Identification of a P2X7 Receptor in GH_4C_1 Rat Pituitary Cells: A Potential Target for a Bioactive Substance Produced by *Pfiesteria piscicida*

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We examined the pharmacologic activity of a putative toxin (pPfTx) produced by *Pfiesteria piscicida* by characterizing the signaling pathways that induce the c-*fos* luciferase construct in GH_4C_1 rat pituitary cells. Adenosine-5´-triphosphate (ATP) was determined to increase and, at higher concentrations, decrease luciferase activity in GH_4C_1 rat pituitary cells that stably express c-*fos* luciferase. The inhibition of luciferase results from cytotoxicity, characteristic of the putative *P. piscicida* toxin (pPfTx). The actions of both pPfTx and ATP to induce c-*fos* luciferase were inhibited by the purinogenic receptor antagonist pyridoxalphosphate-6-azophenyl-2´,4´-disulfonic acid (PPADS). Further characterization of a P2X receptor on the GH_4C_1 cell was determined by the analog selectivity of P2X agonists. The P2X1/P2X3 agonist α,β-methylene ATP (α,β-MeATP) failed to increase or decrease c-*fos* luciferase. However, the P2X7 agonist 2´,3´-(4-benzoyl)benzoyl ATP (BzATP), which had a predominant cytotoxic effect, was more potent than ATP. Immunoblot analysis of GH_4C_1 cell membranes confirmed the presence of a 70-kDa protein that was immunoreactive to an antibody directed against the carboxy-terminal domain unique to the P2X7 receptor. The P2X7 irreversible antagonist oxidized-ATP (oxATP) inhibited the action of ATP, BzATP, and pPfTx. These findings indicate that GH_4C_1 cells express purinogenic receptors with selectivity consistent with the P2X7 subtype and that this receptor pathway mediates the induction of the c-*fos* luciferase reporter gene by ATP and the putative *Pfiesteria* toxin. ***Key words:* c-*fos*, GH_4C_1, P2X7, *Pfiesteria*, pituitary, purinergic, toxin. *Environ Health Perspect* 109:457–462(2001). [Online 1 May 2001]**
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A biologic activity isolated from toxic *Pfiesteria piscicida* cultures has been determined to activitate c-*fos* luciferase in GH_4C_1 cells (*1*). *P. piscicida* is a heterotrophic estuarine dinoflagellate discovered in 1991 by Burkholder and co-workers (*2*); it has been implicated as the causative agent of major fish kills and fish disease in the two largest U.S. mainland estuaries (the Albemarle-Pamlico of North Carolina and Chesapeake Bay in Maryland and Virginia) (*3*). *P. piscicida* was first implicated as hazardous to human health following accidental exposure of laboratory workers (*4*). During 1993–1995, environmental exposures were anecdotally reported in North Carolina estuaries (*4,5*), and in 1997 the first clinical evaluations of people shortly after environmental exposure to *P. piscicida* blooms were completed in Maryland (*6*). People who had contact with toxic *P. piscicida* waters or with potential toxic aerosols reported symptoms including dermal lesions and rashes, a burning sensation on contact with water, fatigue, respiratory irritation, diarrhea, severe headaches, and a neurologic syndrome characterized by learning disabilities manifested as short-term memory dysfunction and other cognitive impairment (*4,6*).

GH_4C_1 cells are a rat pituitary cell line that has been used to characterize signaling pathways for a variety of first messengers (*7*). They have also proven useful for the investigation of algal-derived toxins including maitotoxin (*8,9*) and a biologic activity produced by *Pfiesteria piscicida* (*1*). Each also caused an increase in ionic conductances and an elevation of cytosolic free calcium (*9,10*). Downstream events include activation of c-*fos* luciferase and cytotoxicity (*1,11,12*). *In vitro* methods for characterization of algal-derived toxins have relied largely upon functional assays that include receptor-based assays and cell-based toxicity assays (*13*). Cell based assays can be further modified by changing the end point from the mitochondrial indicator for toxicity (MTT dye-based assay) to specific gene induction (*11*). These assays, known as reporter gene assays, use responsive cell lines that stably express reporter gene constructs.

In this study we investigated the signaling pathways that elicit the reporter gene response in GH_4C_1 cells using adenosine-5´-triphosphate (ATP) as a model compound. We identified ATP as a novel first messenger for GH_4C_1 cells that induces c-*fos* luciferase and cytotoxicity. Using a reporter gene assay, we conducted initial characterization of the ATP receptor through analog specificity studies using P2X receptor agonists and antagonists with differing receptor subtype selectivity. We then used two classes of P2X antagonists to examine whether putative *P. piscicida* toxin (pPfTx) activates a P2X pathway in these cells.

Materials and Methods

Stock cultures of stably transfected rat pituitary cells (GH_4C_1) were maintained in Ham's F10 medium supplemented with 15% horse serum, 2.5% fetal bovine serum (FBS), and 200 µg/mL neomycin antibiotic (G418; Gibco Life Technologies, Grand Island, NY). Cultures were incubated at 37°C with 5% CO_2 and 95% air. GH_4C_1 stable transfectants were obtained by cotransfecting plasmids c-*fos*-luc and pSV2-neo (Richard N. Day, University of Virginia, Charlottesville, VA), as previously described (*1*). We purchased rabbit anti-rat P2X7 receptor antibody from Alomone Labs, Ltd., (Jerusalem, Israel).

Toxin isolation. We used an actively growing, fish-killing culture of *P. piscicida* for toxin isolation. Using methods described previously (*14*), we isolated the cultures from a fish kill and toxic *P. piscicida* outbreak in the Neuse Estuary in North Carolina using fish bioassays and water samples taken from the in-progress kill. *P. piscicida* from the fish-killing bioassays was cloned and grown with algal prey under axenic conditions (but with bacterial endosymbionts retained in the *P.*

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piscicida zoospores) (*14,15*). Following Koch's postulates modified for toxic rather than infectious agents, the axenic clonal *P. piscicida* culture (with bacterial endosymbionts) and residual benign algal prey (< 5 cryptomonads/mL) were added to cultures of healthy fish (*n* = 4). Control fish cultures were treated identically, except that they received similar addition of only residual cryptomonad culture without *P. piscicida* (*n* = 4, with each replicate containing three tilapia (*Oreochromis mossambica*). Fish death occurred and was repeated as additional live fish were added to the cultures of *P. piscicida*. In contrast, control fish, which had been maintained identically but with addition of benign algal prey and not *P. piscicida*, remained healthy (*14*).

We identified *P. piscicida* to species at three levels of isolation: from the fish bioassays of water collected from the Neuse fish kill, from the clonal isolate grown with algal prey, and from the subsequent mass-culture with fish. Species identification was completed from analysis of suture-swollen zoospore cells by scanning electron microscopy (*15*). Following standard procedure in the Burkholder/Glasgow laboratory, the species identifications were then cross-confirmed by three independent laboratories. Molecular probe analyses was conducted by D. Oldach [heteroduplex mobility assay to verify both the species identification and uni-dinoflagellate culture status (*16*)] and P. Rublee [fluorescent *in situ* hybridization rDNA probe for *P. piscicida* (*17*)]. Scanning electron microscopy was conducted by K. Steidinger and co-workers (Florida Fish and Wildlife Conservation Commission Florida Marine Research Institute, St. Petersburg, FL) and by H. Marshall and D. Seaborn (Old Dominion University, Norfolk, VA).

We mass-cultured the toxic *P. piscicida* isolate with live tilapia in a biohazard III facility (*14,15*). They were maintained in 15-psu (practical salinity unit) sterile-filtered seawater (water source 8 km off Beaufort, NC, diluted using deionized water), or in 15-psu water made using Instant Ocean salts (Aquarium Systems, Mentor, OH) and deionized water. Toxic samples were taken from cultures that were actively killing tilapia at the time of collection. The toxic seawater medium was passed through a preparative C18 column and flushed with fresh water to remove the excess salts. The toxic material was then eluted from the column with 100% methanol. This methanol elutant was concentrated and passed through a silica gel column using an elutropic scheme of increasing polarity. We screened fractions for cytotoxicity and reporter gene activity using GH_4C_1 cells as previously described (*1*). The greatest *P. piscicida* activity was found to elute in the later, more polar fractions. These fractions were evaporated to dryness and then were placed under high vacuum to remove any remaining organic solvents that would interfere with the bioassays. The dry residue was taken up in standard volumes of methanol as the carrier solvent for further analysis. The active fraction was determined not to contain ATP by difference in chromatographic retention and by lack of ATP activity using an ATP-dependent *in vitro* luciferase assay, described below under "ATP Assay." Because the chemical structure of the bioactive substance has not yet been determined in the absence of a sufficient quantity of toxic culture to enable purification, the bioactive substance is referred to here as putative *P. piscicida* toxin (pPfTx).

Reporter gene assay. GH_4C_1 c-*fos*-luc cells were seeded in a 96-well clear-bottom white plate (Corning Costar, Cambridge, MA) at a density of 30,000 cells/well in 100 mL culture media and allowed to incubate overnight to ensure cell attachment. Cells treated with pPfTx were incubated for 4 hr and those with ATP were incubated for 10 hr. All incubations were performed at 37°C with 5% CO_2 and 95% air. In experiments where oxidized-ATP (oxATP) was used, pretreatment of one group of wells with 400 mM oxATP was initiated 1 hr before cell treatment with increasing concentrations of either ATP, 2´,3´-(4-benzoyl)benzoyl ATP (BzATP) or pPfTx. After incubation, the experimental media was removed and 20 mL cell lysis buffer [1% Triton X-100, 5 mM Tris, 0.4 mM *trans*-1,2-diaminocyclohexane-*N,N,N´,N´*-tetraacetic acid (CDTA), 10% glycerol, pH 7.8, and 1 mM dithiothreitol (DTT)] was added to each well. Lysis was allowed to proceed at room temperature for 20 min; we then measured solubilized luciferase protein activity using a luminometer (LumiStar; BMG LabTechnologies, Durham, NC). The luminometer was programmed to inject each well with 20 µL of Luciferase Assay Reagent (Promega, Madison, WI), and read the luminescence generated for 10 sec.

Immunostaining. We performed immunostaining for P2X7 receptors using cell homogenates of GH_4C_1 cells on Western transfers. GH_4C_1 cells were removed from 100-mm dishes with PBS-EDTA and resuspended in PBS containing protease inhibitors (4 µM phenylmethylsulfonyl fluoride and 2 µg/mL each of pepstatin, leupeptin, trypsin inihibitor, and aprotinin). Cells were lysed by freeze-thawing and then sonicated at 50 W, three pulses of 20 sec on ice. The lysates were centrifuged at 10,000 × *g* for 20 min, and supernatants were separated by 7.5% SDS-polyacrylamide gel electrophoresis. Separated proteins were then transferred to nitrocellulose and incubated with 1:200 rabbit anti-rat P2X7 antibody

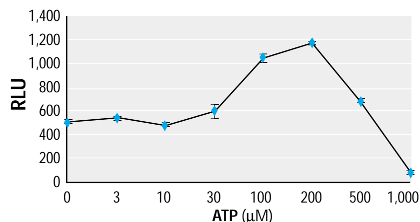

Figure 1. Induction of c-*fos* luciferase in GH_4C_1 cells treated with increasing concentrations of ATP. RLU, relative light units. See "Materials and Methods" for details. Each point represents the mean ± SE of three wells for an experiment repeated three times with similar results.

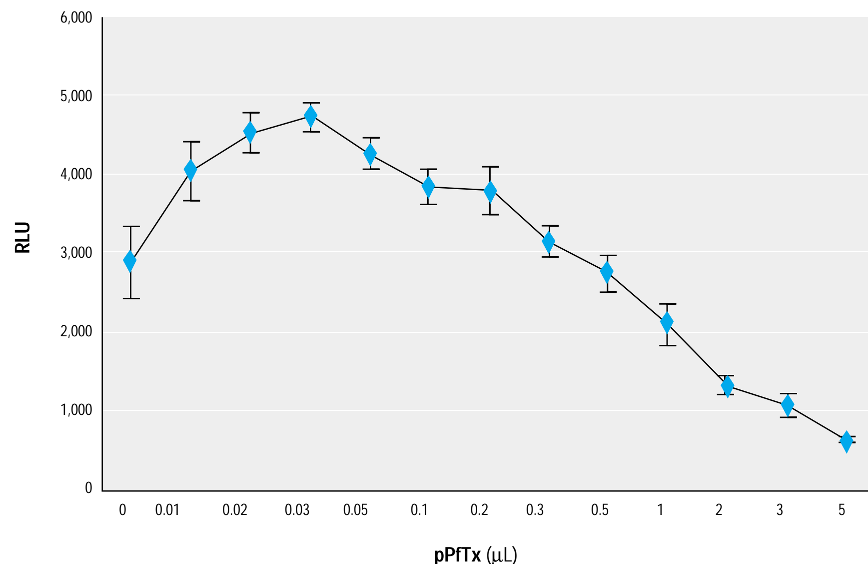

Figure 2. Induction of c-*fos* luciferase in GH_4C_1 cells treated with increasing concentrations of pPfTx. See "Materials and Methods" for details. RLU, relative light units. Each point represents the mean ± SE of three wells for an experiment repeated twice with similar results.

(*18*). Transfers were washed in TBS 0.1% Triton-X100 between antibody incubations. The detection was electrogenerated chemiluminscence according to the manufacturer (Amersham, Buckinghamshire, UK) for 5 min. The transfers were then exposed to Hyperfilm-ECL (Sigma, St. Louis, MO) for 60 sec and developed with an X-ray processor. The corresponding blocking peptide (P2X7 576–595 peptide) at 10 µg/mL was incubated with the same antibody solution for 1 hr at 23°C. Transfers were then probed and developed as described above.

ATP assay. We used the ATP Bioluminescent Assay Kit (Sigma, St. Louis, MO) to determine the amount of ATP present in pPfTx samples. The assay kit contained ATP Standard (2.0 µmol ATP), ATP Assay Mix Dilution Buffer ($MgSO_4$, DTT, EDTA, bovine serum albumin, tricine buffer salts), and ATP Assay Mix (luciferase, luciferin, $MgSO_4$, DTT, EDTA, bovine serum albumin, tricine buffer salts). We made serial dilutions from the ATP Standard after it had been diluted to a concentration of 40 µM in double-distilled H_2O. We plated 40 µL of each serial dilution in triplicate in a 96-well plate. pPfTx was plated in triplicate alongside the ATP Standard. We added 40 µL ATP Assay Mix diluted 1:25 with ATP Assay Dilution Buffer to each well; the luminescence was generated by the catalyzing activity and measured by a luminometer.

Results

Effect of ATP and pPfTx on c-fos luciferase. We examined ATP for its ability to mimic the action of pPfTx in GH_4C_1 cells. A biphasic luciferase response was generated from the induction of the GH_4C_1 cells with increasing concentrations of ATP (Figure 1). We observed a half-maximal effect at 30 mM, with a maximal effect occurring at 200 mM. Concentrations of ATP that exceeded 200 mM caused a concentration-dependent inhibition of c-*fos* luciferase. This decrease is associated with cytotoxicity as determined by MTT cytotoxicity assay. A similar biphasic c-*fos* luciferase response was generated by the addition of serial dilutions of pPfTx to GH_4C_1 cells (Figure 2). These results indicate that pPfTx mimics the action of ATP to induce c-*fos* luciferase and cytoxtoxicity in GH_4C_1 cells and lead us to conduct preliminary characterization of the ATP receptor on GH_4C_1 cells.

Analog characterization of the ATP receptor in GH_4C_1 cells. The preliminary characterization of the ATP receptor was determined by conducting analog selectivity studies. The primary analogs tested that were effective in this study are shown in Figure 3. We first tested the moderately selective P2 antagonist, pyridoxalphosphate-6-azophenyl-2´,4´-disulfonic acid (PPADS). PPADS caused concentration-dependent inhibition of c-*fos* luciferase in the presence and absence of added ATP (Figure 4). The inhibition of c-*fos* luciferase by PPADS was not associated with cytotoxicity. We next examined the P2X1 and P2X3 subtype selective agonist α,β-methyleneadenosine 5´-triphosphate (α,β-MeATP). α,β-MeATP failed to increase or decrease c-*fos* luciferase activity (Figure 5). Taken together, these results indicate that if P2X receptors mediate the effects of ATP on c-*fos* luciferase in GH_4C_1 cells, the receptor is not of the P2X1 or P2X3 subtype.

We next examined a second antagonist, oxATP, which is an irreversible P2X antagonist with moderate selectivity for P2X7 receptors. Pretreatment with 400 µM oxATP inhibited the majority of the effect of ATP to increase c-*fos* luciferase, and fully inhibited the effects of ATP to decrease luciferase (Figure 6). oxATP, unlike PPADS, did not decrease c-*fos* luciferase activity. We next tested the action of an agonist, BzATP, that shows selectivity for P2X7 receptors. BzATP did not increase c-*fos* luciferase activity, but it caused concentration-dependent inhibition of c-*fos* luciferase activity (Figure 7). The half-maximal effect of BzATP was nearly 10 times lower than the half-maximal effect of ATP to inhibit c-*fos* luciferase. This is consistent with an action on P2X7 subtype purinoreceptors. The failure of BzATP to induce c-*fos* luciferase was unexpected. It is possible that

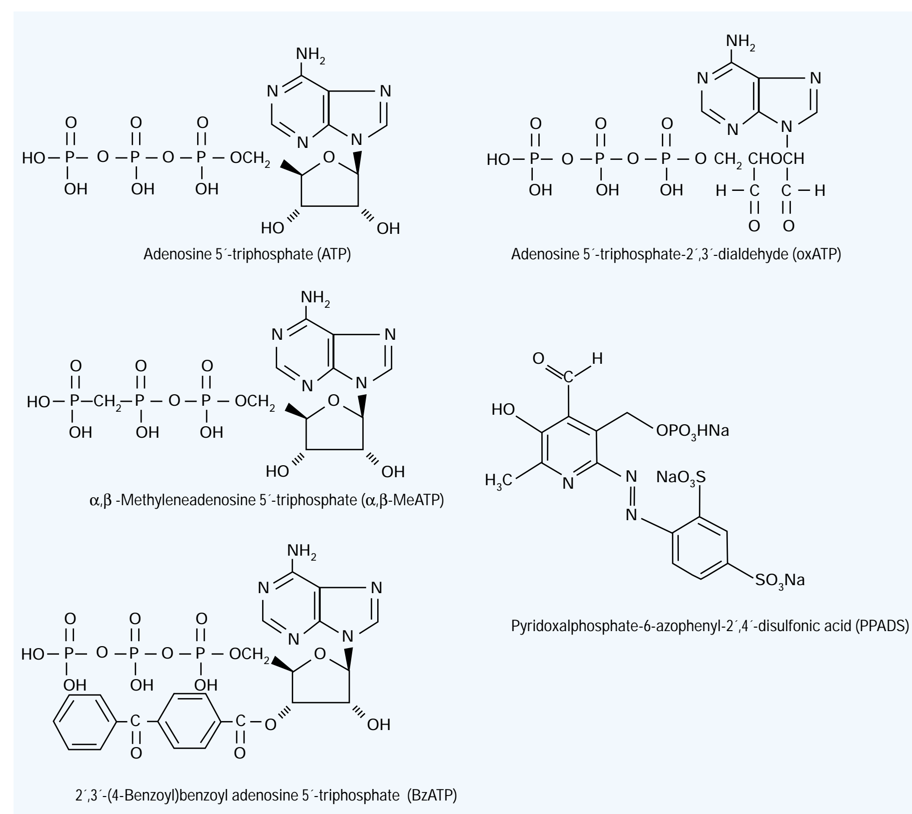

Figure 3. Structures for ATP and selected analogs.

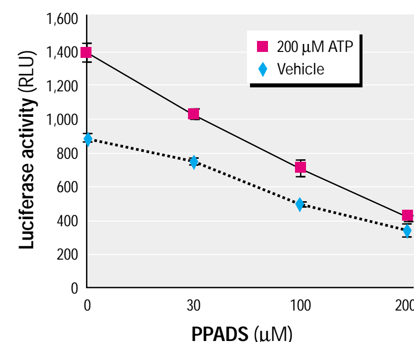

Figure 4. PPADS inhibition of ATP induction of c-*fos* luciferase in GH_4C_1 cells of PPADS. RLU, relative light units. See "Materials and Methods" for details. Each point represents the mean ± SE of three wells for an experiment repeated once with similar results.

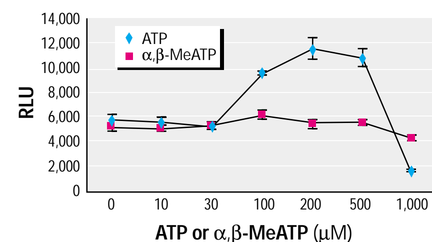

Figure 5. Effect of ATP and α,β-MeATP on c-*fos* luciferase in GH_4C_1 cells. RLU, relative light units. See "Materials and Methods" for details. Each point represents the mean ± SE of three wells for an experiment repeated once with similar results.

BzATP only affected the second component of the biphasic response (i.e., cytotoxicty but not induction of the reporter gene). An alternative possibility is that BzATP induces both responses but has greater efficacy for cytotoxicity. We addressed this question by testing BzATP at the shorter incubation period of 4 hr and found that BzATP did cause a biphasic response (data not shown). We also examined the effect of oxATP on the action of BzATP. Pretreatment of 400 mM oxATP fully inhibited the effect of BzATP to decrease luciferase (Figure 7), which is consistent with an effect mediated by purinogenic receptors of the P2X7 class.

Identification of the P2X7 receptor by immunoblotting. We examined the presence of the P2X7 receptor by immunostaining Western transfers of GH_4C_1 cell membranes. GH_4C_1 cells expressed an approximate 70-kDa band that was immunoreactive to a rabbit antibody directed to the intracellular carboxyl terminal domain, unique to the P2X7 class of purinergic receptors (Figure 8). We examined the specificity of the staining of the 70-kDa band by preabsorption of the primary antiserum with 10 µg/mL of the carboxyl terminal peptide sequence 575–595. No immunostaining of the 70-kDa band was evident under matched conditions (data not shown). This result provides an additional line of evidence for the presence of P2X7 receptors on GH_4C_1 cells.

Testing pPfTx for ATP activity. We examined the role of P2X7 receptors in the action of the pPfTx. We sought to determine whether the pPfTx contained any activity attributable to ATP. We used an ATP-dependent luciferase assay to quantify ATP. The sensitivity of the assay was 40 nM; 4 µM ATP generated a 100-fold increase in response (Figure 9). pPfTx, given in an amount that caused a maximal induction of c-*fos* luciferase in the reporter gene assay, failed to mimic any effect of ATP to activate the luciferase enzyme directly. The pPfTx contained < 40 nM ATP, but by the reporter gene assay, it contained 200 µM ATP equivalents, indicating that the effect of pPfTx in the reporter gene assay is not attributable to ATP.

Effect of P2X antagonists on pPfTx induction of c-fos luciferase. We used two P2 antagonists of differing selectivity to examine the role of P2X receptors in the action of pPfTx to induce c-*fos* luciferase and cytotoxicity. PPADS, a P2 agonist, given at 200 µM inhibited both the activity of pPfTx and ATP (Figure 10). oxATP, an irreversible P2X antagonist that has selectivity for P2X7 receptors, was added at a concentration of 400 µM as a pretreatment 1 hr before the addition of increasing concentrations of both ATP and pPfTx (Figure 11). oxATP inhibited the luciferase induction of both ATP and pPfTx, suggesting that both substances induce c-*fos* luciferase and cytotoxicity by a common mechanism involving a P2X7 subtype receptor.

Discussion

ATP has a dual role as both an energy source for enzymatic reactions and as a first messenger for several classes of G protein-coupled receptors and ligand-gated ion channels. ATP was found to induce c-*fos* luciferase in GH_4C_1 cells with a characteristic biphasic response. pPfTx also induced c-*fos* luciferase in a similar manner. These results suggest that the pPfTx is either ATP or an ATP agonist. We examined whether pPfTx was ATP using two lines of evidence. The first was that ATP and pPfTx do not share common

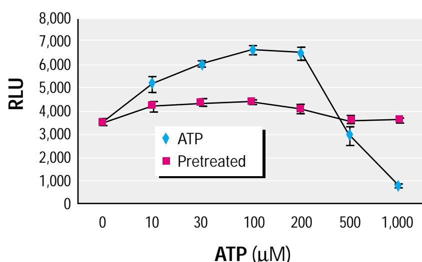

Figure 6. oxATP inhibition of ATP induction of c-*fos* luciferase in GH_4C_1 cells pretreated with 400 µM oxATP for 1 hr. RLU, relative light units. See "Materials and Methods" for details. Each point represents the mean ± SE of three wells for a representative experiment repeated once with similar results.

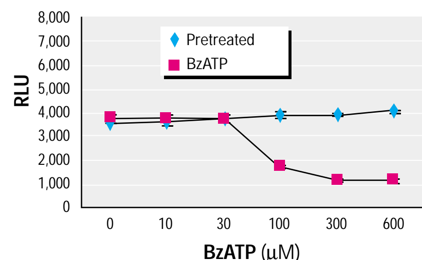

Figure 7. BzATP inhibition of c-*fos* luciferase and antagonism by oxATP in GH_4C_1 cells pretreated either in the absence or presence of 400 µM oxATP for 1 hr, followed by treatment with increasing concentrations of BzATP. RLU, relative light units. See "Materials and Methods" for details. Each point represents the mean ± SE of 3 wells for a representative experiment, repeated once with similar results.

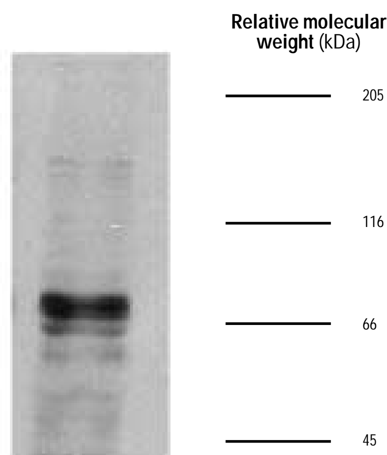

Figure 8. Western blot analysis of P2X7 receptor in GH_4C_1 cells. See "Materials and Methods" for details. Preabsorption of the antiserum with the 575–595 peptide of the P2X7 receptor prevented immunostaining of the 70 kDa band (not shown). This experiment was repeated three times with similar results.

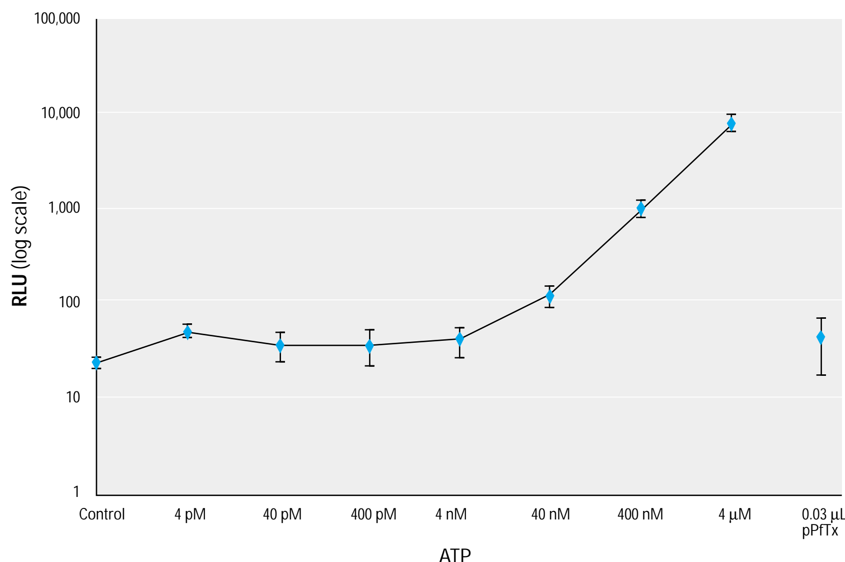

Figure 9. Inability of pPfTx to catalyze isolated luciferase enzyme in the presence of cofactors. The catalyzing activity of pPfTx was compared to the ATP standard curve. RLU, relative light units. See "Materials and Methods" for details. Each point represents the mean ± SE of three wells for an experiment repeated once with similar results.

chromatographic retention properties (data not shown). The second was that pPfTx could not mimic the action of ATP to catalyze isolated luciferase enzyme in the presence of cofactors. Taken together, these results indicate that pPfTx is not ATP, but rather an ATP agonist.

ATP activates receptors of the purinogenic P2 class (*19*). P2 receptors are divided into two classes—P2X and P2Y—based on molecular structure. P2X receptors are ATP-activated ion channels, and P2Y receptors are ATP-activated G protein-coupled receptors. We began by examining analog selectivity for induction of c-*fos* luciferase by ATP using P2 agonists and antagonists of differing selectivity. PPADS, a P2 antagonist of moderate selectivity for P2X receptors (*19*), caused concentration-dependent inhibition of ATP induction of c-*fos* luciferase in GH_4C_1 cells with a half-maximal effect (100 μM), which is consistent with an action on P2 receptors. Further examination with α,β-methylene ATP (α,β-MeATP), both a P2X1- and P2X3-selective ATP agonist (*19,20*), indicated that α,β-MeATP did not affect c-*fos* luciferase, providing additional evidence that the pathway of activation was neither a P2X1 or P2X3 receptor. A second antagonist, oxATP, which is irreversible and more selective for P2X receptors (*21*), inhibited nearly fully the actions of ATP on c-*fos* luciferase in GH_4C_1 cells. Because oxATP has been reported to have some degree of selectivity for P2X7 receptors, we tested an agonist, BzATP, which has selectivity for P2X7 receptors (*21–23*). BzATP failed to increase c-*fos* luciferase in GH_4C_1 cells, but it did cause a concentration-dependent inhibition of luciferase activity. BzATP was nearly 10 times more potent at inhibiting c-*fos* luciferase than ATP. BzATP induces greater maximal ion conductance than ATP in cells expressing P2X7 receptors; this may be the basis for the greater efficacy of BzATP for cytotoxicity (*21*). The action of BzATP was fully inhibited by oxATP. Taken together, these analog selectivity experiments with BzATP and oxATP are consistent with the presence of P2X7 receptors on GH_4C_1 cells. The presence of P2X7 receptors on GH_4C_1 cells was additionally supported by the immunostaining of Western transfers of GH_4C_1 cell membranes using an antibody specific to the unique carboxy terminal domain of the P2X7 receptor.

ATP-activated ion channel receptors were originally identified in mast cells (*24*) and later found in other myeloid-derived cells, including macrophages and microglia (*25,26*). These channels, designated P2Z, were subsequently found to be of the P2X class and renamed P2X7 (*27*). Although the P2X7 receptor subtype is found largely in cells of immune origin, it has also been identified in cell lines and primary cell cultures of nonimmune origin (*18,28,29*). The role for a P2X7 receptor in GH_4C_1 cells has not been determined; however, it is well known that this cell lineage (growth hormone-producing cells) mediates local inflammatory responses in the pituitary gland and may modulate the hypothalamic pituitary axis during systemic inflammatory reactions (*30*).

The antagonists PPADS and oxATP, which we used to characterize the P2X receptor in GH_4C_1 cells, were also useful in examining the action of pPfTx. The pathway(s) leading to the biphasic effect on c-*fos* luciferase of pPfTx in GH_4C_1 cells appears to be mediated by the same receptor that mediates the response to ATP. Both PPADS and oxATP inhibited pPfTx induction of c-*fos* luciferase. These results are consistent with pPfTx acting as a P2X7 receptor agonist. Although these results do not prove that P2X7 is the initial cellular target for the putative toxin, they do indicate that this receptor is necessary in the signal transduction pathway. At this point it is not possible to exclude effects of pPfTx on additional receptors, including other P2X receptor subtypes. This may be most readily determined using expression systems for various cloned receptors.

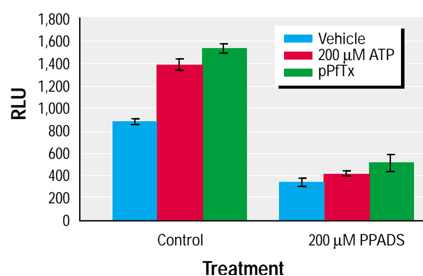

Figure 10. PPADS inhibition of pPfTx induction of c-*fos* luciferase in GH_4C_1 cells treated with ATP or pPfTx. RLU, relative light units. See "Materials and Methods" for details. The ATP and pPfTx treatments differed (Tukey multiple comparison test, $p < 0.05$) from the vehicle [Milli-Q-treated water (Millipore, Bedford, MA), final concentration 2.5%] for the control group but not for the PPADS group. Each point represents the mean ± SE of three wells for an experiment repeated once with similar results.

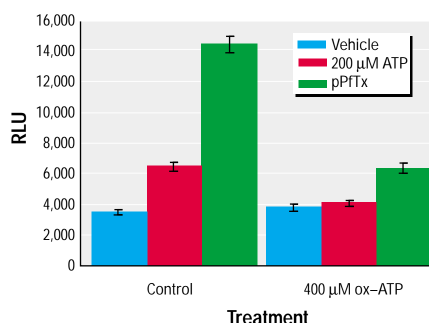

Figure 11. oxATP and PPADS inhibition of ATP and pPfTx induction of c-*fos* luciferase in GH_4C_1 cells pretreated with 400 mM oxATP for 1 hr then treated with increasing concentrations of ATP and pPfTx. RLU, relative light units. See "Materials and Methods" for details. The oxATP treatment did not differ from the vehicle [Milli-Q-treated water (Millipore, Bedford, MA), final concentration 2.5%] but did cause a significant (Tukey multiple comparison test, $p < 0.05$) difference in pPfTx induction of c-*fos* luciferase. Each point represents the mean ± SE of three wells for an experiment repeated once with similar results.

It remains to be determined whether the P2X7 agonist activity isolated from *P. piscicida* is responsible for the wildlife effects associated with this organism. Macrophages and mast cells express P2X7 receptors, which have been suggested to have a role in inflammation. The entry of monocytes into peripherial tissues precedes their differentiation into activated macrophages, a process that involves the action of interferon-γ, which in turn leads to expression of P2X7 receptors (*31*). In activated macrophages, P2X7 receptors mediate chronic inflammatory responses normally driven by ATP. The responses include fusion of macrophages into multinucleated giant cells and several inflammatory responses that result from production of interleukin 1-β, including release of prostaglandins, production of matrix, and chemoattraction of neutophils (*32–34*). These responses are characteristic of granulatomatous lesions found in fish that are associated with toxic *P. piscicida* (*9,35*). Because *P. piscicida* has the capacity to phagocytize blood cells and cause tissue injury (*2*), it may initiate an acute inflammation that is potentiated to a chronic response by pPfTx, behaving as a potent ATP mimic at P2X7 receptors on activated macrophages.

Whether the P2X7 agonist activity isolated from *P. piscicida* contributes to the human neurocognitive effects associated with this organism is less obvious. P2X7 receptors in the central nervous system have been best characterized in microglia (*31,36*). Microglia are the central nervous system counterpart to tissue macrophages and they normally provide a defensive inflammatory response to infections and tissue damage (*37*). However, inappropriate activation of microglia can elicit neurotoxic effects that may include release of excitotoxic amino acids and cytolytic and inflammatory agents (*37*). One approach used to study the effects of *Pfiesteria* on neurocognitive impairment is a rat model using radial arm-maze testing (*38*).

Our results indicate that the cytotoxic effect originally described for a putative *P. piscicida* toxin is mediated by a P2X7 receptor. Based on the current understanding of the role of P2X7 receptors in disease and the observed effects directly attributable to exposure to *P. piscicida* toxins, P2X7 receptor-mediated

chronic inflammation may provide a basis to better understand the animal and human toxicity associated with this organism.

REFERENCES AND NOTES

1. Fairey ER, Edmunds JSG, Deamer-Melia NJ, Glasgow H Jr, Johnson FM, Moeller PR, Burkholder JM, Ramsdell JS. Reporter gene assay for fish-killing activity produced by *Pfiesteria piscicida*. Environ Health Perspect 107:711–714 (1999).
2. Burkholder TJ, Noga EJ, Hobbs CW, Glasgow HB, Smith SA. New "phantom" dinoflagellate is the causative agent of major estuarine fish kills. Nature 358:407–410 (1992).
3. Burkholder JM, Glasgow HB Jr. *P. piscicida* and *P. piscicida*-like dinoflagelletes: behavioral impacts and environmental controls. Limnol Oceanogr 42:1052–1075 (1997).
4. Glasgow HB, Burkholder JM, Schemechel DE, Tester PA, Rublee PA. Insidious effects of a toxic estuarine dinoflagellate on fish survival and human health. J Toxicol Environ Health 46:501–522 (1995).
5. Burkholder JM. Implications of harmful marine microalgae and heterotrophic dinoflagellates in management of sustainable marine fisheries. Ecol Appl Suppl 8:S37–S62 (1998).
6. Grattan LM, Oldach D, Perl TM, Lowitt MH, Matuszak DL, Dickson C, Parrot C, Shoemaker RC, Kauffman CL, Wasserman MP, et al. Learning and memory difficulties after environmental exposure to waterways containing toxin-producing *P. piscicida* or *P. piscicida*-like dinoflagellates. Lancet 352:532–539 (1998).
7. Tashjian AHJ. Clonal strains of hormone-producing pituitary cells. Methods Enzymol 58:527–535 (1979).
8. Xi D, Van Dolah FM, Ramsdell JS. Maitotoxin activates type L-voltage dependent calcium channels and induces a calcium-dependent membrane depolarization in GH_4C_1 pituitary cells. J Biol Chem 267:25025–25031 (1992).
9. Xi D, Kurtz DR, Ramsdell JS. Maitotoxin induces calcium entry by nimodipine sensitive and insensitive pathways in GH_4C_1 pituitary cells. Biochem Pharmacol 166:49–56 (1996).
10. Melo AC, Ramsdell JS. Unpublished data.
11. Fairey, ER, Ramsdell JS. Reporter gene assays for algal-derived toxins. Nat Toxins 7:415–421 (1999).
12. Van Dolah FM, Ramsdell JS. Maitotoxin, a calcium channel activator, inhibits cell cycle progression through the G1/S and G2/M transitions and prevents CDC2 kinase activation in GH_4C_1 cells. J Cell Physiol 2:189–196 (1994) .
13. Van Dolah FM, Ramsdell JS. *In vitro* detection methods for algal toxins: Conceptual approaches and recent developments. JAOAC Int (in press).
14. Burkholder JM, Glasgow HBJ. Trophic controls on stage transformation of a toxic ambush-predator dinoflagellate. J Eukaryot Microbiol 44:200–205 (1997).
15. Glasgow HB Jr. The Biology and Impacts of Toxic *P. piscicida* Complex Species [PhD Thesis]. Raleigh, NC:North Carolina State University, 2000.
16. Oldach DW, Delwiche CF, Jakobsen KS, Tengs T, Brown EG, Kempton JW, Schaefer EF, Bower H, Glasgow HB Jr, Burkholder JM, et al. Heteroduplex mobility assay guided sequence discovery: elucidation of the small subunit (18S) rDNA sequence of *Pfiesteria piscicida* from complex algal cluture and environmental sample DNA pools. Proc Natl Acad Sci USA 97:4304–4308 (2000).
17. Rublee PA, Kempton J, Schaefer E, Burkholder JM, Glasgow HB Jr, Oldach D. PCR and FISH detection extends the range of *Pfiesteria piscicida* in estuarine waters. VA J Sci 50:325–336 (1999).
18. Collo G, Neidhart S, Kwaashima E, Kosco-Vilbois M, North RA, Buell G. Tissue distribution of the P2X7 receptor. Neuropharmacology 36:1277–1283 (1997).
19. Burnstock G. P2 purinoceptors: historical perspective and classification. Ciba Found Symp 198:1–28 (1996).
20. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 50:413–478 (1998).
21. MacKenzie AB, Surprenant A, North AR. Functional and molecular diversity of purinergic ion channel receptors. Ann NY Acad Sci 868:716–729. (1999).
22. Murgia M, Hanau S, Pizzo P, Rippa M, Di Virgilio F. Oxidized ATP: an irreversible inhibitor of the macrophage purinergic P2Z receptor. J Biol Chem 268:8199–8203 (1993).
23. Michel AD, Chessel IP, Humphrey PPA. Ionic effects on human recombinant P2X7 receptor function. Naunyn-Schmiedeberg's Arch Pharmacol 359:102–109 (1999).
24. Cockcroft S, Gomberts BD. ATP induces nucleotide permeability in mast cells. Nature 279:541–542 (1979).
25. Ferrari D, Villalba M, Chiozzi P, Falzoni S, Ricciardi Castagnoli P, Di Virgilio F. Mouse microglial cell express a plasma membrane pore gated by extracellular ATP. J Immunol 156:1531–1539 (1996).
26. Steinberg TH, Newman AS, Swanson JA, Silverstein SC. ATP4-permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. J Biol Chem 262:8884–8888 (1987).
27. Surprenant A, Rassendren F, Kawashima E, North AR, Buell G. The cytolitic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). Science 272:735–738 (1996).
28. Michel AD, Chessell IP, Hibell AD, Simon J, Humphrey PPA. Identification and characterization of an endogenous P2X7 (P2Z) receptor in CHO-K1 cells. Br J Pharmacol 125:1194–1202 (1998).
29. Tenneti L, Bibbons SJ, Talamo BR. Expression and transsynaptic regulation of P2X4 and P2Z receptors for extracellular ATP in parotid acinar cels. J Biol Chem 273:26799–26808 (1998).
30. Ray D, Melmed S. Pituitary cytokine and growth factor expression and action. Endocr Rev 18:206–228 (1997).
31. Humphreys BD, Dubyak GR. Induction of the P2z/P2X7 nucleotide receptor and associated phospholipase D activity by lipopolysaccharide and IFN-gamma in the human THP-1 monocytic cell line. J Immunol 157(12):5627–5637 (1996).
32. Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Melchiorri L, Baricordi OR, Di Virgilio F. Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages. J Immunol 159(3):1451–1458 (1997).
33. Falzoni S, Munerati M, Ferrari D, Spisani S, Moretti S, Di Virgilio F. The purinergic P2Z receptor of human macrophage cells. Characterization and possible physiological role. J Clin Invest 95(3):1207–1216 (1995).
34. Di Virgilio F. The P2Z purinoreceptor: an intriguing role in immunity, inflammation and cell death. Immunol Today 16:524–528.
35. Noga EJ, Khoo L, Stevens JB, Fan Z, Burkholder JM. Novel toxic dinoflagellete causes epidemic disease in esturarine fish. Mar Pollut Bull 32:219–224 (1996).
36. Di Virgilio F, Sanz JM, Chiozzi P, Falzoni S. The P2Z/P2X7 receptor of microglial cells: a novel immunomodulatory receptor. Prog Brain Res 120:355–368 (1999).
37. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19:312–318 (1996).
38. Levin ED, Schmechel DE, Burkholder JM, Glasgow HB Jr, Deamer-Melia NJ, Moser VC, Harry GJ. Persistent learning deficits in rats after exposure to *Pfiesteria piscicida*. Environ Health Perspect 105:1320–1325 (1997).