SEARCH FOR RECEPTOR MEDIATED PROCESSES IN *AMOEBA PROTEUS*

by

Sanae Hashimoto

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, 2006

This paper was prepared

under the direction of

Professor Susan Barry

for eight credits.

ACKNOWLEDGEMENTS

I would not have been able to complete my research without support from the following people.

Professor Barry. Thank you for encouraging for my scientific curiosity. You have taught me the joy of scientific research.

Professor Curtis Smith. Thank you for creating a fantastic cell stimulator. Your invention made my research more exciting.

Marian Rice. Thank you for the information on *Tetrahymena pyriformis* deciliation and for technical support for using the microscope and software.

Jennifer Burwell. Thank you for helping me to improve my writing through day and night.

Professor Bacon, Professor Susan Smith, and Professor Will Millard. Thank you for participating on my honors thesis committee.

TABLE OF CONTENS

List of Figures……………..…………………..…………………..…………..v

List of Table…………………………………………………………………..vi

Abstract………………………………………………………………………vii

Introduction……………………………………………………………………1

Materials and Methods………………………………………………………19

Results…………………………………………………………………………..26

Discussion……………………………………………………………………34

Literature Cited………………………………………………………….....52

LIST OF FIGURES

Figure 1. Model of FcγRIIA receptor mediated signaling pathways in PMN……………………………………..……..…………...7

Figure 2. Model mechanisms of pinocytosis in amoebae…………….……16

Figure 3. Cell stimulator…………………………………………………23

Figure 4. Shape of representative *A. proteus* in *T. pyriformis* medium and Pringsheim's solution…………….…………………..………...27

Figure 5. Circularity of *A. proteus* after a 5 minutes exposure to either 0.3 ml control solution or *T. pyriformis* medium……………………28

Figure 6. Number of *A. proteus* that phagocytosed either ciliated (control) or deciliated *T. pyriformis* within a 5 minute period…..………29

Figure 7. Number of *A. proteus* that phagocytosed either non heated (control) or heated *T. pyriformis* within a 5 minute period…..30

Figure 8. Percentages of *A. proteus* that phagocytosed tetrahymena in a comparison of the data between exposure to heated *T. pyriformis* from experiment II and deciliated tetrahymena from experiment III……………………..……..………………...31

Figure 9. Number of *A. proteus* that phagocytosed the micropipette within a 5 minute period under each of four conditions…………………………………………………33

LIST OF TABLE

Table 1. Summery of the Experiments……………………………………….25

ABSTRACT

A single-cell organism, *A. proteus* ingests food such as *Tetrahymena pyriformis* by phagocytosis. Specific factors that trigger the phagocytosis are not known. A previous study suggests that phagocytosis of *A. proteus* might be triggered both by chemical and mechanical stimuli presented by the target cells or food organisms.

I designed experiments to determine whether phagocytosis of *A. proteus* was induced by chemoreceptors, mechanoreceptors, or both. The behavior of *A. proteus* was observed during applications of each condition for five minutes: *T. pyriformis* medium, heated (killed) *T. pyriformis*, deciliated *T. pyriformis*, a mixture of deciliated and ciliated *T. pyriformis*, or micropipettes filled with *T. pyriformis* medium, filled with control solution and vibrated at 4 Hz, or filled with *T. pyriformis* medium and vibrated at 4Hz. The experimental results indicate that stimulation of chemoreceptors may initiate phagocytosis. However, the experiments for mechanoreceptors show contradictory results. Finally, the experiments to test for a synergistic effect between chemo- and mechanoreceptors imply that stimulation of the mechanoreceptors may not enhance the chemoreceptor mediated phagocytosis by of *A. proteus*.

INTRODUCTION

Amoeba proteus is a single-cell eukaryote that generates its movement by cytoplasmic streaming (Janson and Taylor 1993). *A. proteus* recognizes a prey organism such as *Tetrahymena pyriformis*, moves toward it, surrounds it with its pseudopodia in order to engulf the prey, and captures it into a food vacuole which is formed by fusion of the outreaching pseudopodia (Prusch and Minck 1985). This behavior is called phagocytosis (Prusch et al. 1989). A previous study suggests that phagocytosis by *A. proteus* may be triggered both by chemical and mechanical stimuli presented by the target cells or food organisms (Prusch and Minck 1985). Extracellular signals such as chemical molecules or physical stimuli bind to, or change, the conformation of protein receptors on the surface of a target cell membrane (Krauss 2001). The receptors transduce the signals and initiate a sequence of intracellular signaling processes (Krauss 2001). This activates a specific biochemical pathway in the target cell, which changes the cell's behavior (Krauss 2001). Certain stimuli such as pH (Korohoda et al. 1997), light (Grebecki et al. 1981; Grebecki and Klopocka 1981; Prusch 1986), and chemicals (Prusch and Minck 1985; Prusch and Joseph 1987; Prusch et al. 1989) change *A. proteus* cytosolic behaviors. The specific mechanisms, however, which initiate the cytoplasmic movement toward the prey and phagocytosis, are not well understood (Prusch et al. 1989). Single-celled eukaryotes, such as *A. proteus* may possess phylogenically old sensory motor processes (Gebbie et al. 2004),

and only eukaryotes use Ca^{2+} as second messengers to control cellular motilities (Hille 2001). It is important to research receptor-mediated processes in *A. proteus* in order to understand the universal cell signaling transduction mechanisms. My research objective was to understand what kind of sensory receptors are involved in phagocytosis by *A. proteus*. Moreover, I attempted to understand whether *A. proteus* phagocytosis occurred by activation of chemoreceptors, mechanoreceptors, or both types of receptors. Before introducing my hypothesis, I will provide background information on 1) general movements of *A. proteus*, 2) chemo- and mechanoreceptors mediated processes, and 3) past studies of *A. proteus* sensory mechanisms.

1) General Movements of A. proteus

Cytoplasmic Streaming in A. proteus

Amoeba proteus moves by altering the viscosity of the cortical actin network in its cytoplasm, which consists of ectoplasm, a gel-like outer layer, and endoplasm, a fluid-like inner layer (Janson and Taylor 1993). When an amoeba extends a pseudopodium forward, the ectoplasm and the endoplasm continuously transform into each other in the cell cytoplasm (Soden 2005). In the tail, the ectoplasm gel increases its fluidity, moves into the cell's center, and becomes new endoplasm (Janson and Taylor 1993). On the other hand, in the center of the cell, the endoplasm migrates into the tip, becomes new ectoplasm, and forms an extended pseudopodium (Janson and Taylor 1993).

This cytoplasmic fluidity may be regulated by the concentration of Ca^{2+} in *A. proteus* (Gollnick et al. 1991; Barry et al. 2005; Soden 2005). In

general, high concentrations of Ca^{2+} in the tail regions have been observed among moving *A. proteus* (Kuroda et al. 1988). Past studies showed that low external Ca^{2+} concentration decreased *A. proteus*' movements, whereas high external Ca^{2+} concentration enhanced its movements (Gollnick et al. 1991). However, the movement of *A. proteus* via altering cytoplasmic fluidities may be dependent not only on the extracellular Ca^{2+} influx, but also on an efflux into the cytoplasm of internal Ca^{2+} which is stored in the endoplasmic reticulum (ER) of the cell (Barry et al. 2005; Soden 2005). Activation of the inositol 1,4,5- trisphosphate (IP_3) receptors triggers the internal Ca^{2+} releases from ER and facilitates *A. proteus* movement (Barry et al. 2005; Soden 2005). Inhibition of IP_3 receptors by
2- aminoethoxydiphenyl borate slows down *A. proteus*' movements (Barry et al. 2005; Soden 2005).

The mechanism of cytosolic movement of *Amoeba proteus* has been widely studied, however, the specific factor (s), which triggers the movement is not known (Prusch et al. 1989). In contrast, the response of *Dictyostelium discoideum* amoebae to the specific chemoattractant, cyclic AMP, has been widely studied (Tani and Naitoh 1999).

Endocytosis in A. proteus: Phagocytosis and Pinocytosis

Diverse cells exhibit endocytosis to uptake extracellular molecules in their surrounding environments (Prusch et al. 1989; Smythe and Warren 1991). Endocytosis consists of phagocytosis and pinocytosis (Vogel et al. 1980; Prusch et al. 1989). Phagocytosis is the process that cells recognize and

engulf large particles (Ito et al. 1981; Prusch et al. 1989; Cohen et al. 1994; Caron and Hall 1998). Pinocytosis is described as "cell drinking" (Brandt and Pappas 1960), which cells uptake solute material (Prusch 1986; Prusch et al. 1989).

Pinocytosis and phagocytosis in *A. proteus* share similar mechanisms. Both are initiated when surface receptors are activated, in this process Ca^{2+} plays an important role (Prusch and Minck 1985). However, signaling molecules that bind to the receptors are specific to either process (Prusch and Minck 1985). For example, in *A. proteus*, glutathione induces phagocytosis but does not induce pinocytosis, and gelatin initiates pinocytosis but does not initiate phagocytosis (Prusch and Minck 1985). The study suggests receptor-mediated processes, which involved in phagocytosis and pinocytosis of *A. proteus*, are initiated by different chemicals (Prusch and Minck 1985; Prusch and Joseph 1987).

The purpose of this research was to understand the receptor-mediated processes that induce phagocytosis of *T. pyriformis* in *A. proteus*. Phagocytosis events in *A. proteus* involve recognition of stimuli from *T. pyriformis*, alteration of actin fluidities in cytoplasm, pseudopodia extensions, adhesion to *T. pyriformis*, engulfment of the prey by pseudopodia, and fusion of the pseudopodia into a food vacuole (Prusch et al. 1989; Shiratsuchi and Basson 2004). Pseudopodia extension is associated with cell polarity, and it creates directionality in *A. proteus* cell shape. Thus, cell polarity is essential to phagocytose a target. Activation of receptors on the extracellular surface of *A. proteus* initiates these processes of phagocytosis (Prusch et al. 1989).

All steps of phagocytosis result from rearrangements of the actin cytoskeleton (Samaj et al. 2004). To understand the mechanisms of *A. proteus* phagocytosis, I will describe model receptor mediated mechanisms such as the phagocytosis mechanisms of human neutrophils and macrophages (Aderem and Underhill 1999; Raeder et al. 1999; Mayer-Scholl et al. 2004; Shiratsuchi and Basson 2004).

2) Chemo- and Mechanoreceptor Mediated Processes

Human Neutrophils: Chemoreceptor-Mediated Processes

Human neutrophils, polymorphonuclear leukocytes (PMN), respond to microbial invasion in human bodies; they migrate to the site of the infection through the blood stream, engulf the pathogens by phagocytosis, and kill the pathogens in phagolysosomes that are rich in antibacterial peptides, enzymes, and reactive oxygen species (Mayer-Scholl et al. 2004). PMNs conduct the Fc-receptor-mediated phagocytosis, and this is the most well studied phagocytosis mechanism (Beningo and Wang 2002).

On the plasma membrane of PMN, Fc receptors are exposed to the extracellular environment (Gresham et al. 1990). PMNs have different classes of Fc receptors that are involved in their phagocytosis events: FcγRI, FcγRIIA, and FcγRIIIB (Raeder et al. 1999). For example, when FcγRIIA receptors on PMN bind to the Fc domains of target substances, such as antibody-coated erythrocytes (EIgG), Src-like tyrosine kinases (Syk) are phosphorylated and activate intracellular signaling cascades (Raeder et al. 1999). Two known examples of these intracellular signaling pathways,

mitogen-activated protein kinase (MAP kinase) and phosphatidylinositide 3-kinase (PI 3-kinase) pathways are activated by the ligand bindings to FcγRIIA receptors in PMN (Raeder et al. 1999; figure 1). EIgG binds to the receptor and increases phosphorylation of tyrosines and activation of enzymes in Syk (Raeder et al. 1999). This initiates generation of diacylglyceride from phospholipase D through removal of its phosphate by phosphatidic phosphohdrolase (Raeder et al. 1999). Then, diacylglyceride catalyzes translocation of protein kinase Cδ (PKCδ) and Raf-1 from the cytoplasm to the plasma membrane, which activates PI 3 kinase (Raeder et al. 1999).

When Syk activation was inhibited by piceatannol, translocation of protein kinase Cδ (PKCδ) and Raf-1 from cytoplasm to plasma membrane did not occur, and phagocytosis was inhibited (Raeder et al. 1999). Piceatannol also stopped PI 3 kinase activities (Raeder et al. 1999). When the PI 3 kinase inhibitor wortmannin was applied, phagocytosis of PMN did not occur (Raeder et al. 1999). However, PKCδ and Raf-1 translocations were not affected by wormannin; this indicated that the MAP kinase pathway was independent from PI 3 kinase pathway (Raeder et al. 1999). Moreover, to induce phagocytosis, activation of both pathways was required (Raeder et al. 1999).

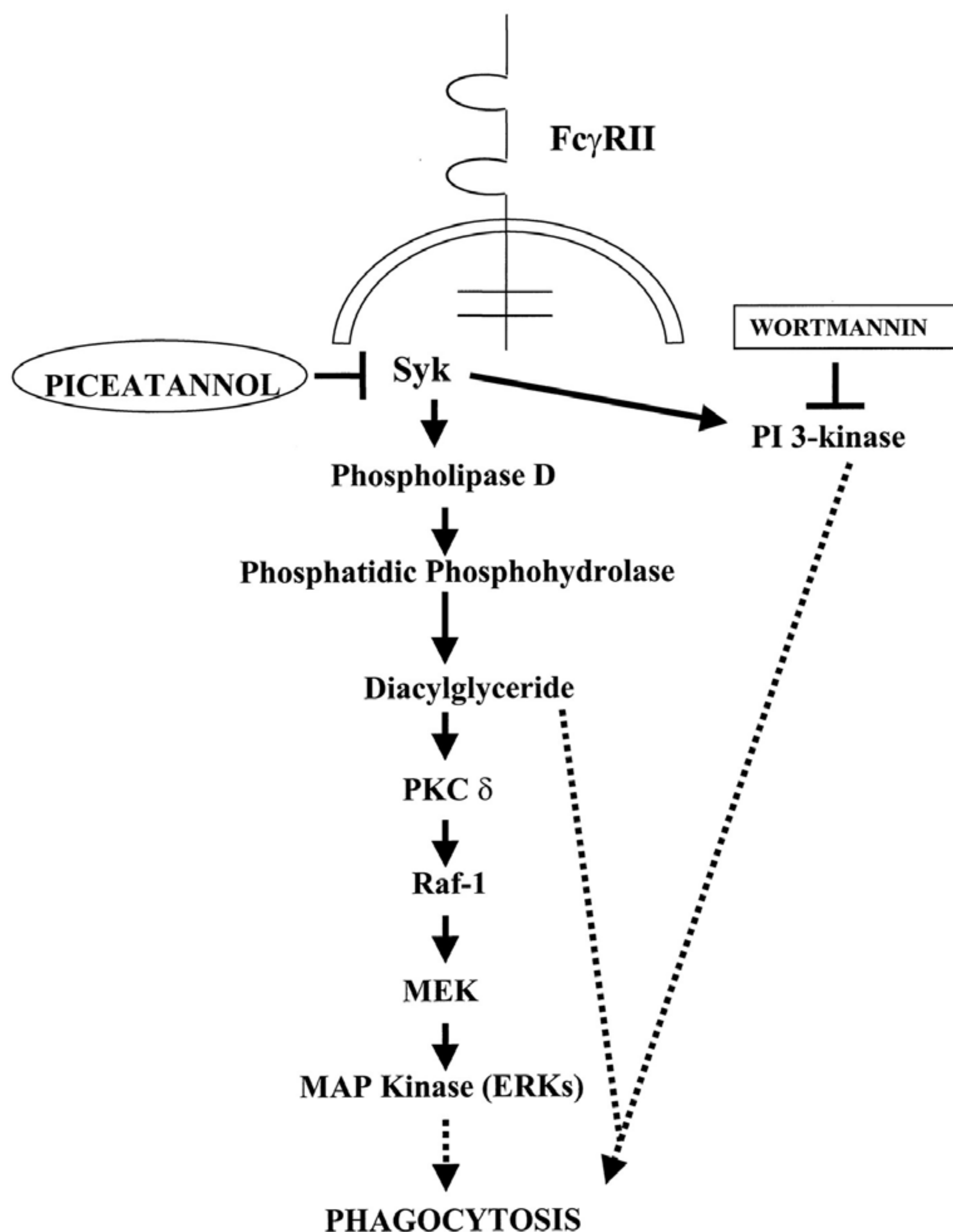


Figure 1. Model of FcγRIIA receptor mediated signaling pathways in PMN created by Reader et al. (1999). Both MAP kinase and PI 3 kinase pathways play roles in inducing phagocytosis. Ligand binding activates the receptor, and this increases enzyme Syk activities. Syk activity initiates the MAP kinase signaling pathway: the signals are passed to MAP kinase via phospholipase D, phosphatidic phosphohdrolase, diacylglyceride, PKC δ, Raf-1, and MEK. Syk activity also initiates PI 3 kinase pathway. Piceatannol inhibits activation of Syk and phagocytosis. Wortmannin inhibits activation of PI 3-kinase and phagocytosis.

Downstream of MAP kinase Pathway

Recent studies demonstrated a strong link between the MAP kinase signaling pathways and the regulation of the actin cytoskeleton (Samaj et al. 2004). Cyclic AMP (cAMP) stimulates G protein binding receptors such as CAR1 and 3, and application of cAMP to *Dictyostelium* amoeba initiates actin nucleation and polymerization in the cytoskeleton (Wang et al. 1998). The mutant *Dictyostelium* has a mutation in the DdERK2 gene, and therefore lacks MAP kinase, showed fewer and slower chemotactic events in association with the chemoattractant, cAMP (Wang et al. 1998).

Downstream of PI 3 kinase Pathway

The activation of target proteins via specific signaling pathways regulates particular cell behaviors (Krauss 2001). According to Stephens et al. (2002), immediately after stimulation of the PMN receptors, a phagocytic cup was formed by actin polymerizations, and these processes were dependent on Syk phosphorylations, but not on PI 3 kinase activities. On the other hand, pseudopodia extensions are mainly controlled by "rearrangements" rather than polymerization of the actin cytoskeleton (Stephens et al. 2002). This rearrangement of actin is regulated exclusively by PI 3 kinase activities that lead to phosphatidylinositol 3,4,5-trisphosphate [PI (3,4,5) P_3] formation (Stephens et al. 2002). Then, [PI (3,4,5) P_3] may associate with BTK tyrosine kinase which then activates phospholipase C-γ (PLC-γ) which then cleaves PI (4,5) P_2 into both inositol 1,4,5,triphosphate (IP_3) and diacylglycerol (Vossebeld et al. 1997; Stephens et al. 2002). IP_3 is known to

bind to Ca^{2+} channels in the ER. This leads to a release of Ca^{2+} into the cytoplasm of the PMN (Berridge and Irvine 1989). The study of Vossebeld et al. (1997) showed that binding of EIgG to FcγRIIA receptors in PMNs increased both intracellular IP_3 and Ca^{2+} concentration. Intracellular Ca^{2+} concentration may also be largely involved in phagocytosis of *A. proteus* (Prusch and Joseph 1987; Prusch et al. 1989).

Mechanical Stimuli and Cells

Whether the cells exist as unicellular organisms or make up multicellular organisms, all cells respond to extracellular mechanical stimuli (Apodaca 2002). Some examples of mechanical stimuli are strain stresses, fluid pressures, and vibrations (Apodaca 2002). Cell secretions and morphological changes are well-known examples of cell responses to these mechanical stimuli (Malek and Izumo 1996; Gudi et al. 1998). Recently, phagocytosis has been recognized as one cell function that reacts to mechanical stimuli (Shiratsuchi and Basson 2005).

In cardiac fibroblasts, strain stresses have an impact on the secretion of cardiac extracellular matrix via activation of GTP-binding proteins (Gudi et al. 1998). Gudi et al. (1998) stretched cardiac fibroblasts at various rates and with various forces; they found that a specific rate and strength of stretch induced the activation of particular types of G proteins, $G_{\alpha q}$ and $G_{\alpha il}$. In addition, activation of the IP_3 pathway causes an increase in intracellular Ca^{2+} concentration (Gudi et al. 1998).

Mechanosensitive channel receptors are well studied in vertebrate

hair cells in the auditory system (Garcia-Anoveros and Corey 1997). Hair cells have cilia on the apical side of the cell, and each cilium is connected by tip links (Garcia-Anoveros and Corey 1997). The tip link connects an ion channel in one cilium to a neighboring cilium (Garcia-Anoveros and Corey 1997). When the cilia are physically moved by sound waves, the tip link pulls the gate of the ion channel, and opens the channel (Garcia-Anoveros and Corey 1997). The channel opening creates an influx of cations such as Ca^{2+} and K^{+} which leads to depolarization of the cell and neurotransmitter release (Garcia-Anoveros and Corey 1997).

Bone cells (MC3T3-El) respond to vibrations and change bone densities and structures via cell secretions (Bacabac et al. 2006). Transmission of mechanical stimuli in bone cells may take two pathways (Mikuni-Yakagaki 1999). First, young osteocytes directly induce matrix synthesis by responding to mechanical stimuli (Mikuni-Yakagaki 1999). Second, osteoblasts respond to paracrine signals, such as nitric oxide (NO), that are released by the mechanically stimulated osteocytes (Mikuni-Yakagaki 1999). Bacabac et al. (2006) tested this latter response. They applied a wide range of vibration stresses (5-100 Hz) for 5 minutes to bone cells, and examined their production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) and their expression of cyclooxygenase-2 (Cox-2) mRNA (Bacabac et al. 2006). Their results showed the production of NO, PGE_2 and mRNA expression of Cox-2 increased after the vibration stresses (Bacabac et al. 2006). The enzyme Cox-2 plays an important role in PGE_2 production of the bone cells in response to mechanical stimuli (Bacabac et al. 2006). In vivo, the

release of NO and PGE_2 from the bone cells is essential to form new bone structures (Bacabac et al. 2006).

Malek and Izumo (1996) showed that fluid shear stress (FSS) induced morphological changes in aortic endothelium cells. FSS changed the conformation of focal adhesion associated proteins and mechanosensitive channels in the cell cytoskeleton and initiated signal transduction in the cells (Malek and Izumo 1996). Transmission of the mechanical stimuli activates changes in microtubule and microfilament networks (Malek and Izumo 1996). Modification of the F-actin network of the cells depends on tyrosine kinase activities, internal Ca^{2+} concentrations, and interaction with microtubules (Malek and Izumo 1996). Inhibition of any of the functions prohibited the cell shape change produced by FSS (Malek and Izumo 1996). One type of focal adhesion associated protein is the integrin receptor, and the receptor may play an important role in the cell's response to mechanical stimuli (Maniotis et al. 1997).

Integrin Receptors

Maniotis et al. (1997) demonstrated that integrin receptors on the cell surface, cytoskeletal filaments, and the nucleus are physically connected, or "hard-wired", to each other. In contrast, transmembrane metabolic receptors are not physically connected either in the cytoskeleton or the nucleus (Maniotis et al. 1997). First, Maniotis et al. (1997) exposed bovine capillary endothelial to microbeads coated with either synthetic arginine-glycine-aspartate (RGD) peptide in fibronectin, which binds to

integrin receptors, or acetylated low density lipoproteins (AcLDL), which bind to transmembrane metabolic receptors (Maniotis et al. 1997). When the RGD bead/integrin receptor was physically pulled, the orientation of the cytoplasmic filament was modified, and the cell nucleus was distorted toward the pulling direction (Maniotis et al. 1997). When the AcLDL bead/transmembrane metabolic receptor was pulled, the bead-receptor complex was detached from the surface and did not have an influence on the nucleus (Maniotis et al. 1997).

Integrin is known to be involved in a variety of cell functions such as cellular gene expression, growth, differentiation, apoptosis, and proliferation (Giancotti and Ruoslahti 1999; Carson and Wei 2000). For example, Carson and Wei (2000) suggest that gene expression of skeletal muscle hypertrophy is induced by mechanical stimuli via integrin-mediated signaling pathways. The intensity of the mechanical stimuli altered the rate of protein synthesis of skeletal muscle cells (Carson and Wei 2000). Activation of integrin receptors on the cell membrane by fluid shear stress facilitates the formation of integrin clusters (Fluck et al. 1999). The integrin cluster initiates formation of focal adhesion complexes that serve as cell-matrix junctions (Fluck et al. 1999). In the focal adhesions, phosphorylation of focal adhesion kinase (FAK) and tyrosine kinase initiate downward signal transductions such as paxillin activation by FAKs (Fluck et al. 1999; Juliano 2002). Fluck et al. (1999) found FAK and paxillin concentrations were increased in hypertrophied skeletal muscle. Similar integrin modulated pathways via activation of Rho-A proteins may alter expression of genes by triggering the transcription

factor, serum response factor (SRF) that controls growth of the skeletal muscle cells (Carson and Wei 2000).

Integrin Receptors on Amoebae

Although integrin receptors have been yet not found in *A. proteus*, past studies implied the presence of integrin receptors in different protozoa and other organisms. Han et al. (2004) found receptors, which exhibited similar functions as integrin in the pathogenic amoeba, *Naegleria fowleri*. In *N. fowleri*, the activities of PKC increased as its adhesion to fibronectin in ECM increased (Han et al. 2004). On the other hand, when PKC inhibitors were exposed to the cell, *N. fowleri*'s adhesion to fibronectin decreased (Han et al. 2004). *Entamoeba histolytica* is an intestinal parasite that causes illness in humans; it attaches to collagen type I in host tissues thorough integrin-FAK complexes (Robledo et al. 2005). The mutant *E. histolytica* (BG-3), which has abnormally high FAK and MAP kinase (Erk2) activities, showed less adherence to collagen type I than the control (Robledo et al. 2005). In *Dictyostelium discoideum* amoeba (slime mold), the talin part of focal adhesion complex is necessary to perform phagocytosis (Gebbie at al. 2004).

Macrophage: Mechanoreceptor-Mediated Phagocytosis

Shiratsuchi and Basson (2004) applied various pressures (20-100 mmHg) to PMA-differentiated macrophage-like THP-1 cells. When the cells were exposed to higher pressure (100 mmHg) they induced significantly more phagocytosis of serum-opsonized latex beads than lower pressure (20 mmHg)

(Shiratsuchi and Basson 2004). Interestingly, pressures enhanced the THP-1 cells' phagocytosis behaviors by inhibiting phosphorylations of focal adhesion kinase, FAK, and MAP kinase, ERK (Shiratsuchi and Basson 2004). In addition, applications of Cy3-conjugated luciferase SiRNA which reduces gene expression of FAK and application of PD-98059 which inhibits the activation of ERK significantly increased the THP-1 cells' phagosytosis behaviors (Shiratsuchi and Basson 2004). In macrophages, intracellular Ca^{2+} concentration increases before both non-Fc and Fc-receptor-mediated phagocytosis, and this phenomenon is not observed in cells that do not induce phagocytosis (Hishikawa et al. 1991). Non-Fc-receptor-mediated phagocytosis is affected by removal of extracellular Ca^{2+} concentration, whereas Fc-receptor-mediated phagocytosis is not (Hishikawa et al. 1991). Moreover, Fc-receptor-mediated phagocytosis is not affected by protein kinase C (PKC) inhibitors (Hishikawa et al. 1991).

Calcium and A. proteus Phagocytosis

As I mentioned above, intracellular Ca^{2+} concentration is positively correlated with cytoplasmic motility in *A. proteus*. Intracellular Ca^{2+} concentration may also be involved in phagocytosis of *A. proteus* (Prusch and Joseph 1987; Prusch et al. 1989). For example, the Ca^{2+} channel inhibitors, verapamil and flunarizine, interfered with the phagocytosis behaviors of amoebae (Prusch and Joseph 1987; Prusch et al. 1989). An increase in external Ca^{2+} concentration was positively correlated with the phagocytosis rate of amoebae (Prusch and Minck 1985; Prusch and Joseph 1987).

Phagocytosis behaviors of *A. proteus*, induced by exposure of PGE_2 and NFMLP, were dependent upon extarcellular Ca^{2+} (Prusch et al. 1989). Extracellular Ca^{2+} concentrations also influenced phagocytosis of the reduced-glutathione beads by *A. proteus* (Prusch and Minck 1985).

Ca^{2+} Regulation of Actin Cytoskeleton

Ca^{2+} regulates actin cytoskeleton dynamics (Furukawa et al. 2002; Barry et al. 2005; Soden 2005). Specific proteins, which are Ca^{2+} sensitive, are found in phagocytic cups and pseudopodia (Furukawa et al. 2002). The proteins can link F-actin into bundles when sufficient Ca^{2+} is available in the cell, and this increases contractility of the cytoskeleton (Furukawa et al. 2002). Taylor et al. (1980) demonstrated an important role for Ca^{2+} in the pinocytosis of amoeba (figure 2). They suggested that phagocytosis in amoebae also use this Ca^{2+} regulated actin contractile system (Taylor et al. 1980).

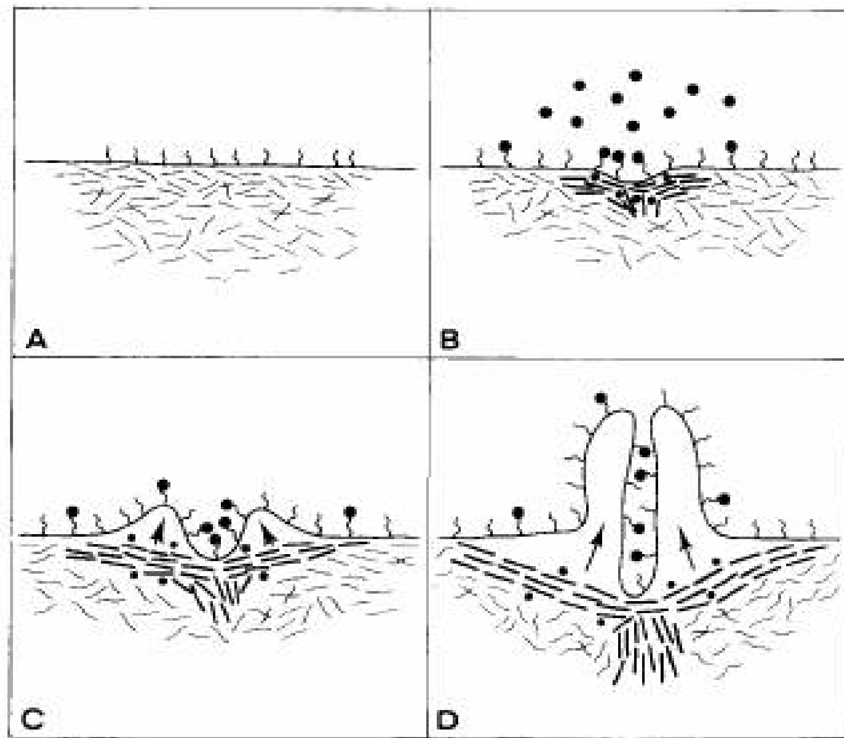


Figure 2. Model mechanisms of pinocytosis in amoebae crated by Taylor et al. (1980). A. Cross section of the cell membrane. The fur like structure on the membrane is a chemoreceptor. The cortex is in a non-contractile state. B. When ligands (bigger dots) bind to the chemoreceptors, the intracellular Ca^{2+} concentration (smaller dots) increases via an activation of signaling cascades. F-actin is cross-linked into bundles (heavy lines). C. The contractile bundles are attached to the membrane and pulled down, while the contractile cortex is pushed into the environment. D. Pinocytotic channel is formed.

3) Past Studies of A. proteus Sensory Motor Mechanisms

Chemical Stimuli and A. proteus Phagocytosis

Water-soluble ligands, which are secreted by prey organisms such as *Tetrahymena pyriformis*, may trigger *A. proteus* phagocytosis of the prey via binding to the receptors on the cell surface (Prusch and Joseph 1987). The corresponding chemotactic mechanisms are observed in various cells such as the ligands binding to FcγRIIA receptors in PMNs (Prusch and Joseph 1987). In Prusch and Joseph's experiment (1987), a micropipette that was filled with

10^{-9} to 10^{-5} M tripeptide n-formyl-methionyl-leucyl-phenylalanine (NFMLP) solution, was placed near an amoeba and its chemotactic behavior was observed. *A. proteus* streamed toward the micropipette (Prusch and Joseph 1987). NFMLP induced phagocytosis at low concentrations (10^{-9} M) (Prusch and Joseph 1987; Prusch et al. 1989). *A. proteus* also initiated phagocytosis when it was exposed to the fatty acids prostaglandin E_2, PGE_2 (Prusch et al. 1989).

When agarose beads with glutathione attached to their surfaces were placed near *A. proteus*, the amoebae phagocytosed the beads (Prusch and Minck 1985). On the other hand, *A. proteus* did not engulf plain agarose beads (Prusch and Minck 1985). In order for glutathione to effectively induce phagocytosis, the -SH group had to be exposed (Prusch and Minck 1985). Thus, glutathione should be in its reduced form while on the surface of the beads (Prusch and Minck 1985).

Application of Ciliated or Deciliated T. pyriformis to A. proteus

According to Prusch and Minck (1985), phagocytosis of the de-ciliated *T. pyriformis* occurred five to ten minutes more slowly than phagocytosis of ciliated *T. pyriformis* (Prusch and Minck 1985). *A. proteus* engulfed similar quantities of the ciliated and deciliated cells (Prusch and Minck 1985). When *A. proteus* was exposed to the cilia alone, the amoeba extended its pseudopodia toward the cilia, but did not engulf them (Prusch and Minck 1985). Chemoattractants may be on both the cell body and cilia of *T. pyriformis* (Prusch and Minck 1985).

In my experiments, I applied both chemical and mechanical stimuli to *A. proteus* in order to test for the presence of chemo- and mechanoreceptors. Because *A. proteus* engulfed both ciliated and deciliated *T. pyriformis* (Prusch and Minck 1985), I hypothesized that phagocytosis in *A. proteus* is induced mainly through activation of chemoreceptors by molecules presented by *T. pyriformis*. In addition, because *A. proteus* engulfed the immotile deciliated *T. pyriformis* at a slower rate than motile ciliated *T. pyriformis* (Prusch and Minck 1985), I also hypothesized that ciliary movements of *T. pyriformis* will enhance the chemoreceptor-induced phagocytosis by activating its mechanoreceptors on *A. proteus*.

MATERIALS AND METHODS

Amoeba proteus Culture

Amoeba proteus were obtained from Carolina Biological Supply and cultured in Pringsheim's solution (0.11 mM Na_2HPO_4, 0.35 mM KCl, 0.85 mM $Ca(NO_3)_2$, 0.08mM $MgSO_4$, 0.007 mM $FeSO_4$, pH 7.0). They were fed twice a week with *Tetrahymena pyriformis* which were recultured weekly into a sterile 1% proteose peptone solution. The cell density of *A. proteus* was approximately 14 cells/ml, while the cell density of *T. pyriformis* was approximately 1.2 X 10^4 cells/ml.

I. T. pyriformis Medium Experiment: Test for Chemoreceptors.

There were two conditions. 1) In control condition, an 0.5 ml culture solution of *A. proteus* was exposed to 0.3 ml of Pringsheim's solution (control). 2) In the experimental condition, an 0.5 ml culture solution of *A. proteus* was exposed to 0.3 ml of *T. pyriformis* medium. The amoebae were observed for 5 minutes after the application of each solution under the phase contrast microscope (Nikon Eclipse TE 2000–U) with a total magnification of 150X. Both conditions contained 30 amoebae, and a total of 60 amoebae were observed.

II. Deciliated Tetrahymena pyriformis Experiment: Test for Mechanoreceptors

Application of Deciliated or Ciliated T. pyriformis

A. proteus was placed onto a slide with a well of the following dimensions (40 mm X 12 mm X 5 mm). There were three conditions. 1) In the control condition, an 0.3 ml sample of ciliated (motile) *T. pyriformis* was added to an 0.5 ml solution of *A. proteus*. The amoebae had not been fed for three days. 2) To test for effects of deciliated (immotile) *T. pyriformis*, an 0.3 ml of deciliated *T. pyriformis* were applied to an 0.5 ml *A. proteus*. The amoebae had not been fed for three days. 3) In the application of both ciliated and deciliated *T. pyriformis*, 0.25 ml each of ciliated and deciliated *T. pyriformis* were applied to an 0.5 ml solution of *A. proteus*. The amoebae had not been fed for three days.

Deciliation of T. pyriformis

The cilia were removed from the *T. pyriformis* based on the methods described by Thompson et al. (1974). A 10 ml sample of 25 mM dibucaine was added to a 10 ml culture of *Tetrahymena pyriformis*. *T. pyriformis* ceased all movement within 10 minutes. The culture was centrifuged in a rotor for 30 seconds at 1,500 rpm at room temperature so that the cells were pelleted at the bottom of the tube. The cells were resuspended in 10 ml of spring water and centrifuged for 30 seconds at 1,500 rpm in the rotor in order to isolate the cells from the solution. Finally, deciliated *T. pyriformis* were resuspended in 10 ml of Pringsheim's solution.

Observation and Recording

After the application of *T. pyriformis*, the behavior of *A. proteus* was observed for 5 minutes through a phase-contrast microscope (Nikon Inversed Microscope Diaphto-TMD) with a total magnification of 150X. Both ciliated (control) and deciliated conditions contatined 20 amoebae each for a total of 40 amoebae. In the mixed condition, 15 amoebae were observed.

III. Application of Heated T. pyriformis: Test for Chemo- and Mechanoreceptors.

After the procedures for ciliated *T. pyriformis* preparation, 10 ml of the culture was heated to boiling. Then, the culture was cooled down to room temperature. 0.3 ml of heated and non-heated (control) *T. pyriformis* were applied to an 0.5 ml culture solution of *A. proteus*. The amoebae had not been fed for three days.

Observation

The behavior of *A. proteus* was observed for 5 minutes through a phase-contrast microscope (Nikon Inversed Microscope Diaphto-TMD) with a total magnification of 150X 5 minutes after the application of heated or non-heated *T. pyriformis*. In each condition, 15 amoebae were randomly chosen and observed.

IV. Micropipette Experiment: Test for Chemo- and Mechanoreceptors.

A. proteus culture was placed onto a slide with a well of the following

dimensions (40 mm X 12 mm X 5 mm). There were four conditions in the experiment. 1) In control condition, a micropipette filled with Pringsheim's solution was placed near an amoeba. 2) To test for chemoreceptors, a micropipette filled with *T. pyriformis* medium was placed near an amoeba. 3) To test for mechanoreceptors, a micropipette filled with Pringsheim's solution was placed near an amoeba and vibrated at 4 Hz. 4) To test for a synergistic effect between chemo- and mechanoreceptors, a micropipette filled with *T. pyriformis* medium was placed near an amoeba and vibrated at 4 Hz. In all conditions, the distance between the micropipette and the amoeba was kept constant (58 μm), and the tip of micropipette (diameter: 17 μm) was always placed by the amoeba's extending pseudopodia.

T. pyriformis Medium

T. pyriformis culture medium contains proteose peptone which is toxic to *A. proteus*. To remove the culture medium, *T. pyriformis* culture was placed in a plastic tube and centrifuged in a rotor for 30 seconds at 1,500 rpm at room temperature. Then, the culture medium remained in the supernatant and was poured out while the *T. pyriformis* cells were pelleted at the bottom of the tube. The cells were resuspended in 10 ml of spring water and centrifuged in a rotor for 30 seconds at 1,500 rpm. Again, the supernatant was removed, and only *T. pyriformis* cells remained on the bottom of the tube. The cells were resuspended in 10 ml of Pringsheim's solution for two days. Finally, *T. pyriformis* medium was centrifuged for five minutes at 1,500 rpm in the rotor; the supernatant-containing medium without the cells was transferred

to a new tube and used as *T. pyriformis* medium.

Cell Stimulator

Professor Curtis Smith at Mount Holyoke College in South Hadley, Massachusetts created the cell stimulator. Part of an earphone was modified and connected to a micropipette and a stimulator. The frequency of the stimulator pluses regulated the frequency of the earphone vibration. A micropipette was attached with sealing wax to the earphone and vibrated at the stimulator pulse frequency. An oscilloscope was also connected to the stimulator to record the frequency of the vibration (figure 3). The stimulator frequency was set to at 4 Hz.

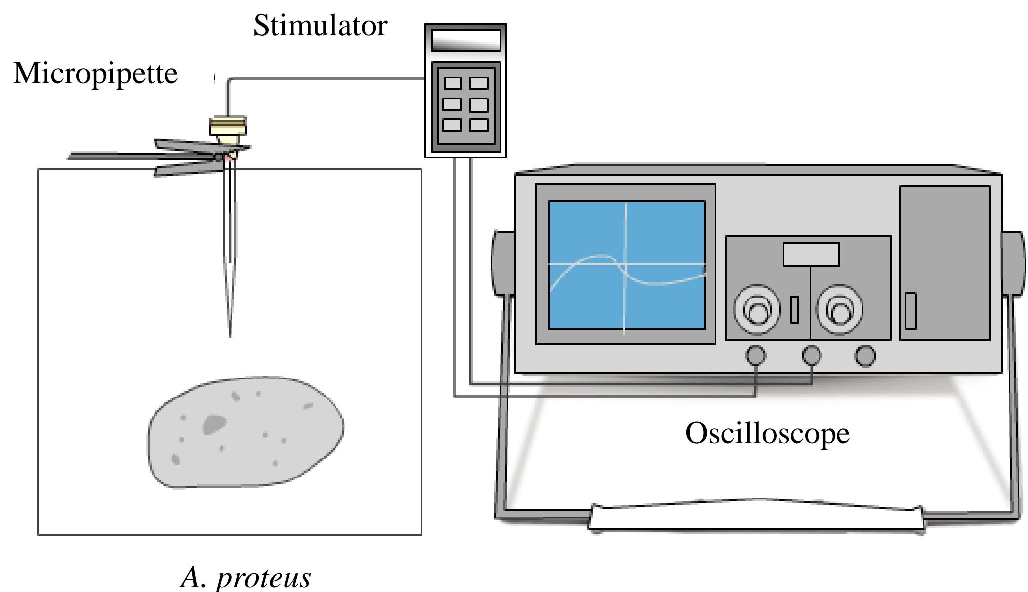


Figure 3. Cell stimulator. Part of an earphone was modified and connected to a glass micropipette and a stimulator. A micropipette was attached with sealing wax to the earphone and vibrated at the stimulator pulse frequency. An oscilloscope was also connected to the stimulator to record the frequency of the vibration.

Observation and Recording

The behavior of the *A. proteus* was observed for 5 minutes through a phase-contrast microscope (Nikon Inversed Microscope Diaphto-TMD) with a total magnification of 150X. The observation was also video taped using a NEC video camera mounted on the microscope. Each condition contained 30 amoebae, for a total of 120 amoebae tested.

Data Analysis

In all experiments, statistical analysis was done by comparing differences between the control group and the experimental group (Student's t-test).

Summery of the Experiments

Table 1. Summery of the Experiments.

	Conditions	Test for Chemo-Receptors	Test for Mechano-Receptors
1. *T. pyriformis* Medium Experiment	Application of *T. pyriformis* Medium: Medium or Control	√	
2. Deciliated *T. pyriformis* Experiment	Application of *T. pyriformis* Cells: Deciliated, Ciliated (control), or a Mixture of Deciliated and Ciliated.		√
3. Application of Heated *T. pyriformis*	Application of *T. pyriformis* Cells: Heated or Non-Heated (Control)		√
4. Micropipettes Experiment	Application of a Micropipette: Filled with *T. pyriformis* Medium	√	
	Application of a Micropipette: 4 Hz Vibration + Filled with Control Solution		√
	Application of a Micropipette: 4 Hz Vibration + Filled with *T. pyriformis* Medium	√	√
	Application of a Micropipette: Filled with Control Solution		

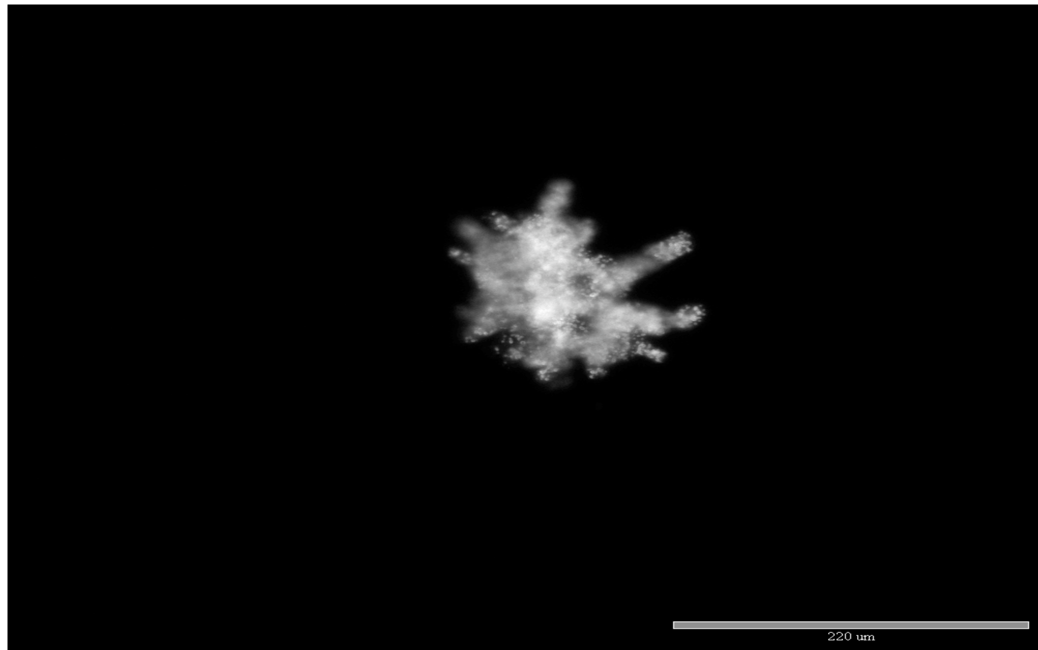
RESULTS

I. Application of T. pyriformis Medium to A. proteus

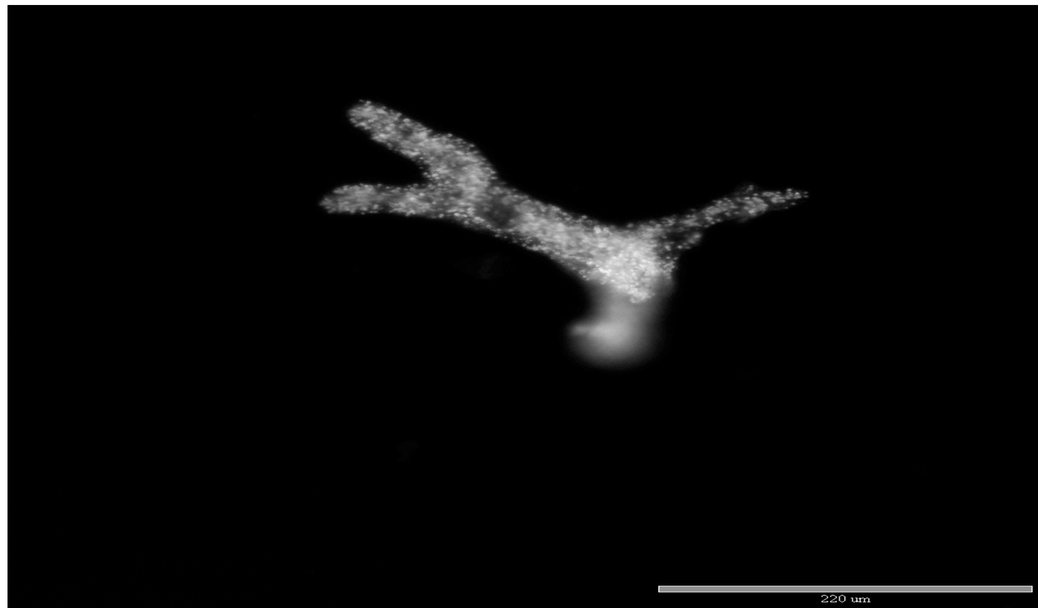
When *A. proteus* was exposed to *T. pyriformis* medium for 5 minutes, they stopped cytoplasmic streaming and became round (figure 4). The circularity of the cell was calculated according to the formula below by ImageJ software.

Circularity: $\dfrac{4\pi\,(\pi r^2)}{(2\pi r)^2}$

According to the formula, the value of a complete circle is one, and a less circular shape has a value less than one; the smaller the value, the less circular the object will be. The circularity of *A. proteus* was 0.57 and 0.29 in *T. pyriformis* and control condition respectively (figure 5); *T. pyriformis* medium conditions induced significantly more circularity among amoebae than the control condition (t-test, $t = 8.29$, d.f. = 58, $p < 0.005$). In *T. pyriformis* medium, some of the amoebae alternately extended and retraced small pseudopodia that did not contribute to their cell locomotion. On the other hand, in control condition, amoebae did not change their behavior after application of Pringsheim's solution. Before and after the application of Pringsheim's solution, they showed cytoplasmic streaming in non-specific directions.



4.1



4.2

Figure 4. Shape of a representative *A. proteus*. 4.1 Darkfield image of *A. proteus* was exposed to *T. pyriformis* medium for 5 minutes; it stopped cytoplasmic streaming and became spherical. *A. proteus* alternately extended and retracted small pseudopodia that did not contribute to any cell locomotion. 4.2 *A. proteus* was exposed to control solution for 5 minutes; it showed locomotion and polarity. Observations were done under the phase contrast microscope (150X). Bar = 220 μm.

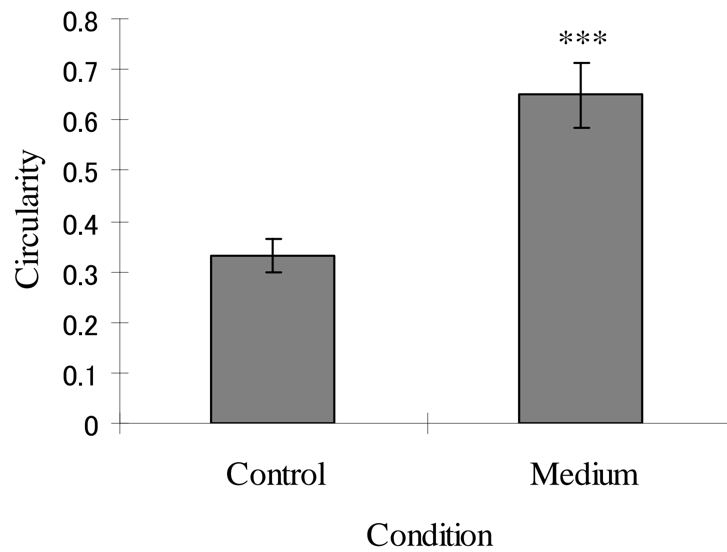


Figure 5. Circularity of *A. proteus* after a 5 minutes exposure to either 0.3 ml control solution or *T. pyriformis* medium. Each condition had 30 amoebae. The observations were done under the phase contrast microscope (150X). Circularities were calculated by using ImageJ software. Bars are ± standard error. *** = significant difference, $p < 0.005$.

II. Application of Deciliated and Ciliated T. pyriformis to A. proteus

According to figure 6, *A. proteus* phagocytosed both ciliated (control) and deciliated *T. pyriformis*. 1) 15 out of 20 *A. proteus* phagocytosed ciliated *T. pyriformis*. 2) 10 out of 20 *A. proteus* phagocytosed deciliated *T. pyriformis*. Thus, there was 25% less phagocytosis of deciliated than ciliated *T. pyriformis*. However, the difference between the two conditions was not statistically significant. 3) In the mixture containing even numbers of ciliated and deciliated *T. pyriformis*, 100% (n = 15) of *A. proteus* engulfed ciliated *T. pyriformis*, and no deciliated cells were engulfed.

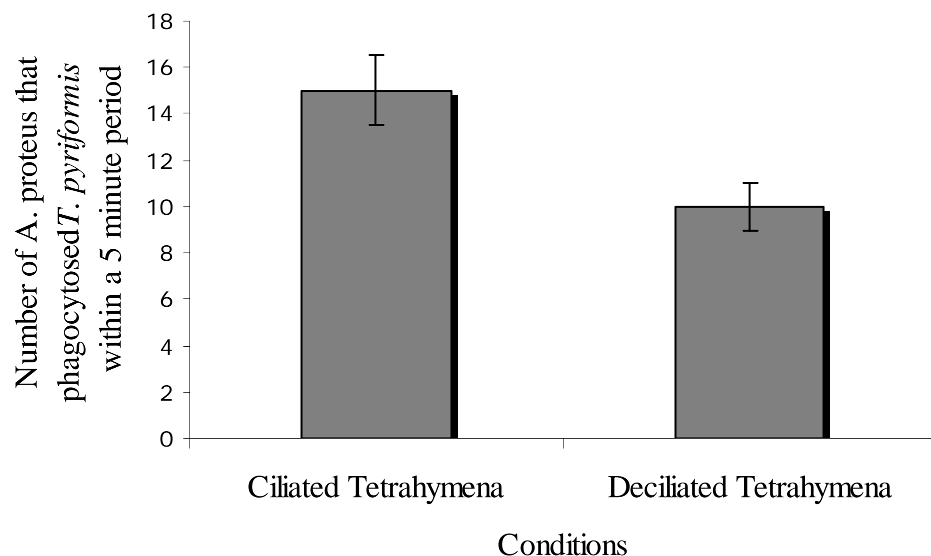


Figure 6. Number of *A. proteus* that phagocytosed either ciliated (control) or deciliated *T. pyriformis* within a 5 minute period. Each condition had 20 amoebae. The observations were done under the phase contrast microscope (150X). There was no statistical difference between the conditions. Bars are ± standard error.

III. Application of Heated T. pyriformis to A. proteus

Five minutes after application of heated *T. pyriformis*, 4 out of 15 *A. proteus* initiated phagocytosis of heated *T. pyriformis* (figure 7). In contrast, 12 out of 15 *A. proteus* induced phagocytosis of non-hearted *T. pyriformis* (control). The difference between the two conditions was statistically significant (t-test, $t = 3.35$, d.f. = 28, $p < 0.005$).

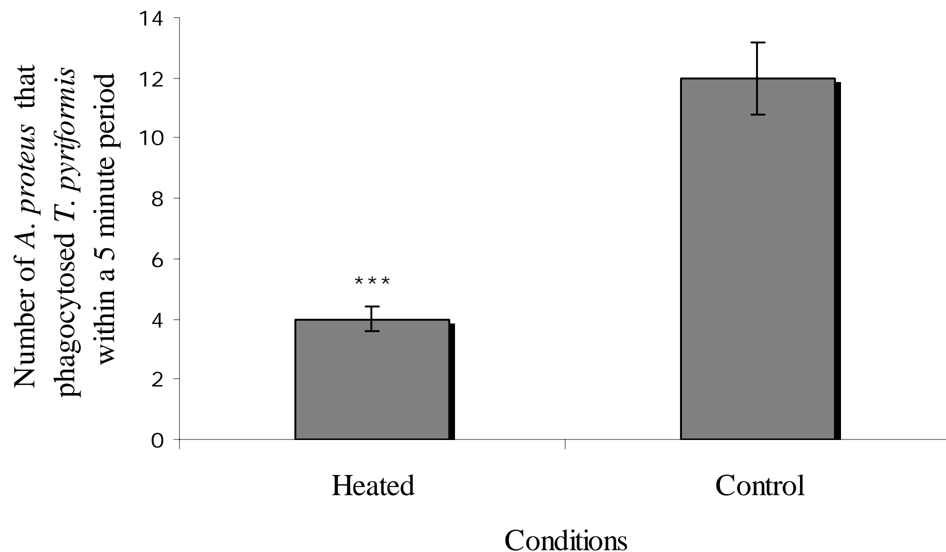


Figure 7. Number of *A. proteus* that phagocytosed either non heated (control) or heated *T. pyriformis* within a 5 minute period. Each condition had 15 amoebae; the heated *T. pyriformis* condition showed 53% less phagocytosis events than control. The observations were done under the phase contrast microscope (150X). Bars are ± standard error. *** = significant difference, $p < 0.005$.

In experiment II and III, the motility of *T. pyriformis* was reduced by different methods: killing by heat application and removing cilia. *A. proteus* phagocytosed 23% fewer heated *T. pyriformis* than deciliated *T. pyriformis* (t-test, $t = 1.39$, d.f. = 33, $p < 0.1$). In control conditions, both in experiment II and III, about 80% of the total *A. proteus* phagocytosed untreated *T. pyriformis*.

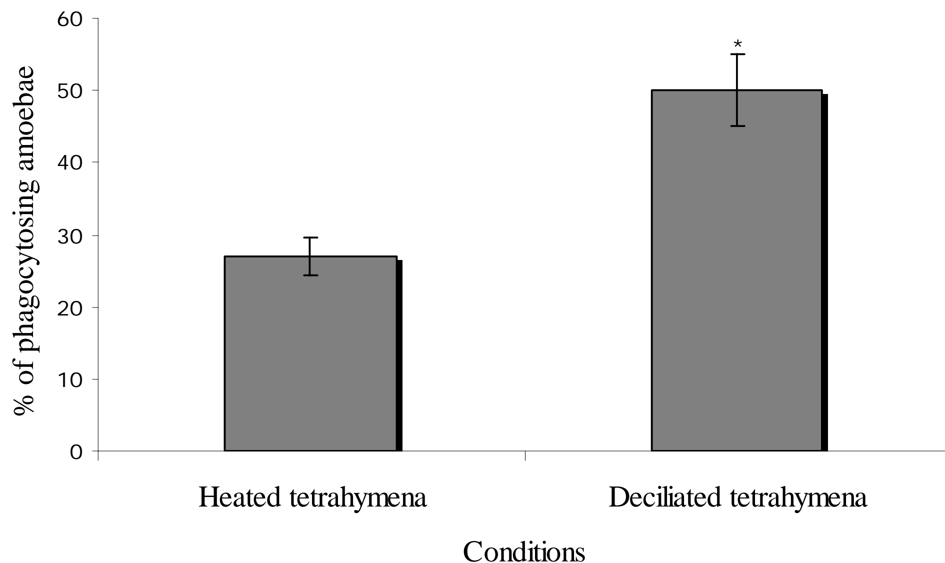


Figure 8. Percentages of *A. proteus* that phagocytosed *T. pyriformis* in a comparison of the data between heated tetrahymena from experiment II and deciliated tetrahymena conditions from experiment III. In control conditions, both in experiment II and III, about 80% of *A. proteus* phagocytosed untreated *T. pyriformis*. In experiment II, 27% of *A. proteus* phagocytosed heated *T. pyriformis*. In experiment III, 50% of *A. proteus* phagocytosed deciliated *T. pyriformis*. *A. proteus* phagocytosed 23% less heated *T. pyriformis* than deciliated *T. pyriformis*. Bars are ± standard error. * = significant difference, $p < 0.1$.

IV. Application of Micropipette to A. proteus

According to figure 9, *A. proteus* phagocytosed the microelectrode in all four conditions. 1) In control condition, 2 out of 30 *A. proteus* showed phagocytosis events. 2) 12 out of 30 *A. proteus* phagocytosed the micropipette that was filled with *T. pyriformis* medium but not vibrated. This 33% increase in phagocytosis events was statistically significant (t-test, $t = 3.27$, $d.f. = 58$, $p < 0.005$). 3) 6 out of 30 *A. proteus* phagocytosed the micropipette that was filled with Pringsheim's solution but vibrated at 4Hz. 4) 6 out of 30 *A. proteus* phagocytosed the micropipette that was filled with *T. pyriformis* medium and vibrated at 4Hz. The vibration condition and the *T. pyriformis* medium and vibration condition showed the same 13% increase in the number of phagocytosis events but this increase was not statistically significant.

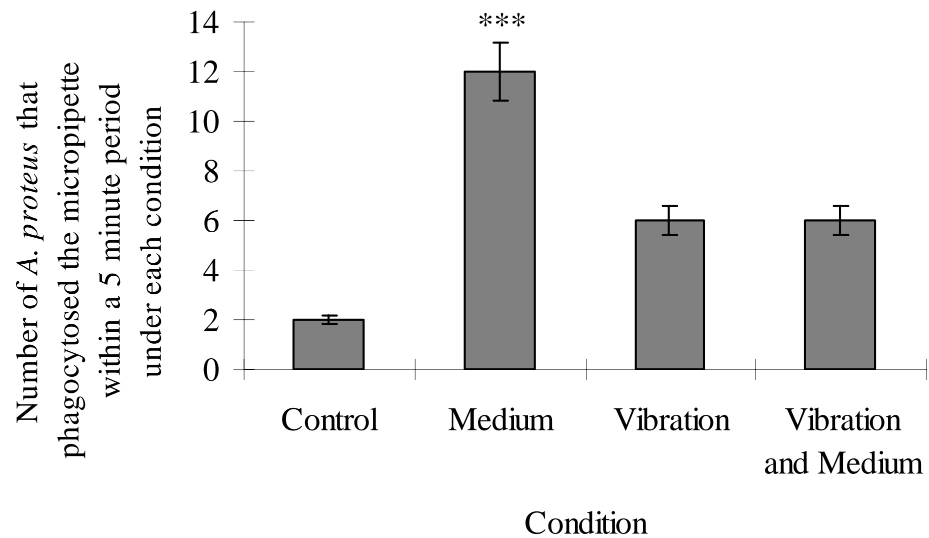


Figure 9. Number of *A. proteus* that phagocytosed the micropipette within a 5 minute period under each of four conditions: the micropipette filled with control solution (control), micropipette filled with *T. pyriformis* medium (medium), micropipette filled with control solution and vibrated at 4 Hz (vibration), and micropipette filled with *T. pyriformis* medium solution and vibrated at 4 Hz (vibration plus medium). The medium condition showed 33% more phagocytosis events than control. Both vibration and vibration plus medium conditions showed 13% more phagocytosis events than control. Each condition had 30 amoebae. The observations were done under the phase contrast microscope (150X). Bars are ± standard error. *** = significant difference, $p < 0.005$.

DISCUSSION

In my experiments, I applied both chemical and mechanical stimuli to *A. proteus* in order to test for the presence of chemo- and mechanoreceptors in *A. proteus*. My experimental results support my first hypothesis that phagocytosis of *A. proteus* is mainly induced through activation of chemoreceptors by molecules presented by *T. pyriformis*. However, not all results supported my second hypothesis that the mechanical movements of *T. pyriformis* will enhance the chemoreceptor-induced phagocytosis of *A. proteus* by activating the amoeba's mechanoreceptors.

I. Application of T. pyriformis Medium to A. proteus.

In this experiment, the importance of chemical stimuli regarding *A. proteus*' phagocytotic behavior is tested. According to my hypothesis, I expect to observe some behavioral changes in *A. proteus* when *T. pyriformis* medium (chemical stimulus) is present, because *A. proteus*' chemoreceptors are activated. As I predicted, the results show behavioral changes in *A. proteus* upon application of the medium.

When *A. proteus* is exposed to *T. pyriformis* medium for five minutes, *A. proteus* slows cytoplasmic streaming and becomes round (figure 4.1 and 5). Some of the amoebae alternately extend and retract small pseudopodia that do not contribute to any cell locomotion. However, in the control condition, *A. proteus* does not change their behavior after the application of Pringsheim's

solution; before and after the application of Pringsheim's solution, they show cytoplasmic streaming and locomotion in non-specific directions (figure 4.2 and 5). The results show that *A. proteus* changes its appearance from the polar, motile state to the nonpolar, immotile state after the *T. pyriformis* medium is applied. Some chemical substances in the *T. pyriformis* medium may stimulate chemoreceptors on *A. proteus* and cause these behavioral changes.

In the control condition, in the absence of *T. pyriformis* medium, *A. proteus* randomly moves by cytoplasmic streaming. A past study by Prusch et al. (1989) showed similar results. They observed that *A. proteus* randomly streamed in non-specific directions in control condition (Prusch et al. 1989). Erban and Othmer (2004) stated that many unicellular organisms move randomly until they detect signals from food sources. Because there is no food source or chemical gradient in the control condition, *A.* proteus' random movement is possibly in search of a food source.

A. proteus stops cytoplasmic streaming and becomes round when exposed to the *T. pyriformis* medium (chemical stimuli). There are several possible explanations for this change in behavior. First, *A. proteus* may lose cell polarity in high concentrations of environmental chemoattractants. In lower concentrations of glutathione solution (10^{-6} M), cell polarity was maintained, and *A. proteus* initiated psedudopodia extension and phagocytosis (Prusch and Minck 1985). However, a high concentration of glutathione (10^{-3} M) induced a loss in cell polarity and a spherical shape in *A. proteus* (Prusch and Minck 1985). According to Prusch and Joseph (1987), the

inhibition of phagocytosis in a higher chemical concentration occurred because all chemical-sensitive receptors on the *A. proteus'* membrane were occupied by ligands. In other words, polarity is created in *A. proteus* when ligands bind to the membrane receptors on one side of the amoeba, but not on the other side. When all chemoreceptors are bound to a ligand, none of the receptors are empty. For this reason, a chemical gradient is necessary to start the phagocytosis process (Prusch and Joseph 1987). For example, *Dictostelium* amoebae cannot respond to chemotactic agents unless the agents disperse asymmetrically in their environment (Noegel and Schleicher 2000). In my experiment, the amount of chemoattractants in *T. pyriformis* medium is possibly high enough to occupy all of the chemoreceptors on the *A. proteus*' membrane. Therefore, there is no chemical gradient to detect the chemoattractants by *A. proteus*. Thus, there is no polarity in the cell, and *A. proteus* phagocytosis does not occur. Moreover, *A. proteus* alternately extends and retracts small pseudopodia that do not contribute to any cell locomotion (figure 4.1). Again, this behavior may be attributed to the lack of a chemical gradient in the environment, because there was no single polarity for these cells.

Secondly, the chemical condition in the environment may influence specific stages of phagocytosis. The normal stages of phagocytosis proceed as follows: 1) recognition of stimuli from a phagocytotic target, 2) alteration of actin fluidities in cytoplasm, 3) pseudopodial extensions, 4) increasing adhesion to the target, 5) engulfment of the target by pseudopodia, and 6) fusion of the pseudopodia into a food vacuole (Prusch et al. 1989; Shiratsuchi

and Basson 2004). After phagocytosis, when *A. proteus* is full from eating enough prey organisms, it tends to become round (Prof. Barry, personal communication). Prusch and Minck (1985) also pointed out that 15 to 20 minutes after phagocytosis, *A. proteus* became spherical and stopped its movement. In lower concentrations, the application of NFMLP solution to *A. proteus* initiated phagocytosis (Prusch and Joseph 1987). The exposure to a high concentration of NFMLP solution induced only the formation of food vacuoles in the amoeba cell, but no other phagocytosis activity (Prusch and Joseph 1987). I did observe large vacuoles forming when amoebae were exposed to *T. pyriformis* medium although I did not quantify these observations. These observations may indicate that when more chemoreceptors are bound to ligands, *A. proteus* will exhibit latter and/or post-phagocytotic behaviors. If this hypothesis is true, phagocytosis in *A. proteus* is a highly chemoreceptor-dependent process. A small concentration of chemoattractants in the environment will bind fewer chemoreceptors and may cause locomotion toward the target and the initial phases of phagocytosis. On the other hand, as a target comes closer to the amoeba, its chemoattractants become more concentrated and bind more chemoreceptors. This may trigger later stages of phagocytosis. Future studies could involve the application of different concentrations of *T. pyriformis* medium to *A. proteus* and observations of behavioral and morphological changes.

II. Application of Deciliated or Ciliated T. pyriformis to A. proteus.

In this experiment, the importance of mechanical stimuli regarding *A.*

proteus' phagocytotic behavior was tested. According to my hypothesis, I expected to observe a smaller number of phagocytotic events when deciliated *T. pyriformis* was applied to *A. proteus* as opposed to ciliated *T. pyriformis*. The movements of *T. pyriformis* in the control condition may stimulate more mechanoreceptors in *A. proteus* than immotile deciliated *T. pyriformis*. For the same reason, I predicted that *A. proteus* would phagocytose more ciliated *T. pyriformis* than deciliated *T. pyriformis* when an even number of types of cell are applied to *A. proteus* at the same time.

The results are the following (figure 6): First, the deciliated *T. pyriformis* induces 25% less phagocytosis in *A. proteus* than ciliated *T. pyriformis* although this difference is not statistically significant. Second, in the presence of equal numbers of ciliated and deciliated *T. pyriformis*, 100% (n = 15) of *A. proteus* phagocytoses ciliated *T. pyriformis* in a five minute period while no deciliated cells were engulfed.

Against my prediction, the first result indicates that stimulation of mechanoreceptors via the movements of *T. pyriformis* may not be a critical factor in initiating phagocytosis in *A. proteus* because amoebae phagocytose both ciliated and deciliated *T. pyriformis* at similar rates. However, it is possible that the data in the first result do not show differences in the two conditions because of a ceiling effect.

Prusch et al. (1989) pointed out that amoebae phagocytose deciliated and ciliated *T. pyriformis* in a time dependent manner (Prusch et al. 1989). In their study, *A. proteus* phagocytose the same total number of ciliated and deciliated *T. pyriformis* but at different rates (Prusch and Minck 1985). *A.*

proteus took five minutes to engulf deciliated *T. pyriformis* but only three minutes to engulf the ciliated *T. pyriformis* (Prusch and Minck 1985). In my experiment, the *A. proteus*' phagocytotic behavior is observed for five minutes in all conditions. This observation time may create a ceiling effect in my results. In other words, the amoebae phagocytose fewer deciliated *T. pyriformis* than ciliated *T. pyriformis*, during a time period of less than five minutes.

Moreover, there is the possibility that *T. pyriformis* grew back their cilia during the experiment and created fine ciliary vibrations which stimulated the mechanoreceptors in *A. proteus*. According to Thompson et al. (1974), when *T. pyriformis*' cilia were removed by dibucaine application, its cilia were regenerated within thirty minutes. My observations of *A. proteus* in the deciliated experiments took at least 100 minutes. It is possible that ciliary vibrations from *T. pyriformis* too small to be detected by the experimenter stimulate mechanoreceptors on *A. proteus*.

As I expected, in the second result, the amoebae exclusively phagocytose ciliated *T. pyriformis* when there were both ciliated and deciliated *T. pyriformis* present at same time. These data imply that mechanoreceptor stimulation by the movements of *T. pyriformis* may be an important factor for inducing phagocytosis in *A. proteus*. This result agrees with Prusch and Minck's study (1985) that *A. proteus* phagocytoses ciliated *T. pyriformis* faster than deciliated *T. pyriformis*. Then, how do ciliated *T. pyriformis* induce faster phagocytosis by *A. proteus*? To answer this question, the influence of ciliary beating by *T. pyriformis* may have to be reconsidered.

First, ciliary beating of *T. pyriformis* may create vibrations in the aquatic environment, and the vibrations may stimulate mechanoreceptors on *A. proteus*. According to past studies, possible mechanoreceptors on *A. proteus* may include G protein binding receptors (Gudi et al. 1998), mechanosensitive channel receptors (Malek and Izumo 1996), and integrin like receptors (Han et al. 2004).

Activation of G protein binding mechanoreceptors may induce rapid phagocytosis of moving prey in *A. proteus*. Rac is one of the G proteins (Gomperts et al. 2002), and Rac-like proteins have been known to control actin polymerization in *A. proteus* (Klopocka et al. 2005). In macrophages, Rac is associated with Fc receptor, transduces mechanical stimuli into intracellular signals, and induces phagocytosis as a cellular response (Beningo and Wang 2002). Rac has been known to phosphorylate p21-activated-kinase (PAK), which activates proteins, such as myosin II, in cytoskeletons (Klopocka et al. 2005). For example, inhibition of Rac-like proteins in *A. proteus* prevented cytoplasmic streaming (Klopocka and Redowicz 2003, 2004). Klopocka and Redowicz (2004) concluded that inhibition of Rac-like proteins increased actin-myosin II interactions in *A. proteus*, which created "hypercontracted" cell membranes and inhibited *A. proteus* movements (Klopocka and Redowicz 2004).

Even though, specific receptors that activate Rac are not yet identified in *A. proteus* (Klopocka and Redowicz 2003, 2004; Klopocka et al. 2005), Rac binding mechanoreceptors may play an important role in *A. proteus*' phagocytosis.

Activation of mechanosensitive ion channel receptors may induce rapid phagocytosis of moving prey in *A. proteus*. For example, mechanosensitive K^+ channel receptors have been found in macrophages (Martin et al. 1995). The K^+ channel receptors increased K^+ current, when they were exposed to a pressure of 10 mmHg (Martin et al. 1995). When the channels were blocked, the macrophage's cytoskeleton was disrupted by the changing distribution of actin, tubulin and vimentin (Martin et al. 1995). The channel activities increased when the macrophages touched a target (Martin et al. 1995). In addition, Shiratsuchi and Basson (2004) found that application of pressure increased phagocytotic activities in macrophage-like cells. The movements of *T. pyriformis* may activate mechanosensitive ion channel receptors in *A. proteus* and induce fast phagocytotic responses.

Activation of integrin receptors may induce rapid phagocytosis of moving prey in *A. proteus*. Integrin-like receptors have been found in *N. fowleri* and *E. histolytica*, which are pathogenic amoebae (Han et al. 2004; Robledo et al. 2005). Moreover, integrin receptors can be associated with the Rho family of G proteins such as Rac and regulate actin reorganization and cell motilities (Aplin et al. 1998). Activation of Rac may transduce mechanical stimuli into intracellular signals and induce phagocytosis as a cellular response (Beningo and Wang 2002). However, further investigations require an understanding of the relationship between *A. proteus*' phagocytosis and integrin receptors.

Second, cilia give *T. pyriformis* an ability to reach its target. The ciliated *T. pyriformis* may induce more phagocytosis in *A. proteus* because it

can move toward the amoeba. *T. pyriformis* appeared to graze the surface of *A. proteus* (Prusch and Minck 1985). The surface membrane of *A. proteus* may harbor microorganisms that are attractive to *T. pyriformis* and this makes it easy for the amoeba to reach *T. pyriformis* (Prusch and Minck 1985). Even, random movements of *T. pyriformis* may facilitate phagocytosis behavior in *A. proteus* via increasing direct contacts between *A. proteus* and *T. pyriformis*. According to Christiansen and Marshall (1965), *Paramecium aurelia* moves randomly in a medium and when they bump into a giant amoeba *Chaos Chaos* by chance, the tetrahymena cilia are sometimes mildly trapped by the "sticky surface" on the amoeba. The adherence to the amoeba surface is not persistent, though it increases the likelihood of prolonged contact between *P. aurelia* and the surface of the amoeba, and may allow the amoeba to engulf the prey (Christiansen and Marshall 1965).

Finally, other chemical attractants on the cilia may be a key factor in inducing phagocytosis of ciliated *T. pyriformis* by *A. proteus*. The chemical attractants that induce phagocytosis in *A. proteus* are both on the cell surface and cilia of *T. pyriformis* (Prusch and Minck 1985). Deciliated *T. pyriformis* may induce less phagocytotic activities than ciliated *T. pyriformis* because the removal of cilia from the cell may reduce the total amount of chemical attractants.

III. Application of Heated T. pyriformis to A. proteus.

In this experiment, the role of mechanical stimuli in *A. proteus*' phagocytotic behavior was tested. According to my hypothesis, I expected to

observe a smaller number of *A. proteus*' phagocytosis events when heated, killed *T. pyriformis* was applied to *A. proteus* than when control motile *T. pyriformis* was applied. The movements of *T. pyriformis* in the control condition stimulate more mechanoreceptors in *A. proteus* than immotile *T. pyriformis*.

As I expected, the results (figure 8) show that within a five-minute period, *A. proteus* phagocytosed 53% fewer heated (killed) *T. pyriformis* than non-heated (live) *T. pyriformis* (control). This difference is statistically significant indicating that immotile *T. pyriformis* induce less phagocytosis than motile cells. This supports my hypothesis that mechanical stimuli facilitate phagocytotic behaviors in *A. proteus* via mechanoreceptor stimulation.

In addition, the application of heat may have changed the rigidity of the *T. pyriformis* cells. Too soft or too stiff *T. pyriformis* cells may activate fewer mechanoreceptors on the amoeba membrane and prevent the initiation of phagocytotic events. In Beningo and Wang's experiment (2002), macrophages were exposed to polyacrylamide microbeads that had identical chemical properties but different rigidities, stiff or soft. The macrophages were six times more likely to phagocytose the stiff beads than the soft beads (Beningo and Wang 2002).

A comparison of experiment II and III shows that *A. proteus* phagocytosed 23% fewer heated (killed) *T. pyriformis* than deciliated *T. pyriformis* (figure 8). Both treatments, heat and deciliation, created an immotile *T. pyriformis*, but the heated *T. pyriformis* induced less phagocytosis.

Heating kills *T. pyriformis* and takes away its movements completely.

As I mentioned before, deciliated *T. pyriformis* may regenerate their cilia and regain mobility during the experiment. Thus, they may induce more phagocytosis than heated *T. pyriformis*.

Likewise, only living *T. pyriformis* secrete chemical attractants into the environment. Therefore, application of heated, killed *T. pyriformis* may induce less phagocytosis by *A. proteus* than deciliated living *T. pyriformis*. For example, a different tetrahymena species, *Tetrahymena thermophila* secretes a variety of enzymes into the environment (Herrmann et al. 2006), which may act as chemical attractants.

If chemical attractants are present on the surface of *T. pyriformis*, then application of heat may denature the chemical attractants. For example, application of fatty acids such as prostaglandin E_2 (PGE_2) induces phagocytosis in *A. proteus* (Prusch et al. 1989). The melting point of PGE_2 is 64-71°C (Pfizer Inc.). Since heated *T. pyriformis* are cooked in about 100°C, some chemical attractants on the surface of *T. pyriformis*, which may be similar to PGE_2, are denatured and may not have been bound

IV. Application of Micropipettes to A. proteus.

In this experiment, the relative role of chemical and mechanical stimuli to phagocytotic behavior is tested. According to my hypothesis, I expected to observe the largest number of *A. proteus*' phagocytosis events in the test involving synergistic effects of activation of chemo- and mechanoreceptors. In this test, the micropipette is filled with *T. pyriformis* medium, vibrated at 4 Hz, and placed near the amoeba. Then, the second

largest number of phagocytotic events should be observed when the micropipette is filled with *T. pyriformis* medium and placed near *A. proteus* but is not vibrated. Finally, the smallest number of phagocytotic events should be observed when in the chemically inert micropipette is vibrated at 4 Hz and placed near *A. proteus*.

However, the results indicate the medium plus vibration condition did not produce the greatest number of phagocytotic events (figure 9). This condition induced 13% more phagocytosis events by *A. proteus* than the control condition but this increase is not statistically significant. Instead, the chemoreceptor stimulation induces the most phagocytosis events in *A. proteus*. Application of the non-vibrating micropipette filled with *T. pyriformis* medium induces 33% more phagocytosis than control, and this difference is statistically significant. The chemically inert micropipette, which vibrates at 4 Hz, induces 13% more phagocytotic events in *A. proteus* than the control condition, but this increase is not statistically significant. This condition induced the same number of phagocytosis events in *A. proteus* as *T. pyriformis* medium plus vibration condition.

The experimental results support my first hypothesis that phagocytosis of *A. proteus* is mainly induced through activation of chemoreceptors because the *T. pyriformis* medium condition induces the greatest number of *A. proteus*' phagocytosis events. The vibration condition induces less phagocytosis than this condition. However, my second hypothesis that stimulation of mechanoreceptors will enhance the chemoreceptor-induced phagocytosis by *A. proteus* is not supported because *T.*

pyriformis medium plus vibration condition induced less phagocytosis than *T. pyriformis* medium condition alone.

In my experiments, *T. pyriformis* medium significantly increased phagocytosis by *A. proteus*. The past studies suggest possible chemical attractants, which may be present in *T. pyriformis* medium. The studies of Prusch and Minck (1985) and Prusch et al. (1989) indicated the possible molecules that induce phagocytosis among *A. proteus*: glutathione, *N*-formyl-Met-Leu-Phe (NFMLP), and prostaglandin E_2 (PGE_2). These molecules in *T. pyriformis* medium may stimulate chemoreceptors on the amoeba cells and initiate phagocytosis.

Glutathione consists of three amino acids: glutamate, cysteine, and glycine, and it is known to bind glutamate receptors (Janaky at al. 1999). There are metabotropic (mGluR) and ionotroic (iGluR) glutamate receptors (Janaky at al. 1999). In neurons, the mGluR is coupled to the G protein. When it is activated, it depolarizes the neuron by inhibiting GABA receptors (Cao 2005). Depolarization of the cell activates iGluRs such as the NMDA receptors (Cao 2005). The cell depolarization removes Mg^{2+} from the NMDA receptor channel and increases Ca^{2+} permeability into the cell (Cao 2005). Therefore, the stimulation of glutamate receptors contributes to an increase in the intracellular Ca^{2+} concentration.

In human neutrophils, the tripeptieds, NFMLP binds to fMet-Leu-Phe receptors, which associate with two kinds of G proteins, stimulatory (Gs) or inhibitory (Gi) (Lad et al. 1985). These G proteins influence the activity of the enzyme adenylate cycase that catalyzes cyclic AMP formations from ATP

(Lad et al. 1985). Adenylate cyclase is not only regulated by G proteins but is also regulated by Ca^{2+} and phosphrylation by PKC (Gomperts et al. 2002).

Prostaglandin E_2 is a fatty acid that binds to the G protein binding receptors, E-prostanoid (EP) 1, EP2, EP3, and EP4 (Aronoff et al. 2004). When PGE_2 binds to EP1, the intracellular Ca^{2+} concentration increases (Aronoff et al. 2004). When PGE_2 binds to EP2 and 4, the intracellular cAMP concentration increases (Aronoff et al. 2004). When PGE_2 binds to EP3, the intracellular cAMP concentration decreases (Aronoff et al. 2004).

In rat alveolar macrophages (AM), PGE_2 acts as a stimulator or an inhibitor of phagocytosis (Aronoff et al. 2004). Aronoff et al. (2004) found that the external application of PGE_2 to the AM decreased Fc-receptor-mediated phagocytosis. Meanwhile, inhibition of the internal PGE_2 synthesis in the AM increased Fc-receptor-mediated phagocytosis (Aronoff et al. 2004). In addition, the treatment of PGE_2 to AM increased the intracellular cAMP concentration (Aronoff et al. 2004). Western blot analysis showed that AM contains E2 and E3 receptors which control cAMP concentrations (Aronoff et al. 2004).

These ligand-receptor interactions may activate signaling cascades in *A. proteus* and lead to its behavioral response, phagocytosis.

Four Hz micropipette vibrations do not provoke phagocytosis by *A. proteus*. The results indicate that mechanical stimuli may not play an important role in phagoctosis or may have to be very specific to activate mechanoreceptors in *A. proteus*. According to Gudi et al. (1998), activation of G protein in the rat cardiac fibroblasts required both a specific force and

rate of stretch. In fibroblasts, six percent maximum strain induced 4.7 fold more G protein activation than three percent maximum strain (Gudi et al. 1998). Fibroblasts that were stretched at a higher strain rate, for 10 seconds duration, induced significantly more G protein activation than when stretched at a lower strain rate even for 60 seconds (Gudi et al. 1998). Moreover, specific G proteins, $G_{\alpha q}$ and $G_{\alpha il}$, but not $G_{\alpha i3}$, were activated by the strains (Gudi et al. 1998). In Bacabac et al.'s study (2006), the production of NO by bone cells was positively correlated with the frequencies of vibration; the cells in100 Hz condition produced the highest amount of NO. On the other hand, the production of PGE_2 by bone cells was negatively correlated with the frequencies of vibration; the cells in 5 Hz condition produced the highest amount of PGE_2 (Bacabac et al. 2006). The mRNA expression of enzyme Cox-2 which catalyses PGE_2 in the cells was positively correlated with the frequencies of vibration; the cells in 100 Hz condition showed the highest mRNA expression of Cox-2 after 2.5 hours of incubation (Bacabac et al. 2006). When macrophages were exposed to a high pressure, 100 mmHg, they induced significantly more phagocytosis of serum-opsonized latex beads than when exposed to lower pressure, 20 mmHg (Shiratsuchi and Basson 2004). Similarly, indicated that initiation of *A. proteus*' phagocytosis may require very specific range of the vibrations. For example, cilia of *Tetrahymena thermophila* beat at about 35 Hz (Hennessey et al. 2002). If cilia of *T. pyriformis* beat at a similar frequency, vibrations of the micropipette in my experiment, 4 Hz, may be too weak to activate mechanoreceptors on *A. proteus*.

Furthermore, direct contact of the mechanoreceptor may be required for phagocytosis in *A. proteus*. In an interaction between a giant amoeba *Chaos Chaos* and its prey organism *Paramecium aurelia*, the amoeba had to contact *P. aurelia* directly for about two seconds or more to promote phagocytosis (Christiansen and Marshall 1965). Korn and Weisman's study (1967) suggested that mechanical contact was essential for *Acanthamoeba* to engulf prey organisms because the amoeba showed phagocytosis toward chemically inert latex beads when it had physical contact with the beads. Future studies could involve observations of *A. proteus* when the vibrating micropipette is placed near or contacts the amoeba's cell surface.

A. proteus shows less phagocytosis of a vibrating micropipette filled with *T. pyriformis* medium than a non-vibrating medium-filled pipette. This result is hard to interpret. The vibrations of the micropipette may disperse the chemical attractants in the medium, thereby crating less of a chemical gradient in the environment, stimulating fewer chemoreceptors, and reducing phagocytosis by *A. proteus*.

Conclusion

My experiments were designed to investigate, whether or not *A. proteus* is induced to phagocytose as a result of activation of chemoreceptors, mechanoreceptors, or both types of receptors.

The results from experiments involving, application of *T. pyriformis* medium, deciliated *T. pyriformis*, and micropipettes filled with *T. pyriformis* medium point to the involvement of chemoreceptors in stimulating

phagocytosis. Ligands, which are present on or secreted by prey organisms, may bind to the chemoreceptors, trigger chemoreceptor mediated signal transduction, and induce phagocytosis by *A. proteus*.

The results from the experiments to test for mechanoreceptor in *A. proteus* are more complicated to interpret. The results of experiments involving application of heated and deciliated and ciliated *T. pyriformis* support an involvement of mechanoreceptors in phagocytosis. Movement of the prey organisms may activate the mechanoreceptors, trigger mechanoreceptor mediated signal transduction, and induce phagocytosis by *A. proteus*. However, other experiments such as, application of deciliated *T. pyriformis* and the vibrating micropipette no involvement of mechanoreceptors in phagocytosis by *A. proteus*.

Moreover, application of the vibrated micropipette filled with *T. pyriformis* medium to the amoebae tests for the combined effect of activation of chemo- and mechanoreceptors on phagocytosis. The results imply no enhancement of chemoreceptor-mediated phagocytosis by the stimulation of mechanoreceptors. These studies provide some insight into the sensory world of *A. proteus*.

I observed the interactions between *A. proteus* and *T. pyriformis* for a year, and I often saw interesting behaviors displayed by the two types of cells. When high densities of *T. pyriformis* are released into *A. proteus* culture, *T. pyiformis* bumps into the amoebae by random ciliary movements. Then, *A. proteus* phagocytoses them in a few seconds. On the other hand, when low densities of *T. pyriformis* are released into the *A. proteus* culture, the amoebae

have to sense them perhaps with their chemoreceptors and then chase them by locomotion because the random bumping of *T. pyriformis* decreases. This kind of phagocytosis takes a longer time, and the amoebae sometimes fail to phagocytose *T. pyriformis* at all. Based on these anecdotal observations, physical touch of *T. pyriformis* may play a very important role for successful phagocytosis.

Moreover, phagocytosis involves a great deal of the amoeba's cytoplasm. When *A. proteus* needs to use cytoplasm to extend pseudopodia for locomotion to reach *T. pyriformis,* there is less cytoplasm available for phagocytosis. This may lead *A. proteus* to take a longer time to engulf the target. The random bumping of *T. pyriformis* reduces the necessity for *A. proteus* to use its cytoplasmic extensions for locomotion, thus making more cytoplasm available for efficient phagocytosis.

In conclusion, chemoreceptors may be important for sensing *T. pyriformis* and perhaps other prey at a distance. If the amoebae are not locomoting toward the target, however, they are more readily able to phagocytose prey that is detected by direct contact via mechanoreceptors.

LITERATURE CITED

Aderem A, Underhill D. 1999. Mechanisms of phagocytosis in macrophages. Ann Rev Immunol 17: 593-623.

Aplin AE, Howe A, Alahari SK, Juliano RL. 1998. Signal transduction and signal modulation by cell adhesion receptors: The role of integrins, cadherins immunoglobulin-cell adhesion molecules, and selectins. Pharma Rew 50: 197-263.

Apodaca G. 2002. Modulation of membrane traffic by mechanical stimuli. Am J Physiol Renal Physiol 282: F179-190.

Aronoff DM, Canetti C, Peters-Golden M. 2004. Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. J Immunol 173: 559-565.

Bacabac RG, Smit TH, Van Loon JJWA, de Blieck-Hogervorst JMA, Semeins CM, Doulabi BZ, Helder M, Klein-Nulend J. 2006. Bone cell responses to high-frequency vibration stress: does the nucleus oscillate within the cytoplasm? In: Bone Cell Mechanosensitivity and Microgravity. P105-125. The Dutch Experiment Support Center. Home page <http://www.desc.med.vu.nl/Frames.htm>. Accessed 2006 Apr 3.

Barry SR, Broughton ED, Soden EM. 2005. 2-APB, an IP3 receptor blocker, inhibits cytoplasmic streaming in Amoeba proteus. (Submitted paper).

Beningo KA, Wang Y. 2002. Fc-receptor-mediated phagocytosis is regulated by mechanical properties of the target. J Cell Sci 115: 849-856.

Berridge MJ, Irvine RF. 1989. Inositol phosphates and cell signaling. Nature 341: 197-205.

Brandt P, Pappas GD. 1960. An electron microscopic study of pinocytosis in amoeba. J Biophys Biochem Cytol 8: 675-678.

Cao J. 2005. Doctoral dissertation: The regulation and role of stress-activated protein kinases (p38 and JNK) in neuronal cell death. Finland: Kuopio University.

Caron E, Hall A. 1998. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science 282: 1717-1721.

Carson JA, Wei L. 2000. Integrin signaling's potential for mediating gene expression in hypertrophying skeletal muscle. J Appl Physiol 88: 337-343.

Christiansen RG, Marshall JM. 1965. A study of phagocytosis in the amoeba *Chaos Chaos*. J Cell Biol 25:443-457.

Cohen CJ, Bacon R, Clarke M, Joiner K, Mellman I. 1994. *Dictyostelium* discoideum mutanta with conditional defects in phagocytosis. J Cell Biol 126: 955-966.

Erban R, Othmer HG. 2004. From individual to collective behavior in bacterial chemotaxis. SIAM J Appl Math 65: 361-391.

Fluck M, Carson JA, Gordon SE, Ziemiecki A, Booth FW. 1999. Focal adhesion proteins FAK and paxillin increase in hypertrophied skeletal muscle. J Physiol 277: C152-162.

Furukawa R, Maselli A, Thomson SAM, Lim RWL, Stokes JV, Fechheimer M. 2003. Calcium regulation of actin crosslinking is important for function of the actin cytoskeleton in Dictyostelium. J Cell Sci 116: 187-196.

Garcia-Anoveros J, Corey DP. 1997. The molecules of mechanosensation. Ann Rew Neurosci 20: 567-94.

Gebbie L, Benghezal M, Cornillon S, Froquet R, Cherix N, Malbouyres M, Lefkir Y, Grangeasse C, Fache S, Dalous J, Bruckert F, Letourneur F, Cosson P. 2004. Phg2, a Kinase involved in adhesion and focal site modeling in *Dictyostelium*. Mol Biol Cell 15: 3915-3925.

Giancotti FG, Ruoslahti E. 1999. Integrin Signaling. Science 285: 1028-1032.

Gollnick F, Meyer R, Stockem W. 1991. Visualization and measurement of calcium transients in *Amoeba proteus* by furan- 2 fluorescence. Eur J Cell Biol 55:262-271.

Gomperts BD, Kramer IM, Tatham PER. 2002. GTP-binging proteins and signal transduction. In: Signal Transduction. California: Academic Press p71-126.

Grebecki A. 1981. Effects of localized photic stimulation on amoeboid movement and their theoretical implications. Eur J Cell Biol 24:163-175.

Grebecki A, Klopocka W. 1981. Functional interdependence of pseudopodia in *Amoeba proteus* stimulated by light-shade difference. Cell Sci 50: 245-258.

Gresham HD, Zheleznyak A, Mormol JS, Brown EJ. 1990. Studies on the molecular mechanisms of human neutrophil Fc receptor-mediated phagocytosis. J Biol Chem 265: 7819-7826.

Gudi SRP, Lee AA, Clark CB, Frangos JA. 1998. Equibiaxial strain and strain rate stimulate early activation of G proteins in cardiac fibroblasts. Am J Physiol 274: C1424-1428.

Han K, Lee H, Shin MH, Shin H, Im K, Park S. 2004. The involvement of an integrin-like protein and protein kinase C in amoebic adhesion to fibronectin and amoebic cytotoxicity. Parasitol Res 94: 53-60.

Hennessey TM, Kim DY, Oberski DJ, Hard R, Rankin SA, Pennock DG. 2002. Inner Arm dynein 1 is essential for Ca^{++}-dependent ciliary reversals in *Tetrahymena thermophila*. Cell Motil Cytoskeleton 53: 281-288.

Herrmann L, Erkelenz M, Aldag I, Tiedtke A, Hartmann MWW. 2006. Biochemical and molecular characterization of *Tetrahymena thermophila* extracellular cysteine proteases. BMC Microbiol 6: 1471-2180.

Hichikawa T, Cheung JY, Yelamarty RV, Knutson DW. 1991. Calcium Transients during Fc receptor-mediated and nonspecific phagocytosis by murine peritoneal macrophages. J Cell Biol 115: 59-66.

Hille, B. 2001. Evolution and origins. In: Ion Channels of Excitable Membranes. 3rd ed. Massachusetts: Sinauer Associates, INC. p 693-722.

Ito T, Ueda MJ, Okada TS, Ohnishi S. 1981. Phagocytosis by macrohages. II. the dissociation of the attachment and ingestion steps. J Cell Sci 51: 189-201.

Janson LW, Taylor DL. 1993. In vitro models of tail contraction and cytoplasmic streaming in amoebaid cells. J Cell Biol 123:345-356.

Juliano RL. 2002. Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. Ann Rev Pharmacol Toxicol 42: 283-323.

Klopocka W, Redowicz MJ. 2003. Effect of Rho family GTP-binding proteins on *Amoeba proteus*. Protoplasma 220: 163-172.

Klopocka W, Redowicz MJ. 2004. Rho/Rho-dependent kinase affects locomotion and actin-myosin II activity of *Amoeba proteus*. Protoplasma 224: 113-121.

Klopocka W, Moraczewska J, Redowicz MJ. 2005. Characerisation of the Rac/PAK pathway in *Amoeba proteus*. Protoplasma 225: 77-84.

Korn ED, Weisman RA. 1967. Phagocytosis of latex beads by *Acanthamoeba*. II. Electron microscopic study of the initial events. J Cell Biol 34:219-227.

Krauss G. 2001. Structure of signaling pathways. In: Biochemistry of Signal Transduction and Regulation. 2nd ed. New York: John Wiley and Sons. p 121-147.

Kuroda K, Yoshimoto Y, Hiramoto Y. 1988. Temporal and spatial localization of Ca^{2+} in moving *Amoeba proteus* visualized with aequorin Protoplasma. 144:64-67.

Lad PM, Olson CV, Smiley PA. 1985. Association of the N-formyl-Met-Leu-Phe receptor in human neutrophils with a GTP-binding protein sensitive to pertussis toxin. Proc Natl Acad Sci USA 82: 869-873.

Malek AM, Izumo S. 1996. Mechanism of endothelial cell shape change and cytoskeletal remodeling in response to fluid shear stress. J Cell Sci 109: 713-726.

Maniotis AJ, Chen CS, Ingber DE. 1997. Demonstration of mechanical connections between integrins, cytoskeletal filaments, and uncleoplasm that stabilize nuclear structure. Proc Natl Acad Sci US 94: 849-854.

Martin DK, Bootcov MR, Campbell TJ, French PW, Breit SN. 1995. Human macrophages contain a stretch-sensitive potassium channel that is activated by adherence and cytokines. J Membr Biol 147: 305-315.

Mayer-Scholl A, Averhoff P, Zychlinsky A. 2004. How do neutrophils and pathogens interact? Curr Opini Microbiol 7: 62-66.

Mikuni-Takagaki Y. 1999. Mechanical responses and signal transduction pathways in stretched osteocytes. J Bone Miner Metab 17: 57-60.

Noegel AA, Schleicher M. 2000. The actin cytoskeleton of *Dictyostelium*: a story told by mutants. J Cell Sci 113: 759-766.

Pfizer Inc. Handout for product information: Prostin E2®. Home page <http://www.pfizer.com/pfizer/main.jsp>. Accessed 2006 Apr 3.

Prusch RD. 1986. Calcium and initial surface binding phase of pinocytosis in *Amoeba proteus*. Am Physiol Soc.

Prusch RD, Goette S, Haberman P. 1989. Prostaglandis may play a signal-copling role during phagocytosis in *Amoeba proteus*. Cell Tissue Res 255:553-557.

Prusch RD, Joseph BC. 1987. Peptide stimulation of phagocytosis in *Amoeba proteus*. Cell Tissue Res 250:589-593.

Prusch RD, Minck DR. 1985. Chemical stimulation of phagocytosis in *Amoeba proteus* and the influence of external calcium. Cell Tissue Res 242:557- 564.

Prusch RD, Vedovatti PA. 1989. Calcium movements associated with peptide induced phagocytosis in *Amoeba proteus*. J Comp Physiol B 159:287-292.

Raeder EMB, Mansfield PJ, Hinkovska-Galcheva V, Shayman JA, Boxer LA. 1999. Syk activation initiates downstream signaling events during human polymorphonuclear leukocyte phagocytosis. J Immunol 163: 6785-6793.

Robledo T, Cruz-Vera J, Reyes M, de la Garza M, Ortega A, Salazar EP. 2005. *Entamoeba histolytica*: focal adhesion kinase and Erk phosphrylation are altered in the cytoskeleton mutant BG-3. Parasitol Res 95: 231-235.

Samaj J, Baluska F, Hirt H. 2004. From signal to cell polarity: mitogen-activated protein kinases as sensors and effectors of cytoskeleton dynamicity. J Exp Botany 55: 189-198.

Samaj J, Baluska F, Voigt B, Schlicht M, Volkmann D, Menzel D. 2004. Endocytosis, actin cytoskeleton, and signaling. Plant Phyciol 135:1150-1161.

Shiratsuchi H, Basson MD. 2005. Activation of p38 MAPKα by extracellular pressure mediates the stimulation of macrophage phagocytosis by pressure. Am J Physiol Cell Physiol 2008: C1083-1093.

Shiratsuchi H, Basson MD. 2004. Extracellular pressure stimulates macrophage phagocytosis by inhibiting a pathway involving FAK and ERK. Am J Physiol Cell Physiol 286: C1358-1366.

Smythe E, Warren G. 1991. The mechanism of receptor-mediated endocytosis. Eur J Biochem 202: 689-699.

Soden ME. 2005. Senior honors Thesis: The role of calcium in amoeboid movement: Results with fluorescent indicator dyes. Mount Holyoke College.

Stephens L, Ellson C, Hawkins P. 2002. Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. Curr opini Cell Biol 14: 203-213.

Tani, T, Naitoh, Y. 1999. Chemotactic responses of *Dictyostelium discoideum* amoebae to a cyclic AMP concentration gradient: evidence to support a spatial mechanism for sensing cyclic AMP. J Exp Biol 202:1-12.

Taylor DL, Blinks JR, Reynolds G. 1980. Contractile basis of ameboid movement VIII. Aequorin luminescence during ameboid movement, endocytosis, and capping. J Cell Biol 86: 599-607.

Thompson GA, Baugh LC, Walker, FL. 1974. Nonlethal deciliation of tetrahymena by local anesthetic and its utility as a tool for studying cilia regeneration. J Cell Biol 61:253-257.

Vogel GV, Thilo L. Schwarz H, Steinhart R. 1980. Mechanism of phgocytosis in *Dictyostelium discoideum*: Phgocytosis is mediated by different recognition sites as disclosed by mutants with alterd pagocytotic properties. J Cell Biol 86: 456-465.

Vossebeld PJM, Homburg HE, Schweizer RC, Ibarrola I, Kessler J, Koenderman L, Roos D, Verhoeven AJ. 1997. Tyrosine phosphorylation-dependent activation of phosphatidylionsitide 3-kinase occurs upstream of Ca^{2+}-signalling induced by Fcγ receptor cross-linking in human neutrophils. Biochem J 323: 87-94.

Wang Y, Liu J, Segall JE. 1998. MAP kinase function in amoeboid chemotaxis. J Cell Sci 111: 373-383.