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THE ROLE OF PLASMA MEMBRANE CALCIUM ATPASE AND ITS ASSOCIATION WITH LIPID RAFTS IN CHEMOATTRACTION IN PARAMECIUM

A Thesis Presented

by

Yunfeng Pan

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirement for the Degree of Master of Science Specializing in Biology

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Abstract

Paramecium, a unicellular ciliate, can be attracted by various chemical stimuli. Chemoattractants such as glutamate, folate, cAMP, and acetate activate different receptor mediated signal transduction pathways. The final event in these signal transductions is a hyperpolarization of membrane potential, which makes Paramecium swim smoothly and fast. There is evidence that the effector of this hyperpolarization is the plasma membrane calcium ATPase (PMCA), that when activated, expels Ca^{2+} from the cell. In Paramecium three PMCA isoforms, named PMCA2, 3, and 4, have been cloned. PMCA2 is associated with lipid rafts, which is demonstrated by its resistance to cold detergent solubilization and distribution in sucrose density gradients in ultracentrifugation. PMCA3 and 4 are not associated with lipid rafts. On the cell surface, PMCAs are localized to the bases of cilia. Sterol-depletion by methyl-ß-cyclodextrin (MßCD) treatment disrupts the distribution of PMCA2 in sucrose density gradients and ciliary base-localization on the cell surface. MßCD treatment also decreases the chemoattraction to glutamate and cAMP. This indicates that PMCA2 and its association with lipid rafts are essential in the chemoattraction signal transduction pathways. Based on these results, a model of membrane domains incorporating three signal transduction pathways is proposed.
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Chapter 1

Comprehensive Literature Review
I. Paramecium

A. General introduction of Paramecium

Paramecium is a unicellular ciliated Protozoa, living in fresh water. Paramecium employs both typical eukaryotic organelles such as mitochondria, ribosome, endoplasmic reticulum, Golgi apparatus, an endocytic system and lysosome system, and nuclear systems and some specialized structures such as the contractile vacuole complexes, the alveolar system, the trichocysts, the buccal cavity and the cytoproct in growth, metabolism, catabolism and reproduction to maintain life (Allen, 1988).

The life cycle of Paramecium consists of both asexual phase, binary fission, and sexual phase, conjugation and autogamy (Vivier, 1974).

Paramecium is 80 μm - 350 μm in length and 40 μm - 80 μm in width according to species (Vivier, 1974). The cell body is coated with 5000-6000 cilia (Preston and Saimi, 1990), each of which is covered by a membrane. Although the ciliary membranes and the somatic membranes are physically continuous, the molecular components and biochemical composition are different, which reflects the specialized functions of these two surfaces (Allen, 1988). The cilia have important roles in the life of Paramecium due to their responsibilities for locomotion in water, ingestion of food and conjugation.

Like other ciliates, Paramecium has a dual nuclear system. There is one larger macronucleus and variable number of small micronuclei. They share a common cytoplasm. Only the macronucleus is active during transcription and
essential for the survival and reproduction of the cell. It contains genomic DNA, produces both ribosomal RNA and messenger RNA for protein synthesis, and governs the cytoplasmic and nuclear physical activities. The micronucleus serves as a germinal nucleus, which generate gametic nuclei to form zygotic nucleus after conjugation (Mikami, 1988).

*Paramecium* uses TGA as a stop codon in protein translation, and the other two universal stop codons TAA and TAG encode glutamine or glutamic acid respectively (Freiburg, 1988).

One special property of *Paramecium* is that it contains an excitable membrane that can be depolarized and hyperpolarized. The change in membrane potential results in observable changes of *Paramecium* swimming pattern. When the cell membrane is depolarized to threshold, an action potential occurs. Consequently, *Paramecium* swims backward transiently during the depolarization. After repolarization the cell swims forward again in a new direction. When the cell membrane is hyperpolarized, the *Paramecium* swims fast and smoothly with few turns (Preston and Saimi, 1990).

Another property of *Paramecium* is chemosensory response. *Paramecium* is capable to detect changes in a variable environment including chemicals, temperature, pH, ionic composition and food (bacteria). When *Paramecium* senses harmful chemicals, unfavorable ion concentration, or extreme pH condition, it swims backward for a short distance, followed by swimming forward again in a new direction. This response is called chemorepulsion. When the
*Paramecium* senses bacterial metabolites such as glutamate, folate or acetate that signal its food source, it increases swimming speed toward these chemicals. This response is called chemoattraction (Van Houten, 1998). Chemical stimuli resulting in repulsion or attraction of *Paramecium* are based on changing the membrane potential of the cell. During chemorepulsion, membrane potential is depolarized; and during the chemoattraction, is hyperpolarized.

**B. Membrane Potential and Swimming Behavior**

*Paramecium* lives in fresh water. In the laboratory, the condition of fresh water with low ion concentration is mimicked by a solution containing 1 mM K\(^+\) and 1 mM Ca\(^{2+}\), resulting in a resting membrane potential of \(-30\) to \(-40\) mV (Machemer, 1988). The resting membrane potential of *Paramecium* is maintained through two essential ion concentration batteries, Ca\(^{2+}\) and K\(^+\). Ca\(^{2+}\) has an inward-directed concentration gradient making for Ca\(^{2+}\) influx. K\(^+\) has an outward-directed concentration making for K\(^+\) efflux (Machemer, 1988). Any changes of Ca\(^{2+}\) or K\(^+\) in extracellular milieu cause a dramatic and direct effect on the resting membrane potential and upon then swimming behavior.

The membrane potential controls the activity of ion conductance and enzymes in the cilia, which finally determine the patterns of ciliary beating. The swimming behavior of *Paramecium* depends on two characteristic elements of cilia: the beating frequency and the beating direction. The membrane potential can affect them both.
When the membrane potential undergoes a sufficient depolarization in response to certain cations, mechanical stimulation or organic repellent, a Ca\(^{2+}\) action potential is triggered resulting from activation of voltage-gated Ca\(^{2+}\) channels that are localized in the ciliary membrane (Preston and Saimi, 1990). Ca\(^{2+}\) enters the cell through voltage-gated Ca\(^{2+}\) channels to increase the internal free Ca\(^{2+}\) from 10\(^{-8}\) M to 10\(^{-6}\) M (Wright, 1990). The increase of Ca\(^{2+}\) causes the axoneme to beat more rapidly in reverse direction, but the mechanism is still unknown (Preston and Saimi, 1990). Consequently, *Paramecium* swims backward. This voltage-gated Ca\(^{2+}\) channel in *Paramecium* shares the similarity with those in higher organisms in that it can be inactivated by the influxed Ca\(^{2+}\). But it is sensitive to an anticalmodulin agent, W-7 [N-(6 aminohexyl)-5-chloro-1-naphthalenesulphonamide] that inhibits the voltage-gated Ca\(^{2+}\) current reversibly as well as backward swimming (Hennessey and Kung, 1984). The duration of depolarization and backward swimming is normally transient. After each action potential, the cell will be repolarized by K\(^{+}\) conductance through voltage-gated K\(^{+}\) channels, and the increased Ca\(^{2+}\) will be removed. The membrane potential returns to the resting level and *Paramecium* swims forward again. If the depolarization-triggered Ca\(^{2+}\) action potential occurs in Na\(^{+}\)-containing medium, the depolarization will be prolonged by the influx of Na\(^{+}\) through the Ca\(^{2+}\)-dependent Na\(^{+}\) channels, which leads a longer backward swimming. This results in the activation of Ca\(^{2+}\)-dependent K\(^{+}\) channels that also make a contribution in the repolarization of the membrane potential (Ramanathan et al., 1988).
Paramecium also responds to the hyperpolarization of membrane potential. As a result of hyperpolarization of membrane potential resulting from a K⁺ efflux, the cilia beat more frequently in a posterior direction and the cell swims forward fast and smoothly with few turns. Hyperpolarization activates voltage-gated K⁺ channels (Preston and Saimi, 1990). The biochemical consequence of hyperpolarizing membrane potential is the activation of adenylyl cyclase and the increase of the concentration of intraciliary cAMP, which stimulates cAMP-dependent protein kinases to phosphorylate a number of ciliary proteins (Bonini et al., 1986). With these phosphorylated ciliary proteins, the cilia beat with increased frequency in a more posterior direction, and the cell swims forward faster. During chemoattraction, Paramecium exhibits the hyperpolarization of membrane potential.

C. Chemoattraction of Paramecium

Paramecium can be attracted by different types of chemical stimuli. Chemicals such as acetate, biotin, cyclic AMP, folate, glutamate, and NH₄Cl are known as chemoattractants of Paramecium. These chemoattractants are bacterial metabolites, which signal the food source of Paramecium. It is proposed that there are three signal transduction pathways for chemoattractants, all of which can lead to hyperpolarization of membrane potential and change in swimming pattern (Van Houten, 1998). These three signal transduction pathways in chemoattraction are illustrated in figure A (Fig. A)
In pathway 1, the attractants can be acetate, biotin, cyclic AMP or folate. Through binding kinetics studies, behavioral studies and electrophysiology studies, it is found that there are specific binding sites, namely receptors, on the cell surface for each attractant (Sasner and Van Houten, 1989; Van Houten et al., 1991; Bell et al., 1998). Recently, the genes of the cyclic AMP receptor and the folate receptor in Paramecium have been cloned (Valentine et al., 2005). Binding of each attractant to its receptors can lead Paramecium to hyperpolarize 8-10 mV (Van Houten, 2000). It is believed that a Ca$^{2+}$ conductance generated by the plasma membrane Ca$^{2+}$ pump (PMCA) may contribute to this sustained hyperpolarization. Neither Na$^+$ nor K$^+$ current is involved, because there is no reversal potential or removal of Na$^+$ or K$^+$ from extracellular medium has no effect on the attractant-induced hyperpolarization (Preston and Van Houten, 1987). This result also excludes a Na$^+$/Ca$^{2+}$ exchanger to generate the Ca$^{2+}$ conductance (Preston and Van Houten, 1987). It has been demonstrated that a Ca$^{2+}$ ATPase possesses all the peculiar PMCA properties in cell pellicle membranes (Wright and Van Houten, 1990). Genes of three PMCA isoforms have been cloned (Elwess and Van Houten, 1997; Gannon-Murakami, 2004). There are two pieces of evidence to support the proposal that the Ca$^{2+}$ conductance is generated by the activation of PMCA to hyperpolarize membrane potential during chemoattraction (Wright et al., 1992). First, Li$^+$ can inhibit Ca$^{2+}$ pump ATPase activity. Paramecium treated with 10 mM LiCl for 1 hour shows decreased rate of Ca$^{2+}$ efflux (Wright et al., 1992). Li$^+$ treatment also disrupts the
chemoattraction to acetate, cyclic AMP, or folate (Wright et al., 1992). Second, a mutant of Paramecium, K-shy, has a defective Ca^{2+} extrusion mechanism (Evans et al., 1987) is not attracted by the same attractants (Wright et al., 1992). However, second messengers such as cAMP, cGMP or IP_{3} have been not identified in this pathway. The mechanism of coupling receptors to Ca^{2+} pumps is still unknown.

In pathway 2, the chemoattractant is glutamate. The glutamate chemoreceptor has been characterized through binding assays and behavioral studies (Yang, 1995). The glutamate receptor gene has been cloned (Jacobs, 2007). After glutamate binds to a surface receptor, a transient K^{+} conductance and sustained hyperpolarization are observed (Preston and Usherwood, 1988). Additionally, the intracellular cAMP level increases by 3-fold in 30 milliseconds and by 7-fold in 150 milliseconds (Yang, 1997). One K^{+} channel in Paramecium is associated with an adenylyl cyclase, which converts ATP to cAMP (Shultz et al., 1992). A mutant of Paramecium, restless, which is oversensitive to low extracellular K^{+} and lacks control of K^{+} resting conductance, has a defect in cAMP formation (Saimi and Kung, 1987; Shultz et al., 1992). These results indicate that a hyperpolarization-activated K^{+} conductance directly regulates the cAMP formation in Paramecium. It is possible that the increased cAMP activates cAMP-dependent protein kinases (PKA), which is known to activate the PMCA in mammalian system (James, 1989). The existence of PKA in Paramecium has been proven (Hochstrasser and Nelson, 1989). In Paramecium there is no direct
evidence that PKA phosphorylates the PMCA \textit{in vivo}. However, addition of cAMP or PKA increases the Ca$^{2+}$ ATPase activity on cell membrane preparations (Yang, 1997). The calmodulin-binding domains (CBD) in \textit{Paramecium} PMCA$s$ are substrates of PKA \textit{in vitro} (Gannon-Murakami, 2004). Furthermore, the general kinase inhibitors H7 and H8 were shown to disrupt the chemoattraction to glutamate (Yang, 1997). All these results strongly indicate that glutamate signals by increasing the intracellular cAMP and activating PKA, which phosphorylates the CBDs of PMCA and activates the Ca$^{2+}$ pump to generate a sustained hyperpolarization conductance by extruding Ca$^{2+}$.

In pathway 3, the only identified stimulus is NH$_4$Cl. No receptor is required in this pathway. NH$_4$Cl can be equilibrated with NH$_3$ to diffuse across the membrane and rapidly alkalinize the inside of cells, which can be monitored by using a sensitive fluorescent dye (Davis et al., 1998). It is proposed that the hyperpolarization in this pathway results from the activation of a pH sensitive cation channel to generate the hyperpolarization conductance (Van Houten, 1998).
II. Plasma Membrane Ca\(^{2+}\) ATPase

A. General properties of PMCA

Plasma membrane Ca\(^{2+}\)-ATPases (PMCA or Ca\(^{2+}\) pumps) are well characterized Ca\(^{2+}\) expulsion system in eukaryotic cells. Working along with Na/Ca exchangers, PMCAs extrude from the cell the elevated intracellular Ca\(^{2+}\) that occurred during Ca\(^{2+}\) signaling. Due to the high affinity for Ca\(^{2+}\) (the K\(_m\) can be 0.2 \(\mu\)M at the activated state), PMCAs can interact with Ca\(^{2+}\) at very low concentration, which makes PMCAs responsible for continuous and accurate regulation of intracellular Ca\(^{2+}\) and maintenance of Ca\(^{2+}\) homeostasis (Carafoli, 1991a and b).

PMCA is a member of the P-type family of ion-transporting ATPases, which are attributed by the formation of a phosphoenzyme intermediate during the reaction cycle. The mechanism of Ca\(^{2+}\) transport by PMCAs is illustrated in figure B (Fig. B). In the presence of Ca\(^{2+}\), an aspartic acid residue in PMCA will be phosphorylated by ATP to form the aspartyl phosphate intermediate. This reaction can be accelerated by the presence of Mg\(^{2+}\). There are two conformers of the PMCA intermediate that are termed as E1 (Ca\(^{2+}\) Bound) and E2 (Ca\(^{2+}\) free) in the catalytic reaction cycle. In the state of E1, the Ca\(^{2+}\) binding site is toward the cytosolic side; in the state of E2 that is toward the extracellular side. The translocation of Ca\(^{2+}\) across the plasma membrane is correlated to the conformational transition from the high-energy form E1~P to the low-energy form E2-P. The stoichiometry of Ca/ATP per reaction cycle is one. There are two
classic inhibitors of PMCA. La\(^{3+}\) inhibits PMCA by stabilizing the phosphoenzyme resulting from inhibiting the hydrolysis of aspartyl phosphate. Vanadate inhibits PMCA by blocking the formation of the aspartyl phosphate (Jencks, 1989; Carafoli, 1991a and b; Stokes, 2003).

PMCA consists of 10 transmembrane domains (TM1-TM10) (Wang, 1992). Both NH\(_2\)-terminus and COOH-terminus are in the cytosol. Connecting TM2 and TM3 is the first intracellular loop, called an actuator domain, which contains one acidic phospholipid sensitive region. The second intracellular loop, containing the catalytic domain, connects TM4 and TM5, which include the aspartyl phosphate site (aspartic acid residue), and the ATP binding site (lysine residue) (Wang, 1992; Monteith, 1995; Penniston, 1998). Most of the regulatory domains reside in the region between TM10 and COOH-terminus, which includes calmodulin binding domain, protein kinase activating sites, PDZ binding domains and protease cleavage sites (Carafoli, 1994; Strehler, 2001).

PMCA are encoded by 4 separate genes in mammalian systems. Four major PMCA isoforms (PMCA1-4) are expressed in cells (Monteith, 1995; Strehler, 2001). PMCA1 and PMCA4 are widely expressed in most adult tissues; however, PMCA2 and PMCA3 have a limited tissue distribution. PMCA2 is predominantly expressed in brain and lactating mammary glands (Strehler, 2004). The expression of PMCA3 is abundant in rat skeletal muscles. Extra PMCA variants are generated through alternative mRNA splicing of the 4 primary PMCA isoforms. There are two splicing sites in the PMCA gene transcript. One (splicing
site A) is located in the first cytosolic loop, just before the acidic phosphoslipid activating site; the other (splicing site C) is in the calmodulin binding domain (Strehler, 2001).

B. Regulation of PMCA

As an intracellular Ca\(^{2+}\) tuner, being essential in exporting the increased Ca\(^{2+}\) and maintaining the low resting level of intracellular Ca\(^{2+}\), PMCAs are multifariously regulated in vivo. Different methods of regulating PMCAs include interactions with calmodulin, phospholipids, protein kinases, G-proteins, oligomerization and localization in lipid rafts (Wang et al., 1992; Carafoli, 1994; Monteith, 1995).

a. Activation by Calmodulin

The activation of PMCA by calmodulin has been comprehensively studied. In the inactive state, PMCA is autoinhibited by the interaction between the calmodulin binding domain and two regions in actuator and catalytic domains respectively (Enyedi1989; Falchetto, 1991; Falchetto et al., 1992). Due to the blockade of COOH-terminal domain at the catalytic domain in the second intracellular loop, the catalytic reaction cannot occur. When calmodulin binds to the COOH-terminal autoinhibitory domain, the intramolecular interaction is dissociated and the autoinhibition is released resulting in the active state of PMCA (Carafoli, 1992; Strehler 2001). The interaction with calmodulin increases Ca\(^{2+}\) affinity, which deceases \(K_m\) of Ca\(^{2+}\) from 10-20 \(\mu\)M to 0.4-0.7\(\mu\)M, and raises
the maximum velocity ($V_{\text{max}}$) of the catalytic reaction by tenfold (Wang, 1992; Carafoli, 1994).

One alternative splicing site resides in the calmodulin binding domain. The alternative splicing affects the activation of PMCA by calmodulin (Strehler 2001). The calmodulin sensitivity of PMCA is isoform-dependent. PMCA2a shows the highest affinity of calmodulin whose dissociation constant ($K_d$) is $\sim 2$ nM (Elwess et al., 1997). Among PMCA4 isoforms, the $K_d$ of isoform 4b is 7.6 nM and that of isoform 4a is 125 nM (Enyedi, 1994; Caride et al., 1999).

b. Activation by acidic phospholipids

Besides the activation by calmodulin, PMCA can be stimulated by acidic phospholipids (APL), particularly phosphotidylinositol 4-phosphate and phosphotidylserine, and long-chain polyunsaturated fatty acids such as oleic acids (Carafoli, 1991a and b; Wang, 1992; Monteith, 1995). There are two acidic phospholipid binding regions in PMCA. One is located at the splicing site A in the actuator domain; the other is located at the splicing site C in the calmodulin binding domain (Brodin et al., 1992; Filoteo et al., 1992). The binding of APL to the calmodulin binding domain can make PMCA insensitive to calmodulin (Enyedi, 1987). The activation is like the activation by calmodulin increasing the $V_{\text{max}}$ by releasing the autoinhibition. The activation by APL in the actuator domain is calmodulin-independent, which can further activate PMCA by decreasing the $K_d$ of calmodulin activated PMCA from 0.4 μM to 0.2 μM (Wang, 1992; Monteith 1995; Strehler 2001).
c. Regulation by protein kinases

PMCA can be activated by cAMP-dependent protein kinase (PKA) (Caroni, 1981). In vitro, PKA phosphorylates a serine that is located between calmodulin binding domain and COOH-terminus. The phosphorylation by PKA can reduce the $K_m$ of the purified PMCA from 10 $\mu$M to 1.4 $\mu$M and raise the $V_{max}$ about twofold (James, 1989).

Protein kinase C can also phosphorylate PMCA (Smallwood, 1988). Threonine and serine residues from both calmodulin binding domain and its downstream region are the targets of the PKC (Wang et al., 1991; Enyedi, 1996). Phosphorylation within calmodulin binding domain reduces the binding of calmodulin. However, the downstream phosphorylation has no interference with calmodulin binding, which partially activate PMCA by increasing the $V_{max}$ of PMCA (Hofmann, 1994; Monteith, 1995). Tyrosine phosphorylation is also found on PMCA in human platelets. But this phosphorylation down-regulates PMCA activity (Dean, 1997).

d. Regulation by G-proteins

In liver cells, PMCA activity is indirectly regulated by G-proteins (Monteith, 1995). All the data suggests that the G-protein regulation results in inhibition of PMCA activity. PMCA can be inhibited by several hormones such as mini-glucagon, calitonin and human parathyroid hormone (Jouneaux, 1994). G-protein $\beta\gamma$ subunits increase the glucagon-sensitivity of PMCA resulting in inhibition of the basal activity of PMCA (Lotersztajn, 1992). In addition, $G_s\alpha$
subunit can transduce the signal of hormonal inhibition to PMCA (Jouneaux, 1993). Another G-protein regulated inhibition of PMCA results from the G-protein activated phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5 biphosphate (PIP$_2$) which can partially activate PMCA (Monteith, 1995; Strehler 1995).

e. Regulation by oligomerization

PMCA can be activated by oligomerization (Kosk-Kosicka, 1988). The self-association (dimerization) of PMCA is mediated through the calmodulin-binding domain. When the concentration of PMCA reaches 25 nM, the activation of PMCA is calmodulin-independent (Kosk-Koska, 1989; Wang, 1992; Monteith, 1995). A Ca$^{2+}$-dependent protease, calpain, can truncate the calmodulin domain in PMCA, resulting in a constitutively active form of PMCA. This truncated PMCA cannot form the dimer any longer (Vorherr, 1991).

f. Localization in lipid rafts

PMCA was first found localized in caveolae (Fujimoto, 1993), a subtype of lipid rafts. In proximal kidney tubules, the activation of PMCA is dependent on the exclusive localization of PMCA in caveolae (Tortelote, 2004). Recently, in primary cultured neurons, PMCA was localized in lipid rafts (Sepulveda, 2006). The localization in lipid rafts raises the total PMCA activity compared with the non-raft PMCAs. Raft-disruption by cholesterol depletion can reduce the raft-associated PMCA activity (Jiang, 2007). All these results indicate that the
localization in lipid rafts can be a new regulatory mechanism of PMCA activity and further intracellular Ca\(^{2+}\) signaling.

**C. Protein-protein interactions of PMCA**

Protein-protein interactions of PMCA have been studied extensively. The b splicing isoforms of PMCA contain PDZ (PSD-95/Dlg/ZO-1)-binding domain at COOH-terminus that can interact with PDZ-domain containing proteins (Strehler, 2001). A few protein interaction partners of PMCAS have been identified such as nitric oxide synthase I (NSO I) (Schuh, 2001), Na\(^{+}/H^{+}\) exchanger regulatory factor 2 (NHERF2) (DeMarco, 2002), cytoskeleton (Zabe, 2001), Ca\(^{2+}/\)calmodulin – dependent membrane associated kinase (CASK) (Schuch, 2003) and membrane-associated guanylate kinase (MAGUK) (Kim, 1998; DeMarco, 2001). The interaction of PMCA with NSO I or NHERF 2 may provide cross-talk between Ca\(^{2+}\) signaling and other cellular signal transductions. While the interaction with cytoskeleton, CASK, or MAGUK can recruit PMCA in a specific domain in plasma membrane to regulate the local Ca\(^{2+}\) signaling in a microenvironment.

In addition to PDZ-binding domain at COOH-terminus, other domains in PMCA also participate in protein interactions. The catalytic domain in the second intracellular loop of PMCA4b interacts with tumor suppressor RAS-associated Factor 1(Armesilla, 2004). This interaction indicates that PMCA may be involved Ca\(^{2+}\) regulation in apoptosis signaling. Another protein interaction domain in
PMCA is NH$_2$-terminus of PMCA4, which interacts with 14-3-3 ε protein. This interaction inhibits PMCA activity (Rimessi, 2005).

D. PMCA in Paramecium

PMCA activity was first biochemically characterized in Paramecium. In the presence of Mg$^{2+}$, the K$_m$ of Ca$^{2+}$ is 90 nM (Wright, 1990). Three PMCA isoforms (2-4) have been cloned, and 20 more were found in annotated Paramecium genome (Genoscope). The calculated molecular weight of the three cloned PMCA isoforms (2-4) are listed in table A. Paramecium PMCAs share the characteristic properties with the counterparts in mammalian cells. However, Paramecium PMCAs contain a shorter calmodulin domain (16 amino acids) compared with that (28 amino acids) of mammalian PMCAs (Elwess, 1997; Penniston, 1998). The protein sequences of calmodulin-binding domain (CBD) from PMCA2, 3 and 4 show a very high homology. Their alignment is showed in table B. Two serines from calmodulin-binding domain of Paramecium PMCAs can be phosphorylated by PKA and PKC in vitro (Gannon-Murakami, 2004). An antibody has been generated against the CBD form PMCA2, which is referred to as anti-CBD. Since the CBDs show high homology among PMCA isoforms, several CBDs from different PMCA isoforms were tested to determine whether these CBDs can be recognized by anti-CBD in Western Blot. It has been found that CBDs from PMCA2, 3,4,18 and possibly 19 (PMCA18 and 19 have the same protein sequence of CBD) can be recognized by anti-CBD. The results are
summarized in table C (table C). Based on the putative amino acid sequence, the PMCA protein structure is developed (Fig. C). The PMCAs may be involved in chemoattraction in *Paramecium* by regulating Ca\(^{2+}\) efflux to generate the attractant-stimulated hyperpolarization. It has been found that PMCA2 overexpression leads to defective Ca\(^{2+}\) homeostasis and a defective chemoattraction to folate. PMCA3 overexpression results in not only the defective Ca\(^{2+}\) homeostasis, but also a significant disruption in chemoattraction to glutamate, acetate and folate (Dr. Yano, personal communication). All these results implicate PMCAs as an important element in the chemoattraction in *Paramecium*. 
III. Lipid Rafts

A. General concept of lipid rafts

Lipid rafts are small and specialized domains in cell membranes. The size of lipid rafts varies in studies using different biochemical and biophysical method, but the generally accepted size is 10-200 nm (Pike, 2006). Lipid rafts are compositionally heterogeneous. Lipid rafts contain high levels of saturated fatty acids, sterol, and sphingolipids. The extended saturated fatty acid chain and intercalated cholesterol make membranes a tightly packed structure in liquid-order phase compared to membranes filled with unsaturated fatty acid chain that are more fluid and liquid-disordered (Simons, 1997; Zurzolo, 2003; Munro, 2003). This special composition and structure of lipids makes lipid rafts resistant to detergent (Triton X-100) solubilization at 4°C (Simons, 1997). Lipid rafts are also referred to as detergent-resistant membrane (DRM) (Munro, 2003). The traditional method to recover lipid rafts is floatation of lipid rafts in a 5% to 40% linear sucrose density gradient through ultracentrifugation (Brown, 1992).

There is a specialized subtype of cholesterol-enriched lipid rafts, which forms “flask shaped” invaginations of membranes. These invaginated lipid rafts are termed caveolae (Simon, 1997; Anderson, 1998). The formation of caveolae is due to the self-association of caveolin that is a cholesterol-bound and acylated integral membrane protein (Simons, 1997).

Since cholesterol is the essential component of lipid rafts, any manipulation of content of cholesterol in membrane can affect the formation and
function of lipid rafts (Zajchowski, 2002). In studies, methyl-β-cyclodextrin (MβCD) that depletes cholesterol from membrane is a widely used drug to reduce the cholesterol level in membrane, which consequently disrupts lipid rafts (Zajchowski, 2002, Wang, 2006). MβCD is a cyclic oligosaccharide containing 7 glucopyranose units. MβCD works like a cholesterol chelator, which binds cholesterol and sequesters them out of membrane (Zidovetzki, 2007).

In addition to caveolin, there are different types of proteins that are preferentially associated with lipid rafts. The exemplary raft-associated proteins target into lipid rafts through various mechanisms. Some raft-associated proteins partition into lipid rafts through protein-lipid interaction, like hedgehog (cholesterol-bound), and annexin (phospholipids-bound) (Rajendran, 2005; Wang, 2006). Others through different post-translational modifications like folate receptors (glycosylphosphotidylinosito (GPI)-anchored), Src-family tyrosine kinases (dually acylated), flotillin (palmitoylated and myristoylated). These proteins can be used as markers for lipid rafts (Rajendran, 2005; Wang, 2006).

Lipid rafts can be associated with cytoskeleton proteins (Ikonen, 2001), which brings a new concept of lipid rafts. Lipid rafts float in top fractions in the linear sucrose density gradient due to their lighter density. When lipid rafts are associated cytoskeleton, lipid rafts show high buoyant density and float in the bottom fractions with high dense sucrose gradient (Nebal, 2002). These cytoskeleton-attached lipid rafts are referred as DRM with high buoyant density (DRM-H). Cytoskeleton protein F-actin and other scaffold proteins, such as fodrin,
α-actinin, vimentin, and supervillin, form a complex of membrane skeleton structure beneath lipid rafts, referred as membrane skeleton (Luna, 2005).

Lipid rafts are temporally and spatially dynamic (Pike, 2006). The formation of lipid rafts is mediated by the interactions between the incorporated lipids and proteins to cope with the cellular physiological activities.

**B. Function of lipid rafts**

Lipid rafts play roles in various cellular physiological activities. One of the essential and relatively well established roles of lipid rafts is mediating receptor-coupled signal transductions in cells, such as growth factor receptors, G-coupled receptors, and immune receptors (Zajchowski, 2002).

Several growth factor receptors have been demonstrated to have their signaling correlated with lipid rafts. Insulin receptor is preferentially localized in caveolae. Non-raft-associated insulin receptor will also be translocated in caveolae after ligand (insulin) stimulation (binding) (Pike, 2005). NGF receptors are also highly concentrated in caveolae, but their caveola-localization is constitutive and independent on ligand binding (Pike, 2003). EGF receptors and PDGF receptors are originally localized in lipid rafts. After the ligand binding, these receptors will be excluded from lipid rafts, and undergo endocytosis through clathrin-coated pits (Zajchowski, 2002). Together with receptors, signaling effectors such as Src-family kinases, adaptor proteins, MAP Kinases
are also recruited in lipid rafts to fulfill the signal transduction (Simons, 2000; Pike, 2005).

A great number of G protein-coupled receptors (GPCR) such as acetylcholine receptor, adrenergic receptor, dopamine receptor, Neurokinun receptor, purinergic receptor and serotonin receptor have been shown to localized in lipid rafts by anatomical and biochemical methods (Pike, 2003; Allen, 2006). After ligand stimulation, their translocation into lipid rafts make them readily interact with G proteins, Gαs, Gαq, Gβγ, and G-protein effectors, adenylyl cyclase and phopholipase C, which are concentrated in lipid rafts as well (Allen, 2006).

Lipid rafts are also involved in immune receptor signaling (Zajchowski, 2002). The typical example is B-cell receptor (BCR), whose translocation into lipid rafts is ligand (antigen) mediated. In the resting B-cells, individual BCR reside outside of lipid rafts. Once cross-linked by antigens, the oligomerized BCR will be translocated into lipid rafts, where they are phosphorylated by raft-associated Src-family kinase Lyn, commencing the signaling transduction. Furthermore, the separated lipid rafts with the activated BCRs will aggregate with the aid of cytoskeleton to form immune synapse in B-cells (Pierce, 2002). Other immune receptor signaling like T-cell receptor (TCR) and IgE receptor are similar to BCR signaling carried on in lipid rafts (Simons, 2000).

In summary, in signal transduction, lipid rafts serve as platforms that recruit receptors and effectors into a favorable condition to readily interact with
each other and efficiently transducer signals. By incorporating and excluding receptors and effectors, lipid rafts can temporally and spatially monitor signal transductions in a precise manner.

Lipid rafts are involved in membrane transport (Ikonen, 2001). Some proteins are endocytosed through translocation into lipid rafts (Parton, 2003). The endocytosis of cross-linked GPI-anchored proteins is carried on by internalization of caveolae other than clathrin-coated pits (Rajendran, 2005). EGF receptors and PDGF receptors first move out of lipid rafts after ligand binding (stimulation), then internalized in clathrin-coated pits (Zajchowski, 2002). Lipid rafts serve as a switch to turn off (down-regulating) EGF receptor and PDGF receptor signaling. Lipid rafts also mediate protein sorting. In the ER system and Golgi apparatus, raft structures are identified (Helms, 2004). In polarized epithelial cells, the association of apical GPI-anchored proteins with lipid rafts starts in the Golgi apparatus (Simons, 1997). The same sorting of GPI-anchored proteins in yeast happens in ER (Ikonen, 2001).

Lipid rafts may also be involved in governing the membrane potential and Ca\(^{2+}\) homeostasis. Voltage gated K\(^{+}\) channel (K\(_{\text{v}1.5}\)) is localized in caveolae. K\(_{\text{v}1.5}\) is a substrate of Src-family tyrosine kinases. Phosphorylation decreases its conductance (Marten, 2004). In addition, PMCA, Na/Ca exchange I (NCX I), and voltage-dependent Ca\(^{2+}\) channels are co-localized in caveolae in smooth muscle. It is proposed that caveolae with Ca\(^{2+}\) handling proteins work along with
peripheral Ca\(^{2+}\) store (sacoplasmic reticulum) to control local Ca\(^{2+}\) concentration (Daniel, 2006).

C. Lipid rafts in Paramecium

A model of lipid rafts has been characterized by biochemical criteria (Chandran, 2004). *Paramecium* lipid rafts share the general raft-properties: (i) they are resistant to cold, non-ionic detergent (Triton X-100) extraction, and (ii) enriched with cholesterol, glycosphingolipids, and GPI-anchored proteins (Chandran, 2004). In this model, it is proposed that lipid rafts are organized into two types according to the different buoyant densities in sucrose or Optiprep gradients. Ganglioside Gm, CaM-binding membrane-bound proteins and GPI-anchored surface antigens are the main components in the light-density lipid rafts. High-density lipid rafts are enriched with GPI-anchored folate binding proteins and PMCAs as well as GPI-anchored surface antigens (Chandran, 2004). Lipid rafts in *Paramecium* may be involved in chemosensory signal transductions. Depletion of cholesterol by MβCD can disrupt the chemoattraction to glutamate and folate. Lipid rafts may also serve as platforms to recruit receptors and downstream effectors in chemoattraction signal transductions.
Figure A: The three proposed signal transduction pathways in chemoattraction in *Paramecium*.

Pathway 1: The attractants can be acetate, biotin, folate, or cAMP. These attractants bind to their specific receptors on the cell surface and activate PMCA. The detail of this signal transduction pathway is still not clear.

Pathway 2: The attractant is glutamate. Glutamate binds to the glutamate receptor on the cell surface, which activates a $K^+$ conductance and adenylyl cyclase. Adenylyl cyclase converts ATP to cAMP, which activates protein kinase A (PKA). PKA activates PMCA by phosphorylation.

Pathway 3: The attractant is NH$_4$Cl. It diffuses across the membrane. A pH sensitive cation channel will be activated.
Figure B: The reaction cycle of the plasma membrane Ca\(^{2+}\) ATPase.

In the state of E1, the Ca\(^{2+}\) binding site is toward the cytosolic side; in the state of E2 that is toward the extracellular side. The translocation of Ca\(^{2+}\) across the plasma membrane is correlated to the conformational transition from the high-energy form E1~P to the low-energy form E2-P.
Figure B

E1 \(\xrightarrow{\text{ATP}}\) E1-ATP \(\xrightarrow{\text{Mg}^2+}\) E1-\(\text{Ca}^{2+}\sim\text{P}\) \\
\(\xleftarrow{\text{Ca}^{2+}}\) \(\xrightarrow{\text{ADP}}\) \(\xleftarrow{\text{Mg}^2+}\) \\
E2 \(\text{E2-P}\) \\
\(\xrightarrow{\text{Ca}^2+}\)
**Figure C: The proposed protein structure of *Paramecium PMCA.***

1-10, transmembrane helices; D-- P, aspartate acylphosphate site; K, lysine ATP binding site; P, proline hinge site. E (glutamate), N (aspargine) and D (aspartate) in transmembrane domains form a Ca2+ binding site. CBD, calmodulin binding site; SS, double serines phosphorylated by PKA and PKC in vitro.
Table A: The calculated molecular weight of the three cloned PMCA isoforms (2-4).

<table>
<thead>
<tr>
<th>Isoform</th>
<th>PMCA2</th>
<th>PMCA3</th>
<th>PMCA4</th>
</tr>
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<tr>
<td>M.W.</td>
<td>129 KD</td>
<td>125 KD</td>
<td>125 KD</td>
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</table>
Table B: The alignment of protein sequences of calmodulin-binding domains (CBDs) from PMCA2, 3 and 4.

<table>
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<th></th>
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<th>CBD4</th>
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<td>KPSG</td>
<td>KPSG</td>
</tr>
<tr>
<td></td>
<td>ILELRRGSSLR</td>
<td>VLELRRGSSLR</td>
<td>ILELRRGSSVR</td>
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Table C: Calmodulin-binding domains (CBDs) of different PMCA isoforms can be recognized by anti-CBD. +: yes; -: no; ?: possibly.

<table>
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<tr>
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<th>CBD4</th>
<th>CBD12</th>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>
Project objectives

Hypothesis

In *Paramecium*, there are three signal transduction pathways involved in chemoattraction, which have been described in detail in previous section (Fig. A).

In pathway 1 and 2, it is believed that the sustained membrane potential hyperpolarization is due to the activation of PMCA, which generate the hyperpolarizing conductance by extruding Ca\(^{2+}\) from cells during chemoattraction.

Based on the preliminary results, one hypothesis is developed: *Paramecium* PMCA and its association with lipid rafts are essential in the chemoattraction signal transduction.

Specific aims

This hypothesis is tested under 3 specific aims.

Specific aim 1

To identify the localization of PMCA on the cell surface membrane. The pellicle proteins will be analyzed by Western Blot to demonstrate that PMCAs are present in the pellicle. The localization of PMCA on the cell surface will be determined by immunomicroscopy studies of *Paramecium*.

Specific aim 2

To determine the association of PMCA with lipid rafts in *Paramecium*. First, the Triton X-100 solubility of pellicle proteins will be studied. Second, the Triton
insoluble pellicle proteins will be analyzed by sucrose gradient ultracentrifugation to demonstrate that PMCA is associated with lipid rafts. Third, the sterol in the cell membrane will be decreased by methyl-β-cyclodextrin (MβCD) treatment. Through the sterol-depletion, the lipid rafts will be disrupted in the cell membrane. Fourth, the Triton-insoluble pellicle proteins from sterol-depleted cells will be analyzed by sucrose gradient ultracentrifugation to study the effect of MβCD treatment on the distribution of raft-associated proteins in sucrose gradients. In addition, the effect of MβCD treatment on the localization of membrane proteins will also be studied by immunomicroscopy.

**Specific aim 3**

To study the effect of PMCA down-regulation or raft-disruption on the chemoattraction of *Paramecium*. PMCA will be down-regulated by RNAi feeding method. The lipid rafts in *Paramecium* will be disrupted by MβCD treatment. The chemoattraction of the PMCA down-regulated cells and the raft-disrupted cells will be studied in T-maze assays.
Chapter 2

The Role of Plasma Membrane Calcium ATPase (PMCA) and its Localization in Lipid Rafts in Chemoattraction in *Paramecium*
Introduction

*Paramecium tetraurelia* is a unicellular ciliate. It can be attracted towards different types of chemical stimuli, which are referred to as chemoattractants. Usually, these attractants are bacterial metabolites, which indicate the food source (bacteria). Chemoattractants can fall into three signal transduction pathways. In pathway I, the only attractant is glutamate. In pathway II, the attractants can be biotin, acetate, folate or cyclic AMP among others. In pathway III, NH$_4$Cl is the only one that has been identified (Van Houten, 1998). All these three signal transduction pathways result in a hyperpolarization of membrane potential in *Paramecium*. During hyperpolarization, cilia beat more frequently and more directly toward posterior, making the cell forward swimming fast and smooth (Preston and Saimi, 1990). In the chemoattraction signal transduction pathway I and II, the signal transduction to membrane hyperpolarization is receptor mediated. On the cell surface membrane, receptors of glutamate, cAMP, folate, or biotin respectively have been characterized (Sasner and Van Houten, 1989; Van Houten et al., 1991; Bell et al., 1998). The effect of hyperpolarization of membrane potential results from the activation of plasma membrane Ca$^{2+}$ ATPase (PMCA), which generates a hyperpolarizing conductance by extruding Ca$^{2+}$ from the cell (Van Houten, 1998).

Three PMCA isoforms (named PMCA2-4) in *Paramecium* have been cloned (Gannon-Murakami, 2004). Twenty more isoforms were found in the
annotated *Paramecium* genome (Genoscope). *Paramecium* PMCA2-4 shares the properties with mammalian counterparts (Wright, 1990; Elwess, 1997; Gannon-Murakami, 2004).

Lipid rafts are particular microdomains in cell membranes, which are recognized by enrichment of cholesterol, sphingolipids, and saturated fatty acids, and resistance of cold detergent solubilization (Simons, 1997; Munro, 2003). One critical role of lipid rafts in cellular physiological activities is serving as a platform in signal transduction, which can recruit receptors and downstream effectors in a relatively proximal distance to make signals transduce efficiently or exclude them from rafts to terminate the signal transduction according to the physiological necessity (Pike, 2003; Zajchowski, 2002). Lipid rafts can regulate signal transductions in spatially and temporally precise manner. In *Paramecium*, lipid rafts have been identified, which share the defining characteristics (Chandran, 2004).

PMCA can be localized to lipid rafts. In kidney epithelial cells PMCA is predominantly localized in caveolae. Its activity is caveola-dependent (Tortelote, 2004). In addition, PMCA can be selectively associated with lipid rafts. In primary cortical neurons, the activity of PMCA is dependent on its localization into lipid rafts. Raft-disruption through cholesterol depletion can reduce the activity of the PMCA, but has no effect on that from outside of lipid rafts. The localization in lipid
rafts provides a new regulatory mechanism to activate PMCA (Jiang, 2007). It also implicates that lipid rafts may participate in the regulation of Ca\(^{2+}\) signaling by incorporation of PMCA.

Here we report that in *Paramecium*, PMCA2, together with GPI-anchored surface antigens and *Paramecium* calmodulin-binding membrane-bound proteins (PCM) partition into lipid rafts. These proteins can be recovered by floatation in sucrose density gradients through ultracentrifugation. *Paramecium* lipid rafts are associated with cytoskeleton proteins such as tubulin and actin. In contrast, PMCA3 and 4 are not localized in lipid rafts. On the cell surface membrane, PMCA3 and 4 are not localized in lipid rafts. On the cell surface membrane, PMCAs are localized at the bases of cilia. Sterol depletion by Methyl-β-cyclodextrin disrupts the distribution of PMCA2 in a sucrose density gradient and the ciliary base-localization. In behavioral studies, the MβCD treated paramecia show decreased chemoattraction to glutamate and cAMP. These results implicate that PMCA and its association with lipid rafts play roles in chemoattraction signal transduction. A revised model of 3 types of membrane domains is proposed to explain the chemoattraction signal transduction in *Paramecium*. 
Material and Methods

Cell Culture: *Paramecium tetraurelia* strain 51-s (sensitive to killer) was cultured at 28°C in wheat grass (Pines, Lawrence, KS) medium (Sasner and Van Houten 1989) supplemented with 1mg/l stigmasterol, and Na$_2$HPO$_4$·7H$_2$O. The medium was inoculated with *Klebsiela pneumoniae* 24 hours before addition of *P. tetraurelia*. The cells were cleaned for 1 hour in 10 ml of Dryl’s solution (1 mM NaH$_2$PO$_4$·H$_2$O, 1 mM Na$_2$HPO$_4$·7H$_2$O, 2 mM sodium acetate, 1.5 mM CaCl$_2$, pH6.8) with 100 µl of gentamicin reagent solution and transferred into a new inoculated 1.5 l of "Improved" wheat medium (wheat grass medium supplemented with 0.67 g/l protease peptone, Na$_2$HPO$_3$, NaH$_2$PO$_3$, and Tris-HCl to buffer pH). The cells were grown at 28°C until in logarithmic phase.

Crude pellicle preparation: Cells from 6 liters of “Improved” wheat grass medium were harvested by filtering the medium through Kimwipes tissue paper and cheesecloth and concentrated by centrifugation at 820xg in the IEC-CLINICAL continuous flowing centrifuge (Chemical Model) and pelleted by centrifugation at 350xg for 2 minutes in pear-shaped centrifuge tubes (IEC HN-DII centrifuge). The cell pellet was washed twice by centrifugation for 2 minutes in 125 ml ice-cold HM (homogenization) buffer (20 mM Tris-base, 20 mM Maleic acid, and 1 mM Na$_2$EDTA pH 7.8). The cell pellet was washed one more time in 125 ml freshly prepared HMI buffer (HM buffer with protease inhibitors, 1.25 ml of 10 mM phenylmethylsulfonyl fluoride (PMSF), 1.25 ml of 100 mM iodoacetic acid,
125 μl of 1 μg/ml leupeptin, 125 μl of 1 μg/ml pepstatin). After centrifugation, the cell pellet was transferred to a cold 15 ml Potter/Elvehjem homogenizer. The cells were homogenized with the pestle in an electric drill chuck to deciliate and rupture the cells. Cell disruption was checked using phase-contrast microscopy and continued until more than 75% of the cells were ruptured. The homogenate was centrifuged at 3440xg for 5 minutes at 4°C in JA-17 rotor (Beckman J2-21 centrifuge). The pellet was washed with ice-cold HMI buffer by vigorous mixing for 30 seconds and centrifuged at the same condition to discard the supernatant. The washes and centrifugation were repeated for 6-8 times until the supernatant was clear. After last wash, the pellet was resuspended in 2 ml membrane buffer (50 mM Tris, 50 mM KCl, 5 mM MgCl₂, 1 mM EGTA, pH 7.4) with protease inhibitors. The protein concentration of the pellicle preparation was measured with Micro BCA Protein Assay (Pierce) using bovine serum albumin as standard.

**Sodium Carbonate Extraction of Pellicle Proteins:** Pellicle preparations (500 μg) were suspended in 1 ml 0.1 M Na₂CO₃ (pH 11.5) and rocked at 4°C for 1 hour. The sample was centrifuged at 13,100xg at 4°C for 8 minutes (Eppendorf centrifuge 5415c) to pellet the protein. The pellet was resuspended in one volume of 2x SDS/sample buffer (0.5 M Tris-HCl, 20% glycerol, 4% SDS, 0.2 M DTT, 0.001% bromophenol blue, pH 6.8) and analyzed by SDS-PAGE and Western blotting.
**Triton X-100 Solubilization of Pellicle Proteins:** The crude pellicle preparation (20 mg) was suspended in 4 ml membrane buffer with 80 µl Triton X-100 (final concentration 1%) and protease inhibitors (0.04 ml of 10 mM phenylmethylsulfonyl fluoride (PMSF), 0.04 ml of 100 mM iodoacetic acid, 40 µl of 1 µg/ml leupeptin, 40 µl of 1 µg/ml pepstatin) and solubilized at 4°C with rocking for 1 hour. The solubilized pellicle preparation was centrifuged at 29,400xg for 10 minutes at 4°C in a J-17 rotor (Beckman J2-21 centrifuge) to obtain the Triton X-100-insoluble pellet that was used in lipid rafts analysis. The solubilized proteins in the supernatant were precipitated by one volume of cold 100% acetone at 4°C for 15 minutes. The samples were centrifuged at 13,100xg at 4°C for 10 minutes (Eppenndorf centrifuge 5415c) to pellet the proteins. The pellet from the supernatant was resuspended in one volume of 2x SDS/sample buffer and analyzed by SDS/PAGE and Western blotting.

**Sucrose gradient centrifugation lipid raft preparation:** The Triton X-100-insoluble pellet was suspended with 0.5 ml membrane buffer and 1.5 ml ice-cold 80% (w/v) sucrose TNE (10 mM Tris-base, 100 mM NaCl, and 1 mM sodium EDTA) solution to get a final concentration of 40% (w/v) sucrose in the suspension. 500 µl of this suspension was overlaid with 4 ml 5-40% continuous linear sucrose gradient, which was made with a continuous gradient maker and a Buchler Duostaltic pump, in each of six ice-cold 4.5 ml Ultra-clear (11×60 mm) centrifuge tubes (Beckman). The tubes were balanced by adding 5% sucrose...
TNE solution in the remaining space in the tube. The Ultra-clear tubes were placed in Beckman SW60 Ti swinging bucket rotor and centrifuged at 160,000xg at 4°C for 4 hours (Beckman L8-70M Ultracentrifuge). The supernatants from each of the six Ultra-clear tubes were removed in nine fractions of 500 µl each. The similar fractions from the 6 tubes were pooled together. The 9 fractions were dialyzed in MilliQ H₂O at 4°C for 24 hours, and precipitated in 4 times of the volume with 100% acetone at -20°C overnight, and centrifuged at 15,200xg at 4°C for 15 minutes in J-17 rotor (Beckman J2-21 centrifuge) to pellet the proteins. The pellets from each fraction were washed with 90% acetone, kept at 4°C for 10 minutes, and centrifuged again. The supernatants were discarded and the pellets were air-dried on ice. The pellets were suspended in 50 µl membrane buffer and 10 µl 6× SDS/sample buffer.

**SDS-PAGE Electrophoresis and Western blotting:** 7% SDS acrylamide separating gel or 7%--15% gradient gel, with 3.9% stacking gel was used to separate proteins. The gels were run at stable current of 30 mA in Hoeffer Might Small system. All the samples were boiled for 5 minutes before being loaded in the gel. The gels were transblotted at 250 mA and 390 mA for 45 minutes respectively onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in Tris-Buffer Saline with Tween-20 (TBS-T; 0.16 M Tris-HCl, 0.04 M Tris-base, 1.4 M NaCl and 1% Tween-20 pH7.5) for 1 hour or with addition of 2% telost gelatin and 3% goat serum in the dry milk TBS-T for 1 hour.
The blots were probed for the proteins of interest with different primary antibodies (rabbit anti-surface antigens 1:5,000, rabbit anti-CBD (calmodulin binding domain) 1:5,000, commercial rabbit anti-FBP 1:2,000, rabbit anti-pFBP (*Paramecium* folate receptor) 1:10,000, mouse anti-tubulin 1:10,000 or rabbit anti-actin 1:5,000) overnight. The blots were washed in TBS-T 3 times (15 minutes×1, 5 minutes×3) and incubated with alkaline phosphatase conjugated secondary antibody (goat anti-rabbit IgG, Sigma), 1:10,000 diluted in TBS-T. Then the blots were washed again in TBS-T 3 times (15 minutesx1, 5 minutesx3). Finally, the blots were developed in 10 ml of nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP).

Some blots were probed using enhanced chemiluminescence (ECL, Amersham) following the manufacture's instructions. The blots that were blocked with 5% non-fat dry milk in TBS-T for 1 hour were probed with primary antibody of interest (rabbit anti-N terminus of PMCA3 1:5,000 or mouse anti-HA 1:5,000) overnight. The blots were washed in TBS-T 3 times (15 minutes×1, 5 minutes×3). The blots were incubated with a suitable secondary antibody (goat anti-rabbit or goat anti-mouse IgG 1:5,000, Amersham) conjugated with horseradish peroxidase. The blots were washed again in TBS-T 3 times (15 minutesx1, 5 minutesx3). The blots were incubated in equal volumes of reagent 1 and 2 (ECL Kit Amersham) for 1 minute. The blots were exposed to films (Kodak BioMax XAR Film) for 10 seconds to 2 hours. The exposed films were developed in D-19 developer (Kodak) and fixed in rapid fixer (Kodak).
**Stripping and reprobing blots:** The blots that had been developed with ECL were stripped of the primary and secondary antibodies by incubating the blots in the stripping buffer (Pierce) at 37°C for 45 minutes with rocking. The blots were rinsed in TBS-T to remove the stripping buffer. Then the blots were blocked, reprobed with the primary antibody of interest, incubated with alkaline phosphatase conjugated secondary antibody and developed in NBT/BCIP as described above.

**Overexpression of HA-tagged PMCA2 in transformed cells:** The transformed cell line is a gift from Dr. Junji Yano. The transformed cells containing the HA-tagged PMCA2 construct were cultured in wheat grass medium containing 200 μg/ml paramomysin at 22°C. The paramomysin can screen the transformed cell which containing the HA-tagged PMCA2 construct in the macronucleus during cell division. The transformed cells are harvested and used in pellicle preparation as described before.

**Immunofluorescence Microscopy:** Cells were harvested by centrifugation at 350xg for 2 minutes in pear-shaped centrifuge tubes (IEC HN-DII centrifuge). The cell pellet was washed in 10 ml Dryl’s solution by centrifugation (IEC-CLINICAL centrifuge Clinical Model) at 1050xg for 2 minutes. Cells were permeabilized in 10 ml PHEM solution (60 mM PIPES, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂ and 0.05% Triton X-100) for 5 minutes. Cells were fixed in
10 ml PBS (1.85 mM NaH₂PO₄·H₂O, 8.4 mM Na₂HPO₄, and 150 mM NaCl pH 7.4) containing 2% freshly made paraformaldehyde for 1 hour. Each time cells were pelleted by centrifugation and the supernatant was discarded. Cells were washed 3 times by centrifugation in 10 ml blocking solution (PBS containing 10 mM EGTA, 2 mM MgCl₂, 0.1 Tween-20 and 1% albumin from bovine serum). Cells were pelleted by centrifugation, resuspended in PBS-T and aliquoted 100 µl each as one sample. Each sample of cells was incubated with the different primary antibodies (rabbit anti-CBD 1:50, rabbit anti-FBP 1:50, or rabbit anti-AB 1:50 and mouse anti-1D5 1:100) diluted in PBS-T (PBS with 0.1% Tween-20) at room temperature for 1 hour or at 4°C overnight. Cells were washed 3 times by centrifugation in 1 ml PBS-T. Cells were incubated with the secondary antibodies goat anti-rabbit IgG conjugated with Alexa Fluor 488 and goat anti-mouse IgG conjugated with Alexa Fluor 568 (Molecular Probes) 1:200 diluted in PBS-T at room temperature for 1 hour. Cells were washed 5 times by centrifugation in 1 ml PBS-T. Cells that were only incubated with the secondary antibodies (goat anti-rabbit IgG conjugated with Alexa Fluor 488 and goat anti-mouse IgG conjugated with Alexa Fluor 568) served as a negative control. Finally each sample of the immunostained cells were suspended in the 100 µl mounting medium (Vector Shield). 5 µl of the cell suspension was put on slide with cover slip on top and observed under Confocal Microscope or DeltaVision Microscope.
**Antibodies:** Anti-CBD is an affinity purified rabbit polyclonal antibody, which is against KLH peptide (KPSFILELRRGSSLRRK) (Biosource Caramillo, CA). Anti-N-terminus 3, a rabbit polyclonal antibody, is made against a GST- fusion protein with the sequence of MNQSALKTDVNVTVEEMRLNMG, which was then purified to remove anti-GST (Bethyl Lab Montgomery, TX). Anti-1D5, a mouse monoclonal antibody, is purchased from Synaptic System (Göttingen Germany). Anti-tubulin, a mouse monoclonal antibody, is purchased from Sigma (St. Louis, MO). Anti-folate binding protein (FBP), a rabbit polyclonal antibody, is purchased from Biogenesis (Brentwood, NH). Anti-pFPB, a rabbit polyclonal antibody, is made against a GST-fusion protein with the sequence of 100 amino acids from the initiation site of N-terminus of *Paramecium* folate binding protein (Weeraratne, 2004) by Lampire Biological Lab Inc.(Pipersville, PA). Anti-surface antigens, a rabbit polyclonal antibody, donated by Dr. Jim Forney, is made against the salt-ethanol wash proteins of *Paramecium*, which expresses surface antigen A or B. Anti-actin is a rabbit polyclonal antibody, donated by Dr. Helmut Plattner.

**Sterol Depletion:** Cells were harvested and pelleted as described above. The pelleted cells were incubated in 2mM NaCl chemokinesis buffer (1 mM calcium citrate, 1 mM Tris-base and 2 mM NaCl pH 7.05) containing 5 mg/ml methyl-β-cyclodextrin (MβCD) at 28°C for 2 hours. Cells that were incubated in 2mM NaCl chemokinesis buffer without MβCD served as a control. Cells were collected and
used in crude pellicle preparation as described above. The cholesterol levels in pellicle were measured in cholesterol assay.

**Cholesterol Assay:** Following the manufacturer’s protocol, 50 μl of cholesterol reference standard with different concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8 μg/ml) were made in 1x Reaction Buffer (Amplex Red Kit, Molecular Probes). 50 μl of 10 μM hydrogen peroxide served as a positive control. Pellicle containing 60 μg protein diluted in 1x Reaction Buffer to the final volume of 50 μl was taken as one sample. The cholesterol reference standard, the hydrogen peroxide and samples were loaded in 96-well microtiter plate. The reactions were started by adding 50 μl of 300 μM Amplex Red Reagent (20 mM Amplex Red reagent, 2 U/ml Horseradish peroxide, 2 U/ml cholesterol oxidase and, 0.2 U/ml cholesterol esterase) and incubated at 37°C for 30 minutes protected from light. The data were collected by a fluorescence microtiter plate reader (wavelength of excitation at 530 nm, emission at 590 nm). The standard curve was plotted by Microsoft Excel. The concentrations of cholesterol in each sample were obtained from the standard curve and equation.

**Protein Assay:** 100 μl of various concentrations (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 mg/ml) of bovine serum albumin (BSA) were made by diluting 2 mg/ml BSA stock in MilliQ H₂O to the final volume of 100 μl. Pellicle were 1:10 diluted to the final volume of 100 μl. 2 ml of BCA protein assay reagents A and B at the ratio of
98(A):2(B) (Pierce) were added to the standards and the samples respectively. The reactions were incubated at 37°C for 30 minutes. The absorbance of the reactions was measured at 562 nm in Spectrophotometer (Agilent 8453). The standard curve was plotted and the protein concentration in samples was obtained by UV-visible Chemstation Software.

**Phosphatase treatment:** The crude pellicle was suspended in 1 ml of Tris/MgCl$_2$ Buffer (50 mM Tris·Cl, 1 mM MgCl$_2$ pH7.5). The protein concentration was obtained through Protein Assay. 500 ug of protein was taken as a sample and diluted with Tris/MgCl$_2$ Buffer to a final volume of 250 ul. Samples were incubated at 30°C for 15 minutes. 100U or 150U of calf intestine alkaline phosphatase and that are boiled for 5 minutes were added to individual sample and incubate them at 30°C for 15 minutes. After incubation, samples were centrifuged at 13,100xg at 4°C for 10 minutes. Supernatants were discarded. The phosphate-treated pellicle was extracted by Na$_2$CO$_3$ as described before.

**Paramecium Genomic DNA Isolation:** Paramecia were grown in 100 ml wheat grass medium at 28°C till stationary growing phase. Cells were harvested by filtering through Kimwipes tissue and pelleted by centrifugation at 350xg for 2 minutes in IEC HN-DII centrifuge. Cells were washed twice in 10 ml Dryl’s solution (1 mM NaH$_2$PO$_4$·H$_2$O, 1 mM Na$_2$HPO$_4$·7H$_2$O, 2 mM sodium acetate, 1.5 mM CaCl$_2$, pH6.8). Each time the cells were pelleted by centrifugation at 200xg.
for 2 minutes in the IEC Clinicla Centrifuge. The cell pellet was suspended in 100 
µl Drl’s solution and transferred into 2 ml Microfuge tube. To the cell suspension,
200 µl Denaturing solution (Promega) was added, mixed well by inversion
several times and followed by addition of 30 µl 3 mM sodium acetate (pH 5.2),
and mixed well. An equal volume of phenol/chloroform/isoamyalcohol (25:24:1)
was added to the sample, mixed well and kept on ice for 10 minutes. The sample
was centrifuged at 16,000xg for 20 minutes in the Eppenddorf centrifuge 5414C
at 4°C. The aqueous phase (upper layer) was transferred to a new 1.5 ml
Microfuge tube, followed by the addition of same volume of 100% isopropanol
and mixed well. The sample was kept at -20°C for 30 minutes to precipitated
DNA. The sample was centrifuged at 16,000xg for 20 minutes at 4°C to pellet
DNA. The DNA pellet was washed 3 times with 500 µl 70% ethyl alcohol. Each
time, the sample was centrifuged at 16,000xg for 5 minutes at 4°C, to discard the
supernatant. The sample was placed in fume hood for 20 minutes to vaporize the
remaining ethanol. The genomic DNA was dissolved in 50 µl of sterilized MilliQ
H₂O and stored at -20°C for future use. The concentration of the DNA was
measured by using the Nanodrop quantitation (Nanodrop).

**Generation of RNAi construct:** 1003 and 1002 bases of gene fragments from
the initiation site of ATG of PMCA2 (AY057227) and PMCA3 (AY057226) genes
respectively were amplified by PCR using following primers and genomic DNA
as template.
The primers of PMCA2 RNAi construct:

Forward primer: 5' (+1) ATG TAA TCT CCA AAT AAA AGT GC 3'
Reverse primer: 5' (+1003) CTG AGA TCA ATC CAA ACT TAC C 3'

The primers of PMCA3 RNAi construct:

Forward primer: 5' (+1) ATG AAAT CAA TCG GCT TTA AAA AC 3'
Reverse primer: 5' (+1002) CAC AAT CAA TAT GGC AGC ATA 3'

The PCR was applied under the following condition:

Stage 1: 94°C for 2 minutes; Stage 2: 94°C for 1 minute, 50°C for 2 minutes, and 72°C for 2 minutes, 30 cycles; Stage 3: 72°C for 15 minutes, hold at 4°C.

The amplified PMCA2 or PMCA3 gene fragments were cloned into TOPO plasmid vector (Invitrogen) and transformed into OneShot chemically competent cells under the manufacture’s instruction.

The plasmids with insert of PMCA2 or PMCA4 gene fragment were isolated by using Wizard Plus Mini-Prep Kit (Promega) under the manufacture’s instruction. The TOPO plasmid with insert was sequence by Vermont Cancer Center Sequencing Facility.

The TOPO plasmids with the insert were digested with restriction enzymes Hind III and Xho I (Invitroge) to release the PMCA2 or PMCA3 gene fragment. At the same time, the Litmus 28i plasmid vector was digested by the same restriction enzyme to linearize the vector. The products of digested TOPO plasmids were run in a 1.2% agarose gel to separate the insert of gene fragment.
form the plasmids. The separated gene fragments were purified by using PerfectPrep Gel Cleanup Kit (Eppendorf) under manufacture’s instruction.

The purified gene fragments were ligated to the linearized Litmus 28i vector by using the Clonable Ligation 2X Premix (Novagen) following the manufacture’s instruction. The ligated products were transformed into Ecoli. HT 115 competent cells. The Litmus 28i empty vector was also transformed into Ecoli. HT115 competent cells to serve as a control. The positive colony was picked to grow in 5 ml LB culture with 5 μl of 100 μg/ml Ampicillin at 37°C overnight. 275 μl of HT115 cell culture was mixed with 25 μl of sterilized glycerol to make a glycerol stock that was kept at -80°C for future use.

**RNAi Feeding:** The 300 μl glycerol stocks of HT115 cells with PMCA2 or 3 RNAi construct or Litmus 28i empty vector were grown in 5 ml LB culture with 5 μl of Ampicillin in the shaking incubator at 37°C overnight. 1 ml of the overnight culture was added to 50 ml of LB with 50 μl of Ampicillin and grown in the shaking incubator at 37°C. When the O.D.₅₉₅ₙ₅ value reached 0.4, the cultures were induced with 125 μl of 50 mg/ml isopropyl-β-D-thiogalactopyranoside, dioxane free (IPTG) and kept under the same condition. The induced HT115 bacteria were harvested by centrifugation at 2,200xg for 10 minutes in JA-17 rotor (Beckman J2-21 centrifuge). The bacterial pellet was suspended in 100 ml Paramecium wheat grass culture with the100 μl of 100 μg/ml Ampicillin, 250 μl of 50 mg/ml IPTG and 100 μl of 8 mg/ml of Stigmasterol. Next, 50 to 100 paramecia
that were cleaned in Dryl’s solution at room temperature were introduced to the RNAi cultures grown at 28°C for 72 hours.

Every 24 hours, the supplement of 100 µl of 100 µg/ml Ampicillin, 250 µl of 50 mg/ml IPTG and 100 µl of 8 mg/ml of Stigmasterol was added. After 72-hour growth, paramecia were harvested and cleaned in 2mM NaCl chemokinesis buffer for T-Maze assay.

**T-maze Assay:** The T-maze assays were performed as previously described (Van Houten et al., 1982). One arm of the T-maze was filled with 2mM NaCl chemokinesis buffer as the control arm. The other arm was filed with chemokinesis buffer with indicated salt solution (2mM Na-glutamate, 2mM Na-acetate, 1 mM of Na₂-folate or 2mM NH₄Cl) as the test arm. Paramecia containing PMCA2 or PMCA3 RNAi construct or empty Litmus 28i vector were loaded in the stopcock. The stopcock was turned on allowing paramecia to swim to each arm. After 30 minutes, the stopcock was closed and the paramecia from each arm were collected separately. Cells in 1.5 ml of aliquot from each arm were counted under bright field microscope. The value of Index of Chemoattraction (I \textsubscript{che}) was calculated through the formula of \[ \text{I}_{\text{che}} = \frac{\text{cell number in test arm}}{\text{sum of cell number in both test and control arms}}. \] Any I\textsubscript{che} higher than 0.5 indicates chemoattraction.
Results

The presence of PMCA in Pellicle

The pellicle proteins from wild type Paramecium were extracted with 0.1 M Na₂CO₃ and analyzed through 7% SDS-PAGE and Western Blot. An antibody against the calmodulin-binding domain (CBD) of PMCA2 was used to immunoprobe the blot. This recognizes three proteins around the region of molecular weight of 115.5 KD (Fig 1). The calculated molecular weights of the three bands are 125KD, 113KD, and 106KD. Anti-CBD is an affinity purified antibody that was generated against the calmodulin binding domain of PMCA2. It has been shown that this antibody can recognize the expressed CBD-fusion protein of PMCA2, 3, and 4 (Gannon-Murakami, 2004). The alignment of CBD from PMCA2, 3 and 4 is shown in figure 1. We conclude that PMCA2, 3, and 4 are present in pellicle, but it is unknown which band corresponds to which isoform among PMCA2, 3 and 4.

Determination of PMCA isoform 2

To figure out which of PMCA isoforms correspond to the three protein bands on the Western blots, transformed cells containing overexpressed HA-tagged PMCA2 were used. First, Western blots from the pellicle were immunoprobed with anti-CBD or anti-HA. One protein band was recognized by both of these two antibodies respectively at the same molecular weight region relative to the protein marker 115.5 KD on blot (Fig. 2A). This band corresponded
to the upper protein band among the three that were recognized by anti-CBD in wild-type cells. Second, Western blots from the pellicle were immunoprobbed by anti-HA and developed through ECL. One protein band above the 115.5 KD protein marker was recognized by anti-HA on the ECL film. However, on the ECL film, anti-HA recognized some proteins at lower molecular weight region. The same blot was stripped and reprobed by anti-CBD. When the ECL film was overlaid on the blot, it was clear that the protein band recognized by anti-HA exactly overlaps that recognized by the anti-CBD (Fig. 2B). It was concluded that among the three protein bands recognized by anti-CBD, the top one is PMCA2.

**Identification of the two lower protein bands**

To identify the two lower protein bands, another antibody, anti-N terminus 3 (anti-N3), was used. In similar experiments, the pellicle proteins from wild type cells on blot were immunoprobbed by anti-N3 and developed through ECL. Then the blot was stripped and reprobed with anti-CBD. The two protein bands recognized by anti-N3 on the ECL film exactly overlapped the two lower bands recognized by anti-CBD (Fig. 3A). The antibody of anti-N3 was generated against the NH$_2$-terminal peptide of PMCA3, which has a high homology with the NH$_2$-terminal peptide of PMCA4. The alignment of N-terminal peptide from PMCA3 and PMCA4 is shown in figure 3A. So it is possible that anti-N3 recognized both PMCA3 and PMCA4.
The specificity of anti-N3 was also tested through preabsorbing the antibody with a fusion protein of GST-tagged NH$_2$-terminal peptide of PMCA3. In similar experiment, the pellicle proteins from wild-type cells on blot were immuoprobed with the preabsorbed anti-N3 and developed through ECL. On the ECL film, compared with the control experiment using the mock absorbed anti-N3, the preabsorbed anti-N3 weakly recognized two protein bands that were strongly recognized by the mock-absorbed (Fig. 3B). It was concluded that the two lower protein bands recognized by anti-CBD were PMCA3 or PMCA4.

One possible reason for the two protein bands being recognized by anti-N3 could be due to phosphorylation. Two serines in calmodulin-binding domain of PMCA3 can be phosphorylated by protein kinases (Gannon-Murakami, 2004). To determine the phosphorylation state, the pellicle proteins were treated with phosphatase before Na$_2$CO$_3$ extraction. In this experiment, 500 μg pellicle proteins were treated with 100 Units or 150 Units of calf intestine alkaline phosphatase. However, anti-N3 still recognized two protein bands in the phosphatase treated pellicle (Fig. 3C). But a positive control that can confirm that the phophatase was active in the experiment was lacking. Therefore it is not possible to conclude that the two protein bands recognized by anti-N3 results form to phosphorylation.

**Localization of PMCAs on cell surface**
The localization of PMCAs was studied by the deconvolution microscopy. An antibody of anti-1D5, which recognizes tubulin at the ciliary basal body, was used as a marker (Libusova, 2005). The permeablized wild-type cells were double immunostained with anti-CBD and anti-1D5. PMCAs, labeled by anti-CBD, were localized at the bases of cilia on the cell surface (Fig. 4A). PMCAs were also found localized in cilia, especially at the ciliary tips (Fig. 4B). On the ciliary tips, PMCAs show a strong expression. There was no staining observed in the cytoplasm (data not shown).

Triton X-100 solubility studies

The pellicle proteins from wild-type cells were solubilized with 1% Triton X-100 at 4°C for 1 hour. Both Triton-soluble and insoluble pellicle proteins were analyzed by SDS-PAGE and Western Blot. The blot was immunoprobed by anti-N3 and developed by ECL. After stripping, the blot was reprobed with anti-CBD. The protein bands (PMCA3 or PMCA4) recognized by anti-N3 on ECL film appeared in the supernatant (Triton-soluble) fraction. These two bands can completely overlap those bands recognized by anti-CBD on the blot in the same fraction. One more protein band (PMCA2) that appeared in the pellet (Triton-insoluble) fraction was recognized anti-CBD (Fig. 5). It was concluded that PMCA2 cannot be solubilized by cold Triton X-100 and stays in the insoluble fraction. But PMCA3 or 4 can be readily solubilized by cold Triton X-100. These
results implied that PMCA2 may be associated with the cytoskeleton or lipid rafts or both due to its insolubility in cold Triton X-100.

**Analysis of Triton-insoluble pellicle proteins**

Proteins that cannot be solubilized by cold detergent may be associated with lipid rafts. These raft-associated proteins can be recovered by floatation in sucrose density gradient. To find out whether PMCA2 was associated with lipid rafts, the Triton-insoluble pellicle proteins were recovered in 5%-40% sucrose linear gradient through ultracentrifugation. Every 500 μl of supernatant was taken as one fraction. A total of 9 fractions were taken. Proteins from each fraction were analyzed through 7%-15% gradients SDS-PAGE and Western Blot. The blots were cut into several pieces according to the molecular weight and immunoprobed with several different antibodies. GPI-anchored surface antigens, that are markers for raft proteins, and *Paramecium* calmodulin-binding membrane associated protein (PCM) distributed in all fractions (fraction1-9) (Fig. 6A and Fig. 6C). PMCA2 distributes in fraction 3-8 (Fig. 6B). The blots in figure 6A and 6B were from the same experiment. In this experiment ~29 mg pellicle proteins were used in the preparation. The blot in figure 6C was from another experiment. In this experiment, ~30 mg pellicle proteins were used in preparation.

Lipid rafts can be associated with cytoskeleton proteins. To find whether lipid rafts in *Paramecium* are associated lipid rafts, some blots were also probed with antibodies against cytoskeleton proteins. Tubulin distributed in all fractions.
(Fig. 6D) and actin distributes in lower fractions (fraction 6-9) (Fig. 6E). Therefore GPI-anchored surface antigens, PCM, and PMCA2 were localized in lipid rafts in Paramecium. Cytoskeleton proteins can be associated with lipid rafts. The blots in figure 6D and 6E were from the same experiment. In this experiment, ~31 mg pellicle proteins were used in preparation.

**Effects of sterol-depletion on raft-associated proteins**

Cholesterol is an essential component of mammalian lipid rafts (Simons, 2000). Reduction of sterol levels can disrupt lipid rafts. Methyl-β-cyclodextrin (MβCD) is a widely used drug that binds to sterols (including cholesterol) and sequesters them out of membrane to decrease the cholesterol level. To reduce the cholesterol level in Paramecium, cells were treated with 5mM MβCD at 28°C for 2 hours. After treatment, the cholesterol level from pellicle was measured in cholesterol assay. Compared with mock treated (control) cells, the cholesterol level in the pellicle of MβCD treated cells was reduced by 42 ± 1.4 percent (Fig. 7).

To figure out the effects of sterol-depletion on raft-associated proteins, the pellicle proteins from MβCD treated cells were also analyzed through sucrose density gradient ultracentrifugation. The distribution of GPI-anchored surface antigens was limited to fraction 6 to 9 (Fig. 8A). The distribution of PMCA2 was completely disrupted. PMCA2 was not in the sucrose gradient fractions (Fig. 8B). The proteins in the pellet (fraction 10) after sucrose gradient ultracentrifugation
were also analyzed by Western Blot. Surface antigens were present in the pellet (fraction 10) of both mock treated cells or MβCD treated cells after sucrose gradient ultracentrifugation (Fig. 8G). Surprisingly, PMCA2 was not present in the pellet (fraction 10) of either mock treated cells or MβCD treated cells (Fig. 8F). However, PMCA2 was still present in the pellicle from the MβCD treated cells (Fig. 9A). In addition, MβCD treatment had no effect on the detergent-solubility of PMCA2 or another GPI-anchored protein of folate receptors. PMCA2 and GPI-anchored folate receptors were still Triton-insoluble and stayed in the pellet fraction (Fig. 9B). Interestingly, PCM still distributed in all fractions from 1 to 10 (Fig. 8C). MβCD treatment did not affect the distribution of PCM in sucrose gradient fractions. Similar to the GPI-anchored surface antigens, the distribution of tubulin was also restricted to lower sucrose gradient fractions (fraction 6-9) (Fig. 8D). The distribution of actin in sucrose gradient was slightly changed into fraction 7-9 (Fig. 8E). The blots from figure 8A and 8B were from one experiment. In this experiment, ~32 mg pellicle proteins were used in preparation. The blot in figure 8C was from another experiment. In this experiment, ~28 mg pellicle proteins were used in preparation. The blots in figure 8D and 8E were from same experiment. In this experiment, ~33 mg pellicle proteins were obtained in preparation.

The effects of MβCD treatment on the localization of GPI-anchored surface antigens and PMCA2 in permeabilized cells were studied by deconvolution immunomicroscopy. It was found that GPI-anchored surface
antigens were localized at the bases of cilia on the cell surface. MβCD treatment affects their localization pattern (Fig. 10A). In the MβCD treated cells, GPI-anchored surface antigens were still on the cell surface membrane, but the ciliary-base staining pattern was disrupted. In the MβCD treated cells, GPI-anchored surface antigens were not localized at the bases of cilia. Similarly, PMCAs were also localized at the bases of cilia on the cell surface. MβCD treatment dramatically disrupted the ciliary base-localization of PMCAs. PMCAs distributed across the surface membrane (Fig. 10B).

In addition, in the unpermeabilized mock treated cells, surface antigen immunostaining showed a different pattern. Surface antigens were localized on the ridge of the cortical units on cell surface (Fig. 10C). The cortical unit was a parallelogram pattern with delineated ridges which consists of plasma membrane, alveolar sacs and epiplasmic layers (Hufnagel, 1969). However, in the unpermeabilized MβCD treated cells, the cortical unit-stain pattern showed a typical change (Fig. 10C). Surface antigens showed a plaque-staining pattern on the cell surface membrane.

**Chemoattraction studies of RNAi treated cells**

RNAi feeding method was applied to down-regulate PMCA2 or PMCA3 in *Paramecium*. Unfortunately, both RNAi-PMCA2 and RNAi-PMCA3 cells had no detectable reduction at mRNA level or protein level of PMCA2 and PMCA3 (data not shown). But their chemoattractions were still studied in T-Maze assays.
Compared with control cells, both RNAi-PMCA2 and RNAi-PMCA3 cells showed decreased chemoattraction toward glutamate only. No change was seen toward other attractants (Table 1).

**Chemoattraction studies of MβCD treated cells**

The effect of MβCD treatment on chemoattraction of *Paramecium* was studied in T-Maze assays. MβCD treatment decreased the chemoattraction toward glutamate. Unexpectedly, the MβCD treated cells showed a chemorepulsion to cAMP compared with the chemoattraction of control cells toward cAMP (Table 2).
Discussion

The presence of PMCAs in pellicle

The pellicle is a membrane structure, consisting of plasma membrane, tightly associated alveolar sacs and an epiplasmic layer. A putative Ca\(^{2+}\)-transporting ATPase possessing properties of PMCAs was first biochemically characterized in the pellicle of *Paramecium* (Wright, 1990). Wright reported that the Ca\(^{2+}\)-ATPase activity is predominantly in plasma membrane, not in cilia. In current work, we show that PMCAs are present in pellicle through fractionation and Western Blot analysis of pellicle proteins. Recently it was reported that PMCAs were present in cilia through the Western blot analysis of ciliary proteins (Ray, personal communications). These results are supported by our immunomicroscopy studies, in which, immunolabeled PMCAs were present in cilia. An antibody anti-CBD was used in both Western Blot analysis and immunomicroscopy studies. Anti-CBD is an affinity-purified antibody that is generated by against the calmodulin-binding domain (CBD) of PMCA2.

With Western blot anti-CBD recognizes three protein bands. In immunostaining images, this antibody labels PMCAs on the surface membrane and cilia. On the cell surface PMCAs are localized at the base of cilia. Although anti-CBD is against CBD of PMCA2, it can also recognize CBD of PMCA3 and PMCA4 (Gannon-Murakami, 2005). So, it is not surprising that anti-CBD recognizes three protein bands. The calculated molecular weights of these three bands based on their mobility on the gel are 125KD, 113KD and 106 KD. These
three bands may represent PMCA2, 3 and 4. On the blot, the molecular weight of three bands is around 115.5 KD, which is a little different from the calculated molecular weight based on the putative protein sequence (PMCA2 129 KD; PMCA3 and PMCA4 125 KD). But it is usual that the mobility of protein on SDS-PAGE does not show the exact molecular weight due to numerous factors such as gel concentration, the amount protein loaded, and denaturing degree.

**Identification of three PMCA isoforms**

Since three PMCA isoforms are recognized by anti-CBD in Western Blot, we tried to determine which isoform each protein band represents. Overexpression of HA-tagged PMCA2 in transformed cell was used to identify PMCA2. Interestingly, overexpression of one isoform (PMCA2) can affect the expression of the other two isoforms (PMCA3 and PMCA4) (Dr. Yano, personal communication). So in the Western Blot analysis of pellicle proteins from transformed cells, anti-CBD and anti-HA both recognize the same protein band, which is the highest molecular weight band among the three protein bands according to the molecular weight marker. So it is concluded that the upper protein bands among those three represents PMCA2.

To determine the presence of the PMCA3, another antibody, anti-N3 was used in Western Blot analysis. This antibody is generated against the N-terminal peptide of PMCA3, but recognizes two lower proteins bands. The specificity of anti-N3 is tested by absorbing the antibody with a fusion protein of GST-tagged
N-terminal peptide of PMCA3. In the test, anti-N3 shows a high specificity of the
two lower protein bands. One explanation of these two bands is due to
phosphorylation, because it has been found that two serines in CBD of PMCA3
are the substrates of protein kinases. But this possibility can be ruled out by the
phosphatase treatment. Anti-N3 still recognizes two lower protein bands in the
phosphatase-treated pellicle. But one limit of this experiment is that there is no
positive control to confirm the phosphatase is active in the experiment. However,
it is found that N-terminal peptides of PMCA3 and PMCA4 share a high
homology. In addition, anti-N3 is generated against the N-terminal peptide of
PMCA3. So it is possible that anti-N3 recognizes both PMCA3 and PMCA4.

**Down-regulation of PMCA2 or PMCA3**

It has been demonstrated that during chemoattraction, the
hyperpolarization of membrane potential is sustained by the activation of PMCA to
generate the hyperpolarizing conductance (Van Houten, 1998). So the effects
of down-regulation of PMCA2 or PMCA3 were studied for their effects in
attraction. RNAi feeding method was applied to down-regulate the expression
level of PMCA2 or PMCA3. Unfortunately, there were no detectable changes of
either mRNA level or protein level. This may be due the high message level of
PMCA2 or 3, and the introduced siRNA cannot degrade the target mRNA
efficiently. But PMCA2 or PMCA3 RNAi fed cells show a decreased
chemoattraction toward glutamate, and no effect on other attractant. One
The phenomenon of siRNA, called off-target effect, may be the explanation for the change in chemoattraction. The off-target effect is that the siRNA binds to unspecific mRNA targets through partial complementarity and causes the mRNA degradation and the protein expression level decrease. This off target effect can also result in phenotype change (Jackson et al., 2006). In this case the siRNA does not bind to the target mRNA (transcript of PMCA2 or PMCA3), but binds to mRNAs of other proteins, one of which is likely one component of glutamate chemoattraction signal transduction.

The association of PMCA2 with lipid rafts

More and more signaling molecules, such as receptor proteins and their effector proteins, have been found preferentially localized into lipid rafts. These proteins use lipid rafts as platforms to assemble into signal transduction complexes and work efficiently. Lipid rafts can also regulate signal transduction through recruiting or expelling signaling components. In this work, it is demonstrated that PMCA2 is localized in lipid rafts, but PMCA3 or PMCA4 are not. The raft-association of PMCA2 is highly dependent on the sterol content in cell membrane as shown by MβCD treatment which disrupts the distribution of PMCA2 in sucrose gradient and alters localization at the ciliary bases. MβCD treated cells also show a decreased chemoattraction toward glutamate. In addition, after the MβCD treatment PMCA2 is not present in the pellet (fraction 10) in the sucrose gradient ultracentrifugation, since there are no proteins can be
recognized by anti-CBD around the region of 115.5 KD. This result is different from the theoretical postulation that if PMCA2 does not distribute in the sucrose gradient, it should stay in the pellet (fraction 10) after the sucrose gradient ultracentrifugation. It may be due to the mechanical damage on the epitope of PMCA2 in the sucrose gradient ultracentrifugation. But in the Na$_2$CO$_3$ extraction, PMCA2 still stay in the pellicle.

It has been reported that the raft-association of PMCA can be isoform specific, and certain isoforms may play specific roles in cells. In cerebellum membrane, only PMCA4 is associated with lipid rafts (Sepulveda, 2006). In hair bundle cells, PMCA2 continuously extrudes Ca$^{2+}$ resulting in a localized high extracellular Ca$^{2+}$ environment to maintain the inside-negative membrane potential (Prasad, 2004). Our data suggests that PMCA2 may serve specifically in glutamate chemoattraction signal transduction to hyperpolarize the membrane potential. Its association with lipid rafts helps PMCA2 fulfill the role.

**Lipid rafts in *Paramecium***

Lipid rafts have been previously characterized in *Paramecium* (Chandran, 2004). Raft-associated proteins can be recovered in sucrose density gradient through ultracentrifugation. This traditional method was applied to analyze the pellicle proteins in *Paramecium*. Besides PMCA2, GPI-anchored surface antigens and PCM are also localized in lipid rafts. In sucrose density gradient, these two proteins distribute across all the factions. They can serve as raft-
marker proteins in studies. It has been reported that some lipid rafts are associated with cytoskeleton proteins. The cytoskeleton-associated lipid raft is termed detergent-resistant membrane with high buoyant density (DRM-H) (Nebl et al., 2002). In Paramecium, lipid rafts are also associated with cytoskeleton proteins. It is found that tubulin distributes across all the fractions. Actin distributes in fraction 6 to 9. These results imply that in Paramecium, cytoskeleton proteins participate in stabilizing lipid rafts.

MβCD treatment shows different effects on these raft-associated proteins. After MβCD treatment, the distribution of GPI-anchored surface antigens and tubulin appear only in high buoyant density fractions in sucrose gradient. PMCA2 does not show up in the sucrose gradient at all, but the distribution of PCM is not affected by MβCD treatment. So MβCD treatment does not affect the distributions of every raft proteins in sucrose gradients. These results indicate that in Paramecium, lipid rafts may fall into two types, MβCD sensitive or insensitive. The stabilization of MβCD-sensitive rafts is highly dependent on cholesterol level in membrane. Any change of cholesterol can disrupt this type of lipid rafts. In Paramecium, MβCD treatment can cause about 40% decrease of cholesterol in pellicle. PMCA2 may exclusively localize in the MβCD-sensitive lipid rafts. MβCD treatment can disrupt the distribution of PMCA2 in sucrose density gradients and the ciliary base-localization on cell surface. In addition, cAMP receptor or its effector may be localized in the MβCD-sensitive lipid rafts too, since in chemoattraction studies, MβCD-treated cells show chemorepulsion.
to cAMP. The MβCD-insensitive lipid rafts may have a high level of GM1 ganglioside, which can compensate the reduction of cholesterol to preserve the lipid rafts (Jiang et al., 2007). PCM may be localized in the MβCD-insensitive lipid rafts. MβCD treated cells show no effect on the folate chemoattraction, so the GPI-anchored folate receptor may be localized in this type too. GPI-anchored surface antigens and tubulin may be localized in both types of rafts, as their distribution in sucrose gradient is partially affected by MβCD treatment.

Summary

In this research, PMCA isoform 2 is identified in Western Blot analysis of pellicle proteins. Further, it is determine that PMCA2 is associated with lipid rafts. It is also found that GPI-anchored surface antigens and PCM are localized in lipid rafts too. Lipid rafts are attached with cytoskeleton proteins including tubulin and actin. Based on the results of effects of MβCD treatment on raft-associated proteins, it is proposed that in Paramecium, there are two types of rafts, MβCD sensitive or insensitive. The effect of PMCA2 in chemoattraction toward glutamate may highly depend on its association with MβCD sensitive lipid rafts. Paramecium may utilize these two types of lipid rafts to recruit signaling molecules with different properties to regulate the chemoattraction signal transduction tightly. Based on the previous and current results, a working model of membrane domains incorporating three signal transduction pathways is proposed (Fig.11). In this model, in pathway 1, glutamate is the only attractant.
After glutamate binds to its receptor, adenylyl cyclase and a K⁺ channel are activated. Adenylyl cyclase converts ATP into cAMP, which consequently activates PKA. PKA can activate raft-associated PMCA2 to generate the sustained hyperpolarization conductance. In pathway 2, the attractants are more numerous including cAMP, folate, and acetate. The signaling components in this pathway are still not clarified. In this pathway, the non raft-associated PMCA(s) may be activated to generate the sustained hyperpolarization conductance. In the pathway 3, there are two equilibria of NH₄Cl. At extracellular side, NH₃ diffuses across the membrane. At the intracellular side, the alkaline pH activates an unknown cation channel. The efflux of cation generates the hyperpolarization conductance.
Chapter 3

Future Directions
In *Paramecium*, PMCAs are localized in the pellicle, especially at bases of cilia. PMCA2 is insoluble in cold Triton X-100, but PMCA3 and/or PMCA4 is soluble in cold Triton X-100. These results imply that PMCA2 may be associated with cytoskeleton or present in lipid rafts or both; PMCA3 and PMCA4 may not be present in lipid rafts or associated lipid rafts. In the lipid raft analysis of pellicle proteins, PMCA2 may be present in MβCD sensitive lipid rafts. PCM may be present in MβCD insensitive lipid rafts. GPI-anchored surface antigens may be present in both MβCD sensitive and insensitive lipid rafts. Lipid rafts in *Paramecium* are associated with cytoskeleton proteins such as tubulin and actin. Sterol-depletion disrupts the localization of surface antigens and PMCAs to bases of cilia. It also disrupts the distribution of PMCA2 in the sucrose gradients, but has effect on the distribution of PCM in the sucrose gradients. Sterol-depletion disrupts the chemoattraction to glutamate and cAMP. RNAi-PMCA2 and RNAi-PMCA3 transformed cells show reduced chemoattraction to glutamate.

In the further studies, the localization of PMCA3 and PMCA4 need to be identified. Generation of isoform specific antibodies would be helpful in this work. Anti-N3 recognizes two protein bands on the blots. This could be due to the protein kinase phosphorylation. To figure out this problem, in the experiment of phosphatase treatment, one positive control is needed to prove that the phosphatase is active in the experimental system. Additionally, the three protein
bands that are recognized by anti-CBD can be analyzed in Mass Spectrometry to support the findings. After MβCD treatment, PMCA2 is still Triton insoluble. It implies that PMCA2 may be associated with cytoskeleton proteins. The PMCA2 protein interaction partner may be important in PMCA2 raft-targeting, furthermore, makes PMCA2 serve in a specific signal transduction pathway such as glutamate pathway in chemoattraction. PMCA3 and/or PMCA4 are not associated in lipid rafts. These two isoforms may serve in raft-independent signal transductions in chemoattraction. It is interesting to study the raft-associated and non-raft-associated PMCs in chemoattraction to clarify the role of lipid rafts in the chemosensory signal transductions. Additionally, 20 more PMCA isoform genes are found in annotated *Paramecium* genome. These results imply that PMCA isoforms may serve different physiological roles in *Paramecium*. It is significant to find out that the specific roles of these 23 PMCA isoforms in *Paramecium*. Since RNAi-PMCA2 and RNAi-PMCA3 transformed cells show reduced chemoattraction to glutamate, but there were no detectable change at both mRNA and protein level of PMCA2 or PMCA3. A more specific and efficient siRNA constructing would be very helpful in the studies of the roles of 23 PMCA isoforms in *Paramecium*. Beside PMCA2, some receptors and their down-stream effectors may partition in to lipid rafts. cAMP receptor may be a possible candidate to be studied, because MβCD treatment dramatically disrupted chemoattraction toward cAMP. GPI-anchored folate receptor has been characterized in *Paramecium* (Weeraratne, 2004). GPI-anchored proteins are
preferentially localized in lipid rafts. So *Paramecium* folate receptor can be another candidate for studying lipid rafts function in chemoattraction. Finally, it is important to find out the link between surface receptors and PMCAs in signal transduction. There may be several components between receptors and effectors. Like in pathway 1, after glutamate binds to its receptor, adenylyl cyclase may be activated. This membrane-bound adenylyl cyclase may also be associated lipid rafts to serve in the glutamate signal transduction pathway. To clarify each component in the signal transduction pathways can be helpful to understand the signal transduing mechanisms in chemoattraction.
References


Figure 1: The presence of PMCA in pellicle proteins.

The pellicle proteins from wild type cells were extracted by 0.1 M Na₂CO₃ and analyzed through Western Blot. The blot was immunoprobed by anti-CBD (Primary, 1:5,000) and alkaline phosphatase conjugated rabbit anti-IgG (Secondary, 1:10,000).

Blue arrows point molecular weight. Black arrows point proteins.
Figure 1

115.5 KD

Anti-CBD

PMCA2  KPSG ILELRRGSSLRKK
PMCA3  KPSG VLELRRGSSI RRS
PMCA4  KPSG ILELRRGSSVRRS
**Figure 2: Determination of PMCA isoform 2.**

The pellicle proteins from transformed cells containing HA-tagged PMCA2 are analyzed through Western Blot.

A, Left, the blot was immunoprobed by anti-CBD (primary, 1:5,000). Right, the blot was immunoprobed by anti-HA (primary, 1:5,000). The secondary antibody of alkaline phosphatase conjugated goat anti-rabbit IgG was diluted 1:10,000.

B, Left. The blot was immunoprobed by anti-HA (primary, 1:5,000) and horseradish peroxidase conjugated goat anti-rabbit IgG (secondary 1:5,000 diluted) and developed through ECL. Middle, The blot was stripped and reprobed by anti-CBD (primary, 1:5,000) and alkaline phosphatase conjugated goat anti-rabbit IgG (secondary, 1:10,000). Right, The ECL film was overlaid on the blot.

Blue arrows point molecular weight. Black arrows point proteins.
Figure 2

A. 115.5 KD

PMCA2

Anti-CBD

B. 115.5 KD

PMCA2

Overlapped

Anti-CBD

Anti-HA
Figure 3: Identification of two lower protein bands.

A. The pellicle proteins from wild type cells were analyzed through Western Blot. Left, the blot is immunoprobed by anti-N3 (primary, 1:5,000) and horseradish peroxidase conjugated goat anti-rabbit IgG (secondary 1:5,000) and developed by ECL. Middle, The bolt was stripped and reprobed by anti-CBD (primary, 1:5,000 diluted) and alkaline phosphatase conjugated goat anti-rabbit IgG (secondary, 1:10,000). Right, The ECL film was overlaid on the blot.

B. The specificity test of anti-N3. Left, the blot was immunoprobed by intact anti-N3. Right, the blot was immunoprobed by the preaborbed anti-N3.

C. The pellicle proteins were treated by phosphatase, and analyzed through Western Blot. The blot was immunoprobed by anti-N3 and (primary, 1:5,000) and horseradish peroxidase conjugated goat anti-rabbit IgG (secondary 1:5,000) and developed by ECL. Lane 1, control, no phosphatase. Lane 2, 100 Units phosphatase. Lane 3, 150 Units phosphatase. Lane 4, 100 Units phosphatase boiled for 5 minutes. Lane 5, 150 Units phosphatase boiled for 5 minutes.

Blue arrows point molecular weight. Black arrows point proteins.
Figure 3B

anti-N terminus 3

anti-N terminus 3 preabsorbed with a fusion protein of GST-tagged N-terminal peptide of PMCA3
Figure 4: Localization of PMCAs.

A. Cell surface was labeled by anti-CBD (1:50). PMCAs are localized to the bases of cilia.

B. Cilia were labeled by anti-CBD (1:50). Anti-1D5 (1:100) that labels tubulin at basal body is used as a marker.

Images were taken under the objective lens with 60X magnification.
Figure 5: Triton X-100 solubility study of PMCAs.

Pellicle proteins (500 ug) were solubilized by 1% triton X-100 at 4°C for 1 hour.

Left. The blot was immunoprobed by anti-N3 (primary, 1:5,000) and horseradish peroxidase conjugated goat anti-rabbit IgG (secondary 1:5,000 diluted) and developed by ECL. Right. After being stripped, the blot was reprobed by anti-CBD (primary, 1:5,000) and alkaline phosphatase conjugated goat anti-rabbit IgG (secondary, 1:10,000) and developed by NBT/BCIP.

Blue arrows point molecular weight. Black arrows point proteins.

P: Triton insoluble; S: Triton soluble.
**Figure 5**

- **P**: Insoluble pellet (Triton insoluble)
- **S**: Supernatant (Triton soluble)
Figure 6: Distributions of pellicle proteins in sucrose density gradient.

The pellicle proteins (~30 mg) were extracted with 1% Triton X-100 and recovered in 5%-40% linear sucrose density gradient. Lane 1-9 represents 9 factions of sucrose density gradient. Lane M is protein marker.

A. The blot was immunoprobed by anti-surface antigens (1:5,000 diluted). GPI-anchored surface antigens (220-250 KD) distributed in all 9 fractions.

B. The blot was immunoprobed by anti-CBD (1:5,000). PMCA2 (125 KD) distributes in fraction 3 to 8.

C. The blot was immunoprobed by commercial anti-FBP (1:2,000). PCM (65 KD) distributes in all 9 fractions.

D. The blot was immunoprobed by anti-tubulin (1:10,000 diluted). Tubulin (54 KD) distributes all 9 fractions.

E. The blot was immunoprobed by anti-actin, Actin (40 and 43 KD) distribute in fraction 6 to 9.

Blue arrows point molecular weight. Black arrows point proteins.

The blots represent 2—4 experiments.
Figure 6

A 310 kDa
B 115 kDa
C 54 kDa
D 49 kDa
E 37 kDa

1 2 3 4 5 M 6 7 8 9

Surface antigens (GP: anchored)
PMCA2
FCM
Tubulin
Actin
Figure 7: Sterol depletion in pellicle.

The cholesterol level in pellicle was measured through cholesterol assays. The cholesterol level in pellicle from MβCD treated and mock treated cells were compared. The value is average of 3 measurements.

Cholesterol level in MβCD treated cells shows 42%±1.4 decrease compared with mock treated cells.
Figure 8: Distributions of pellicle proteins from MβCD treated cells in sucrose density gradient.

The pellicle proteins from MβCD treated cells (~30 mg) were extracted with 1% Triton X-100 and recovered in 5%-40% linear sucrose density gradient. Lane 1-9 represents 9 factions of sucrose density gradient. Lane M is protein marker. Blue arrows point the molecular weight of protein marker. Black arrows point the proteins immunoprobed by different antibodies.

A. The blot was immunoprobed by anti-surface antigens (1:5,000). GPI-anchored surface antigens on (220-250 KD) distribute in fraction 6 to 9.

B. The blot was immunoprobed by anti-CBD (1:5,000). PMCA2 does not distribute sucrose density gradient.

C. The blot was immunoprobed by commercial anti-FBP (1:2,000). PCM (65 KD) distribute in all 9 fractions.

D. The blot was immunoprobed by anti-tubulin (1:10,000). Tubulin (54 KD) distributes fraction 7 to 9.

E. The blot was immunoprobed by anti-actin (1:5,000), Actin (40 and 43 KD) distribute in fraction 7 to 9.

F. The blot was immunoprobed by anti-CBD (1:5,000). PMCA2 is not present in the pellet (fraction 10) after sucrose gradient ultracentrifugation.
G. The blot was immunoprobed by anti-surface antigens (1:5,000). Surface antigens are present in the pellet (fraction 10) after sucrose gradient ultracentrifugation.

Blue arrows point molecular weight. Black arrows point proteins.

The blots represent 2—4 experiments.
Figure 9: Western Blot analysis of pellicle proteins from MβCD treated cells.

A. The pellicle protein from MβCD treated and mock treated cells were extracted by 0.1 M Na₂CO₃ at 4°C for 1 hour and analyzed through Western Blot. The blots are immunoprobed by anti-CBD (Primary, 1:5,000 diluted) and alkaline phosphatase conjugated rabbit anti-IgG (Secondary, 1:10,000).

B. The pellicle protein from MβCD treated and mock treated cells were solubilized by 1% Triton X-100, and then analyzed by Western Blot. The Blots are immunoprobed by anti-CBD (primary, 1:5,000) or anti-pFBP (primary 1:2,000). The secondary antibody of alkaline phosphatase conjugated goat anti-rabbit IgG was diluted 1:10,000.

Blue arrows point the molecular weight. Black arrows point the proteins.
Figure 9B

P Insoluble pellet (Triton insoluble)
S Supernatant (Triton soluble)
Figure 10: The effects of MβCD treatment on the localization of GPI-anchored surface antigens and PMCAs.

A. The cells were permeabilized by 0.05% Triton X-100 and double immunostained by anti-surface antigens (1:100) and anti-1D5 (1:100). In mock treated cells, GPI-anchored surface antigens are localized at the basis of cilia. In MβCD treated cells, their localization pattern is changed.

B. The cells were permeabilized by 0.05% Triton X-100 and double immunostained by anti-CBD (1:50 diluted) and anti-1D5 (1:100). In mock treated cells, PMCAs were localized at the basis of cilia. In MβCD treated cells, PMCAs distributed across the cell surface membrane.

Images in A and B were taken under objective lens with 60X magnification.

C. The cells non-permeabilized. Both mock and MβCD treated cells were immunostained by anti-surface antigen A (1:100 diluted). This antibody labeled the surface antigen on cell surface membrane. But the staining patterns were different between the mock and MβCD treated cells.

Images in C were taken under objective lens with 100X magnification.
Figure 10A

Mock treated

Anti-Surface antigens

Anti-1D5

Merged

Anti-1D5

Merged

Anti-Surface antigens

5 μm

5 μm
Table 1: Chemoattraction studies of RNAi cells.

The chemoattraction of RNAi cells were studied through T-Maze assays. The chemoattractant solutions are 2mM Na-L-glutamate, 2 mM Na-Acetate, 2mM NH₄Cl, 1mM Na₂-folate, or 2mM Na-cAMP. The control solution is 2mM NaCl. The chemoattraction of RNAi cells toward 2mM Na-L-glutamate is statistically significantly decreased compared with control cells at the P<0.05 level in Mann-Whitney U test.

<table>
<thead>
<tr>
<th>Chemoattractant</th>
<th>Control</th>
<th>PMCA2 RNAi</th>
<th>PMCA3 RNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate n=21</td>
<td>0.83±0.06</td>
<td>0.71±0.05**</td>
<td>0.70±0.09**</td>
</tr>
<tr>
<td>Acetate n=9</td>
<td>0.67±0.05</td>
<td>0.67±0.06</td>
<td>0.62±0.08</td>
</tr>
<tr>
<td>Folate n=9</td>
<td>0.84±0.12</td>
<td>0.86±0.08</td>
<td>0.80±0.09</td>
</tr>
<tr>
<td>NH₄Cl n=9</td>
<td>0.84±0.08</td>
<td>0.87±0.07</td>
<td>0.83±0.08</td>
</tr>
<tr>
<td>cAMP n=6</td>
<td>0.72±0.01</td>
<td>0.75±0.02</td>
<td>0.72±0.02</td>
</tr>
</tbody>
</table>

** Statistically significantly different from control at the P<0.05 level, Mann-Whitney U test
n=number of T-Mazes
Table 2: Chemoattraction studies of MβCD treated cells.

The chemoattraction of MβCD treated cells are studied through T-Maze. The chemoattractant solutions are 2mM Na-L-glutamate, 2 mM Na-Acetate, 2mM NH₄Cl, 1mM Na₂-folate, or 2mM Na-cAMP. The control solution is 2mM NaCl. The chemoattraction of MβCD treated cells toward 2mM Na-L-glutamate or 2mM Na-cAMP are statistically significantly decreased compared with mock treated cells at P<0.05 level in Mann-Whitney U test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NaClAc</th>
<th>Folate</th>
<th>Glutamate</th>
<th>NH₄Cl</th>
<th>cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.88±0.04</td>
<td>0.90±0.02</td>
<td>0.87±0.01</td>
<td>0.97±0.01</td>
<td>0.90±0.06</td>
</tr>
<tr>
<td>MβCD</td>
<td>0.87±0.03</td>
<td>0.87±0.02</td>
<td>0.61±0.07</td>
<td>0.98±0.01</td>
<td>0.38±0.05</td>
</tr>
</tbody>
</table>

Data are averages of 16-18 T-mazes ± S. D. Red data are statistically significantly different from control at P<0.05 level in Mann-Whitney U test.
Figure 11: A working Model of 3 signal transduction pathways in chemoattraction in Parameicum.

In this model, in the pathway 1, glutamate is the only attractant. After glutamate binds to receptor, adenylyl cyclase and a K⁺ channel are activated. Adenylyl cyclase converts ATP into cAMP, which consequently activates PKA. PKA can activate raft-associated PMCA2 to generate the sustained hyperpolarization conductance. In the pathway 2, the attractants are more numerous including cAMP, folate, and acetate. The signaling components in this pathway are still not clarified. In this pathway, the non raft-associated PMCA(s) may be activated to generate the sustained hyperpolarization conductance. In the pathway 3, there are two equilibria of NH₄Cl. At extracellular side, NH₃ diffuses across the membrane. At the intracellular side, the alkaline pH activates an unknown cation channel. The efflux of cation generates the hyperpolarization conductance.