Extracellular adenosine 5'-triphosphate (ATP) acting on purinergic (P2) receptors regulates a variety of functions, using transduction pathways that are dependent on the type of the P2-receptor and on the type of cells where the action occurs (1,2). For example, in the nervous system, ATP is co-released with neurotransmitters by exocytosis to modulate the synaptic transmission via the activation of P2X-receptor cation channels (3). Macrophages and mast cells express a different functional type of P2X-receptor that changes its ionic selectivity when exposed to extracellular ATP (4). In this type of receptor, first designated P2Z and later recognized as the assemblage of subunits, each one with two transmembrane domains (12,13), whereas P2Y has the typical structure of a G-protein-coupled receptor with seven membrane-spanning domains (13). Seven types of the P2X class of P2 receptors have been cloned (P2X1-P2X7) and are distinguished pharmacologically in two groups on the basis of sensitivity to the ATP congener 2'-3'-O-(benzoyl-4-benzoyl)-ATP (BzATP) than to ATP itself, and revealed a sensitivity to the ionic environment in the kinetics of cell permeabilization (21). The finding that the replacement of extracellular ionic sodium by the larger ion N-methyl-D-glucamine (NMDG) facilitates the permeabilization mode of the activated receptor, causing a delay or inhibition of cell lysis (5), has been widely used in the functional characterization of the P2X7 (22).

It is well known that toxins synthesized by a variety of organisms can selectively bind to ion conduction channels, altering their functional properties and leading to toxicity. The heterotrophic estuarine dinoflagellate Pfiesteria piscicida Steidinger & Burkholder was discovered in 1991 by Burkholder and co-workers (22) and 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) (23). A second toxic Pfiesteria species has subsequently been identified (among a number of organisms morphologically similar to P. piscicida, termed “Pfiesteria-like” or “Pfiesteria look-alike” organisms) from fish-kill/fish-disease events in aquaculture and coastal areas from Delaware to the Gulf of Mexico (23). The health hazards attributed to this organism (24–26) led to the need to characterize bioactive substances produced by P. piscicida capable of causing adverse effects. Research to fully characterize the chemical structure and biological effects of this putative toxin is a current challenge. P. piscicida produces a bioactive substance that induces cytotoxicity in GH4C1 rat pituitary cells and at nontoxic concentrations inhibits i

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concentrations induces a c-fos luciferase reporter gene (27). The expression of c-fos in neural cells is often associated with elevated cytosolic free calcium [Ca\textsuperscript{2+}] (28).

Recently we identified the presence of P2X7 receptor expression in GH\textsubscript{4}C\textsubscript{1} rat pituitary cells and provided evidence for a role of this receptor type to mediate the action of the putative Pfiesteria toxin (pPfTx) in the induction of c-fos luciferase (20). Here we investigated the ionic properties of the P2X7 receptor in GH\textsubscript{4}C\textsubscript{1} cells, and the activation of these characteristic ionic conductances by the pPfTx. We found that GH\textsubscript{4}C\textsubscript{1} cells have ionotropic purinergic receptors with pharmacologic and functional properties consistent with the P2X7 subtype and that pPfTx mimics the kinetics of cell permeabilization by ATP.

**Material and Methods**

**Cell Culture**

Clonal GH\textsubscript{4}C\textsubscript{1} cells were maintained in monolayer cultures in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum (F10+) in a water-saturated atmosphere of 5% CO\textsubscript{2} and 95% air at 37 ± 1°C. Before experiments, cells were harvested from one donor culture dish with 0.02% EDTA and reseeded on glass coverslips at a density of approximately 200,000 cells/cm\textsuperscript{2}. The cells were grown for 2–5 days, with change of medium each second day. Only cultures with less than 10 passages were used.

**Chemicals**

Culture medium and sera were purchased from Gibco (Grand Island, NY, USA). ATP, oxidized ATP (oxATP), and BzATP were purchased from Sigma (St. Louis, MO, USA). Piridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) was from Research Biochemical International (Natick, MA, USA). The fluorescent dyes Fura-2/AM, Fura-2 pentapotassium salt and YO-PRO-1 (quinolinium, 4-[(3-methyl-2(3H)-benzoxazolylidenne)methyl]-1-[3-(triethylammonio)propyl]-3-diiodide) were from Molecular Probes (Eugene, OR, USA). Putative toxin was isolated from fish-killing cultures of P. plicicida precisely as described (20).

**Calcium Analysis**

GH\textsubscript{4}C\textsubscript{1} cells plated in monolayer on glass coverslips were loaded with Fura-2 just before the experiment. Cells were washed twice with Hepes-buffered saline Hanks' balanced salt solution (HBSS)-II (Hepes 20, D-glucose 10, NaCl 118, KCl 4.6, CaCl\textsubscript{2} 0.4 mM, pH 7.2) and incubated with 2 µM Fura-2/AM in the same buffered saline in a water-saturated atmosphere of 5% CO\textsubscript{2} at 37 ± 1°C for 30 min. Cells were then washed twice, incubated at room temperature for 20 min, then mounted in a flow-through chamber (FCSII; Bioptechs Inc., Butler, PA, USA). Experiments were performed with an inverted fluorescence microscope [Zeiss 100, oil immersion Zeiss F-Fluor 40× lens (Carl Zeiss, Inc., Thornwood, NY, USA)], using the Attofluor ratio imaging analysis system (Atto Instruments, Rockville, MD, USA). Temperature in the chamber was controlled at 37 ± 1°C and HBSS-II was pumped in a laminar flow over the cells at a constant rate of 100 µL/min. Calcium concentration was calculated by the method of Grynkiewicz (29). Excitation wavelengths were 340 and 380 nm, and emission intensity was measured at 510 nm. The cytosolic free calcium concentration in single cells was monitored in real time. At least 30 cells were monitored simultaneously in each experiment. The values of cytosolic free calcium from each cell were averaged after the experiment. We used the in vitro method of calibration modified from the Attofluor manual. This method uses two calibration points, a maximum and a minimum correspondent respectively to a saturated calcium solution (10 mM CaCl\textsubscript{2}) and a solution calcium free (with 10 mM EGTA). One µM Fura-2 pentapotassium was added to each solution, which was used to calibrate the system before each experimental session.

**Cell-Permeabilization Analysis**

The dye YO-PRO-1, a fluorescent analog of propidium iodide of 629 Da molecular weight, was used to detect cell permeabilization (5). GH\textsubscript{4}C\textsubscript{1} cells plated in monolayer on glass coverslips were washed twice with HBSS-II and mounted in the flow-through chamber. The experiments were performed using an inverted microscope and the Attofluor imaging system (described for the calcium analysis experiments) but in a single wavelength mode of analysis. A modified Hepes-buffered saline solution, made with NMDG substituting for NaCl (Hepes 20, D-glucose 10, NMDG 118, KCl 4.6, CaCl\textsubscript{2} 0.4 mM, pH 7.2) and henceforth designated HBSS-YO, was used in the permeabilization assays (5). HBSS-YO was pumped in a laminar flow at the rate of 100 µL/min. Test reagents were diluted in 200 µL HBSS-YO and injected in one of the inflows (see flow-through description below). The flow of the buffered saline was constant throughout the experiments. The excitation wavelength was 460 nm, and emission was measured at 510 nm. At least 30 cells were individually monitored simultaneously in real time during each experiment. Experiments with BzATP were conducted five times, ATP four times, and pPfTx five times. For the experiments where the inhibition by oxATP was tested, cells were preincubated with 300 µM of oxATP in F10+ for 40 min in a water-saturated atmosphere of 5% CO\textsubscript{2} at 37 ± 1°C. Cells were then washed twice and mounted in the flow-through chamber, and the experiments were performed as above.

**Flow-Through System**

All of our experiments were completed in a closed chamber (FCSII; Bioptechs Inc.) through which a constant laminar flow of the Hepes-buffered saline was maintained over the cells. The inflow was fed by one of two
P2X<sub>7</sub> receptors, we next investigated ATP, the physiologic agonist of the P2 receptors. The mean activity of 60 cells that were monitored simultaneously was determined after normalization by reducing the background level, which was also measured simultaneously (Figure 3). During the periods indicated (Figure 3), the cells were exposed to 5 µM YO-PRO-1 only, or to 5 µM YO-PRO-1 with 200, 400, or 600 µM ATP. There was a concentration-dependent elevation of light emission from the cells, demonstrating that ATP had permeabilized GH<sub>4</sub>C<sub>1</sub> cell membranes to the YO-PRO-1 dye. A small response (an increase of 2-fold the initial level) was obtained with 200 µM ATP. When the cells were stimulated with 400 or 600 µM ATP, there was a 10-fold increase in activity.
relative to the level of the emission plateau before each treatment.

To reexamine whether P2 receptors mediate the ATP permeabilization of GH4C1, we co-administered PPADS, an antagonist of P2 receptors, and ATP (10). GH4C1 cells were first treated with 300 mM ATP, leading to accumulation of YO-PRO-1. PPADS given at 300 µM caused a small increase emission, and the addition of 300 µM ATP led to a small increase in dye accumulation. However, this partial inhibitory effect was reversible, and after the antagonist was washed out, led to further accumulation of YO-PRO-1 in the cells (Figure 4). We next investigated the action of oxATP, an irreversible and more selective P2X antagonist (30,32) in GH4C1 cells. Cells were preincubated with 300 µM oxATP for 30 min in HBSS-II buffered saline, washed twice, and mounted in the flow-through chamber using the Hepes buffer with NMDG substituting NaCl (HBSS-YO), as in the previous experiments. Pretreatment with oxATP fully inhibited the accumulation of YO-PRO-1 in GH4C1 cells exposed to 300 and 600 µM ATP (Figure 5).

To evaluate the role of P2X7 as a potential target in mediating the toxic effects of Pfiesteria, we used a partially purified fraction from a toxic culture of the dinoflagellate. The action of pPfTx on cytosolic free calcium was investigated in GH4C1 cells loaded with the fluorescent calcium chelator Fura-2. Cells were mounted on an open coverslip in an inverted fluorescence microscope, and individual cells were monitored under static incubation conditions. With each incremental addition of the pPfTx caused a gradual sustained elevation of [Ca2+]i, that reached near plateau level in about 90 sec (Figure 6). Enhanced effects were observed with further additions, which eventually reached very high values consistent with cytotoxicity. We next examined the effect of the pPfTx on GH4C1 cell permeability to YO-PRO-1. Cells were mounted in the flow-through chamber and were exposed to 200 µL of 1 µL/100 µL, 2 µL/100 µL, or 4 µL/100 µL pPfTx. The pPfTx fraction induced the accumulation of YO-PRO-1 in GH4C1 cells in a concentration-dependent manner. The intensity of emission was monitored simultaneously in 60 cells (Figure 7). Exposure of the cells to 1 µL/100 µL caused a small elevation of emission from 1 to 5 RFU. Exposure to 2 µL/100 µL was repeated, and in both exposures the average cell emission was elevated with a reproducible effect that doubled the effect produced by exposure to 1 µL/100 µL. Exposure of GH4C1 cells to 4 µL/100 µL led to an increase of average cell emission to nearly 58 RFU, an effect 5-fold higher than that observed from exposure to 2 µL/100 µL (Figure 7). When we used GH4C1 cells that previously had been incubated with 300 mM oxATP, the accumulation of YO-PRO-1 in GH4C1 cells exposed to 4 µL/100 µL pPfTx was inhibited (Figure 8). Further exposure to 400 µM ATP also failed to induce the accumulation of dye in these cells.

**Discussion**

This research provides evidence for a P2 receptor with the functional characteristics of the P2X7 type in GH4C1 cells. We used two signal transduction measurements to identify this receptor. One was the ability of the selective agonist BzATP to elevate the cytosolic free calcium. The other was the subsequent accumulation of the fluorescent dye YO-PRO-1 due to the permeabilization of the cells to larger ions. In GH4C1 cells, BzATP
induced a rapid calcium response that elevated cytosolic free calcium 2-fold. After the agonist was washed out, the effect persisted for at least 3 min. When GH4C1 cells were exposed to BzATP in the presence of the fluorescent dye YO-PRO-1, a gradual accumulation of the dye in the cells was observed. The comparison between the effect of BzATP on the concentration of cytosolic calcium, and on the accumulation of YO-PRO-1 in the cells, corresponds to the dynamics of pore formation characteristic of the purinoceptor P2X7 (8,12,18). Once activated by an agonist, P2X7 forms a nonselective cation channel, a characteristic of the ionotropic P2X class of P2 receptors. The influx of calcium ions causes the elevation of the cytosolic free calcium. P2X7 differs from the other members of P2X receptors in that its activation leads to the cell membrane permeability to larger ions (6). This characteristic has been studied using fluorescent dyes, particularly YO-PRO-1 (a derivative of propidium iodide), which are ions of large molecular weight with affinity to nucleic acids (5). The sensitivity to the selective agonist BzATP and the accumulation of YO-PRO-1 in GH4C1 cells provides evidence for the presence in these cells of an ionotropic purinoceptor with the functional characteristics of P2X7.

To further characterize the induction of cell permeability in GH4C1 cells by the activation of P2 receptors, we exposed cells to increasing concentrations of ATP. We observed that ATP induces the permeability of GH4C1 cells to YO-PRO-1 in a concentration-dependent manner. The reversibility of the ATP-induced permeability of the cells was also shown in this experiment, as the cells did not accumulate YO-PRO-1 when they were exposed to the dye both in the absence of ATP and after observed induction by 400 µM of the agonist. We next used two classes of antagonists of P2 receptors to inhibit the accumulation of YO-PRO-1 in GH4C1 cells in response to ATP: PPADS, a reversible antagonist of P2 receptors, and oxATP, a selective and irreversible inhibitor of P2X7 (6). PPADS partially inhibited cell permeability to YO-PRO-1 by ATP, and this partial inhibition was reversed after the antagonist was washed out. To investigate the effect of the P2X7-selective antagonist oxATP, we pretreated cells with the antagonist (30,31). Pretreatment of GH4C1 cells with oxATP fully inhibited the accumulation of YO-PRO-1 during exposure to 300 and 600 µM ATP. The ability of a selective antagonist to prevent the accumulation of YO-PRO-1 provided a second line of evidence for functional P2X7 receptors in GH4C1 cells.

We next investigated the role of the receptor functionally characterized in this work as a potential target for the toxicity of the dinoflagellate Pfiesteria. A bioactive substance has been identified in toxic Pfiesteria cultures with cytotoxic selectivity to GH4C1 cells based upon a panel of different cell types (27). We report here that the pPfTx elevates cytosolic free calcium in Fura2-loaded GH4C1 cells. The elevation of calcium is sustained and is consistent with activation of a membrane channel that gates calcium. The time course of elevated cytosolic free calcium is consistent with activation of P2X7 receptors (32). Calcium is a second messenger, and one of its downstream signaling events is among those factors that induce the immediate response gene c-fos. For that reason, the sustained elevation of cytosolic free calcium by pPfTx is consistent with the induction of the c-fos expression. Figures 6 and 7 show the time course of calcium elevation in the presence of pPfTx and the sustained permeabilization of the cells, respectively.
luciferase construct in GH4C1 cells, a downstream response used to originally identify the action of this substance (20, 27). Activation of P2X7 receptors leads to strong induction of additional transcription factors as well, including nuclear factor (NF)-kappaB and the nuclear factor of activated T cells (NFAT), both of which are associated with cytokine production (34, 35). NFAT is induced rapidly and requires the entry of extracellular calcium. NFAT has a unique DNA binding region, yet binds cooperatively with the Fos/Jun heterodimer to the regulation region of the interleukin-2 gene (34). By contrast, NF-kappaB is induced more slowly and may require more downstream signaling events (35). Subsequent to elevating cytosolic free calcium, pPfTx induced the permeabilization of GH4C1 cells to YO-PRO-1 and its inhibition by oxATP, which is a specific antagonist of the P2X7 purinergic receptor. Data are given as the mean RFU ± 1 SD of the activity from 60 cells individually measured simultaneously in a single real-time experiment, after subtracting the background light intensity which was also monitored simultaneously from five selected areas free of cells in the field of view. We used 400 µM ATP as a positive control. Test chemicals were always delivered simultaneously with 5 µM YO-PRO-1 in 200 µL buffer at the same flow rate.

Figure 8. Permeabilization of GH4C1 cells by the pPfTx to the fluorescent dye YO-PRO-1 and its inhibition by oxATP, which is a specific antagonist of the P2X7 purinergic receptor. Data are given as the mean RFU ± 1 SD of the activity from 60 cells individually measured simultaneously in a single real-time experiment, after subtracting the background light intensity which was also monitored simultaneously from five selected areas free of cells in the field of view. We used 400 µM ATP as a positive control. Test chemicals were always delivered simultaneously with 5 µM YO-PRO-1 in 200 µL buffer at the same flow rate.

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