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Reversible Inhibition of *Chlamydia trachomatis* Infection in Epithelial Cells Due to Stimulation of P2X₄ 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 irreversibly bacterial infection through ligation of P2X₇ receptors. We show here that the proinflammatory mediator, ATP, is released from *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 P2X₄ receptors. A specific role for P2X₄ was confirmed using cells overexpressing P2X₄. 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.

Danger signals comprise a varied group of extracellular molecules which indicate a potentially harmful physiological state and for which specific sensory mechanisms exist (38, 40, 51, 55, 56). Well-known danger signals include extracellular ATP, adenosine, uric acid crystals, the chromatin component HMGB1, and heat shock proteins. However, the level of danger posed elicits different cellular responses. For example, ATP is released from resting cells (nanomolar range) (5, 37), stressed and dying cells (micromolar range) (23), and physically compromised cells (millimolar range), although actual concentrations depend heavily on cell type and environment. Cells in the adjacent tissue must then decide whether to modify their own behavior in response to the extracellular signal, and whether to amplify the response by secreting other signaling molecules such as cytokines.

The cells sense extracellular ATP and other nucleotides and nucleosides via a family of membrane receptors called purinergic receptors. These receptors are subdivided by function and homology into three classes: P2X are ligand-gated ion channels sensitive to ATP, P2Y are G-protein-coupled receptors stimulated by adenine and uracil nucleotides, and P1 receptors are also G protein coupled but are sensitive to adenosine (11, 45). The tissue distribution and sensitivity to the ligands vary significantly within each family (12), such that slightly different concentrations of ligand can have different effects at a single cell type, and the same concentration of ligand can have contrasting effects on different cell types.

While purinergic receptors have been best characterized in the central nervous system, the receptors are expressed nearly ubiquitously. The activity of purinergic receptors is also modulated by ecto-enzymes such as nucleotidases and adenosine deaminase, which regulate the concentration of extracellular nucleotides. Thus, immunomodulatory adenosine is generated by regulatory T cells (9, 22) and during inflammation-induced hypoxia (24) via enzymatic processing of released nucleotides by CD39 (ecto-apyrase, which processes ATP and ADP to AMP) and CD73 (ecto-5'-

nucleotidase, which converts AMP to adenosine). In fact, there is accumulating evidence that purinergic signaling plays an essential role in immune function (10). However, while the contribution of purinergic signaling to the function of committed immune cells is becoming well accepted, less is known about the direct effects of extracellular ATP and other danger signals on infected host cells. In this regard, it would be especially useful to understand the effects of danger signals on the main cell types that are infected by most pathogens, namely, mucosal epithelial cells. The expression and tissue distribution of purinergic receptors has been described for many kinds of epithelial cells (1, 2, 7, 19, 29, 30, 42, 54, 57, 62, 63, 66), but functional characterization of these receptors in the context of infection of epithelial cells is lacking.

Chlamydiae are obligate intracellular bacteria that initially infect mainly epithelial mucosa and are inherently sensitive to the regulatory mechanisms and metabolism of the host cell (31, 64). Different strains of *Chlamydia trachomatis* are responsible for infections of genital and ocular tissue in humans (27, 52, 59), and *Chlamydia pneumoniae* is a common cause of community-acquired pneumonia in humans and is associated with an increased risk for atherosclerosis (13). All *Chlamydia* species are thought to initiate infection by entering into, surviving within, and multiplying within mucosal epithelial cells by conserved mechanisms involving a unique biphasic developmental cycle (3, 31, 44, 50, 64). The extracellular form of *Chlamydia*, the elementary body (EB), is

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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 to 72 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 *in vivo* lower-genital-tract infections were 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 P2X₇ (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, ATP γ S, AMP-CPP (α -methylene ATP), apyrase, PPADS, suramin, U73122, and ivermectin 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 chlamydiae 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 μ M 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 μ l containing 1 μ l 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 (C_T) 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'-CCCGAGTCGGCATCTAATAC-3' for the forward primer and 5'-CTACGCATTTCCACCGCTACA-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 rRNA 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

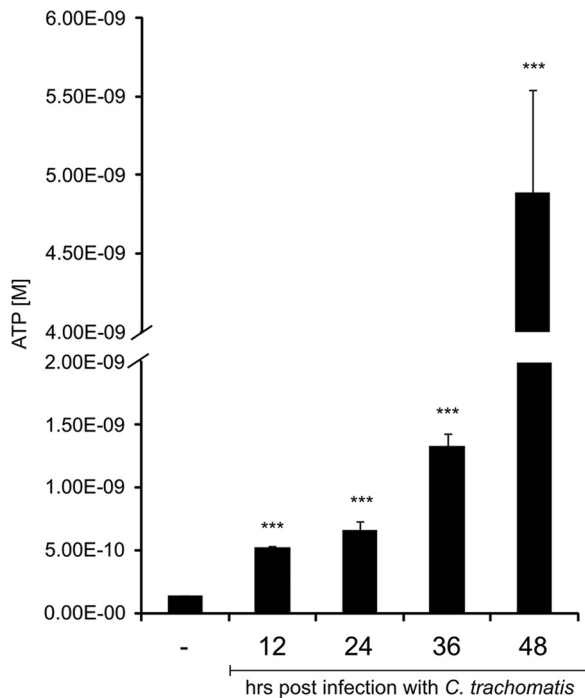


FIG 1 ATP release from *Chlamydia*-infected cells. Supernatants from HeLa cells infected with *Chlamydia* at an MOI of 1.0 at indicated time points were evaluated with a bioluminescent ATP detection assay, and the values represent the ATP concentration in 1 ml of supernatant over approximately 350,000 cells. Infected cells release ATP at significantly elevated levels throughout the course of infection. The means + the standard errors of the mean (SEM) of three independent experiments are shown. ***, $P < 0.001$ for infected cells compared to uninfected cells.

ADP, or ATP, dramatically influenced the ability of *Chlamydia* to grow in epithelial cells (Fig. 2A). Application of the less-hydrolyzable form of ATP (ATP γ S) 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 μ M, 30 min apart, six additions) also induced significant inhibition of intracellular chlamydial growth at concentrations as low as 5 μ M (Fig. 2C).

The purinergic receptor P2X₄ mediates the response to micromolar ATP. We previously reported that millimolar concentrations of extracellular ATP inhibit chlamydial infection in macrophages and epithelial cells through ligation of P2X₇ (20, 21). We therefore used HEK cells, which do not normally express P2X receptors, and P2X₇-overexpressing HEK cells to show that ligation of P2X₇ by millimolar 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 P2X₄-overexpressing HEK cells were treated with low concentrations of ATP, we observed an inhibition of infection (Fig. 3B), implying that P2X₄ 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 μ M) 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 μ M), 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 μ M 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 application of 20 μ M ATP to infected cells did not impact chlamydial development, but pretreatment with 5 μ M ivermectin rendered the cells sensitive to 20 μ M 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, in 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).

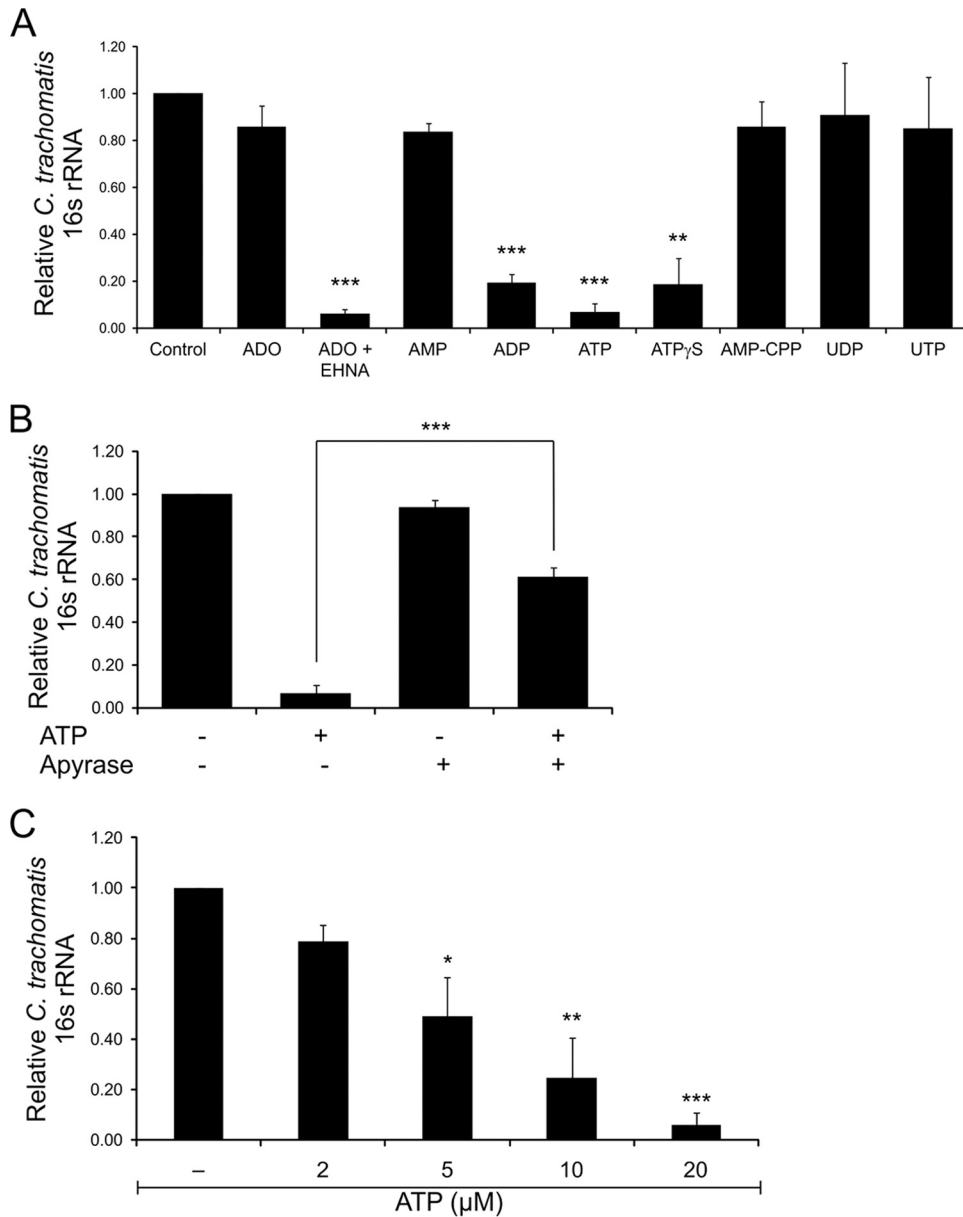


FIG 2 Extracellular ADP and ATP inhibit chlamydial growth. (A) HeLa cells were infected with *Chlamydia*, followed by treatment with indicated purinergic receptor agonists (100 μM each) at 1 hpi. (B) The cells were treated or not with 2.5 U of apyrase/ml at 1 hpi, followed by ATP (100 μM) or not 15 min later. (C) The cells were treated with the indicated ATP concentrations starting at 1 hpi every half-hour through six additions. The total RNA was harvested at 24 hpi in each experiment for quantification of chlamydial 16S rRNA. The values shown are relative to control values for each experiment and are means + the SEM of three independent experiments ($n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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). Fur-

thermore, 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

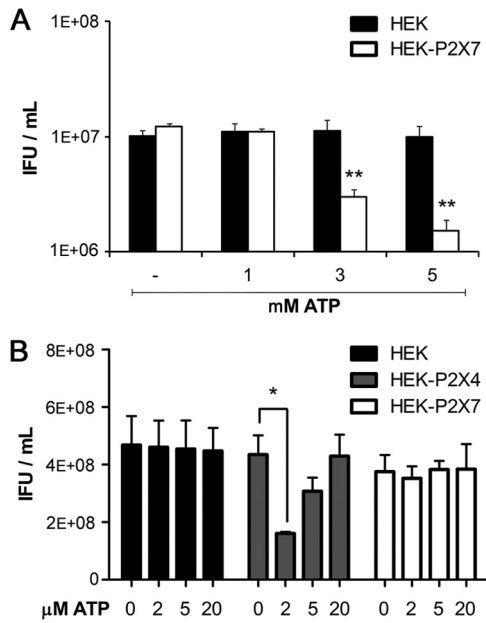


FIG 3 Stimulation of P2X₇ mediates chlamydial inhibition at high, but not low, ATP concentrations, but P2X₄ ligation inhibits infection at low ATP concentrations. HEK-293 cells overexpressing, or not expressing, P2X₇ or P2X₄ were infected with *C. trachomatis* serovar L2 at an MOI of 1, followed by treatment with high (A) or low (B) concentrations of ATP at 24 hpi. The medium was changed at 28 hpi, and the samples were harvested at 30 hpi for quantification of reinfectious yield (IFU/ml) on new cell monolayers. The histogram values show means + the SEM from three independent experiments ($n = 3$; **, $P < 0.01$).

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⁺ (36), 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

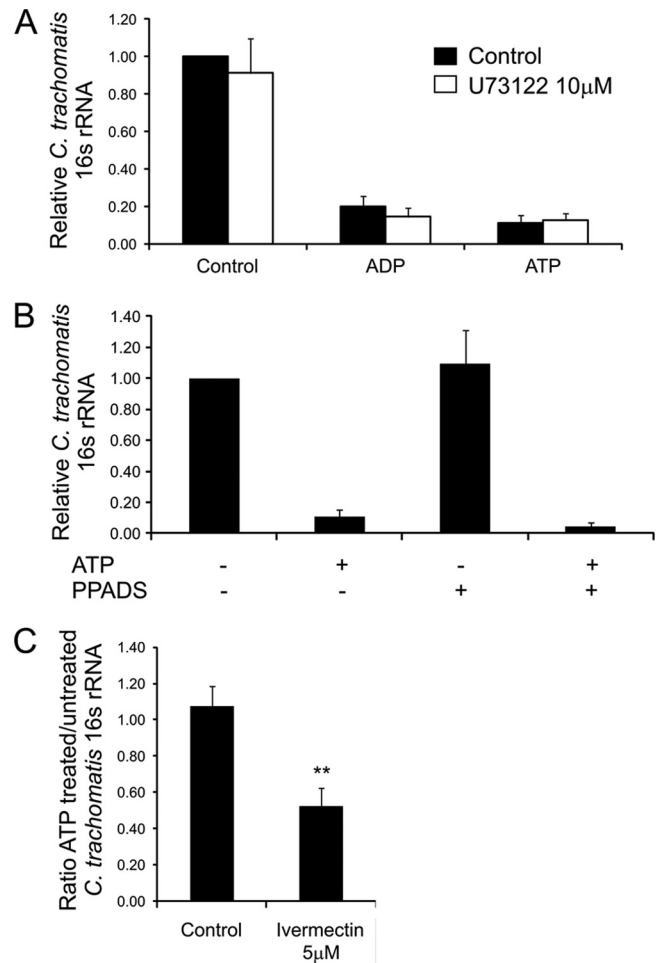


FIG 4 P2X₄-like profile for ATP-mediated chlamydial inhibition. (A) HeLa cells were infected with *Chlamydia*, followed by treatment with the PLC inhibitor U73122 (10 μM) at 1 hpi, followed 10 min later by treatment with 100 μM ATP or ADP. (B) HeLa cells were infected with *Chlamydia*, followed by treatment with the P2X receptor antagonist PPADS (100 μM) at 1 hpi, followed 10 min later by treatment with 100 μM ATP. (C) HeLa cells were infected with *Chlamydia*, followed by treatment with ivermectin (5 μM) at 1 hpi, followed 10 min later by treatment with 20 μM ATP. Total RNA was harvested at 24 hpi in each experiment for quantification of chlamydial 16S rRNA. The values shown are relative to control values for each experiment and are means + the SEM of at least three independent experiments ($n = 3$ for panels A and B and $n = 5$ for panel C; **, $P < 0.01$).

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-

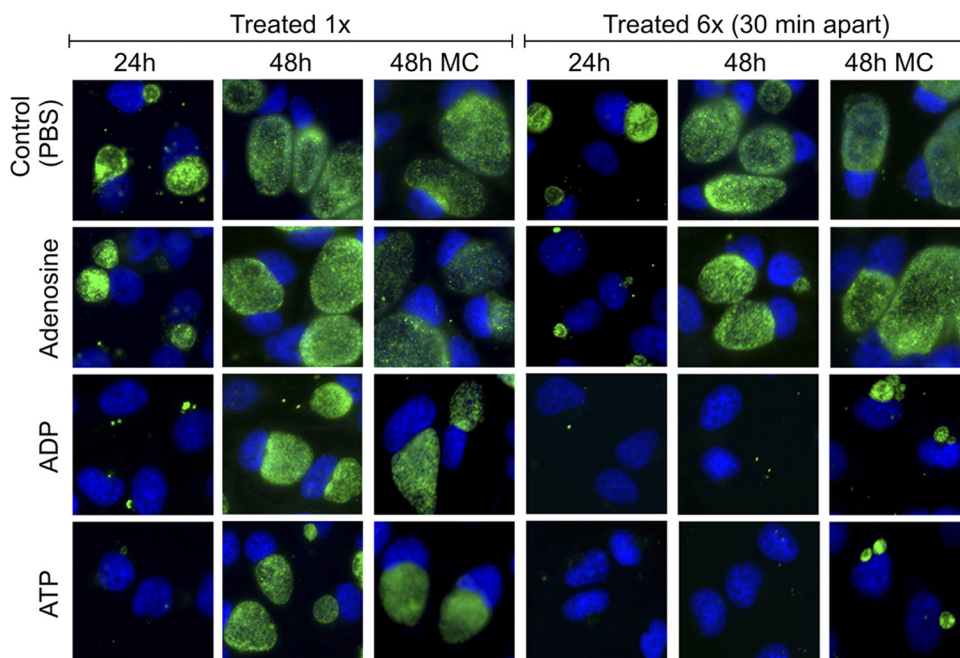


FIG 5 Micromolar extracellular ATP reversibly inhibits chlamydial infection. HeLa cells were infected with *Chlamydia*, followed by treatment with the indicated purinergic receptor agonists (100 μ M each) once at 1 hpi or six times starting 1 hpi (30 min apart). The samples were prepared for immunofluorescence 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 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).

istic sustained phases of Ca^{2+} influx in response to low extracellular ATP stimulus ($<4 \mu\text{M}$) but not in response to higher levels of extracellular ATP ($>10 \mu\text{M}$) (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). P2X₇ 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 P2X₄: 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).

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