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Reversible Inhibition of \textit{Chlamydia trachomatis} Infection in Epithelial Cells Due to Stimulation of P2X_4 Receptors

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Bacterial infections of the mucosal epithelium are a major cause of human disease. The prolonged presence of microbial pathogens stimulates inflammation of the local tissues, which leads to changes in the molecular composition of the extracellular milieu. A well-characterized molecule that is released to the extracellular milieu by stressed or infected cells is extracellular ATP and its ecto-enzymatic degradation products, which function as signaling molecules through ligation of purinergic receptors. There has been little information, however, on the effects of the extracellular metabolites on bacterial growth in inflamed tissues. Millimolar concentrations of ATP have been previously shown to inhibit reversibly bacterial infection through ligation of P2X_7 receptors. We show here that the proinflammatory mediator, ATP, is released from \textit{Chlamydia trachomatis}-infected epithelial cells. Moreover, further stimulation of the infected cells with micromolar extracellular ADP or ATP significantly impairs the growth of the bacteria, with a profile characteristic of the involvement of P2X_4 receptors. A specific role for P2X_4 was confirmed using cells overexpressing P2X_4. The chlamydiae remain viable and return to normal growth kinetics after removal of the extracellular stimulus, similar to responses previously described for persistence of chlamydial infection.
infectious and is considered to be metabolically inactive. The EBs are internalized into host epithelial cells via small vacuoles which resemble endosomes, the majority of which evade fusion with host cell lysosomes. The EBs differentiate into metabolically active reticulate bodies (RBs), which are not infectious. The RBs proliferate within the same membrane-bound vacuole and, after several divisions, differentiate back into EBs. After 48 h, the EBs are released from the infected cell and can begin a new cycle of infection.

We have previously demonstrated that P2X7 receptor ligation in Chlamydia-infected macrophages with millimolar ATP concentrations leads to fusion of chlamydial vacuoles with lysosomes and irreversible reduction in infectious activity (20) and that P2X7 ligation in infected epithelial cell lines also inhibits infection (21). Inflammation and the intensity of infection of C. trachomatis was also increased in P2X7-deficient mice, suggesting a role for this receptor during the host response to infection (21). More recently, we showed that prolonged exposure to adenosine dramatically inhibited growth of C. trachomatis in epithelial cells, which was mediated by the P1 purinergic receptor A2b (47). We demonstrate here that stimulation with micromolar concentrations of adenine nucleotides (ADP or ATP) induces significant reversible inhibition of C. trachomatis development in cervical epithelial cells through stimulation of the purinergic receptor P2X4.

MATERIALS AND METHODS

Cells, bacteria, and reagents. HeLa 229 cervical epithelial cells (American Type Culture Collection, Manassas, VA) or HEK293 expressing mouse P2X4 (28) or HEK293 cells transfected with TransIT-293 (Mirus) expressing human P2X4 (Invitrogen) were cultured in a humidified incubator at 37°C with 5% CO2 in Dulbecco modified Eagle medium (DMEM–F-12; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum. The LGV/L2 strain of C. trachomatis was from Roger Rank (University of Arkansas, Little Rock, AR). Adenosine, EHNA, AMP, ADP, ATP, UDP, UTP, ATPyS, AMP-CPP (β,γ-methylene ATP), apyrase, PPADS, suramin, U73122, and ivere hypnotecet were from Sigma (St. Louis, MO).

Cell culture and infection. HeLa cells growing at 70% confluence in tissue culture plates (Costar) were infected with the LGV/L2 serovar of C. trachomatis as previously described (46). Unless otherwise noted, cells were infected at a multiplicity of infection (MOI) of 1.0 and incubated at 37°C under 5% CO2 with treatments and medium changes at the indicated times. The infectious activity was determined by titrating chlamydialae obtained from infection on a fresh monolayer of HeLa cells and quantifying inclusions, as previously described (46).

ATP measurement. ATP release was quantified using an ATP bioluminescent assay kit (Sigma) according to the manufacturer’s instructions with a 10-fold dilution of ATP assay mix solution. Whole supernatant (1 ml) from cells grown in 12-well plates, and infected as indicated, were diluted 3-fold and mixed in molecular biology-grade water, and the sample was analyzed within 30 s on a Sirius luminometer (Berthold Detection Systems, Pforzhold, Germany). Standards and sample ATP concentrations were determined by fit to a standard curve.

Microscopy. HeLa cells were grown on coverslips, and after the indicated experimental conditions were fixed with ice-cold methanol for 10 min. Cells were stained with C. trachomatis genus antibodies from Argene (North Massapequa, NY) and Hoechst (Sigma) and were observed on a wide-field fluorescence microscope (Leica, Deerfield, IL). For intracellular Ca2+ observation, the cells were loaded with Fluo-4 AM (Invitrogen) to a final concentration of 5 μM with a 0.02% final concentration of Pluronic (Invitrogen) for 1 h at 37°C before rinsing and the addition of fresh phenol-red free Dulbecco modified Eagle medium with or without U73122 (PLC inhibitor) for 10 min before observation on a wide-field fluorescence microscope.

Quantitative PCR with SYBR green. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The total RNA was quantified by measuring optical density with an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). A total of 400 ng of total RNA was reverse transcribed at 42°C using TaqMan reverse transcriptase (Applied Biosystems) and oligo(dT) according to the manufacturer’s recommendations.

For each transcript, a standard curve was constructed using the purified PCR product generated for each specific primer pair. Each PCR utilized Brilliant SYBR Green master mix (Stratagene) and consisted of 25 μl containing 1 μl of cDNA and a 100 nM final concentration of each primer. A nontemplate negative control to check for primer dimerization was run for each primer pair. The real-time qPCR was run on an MX3000p (Stratagene). The cycling conditions were 1 cycle of denaturation at 95°C for 10 min, followed by 40 cycles of amplification (95°C for 30 s, 55°C for 1 min, and 72°C for 30 s), where the fluorescence was automatically measured during PCR, and finally 1 cycle of product melting (95°C for 1 min, 55°C for 30 s, and 95°C for 30 s). The baseline adjustment method of the Mx3000 software was used to determine the threshold cycle (Ct) in each reaction. A melting curve was constructed for each primer pair to verify the presence of one gene-specific peak and the absence of primer dimerization. All samples were amplified in triplicates, and the mean was used for further analysis. The primer sequences for C. trachomatis 16S rRNA the primers were 5′-CCCGAGTGGCGATCATAATAC-3′ for the forward primer and 5′-CTACGACCTTCACCACCTACA-3′ for the reverse primer.

Statistical analysis. The statistical analysis was performed using Instat software (GraphPad Software, Inc., La Jolla, CA) utilizing the Student t test and was considered significant at P < 0.05.

RESULTS

ATP is released from infected epithelial cells. ATP is released from cells in a variety of circumstances (5, 23, 37, 39), but the possibility that the extracellular ATP concentration may increase during Chlamydia infection in vitro has not been previously investigated. We thus used a luciferase/luciferin bioluminescence detection assay to determine whether epithelial cells release ATP during infection with C. trachomatis. Cells infected with C. trachomatis at an MOI of 1.0 released significant amounts of ATP by 12 and 24 h postinfection (hpi), whereas higher amounts were found at 36 hpi (Fig. 1) when there were no measurable levels of host cell apoptosis (trypan blue exclusion assay [data not shown]). Much higher levels of ATP were released at 48 hpi, at which time fewer than 5% of the cells were dying (not shown). The volumes analyzed were from whole supernatant (1 ml) over 350,000 infected HeLa cells, showing that nanomolar concentrations of extracellular ATP were produced. However, concentrations at the cell surface near release would be expected to be considerably higher, especially in the denser cellular matrix found in tissues.

Extracellular nucleotides inhibit C. trachomatis development. We next evaluated the effect of various agonists of purinergic receptors on the development of Chlamydia within infected host cells. Single applications of the P1 receptor agonist adenosine did not influence chlamydial 16S RNA accumulation, and neither did AMP for which a receptor has not been identified, although cotreatment with an adenosine deaminase inhibitor, erythro-9- (2-hydroxy-3-nonyl) adenine (EHNA), allowed adenosine to modify the growth of Chlamydia, as we reported previously (47). Similarly, uracil nucleotides, which stimulate P2Y receptors on epithelial cells (P2Y2, P2Y4, and P2Y6) (11, 63) did not modulate chlamydial growth. However, exposure to extracellular...
ADP, or ATP, dramatically influenced the ability of *Chlamydia* to grow in epithelial cells (Fig. 2A). Application of the less-hydrolysable form of ATP (ATPγS) had a similar effect, and the administration of apyrase (2 U/ml) largely abrogated the effect of extracellular ATP (Fig. 2B), indicating that the adenine nucleotides were responsible for inhibition of chlamydial infection, and not products of their extracellular metabolism. Adding lower quantities of extracellular ATP repeatedly to mimic prolonged exposure (2 to 20 μM, 30 min apart, six additions) also induced significant inhibition of intracellular chlamydial growth at concentrations as low as 5 μM (Fig. 2C).

The purinergic receptor P2X₄ mediates the response to micromolar ATP. We previously reported that micromolar concentrations of extracellular ATP inhibit chlamydial infection in macrophages and epithelial cells through ligation of P2X₄ (20, 21). We therefore used HEK cells, which do not normally express P2X receptors, and P2X₄-overexpressing HEK cells to show that ligation of P2X₄ by micromolar concentrations of ATP during infection leads to significant reductions in the reinfectious yield (Fig. 3A), a finding consistent with our previous results. However, administration of low concentrations of ATP to these cells did not influence the course of infection (Fig. 3B), suggesting that the P2X₇ receptor was not involved in the response to micromolar ATP in cervical epithelial cells. Conversely, when P2X₇-overexpressing HEK cells were treated with low concentrations of ATP, we observed an inhibition of infection (Fig. 3B), implying that P2X₇ can mediate effects of low ATP on chlamydial infection. The inhibition of infection disappeared at higher concentrations of ATP (Fig. 3B), for reasons which were not investigated, although functional saturation at high ATP concentrations has been previously observed for P2X₇ (18).

To investigate the possibility that an ADP- or ATP-sensitive P2Y receptor might also be involved, we evaluated the effect of phospholipase C (PLC) inhibition on chlamydial infection in cells treated with ADP or ATP. P2Y receptors mediate intracellular calcium release primarily via activation of PLC (16), but epithelial cells pretreated with the PLC inhibitor U73122 (10 μM) were fully responsive to extracellular ADP or ATP (Fig. 4A). To confirm that PLC activation by P2Y receptors was sensitive to U73122 under these conditions, epithelial cells were loaded with the nonratiometric Ca²⁺ sensitive dye Fluo-4 AM, and calcium responses to nucleotides were observed on a fluorescence microscope. A large release of intracellular Ca²⁺ occurred after stimulation with UTP (100 μM), which was completely inhibited in cells pretreated with U73122 (not shown).

Pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and suramin antagonize most members of the P2X receptor family efficiently, although their activity at the P2X₄ receptor is less clear (45). Neither PPADS (Fig. 4B) nor suramin (data not shown) pretreatment of cervical epithelial cells (100 μM each) inhibited the effect of ATP or ADP on infection. In fact, PPADS seemed to slightly potentiate the response, which has been reported previously for the mouse P2X₄ receptor (60), but not for the human P2X₄ receptor. The most distinguishing pharmacological approach to investigating P2X₄ receptors is potentiation by the macrocyclic lactone ivermectin (45). A single applications of 20 μM ATP to infected cells did not impact chlamydial development, but pretreatment with 5 μM ivermectin rendered the cells sensitive to 20 μM ATP (Fig. 4C), inducing a significant reduction in chlamydial growth. These results further strengthen the interpretation that low concentrations of ATP inhibit chlamydial growth in cervical epithelial cells through ligation of P2X₄.

**Chlamydiae remain viable after stimulation with extracellular nucleotides, persisting in cells until nucleotide stimulation is removed.** Immunofluorescence microscopy confirmed that chlamydial growth is severely retarded by exposure to extracellular ADP or ATP (Fig. 5). Single application of these nucleotides led to nearly complete suppression of chlamydial inclusion growth by 24 hpi, and no inclusions were apparent through 24 h in cells exposed to repeated applications. Chlamydiae were able to return to nearly full growth by 48 hpi after single applications of ADP or ATP, but not in cells that had undergone repeated exposure to nucleotides, unless the cell culture medium was changed at 24 hpi. In addition, cells treated repeatedly with nucleotides, and for which no inclusions were apparent at 48 hpi, a medium change at 48 hpi allowed the growth of chlamydiae by 72 hpi (data not shown). Repeated stimulation with adenosine also diminished growth significantly through 24 h, but not to the same extent as ADP or ATP. These data suggest that *C. trachomatis* remains viable within host cells for extended times following extracellular ADP or ATP stimulation and are able to reengage their developmental program when growth conditions are no longer prohibitive. Reversible inhibition of chlamydial infection through P2X₄ ligation thus resembles responses previously described for persistence of chlamydial infection (4, 15, 43, 53).
DISCUSSION
Chlamydia species infect primarily epithelial cells, eliciting tissue-damaging inflammatory responses from human hosts (6). An adaptive immune response is critical for ultimately controlling infection, but innate immune cells and the response of the infected epithelium play critical roles in directing the host response (48, 49, 58). C. trachomatis-infected epithelial cells can produce proinflammatory cytokines such as interleukin-6 (IL-6), IL-8, tumor necrosis factor alpha, and granulocyte-macrophage colony-stimulating factor (33, 48), and here we show that C. trachomatis-infected epithelial cells release significant amounts of extracellular ATP, which is also a proinflammatory mediator (10, 26, 41). Furthermore, we found that increasing the concentration of extracellular ATP can negatively modulate the growth kinetics of Chlamydia during infection of epithelial cells. Thus, epithelial cells are active players in limiting secondary cycles of infection while an adaptive response is established by secreting both cytokines and ATP.

Extracellular ATP has been thoroughly characterized as a signaling molecule in the central nervous system (11) and is beginning to attract increasing attention due to its contribution to signaling in committed immune cells (10, 26, 61). However, few studies have investigated a potential role of ATP in modulating directly the response of the preferred target of many important
pathogens, mucosal epithelial cells. P2X7 stimulation in Chlamydia-infected macrophages leads to inhibition of chlamydial growth, in part by inducing fusion of chlamydial inclusions with lysosomes (20). Treatment of C. trachomatis-infected epithelial cells extracellular ATP and P2X7 agonists likewise leads to the inhibition of intracellular growth (21). Of interest, P2X7 has also been reported to mediate inhibition of growth of another critically important intracellular bacterium, Mycobacterium tuberculosis, in macrophages (34–36). However, other evidence already suggested the involvement of other purinergic receptors besides P2X7. The most potent P2X7 agonist, 2(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP), was less effective than ATP in inhibiting mycobacterial infection (34), and the antimicrobial effects of ATP were amplified by addition of extracellular Zn2+/H11001, which has been subsequently shown to potentiate stimulation of P2X4 receptors and partially block signaling through P2X7 (45).

Given the expression of P2X4 and likely other ATP-sensitive receptors in macrophages (17), it would not be surprising to discover that cooperation between various purinergic receptors may have contributed to the final effect of ATP in cervical epithelial cells.

Previous studies investigating the effects of extracellular ATP on pathogens have utilized very high concentrations of ATP, while micromolar extracellular ATP could be a more consistent feature of infection-induced inflammation. We show here that micromolar concentrations of ATP do reversibly inhibit C. trachomatis growth in cervical epithelial cells. This effect was not sensitive to receptor antagonization by PPADS or suramin or the inhibition of PLC and was potentiated by ivermectin, which suggests a role for the P2X4 receptor. This interpretation was strengthened by the observation that low concentrations of ATP inhibit chlamydial infection in HEK overexpressing P2X4, but not in HEK cells overexpressing P2X7. Given the prominent expression profile of P2X4 in mucosal epithelial cells and that elevated extracellular ATP is a key feature of many pathologies, it would be of interest to determine whether P2X4 modulates the host response to infection by other intracellular pathogens. Although ADP is not commonly associated with stimulation of P2X receptors, it has been shown to have activity at P2X4 receptors (14, 32). Since ADP induces reversible morphological changes in Chlamydia-infected cells that are similar to those observed with ATP (Fig. 5), it is likely that ADP-mediated inhibition of infection could also take place via P2X4.

RNA interference (RNAi) of P2X4 receptor expression has been previously shown to mediate inhibition of P2X4-character-
P2X4 in cervical epithelial cells did not show an effect on the ability of low concentrations of ATP to inhibit chlamydial infection (not conjugated anti-Chlamydia change of medium. The images are all from a single experiment but are representative of two independent experiments. Green, fluorescein isothiocyanate-growth through 24 hpi, and repeated exposure is inhibitory through 48 hpi, However, chlamydiae remained viable and reinitiated development following the change of medium. The images are all from a single experiment but are representative of two independent experiments. Green, fluorescein isothiocyanate-conjugated anti-Chlamydia antibody; blue, Hoechst staining (DNA).

FIG 5 Micromolar extracellular ATP reversibly inhibits chlamydial infection. HeLa cells were infected with Chlamydia, followed by treatment with the indicated purinergic receptor agonists (100 μM each) once at 1 hpi or six times starting 1 hpi (30 min apart). The samples were prepared for immunofluorescence microscopy at 24, 48, or 48 hpi after a medium change 24 h after stimulation with agonists (48h MC). Single applications of extracellular ADP or ATP inhibit purinergic receptor agonists (100 μM) but not in response to higher levels of extracellular ATP (>10 μM) (65), which could indicate that reduced populations of these receptors would still be functional at the moderate extracellular ATP concentrations that were used in the present study (5 to 100 μM). In fact, partial RNAi depletion of P2X4 in cervical epithelial cells did not show an effect on the ability of low concentrations of ATP to inhibit chlamydial infection (not shown). P2X4 depletion by RNAi was not attempted in the cervical epithelial cells due to the expression of a truncated, partially inactive form of P2X7 in HeLa cells (25).

Finally, in the context of chlamydial infection, it is worthwhile noting the tissue distribution of P2X7; vaginal epithelia had the greatest abundance of P2X4 among rat tissues (8), and P2X4 was the most abundantly expressed purinergic receptor in a cancer cell line of human cervical epithelial cells (63).

ACKNOWLEDGMENTS
This project was supported by National Institutes of Health grant R01 DE019444, a Presidential Endowed Chair from the University of California, Else Kroener-Fresenius Stiftung, and the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil, the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

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