External GTP alters the motility and elicits an oscillating membrane depolarization in *Paramecium tetraurelia*

**behavior/electrophysiology/signal transduction/purinergic**

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**Abstract** *Paramecium*, a unicellular ciliated protist, alters its motility in response to various stimuli. Externally added GTP transiently induced alternating forward and backward swimming interspersed with whirling at a concentration as low as 0.1 μM. ATP was 1000-fold less active, whereas CTP and UTP produced essentially no response. The response to the nonhydrolyzable GTP analog guanosine 5′-[β,γ-imido]triphosphate and guanosine 3′-diphosphate 5′-diphosphate was indistinguishable from that to GTP. This behavioral response was correlated with an unusual transient and oscillating membrane depolarization in both wild-type cells and the mutant pawn B, which is defective in the voltage-dependent Ca2+ current required for action potentials. This is a specific effect of external GTP on the excitability of a eukaryotic cell and, to our knowledge, is the first purinergic effect to be discovered in a microorganism.

*Paramecium tetraurelia* normally swims forward except for occasional brief periods of backward swimming or whirling, a randomly directed motion (1). However, many stimuli, thermal, electrical, mechanical, or chemical, can alter the swimming speed and the frequency and duration of backward swimming and whirling events (2–6). These responses are normally transient. Cells return to their prestimulus behavior even in the continued presence of the stimulus, a form of sensory adaptation. The combination of behavioral response and subsequent adaptation can result in attraction to or repulsion from a stimulus (6). These swimming behaviors generally have clear, well-studied, and readily measurable electrophysiological correlates that can aid in unraveling a signal-transduction pathway. For example, increased swimming speed is correlated with membrane hyperpolarization, whereas decreased swimming speed is correlated with membrane depolarization. Strong depolarizations can elicit graded Ca2+-based action potentials, resulting in whirling and backward swimming due to increased intraciliary Ca2+ (3, 4).

To quantify these swimming behaviors, we have developed a computerized motion analysis assay that measures the percentage of total path time spent whirling and undergoing transitions between forward and backward swimming (defined as percent directional changes (PDCs) (1)). While using this assay to quantify the behavioral effects of externally added nucleotides, we found that guanine nucleotides specifically and potently altered the swimming behavior of paramecia. In addition, while attempting to corroborate the GTP-induced behavior of the cell with changes in membrane potential, we discovered an electrophysiological response.

**Materials and Methods**

**Materials.** The guanosine 3′-diphosphate 5′-diphosphate and guanosine 3′-diphosphate 5′-triphosphate were obtained from Boehringer Mannheim. All other compounds were obtained from Sigma, the Tris salt being used unless otherwise indicated.

**Cell Growth and Harvesting.** *P. tetraurelia* d4-51, behaviorally wild type, was grown in wheat grass medium [prepared in the manner of Cerophyl medium (7)] to late logarithmic phase at room temperature (~25°C). Cells were washed four times in a resting buffer (10 mM Hapes/0.4 mM KCl/0.1 mM NaCl/0.5 mM MgCl2/10 μM CaCl2/0.1 mM Na2EDTA) unless otherwise noted [most behavioral work on *Paramecium* uses higher concentrations of KCl (4 mM) and CaCl2 (1 mM), which when tested did not alter the response to GTP]. The pH was adjusted to 7.5 by adding Tris base. After the final wash, the cells were allowed to adapt to this buffer for at least 1.5 hr before being used for behavioral studies. All experiments were carried out at room temperature.

**Behavioral Assays.** *Population assay.* The behavior of cells in a 6-μl drop was recorded on videotape for 15 sec starting ~2 sec after the addition of an equal volume of a 2-fold concentration of the indicated nucleotide. Blank additions of resting buffer were always given to control for mechanical stimulation. The concentration of cells was adjusted so that on average ~10 cells were on the screen at any given time. Cells were observed on a monitor [RCA (Lancaster, PA) TC1209] connected through a videocassette recorder (JVC BR-3100U) to a video camera (RCA TC1020) mounted on a microscope (Zeiss Standard). A Zeiss Planachromat 2.5×/0.08 objective was used without a condenser. The field of view displayed on the monitor was 2.1 mm by 3.3 mm. All cell movement was in the plane of the microscope slide; movement perpendicular to the slide was not observed due to the thin layer of fluid.

The video-recorded cell images were processed into a series of cell outlines by a video processor (VP110; Motion Analysis, Santa Rosa, CA) at a rate of 15 frames per sec. All other analysis was done by computer software (EXPERT VISION Version 2.01, Motion Analysis). Briefly, the center (centroid) of each video image was calculated from the cell outlines. Cell paths were then constructed from the centroids and smoothed twice to remove video noise.

Angular changes in the path direction, calculated from every set of three consecutive points in the path, determined the amount of deviation in degrees from straight-line swimming. For example, a cell moving directly forward will give a value of 0° and a cell that exactly reverses its direction will give a value of 180°. Right or left turns always register as positive changes in path direction. Changes in path direction greater than or equal to 17° were registered as “directional changes” (1):

**Abbreviations:** PDC, percent directional changes; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate.

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The behavioral effects of GTP and all test compounds were expressed as PDC. A directional change will register anytime a cell is whirling or making a transition between forward and backward swimming. The backward swimming time, although increased by GTP, cannot be directly measured here because all the cells do not swim backward at the same time and for the same duration, as they do in the presence of a strong depolarizing stimulus such as K+ addition (1). The user program is available from the authors. All data were graphed on SIGMAPLOT Version 3.1 (Jandal, Corte Medera, CA).

ED50 values were calculated by the following method: The PDC halfway between the maximum response and the blank addition response (PDC50) was determined, and the two experimentally obtained PDC responses bracketing the PDC50 were used to construct a line. The ED50 value was then calculated from the position of the PDC50 on this line.

Single cell assay. A single cell was added to 200 µl of resting buffer containing 10 µM GTP on a microscope slide at time 0. The cell was followed visually through a dissecting microscope and all backward swimming periods (not including whirling) were manually registered on a computer (software available on request).

Chemosresponse assay. Logarithmic-phase cells were concentrated directly in their wheat grass growth medium by centrifugation (500 × g) and then 400 µl of this cell suspension was placed into the bottom of a 6 mm × 50 mm borosilicate glass tube (five tubes total). GTP solutions, made in the supernatant collected from the cells, were carefully layered on top of the cell suspensions with a glass micropipet. If GTP were a repellent, then it should inhibit the natural upward movement of the cells, called negative geotaxis (5). Because there were no barriers to diffusion of the GTP, the effect only lasted for a few minutes, during which photographs were taken. The PDC response of cells in wheat grass solution (data not shown) is similar to that seen in the resting buffer (see Fig. 2).

Electrophysiology. Cells in resting buffer were impaled with a single electrode containing 0.5 M KCl. After the membrane potential stabilized, GTP or UTP at various concentrations was perfused into the chamber containing the impaled cell. The free-running membrane potential was then recorded.

RESULTS

The swimming behavior of cells responding to a strong GTP stimulus generally consisted of whirling, backward swimming, more whirling, and then a return to forward swimming (Fig. 1A). This behavioral motif was repeated many times until adaptation to the stimulus occurred, at which time the cell resumed predominantly forward motion. This series of backward swimming events was quantified by observing single cells under the microscope (Fig. 1B). Immediately after the cell was presented with the stimulus, periods of backward swimming followed in rapid succession, typically for ≈30 sec after GTP addition. The frequency then gradually decreased (30 sec to 5 min), after which there was no more backward swimming. In a simple and qualitative chemoresponse assay (Fig. 1C), GTP acted as a repellent to a population of cells. In this assay, the repellent acted by counteracting the natural tendency to swim upward, called negative geotaxis. When no GTP was present in the top layer of the tube, cells swam rapidly upward and collected near the top of the tube. GTP in the top layer of the tube prevented this accumulation of cells in a concentration-dependent fashion. Repulsion by 0.1 mM GTP resulted in a top layer almost devoid of cells. Even at 0.1 µM GTP slight repulsion was evident, although this effect was not always reproducible and usually lasted less than a minute. This repellent effect of GTP was also apparent using a different method to measure
Paramecium behavior, the modified T-tube chemoresponse assay (data not shown and ref. 8).

Of the four common nucleotides tested (ATP, GTP, CTP, and UTP), only GTP evoked a PDC response in the computer-assisted behavioral assay at concentrations <100 \( \mu \text{M} \) (Fig. 2A). The response to GTP was detectable at 0.1 \( \mu \text{M} \), and the maximal response occurred in the range of 10 \( \mu \text{M} \)-100 \( \mu \text{M} \) GTP, from which an ED\(_{50}\) value of 0.12 \( \mu \text{M} \) was calculated. GTP showed little or no activity at 0.01 \( \mu \text{M} \) (data not shown); hence, all subsequent dose–response curves began at 0.1 \( \mu \text{M} \). A response to ATP was observed only at 100 \( \mu \text{M} \), the highest concentration tested. The pyrimidine nucleotides CTP and UTP elicited no detectable response above background at concentrations between 0.1 \( \mu \text{M} \) and 100 \( \mu \text{M} \), although they did elicit an increased PDC at 1 mM (data not shown). These results indicate the overall preference for purines and the strong specificity for GTP.

To determine whether the phosphate chain of GTP was essential for the behavioral response, GDP, GMP, and guanosine were compared to GTP in the computer-assisted assay (Fig. 2B). The response elicited by each compound decreased with each phosphate group removed. ED\(_{50}\) values for GDP and GMP were not calculated because the PDC response never became clearly saturated. Guanosine gave essentially no response. The response to guanosine tetraphosphate, also decreased relative to GTP, is shown in Table 1.

Several other GTP analogs were tested for their ability to mimic GTP in eliciting a behavioral response (Table 1). The nonhydrolyzable GTP analogs guanosine 5'-[\( \gamma \)-thio]triphosphate and guanosine 5'-[\( \beta \),\( \gamma \)-imido]triphosphate (p[NH]ppG) were similar to GTP in their ability to induce directional changes, showing maximal responses (relative to GTP) of 79% and 87%, respectively. There was no increase in the relative ED\(_{50}\) of guanosine 5'-[\( \gamma \)-thio]triphosphate, and only a slight increase in the relative ED\(_{50}\) of p[NH]ppG to 2.9. The nonhydrolyzable analog guanosine 5'-[\( \beta \),\( \gamma \)-methylene]triphosphate was not as effective in eliciting a behavioral response, giving a maximal response of 72% compared with GTP, and an ED\(_{50}\) value 8 times greater than GTP. cGMP elicited a very poor maximal response of 20% and a much higher relative ED\(_{50}\) value of 20. The response to 2'-deoxy-GTP showed a 9-fold increase in the relative ED\(_{50}\) value and a small decrease in maximal response to 71%. Other modi-

![Fig. 2](image)

**Fig. 2.** Effect of various nucleotides and nucleosides on the swimming behavior of Paramecium as measured by motion analysis. (A) Response to GTP, ATP, CTP, and UTP. (B) Response to GTP, GDP, GMP, and guanosine. Each point on the dose–response curves represents the averaged PDC of three experiments. Each experiment, which involved \( \approx 10 \) cells, represents the averaged PDC over a 15-sec period after the addition of the stimulus. The error bars represent the standard deviation.

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Maximal response (relative to GTP), %</th>
<th>ED(_{50}), ( \mu \text{M} )</th>
<th>ED(_{50}) relative to GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li(_4) GTP(\gamma \text{S})</td>
<td>79</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Na(_2)[NH]ppG</td>
<td>87</td>
<td>0.3</td>
<td>2.9</td>
</tr>
<tr>
<td>Na(_2)[CH(_2)]ppG</td>
<td>72</td>
<td>0.7</td>
<td>8.0</td>
</tr>
<tr>
<td>ppppG</td>
<td>77</td>
<td>0.7</td>
<td>7.4</td>
</tr>
<tr>
<td>cGMP</td>
<td>20</td>
<td>5.7</td>
<td>20</td>
</tr>
<tr>
<td>2'-deoxy-GTP</td>
<td>71</td>
<td>0.8</td>
<td>8.7</td>
</tr>
<tr>
<td>8-N(_2)-GTP</td>
<td>37</td>
<td>7.3</td>
<td>87</td>
</tr>
</tbody>
</table>

Maximal response = \((\text{maximum analog response} - \text{control response}) \times 100\)/(maximum GTP response – control response). ED\(_{50}\) relative to GTP = ED\(_{50}\) (analog)/ED\(_{50}\) (GTP). GTP\(\gamma \text{S}\), guanosine 5'-[\( \gamma \)-thio]triphosphate; p[CH\(_2\)]ppG, guanosine 5'-[\( \beta \),\( \gamma \)-methylene]triphosphate; ppppG, guanosine 5'-tetraphosphate. PDC response to each analog was measured, along with the response to GTP, in the concentration range from 0.1 \( \mu \text{M} \) to 0.1 mM (as in Fig. 2). The response at 1 mM nucleotide was not included because most compounds gave a response at this concentration, indicating a lack of specificity. The following compounds were also tested and produced no detectable response: Na\(_2\)HPO\(_4\), Na\(_2\)PO\(_4\), Na\(_3\)P\(_3\)O\(_10\), adenosine 5'-[\( \beta \),\( \gamma \)-methylene]triphosphate, ITP, XTP, and 7-methyl-GTP. The compounds guanosine 3'-diphosphate 5'-diphosphate and guanosine 3'-diphosphate 5'-triphosphate produced a detectable response only at 0.1 mM.

![Fig. 3](image)

**Fig. 3.** Behavioral response and adaptation to GTP and the nonhydrolyzable analog p[NH]ppG. Each time point represents the averaged PDC from eight experiments. The points are calculated by counting the number of cells undergoing directional changes within each 1/15-sec video frame, then dividing by the total number of cells within that frame, and multiplying by 100. Blank additions of resting buffer were also given but are omitted for clarity (average PDC = 1.2). (A) Addition of GTP to a final concentration of 10 \( \mu \text{M} \). (B) Addition of the nonhydrolyzable GTP analog p[NH]ppG to a final concentration of 10 \( \mu \text{M} \).
fications of the ribose ring, the polyphosphorylated compounds guanosine 3'-diphosphate 5'-diphosphate and guanosine 3'-diphosphate 5'-triphosphate, elicited no significant response. Substitutions in the guanine ring especially reduced the response: an azide group at position 8 resulted in an 87-fold increase in $ED_{50}$ value and a large decrease in the maximal response to 37%. Substitutions at position 2, ITP and XTP, or at position 7, 7-methyl-GTP, caused a complete loss of activity. As an additional control, the inorganic phosphates alone, ortho-, pyro-, and triphosphate, produced no detectable response. We conclude from these results that the behavioral response to GTP is very specific with regard to the structure of the guanine ring but less stringent with regard to modifications of the phosphate chain.

The time course of adaptation to 10 $\mu$M GTP is shown in Fig. 3A. The initial response was mostly backward swimming (the rise in the curve), then a mixture of whirling and backward swimming (the peak of the curve), and finally a decay in the response over a period of ~60 sec to a mixture of forward swimming and whirling with occasional periods of backward swimming. Even after 60 sec, the cells maintained a higher PDC than cells not treated with GTP, a response that persisted for many minutes. The adaptation response to p[NH]ppG showed no striking difference when compared to that of GTP (Fig. 3B), indicating that hydrolysis of the terminal phosphate of GTP is not necessary to adapt to the signal on this time scale and that adaptation is probably due to an intracellular process.

The addition of GTP to a cell induced a series of membrane depolarizations, each lasting from 4 to 8 sec and spaced at average intervals of ~15 sec (Fig. 4A). The frequency and duration of these oscillating depolarizations generally correlated with the observed episodes of backward swimming in a typical cell (see Fig. 1B). Over a period of minutes, the frequency of the depolarizations generally diminished; by ~5 min, they no longer occurred (data not shown). This electrophysiological response also occurred in pawn B, a mutant deficient in the ciliary voltage-dependent Ca$^{2+}$ current necessary for the firing of Ca$^{2+}$-based action potentials (Fig. 4A) (9). This defect in ion-channel activity renders pawn B unable to swim backward in response to depolarizing stimuli such as increased [K$^+$]. Pawn B also responded poorly to depolarizing GTP stimuli in the concentration range 0.1 $\mu$M to 0.1 mM, displaying only whirling and no backward swimming (data not shown). Deciliated wild-type cells also showed the oscillating depolarizations, indicating that no ciliary channels are required (data not shown). The amplitude of the oscillating depolarizations in wild-type cells ranged up to 8 mV and was graded with the concentration of GTP (Fig. 4B). In control experiments with UTP, no oscillation of membrane potential was observed at concentrations up to 10 $\mu$M, with only a slight response at 100 $\mu$M. This specificity for GTP over UTP is consistent with the behavioral response (see Fig. 2A).

**DISCUSSION**

The first purinergic effect discovered, the sensitivity of blood pressure to adenosine and AMP, was described in 1929 (10). Since then, many other mammalian tissue and cell types have been found to be responsive to adenine nucleotides (11). To our knowledge, the data presented here provide the first evidence of purinergic sensitivity in a microorganism. This response is unusual in that it is GTP-specific rather than ATP-specific. Both ATP and ADP elicit purinergic responses in mammalian cells. *Paramecium* exhibited a similar specificity; the response to GDP was only slightly decreased relative to GTP. These results demonstrate a class of purinergic effects outside the present pharmacologically defined categories (11).

Is there a physiological role of GTP in the behavior of *Paramecium?* Although we routinely measure the response to GTP with a highly sensitive computer assay of motility, the response is also easily detectable in a simple chemoresponse assay. GTP at low concentrations is, therefore, capable of altering the swimming behavior of populations of paramecia, and this effect may have a significant role in the biology of *Paramecium.* But what is the relevant source of this repellent signal? It is possible that GTP, released from lysed paramecia, is used as a signal for cell death brought on by predators or noxious chemicals. Cell cytoplasm, which contains >0.1 mM GTP (ref. 12 and K.D.C., unpublished observation), would release enough repellent signal to cause other cells to evacuate the local area. By analogy with vertebrate secretory cells that release ATP (13), we have also considered the possibility that secretory organelles of *Paramecium* (trichocysts) might release GTP as a means of cell–cell communication. Although, in preliminary experiments, we failed to find GTP in the material secreted during trichocyst exocytosis, rapid extracellular hydrolysis of GTP may have occurred. *Paramecium,* like some animal cells (14), has a potent ectonucleoside triphosphatase activity (K.D.C., unpublished data). Whereas this activity is clearly not necessary for the short-term response or adaptation (see Fig. 3), it may be important in longer-term degradation of the GTP signal. This signal degradation is reminiscent of the action of the acetylcholinesterase present on nerve cells (15).
Are the GTP-induced changes in behavior mediated by an extracellular receptor? The behavioral effect is highly specific for GTP and occurs at a submicromolar concentration. In addition, electrophysiological measurements showed a depolarization of the membrane in wild-type cells that was also GTP-specific and occurred in the same concentration range. This high degree of specificity is suggestive of a receptor-mediated phenomenon. This putative receptor is probably external for the following reasons: (i) The response to GTP addition is rapid; a submicromolar addition resulted in a response within a few seconds (data not shown), whereas the addition of 10 μM caused an immediate response. (ii) The inherent membrane impermeability of a highly charged molecule such as GTP and the slow internalization of an extracellular molecule through normal digestive processes (16) would make this rapid response unlikely if the receptor were internal. Even if there were rapid internalization of the external GTP, the low extracellular concentration of GTP needed to alter behavior (0.1 μM) could hardly alter the much higher intracellular concentration of GTP. It is conceivable that Paramecium has an internal receptor-containing compartment with a low GTP concentration and a method for rapid uptake of GTP, but there is no direct evidence for either.

The GTP-induced oscillation in membrane potential that we report here is unusual in several respects. The depolarization and repolarization are much slower than for the well-known Ca2+-based action potentials that result from Ba2+ addition (17); each GTP-induced oscillating depolarization lasts for several seconds, whereas the action potentials elicited by Ba2+ last only a few milliseconds. It is possible that the relatively slow oscillations reflect metabolic changes such as second messenger generation within the cell and not simply the voltage- or ion-dependent opening of ion channels that underlies the much faster action potentials in Paramecium. The backward swimming and whirling that occur in response to GTP addition in intact wild-type cells are very likely triggered by periodic increases in intracellular Ca2+, the only second messenger known to bring about backward swimming in Paramecium. This Ca2+ most likely enters the cilia from outside the cell through the voltage-dependent Ca2+ channels (9); paw B cells, which exhibit the GTP-induced oscillating membrane depolarizations but are deficient in this Ca2+ current, show only a weak behavioral response to GTP. This also indicates that although the magnitude of the depolarizations is probably sufficient to trigger Ca2+ current through these voltage-dependent channels, the membrane potential oscillations do not require these ciliary Ca2+ channels. The current(s) responsible for the oscillating depolarizations is unknown but may be due to a receptor-operated channel that must be located on the cell body.

Even though the biological significance of the behavioral response to GTP is not yet understood, the sensitivity and selectivity that Paramecium exhibits in response to GTP indicate that it likely plays a physiologically important role in the behavior of the cells. The discovery of a purinergic receptor in a microorganism that is readily amenable to biochemical, electrophysiological, and genetic analyses provides a means to elucidate the signal transduction pathway between activation of an external receptor and the regulation of the various well-studied ion channels of Paramecium.

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