Regulation of the contractile vacuole in *Dictyostelium* amoebae

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ABSTRACT

The contractile vacuole (CV) is a protozoan organelle network that is responsible for osmoregulation by actively expelling water from the cell in hypotonic environments. However the mechanism by which the CV interacts with the plasma membrane to discharge its contents is poorly understood. While little is known about the biogenesis of this organelle, knockout studies in *Dictyostelium discoideum* have demonstrated the requirement of several proteins such as clathrin for normal CV formation. However, clathrin-mediated endocytosis may not be the directly responsible for the organization and recycling of CV components. With fluorescence microscopy and fluorescently-labeled proteins, 1-butanol, a reversible inhibitor of clathrin function, was applied to observe the direct role of clathrin in CV function. The involvement of actin was also examined by blocking polymerization with latrunculin. While actin inhibition does not immediately perturb CV function, 1-butanol treatment results in a rapid widespread disruption of the CV network. Because 1-butanol is a known PLD inhibitor, phosphatidic acid, the immediate product of PLD, was applied in the presence of 1-butanol. Phosphatidic acid resulted in only modest recovery, suggesting the fine balance in signaling required for appropriate CV function. These results implicate the role of PLD activity in CV assembly and maintenance. Understanding the pathway by which CV function is regulated will yield insights into the function of proteins in the endocytic pathway as well as provide a framework for similar studies in human macrophages.

INTRODUCTION

All organisms need to take up extracellular nutrients or regulate membrane components including receptors. However the plasma membrane serves as an effective barrier that separates the intra- and extra-cellular environments. In order to acquire the necessary components located outside or on the membrane, cells utilize the process of endocytosis. Endocytosis, or the uptake of materials without crossing the membrane, can be divided into four different types: clathrin-mediated endocytosis, cavoelae, macropinocytosis, and phagocytosis (1, 2). Pinocytosis and phagocytosis are primarily used for liquid and large matter respectively (1, 3). Cavoelae, a clathrin-independent pathway, has been implicated in signal transduction. However, clathrin-mediated endocytosis remains one of the most well studied pathways, as it is the predominant pathway for the concentration and recycling of membrane receptors.

While the endocytic pathway is crucial for regulating how cells respond to their environment, many single-celled organisms (i.e. *Dictyostelium discoideum*) can also have permeable membranes that allow wastes and nutrients to go in and out of the cell by simple diffusion. To maintain water balance, many have developed a unique and specialized organelle known as the contractile vacuole (CV) (4, 5). The CV is a complex organelle responsible for protozoan osmoregulation that enables organisms to expel water and survive especially under stressful hypotonic environments. The CV membrane has been shown to be distinct with vacuolar-type proton pumps yet no actin filaments (part of the cytoskeleton), while the plasma membrane is completely the opposite (6, 7). However the mechanism by which they fuse to the plasma membrane and discharge their contents is still poorly understood. Not much has been discovered

about CV biogenesis except that proteins found on it are carried through the Golgi apparatus before reaching the CV (5). By understanding the regulation and maintenance of this organelle, we may be able to more clearly understand the endocytic pathway and how other membrane-bound compartments are able to maintain their identities in more complex organisms.

Dictyostelium discoideum are haploid soil-living amoeba that grow rapidly in culture and can feed on bacteria. As a model organism, they have been widely used to study membrane trafficking and intracellular transport (8, 9). Genetic knockouts can easily be generated by homologous recombination, allowing scientists to characterize the specific function of gene products and use them as tools to study other proteins. Many genes are homologous to human genes and therefore many of its proteins have homologs in humans (10, 11). They also have a comparable cytoskeletal composition to human macrophages, so that many of the processes that rely on these components such as phagocytosis, vesicle sorting, and other endocytotic events are also analogous (10, 12). *Dictyostelium* can provide significant insight into the endocytic pathway of eukaryotic cells which are more complex and harder to differentiate.

In endocytosis, membrane components such as receptors or ion channels are brought into the cell and concentrated at endosomes. From the endosomes, components can either be recycled back to the cell membrane or transported to the lysosome for degradation. Clathrin-mediated endocytosis (CME) is the primary method that cells are able to regulate their membrane protein and lipid compositions (13). In particular, CME is responsible for the transport of materials in clathrin-coated vesicles from the Golgi apparatus, endosomal compartments, and cell membrane (14).

Consequently, CME is particularly important in regulating how cells constantly interact and respond to their environment. Clathrin, the major protein component of vesicles in CME, is a triskelion structure composed six polypeptides: 3 heavy chain and 3 light chains (13, 15). Proteins coats like clathrin serve two primary purposes: (1) to concentrate membrane components and (2) to aid in vesicle structure formation. At the membrane, triskelions organize in a basketlike structure to form clathrin-coated pits (1, 13, 15).

While clathrin can spontaneously come together in solution, adaptor proteins are required for clathrin assembly within the cell. Adaptor proteins such as AP-2 are the specific connections between clathrin and the plasma membrane, particularly various cargo receptors (15). Because different adaptor proteins and accessory proteins bind to different proteins, select membrane components can be transported in each clathrin-coated vesicle. Previous studies in the laboratory showed that knockouts in clathrin heavy chain, light chain, as well as adaptor proteins all exhibited impaired osmoregulation and/or abnormal CV network (16-18). However, since clathrin and its accessory proteins are important for many other trafficking pathways, it remained possible that the requirement of clathrin for CV function was not direct. Applying drugs that affect clathrin function in a rapid and reversible way may help to determine the role of clathrin in CV function.

Phosphoinositides (PIs) have also been implicated in CME by regulating the timing and localization of adaptor protein recruitment (19). PIs are formed by different PI kinases that are activated by small GTPases. For example, PI(4)P 5-kinases form phosphatidylinositol-(4,5)-bisphosphates (PIP_2), the direct membrane partner of AP-2

(20). A major player in exo- and endocytosis, PIP_2 has been shown to be particularly important in secretory granules and neurotransmitter release (19). Furthermore, knock outs of PI(4)P 5-kinases showed decreased PIP_2 levels in human cells, while overexpression significantly enhanced the formation of clathrin-coated pits at the membrane (21). However, the role of other membrane enzymes and cytoskeleton proteins such as actin in endocytic pathways are less well defined and have only begun to be elucidated.

Phospholipase D (PLD), an enzyme recruited to the plasma membrane, has been suggested to play a role in CME (22-24). Studies have shown that transferrin receptor internalization, a canonical CME pathway, is inhibited in the presence of 1-butanol, while reduced internalization of G-protein-coupled receptors was observed in PLD mutant lines (22, 25, 26). *In vivo*, PLD forms phosphatidic acid (PA) by using water to hydrolyze phosphatidylcholine. PLD has been found to be activated by PIP_2 synthesis, while also promoting the activity of PI(4)P 5-kinase which produces PIP_2 (22). Taken together, these observations strongly suggest a role for PLD in CME by regulating the synthesis of PIP_2.

1-butanol, a primary alcohol, can be substituted for water at the catalytic site of PLD to form phosphatidyl-butanol instead of phosphatidic acid (23). Studies that have applied 1-butanol to live cells show a direct dependence of AP-2 to PIP_2 for CME (22). Other scientists have shown that actin-based motility was completely inhibited in a reversible reaction by 1-butanol, although actin polymerization still occurred (27). By blocking the assembly of clathrin-coated vesicles, 1-butanol serves as a powerful tool to dissect apart the endocytic machinery.

Actin, a major cytoskeletal protein, has also been shown to play an important role in CME (8). Mutants deficient in actin proteins demonstrate severely impaired CME. Actin and endocytic proteins have been shown to co-localize at the membrane (22, 28, 29). However, current studies have been unable to elucidate its exact function in CME mainly due to the absence of structural studies that focus on the mechanism of assembly and disassembly. A previous study by Heuser (2006) indicated that under harsh conditions in the presence of latrunculin, the CV would collapse and discharge its contents through complete fusion with the plasma membrane in an exocytotic event. This seemingly disastrous effect surprisingly does not result in mixing of the CV and plasma membranes (7). Latrunculin and cytochalasin, inhibitors of actin polymerization, may provide insights into how actin is involved in CME and CV maintenance.

My aim is to understand the mechanisms of membrane trafficking and how different organelles, such as the contractile vacuole, are able maintain distinct membrane components in a simple model organism. To study the effect of drugs on clathrin and CV function, I use fluorescently-labeled proteins and both fixed and light microscopy with fluorescence. Latrunculin A blocks budding of clathrin-coated vesicles but does not impair CV function in hypotonic conditions, suggesting that actin is not immediately required for osmoregulation. Interestingly, 1-butanol blocks coated vesicle assembly and completely disrupts the CV network within minutes. Because 1-butanol is a known PLD inhibitor, phosphatidic acid, the immediate product of PLD, is applied in the presence of 1-butanol to rescue normal CV function. By itself, phosphatidic acid also disrupts the CV network, suggesting the fine balance in PLD activity required for

appropriate osmoregulation. PLD mutants also exhibit similar results. This has led to my current hypothesis that regulation of PLD activity is important for CME and may play a role in CV assembly and maintenance. By doing a systematic disruption of candidate pathways and observing how the endocytic pathway responds, we can begin to elucidate the complexities of membrane trafficking.

RESULTS

Dictyostelium cells were transformed with Dajumin-GFP to visualize the cells' contractile vacuoles, as Dajumin-GFP localizes to the CV membrane (See Fig. 1). In addition, the cells were placed in water rather than isotonic low fluorescence axenic media to stimulate osmoregulation and observe if there are any defects in the structure or function of the CVs. In water, the AX2 wild-type (control) cells exhibit clearly labeled CV networks with swollen bladders and tubules. The phospholipase D mutants (A, B, C, and A/C double) exhibited the similar phenotypes under the same conditions. The CVs are similar in size and do not appear to have any obvious osmoregulation defects.

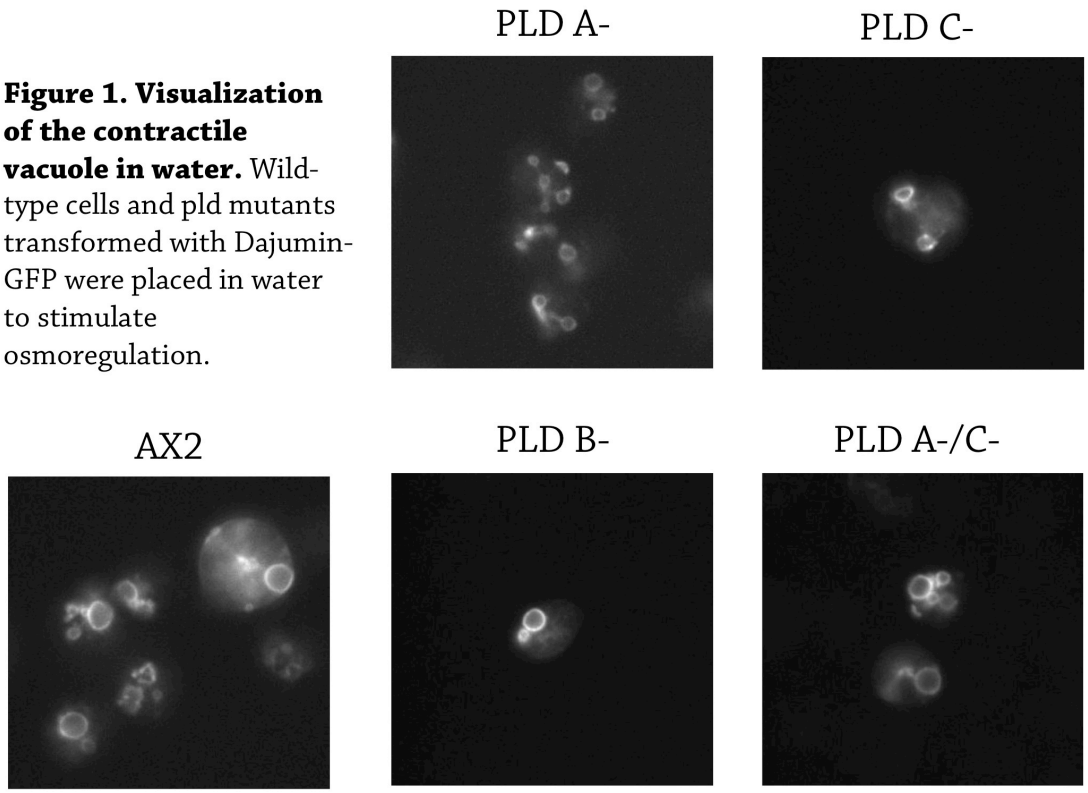

Figure 1. Visualization of the contractile vacuole in water. Wild-type cells and pld mutants transformed with Dajumin-GFP were placed in water to stimulate osmoregulation.

To ensure that the effects of 1-butanol were specific, particularly due to the disruption of the phospholipase D pathway, other alcohols were also applied to AX2 wild type cells in water (See Fig. 2). Ethanol, another short chain primary alcohol, could also replace water in the nucleophilic reaction at PLD's catalytic site. However unlike 1-butanol, ethanol does not appear to have an effect on the CV. Additionally, t-butanol, a structural isomer of butanol, was applied to ensure that the 1-butanol effect was not related to any nonspecific toxicity to alcohols. Similarly, cells do not exhibit any obvious defects in the presence of t-butanol. Therefore, the effects of 1-butanol on the CV are specific.

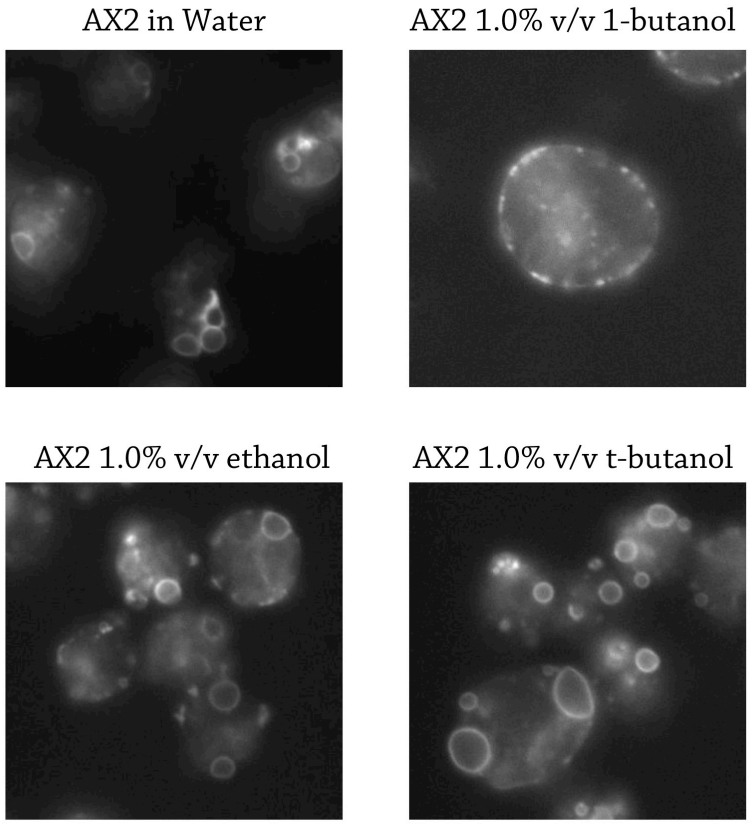

Figure 2. Effect of alcohols on the contractile vacuole. Wild-type cells were placed in different alcohols. Ethanol served primary alcohol control, while t-butanol served as a general nonspecific alcohol control. Both alcohols did not result in the phenotype observed when the cells were placed in 1-butanol.

Because earlier reports had utilized 1-butanol, a primary alcohol, in studies of AP-2 and PIP_2, I applied 1-butanol to Dajumin-GFP transformed AX2 wild type cells (see Fig. 3) to test the involvement of clathrin-mediated endocytosis and CV maintenance (22). 1-butanol has been shown to be an inhibitor of clathrin-coated pit assembly (27). No visible differences were observed at 0.25% v/v 1-butanol. With 0.5%, the CVs were noticeably smaller compared to the wild-type. When 1.0% v/v 1-butanol was added, the CV network was entirely disrupted within several minutes. Small GFP-labeled vacuoles appear to clump near the plasma membrane. However, it is important to note that the CV membrane components are still distinct from the plasma membrane. There is no

mixing of the two membranes. At increasing concentrations (2.5%), the same effect is observed, however cell death increased significantly compared to lower concentrations. Therefore 1.0 v/v% is chosen as a good working concentration.

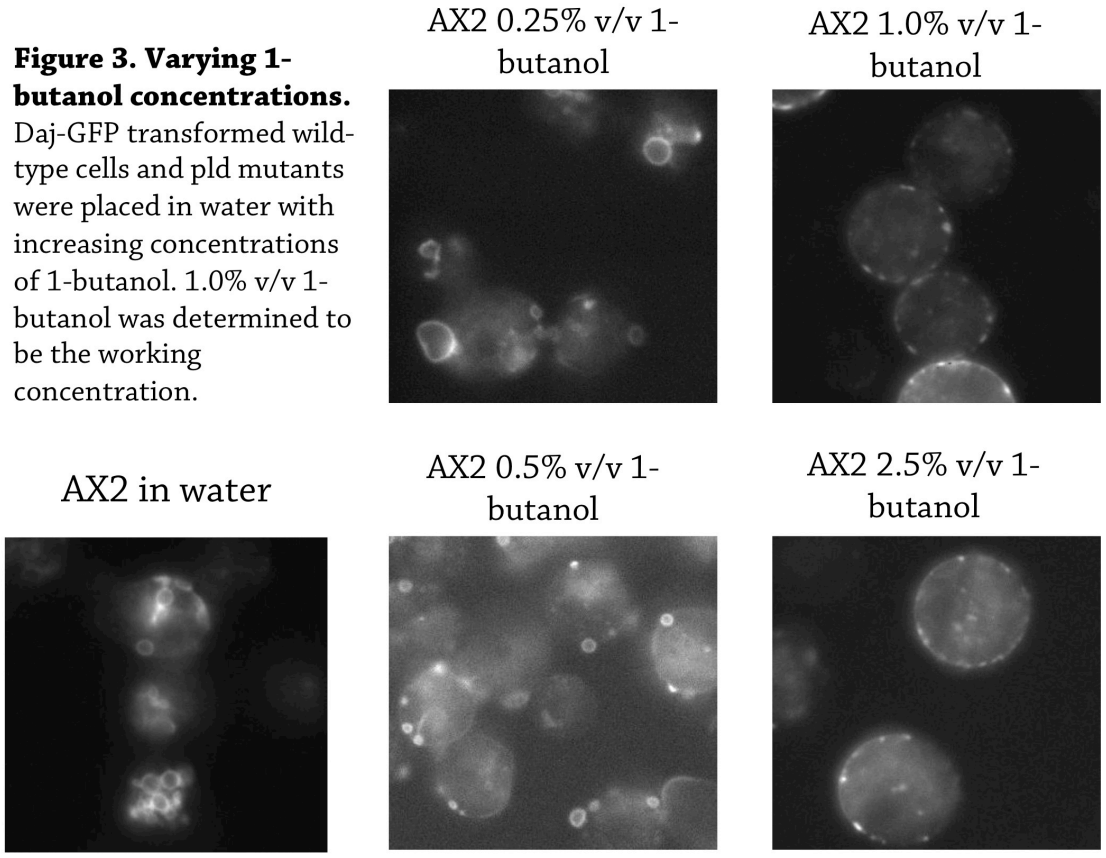

Figure 3. Varying 1-butanol concentrations. Daj-GFP transformed wild-type cells and pld mutants were placed in water with increasing concentrations of 1-butanol. 1.0% v/v 1-butanol was determined to be the working concentration.

When 1-butanol is applied, the CV membrane components aggregate near but not at the membrane (See Fig. 4). After 10 minutes in the presence of 1-butanol, the cells are able to reform some of the CV network. The smaller vacuoles leave the plasma membrane, larger vacuoles appear, and even bladders begin to form. Therefore, cells are able to find ways to recover from PLD stress, exemplifying the cell's overlapping and complex signaling pathways. When 1-butanol is washed off and replaced with water, cells rapidly reform their CVs. Cells exhibit swollen CVs and GFP label is localized solely

to the CV membranes. Thus, the effects of 1-butanol are readily reversible upon removal of the inhibitor.

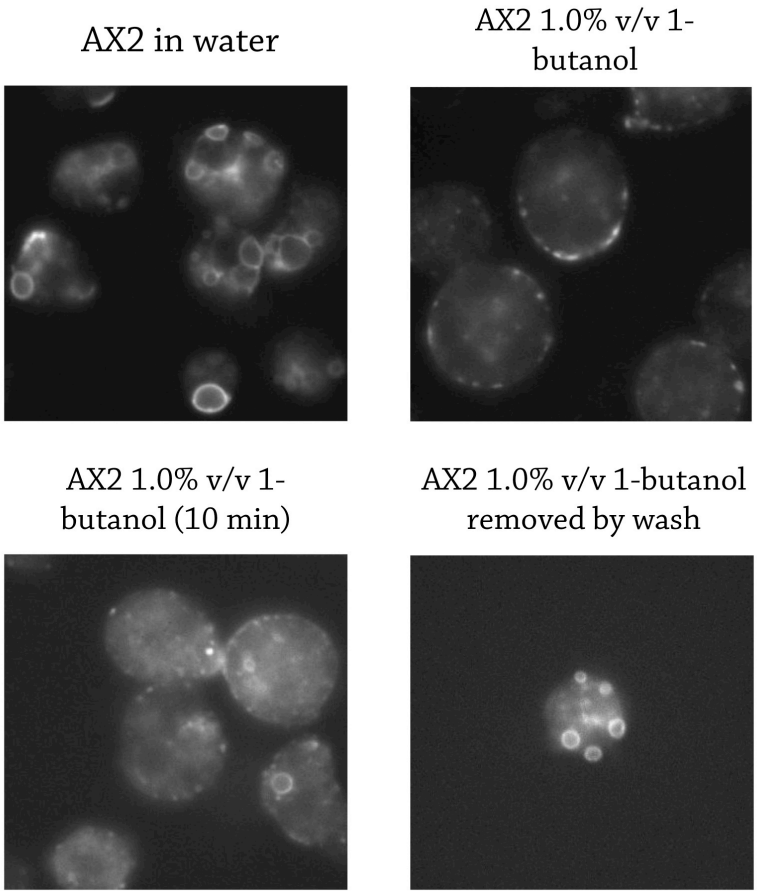

Figure 4. Reversible effect of 1-butanol. Wild type cells were placed in 1-butanol in achieve disruption of the CV network. Even in the presence of 1-butanol, cells were able to reform some vacuoles after 10 min. Immediately after removal of the alcohol, vacuoles reappear.

In a transphosphatidylation reaction specific to PLD, 1-butanol replaces water to nucleophilically attack phosphatidylcholine to produce phosphatidylbutanol instead of phosphatidic acid (24). With the exogenous introduction of 1-butanol, the cells' CV networks are destroyed and seemingly reform as small vacuoles that remain physically and chemically distinct from the plasma membrane. Therefore a synthetic water-soluble and membrane-permeable analog of phosphatidic acid, dioctanoyl-PtdOH, was

applied in an effort to recover signaling in the PLD pathway (See Fig. 5). Previous studies had shown that 100 uM dioctanoyl-PtdOH was adequate to recover cell mobility inhibited by 1-butanol (27). When presented with both 1-butanol and dioctanoyl-PtdOH simultaneously, cells exhibit an abnormal phenotype similar to that of 1-butanol treatment only. However, a significant number of clearly labeled vacuoles still exist. When dioctanoyl-PtdOH is applied immediately after the effects of 1-butanol have already occurred, cells begin to reform some CVs near the membrane as smaller vacuoles seemingly merge to become larger compartments. However, it is clear that a direct application of the downstream PLD products is not sufficient for the cell to regain full recovery of its CV network. 1-butanol may have other nonspecific effects on the cell, and other pathways may also have been disrupted.

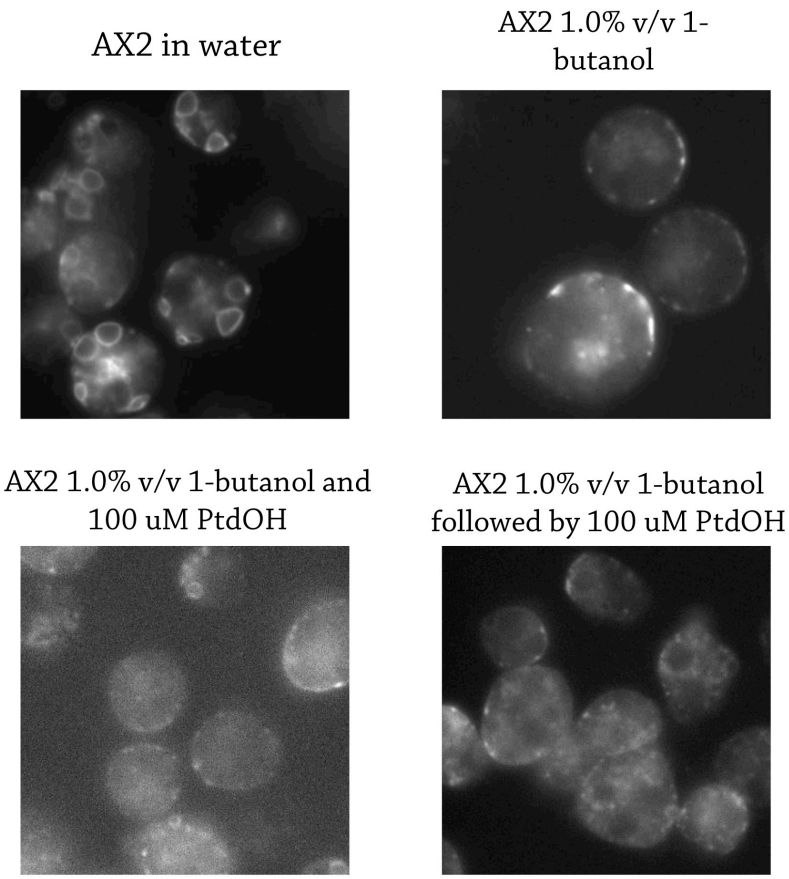

Figure 5. Addition of soluble phosphatidic acid analog to 1-butanol-treated cells. The contractile vacuole was attempted to be rescued using a PLD downstream signaling molecule. Although recovery was modest, the 1-butanol effect was not as dramatic in the presence of PtdOH and some vacuoles begin to reform.

In *Dictyostelium*, there are three genes encoding for phospholipase D named *PLDA*, *PLD B*, and *PLDC* (11). Because several PLD mutants had been acquired, the effects of 1-butanol due to a specific PLD may be explored. In the presence of 1-butanol, the pldA, pldB, and pldC mutants do not exhibit a similar phenotype as treated wild type cells (See Fig. 6). Rather than small vacuoles evenly distributed beneath the plasma membrane, the CVs seemed to have flattened against the plasma membrane. Due to the limits of fluorescence microscopy, it is not possible to observe the plasma membrane interface more closely. However, the thickness of GFP label near the membrane

suggests that the CVs have become flattened sacs. In the plda/pldC double mutant, a similar phenotype to the single mutants is observed. The similarities of 1-butanol effects on the mutants suggest redundancy of signaling activity between the three phospholipases.

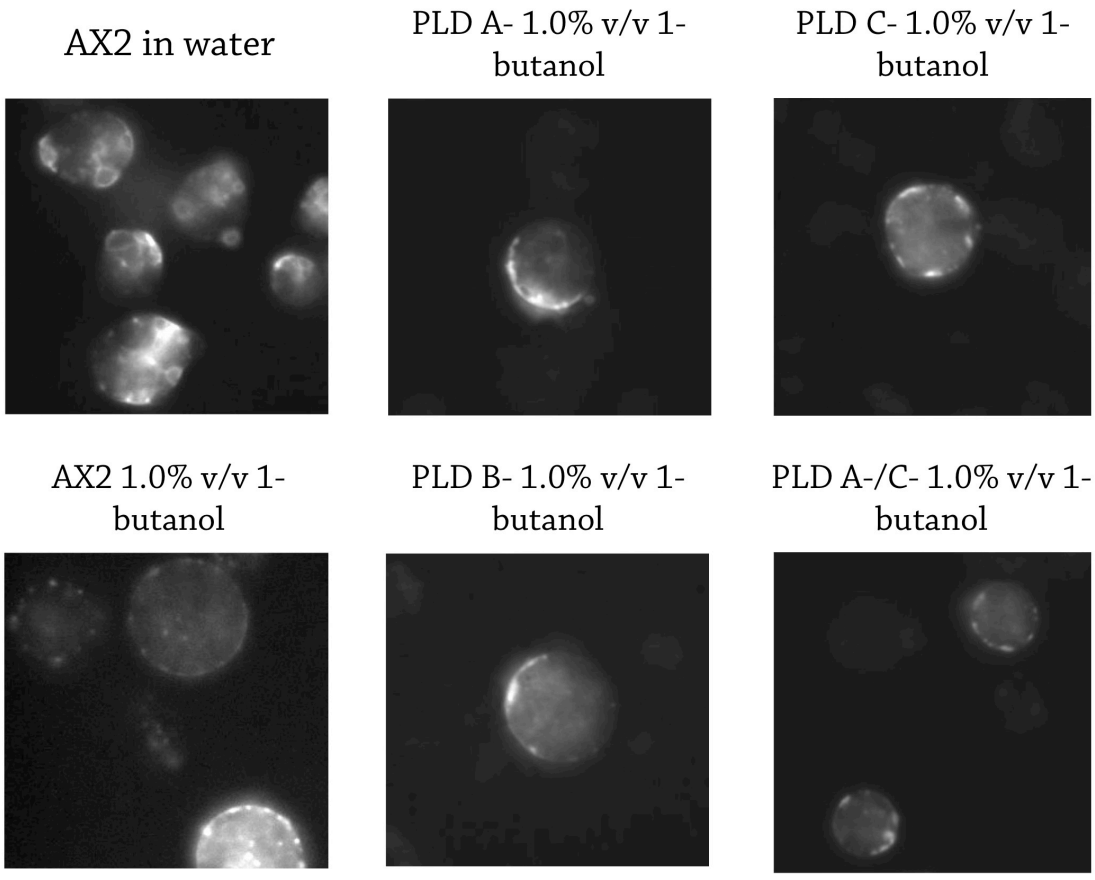

Figure 6. Effect of 1-butanol on pld mutants. When treated with 1-butanol, pld mutants exhibit a unique phenotype different from treated wild type cells. The CV bladders appear to have flattened against the plasma membrane rather than dispersed into smaller vacuoles.

Previous studies have reported that inhibition of actin polymerization caused collapse of the CV onto the plasma membrane (7). Actin inhibitors, cytochalasin A and latrunculin A were exogenously applied to replicate such findings. However, neither inhibitor showed any difference compared to untreated wild type cells in water even at

relatively high concentrations (See Fig. 7). Therefore, actin inhibition does not appear to have a direct role in CV maintenance and structure. However, when cells are presented with both latrunculin and 1-butanol, a unique phenotype is observed. Instead of the dispersed 1-butanol phenotype, the majority of CV membrane components appear to have aggregated to one side of the cell. When 1-butanol is applied after latrunculin, a similar phenotype is observed however it is not as drastic. Some CVs still appear to be intact. Therefore, actin may play a role in the recovery and reassembly of the CV network after it is disrupted due to stress.

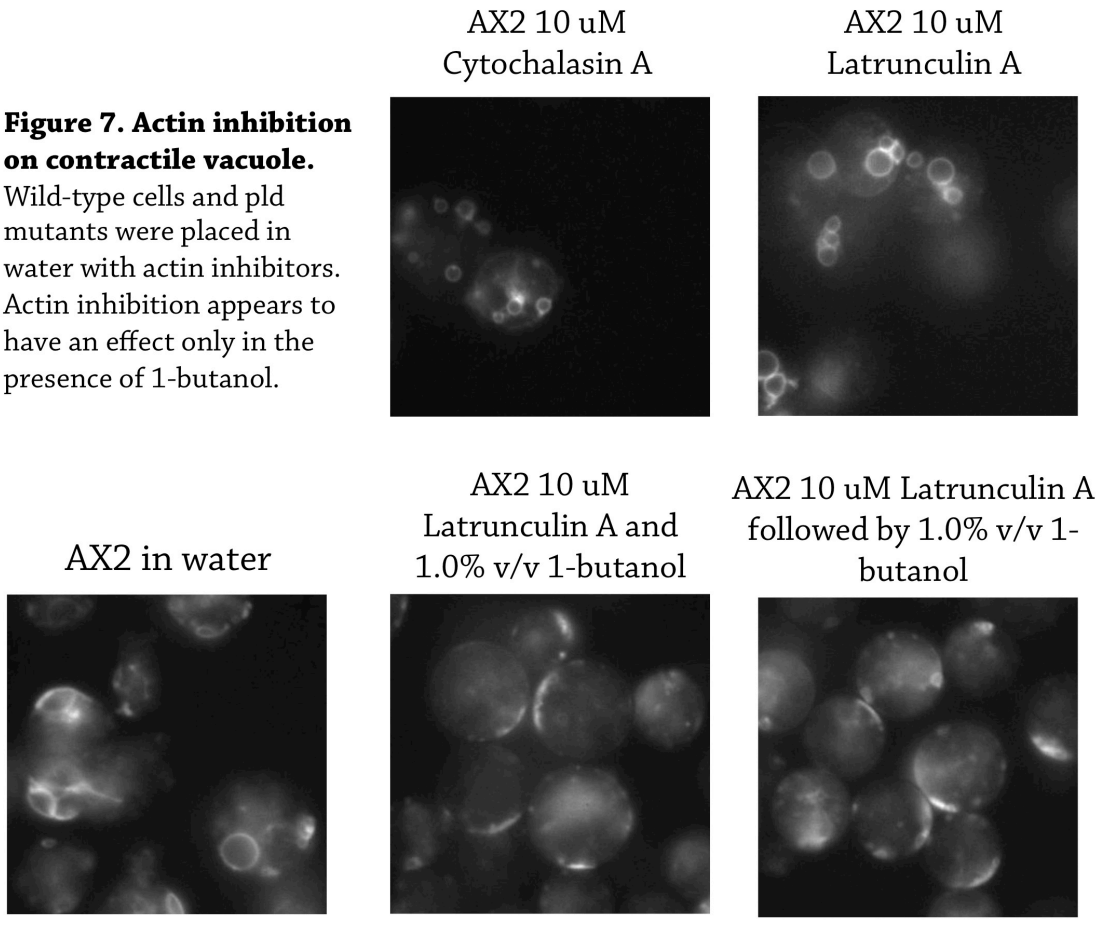

Figure 7. Actin inhibition on contractile vacuole. Wild-type cells and pld mutants were placed in water with actin inhibitors. Actin inhibition appears to have an effect only in the presence of 1-butanol.

Wild type *Dictyostelium* and pld mutants were transformed with CLC-GFP to visualize clathrin (light chain) in the cell (See Fig. 8). In wild type cells, punctae of CLC-

GFP are clearly visible dispersed throughout the cell. At the membrane, clathrin-coated pits form at pre-mature vesicles. Within the cell, clathrin-coated vesicles are responsible for membrane trafficking at the Golgi apparatus. In the pld mutants, the clathrin appears to be more widely distributed throughout the cell. Furthermore, the punctae appear to be smaller, and a significant amount of clathrin appears to aggregate at the Golgi. Otherwise, the observed phenotypes are unremarkable.

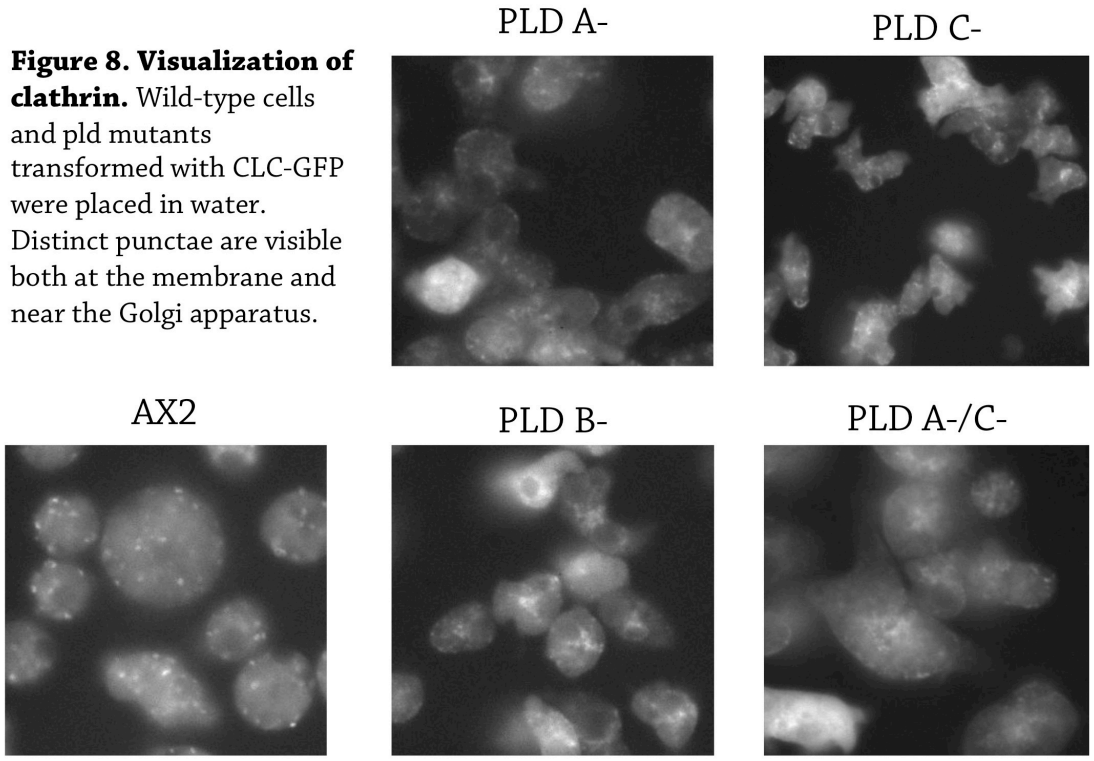

Figure 8. Visualization of clathrin. Wild-type cells and pld mutants transformed with CLC-GFP were placed in water. Distinct punctae are visible both at the membrane and near the Golgi apparatus.

1-butanol inhibits the formation of phosphatidic acid, which stimulates PI(4)P 5-kinase to generate PIP_2 which binds to clathrin adaptor protein, AP-2. In wild type cells, the presence of 1-butanol causes clathrin to become soluble and no distinct punctae are visible as expected (See Fig. 9). Without the binding of adaptor proteins, the clathrin coats cannot assemble. Likewise, the phospholipase mutants show similar phenotypes where the majority of the clathrin has become solubilized within the

cytoplasm. Interestingly, the pldA and pldA/pldC double mutants still exhibit some punctae. Therefore, the majority of 1-butanol's effects may be due to pldA.

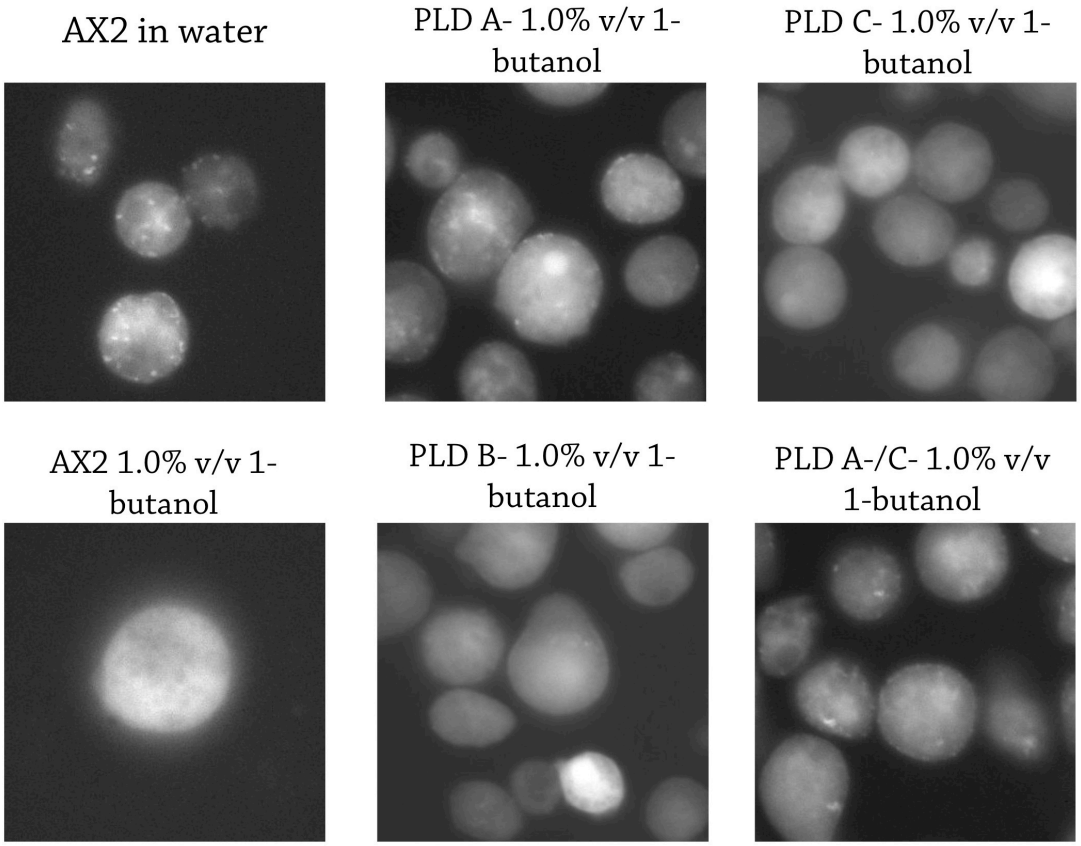

Figure 9. Effect of 1-butanol on clathrin localization. When wild type cells and pld mutants transformed with CLC-GFP are treated with 1-butanol, majority of the punctae disappear and clathrin becomes dispersed throughout the cell. However, pldA and pldA/pldC double mutants retain some distinct punctae.

While actin inhibition did not have a direct or obvious effect on the *Dictyostelium* CV network, latrunculin blocks the budding of clathrin-coated vesicles (See Fig. 10). When latrunculin is applied to wild type cells, many clathrin punctae are observed frozen at the membrane. There is also a significant amount of clathrin aggregated at the Golgi. However, actin inhibition in the pld mutants results in a more dramatic effect. Rather than punctae, a ring of fluorescence is observed at the plasma membrane. Furthermore, clathrin not at the membrane appears to have solubilized and

become disorganized. In the pldA/pldC double mutant, the latrunculin effect is reduced and resembles more of the wild type phenotype. Distinct punctae are observed both at the membrane and within the cell.

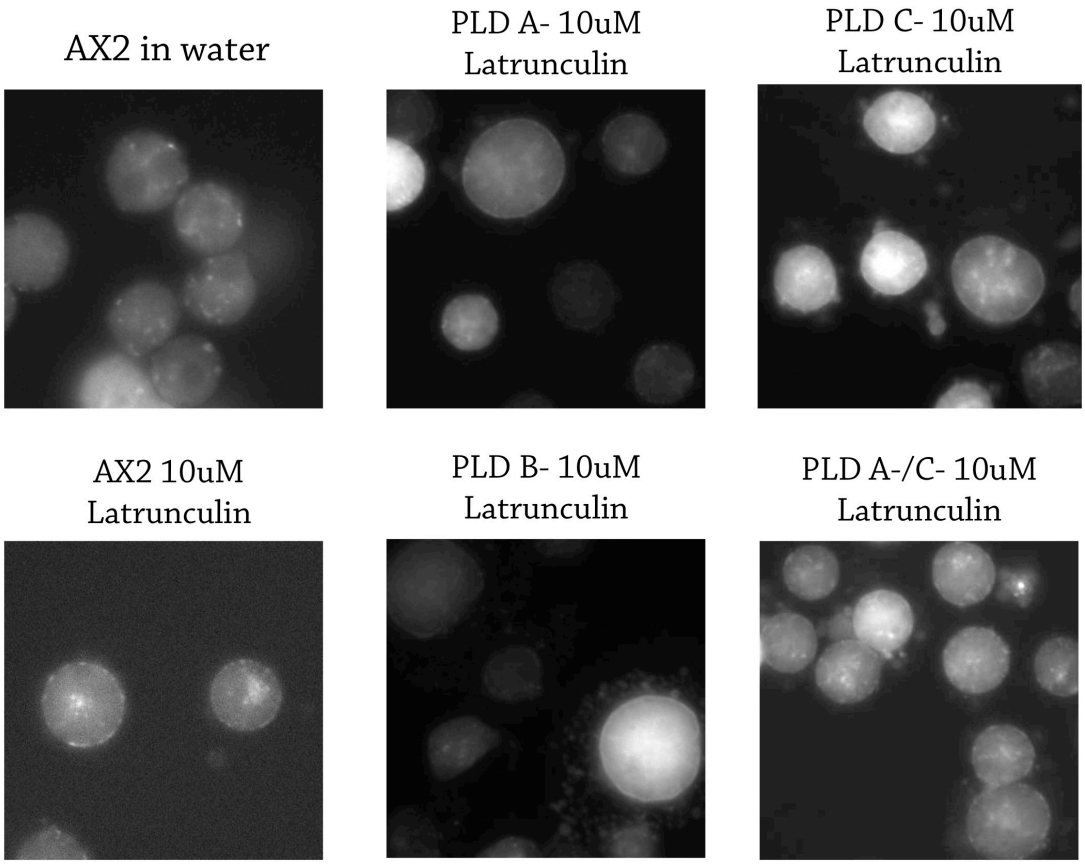

Figure 10. Effect of actin inhibition on clathrin localization. When wild type cells and pld mutants transformed with CLC-GFP are treated with latrunculin, clathrin becomes frozen at the membrane. In treated wild type cells, distinct punctae are still visible. However in pld mutants, the punctae are less visible and clathrin appears to be more evenly distributed at the membrane.

DISCUSSION

The endocytic pathway is the primary method that the cell is able to transport matter as well as respond to the environment. While older studies had to rely on gross morphological studies, the advent of genetic techniques such as knockouts allowed for the elucidation of specific pathways. There must remain an appreciation for the complexity of the endocytic pathway, and the degree of redundancy and overlap there is between pathways. Different molecules and cellular components often have multiple roles in the cell. All these processes must be carefully regulated and controlled, depending on the status and environment that the organism is in.

The contractile vacuole is a tightly regulated compartment that is essential for osmoregulation and cell survival in hypotonic environments. However, many questions remain about the biogenesis and maintenance of this organelle. The purpose of this study was to begin to unravel some of the mechanisms through which the CV maintains its biochemical and structural integrity by applying various drugs that inhibit different cellular process to wild-type and knockouts. Because phospholipase D has been implicated in actin-based mobility and clathrin-mediated endocytosis through generation of PIP_2, the enzyme was a strong candidate for understanding the CV.

Phospholipase D mutants did not appear to have any significant morphological defects and appeared to have functioning CVs (see Table 1). In the specific presence of 1-butanol, the CV network was immediately fractured into small vacuoles plastered near the plasma membrane. In treated PLD mutants, the CV appeared to have flattened against the membrane. The dose-dependent effects were reversible within 10 minutes or immediately after the removal of the inhibitor, suggesting the robustness of the

organism and possibility of alternative pathways. The addition of dioctanoyl-PtdOH, product of PLD activity, only partially rescued the untreated phenotype. Two potential reasons for this result include other nonspecific effects of 1-butanol or localization of PLD signaling. Widespread application of PLD product on the cell may not be appropriate. This may exemplify the old adage: "At the right place at the right time." Interestingly actin inhibition did not have any visible or immediate effects on CV function as previously reported. However in the presence of both inhibitors, all CV components become aggregated on one side of the cell. This suggests that actin and PLD both play a role in CV maintenance, however their roles are likely separate and occur at different stages.

Table 1. Summary of stress on the contractile vacuole

Contractile Vacuole by Dajumin-GFP					
	AX2 (Wild Type)	PLD A-	PLD B-	PLD C-	PLD A-/C-
Water	Clearly visible, large, swollen CVs	Clearly visible CVs			Large, swollen CVs
10 uM Latrunculin	No effect				
1.0% v/v 1-butanol	CV components (small vesicles) near plasma membrane				

	AX2 (Wild Type)
10 uM Latrunculin and 1.0% v/v 1-butanol simultaneously	Aggregation of CV components near the plasma membrane
10 uM Latrunculin followed by 1.0% v/v 1-butanol	Aggregation of CV components, appearance of some larger vacuoles

Similarly, clathrin localization and levels appeared normal in both wild-type and PLD mutants (see Table 2). 1-butanol inhibits the formation of phosphatidic acid and consequently the generation of PIP_2. Because of the lack of adaptor proteins, 1-butanol prevents the recruitment of clathrin to the membrane, blocking CME. Although it does

not significantly affect CV structure, actin inhibition blocked CME by preventing the budding of clathrin-coated vesicles. Therefore CV maintenance is likely to be at least partially regulated by PLD activity, while actin may be required for more downstream events that are not specific to the CV. Examining the effects of downstream PLD products such as PIP_2 on mutant cells could further elucidate the CV biogenesis pathway.

Table 2. Summary of stress on the clathrin light chain localization

Clathrin Light Chain by CLC-GFP					
	AX2 (Wild Type)	PLD A-	PLD B-	PLD C-	PLD A-/C-
Water	Distinct punctae	Small punctae concentrated near Golgi			
10 uM Latrunculin	Punctae frozen near plasma membrane	Ring of fluorescence around plasma membrane, no punctae visible			
1.0% v/v 1-butanol	No punctae visible	Few punctae visible	No punctae visible		Few punctae visible

Previous studies in the lab have shown that using clathrin null cells will not result in formation in contractile vacuole at all, further implicating the role of clathrin in CV biogenesis and maintenance. Future studies include using the adaptor proteins which are only important in parts of the endocytic pathway. For example, AP-2 is associated with initial formation of vesicles from the plasma membrane. AP-3 which has not been clearly localized, but shown to be required for recycling endosomes can be rendered nonfunctional in μ3 null cells which have already been successfully generated in the laboratory. Total internal reflection fluorescence microscopy (TIRFM) will also be a powerful tool to observe events at the plasma membrane. Current light and epifluorescence microscopy methods do not have high enough resolution to distinguish what is at or below the plasma membrane. TIRFM will also provide insight into how the inhibitors are morphologically affecting the CV.

If contractile vacuole proteins that are displaced on the plasma membrane are indeed internalized by a clathrin AP-2-mediated process and sorted out of the lysosomal pathway, then AP2 mutants when treated with 1-butanol will not be able to recover Dajumin-GFP to reform the CV. This is because AP-2 links clathrin to receptors in coated vesicles from the membrane. However it is possible that the μ3 null cells show that the Dajumin-GFP is not recovered. This would indicate that AP-3 plays a role in sorting of contractile vacuole proteins, and the incorrectly placed proteins are internalized through the recycling endosome pathway. If both of these mutants show that the contractile vacuole is able to reform even after treatment with 1-butanol, the contractile vacuole proteins are perhaps regrouped through a different endocytic pathway.

It is clear that additional studies are required to further understand the mechanism by which the CV is able to maintain its structure and function. The results of this study provide biological evidence as well as some biochemical tools that easily and rapidly affect the CV in a reversible manner. The main advantage of *Dictyostelium* lies in the ease at which genetic and morphological studies can be carried out. The endocytic pathways in mammalian cells and yeast are significantly trickier to dissect due to the intricacy and difficulty in visualization. Furthermore, many aspects of *Dictyostelium* resemble that of human macrophages including morphology and biochemistry (12). Understanding the pathway by which CV function is regulated will yield insights into the function of proteins that show a similar phenotype in their null mutants. It could also be a starting point in the treatment of human diseases caused by protists such as trypanosome, which have a similar CV network (30). Therefore,

additional studies in this model organism will definitely provide crucial insights and a framework for studies on the endocytic pathway in more complex systems.

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