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# PPNDS is an agonist, not an antagonist, for the ATP receptor of *Paramecium*

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**Summary**

***Paramecium*** **represents a simple, eukaryotic model system to study the cellular effects of some neuroactive drugs. They respond to the agonist β,γ-methylene ATP with a transient depolarizing receptor potential, $Ca^{2+}$-based action potentials and repetitive bouts of forward and backward swimming called 'avoiding reactions' (AR).** ***In vivo*** **[$^{32}$P]ATP binding assays showed saturable [$^{32}$P]ATP binding with an apparent $K_d$ of approximately 23 nmol $l^{-1}$. Prolonged (15 min) exposure to 25 μmol $l^{-1}$ β,γ-methylene ATP caused behavioral adaptation and losses of AR, ATP receptor potentials and [$^{32}$P]ATP binding. While screening various ATP receptor inhibitors, we found that the P2X1 'antagonist' pyridoxal-phosphate naphthylazo-nitro-disulfate (PPNDS) is actually an agonist, producing the same responses as β,γ-methylene ATP. [$^{32}$P]ATP binding assays suggest that both agonists may bind to the same site as [$^{32}$P]ATP. Cross-adaptation is also seen between PPNDS and β,γ-methylene ATP in terms of losses in AR, depolarizing receptor potentials and [$^{32}$P]ATP binding. We conclude that the inhibition caused by PPNDS in** ***Paramecium*** **is due to agonist-induced desensitization. Either this represents a unique new class of ATP receptors, in which PPNDS is an agonist instead of an antagonist, or PPNDS (and other drugs like it) may actually be an agonist in many other cell types in which prolonged exposure is necessary for inhibition.**

Key words: PPNDS, ATP receptor, P2X1 antagonist, adaptation, desensitization, *Paramecium*, β,γ-methylene ATP.

## Introduction

*Paramecium* are motile, eukaryotic unicells that are used as simple model systems for studying the kinds of chemosensory transduction and sensory adaptation mechanisms that are common to neuronal cells. In this respect, *Paramecium* are often thought of as 'free-swimming sensory cells' (Machemer and dePeyer, 1977). As their swimming behaviors have been well correlated with electrophysiological changes (Eckert, 1972; Saimi and Kung, 1987; Hennessey, 1989; Machemer, 1988a,b; Hinrichsen and Schultz, 1988), behavioral bioassays are used as quick and convenient estimates of membrane ion channel functions. Confirmation of actual membrane conductance changes is easily obtained by intracellular membrane potential measurements and voltage clamp procedures (Saimi and Kung, 1987; Machemer, 1988b). Biochemical and molecular genetic approaches are also routinely used because these cells can be grown as pure clonal cultures in axenic media. The combination of behavioral mutant screening (Kung, 1971; Saimi and Kung, 1987) and genetic transformations by microinjection (Kung et al., 2000) enables both 'forward' and 'reverse' genetic approaches to understanding receptor-operated ion channel activation and regulation. Because of the ease and relevance of these behavioral bioassays, these cells can be useful in pharmacological screening for drugs that may affect membrane receptors, ion channel functions, chemosensory transduction and adaptation (Hennessey, 1989).

In *Paramecium*, there are many types of depolarizing stimuli that elicit graded, somatic depolarizations (receptor potentials) and consequent $Ca^{2+}$-based action potentials (Eckert, 1972). These stimuli include ionic stimuli (Machemer, 1988a,b), anterior mechanosensory stimuli (Ogura and Machemer, 1980), heat (Hennessey et al., 1983) and chemorepellents (Francis and Hennessey, 1995) such as oxidants (Hennessey et al., 1994), GTP (Clark et al., 1992), polycations (Hennessey et al., 1995) and ATP (Kim et al., 1999). These depolarizing receptor potentials are initiated on the body (somatic) membrane and are conducted passively to the voltage-dependent ciliary $Ca^{2+}$ channels for action potential generation (Eckert, 1972; Machemer, 1988a,b). When an action potential is produced, the $Ca^{2+}$ influx causes elevation of intraciliary $Ca^{2+}$ and $Ca^{2+}$-dependent ciliary reversal. Therefore, a sufficiently strong depolarizing stimulus will cause ciliary reversal and backward swimming. Some depolarizing stimuli (such as $Ba^{2+}$, $Na^{+}$, lysozyme, oxidants and ATP) can cause trains of action potentials and repetitive bouts of backward and forward swimming called 'avoiding reactions' (AR; Jennings, 1976). These chemicals act as chemorepellents because the avoiding reactions re-orient the swimming direction and result in movement away from the depolarizing stimulus in a manner very similar to the 'tumble' response of motile bacteria to chemorepellents (Adler, 1987; Tso and Adler, 1974; Koshland,

1988). The avoiding reactions are easily observed under a simple dissecting microscope and serve as the basis for many behavioral bioassays.

We have previously described ATP-induced AR, depolarizing receptor potentials, external [$^{32}$P]ATP binding, chemorepulsion and chemosensory adaptation in the ciliate *Tetrahymena* (Kim et al., 1999). We define chemosensory adaptation as a decrease in responsiveness to a ligand as a function of time of exposure to an appropriate concentration of that ligand. This is different from the 'adaptation', 'accommodation' or 'acclimatization' responses previously described in *Paramecium* because they involve long-term exposure to a high $K^+$ stimulus and testing in a different stimulus such as $Ba^{2+}$, $Mg^{2+}$ or heat (Schusterman et al., 1978).

Chemosensory adaptation has been shown in responses to 15 min exposure to micromolar quantities of GTP, ATP and polycationic chemorepellents [such as lysozyme and PACAP (pituitary adenylate cyclase activating peptide)] in *Tetrahymena* (Kuruvulla et al., 1997; Kim et al., 1999; Mace et al., 2000), and similar adaptation has been shown to lysozyme and GTP in *Paramecium* (Kim et al., 1997). Our working model is that there are three separate chemorepellent receptors and associated pathways (the GTP, 'polycation' and ATP reception systems), because cross-adaptation is not seen among the three systems. For example, adaptation to 10 μmol $l^{-1}$ GTP for 15 min causes a loss of GTP-induced AR, GTP receptor potentials and surface [$^{32}$P]GTP binding in *Paramecium* without affecting [$^{3}$H]lysozyme binding, lysozyme receptor potentials and lysozyme-induced AR (Kim et al., 1997). In *Tetrahymena*, no cross-adaptation was seen between the ligand binding, behavior or receptor potentials associated with the responses to the three representative chemorepellents GTP, ATP and lysozyme (Kuruvilla et al., 1997; Kim et al., 1999). Cross-adaptation is seen when two ligands utilize the same receptor, causing loss of responsiveness to both ligands following adaptation to one of them. Such cross-adaptation has been shown in the responses to lysozyme and PACAP in *Tetrahymena*, suggesting that they both activate the same 'polycation receptor' (Mace et al., 2000) or its adaptation pathway. Therefore, behavioral cross-adaptation can be used as a first-screen bioassay to see whether two ligands may activate either the same receptor or a common receptor adaptation pathway.

In vertebrates, ATP receptors are involved in neurotransmission, regulation of blood flow and sensory transduction of signals for pain, tissue stretch and temperature (Burnstock, 1996, 2000; Cook et al., 1997; Dubyak and El-Moatassim, 1993; Harden et al., 1995; Ralevic and Burnstock, 1998). Although ATP can be released from many types of cells, an extracellular ATP signal often represents cell lysis and acts as a signal for cell and organ damage and abnormal stretching or distention. It is this type of cell-lysis-produced ATP that has recently been studied as a pain (nociceptive) signal (Burnstock, 1996; Cook et al., 1997; McCleskey and Gold, 1999; Souslova et al., 2000). Response adaptation and receptor desensitization has been shown in several types of ATP receptor systems (Burnstock, 2000), and agonist-induced receptor internalization of native P2X1 has recently been demonstrated in a vertebrate preparation (Ennion and Evans, 2001). We have proposed that ATP receptors in the ciliates *Paramecium* and *Tetrahymena* are also acting as cell lysis detectors, providing a general 'blood in the water' danger signal so that these cells can avoid whatever the situation was that caused nearby cell lysis. Therefore, the ciliate ATP receptors are chemorepellent receptors.

There are many types of external purinergic receptors in vertebrates. In general, responses to external purines are categorized as either P1 or P2, with P1 referring primarily to responses to ligands such as adenosine while P2 ligands are generally nucleoside triphosphates such as ATP (Abbracchio et al., 1993; Khakh et al., 2001). The P2X receptors are also ion channels (ionotropic), while P2Y receptors are G-protein coupled (metabotropic) (Khakh et al., 2001). There are currently seven different P2X receptor genes cloned, and each of these subtypes of receptors (P2X1–P2X7) has indicative agonist and antagonist specificities (Khakh et al., 2001). The non-hydrolyzable ATP analog β,γ-methylene ATP is often used as the preferred agonist in ATP receptor studies because ecto-ATPases and other extracellular hydrolases are often present to hydrolyze external ATP. It has been suggested that ecto-ATPases may be involved in the deactivation of purinergic agonists in much the same way as acetylcholinesterase inactivates acetylcholine signals (Kennedy et al., 1997; Hennessey et al., 1997). While ATP is a potent agonist for P2X receptors, the non-hydrolyzable analogs, such as β,γ-methylene ATP, are often much more potent (Burnstock, 2000; Khakh et al., 2001).

To determine whether the properties of the ATP receptors of *Paramecium* and *Tetrahymena* are similar to other known ATP receptors, we followed a classical pharmacological approach to see if these receptors fit into any of the known ATP receptor classifications. As the drug pyridoxal-phosphate naphthylazo-nitro-disulfate (PPNDS) has been described as a specific P2X1 receptor antagonist (Lambrecht et al., 2000), we tested it to see if it would block the responses of *Paramecium* to external ATP and its analogs. Surprisingly, PPNDS produced the same depolarizing responses as β,γ-methylene ATP, suggesting that PPNDS is not an ATP receptor antagonist in *Paramecium* but an agonist. We propose that the ability of PPNDS to cause inhibition of ATP responses is due, at least in *Paramecium*, to time-dependent, agonist-induced ATP receptor adaptation.

## Materials and methods

### *Cell cultures*

The trichocyst non-discharge mutant, nd6, of *Paramecium tetraurelia* (Sonneborn, 1975) was used primarily in this study to avoid trichocyst discharge during electrophysiological recording. Cells were grown to late log phase (4 days) in the axenic medium of Soldo and Van Wagtendonk (1969).

*Chemicals and solutions*

The '*Paramecium* wash buffer' solution contained 1.0 mmol $l^{-1}$ $CaCl_2$, 1.0 mmol $l^{-1}$ MOPS, 0.5 mmol $l^{-1}$ $MgCl_2$, pH 7.2 with Tris-base. As PPNDS is a tetrasodium salt, cells were preincubated for 15 min in the wash buffer solution with the same $Na^+$ concentration before exposure to PPNDS to adapt them to the $Na^+$. Similar $Na^+$ balancing was done with β,γ-methylene ATP because it is a disodium salt. The solution used for the *in vivo* [$^{32}$P]ATP binding assays contained 10 mmol $l^{-1}$ Tris-base, 0.5 mmol $l^{-1}$ MOPS, 1.0 mmol $l^{-1}$ tartrate, 10 μmol $l^{-1}$ EGTA, pH 7.2. PPNDS was obtained from Tocris Cookson Inc., Ellisville, MO, USA, but all other chemicals were purchased from Sigma-Aldrich, St Louis, MO, USA.

*Behavioral assays*

The chemorepellent behavioral assay used was the same as previously described (Kim et al., 1997). In this assay, individual cells were manually transferred to a test solution using a micropipet and observed under the dissecting microscope for swimming behavior. Cells showing any significant backward swimming events ('avoiding reactions') during the first 5 s after transfer were scored as positive responders. The avoiding reaction is defined by at least one backward movement of at least one body length. Ten cells were observed for each trial, and the mean ± S.D. was calculated for three trials (30 cells total) and expressed as the percentage of cells showing avoiding reactions (% AR).

In the adaptation experiments (as in all experiments), cells were first pre-incubated in the appropriate wash solution for 15–30 min before testing. Cells were then placed in either 25 μmol $l^{-1}$ β,γ-methylene ATP (a non-hydrolyzable analog of ATP) or 100 μmol $l^{-1}$ PPNDS for varying amounts of time. Cells were transferred to the same solution without the repellent for 20 s (as a brief wash) and then transferred into the original test solution or a new test solution to assay for repellent responses.

*Electrophysiology*

One-electrode intracellular membrane potential measurement procedures were similar to those described previously (Kim et al., 1997). The electrophysiological recordings were taken in the behavioral solutions detailed above with 500 mmol $l^{-1}$ KCl electrodes (resistances of 100–200 mΩ). The membrane potentials were recorded under continuous perfusion conditions at a rate of 15–20 ml $min^{-1}$, and bath volume was approximately 1 ml. In the adaptation experiments, a cell was impaled in the repellent solution and exposed to repellent perfusion for varying times. After a 15–20 s buffer perfusion wash, the cell was re-exposed to the repellent perfusion.

In vivo *binding assays*

The *in vivo* [$^{32}$P]ATP binding assays were performed in a solution without added $Ca^{2+}$ to prevent ATP hydrolysis by the $Ca^{2+}$-dependent ecto-ATPase (Smith et al., 1997; Hennessey et al., 1997). In the [$^{32}$P]ATP binding assay, 0.2 ml of packed cells were washed three times by centrifugation (700 ***g*** for 1 min) in the solution and then 100 μl aliquots were withdrawn for binding assays. Each 100 μl aliquot was mixed with [$^{32}$P]ATP and binding solution to a final volume of 200 μl. This was vortexed gently and two 20 μl samples removed and added to 2.5 ml of Ecoscint scintillation fluid for scintillation counting. The average of these readings represented the sum of the bound and free [$^{32}$P]ATP. The remaining sample was pelleted by centrifugation (500 ***g*** for 1 min) and 20 μl of the supernatant was removed for scintillation counting. This represented the amount of free [$^{32}$P]ATP. The amount bound was determined by subtraction because [bound] = [bound + free] – [free]. In the binding assay involving PPNDS, 0.2 ml of packed cells were washed three times by centrifugation in this solution and diluted to a final volume of 2.0 ml. After a 15 min incubation in this binding solution, PPNDS was added to a concentration of 100 μmol $l^{-1}$. Cells were incubated in 100 μmol $l^{-1}$ PPNDS for various times, and 100 μl aliquots were withdrawn and quickly washed by centrifugation in 1 ml of binding solution. The resulting cell pellet was vortexed with [$^{32}$P]ATP and binding solution. The resulting mixture was pelleted by centrifugation (500 ***g*** for 1 min). This pellet was extracted and washed *via* centrifugation in binding solution then placed into 2.5 ml Ecoscint for scintillation counting. This reading represented the amount of [$^{32}$P]ATP bound to cells. In the competitive inhibition studies, [$^{32}$P]ATP binding was performed in the presence of a 50-fold concentration excess (compared with the concentration of [$^{32}$P]ATP present) of either cold (unlabelled) β,γ-methylene ATP or PPNDS. This binding mixture was pelleted by centrifugation (500 ***g*** for 1 min) and the pellet was extracted and placed into 2.5 ml Ecoscint for scintillation counting.

## Results

β,γ-methylene ATP is an effective, non-toxic, chemorepellent in *Paramecium*. This non-hydrolyzable ATP analog caused repetitive avoiding reactions (AR) at micromolar concentrations, indicative of a depolarizing chemorepellent (Fig. 1B). It is important to note that these responses were immediate, the cells showing ATP-induced AR as soon as they came out of the transfer pipet. The $EC_{50}$ value (the concentration at which 50% of the cells tested showed AR) was approximately 12 μmol $l^{-1}$ for β,γ-methylene ATP, and maximal responses were seen at approximately 25 μmol $l^{-1}$ (Fig. 1C). The electrophysiological responses to 25 μmol $l^{-1}$ β,γ-methylene ATP were transient receptor potentials (Fig. 2) that showed a mean amplitude of 13.7±0.6 mV and a mean duration of 26.5±7.2 s ($N$=10 each). The faster spikes, which are often seen riding on top of the receptor potential (see Fig. 2, Cell 1), are consistent with the graded, $Ca^{2+}$-based action potentials associated with the AR (Eckert, 1972). It was often possible to observe visible jerks of the cell on the electrode correlating with the appearance of these spikes, verifying that they represent $Ca^{2+}$-based action potentials.

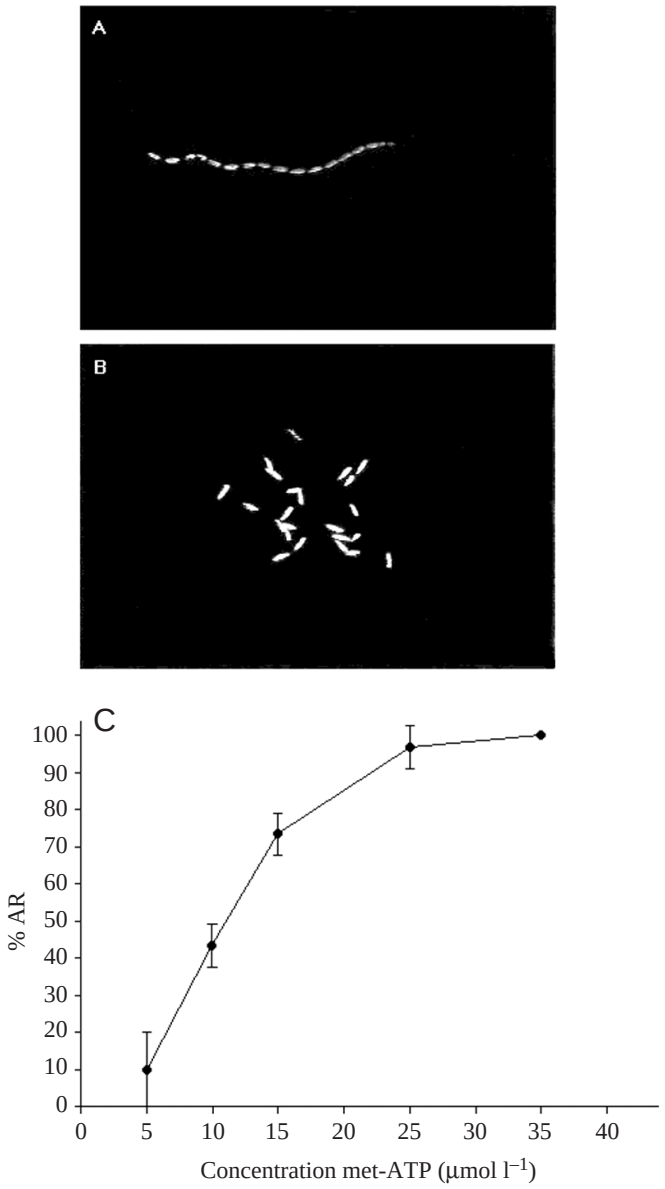


Fig. 1. Transfer of *Paramecium* to β,γ-methylene ATP (met-ATP) causes immediate avoiding reactions (AR). Digital video imaging shows the swimming path of a cell to be relatively straight in the control solution (A), while a different cell in 25 μmol $l^{-1}$ β,γ-methylene ATP shows repetitive jerking back and forth, indicative of AR (B). The video capture rate was approximately 16 frames $s^{-1}$. (C) The percentage of cells showing AR (% AR) increased in a concentration-dependent manner, with 100% of the cells showing AR at concentrations of β,γ-methylene ATP above 25 μmol $l^{-1}$ and an $EC_{50}$ of approximately 12 μmol $l^{-1}$. Each point represents the mean ± S.D. of three trials.

*Paramecium* showed saturable external [$^{32}$P]ATP binding in the *in vivo* binding assays (Fig. 3A). A Scatchard plot analysis of amount bound and free (unbound) [$^{32}$P]ATP suggested a single set of external binding sites within the concentration range of 5–30 nmol $l^{-1}$ (Fig. 3B). The apparent $K_d$ was 23.1 nmol $l^{-1}$, and the $B_{max}$ was 112 pmol $l^{-1}$. Taking into consideration the number of cells in the assay, the number of functional surface receptors was estimated to be approximately $7.023\times10^5$ receptors $cell^{-1}$.

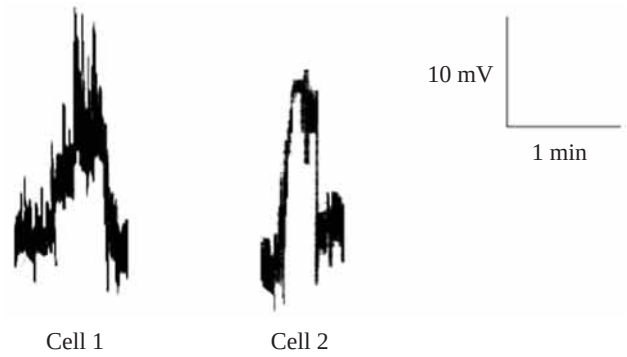


Fig. 2. The electrophysiological response of *Paramecium* to 25 μmol $l^{-1}$ β,γ-methylene ATP was a transient depolarization. This is the β,γ-methylene ATP-receptor potential. As the response was somewhat variable, two different cells are shown. The very fast spikes seen in the first trace (Cell 1) are the $Ca^{2+}$-based, graded action potentials common to these cells but they are less evident in the second trace (Cell 2). In both traces, the cells were exposed to 25 μmol $l^{-1}$ β,γ-methylene ATP during the entire time. The recording medium contained 1 mmol $l^{-1}$ $CaCl_2$, 1 mmol $l^{-1}$ MOPS, 0.5 mmol $l^{-1}$ $MgCl_2$ and pH 7.2 with Tris-base. The recording electrode contained 500 mmol $l^{-1}$ KCl.

Continued exposure of cells to 25 μmol $l^{-1}$ β,γ-methylene ATP for 15 min resulted in a time-dependent behavioral adaptation (Fig. 4A). This type of long-term adaptation was seen by exposing cells to 25 μmol $l^{-1}$ β,γ-methylene ATP for varying amounts of time, transferring the cells to the wash buffer for 20 s and then retesting the cells in 25 μmol $l^{-1}$ β,γ-methylene ATP. This is different from the short-term adaptation that was seen when cells were observed continuously after transfer to 25 μmol $l^{-1}$ β,γ-methylene ATP for several minutes (C. R. Wood and T. M. Hennessey, manuscript in preparation). Short-term adaptation was seen as a decrease in the frequency of AR within the first 10–30 s after transfer to β,γ-methylene ATP and it roughly correlated with the duration of the receptor potential. For example, a cell transferred to 25 μmol $l^{-1}$ β,γ-methylene ATP would show immediate, repetitive AR but the frequency of AR decreased over time until, after approximately 30 s, the cell was swimming forward most of the time with very few AR. This is short-term adaptation. However, if this cell (which had been exposed to 25 μmol $l^{-1}$ β,γ-methylene ATP for 30 s) was transferred to wash buffer for 20 s and re-exposed to 25 μmol $l^{-1}$ β,γ-methylene ATP, it would show AR because it had not been in β,γ-methylene ATP long enough to initiate long-term adaptation. Electrophysiological analysis confirmed that receptor potentials could still be generated upon re-exposure to 25 μmol $l^{-1}$ β,γ-methylene ATP even after 8 min of adaptation but the amplitudes were significantly decreased (Fig. 4B). Exposing cells to 25 μmol $l^{-1}$ β,γ-methylene ATP for 15–20 min caused almost a complete loss of both AR (Fig. 4A)

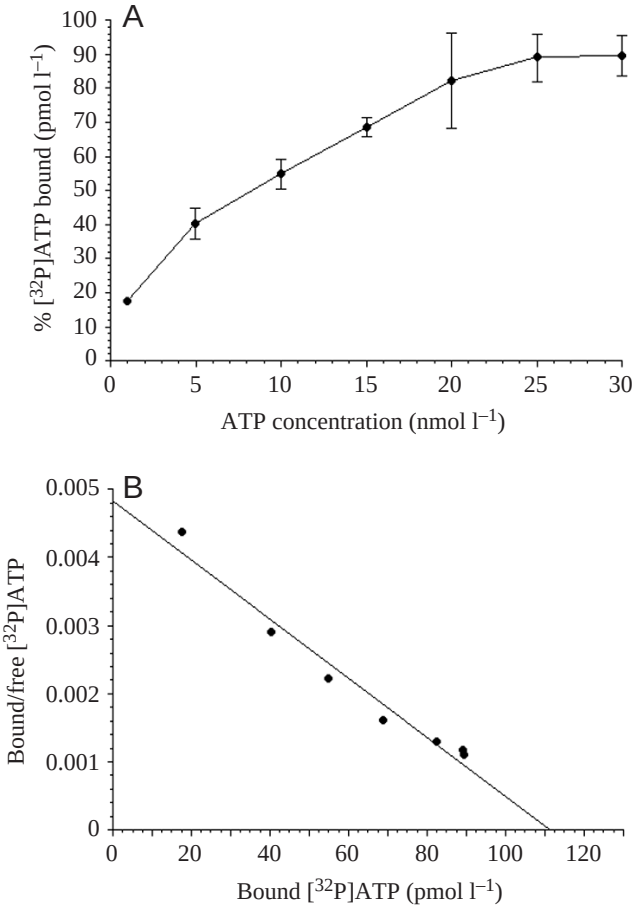


Fig. 3. *In vivo* [$^{32}$P]ATP binding assays were used to show saturable, external binding in the range of 1–30 nmol $l^{-1}$ [$^{32}$P]ATP (A). Each point represents the mean ± S.D. of at least three trials. A Scatchard plot of this data infers a single class of external [$^{32}$P]ATP binding sites with an apparent $K_d$ of approximately 23 nmol $l^{-1}$ and a $B_{max}$ of 110 pmol $l^{-1}$ (B). The estimate of the number of surface [$^{32}$P]ATP binding sites is approximately $7.023\times10^5$ receptors $cell^{-1}$.

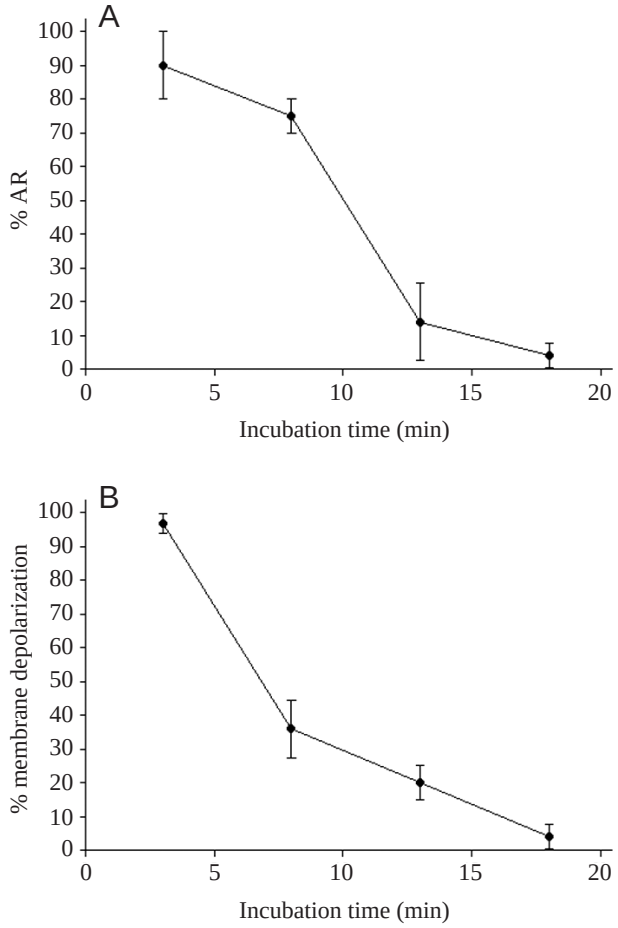


Fig. 4. Behavioral adaptation to 25 μmol $l^{-1}$ β,γ-methylene ATP is seen as a time-dependent decrease in β,γ-methylene ATP avoidance response (% AR) (A) and in the amplitude of the β,γ-methylene ATP-receptor potential (B). In both cases, it takes approximately 15 min for adaptation to be complete. Therefore, adaptation can be defined as a time-dependent loss of either the β,γ-methylene ATP-AR or the β,γ-methylene ATP receptor potential. Each point represents the mean ± S.D. of three trials.

and the receptor potential (Fig. 4B), defining conditions for long-term adaptation.

In summary, 25 μmol $l^{-1}$ β,γ-methylene ATP can elicit a reliable, transient receptor potential that is the basis for the ATP-induced AR. Long-term behavioral and electrophysiological adaptation is complete after 15–20 min of exposure to 25 μmol $l^{-1}$ β,γ-methylene ATP.

As there are many types of ATP receptors and they are often characterized by their sensitivities to agonists and antagonists, we decided to see whether a specific P2X1 inhibitor, PPNDS (Lambrecht et al., 2000), could eliminate the ATP responses of *Paramecium*. Instead of inhibiting the type of responses we were assaying, PPNDS caused the same responses as β,γ-methylene ATP. PPNDS produced the same kind of AR as β,γ-methylene ATP in the concentration range commonly used in the literature for inhibition of vertebrate P2X1 responses (Lambrecht et al., 2000). The $EC_{50}$ for PPNDS was approximately 70 μmol $l^{-1}$, and maximal responses were seen at approximately 120 μmol $l^{-1}$ (Fig. 5A). Addition of 100 μmol $l^{-1}$ PPNDS to β,γ-methylene-ATP-containing solutions did not produce any immediate inhibition of AR because PPNDS itself produced the same AR as β,γ-methylene ATP.

PPNDS also produced similar transient depolarizations (Fig. 5B) with similar amplitudes and durations to those seen with β,γ-methylene ATP. The 'receptor potentials' induced by 100 μmol $l^{-1}$ PPNDS had a mean amplitude of 13.2±2.2 mV and a mean duration of 29.2±7.3 s. Using a standard *t*-test, there was no significant difference between the amplitudes ($t$=0.46, $P$>0.1, d.f.=4) or the durations ($t$=0.38, $P$>0.1, d.f.=4) of the β,γ-methylene ATP-induced depolarizations and the PPNDS-induced depolarizations. Exposure of cells to PPNDS for 15–20 min produced long-term adaptation as represented by a time-dependent loss of both the PPNDS receptor potential (Fig. 5C) and the PPNDS AR (Fig. 6B). Therefore, the behavioral and

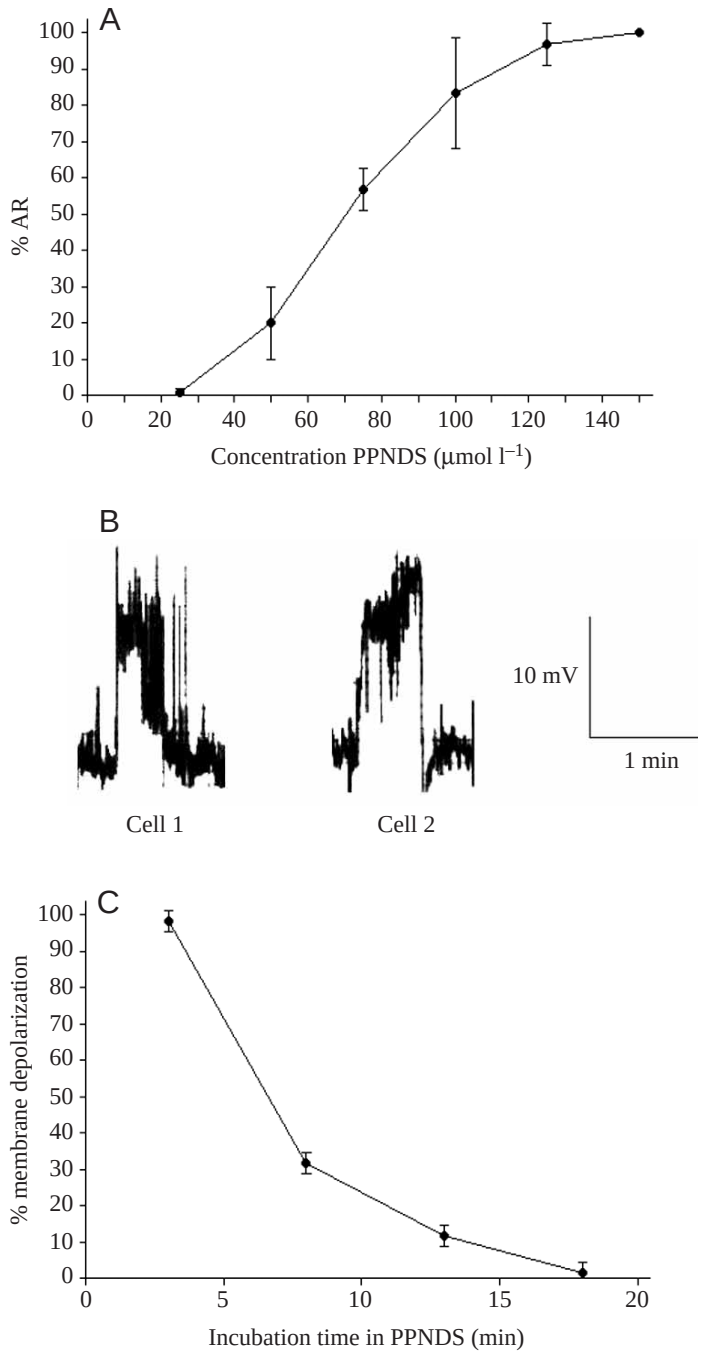


Fig. 5. PPNDS (pyridoxal-phosphate naphthylazo-nitro-disulfate) also caused avoidance responses (AR) and transient receptor potentials. PPNDS caused 100% AR at approximately 120 μmol $l^{-1}$ and the $EC_{50}$ was approximately 70 μmol $l^{-1}$ (A). The transient receptor potential (B) caused by 100 μmol $l^{-1}$ PPNDS was similar in amplitude and duration to those seen with 25 μmol $l^{-1}$ β,γ-methylene ATP (compare with Fig. 2). Two different cells are shown to demonstrate the variability of the responses. The amplitude of the PPNDS receptor potential decreased as a function of time of exposure to 100 μmol $l^{-1}$ PPNDS (C). The behavioral bioassays and the recording solution were the same as the solutions used for Figs 1, 2. Each point represents the mean ± S.D. of three trials.

electophysiological responses to PPNDS are similar to the responses to β,γ-methylene ATP and they both show the same type of long-term adaptation.

Behavioral cross-adaptation was observed for β,γ-methylene ATP and PPNDS. Cells that were behaviorally adapted to 25 μmol $l^{-1}$ β,γ-methylene ATP for 15 min lost AR after retesting in either 100 μmol $l^{-1}$ PPNDS or 25 μmol $l^{-1}$ β,γ-methylene ATP (Fig. 6A). Similarly, cells that were behaviorally adapted to 100 μmol $l^{-1}$ PPNDS lost AR after retesting in either 100 μmol $l^{-1}$ PPNDS or 25 μmol $l^{-1}$ β,γ-methylene ATP (Fig. 6B). By contrast, cells that were behaviorally adapted to β,γ-methylene ATP or PPNDS still retained full responsiveness to another non-toxic, depolarizing chemorepellent, 10 μmol $l^{-1}$ GTP (Clark et al., 1992). These adapted cells also showed normal AR in the standard ionic depolarizing test solutions (Saimi and Kung, 1987) such as 40 mmol $l^{-1}$ $K^+$, 10 mmol $l^{-1}$ $Na^+$ and 8 mmol $l^{-1}$ $Ba^{2+}$. This suggests that the adaptation is specific for the ATP-sensing pathway in these cells and is not due to an overall desensitization.

Behavioral adaptation to 100 μmol $l^{-1}$ PPNDS was associated with a time-dependent decrease in external [$^{32}$P]ATP binding sites (Fig. 7A). Adaptation to 25 μmol $l^{-1}$ β,γ-methylene ATP for 15 min caused a similar loss of external [$^{32}$P]ATP binding (Fig. 7B). This showed that adaptation to either β,γ-methylene or PPNDS caused the loss of external [$^{32}$P]ATP binding. These changes in [$^{32}$P]ATP binding are not permanent but reversible. Cells were adapted to 100 μmol $l^{-1}$ PPNDS for 15 min and were then transferred to the same solution without PPNDS for an additional 15 min. These 'de-adapted' cells regained nearly 100% [$^{32}$P]ATP binding, as did cells that were previously adapted to β,γ-methylene ATP for 15 min and then washed free of β,γ-methylene ATP and 'de-adapted' (Fig. 7B). Similar reversibility ('de-adaptation') was seen in the behavioral and electrophysiological changes described when adapted cells were incubated in the ligand-free wash buffer for 15 min. We therefore do not consider this time-dependent, PPNDS-induced loss of [$^{32}$P]ATP binding to be true antagonist-type inhibition because the well-known ATP receptor agonist β,γ-methylene ATP has the same effect. Both β,γ-methylene ATP and PPNDS are agonists in this system and prolonged (15 min) exposure to either ligand causes the same behavioral adaptation for the same reasons.

If both β,γ-methylene ATP and PPNDS activate the same receptor, they may bind to the same active site. To test this hypothesis, we added a 50-fold excess of PPNDS to the *in vivo* [$^{32}$P]ATP binding mixture and immediately measured the amount of [$^{32}$P]ATP bound. Excess PPNDS caused an immediate decrease in [$^{32}$P]ATP binding to whole cells (Fig. 8), suggesting that PPNDS may compete with [$^{32}$P]ATP for binding to the ATP receptor. Similarly, cold (non-radioactive) β,γ-methylene ATP caused immediate inhibition of [$^{32}$P]ATP binding when added in a 50-fold excess (Fig. 8). These observations are consistent with the idea that β,γ-

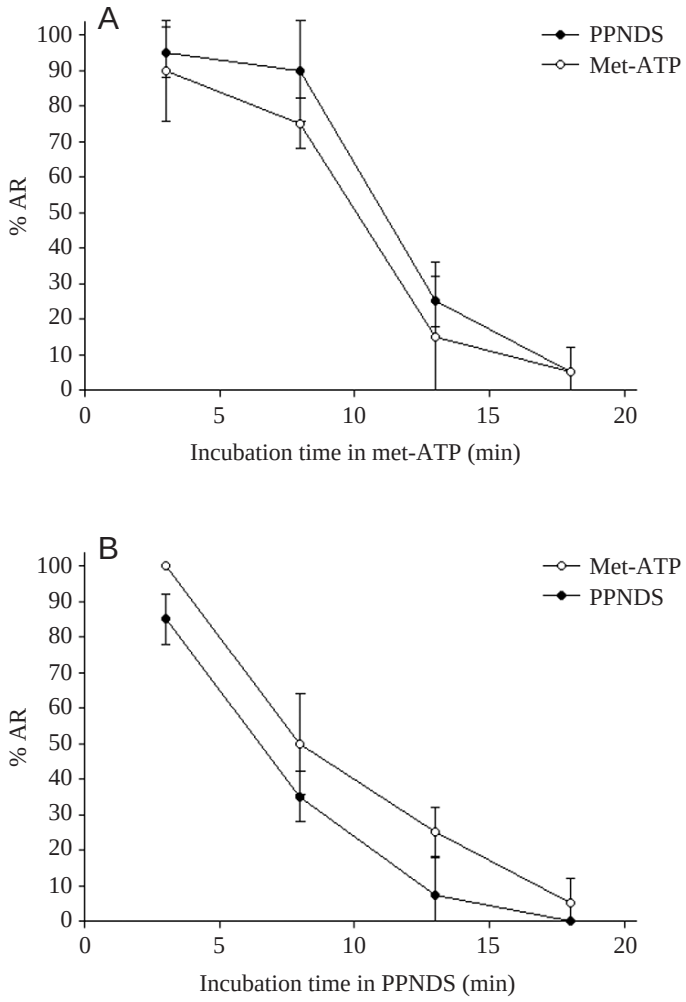


Fig. 6. Behavioral cross-adaptation seen as a function of time in either 25 μmol $l^{-1}$ β,γ-methylene ATP (met-ATP) or 100 μmol $l^{-1}$ PPNDS (pyridoxal-phosphate naphthylazo-nitro-disulfate). Cross-adaptation between two ligands is seen as the loss of responsiveness to one ligand following exposure to the other ligand and *vice versa*. (A) Cells were adapted to 25 μmol $l^{-1}$ β,γ-methylene ATP and then retested in either 25 μmol $l^{-1}$ β,γ-methylene ATP (open circles) or 100 μmol $l^{-1}$ PPNDS (filled circles). Cross-adaptation was seen in response to either ligand with similar time courses. (B) Cross-adaptation was also demonstrated by incubation in 100 μmol $l^{-1}$ PPNDS and retesting in either 25 μmol $l^{-1}$ β,γ-methylene ATP (open circles) or 100 μmol $l^{-1}$ PPNDS (filled circles). Each point represents the mean ± S.D. of three trials.

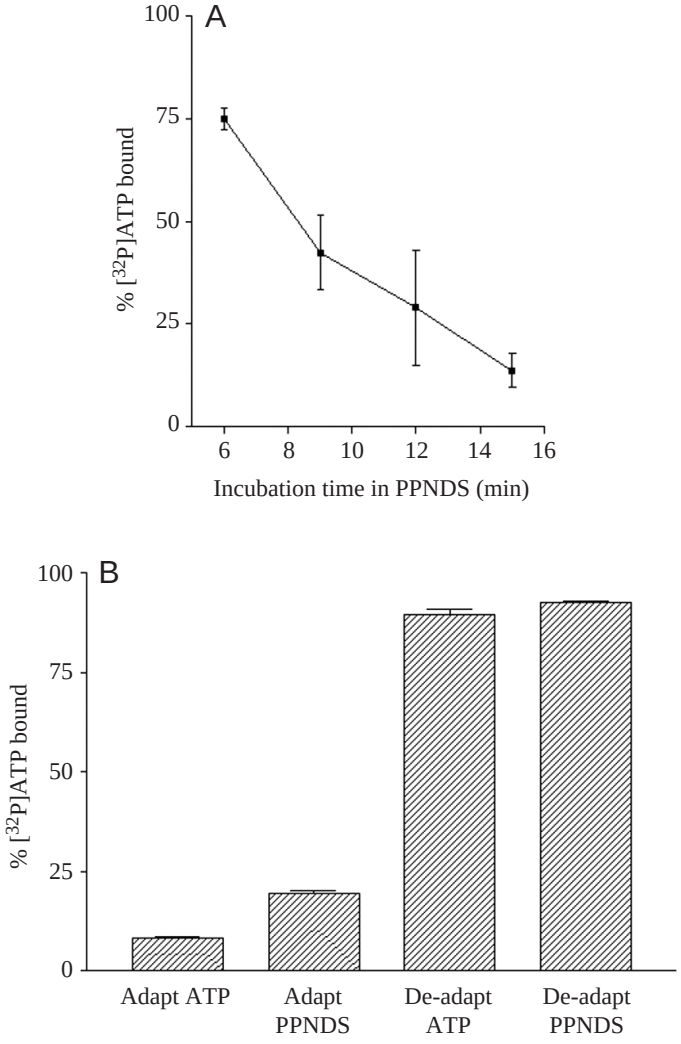


Fig. 7. Behavioral adaptation to either 100 μmol $l^{-1}$ PPNDS (pyridoxal-phosphate naphthylazo-nitro-disulfate) or 25 μmol $l^{-1}$ β,γ-methylene ATP is associated with a reversible loss of external [$^{32}$P]ATP binding. (A) When assayed as a function of time, the % [$^{32}$P]ATP bound to live cells decreased over 15 min exposure to 100 μmol $l^{-1}$ PPNDS. (B) When the % [$^{32}$P]ATP bound to live cells was assayed after a 15 min exposure to either 25 μmol $l^{-1}$ β,γ-methylene ATP or 100 μmol $l^{-1}$ PPNDS, both caused dramatic decreases in [$^{32}$P]ATP binding. When cells were adapted to either 25 μmol $l^{-1}$ β,γ-methylene ATP or 100 μmol $l^{-1}$ PPNDS, washed free of the adapting ligand and then 'de-adapted' in wash buffer for 15 min, the [$^{32}$P]ATP binding returned to normal levels. This showed that the adaptation was reversible. Each point represents the mean ± S.D. of three trials.

methylene ATP and PPNDS may compete for [$^{32}$P]ATP binding to the active site of the ATP receptor; however, future purification of the ATP receptor is necessary to confirm such competitive inhibition. In this respect, both β,γ-methylene ATP and PPNDS are inhibitors of [$^{32}$P]ATP binding when they are present in the *in vivo* [$^{32}$P]ATP assay, but it must be remembered that cells were washed free of external β,γ-methylene ATP and PPNDS in all previous experiments where long-term, time-dependent adaptation was seen (see Figs 4–8).

## Discussion

As we had previously characterized the ATP avoidance behavior, depolarizing receptor potential, *in vivo* external [$^{32}$P]ATP binding and chemosensory adaptation in *Tetrahymena* (Kim et al., 1999), our first goal was to verify that similar responses were reliably seen in *Paramecium*. Although electrophysiological analysis of ATP responses is possible in *Tetrahymena* (Kim et al., 1999; Hennessey and

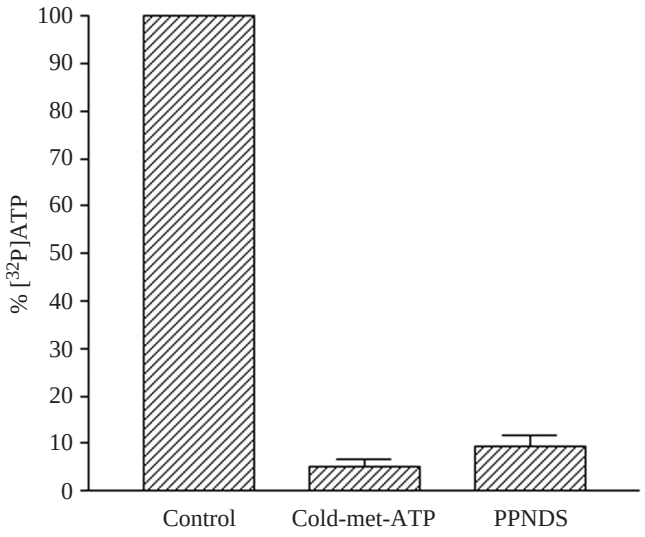


Fig. 8. The amount of [$^{32}$P]ATP bound to live cells was reduced dramatically by including a 50-fold excess of either β,γ-methylene ATP (met-ATP) or PPNDS (pyridoxal-phosphate naphthylazo-nitro-disulfate) in the binding assay. The control column represents the amount of [$^{32}$P]ATP bound in the absence of any extra added ligand, and each of the other columns is normalized to those counts. Each point represents the mean + S.D. of three trials.

Kuruvilla, 1999), we chose *Paramecium* for further pharmacological and electrophysiological studies because their larger size enables easier and more reliable electrophysiological results and greater ease of viewing in behavioral assays. As with *Tetrahymena* (Kim et al., 1999), *in vivo* [$^{32}$P]ATP binding assays with *Paramecium* showed [$^{32}$P]ATP binding kinetics that suggest a single class of binding sites within the concentration range tested and an apparent $K_d$ in the nanomolar range (Fig. 4), although *Paramecium* were generally less sensitive to β,γ-methylene ATP in behavioral and electrophysiological assays compared with *Tetrahymena* (Kim et al., 1999). However, the β,γ-methylene ATP-induced receptor potential was larger and more clearly transient in *Paramecium* (Fig. 2) than in *Tetrahymena* (Kim et al., 1999).

Using *Paramecium*, we have shown that long-term behavioral adaptation to β,γ-methylene ATP occurs over a time span of approximately 15 min (Fig. 4). While the initial response of a cell to 25 μmol l$^{-1}$ β,γ-methylene ATP is a depolarizing receptor potential (and consequent AR), cells adapted for 15 min in 25 μmol l$^{-1}$ β,γ-methylene ATP swim forward and show no β,γ-methylene ATP-induced receptor potentials, even after a 20 s wash and retest. As with *Tetrahymena* (Kim et al., 1999), behavioral adaptation to β,γ-methylene ATP in *Paramecium* is correlated with a loss in extracellular [$^{32}$P]ATP binding (Fig. 7). The loss of [$^{32}$P]ATP binding prevents the generation of the β,γ-methylene ATP-induced receptor potential, and the result is no β,γ-methylene ATP-induced AR. We conclude that *Paramecium* offer a unique opportunity to use quick, easy and humane behavioral bioassays to screen the effects of purinergic agonists and antagonists and then follow these behavioral screens with further electrophysiological and [$^{32}$P]ATP binding analysis.

The current literature suggests that one of the most specific anti-P2X1 antagonists to use for such a purpose is PPNDS (Lambrecht et al., 2000), a derivative of PPADS (pyridoxal-phosphate-6-azophenyl-2,4-disulfonic acid) with reportedly higher specificity and potency than PPADS (Khakh et al., 2001). It has been common in such studies to also expose a cell to a variety of agonists and antagonists and to categorize the receptor on the basis of the spectrum of sensitivities to these ligands (Khakh et al., 2001). If PPNDS is an antagonist of the ATP receptor of *Paramecium*, it should inhibit β,γ-methylene ATP-induced AR.

PPNDS, like β,γ-methylene ATP, is an effective agonist for the ATP responses of *Paramecium*. PPNDS causes the same depolarizing responses as β,γ-methylene ATP. The behavioral $EC_{50}$ is higher for PPNDS than for β,γ-methylene ATP and it requires >100 μmol l$^{-1}$ PPNDS for maximal stimulation. This is the approximate PPNDS concentration used in the previous inhibition studies (Lambrecht et al., 2000). PPNDS at 100 μmol l$^{-1}$ produces transient, depolarizing receptor potentials (Fig. 5B) that are indistinguishable from those elicited by 25 μmol l$^{-1}$ β,γ-methylene ATP (Fig. 2) in both amplitude and duration.

Prolonged (15 min) exposure to 100 μmol l$^{-1}$ PPNDS produces chemosensory adaptation, seen as a loss of PPNDS-induced AR and PPNDS receptor potentials. Similarly, adaptation to β,γ-methylene ATP causes adaptation and loss of β,γ-methylene ATP-induced AR and β,γ-methylene ATP receptor potentials. This type of loss in responsiveness has also sometimes been called desensitization or downregulation in the sensory literature but we chose the term adaptation to encompass the behavioral, electrophysiological and receptor binding events. The ATP receptor in rat vas deferens, which is a P2X1-like receptor, undergoes similar agonist-induced desensitization (Ennion and Evans, 2001).

Cross-adaptation is seen between PPNDS and β,γ-methylene ATP responses, suggesting that they may be activating the same receptor. Cells that have been adapted to 100 μmol l$^{-1}$ PPNDS also lose responsiveness to β,γ-methylene ATP (and *vice versa*). The time courses of losses in β,γ-methylene ATP-induced AR are similar for both PPNDS and β,γ-methylene ATP adaptation (Fig. 6A). The opposite is also true (Fig. 6B). Adaptation to either PPNDS or β,γ-methylene ATP causes loss of external [$^{32}$P]ATP binding (Fig. 7A), which is reversible by a process we call 'de-adaptation' (Fig. 7B). Therefore, prolonged (15 min) exposure to 100 μmol l$^{-1}$ PPNDS causes a loss of responsiveness to β,γ-methylene ATP because of cross-adaptation of these responses. This is similar to the cross-adaptation we have seen between ATP and β,γ-methylene ATP in both *Paramecium* (C. R. Wood and T. M. Hennessey, unpublished observations) and *Tetrahymena* (Kim et al., 1999). This adaptation was specific for ATP and PPNDS responses because we did not see any cross-adaptation between PPNDS and GTP responses or β,γ-methylene ATP and GTP responses in either *Tetrahymena* (Kim et al., 1999) or the present study.

*Paramecium* that were adapted to either PPNDS or β,γ-methylene ATP were fully responsive to the other chemorepellents GTP and lysozyme as well as to ionic stimuli (data not shown). This is consistent with our previous hypothesis that there are at least three separate chemorepellent pathways in *Paramecium* and each pathway shows independent adaptation without affecting the sensitivities of the other two pathways. We further proposed (Kim et al., 1997; Kuruvilla et al., 1997) that behavioral cross-adaptation is a convenient way to test whether different ligands may activate the same receptor (or receptor pathway).

If two ligands activate the same receptor by binding to the same site, they should also compete for binding. We have shown that a 50-fold excess of either β,γ-methylene ATP or PPNDS can virtually eliminate all external [$^{32}$P]ATP binding in our *in vivo* assay (Fig. 8) but a 50-fold excess of GTP will not. These results support the hypothesis that both PPNDS and β,γ-methylene ATP are agonists that share a common binding site on the ATP receptor of *Paramecium* but GTP does not. The question of whether PPNDS is a true competitive inhibitor for ATP binding to the ATP receptor will be best approached when the ATP receptor of *Paramecium* is purified for *in vitro* binding assays.

Preliminary observations in low $Ca^{2+}$ solutions suggest the $EC_{50}$ for ATP to be approximately 200 μmol $l^{-1}$ (S. Zdep, personal communication), which is much higher than the measured $K_d$ value for [$^{32}$P]ATP. This discrepancy may be due to the fact that $Ca^{2+}$ is included in the behavioral assays (because it is necessary to show AR) but not in the binding assays. $Ca^{2+}$ can activate the $Ca^{2+}$-dependent ecto-ATPase and raise the $EC_{50}$ because of ATP hydrolysis (Hennessey et al., 1997). For this reason, $Ca^{2+}$ is omitted from the binding assays to inhibit this ecto-ATPase (which has no known inhibitor in *Paramecium*). However, such a large discrepancy between the $EC_{50}$ and $K_d$ values for ATP suggests that the binding data may not always fit quantitatively with the behavioral data for other reasons that we have yet to resolve.

We conclude that PPNDS is an effective ATP receptor agonist (activator) in *Paramecium* and it is not an antagonist in this system. PPNDS causes the same depolarizing receptor potentials and avoiding reactions as the well-known ATP receptor agonist β,γ-methylene ATP, and cross-adaptation is seen between these two agonists. The *in vivo* [$^{32}$P]ATP binding results are consistent with the hypothesis that PPNDS and β,γ-methylene ATP both compete for the same ATP binding site on the ATP receptor.

As the literature describes PPNDS as an antagonist in vertebrate cells, it is possible that PPNDS only has agonist activity in *Paramecium* because of a difference in the structure of the ciliate ATP receptor. Considering the long evolutionary distance between the ciliates and vertebrates, such a difference in agonist specificity cannot be ruled out. If so, this ciliate ATP receptor may represent a new classification of ATP receptors. This class would be easily recognized by the fact that PPNDS acts as an agonist instead of an antagonist. However, it is possible that PPNDS may also be an agonist in other systems where preincubation with 100 μmol $l^{-1}$ PPNDS for ≥15 min is necessary for inhibition of an ATP-dependent response. In some of these cases, it is possible that the loss of ATP responses seen after prolonged exposure to PPNDS may be due to the same agonist-induced adaptation (or 'desensitization') that might be caused by any agonist for this receptor (such as β,γ-methylene ATP) rather than by the action of a true antagonist.

The distinction between ATP receptor antagonism and agonist-induced adaptation is not a trivial matter when considering the mechanism of action of new, experimental drugs. An example is the type of vertebrate ATP receptor that is a specific type of pain receptor (Cook et al., 1997). For these receptors, ATP released from lysed cells causes pain by activation of ATP receptors on specialized nerve endings. This type of preparation may prove valuable for screening drugs that may act as local analgesics by blocking the ATP receptors. However, it would be important to know that the drug does not cause the pain that you want to prevent. Although sensory adaptation could eventually result in analgesia by prolonged exposure to an agonist, the action of a true antagonist might be preferred.

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