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Purinergic Receptors Are Required for HIV-1 Infection of Primary Human Macrophages

Joy E. Hazleton,* Joan W. Berman,*† and Eliseo A. Eugenin*‡,§

Macrophages play a significant role in HIV infection, viral rebound, and the development of AIDS. However, the function of host proteins in viral replication is incompletely characterized in macrophages. Purinergic receptors P2X and P2Y are major components of the macrophage immune response to pathogens, inflammation, and cellular damage. We demonstrate that these receptors are necessary for HIV infection of primary human macrophages. Inhibition of purinergic receptors results in a significant reduction in HIV replication in macrophages. This inhibition is independent of viral strain and is dose dependent. We also identify that P2X1, P2X2, and P2Y1 receptors are involved in viral replication. We show that P2X1, but not P2X2 or P2Y1, is necessary for HIV entry into macrophages. We demonstrate that interaction of the HIV surface protein gp120 with macrophages stimulates an increase in ATP release. Thus, we propose that HIV’s binding to macrophages triggers a local release of ATP that stimulates purinergic receptors and facilitates HIV entry and subsequent stages of viral replication. Our data implicate a novel role for a family of host proteins in HIV replication in macrophages and suggest new therapeutic targets to reduce the devastating consequences of HIV infection and AIDS. The Journal of Immunology, 2012, 188: 4488–4495.

Macrophages are critical for HIV infection and spread within the host. They are the first cells to become infected and serve as a viral reservoir (1–5). Macrophage infection does not result in cell death, and HIV-infected macrophages can persist for long periods of time in host tissues, even in the presence of combined antiretroviral therapy (6–9). Infected macrophages in the CNS are also important mediators of HIV-associated neurocognitive disorders, secreting inflammatory mediators and neurotoxic proteins that result in CNS dysfunction (10, 11).

HIV infects macrophages by binding of the envelope protein gp120 to CD4 and then CCR5 receptors and subsequent fusion with the host cell membrane (12–14). Binding of gp120 results in increased intracellular calcium and G protein signaling (15–17) that involve opening of nonselective cation channels and calcium-activated potassium channels (18). However, additional specific host proteins that participate in this process need further evaluation.

Purinergic receptor activation by extracellular ATP and its byproducts, including ADP and UTP, and are classified into three groups: adenosine receptors (P1), ATP-gated cation channels (P2X), and G protein-coupled receptors (P2Y). Activation of P2X or P2Y receptors causes an increase in intracellular calcium (19). Results from our studies and those of other investigators indicate that macrophages predominantly express P2X1, P2X4, P2X7, P2Y1, P2Y2, P2Y5, P2Y6, and P2Y12 receptors (20, 21, data not shown). P2X and P2Y receptors in macrophages are important mediators of the response to host injury, recognizing extracellular ATP as a damage signal that induces inflammation through cytokine release and superoxide formation (22–24).

Recent studies showed that ATP release and purinergic receptor signaling are activated in response to infectious agents (25, 26). We demonstrate that purinergic receptors expressed in primary human macrophages are required for efficient viral replication. More specifically, we show that P2X1 receptors are required for HIV entry into macrophages. Our data indicate that HIV gp120’s binding to macrophages results in ATP release that then may stimulate autocrine P2X and/or P2Y receptors, resulting in HIV entry and mediating subsequent stages in the viral life cycle. Our data provide evidence of a novel function of P2X and P2Y receptors, in that they are required for HIV replication in macrophages, suggesting new therapeutic targets to limit HIV infection.

Materials and Methods
Reagents
Oxidized ATP (oATP) and brilliant blue G (BBG) were from Sigma-Aldrich (St. Louis, MO), A-740003, MRS 2179, NF 279, and suramin were from Tocris Bioscience (Ellisville, MO). To assure that our antagonists were used at the concentration that blocks purinergic receptors, we tested different concentrations of the inhibitors A-740003 (10, 50, 75, and 100 μM), MRS 2179 (0.1, 1, 10, and 100 μM), NF 279 (10, 50, 75, and 100 μM), and BBG (10, 50, and 100 μM). We determined that 100 μM and lower concentrations of these antagonists reduced viral replication in macrophages (Supplemental Fig. 1). We also did not detect any toxic effect of these antagonists in macrophages. Monomeric gp120Hab (R5-tropic) and the plasmids for β-lactamase virus production, pWT/Hab, and pMM310 were from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Georgetown, MD). Monomeric gp120SF162 (R5-tropic) was a generous gift from Dr. Leo Stamatatos (Seattle Biomedical Research Institute, Seattle, WA). RPMI 1640 and penicillin/streptomycin (P/S) were from Life Technologies/Invitrogen (Grand Island, NY). Human AB serum and FBS were from Lonza (Walkersville, MD). HEPES was from USB (Cleveland, OH).
Monocyte isolation and macrophage culture

Human monocytes were isolated from leukopaks obtained from the New York Blood Center (New York, NY). PBMCs were isolated by differential centrifugation using a Ficoll gradient (GE Healthcare, Piscataway, NJ), and CD14+ cells were isolated from PBMCs using CD14 mAbs that were then bound by magnetic nanoparticles and separated using a magnetic field (Stem Cell Technologies, Vancouver, BC, Canada). Isolated CD14+ cells were cultured adherently for 6 d in the presence of 10 ng/ml M-CSF (PeproTech, Rocky Hill, NJ) in RPMI 1640 with 10% FBS, 5% human AB serum, 1% P/S, and 10 mM HEPEs to differentiate the cells into macrophages.

HIV replication

After 6 d in culture, macrophages in 48-well plates (at a density of 10⁵ cells/well) were inoculated with 2–20 ng/ml HIVADA, 2 ng/ml HIVYU-2, or 0.2–5 ng/ml HIVBaL for 24 h in the presence or absence of purinergic receptor inhibitors. Five wells were inoculated per condition. After 24 h, cells were washed with PBS and then cultured with media. Supernatants were collected, and medium was changed every 24 h until 7 d postinoculation (d.p.i.). When the inhibitors αATP, A-740003, MRS 2179, NF 279, BBG, or suramin were used, they were added when the medium was changed every 24 h. Viral replication was analyzed by HIV p24 ELISA (Advanced Bioscience Laboratories, Kensington, MD), according to the manufacturer’s instructions.

To determine whether any of the inhibitors used resulted in cell death, we assessed cell viability using a live/dead assay. Macrophages were grown in 35-mm MatTek (Ashland, MA) dishes at a density of 10⁵ cells/dish for 6 d and then treated with 100 μM αATP, A-740003, MRS 2179, NF 279, BBG, or suramin for 24 h. Cells were then washed with PBS and inoculated with 400 μl of HIVBaL-containing hemin (1 μg/ml heme, 0.5 μM Calcein AM (excitation 494 nm, emission 517 nm) for 30–45 min, according to the manufacturer’s instructions (LIVE/DEAD Viability/Cytotoxicity Kit; Invitrogen, Eugene, OR). Cells were imaged using a Zeiss AxioObserver.D1 with an LD Plan-Neofluar 20 x0.4 objective lens. Images were collected at room temperature through a Zeiss AxioCam MRm camera using AxioVision software. None of the antagonists caused any decrease in cell viability.

Immunofluorescence

After 6 d in culture, macrophages cultured in 35-mm MatTek dishes at a density of 10⁵ cells/dish were inoculated with 20 ng/ml HIVADA, for 24 h in the presence or absence of αATP. Two MatTek dishes were inoculated per condition. After 24 h, cells were washed with PBS and cultured with medium. Fresh αATP was added, and the medium was changed every 24 h until 7 d.p.i. Cells were then fixed using 4% paraformaldehyde and permeabilized using 0.2% Triton X-100. Cells were washed with PBS and blocked using 0.7% fish gelatin, 50 μM EDTA, 1% horse serum, and 1% globulin-free albumin. The following antibodies were used: mouse monoclonal HIV p24 Ab at a 1:50 dilution (Abcam, Cambridge, MA) or mouse myeloma IgG1 (Sigma) as a negative control. Secondary Ab staining was with anti-mouse IgG F(ab)₂ conjugated to FITC (Sigma). Actin was stained using Texas Red-X conjugated to phalloidin (Invitrogen). ProLong Gold Antifade reagent containing DAPI was added to all cells to stain for nuclei. Cells were imaged using a Zeiss AxioObserver.D1 with an LD Plan-Neofluor 20 x0.4 air objective lens. Images were collected at room temperature with a Zeiss AxioCam MRm camera using AxioVision software. There was no image variability in the amount of HIV p24 produced (J.E. Hazleton, Rocky Hill, NJ) in RPMI 1640 with 10% FBS, 5% human AB serum, 1% P/S, and 10 mM HEPEs to differentiate the cells into macrophages.

HIV entry assay

Primary human macrophages were cultured in black 96-well plates with a clear bottom (Corning, Corning, NY) at a density of 43,000 cells/well. After 7–8 d in culture, cells were inoculated with β-lactamase–containing HIVBaL at a multiplicity of infection (MOI) of 0.01, 0.005, or 0.001 in the presence or absence of the purinergic receptor inhibitors αATP, NF 279, A-740003, or MRS 2179 for 4 h. β-lactamase–containing HIVBaL was generated as described previously (27). Briefly, HEK-293T cells were transfected using calcium phosphate with two plasmids: one containing the HIVBaL genome, pWT/BaL (NIH repository) and the other containing β-lactamase fused to HIV vpr, pMM310 (NIH repository). Cells were transfected for 16 h, and supernatant was collected 24 and 48 h following transfection. This resulted in production of complete viral particles containing active β-lactamase. Viral stocks were generated by sucrose-gradient purification of the transfection supernatants.

After viral inoculation of primary human macrophages, cells were washed with phenol red-free DMEM (Life Technologies) and then loaded with CCFL-AM for 6 h at room temperature using the GeneBLAzer In Vivo Detection Kit (Invitrogen, Carlsbad, CA). CCFL-AM fluoresces green as the result of an internal fluorescence resonance energy transfer; however, when cleaved by β-lactamase, this interaction is lost, and the molecule fluoresces blue. Cells were imaged using an Olympus IX70 inverted fluorescent microscope with a Plan 10×/0.25 air objective lens. Images were collected at room temperature through an Olympus E-620LS camera attached to the microscope through an OM adapter MF-1. Images were collected directly onto a memory card, and there was no image processing following image collection. Images were collected for eight fields in four wells per condition, and percentage of cells positive for viral entry was determined by counting the number of blue cells (cells that virus had entered) compared with the total number of blue and green cells.

ATP-release assay

Macrophages were cultured in 96-well plates at a density of 30,000–43,000 cells/well. After 6–8 d in culture, medium was changed to phenol red-free RPMI 1640 with 1% P/S and 10 mM HEPEs. Cells were then inoculated with media or 20–60 nM gp120BaL. (n = 2) or gp120gp162, (n = 8) for 2, 5, 10, 15, 30, or 45 min. Supernatants were collected, and ATP concentrations were determined using the ATPLite luminescence assay system (PerkinElmer, Waltham, MA) by combining 100 μl sample with 100 μl ATP reagent. Luminescence was measured using a GloMax 96 Microplate Luminometer (Promega, Madison, WI) or an EnVision plate reader (PerkinElmer, Waltham, MA). The concentration of ATP released was determined by comparing sample luminescence to a standard curve of 0.39–100 nM ATP using the ATP standard provided by the manufacturer. As a positive control for ATP release, pipetting up or down to mechanically activate the release of ATP was performed.

Statistical analysis

Statistical analyses were used to determine significance of data from all experiments. Significance was assessed by determining the validity of the null hypothesis that states that all treated groups were the same as their respective controls, which were set to 1 [i.e., the null hypothesis was that the ratio of (HIV+ treatment)/(HIV only) = 1 or gp120 treated/untreated = 1]. GraphPad Prism software (version 5.0b) was used to test the null hypothesis by comparing the relative value to a theoretical mean of 1 using a two-tailed one-sample t test with a 95% confidence interval.

Results

Purinergic receptors are necessary for HIV replication in human macrophages

To determine whether purinergic receptors are required for HIV replication, we infected primary human macrophages with HIV in the presence or absence of the P2X receptor inhibitor αATP and examined production of the viral capsid protein HIV p24 in the medium every 24 h postinoculation. αATP inhibits mainly P2X₇, as well as P2X₁ and P2X₂, receptors (28, 29). Treatment with 100 μM αATP significantly blocked HIV replication in macrophages up to 7 d.p.i., the last time point examined (Fig. 1A, representative donor). Thus, we focused our studies on these purinergic receptors. This effect was consistent in 10 donors tested, despite donor variability in the amount of HIV p24 produced (J.E. Hazleton, J.W. Berman, and E.A. Eugenio, unpublished observations). Treatment with 100 μM αATP did not reduce cell viability, as determined by a live/dead cell assay (see Materials and Methods).

Inhibition of HIV replication was independent of viral strain, because treatment of macrophage cultures with 100 μM αATP significantly inhibited replication of three R5-tropic strains: HIVADA, HIVBaL, and HIVYU-2 (Fig. 1B, n = 10, Fig. 1C, n = 3). HIVADA replication was reduced 92–99%, depending upon the d.p.i. (***p < 0.0001 at every d.p.i. relative to HIV only, Fig. 1B); HIVBaL replication was reduced 89–97% (**p < 0.001–0.0005, relative to HIV only and according to the respective d.p.i., Fig. 1C), and for HIVYU-2, αATP reduced replication by >99% on every d.p.i. (**p < 0.001 at every d.p.i. relative to HIV only, Fig. 1C). The inhibition of replication with αATP treatment for each donor was dose dependent, with 10 μM αATP inhibiting HIVADA p24 production to a lesser extent than 100 μM, 50–85% inhibition relative to untreated HIVADA p24 production.
only depending upon d.p.i. (p = 0.0004–0.04, Fig. 1B), and 1 μM oATP showing no effect (Fig. 1A, 1B). Treatment with suramin, a nonspecific general P2X and P2Y antagonist, for 24 h. Supernatants were collected, and fresh oATP or suramin was added every 24 h until 7 d.p.i. HIV p24 levels in the supernatant were determined by ELISA. (A) Replication curve for one representative donor infected with HIVADA. HIV p24 production with oATP relative to HIV only is summarized for multiple donors for HIVADA (B, n = 5 for 1 and 10 μM oATP, n = 10 for 100 μM oATP), HIVRNA, or HIVYU-2 (C, n = 3, 100 μM oATP). (D) Replication curve for one representative donor infected with HIVADA and treated with suramin. Three donors all showed 100% inhibition of replication with suramin at every time point tested. All data are mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, relative to HIV only.

Inhibition of purinergic receptors decreases the percentage of HIV-infected macrophages

To determine whether the reduction in viral replication with purinergic receptor antagonists was due to a decrease in the number of cells infected with HIV or to a decreased release of virus from the same number of infected cells, macrophages were infected for 7 d in the presence or absence of oATP (100 μM); stained for HIV p24 (green, FITC), actin (red, phalloidin-Texas Red), and nuclei (blue, DAPI); and examined by fluorescence microscopy. In the HIVADA-infected cultures we detected positive intracellular HIV p24 staining and frequent formation of multinucleated giant cells (Fig. 2A), which result from fusion of HIV-infected cells (30). Inhibition of purinergic receptors with oATP resulted in a significant 92% decrease in the percentage of HIV p24+ cells, and very few multinucleated giant cells were detected in these cultures compared with HIV-infected cultures (Fig. 2B). HIV p24+ cells were quantified by calculating the percentage of nuclei in cells with green cytoplasmic staining relative to total number of nuclei. Data from two additional donors were quantified and are illustrated in Fig. 2D (n = 3, **p = 0.0037, HIVADA + oATP relative to HIV only). The use of an IgG1 negative control Ab in HIV-infected cultures did not result in any nonspecific staining (Fig. 2C).

P2X, P2X7, and P2Y1 purinergic receptors participate in HIV replication in macrophages

Macrophages express P2X1, P2X4, P2X7, P2Y1, P2Y2, P2Y4, P2Y6, and P2Y12 receptors (20, 21). Our combined data obtained

FIGURE 1. Blocking P2 receptors inhibits HIV replication in a dose-dependent manner. This inhibition is independent of viral strain. Primary human macrophages were inoculated with 2 ng/ml of HIVADA (A, B, D), 0.2 ng/ml HIVADA, or 2 ng/ml HIVYU-2 (C) in the presence or absence of oATP (A–C), a P2X receptor inhibitor, or suramin (D), a nonspecific general P2X and P2Y antagonist, for 24 h. Supernatants were collected, and fresh oATP or suramin was added every 24 h until 7 d.p.i. HIV p24 levels in the supernatant were determined by ELISA. (A) Replication curve for one representative donor infected with HIVADA. HIV p24 production with oATP relative to HIV only is summarized for multiple donors for HIVADA (B, n = 5 for 1 and 10 μM oATP, n = 10 for 100 μM oATP), HIVRNA, or HIVYU-2 (C, n = 3, 100 μM oATP). (D) Replication curve for one representative donor infected with HIVADA and treated with suramin. Three donors all showed 100% inhibition of replication with suramin at every time point tested. All data are mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, relative to HIV only.

FIGURE 2. Inhibition of P2 receptors decreases the percentage of HIV-infected cells. Primary human macrophages were inoculated with 20 ng/ml HIVADA (A, C) or 20 ng/ml HIVADA and 100 μM oATP (B) for 24 h. The cells were washed, and medium with fresh oATP was replaced every 24 h until 7 d.p.i., when the cells were fixed, permeabilized, and stained for actin (phalloidin-Texas Red, red), nuclei (DAPI, blue), and HIV p24 (green, FITC); (A, B) or mouse IgG1 control (C) (FITC, green). Images were taken of 15 fields per condition, and the percentage of HIV p24+ cells was determined by counting the number of nuclei with green cytoplasmic staining relative to total number of nuclei (n = 3). Scale bars, 50 μM. Inset, green channel only. (D) Results are shown as the mean ± SD. **p = 0.0037, relative to HIV only.
using oATP, which mostly inhibits P2X1, P2X2, and P2X7 (28, 29); BBG, a specific P2X7 antagonist (Supplemental Fig. 1); and suramin, a general P2X and P2Y inhibitor focused our research on P2X1 and P2X7 receptors. We also examined P2Y1 receptors because they have been identified in macrophages. Additionally, Western blot analyses indicated that macrophages expressed all of these purinergic receptors in control and HIV-infected conditions (data not shown). To determine the purinergic receptors involved in HIV replication in macrophages, we used the following specific pharmacologic P2 receptor antagonists: NF 279 (a specific P2X1 antagonist), A-740003 (a specific P2X7 antagonist), and MRS 2179 (a specific P2Y1 antagonist). At 100 μM, all three inhibitors significantly reduced viral replication (Fig. 3A, representative donor). As described in Materials and Methods, different concentrations of these antagonists were tested. Inhibition was dose dependent, with 100 μM resulting in the greatest inhibition (Supplemental Fig. 1). Treatment with 100 μM NF 279, A-740003, or MRS 2179 did not alter cell viability during the time course analyzed (see Materials and Methods). Quantification of viral replication from macrophages isolated from three independent donors indicated that NF 279 resulted in 90–98% inhibition of HIV p24 production on days 2–7 postinoculation, and MRS 2179 inhibited HIV p24 production 60–70% compared with HIV-infected cultures without the inhibitors (Fig. 3B). The P2X7 antagonist A-740003 inhibited HIV p24 production 70–91% relative to HIV plus DMSO (A-740003 vehicle). BBG treatment of macrophage cultures infected with HIV inhibited viral replication 54–90% relative to cultures infected with HIV only (Supplemental Fig. 1). Therefore, at least three purinergic receptors, P2X1, P2X7, and P2Y1, are required for HIV replication in macrophages. Our studies cannot discount the participation of P2X2 and P2X4 receptors, because no specific antagonists for those receptors are available. Both receptors are expressed in human macrophages, as shown by Western blot analyses (data not shown).

Purinergic receptors are required for HIV entry into macrophages

To determine whether purinergic receptors are required for viral entry, we examined the effect of oATP on HIV entry into human macrophages. Macrophages were inoculated with β-lactamase–containing HIV_BaL at an MOI of 0.001–0.01 for 4 h in the absence (A) or presence (B) of 100 μM oATP, followed by loading with CCF2-AM, which fluoresces green (excitation 409 nm, emission 520 nm). When viral entry occurs, β-lactamase cleavage of CCF2-AM results in blue fluorescence (excitation 409 nm, emission 447 nm, representative cells indicated by arrows). (C) There was no nonspecific blue fluorescence detected in uninfected control cells. (D) Percentage of cells with viral entry in the presence of oATP relative to HIV only (mean ± SD; n = 5). The percentage of cells with viral entry decreased 57% with oATP treatment. Original magnification ×10. ***p = 0.0009.
Macrophages were inoculated with β-lactamase–containing viral particles and loaded with CCF2-AM, which fluoresces green, but the molecule fluoresces blue when cleaved by β-lactamase. Therefore, in this assay, cells into which HIV entered fluoresced blue, whereas all other cells are green. The percentage of viral entry was determined by assessing the number of blue cells relative to the total number of cells. HIV infection of macrophages for 4 h with β-lactamase–containing HIV\textsubscript{BaL} resulted in 4–30% viral entry, depending upon the donor (Fig. 4A, representative donor). When cells were inoculated with β-lactamase containing HIV\textsubscript{BaL} in the presence of the purinergic receptor blocker oATP (100 μM), there was a reduction in viral entry (Fig. 4B, representative donor). Quantification of HIV entry into macrophages from five independent donors indicated that oATP significantly reduced viral entry by 57% (Fig. 4D, ***p = 0.0009, relative to HIV only). In uninfected cultures, there were no blue cells, indicating no nonspecific blue fluorescence (Fig. 4C). The majority of our previous experiments was performed using HIV\textsubscript{ADA}. However, we did not perform entry assays with HIV\textsubscript{ADA} because there is no molecular clone of this strain, which is required for the production of β-lactamase–containing viral particles.

\textit{P2X\textsubscript{1} receptors, but not P2X\textsubscript{7} or P2Y\textsubscript{1} receptors, facilitate HIV entry}

To determine whether the purinergic receptors P2X\textsubscript{1}, P2X\textsubscript{7}, and/or P2Y\textsubscript{1} that facilitate viral replication also participate in viral entry, we used the β-lactamase HIV entry assay to examine HIV entry into macrophages in the presence of 100 μM NF 279 (a P2X\textsubscript{1} antagonist), A-740003 (a P2X\textsubscript{7} antagonist), and MRS 2179 (a P2Y\textsubscript{1} antagonist). Concomitant treatment with the P2X\textsubscript{1} antagonist resulted in a 98% reduction in HIV\textsubscript{BaL} entry (Fig. 5A–C, n = 3, ***p < 0.0001). Neither the P2X\textsubscript{7} antagonist (Fig. 5D–F, n = 3) nor the P2Y\textsubscript{1} antagonist (Fig. 5G–I, n = 3) inhibited viral entry, despite the fact that P2X\textsubscript{7} antagonism resulted in a 70–91% reduction in viral replication, and P2Y\textsubscript{1} antagonism resulted in 60–70% inhibition of replication (Fig. 3B). This suggests that P2X\textsubscript{1} participates in viral entry, whereas P2X\textsubscript{7} and P2Y\textsubscript{1} regulate later stages of the viral life cycle.

\textit{HIV gp120 induces ATP release from macrophages}

We demonstrated that purinergic receptors play an important role in HIV replication, and P2X\textsubscript{1} receptors regulate HIV entry into macrophages. Activation of these receptors requires binding by an extracellular ligand, such as ATP. To determine whether HIV’s binding to CD4 and CCR5 on macrophages directly stimulates ATP release, we measured levels of extracellular ATP following treatment with HIV gp120, the surface protein that initiates viral binding and fusion. ATP in the supernatant was quantified using a chemiluminescent assay. Treatment of primary human macrophage cultures with gp120 (SF162 or BaL, 20–60 nM, both CCR5-tropic) resulted in a significant increase in ATP release (Fig. 6A, representative donor with an increase in ATP release at 10 min). No significant release was detected before 5 min of treatment. Peak ATP release always occurred within 5–15 min of gp120 treatment.

\textbf{FIGURE 5.} P2X\textsubscript{1} receptors are necessary for HIV entry into primary human macrophages. Macrophages were inoculated with β-lactamase–containing HIV\textsubscript{BaL} at an MOI of 0.001–0.005 for 4 h in the absence (A, D, G) or presence of 100 μM NF 279, a P2X\textsubscript{1} antagonist (B), 100 μM A-740003, a P2X\textsubscript{7} antagonist (E), or 100 μM MRS 2179, a P2Y\textsubscript{1} antagonist (H). Viral inoculation was followed by loading the cells with CCF2-AM, which fluoresces green. When viral entry occurs, β-lactamase cleavage of CCF2-AM results in blue fluorescence. (C, F, and I) Percentage of cells with viral entry in the presence of antagonist relative to HIV only (mean ± SD; n = 3). (C) The percentage of cells with viral entry decreased 98% with NF 279 treatment. Viral entry did not change with A-740003 (F) or MRS 2179 (I) treatment. Original magnification ×10. ***p < 0.0001.
treatment, depending upon the donor. A 2.8-fold increase in ATP release with gp120 treatment was detected for the peak response time to gp120 relative to vehicle-treated cultures (Fig. 6B, n = 10, *p = 0.0387).

Thus, we propose that HIV’s binding to CD4 and CCR5 results in an autocrine release of ATP. This extracellular ATP binds to P2X1 receptors, resulting in signaling that is necessary for HIV entry into macrophages (see proposed model, Fig. 6C). Other purinergic receptors, including P2X7 and P2Y1, likely participate in later stages of the viral life cycle and may be activated following accumulation of ATP or conversion of ATP to ADP (Fig. 6C). Activation of these receptors requires ADP or higher levels of extracellular ATP.

Discussion

We demonstrate a novel function for purinergic receptors as necessary mediators of HIV replication in macrophages. Selective P2X1, P2X7, and P2Y1 antagonists all blocked viral replication, but only the P2X1 antagonist inhibited viral entry. P2X7 and P2Y1 receptors likely participate in later stages of the viral life cycle. We also demonstrate that gp120’s binding to primary human macrophages results in local ATP release within 5–15 min, facilitating an autocrine activation of purinergic receptors.

There are three classes of purinergic receptors: P1, adenosine receptors, and P2X and P2Y, ATP receptors. P2X (1–7) receptors are ligand-gated cation channels, predominantly resulting in increased calcium flux from the extracellular space. P2Y (1–12) receptors are G protein coupled. The majority of P2Y receptor subtypes signal through IP3 to cause an increase in intracellular calcium levels from intracellular stores (19). We identified a role for P2X1, P2X7, and P2Y1 receptors in HIV replication. Each of these receptors participates in inflammation and the host response to pathogens. P2X1 receptors facilitate neutrophil chemotaxis, increasing neutrophil recruitment to sites of inflammation, and participate in thrombin-induced platelet activation (32, 33). P2Y1 receptors are required for inflammatory hyperalgesia, Chlamydia pneumoniae-induced platelet aggregation, and chemokine secretion by keratinocytes (34–36). P2X7 receptors have a wide range of inflammatory functions, including cytokine secretion, particularly IL-1β secretion, cell-to-cell fusion, and superoxide formation (22–24, 37). P2X7 receptors also regulate the host response to Toxoplasma gondii, Chlamydia trachomatis, and Mycobacterium tuberculosis (38–40). Thus, our data indicate a novel role for P2 receptors in facilitating HIV replication, contributing to our understanding of the significance of these receptors in mechanisms of infection and in the immune system.

We showed that P2X1 receptor is the predominant subtype required for HIV entry and that P2X7 and P2Y1 are involved in later stages of the viral life cycle. HIV entry involves binding of gp120 to host CD4 and subsequent conformational changes that enable binding to the coreceptor CCR5 or CXCR4. Binding of these proteins leads to fusion with the cell membrane at the cell surface or, in some circumstances, within an endosomal compartment. The role of other host cell proteins involved in viral entry is not well characterized, but studies indicate that viral binding initiates a signaling cascade that may be necessary for entry. Treatment of

FIGURE 6. HIV gp120 induces an increase in ATP release from primary human macrophages. Cells were inoculated with 20–60 nM monomeric R5-tropic gp120 (n = 2) or gp120 (n = 8) for 2, 5, 10, 15, 30, or 45 min. ATP levels in the supernatant were quantified using a luminescence assay. ATP concentration increased at 5, 10, or 15 min, depending upon the donor used. (A) ATP release for one representative donor with peak at 10 min. Fold change in ATP release was calculated for the time when there was maximal increase. (B) Peak fold change in ATP release (mean ± SD). HIV gp120 significantly increased ATP release 2.8-fold (n = 8). Mechanical stress (repeated pipetting of media) to the macrophage cultures was used as a positive control. (C) Proposed model for purinergic receptor involvement in HIV replication. HIV’s binding to CD4 and CCR5 (1) induces signaling (2), potentially through Goq proteins that lead to ATP release (3). Extracellular ATP binds to and activates P2X1 receptors, causing calcium influx (4) that facilitates HIV entry (5). ATP release continues; over time enough ATP accumulates to activate P2X7 receptors, leading to further calcium influx and downstream signaling events that facilitate later stages in the HIV life cycle (6). With time, ATP will also be converted to ADP, activating P2Y1 receptors, which may signal through Goq to increase intracellular calcium and cause signaling that facilitates later stages in the HIV life cycle (7). *p = 0.0387.
macrophages with HIV gp120 induces CCR5-dependent calcium influx (16, 18). Studies with cell lines indicate that gp120 signals through CCR5 to activate Goq and initiates signaling involving phospholipase C, protein kinase C, Pyk2, and Ras (15). This signaling leads to Rac1-mediated rearrangements in the actin cytoskeleton that facilitate membrane fusion and may be necessary for viral entry (15). We demonstrated that signaling induced by gp120’s binding also stimulates ATP release. We propose that subsequent autocrine activation of P2X1 receptors by extracellular ATP results in facilitation of infection.

We demonstrated an ATP release induced by gp120’s binding to macrophages. The cellular mechanisms for ATP release in primary human macrophages have not been well characterized, but a number of potential mechanisms have been identified in other cell types. Pannexin and connexin hemichannels are proteins through which extracellular ATP is released in many cells, including those of the immune system (41–44). Recently, it was demonstrated that T cell lines release ATP through opening of pannexin-1 hemichannels upon HIV infection (45) or during cell to cell fusion (37). These large-pore channels are activated by signals of cellular stress, including increased intracellular calcium (46), which occurs in macrophages following gp120’s binding. These hemichannels are likely activated following gp120’s binding and may represent a conduit for ATP release. Studies in our laboratory are currently examining the role of hemichannel activation in ATP release and HIV entry in macrophages. Other potential mechanisms of ATP release include voltage-dependent anion channels or exocytosis from ATP-containing vesicles (47).

Regardless of the mechanism, it appears that the initial ATP release that occurs with gp120’s binding activates P2X7 and that this receptor specifically mediates viral entry rather than P2X1 and P2Y1, which we also found to be involved in viral replication. P2X1 is highly responsive to ATP, being activated by low nanomolar concentrations, whereas ATP is a relatively poor agonist of P2X7, and ADP is the predominant agonist of P2Y1 (48–50). Therefore, P2X1 is likely activated initially by ATP, whereas activation of the other receptors may be delayed, requiring an accumulation of ATP or its conversion to ADP by ectonucleotidases. In agreement, our apyrase data indicated no reduction in HIV entry or replication in macrophages. This may be due to the fact that apyrase breaks down ATP to ADP and subsequently to adenosine, and all of these products also activate purinergic and adenosine receptors. Thus, we propose that not only ATP, but perhaps also products generated from ATP cleavage, are required for HIV entry and replication. We are currently examining the kinetics of purinergic receptor activation and the role of P2X1 and P2Y1 receptors in later stages of viral replication, including viral transcription and budding.

Our findings that purinergic receptors are necessary for HIV entry and later stages in viral replication have therapeutic implications. The nearly complete inhibition of viral replication by multiple purinergic receptor antagonists suggests that these receptors may be appropriate targets for therapy. A number of purinergic receptor antagonists are already in animal model or human testing for treatment of neuropathic pain, inflammatory disease, and potentially depression (31, 51–54). Three P2X1 receptor antagonists, AZD9056, CE-224535, and EVT-401, are in clinical trials for treatment of rheumatoid arthritis (53). Studies using oATP, which we found to be a potent inhibitor of HIV replication and of viral entry, in an in vivo mouse model demonstrated that it blocks purinergic receptors systemically and can prevent the onset of diabetes and inflammatory bowel disease (54). BBG, another antagonist that we showed to be an inhibitor of viral replication, has also been tested in vivo in a rat model of traumatic spinal cord injury. This study demonstrated recovery of motor function and limited inflammation following spinal cord injury without any toxicity of BBG (31). Our findings indicate that these therapeutic treatments should also be considered as treatment regimens in HIV-infected individuals.

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Disclosures

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References