Identification of a P2X7 Receptor in GH4C1 Rat Pituitary Cells: A Potential Target for a Bioactive Substance Produced by Pfiesteria piscicida

Karen L. Kimm-Brinson,1 Peter D. R. Moeller,1 Michèle Barbier,1 Howard Glasgow, Jr,2 JoAnn M. Burkholder,2 and John S. Ramsdell1

1Marine Biotoxins Program, Center for Coastal Environmental Health and Biomolecular Research, National Oceanic and Atmospheric Administration-National Ocean Service, Charleston, South Carolina, USA; 2Department of Botany, North Carolina State University, Raleigh, North Carolina, USA

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 GH4C1 rat pituitary cells. Adenosine-5'-triphosphate (ATP) was determined to increase and, at higher concentrations, decrease luciferase activity in GH4C1 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 purinergic receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS). Further characterization of a P2X receptor on the GH4C1 cell was determined by the analog selectivity of P2X agonists. The P2X1/P2X3 agonist c,β-methylene ATP (c,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 GH4C1 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 GH4C1 cells express purinergic 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, GH4C1, 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 activate c-fos luciferase in GH4C1 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 anecdotal 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).

GH4C1 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 (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 GH4C1 cells using adenosine-5'-triphosphate (ATP) as a model compound. We identified ATP as a novel first messenger for GH4C1 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 (GH4C1) 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% CO2 and 95% air. GH4C1 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 N.W. Estuary in North Carolina using fish biosaas and water samples taken from the in-progress kill. P. piscicida from the fish-killing biosaas was cloned and grown with algal prey under axenic conditions (but with bacterial endosymbionts retained in the P.
piscida 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 species identification and uni-dinoflagellate (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 (water source 8 km off Beaufort, NC, 0.4 mM trans-1,2-dilinocyclohexane-N,N,N,N'-tetaacetic 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 Lab Technologies, 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 GH4C1 cells on Western transfers. GH4C1 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 inhibitor, 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.
The transfers were washed in TBS 0.1% Triton-X100 between antibody incubations. The detection was electrogenerated chemiluminescence 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 pPTT x samples. The assay kit contained ATP Standard (2.0 µmol ATP), ATP Assay Mix (luciferase, luciferin, bovine serum albumin, tricine buffer salts), and ATP Assay Mix diluted 1:25 with ATP Assay Buffer. ATP Standard was added 40 µL of dilutions from the ATP Standard after it had been incubated with the same antibody solution for 1 hr at 23°C. Transfers were then probed and developed as described above.

**Results.**

**Effect of ATP and pPTT x on c-fos luciferase.** We examined ATP for its ability to mimic the action of pPTT x in GH4C1 cells. A biphasic luciferase response was generated by the catalyzing activity of ATP (Figure 1). With increasing concentrations of ATP, the inhibition of 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 pPTT x to GH4C1 cells (Figure 2). These results indicate that pPTT x mimics the action of ATP to induce c-fos luciferase and cytotoxicity in GH4C1 cells and lead us to conduct preliminary characterization of the ATP receptor on GH4C1 cells.

**Analog characterization of the ATP receptor in GH4C1 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 αβ -methyleneadenosine 5’-triphosphate (αβ-M eATP). αβ-M eATP 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 GH4C1 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 activity 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 purinoceptors. The failure of BzATP to induce c-fos luciferase was unexpected. It is possible that...
BzATP only affected the second component of the biphasic response (i.e., cytotoxicity 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 purinergic 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 GH4C1 cell membranes. GH4C1 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 GH4C1 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 mM 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 GH4C1 cells with a characteristic biphasic response. pPfTx also induced c-fos luciferase and cytotoxicity by 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
The treatments differed (Tukey multiple comparison test, p < 0.05) from the vehicle (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. BzATP, which has selectivity for P2X7 receptors, was also useful in examining the action of pPfTx. The pathways leading to the biphasic effect on c-fos luciferase of pPfTx in GH4C1 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 pFfTx 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 pFfTx on additional receptors, including other P2X receptor subtypes. This may be most readily determined using expression systems for various cloned receptors.

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 peripheral 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-1β, including release of prostaglandins, production of matrix, and chemotraction of neutrophils (32–34). These responses are characteristic of granulomatous lesions found in fish that are associated with toxic *P. piscicida* (9,35). Because *P. piscicida* has the capacity to phagocyte blood cells and cause tissue injury (2), it may initiate an acute inflammation that is potentiated to a chronic response by pFfTx, 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. Fairer ER, Edmunds JS, Deamer-Melia NJ, Glasgow H Jr, Johnson FM, Meller PR, Burkholder JM, Ramsdell JS. Reporter gene assay for fish-kill activity produced by Pfiesteria piscicida. Environ Health Perspect 107:713–714 (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 dinoflagellates: 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, P. piscicida-like dinoflagellates: implications for sustainable marine fisheries. Ecol Appl Suppl 8:S37–S62 (1998).

6. Oldach DW, Delwiche CF, Kobbsen KS, Tengs T, Brown EG, Kempton JW, Schafer 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 culture and environmental sample DNA pools. Proc Natl Acad Sci USA 97:4304–4308 (2000).

7. Rublee PA, Kempton J, Schafer E, Burkholder JM, Glasgow HB Jr, Oldach D. PCR and FISH detection extends the range of Pfiesteria piscicida in estuarine waters. VA J Sci 50:325–337 (1999).

8. Collo G, Neidhart S, Kwaashima E, Kosco-Vilbis M, North RA, Buell DG. Tissue distribution of the P2X7 receptor: Neuromorphometry. Lab Invest 77:1277–1283 (1997).

9. Burnstock G. P2 purinoceptors: historical perspective and classification. Ciba Found Symp 198:1–28 (1996).

10. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 50:413–478 (1998).

11. Michel AD, Chessel IP, Humphrey PPA. Identification and characterization of an endogenous P2X7 (P2Z) receptor in CHO-K1 cells. J Biol Chem 273:26799–26808 (1998).

12. Di Virgilio F. The P2 purinoceptor: an intriguing role in immunity, inflammation and cell death. Immunol Today 16:524–528 (1995).

13. Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. Tissue distribution of the P2X7 receptor. J Neuroimmunol 156:1531–1539 (1996).

14. 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).

15. 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 P2X7 receptor of mouse macrophages. J Immunol 159:1451–1458 (1997).

16. Oldach DW, Delwiche CF, Kobbsen KS, Tengs T, Brown EG, Kempton JW, Schafer 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 culture and environmental sample DNA pools. Proc Natl Acad Sci USA 97:4304–4308 (2000).

17. Rublee PA, Kempton J, Schafer E, Burkholder JM, Glasgow HB Jr, Oldach D. PCR and FISH detection extends the range of Pfiesteria piscicida in estuarine waters. VA J Sci 50:325–337 (1999).

18. Tashjian AH Jr. Clonal strains of hormone-producing pituitary cells. Methods Enzymol 58:527–535 (1979).

19. Michel AD, Chessel IP, Humphrey PPA. The purinergic P2Z receptor of human microglial cell express oxinduced ATP: an irreversible inhibitor of the macrophage uricase activity. Br J Pharmacol 125:1194–1202 (1998).

20. Tashjian AH 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).

21. 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 P2X7 receptor of mouse macrophages. J Immunol 159:1451–1458 (1997).

22. 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 P2X7 receptor of mouse macrophages. J Immunol 159:1451–1458 (1997).

23. Michel AD, Chessel IP, Humphrey PPA. Identification and characterization of an endogenous P2X7 (P2Z) receptor in CHO-K1 cells. Br J Pharmacol 125:1194–1202 (1998).

24. 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 P2X7 receptor of mouse macrophages. J Immunol 159:1451–1458 (1997).

25. 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 P2X7 receptor of mouse macrophages. J Immunol 159:1451–1458 (1997).

26. 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 P2X7 receptor of mouse macrophages. J Immunol 159:1451–1458 (1997).