Modulation of P2Z/P2X₇ receptor activity in macrophages infected with Chlamydia psittaci

ROBSON COUTINHO-SILVA, JEAN-LUC PERFETTINI, ALICE DAUTRY-VARSAT, AND DAVID M. OJCIUS

1Unité de Biologie des Interactions Cellulaires, Centre National de la Recherche Scientifique, Unité de Recherche Associée 1960, Institut Pasteur, 75724 Paris Cedex 15, France; and 2Laboratorio de Imunobiofisica, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21941-590 Rio de Janeiro, Brazil

Received 17 May 2000; accepted in final form 8 August 2000

Coutinho-Silva, Robson, Jean-Luc Perfettini, Pedro M. Persechini, Alice Dautry-Varsat, and David M. Ojcius. Modulation of P2Z/P2X₇ receptor activity in macrophages infected with Chlamydia psittaci. Am J Physiol Cell Physiol 280: C81–C89, 2001.—Given the role that extracellular ATP (ATPᵩ)–mediated apoptosis may play in inflammatory responses and in controlling mycobacterial growth in macrophages, we investigated whether ATPᵩ has any effect on the viability of chlamydiae in macrophages and, conversely, whether the infection has any effect on susceptibility to ATPᵩ–induced killing via P2Z/P2X₇ purinergic receptors. Apoptosis of J774 macrophages could be selectively triggered by ATPᵩ, because other purine/pyrimidine nucleotides were ineffective, and it was inhibited by oxidized ATP, which irreversibly inhibits P2Z/P2X₇ purinergic receptors. Incubation with ATPᵩ, but not other extracellular nucleotides inhibits the growth of intracellular chlamydiae, consistent with previous observations on ATPᵩ effects on growth of intracellular mycobacteria. However, chlamydial infection for 1 day also inhibits ATPᵩ–mediated apoptosis, which may be a mechanism to partially protect infected cells against the immune response. Infection by Chlamydia appears to protect cells by decreasing the ability of ATPᵩ to permeabilize macrophages to small molecules and by abrogating a sustained Ca²⁺ influx previously associated with ATPᵩ–induced apoptosis.

apoptosis; bacteria; immunity; purinergic receptors

THE CHLAMYDIA SPECIES are the causative agents of several significant diseases in humans and a wide variety of animals. Over 600 million humans are estimated to be infected with C. trachomatis strains, which invade primarily epithelial cells of the eyes and genital tract, leading to conjunctivitis and trachoma and causing sexually transmitted diseases that result in sterility (4, 14, 30, 59). The chlamydiae are obligate intracellular bacteria that exist in two developmental forms. The metabolically inert elementary bodies (EB) are internalized by epithelial cells into vacuoles that avoid fusion with host-cell lysosomes and that differentiate a few hours after infection into the metabolically active reticulate bodies (RB). The RB proliferate within a membrane-bound inclusion and, after 1 day of infection, begin differentiating back into EB. The EB are released from the infected cell after about 2 days of infection, allowing a new infection cycle to begin (4, 39).

In addition to infecting epithelial cells, C. trachomatis and C. psittaci also infect macrophages in vitro and in vivo (31).

Macrophages and other eukaryotic cells die mainly through one of two mechanisms, necrosis or apoptosis (8, 15, 55, 64). Necrosis is often referred to as accidental cell death and is caused through irreversible damage of the plasma membrane. Perforin, produced by cytotoxic lymphocytes, and complement kill cells by destroying the integrity of the plasma membrane, leading to osmotic swelling and necrosis (7, 35, 36). A perforin-dependent mechanism, however, does not appear to be necessary for controlling Chlamydia infections (51, 56). Apoptosis, or programmed cell death, is distinguished from necrosis by morphological and biochemical criteria. Apoptosis is associated with nuclear and cytosolic condensation and with fragmentation of chromatin and DNA. Until recently, caspase activation was often used as a criterion for defining apoptosis, although caspase-independent pathways have also been identified (8, 15, 55). Apoptosis is induced by, among other signals, the Fas ligand or antibodies against Fas (41), glucocorticoids (63), beauvericin and valinomycin (48, 65), staurosporine (37), and extracellular ATP (ATPᵩ) (1, 2).

The Fas receptor is constitutively expressed on many cells, including peripheral blood monocytes. Treatment with monoclonal antibodies directed against Fas in vitro and in vivo induces caspase-dependent apoptosis of cells expressing the Fas receptor (41). Chlamydia protects infected cells against apoptosis induced by external ligands such as anti-Fas antibodies by preventing cytochrome release from mitochondria and...
caspase activation (20), but this protection cannot prevent apoptosis caused by the Chlamydia infection itself, which does not rely on known caspases (47). Consistent with these results, infection by C. trachomatis causes apoptosis in the genital tract in vivo (49), and Fas-induced apoptosis is not critical for clearance of the bacteria from the same tissues (51).

Caspases are also activated during ATP<sub>o</sub>-mediated apoptosis but are not required for this cell death pathway (17). ATP<sub>o</sub> is thought to be involved in inflammatory responses (2, 16, 19), and the ability of ATP<sub>o</sub> to trigger apoptosis has been demonstrated in a number of cell types, including macrophages, thymocytes, and dendritic cells (1, 9, 13, 16, 68). ATP<sub>o</sub> has its effect primarily via nucleotide receptors, which are distributed throughout the body and belong to several families: P2X receptors, which are ligand-gated ion channels; P2Y and P2U receptors, which are G protein-coupled receptors; and P2Z receptors, which are ligand-gated ion channels that can be activated by ATP<sub>o</sub> (1, 19, 23). The different receptors can be distinguished on the basis of the behavior of different agonist and antagonist nucleotides (19, 23), and the corresponding genes have been cloned (23, 62). The P2Z/P2X<sub>7</sub> receptor, which represents the P2Z receptor previously characterized on macrophages (62), generates a pore that is reversibly permeable to hydrophilic molecules smaller than 900 Da (16). Macrophages and dendritic cells have been shown to express the genes for both the P2Y and P2Z/P2X<sub>7</sub> receptors (10, 13, 44).

Interestingly, induction of apoptosis in Mycobacterium tuberculosis-infected macrophages with ATP<sub>o</sub> is associated with killing of the intracellular mycobacteria (33, 38). In studies with monocytes infected with bacillus Calmette-Guerin, both H<sub>2</sub>O<sub>2</sub> and ATP<sub>o</sub> killed the monocytes, but only ATP<sub>o</sub> treatment killed the mycobacteria (38). In a comparison with other ligands that can trigger lysis of macrophages, including complement-mediated cytolysis, Fas ligation, and CD69 activation, only ATP<sub>o</sub> treatment led to death of both host cells and intracellular mycobacteria (33).

We therefore evaluated the effects of ATP<sub>o</sub> treatment on the viability of chlamydiae in infected macrophages. Consistent with the results on mycobacteria-infected cells, ATP<sub>o</sub> inhibited the infectious activity of the chlamydiae. Moreover, we found that Chlamydia infection also partially inhibits ATP<sub>o</sub>-induced macrophage death. Because this inhibition would presumably operate on a level different from that previously reported for inhibition of caspase-dependent pathways, we investigated the effects of the infection on the activity of the P2Z/P2X<sub>7</sub> purinergic receptor. We found that the permeabilizing activity of the receptor decreases partially after infection. Thus the immune response may use an ATP<sub>o</sub>-dependent mechanism to control Chlamydia infections, but the bacteria themselves may attempt to limit the effectiveness of the immune response by protecting the infected cells against ATP<sub>o</sub>-mediated apoptosis.

**METHODS**

**Cells and materials.** The human cervical adenocarcinoma cell line HeLa 229 and the mouse macrophage cell line J774 were from American Type Culture Collection. The cells were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> in Dulbecco's modified minimal essential medium (GIBCO BRL) for HeLa or RPMI 1640 (for J774) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL) and 2 mM l-glutamine. The J774 cells were purchased in 1988 and maintained in culture almost continuously since then. The Chlamydia strain used here, the guinea pig inclusion conjunctivitis serovar of C. psittaci (4), was obtained from Dr. Roger Rank (University of Arkansas). FITC-labeled anti-Chlamydia monoclonal antibodies (MAB) were from Argene (Varrilhes, France).

**Preparation of chlamydiae and infection of macrophages.** The chlamydiae were grown in infected HeLa cell monolayer cultures as described (46). For infections, adherent J774 cells were typically grown on coverslips or on 75-cm<sup>2</sup> tissue culture flasks (Costar) until 60–70% confluence and then incubated with chlamydiae in cell culture medium for the indicated times at 37°C in 5% CO<sub>2</sub>. The Chlamydia preparation was used at a multiplicity of infection (MOI) of 4.0. For chloramphenicol inhibition experiments, the cells were incubated with 68 μg/ml chloramphenicol for 30 min at 37°C before bacteria were added, and the antibiotic was maintained with the cells during the duration of the infection.

**Measurement of nucleotide-induced apoptosis by cytofluorometry.** Macrophages were first grown on 75-cm<sup>2</sup> tissue culture flasks (Costar) until 60–70% confluence and then incubated with ATP, ATP analogs, or nucleotides with inhibitors in PBS for 30 min at 37°C in 5% CO<sub>2</sub>, and the medium was then removed and replaced with cell culture medium. The cells were then incubated for the indicated times (usually 6 h) at 37°C in 5% CO<sub>2</sub>. Cells were incubated with the P2Z/P2X<sub>7</sub> blocker oxATP for 2 h, and oxATP was then removed from the medium before ATP was added. For experiments with infected cells, macrophages were first infected with C. psittaci for 24 h, as indicated, and then incubated with nucleotides at 37°C for an additional 6 h.

Quantitative measurements of apoptosis were performed by cytofluorometry of detergent-permeabilized propidium iodide (PI)-stained cells, as described previously (18, 43). Both adherent cells and cells in the supernatant were collected for analysis. The cells were transferred into 12 × 75-mm FALCON 2052 FACS tubes (Becton Dickinson, San Jose, CA). Data from 10,000 J774 cells were collected on a FACSscan flow cytometer (Becton Dickinson) with an argon laser tuned to 488 nm.

**DNA fragmentation assay.** After J774 cells were incubated with 5 mM ATP, ADP, AMP, UDP, UTP, benzoylbenzoyl ATP (BzATP), oxidized ATP (oxATP), and adenosine 5‘-O-(3-thiotriphosphate) were purchased from Sigma (St. Louis, MO). The reagents were prepared as stock 100 mM solutions in PBS and stored at −20°C until use. Lucifer Yellow (LY) was from Molecular Probes (Eugene, OR).

**Measurement of nucleotide-induced apoptosis by cytofluorometry.** Macrophages were first grown on 75-cm<sup>2</sup> tissue culture flasks (Costar) until 60–70% confluence and then incubated with ATP, ATP analogs, or nucleotides with inhibitors in PBS for 30 min at 37°C before bacteria were added, and the antibiotic was maintained with the cells during the duration of the infection.

**Measurement of nucleotide-induced apoptosis by cytofluorometry.** Macrophages were first grown on 75-cm<sup>2</sup> tissue culture flasks (Costar) until 60–70% confluence and then incubated with ATP, ATP analogs, or nucleotides with inhibitors in PBS for 30 min at 37°C before bacteria were added, and the antibiotic was maintained with the cells during the duration of the infection.

**Quantitative measurements of apoptosis were performed by cytofluorometry of detergent-permeabilized propidium iodide (PI)-stained cells, as described previously (18, 43). Both adherent cells and cells in the supernatant were collected for analysis. The cells were transferred into 12 × 75-mm FALCON 2052 FACS tubes (Becton Dickinson, San Jose, CA).** Data from 10,000 J774 cells were collected on a FACSscan flow cytometer (Becton Dickinson) with an argon laser tuned to 488 nm.

**DNA fragmentation assay.** After J774 cells were incubated with 5 mM ATP, ADP, AMP, UDP, or UTP as described above and then incubated for an additional 6 h at 37°C in a CO<sub>2</sub> incubator, both adherent cells and cells in suspension (1–3 × 10<sup>6</sup>) were collected and centrifuged (270 g for 5 min). The pellet was then lysed with 0.6% SDS, 10 mM EDTA, 10 mM Tris, and 20 μg/ml RNase A, pH 7.5, for 1 h at 37°C in 3-mL aliquots. Three hundred microliters of 5 M NaCl were added, and the preparation was incubated for 1 h on ice and finally centrifuged for 30 min at 13,000 g. The supernatant, containing the DNA, was extracted with phenol-chloroform-isomyl
alcohol (25:24:1), and low-molecular-weight DNA was precipitated with ethanol. The same quantity of DNA sample was loaded per gel well. Samples were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

**Measurement of infectious activity of chlamydiae.** Macrophages that had been infected with *C. psittaci* at an MOI of 4.0 for 24 h were incubated with 5 mM ATP, UTP, ADP, or AMP in PBS for 30 min at 37°C in 5% CO₂, and the medium was then removed and replaced by cell culture medium. The cells were incubated for an additional 6 h at 37°C in 5% CO₂. The cells and supernatant were then combined and centrifuged for 60 min at 12,000 rpm in a Sorvall type GSA rotor. The pellet was resuspended in ice-cold culture medium with a 21-gauge 2-ml syringe to dissociate aggregates, giving the final suspension of *Chlamydia* used to infect HeLa cells. Serial dilutions of the chlamydial preparation were used to infect HeLa cells on coverslips for 72 h, and the chlamydial vacuoles were revealed by fixing the cells with paraformaldehyde, permeabilizing with saponin, and incubating with FITC-conjugated anti-*Chlamydia* MAb, as previously described (45). Samples were examined with a Zeiss microscope (Axiophot Zeiss) attached to a cooled charge-coupled device camera (Photometrics), and images were acquired and analyzed with the IPLab spectrum program (Signal Analytics, Vienna, VA). The bacteria recovered from macrophages did not give rise to large inclusions. Thus, to define the relative size (in square pixels) of noninfectious bacteria, HeLa cells were also incubated with ultraviolet-inactivated chlamydiae, prepared as previously described (47); all fluorescent particles with a size larger than the noninfectious bacteria were then counted as infectious vacuoles. At least 10 separate fields containing an average of 200–300 HeLa cells were counted per sample, and the experiment was repeated on three separate occasions.

**Demonstration of permeabilization by fluorescence microscopy.** Cell permeabilization was assessed by observing the differential uptake of LY in infected or uninfected macrophages growing on coverslips that had been treated with 5 mg/ml LY with or without 5 mM ATP at 37°C for 10 min. Coverslips were then rapidly rinsed with PBS at room temperature, and samples were examined immediately with the Zeiss microscope. Acquired images were then analyzed with the IPLab spectrum program.

To distinguish fluorescently positive (permeabilized) cells from nonfluorescent (nonpermeabilized) cells, a threshold of fluorescence intensity was defined with the use of a sample that had been exposed to LY in the absence of ATP. To identify permeabilized cells in images, the IPLab spectrum program was used to quantify the number of cells per field that had fluorescence intensities higher than the threshold level. At least 50 cells per sample were analyzed, and the percentage of permeabilized (LY positive) cells in the different samples was calculated.

**Intracellular Ca²⁺ Measurements.** Cells were loaded with 6 μM fura 2-AM (Molecular Probes) for 1 h at room temperature in RPMI culture medium. The cells were then washed and perfused with PBS supplemented with 1 mM CaCl₂ by using a three-compartment superfusion chamber whose bottom is formed by the coverslip containing the cells (26). Intracellular Ca²⁺ concentrations in groups of 20–40 cells were monitored continuously at 37°C with a fluorescence photometer (Photon Technology, Princeton, NJ). Fura 2 was excited alternatively at 340 and 380 nm, and the emission at 510 nm was measured. The ratio measurement, which is proportional to the logarithm of the intracellular Ca²⁺ concentration, was determined every 100 ms.

**RESULTS**

**Apoptosis of macrophages due to treatment with extracellular nucleotides.** The effects of ATPo on the viability of J774 macrophages were characterized after a 30-min incubation of the macrophages with ATPo, followed by a 6-h incubation in the absence of ATPo. Apoptosis was then assayed by measuring nuclear condensation in PI-stained macrophages, as described in MATERIALS AND METHODS. There was very little effect at ATPo concentrations below 2 mM, but over one-third of the cells were apoptotic after exposure to 5 mM ATPo (Fig. 1). The concentration dependence of ATPo-induced apoptosis in J774 cells is thus consistent with previous reports on the ability of ATPo to trigger apoptosis in macrophages, microglial, mesangial, and dendritic cells (13, 16, 22, 42, 57). Previous studies have also confirmed the apoptotic nature of macrophage cell death by showing DNA fragmentation and morphological features of apoptosis due to ATPo treatment (33, 38, 42).

ATPo-induced apoptosis of macrophages has been attributed to activation of the P2Z/P2X7 purinergic receptor. The nucleotides ADP, AMP, and UTP are able to engage other members of the P2 receptor family, but not P2Z receptors (16, 19, 23, 58). To evaluate whether a P2Z/P2X7 receptor is responsible for the apoptosis of J774 cells, the cells were incubated with 5 mM ATPo, ADPo, AMPo, or UTPo, and apoptosis was measured by cytofluorometry. Only ATPo was able to induce a significant level of apoptosis (Fig. 2).

Finally, because DNA fragmentation of the dying cell is a hallmark of apoptosis (15, 64), the apoptotic nature of ADPo-induced death was confirmed by incubating J774 cells with ATPo or other extracellular nucleotides and determining whether the DNA of the cells was fragmented. As shown in Fig. 3, there is essentially no spontaneous fragmentation in J774 cells incubated with control buffer, and incubation with 5 mM ADP, AMP, UDP, or UTP did not have an appreciable effect on the fragmentation. However, there was a large

![Fig. 1. Concentration dependence of extracellular ATP (ATPo)-mediated apoptosis of macrophages. J774 cells were incubated with indicated concentrations of ATPo for 30 min. The supernatant was then removed and replaced with cell culture medium, and the cells were incubated in the CO2 incubator at 37°C for an additional 6 h.](http://apccell.physiology.org/Downloadedfrom)
amount of DNA fragmentation after incubation with 5 mM ATP.

Effect of P2Z/P2X7 receptor agonists and antagonists on macrophage apoptosis. The specificity of the receptor was confirmed by evaluating the effect of various agonists and antagonists of the P2Z/P2X7 receptor. BzATP is a selective agonist of the receptor (19), and Fig. 4A shows that it induces apoptosis of J774 cells. BzATP is believed to induce permeabilization of cells better than ATP, but both nucleotides trigger apoptosis (13, 69).

Conversely, oxidized ATP at low concentrations is known to block ATPo-mediated apoptosis of macrophages and permeabilization of J774 cells (40). As expected, oxidized ATP also inhibits irreversibly ATPo-dependent apoptosis of J774 cells (Fig. 4B). Because the active form of ATP that binds to the P2Z/P2X7 receptor is the tetrabasic ATP$^{4-}$ (60, 61), the receptor is also antagonized by Mg$^{2+}$, which chelates ATP$^{4-}$. Consistent with these findings, ATPo-induced apoptosis of J774 cells is inhibited reversibly by Mg$^{2+}$ (Fig. 4B).

Effect of ATPo on viability of intracellular chlamydiae. It has previously been shown that intracellular mycobacteria are killed if the host macrophages undergo apoptosis induced by ATPo, but not if induced by ligation of surface CD95, while treatment of extracellular mycobacteria with ATPo has no toxic effect (33, 34). To determine if ATPo might have any effect on the viability of intracellular chlamydiae, we infected J774 macrophages with C. psittaci for 24 h, incubated the infected cells with 5 mM extracellular nucleotides (ATP, UTP, ADP, AMP) for 30 min and, after incubating the infected cells for an additional 6 h at 37°C in the absence of exogenous nucleotide, harvested the bacteria from both the supernatant and adherent infected cells. Infectious activity was then determined by

![Fig. 2. Apoptosis of macrophages due to incubation with ATPo and other extracellular nucleotides. J774 cells were incubated with control (Cont) buffer or 5 mM of the indicated nucleotides, and apoptosis was quantified by cytofluorometry with PI-stained cells, as described for Fig. 1. Values represent means ± SD of 3 separate experiments.](image)

![Fig. 3. Effect of extracellular nucleotides on DNA fragmentation. A very low level of spontaneous fragmentation is seen in the DNA isolated from J774 cells treated with control buffer (PBS) or 5 mM ADP, AMP, UDP, or UTP, while there is prominent fragmentation in cells treated with 5 mM ATP. DNA fragmentation was visualized on a 1.5% agarose gel stained with ethidium bromide, as described in MATERIALS AND METHODS.](image)

![Fig. 4. Effect of P2Z/P2X7 receptor agonists and antagonists on macrophage apoptosis. A: effect of the agonist benzoylbenzoyl ATP (BzATP) on apoptosis. J774 cells were incubated with 2 mM BzATP or 5 mM ATP, as described in MATERIALS AND METHODS. B: effect of the antagonist Mg$^{2+}$ and an irreversible antagonist, oxidized ATP (oxATP), on macrophage apoptosis. J774 cells were incubated with 10 mM MgCl$_2$ alone, 5 mM ATP plus 10 mM MgCl$_2$, 0.3 mM oxATP alone, 5 mM ATP alone, or 0.3 mM oxATP for 2 h followed by 5 mM ATP. Apoptosis was quantified by cytofluorometry, and the apoptosis values were normalized with respect to the value for 5 mM ATP. Normalized values represent means ± SD of 3 separate experiments.](image)
infecting HeLa cells with the bacteria recovered from macrophages and then quantifying infectious units as described in MATERIALS AND METHODS. Of the four nucleotides tested, only ATPo had a significant effect on the ability of the chlamydiae to reinfect new host cells (Fig. 5).

Effect of Chlamydia infection on ATPo-induced apoptosis. It has recently been shown that infection with Chlamydia protects infected cells against apoptosis triggered by external ligands, such as anti-Fas antibodies or staurosporine (20). To evaluate whether Chlamydia infection may also protect against apoptosis induced by ATPo, we infected J774 cells with C. psittaci and then measured the ability of ATPo to induce apoptosis by using the standard 6-h assay described above. Because Chlamydia infection can also lead to apoptosis after 1 day of infection, becoming especially significant after 2 days of infection (47), we infected J774 cells for 24 h before incubating with ATPo. At this length of infection, very little if any apoptosis was measured as a result of the infection itself, compared with apoptosis induced by ATPo (Fig. 6A). However, preinfecting macrophages for 1 day inhibited almost all the apoptosis due to subsequent incubation with ATPo (Fig. 6A). To exclude the possibility that a toxic component of Chlamydia may have inhibited ATPo-mediated apoptosis, in the absence of infection, the chlamydiae and macrophages were incubated in the presence of chloramphenicol, which inhibits protein synthesis of chlamydiae but not the eukaryotic host cell (54). Under these conditions, ATPo induced as much apoptosis as in the complete absence of bacteria (Fig. 6B).

Effect of Chlamydia infection on ATPo-induced permeabilization and Ca\textsuperscript{2+} fluxes. Chlamydia infection was reported to protect cells against apoptosis due to inhibition of caspase activation in infected host cells (20). To investigate whether the infection may also inhibit apoptosis at some level other than the caspases, we have studied the effect of the infection on the activity of the P2Z/P2X\textsubscript{7} receptor.

One of the early events following P2Z/P2X\textsubscript{7} receptor engagement is permeabilization of the plasma membrane via the opening of cation-specific ion channels and nonspecific pores that conduct a range of small solutes of molecular mass <900 (11, 12, 60). We have therefore measured permeabilization of J774 cells to the fluorescent dye LY in the presence and absence of infection. In uninfected macrophages, ~70% of cells were loaded with LY after a 10-min incubation with 5 mM ATPo, as described for Fig. 1. J774 cells that had been infected with C. psittaci for 24 h were incubated with control PBS buffer (Chlam) or 5 mM ATPo (Chlam + ATP). B: uninfected J774 cells were incubated with control PBS buffer or 5 mM ATPo, in the presence of 68 mg/ml chloramphenicol. J774 cells that had been infected with C. psittaci for 24 h in the presence of 68 mg/ml chloramphenicol were then incubated with control PBS buffer (Chlam) or 5 mM ATPo (Chlam + ATP). Apoptosis was quantified by cytofluorometry, and the apoptosis values were normalized with respect to the value obtained with 5 mM ATPo on uninfected cells. Normalized values represent means ± SD of 3 separate experiments.
level of micropinocytosis of LY in infected or uninfected cells (not shown).

As inhibition of permeabilization to LY was partial, compared with the ability of the infection to inhibit most of the ATPo-induced apoptosis (Fig. 6), we also studied the characteristics of Ca\(^{2+}\) fluxes triggered by ATPo in macrophages. We have recently reported that small concentrations of ATPo that do not cause apoptosis are still able to induce a transient influx of Ca\(^{2+}\) in dendritic cells; however, they are not able to maintain the sustained increase in intracellular Ca\(^{2+}\) concentrations observed after incubation with higher, apoptosis-inducing concentrations of ATPo (13). Addition of 5 mM ATPo to uninfected J774 cells at 37°C induced a fast-activating spike of Ca\(^{2+}\) followed by a slowly inactivating plateau in the intracellular Ca\(^{2+}\) concentration (Fig. 8A), similar to the Ca\(^{2+}\) profiles previously measured in peritoneal macrophages (52) and dendritic cells (13). This suggests that a continuous influx of Ca\(^{2+}\) takes place in J774 cells as a consequence of membrane permeabilization. However, the sustained increase was not observed in any of the experiments done with Chlamydia-infected J774 cells (n = 11) (Fig. 8B). We observed that Ca\(^{2+}\) levels return to the basal level after 229 ± 47 s in infected cells, while in uninfected cells the sustained plateau, representing 46 ± 28% (n = 13) of the peak maximum, was present for >10 min. To analyze amplitude changes, we have measured the level of the plateau 5 min after ATPo addition as a percentage of the size of the peak. In infected cells (n = 11), the result was 0.9 ± 0.3%, and in uninfected cells (n = 13), it was 46 ± 28%.

DISCUSSION

A number of macrophage cell lines and primary monocytes and macrophages are known to undergo apoptosis after treatment with ATPo (33, 38, 42), and the J774 macrophage cell line has been extensively used to show that ATPo can permeabilize the plasma membrane through engagement of the P2Z/P2X\(_{7}\) purinergic receptor. Using agonists and antagonists specific for the P2Z/P2X\(_{7}\) receptor, we have shown here that ATPo also induces apoptosis of J774 macrophages through engagement of the same receptor. When Chlamydia-infected J774 cells are incubated with ATPo for 30 min, the bacteria recovered 6 h later have significantly reduced infectious activity. Because other extracellular nucleotides have no effect on Chlamydia viability, we have concluded that ATPo displays its bacteriocytic effect via the P2Z/P2X\(_{7}\) receptor. However, at the present we cannot determine whether the chlamydiae die as an indirect consequence of macrophage apoptosis or whether the P2Z/P2X\(_{7}\) receptor may have an effect directly on the bacteria, independently of host cell apoptosis. In previous studies with macrophages infected with mycobacteria (33, 34, 38), it was shown that apoptosis induced by ATPo, but not by Fas ligation, resulted in loss in viability of most of the intracellular mycobacteria through a process involving active killing, rather than bacteriostasis, of the mycobacteria.
Different species and strains of Chlamydia can infect monocytes and macrophages, although as a rule the infection is less efficient than in epithelial cells (31). It has been reported that the infection of C. trachomatis in human monocytes and macrophages in vitro is not productive but that the bacteria are transcriptionally active and resemble bacteria present in vivo during persistent infections (24). These chlamydiae could therefore serve as a source of bacteria for subsequent infections. Activation of Chlamydia-infected epithelial cells with interferon-γ (IFN-γ) has also been used as an in vitro model for persistence of infection (5, 6).

Most of the damage resulting from Chlamydia infection is due to the inflammatory response of the host, rather than to the infection itself (53). ATPo is also thought to be involved in initiating proinflammatory reactions (16), and it has been shown that the P2Z/P2X7 receptor regulates processing of the inflammatory cytokine interleukin-1β by lipopolysaccharide-treated macrophages (21, 27, 32). During an inflammatory response following infection with Chlamydia, IFN-γ is produced (53), which activates macrophages and increases expression of the P2Z/P2X7 receptor (28, 29). A primary role of P2Z/P2X7-dependent apoptosis may thus be used to lyse macrophages infected with intracellular pathogens such as chlamydiae or mycobacteria during inflammatory responses in such a way that the pathogens are not released from the host cells in viable form.

While the immune system may use inflammatory mediators such as ATPo to eliminate intracellular pathogens, Chlamydia also attempts to minimize the damage to itself by partially inhibiting ATPo-mediated apoptosis. It has recently been reported that chlamydiae can inhibit apoptosis due to external ligands such as Fas by blocking caspase activation (20), and we find that the bacteria can also interfere with apoptosis by decreasing the activity of the P2Z/P2X7 receptor. Because Chlamydia can also directly induce apoptosis (25, 47, 49), the bacteria appear to modulate host-cell apoptosis at several levels simultaneously. Most of these phenomena have been observed in vitro, and a role for the respective activities in vivo now awaits elucidation.

Chlamydia infection inhibits ATPo-induced permeabilization to LY only partially, consistent with the effects of the infection on ATPo-induced apoptosis. In both infected and uninfected macrophages, ATPo was able to trigger a rapid, transient rise in the intracellular Ca2+ concentration. We have recently shown that treatment of dendritic cells with 50 μM ATPo, a concentration that does not induce apoptosis, also causes the short-lived spike in intracellular Ca2+, but without the large, sustained increase observed afterward following treatment with 5 mM ATPo, which does cause apoptosis (13). In infected macrophages, 5 mM ATPo did cause a small, sustained increase in the long-lived component, compared with the basal level in untreated cells, but the sustained increase was significantly larger in uninfected cells. Thus the Ca2+ measurements are also in line with the partial inhibition of ATPo-induced apoptosis observed in infected macrophages.

The effects of infection on P2Z/P2X7 receptor activity have been previously characterized for two pathogens, mycobacteria and Pseudomonas aeruginosa. Mycobacteria inhabit macrophages as their preferential host cell, and ATPo-induced apoptosis of infected cells results in mycobacterial death (33, 38). Because host cell death is detrimental to the survival of the intracellular pathogens, the mycobacteria secrete ATP-scavenging enzymes such as ATPase to minimize the cytotoxic effect of extracellular ATP (66). Conversely, the extracellular pathogen P. aeruginosa secretes as yet uncharacterized factors that increase the sensitivity of macrophages to ATPo-induced cytolysis, presumably by modulating the activity of the P2Z/P2X7 receptor (67). The factors are produced by a mucoid strain of P. aeruginosa isolated from the lungs of a cystic fibrosis patient, but not in a nonmucoid laboratory strain, suggesting that the secreted enzymes may represent virulence factors used in vivo. It is thus interesting that chlamydiae protect the infected macrophage, which may be used as a vehicle for chlamydial persistence, while P. aeruginosa, which does not need to grow within macrophages, secretes factors that kill the same cells.

Finally, the cytotoxic activity of the P2Z/P2X7 receptor requires high doses of ATPo (100 μM–1 mM), raising the obvious question of how these nucleotide concentrations could be achieved in the extracellular space. A number of cell types, including platelets, are known to secrete ATP (19), and ATP is thought to be released from cytotoxic lymphocytes as part of their cytolytic arsenal (3). High local concentrations of ATPo might therefore be attained in the contact zone formed between a cytotoxic lymphocyte and its target cell. ATP can also be released from the cytosol of damaged cells, and cells infected with Chlamydia contain ATP concentrations that are two- to threefold higher than in uninfected cells (46). Transient release of ATP from dying infected cells could therefore engage the P2Z/P2X7 receptor of neighboring macrophages. It has been shown, in fact, that transient exposure to extracellular ATP is sufficient to trigger cytoplasmic Ca2+ fluxes (13, 52) and that cells need to be exposed to ATPo for only short periods of time (15 min) for the cells to die several hours later (27, 38, 50). Because the intracellular ATP concentration in uninfected cells is 5–10 mM, it is thus conceivable that sufficiently high concentrations of ATPo to activate the P2Z/P2X7 receptor could be produced over short periods locally, such as from infected cells during inflammatory reactions.

We are grateful to Philippe Souque and Vandir da Costa for excellent technical assistance.

These studies were supported by funds from the Institut Pasteur, Centre National de la Recherche Scientifique, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro, Pronex, and Fundação Universitaria Jose Bonifacio.
REFERENCES


