence of metabolic inhibitors, cells shortened an average of 11 % during the duration of stimulation (A). When cells were subjected to the same conditions of electrical stimulation in the presence of 0·1 mM 2,4-DNP, they shortened an average of 46 % of their initial length at the end of 16 trials (B). A, control; B, treated with DNP; ○, relaxed length; ●, contracted length.

Table 1. *Values for Pearson correlation coefficients determined from comparisons between the initial length* (L_1)*; the contracted length* (L_2)*, and the return length* (L_3) *in control and experimental trials*

	Comparison	Correlation	Significance
Control	$L_1:L_2$	−0·189	0·028
Control	$L_1:L_3$	0·349	0·001
Control	$L_2:L_3$	−0·076	0·374
KCN	$L_2:L_3$	−0·527	0·001
2,4-DNP	$L_2:L_3$	−0·537	0·020

preventing complete recovery of cells during the intervals reported. Comparisons between the initial length (L_1) and the contracted length (L_2) showed a negative coefficient of correlation (−0·189), indicating that the fully extended cell tended to go to maximum contraction when stimulated, though values for L_2 varied by less than 2 % throughout the experiment. Comparisons between the initial length (L_1) and the return length following contraction (L_3) showed a correlation coefficient of 0·35,

indicating a partial recovery of cell length during pulse intervals. A negative correlation coefficient ($-0{\cdot}076$) was also shown to exist between the contracted length (L_2) and the return length (L_3) (Table 1).

Effects of 2,4-dinitrophenol and electrical stimulation

When cells from the same culture were pretreated with 1·0 mM 2,4-DNP for 5 min before stimulation, there was a 46 % average decrease in length of the cells at the end of 5 min of stimulation (Fig. 1). Determination of the correlation coefficients showed a negative correlation ($-0{\cdot}537$) between the contracted length (L_2) and the return length (L_3) (Table 1).

Effects of KCN and electrical stimulation

Cells treated with 0·1 mM KCN became stationary, stopping all ciliary activity. Repeated electrical stimulation resulted in an average shortening by 33 % of the value of L_1. Scanning micrographs revealed that experimental cells were completely denuded of cilia (Fig. 2D). Comparisons between the values for the contracted length (L_2) and the return length (L_3) showed a negative correlation coefficient of ($-0{\cdot}527$) (Table 1).

Effects of cytochalasin B

Cells subjected to treatment with 0·5 μg/ml cytochalasin B became flattened and elongated an additional 25 % of their initial lengths during the first 10 min of treatment (Fig. 2C). During this interval, the effects of cytochalasin were reversible by dilution. Beyond the initial 10 min of treatment, they became stationary and exhibited asynchronous ciliary activity followed by loss of cilia. At the end of 20 min of treatment, the cells failed to respond to any stimulation to contract, including fixatives.

Effects of colchicine

Cells treated with 0·1 mM colchicine shortened by 45 % of their original lengths within 30 min of treatment, becoming pear-shaped (Fig. 2A). These cells still exhibited normal locomotory activity and would show brief contractures when stimulated electrically or mechanically.

DISCUSSION

It seems unlikely that the whole cycle of contraction and relaxation in contractile cells is independent of metabolic energy. Thus, the experimental approaches presented in this article were intended to identify the active phase of contraction in *Spirostomum* in the light of the findings in *Stentor* and *Vorticella* (Huang & Pitelka, 1973; Weis-Fogh & Amos, 1972). Weis-Fogh & Amos report that the extracted *Vorticella* stalk is able to work in both directions. *Spirostomum* failed to re-extend following stimulation in the presence of metabolic inhibitors. This suggests that re-extension is dependent on the availability of metabolic energy. 2,4-DNP seems most effective as an inhibitor of re-extension, ostensibly by uncoupling mitochondrial oxidative

phosphorylation. KCN had an additional effect on locomotion by destroying ciliar activity. It is presumed that KCN affects other aspects of cellular metabolism than does 2,4-DNP.

Colchicine reputedly solubilizes microtubules by binding to the subunit and shifting the monomer/polymer equilibrium of the tubule subunits in favour of the monomer. Following treatment of *Spirostomum* with colchicine for 30 min, the cells shortened to their contracted dimension without any obvious effects on the locomotory activity of the cell or its ability to exhibit additional contractures when stimulated. The effects of colchicine could be reversed by dilution.

Cytochalasin B, an inhibitor of microfilament-induced motility (Wessels *et al.* 1971) effectively inhibited electrically or chemically induced contraction in *Spirostomum*. In addition, the cell eventually extended to 125 % of its original length. The extension was accompanied by asynchrony and eventual loss of cilia.

There has been evidence from several investigators which suggests that the occurrence of cross-bridges between microtubules in both protozoan and metazoan cells is essential for the maintenance of microtubular arrays (McIntosh, 1974; Tilney, 1971). Intertubular bridges, such as exist in the axostyle of *Saccinobaculus* and in the flagellar microtubules of various sperm cells, have an associated protein, dynein, which displays ATPase activity and may be functionally analogous to myosin as a mechanochemical coupling agent for cell motility (McIntosh, 1974). Gel electrophoretic analysis of the protein composition of homogenized *Spirostomum* shows 2 prominent bands with the same electrophoretic mobility as dynein and tubulin standards (unpublished results). The microtubules in *Spirostomum* could presumably slide over one another during extension as a direct consequence of the work performed when microtubular dynein hydrolyses ATP. Gibbons & Gibbons (1972) reactivated sea-urchin sperm flagella extracted with Triton X-100 with $MgATP^{2-}$. The rate of ATP hydrolysis was reduced when equimolar quantities of $CaATP^{2-}$ were added to the models, both in the presence and absence of $MgATP^{2-}$. Their results suggest that sperm flagellar dynein does not require calcium for motility. Their observations support the *in vitro* studies of Borisy & Olmstead (1972) and Weisenberg (1972) which show that the polymerization of microtubules requires Mg and ATP or GTP, and is accomplished when the calcium concentration in the medium is maintained relatively low. We suggest that calcium might competitively inhibit the hydrolysis of $MgATP^{2-}$.

The results of the present investigation, though not conclusive, show that calcium influx to the cytoplasm following stimulation may have a disruptive effect on cortical microtubules, possibly by inhibiting a dynein-ATP mediated cross-bridging. The presence of metabolic inhibitors blocks the formation of ATP and thus prevents the re-association or sliding of tubules following repetitive stimulation of the cell.

These observations, along with data published by other investigators, support the assumption that contraction in *Spirostomum* is under the control of 2 antagonistic systems which may respond independently to changes in free calcium concentration. This process can be inhibited by specific inhibitors of oxidative phosphorylation and antimitotic agents such as colchicine and cytochalasin which reputedly affect microtubules and microfilaments, respectively.

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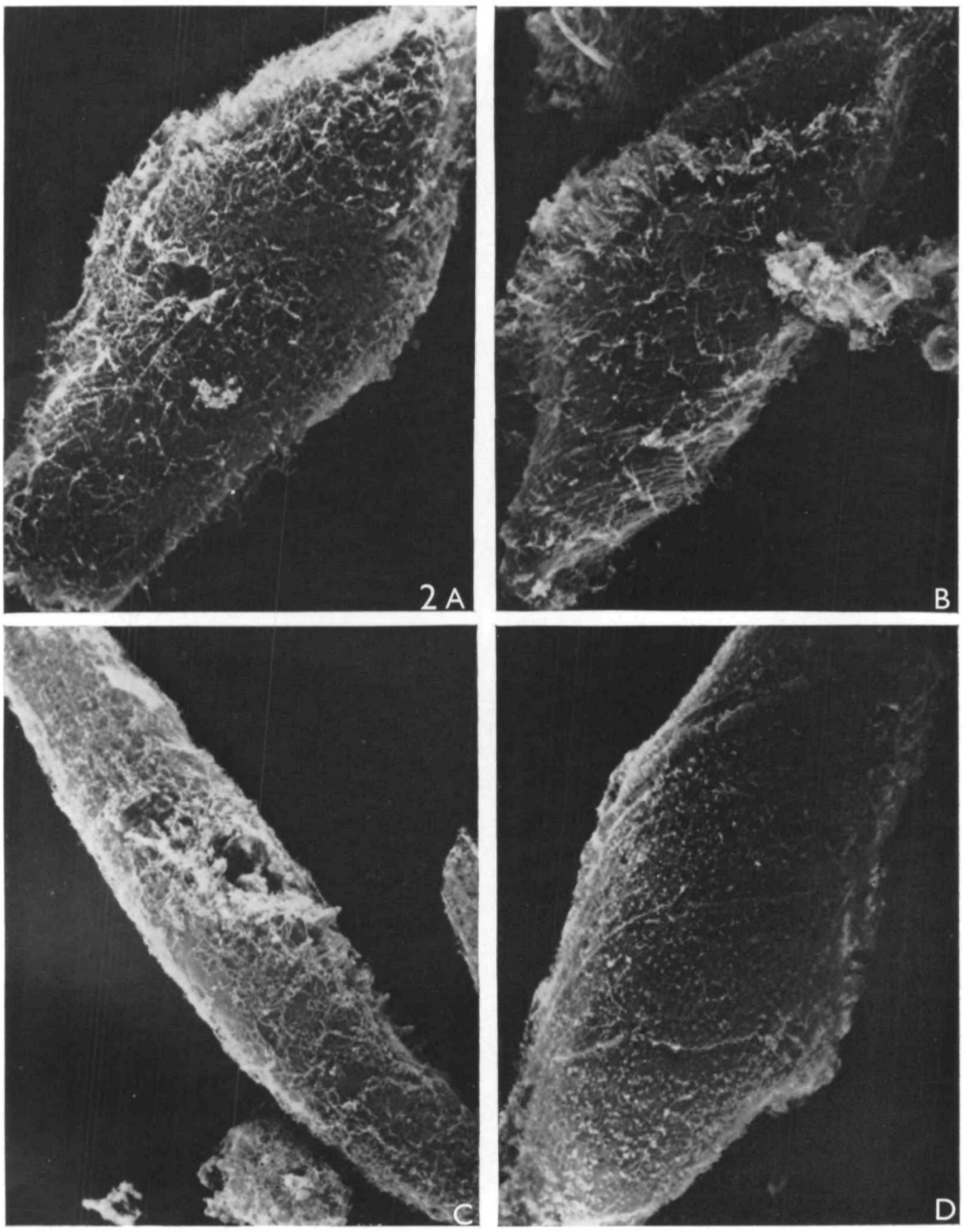

Fig. 2A. Untreated cell. × 500.

Fig. 2B. 0·1 mM colchicine-treated cell in contracted state. × 525.

Fig. 2C. Cell treated with 0·5 μg/ml cytochalasin B. Cell has become elongated by an additional 25 % of its original length. × 400.

Fig. 2D. Deciliated cell following treatment with 0·1 mM KCN. × 525.