THE ROLE OF DIVALENT CATIONS IN THE REGULATION OF MICROTUBULE ASSEMBLY

In Vivo Studies on Microtubules of the Heliozoan Axopodium Using the Ionophore A23187

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ABSTRACT

Low concentrations of calcium and magnesium ions have been shown to influence microtubule assembly in vitro. To test whether these cations also have an effect on microtubules in vivo, specimens of *Actinosphaerium eichhorni* were exposed to different concentrations of Ca^{++} and Mg^{++} and the divalent cation ionophore A23187. Experimental degradation and reformation of axopodia were studied by light and electron microscopy. In the presence of Ca^{++} and the ionophore axopodia gradually shorten, the rate of shortening depending on the concentrations of Ca^{++} and the ionophore used. Retraction of axopodia was observed with a concentration of Ca^{++} as low as 0.01 mM. After transfer to a Ca^{++}-free solution containing EGTA, axopodia re-extend; the initial length is reached after about 2 h. Likewise, reformation of axopodia of cold-treated organisms is observed only in solutions of EGTA or Mg^{++}, whereas it is completely inhibited in a Ca^{++} solution. Electron microscope studies demonstrate degradation of the axonemal microtubular array in organisms treated with Ca^{++} and A23187. No alteration was observed in organisms treated with Mg^{++} or EGTA plus ionophore.

The results suggest that, in the presence of the ionophore, formation of axonemal microtubules can be regulated by varying the Ca^{++} concentration in the medium. Since A23187 tends to equilibrate the concentrations of divalent cations between external medium and cell interior, it is likely that microtubule formation in vivo is influenced by micromolar concentrations of Ca^{++}. These concentrations are low enough to be of physiological significance for a role in the regulation of microtubule assembly in vivo.

Many functions with which microtubules are associated depend on their ordered assembly and disassembly. In vivo studies clearly show that formation and breakdown of microtubules are influenced by temperature, hydrostatic pressure, and D_2O (for review, see reference 48). These

and numerous investigations suggest that the assembly of microtubules from their subunits is controlled by an equilibrium process. Nevertheless, relatively little information is available about the precise nature of the factors governing the assembly and disassembly of microtubules in living cells.

Recently, the successful isolation of microtubule protein and the development of an in vitro system for the study of microtubules have allowed a closer examination of the factors controlling microtubule formation (2, 18, 25, 44, 52). Of particular interest is the observation that low concentrations of Ca^{++} prevent microtubule assembly (52) and cause the disassembly of existing tubules (13, 14). Likewise, isolation of the mitotic apparatus (31) and the assembly of microtubules of isolated spindles (17, 32) are also dependent upon the presence of a chelating agent. These observations led to the suggestion that Ca^{++} might be a regulator of the polymerization of microtubules not only in vitro, but also in the living cell. However, experimental examination of the results obtained in in vitro studies by using in vivo systems seems to be particularly difficult. To clarify whether Ca^{++} has a regulatory function, one should (a) manipulate the concentration of free Ca^{++} in the cytoplasm, or (b) demonstrate a Ca^{++} fluctuation associated with phases of microtubule assembly and disassembly, as suggested by Bryan (3).

In performing experiments which fall into the first category, a major difficulty with which one is confronted is that the cell membrane serves as an effective barrier to Ca^{++} ions (1, 40). In addition, many cells possess a regulatory system for the sequestration of Ca^{++} in a separate intracellular compartment (7, 30). Simply raising the Ca^{++} concentration in the external medium is, in most instances, ineffective (30) or, at higher concentrations, does not exclude nonspecific or even toxic effects.

A unique and versatile probe for the study of divalent cation effects within a physiological concentration range is the ionophorous antibiotic A23187 (29, 34, 53). This substance promotes transmembrane transport of divalent cations with a high affinity for Ca^{++} and Mg^{++} (28, 34). Recently, A23187 has repeatedly been used in the study of Ca^{++}-dependent cellular activities (e.g., references 4, 9, 19, 22, 43, 46).

For the study of the effects of Ca^{++} ions on microtubules in vivo presented in this paper, *Actinosphaerium eichhorni* was used as an experimental organism. The long slender axopodia radiating from the cell body each contain a highly ordered system of microtubules, the axoneme (38, 50). Axonemal microtubules undoubtedly belong to a class of microtubules which are highly labile. Their relative isolation from the cell body and, hence, their high sensitivity to various chemical and physical agents (45, 47, 51) has made them a useful tool in the study of microtubule functions. Since changes in the degree of microtubule polymerization are reflected by changes in the length of the axopodium (which can easily be recorded by light microscopy), this organism is especially well suited for a study of the degradation and reformation of microtubules.

The present investigation deals with the effects of A23187 on the maintenance and reformation of axopodia. In addition, the ultrastructure of axonemes is investigated with special emphasis on the morphology of Ca^{++}-induced microtubule breakdown.

MATERIAL AND METHODS

Maintenance of the Heliozoa

The organisms were cultured in a medium prepared from extracted garden mould: 1 kg of garden mould is boiled in 2 liters of tap water for 1 h. The supernate is decanted, reduced by boiling to 1 liter and kept as a stock solution at 4°C. 2 ml of this solution are diluted with 100 ml of distilled water to give the culture medium. Occasionally, 0.05% Torumin is added to the final medium. *Paramecium caudatum* grown axenically were used as food organisms. The ciliates were concentrated by mild centrifugation, resuspended in distilled water, and again centrifuged. New cultures of *Actinosphaerium* were started every 3-4 wk.

Light Microscopy

Observations and measurements of axopodial length were carried out with a Leitz Orthoplan microscope equipped with Nomarski optics. Single specimens of *Actinosphaerium* were placed in a shallow depression slide with one drop of culture medium or test solution and covered with a cover slip. Before treatment with test solutions containing the ionophore, organisms were allowed to equilibrate for at least 20 min in the corresponding salt solution without ionophore. For each experimental condition, the length of about 10 randomly selected axopodia per cell of at least 15 organisms was measured at appropriate time intervals, with an eyepiece micrometer. To obtain exact measurements, special at-

tention was paid to the fact that these axopodia were in the plane of focus over their whole length. All experiments were carried out at room temperature (21-24°C).

Cold Treatment

Organisms were placed in a refrigerator whose temperature had been adjusted to 2°C. After 3 h the cells were transferred to the different test solutions with and without ionophore at 2°C and were then allowed to equilibrate at room temperature. In these experiments, small petri dishes were used instead of depression slides. Observations and measurements were carried out on an ordinary light microscope.

Solutions

A23187 was obtained from Dr. R. Hamill, Ely Lilly Co., Indianapolis, Ind., and was dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 5 mg/ml. The ionophore was added to the test solutions just before the experiments. The final concentration of DMSO did not exceed 0.1%. In every experiment in which the ionophore was used, the solution used in the corresponding control experiment contained 0.1% DMSO. This concentration of the solvent affects neither the viability of the heliozoa nor axopodial length. Magnesium chloride, calcium chloride, and ethylenebis-(oxyethylene-nitrilo)-tetraacetic acid (EGTA) were dissolved in deionized distilled water. These substances were analytical grade and were obtained from Merck, Darmstadt. All solutions had a pH similar to that of the culture medium (6.4-6.8).

Electron Microscopy

The heliozoa were fixed for electron microscopy by the method of Roth et al. (38) with several slight modifications. The first fixation step involved addition of 12% glutaraldehyde in 0.033 M phosphate buffer (pH 6.6) supplemented with 0.01 mM $MgCl_2$ and 1 mM sucrose to an equal amount of test solution containing the organisms. After about 30-60 s 1% osmium tetroxide in 0.016 M phosphate buffer, 0.005 mM $MgCl_2$, and 0.5 mM sucrose was added directly to this medium. In this second fixation step which lasted for 30-40 min the final concentrations were: 3% glutaraldehyde, 0.5% osmium tetroxide, 0.016 M phosphate buffer, 0.005 mM $MgCl_2$, and 0.5 mM sucrose.

After fixation, the organisms were rinsed three times in 0.016 M phosphate buffer and quickly dehydrated in a graded series of ethanol. During the 70% and 90% steps, 1% phosphotungstic acid and 0.5% uranyl acetate was present in the solutions (cf. reference 42). After embedding in an Epon-Araldite mixture, ultrathin sections were cut on an LKB ultrotome III with a diamond knife and were picked up on Pioloform-coated 100-mesh grids. After staining with uranyl acetate and lead citrate

(36), sections were examined with a Hitachi H 500 electron microscope operated at 50 and 75 kV.

RESULTS

Control Organisms, Maintenance of Axopodia

Since the organisms were cultured in a medium of low osmolarity (20-30 mosM), viability was not affected if the heliozoa were maintained in distilled water or salt solutions of low concentration for several hours. In addition, as summarized in Table I, there was no alteration of axopodial length for at least 2 h after organisms had been transferred to distilled water containing 2 mM of $CaCl_2$, $MgCl_2$, or EGTA. Of particular interest is the observation that a solution of 1 μg/ml A23187 in distilled water or EGTA has no effect at all on axopodial length (Table I).

Reformation of Axopodia after Cold Treatment

When specimens of *Actinosphaerium* are kept at 2°C, axopodia extending from the body surface begin to shorten until, at the end of cold treatment after 3 h, only a decreased number of short axopodia with an average length of 30 μm are present. Upon removal from the cold, axopodia reform. Their regrowth is accompanied by an increase in birefringence of the axoneme and, hence, by reformation of axonemal microtubules (51).

In solutions of Ca^{++}, Mg^{++}, or EGTA (0.5 mM), reforming axopodia steadily increase in length (Fig. 1a-c), but at a slightly different rate in the different solutions. The rate of elongation decreases as they get longer (Fig. 2a). If cells are transferred after cold treatment to the same solutions containing 0.5 μg/ml A23187, reformation

TABLE I

Average Length of Axopodia after a 2-h Treatment with Different Solutions

Medium	Axopodial length (μm)	
	0 min	120 min
Culture medium	274 ± 52	268 ± 48
Distilled water	223 ± 60	231 ± 58
2 mM Ca^{++}	230 ± 65	233 ± 60
2 mM Mg^{++}	267 ± 58	275 ± 63
2 mM EGTA	256 ± 43	264 ± 49
Aq. dest. + 1 μg/ml A23187	223 ± 45	211 ± 53
1 mM EGTA + 1 μg/ml A23187	272 ± 48	278 ± 58

Results in columns 2 and 3 are mean ± standard error.

of the axopodia proceeds at a rate comparable to that of control organisms only in 0.5 mM Mg^{++} or EGTA, while it is completely inhibited in Ca^{++} (Fig. 2b). Instead, Ca^{++} causes a transient decrease of the length of the axopodia which are still present after cold treatment.

Degradation of axopodia by
Calcium ions

When heliozoa are exposed to solutions of Ca^{++} at different concentrations in the presence of 0.5 $\mu g/ml$ A23187, axopodia shorten at a rate characteristic for each Ca^{++} concentration (Fig. 3). With the strongest solution (1 mM), axopodial retraction is quite rapid, with 80% shortening within the first 4 min. After this rapid phase of retraction, the length of the axopodia is maintained at about the same level of 50 μm for at least 30 min. With the lower concentrations of Ca^{++}, the ionophore causes gradual axopodial retraction at a slower rate. Complete retraction of all axopodia is never found, although their number is, in most instances, obviously reduced at the end of the experiments. The average length of the remaining axopodia also depends on the Ca^{++} concentration: 50 μm in 0.5 mM, 70 μm in 0.1 mM, and 110 μm in 0.01 mM. The respective plateau values are maintained even after prolonged treatment with the ionophore. Essentially no axopodial shortening is observed in Ca^{++}-free media containing 0.5 mM EGTA (Fig. 3).

If the concentration of the ionophore is varied while the Ca^{++} concentration is maintained at 1 mM, a likewise dose-dependent rate of retraction results (Fig. 4). Shortening of axopodia occurs in the presence of the ionophore at a concentration as low as 0.01 $\mu g/ml$ ($1.9 \cdot 10^{-8}$ M). With 0.1 and 0.01 $\mu g/ml$ A23187, a slow but constant decrease in axopodial length is observed throughout the experiment, indicating that in fact the ionophore concentration is the limiting factor. It is only with high concentrations of both ionophore and Ca^{++} that after a rapid phase of shortening a plateau value of axopodial length (about 50 μm) is reached and maintained for at least 90 min.

MORPHOLOGY OF RETRACTING AXOPODIA: From a morphological point of view, several forms of axopodial retraction can be distinguished (Fig. 5): (I), rapid fragmentation into numerous small vesicles. This cataclysmic collapse which starts at the tip and proceeds proximally is completed within a few seconds. The vesicles remain
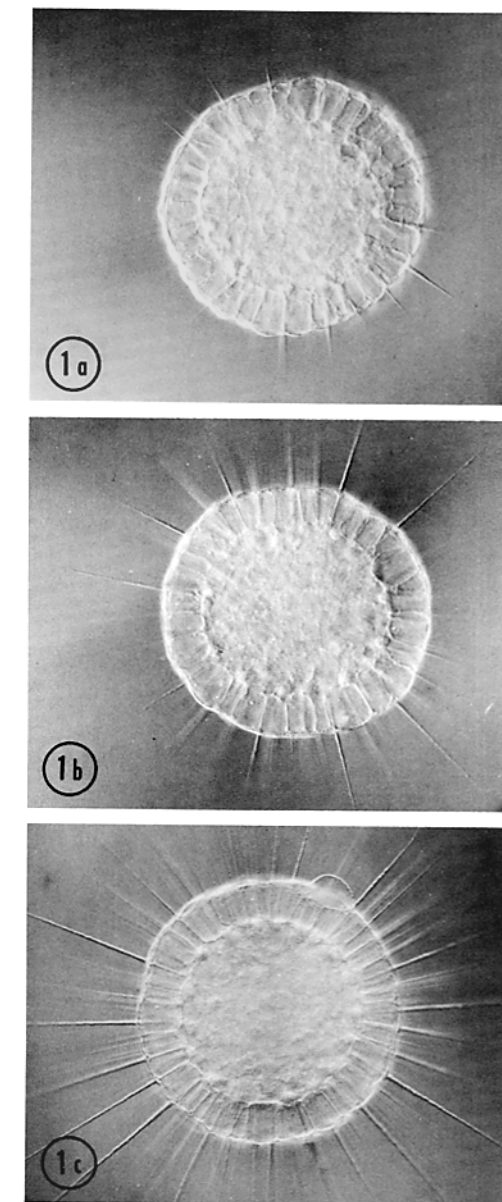

FIGURE 1 Living organism recovering from cold treatment in a solution containing 0.5 mM Mg^{++} and 0.5 $\mu g/ml$ A23187. a, 5 min, b, 30 min, c, 2 h at room temperature. × 100.

separated from the cell body; (II), fragmentation into vesicles which starts at one point along an axopodium and proceeds distally, while the proximal portion remains straight and slowly shortens by other means (IV–VI); (III), kinking in one point. The distal part bends in and gradually fuses with the proximal part; (IV), continuous
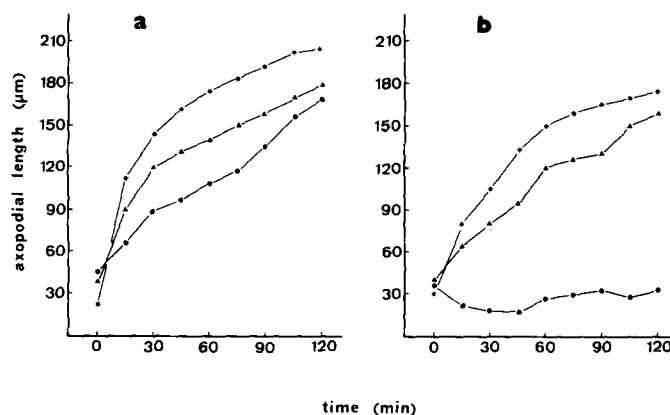
FIGURE 2 Changes in axopodial length during recovery from cold treatment. a, Without ionophore; b, in the presence of 0.5 μg/ml A23187. * 0.5 mM EGTA, ▲ 0.5 mM Mg^{++}, ● 0.5 mM Ca^{++}.
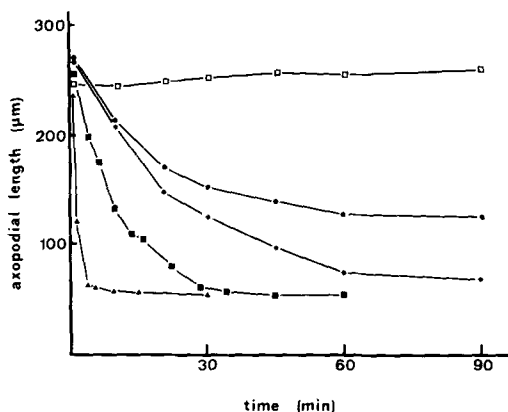

FIGURE 3 Rate of axopodial retraction at varying concentrations of Ca^{++} in the presence of 0.5 μg/ml A23187. ▲ 1 mM Ca^{++}, ■ 0.5 mM Ca^{++}, * 0.1 mM Ca^{++}, ● 0.01 mM Ca^{++}; □ 0.5 mM EGTA in Ca^{++}-free solution.
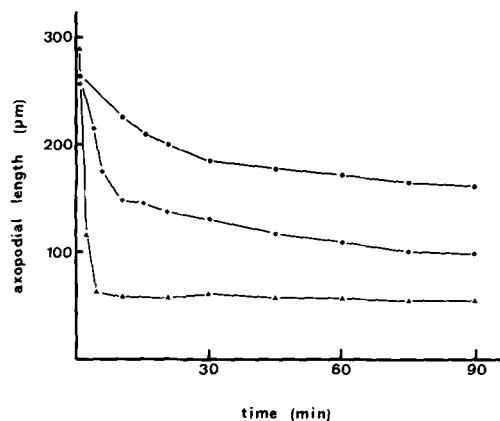
FIGURE 4 Axopodial retraction at varying concentrations of A23187 in the presence of 1 mM Ca^{++}. ▲ 1 μg/ml, * 0.1 μg/ml, ● 0.01 μg/ml A23187.

and relatively rapid "melting." Such an axopodium usually bears a terminal swelling; (V), slow continuous retraction with one or more bulbous swellings along the axopodium which give it a beaded appearance. The swellings move towards the cell body at about the same rate as the axopodium shortens, occasionally a little slower or faster (Fig. 6). During the course of retraction, two swellings may fuse or a new one may be formed at another point (Fig. 6); (VI), slow continuous retraction without any apparent morphological changes. Here, as in (V), shortening is accompanied by thinning of the axopodia.

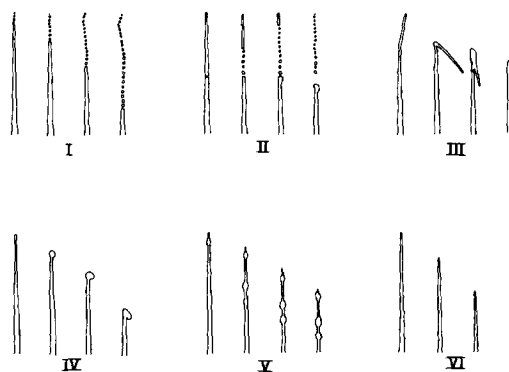During shortening, motion of axopodial particles is almost exclusively directed towards the cell

FIGURE 5 Forms of axopodial retraction during treatment with solutions of Ca^{++} and ionophore. For further description, see text.

body. Shortening of type IV-VI is observed at all concentrations of Ca^{++} and ionophore, while type I-III retraction is particularly abundant at higher doses.

REFORMATION OF AXOPODIA: After exposure to different solutions of Ca^{++} in the presence of A23187, organisms were transferred to a Ca^{++}-free medium containing different concentrations of EGTA. Just after transfer, axopodia started to elongate again (Fig. 7). As in recovery from cold treatment (cf. reference 51), reforming axopodia are straight with a smooth and even surface, while retracting axopodia appear beaded or show other irregularities (see Fig. 5). The rate of re-extension is independent of the EGTA concentration in the recovery medium. During the first 30 min their length increases from about 70 μm to 120 μm. Elongation apparently progresses at a slower rate towards the end of recovery. The

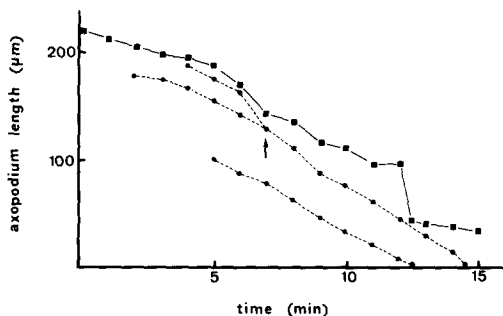

FIGURE 6 Changes of the length of an axopodium (■) and movement of three axopodial swellings (●) during treatment with 0.5 mM Ca^{++} plus 0.5 μg/ml A23187. At the point marked with an arrow, two swellings fuse.

initial length of axopodia is restored after about 2 h. Of particular interest is the observation that the organisms could be brought through at least three cycles of axopodial retraction and re-extension by being placed in ionophore solutions with and without Ca^{++}. Minute regulation of axopodial length was also achieved by short-term exposure to the respective solutions.

Effect of Magnesium Ions

The reaction of heliozoan axopodia to solutions of Mg^{++} was considerably different from the effect of Ca^{++} and depended on the concentrations of Mg^{++} and ionophore used (Fig. 8). With 0.1 mM Mg^{++} and 0.5 μg/ml A23187, the length of axopodia slightly increases and finally is maintained for at least 1 h. A higher concentration of Mg^{++} (1 mM) and a lower one of A23187 (0.1 μg/ml) lead to slight variations of axopodial length around the initial value. High concentrations of both Mg^{++} and ionophore (1 mM and 1 μg/ml, respectively) increase these oscillations with an overall slight tendency towards shortening. The different actions of Ca^{++} and Mg^{++} are further well documented by consecutively applying Ca^{++} and Mg^{++} solutions of the same concentration to a group of organisms in the presence of 0.5 μg/ml A23187 (Fig. 9). While axopodial length is maintained at about the usual level (250-300 μm) for 60 min in 0.5 mM Mg^{++}, Ca^{++} is found to cause 65% shortening within 10 min. If these cells are again transferred to 0.5 mM Mg^{++}, axopodia start to re-extend in the same way as they do in Ca^{++}- and Mg^{++}-free EGTA solution. In contrast to these
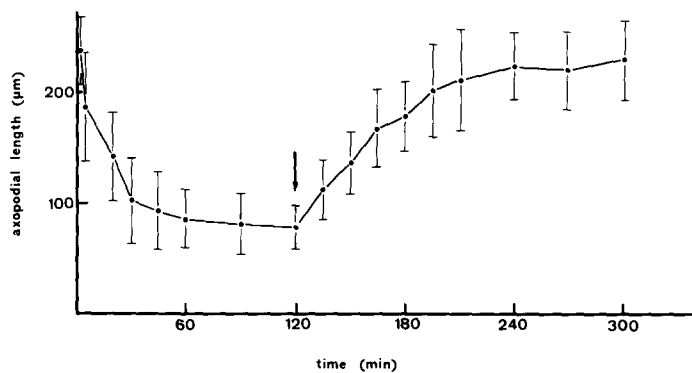

FIGURE 7 Changes in axopodial length during treatment with 0.1 mM Ca^{++} plus 0.5 μg/ml A23187 followed by recovery in 0.1 mM EGTA plus 0.5 μg/ml A23187. At the time marked with an arrow, the organisms were transferred from the Ca^{++} solution to the EGTA solution. Vertical bars indicate standard errors.

recovery experiments, however, the initial length of axopodia is not regained in the presence of Mg^{++}.

Electron Microscope Observations

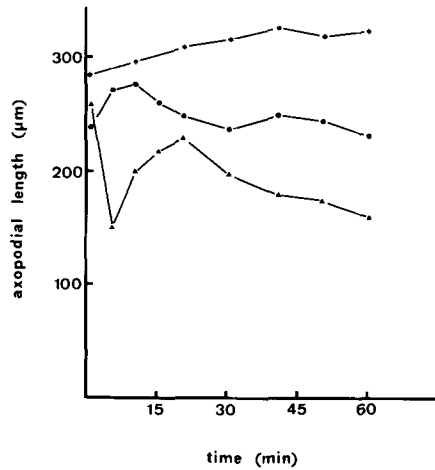Since the fine structure of heliozoa has already been studied extensively (e.g., references 24, 38,

FIGURE 8 Changes in axopodial length during treatment with different concentrations of Mg^{++} and ionophore. ★ 0.1 mM Mg^{++} plus 0.5 µg/ml A23187, ● 1 mM Mg^{++} plus 0.1 µg/ml A23187, ▲ 1 mM Mg^{++} plus 1 µg/ml A23187.

49, 50), only those observations will be described here which are of importance for an understanding of the effects elicited by Ca^{++} and Mg^{++} ions and the ionophore. For comparison between normal and experimentally altered axonemal microtubular patterns, a section through the basal portion of an axoneme of an organism treated with 0.5 mM Mg^{++} and 0.5 µg/ml A23187 for 30 min is shown in Fig. 10. These cells, as well as untreated organisms in culture medium or those treated with EGTA or distilled water plus ionophore (no examples shown), exhibit no gross differences in the fine structure of axonemes, at least in the proximal portions. Stability of the microtubular array appears to be increased in Mg^{++}-treated organisms. The number of missing or incomplete microtubules (C microtubules) is small and their appearance is usually restricted to the peripheral microtubule rows.

The degree of fine-structural alteration of axonemal microtubule patterns produced by Ca^{++} ions and the ionophore depends on the concentrations used. At relatively high levels of Ca^{++} and ionophore (0.5 mM and 0.5 µg/ml, respectively), several steps of axonemal degradation can be discerned. In accordance with rapid axopodial shortening observed light microscopically, destruction of the microtubule array is more severe than with lower concentrations, even in the basal
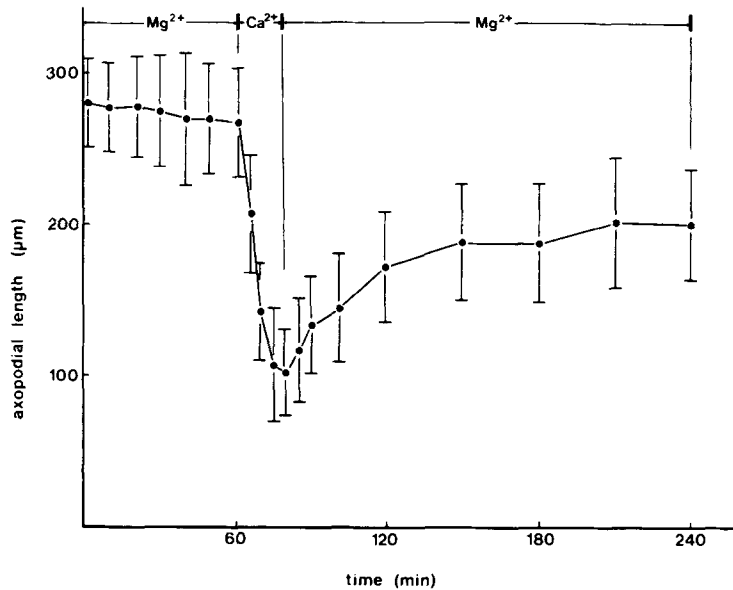

FIGURE 9 Changes of axopodial length of organisms consecutively treated with 0.5 mM Mg^{++}, 0.5 mM Ca^{++}, and, again, 0.5 mM Mg^{++} in the presence of 0.5 µg/ml A23187. Vertical bars indicate standard errors.
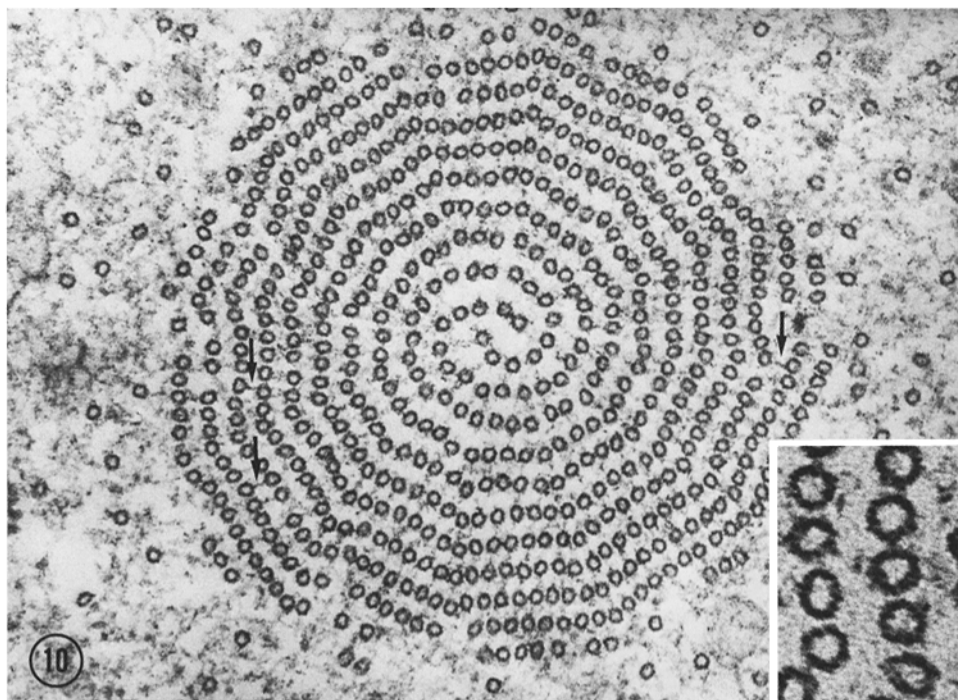
FIGURE 10   Cross section of an axoneme in the cortical region of the cell body. The organism was kept in 0.5 mM Mg^{++} plus 0.5 $\mu g/ml$ A23187 for 30 min. Except for some missing microtubules in the most peripheral rows, the microtubule array is quite perfect. Long links extend between microtubules of adjacent rows (arrows). Several single microtubules are located in the vicinity of the axoneme. × 80,000. *Inset,* Higher magnification showing normal microtubule structure. × 270,000.

portions of axonemes. Organisms fixed during the rapid phase of axopodial retraction show a gradual increase of the number of incomplete or missing microtubules (Figs. 11 and 12). Frequently, flocculent material appears in association with degrading axonemes (Fig. 12). Disruption of the microtubule array is even more evident in cross sections of axopodia obtained at a distance of 40-60 μm from the cell body. Besides irregularly packed microtubules more frequently observed at a lower dose of Ca^{++}, a considerable number of axopodia exhibit only few microtubules occasionally grouped in small rows (Fig. 13). These tubules are embedded in a fibrous matrix which presumably consists of the degradation products of depolymerized microtubules. Several micrometers more distally, the axopodium shown in Fig. 13 ends in a bulbous swelling (Fig. 14). Apart from some vesicles with electron-opaque content believed to be involved in prey capture or quieting (50), the lumen of this swelling is almost entirely filled with fibrous material. No microtubules are present.

The axopodium of Figs. 13 and 14 presumably belongs to the degradation type (IV) schematized in Fig. 5.

Axonemes of organisms fixed after 30 min in 0.1 mM Ca^{++} plus 0.5 $\mu g/ml$ A23187 are, as a rule, not so severely affected as they are at the higher concentration of Ca^{++}. Axopodial axonemes mostly show more or less complete disruption of the microtubule pattern with an apparently high number of still intact microtubules. In the basal region where stability to degradation seems highest, only slight disturbances of the microtubule pattern are evident. Groups of microtubules may be missing, or one-half of an axoneme may be more affected than the other half so that an asymmetric array results (Fig. 15). Few C-shaped microtubules are observed. Often, however, the axonemal structure is quite comparable to that of organisms not treated with Ca^{++} and ionophore.

During recovery from treatment with Ca^{++} and A23187, axonemes with a regular microtubule array reappear. In this respect, no differences in
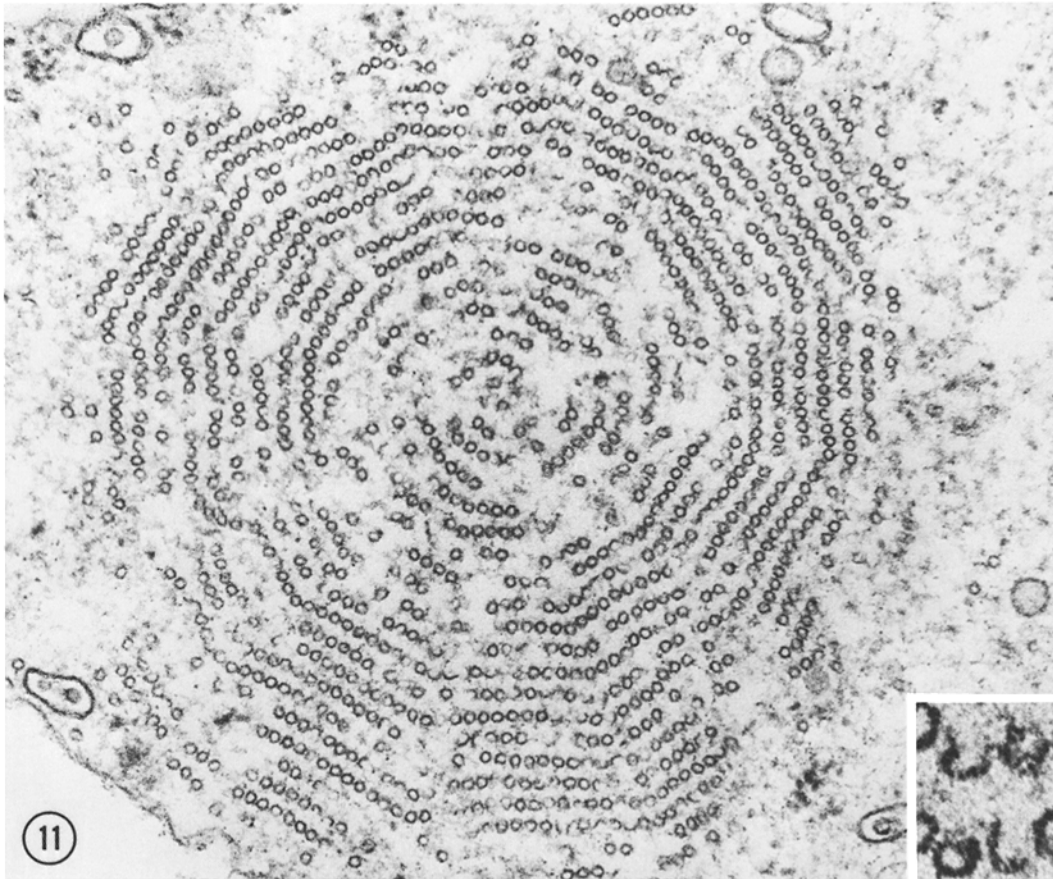
Figure 11   Cross section of an axoneme with numerous missing or C-shaped microtubules, particularly in the central zone. 0.5 mM Ca^{++} plus 0.5 μg/ml A23187, 10 min. × 65,000. *Inset,* Higher magnification showing disruption of the microtubule structure. × 270,000.

structure between these axonemes and axonemes recovering after degradation induced by other agents (e.g., references 39, 45, 51) could be detected.

DISCUSSION

The experiments described in this paper were undertaken to investigate the ability of Ca^{++} ions to affect microtubules in vivo. It was intended to influence the level of intracellular Ca^{++} in a cell system that allows easy and direct testing of the Ca^{++} effects on microtubules. This goal has been approached by using the divalent cation ionophore A23187 in experiments performed with the heliozoan *Actinosphaerium eichhorni.* The study demonstrates shortening of axopodia which depends on the concentrations of Ca^{++} and ionophore used

and axopodial re-extension in a Ca^{++}-free solution. Since the formation of axopodia correlates with the presence of microtubules (51), the results suggest that the formation of axonemal microtubules can be regulated by manipulating the Ca^{++} concentration.

There are at present few reports which indicate, in vivo, a relationship between intracellular Ca^{++} levels and microtubule assembly. Gallin and Rosenthal (11) demonstrated rapid Ca^{++} release and a shift of Ca^{++} out of the cytoplasm into a granule fraction (perhaps mitochondria) associated with microtubule assembly during human granulocyte chemotaxis. Other (indirect) evidence comes from the work of Petzelt (26) who demonstrated a Ca^{++}-activated ATPase during the first cell divisions of sea urchin eggs. Petzelt and Ledebur-
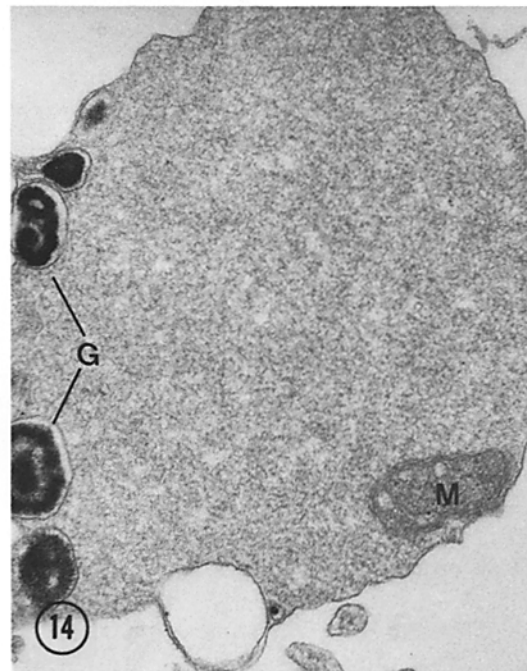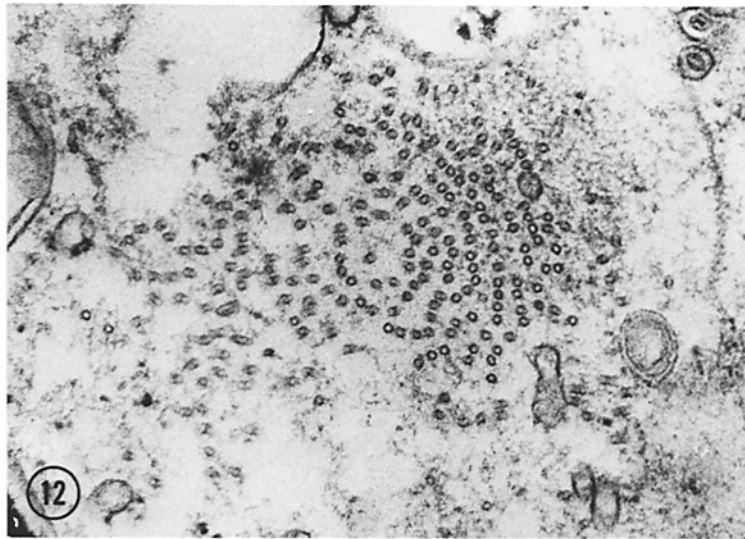
FIGURE 12   Cross section of an axoneme in the periphery of the cell body. The regular pattern is almost completely lost. The remaining microtubules are embedded in fibrous material. 0.5 mM Ca^{++} plus 0.5 µg/ml A23187, 10 min. × 54,000.

FIGURE 13   Cross section of an axopodium at a distance of about 40–60 µm from the body surface of the organism. A few microtubules, occasionally arranged in small rows, are embedded in a fibrous matrix. No axonemal microtubule pattern can be recognized. 0.5 mM Ca^{++} plus 0.5 µg/ml A23187, 10 min. × 55,000.

FIGURE 14   Same axopodium as previous figure, but several micrometers more distally where it ends in a bulbous swelling. The space of this swelling is almost entirely filled with fibrous material of moderate electron density. Some dense granules (G) and a mitochondrion (M) are present near the limiting membrane. 0.5 mM Ca^{++} plus 0.5 µg/ml A23187, 10 min. × 22,000.
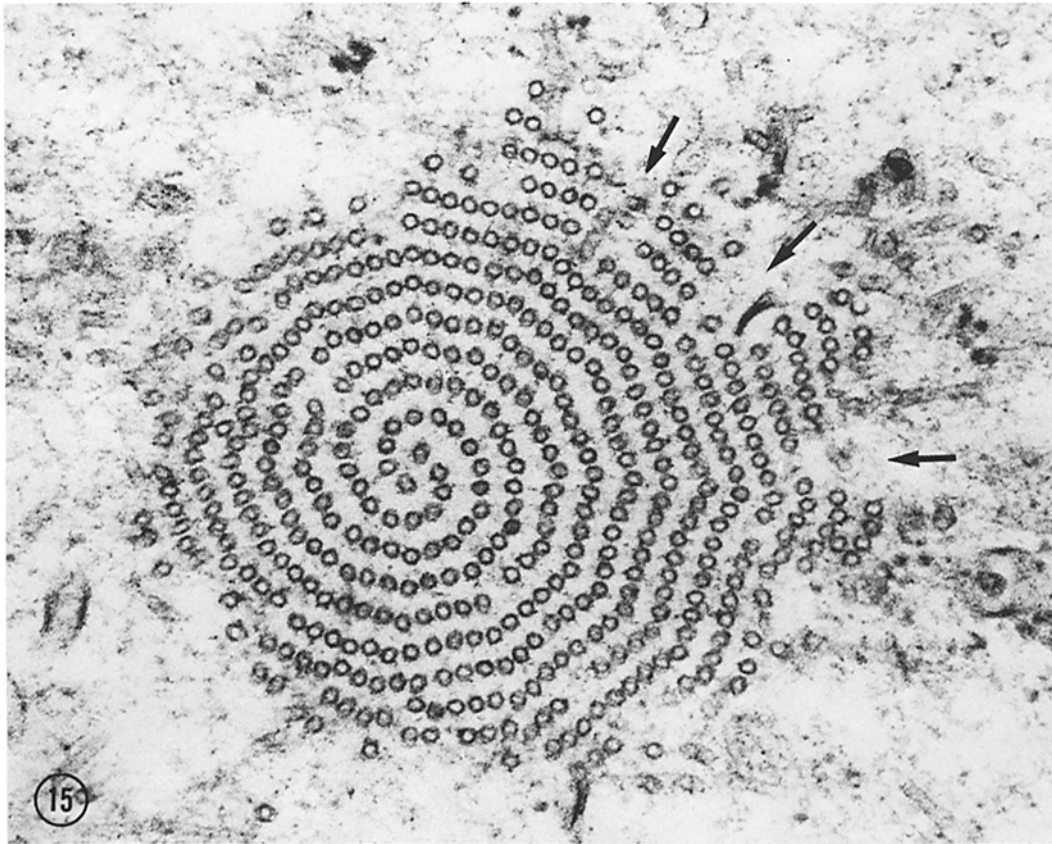
Figure 15   Asymmetric microtubule array after 30 min of treatment with 0.1 mM Ca⁺⁺ plus 0.5 μg/ml A23187. Along the horizontal axis of this micrograph, the number of complete microtubule rows are: from center to left five; from center to right nine. It is typical of treatment with 0.1 mM Ca⁺⁺ that microtubules near the border lines of the 12 sectors in the periphery are missing (arrows). × 82,000.

Villiger (27) further suggested that this enzyme may function in the assembly of the mitotic apparatus by lowering the Ca⁺⁺ concentration to a level where polymerization of microtubules is possible. This may be achieved by a shift of Ca⁺⁺ from the cytoplasm into a Ca⁺⁺-sequestering vesicle fraction (20). In a study of the effects of Ca⁺⁺ on the degeneration of amputated axons in tissue culture, Schlaepfer and Bunge (41) noted preservation of axoplasmic microtubules only in the presence of EGTA (which reduces the estimated Ca⁺⁺ concentration below 0.3 μM), while with 25–50 μM Ca⁺⁺ the microtubules were disrupted or markedly distorted.

Recent studies on the requirements of in vitro polymerization of microtubules show that, among other factors influencing assembly, Ca⁺⁺ is an effective inhibitor of microtubule formation (13, 14, 21, 44, 52). In addition, some in vitro observa-

tions link microtubule assembly to Ca⁺⁺-sequestering organelles such as mitochondria or certain vesicle fractions. Fuller et al. (10) added isolated mitochondria from rat liver to bovine brain tubulin and demonstrated that Ca⁺⁺ concentrations which inhibit microtubule assembly can be taken up by the mitochondria. Reassembly does not occur when the mitochondria are poisoned by various agents. In other experiments, Triton X-100 or caffeine has been added to crude supernates of chick embryo brain (3) known to contain a vesicle fraction which sequesters Ca⁺⁺ (23). These agents inhibit microtubule assembly in the crude supernates, most probably by causing the vesicles to release stored Ca⁺⁺. This effect can be partially reversed by the addition of EGTA. Polymerization of purified tubulin free of vesicles, on the other hand, is not affected by these agents (3).

For the concentration of Ca⁺⁺ which effectively

inhibits microtubule assembly in vitro, differing values are reported. The initial observation of Weisenberg (52) suggests inhibition at 6 μM. Lee et al. (21) and Haga et al. (14) reported inhibition at an effective concentration of Ca^{++} around 30 μM, while Olmsted and Borisy (25) observed inhibition only with concentrations above 1 mM. In addition, the concentration of Mg^{++} present in the medium influences the level of Ca^{++} required for blockage of microtubule assembly (25, 37).

The in vivo studies reported here do not allow precise values to be given for the Ca^{++} concentration that is effective in inhibition since the reaction conditions inside a living cell cannot be clearly defined. However, a Ca^{++} concentration as low as 10 μM has a degrading effect on the heliozoan axopodium if the ionophore is present. As pointed out by Reed and Lardy (34), A23187 acts as a free mobile carrier which catalyzes equilibration of divalent cations between external medium and cell interior. Under the assumption that, in the presence of the ionophore, the Ca^{++} concentrations within the axopodium and in the medium tend to equilibrate, it seems possible that microtubule formation in vivo is influenced by micromolar concentrations of Ca^{++}. This concentration is low enough to be of physiological significance.

The present investigation does not show whether Ca^{++} also regulates formation of axonemal microtubules of heliozoa under natural conditions. Davidson (5) reported rapid shortening of axopodia upon mechanical stimulation in the marine centrohelidan heliozoan *Heterophrys marina*. This retraction is inhibited in Ca^{++}-free seawater, suggesting that extracellular Ca^{++} is involved. Similarly, a Ca^{++} influx into the cytoplasm of the axopodium is supposed to promote rapid breakdown of the axonemes in *Actinophrys sol* (C. D. Ockleford, personal communication). On the other hand, in the ameba, another one-celled organism, a Ca^{++}-sequestering system has been demonstrated which probably is involved in the regulation of contractile processes (35). In addition, cells from various sources have been shown to accumulate Ca^{++} in certain compartments (e.g., references 6, 12, 15, 16). Despite the lack of direct evidence for a comparable system in heliozoa, the recent demonstration of contraction of isolated heliozoan cytoplasm induced by manipulation of the concentration of Ca^{++} ions (8) suggests a regulating mechanism based on local release or uptake of Ca^{++}. Further experiments are needed to clarify whether extracellular or in-

tracellular Ca^{++} sources are involved. In any case, the experiments with the ionophore demonstrate a high Ca^{++} sensitivity of heliozoan microtubules.

The ultrastructural studies of degrading axonemes show structural modifications of the microtubular array during Ca^{++}-induced axopodial retraction. The degree of disturbance of the microtubule pattern correlates with the rate of axopodial shortening observed light microscopically and, hence, with the concentration of Ca^{++} used. A relatively high concentration (0.5 mM) causes axonemal anomalies comparable to those produced by other agents, e.g., colchicine (47), low temperature (51), cupric ions (39), and urea (45). These anomalies include missing or C-shaped microtubules and the accumulation of fibrillar material, presumably subunits of depolymerized microtubules. With a lower concentration (0.1 mM), degradation of the microtubule array is not so dramatic as with the higher concentration; in most instances, there seems to be a controlled, gradual disassembly of microtubules which starts at the periphery of the axoneme. As a consequence, axopodia concomitantly become thinner as they shorten. Nevertheless, the precise sequence of axonemal decomposition occurring during axopodial retraction is not yet entirely clear.

Unlike Ca^{++}, Mg^{++} at low concentrations not only increases the rate of assembly in vitro, but is absolutely required for the assembly process. Presumably, a Mg^{++}-GTP complex is the substrate involved in assembly (25). However, Mg^{++} also inhibits at concentrations greater than 1 mM (2, 37). The results obtained here demonstrate a slight increase in axopodial length at low Mg^{++} concentrations (0.1 mM), suggesting a stabilization of the axoneme. This observation is corroborated by the electron microscope observations which show that microtubular arrays are most perfect in Mg^{++}-treated organisms (Fig. 10). Higher concentrations, on the other hand, cause successive phases of axopodial shortening and re-extension with a slight tendency towards shortening. These results are in agreement with the view that Mg^{++} favors assembly at low concentrations and is inhibitory at higher concentrations. Possibly, Mg^{++} can replace Ca^{++} in blocking microtubule assembly if used at high concentrations.

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