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Chemotaxis and Calcium Responses of Phagocytes to Formyl Peptide Receptor Ligands Is Differentially Regulated by Cyclic ADP Ribose

Santiago Partida-Sánchez,* Pablo Iribarren,† Miguel E. Moreno-García,* Ji-Liang Gao,‡ Philip M. Murphy,† Norman Oppenheimer,§ Ji Ming Wang,‡ and Frances E. Lund2*

Cyclic ADP ribose (cADPR) is a calcium-mobilizing metabolite that regulates intracellular calcium release and extracellular calcium influx. Although the role of cADPR in modulating calcium mobilization has been extensively examined, its potential role in regulating immunologic responses is less well understood. We previously reported that cADPR, produced by the ADP-ribosyl cyclase, CD38, controls calcium influx and chemotaxis of murine neutrophils responding to fMLF, a peptide agonist for two chemoattractant receptor subtypes, formyl peptide receptor and formyl peptide receptor-like 1. In this study, we examine whether cADPR is required for chemotaxis of human monocytes and neutrophils to a diverse array of chemoattractants. We found that a cADPR antagonist and a CD38 substrate analogue inhibited the chemotaxis of human phagocytic cells to a number of formyl peptide receptor-like 1-specific ligands but had no effect on the chemotactic response of these cells to ligands selective for formyl peptide receptor. In addition, we show that the cADPR antagonist blocks the chemotaxis of human monocytes to CXCR4, CCR1, and CCR5 ligands. In all cases, we found that cADPR modulates intracellular free calcium levels in cells activated by chemokines that induce extracellular calcium influx in the apparent absence of significant intracellular calcium release. Thus, cADPR regulates calcium signaling of a discrete subset of chemoattractant receptors expressed by human leukocytes. Since many of the chemoattractant receptors regulated by cADPR bind to ligands that are associated with clinical pathology, cADPR and CD38 represent novel drug targets with potential application in chronic inflammatory and neurodegenerative disease.

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A deoxynosine 5′-diphosphate ribosyl cyclases (cyclases), such as the mammalian ecto-enzyme CD38, transform NAD+ into several products including the calcium-mobilizing metabolite cyclic ADP-ribose (cADPR)3 (1, 2). Since CD38 is a member of a highly conserved family of cyclases isolated from plants, invertebrates, and vertebrates (2), it has been hypothesized that CD38, via its production of cADPR, is likely to be an important regulator of calcium-based signal transduction. cADPR modulates the level of intracellular free calcium in cells in at least two ways. In combination with free cytosolic calcium, cADPR induces intracellular calcium release from ryanodine receptor-gated stores by a process referred to as calcium-induced calcium release (3). In addition, cADPR has been shown to regulate the influx of extracellular calcium (4, 5), possibly by activating the store-operated calcium release-activated calcium current channels (I_{SOCA}) (6). Although it is very clear that cADPR modulates intracellular free calcium levels in cells, less is known about which receptors rely on cADPR for signaling. In addition, very little is understood about the role(s) for cADPR in regulating important cellular processes such as development, growth, and differentiation.

To address which receptors utilize cADPR for signaling, several laboratories have now synthesized a number of different cADPR inhibitors including 8-Br-cADPR, a potent cADPR antagonist (7) and N(8-Br-A)D+, a NAD+ analogue that can be cyclized by CD38 into the cADPR antagonist 8-Br-cADPR (5). These antagonists have recently been successfully used to identify receptors such as the muscarinic receptor that mobilizes calcium in a cADPR-dependent fashion (8, 9). To assess the in vivo signaling role of cADPR, we produced mice that lack CD38 (10), one of the two known mammalian ADP-ribosyl cyclases (2). Using bone marrow neutrophils isolated from the CD38 knockout (KO) mice, we demonstrated that calcium signaling induced upon ligation of the classical chemoattractant formyl peptide receptor (FPR) is dependent on CD38 and cADPR (5). Importantly, we also found that chemotaxis of mouse neutrophils to the FPR ligand fMLF is regulated by cADPR and CD38 (5). Furthermore, we showed that pretreatment of normal mouse neutrophils with either 8-Br-cADPR or N(8-Br-A)D+ inhibited the chemotactic response of these normal neutrophils to fMLF (5, 11). Together, these data showed that cADPR and the ADP-ribosyl cyclase CD38 modulate FPR-induced signal transduction and control the chemotactic responses of mouse neutrophils to fMLF.

The G protein-coupled FPR is one of the founding members of the chemoattractant receptor superfamily (12–15). Like many of...
the chemotactic receptors, FPRs are constitutively expressed by myeloid lineage cells such as neutrophils, monocytes/macrophages, and dendritic cells (15). The prototype FPR ligand fMLF induces a number of biologic activities in myeloid cells, including chemokinesis, chemotaxis, cytokine production, and superoxide generation (13, 14, 16–18). Since N-formylated peptides are derived from bacterial (19–21) or mitochondrial proteins (22), investigators initially proposed that the primary function of FPRs is to promote the traffic of phagocytes to sites of infection and tissue damage where they can exert their antibacterial effector functions and also clear cell debris. Indeed, mice deficient in one of the known FPRs, mouse FPR (mFPR)-like 1, have been shown to be more susceptible to bacterial infections (23).

Recently, it has become clear that regulation of inflammatory processes by FPRs is more complex than originally assumed. In addition to the “classical” high-affinity FPR, it is now known that human myeloid cells also express a second related receptor, referred to as FPR-like 1 (FPRL1) (24). FPRL1, unlike FPR, is a low-affinity receptor for fMLF and is only activated by high (micromolar) concentrations of fMLF (25). A wide variety of new ligands for the different FPRs have recently been identified. These include peptide library-derived agonists that activate FPR and/or FPRL1 such as MMK1 (26), W (27), and A5 (28) peptides. FPR and FPRL1 can also be activated by peptides derived from host and pathogen proteins. In particular, FPR is activated by the T20 peptide derived from gp41 of HIV-1 (29), while FPRL1 can be activated by F peptide and V3 peptide derived from HIV-1 gp120 (30, 31). Likewise, FPR binds a peptide derived from the endogenous protein annexin 1 (lipocortin) (32), while FPRL1 binds serum amyloid A (SAA) (33), β amyloid peptide (34), prion protein peptide (35), and the lipid metabolite lipoxin A₄ (36).

Given that several of the naturally occurring FPR/FPRL1 ligands are associated with the pathology that accompanies diseases such as amyloidosis, Alzheimer’s disease, and prion disease, it has been speculated that cells responding to FPR/FPRL1 ligands may contribute to the inflammatory pathology observed in the diseased tissues (18, 37, 38). Since cADPR regulates fMLF-induced calcium signaling in mouse neutrophils (5), we hypothesized that this metabolite might also regulate signaling through FPRs in human cells. To determine whether cADPR regulates signaling through FPR, FPRL1, or both receptors, we have now examined the effect of cADPR antagonists or CD38 substrate analogues on the calcium and chemotactic responses of human neutrophils and monocytes to FPR and FPRL1-specific ligands as well as to a number of additional inflammatory and homeostatic chemokines. Our results demonstrate that cADPR regulates calcium mobilization and chemotaxis of human neutrophils and monocytes that have been stimulated with a discreet, biochemically distinct subset of chemotactic receptors.

Materials and Methods

Mice, cell lines, and NAD⁺/cADPR analogues

mFPR-deficient mice (backcrossed to C57BL/6j for six generations) were produced as previously described (23) and wild-type C57BL/6j control animals were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in the Trudeau Institute Animal Breeding Facility in accordance with all Trudeau Institute Institutional Animal Care and Use Committee guidelines. The N9 marine microglial cell line (39) was a kind gift from Dr. P. Ricciardi-Castagnoli (Università Degli Studi di Milano-Bicocca, Milan, Italy). The cells were grown in IMDM supplemented with 5% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 mM 2-ME. The cADPR antagonist 8-Br-cADPR was synthesized according to the protocol of Abdallah et al. (40).

Isolation of neutrophils and monocytes

Mouse bone marrow neutrophils were prepared by flushing bone marrow from wild-type or CD11c-deficient and C57BL/6j mice and then positively selecting the neutrophils using biotinylated GR-1 (BD Pharmingen, San Diego, CA) and MACS Streptavidin Microbeads (Miltenyi Biotech, Auburn, CA). Purity was >95% as assessed by FACS. Human leukocytes were isolated from fresh peripheral blood donated by healthy volunteers in accordance with the Trudeau Institute Institutional Review Board regulations (samples kindly provided by the Blood Donor Center, Champlain Valley Plattsburgh Hospital, Plattsburg, NY). Neutrophils were purified (>95% purity) using a one-step Ficol gradient (Robbins Scientific, Sunnyvale, CA). Monocytes were isolated by enriching for mononuclear cells using the one-step Ficol density gradient centrifugation method and then purified (>95%) by MACS using a CD14 monocyte isolation kit (Miltenyi Biotech). All purified cells were washed and resuspended in HBSS supplemented with 1% FBS.

Analysis of CD38 expression and cyclase activity in human peripheral blood leukocytes

Human peripheral blood leukocytes were isolated from whole fresh peripheral blood by Ficol density gradient centrifugation and then assessed for CD38 expression by FACS. Cell suspensions were stained with mouse anti-human CD38-biotin (Caltag Laboratories, Burlington, CA) or biotinylated mouse anti-isotype control Ab (Zymed Laboratories, San Francisco, CA) and anti-human CD15-FTTC (BD Biosciences, San Jose, CA) and then streptavidin-allophycocyanin (BD Biosciences), and were then analyzed by flow cytometry using a FACS Calibur (BD Biosciences).

To measure CD38-dependent ADP ribosyl cyclase activity in neutrophil lysates, purified human peripheral blood neutrophils (1.5×10⁶ cells) were disrupted using 2 mM EDTA, 50 μg/ml leupetin, 1 μg/ml pepstatin, 50 μg/ml PMSF, and 1% Triton X-100 (v/v; Sigma-Aldrich). Solubilized proteins were recovered from the lysate by centrifugation and were incubated with biotinylated mouse anti-human CD38 (Caltag Laboratories) or a biotinylated mouse IgG1 Ab (Zymed Laboratories) along with streptavidin agarose beads (Sigma-Aldrich). The precipitated protein-bead complexes were extensively washed and then resuspended in 40 μl of HBSS. CD38-dependent GDP-ribosyl cyclase activity was determined by measuring accumulation of the fluorescent product, cyclic GDP-ribose (cGDPR), as previously described (41). Aliquots of the protein-bead complex (10 μl) were placed in individual wells of opaque 96-well plates (Corning, Rochester, NY) containing 80 μl of HBSS/well and were allowed to settle for 30 min. NOD⁻ (10 μl, 40 μM final concentration) was added to each well and the plates were incubated for 20 min at 37°C. Cyclase activity was then determined by monitoring the accumulation of cGDPR in the reaction using a SpectraMax Gemini XS microplate fluorometer ( Molecular Devices, Sunnyvale, CA) that was calibrated for an excitation wavelength of 300 nm and an emission wavelength of 415 nm. Relative fluorescence units are reported.

Chemotacticants

The chemokines used in this study were obtained from either Sigma-Aldrich (IL-8 and CA5) or R&D Systems (macrophage-inflammatory protein [MIP] Ια and RANTES; Minneapolis, MN). Chemotactic peptides used in this study included the following FPRL1 agonists: the synthetic A5, W, and MKK-1 peptides (26–28), αmyloid β₄₄ peptide (42), HIV-derived F peptide (HIV-1, gp120 C4-V4 region) (30), HIV-derived V3 peptide (HIV-1, gp120 V3 region) (31), and SAA polypeptide (33). In addition, the study used the following FPRL1 agonists: HIV-derived T20 peptide (HIV-1 gp41) (29) and fMLF. N-formylated fMLF peptide was purchased from Sigma-Aldrich. A5 peptide and F peptide were purchased from New England Peptide (Witchich, MA). Amyloid β₄₄ peptide was purchased from Peninsular Laboratories (San Carlos, CA) and V3 peptide was obtained from Global Peptide Services (Fort Collins, CO). T20 peptide, MMK-1 peptide, and W peptide were synthesized and purified by the Department of Biochemistry, Colorado State University (Fort Collins, CO). The amino acid composition of all peptides was verified by mass spectrometry and the purity was shown to be >95%. No endotoxin was detected in the solubilized peptides.

Chemotaxis assays

Chemotaxis assays with mouse neutrophils and human neutrophils and monocytes were performed using 24-well Transwell plates (Costar, Cambridge, MA) with a 3-μm (for neutrophil chemotaxis assays) or a 5-μm (for monocyte chemotaxis assays) pore size polycarbonate filter. Chemotaxis assays for N9 cells were performed with 48-well chemotaxis chambers (NeuroProbe, Cabin John, MD) using polycarbonate filters with an 8-μm
pore size. Chemoattractants were diluted in HBSS and placed in the lower chamber, upper chamber, or both upper and lower chambers of the Transwell. In most experiments, cells were first pretreated for 15–20 min with either 8-Br-cADPR (0–100 μM; Sigma-Aldrich) or N-(8-Br-ADP)2 (500 μM) and then added to the upper chamber of the Transwell in the continued presence of the drug. For neutrophil assays, 1 × 10⁶ cells/Transwell were incubated at 37°C for 45 min, whereas for monocyte and microglial cell assays 1 × 10⁵ cells/Transwell were incubated at 37°C for 90 min. The transmigrated cells were collected from the lower chamber, fixed, and counted on a flow cytometer. To determine the absolute number of cells in each sample, a standard number of 20 μm size fluorescent microspheres (Polysciences, Warrington, PA) was added to each tube and counted along with the cells. The total number of transmigrated cells = the number of counted neutrophils × the total number of beads/the number of beads counted. In some cases the results are expressed as the mean ± SD of the chemotaxis index (CI). The CI represents the fold increase in the number of untreated or inhibitor-pretreated cells that migrated in response to the chemoattractant divided by the basal migration of untreated or antagonist pretreated cells migrating in response to control medium.

**Intracellular calcium mobilization**

Purified human peripheral blood neutrophils or monocytes were resuspended in cell-loading medium (HBSS plus 1 mM Ca²⁺ plus 1 mM Mg²⁺ plus 1% FBS plus 4 mM phenolred) at 1 × 10⁶ cells/ml and loaded with a mixture of the calcium-sensitive dyes Fluo-3 AM (4 μg/ml) and Fura-Red AM (10 μg/ml; Molecular Probes, Eugene, OR). The cells were incubated at 37°C for 30 min, washed twice, and resuspended in cell-loading medium at 1 × 10⁶ cells/ml. Cells were preincubated in the presence or absence of 8-Br-cADPR (100 μM; Sigma-Aldrich) for 20 min and then stimulated with various chemokines and chemoattractants in calcium-containing or calcium-depleted (plus 2 mM EGTA) medium. The accumulation of intracellular free calcium was assessed by FACS over the next 7 min by measuring the fluorescence emission of Fluo-3 in the FL-1 channel and Fura-Red in the FL-3 channel. Data were analyzed by FACS analysis software FlowJo 4.0 (Tree Star, San Carlos, CA) using the kinetic platform. The relative intracellular free calcium levels were expressed as the ratio between Fluo-3 and Fura-Red mean fluorescence intensity over time.

**Statistical analysis**

Data sets were analyzed using GraphPad Prism version 3.0a for Macintosh (GraphPad Software, San Diego, CA). Student’s t test analyses were applied to the data sets to determine statistically significant differences between groups. Differences were considered significant when p values were <0.05.

**Results**

Chemotaxis of mouse bone marrow neutrophils to mFPR1 and mFPR2 agonists is regulated by cADPR

Murine neutrophils express at least two functional fMLF receptors (mFPR1 and mFPR2) that can be activated by a number of different agonists, including fMLF, HIV-derived peptides, SAA polypeptide, and amyloid β peptide (14, 15). We previously showed that 8-Br-cADPR, a competitive antagonist of cADPR that acts by blocking cADPR-induced calcium mobilization (7), also inhibits chemotaxis of mouse bone marrow neutrophils to fMLF (5, 43). To test whether 8-Br-cADPR inhibits the migration of mouse neutrophils to other mFPR1 or mFPR2 ligands, we measured the chemotactic response of 8-Br-cADPR-treated neutrophils isolated from the bone marrow of C57BL/6 and mFPR1-deficient mice (Ref. 23; mFPR1 KO) to peptides that activate mFPR1 and/or mFPR2. In agreement with previous studies (44), we found that HIV-derived T20 peptide induced a strong migratory response in C57BL/6 neutrophils and essentially no response in mFPR1 KO neutrophils (Fig. 1), indicating that T20 peptide preferentially activates mFPR1. In contrast, fMLF, A5 peptide, and the HIV-derived F peptide induced the chemotaxis of mFPR1 KO neutrophils (Fig. 1B), indicating that these ligands can activate mFPR2 in mouse bone marrow neutrophils. However, all peptides including fMLF, A5 peptide, and F peptide induced even greater migration of C57BL/6/6 neutrophils compared with mFPR1 KO neutrophils (cf Fig. 1, A vs B), suggesting that these ligands, at least at the concentrations used in this experiment, may activate both mFPR1 and mFPR2 in mouse bone marrow neutrophils. Interestingly, 8-Br-cADPR treatment blocked the migration of both C57BL/6/6 and mFPR1 KO neutrophils to all of the mFPR1 and mFPR2 ligands tested (Fig. 1). This 8-Br-cADPR-mediated inhibition of chemotaxis to mFPR1 and mFPR2 ligands was specific for FPR ligands as 8-Br-cADPR treatment had no effect on the chemotaxis of mouse neutrophils to the CXCR2 agonists IL-8 and MIP-2 (data not shown and Ref. 5). Together, these data show that a cADPR antagonist can be used to inhibit the migration of mouse bone marrow neutrophils to a variety of FPR ligands and suggest that cADPR regulates the signaling of both mFPR1 and mFPR2 in mouse neutrophils.

**FIGURE 1.** Chemotaxis of mouse bone marrow neutrophils to mFPR1 and mFPR2 ligands is dependent on cADPR. Bone marrow neutrophils isolated from either C57BL/6J (A) or mFPR1-deficient mice (B) were preincubated for 15 min in medium () or medium containing 8-Br-cADPR (100 μM, ). Cells were then placed in the upper wells of chemotaxis chambers that contained medium or peptide chemoattractants that activate mFPR1 (HIV-derived T20 peptide) or mFPR1 and mFPR2 (fMLF, HIV-derived F peptide, and A5 peptide) in the lower wells of the chamber. Transmigrated cells were collected after 45 min and enumerated by flow cytometry. The results are expressed as the mean ± SD of triplicate wells for each experimental condition and are representative of at least four similar experiments. Values of p were determined by Student’s t test. *, p = 0.02; **, p = 0.001; ***, p = 0.0003.
Human neutrophils express a functional ADP-ribosyl cyclase

The cADPR antagonist 8-Br-cADPR inhibited the chemotaxis of mouse neutrophils to a number of different FPR ligands (Fig. 1). These data suggested that compounds that block cADPR-dependent signaling could be used therapeutically to modulate inflammatory responses mediated by the potentially pathogenic FPRL1 ligands. Since CD38 is the primary and best-characterized mammalian ADP-ribosyl cyclase (cyclase) (11, 45, 46), we first performed FACS analysis to determine whether CD38 is expressed by different subpopulations of human peripheral blood leukocytes. As has been previously reported (47), only a subset of the CD15<sup>high</sup> lymphocytes expressed CD38 while essentially all of the CD15<sup>low</sup> monocytes expressed CD38 (Fig. 2A). Although it has been previously suggested that CD38 is not expressed by human neutrophils (47), we found that the majority of the highly granular CD15<sup>high</sup>-expressing neutrophils expressed CD38, albeit at lower levels compared with the CD38-expressing monocytes and lymphocytes (Fig. 2A).

Primary human lymphocytes and monocytes have been previously shown to exhibit CD38-dependent ADP-ribosyl cyclase activity (48, 49); however, it has not been tested whether primary human neutrophils express a functional cyclase. To test whether the cyclase reaction could be catalyzed by neutrophil-derived CD38, we immunoprecipitated CD38 from lysates of purified peripheral blood neutrophils and then incubated the purified CD38 protein with a synthetic substrate, nicotinamide guanine dinucleotide (NGD<sup>+</sup>). Cyclases, such as CD38, utilize NGD<sup>+</sup> as a substrate and catalyze production of the highly fluorescent cyclic product cGDPR, which can be detected using a fluorometer (41). As shown in Fig. 2B, CD38 isolated from human neutrophils rapidly produced cGDPR when incubated with NGD<sup>+</sup>. Similar results were obtained upon analysis of polymorphonuclear cell (PMN) samples from multiple donors (data not shown) indicating that human neutrophils, like human lymphocytes and monocytes, express CD38 and can produce cyclic metabolites such as cADPR.

The cADPR antagonist 8-Br-cADPR blocks the chemotaxis of human neutrophils to FPRL1-specific ligands

Human neutrophils express at least two FPRs, the high-affinity fMLF-binding receptor FPR and the low-affinity fMLF-binding receptor FPRL1 (15). The human FPRL1 is considered to be an orthologue of the mFPR2 and both have a similar low affinity for fMLF (25, 50–52). Likewise, the human FPR is thought to be most closely related to the mFPR1, although the affinity of the mFPR1 for fMLF is 100- to 500-fold lower than the affinity of the human FPR for fMLF (25, 50–52). Using cells transfected with either FPR or FPRL1, it has been demonstrated that some agonists, such as fMLF and T20 peptide, specifically activate FPR while others, like A5 and amyloid β peptide, preferentially activate FPRL1 (reviewed in Refs. 14 and 15). Since the cADPR antagonist 8-Br-cADPR blocked the chemotaxis of mouse neutrophils to all of the FPR ligands that we tested, including agonists that are known to specifically activate human FPR or FPRL1, we predicted that 8-Br-cADPR would also inhibit the chemotaxis of human neutrophils to all of the different FPR- and FPRL1-binding ligands. To test this hypothesis, we incubated human peripheral blood neutrophils in the presence or absence of 8-Br-cADPR and then measured the migration of these cells to fMLF and to the synthetic FPRL1-specific agonist A5 peptide (28). Interestingly, treatment of human neutrophils with 8-Br-cADPR had no effect on the migration of these cells to nanomolar concentrations of fMLF (Fig. 3A). However, the cADPR antagonist did inhibit the migration of human neutrophils to the A5 peptide (Fig. 3B) in a dose-dependent manner (Fig. 3C). Thus, the cADPR antagonist blocked chemotaxis of human PMNs to a FPRL1-specific ligand but not to a FPR-specific ligand.

Since the cADPR antagonist inhibited neutrophil migration to the A5 peptide, a FPRL1-specific ligand, but had no effect on chemotaxis to very low concentrations of fMLF, we postulated that the cADPR antagonist inhibited FPRL1 dependent, but not FPR
dependent, signaling in human neutrophils. To test this hypothesis, we incubated human neutrophils in the presence or absence of 8-Br-cADPR and then performed migration assays using IL-8 and a variety of different chemoattractants with demonstrated specificity for either FPR or FPRL1. As shown in Fig. 4A and Table I, we found that 8-Br-cADPR treatment of human neutrophils had no effect on migration of the cells to IL-8. However, unlike what we had observed with murine neutrophils (Fig. 1), 8-Br-cADPR treatment of human neutrophils had minimal effect on the chemotactic response of the cells to fMLF or T20 peptide (Fig. 4A and Table I). Strikingly, however, the migration of human neutrophils to all of the FPRL1 ligands tested, including HIV-derived F peptide, HIV-derived V3 peptide, amyloid β peptide, MMK-1 peptide, and A5 peptide, was significantly inhibited by 8-Br-cADPR treatment (Fig. 4A and Table I). Taken together, these data indicate that the cADPR antagonist 8-Br-cADPR specifically inhibits the migration of human neutrophils to a FPRL1-specific ligand but has no effect on chemotaxis to fMLF, a high-affinity FPR ligand. Human peripheral blood neutrophils were preincubated in medium or medium containing 8-Br-cADPR (100 μM) for 15 min and then placed in the upper wells of the chemotaxis chamber. The lower wells of the chambers contained increasing concentrations of fMLF (A) or the FPRL1-specific ligand A5 peptide (B). Transmigrated cells were collected after 45 min and enumerated by flow cytometry. The results are expressed as the mean ± SD of triplicate wells for each experimental condition and are representative of at least four similar experiments using neutrophils from different donors. Values of p were determined by Student’s t test. *p = 0.001. C. Human peripheral blood neutrophils were preincubated in medium containing increasing concentrations of 8-Br-cADPR (1–100 μM) for 15 min and were then tested in a chemotaxis Transwell assay using 1 μM A5 peptide as the chemoattractant. One micromolar 8-Br-cADPR inhibited chemotaxis of neutrophils to A5 peptide by 50%.

FIGURE 3. A cADPR antagonist inhibits chemotaxis of human neutrophils to a FPRL1-specific ligand but has no effect on chemotaxis to fMLF, a high-affinity FPR ligand. Human peripheral blood neutrophils were preincubated in medium or medium containing 8-Br-cADPR (100 μM) for 15 min and then placed in the upper wells of the chemotaxis chamber. The lower wells of the chambers contained increasing concentrations of fMLF (A) or the FPRL1-specific ligand A5 peptide (B). Transmigrated cells were collected after 45 min and enumerated by flow cytometry. The results are expressed as the mean ± SD of triplicate wells for each experimental condition and are representative of at least four similar experiments using neutrophils from different donors. Values of p were determined by Student’s t test. *p = 0.001. C. Human peripheral blood neutrophils were preincubated in medium containing increasing concentrations of 8-Br-cADPR (1–100 μM) for 15 min and were then tested in a chemotaxis Transwell assay using 1 μM A5 peptide as the chemoattractant. One micromolar 8-Br-cADPR inhibited chemotaxis of neutrophils to A5 peptide by 50%.

FIGURE 4. Calcium mobilization and migration of human neutrophils to multiple FPRL1 ligands is dependent on cADPR. A. Migration of human neutrophils to IL-8, FPR-specific ligands (fMLF and T20 peptide), and FPRL1-specific ligands (MMK-1 peptide, HIV V3 and F peptides, amyloid β1-42 peptide, and A5 peptide) was measured using Transwell chambers. Human peripheral blood neutrophils were preincubated in medium or medium containing 8-Br-cADPR (100 μM) for 15 min and then placed in the upper well of the chemotaxis chamber. The cells that migrated to the bottom chamber in response to the chemotactic stimulus were collected after 45 min and then quantitated by flow cytometry. The results are expressed as the mean ± SD of the CI, which represents the fold increase in the number of untreated or 8-Br-cADPR-pretreated cells that migrated in response to the chemoattractant divided by the basal migration of untreated or 8-Br-cADPR-treated cells to control medium. The data are averaged from a minimum of three independent experiments using neutrophils from different donors and were performed in triplicate for each experimental condition. Values of p were determined by Student’s t test. *p = 0.001; **p = 0.0004; ***p = 0.0001. B. Peripheral blood human neutrophils were loaded with fluorescent calcium-detecting dyes Fluo-3 and Fura-Red and preincubated in the presence or absence of 8-Br-cADPR (100 μM) for 15 min. Cells were then stimulated with IL-8 or peptides that preferentially activate either FPR (fMLF and HIV T20 peptide) or FPRL1 (A5 and MMK-1 synthetic peptides) in medium containing extracellular calcium (blue line and red line) or calcium-free (plus 2 mM EGTA) medium (green line). Intracellular free calcium levels were measured by FACS for 7 min. Data are from a representative experiment of a total of at least three similar experiments using neutrophils isolated from different donors.
of human neutrophils to FPRL1-specific, but not FPR-specific, ligands.

cADPR regulates calcium mobilization in FPRL1-stimulated human neutrophils

Since cADPR is a known calcium-mobilizing metabolite (2), we predicted that cADPR might regulate intracellular free calcium levels in FPRL1-stimulated human neutrophils. To test this hypothesis, we measured the accumulation of intracellular free calcium in primary human neutrophils stimulated with IL-8, FPR-specific agonists, or FPRL1-specific agonists (Fig. 4B). As expected, calcium mobilization in response to IL-8 was essentially identical between 8-Br-cADPR-treated and control neutrophils (Fig. 4B). Likewise, 8-Br-cADPR treatment had minimal effect on the calcium response of human neutrophils stimulated with the FPR-specific ligands fMLF and T20 peptide (Fig. 4B). In contrast, the calcium response of 8-Br-cADPR-treated neutrophils to the FPRL1-specific ligands, A5 and MMK-1 peptides, was reduced compared with the neutrophils that were not pretreated with 8-Br-cADPR (Fig. 4B). Interestingly, calcium mobilization in FPRL1-stimulated neutrophils was completely dependent on the presence of external calcium because the calcium signal was abolished when external calcium was depleted from the medium using EGTA (Fig. 4B). This is in contrast to what we observed when the cells were stimulated with IL-8, T20 peptide, or fMLF, as a substantial calcium response was seen even when extracellular calcium was depleted from the medium (Fig. 4B). Thus, these data indicate that FPR and FPRL1 induce distinct calcium responses, with FPR inducing a calcium response that occurs independently of cADPR and is largely, although not exclusively, due to intracellular calcium release, while FPRL1 induces a calcium response that is regulated by cADPR and is primarily due to extracellular calcium influx.

cADPR antagonists block FPRL1-mediated chemotaxis, but not FPRL1-induced chemokinesis

Treatment of human neutrophils with the cADPR antagonist did not completely block migration of the neutrophils to the FPRL1 ligands (e.g., Fig. 4A). However, we have previously shown that 8-Br-cADPR specifically blocked the chemotaxis (chemoattractant-induced directional migration) of murine bone marrow neutrophils to fMLF, but had minimal effect on fMLF-induced chemokinesis (chemoattractant-induced nondirectional migration) (5).

Therefore, we concluded that cADPR-dependent signaling is most critical for mediating directional movement toward FPR ligands. To determine whether cADPR is required for the directional movement of FPRL1-stimulated human neutrophils, we incubated peripheral blood neutrophils in the presence or absence of 8-Br-cADPR and then performed a Transwell checkerboard assay to measure basal migration, chemokinesis, and chemotaxis. As shown in Fig. 5, A and B, pretreatment of human neutrophils with 8-Br-cADPR had no effect on basal migration, chemokinesis, or chemotaxis of neutrophils stimulated with the FPR ligands, T20 peptide, and fMLF. Likewise, treatment of neutrophils with 8-Br-cADPR had no effect on the basal migration or migration due to chemokinesis of neutrophils activated with the FPRL1 ligands, V3 peptide, F peptide, amyloid β peptide, and A5 peptide (Fig. 5, C–F). In striking contrast, 8-Br-cADPR treatment inhibited the directional migration of the neutrophils responding to the FPRL1 ligands and reduced the migration that can be specifically attributed to chemotaxis by ~90% (Fig. 5, C–F). Therefore, these data demonstrate that the cADPR antagonist specifically blocks the directional movement of human neutrophils that are responding to FPRL1-specifc signals.

The NAD⁺ analogue N(8-Br-A)D⁺ inhibits chemotaxis of FPRL1-activated neutrophils

Our experiments demonstrate that treatment of human neutrophils with a cADPR antagonist blocks the directional migration of these neutrophils to a number of FPRL1-specific ligands including inflammatory mediators such as amyloid β peptide. These data strongly suggest that compounds that either inhibit the activity of the cyclase(s) that produce cADPR or that alter the products that are produced by these cyclase(s) could also be used to block the migration of neutrophils responding to FPRL1-specific agonists. One easily synthesized compound that can be used to alter product formation by ADP-ribosyl cyclases is the NAD⁺ analogue N(8-Br-A)D⁺ (40). ADP-ribosyl cyclases, like CD38, utilize this NAD⁺ analogue as a substrate, but instead of producing cADPR, cells incubated with this substrate produce 8-Br-cADPR, the cADPR antagonist (7). In previous experiments using mouse neutrophils, we showed that treatment of neutrophils with N(8-Br-A)D⁺ inhibited the chemotactic response of the neutrophils to fMLF in a CD38- and cADPR-dependent manner (5, 11).

Since human neutrophils express CD38 and are dependent on cADPR for FPRL1-induced chemotactic responses, we predicted

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Table I. Migration of human neutrophils to FPRL1 ligands is inhibited by a cADPR antagonist

| Preincubation of Chemoattractant | No Attractant | 8-Br-cADPR | No Attractant | 8-Br-cADPR |
|--------------------------------|--------------|------------|--------------|------------|
| A5 peptide                     | 6,101 ± 737  | 6,877 ± 3,380 | 161,289 ± 12,353 | 76,272 ± 11,893* |
| Amyloid β                      | 6,092 ± 1,956 | 10,731 ± 997 | 69,532 ± 4,727  | 26,491 ± 2,107** |
| HIV V3                         | 1,775 ± 278  | 1,515 ± 1,540 | 12,395 ± 242    | 4,718 ± 1,179**  |
| HIV F                          | 3,493 ± 20   | 4,545 ± 1,836 | 13,761 ± 1,352  | 6,574 ± 719*     |
| MMK-1                          | 3,625 ± 130  | 3,404 ± 130  | 109,122 ± 4,808 | 67,323 ± 1,168** |
| HIV T20                        | 2,084 ± 176  | 2,899 ± 422  | 118,109 ± 4,011 | 127,470 ± 4,204  |
| fMLF                           | 1,732 ± 307  | 2,250 ± 840  | 186,265 ± 11,714| 185,900 ± 37,785 |
| IL-8                           | 4,442 ± 189  | 5,000 ± 1,108| 190,126 ± 29,459| 175,434 ± 8,906  |

* Human peripheral blood neutrophils were preincubated for 15 min in medium alone or 100 μM 8-Br-cADPR and then assessed in Transwell migration assays.

** The chemoattractants (or media control) were placed in the bottom well of the Transwell chambers at the concentrations listed in Fig. 4 legend.

The number of neutrophils that migrated to the bottom chamber in 45 min was determined by FACS and the mean and SD of triplicate wells for each condition are shown. The results are representative of at least four separate experiments for each chemoattractant analyzed.

* p = 0.001; ** p < 0.0004, Student’s t test.
that treatment of human neutrophils with \(N(8\text{-Br-A})\text{D}^+\) would inhibit the capacity of these cells to respond to chemotactic gradients of FPRL1-specific agonists. To test this prediction, we preincubated human peripheral blood neutrophils with \(N(8\text{-Br-A})\text{D}^+\) and then assessed the chemotactic potential of these cells in checkerboard assays using FPR and FPRL1 agonists as the chemotacticants. As shown in Fig. 6A, treatment of neutrophils with \(N(8\text{-Br-A})\text{D}^+\) had absolutely no effect on the chemokinetic or chemotactic

![FIGURE 6. A substrate analogue of CD38 inhibits FPRL1- but not FPR-induced chemotaxis of human neutrophils. Human peripheral blood neutrophils were preincubated in medium (□) or in medium containing \(N(8\text{-Br-A})\text{D}^+\) (500 \(\mu\text{M}, \text{■}\)) for 15 min and then placed in the upper wells of chemotaxis chambers. Checkerboard assays (as described in Fig. 5 legend) were performed using FPR-specific (fMLF, A) or FPRL1-specific (amyloid \(\beta_{1-42}\) peptide, B and C, respectively) ligands. The cells that migrated to the bottom chamber in 45 min were collected and enumerated by flow cytometry. The mean ± SD of the triplicate wells is shown. The data are representative of three independent experiments using neutrophils from different donors. Values of \(p\) were determined by Student’s \(t\) test. *, \(p < 0.001\).](http://www.jimmunol.org/)
response of the cells to the FPR ligand fMLF. In addition, the NAD$^+$ analogue did not affect the chemokinetic response of the neutrophils to the FPRL1 ligands amyloid β peptide and HIV-derived V3 peptide (Fig. 6, B and C). However, the chemotactic response of neutrophils responding to FPRL1 ligands was dramatically reduced when the cells were preincubated with N(8-Br-A)ID$^+$ (Fig. 6, B and C). Together, these results show that both NAD$^+$ analogues and cADPR antagonists can be used to block the directional migration of human neutrophils to inflammatory chemotactants that activate FPRL1 but not FPR.

8-Br-cADPR inhibits FPRL1/mFPR2-mediated chemotaxis of multiple cell types

Neutrophils are not the only cells that express FPRL1 and migrate directionally in response to FPRL1 ligands. Indeed, FPRL1 is constitutively expressed by monocytes (15) and is inducibly expressed by microglial cells (39), which reside in the CNS and are of myeloid origin. Since FPRL1-dependent migration is regulated by cADPR in neutrophils, we postulated that the cADPR antagonist 8-Br-cADPR could also be used to block the migration of other cell types to FPRL1 ligands. To examine this possibility, we tested the effect of 8-Br-cADPR treatment on migration of human monocytes in response to FPRL1-specific ligands. First, we assessed the chemotactic response of 8-Br-cADPR-treated human peripheral blood monocytes to FPR and FPRL1-specific ligands. As previously reported, peripheral blood monocytes migrated in response to a variety of FPR and FPRL1 ligands including HIV-derived peptides (Fig. 7A), amyloid peptides (Fig. 7B), and synthetic peptides (Fig. 7, A and B). Treatment of monocytes with 8-Br-cADPR had no effect on the chemotactic responses of these cells to the FPR ligands, fMLF and HIV-derived T20 peptide (Fig. 7, A and B). In contrast, the migration of the 8-Br-cADPR-treated monocytes was significantly reduced in response to the synthetic FPRL1 ligands MMK-1 and A5 peptides, the HIV-derived F peptide, and the amyloid peptides SAA and amyloid β peptide (Fig. 7, A and B). Therefore, in complete accordance with our data using peripheral blood neutrophils, we found that the cADPR antagonist inhibited the chemotaxis of primary human peripheral blood monocytes to a number of different FPRL1-specific ligands.

Next, we examined whether cADPR controls the migration of mouse myeloid-derived microglial cells to mFPR1/mFPR2 ligands. To do so, we used a murine microglial cell line, N9, that expresses typical markers of resting mouse microglia and are frequently used for functional analyses of microglial cells (18, 39, 53). Importantly, when N9 cells or normal mouse microglial cells are stimulated with LPS the ADP-ribosyl cyclase CD38 is expressed at low levels on the plasma membrane (data not shown), and mFPR2, the mouse orthologue of human FPRL1, is also up-regulated (18, 39). As expected, after 24 h of LPS treatment, the N9 cells expressed mFPR2 and could migrate in response to several mFPR2 ligands including the synthetic W and A5 peptides and high micromolar concentrations of fMLF (Fig. 7C). However, when the N9 cells were first pretreated with the cADPR antagonist 8-Br-cADPR, the cells were significantly impaired in their ability to migrate in response to any of the agonists (Fig. 7C). Interestingly, the cADPR antagonist had no effect on the chemotaxis of the microglial cells to another chemoattractant, C5a (Fig. 7C). Taken together, these data indicate that the cADPR antagonist specifically blocks FPRL1/mFPR2-dependent chemotaxis in myeloid-derived monocytes and microglial cells.

FIGURE 7. cADPR controls the migration of monocytes and myeloid-derived microglial cells to FPRL1-specific ligands. A and B, Human peripheral blood monocytes were preincubated with medium (□) or medium containing 8-Br-cADPR (100 μM, ■) for 15 min and then placed in the upper wells of chemotaxis chambers. The lower wells of the chambers contained peptides that preferentially activate FPR (fMLF and HIV T20 peptide) or FPRL1 (A5 peptide, amyloid β1-42 peptide, HIV-derived F and V3 peptides, SAA polypeptide, and MMK-1 peptide). Transmigrated cells were collected after 90 min and were enumerated by flow cytometry. The mean ± SD of the triplicate wells is shown. The data are representative of three independent experiments using neutrophils from different donors. C, N9 microglial cells were stimulated with 300 ng/ml LPS for 48 h and then preincubated with medium (□) or with 100 μM 8-Br-cADPR (■). Cell migration in response to the synthetic W peptide (10$^{-6}$ M), fMLF (10$^{-5}$ M), the synthetic A5 peptide (50 μg/ml), or C5a (100 ng/ml) was then determined by counting the number of cells present on the Transwell filter (number of cells per four high-power fields). The mean ± SD of the triplicate wells is shown. The data are representative of two independent experiments. Values of $p$ were determined by Student’s $t$ test. *, $p = 0.02$; **, $p = 0.001$; ***, $p = 0.0004$.

Treatment of monocytes with a cADPR antagonist blocks calcium influx and chemotaxis to homeostatic and inflammatory chemokines

The data from the experiments with human leukocytes indicated that cADPR is required for FPRL1-dependent chemotaxis but is...
monocytes to fMLF (FPR-specific ligand), SDF-1 (CXCR4 ligand), and to RANTES and MIP-1α.

Interestingly, migration of CCR1-expressing murine neutrophils to the CXCR4 ligand stromal cell-derived factor 1 (SDF-1) (Fig. 8B) or medium containing 8-Br-cADPR (100 μM, M) for 15 min and then placed in the upper well of the chemotaxis chamber. The cells that migrated to the bottom chamber in response to the chemotactic stimulus were collected after 90 min and then quantitated by flow cytometry. The mean ± SD of the triplicate wells is shown. The data are representative of at least three independent experiments using neutrophils from different donors. Values of p were determined by Student’s t test. *, p = 0.04; **, p = 0.001; ***, p < 0.0003. B, Peripheral blood human monocytes were loaded with fluorescent calcium-detecting dyes Fluo-3 and Fura-Red and preincubated in the presence (red line) or absence of 100 μM 8-Br-cADPR (blue and green lines) for 15 min. Cells were then stimulated with fMLF, SDF-1, or MIP-1α in medium containing extracellular calcium (blue line and red line) or calcium-free (plus 2 mM EGTA) medium (green line). Intracellular free calcium levels were measured by FACS for 7 min. Data are from a representative experiment of a total of at least three similar experiments using neutrophils isolated from different donors.

FIGURE 8. Calcium influx and chemotaxis of human monocytes to CCR5 and CXCR4 ligands is regulated by cADPR. A, Migration of human monocytes to fMLF (FPR-specific ligand), SDF-1 (CXCR4 ligand), and to RANTES and MIP-1α (CCR1/CCR5 ligands) was measured using Transwell chambers. Human peripheral blood monocytes were preincubated in medium (C) or medium containing 8-Br-cADPR (100 μM, M) for 15 min and then placed in the upper well of the chemotaxis chamber. The cells that migrated to the bottom chamber in response to the chemotactic stimulus were collected after 90 min and then quantitated by flow cytometry. The mean ± SD of the triplicate wells is shown. The data are representative of at least three independent experiments using neutrophils from different donors. Values of p were determined by Student’s t test. *, p = 0.04; ***, p = 0.001; ***, p < 0.0003. B, Peripheral blood human monocytes were loaded with fluorescent calcium-detecting dyes Fluo-3 and Fura-Red and preincubated in the presence (red line) or absence of 100 μM 8-Br-cADPR (blue and green lines) for 15 min. Cells were then stimulated with fMLF, SDF-1, or MIP-1α in medium containing extracellular calcium (blue line and red line) or calcium-free (plus 2 mM EGTA) medium (green line). Intracellular free calcium levels were measured by FACS for 7 min. Data are from a representative experiment of a total of at least three similar experiments using neutrophils isolated from different donors.

not needed for CXCR1/2 (IL-8 receptors), FPR, or C5a receptor-dependent chemotaxis. These data suggested that cADPR must regulate signaling through only a subset of the chemotactic receptors expressed by monocytes. Human peripheral blood monocytes are reported to express a number of additional chemokine receptors including CC1, CC2, CCR5, and CXCR4 (31, 54). To test whether cADPR regulates signaling through some of these other chemokine receptors, we measured calcium and chemotactic responses in 8-Br-cADPR-treated human monocytes that were stimulated with CCR1/CCR5 and CXCR4 agonists (Fig. 8). As expected, the chemotactic response of 8-Br-cADPR-treated human monocytes stimulated with the FPR ligand fMLF was completely normal (Fig. 8A). Likewise, the fMLF-induced calcium response was unaffected by the presence of 8-Br-cADPR (Fig. 8B). In contrast, 8-Br-cADPR treatment did block the migration of monocytes in response to the CCR1/CCR5 ligands RANTES and MIP-1α and to the CXCR4 ligand stromal cell-derived factor 1 (SDF-1) (Fig. 8A).

Interestingly, migration of CCR1-expressing murine neutrophils to MIP-1α was also found to be cADPR and CD38 dependent (data not shown). Although 8-Br-cADPR treatment did not completely inhibit calcium mobilization in SDF-1- or MIP-1α-stimulated monocytes, the response was significantly reduced (Fig. 8B). When the cells were stimulated in calcium-free medium, no calcium mobilization was observed after SDF-1 and MIP-1α stimulation (Fig. 8B). Thus, as we observed after stimulation with FPR1-specific ligands (see Fig. 4B), calcium mobilization induced by ligation of CCR1/CCR5 and CXCR4 is almost exclusively due to extracellular calcium influx. Taken together, cADPR appears to modulate calcium influx in cells that have been activated by chemotactic receptors that induce calcium mobilization via a calcium influx-dependent mechanism. This cADPR-regulated calcium influx signal is required for FPR1-, CCR1/CCR5-, and CXCR4-induced chemotaxis but is not obligate for FPR2-, CX3CR1/2-, or C5aR-mediated chemotaxis.

Discussion

The data presented in this manuscript demonstrate that cADPR regulates calcium signaling in a discreet subset of chemokine and chemoattractant receptors. The chemokine receptors that utilized cADPR for signaling could be distinguished from the other non-cADPR-dependent receptors because the receptors that utilized cADPR mobilized calcium in a unique fashion. Typically, it is believed that engagement of chemokine receptors leads to the generation of the calcium-mobilizing second messenger inositol trisphosphate (IP3) which in turn leads to release of intracellular calcium from IP3 receptor-gated stores in the endoplasmic reticulum (55). The initial, very rapid, short-lived release of calcium is often, although not always, accompanied by a delayed and sustained influx of extracellular calcium (55). This classical calcium response to chemotactic receptor engagement was observed after engagement of receptors such as C5aR (data not shown), CCR1/2 (Fig. 4), and the human high-affinity FPR (Fig. 4); receptors that all signal in a cADPR-independent fashion. In striking contrast and in agreement with what we previously observed with mFPR receptors (5), engagement of the chemotactic receptors that do utilize cADPR, such as FPR1L, CXCR4, CCR1, and CCR5, resulted in minimal intracellular calcium release that was accompanied by a very strong influx of extracellular calcium (Figs. 4 and 8).

Although it is not known how cADPR regulates extracellular calcium influx in any cell type, it has been suggested that cADPR regulates the activation of L-type calcium channels and store-operated Inac channels (6, 56). Interestingly, human neutrophils have been reported to express L-type channels as well as Inac channels (57, 58). Regardless of the exact mechanism by which cADPR regulates calcium influx in leukocytes, the data are clear that a specific cADPR antagonist partially inhibits the calcium response and blocks chemotaxis of human leukocytes responding to FPR1L, CXCR4, and CCR1/CCR5 ligands (i.e., Fig. 4).

These cADPR-dependent chemoattractant receptors are not the first chemoattractant receptors to be identified that mobilize calcium primarily by a calcium influx-dependent mechanism. Indeed, ligation of the platelet-activating factor receptor, CCR2, and CX1, CR1 induces minimal intracellular calcium release and strong calcium influx (59–62). Interestingly, no one has yet identified the
Despite the biochemical and functional differences between mFPR1 and human FPR, it is clear from the data presented here that a cADPR antagonist as well as a CD38 substrate analogue can be used to inhibit the chemotactic response of human and mouse neutrophils to a diverse array of mFPR2 and human FPR1-specific ligands including several synthetic peptides, multiple HIV gp120-derived peptides, and at least two different amyloidogenic peptides (Figs. 4 and 6). This result was not limited to neutrophils as we also found that chemotaxis of human monocytes and mouse myeloid-derived microglial cells to these same ligands was inhibited by the cADPR antagonist (Fig. 7). Therefore, based on these data, we suggest that the signal transduction pathways engaged upon mFPR2/FPR1 ligand in all three cell types are similarly dependent on cADPR. Furthermore, we predict that cADPR antagonists should be effective inhibitors of FPR1-dependent chemotaxis of all myeloid-derived human cells. Finally, given that a number of the FPR1-specific ligands are present in diseased tissue and are postulated to exacerbate the inflammatory response within these tissues (38), we predict that compounds like 8-BrcADPR and N(8Br-A)D3 are likely to be useful for assessing the impact of FPR1-induced inflammatory cell recruitment on the progression of pathology within the diseased tissues.

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References
1. Howard, M., J. C. Grimaldi, J. F. Bazan, F. E. Lund, L. Santos-Argumedo, R. M. E. Parkhouse, T. F. Walseth, and H. C. Lee. 1993. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. Science 262:1056.
2. Lee, H. C. 1993. Potentiation of calcium- and caffeine-induced calcium release by cyclic ADP-ribose. J. Biol. Chem. 268:293.
3. Guse, A. H., C. P. da Silva, I. Berg, A. L. Skapenko, K. Weber, P. Heyer, M. Hohenegger, G. A. Ashamu, H. Schultz-Koops, B. V. L. Potter, and G. W. Mayr. 1999. Regulation of calcium signaling in T lymphocytes by the second messenger cyclic ADP-ribose. Nature 398:70.
4. Partida-Sanchez, S., D. A. Cockayne, S. Monard, E. L. Jacobson, N. Oppenheimer, B. Garry, K. Kussel, S. Goodrich, M. Howard, A. Harmsen, et al. 2001. Cyclic ADP-ribose production by CD38 regulates intracellular calcium mobilization in chemoattractant-activated leukocytes and that these antagonists modulate the extracellular calcium influx induced by a selective group of chemoattractants and regulate only a subset of the biologic functions induced upon binding of these chemoattractants.

Our observation that two highly related human chemoattractant receptors, FPR and FPR1, can be distinguished by their reliance on cADPR for calcium signaling is of potential therapeutic importance. FPR1-dependent calcium mobilization is primarily due to extracellular calcium influx and utilizes cADPR, whereas FPR-dependent calcium mobilization is due in large part to intracellular calcium release and is cADPR independent. This result was initially somewhat surprising to us since we had previously shown that CD38 is necessary for chemotaxis of mouse neutrophils to the prototypic FPR receptor ligand fMLF (5). Indeed, the mFPR1 and human FPR are believed to be orthologues of one another and were presumed to signal through similar mechanisms (14, 15). However, the data presented here, as well as previous published data, indicate that there are significant differences between these two receptors. First, the affinity of fMLF for human FPR is quite high (nanomolar range) whereas the affinity of fMLF for the mFPR1 is ~400 times lower (51). Second, mFPR1 expressed by mouse bone marrow neutrophils appears to be a more promiscuous receptor as it can be activated by high concentrations of ligands such as HIV-derived F peptide and the synthetic A5 peptide (Fig. 1), which do not efficiently activate the high-affinity human FPR (15, 28). Third, chemotaxis induced upon mFPR1 engagement is inhibited by the cADPR antagonist (Fig. 1), whereas chemotaxis induced upon ligation of the human FPR is not affected by the cADPR antagonist (Fig. 3). Finally, fMLF only weakly induces extracellular calcium influx in human peripheral blood neutrophils (Fig. 4), while it strongly induces calcium entry in fMLF-stimulated mouse bone marrow neutrophils (5). Thus, while mFPR1 is most homologous at a structural level to human FPR (52), its affinity for fMLF, its reliance on cADPR, and the pattern of calcium mobilization induced by receptor engagement appears more similar to that seen with the human FPR1 and mFPR2 receptors.
Adenylyl cyclase (N-formylpeptide receptor) agonist activity measurement.