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Calcium metabolism in Volvox Carteri

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Calcium Metabolism in 
*Volvox Carteri*

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Calcium Metabolism in *Volvox Carteri*

A thesis submitted to the Chemistry Department for graduation with Honors in Chemistry

Approved by:

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Vita

Bridget Anne Katherine Neville, daughter of Susan Gaudet Neville and Timothy Francis Neville, was born on August 14, 1975 at 1:02 PM in Springfield, Massachusetts. She grew up in Enfield, Connecticut and attended Enrico Fermi High School, graduating as Salutatorian in June of 1993.

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Abstract

Calcium metabolism is involved in many ways in the cellular activities of the colonial green alga, *Volvox carteri*. Various drugs that are known in other systems to cause specific changes in calcium metabolism, gave rise to various visible metabolic effects in this study. These effects provide evidence for calcium's role in *Volvox* growth and development and in its cytoskeleton. Another way that calcium exerts its effect on *Volvox* is by acting as a second messenger in a G-protein/inositol phosphate signal transduction pathway.

This study also supports the fact that cytoskeletal components are important for development in *Volvox*. Both microtubules and microfilaments are necessary for mitosis. Microtubules are necessary for cell shape changes that occur at the beginning of inversion. Microfilaments are necessary to complete the inversion process.
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Introduction

Calcium's roles in living things are numerous. For example, a typical human body contains 1-2 kg (2.2-4.4 lb.) of calcium, with roughly 99 percent of it deposited in the skeleton (Martini, 1995). One of its functions, though, in most living systems is as a second messenger in signaling pathways. Second messengers are intracellular signaling molecules that experience a short-lived increase or decrease in their concentration due to the binding of a ligand to a cell-surface receptor (Lodish, 1995). Other second messengers include cAMP, inositol 1,4,5-trisphosphate (IP₃), and 1,2-diacylglycerol. These messengers control uptake and use of glucose, storage and mobilization of fat, secretion of cellular products, proliferation, differentiation, and survival of cells (Lodish, 1995).

Localized increases in the cytosolic level of free \( \text{Ca}^{2+} \) are critical to its function as a second messenger. Small increases in the level of cytosolic \( \text{Ca}^{2+} \), which often are mediated by a rise in IP₃, trigger many cellular responses (Lodish, 1995). \( \text{Ca}^{2+} \) aids in the degradation of glycogen to glucose 1-phosphate in both liver and muscle cells and contraction in smooth or striated muscle cells. Other roles of calcium in the human body consist of forming the crystalline component of bone, controlling neural activity, being involved in blood clotting, and acting as a cofactor for enzymatic reactions (Martini, 1995). In algae such as *Chlamydomonas*, calcium plays a role in the movement of flagella. Changes in \( \text{Ca}^{2+} \) concentration mediate the switching between a symmetric waveform, which pushes the cell through the medium, and an asymmetric waveform, which pulls the cell along (Lodish, 1995). A recent study concluded that mastoparan (a wasp venom peptide known to affect flagella) induces the release of an intracellular pool of \( \text{Ca}^{2+} \)
whereas extracellular addition of acid induces an influx of extracellular \( \text{Ca}^{2+} \) to activate the machinery of deflagellation (Quarmy and Hartzell, 1994). The vast array of uses of calcium in living organisms makes it interesting to study the mechanisms by which it is used.

![Diagram](image)

Figure 1. A general model for signal transduction.

**A General Model For Signal Transduction**

There is a general model for calcium’s involvement in signal transduction (Figure 1). A hormone or other extracellular signal binds to its cell-surface receptor, causing the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (\( \text{PIP}_2 \)), which is an example of one of several inositol

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*Note: The diagram and text are integrated to provide a comprehensive understanding of the content.*
phospholipids found in the cytosolic leaflet of the plasma membrane. This hydrolysis is done by a plasma-membrane enzyme phospholipase C (PLC), yielding two products: 1, 2-diacylglycerol (DAG), that remains in the membrane, and inositol phosphate (IP$_3$), which is cytosolic. The hormone-induced stimulation of PLC activity and subsequent generation of IP$_3$ is mediated by G protein-linked receptors.

Following hormone stimulation of a target cell, IP$_3$ is formed and diffuses to the endoplasmic reticulum (ER) surface, where it binds to a specific IP$_3$ receptor on a Ca$^{2+}$ channel protein composed of four identical subunits, each containing an IP$_3$-binding site. IP$_3$ binding induces the opening of the channel allowing Ca$^{2+}$ ions to exit from the ER into the cytosol (Lodish, 1995). It is important to note that the plasma membrane also contains these IP$_3$-regulated Ca$^{2+}$ channels. Released Ca$^{2+}$ binds to the ER IP$_3$ receptor, causing a conformational change that induces the opening of adjacent plasma membrane Ca$^{2+}$ channels (Garrett and Grisham, 1995). The release of the Ca$^{2+}$ from the ER is short-lived because a sustained release would be toxic to the cells.

The short-lived release of calcium is translated into intracellular responses by calcium-binding proteins, which in turn regulate many cellular responses. One group is the calcium-modulated proteins, including calmodulin and many others, all of which have a common structural feature, the EF hand. This hand is made up of two alpha helices joined by a loop of 12 amino acids. Calcium binds to the EF hand through coordination by six carbonyl oxygens contributed by a glutamate and three aspartates, by a carbonyl oxygen from a peptide bond, and by the oxygen of a coordinated water molecule (Garrett and Grisham, 1995). This binding allows calcium to bind with its target protein(s), such as protein kinase C (PKC). Calcium can exert its
effects by binding with PKC or through its interaction with calmodulin, which binds with other
target proteins besides PKC.

Protein kinase C (PKC) is an example of a cellular transducer, which translates a hormonal
message and the signals of the second messengers into the protein phosphorylation events that
control growth and development. It has four conserved binding domains, including one for DAG
and another for calcium. Binding of DAG to PKC causes conformational changes that increase
the affinity of PKC for calcium, allowing PKC to become active. When it is active, PKC
phosphorylates target proteins at serine and threonine residues. This phosphorylation is what
causes the cellular responses to occur (Garren and Grisham, 1995).

One way to understand the signal transduction pathway and its cellular responses in
*Volvox* is through the use of drugs directed at particular participants in that pathway. The first
step in this understanding is to confirm calcium's role in the pathway. This will be done by
treating *Volvox* cells with one of three drugs: calcium ionophore A23187, glyburide, or verapamil.

*Calcium Ionophore, A23187*

An ionophore is a toxin produced by microorganisms that facilitates ion transport across
membranes (Garren and Grisham, 1995). Ionophores are hydrophobic molecules that increase
membrane permeability of specific inorganic ions, enabling the ion to penetrate the hydrophobic
interior of the lipid bilayer. A calcium ionophore, then, is a molecule that facilitates transport of
$\text{Ca}^{2+}$ ions across membranes. Calcium ionophores, in general, are used to artificially raise the level
of intracellular calcium, allowing for equilibrium across membranes. More specifically, they
shield the charge of calcium to make it easier for it to enter the cell (note that all drugs used in this
project are described and referenced in the Calbiochem *Signal Transduction Catalog & Technical*
Resource 1996/1997). A23187, an example of a calcium ionophore, will be used in this study to investigate calcium’s role in the *Volvox* signal transduction pathway.

**Glyburide**

Glyburide indirectly affects calcium metabolism as a potassium ATPase inhibitor. An ATPase is an ATP-powered pump that transports ions against their electrochemical gradients across a membrane. A potassium ATPase in the plasma membrane indirectly pumps calcium out of the cell (Lodish, 1995) while pumping $K^+$ ions in. If the pump is inhibited, $Ca^{2+}$ ions accumulate inside the cell, not able to get out. Glyburide causes this $Ca^{2+}$ accumulation by selectively blocking ATP-sensitive potassium channels. The reduced potassium conductance causes membrane depolarization and influx of calcium through voltage sensitive calcium channels. If calcium plays a role in the *Volvox* signal transduction pathway, addition of glyburide to the organisms will cause the cells to experience an altered cellular response to the intracellular $Ca^{2+}$ ion influx.

**Verapamil**

Another drug that affects the intracellular calcium level is verapamil. It is another ATPase inhibitor, but instead of allowing calcium to pass through a channel that is normally closed, it blocks calcium channels (principally the L-type which are activated by high voltages). Presumably, this blockage will prevent calcium from entering the *Volvox* cells. This lack of intracellular calcium should affect the cells’ “normal” responses.
Involvement of Inositol Phosphate in the Pathway

If calcium plays a role in *Volvox* signal transduction, it probably is regulated by another second messenger like cAMP or IP$_3$. This study will try to determine if IP$_3$ is indeed that second messenger. Two drugs will be used to investigate the involvement of IP$_3$ in the *Volvox* signal transduction pathway: MAS7 and neomycin sulfate.

**MAS7**

MAS7, a mastoparan analog with five-fold greater potency than mastoparan, is a direct activator of G-proteins. Mastoparan is a wasp venom peptide that activates a phospholipase C-coupled G protein, leading to the production of IP$_3$, which opens an intracellular Ca$^{2+}$ channel. This releases Ca$^{2+}$ into the cytosol, thereby triggering cellular responses (Quarmby and Hartzell, 1994). If MAS7 causes the *Volvox* cells to experience altered metabolic effects, IP$_3$ is probably a participant in the signal transduction pathway.

**Neomycin Sulfate**

Neomycin sulfate is an inhibitor of phospholipase C, an amplifier in the G-protein/IP$_3$ signal transduction pathway. Inhibition of phospholipase C prevents the hydrolysis of PIP$_2$ to DAG and IP$_3$. This affects calcium regulation and causes metabolic effects in the cells because IP$_3$ cannot signal calcium regulation. If IP$_3$ is involved in the *Volvox* signal transduction pathway, altered metabolic effects will occur upon addition of neomycin sulfate.
A Background on Volvox Carteri

The green alga Volvox carteri is a simple multicellular organism having two types of cells, somatic and reproductive (or gonidia). Between 2000 and 4000 pear-shaped somatic cells are arranged into a hollow sphere, with about 16 gonidia located in the posterior region of the spheroid. The asexual development of these spheroids is through successive divisions of the gonidia (Figure 3). In the developing asexual embryo, differentiation into somatic and reproductive cells is usually seen at the division from 32-64 cells. At this division, 16 cells of the embryo undergo unequal cleavage, forming a small somatic and a large gonidial initial. All cells are joined by numerous short cytoplasmic bridges which are believed to be the result of incomplete
cytokinesis (Viamontes and Kirk, 1979). While cell division ceases in the gonidial initials, the remaining cells (somatic initials) continue their synchronous divisions. At the termination of cell divisions, the many somatic cells form a hollow sphere with the gonidia conspicuously placed on the outer surface (Sumper, 1979). This is what is known as pre-inversion (also termed the "Mexican hat" or "sombrero" formation). At this pre-inverted state, the embryo is inside-out with respect to the adult conformation. This orientation is reversed shortly after the end of cleavage when the embryo undergoes "inversion" and turns inside-out through its phialopore, which is a pair of intersecting slits at one pole of the embryo (Viamontes and Kirk, 1977). The young spheroid now enters a period of expansion. This brings the organism into the second day of its life cycle. Formation of a gelatinous matrix of the colony begins and the spacing between the cells increases. Finally, the mature daughter colonies are released from the parent colony (Sumper, 1979). This last event is called "hatching." The total reproductive process takes 48 hours (Figure 2).
Figure 3. The first day of the *Volvox* life cycle. (a) shows the pre-cleavage stage where gonidia have not yet started to divide. (b) is gonidia at the 4-cell stage. (c) shows the 8-cell stage. (d) is asymmetric division or the 16-32 cell stage. (e) shows pre-inversion. (f) is the inversion stage.

**Inversion**

Of all the stages of development, the one very often studied is inversion. It is a relatively simple event, but it bears certain striking parallels with other morphogenic events (such as gastrulation and neurulation) in more complex embryos like vertebrates (Viamontes and Kirk, 1977). There is much promise for *Volvox* inversion as a model system demonstrated by the availability of many mutant lines with blocked inversion (Sessoms and Huskey, 1973; Huskey et al., 1979). Many of these mutants appear to be blocked in various specific stages of morphogenesis and to have no other discernible defects. Viamontes and Kirk (1977) hoped that studying these mutants would lead to discovery of the wild-type alleles that control the various
steps of inversion. But before the defects in the mutants can be studied, it is necessary to know how the normal events of inversion occur.

The inversion process is made up of three events. According to Viamontes and Kirk (1977), the first event of inversion is signaled by a change in shape of the somatic cells, from pear shape to spindle shape, due to elongation along their flagellar-chloroplast axes and contraction in the plane of the spheroid wall. This ultimately leads to a decrease in the overall diameter of the spheroid and the opening of the phialopore. The second and major event of inversion is the generation of negative curvature, which consists of an elongation of the cells (to produce a classical "flask" shape) and migration of cytoplasmic bridges to the outermost ends of flask cells. According to Viamontes, Fochtmann and Kirk (1979), the elongation is driven by microtubules. They also state that an elastic snap-through mechanism, bringing the gonidia inside the somatic cell layer, occurs to make inversion complete; this mechanism requires microfilaments (Figure 4). Thus, it is an objective of this study to investigate the role calcium has during inversion, especially its involvement with microtubules and microfilaments during inversion.

Figure 4. A Volvox completes inversion by bringing its gonidia from the outside of the juvenile (bottom of figure) to the inside (top).

The Cytoskeleton

Microtubules and microfilaments play different but important cellular roles. They make up the cytoskeleton which provides support, protection, and movement to the cells.
Microtubules

Microtubules are polymers of globular tubulin subunits arranged in a cylindrical tube measuring 24 nm in diameter (Lodish, 1995). They are responsible for many cellular movements, which result from the polymerization or depolymerization of the microtubules or the actions of their motor proteins, dynein and kinesin. Motor proteins are cytosolic proteins that bind to microtubules in the presence of ATP and are responsible for movement of intracellular components along microtubules (Lodish, 1995). Examples of those movements are alignment and separation of chromosomes during meiosis and mitosis, beating of cilia and flagella, and the transport of membrane vesicles in the cytoplasm. Sometimes though, microtubules serve purely structural purposes in the cell (Lodish, 1995). For example, this study hopes to identify microtubules (and/or microfilaments) in cytoplasmic bridges that exist between dividing Volvox cells. The stability of cytoplasmic microtubules changes in response to a change in the environment surrounding a cell. For example, in a confluent culture of mammalian cells, if some of the cells are scraped from the culture dish, the cells remaining at the edge of the “wound” begin to move into the open area and eventually fill in the wound. This movement is accompanied by a rearrangement in the microtubule cytoskeleton, which changes from a symmetric arrangement in the “resting” cells at the border of the wound into an array in which most of the microtubules radiate toward the open wound (Lodish, 1995). To test the stability of the Volvox cytoplasmic microtubules (if there are any), a change in the environment will be created by adding the anti-cancer drug, colchicine. This drug disrupts microtubules and inhibits tubulin polymerization. Treating Volvox with colchicine will help identify microtubules (if they exist) in the cytoplasmic bridges. It will also confirm their involvement during the second (and maybe also first) event of
inversion. The results of colchicine addition should also confirm the Volvox cell’s need of microtubules to progress through mitosis.

**Microfilaments**

Microfilaments are composed of bundles or networks of actin, which is a globular protein. The globular monomers, called G-actin, polymerize into filaments 7-9 nm in diameter, called F-actin. Bundles of filaments support fingerlike projections of membrane, while networks of filaments stiffen plasma membranes (Lodish, 1995). Cytochalasin B is a drug that affects microfilaments by blocking their formation. It shortens actin filaments by blocking monomer addition at the fast growing end of polymers. Microfilaments have two ends, a (+) and a (−) end, the (+) growing faster than the other. Cytochalasin B will help identify microfilaments (if they exist) in the cytoplasmic bridge make-up. It will also confirm microfilaments’ role in the snap-through mechanism of inversion. Lastly, addition of cytochalasin B will confirm the need of microfilaments for mitosis progression.

**Calcium and the Cytoskeleton**

Calcium’s role in the cytoskeleton is not entirely known. What is known, though, is that tip growth of actin filaments (microfilament capping and severing proteins) is regulated by two signaling pathways, one mediated by calcium and one by PIP$_2$. A recent study by Diedrik Menzel on Acetabularia (a giant unicellular green alga) supports the fact that calcium is important for tip growth of actin filaments (Menzel, 1996). In the absence of calcium, severing proteins such as gelsolin are not bound to actin, but in the presence of calcium, a severing protein binds an actin filament, breaking it into pieces. The severing protein remains tightly bound to the newly
created (+) end of the filament, where it blocks monomer growth. This action of severing proteins can convert a gel of long actin filaments cross-linked by a protein such as filamin into a solution of short actin filaments (Lodish, 1995). Knowing calcium's importance with microfilaments is just the beginning of an understanding to calcium's role with the rest of the cytoskeleton.

Not much is known about calcium's involvement with microtubules. As mentioned previously, the movement of flagella (a structure made up of microtubules) in *Chlamydomonas* (an alga closely related to *Volvox*), is sensitive to very small changes in Ca\(^{2+}\) concentration (Lodish, 1995). Although these changes affect movement, the calcium does interact with the microtubules, so it is possible for an interaction to occur for other purposes. It has been suggested that tubulin (the building block of microtubules) is a GTP-binding protein that shares a weak homology with signal-transducing G proteins (Mandelkow, 1995). This provides new evidence that calcium is involved in regulating microtubules.

**Objective**

The major goal of this study is to interpret the altered metabolic effects caused by the different drug treatments and use them to postulate roles for calcium in *Volvox carteri*. Is calcium involved in a *Volvox* signal transduction pathway? If it is, is this regulated by IP\(_3\)? Does calcium play a role in the activity of the *Volvox* cytoskeleton? Which cytoskeletal components make up the cytoplasmic bridges? Are microtubules required for the second event of inversion to occur? Are microfilaments required for the snap-through mechanism to take place?
Materials and Methods

Growth Conditions

*Volvox carteri* was used for all the experiments reported here. Stock cultures were maintained in synchronous asexual growth and development in standard *Volvox* medium (SVM) using a 16 hour light and 8 hour dark cycle (Kirk and Kirk, 1978). Under these conditions, the entire life cycle takes 48 hours, and inversion occurs only in the dark period.

Drug Treatments

Glyburide and calcium ionophore A23187 stock solutions were dissolved in dimethylsulfoxide (DMSO), and verapamil-hydrochloride was dissolved in distilled water to a concentration of 1 mM. Final drug concentrations in SVM were as follows: glyburide, 131.2 μM; calcium ionophore A23187, 91.4 μM; verapamil-hydrochloride, 290.9 μM.

Colchicine stock solution was dissolved in 95% ethanol to a concentration of 2 mg/mL. Cytochalasin B stock solution was dissolved in DMSO to a concentration of 100 μg/mL. Final concentrations in SVM were as follows: colchicine, 0.3 mg/mL and cytochalasin B, 16.7 μg/mL.

Neomycin sulfate and MAS7 stock solutions were dissolved in de-ionized water to concentrations of 6.83 mM and 100 μM respectively. Final concentrations in SVM were as follows: neomycin sulfate, 890.9 μM and MAS7, 13.04 μM.

All final drug concentrations in SVM used were in each case close to the minimum effective concentration (nonlethal). This was determined through much trial and error. Various dilutions were made of each drug to test on the *Volvox* until a concentration was found that had an effect (like visible cell degradation), but did not kill them right away. *Volvox* death can be distinguished from life by the loss of their green coloring. Treatment times ranged from 1-2 hours.
for each drug. Drug treatments were done at various times during the day in which the *Volvox*
were cleaving to obtain results from each stage of the reproductive process. The appropriate
drug volume (of nonlethal concentration) was added to 5 mL of SVM in a six-well plate. After
the 1-2 hour treatment at roughly 32 °C, samples were prepared and analyzed using a compound
microscope and pictures were taken. All chemicals were obtained from Sigma, Calbiochem and
Fisher Chemicals, Inc.

**Results**

*Calcium Directed Drugs*

In general, when the drugs were added, mitosis stopped immediately. *Volvox* were
arrested in the particular stage (whether symmetric or asymmetric cleavage) they were in upon
receiving the drug. For example, if the drug was added during the 8-cell stage, an hour later, it
would still be in the 8-cell stage. In addition to this arrested development, spaces appeared
between dividing cells (the cytoplasmic bridge site). Normally, adjacent cells are in direct contact
with no visible space. Also, many cells experienced degradation in response to the drug
treatment. The somatic cell layer was broken and eye spots lost their reddish-orange pigment.
Verapamil-treated *Volvox* exhibited the clearest and most defined spacing between cells,
compared to calcium ionophore A23187-treated and glyburide-treated *Volvox* (Figure 5).
Figure 5. (a) shows gonidia arrested at the 8-cell stage by 290.9 μM verapamil. (b) shows arrest at 8-cell stage by 91.4 μM A23187. (c) is the arrest at the 8-cell stage by 131.2 μM glyburide.

**Inositol Phosphate Directed Drugs**

The results with neomycin sulfate and MAS7 were similar to the other drug treatments. Spaces were again seen between the dividing cells. Degradation of the individual cells occurred as well (much more than with the other drugs) of the individual cells. Arrested mitosis was also seen. Treatment with neomycin sulfate was a little different compared to treatment with other drugs, in that it caused the somatic cells to condense. This made the organism appear darker green when viewed through the microscope (Figure 6).

Figure 6. (a) shows gonidia arrested at the 16-32 cell stage by 890.9 μM neomycin sulfate. (b) shows arrest at 16-32 cell stage by 13.04 μM MAS7.
The effects of both drugs on inversion were very similar to those on the rest of division; it was arrested. This arrest occurred regardless of when the drugs were added: pre-inversion or either step of inversion. Thus, the organisms were arrested at whatever stage they were in when the drugs were added. Overall though, MAS7 and neomycin sulfate caused the most degradation of the cells, sometimes making it difficult to recognize the particular symmetrical cleaving stage the organism was in.

Figure 7. Gonidia arrested in inversion by 890.9 µM neomycin sulfate.

Cytoskeletal Directed Drugs

The visual effects of colchicine and cytochalasin B were very similar to the effects of the other two types of drugs. Upon addition of the drug, arrested development occurred. Also, spaces appeared between individual cells of the cleaving embryo (Figure 8). The spaces were more defined in the Volvox that were treated with colchicine compared to those treated with cytochalasin B. Both drugs caused some degradation of the cells as well. Colchicine and cytochalasin B both gave rise to the loss of the reddish-orange color from the somatic cell eye spots.
Figure 8. (a) shows gonidia arrested at the 4-cell stage by 16.7 μg/mL cytochalasin B. (b) shows arrest at 16-32 cell stage by 0.3 mg/mL colchicine.

The difference between the effects of colchicine and cytochalasin B became apparent when looking at the inversion process. *Volvox* that were treated with colchicine during pre-inversion arrested there. They did not move on to that part of inversion where the cells elongate. Those that were treated with cytochalasin B during pre-inversion went through inversion an hour later. When cytochalasin B was added during inversion, an hour later, when the “drugless” *Volvox* had already inverted, the “drugged” ones had not. Colchicine-treated organisms continued through and completed inversion when the drug was added after cell elongation.

*Unusual Results*

At various times during the cell cycle, a drug was added that resulted in a *Volvox* that looked like its presumptive gonidia had either disappeared or were fusing together. It was not possible to tell if cell division of these presumptive gonidia had stopped. This happened with both verapamil, a calcium channel blocker and neomycin sulfate, a phospholipase C.
inhibitor. It happened when verapamil was added at various stages, such as the 4-8 cell stage, the 16-32 cell stage, pre-inversion and inversion. In some instances, this phenomenon occurred in some juveniles but not in others that were located in the same larger organism. When neomycin sulfate was added during the 8-16 cell stage, for example, an hour later, it would appear as if the cells of the embryo were arrested and fused together. They all looked very similar, actually resembling a gonidium that had not yet begun to divide.

Discussion

Growth, Development and Reproduction

Presumed changes in the levels of intracellular calcium inhibited mitosis in *Volvox carteri*. Whether the change was an influx of calcium into the cell (caused by calcium ionophore A23187 and glyburide) or a decrease in intracellular calcium levels (caused by verapamil), the results were very similar. The fact that mitosis was arrested upon drug addition demonstrated that a certain level of intracellular calcium is required for mitosis to proceed normally. It is known that the concentration of free calcium ions in the cytosol usually is kept below 0.2 µM (Lodish, 1995). Calcium ATPases pump cytosolic calcium ions across the plasma membrane to the cell exterior or into the lumen of the endoplasmic reticulum or other intracellular vesicles that store calcium ions. This study has also shown that it is not only increases in cytosolic calcium, but decreases as well, that trigger those responses. Thus, *Volvox* must have specific times for calcium regulation through specific intracellular concentration changes. Any deviation from those specifics causes problems with growth, development, and reproduction.
Although the results show that calcium is involved in mitosis, the question of how is still unanswered. Because the results with MAS7 and neomycin sulfate were very similar in that they arrested mitosis, it can be concluded that inositol phosphate also plays a role in mitosis. In the general model for signal transduction, IP$_3$, as a second messenger, affects the regulation of calcium, which has metabolic effects on the cell. The results of this study suggest that the G-protein/IP$_3$ pathway is indeed the main means of signal transduction for mitosis in \textit{Volvox}. Some extracellular signal probably is indirectly involved with the regulation of \textit{Volvox} mitosis. Thus, the IP$_3$ signaling pathway may adjust the calcium concentration in the cell, and a certain range of calcium concentration might be necessary for mitosis. This explains the similarities between the two drug treatments (calcium drugs and IP$_3$ drugs). More experiments need to be done to clarify the role of this signaling pathway in the process of mitosis.

\textit{Cytoskeleton}

Since colchicine and cytochalasin B-treated \textit{Volvox} also experienced arrested development during mitosis, it is confirmed that the polymerization and depolymerization of microtubules and microfilaments are required for \textit{Volvox} to go through mitosis.

Microtubules are extremely important in mitosis in directing chromosomal movement during cell division. They form the scaffold of the mitotic cell; they comprise the tracks along which the chromosomes move and they ensure that the chromosomes are apportioned appropriately to the daughter cells (Lodish, 1995). They probably aid movement directly as well. Also, during metaphase, microtubules, which comprise the spindle, help line up the chromosomes on the metaphase plate. The exposure of a dividing cell to colchicine causes the rapid disappearance of the mitotic spindle, indicating that a chemical equilibrium is maintained.
through continual exchange of subunits between the spindle microtubules and the pool of free tubulin (Alberts, 1994). If this equilibrium is not maintained, the chromosomes cannot line up on the metaphase plate, and the cell cannot move on to anaphase (Alberts, 1994). Thus, it can be seen that when colchicine is added, mitosis will be arrested.

Likewise microfilaments are necessary for the progression of mitosis. During the last step of mitosis, a contractile ring assembles at the equator of the dividing cell. This ring is a contractile bundle of actin filaments. It has been shown through fluorescence microscopy that during mitosis, actin and myosin accumulate at the equator of a cell, midway between the poles of the spindle. That is where they align into a bundle which encircles the cell (thus a ring). As mitosis continues, the diameter of this ring decreases and the cell is pinched into two parts by what is known as the cleavage furrow (Volvox divide more like mammal cells than plant cells). So, when a drug is added that inhibits the polymerization and depolymerization of microfilaments (like cytochalasin B), this ring can not be formed (Lodish, 1995). That lack of ring formation would prevent mitosis from continuing.

The controlling mechanisms of this ring formation are not completely known. It is proposed, though, that calcium is involved. Evidence for calcium's involvement is that calcium-dependent phosphorylation (as described earlier in the signal transduction pathway) of myosin-II (an actin motor protein) both increases the ring's interaction with actin and promotes myosin's assembly into short bipolar filaments (Alberts, 1994).

It can also be said that calcium is involved with microtubules. Calcium could bind to microtubules/tubulin and aid in the spindle action of the mitotic cell. If calcium is indeed responsible for such cytoskeletal control, it is not explicitly clear in this study. What is clear,
though, is that (a particular concentration range of) calcium, microtubules and microfilaments are necessary for mitosis to proceed as usual.

**Cytoplasmic Bridges**

Results using colchicine and cytochalasin B treatment suggest that the linkages between dividing cells consist of both microfilaments and microtubules. Both drug treatments yielded spaces between the embryonic cells, demonstrating the lack of that connection or simply a part of it missing. Because of the very small size of these bridges (too small to see at 200X total magnification), not much is known about them. It appears they are too small to study. As to how they are formed and exist, there are various possibilities. Since both microtubules and microfilaments appear to be involved, there could be communication between the microtubules and microfilaments or they could be doing two different things. It could also be possible that the microtubules and microfilaments are on the inside of the cells and help to establish the bridges (instead of being part of the bridge make-up). The results of this study suggest that calcium is involved somehow with the cytoplasmic bridges, demonstrated by the increased spaces between dividing gonidia cells upon addition of calcium directed drugs like A23187, glyburide and verapamil. But, even though calcium is involved with these bridges, it is not necessarily the main controller of their formation or existence. IP₃ could be that controller, for example, or some other signal.

The results from the IP₃ drugs demonstrated that altering IP₃ metabolism disrupted normal intracellular connections. Addition of MAS7 and neomycin sulfate resulted in the similar spacing as with the calcium drugs and the cytoskeletal drugs. Again, it is likely that changes in IP₃ concentration alter the formation and existence of the cytoplasmic bridges. However, more
inversion needs to be done to find the exact control mechanism, whether it be calcium, IP₃, or both.

**Inversion**

According to our hypothesis, there are three distinct, cytoskeletal-related events that occur as part of the inversion process. The first event of inversion is a cell shape change probably aided by microtubules, which ultimately leads to a decrease in the overall diameter of the spheroid and the opening of the phialopore. The second event is where microtubules aid in the generation of negative curvature. The elastic snap-through mechanism, bringing the gonidia inside the somatic cell layer occurs as the third and last event to make inversion complete. It has been hypothesized this event requires microfilaments. As shown in the results, the hypotheses concerning the cytoskeletal-related events of inversion were correct.

Microtubules were required for the first and second cytoskeletal related event. This was demonstrated through use of colchicine. When it was added during pre-inversion, the organism did not even begin inversion. This meant that the polymerization and depolymerization of microtubules was necessary for that transition. When the same drug was added during inversion (after the formation of negative curvature), the process continued to completion, which also supports the hypothesis (Figure 9). The exact mechanism of how microtubules are involved in this event is not completely known, but further studies are hopefully being conducted to arrive at the answer.

There are also more studies being done on the involvement of microfilaments in the final event of inversion (the third cytoskeletal event). The results of this paper support the hypothesis that microfilaments are required for the snap-through mechanism to occur. This was
demonstrated through the use of cytochalasin B. When the drug was added during pre-inversion, the organism began inversion and continued up through and including the negative curvature, but never completed it; a snap-through of the gonidia was never achieved. Further support comes from experiments where the drug was added at any point during inversion and the organisms did not complete the process (Figure 9). Thus, somehow, microfilaments are necessary for the transition bringing the gonidia from the outside of the juvenile Volvox to the inside. Again, the exact mechanism for this snap-through event is not known.

![Figure 9. (a) shows gonidia arrested in pre-inversion by 0.3 mg/mL colchicine. (b) shows Volvox treated with 0.3 mg/mL colchicine proceeding through the snap-through mechanism. (c) shows Volvox treated with 16.7 µg/mL cytochalasin B not proceeding through the snap-through mechanism.](image)

**Unexplained Results**

It still is not easily explained why at certain times during development, and with certain drugs, the gonidia appeared as though they were fusing together. It also is not clear whether the cells were dividing or not. Sometime after this phenomenon was first discovered, more
experiments were performed with verapamil and the results could not be duplicated. It is possible that the phenomenon was due to an error in the effective drug concentration. Although 5 mL of Volvox in SVM were added to the dish wells for each trial, it is impossible to get the exact same number of organisms in the well. Thus, the apparent fusion could be due to differences in the effective drug concentration per organism. More research in this area definitely needs to be done to either rule out the possibility of error or to identify the valid drug effects.

More research also needs to be done to investigate the effects verapamil has on Volvox when added during inversion. It is very unusual for an organism to "reverse" its development. There is probably another explanation for Volvox's response to verapamil at that stage in its development.
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