J. Eukaryot. Microbiol., 50(5), 2003 pp. 349–355

G-Protein Modulators Alter the Swimming Behavior and Calcium Influx of *Paramecium tetraurelia*

JOSÉ DE ONDARZA,[a] STEVEN B. SYMINGTON,[b] JUDITH L. VAN HOUTEN[c] and J. MARSHALL CLARK[b]

[a]*Plattsburgh State University of New York, Department of Biological Sciences, Plattsburgh, New York 12901 USA, and*
[b]*University of Massachusetts, Molecular and Cellular Biology Program, Amherst, Massachusetts 01003 USA, and*
[c]*University of Vermont, Department of Biology, Burlington, Vermont 05405 USA*

ABSTRACT. To assess the potential role of G-proteins in chemokinesis, *Paramecium tetraurelia* was pre-incubated with the G-protein modulator pertussis toxin. Pertussis toxin pretreatment significantly reduced *Paramecium* chemoattraction to sodium acetate and ammonium chloride in T-maze behavioral assays and depressed the frequency of avoidance reactions, indicating that heterotrimeric G-proteins may be involved with the motility response. To determine whether G-proteins exert their effect via the ciliary voltage-sensitive calcium channel, we examined responses of *P. tetraurelia* to the potent voltage-sensitive calcium channel agonist, deltamethrin. Pertussis toxin preincubation significantly reduced the toxic effects of deltamethrin exposure as determined by survival under depolarizing conditions and reduced the duration of backward swimming episodes in behavioral bioassays. Furthermore, non-hydrolyzable analogs of guanine nucleotides altered deltamethrin-stimulated calcium influx via calcium channels in isolated ciliary vesicles. Heterotrimeric G-protein subunits were subsequently detected in ciliary vesicles of *P. tetraurelia* by antibodies produced against Gα and Gβ subunits, and by ^{32}P-ADP-ribosylation, indicating that proteins of the appropriate molecular weight are the target of pertussis toxin in these vesicles. These findings provide additional evidence that heterotrimeric G-proteins are associated with ciliary vesicles and that they play a role in the modulation of swimming behavior and the toxic action of deltamethrin in *Paramecium*.

Key Words. Avoidance reactions, backward swimming, calcium channel regulation, chemoresponse, ciliate, deltamethrin, insecticide, pertussis toxin, pyrethroids.

THE freshwater ciliate, *Paramecium tetraurelia*, uses chemosensory cues in locating its bacterial food source, typically by responding to gradients of bacterial metabolites, such as cyclic adenosine monophosphate (cAMP), glutamate, folate, acetate or biotin (Van Houten 1992). In most of these instances, alterations in membrane potential and intracellular free calcium concentrations ($[Ca^{2+}]_i$) initiate the behavioral responses leading to chemoattraction: an increase in linear swimming speed and a decrease in the frequency of turning (reviewed in Kung and Saimi 1982; Van Houten 1978). The intracellular mediators of this chemosensory signaling cascade are not yet fully defined, but are thought to include surface membrane receptors, calmodulin, and a plasma membrane Ca^{2+}-ATPase (Van Houten 1992; Wright et al. 1993). In addition, both intracellular cAMP and cyclic guanosine monophosphate (cGMP) have been implicated in modulating calcium ion fluxes in *Paramecium* (reviewed in Plattner and Klauke 2001). Heterotrimeric G-proteins, whose role in the chemosensory transduction of *Dictyostelium* and *Saccharomyces* has been firmly established (Dietzel and Kurjan 1987; Schenk et al. 1991), remain an elusive element in these responses in *Paramecium*.

There is a small but growing body of evidence to support the presence of G-proteins in *Paramecium* and other ciliates. Partial G-protein alpha-subunit (Gα) sequences have been identified in the ciliate *Stentor* (Fabczak et al. 1993; Marino et al. 2001), while Forney and Rodkey (1992) identified a highly conserved consensus tryptophan–aspartate (WD) sequence motif for the beta-subunit of G-proteins (Gβ) in the macronuclei of several species of *Paramecium*. Fraga and Hinrichsen (1994) used a PCR homology approach in *Paramecium* to identify cDNA transcripts that had deduced amino acid sequence similar to low molecular weight G-proteins. Furthermore, there is evidence that implicates G-proteins in the regulation of the ciliary calcium channel. Bernal et al. (1991) reported that non-hydrolyzable analogs of guanine nucleotides altered *Paramecium* backward swimming behavior and prolonged the duration of whole cell calcium action potentials when injected into cells. Clark et al. (1993) reported that extracellular GTP-γ-S prolonged backward swimming in *Paramecium* and induced whole cell membrane depolarization. Collectively, these results indicate that G-proteins are present in *Paramecium* species and may be involved with *Paramecium*'s chemoresponse to external stimuli.

The discovery that the pyrethroid insecticide deltamethrin is extremely toxic to the fresh-water ciliate *P. tetraurelia* at concentrations as low as 10^{-10} M under depolarizing conditions (Clark et al. 1995) provided a useful tool for studying modulation of the voltage-dependent calcium channel (VDCC). In behavioral bioassays, deltamethrin-treated cells exhibited an increase in backward swimming, a well-characterized avoidance response controlled by the ciliary VDCC. The non-toxic 1S isomer of deltamethrin had no significant effect on either mortality or avoidance behavior of *P. tetraurelia. Pawn B* mutants, which lack a functional VDCC, likewise were unaffected by deltamethrin. Intracellular recordings of whole cells showed that exposure to deltamethrin at concentrations as low as 1 nM resulted in membrane destabilization, an increased number of spontaneous action potentials, and membrane depolarization, establishing that the toxic effect of deltamethrin was stereospecific, dose-dependent and enhanced by depolarization (Clark et al. 1995). Thus CS-syndrome pyrethroids (those causing choeoathetosis-salivation), specifically deltamethrin, act as potent calcium channel agonists in *P. tetraurelia*. Whole cell radioisotope and fluorescent dye studies subsequently demonstrated that deltamethrin-induced mortality and backward swimming behavior was directly correlated to deltamethrin-enhanced Ca^{2+} influx (Symington et al. 1999).

In light of these observations, we investigated the role that heterotrimeric G-proteins may play in modulating the VDCC of *P. tetraurelia* by examining the effect of the G-protein modulators pertussis toxin and the non-hydrolyzable GTP analogues GDP-β-S and GTP-γ-S on normal and deltamethrin-exposed *P. tetraurelia* cells. The current studies present evidence of G-protein involvement in the swimming behavior of *P. tetraurelia*, presumably via modulation of the ciliary voltage-sensitive calcium channel.

MATERIALS AND METHODS

Chemicals. Pertussis toxin, GTP-γ-S, GDP-β-S, purified G-protein standards, and anti-$G\alpha_{COM}$ antibodies were purchased from Calbiochem (La Jolla, CA). Fura-2 penta-potassium salt (fura-2) was purchased from Molecular Probes, Inc. (Eugene, OR). 1R-deltamethrin [3-(2,2-dibromoethenyl)-2,2 dimethyl-

Corresponding Author: J. de Ondarza–Telephone number: 518-564-5156; FAX number: 518-564-3175; E-mail: jose.deondarza@plattsburgh.edu.

cyclopropanecarboxylic acid cyano (3-phenoxyphenyl)-methyl ester] was a generous gift from Prof. D. M. Soderland (Cornell University, Geneva, NY). The anti-Gβ-antibody (sc-261) was purchased from Santa Cruz Biotech (Santa Cruz, CA) and SDS-PAGE precast gels from FMC Bioproducts (Rockland, ME). All other chemicals and reagents were purchased from the Sigma Chemical Company (St. Louis, MO).

Whole cell chemoattraction and turning frequency bioassays. *Paramecium tetraurelia* (strain 51-S) were cultured in wheat grass medium supplemented with $NaHPO_4$ and stigmasterol and inoculated with *Klebsiella pneumoniae* (Van Houten et al. 1991). Log-phase *P. tetraurelia* were incubated overnight in wheat grass medium containing 0.1% dimethylsulfoxide (DMSO) and with or without 100 ng/ml of Pertussis Toxin (PTX). Cells were washed in control buffer (5 mM NaCl, 1 mM $Ca(OH)_2$, 1 mM citric acid, 1.3 mM Tris base, pH 7.2) and assayed for chemoattraction according to the methods of Van Houten (Van Houten et al. 1982). Chemoattraction assays were performed with the control and test buffers in which the attractant ammonium chloride (5 mM) or sodium acetate (5 mM) was used in place of NaCl. Cells were counted after 10 or 30 min.

To determine the frequency of avoidance reactions, PTX-treated and untreated *P. tetraurelia* cells were washed in control buffer and transferred to 100 μl of either control or acetate test buffers (5 mM sodium acetate substituted for NaCl) on a glass slide. Swimming behavior was recorded on videotape and analyzed with a Celltrak Motion Analysis System (Motion Analysis Corp., Santa Rosa, CA). For motion analysis, linear swim speed (mm/s) and percentage of directional change, a measure of turning frequency, were calculated (Davis, Fiekers, and Van Houten 1998).

Whole cell behavioral and mortality bioassays. Behavioral assessments of voltage-sensitive calcium channel ligands on swimming behavior were performed using the avoidance reaction behavior assay as previously described (Clark et al. 1995). Individual cells were transferred into carbowax-treated (5.0% polyethylene glycol (PEG) 20,000) watch glasses (26 mm diam.) containing 500 μl of K^+-depolarization buffer (1 mM $CaCl_2$, 1 mM Tris base, 1 mM citric acid, 20 mM KCl, pH 7.2) and swimming behavior was observed during K^+-induced membrane depolarization (that induces avoidance reactions) under a light microscope. For pretreatment experiments, cells were transferred into a resting solution containing 0.1% DMSO with or without 100 ng/ml of PTX and incubated overnight. The avoidance reaction behavior bioassay was initiated by transferring the cells into K^+-depolarizing buffer and backward swimming duration measured until cells resumed forward swimming. Mortality (LT_{50}) was determined by measuring the time for half of the cells to stop all motion and sink to the bottom of the watch glass.

Ca^{2+} influx into calcium channel-containing vesicles from cilia. Vesicles of ciliary membrane containing calcium channels were prepared as described by Thiele and Schultz (1981). Prior to vesicle formation, 1.5 mM fura-2 and 15 μM of a non-hydrolyzable analogue of guanine nucleotides (GTP-γ-S or GDP-β-S) were added to the ciliary solution to allow incorporation of the analogues into the vesicles during their formation. Vesicle flux analysis (nmoles Ca^{2+}/mg protein) and statistical calculations were performed as previously reported (Symington et al. 1999). Protein concentrations were determined as described by Smith et al. (1985).

Detection of G-protein subunits. Putative G-protein-like subunits were detected as described by Chen and Manning (1999). Ciliary vesicles were purified as described in Thiele and Schultz (1981) and vesicles resuspended in HME buffer (20 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4) containing protease inhibitors (2 μg/ml pepstatin, 2 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride). G-protein standards (0.25 μg) or ciliary vesicles (50–100 μg) were electrophoresed on a 10% polyacrylamide gel (25 mA, ~1.5 h) and transferred to a nitrocellulose membrane in transfer buffer (192 mM glycine, 25 mM Tris base, 20% methanol). The membrane was incubated for 1 h at room temperature in Tris-buffered saline (TBS-T: 20 mM Tris base, 500 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% non-fat dry milk. The membrane was washed five times in TBS-T buffer (twice for 30 s, once for 15 min, and twice for 5 min) to rinse blocking buffer. The membrane was incubated overnight at 4 °C in either anti-Gα-antibody (1:1000) or anti-Gβ-antibody (1:1000) in antibody buffer (20 mM Tris base, 500 mM NaCl, 1% gelatin, pH 7.5). Blots were washed as described above in 10 ml of TBS-T buffer and incubated for 2 h at room temperature in antibody buffer containing anti-IgG horseradish peroxidase-conjugated secondary antibody (1:2000). Blots were washed again as described above to remove secondary antibody and incubated for 1 min with enhanced chemiluminescence (ECL) assay reagents (Amersham Biosciences, Piscataway, NJ) prior to exposing X-ray film following the manufacturer's directions.

PTX-catalyzed ADP-ribosylation of Gα associated with ciliary vesicles was performed as described by Woolkalis (1999). PTX was first activated in PTX activation solution (10 mM HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]), pH 8, 4 mM dithiothreitol (DTT), 0.2 mg/ml bovine serum albumin, 0.025% sodium dodecyl sulfate (SDS), and 20 μg/ml PTX) and incubated at 30 °C for 30 min prior to the initiation of the labeling reaction. The PTX labeling mixture was prepared by adding 4 μg of purified bovine brain G-protein mix or 50–100 μg of ciliary vesicles in 25 μl of PTX assay solution (10 mM HEPES, pH 8, 1 mM DTT, 1 mM EDTA, 10 μM GDP-β-S, and 5 μM [^{32}P]NAD at 20,000 cpm/pmol) . Five μl of PTX activation solution were added to the PTX labeling solution and the mixture incubated at 30 °C for 60 min. The ADP-ribosylation reaction was terminated by the addition of 6 μl of 6× protein loading buffer. Protein samples were heated at 100 °C for 3 min and electrophoresed for 90 min at 125 V on a 10% tris-glycine precast protein gel. The bottom of the gel (protein < 20 kDa) was excised to removed any of the unbound ^{32}P-NAD and protein bands were detected by Coomassie gel stain (50% methanol, 0.05% Coomassie brillant blue R250, 10% acetic acid) and washed in Coomassie destain solution (5% methanol and 7% acetic acid). The gel was placed on 2 sheets of Whatman #3 filter paper and dried using a Bio-Rad 483 slab gel drying system. The dried gel was then exposed to X-ray film and developed according to manufacturer's instructions for visual inspection.

RESULTS

Whole cell behavioral and mortality assays. *Paramecium tetraurelia* tend to accumulate in the attractant in a T-maze with a control solution (sodium chloride) and a chemoattractant, such as ammonium chloride or sodium acetate. Using such assays, we found that 72–81% of the DMSO-treated (control) cells are found in the test arm of the T-maze (Fig. 1), values that were consistent with those previously published (Van Houten 1978). Pre-treatment of *P. tetraurelia* with 100 ng/ml of PTX significantly diminished this chemoresponse ($P < 0.05$) in T-maze assays of 10 min and 30 min duration (Fig. 1). Calculated indices of chemoattraction (I_{CHE}) were decreased by 0.11 to 0.12 compared to control cells for responses to ammonium chloride and acetate, respectively. This finding suggests that G-proteins

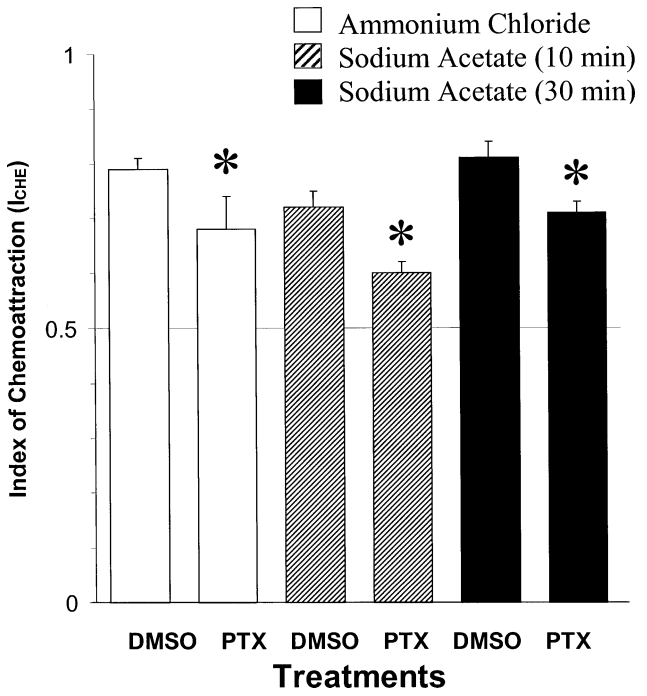

Fig. **1**. Effect of pertussis toxin pretreatment (PTX) on chemoattraction of *Paramecium tetraurelia* to sodium acetate or ammonium chloride. An index of chemoattraction (I_{CHE}) value > 0.5 indicates attraction, and a value < 0.5 indicates repulsion. Results represent Mean $\pm$ Standard Error for between 9 and 19 T-maze assays. * indicates that PTX treatment is significantly different from dimethylsulfoxide (DMSO) control (Mann-Whitney U test, $P < 0.05$).

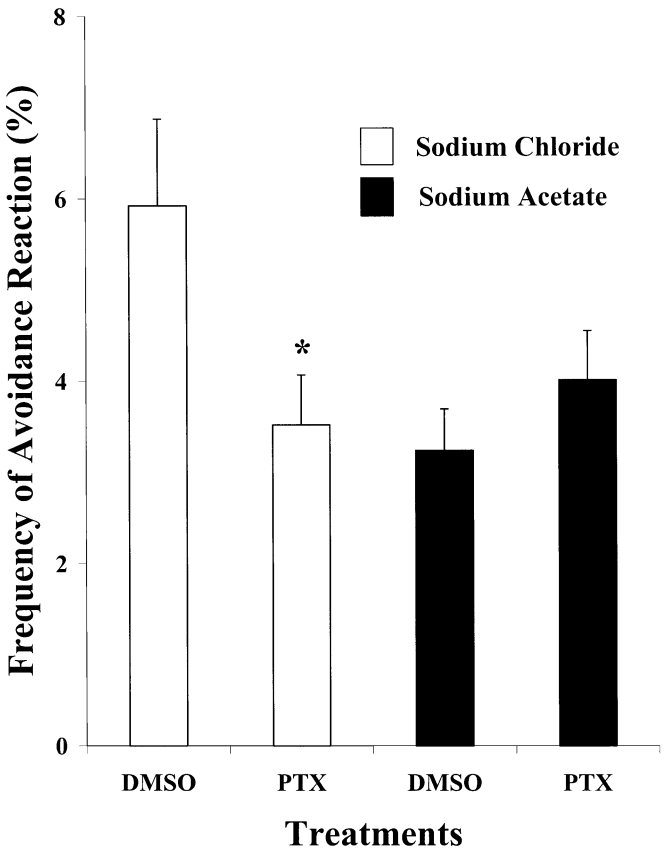

Fig. **2**. Effect of pertussis toxin pretreatment (PTX) on *Paramecium tetraurelia* swimming behavior. Results represent means for 150–200 cells. Standard errors for % data are reported as per Davis, Fiekers, and Van Houten (1998). * indicates that PTX treatment is significantly different from dimethylsulfoxide (DMSO) control (Mann-Whitney U test, $P < 0.05$).

of the G_o/G_i subclasses, known to be pertussis toxin substrates, might play a role in *P. tetraurelia* chemoresponse.

To investigate which component of *P. tetraurelia* swimming behavior (swim speed or turning frequency) was altered by pertussis toxin, cells were videotaped and their paths analyzed using motion-tracking software (modified by Kevin Clark). Linear swim speed was calculated to be 0.48 ± 0.02 mm/s (average $\pm$ standard error of the mean of 50–80 paths) in control and 0.52 ± 0.08 mm/s in PTX-treated cells, indicating no significant difference (Student's *t*-test, $P = 0.90$). However, PTX-treated cells showed a significant decrease in percent change in direction, that is they turned significantly less often than untreated cells ($P < 0.05$, Fig. 2). Turns (avoidance reactions) are produced by calcium action potentials at the ciliary voltage-sensitive calcium channel, suggesting the possibility that PTX-sensitive G-proteins may be involved in the regulation of this channel. The PTX effect on turning frequency was absent in cells exposed to the attractant sodium acetate ($P > 0.05$).

To further investigate the involvement of heterotrimeric G-proteins in the modulation of Ca^{2+} conductance, we used the pyrethroid insecticide deltamethrin. Standard mortality and behavioral bioassays were adapted to evaluate *P. tetraurelia* cells exposed to deltamethrin in the presence or absence of PTX pretreatment. Cell death in deltamethrin-exposed *P. tetraurelia* is caused by excessive Ca^{2+} influx, with an LT_{50} value (average time for 50% of exposed cells to be killed) of 62 s (Table 1). Pre-treatment of *P. tetraurelia* with 100 ng/ml of PTX significantly ($P < 0.05$) delayed the time of cell death by 23% (LT_{50} of 80 s), indicating a protective action of PTX against deltamethrin-induced toxicity.

Since deltamethrin also prolongs the duration of backward swimming in *P. tetraurelia*, we measured backward swimming times in the presence of deltamethrin with and without PTX pre-treatment (Table 2). (In these experiments, 1% ethanol was included with DMSO in controls because ethanol is the vehicle for deltamethrin [Table 2].) There is no significant difference between the backward swimming of cells in DMSO plus ethanol with or without pre-treatment with PTX. Cells swim back-

Table **1**. Effect of pertussis toxin (PTX) on mortality of *Paramecium tetraurelia* in the presence and absence of deltamethrin.

Treatment	LT_{50} value (sec)	95% Confidence interval
DMSO Pretreatment		
Ethanol	9.2×10^4	$8.1 \times 10^4 \geq x \geq 1.1 \times 10^5$
10^{-7} M Deltamethrin	62[a]	$60 \geq x \geq 64$
PTX Pretreatment		
Ethanol	1.4×10^6	$1.3 \times 10^6 \geq x \geq 1.3 \times 10^6$
10^{-7} M Deltamethrin	80[a,b]	$75 \geq x \geq 85$

LT_{50} is the median lethal time that results in death of 50% of the test population. Ethanol (1%) is the vehicle for deltamethrin and is used at this concentration in the controls.

[a] Indicates significant difference between deltamethrin treatment and ethanol control (Student's *t*-test, n = 30, $P < 0.01$).

[b] Indicates significant difference between DMSO and PTX pretreatment in the presence of 10^{-7} M deltamethrin (Student's *t*-test, n = 30, $P < 0.05$).

Table **2**. Effect of pertussis toxin (PTX) on the backward swimming behavior of *Paramecium tetraurelia* in the presence and absence of deltamethrin.

Treatment	Backward swim time (sec)	Standard error
DMSO Pretreatment		
Ethanol	1.1	0.5
10^{-7} M Del-tamethrin	53.9[a]	7.7
PTX Pretreatment		
Ethanol	0.6	0.3
10^{-7} M Del-tamethrin	35.7[a,b]	3.5

Ethanol (1%) is the vehicle for deltamethrin and is used at this concentration in the controls.

[a] Indicates significant difference between deltamethrin treatment groups and their respective ethanol controls (Student's *t*-test, n = 30, $P < 0.01$).

[b] Indicates significant difference between DMSO and PTX pretreatments in cell exposed to 10^{-7} M deltamethrin (Student's *t*-test, n = 30, $P < 0.05$).

ward for a long period of time when exposed to deltamethrin, regardless of the pre-treatment, but the duration of backward swimming is significantly less after pre-treatment with PTX (53.9 ± 7.7 s without pretreatment and 35.7 ± 3.5 s with pretreatment) (Table 2).

Effect of deltamethrin and modified guanosine nucleotides on Ca^{2+} influx in ciliary vesicles. To more closely examine the effects of G-protein modulators on calcium flux in *Paramecium*, we prepared ciliary membrane vesicles and loaded them with the calcium-sensitive dye fura-2 and GTP analogues (Fig. 3). Deltamethrin treatment of vesicles elicits an increase in $[Ca^{2+}]_i$ rapidly following treatment (60–200 s). Similarly, exposure of calcium orange-loaded *P. tetraurelia* cultures to deltamethrin resulted in intracellular calcium accumulation (data not shown). We then measured calcium entry into ciliary vesicles loaded with the GTP analogues GTP-γ-S or GDP-β-S and subsequently exposed to deltamethrin (Fig. 4). Treatment with 10^{-7} M deltamethrin alone stimulated Ca^{2+} influx by approximately 12-fold compared to the ethanol (1%) control. In the presence of the internalized non-hydrolyzable GTP analogue GTP-γ-S, deltamethrin treatment resulted in a significantly enhanced (100-fold) increase in Ca^{2+} influx compared to the corresponding ethanol control (Fig. 4) ($P < 0.05$). In contrast, deltamethrin evoked only a 6-fold influx of Ca^{2+} into vesicles that contained GDP-β-S as compared to ethanol controls, which was approximately half of the the accumulation in the presence of deltamethrin alone (not statistically significant at the $P < 0.05$ level) (Fig. 4). Ethanol control values were not significantly different from values obtained in the presence of non-hydrolyzable GTP analogs ($P > 0.05$), indicating that the GTP analogues alone could not effectively stimulate calcium influx in the absence of deltamethrin.

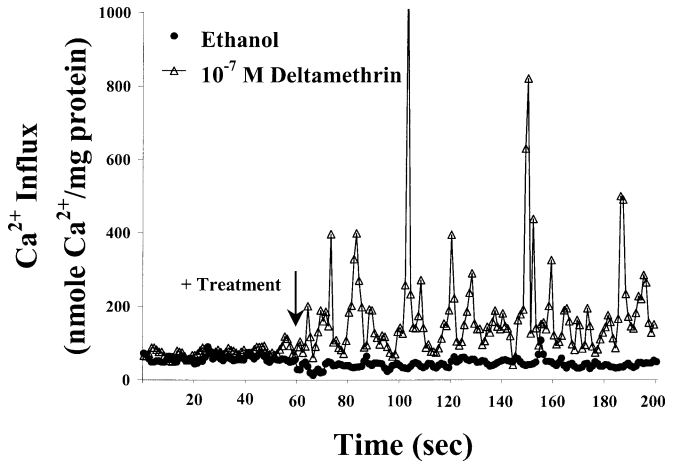

Fig. **3**. Representative recording of internal free $[Ca^{2+}]$ of ciliary vesicles purified from wild-type cells of *Paramecium tetraurelia* as measured by fura-2. Closed circles (●) represent ciliary vesicles treated with 1% ethanol. Open triangles (△) represent ciliary vesicles treated with 10^{-7} M deltamethrin.

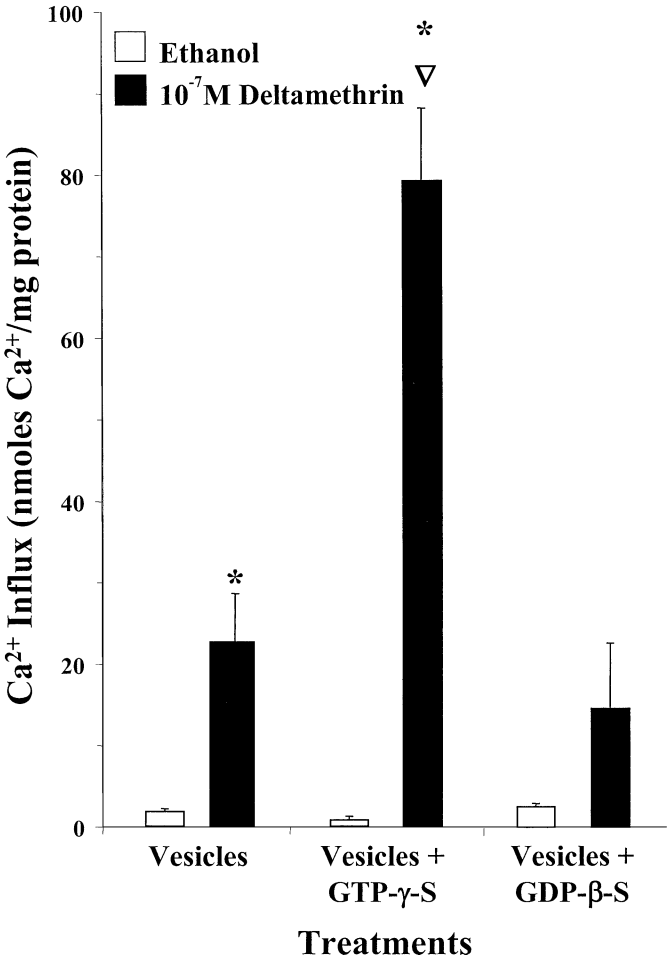

Fig. **4**. Effect of non-hydrolyzable analogs of guanine nucleotides on deltamethrin (10^{-7} M)-stimulated Ca^{2+} influx into membrane vesicles containing calcium channels, purified from wild-type *Paramecium tetraurelia*. * indicates deltamethrin treatment is significantly different from the respective ethanol control group (Student's *t*-test, $P = 0.06$ for vesicles only and P = 0.007 for vesicles + GTP-γ-S, n = 3) . ∇ indicates treatment value is different from control group in the absence of non-hydrolyzable analogs of guanine nucleotides (Student's *t*-test, $P = 0.028$, n = 3).

Detection of heterotrimeric G-protein subunits. In order to demonstrate the presence of heterotrimeric G-protein subunits in *P. tetraurelia*, we prepared ciliary vesicles, separated the proteins by SDS-PAGE and electroblotting, and developed the Western blots using anti-Gα and anti-Gβ antibodies (Fig. 5). Two distinct protein bands, with estimated molecular weights of 41 and 42 kDa, were detected using an antibody raised against the conserved GTP-binding motif of heterotri-

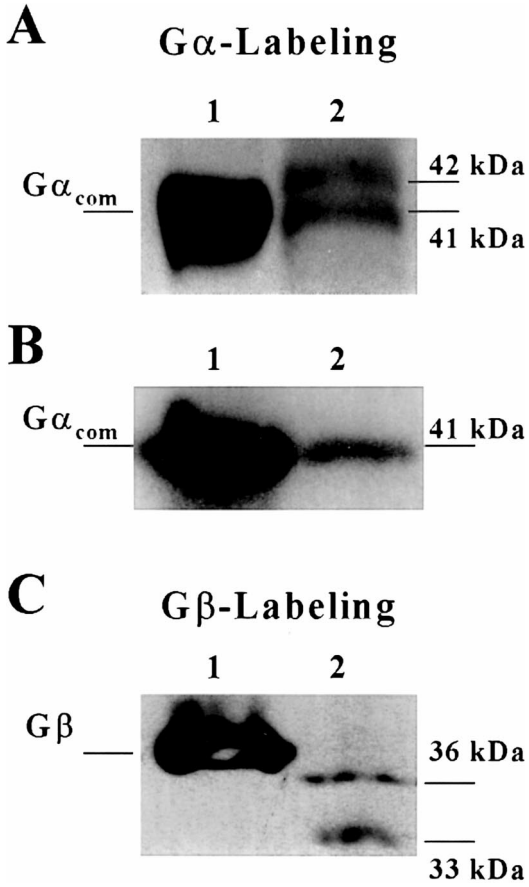

Fig. **5**. Identification of G-protein subunits in ciliary membrane vesicles purified from wild-type cells of *Paramecium tetraurelia*. **A**. Western-blot identification of two Gα-like proteins detected by anti-Gα-protein, an antibody that recognizes a conserved region within the GTP-binding domain of Gα-subunits. Lane 1: Heterotrimeric G-protein standard mixture (250 ng). Lane 2: 100 μg of ciliary membrane vesicles. **B**. Pertussis toxin (PTX) catalyzed ADP-ribosylation of purified cilia isolated from *P. tetraurelia*. Lane 1: 4 μg of purifed G-protein standard mix ($G\alpha_{com}$, Calbiochem #371736). Lane 2: 50 μg of purified ciliary vesicles. **C**. Western-blot identification of two Gβ-like proteins detected by anti-Gβ-protein, an antibody that recognizes the N-terminus of Gβ-subunits. Lane 1: Heterotrimeric G-protein standard mixture (250 ng). Lane 2: 100 μg of ciliary vesicles.

meric G-protein alpha subunits (Fig. 5A). These molecular weights are consistent with those of PTX-sensitive G_o or G_i subclasses of Gα subunits of other organisms (Chen and Manning 1999). Additionally, two distinct bands, with estimated molecular weights of 33 and 36 kDa, were subsequently detected using an antibody to the N-terminus of a G-protein β-subunit (Fig. 5C). To determine whether the putative Gα-subunit detected in these vesicles can be modified by pertussis toxin, a ^{32}P-ADP-ribosylation assay using ciliary vesicles was performed (Fig. 5B). PTX treatment of the vesicle protein fraction labeled a 41-kDa protein band that is consistent in size with the smaller of the two protein bands detected by immunoblotting, strongly supporting our contention that heterotrimeric G-proteins are present in *Paramecium* and that these proteins are the site of action of PTX in our studies.

DISCUSSION

Although genes for heterotrimeric G-proteins have been cloned, their proteins isolated, and a signal transduction role well defined in several eucaryotic microorganisms (Van Houten 1992), their presumed role in *Paramecium* is less clear. G-proteins mediate a multitude of signal transduction events in the metazoa, including many of the chemosensory (smell and taste) modalities (Restrepo, Teeter, and Schild 1995). Since heterotrimeric G-proteins have been shown to transduce the chemosensory cues for mating in yeast (Dietzel and Kurjan 1987) and cAMP-mediated aggregation in slime molds (Schenk et al. 1991), we hypothesized that sensory transduction pathways in *Paramecium* might involve such G-proteins as well. *Paramecium* responds to a wide range of chemical signals, including acetate, glutamate, folate, lactate, cAMP (all attractants), GTP, inositol monophosphate, and lysozyme (all repellants). Nonetheless, candidate chemoreceptors of the 7-transmembrane-domain receptor family, known to be coupled to heterotrimeric G-proteins, and heterotrimeric G-proteins themselves have yet to be positively identified in *Paramecium*. We therefore examined the response of *P. tetraurelia* to various G-protein modulators, including pertussis toxin (PTX), GTP-γ-S and GDP-β-S, and performed immunoblots with antibodies against conserved G-protein motifs. Our results demonstrate the presence of heterotrimeric G-protein α-subunits, presumably of the G_o/G_i family, and β-subunits, in the cilia of *P. tetraurelia* and provide evidence that they may be involved in the regulation of the ciliary voltage-sensitive calcium channel.

Previous studies have pointed toward a functional role of heterotrimeric G-proteins in *Paramecium multimicronucleatum* cold-transduction (Nakaoka et al. 1997), *Paramecium bursaria* phototransduction (Shinozawa et al. 1996), and *Paramecium calkinsii* swimming behavior (Bernal, Kelsey, and Ehrlich 1991), primarily based on biochemical studies using non-hydrolyzable GTP analogues. Unfortunately, aside from two WD-repeat sequences with homology to G-protein β-subunits (Dessen et al. 2001; Forney and Rodkey 1992), no G-protein gene sequences have been identified for *Paramecium*, although sequencing of genes from the recently described indexed genomic library may yield more pertinent data (Dessen et al. 2001; Keller and Cohen 2000). A scarcity of immunodetection data for G-protein subunits had led to speculation that such heterotrimeric G-proteins may even be absent from ciliates, such as *Paramecium*. We have now detected two putative Gα-subunits in *Paramecium* ciliary vesicles using an antibody against the highly conserved GTP-binding region of Gα subunits. The estimated molecular masses of these proteins are 40 kDa and 41 kDa, well within the typical 39–42 kDa range for G_o and G_i subtypes. These proteins differ in size from the putative 57-kDa G-protein identified by immunoblotting in *P. bursaria* (Shinozawa et al. 1996), the 39-kDa band found in *Stentor* (Fabczak et al. 1993), and the 51- and 59-kDa bands identified in *Tetrahymena* (Renaud et al. 1995), suggesting that multiple G-protein subtypes exist in ciliates. Indeed, at least four different genes with homology to Gα subunits have been partially cloned in *Stentor* (Marino, Sherman, and Wood 2001), of which one apparently encodes a PTX-sensitive subtype. In addition to the α-subunit, we have also detected a putative Gβ-subunit, measuring 36 kDa, in *Paramecium* ciliary vesicles. It is likely that the preparation of purified ciliary vesicles made it possible to detect G-protein subunits where other cell preparations have failed. Previous attempts to immunodetect Gα subunits in *Paramecium* pellicles yielded a ~ 50-kDa band in a Triton-insoluble fraction (JdO, unpubl. data), but attempts to purify this protein were unsuccessful.

In order to address the functional role that G-proteins play in *Paramecium*, we pre-treated cells with PTX, a known modulator of G_o/G_i subunits prior to evaluating their chemoresponse to sodium acetate or ammonium chloride. These chemicals are

attractants and exert their effect on *Paramecium* by altering turning frequency (F_{AR}) as well as linear swimming speed (Van Houten 1982). We used these two stimuli because acetate appears to require a surface receptor while ammonium does not (Davis et al. 1998). Both stimuli require that the cells have the ability to execute a turn (avoidance reaction) or the cells will not be attracted (Van Houten 1978). Our behavioral studies showed that PTX does indeed significantly reduce *Paramecium* chemoattraction (I_{CHE}) to acetate and ammonium chloride, indicating that the site of the inhibition of chemoresponse is at the mechanism of the cell turn, which is known to be initiated by opening of the ciliary calcium channels. PTX depressed F_{AR} by approximately 50%, reducing the ability of the cells to distinguish between control solutions and chemoattractants. Indeed, F_{AR} of PTX-treated cells in NaCl buffer was similar to that of cells in sodium acetate (Fig. 2). Swim speed, however, which is inversely proportional to membrane potential (i.e. at more negative potentials, the cell swims faster over small changes from resting potential), was not changed significantly by PTX pretreatment, suggesting that PTX does not directly alter membrane potential. Secondly, chemoattraction to ammonium chloride is thought to occur via changes in intracellular pH (Van Houten 1992) without the need of a chemoreceptor, again discounting the idea that the PTX-sensitive G-protein in *Paramecium* is simply a link between chemoreceptor and effector. We hypothesized therefore that PTX exerts its effects, directly or indirectly, on the ciliary voltage-sensitive calcium channels, by interfering with activation or dissociation of the G-protein heterotrimer. Since chemoresponse was only partially reduced by PTX (Fig. 1), and since PTX treatment did not further reduce F_{AR} in cells already exposed to an attractant (Fig. 2), we posit that the PTX-sensitive G-proteins in *Paramecium* likely only modulate channel activity.

It was previously shown that treatment of *Paramecium calkinsii* with GTP-γ-S, an analogue that activates G-proteins, results in increased backward swimming, prolonged Ca^{2+} action potentials, and an increase in the overall calcium current (Bernal and Ehrlich 1993; Bernal, Kelsey, and Ehrlich 1991). GDP-β-S, an analogue that maintains the G-protein in the inactive heterotrimeric form, reduced backward swimming, Ca^{2+} action potential duration, and the inward calcium current (Bernal and Ehrlich 1993; Bernal, Kelsey, and Ehrlich 1991). By binding to G-proteins, GTP-γ-S liberates activated Gα subunits and Gβγ complexes, whereas GDP-β-S would sequester Gα and Gβγ. The physiological effects of G-protein modulation could therefore be mediated by either Gα or by Gβγ. For instance, Gβγ complexes have been shown to regulate several different intracellular targets, including voltage-sensitive calcium channels (Clapham and Neer 1997).

Given the possible involvement of G-proteins in the regulation of a calcium channel in *Paramecium*, further characterization of heterotrimeric G-proteins and calcium channels in this ciliate is indicated. The VDCC in *Paramecium* has pharmacological similarities to the mammalian E class, T-type calcium channel (Bernal et al. 1991; Ehrlich et al. 1988; Symington et al. 1999). Molecular sequencing will probably be necessary to distinguish among potential channel types and to clarify the mode of action of G-proteins on the *Paramecium* calcium channel.

Further dissection of the possible effects of G-protein modulators on the VDCC was made possible by the finding that pyrethroid insecticides are potent agonists of this channel in *Paramecium* (Clark et al. 1995). Deltamethrin-evoked Ca^{2+} influx produced long bouts of backwards swimming and eventually killed the cell (Table 1, 2). PTX pretreatment of the cells both delayed cell death and reduced the duration of backward swimming evoked by deltamethrin, suggesting that G-protein modulators that prevent dissociation of the heterotrimer reduce the pyrethroid-mediated calcium influx through the VDCC. Our studies also demonstrated a potent synergistic effect of GTP-γ-S on deltamethrin-evoked calcium influx through the VDCC in ciliary vesicles, while GDP-β-S depressed calcium entry (Fig. 4). Again, G-protein modulators such as GDP-β-S, which traps G-proteins in the inactive trimeric form, ameliorated the effects of deltamethrin, while the GTP-γ-S, known to liberate Gα and Gβγ, had the opposite effect. We therefore propose that either Gα (putatively of the $G_{i/o}$ subclass) or Gβγ facilitates calcium influx through the voltage-dependent calcium channel in *Paramecium*, and that the sequestration of G-protein subunits is responsible for the reduced calcium entry through this channel. This possibility is intriguing since binding studies using tritiated pyrethroid analogues have shown that pyrethroids predominantly bound to a 36-kDa membrane protein in rat brain that was identified as the β-subunit of G protein from the rod outer segments of rat retina (Rossignol 1991, 1995). This implicates Gβγ complexes as potential targets of pyrethroids. Alteration of the normal interaction between the α_1-subunit of the calcium channels and Gβγ could be one possible mechanism that couples the toxicological action of deltamethrin at the ciliary voltage-gated calcium channel, resulting in increased Ca^{2+} influx, backward swimming and cell death by osmotic lysis (Clark et al. 1995). Pretreatment of pyrethroid-exposed cells with G-protein modulators that reduce free Gβγ complexes (GDP-β-S, PTX) thus reduces the effects of deltamethrin (Table 1, 2 and Fig. 4), while GTP-γ-S, in freeing up Gβγ, increases its effect (Fig. 4). Nevertheless, a complete pharmacological and molecular characterization of the ciliary calcium channel from *Paramecium* is necessary to establish such relationships.

We also have observed that phospholipase C (PLC) activity is increased by exogenously added Gβγ in bioassays using disrupted ciliary vesicles, and that activation of PLC activity in ciliary vesicles was greatly increased by the toxic 1R isomer of deltamethrin (Symington, Zhang, and Clark 1999). These results not only support our current contention that G-proteins are present in *Paramecium*, but suggest yet another potential pathway for the toxic effects of deltamethrin.

In order to more fully characterize the role of G-proteins in *Paramecium*, however, the genes encoding these proteins need to be identified and cloned. Currently, work is in progress to obtain protein microsequences by MALDI-TOF and to amplify and clone the genes for these G-protein subunits.

ACKNOWLEDGMENTS

This work was supported in part by NIH DC01819-04, DC 00721, and by USDA CSREES Regional Research Project NE-180.

LITERATURE CITED

Bernal, J. & Ehrlich, B. E. 1993. Guanine nucleotides modulate calcium currents in marine *Paramecium*. *J. Exp. Biol.*, **176**:117–133.

Bernal, J., Kelsey, A. M. & Ehrlich, B. E. 1991. GTP-gamma-S increases the duration of backward swimming behavior and the calcium action potential in marine *Paramecium*. *J. Exp. Biol.*, **155**:505–518.

Carbone, E., Carabelli, V., Cesetti, T., Baldelli, P., Hernández-Guijo, J. M. & Giusta, L. 2001. G-protein- and cAMP-dependent L-channel gating modulation: a manyfold system to control calcium entry in neurosecretory cells. *Pflügers Arch.*, **442**:801–813.

Chen, C. A. & Manning, D. R. 1999. Immunological techniques of G-protein analysis. *In:* Manning, D. R. (ed.), G-proteins: Techniques of Analysis. CRC press, Boca Raton. p. 99–121.

Clapham, D. E. & Neer, E. J. 1997. G-proteins βγ subunits. *Annu. Rev. Pharmacol. Toxicol.*, **37**:167–203.

Clark, K. D., Hennessey, T. M. & Nelson, D. L. 1993. External GTP

alters the motility and elicits an oscillating membrane depolarization in *Paramecium tetraurelia*. *Proc. Natl. Acad. Sci. USA,* **90**:3782–3786.

Clark, J. M., Edman, S. J., Nagy, S. R., Conhoto, A., Hecht, F. & Van Houten, J. 1995. Action of DDT and pyrethroids on the calcium channel in *Paramecium tetraurelia*. *In:* Clark, J. M. (ed.), Molecular Action of Insecticides on Ion Channels. American Chemical Society, Washington DC.,**591**:173–190.

Davis, D., Fiekers, J. & Van Houten, J. L. 1998. Intracellular pH and chemoresponse to NH_4^+ in *Paramecium*. *Cell Motil. Cytoskeleton,* **40**:107–118.

Dessen, P., Zagulski, M., Gromadka, R., Plattner, H., Kissmehl, R., Meyer, E., Bétermier, M., Schultz, J. E., Linder, J. U., Pearlman, R. E., Kung, C., Forney, J., Satir, B. H., Van Houten, J. L., Keller, A.-M., Froissard, M., Sperling, L. & Cohen, J. 2001. *Paramecium* genome survey: a pilot project. *Trends Genet.,* **17**:306–308.

DeWaard, M., Liu, H., Walker, D., Scott, V. E., Gurnett, C. A. & Campbell, K. P. 1997. Direct binding of the G-protein beta-gamma complex to voltage-dependent calcium channels. *Nature,* **385**:446–450.

Dietzel, D. & Kurjan, J. 1987. The yeast SCG1 gene: A Gα-like protein implicated in the a- and α-factor response pathway. *Cell,* **50**:1001–1010.

Ehrlich, B. E., Jacobson, A. R., Hinrichsen, R., Sayre, L. M. & Forte, M. A. 1988. *Paramecium* calcium channels are blocked by a family of calmodulin antagonists. *Proc. Natl. Acad. Sci. USA,* **85**:5718–5722.

Fabczak, H., Park, P. B., Fabczak, S. & Song, P. S. 1993. Photosensory transduction in ciliates. II. Possible role of G-protein and cGMP in *Stentor coeruleus*. *Photochem. Photobiol.,* **57**:702–706.

Forney, J. & Rodkey, K. 1992. A repetitive DNA sequence in *Paramecium* macronuclei is related to the beta subunit of G proteins. *Nucleic Acids Res.,* **20**:5397–5402.

Fraga, D. & Hinrichsen, R. D. 1994. The identification of a complex family of low-molecular-weight GTP-binding protein homologues from *Paramecium tetraurelia* by PCR cloning. *Gene,* **147**:145–148.

Keller, A.-M. & Cohen, J. 2000. In indexed genomic library for *Paramecium* complementation cloning. *J. Eukaryot. Microbiol.,* **47**:1–6.

Kung, C. & Saimi, Y. 1982. The physiological basis of taxes in *Paramecium*. *Annu. Rev. Physiol.,* **44**:519–534.

Marino, M. J., Sherman, T. G. & Wood, D. C. 2001. Partial cloning of putative G-proteins modulating mechanotransduction in the ciliate *Stentor*. *J. Eukaryot. Microbiol.,* **48**:527–536.

Nakaoka, Y., Tanaka, T., Kuriu, T. & Murata, T. 1997. Possible mediation of G-proteins in cold-sensory transduction in *Paramecium multimicronucleatum*. *J. Exp. Biol.,* **200**:1025–1030.

Plattner, H. & Klauke, N. 2001. Calcium in ciliated protozoa: sources, regulation, and calcium-regulated cell functions. *Internat. Rev. Cytol.,* **201**:115–208.

Renaud, F. L., Colon, I., Lebron, J., Ortiz, N., Rodriguez, F. & Cadilla, C. 1995. A novel opioid mechanism seems to modulate phagocytosis in *Tetrahymena*. *J. Eukaryot. Microbiol.,* **42**:205–207.

Restrepo, D., Teeter, J. H. & Schild, D. 1995. Second messenger signaling in olfactory transduction. *J. Neurobiol.,* **30**:37–40.

Rossignol, D. P. 1991. Analysis of pyrethroid binding by use of a photoreactive analogue; possible role for GTP-binding proteins in pyrethroid activity. *Pest. Biochem. Physiol.,* **41**:103–120.

Rossignol, D. P. 1995. Possible role for guanosine 5′-triphosphate binding proteins in pyrethroid activity. *In:* Clark, J. M. (ed.), Molecular Action of Insecticides on Ion Channels. American Chemical Society, Washington DC, **591**:149–161.

Schenk, P. W., Van Es, S., Kesbeke, F. & Snaar-Jagalska, B. E. 1991. Involvement of cyclic AMP cell surface receptors and G-proteins in signal transduction during slug migration in *Dictyostelium discoideum* by cAMP. *Dev. Biol.,* **145**:110–118.

Shinozawa, T., Hashimoto, H., Fujita, J. & Nakaoka, Y. 1996. Participation of GTP-binding protein in the photo-transduction of *Paramecium bursaria*. *Cell Struct. Funct.,* **21**:469–474.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.,* **150**:76–85.

Symington, S. B., Zhang, A. & Clark, J. M. 1999. The action of pyrethroids on the voltage-sensitive calcium channel of *Paramecium tetraurelia*. *Pest. Sci.,* **55**:1035–1037.

Symington, S. B., Zhang, A., Karston, W., Van Houten, J. & Clark, J. M. 1999. Characterization of the action of pyrethroids on the ciliary calcium channel in *Paramecium tetraurelia*. *Pest. Biochem. Physiol.,* **65**:181–93.

Thiele, J. & Schultz, J. E. 1981. Ciliary membrane vesicles of *Paramecium tetraurelia* contain the voltage-sensitive calcium channel. *Proc. Natl. Acad. Sci., USA,* **78**:3688–3691.

Van Houten, J. 1978. Two mechanisms of chemotaxis in *Paramecium*. *J. Comp. Physiol.,* **127**:167–174.

Van Houten, J. 1992. Chemosensory transduction in eukaryotic organisms. *Ann. Rev. Physiol.,* **54**:639–663.

Van Houten, J., Martel, E. & Kasch, T. 1982. Kinetic analysis of chemokinesis of *Paramecium*. *J. Protozool.,* **29**:226–230.

Van Houten, J. L., Cote, B. L., Zhang, J., Baez, J. & Gagnon, M. L. 1991. Studies of the cyclic adenosine monophosphate chemoreceptor of *Paramecium*. *J. Memb. Biol.,* **119**:15–24.

Woolkalis, M. J. 1999. ADP-ribosylation of G proteins with cholera toxin or pertussis toxin. *In:* Manning, D. R. (ed.). G Protein: Techniques of Analysis. CRC Press, Boca Raton, FL. p. 173–192.

Wright, M. V., Elwess, N. & Van Houten, J. 1993. Ca^{2+} transport and chemoreception in *Paramecium*. *J. Comp. Physiol.,* **163**:288–296.

Zamponi, G. W., Bourinet, E., Nelson, D., Nargeot, J. & Snutch, T. P. 1997. Crosstalk between G proteins and protein kinase C mediated by the calcium channel α_1 subunit. *Nature,* **385**:442–446.

Received 03/11/03, 06/26/03; accepted 06/26/03