

Review

Responses of the ciliates *Tetrahymena* and *Paramecium* to external ATP and GTP

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Received 31 August 2004; accepted in revised form 13 October 2004

Key words: adaptation, ATP, binding, ecto-ATPase, electrophysiology, GTP, mutants, *Paramecium*, *Tetrahymena*

Abstract

The unicellular ciliates *Paramecium* and *Tetrahymena* are the simplest eukaryotic cells to show reliable depolarizing responses to micromolar concentrations of external ATP and GTP. Their simplicity allows for combined analysis of swimming behavior, electrophysiology, receptor binding, behavioral mutant and drug screens as well as molecular genetic approaches such as RNAi and gene knockouts experiments. ATP and GTP are depolarizing chemorepellents in both ciliates, producing measurable receptor potentials and Ca^{2+} -based action potentials that are correlated with jerking behaviors called avoiding reactions (AR). GTP also causes repetitive continuous ciliary reversals (CCR) and oscillating plateau depolarizations in *Paramecium*. Both ciliates show high affinity, saturable external binding of ^{32}P -GTP and ^{32}P -ATP but GTP does not compete for ATP binding and *vice versa*. Chemosensory adaptation occurs after continued exposure (15 min) to these ligands, producing a loss of external binding and forward swimming. However, cells adapted to ATP still bind and respond to GTP and GTP-adapted cells still bind and respond to ATP. This, combined with pharmacological analyses, suggests that there are two separate receptor systems: A metabotropic ATP receptor pathway and a different, novel GTP receptor pathway. A *Paramecium* mutant (*ginA*) lacks the GTP-induced oscillating depolarizations but does show AR in GTP, unveiling isolated GTP-receptor potentials for study. An ecto-ATPase is also present that may be involved in inactivation of ATP and GTP signals. Gene knockout experiments are currently underway to determine the roles of the ecto-ATPase and a putative 7-transmembrane spanning receptor in these responses.

Introduction

The ciliates *Paramecium* and *Tetrahymena* are excellent model systems for eukaryotic sensory transduction studies. The advantages for using these ciliates for cellular sensory transduction studies are that combined behavioral, electrophysiological, biochemical and genetic (both forward and reverse) approaches can all be used in these simple unicells. Behavioral bioassays are used to estimate the physiological state of the cell as well as for behavioral mutant screens and selections [33, 48, 21] and drug screening [45]. Since *Paramecia* are large (up to 250 μm across) they can be easily used for behavioral observations, behavioral mutant screens and electrophysiological analyses. However, the smaller *Tetrahymena* (about 50 μm long) have the advantages of higher density cultures and the ability to generate transgenic lines and stable gene knockout mutations because of their relatively high rate of homologous recombination [15]. For example, *Tetrahymena* can be grown in a simple proteose peptone, axenic

media to a density of over 500,000 cells/ml with a doubling time of less than 2 h. For more information, see the web pages for *Paramecium* at: http://www.genoscope.cns.fr/externe/English/Projets/Projet_FN/organisme_FN.html and for *Tetrahymena* at: <http://bama.ua.edu/~hsmithso/prof/tweb.shtml>. A unique forward genetics approach, involving antisense ribosome mutagenesis, has also been developed in *Tetrahymena* [5]. This approach is conceptually similar to the use of transposons for tagged mutagenesis, mutant screening and selections in *Drosophila* because it allows for rapid identification of the sequences responsible for new mutant phenotypes. An example of reverse genetics in *Tetrahymena* is seen in the generation of a behavioral mutant that cannot swim backwards because of a specific dynein knockout [22]. Functional genomic information can be approached in *Paramecium* with the reverse genetic procedures of RNAi-by-feeding [14], homology-dependent gene silencing [46] and with electroporation, particle bombardment and microinjection transformation procedures [34]. These procedures complement the classical forward genetics approaches that have been so successful in generating important behavioral mutants in *Paramecium* [33, 48]. The combined advantages of these two ciliates offers a strong 'genetic dissection' approach to identifying the functional components of sensory trans-

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duction and adaptation pathways such as those involved in purinergic responses.

ATP and GTP are depolarizing chemorepellents in the ciliates *Paramecium* and *Tetrahymena* [6, 13, 30, 31, 35, 36]. These cells actively avoid micromolar concentrations of external ATP and GTP by producing avoiding reactions (AR). To view movies of these responses, see: <http://www.nsm.buffalo.edu/Bio/Research/ciliates/>. Avoiding reactions are seen as repetitive bouts of backward and forward jerks which serve to re-orient the cells and bias their swimming direction away from the condition that elicited the response [28], much in the way that tumbling frequency regulates chemotaxis in bacteria [2]. For example, as a *Paramecium* approaches an increasing concentration gradient of a depolarizing chemorepellent, the frequency of ARs increases. Each AR reorients the cell to swim off in a new direction. If the direction takes them down the concentration gradient, the frequency of ARs decreases and they spend more time swimming straight and away from the repellent. If they venture in a path that leads them back up the gradient, AR frequency increases. This is defined as more of a chemokinetic response than a chemotaxis [53, 54] because it is a 'biased random walk' instead of an oriented movement. Chemokinesis to repellents can also be mediated by changes in swim speed, with cells slowing down when approaching the repellent and speeding up when swimming away from it. Chemical stimuli that cause changes in AR frequency are considered to be 'type I chemorepellents' and those that modulate swim speed are referred to as 'type II chemorepellents' [53]. High concentrations (mM) of ions, acids, bases and other compounds are considered to be type I chemorepellents. ATP and GTP differ from these classic type I repellents because they are non-toxic and are believed to involve high affinity, externally facing, membrane receptors. While ATP only elicits AR, GTP also produces a longer response in *Paramecium* called continuous ciliary reversal (CCR) [6]. A CCR is seen as prolonged backward swimming lasting from seconds to minutes. When *Paramecia* are exposed to GTP for more than 10 s, they show repetitive bouts of CCR which are correlated with oscillating plateau depolarizations that last as long as the CCR [6, 7]. Since ATP and GTP are not toxic to these cells, we suggest that they act as depolarizing signaling molecules, much in the way that an excitatory neurotransmitter or nociception (chemical pain) signal would.

We propose that since ATP and GTP are normally at high concentrations inside of cells, the external detection of these compounds by these ciliates may represent nearby cell lysis and whatever caused that lysis may be a condition worth avoiding. ATP has been shown to be a cytoplasmic indicator for cell lysis in animal cells and is released as a pain signal in nociception [8, 10]. Nociception is the signaling of tissue damage or chemical irritation, typically perceived as pain or itch. In both cases (chemorepulsion and nociception) ATP is a cytoplasmic indicator of nearby cell lysis. For the ciliates, ATP and GTP can be 'blood-in-the-water' signals to represent a dangerous situation that these cells should avoid. This is supported by the

observations that a fresh cytoplasmic fraction from either *Paramecium* or *Tetrahymena* elicits chemorepellent responses in both of these ciliates (personal observation). In animal cells, ATP is also released by exocytosis of nucleotide-containing granules and efflux through membrane transport proteins [9]. Therefore, it is also possible that ATP and/or GTP may be released from the ciliates as an intercellular communication strategy. However, regulated release of these compounds has yet to be documented in either of these ciliates.

Behavioral responses to external ATP or GTP

Swimming behavior is used as a convenient bioassay to estimate the sensitivities of these ciliates to external stimuli. ATP, GTP and their non-hydrolyzable analogs produce AR in *Tetrahymena* in a concentration-dependent manner and this can be quantitated by using the AR assay (Figure 1A). *Tetrahymena* have been shown to be more sensitive to GTP and β - γ methylene ATP than ATP [31] (Figure 1B) and we propose that this difference is due to the presence of an ecto-ATPase which hydrolyzes ATP much better than GTP [50]. ADP and GDP are far less effective as stimuli, AMP and GMP are even less effective and adenosine and guanosine are completely ineffective in generating AR (personal observations).

The initial response of *Paramecium* to external ATP and GTP is also AR but the main behavioral difference is that *Paramecium* will also enter into repetitive bouts of prolonged backward swimming (CCR) after a few seconds in the continued presence of GTP [6]. ATP does not normally elicit repetitive CCR in *Paramecium*, making this a unique response to GTP. The GTP responses of *Paramecium* have been quantitated by a computerized motion analysis assay that measures the percent directional changes (PDC) of many cells at once [6]. This assay has been used to show that *Paramecium* are more responsive to GTP than ATP and they are non-responsive to the pyrimidines CTP and UTP. *Paramecium* do show lower concentration-dependent responses to GDP and GMP in this assay but they do not respond to guanosine [6]. It is important to note that the AR assay looks at immediate responses within the first 5 s after cells are first exposed to the stimulus while the PDC assay ignores the first 5–10 s because of cell movements caused by addition of cells to the observation slide. Also, the GTP-AR frequency drops often off within the first 5–10 s while the repetitive GTP-CCR often take 10–15 s to develop. Therefore, the AR assay reports on immediate effects of GTP while the PDC assay has a 5–10 s delay before the repetitive CCR can be quantitated. This may be one of the reasons why ATP responses can be seen in the AR assay at concentrations where there are no detectable responses in the PDC assay. In the AR assay with *Paramecium*, the EC₅₀ is about 0.01 μ M for GTP [31], 10.0 μ M GDP (personal observation) and 12 μ M for ATP [56], while the PDC assay showed EC₅₀ values of 0.12 μ M for GTP [6]. EC₅₀ values for other nucleotides were not determined by the PDC assay because

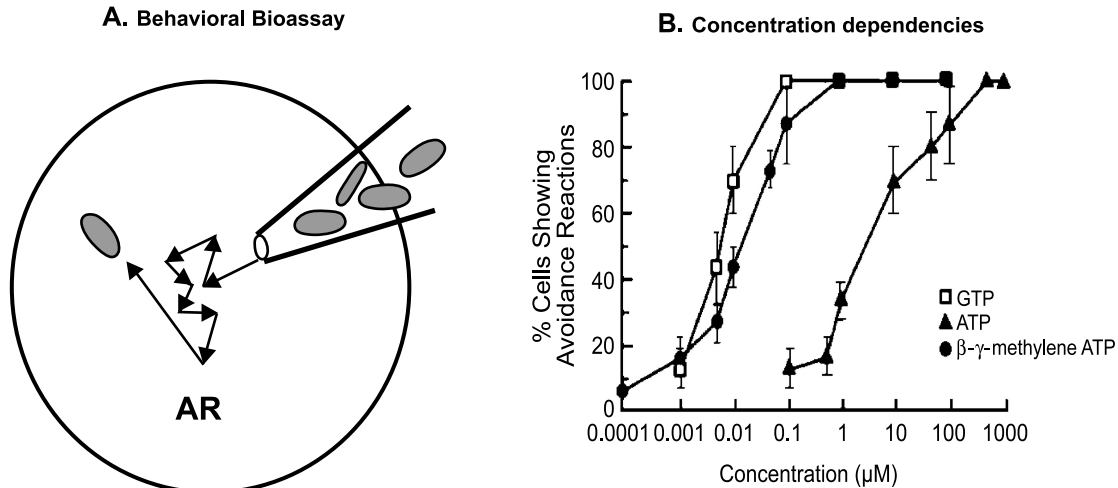


Figure 1. (A) The behavioral bioassay for avoiding reactions (AR) involves using a micropipet to transfer cells into a test solution and then observing their swimming behavior under a dissecting microscope. Individual cells are scored as either showing an AR or not. (B) The responsiveness can be quantitated by repeating this observation many times. For example, if 10 cells are observed and eight cells show AR, the percent cells showing AR = 80%. For each data point in B, 3 blocks of 10 cells each were pooled so the mean \pm SD has an $n = 3$. The EC_{50} (concentration where the ligand is 50% effective) is about 8.0 nM for GTP (open squares), about 10.0 nM for β - γ methylene ATP (closed circles) and 5.0 μ M for ATP (closed triangles) in these cells. The data shown in B were obtained with *Tetrahymena* and the test solution contained 10 mM Tris-base, 0.5 mM MOPS, 1.0 mM disodium tartarate, 50 μ M $CaCl_2$ and pH = 7.0. (From Kim et al. [31].)

they did not saturate. The responses of *Tetrahymena* and *Paramecium* to ATP and GTP have also been quantitated by a capillary tube assay [13] and by the three-way stopcock assay for chemokinesis [53], but these assays cannot be used to measure the initial responses because they take 5–30 min for the cells to distribute within the assay apparatus.

Since the AR assay is so quick and easy, it has been used to screen for drugs that affect the responses of *Tetrahymena* to ATP and GTP. In *Paramecium*, we found that a drug that is often used as an inhibitor of P2X type ATP receptors in vertebrates, PPNSD, is actually an agonist for the ATP receptors [56]. Suramin, another common ATP receptor antagonist, is also an agonist in *Paramecium* (C. Wood, personal communication). The ATP responses of *Tetrahymena* are inhibited by GDP- β -S, pertussis toxin, Calphostin C and Rp-cAMPS, suggesting that the ciliate ATP receptor may be a metabotropic, P2Y-like receptor [45]. However, these inhibitors have no effect on the responses of *Tetrahymena* to GTP. Since the effects of external GTP on internal Ca^{2+} levels in rat PC12 cells is also pertussis toxin-sensitive, it has been suggested that P2Y-like receptors may also be involved in this response [17]. Tyrosine kinase inhibitors have been shown to affect the GTP responses but not the ATP responses in *Tetrahymena* [35], supporting the idea that responses to GTP involve a sensory transduction pathway that is different than the one for ATP reception.

The PDC assay has also been used to identify drugs and mutations that affect the GTP responses of *Paramecium* and to show that Mg^{2+} and/or Na^+ are necessary to show the GTP-induced CCR. Several inhibitors of sarcoplasmic/endoplasmic reticulum Ca^{2+} -dependent ATPase (BHQ, CPA and thapsagargin) have been shown to inhibit GTP-CCR in *Paramecium* [55], suggesting that sequestration

and/or release of Ca^{2+} from internal stores may be involved in the repetitive CCRs seen in response to GTP. Addition of 10 μ M XTP (xanthosine triphosphate) was also shown to inhibit the GTP-CCR in *Paramecium* but XTP itself did not produce CCR [38]. The involvement of a Ca^{2+} -dependent Na^+ conductance and Ca^{2+} -dependent Mg^{2+} conductance in the GTP-CCR was first seen in the fact that either 0.5–1.0 mM Mg^{2+} or 4–8 mM Na^+ must be present in the external solution to see GTP-CCR in the PDC assay [7]. One behavioral mutant of *Paramecium* that lacks the Ca^{2+} -dependent Na^+ conductance (fast-2), only shows GTP-CCR in Mg^{2+} -containing solutions and not in Na^+ solutions (because it has the necessary Mg^{2+} conductance) while another mutant that lacks the Ca^{2+} -dependent Mg^{2+} conductance (eccentric) only produces GTP-CCR in Na^+ -containing solutions and not in Mg^{2+} -containing solutions [7]. This information supports the model that external GTP elicits a metabotropic activation of a pathway or pathways that leads to oscillating elevations in intracellular Ca^{2+} levels. These Ca^{2+} oscillations are mirrored in the activation of Ca^{2+} -dependent Mg^{2+} and/or Na^+ conductances which cause sustained, oscillating depolarizations in the presence of sufficient external Mg^{2+} and/or Na^+ . It is these Ca^{2+} -dependent oscillations that are correlated with the repetitive GTP-CCR, as described below.

Electrophysiological responses to external ATP and GTP

Electrophysiologically, each avoiding reaction (AR) is accompanied by a Ca^{2+} -based action potential. The upstroke of the action potential is due to the activation of a ciliary, voltage-dependent Ca^{2+} channel and the down-

stroke is due to a combination of Ca^{2+} -dependent Ca^{2+} channel inactivation and activation of a voltage-dependent K^+ channel [12]. In *Paramecium*, intraciliary Ca^{2+} has been shown to be the link between somatic depolarizations (receptor potentials), action potentials and changes in swimming behavior [12, 33]. Somatic depolarizations cause ciliary voltage-dependent Ca^{2+} -channels to open. This produces graded Ca^{2+} -based action potentials and consequent inward Ca^{2+} currents. As intraciliary free Ca^{2+} rises, the beat frequency slows. When the free Ca^{2+} exceeds 10^{-6} M the cilia reverse their direction of beat [12, 37, 40]. Therefore, if a strong enough somatic depolarization is generated, the cell swims backwards. Swimming behavior is therefore used as a convenient bioassay for estimating the electrophysiological state of these ciliates, screening for compounds which affect their electrophysiological properties and screening for behavioral mutants [21, 48]. Intracellular electrophysiological measurements in *Tetrahymena* have shown that their responses to depolarizing stimuli are due to electrophysiological and ionic changes that are generally similar to those of *Paramecium* [23]. A non-excitable behavioral mutant, called 'tnr' (*Tetrahymena* non-reversal), has even been described electrophysiologically in *Tetrahymena* [52] and it is similar to the well described 'pawn' [33] and 'cmr' (caudatum non-reversal) [51] mutants of *Paramecium*. This has helped to establish that *Tetrahymena* is also a suitable tool for studies of membrane excitation [43].

As in other types of sensory cells, depolarizing receptor potentials are necessary to generate action potentials and there is an anatomical distinction between where the receptor potentials and action potentials are generated. In *Paramecium*, transduction of thermal [26], mechanical [42] and chemical [54] stimuli has been shown to occur on the body (somatic) plasma membrane because the graded, sensory receptor potentials can be recorded from deciliated cells. The ciliary plasma membrane is not necessary for generating these sensory receptor potentials. In terms of the chemoresponses, classical type-I chemorepellents depolarize cells and increase the frequency of directional changes [53] but their receptors have not been identified. Heat [26] and anterior mechanical stimulation [42] also generate depolarizing receptor potentials and AR but their receptors are also unknown. Sensory receptor potentials have also been recorded in intact *Tetrahymena* in response to mechanical [43] and chemorepellent [23] stimulation. We propose that activation of either the external ATP or GTP receptors causes a change in somatic membrane ion conductance to depolarize the cell to a threshold level, generate action potentials and AR and result in chemorepulsion. GTP, ATP and β - γ -methylene ATP-induced depolarizations are shown for *Tetrahymena* with small, graded action potentials riding on top of prolonged but transient depolarizing receptor potentials (Figure 2).

All of the conductances responsible for the ATP-induced receptor potentials and GTP-induced receptor potentials have not yet been described, but we have suggested that initial responses to either ATP or GTP may involve a receptor-operated Ca^{2+} conductance [7, 24] and Ca^{2+} -

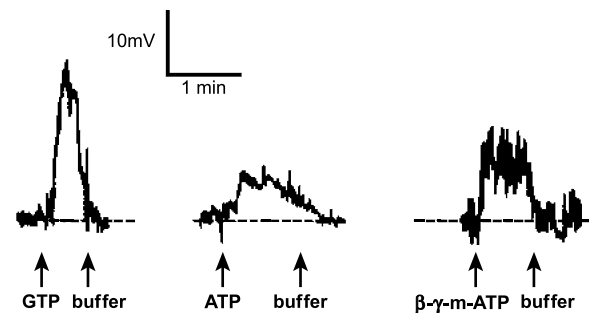


Figure 2. Sustained, reversible depolarizations were seen in *Tetrahymena* in response to either 10 μM GTP, 100 μM ATP or 10 μM β - γ -methylene ATP. These cells were recorded under Cs-TEA conditions (2.0 M CsCl electrodes) in a buffer containing 1.0 mM CaCl_2 , 1.0 mM MOPS, 10 mM TEA-Cl, pH 7.2. In each trace, the ligand was added (at the first arrow) and removed by perfusion of the bath. (From Hennessey and Kuruvilla [23].)

activated Na^+ and/or Mg^{2+} conductances [7]. The initial Ca^{2+} signal may be amplified by release of Ca^{2+} from internal stores. However, it is also formally possible that there is a receptor-mediated release of Ca^{2+} from internal stores without Ca^{2+} influx. External GTP has been shown to cause Ca^{2+} -induced Ca^{2+} release from internal stores in rat PC12 cells and elevations in internal Ca^{2+} levels [18, 44]. Either way, these receptor potentials (see Figures 2 and 4) are primarily due to Ca^{2+} -dependent Na^+ and/or Mg^{2+} conductances (if sufficient Na^+ and/or Mg^{2+} is present in the stimulating solution) because GTP- and ATP-induced receptor potentials, AR and CCR, are not seen in Ca^{2+} only solutions in *Paramecium*. Also, the *Paramecium* mutant that lacks the Ca^{2+} -dependent Mg^{2+} conductance (eccentric) does not show a measurable receptor potential in response to either ATP or GTP in Mg^{2+} -containing solutions but does when sufficient Na^+ is present [7]. Similarly, the mutant that lacks the Ca^{2+} -dependent Na^+ conductance (fast-2), only shows ATP- and GTP-induced receptor potentials in Mg^{2+} -containing solutions and not in Na^+ solutions [7].

External binding of ATP and GTP

High affinity binding to external receptors can be assayed with these cells because they can be grown in high-density, axenic, clonal cultures where there is only one cell type present in the assay. For example, *in vivo* ^{32}P GTP surface binding was clearly saturable in *Tetrahymena* (Figure 3A) and this data can be fit by a single line on a Scatchard plot (Figure 3B), suggesting one class of high affinity GTP receptors. The estimated K_D values were about 20 nM for both non-adapted cells de-adapted cells and the B_{max} (estimated maximum number of binding sites from Scatchard plots) values suggest that the number of receptors/cell dropped from about 1.7×10^4 to near zero after adaptation [36]. This is consistent with a loss of surface GTP receptors during adaptation (desensitization). All of these changes were reversible following 14 min in a GTP-free solution. Similar

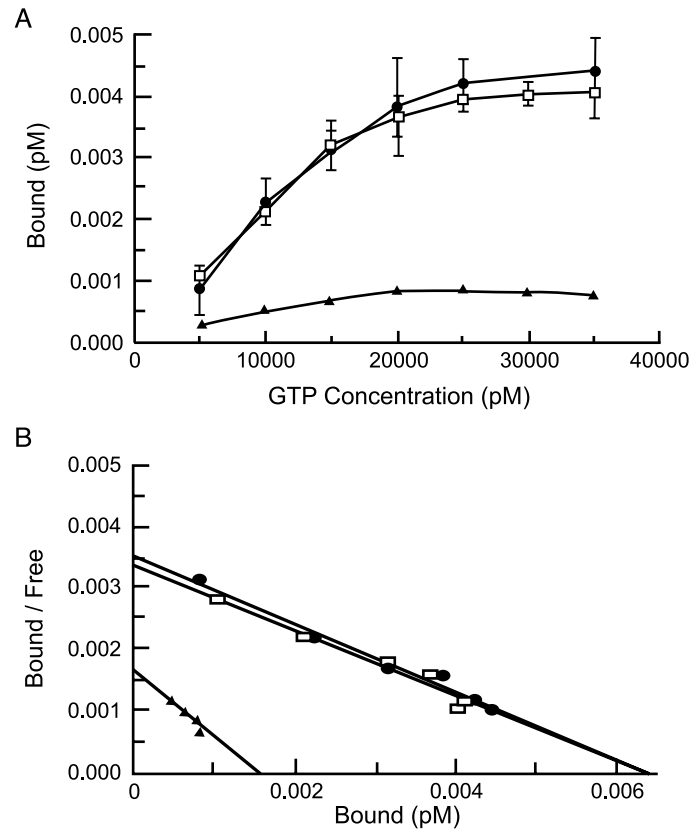


Figure 3. *In vivo* $[^{32}\text{P}]\text{-GTP}$ binding to live, intact *Tetrahymena* showed saturable, high-affinity binding to surface receptors in both control and de-adapted cells but this binding was virtually lost in adapted cells. (A) The amount of $[^{32}\text{P}]\text{-GTP}$ bound to cells increased in a concentration-dependent manner in both control (open squares) and de-adapted (closed circles) cells but adapted cells (closed triangles) showed no measurable binding at any concentration tested. Each point represents the mean \pm SD of three experiments. (B) Scatchard analysis showed that the apparent K_D of control cells (open squares) and de-adapted cells (closed circles) were identical while the B_{max} values were comparable. Adapted cells showed far less binding (closed triangles). (From Kuruvilla et al. [36].)

binding and loss of surface binding sites is seen with ^{32}P ATP in *Tetrahymena* [31] and with ^{32}P ATP and ^{32}P GTP binding in *Paramecium* [30]. In all cases, the unlabeled ('cold') form of the nucleoside triphosphate was a competitive inhibitor of the binding of the radioactive form but GTP did not compete for ^{32}P ATP binding and ATP did not compete for ^{32}P GTP binding. This supports our hypothesis that there are two separate external receptors, one for ATP and the other for GTP. Similar suggestions of at least two different external binding sites for GTP and ATP have also been made in work done with external $^{32}\text{P}\text{-GTP}$ binding in rat PC12 cells [18] and in mouse myoblasts and myotubules [44].

Chemosensory adaptation to the continued presence of ATP or GTP

Chemosensory adaptation is seen in these ciliates as a decrease in responsiveness to a stimulus over time of exposure to that stimulus. There are two different types of adaptation to ATP and GTP in these ciliates. The first is a short-term adaptation that occurs within the first 20–40 s after a cell is transferred to a test solution containing ATP or GTP, depending upon what other ions are present in the test solution. The stimulated cell initially shows repetitive

AR but then regains forward swimming (in the continued presence of the stimulus) due to electrophysiological changes related to the termination of 'type I excitation' [47]. This involves the combined activation of voltage-dependent K^+ conductances and inactivation of the ciliary, voltage-dependent inward Ca^{2+} current to bring the membrane potential and intraciliary Ca^{2+} concentrations back to resting levels. If the cell remains in high enough concentrations of either ATP or GTP for longer periods of time (10–15 min) a long-term adaptation (see Figure 3) is produced due to receptor desensitization and loss of functional, externally facing receptors [30, 31, 36]. This can involve either receptor turnover or modification of the receptor to inactivate it. Behavioral adaptation may also be caused by changes in other parts of the sensory transduction pathway, such as second messengers and their contacts. Behaviorally, the distinction between short-term and long-term adaptation is that short-term adaptation can be reversed by transferring the cell to a repellent-free solution for 20 s while it takes 10–15 min in a repellent-free solution to reverse long-term adaptation. In *Paramecium*, it has been shown that long-term behavioral adaptation to GTP is correlated with a change in frequency of the oscillating depolarizations and decrease in external GTP binding sites [30] while ATP adaptation involves a

decrease in the receptor potential amplitude and a decrease in external ATP binding sites [31]. No cross-adaptation was seen between these two repellent pathways because GTP adapted cells still respond to ATP and *vice versa*. Cells adapted to ATP or GTP had normal responses to ionic stimuli such as 40 mM K⁺, 4.0 mM Ba²⁺ and 10 mM Na⁺. Therefore, cells adapted to ATP or GTP were not generally less excitable but were specifically adapted to the ATP stimulus. These changes were all reversible ('de-adaptation'), following 10 min in an ATP- or GTP-free solution. Similar behavioral and external binding changes have been seen in *Tetrahymena* [36].

Although we do not define it as a form of chemosensory adaptation, responsiveness to external ATP and GTP is also affected by the presence of externally facing ecto-ATPases which can hydrolyze these compounds and inactivate the depolarizing stimulus. In animal cells, it has been shown that soluble ecto-ATPases are involved in the deactivation of purinergic agonists in much the same way as acetylcholinesterase inactivates acetylcholine signals [29]. In this manner, membrane bound ecto-ATPases may regulate the type and amount of purinergic agonists that reach the membrane receptors. An externally facing, membrane bound ecto-ATPase has been described in *Tetrahymena* [50], which can serve to inactivate these purinergic signals. This ecto-ATPase has been shown to be releasable into the surrounding media and the soluble form from *Tetrahymena* has been purified and characterized [50]. We have proposed that this ecto-ATPase may contribute to the ATP sensitivity of *Tetrahymena* to the extent that it may explain why the non-hydrolyzable form of ATP (β - γ methylene ATP) is so much more effective than ATP itself (see Figure 1B). We further suggest that the reason that GTP is more effective than equimolar ATP is because the ecto-ATPase prefers ATP over GTP by 4:1, thus hydrolyzing ATP to a greater extent and lowering its effective concentration near the ATP receptor [50]. This is not due to bulk hydrolysis because this was assayed as immediate responses of single cells in a fresh 1.0 ml volume, suggesting that the agonist must pass by many (ciliary) ecto-ATPases before it can activate the receptor. However, it is also possible that the differences in sensitivities to different ligands is due to the binding selectivity of the receptor.

Two different receptors, one for ATP and one for GTP?

We have suggested that these ciliates may have two different purinergic receptors, one for detecting ATP and the other for GTP. Purinergic receptors are classified as either P1, which prefer adenosine over nucleoside triphosphates or P2 which recognize primarily nucleoside phosphates [1, 3, 4, 19, 41]. However, few of the known vertebrate ATP receptors show any significant responses to micromolar concentrations of external GTP and no dedicated GTP receptor has been purified or cloned. Therefore, either the ciliate GTP receptor is a unique, new type of purinergic receptor or it may be similar to

receptors that exist in some other cell type, such as those that may be involved in the responses of vertebrate cells to external GTP [17, 18, 44]. In general, the P2X class of receptors are thought to be ionotropic receptors (directly activating ion channels) whereas the P2Y class of receptors are metabotropic (requiring second messengers such as cAMP and G proteins) [9]. At least eight (and possibly more) P2Y receptors have been cloned and they all share a predicted seven transmembrane spanning region with considerable homologies [9]. P2X receptors, which act as ligand-gated ion channels to mediate fast transmission in peripheral, sensory and central neurons, have also been cloned (seven different ones at this time) and they contain only two predicted transmembrane spanning regions [41]. Some of these receptors are targeted for down regulation and receptor-mediated endocytosis [11] by phosphorylation (and changes in intracellular second messengers) during desensitization [49]. Drug design for effects on purinergic responses has targeted the P2X [41] and P2Y [9] classes of receptors as well as the ecto-NTPase activities [16]. While the majority of these receptors have been identified by expression cloning in oocytes (because they are so difficult to purify), it is also very difficult to do such heterologous expression in these ciliates because of their different codon usage (for example, the glutamine codon in ciliates is a stop codon in oocytes). Pharmacological results from the lab of Heather Kuruvilla suggests that the ATP responses of *Tetrahymena* involve a metabotropic pathway while the GTP responses may not [45], supporting the idea that there are two separate pathways for purinergic detection in *Tetrahymena*. Behavioral cross-adaptation and *in vivo* binding experiments suggest that there may be two separate purinergic receptors, one for GTP and another for ATP (and methylene ATP) because cells that have been adapted to 10.0 μ M GTP for 10 min have lost their responsiveness to GTP but they are fully responsive to ATP and methylene ATP [30, 36]. Similarly, ATP adapted cells still respond to GTP but they are non-responsive to methylene ATP. External binding studies showed that GTP adapted cells (which have lost their external ³²P-GTP binding) still bind ³²P-ATP and ATP adapted cells bind ³²P-GTP even though they have lost external ³²P-ATP binding sites. Furthermore, cold ATP is not an inhibitor of ³²P-GTP binding (while cold GTP is) and vice versa. There are also differences between electrophysiological responses of ATP and GTP in *Paramecium* in that there are oscillating plateau depolarizations in response to GTP but not to ATP [6].

Genetic dissection of the GTP response in *Paramecium*

An advantage of *Paramecium* is the ability to screen for and select behavioral mutants, identify the genetic defect responsible for the phenotype and use this information to gain insights into the sensory transduction pathway responsible for the phenotype by 'genetic dissection' of the proposed pathway [33]. This forward genetics approach allows the cell to tell us what genes and gene products are important for an identified sensory response. In *Tetrahy-*

mena, a tagged mutagenesis procedure involving antisense ribosome mutants has also been used to generate mutants, screen for a phenotype and identify the gene sequence responsible for that phenotype [5]. This procedure also holds promise as a combined forward and reverse genetics approach to identifying a sensory transduction pathway. In *Paramecium*, mutants were generated by nitrosoguanidine mutagenesis and screened for cells that didn't swim backwards in response to GTP [39]. A mutant was found called *ginA* (GTP-insensitive). This mutant was described as insensitive to GTP by the PDC assay and electrophysiological analysis confirmed that its only defect was in generating the indicative oscillating plateau depolarizations and inward currents that normally correlate with CCR. These *ginA* mutants respond normally to ATP and other depolarizing and hyperpolarizing stimuli. They also had normal Ca^{2+} -dependent Mg^{2+} and Ca^{2+} -dependent Na^{+} currents [39]. It was suggested that the defect in *ginA* might be in either the regulation of intracellular Ca^{2+} in general or, more specifically, in the proposed oscillator responsible for cyclic changes in Ca^{2+} . However, GTP-induced changes in intracellular Ca^{2+} have not yet been reported. An alternative hypothesis is that the mutant is missing the GTP receptor but we found that the *in vivo* external ^{32}P -GTP binding was the same in wild type and *ginA* with estimated K_m values of 473 pM for *ginA* and 367 pM for wild type (M. Kim, personal observations).

We have found that the *ginA* mutant is not insensitive to GTP but since it lacks the oscillating plateau depolarizations and repetitive CCR, it is an excellent mutant for uncovering and isolating the underlying GTP-induced

receptor potential. The initial responses of *ginA* to external GTP are identical to those of wild type because they show the same receptor potential and consequent AR but the difference is that wild type go into repetitive CCR in GTP after 10–15 s while *ginA* do not. This CCR response is apparently not necessary for chemorepulsion because the scores of wild type and *ginA* are the same in the three-way stopcock chemokinesis assay [53] and the GTP-AR assay (personal observation). As shown in Figure 4, wild type show oscillating plateau depolarizations in a recording solution containing 1.0 mM Ca^{2+} , 0.5 mM Mg^{2+} and 2.0 mM Na^{+} when 10 μM GTP is perfused into the recording chamber (Figure 4A). Deciliation of wild type (Figure 4C) eliminates the ciliary Ca^{2+} -based action potentials but does not affect the oscillating depolarizations, showing that they are generated on the body (somatic) membrane. Intact *ginA* mutants retain the depolarizing GTP-induced receptor potential and consequent action potentials (Figure 4B) but they lack the oscillating depolarizations. Deciliation of *ginA* (Figure 4D) unveils the GTP-induced somatic receptor potential by stripping away the oscillating depolarizations and action potentials. The maximal change in membrane potential seen during these transient receptor potentials was 8.8 ± 0.95 mV ($n = 3$). Deciliated *ginA* were also used to look at other isolated receptor potentials and it was found that 10 μM concentrations of ATP produced a maximal change in membrane potential of 9.5 ± 1.7 mV, XTP produced 8.7 ± 1.5 mV and ITP produced 8.3 ± 2.4 mV. GTP, XTP and ITP were very similar in their potencies for eliciting AR in *ginA*, with 100% AR seen at about 0.1 μM for each. The responses of wild type to

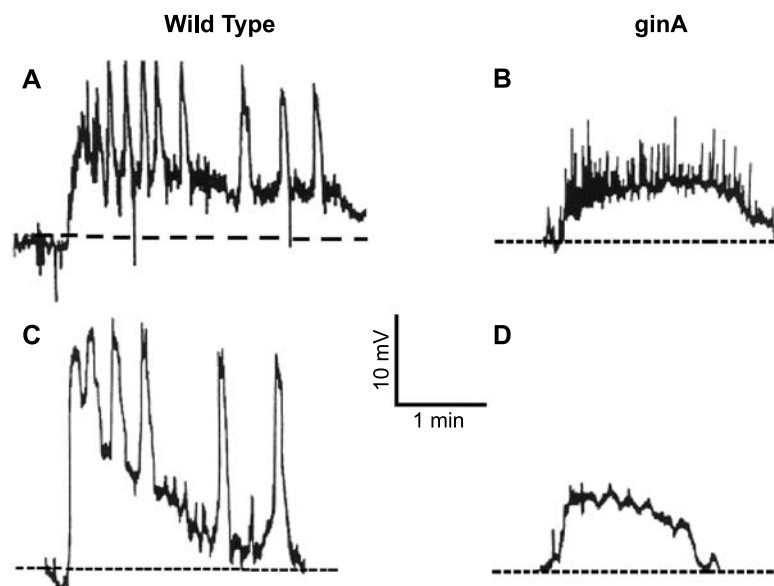


Figure 4. Electrophysiological responses of wild type and mutant *Paramecium* to GTP. The free running membrane potentials were recorded under constant perfusion conditions. The control solution contained 1.0 mM CaCl_2 , 10 mM Tris, 1.0 mM MOPS, 0.5 mM MgCl_2 , 2.0 mM NaCl, pH = 7.2. The single recording electrode contained 500 mM KCl. Perfusion with 10 μM GTP began near the start of each trace and continued during the entire recording period. (A) Intact wild type show repetitive, oscillating plateau depolarizations riding on top of an underlying depolarization when 10 μM GTP is perfused into the recording chamber. Some fast, graded action potentials are seen within the first few seconds. (B) The intact *ginA* mutant does not show any of the oscillating plateau depolarizations but does show action potentials and a transient depolarization. (C) Deciliated wild type show the same responses as intact wild type except the action potentials are lost. (D) Deciliation of *ginA* uncovers the isolated GTP-induced receptor potential. (From Mimekakis et al. [39] and Hennessey et al. [25].)

XTP, ATP and ITP were the same as those of *ginA* because all of these compounds produced AR but not CCR. UTP and CTP produced some AR in *ginA* and WT but it took more than 10 μM of these compounds to produce any noticeable response. At 10 μM concentration, UTP produced only 0.3 ± 0.58 mV depolarization in deciliated *ginA* and CTP produced 0.7 ± 0.58 mV depolarization.

Model for chemosensory transduction of ATP and GTP signals

In the model below (Figure 5), the initial depolarization caused by either ATP or GTP leads to AR but forward swimming (FS) can be regained by a number of methods. Long-term adaptation can cause receptor deactivation and all of the altered parameters return to rest. A receptor antagonist would have the same effect, but no receptor antagonists have yet been found to be effective in these ciliates (personal observation). In the case of GTP stimulation in *Paramecium*, activation of Ca^{2+} -dependent Mg^{2+} and/or Na^{+} conductances is prolonged to convert AR into continuous ciliary reversals (CCR), presumably due to continued elevation in intraciliary Ca^{2+} . The GTP- and ATP-induced depolarizations can be short circuited by the voltage-dependent K^{+} or Ca^{2+} -dependent K^{+} conductances to bring the membrane potential back to rest and this is the most likely mechanism for short-term adaptation. Ca^{2+} -dependent Ca^{2+} channel inactivation can also contribute to the repolarization by decreasing the inward Ca^{2+} current and this also helps to lower the intracellular Ca^{2+} concentrations back to resting levels. It is also possible that the voltage-dependent Ca^{2+} channel inactivation could contribute to a return to resting Ca^{2+} conductance but this process is slow and requires prolonged depolarizations [20]. The intracellular Ca^{2+} concentration is determined by

the relative rates of events that raise this concentration (Ca^{2+} influx and release of Ca^{2+} from internal stores) and those that lower it (Ca^{2+} buffering, sequestration and efflux) so conditions that affect any of these process can affect the extent of AR, duration of CCR and rate of adaptation.

Responses to ATP and GTP in other ciliates

Extracellular nucleotides have been shown to cause Euplotes to change from their normal ellipsoid shape into a 'winged' morph [32]. ATP and adenosine tetraphosphate were the most active (in concentrations of about 100 μM), followed by CTP, UTP and GTP but AMP, adenosine, cAMP, pyrophosphate and tripolyphosphate had no morphogenic activity. It was proposed that this may be due to predator-induced release of these compounds to cause a defensive morphological transformation [32]. A fluorescent analog of GTP has also been shown to bind mainly to the oral area of *Tetrahymena* and also stimulate cell division in starved cells [27]. Therefore, extracellular ATP and GTP could have many types of ligand-induced responses in other ciliates besides chemorepulsion.

Tetrahymena genome database comparisons

Searches of the current *Tetrahymena* Genome Database (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=t_thermophila) did not produce any sequences with high homology matches to any of the cloned P2X-type ATP receptors of vertebrates but an open reading frame has been identified, which we call TP2Y, that has similarities in the conserved regions of the P2Y receptor of rat (P49651). Although the amino acid sequence homology is not very

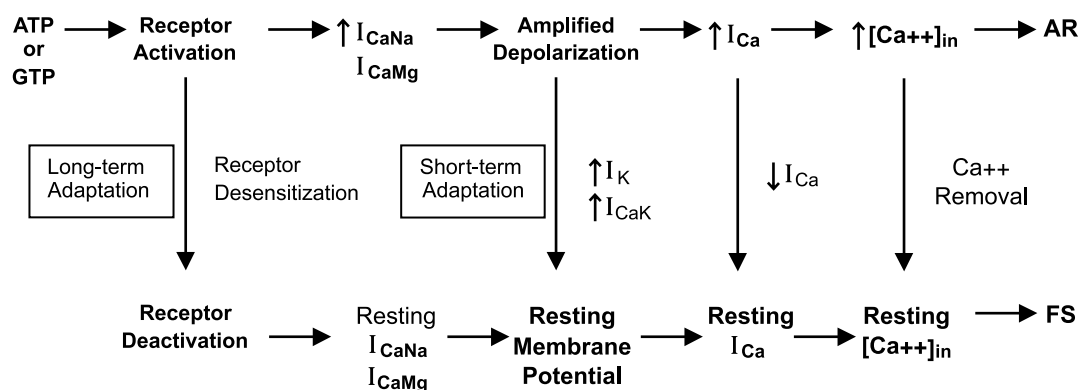


Figure 5. The proposed sensory transduction pathway. Activation of either the ATP receptor or GTP receptor results in increases in the Ca^{2+} -dependent Na^{+} (I_{CaNa}) and/or Ca^{2+} -dependent Mg^{2+} (I_{CaMg}) conductances to amplify the graded, depolarizing receptor potential when sufficient Na^{+} or Mg^{2+} are present in the external solution. Since these are Ca^{2+} -dependent conductances, this could happen by a receptor-activated increase in Ca^{2+} conductance or release of Ca^{2+} from internal stores. However, this mechanism has not yet been identified. Repolarization can occur by activation of the voltage-dependent outward K^{+} current (I_{K}) or the later activating Ca^{2+} -dependent K^{+} current (I_{CaK}) and by inactivation of the inward Ca^{2+} current (I_{Ca}). The depolarizing receptor potential can activate the ciliary, voltage-dependent Ca^{2+} channel to generate an inward Ca^{2+} current (I_{Ca}). The increased Ca^{2+} concentration in the cilia ($[\text{Ca}^{2+}]_{\text{in}}$) triggers ciliary reversal and avoiding reactions (AR). The ciliary reversals can be terminated by short-term adaptation, which brings the membrane potential and intraciliary Ca^{2+} concentration back to resting levels to enable forward swimming (FS). Long-term adaptation terminates the initial receptor activation, allowing all of the conductances to come back to resting levels and the cell to regain forward swimming due to the return of resting Ca^{2+} levels by the Ca^{2+} removal systems.

high overall between the rat P2Y and the *Tetrahymena* sequence we call TP2Y (31% similarity), we propose that it is the protein structures that may be similar enough to generate similar functions in these two distantly related organisms. If TP2Y is a purinergic receptor, we don't know if it is the ATP receptor or the GTP receptor. Since P2Y receptors are metabotropic [9] and the ATP responses of *Tetrahymena* are inhibited by drugs which block P2Y responses in other cell types [45], it is possible that TP2Y codes for the ATP receptor of *Tetrahymena*. A putative ecto-ATPase gene has been identified in *Tetrahymena* that does have high homology to the conserved regions of ecto-ATPases from animals (personal observations), so this enzyme may play a similar role in inactivating external ATP signals. We are currently constructing mutants in TP2Y and the ecto-ATPase in *Tetrahymena* by macronuclear gene knockout (gene disruption) procedures [15] to see if these mutants have altered responses to external ATP and/or GTP.

Acknowledgements

This work was supported by NIH grant DK608860-01 and NSF grant MCB-9983060. I thank Christopher Wood for reading and commenting on the manuscript.

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