Inhibition of Chlamydial Infectious Activity due to P2X₇R-Dependent Phospholipase D Activation

Robson Coutinho-Silva,¹ ² ³ Lynn Stahl,¹
Marie-Noëlle Raymond,⁴ Thomas Jungas,⁴
Philippe Verbeke,⁵ Geoffrey Burnstock,⁵
Toni Darville,⁵ and David M. Ojcius¹ ⁴ ⁵
¹Université Paris 7
Institut Jacques Monod
CNRS UMR 7592
2 place Jussieu
75251 Paris cedex 5
France
² Autonomic Neuroscience Institute
Department of Anatomy and Developmental Biology
Royal Free and University College Medical School
London NW3 2PF
United Kingdom
³ Instituto de Biofísica Carlos Chagas Filho
Universidade Federal do Rio de Janeiro
21949-900 Rio de Janeiro
Brazil
⁴ Université Paris-Sud
Laboratoire de l’activation cellulaire et transduction des signaux
UMR8619
91405 Orsay
France
⁵ Department of Microbiology and Immunology
University of Arkansas for Medical Sciences
Little Rock, Arkansas 72205

Summary

Chlamydia trachomatis survives within host cells by inhibiting fusion between Chlamydia vacuoles and lysosomes. We show here that treatment of infected macrophages with ATP leads to killing of chlamydiae through ligation of the purinergic receptor, P2X₇R. Chlamydial killing required phospholipase D (PLD) activation, as PLD inhibition led to rescue of chlamydiae in ATP-treated macrophages. However, there was no PLD activation nor chlamydial killing in ATP-treated P2X₇R-deficient macrophages. P2X₇R ligation exerts its effects by promoting fusion between Chlamydia vacuoles and lysosomes. P2X₇R stimulation also resulted in macrophage death, but fusion with lysosomes preceded macrophage death and PLD inhibition did not prevent macrophage death. These results suggest that P2X₇R ligation leads to PLD activation, which is directly responsible for inhibition of infection.

Introduction

Chlamydia trachomatis are obligate intracellular bacteria that cause ocular and genital tract infection. Consequences of chronic disease include blindness and female infertility. Occasionally, the organism disseminates from the genital tract to bony joints and causes reactive arthritis (Schachter, 1988). The sexually transmitted biovars of C. trachomatis infect superficial columnar epithelial cells of the genital tract, but some strains can also infect other cells, including macrophages (La Verda and Byrne, 1994; Moulder, 1991).

All Chlamydia species have a characteristic developmental cycle involving two morphologically and functionally distinct forms (Bavoil et al., 2000; Hackstadt, 1999; Moulder, 1991; Wyrick, 2000). The elementary bodies (EB) are metabolically inert and infectious. Following uptake by epithelial cells or macrophages into small entry vacuoles, the EB differentiate into metabolically active reticulate bodies (RB), which proliferate within the same membrane-bound vacuole, called an inclusion. The chlamydiae survive within the host cell by inhibiting fusion between entry vacuoles and lysosomes, through as-yet-uncharacterized mechanisms. After several rounds of division, the RB redifferentiate into EB. The EB exit from the host cell and begin a new cycle of infection in neighboring cells.

There are no unifying rules for predicting how well Chlamydia may survive and replicate in macrophages. Nonetheless, in general, the oculogenital biovars of C. trachomatis do not appear to grow within any type of macrophage, while the lymphogranuloma venereum (LGV) biovar grows in unactivated but not activated macrophages. The 6BC avian strains of C. psittaci proliferate in unactivated but not activated macrophages, while the guinea pig inclusion conjunctivitis (GPIC) strain is not believed to proliferate in any type of macrophage (La Verda and Byrne, 1994). Interferon-γ produced at sites of infection inhibits Chlamydia infection via host-cell indoleamine 2,3-dioxygenase activity, which depletes host-cell concentrations of tryptophan (Beatty et al., 1994), but the molecular basis for control of chlamydial growth by macrophages remains to be fully characterized.

Chlamydiae and their nucleic acids have been detected in synovial tissues of patients with reactive arthritis, and the macrophage is the main host cell where persistent forms are found (Gerard et al., 1998a, 2002; Nanagara et al., 1995; Taylor-Robinson et al., 1992). One potential method of transport of chlamydiae from the genital epithelium to extragenital sites is via peripheral blood monocytes. C. trachomatis serovar K survives in a persistent, transcriptionally active, but nonrelicative form in human monocytes in vitro (Gerard et al., 1998b; Koehler et al., 1997). Intrapерitoneal inoculation of mice with the mouse pneumonitis (MoPn) biovar of C. trachomatis leads to spreading of the infection to liver, lung, and spleen. At the same time, the infection induces NOS in peritoneal macrophages, and an inhibitor of NOS causes the intensity and duration of the infection to increase significantly (Khatseiko et al., 1998). Likewise, in vaginally infected mice, dissemination of the bacteria to spleen and lungs is greater in iNOS-deficient mice than in wild-type mice (Fgiatanseme et al., 1998), and immunosuppressive treatment after apparent resolution of genital tract infection leads to recrudescence of infection in iNOS-deficient mice but not in wild-type mice.

*Correspondence: ojcius@ijm.jussieu.fr
(Ramsey et al., 2001). These results suggest that macrophages are involved in dissemination of infection from the genital tract to remote sites and in establishment of persistent infection.

Extracellular ATP (ATP) is thought to be produced at sites of inflammation (Di Virgilio, 1995). In vitro, ATP has been shown to modify survival of another intracellular pathogen that inhabits macrophages, Mycobacterium tuberculosis. ATP interacts with purinergic P2X receptors (P2X,R), which are expressed on the surface of macrophages and dendritic cells (DC) (Coutinho-Silva et al., 1999; Di Virgilio, 1995, 2001; Mutini et al., 1999). Engagement of the P2X,R leads to macrophage and DC death, and concomitantly maturation and secretion of IL-1β (MacKenzie et al., 2001; Solle et al., 2001). ATP,-induced death of M. tuberculosis-infected macrophages is associated with killing of the intracellular mycobacteria (Fairbairn et al., 2001; Kusner and Adams, 2000; Kusner and Barton, 2001; Lammas et al., 1997; Molloy et al., 1994; Stober et al., 2001). In studies with monocytes infected with bacillus Calmette-Guerin, both H2O2 and ATP, killed the monocytes, but only ATP, treatment killed the mycobacteria (Molloy et al., 1994). In a comparison with other ligands that can trigger death of macrophages, including complement-mediated cytolysis, Fas ligation, and CD69 activation, only ATP, treatment led to death of both host cells and intracellular mycobacteria (Lammas et al., 1997).

ATP, stimulation of the P2X,R is associated with a large increase in the activity of phospholipase D (PLD) (Fairbairn et al., 2001; Humphreys and Dubyak, 1996; Kusner and Adams, 2000; Kusner and Barton, 2001), an enzyme that has been previously linked to leukocyte antimicrobial mechanisms, including phagocytosis and generation of reactive oxidants. PLD activation appears to be directly responsible for killing of intracellular mycobacteria, since a PLD inhibitor decreased significantly the level of P2X,R-mediated killing of virulent strains of M. tuberculosis (Fairbairn et al., 2001; Kusner and Adams, 2000).

At the same time, as macrophage death would be detrimental to the intracellular pathogens, mycobacteria secrete ATP, scavenging enzymes such as ATPase to minimize the cytolytic effect of ATP,. Conversely, ATP, does not affect the growth of extracellular pathogens such as Pseudomonas aeruginosa (Zaborina et al., 1999a, 1999b), which secretes virulence factors that increase the sensitivity of macrophages to ATP,-induced death, presumably via ligation of the P2X,R on the macrophage surface. C. psittaci, like the intracellular mycobacteria, protects J774 macrophages partially from ATP,-induced cell death (Coutinho-Silva et al., 2001). Thus, intracellular mycobacteria and chlamydiae protect the macrophage, which may be used for microbial dissemination, while P. aeruginosa, which does not need macrophages for its propagation, secretes factors that enhance cytolysis of macrophages.

The objectives of this study were to determine if ATP, has an effect on the infectious activity of the MoPn serovar of C. trachomatis in murine peritoneal macrophages, and if the effect requires PLD activity. We show conclusively a role for the P2X,R by characterizing the infection in peritoneal macrophages from wild-type and P2X,R-deficient mice.

Results

Effect of Extracellular Nucleotides and the P2X,R on PLD Activation

ATP, is known to modulate a myriad of cellular responses in macrophages and other cell types, including cytokine secretion, generation of reactive oxygen and nitrogen species, and activation of PLD (Di Virgilio et al., 2001; Gargett et al., 1996; Humphreys and Dubyak, 1996; Kusner and Adams, 2000). The effects of antagonists or agonists of the P2X,R suggested that ATP,-mediated PLD activation relied on stimulation of the P2X,R (Gargett et al., 1996; Humphreys and Dubyak, 1996; Kusner and Adams, 2000), although PLD activity was not tested in P2X,R-/- macrophages.

After incubation with 1 mM ATP, but not 1 mM extracellular UTP, there was a significant increase in the PLD activity of peritoneal macrophages (Figure 1A), as previously observed for human macrophages and lymphocytes (Gargett et al., 1996; Humphreys and Dubyak, 1996; Kusner and Adams, 2000). Similarly, the P2X,R agonist BzATP (200 μM) also induced PLD activation (Figure 1A), suggesting that ATP, activated PLD via the P2X,R.

In the presence of water, PLD produces PA from phosphatidylcholine. In cells pretreated with butan-1-ol, PLD uses preferentially the alcohol as a substrate, producing nonsignaling phosphatidylbutanol (PBut) instead of PA, which might inhibit PLD-dependent responses (Yang et al., 1967). As expected, coincubation with 0.3% butan-1-ol inhibited ATP,-induced PLD activation, as assayed by PA production (Figure 1B).

Effect of Extracellular Nucleotides on PLD Activation in Macrophages from P2X,R-Deficient Mice

To demonstrate directly a role for the P2X,R in ATP,-dependent PLD activation, peritoneal macrophages were isolated from wild-type and P2X,R-/- mice. Incubation of wild-type macrophages with ATP, resulted in a large increase in the activity of PLD, assayed by measuring PBut production in the presence of butan-1-ol, while the same treatment with ATP, had no effect on PLD activity of P2X,R-/- macrophages (Figure 1C). Thus, P2X,R-mediated PLD activation in peritoneal macrophages requires stimulation of the P2X,R.

Effect of ATP, and PLD on Macrophage Death

Previous reports have shown that J774 cells or human or murine macrophages can be induced to die after treatment with ATP, but not other nucleotides, via stimulation of the P2X,R (Chow et al., 1997; Di Virgilio et al., 1998). We confirm that murine peritoneal macrophages are also susceptible to ATP,-induced cell death. When macrophages were treated with ATP, for 2 hr and then incubated in the absence of ATP, for an additional 24 hr, many macrophages became annexin positive (Figure 2A) and permeable to trypan blue (data not shown). In agreement with previous studies with the J774 macrophage cell line (Coutinho-Silva et al., 2001), death of peritoneal macrophages became significant after treatment with ≥2 mM ATP (Figure 2B). There was no macrophage death after a 40 min or 3 hr incubation with ATP, as measured by trypan blue exclusion (data not shown).
Inhibition of Infection by Extracellular ATP

Figure 1. P2X7R Ligation with ATPe Leads to PLD Activation

(A) Peritoneal macrophages from wild-type mice were stimulated for 45 min with ATP, BzATP, or UTP in the presence of 0.3% butan-1-ol. The lipids were extracted, and the amount of PBut was measured as described in Experimental Procedures. PBut is expressed as the % of total radiolabeled lipids.

(B) Peritoneal macrophages from wild-type mice were stimulated with ATPe in the presence or absence of 0.3% butan-1-ol. The lipids were extracted, and the amount of PA was measured as described in Experimental Procedures. PA is expressed as the % of total radiolabeled lipids.

(C) Peritoneal macrophages from wild-type mice (white bar) or P2X7R−/− mice (black bar) were stimulated with ATPe in the presence of 0.3% butan-1-ol. PBut is expressed as the % of total radiolabeled lipids. The experiments were performed on at least 2 separate days, and the values represent the mean and SD of a representative experiment.

Annexin labeling was not used for macrophages incubated with ATPe for up to 3 hr, since early PS surface-exposure following brief P2X7R stimulation is reversible and unrelated to cell death (MacKenzie et al., 2001). As expected, ATPe had no effect on cell death of macrophages isolated from P2X7R−/− mice (Figure 2A).

As ATPe activates PLD via the P2X7R, and the activation could be inhibited with butan-1-ol, we investigated whether PLD activation is required for P2X7R-dependent macrophage death. Incubation of peritoneal macrophages with 5 mM ATP in the presence of 0.3% butan-1-ol had no effect on macrophage death (data not shown), implying that PLD is not involved in P2X7R-dependent macrophage killing.

Effect of C. trachomatis Infection on PLD Activation

Although no putative genes encoding PLD have been identified in the Chlamydia genome (http://chlamydia-www.berkeley.edu:4231), extracts of M. tuberculosis contain PLD activity, and genes homologous to a phospholipase C of P. aeruginosa have been cloned in virulent mycobacterial species (Johansen et al., 1996). In addition, PLD is activated during macrophage phagocytosis of either M. tuberculosis or opsonized zymosan (Kusner et al., 1996).

As we aimed to explore the effects of ATPe-induced PLD activation on the infection by C. trachomatis, we verified whether chlamydial internalization or infection by itself could modify PLD activity. Infected and uninfected wild-type macrophages were therefore incubated with ATP, UTP, or BzATP, and PLD activity was measured. As before, both ATP and BzATP activated the macrophage PLD, whereas UTP had no effect. A previous 24 hr infection with C. trachomatis did not affect PLD activity of untreated cells, nor did it modify PLD activation due to ATP or BzATP treatment (Figure 3). Thus, any effects of PLD on intracellular chlamydiae could be attributable only to ATPe stimulation of the P2X7R.

Effect of P2X7R Stimulation and PLD Activation on Chlamydial Infectious Activity

ATPe-mediated PLD activation has been shown to promote killing of intracellular mycobacteria in macrophages (Fairbairn et al., 2001; Kusner and Adams, 2000). In order to determine whether ATPe-mediated apoptosis affects survival or growth of chlamydiae in infected cells, macrophages were infected with MoPn for 24 hr, the macrophages were treated with the indicated concentration of ATP for 2 hr, and the incubation was allowed to continue in the absence of ATP for an additional 24 hr (Figure 4A) or 4 hr (Figure 4B). The bacteria from supernatant and remaining macrophages were collected and titrated on HeLa epithelial cells. The number of infectious chlamydiae was then measured on HeLa cells by immunofluorescence, using anti-Chlamydia an-
Figure 2. ATPγS-Induced Macrophage Death Requires P2X7R

(A) Peritoneal macrophages from wild-type or P2X7R−/− (KO) mice were incubated with 5 mM ATP as described in Experimental Procedures, and cell death was measured after 24 hr. Cell death was measured by cytofluorimetry with PI-annexin V-FITC double staining, as described in Experimental Procedures. Trypan blue exclusion measurements confirmed that annexin-labeling corresponded to macrophage death at 24 hr.

(B) Peritoneal macrophages from wild-type mice were incubated with the indicated concentration of ATP. Cell death was measured as above, and cells that did not label with annexin V or PI were plotted as a function of the ATP concentration. *, p < 0.05 for 2 mM ATP compared with 0 mM ATP; p < 0.01 for 3 or 5 mM ATP compared with 0 mM ATP. The experiments were performed on 3 separate days, and the values represent the mean and SD of the three experiments.

Figure 3. Infection of Macrophages with C. trachomatis Does Not Affect PLD Activation

Peritoneal macrophages from wild-type mice were infected for 1 day with C. trachomatis at an moi of 0.25 (black bar) or left uninfected (white bar), and were then incubated for 45 min with extracellular ATP, UTP, BzATP, or control buffer in the presence of butan-1-ol. The lipids were extracted, and the amount of PBut was measured as described in Experimental Procedures. PBut is expressed as the % of total radiolabeled lipids. The experiments were performed on 2 separate days, and the values represent the mean and SD of a representative experiment.
Inhibition of Infection by Extracellular ATP

In general, Chlamydia entry vacuoles in epithelial cells maintain a neutral pH by avoiding fusion with host-cell lysosomes (Hackstadt, 1999), and specifically, many vacuoles harboring the MoPn serovar avoid fusion with lysosomes in macrophages (Figure 5A). These results are consistent with previous observations that MoPn can infect alveolar macrophages and macrophages following intraperitoneal infection of mice (Gogolak, 1953; Khatsenko et al., 1998). While some of the chlamydiae that had entered macrophages remained in small vacuoles, most of these also avoided fusion with lysosomes (Figure 5C). Occasionally, small vacuoles that had fused with lysosomes were observed in macrophages that contained a large inclusion (Figure 5A) or only small vacuoles. However, most Chlamydia inclusions contained the LAMP-1 antigen after the infected macrophages were treated with ATP$_e$, for 40 min (Figures 5D and 6), indicating that P2X$_7$R ligation leads to lysosome fusion with mature Chlamydia inclusions. Similarly, most chlamydiae were in acidic compartments following a 3 hr incubation with ATP$_e$ (Figure 5E), suggesting fusion with acidic lysosomes.

Interestingly, most Chlamydia vacuoles expressed the LAMP-1 antigen after a 40 min treatment of infected macrophages with ATP$_e$ (Figure 6), before macrophage death is observed. These results suggest that P2X$_7$R-dependent fusion of Chlamydia vacuoles with host-cell lysosomes is responsible for inhibition of chlamydial growth, independently of macrophage death.

Discussion

C. trachomatis is a major cause of sexually transmitted disease, afflicting 4 million people and costing $2 billion annually in the United States (Pearlman and McNeely, 1994). In women, the manifestations of C. trachomatis infection range from asymptomatic cervicitis to pelvic inflammatory disease, infertility, and ectopic pregnancy (Gerbase et al., 1998). More recently, there has been increasing interest in characterizing infections by C. pneumoniae, which infects endothelial cells and circulating macrophages and is responsible for approximately a tenth of pneumonia cases in industrialized countries, to determine if there is a link between previous infections with C. pneumoniae and increased risk of developing atherosclerosis (Rosenfeld et al., 2000).

Despite extensive work to better characterize the biology of the infection, an effective vaccine against Chlamydia does not exist. Determination of mechanisms that cause genital tract or lung pathology may enable us to develop alternative treatment strategies that prevent the morbidity of chlamydial disease. Any improvement of either pharmacologic or vaccine-based therapies would therefore benefit from a better understanding of the mechanisms that promote natural immunity to Chlamydia. A salient feature of dissemination of invasive

Figure 4. ATP$_e$-Induced Chlamydial Inactivation Requires PLD Activation

(A) J774 macrophages were infected with C. trachomatis for 1 day, and then incubated for 2 hr with the indicated concentration of extracellular ATP. The medium was replaced with cell culture medium and the infection was allowed to proceed for an additional 24 hr. The chlamydiae in cells and supernatant were collected, and the relative infectious activity was measured by titrating the bacteria on HeLa cells, as described in Experimental Procedures. * p < 0.01 for 0.5 mM ATP compared with 0 mM ATP. The experiments were performed on 3 separate days, and the values represent the mean and SD of the three experiments.

(B) J774 macrophages were infected with C. trachomatis for 1 day, and then incubated for 2 hr with extracellular ATP in the presence or absence of butan-1-ol or 2,3-DPG. The medium was replaced with cell culture medium and the infection was allowed to proceed for an additional 4 hr. The chlamydiae in cells and supernatant were collected, and the relative infectious activity was measured as above. * p < 0.02 for 3 mM ATP with 0.1% butan-1-ol or 0.3 mM 2,3-DPG, compared to 3 mM ATP alone. The experiments were performed on 3 separate days, and the values represent the mean and SD of the three experiments.

neal macrophages from wild-type and P2X$_7$R$^{-/-}$ mice with C. trachomatis. While most of the infectious activity was inhibited by treatment of wild-type macrophages with 5 mM ATP, incubation of infected P2X$_7$R$^{-/-}$ macrophages with the same concentration of ATP$_e$ had no effect on chlamydial infectious activity (data not shown), implying that P2X$_7$R-dependent PLD-activation was responsible for the inhibition.

Fusion of Lysosomes and Chlamydia Inclusions due to P2X$_7$R Stimulation

Extracellular ATP kills mycobacteria (Fairbairn et al., 2001; Kusner and Adams, 2000) and induces phagosome-lysosome fusion and acidification of the myco-

bacteria-containing phagosomes (Fairbairn et al., 2001; Kusner and Barton, 2001; Stober et al., 2001). To investigate the mechanism whereby the P2X$_7$R inhibits survival of intracellular chlamydiae, wild-type macrophages were infected for 24 hr with C. trachomatis and then treated with ATP$_e$. Infected macrophages were then fixed and double-stained with anti-Chlamydia antibodies and antibodies against a lysosomal marker, LAMP-1. In general, Chlamydia entry vacuoles in epithelial cells maintain a neutral pH by avoiding fusion with host-cell lysosomes (Hackstadt, 1999), and specifically, many vacuoles harboring the MoPn serovar avoid fusion with lysosomes in macrophages (Figure 5A). These results are consistent with previous observations that MoPn can infect alveolar macrophages and macrophages following intraperitoneal infection of mice (Gogolak, 1953; Khatsenko et al., 1998). While some of the chlamydiae that had entered macrophages remained in small vacuoles, most of these also avoided fusion with lysosomes (Figure 5C). Occasionally, small vacuoles that had fused with lysosomes were observed in macrophages that contained a large inclusion (Figure 5A) or only small vacuoles. However, most Chlamydia inclusions contained the LAMP-1 antigen after the infected macrophages were treated with ATP$_e$, for 40 min (Figures 5D and 6), indicating that P2X$_7$R ligation leads to lysosome fusion with mature Chlamydia inclusions. Similarly, most chlamydiae were in acidic compartments following a 3 hr incubation with ATP$_e$ (Figure 5E), suggesting fusion with acidic lysosomes.

Interestingly, most Chlamydia vacuoles expressed the LAMP-1 antigen after a 40 min treatment of infected macrophages with ATP$_e$ (Figure 6), before macrophage death is observed. These results suggest that P2X$_7$R-dependent fusion of Chlamydia vacuoles with host-cell lysosomes is responsible for inhibition of chlamydial growth, independently of macrophage death.

Discussion

C. trachomatis is a major cause of sexually transmitted disease, afflicting 4 million people and costing $2 billion annually in the United States (Pearlman and McNeely, 1994). In women, the manifestations of C. trachomatis infection range from asymptomatic cervicitis to pelvic inflammatory disease, infertility, and ectopic pregnancy (Gerbase et al., 1998). More recently, there has been increasing interest in characterizing infections by C. pneumoniae, which infects endothelial cells and circulating macrophages and is responsible for approximately a tenth of pneumonia cases in industrialized countries, to determine if there is a link between previous infections with C. pneumoniae and increased risk of developing atherosclerosis (Rosenfeld et al., 2000).

Despite extensive work to better characterize the biology of the infection, an effective vaccine against Chlamydia does not exist. Determination of mechanisms that cause genital tract or lung pathology may enable us to develop alternative treatment strategies that prevent the morbidity of chlamydial disease. Any improvement of either pharmacologic or vaccine-based therapies would therefore benefit from a better understanding of the mechanisms that promote natural immunity to Chlamydia. A salient feature of dissemination of invasive
Immunity

408

Figure 6. P2X-R Stimulation Results in Fusion of Chlamydia Inclusions with Host-Cell Lysosomes

Macrophages were infected with C. trachomatis for 24 hr and then incubated for 0 min, 40 min, or 180 min with ATP. Cells were fixed, and lysosomes (LAMP-1) and Chlamydia inclusions were revealed by immunofluorescence, as described in Experimental Procedures. Samples were examined with a fluorescence microscope, and the number of cells with inclusions that colocalized with LAMP-1 were counted. Values are expressed as the % of cells with inclusions that had fused with lysosomes. The average and SD were calculated from the values obtained from at least fifty microscope fields. The experiment was performed on 2 separate days, and results from a representative experiment are shown.

strains of Chlamydia is the ability of the bacteria to survive within host macrophages (Igietseme et al., 1998; Khatsenko et al., 1998; La Verda and Byrne, 1994; Rosenfeld et al., 2000). However, the specific mechanisms that regulate chlamydial growth within macrophages remain poorly characterized.

Based on reports that ATP$_e$ stimulates killing of intracellular mycobacteria within macrophages (Fairbairn et al., 2001; Kusner and Adams, 2000; Kusner and Barton, 2001; Lammas et al., 1997; Molloy et al., 1994; Stober et al., 2001), we tested the hypothesis that ATP$_e$ could have a similar effect against C. trachomatis in macrophages. We found that ATP$_e$ exerted a profound inhibitory effect on the infectious activity of the MoPn serovar of C. trachomatis through a mechanism requiring PLD activation. Killing of chlamydiae was also dissociable from ATP$_e$-induced macrophage death, since an inhibitor of PLD rescued chlamydiae but did not prevent macrophage death, and the infectious activity of Chlamydia was inhibited at time points when macrophages were still viable.

Characterization of ATP$_e$-mediated killing of C. trachomatis revealed both similarities and differences compared with killing of intracellular bacillus Calmette-Guérin (BCG) or virulent M. tuberculosis (Fairbairn et al., 2001; Kusner and Adams, 2000; Kusner and Barton, 2001; Sikora et al., 1999; Stober et al., 2001). The most

Figure 5. Treatment with Extracellular ATP Leads to Fusion between Chlamydia Inclusions and Host-Cell Lysosomes

J774 macrophages were infected with C. trachomatis for 24 hr and then incubated with control medium or ATP for 40 min or 3 hr, as described in Experimental Procedures. For LAMP-1 localization (A–C), cells were immediately fixed, and lysosomes (LAMP-1) and Chlamydia inclusions were revealed by immunofluorescence, as described in Experimental Procedures. For localization of acidic compartments (E), cells were incubated with LysoTracker during the last 30 min of the experiment.

(A) Image of an infected macrophage revealed by confocal microscopy, in the absence of ATP treatment. Arrowhead points to a productive Chlamydia inclusion that has not fused with lysosomes (LAMP-1 antigen); thin arrows point to small vacuoles that have fused with lysosomes.

(B) Contours of the macrophage in (A) shown in a contrast image.

(C) Image of infected macrophages with small multiple Chlamydia vacuoles that have not fused with lysosomes (LAMP-1 antigen) (arrow), in the absence of ATP treatment.

(D) Image of infected macrophages with Chlamydia vacuoles that have fused with lysosomes (arrow), after 40 min of treatment with ATP. Red, lysosomes (LAMP-1 antigen); green, Chlamydia; yellow, colocalization.

(E) Low magnification image of infected macrophages with Chlamydia vacuoles that have fused with acidic compartments, after 3 hr of treatment with ATP. Red, acidic compartment (LysoTracker); green, Chlamydia; yellow, colocalization.
significant similarity was the dependence on P2X,R for microbial killing. The effects of ATPe on killing of both the BCG bacillus and virulent M. tuberculosis strains were ascribed to P2X,R stimulation based on the effects of different agonists and antagonists of P2X,R. The effects of ATPe were also absent or diminished in P2X,R−/− macrophages (Fairbairn et al., 2001; Sikora et al., 1999; Stober et al., 2001). Although some residual activity against mycobacteria remained, P2X,R is thus the primary receptor for the pathway leading to mycobacterial killing following ATPe, treatment of infected macrophages. The residual activity suggests that additional purinergic receptors may contribute to ATPe-mediated mycobacterial killing.

In contrast, P2X,R appears to be indispensable for ATPe-mediated inhibition of C. trachomatis infectious activity in murine peritoneal macrophages, as no inhibition was observed in macrophages isolated from P2X,R−/− mice. Part of the difference observed between previous reports on mycobacteria and our studies on Chlamydia may be due to macrophage host types, since ATPe-induced mycobacterial killing is more effective in human macrophages than in murine macrophages, the latter being more dependent on P2X,R for ATPe-mediated killing (Stober et al., 2001). Hence, we can not exclude the possibility that additional, P2X,R-independent mechanisms of ATPe-mediated chlamydial killing may be operative in human macrophages.

ATPe treatment led to PLD activation and killing of mycobacteria in both human and murine macrophages (Fairbairn et al., 2001; Kusner and Adams, 2000; Stober et al., 2001). We find that PLD activation is required for inhibition of chlamydial infectious activity, since the chlamydiae were rescued to a large extent when infected macrophages were treated with ATPe in the presence of PLD inhibitors.

PLD activation has been previously associated with lysosomal trafficking and fusion of intracellular organelles (Brown et al., 1998), and ATPe, promotes P2X,R-dependent acidification of phagosomes containing mycobacteria and fusion of phagosomes and lysosomes (Fairbairn et al., 2001; Kusner and Barton, 2001; Molloy et al., 1994). In macrophages infected with mycobacteria, PLD participates in maturation of phagosomes to microbicidal phagolysosomes (Kusner and Adams, 2000), and we propose that a similar mechanism may be operative in Chlamydia-infected macrophages. Chlamydiae normally survive in epithelial cells and macrophages by inhibiting fusion between Chlamydia vacuoles and host-cell lysosomes (Hackstadt, 1999; Schramm et al., 1996; Wyrick, 2000). We observed numerous small Chlamydia vacuoles that are acidic and express the lysosomal marker, LAMP-1, in macrophages that were treated with ATPe, suggesting that P2X,R-dependent PLD activation stimulates fusion between the mature Chlamydia inclusions and lysosomal markers, leading to fragmentation of the inclusion. The fusion was also a rapid event, since inclusions that had avoided interactions with lysosomes for 24 hr would express lysosomal markers within 40 min after ATPe treatment.

For both mycobacteria (Fairbairn et al., 2001) and chlamydiae (this work), inhibition of PLD activity blocked microbial killing, without affecting macrophage death. The time-course of ATPe-mediated killing of mycobacteria and chlamydiae was also more rapid than the induction of macrophage death (Fairbairn et al., 2001). Previous studies have shown that P2X,R agonists do not lead to significant cell death for at least 4 hr after P2X,R activation (Grahames et al., 1999; MacKenzie et al., 2001). These results imply that P2X,R stimulation is required for both microbial inactivation and macrophage death, but the signaling pathways diverge downstream of P2X,R ligation. Thus, for both mycobacteria and chlamydiae, microbial killing is not a direct consequence of host-cell death.

What could be the source of ATPe in vivo? The cytotoxic release of ATPe was observed in macrophages isolated from infected macrophages (Fairbairn et al., 2001; Kusner and Barton, 2001; Molloy et al., 2001). These results imply that P2X7R stimulation is associated with ATPe-mediated inhibition of ATPe-mediated infection should therefore allow the P2X7R to functionally in limiting dissemination of chlamydiae via infected macrophages. Finally, in view of the fact that P2X,R stimulation leads to inhibition of infection by two different pathogens—M. tuberculosis and C. trachomatis—we propose that P2X,R, by activating PLD, could play a general role in regulating infection by bacteria and protozoan parasites that survive in macrophages within membrane-bound vacuoles that avoid fusion with lysosomes.

Experimental Procedures

Mice, Cells, and Materials

The P2X,R−/− mice were described previously (Solle et al., 2001). These animals were maintained on a mixed genetic background (129/Ola x C57BL/6 x DBA/2) backcrossed five times onto C57BL/6. Breeding P2X,R−/− males with P2X,R−/− females was used to maintain the colony of receptor-deficient animals, and genetically comparable wild-type animals were maintained by crossing homozygous animals.

The human cervical adenocarcinoma cell line, HeLa 229, and the mouse macrophage cell lines, J774, were from the American Type Culture Collection (Manassas, VA). The cells were cultured in a humidified incubator at 37°C with 5% CO2 in Dulbecco’s modified minimal essential medium (DMEM) with Glutamax-1 (Life Technologies, Inc.; Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 25 μg/ml gentamicin (cell culture medium for HeLa cells), or RPMI 1640 medium containing 10% FCS, 1 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol,
and 25 μg/ml gentamicin (for J774 cells). The mouse pneumonitis (MoPn) strain of C. trachomatis, obtained originally from American Type Culture Collection, was grown in McCoy cells and purified as previously described (Ramsey et al., 1989). Butan-1-ol, butan-2-ol, ATP, 3-O-(4-benzoylbenzoyl)ATP (BzATP), 2,3-diphosphoglycerate, and UTP were from Sigma (St. Louis, MO). [3H]Myristic acid was purchased from Perkin Elmer Life Science. Other reagents were previously described (Coutinho-Silva et al., 2001).

Isolation of Peritoneal Macrophages

Peritoneal macrophages were recovered from P2X7R−/− and P2X7R+/- mice as described (Albuquerque et al., 1993). In brief, the peritoneal macrophages were obtained by lavage on the intraperitoneal cavity with cold balanced salt solution. Isolated cells were washed with RPMI 1640 medium containing 10% heat-inactivated FCS, 1 mM L-glutamine, and then seeded into 12-well plates (Costar; Corning, NY) at a density of 5×10^4 cells/well. After incubation for 1 hour or overnight at 37°C in a 10% CO₂ humidified incubator, nonadherent cells were removed by vigorous washing and the adherent cells were kept under the same conditions for 24–48 hr, until ready for use. After the 24–48 hr incubation in the incubator, the media were removed, and macrophages were used for infection or treatment with nucleotides.

Measurement of Cell Death

Macrophages from wild-type or P2X7R−/− mice were treated with the indicated concentration of ATP in the presence or absence of 0.3% butan-1-ol in RPMI medium for 2 hr, after which the medium was replaced with RPMI 1640 medium containing 1 mM L-glutamine, 10% heat-inactivated FCS, 25 μg/ml gentamicin, in the absence of butan-2-ol. After an additional 24 hr in the incubator at 37°C, both adherent cells and cells in suspension were collected, washed twice in PBS, and analyzed by cytofluorimetry. Phosphatidylycerine exposure on dying cells was measured by PI staining, as described (Ojcius et al., 1998a). Chlamydial inclusions were identified by staining with rat antibody against murine LAMP-1 (lysosome associated membrane protein-1) (1:100 dilution; from Pharmingen BD Biosciences; Le Pont de Chaix, France), followed by biotinylated F(ab')₂ goat anti-rat Igs (1:200 dilution, from Pharmingen) and revealed by streptavidin-Texas Red (1:100 dilution, from Pharmingen). Compartmental pH was identified with LysoTracker Red DND-99 (Molecular Probes; Eugene, Oregon), by preincubating infected macrophages with 1 μM LysoTracker at 37°C during the last 30 min of the experiment, before washing cells once with PBS and fixing with paraformaldehyde. Samples were examined with either a Zeiss Axiosvert 200M fluorescence microscope (Carl Zeiss; Jena, Germany) attached to a cooled charge-coupled device camera, or a LSM 510 Zeiss confocal microscope. Images were analyzed with Adobe Photoshop software, and Chlamydia inclusions were identified by fluorescence staining. The number of cells with inclusions that colocalized with LAMP-1 was counted in each microscope field, and the average and standard deviation per condition was calculated for the % of cells with colocalizing inclusions in at least fifteen fields, containing an average of ~100 cells per field.

In order to measure effects of extracellular nucleotides on survival of intracellular chlamydiae, J774 macrophages and wild-type and P2X7R−/− macrophages that had been infected with C. trachomatis at an moi of 0.25 for 24 hr were incubated with the indicated concentration of ATP in serum-free RPMI medium for 2 hr at 37°C, in the presence or absence of the indicated concentrations of butan-1-ol; butan-2-ol, or 2,3-DPG (Kusner and Adams, 2000), and the medium was then replaced by fresh cell culture medium. The infection was allowed to proceed for an additional 4 hr or 24 hr at 37°C in the 5% CO₂ humidified incubator. The cells and supernatant were combined and centrifuged for 60 min at 12,000 rpm in a Sorvall type GSA rotor. Macrophages in the pellet were lysed, and the infectious activity of the chlamydiae was measured by resuspending the pellet in ice-cold culture medium, and using serial dilutions of the chlamydial preparation from each well to infect HeLa cells on coverslips for 24 hr, as described (Perfetti et al., 2003). The chlamydial vacuoles were revealed by fixing the cells with methanol and incubating with anti-Chlamydia antibodies, as above.

Acknowledgments

We are grateful to Dr. Christopher Gabel (PGRD, Pfizer Inc. Groton, CT) for generously providing the P2X7R−/− mice, Dr. Jean Kanapalopoulos (Université Paris-Sud) for helpful discussions, and Emmanuelle Perret and Pascal Roux of the Centre d’Imagerie Dynamique for assistance with microscopy. This work was financed by National Institutes of Health grant R01 AI054624, Fondation pour la Recherche Médicale, Université Paris 7, Université Paris-Sud, PTR 94 (Institut Pasteur), and CNRS. R.C.-S. was supported by a fellowship from the Wellcome Trust.

References


