I give permission for public access to my thesis and

for copying to be done at the discretion

of the archives' librarian and/or the College library.

Marta E. Soden

Date

THE ROLE OF CALCIUM IN AMOEBOID MOVEMENT: RESULTS WITH FLUORESCENT INDICATOR DYES

Marta E. Soden

A Paper Presented to the

Faculty of Mount Holyoke College in

Partial Fulfillment of the Requirements for

The Degree of Bachelors of Arts with

Honor

Program in Neuroscience and Behavior

South Hadley, MA 01075

May, 2005

This paper was prepared under the direction of Professor Susan Barry for eight credits.

ACKNOWLEDGEMENTS

I would like to thank Professor Barry for her advice and guidance on this project, and for her unending cheerfulness.

Thanks also to Darcy Broughton for sharing her results and her materials, as well as for her insightful comments and discussion on the subject matter.

Additional thanks to Marian Rice for her assistance with the microscope and imaging software, and to Linda Young for her assistance with everything.

TABLE OF CONTENTS

List of Figures	V
List of Tables	vi
Abstract	vii
Introduction	1
Materials and Methods	16
Results	22
Discussion	37
Literature Cited	55

LIST OF FIGURES

Figure 1. Amoeba proteus
Figure 2. Mechanism of extension at pseudopod tip6
Figure 3. Hyaline cap at the tip of an extending pseudopod6
Figure 4. Example of regions measured21
Figure 5. Pseudocolor image of fluorescence: uroid and wrinkled posterior24
Figure 6. Graphical comparison of fluorescence intensity: uroid versus wrinkled posterior25
Figure 7. Pseudocolor image of fluorescence: pseudopod base and extending pseudopod26
Figure 8. Graphical comparison of fluorescence intensity: pseudopod base versus extending pseudopod27
Figure 9. Graphical comparison of fluorescence intensity: ectoplasm versus endoplasm
Figure 10. Phase and pseudocolor images of ectoplasm/endoplasm comparison
Figure 11. Graphical comparison of fluorescence intensity: normal versus low calcium medium
Figure 12. Pseudocolor images of fluorescence: amoeba in normal calcium and low calcium
Figure 13. Phase images of amoebae mildly and severely affected by U73122
Figure 14. Pseudocolor images of fluorescence: amoebae mildly and severely affected by U73122
Figure 15. Graphical comparison of fluorescence intensity: cells mildly affected by U73122 versus severely affected cells35

LIST OF TABLES

Table 1.	Summary of regional differences in fluorescence	28
Table 2.	Summary of conditional differences in fluorescence	.35

ABSTRACT

The cytoskeleton of the unicellular organism *Amoeba proteus* includes a cortical network of filamentous actin directly beneath and attached to the plasma membrane. The contraction of this layer, occurring primarily in the tail, or uroid, region of amoebae, pushes fluid cytoplasm forward and allows for cytoplasmic streaming and pseudopod extension. Actin fibers in this layer are cross-linked into a gel-like structure via a number of actin binding proteins, and this gel must be partially solated in order for contraction to occur. Calcium interacts with several actin-binding proteins, and elevated calcium levels have been shown to promote the solation and contraction of the actin network.

Moving amoebae were injected with fluorescent calcium indicators in order to visualize local intracellular calcium levels. Results indicate high levels of calcium in the uroid region and at the base of pseudopodia, and low levels of calcium in advancing pseudopodia. Much higher fluorescence was seen in the streaming endoplasm of the amoebae compared to the gelled ectoplasm, supporting the hypothesis that calcium is necessary for maintaining the fluidity of the cytoplasm. In addition, evidence suggests that extracellular calcium and intracellular calcium under the control of a phospholipase C pathway are both critical for normal amoeboid movement.

INTRODUCTION

Cytoskeletal Structure of Amoeba proteus

Locomotion in the unicellular eukaryote *Amoeba proteus* may seem superficially similar to the crawling movements seen in other species and cell types, but it is in fact quite distinct in many ways. For example, movement in the well-studied slime mold *Dictyostelium discoideum* involves only localized cytoplasmic streaming, while *A. proteus* exhibits global streaming. *Dictyostelium* pseudopodia also contain central bundles of actin filaments not found in *A. proteus* (Eckert et al. 1977). Some mammalian cells, such as leukocytes, also exhibit a form of amoeboid locomotion, but these cells extend broad, flat lamellipodia as opposed to the narrow pseudopodia seen in *A. proteus* (Guilford et al. 1995). Though these various types of movement all depend on an actin cytoskeleton, different mechanisms are clearly at work in these different species. It has been necessary, therefore, to develop unique theories regarding the specific processes of movement of *A. proteus*.

The cytoplasm of *A. proteus* is a dynamic fluid that readily alters its viscosity depending on its location within the cell. Though the cytoskeleton of *A. proteus* contains some microtubules and intermediate filaments, it is composed primarily of actin fibers. Directly underneath and attached to the plasma membrane is a gel-like network of filamentous actin and actin binding proteins

known as the ectoplasm (Christiani et al. 1986). This cortical actin layer is distinguishable from the more fluid endoplasm that streams through the center of the amoeba, though these regions are not separated by a physical barrier (figure 1). As an amoeba advances, gelled ectoplasm in the tail, or uroid, region of the amoeba solates into new fluid endoplasm and moves forward as the uroid retracts. At the same time, as endoplasm reaches the tips of pseudopodia it moves to the periphery of the cell and solidifies to form new ectoplasm, a process known as the fountain phenomenon (Haberey 1972).

Theories of Amoeboid Movement

Contraction of the cortical actin network in the ectoplasm has long been believed to enable motile activities such as cytoplasmic streaming and the extension and retraction of pseudopodia. However, much debate occurred regarding which region of the amoeba contracts to provide the force necessary for movement. Proponents of the frontal zone contraction theory argued that as cytoplasm at the tips of advancing pseudopodia contracts into a gel-like structure, it is able to transmit tension towards the posterior end of the cell, pulling the tail forward (Taylor et al. 1973).

A large amount of evidence, however, indicates that the tail of the amoeba is the primary site of contraction. For example, the motor protein myosin, which provides the force of contraction, is especially concentrated in the uroid region (Brix et al. 1990). In addition, the cortical actin layer is thickest in the uroid





Figure 1. Amoeba proteus.

(A) U = uroid; P = extending pseudopod. Dashed arrows indicate direction of cytoplasmic streaming. Bar = $50 \mu m$.

(B) En = endoplasm; Ec = ectoplasm. Note that the endoplasm is densely packed with granules and organelles, while the ectoplasm is relatively clear. Bar = $25 \mu m$. (Stockem et al. 1982), and actin staining demonstrates the existence of dynamic actin structures and distinct fibers in this region as well (Taylor et al. 1980a). Finally, Grebecka and Grebecki (1981) effectively countered the frontal zone contraction theory by demonstrating that the uroid of an amoeba tends to contract steadily, regardless of changes in direction or velocity at the front of the cell and even when the front is inhibited or damaged. Further work demonstrated that, while the uroid is the primary region of contraction, in fact ectoplasm throughout the entire cell contracts to some degree (Grebecki 1984).

The majority of evidence regarding amoeboid movement supports a model known as the generalized cortical contraction theory. This model proposes that amoebae advance because contraction of the ectoplasm, particularly in the uroid region, exerts a pressure that pushes cytoplasm forward toward areas of the cell where the cortical actin layer is not firmly attached to the plasma membrane. The increased flow of cytoplasm towards these areas initiates the formation of pseudopodia and ultimately determines the direction of amoeboid movement (Grebecki 1990). This hypothesis is supported by experiments measuring internal pressure in amoebae, which demonstrated that internal pressure increased to a maximum value directly before pseudopod extension began, and then decreased as cytoplasm streamed forwards into a pseudopod (Yanai et al. 1996).

Mechanism of Pseudopod Extension

By examining highly magnified videos of streaming amoebae, Grebecki (1990) developed a specific model of cortical actin detachment from the plasma

membrane at the tips of pseudopodia (figure 2). According to this model, the advancement of pseudopodia occurs via the cyclic detachment of the actin layer from the plasma membrane and the formation of a new layer in its place. When the cortical layer at the advancing tip of a pseudopod detaches from the membrane, it contracts and moves in towards the center of the cell, forming a structure known as the plasmagel sheet. The global contraction of the ectoplasm and a reduced hydrostatic pressure in the space beyond the retracted sheet pushes cytoplasm, which includes G-actin monomers, through the plasmagel sheet, forming a clear layer at the tip of the pseudopod called the hyaline layer (figure 3). The G-actin monomers then polymerize into a new filamentous actin layer adjacent to the plasma membrane. As the original layer depolymerizes, this new layer contracts and moves inwards to form a new plasmagel sheet. The pseudopod thus advances in a stepwise manner, with forward movement repeatedly pausing and restarting as new actin layers form, detach, contract, and depolymerize. Grebecki's videos show the repeated detachment and retraction of numerous cortical layers, with a new layer forming approximately every two seconds.

Evidence for the Involvement of Calcium

While the specific regions of contraction and the mechanism of pseudopod extension are now fairly well understood, the regulation of this motile system remains somewhat unclear. A great deal of evidence indicates that the control of



Figure 2. Mechanism of extension at pseudopod tip in *A. proteus*. 1: Cortical actin layer is attached to plasma membrane at pseudopod tip. 2: Actin layer detaches and moves inwards, forming plasmagel sheet. 3: Clear cytoplasm, containing G-actin monomers, is pushed through plasmagel sheet, forming hyaline cap. 4: G-actin begins to polymerize into new cortical actin layer. 5: As new cortical layer forms, plasmagel sheet depolymerizes, and granular cytoplasm catches up to pseudopod tip.



Figure 3. Hyaline cap at the tip of an extending pseudopod. Note that granular cytoplasm is held back behind the plasmagel sheet. Bar = 25μ m.

amoeboid movement is largely influenced by changes in local calcium concentrations. The presence of calcium in the external medium is a necessity for locomotion. Adding EGTA, a calcium chelator, to an amoeba's medium causes complete cessation of movement and rounding up of the cell (Hrebenda 1972). Internal calcium levels also profoundly affect the motility of *A. proteus*. In studies using isolated cytoplasm, Taylor et al. (1973) found that a calcium concentration of approximately 1.0 μ M was necessary to induce contraction. This study also showed that the rate and degree of contraction is dependent on the calcium concentration. Kawakatsu et al. (2000) confirmed these results and demonstrated that the detachment of the cortical actin layer from the plasma membrane is under the control of calcium as well.

Calcium Interactions with Actin Binding Proteins

Calcium is also critical for contraction in vertebrate striated muscle, another motor system composed of actin and myosin filaments. In this system, the proteins troponin and tropomyosin act together to block the interaction of myosin and actin when calcium is low. When calcium levels increase, calcium binds to troponin and causes a conformational change that shifts tropomyosin out of the way and enables myosin to bind actin and initiate contraction. No evidence has been found for a troponin and tropomyosin system in amoebae, however. Instead, calcium is believed to exert its effects on the actin network via interaction with a number of actin binding proteins (ABPs), including α -actinin, gelsolin, profilin, vinculin and talin. These proteins perform a number of functions within the cytoskeleton, including acting as cross-bridges between actin fibers and capping the ends of filaments to prevent further polymerization.

Though calcium affects each ABP differently, sometimes directly and sometimes indirectly, the net effect of increasing calcium levels is to promote depolymerization of the actin network. For example, calcium inhibits the crossbridging action of the protein α -actinin (Wachsstock et al. 1994), while it activates the filament-severing and capping protein gelsolin (Isenberg 1991). The ABP profilin can prevent actin polymerization directly by binding to actin filament ends (Isenberg 1991). However, profilin also inhibits actin polymerization by participating in the phosphorylation of G-actin monomers, an action dependent on the presence of calcium (Sonobe et al. 1986). The ability of calcium to influence the activity of a variety of ABPs thus provides a mechanism for calcium to control three major cytoskeletal events necessary for amoeboid movement: contraction of the ectoplasm, detachment of the cortical actin layer from the plasma membrane, and depolymerization of the plasmagel sheet.

Solation-Contraction Coupling

Numerous studies of isolated or synthesized amoeba cytoplasm demonstrated that a minimum amount of gel structure in the ectoplasm is necessary for contraction (Taylor 1977). However, if the actin network is too strongly cross-linked (essentially if it is too solid) the gel will resist the forces provided by myosin motors and contraction will not occur (Janson et al. 1991). However, increasing calcium to the micromolar range partially solates the gelled ectoplasm via the interaction of calcium with ABPs (Janson and Taylor 1993). This weakened gel cannot resist the forces of the myosin molecules, and thus actin filaments slide past each other and contraction occurs. Janson et al. (1991) demonstrated that the rate of contraction in this system depends not only on the amount of myosin present, but also on the degree of solation, with an increase in solation leading to an increased rate of contraction.

This theory, known as the solation-contraction coupling hypothesis, explains the source of motive force in amoebae as well as the phenomenon of cytoplasmic streaming. High calcium levels in the uroid region, the primary area of contraction, first depolymerize the actin network enough to permit contraction, then depolymerize the network completely, enabling the transition from gelled ectoplasm to fluid endoplasm (Janson et al. 1991; Janson and Taylor 1993). Newly formed endoplasm is pushed forwards by the force of contraction until it reaches the tip of an advancing pseudopod, where it resolidifies into the ectoplasm that lines the entire cell. This model is supported by the finding that the endoplasm of the amoeba is most solated in the uroid region and increases in structure as it advances towards the anterior end of the cell (Allen 1973, Taylor 1977).

Detachment of the Actin Layer from the Plasma Membrane

Calcium regulation of ABPs also enables another key event in amoeboid movement: the detachment of the actin network from the plasma membrane. The ABP α -actinin is a membrane associated protein (Isenberg 1991), and is believed to provide at least part of the link between the actin network of the ectoplasm and the plasma membrane itself. Staining for α -actinin reveals that it is present in a thin layer underneath the plasma membrane of amoebae, but it is not found where the actin network is detached from the membrane, i.e. at the plasmagel sheet (Brix et al. 1990). A recent study demonstrated that the dissociation of actin fibers from the plasma membrane in burst *A. proteus* cells depends on the presence of calcium (Kawakatsu et al. 2000). This evidence, coupled with the fact that calcium is known to regulate the affinity of α -actinin for actin (Wachsstock et al. 1994), strongly suggests that calcium plays a significant role in the actin network detachment occurring at pseudopod tips.

Depolymerization of the Plasmagel Sheet:

Calcium interactions with ABPs are also most likely responsible for the depolymerization of successive plasmagel sheets observed by Grebecki (1990). Injecting phalloidin, which prevents actin depolymerization, into *A. proteus* causes the formation of a permanent plasmagel sheet and a hyaline layer surrounding the entire cell periphery (Stockem et al. 1978). This indicates that actin depolymerization is indeed critical for the breakdown of this layer and the

extension of pseudopodia. Therefore, if elevated calcium in the uroid can induce depolymerization and the transition of ectoplasm into endoplasm, it is logical that calcium may act via a similar mechanism at the pseudopod tip to depolymerize the plasmagel sheet.

Possible Sources of Calcium

If calcium regulation of the cytoskeleton is necessary for amoeboid movement, the question then becomes where is this calcium coming from? At least some must be coming from the external medium, since, as mentioned above, removing all calcium from the medium causes a complete cessation of movement. Studies also indicate the presence of a calcium current entering amoebae from the external medium. Using a vibrating calcium-specific electrode, Kutreiber and Jaffe (1990) found a small calcium influx into amoebae, with the largest average influx occurring at the uroid.

Though external calcium is clearly necessary for normal locomotion, release of calcium from internal stores in amoebae seems critical for movement as well. Evidence indicates that this release may be under the control of a phospholipase C (PLC) pathway. PLC is an enzyme associated with the internal plasma membrane that, when activated, cleaves phosphatidylinositol 4,5 bisphosphate (PIP₂) into diacylglycerol and inositol trisphosphate (IP₃). IP₃ then binds to receptors on the endoplasmic reticulum (ER), opening pores that allow for the efflux of calcium into the cytoplasm. Though little is known about the specific internal biochemistry of *A. proteus*, PLC pathways are known to regulate the local changes in intracellular calcium concentrations necessary for a variety of cellular processes in numerous species.

Experiments suggest that this pathway is indeed involved in the intracellular release of calcium in amoebae. Application of 2aminoethoxydiphenyl borate (2APB), a blocker of the IP₃ receptor on the ER, results in the cessation of cytoplasmic streaming, the rounding up of the amoeba, and the formation of numerous small protrusions surrounding the periphery of the cell (S. Barry, personal communication). The PLC inhibitor U73122 elicits a similar effect (Broughton 2005), supporting the theory that PLC-controlled calcium release is an essential component of normal amoeboid movement.

IP₃ is not the only possible trigger of calcium release from internal stores, however. In a process known as calcium-induced calcium release, cytosolic calcium itself can trigger the release of additional calcium from internal stores such as the ER. This phenomenon is well described in numerous cell types, such as neurons (Sandler and Barbara 1999), oocytes (Girard et al. 1992), and cardiac muscle cells (Miller 1974). One model proposes two independent stores of calcium within cells: a "calcium insensitive" store controlled by IP₃ and a "calcium sensitive" store controlled by cytoplasmic calcium (Dupont et al. 1991). This provides a mechanism by which calcium signals may be amplified or calcium waves propagated within a cell. An alternative to calcium-induced calcium release is a process called capacitative calcium entry, whereby the release of calcium from internal stores triggers the opening of calcium channels in the plasma membrane, allowing an influx of calcium (Putney Jr. and Ribeiro 2000). In this case, ER proteins may interact directly with the plasma membrane to cause these calcium channels to open, or it may be that another second messenger is involved in signaling from the ER to the plasma membrane. It is as yet unclear whether either calcium-induced calcium release or capacitative calcium entry occurs in *A. proteus*, though both would provide a connection between the external and internal sources of calcium in amoeboid movement. Calcium-induced calcium release could provide for the amplification of the small calcium current that enters the cell, while capacitative calcium entry could be the mechanism that promotes that current in the first place.

Previous and Current Experiments

In summary, our working model for amoeboid movement is as follows: the alteration of local intracellular calcium concentrations, likely influenced both by external calcium and by a PLC pathway, causes an alteration in the structure of the cortical actin network via interactions with ABPs. Increased calcium causes the partial solation of this network, allowing it to contract and produce a force to move the amoeba forward. Complete solation of the network in the uroid region produces fluid endoplasm that is pushed forwards into advancing pseudopodia, where it resolidifies into gelled ectoplasm. Pseudopodia extend from places in the amoeba where the cortical actin network has detached from the plasma membrane, an action dependent on calcium's inhibition of the ABP α -actinin.

Three previous studies, undertaken in the late 1980's and early 1990's, used fluorescent calcium indicators (aequorin and fura-2) to examine calcium levels in *A. proteus*. Each study presented different results regarding the localization of calcium and how it correlates with movement in amoebae. For example, while two groups found high levels of calcium associated with the very tail end of amoebae (Taylor et al. 1980b; Gollnick et al. 1991), another found high calcium in the posterior region but towards the middle of the cell, not near the edges (Kuroda et al. 1988). In addition, the cytosolic calcium concentrations estimated by each experiment differed considerably, ranging from 10⁻⁸ M to 10⁻⁵ M. Further investigation using calcium dyes is worthwhile not only in an attempt to reconcile previous varying results, but because great improvements in microscope and imaging technology in the past decade allow for a much more precise analysis of calcium localization data.

For this paper, I performed a series of experiments using *A. proteus* cells injected with fluorescent calcium indicator dyes. The fluorescence intensity of these dyes increases when they bind to calcium, with no shift in emittance wavelength, thus indicating intracellular calcium concentrations. These experiments examined regional variations in calcium concentration within the cytoplasm of *A. proteus* in order to confirm the role of calcium in amoeboid

14

movement. In addition, I investigated the effects of extracellular calcium concentrations on intracellular calcium levels in an attempt to gain a better understanding of the differing roles of intra- and extracellular calcium in locomotion, and to examine a possible connection between the two sources. Finally, I examined the effects of a PLC inhibitor (U73122) on intracellular calcium levels in order to confirm the role of a PLC pathway in controlling calcium release and motility in *A. proteus*.

MATERIALS AND METHODS

Culture and Feeding

Amoeba proteus were obtained from Carolina Biological Supply and cultured in Pringsheim's solution (0.11 mM Na₂HPO₄, 0.35 mM KCl, 0.85 mM $Ca(NO_3)_2$, 0.08 mM MgSO₄, 0.007 mM FeSO₄, pH = 7.0). They were fed twice a week with *Tetrahymena elliota*, which were recultured weekly in a sterile 1% proteose peptone solution.

Microinjection

Amoebae were injected with either 0.25 mM calcium orange tetrapotassium salt or 0.25 mM fluo-4 pentapotassium salt (both from Molecular Probes) dissolved in injection buffer (37.5 mM KCl, 2.5 mM PIPES, pH = 7.0). Prior to injection, amoebae were transferred in their culture medium to either a glass coverslip (24 x 50 x 1.5 mm) or a series of interconnected wells with a glass coverslip bottom (for low calcium and U73122 experiments). Micropipettes were pulled from 1.00 mm outer diameter glass on a Sutter Instrument Company P-87 Flaming/Brown micropipette puller and mounted on a micromanipulator. Amoebae were impaled with a micropipette and solutions were injected at a pressure of 65 psi for 2 minutes using a Picospritzer II pressure injection system, made by General Valve Corporation. Multiple attempts were made to measure the precise volume injected by this system, as recommended by the manufacturer, by measuring the size of the droplet expelled from the micropipette. However, because only a very small volume was injected over a relatively long time period, a measurable droplet was not produced, and these attempts were unsuccessful. Though the exact volume of dye injected is unknown, it is estimated to be approximately 10-20% of the volume of an amoeba, or 0.2 to 0.4 nL injected into an approximately 2 nL cell (approximate amoeba volume from Gollnick et al. 1991). Therefore the cytosolic dye concentration is estimated to be between 12.5 and 25 μ M. Injections did not appear to affect the morphology or movement of amoebae.

Fluorescence Microscopy

Multiple cells were injected on one coverslip, which was then transported to the fluorescent microscope, located separately from the injection apparatus. Injected cells were viewed 30 to 90 minutes after injection using a Nikon Eclipse TE 2000-U inverted microscope and a 40x Nikon Plan Fluor lens. Images were taken with a Roper CoolSnapHQ monochrome 1300x1030 cooled digital camera under the control of Universal Imaging Corporation's MetaVue analysis and acquisition software. Cells injected with calcium orange, which is excited maximally at wavelength 549 nm and emits at 576 nm, were viewed using a Chroma 41017 filter set. Cells injected with fluo-4, which is excited maximally at wavelength 494 nm and emits at 516 nm, were viewed using a Chroma 31002a filter set. Fluorescent images were taken immediately following their corresponding phase images. Phase images were exposed for 5 ms, while fluorescent images were exposed for 2-3 s. Multiple images were taken of each cell in rapid succession, as the amoebae were too large to fit in a single frame.

Low Calcium and U73122 Experiments

For low calcium experiments cells were first injected and imaged in normal Pringsheim's solution. Amoebae were then washed three times with a calcium-free Pringsheim's solution (0.11 nM, Na₂HPO₄, 0.35 mM KCl, 10 mM MOPS, pH = 7.0). Cells incubated in each wash for five minutes. The same amoeba was imaged both before and after washing with the calcium-free solution.

U73122 was obtained from BIOMOL Research Labs, Inc. and stored as a 2 mM stock dissolved in 100% ethanol. For U73122 experiments, injected amoebae were first washed three times with a calcium-free Pringsheim's solution and imaged. This solution was then replaced with 1.0 μ M U73122 in calcium-free Pringsheim's solution. Multiple amoebae were imaged approximately 10 minutes after application of U73122.

Fluorescence Intensity Measurements

All data analysis was performed using MetaVue software. Only images of fluo-4 injected cells were quantitatively analyzed (for explanation see Results). Background fluorescence was first subtracted from images, with average background levels being determined individually for each image. Average fluorescence intensity levels were then measured by outlining the region of interest on the phase image and transferring that outline to the corresponding fluorescent image. Regions were defined as follows (figure 4):

- Wrinkled posterior: area containing the wrinkled membrane found at the absolute posterior of the amoeba
- Uroid: area from which the amoeba streams, located directly anterior to the wrinkled posterior
- Base of pseudopod: area on main body of the amoeba from where pseudopod extends
- Extending pseudopod: protrusion off of the main body of the amoeba, containing cytoplasm streaming away from the main body
- Endoplasm: dense, streaming, granular cytoplasm located in the center of the amoeba
- Ectoplasm: generally clear, non-streaming cytoplasm lining the edges of the amoeba

For low calcium and U73122 experiments, measurements were taken of all cytoplasm visible in each image. The threshold function of the MetaVue software was used to exclude regions of extremely bright fluorescence, which consisted of vesicles of sequestered dye (see Results), from intensity measurements. Threshold levels were held constant for all images taken of a single amoeba. Image intensities were recorded as 8-bit pixel values, representing a gray scale ranging from zero (black) to 255 (white).

Data Analysis

Fluorescence intensity measurements of different regions were compared within the same amoeba only, not between different cells. Statistical analyses (Student's t-tests) were performed only for those amoebae with two or more measurements from each region. Statistical analysis of endoplasm/ectoplasm comparisons was performed using a paired subjects t-test. For the low calcium and U73122 experiments, statistical comparisons were made between average overall cytosolic fluorescence of cells in the different conditions.



Figure 4. Example of regions measured. Regions indicated are as follows: WP = wrinkled posterior; U = uroid; B = base of extending pseudopod; P = extending pseudopod. Dashed arrows indicate direction of cytoplasmic streaming. Bar = 50 µm.

RESULTS

Injection of either calcium orange or fluo-4 into amoebae resulted in the almost immediate (within 30 seconds) appearance of roughly spherical, brightly fluorescent spots that flowed with the endoplasm of the cell. According to Sodeik et al. (1989), *A. proteus* can use anion transporter pumps to move molecules such as fluorescent dyes into preexisting vesicles. Thus the spots observed are most likely membrane-bound vesicles of sequestered dye. Dye sequestration was also observed by Gollnick et al. (1991), who attempted to visualize intracellular calcium levels in *A. proteus* using the indicator fura-2. As noted by Gollnick et al., the sequestration of dye does not affect measurements of cytosolic fluorescence. These vesicles of dye were excluded from measurements of average fluorescence intensity.

While some cytosolic fluorescence was seen in amoebae injected with calcium orange, this fluorescence tended to be very dim, and a large amount of the dye was sequestered into vesicles. Calcium orange was originally chosen as an indicator because it is excited by long wavelength light, which is least harmful to amoebae. However, the increase in fluorescence intensity exhibited by calcium orange when binding calcium is relatively low. In addition, according to Thomas et al. (2000), calcium orange is one of the most readily sequestered calcium dyes. Therefore all statistical analyses were performed on cells injected with fluo-4.

This dye is less prone to sequestration, and the fluorescence intensity of fluo-4 increases more than 100 times upon binding to calcium, yielding a more easily measured signal.

Measurements of fluorescence in *A. proteus* cells injected with fluo-4 revealed differences in calcium concentrations between various regions of the cytoplasm. Regions measured were the wrinkled posterior, the uroid, the pseudopod base, and extending pseudopodia themselves. Because amoebae vary in size and cell volume, the concentration of dye in each cell may have varied as well. Therefore, the fluorescence intensities of different regions were only compared with other regions from the same cell.

Average intensity of the uroid was higher than that of the wrinkled posterior for all cells measured (n = 7; see figure 5). However, statistical analyses could only be performed on cells from which multiple images per region were acquired. These analyses showed a significant difference (p < 0.05) for only one of four cells (figure 6). The continual movement of the amoebae, combined with their three-dimensional structure, made it difficult to obtain quality, focused images. Therefore, unfortunately, the number of measurements per region in each cell was fairly small (2 to 6). Thus the absence of a significant difference may be due in part to a lack of power.

Average fluorescence intensity of the pseudopod base was higher than that of extending pseudopodia for all cells measured (n = 6; see figure 7). Statistical analyses of the four cells with multiple images for each region found a significant

difference for three of the four cells (p < 0.05; see figure 8). The pseudopod base was on average more than twice as bright as extending pseudopodia in the same cell. Intensity values for the pseudopod base were similar to those for the uroid regions in the same amoeba.



Figure 5. Pseudocolor image of *A. proteus* injected with fluo-4. Plasma membrane is outlined in red, wrinkled posterior in green, and the uroid in white. Dashed arrows indicate direction of cytoplasmic streaming. Bright yellow spots are vesicles of sequestered dye. Note the much greater fluorescence intensity in the uroid cytoplasm compared to the wrinkled posterior cytoplasm. Bar = $50 \mu m$.



Figure 6. Comparison of fluorescence intensity: Uroid versus wrinkled posterior. The difference in intensity between the uroid and the wrinkled posterior regions in the same cell was significant for only 1 of 4 cells. Higher 8 bit pixel values indicate greater fluorescence. Bars are \pm standard error. ****** = significant difference, p < 0.01.



Figure 7. Pseudocolor image of *A. proteus* injected with fluo-4. Plasma membrane is outlined in red, extending pseudopod in green, and the pseudopod base in white. Dashed arrows indicate direction of cytoplasmic streaming. Bright yellow and orange spots are vesicles of sequestered dye. Note the decreased fluorescence intensity in the cytoplasm of the extending pseudopod compared to the cytoplasm at the base of the pseudopod. Bar = $50 \mu m$.



Figure 8. Comparison of fluorescence intensity: Pseudopod base versus extending pseudopod. In 3 out of 4 cells extending pseudopodia showed significantly less fluorescence than pseudopod bases in the same cell. Bars are \pm standard error. * = significant difference, p < 0.05, ** = significant difference, p < 0.01.

The average intensity of the endoplasm was brighter than that of the ectoplasm for every image analyzed (n = 15 images from 6 cells; see figures 9 and 10). On average, the endoplasm was nearly three times brighter than the ectoplasm in the same cell (p<0.001). Table 1 provides a summary of the fluorescence intensity differences found in different regions of the amoebae.



Figure 9. Comparison of fluorescent intensities: Ectoplasm versus endoplasm. Endoplasm was on average 297% brighter than ectoplasm in the same cell. Bars are \pm standard error. *** = significant difference, p < 0.001.

Region A	Region B	Average Percent Difference in Fluorescence, A to B
Uroid	Wrinkled posterior	54.3% brighter
Base of pseudopod	Extending pseudopod	101.5% brighter
Endoplasm	Ectoplasm	297.3% brighter

Table 1. Summary of regional differences in fluorescence



Figure 10. Corresponding phase (A) and pseudocolor (B) images of *A. proteus* injected with fluo-4. Plasma membrane is outlined in red, and the ectoplasm/endoplasm border in white. Bright white and yellow spots are vesicles of sequestered dye. Note the overall brighter fluorescence in the endoplasm of the cell. Bar = $50\mu m$.

One amoeba was imaged both in normal Pringsheim's solution (0.85 mM Ca^{2+}) and again 10 minutes after rinsing with a calcium-free Pringsheim's solution. Because it is impossible to remove all of the external solution during each wash, and because a large amount of calcium is bound to the external surface of amoebae (up to 18% of total cell calcium [Prusch and Hannafin 1979]), rinsing with a calcium-free solution does not produce a completely calcium-free medium, but instead produces a low calcium medium. Average fluorescent intensity measurements taken of the whole cell decreased by nearly half after rinsing with the calcium-free solution (p<0.01; see figures 11 and 12).



Figure 11. Comparison of fluorescent intensities: Same amoeba in normal (0.85 mM) and low calcium medium. Fluorescence decreased by 48% in the low calcium medium. Bars are \pm standard error. ****** = significant difference, p < 0.01.



Figure 12. Base and extending pseudopodia of *A. proteus* cell injected with fluo-4, imaged in normal calcium medium (A) and in low calcium medium (B). Plasma membrane is outlined in red. Dashed arrows indicate the direction of cytoplasmic streaming. Bright white and yellow spots are vesicles of sequestered dye. Note the decreased overall fluorescence in the calcium-free solution. Bar = 50μ m.

A solution of 1.0 μ M U73122 in calcium-free Pringsheim's was applied to amoebae already in a calcium-free Pringsheim's solution. Application of this phospholipase C inhibitor reduced or eliminated cytoplasmic streaming and caused amoebae to condense into a smaller, more spherical shape. These cells also exhibited numerous small protrusions, composed of both hyaline and granular cytoplasm, extending from the perimeter of the cell (see Broughton 2005 for a detailed analysis of the effects of U73122 on *A. proteus*). These protrusions were static and did not extend or retract like normal pseudopodia.

Measurements of the average cytosolic fluorescence of different amoebae before and after application of U73122 did not reveal an overall difference in intensity. However, while some cells were severely affected by U73122, condensing to a nearly spherical shape and completely ceasing cytoplasmic streaming and movement, other cells were only mildly affected, exhibiting slowed cytoplasmic streaming and partial rounding (figure 13). Comparison of these two degrees of U73122-affected cells revealed significantly higher levels of fluorescence in the mildly affected cells compared to the severely affected cells (n = 11 images from 2 mildly affected cells, 5 images from 2 severely affected cells, p<0.01; see figures 14 and 15). Table 2 provides a summary of the differences in fluorescence intensities between cells in various conditions of calcium concentration and U73122.



Figure 13. Phase images of mildly affected (A) and severely affected (B) A. proteus cells treated with 1.0 μ M U73122. Image A shows only half of the cell, which is streaming slowly. Image B shows the entire cell, which is not streaming. Note the small protrusions surrounding both cells, as well as the highly condensed cytoplasm, particularly in the severely affected cell. Bar = 50 μ m.



Figure 14. Pseudocolor images of mildly affected (A) and severely affected (B) *A. proteus* cells injected with fluo-4 and treated with 1.0μ M U73122. Plasma membrane is outlined in red. Bright white, orange, and yellow spots are vesicles of sequestered dye. Note the lower overall fluorescence in the severely affected cell, including large areas with no detectable fluorescence. Bar = 50 µm.



Figure 15. Comparison of fluorescence intensities: Cells mildly affected by U73122 versus severely affected cells. Severely affected cells, exhibiting no cytoplasmic streaming and highly condensed cytoplasm, showed significantly lower fluorescence than mildly affected cells, exhibiting some streaming and only partially condensed cytoplasm. Bars are \pm standard error. ** = significant difference, p < 0.01.

Table 2. Summary of conditional differences in fluorescence.

		Average Percent Difference
Condition A	Condition B	in Fluorescence, A to B
Normal calcium	Low calcium	92.2% brighter
U73122: mildly	U73122: severely	59.3% brighter
affected	affected	_

While performing *in vitro* calibrations of these indicators is fairly straightforward, these calibrations are largely unreliable because they do not take into account the effect of the cytoplasmic environment on the binding affinity of the indicators (Thomas et al. 2000). Therefore, estimates of the absolute calcium concentration represented by a particular fluorescence intensity cannot be provided at this time.

In summary, fluorescence measurements indicate that calcium levels are high in the uroid, at the base of extending pseudopodia, and in the endoplasm of the cell, while calcium levels are low in the wrinkled posterior, extending pseudopodia, and the ectoplasm of the cell. In addition, washing amoebae with a calcium-free solution reduced overall internal fluorescence by half, implying a corresponding decrease in internal calcium. Finally, amoebae severely affected morphologically by application of the PLC inhibitor U73122 showed lower cytosolic fluorescence than did those cells only mildly affected by this compound.

DISCUSSION

Role of Calcium in Solation and Contraction

The majority of evidence regarding movement in *A. proteus* suggests that calcium plays a critical role in both the solation and the contraction of the cytoplasm. As described above, calcium interacts with ABPs in the cytoskeleton of *A. proteus* to promote actin depolymerization and solation of the cytoplasm. This partial solation, occurring primarily in the uroid of the cell, in turn enables the contraction of the gelled ectoplasm to produce a motive force. My analysis of regional calcium variations in amoebae injected with fluo-4 correlates well with established theories about the role of calcium in amoeboid movement, particularly regarding the role of calcium in the solation and contraction of the cytoplasm.

The highest levels of fluo-4 fluorescence were seen in the uroid region and at the base of extending pseudopodia. If indeed the uroid is experiencing constant, significant contraction, calcium levels would necessarily be elevated in this area when compared to the rest of the cell. The wrinkled posterior of the cell tended to show lower levels of calcium than did the uroid itself, though this difference was only significant for 1 of 4 cells. However, repeating this experiment with a larger number of cells and more measurements per cell may yield a more consistent significant result. If so, this difference could indicate that the wrinkled posterior itself is not an area of active contraction, but that it is instead composed primarily of inactive convoluted plasma membrane that is folded up and retracted as the amoeba advances forward.

Another interpretation of these results is that elevated calcium is not an indication of contraction, but an indication of highly solated cytoplasm. This interpretation fits well with the finding that the fluorescence of fluo-4 was nearly three times brighter in the in the endoplasm of amoebae compared to the ectoplasm, indicating a much higher calcium concentration in the endoplasm. Because calcium is necessary for the contraction believed to occur throughout the ectoplasm, one might expect to see higher calcium concentrations in the ectoplasm. However, though some calcium is needed in contracting regions to partially solate the ectoplasm and enable actin and myosin filaments to slide past one another, even higher levels of calcium are necessary to maintain the complete solation observed in the endoplasm.

According to this hypothesis, the wrinkled posterior may in fact contain contracting ectoplasm, but the uroid itself shows higher levels of calcium because it contains more highly solated endoplasm. This interpretation would account for the observation that new endoplasm is produced in the uroid and streams forward from there. Calcium towards the interior of the uroid would first cause partial solation, allowing for contraction of the ectoplasm in the wrinkled posterior. High calcium would then continue solation, causing the contracting ectoplasm to transition completely into fluid endoplasm. The high levels of calcium seen at the pseudopod base imply that this region, like the uroid, is an area of strong contraction and solation. While contraction of the uroid may provide the primary source of internal pressure that drives cytoplasmic streaming and pseudopod advancement, contraction occurring in the ectoplasm at the base of a pseudopod may provide an extra force that advances streaming in a particular area of the cell. This theory is supported by the finding that actin filaments are heavily concentrated at the base of pseudopodia (Brix et al. 1989). Here, as in the uroid, high levels of calcium in the endoplasm could induce contracting ectoplasm to become fully solated, producing additional endoplasm that streams into the pseudopod.

It is important to realize that amoebae often exhibit a complicated morphology, with multiple pseudopodia extending in different directions. While the localization of myosin almost exclusively in the uroid (Stockem et al. 1982) implies that it is the primary region of contraction, one must remember that these dynamic cells constantly shift their direction of streaming, and a particular location can quickly transition from pseudopod to uroid and back again. In addition, an individual amoeba may be contracting in and streaming from more than one region simultaneously. It is to be expected, therefore, that the distribution of calcium is not a simple gradient from posterior to anterior, but instead that it reflects the polypodial nature of these cells.

The interpretation that high calcium represents high solation fits well with results found by Taylor (1977), who injected 1.0 μ M calcium directly into

different regions of amoebae. Injecting calcium into the ectoplasm caused strong contraction, indicating that this region is highly gelated and contains intact fibers ready to slide past one another. Injecting calcium into endoplasm in the anterior of the cell caused a moderate contraction, indicating some degree of gelation here. A very weak contraction was produced by calcium injected into the uroid endoplasm, demonstrating that this is the least structured and most solated region of the cell, containing few intact actin filaments. Viewed in this light, then, the regional differences in fluorescence seen in fluo-4 cells seem to be a fairly accurate indication of cytoplasm fluidity in a particular area of an amoeba.

From these observations, an amended version of the solation-contraction coupling hypothesis may be proposed. Elevated calcium in the endoplasm of the amoeba seems to maintain the solated state of this region and allow for cytoplasmic streaming. One could imagine, then, these elevated levels of endoplasmic calcium acting on ABPs at the endoplasm/ectoplasm border to induce solation and contraction in the ectoplasm throughout the cell. In the uroid and at the base of a pseudopod, where endoplasmic calcium levels are particularly high, the solation and contraction of the ectoplasm (and the production of new fluid endoplasm) would be particularly high as well.

Role of Calcium in Actin Layer Detachment

Evidence suggests that the ABP α -actinin comprises at least a part of the link between the actin network of the ectoplasm and the plasma membrane itself.

In vitro studies have shown that calcium, via the inhibition of α -actinin, causes the detachment of the actin network from the plasma membrane (Kawakatsu et al. 2000). Therefore, one might predict that experiments with fluorescent calcium indicators would reveal high levels of calcium at the tips of advancing pseudopodia, coinciding with the detachment of the actin network and the formation of the plasmagel sheet. However, this phenomenon was not observed in my imaging of fluo-4 injected amoebae.

It is possible that high calcium was not observed at pseudopod tips because detachment of the actin layer *in vivo* is not actually triggered by calcium, but instead by another process. However, it is more likely that calcium does in fact play a role in this process, but that I was unable to visualize this calcium using the current technique. The amount of calcium required to cause detachment from the plasma membrane may be a very small amount, undetectable with fluo-4. Alternatively, the calcium increase could be large but undetectable because it is concentrated within an extremely small area, i.e. only at the ectoplasm/plasma membrane interface.

My images showed a complete absence of fluorescence in the hyaline cap at the tips of all pseudopodia, implying that the fluorescent indicators may not be able to penetrate the plasmagel sheet. This would prevent visualization of any elevations of calcium occurring beyond this border in the hyaline layer. Also, it is probable that any calcium fluctuations at the pseudopod tip occur very rapidly, and thus the relatively long exposure time used in these experiments (2-3 seconds) may mask any transient calcium increases. This possibility is increasingly likely considering Grebecki's observation that plasmagel sheet formation occurs approximately every 2 seconds (1990). Any calcium fluctuations, then, would most likely last only a fraction of this period, and would be extremely difficult to visualize with the current method. In addition, bright light, such as that used to excite fluo-4, inhibits pseudopod extension when shone on a pseudopod tip (Grebecki 1981). Therefore, this light may temporarily alter the calcium release occurring at the pseudopod tip and prevent the imaging of normal calcium levels there.

Comparison to Previous Studies

Previous attempts to image calcium in amoebae have yielded varying results. Some findings are consistent with results seen here, though a number of discrepancies are also found. Using the indicator aequorin in the giant amoeba *Chaos carolinensis*, Taylor et al. (1980b) found a continuous luminescence in the tails of amoebae and pulses of luminescence lasting between 2 and 5 seconds in the anterior region of the cells. These pulses may or may not have been associated with advancing pseudopodia. A correlation was also found between increased luminescence and increased velocity of movement. A second aequorin study, performed on *A. proteus* by Kuroda et al. (1988) also revealed high luminescence in the posterior of the cells, but unlike in the Taylor et al. study, the A third study (Gollnick et al. 1991) used the calcium indicator fura-2 to visualize internal calcium levels in *A. proteus*. A gradient of calcium was observed, decreasing from the uroid to the advancing front of the cells. These experiments also found decreased calcium in extending pseudopodia and increased calcium in retracting pseudopodia. Elevated fluorescence was also found in along the edges of the cells, particularly in the uroid and intermediate regions of the cell.

The one common result from these three experiments, as well as from my own, is an elevation of calcium in the uroid of advancing amoebae. My results agree with Kuroda et al.'s demonstration of elevated calcium in the inner portion of the cell (i.e. the endoplasm), but contradict the finding of elevated calcium at the cell periphery (Gollnick et al. 1991). The finding of Gollnick et al. may be called into question, however, because this study examined only fluorescent images and not their corresponding phase counterparts. It is very possible, therefore, that what they considered to be the ectoplasm because it is the edge of the fluorescent image is not in fact the edge of the actual cell. In addition, the spatial resolution achieved with these three previous studies, particularly with the aequorin experiments, is much lower than the resolution of my images. Therefore, small differences in fluorescence, for example the difference between the wrinkled posterior and the uroid, may have been undetectable in these earlier studies. Unfortunately, however, my experiments have limitations as well. The sequestration of both calcium orange and fluo-4 placed limitations on the amount of dye present in the cytoplasm. Because this resulted in low overall fluorescence levels, relatively long exposure times were needed to image injected cells. This poses a potential problem, particularly with fluo-4, because the blue light used to excite this indicator can be harmful to amoebae. A long exposure also introduces the possibility of a movement artifact affecting intensity measurements. However, as amoebae normally stream at a rate of only 0.5-1.0 μ m/s (Taylor et al. 1980b), a 2-3 second exposure should not yield serious movement artifact. In addition, this exposure time is only half of that used in the study by Kuroda et al. (1988), where aequorin-injected amoebae were imaged with a 4-6 second exposure. Another limitation of my own experiments is that I was unable to calibrate my indicators, and thus I can not provide an estimate of the actual calcium concentration corresponding to specific fluorescence intensities.

Role of External Calcium:

After washing with a calcium-free solution, the overall cytosolic fluorescence of an amoeba injected with fluo-4 decreased by nearly 50%. This result indicates that calcium entering the cell from the external medium has a significant effect on internal calcium levels. This is consistent with research detecting a steady calcium current that flows into the tails of amoebae. Removing all calcium from the external medium completely inhibits locomotion in amoebae, thus it follows that this influx of external calcium plays an important role in normal movement. What controls this influx, however, and how the calcium ions act once they enter the cell, remains to be determined.

One possible mechanism of action by which this external calcium could influence amoeboid movement is through calcium induced calcium release. This process, described primarily in excitable cells such as neurons and muscle cells, involves cytosolic calcium itself triggering the release of additional calcium from internal stores, such as the ER. If this mechanism is at work in *A. proteus*, the calcium current entering the uroid could trigger a release of calcium from the ER, essentially amplifying the calcium signal and creating the high levels of calcium seen in this region. This amplification may be necessary because *A. proteus* lives in a fresh-water habitat where calcium levels are generally very low.

A second mechanism providing a possible connection between internal and external calcium sources is capacitative calcium entry, whereby the IP₃induced release of calcium triggers the opening of plasma membrane calcium channels. Because this process is more common in nonexcitable cells (Putney Jr. and Ribeiro 2000), it may be the more likely candidate in *A. proteus*, which have no known voltage-gated ion channels (S. Barry, personal communication). Capacitative calcium entry in *A. proteus* could provide a possible mechanism by which the release of calcium by IP₃ causes the influxing calcium current seen in the tails of amoebae. Though the fluorescence decrease observed in the low calcium experiment was quite dramatic, measurements were only taken from one cell, and thus these results must be replicated to confirm their significance. Measuring the internal calcium levels of amoebae placed in varied, precisely known, concentrations of calcium would offer further insight into exactly how the internal and external calcium concentrations are related. Examining the temporal relationship between external and internal calcium changes would be a valuable experiment as well.

Role of PLC:

Application of 1.0 μ M U73122, a phospholipase C inhibitor, also affected internal calcium levels in *A. proteus*. Amoebae exposed to U73122 retract all pseudopodia and become rounded, forming a nearly spherical cell with very dense cytoplasm and numerous small protrusions surrounding the cell periphery. This drastic change in cell shape makes it difficult to compare fluorescence levels between cells before and after application of U73122, since the dye in affected cells becomes concentrated in a much smaller area.

However, not all cells are completely affected by this low concentration of U73122. This allows for a comparison of fluorescence intensities between amoebae that are severely affected, i.e. amoebae that have rounded up and completely ceased streaming, and amoebae that are only mildly affected, i.e. amoebae that exhibit partial rounding and only somewhat inhibited streaming.

Comparing these two classes of cells revealed that the mildly affected cells had significantly higher calcium levels than the severely affected cells. This implies that a PLC pathway is involved in regulation of internal calcium levels in *A. proteus*, since a greater inhibition of this pathway seems to reduce internal calcium. In addition, the fact that the IP₃ receptor blocker 2APB causes morphological effects nearly identical to the effects caused by U73122 indicates that this PLC-controlled calcium release is most likely being caused by the action of IP₃ opening channels in the ER or a similar storage vesicle.

However, the results regarding the effects of U73122 should be viewed as preliminary data only. Because fluorescence measurements were compared between different cells, it is possible that differences in fluorescence intensity were due to variations in the concentration of dye present in each amoeba. In order to verify these results, a much larger number of amoebae should be measured, preferably both before and after application of U73122, or even at different concentrations of the inhibitor. Demonstrating that calcium concentrations return to baseline after the removal of U73122 and the return of normal streaming activity would also help to confirm the role of a PLC pathway in amoeboid movement. Further evidence in support of the involvement of this pathway could be gained by applying 2ABP to amoebae injected with fluo-4 in order to determine whether interfering at a different place in the PLC pathway causes a similar effect on internal calcium.

PIP₂ Interactions with ABPs

Calcium interacting directly with ABPs is not the complete story in the control of amoeboid movement however. A number of other compounds interact with ABPs as well, most notably the substrate of PLC, phosphatidylinositol 4,5-bisphosphate (PIP₂). PIP₂ modulates the activity of α -actinin, gelsolin, profilin, and vinculin (Lin et al. 1997). Though, like calcium, PIP₂ acts on each APB in a different way, its net effect is to promote the assembly of actin filaments. The three-way interactions between ABPs, PIP₂, and calcium are varied and complex, however.

For example, PIP₂ inhibits the ability of gelsolin to bind actin filaments, and Lin et al. (1997) demonstrated that the binding of PIP₂ to gelsolin is promoted by high calcium levels. One proposed model for the interaction of calcium, gelsolin, and PIP₂ is as follows: elevated calcium induces gelsolin to sever and cap actin filaments, but this calcium also induces PIP₂ to bind to gelsolin, releasing the gelsolin from actin filament ends (Lin et al. 1997). This severing action actually promotes actin polymerization by exposing actin filament ends to which more subunits can bind. However activated PLC, which may induce the elevated calcium in the first place, will sever PIP₂ molecules, thus allowing gelsolin to remain bound to actin filaments, promoting depolymerization. Clearly a fine balance exists between calcium, PIP₂, PLC, and gelsolin that is crucial for the control of actin polymerization in amoebae. PIP₂ also interacts with α -actinin, promoting its ability to bind actin filaments and induce cross-linking and gelation (Fukami et al. 1992). PIP₂ regulates the polymerization-inhibiting ABP profilin as well, competing with actin filaments for binding to profilin and thus promoting actin polymerization (Isenberg 1991). Fukami et al. (1994) found large amounts of PIP₂ bound to the actin network throughout the cytoplasm, and demonstrated that a decrease in the amount of PIP₂ can cause the depolymerization of the actin network. Activation of the PLC pathway, therefore, could cause actin depolymerization and solation of amoebae cytoplasm not only by inducing internal calcium release, but also by triggering the hydrolysis of PIP₂ bound to ABPs.

Triggers of Directional Movement

While the role of calcium in amoeboid movement is becoming clearer, the question of what environmental factors trigger pseudopod extension in a particular direction remains largely unanswered. We do have some knowledge of how amoebae respond to various changes in their environment. For example, shining light on any part of an amoeba causes contraction in that region, causing amoebae to stream away from light and into shaded areas (Grebecki 1981). Amoebae also respond to a pH gradient in their external medium, streaming towards regions of lower pH (Koruhoda et al. 1997). In addition, *A. proteus* demonstrates limited chemotaxis in response to prey such as *Tetrahymena* (Allen

1973). However, specific chemo- or photoreceptors that may be present on the external surface of amoebae have not yet been identified.

Regardless of our limited knowledge about the specific mechanism of amoeboid response to light or to various molecules present in the external medium, it is not too difficult to imagine this response involving a PLC or similar pathway. For example, molecules released by prey into the medium could bind to G-coupled receptors on the amoeba plasma membrane, thus activating PLC and producing IP₃. In addition, stimulation of photoreceptors by light has been shown to cause the hydrolysis of PIP₂ and the production of IP₃, which leads to the release of calcium (Brown 1984). In either case, this calcium increase would promote solation of the cytoplasm in the uroid or detachment of the actin layer from the plasma membrane at the pseudopod tip, resulting in pseudopod extension. Though we cannot assume that these specific mechanisms are occurring within *A. proteus*, it seems that some connection must exist between photo- and chemoreceptors and calcium regulated amoeboid movement.

Multiple Mechanisms at Work

Previous work, along with the results of the present study, indicates that calcium acts to produce at least two distinct effects critical to normal amoeboid movement: contraction in the uroid and detachment of the actin network from the plasma membrane at the pseudopod tip. One may ask, therefore, why high calcium at the uroid does not induce detachment of the ectoplasm there, or why high calcium at the tips of pseudopodia does not induce contraction. The answer may lie in the specific localization of calcium within these regions. My results indicate that calcium in the uroid is not elevated directly adjacent to the plasma membrane, but instead in the interior of the cell. In addition, though I was unable to visualize calcium at the tips of pseudopodia, if high calcium is interacting with α -actinin to cause detachment of the cortical actin layer, that calcium is presumably acting directly at the border between the plasma membrane and the actin network. It may be the precise localization of calcium, then, that allows for these two disparate effects at two different regions. While calcium in the uroid may not be close enough to the plasma membrane to cause separation, calcium at the pseudopod tip may be located only adjacent to the plasma membrane, and may be inaccessible to the majority of the ectoplasm. This theory is supported by the finding that injecting a large volume of 1.0 µM calcium into the center of an amoeba causes a general contraction of the cytoplasm, but does not induce detachment of the ectoplasm from the plasma membrane (Taylor 1977).

The key to this difference in calcium localization may lie in the localization of calcium storage vesicles such as the ER. Perhaps the highly crosslinked, gelled structure of the ectoplasm restricts the ER to the less structured endoplasm. Thus in the uroid, where the ectoplasm is particularly thick (Stockem et al. 1982), the ER may be found only in the interior of the cell. IP₃-mediated calcium release, therefore, would only occur in the endoplasm where the ER is located, allowing for contraction, but not actin layer detachment. A cortical actin network has, in fact, been shown to act as a barrier between the ER and the plasma membrane and prevent capacitative calcium entry in human platelet cells (Rosado et al. 2004) and smooth muscle cells (Morales et al. 2005, abstract only). At the tip of a pseudopod, however, where the ectoplasm is up to ten times thinner (Stockem et al. 1982) ER could extend much closer to the plasma membrane. Calcium released via IP₃ at the pseudopod tip would then be close enough to act on ABPs connecting the cortical actin layer to the plasma membrane.

This theory presents a mechanism by which a combination of external stimuli could induce amoeboid movement in a particular direction. For example, shining light on the uroid region may stimulate photoreceptors and lead to IP₃ production. IP₃ would then trigger calcium release from the ER located in the endoplasm, leading to contraction and retraction of the uroid and providing the force needed to move the cell forward. At the same time, chemical stimuli could bind to receptors at the pseudopod tip, activate a PLC pathway, and induce calcium release from the ER directly adjacent to the plasma membrane. This release in turn could trigger capacitative calcium entry, opening channels in the plasma membrane and allowing calcium to flow in. This amplified calcium signal, localized at the interface between the plasma membrane and the cortical actin layer, would then interact with ABPs to enable detachment of this layer and pseudopod extension, providing a mechanism for "steering" the amoeba in a specific direction.

This proposed mechanism is only speculation, though a careful study of ER localization in amoebae could offer support of its validity. Some questions remain unanswered, however, such as why the influxing calcium current detected by Kuhtreiber and Jaffe (1990) does not cause actin layer detachment when it first crosses the plasma membrane. One possibility is that this influx, though relatively constant, is not large enough or concentrated enough to cause detachment. It is also unclear how the morphological differences between the uroid and the pseudopod originate. For example, what causes myosin filaments to localize almost exclusively to the uroid, and what causes the translocation of myosin when a region of the cell shifts from pseudopod to uroid or vice versa? Clearly, though calcium certainly plays a major role, it is not the only factor in the control of amoeboid movement.

Conclusions

The study of amoeboid movement, though fascinating in its own right, also has applications outside the realm of protists. Keller and Eggli (1998) recently made the connection between the mechanism of movement in *A. proteus* and that of a specific type of metastasizing cancer cell, known as a blebbing Walker carcinosarcoma cell. These cells extend "blebs" like amoebae extend pseudopodia, via the detachment of a cortical actin layer from the plasma membrane. As in amoebae, hydrostatic pressure in Walker carcinosarcoma cells pushes cytoplasm through the detached actin layer, and new layers then form at the plasma membrane. The understanding of amoeboid movement, then, may have clinical implications for treating this type of cancer.

In summary, the current results suggest that elevated calcium within *A*. *proteus* is correlated with increased solation of the cytoplasm. High calcium was seen both at the uroid of the cell, as well as at the base of extending pseudopodia. Calcium is believed to act on ABPs at the ectoplasm/endoplasm interface in these regions, inducing solation and contraction of the ectoplasm. Results also indicate that external calcium sources are critical for normal amoeboid movement, and may be connected to internal calcium release via calcium-induced calcium release or capacitative calcium release. Evidence also suggests that the release of calcium from an internal store under the control of a PLC pathway also plays an important role in amoeboid locomotion. How environmental factors influence this release, and thus the direction of movement, remains to be seen.

LITERATURE CITED

- Allen RD. 1973. The behavior of amoebae. In: Pérez-Miravete A, editor. *Behavior of Microorganisms*. NewYork: Plenum Press. p 157-167.
- Brix K, Reinecke A, Stockem W. 1990. Dynamics of the cytoskeleton in *Ameba proteus* III: Influence of microinjected antibodies on the organization and function of the microfilament system. Eur J Cell Biol 51:279-284.
- Broughton DE. 2005. Senior Honors Thesis: A phospholipase C inhibitor, U-73122, affects locomotion and morphology in *Amoeba proteus*. Mount Holyoke College.
- Brown JE, Rubin LJ, Ghalayini AJ, Tarver AP, Irvine RF, Berridge MJ, Anderson RE. 1984. Myo-inositol polyphosphate may be a messenger for visual excitation in *Limulus* photoreceptors. Nature 311:160-163.
- Christiani A, Hugelmeyer P, Stockem W. 1986. Morphological evidence for the existence of a more complex cytoskeleton in amoeba proteus. Cell Tissue Res 246:163-168.
- Dupont G, Berridge MJ, Goldbeter A. 1990. Signal-induced Ca²⁺ oscillations: Properties of a model based on Ca²⁺-induced Ca²⁺ release. Cell Calcium 12:73-85.
- Eckert BS, Warren RH, Rubin RW. 1977. Structural and biochemical aspects of cell motility in amebas of Dictyostelium-discoideum. J Cell Biol 72:339-350.
- Fukami K, Furuhashi K, Inagaki M, Endo T, Hatano S, Takenawa T. 1992. Requirement of phosphatidylinositol 4,5-bisphosphate for alpha actinin function. Nature 359:150-152.
- Fukami K, Endo T, Imamura M, Takenawa T. 1994. Alpha-actinin and vinculin are PIP2-binding proteins involved in signaling by tyrosine kinase. J Biol Chem 269:1518-1522.
- Girard S, Luckhoff A, Lechleiter J, Sneyd J, Clapham D. 1992. Two-dimensional model of calcium waves reproduces the patterns observed in *Xenopus* oocytes. Biophys J 61:509-17.

- Gollnick F, Meyer R, Stockem W. 1991. Visualization and measurement of calcium transients in *Amoeba proteus* by fura-2 fluorescence. Eur J Cell Biol 55:262-271.
- Grebecka L and Grebecki A. 1981. Testing motor functions of the frontal zone in the locomotion of *Ameba proteus*. Cell Biol Int Rep 5:587-594.
- Grebecki A. 1981. Effects of localized photic stimulation on amoeboid movement and their theoretical implications. Eur J Cell Biol 24:163-175.
- Grebecki A. 1984. Relative motion in *Ameba proteus* in respect to the adhesion sites I: Behavior of monotactic forms and the mechanism of fountain phenomenon. Protoplasma 123:116-134.
- Grebecki A. 1990. Dynamics of the contractile system in the pseudopodial tips of normally locomoting amebas, demonstrated in vivo by video enhancement. Protoplasma 154:98-111.
- Guilford WH, Lantz RC, Gore RW. 1995. Locomotive forces produced by single leukocytes in-vivo and in-vitro Am J Physiol-Cell Physiol 268:C1308-C1312.
- Haberey M. 1972. Cinematography of cell membrane behaviour and flow phenomena in *Amoeba proteus*. Acta Protozoologica 11:95-102.
- Hrebenda B. 1972. The role of external calcium in motile phenomena of *Amoeba proteus*. Acta Protozoologica 11:107-112.
- Isenberg G. 1991. Actin binding proteins lipid interactions. J Muscle Res Cell Motil 12:136-144.
- Janson LW, Kolega J, Taylor DL. 1991. Modulation of contraction by gelation/solation in a reconstituted motile model. J Cell Biol 114:1005-1015.
- Janson LW and Taylor DL. 1993. In vitro models of tail contraction and cytoplasmic streaming in amoeboid cells. J Cell Biol 123:345-356.
- Kawakatsu T, Kikuchi A, Shimmen T, Sonobe S. 2000. Interaction of actin filaments with the plasma membrane in amoeba proteus: Studies using a cell model and isolated plasma membrane. Cell Struct Funct 25:269-277.
- Keller H, Eggli P. 1998. Protrusive activity, cytoplasmic compartmentalization, and restriction rings in locomoting blebbing walker carcinosarcoma cells are related to detachment of cortical actin from the plasma membrane. Cell Motil Cytoskeleton 41:181-193.

- Korohoda W, Golda J, Sroka J, Wojnarowicz A, Jochym P, Madeja Z. 1997. Chemotaxis of *Amoeba proteus* in the developing pH gradient within a pocket-like chamber studied with the computer assisted method. Cell Motil Cytoskeleton 38:38-53.
- Kuhtreiber WM, Jaffe LF. 1990. Detection of extracellular calcium gradients with a calcium-specific vibrating electrode. J Cell Biol 110:1565-1573.
- Kuroda K, Yoshimoto Y, Hiramoto Y. 1988. Temporal and spatial localization of Ca²⁺ in moving *Amoeba proteus* visualized with aequorin. Protoplasma 144:64-67.
- Lin KM, Wenegieme E, Lu PJ, Chen CS, Yin HL. 1997. Gelsolin binding to phosphatidylinositol 4,5-bisphosphate is modulated by calcium and pH. J Biol Chem 272:20443-20450.
- Miller DJ. 1974. Evidence for calcium-induced release in intact depolarized frog heart muscle. J Phisiol 242:93-95.
- Morales S, Camello PJ, Rosado JA, Mawe GM, Pozo MJ. 2005. Disruption of the filamentous actin cytoskeleton is necessary for the activation of capacitative calcium entry in naive smooth muscle cells. Cell Signal 17:635-645.
- Prusch RD and Hannafin JA. 1979. Calcium distribution in *Amoeba proteus*. J Gen Physiol 74: 511-521.
- Putney JW Jr, Ribeiro CM. 2000. Signaling pathways between the plasma membrane and endoplasmic reticulum calcium stores. Cell Mol Life Sci 57:1272-1286.
- Rosado JA, Lopez JJ, Harper AGS, Harper MT, Redondo PC, Pariente JA, Sage SO, Salido GM. 2004. Two pathways for store-mediated calcium entry differentially dependent on the actin cytoskeleton in human platelets. J Biol Chem 279:29231-29235.
- Sandler VM and Barbara JG. 1999. Calcium-induced calcium release contributes to action potential-evoked calcium transients in hippocampal CA1 pyramidal neurons. J Neurosci 19:4325-4336.
- Sodeik B, Brix K, Stockem W. 1989. Sequestration of microinjected molecular probes from the cytoplasm of *Amoeba proteus*. Europ J Protistol 25:75-84.

- Sonobe S, Takahashi S, Hatano S, Kuroda K. 1986. Phosphorylation of Amoeba G-actin and its effect on actin polymerization. J Biol Chem 261:14837-14843.
- Stockem W, Weber K, Wehland J. 1978. The influence of microinjected phalloidin on locomotion, protoplasmic streaming and cytoplasmic organization in *Amoeba proteus* and *Physarum polycephalum*. Eur J Cell Biol 18:114-131.
- Stockem W, Hoffmann HU, Gawlitta W. 1982. Spatial organization and fine structure of the cortical filament layer in normal locomoting *Ameba-proteus*. Cell Tissue Res 221:505-519.
- Taylor DL, Condeelis JS, Moore PL, Allen RD. 1973. Contractile basis of ameboid movement I: Chemical control of motility in isolated cytoplasm. J Cell Biol 59:378-394.
- Taylor DL. 1977. The contractile basis of amoeboid movement IV: The viscoelasticity and contractility of amoeba cytoplasm in vivo. Exp Cell Res 105:413-426.
- Taylor DL, WangYL, Heiple JM. 1980a. Contractile basis of ameboid movement VII. The distribution of fluorescently labeled actin in living amebas. J Cell Biol 86:590-598.
- Taylor DL, Blinks JR, Reynolds G. 1980b. Contractile basis of ameboid movement VIII: Aequorin luminescence during ameboid movement, endocytosis, and capping. J Cell Biol 86:599-607.
- Thomas D, Tovey SC, Collins TJ, Bootman MD, Berridge MJ, Lipp P. 2000. A comparison of fluorescent Ca^{2+} indicator properties and their use in measuring elementary and global Ca^{2+} signals. Cell Calcium 28:213-223.
- Wachsstock DH, Schwarz WH, Pollard TD. 1994. Cross-linker dynamics determine the mechanical properties of actin gels. Biophys J 66:801-809.
- Yanai M, Kenyon CM, Butler JP. 1996. Intracellular pressure is a motive force for cell motion in *Amoeba proteus*. Cell Motil Cytoskeleton 33:22-29.