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Chemokine Production by G Protein-Coupled Receptor Activation in a Human Mast Cell Line: Roles of Extracellular Signal-Regulated Kinase and NFAT[1]

Hydar Ali,[2]* Jasimuddin Ahamed,* Cristina Hernandez-Munain,[3‡] Jonathan L. Baron,[†] Michael S. Krangel,[‡] and Dhavalkumar D. Patel[†‡]

Chemoattractants are thought to be the first mediators generated at sites of bacterial infection. We hypothesized that signaling through G protein-coupled chemoattractant receptors may stimulate cytokine production. To test this hypothesis, a human mast cell line (HMC-1) that normally expresses receptors for complement components C3a and C5a at low levels was stably transfected to express physiologic levels of fMLP receptors. We found that fMLP, but not C3a or C5a, induced macrophage inflammatory protein (MIP)-1β (CCL4) and monocyte chemoattractant protein-1 (CCL2) mRNA and protein. Although fMLP stimulated both sustained Ca^{2+} mobilization and phosphorylation of extracellular signal-regulated kinase (ERK), these responses to C3a or C5a were transient. However, transient expression of C3a receptors in HMC-1 cells rendered the cells responsive to C3a for sustained Ca^{2+} mobilization and MIP-1β production. The fMLP-induced chemokine production was blocked by pertussis toxin, PD98059, and cyclosporin A, which respectively inhibit $G_i\alpha$ activation, mitgen-activated protein kinase kinase-mediated ERK phosphorylation, and calcineurin-mediated activation of NFAT. Furthermore, fMLP, but not C5a, stimulated NFAT activation in HMC-1 cells. These data indicate that chemoattractant receptors induce chemokine production in HMC-1 cells with a selectivity that depends on the level of receptor expression, the length of their signaling time, and the synergistic interaction of multiple signaling pathways, including extracellular signal-regulated kinase phosphorylation, sustained Ca^{2+} mobilization and NFAT activation. ***The Journal of Immunology*****, 2000, 165: 7215–7223.**

Chemoattractants, such as formyl containing bacterial peptides (for example, fMLP), and the complement components C3a and C5a (1–3) are among the first mediators generated at sites of bacterial infection. These chemoattractants activate leukocyte chemotaxis and cause mediator release via their interaction with cell surface G protein-coupled receptors (GPCR).[4] fMLP causes the generation of superoxide anions and stimulates the production of IL-8 (CXCL8) in neutrophils and monocytes (4, 5). We hypothesized that leukocytes probably produce other proinflammatory cytokines in response to stimulation by chemoattractants.

The signaling pathways and biological responses activated by fMLP and C5a receptors (FR and C5aR) have been studied in the most detail in human neutrophils. However, very little information is available on the signaling pathways activated by C3aR. In neutrophils, both FR and C5aR couple to the same pertussis toxin (ptx)-sensitive G proteins to activate similar biological responses, such as superoxide generation and the release of proteolytic enzymes (3, 6, 7). The biochemical events that follow FR and C5aR activation in neutrophils include the activation of phospholipase C, phosphoinositol 3-kinase (PI3 kinase), increased association of Src family of protein kinases with $p21^{ras}$, and activation of p42/p44 mitogen-activated protein kinases (MAPK), known as extracellular signal-regulated kinases (ERKs) (6, 8, 9). The transcription factor NF-κB is activated by fMLP and C5a in neutrophils, and this activation is associated with IL-8 production (4, 5). The transcription factor NFAT regulates the activation of cytokine genes in Ag-stimulated T cells and mast cells (10, 11). Whether NFAT is activated by GPCR in leukocytes and what biological response it performs are not known.

The purpose of the present study was to determine whether GPCRs could activate the production of cytokines and to delineate the signaling pathways involved in this process. We have used a human mast cell line, HMC-1 cells, which is a rich source of chemokines (12), as a model for GPCR activation. Although this cell line natively expresses C3aR and C5aR at low levels, it does not express FR. Therefore, we developed this cell line to stably express physiologic levels of FR. We found that fMLP stimulated a sustained Ca^{2+} mobilization, whereas the responses to C3a or C5a were transient. Using this system, we demonstrate that fMLP induces the expression and release of the chemokines macrophage inflammatory protein (MIP)-1β and monocyte chemoattractant protein (MCP)-1. Furthermore, we have dissected the signaling pathways activated by GPCRs in HMC-1 cells and made the novel observations that their ability to stimulate chemokine production

*Department of Pathology, University of Pennsylvania School of Dental Medicine, Philadelphia, PA 19104; and Departments of [†]Medicine and [‡]Immunology, Duke University Medical Center, Durham, NC 27710

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[2] Address correspondence and reprint requests to Dr. Hydar Ali, Department of Pathology, University of Pennsylvania School of Dental Medicine, 4010 Locust Street, Philadelphia, PA 19104-6002. E-mail address: ali@path.dental.upenn.edu

[3] Present address: Basel Institute for Immunology, Grenzacherstrasse 487, 4005 Basel, Switzerland.

[4] Abbreviations used in this paper: GPCR, G protein-coupled receptor; FR, fMLP receptor; HA, hemagglutinin; HMC, human mast cell; MAPK, mitogen-activated protein kinase; MIP, macrophage inflammatory protein; MCP, monocyte chemotactic protein; ptx, pertussis toxin; PI3 kinase; phosphoinositol 3-kinase; cpt-cAMP, 8-(4-chlorophenylthio)-cAMP; RPA, RNase protection assay; CsA, cyclosporin A; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase.

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depends on the level of receptor expression, the length of signaling time, and the synergistic interaction of ERK phosphorylation, sustained Ca^{2+} mobilization and NFAT activation.

Materials and Methods

Materials

FMLP, wortmannin, bisindolylmaleimide (GF 109203X), PD98059, fluphenazine, and A23187 were purchased from Calbiochem (La Jolla, CA). [^{3}H]fMLP (53.6 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [γ-^{32}P]ATP was purchased from ICN Radiochemicals (Irvine, CA). Recombinant C5a and 8-(4-chlorophenylthio)-cAMP (cpt-cAMP) were purchased from Sigma (St. Louis, MO). PMA, indo-1/AM, and pluronic acid were obtained from Molecular Probes (Eugene, OR). Rabbit anit-ERK-1, anti-ERK-2, and anti-phospho-Elk-1 Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit anti-phospho-ERK Ab was purchased from New England Biolabs (Beverly, MA). 12CA5 Ab was obtained from Roche (Indianapolis, IN). Purified C3a was obtained from Advanced Research Technologies (San Diego, CA). The ptx was obtained from List Biologicals (Campbell, CA). The ECL Western blotting analysis kit was purchased from Amersham (Arlington Heights, IL). All tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD).

Cell culture and transfection

The human mast cell line HMC-1 was established from a patient suffering from mast cell leukemia and was provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN). HMC-1 cells were cultured in IMDM supplemented with 10% FCS, glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). For transfection, cells were washed twice in Earle's modified Eagle's medium supplemented with 50 mM HEPES and 20% FBS. Cells were resuspended in the same medium at a density of 5×10^7/ml, and 1×10^7 cells were electroporated (Gene Pulser, Bio-Rad, Hercules, CA) at 250 V and a capacitance of 500 microfarad in the presence of a mammalian expression vector pcDNA3 (10 μg) containing cDNA encoding hemagglutinin (HA)-tagged fMLP receptor (HA-FR) (13). The culture medium was replaced with fresh medium containing 1 mg/ml geneticin (G418) 24 h after transfection. Two weeks after electroporation, the antibiotic resistant cells were analyzed for cell surface expression of HA-FR by flow cytometry, and the top 3% of cells expressing the receptor were sorted by FACS and cultured for use in this study.

Radioligand binding

Binding of [^{3}H]fMLP to intact HMC-1 cells expressing HA-FR was conducted on 1×10^6 cells in 200 μl of HEPES-buffered saline supplemented with 1% BSA at 4°C for 4 h. Cells were collected by vacuum filtration on Whatman GF/C filters (Clifton, NJ) and washed four times with ice-cold saline, then dried filters were counted in scintillation fluid (13).

Calcium measurements

HMC-1 cells (3×10^6) were loaded with 1 μM indo-1/AM in the presence of 1 μM pluronic acid for 30 min at room temperature. Cells were washed and resuspended in 1.5 ml of HEPES-buffered saline. Intracellular Ca^{2+} measurements were conducted in a Perkin-Elmer fluorescence spectrophotometer (model 650-19, Norwalk, CT) with an excitation wavelength of 355 nm and an emission wavelength of 410 nm. Maximum and minimum fluorescence values were determined in the presence of 0.1% Triton X-100 and 20 mM Tris-HCl (pH 8.0)/5 mM EGTA, respectively. Intracellular Ca^{2+} concentrations were calculated using the following formula: $[Ca^{2+}] = K_d\ (F - F_{min})/F_{max\ -}\ F)$ (13, 14).

Analysis of cytokine mRNA expression by RNase protection assay (RPA)

HMC-1 cells (8×10^6/3 ml of complete growth medium) were exposed to fMLP, C3a, or C5a, incubated at 37°C for 1–15 h, collected, centrifuged, and washed with PBS. Total RNA was extracted and quantitated by absorbance at 260 nm. Cytokine mRNAs encoding IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13, IL-14, IL-15, TNF-α, TNF-β, LTβ, IFN-β, IFN-γ, TGF-β1, TGF-β2, TGF-β3, G-CSF, M-CSF, GM-CSF, stem cell factor, leukocyte inhibitory factor, oncostatin M, lymphotaetin, MCP-1, MIP-1α (CCL3), MIP-1β, I-309 (CCL1), IFN, γ-inducible protein of 10 kDa (IP-10, CXCL10), and RANTES (CCL5) were detected using the RiboQuant Multiprobe RPA template sets hCK-1, hCK-3, hCK-4, and hCK-5 (PharMingen, San Diego, CA). ^{32}P-labeled riboprobes were generated according to the manufacturer's recommendations and were hybridized overnight with 10 μg of RNA samples. The hybridized RNA was treated with RNase and purified according to the manufacturer's recommendations. The samples were electrophoresed in 6% polyacrylamide gels, the gels were dried and exposed, and protected fragments were quantified by phosphorimager analysis.

Assay of chemokine protein production by ELISA

For measurement of chemokine protein release, HMC-1 cells were resuspended in fresh complete growth medium (1×10^6/ml), and cells were stimulated with C3a, C5a, or fMLP for 6 h (unless otherwise stated). Supernatants were collected from centrifuged samples and were stored frozen at −80°C until analysis. Chemokine protein levels were quantified by sandwich ELISA using matched Ab pairs. ELISA plates (Costar, Cambridge, MA) were coated with 400 ng/well of capture Ab (14215.41 for MIP-1α (R&D Systems, Minneapolis, MN), 24006.111 for MIP-1β (R&D Systems), 6217.11 for IL-8 (R&D Systems), and B69–2 for MCP-1 (PharMingen)) in PBS overnight at 4°C, blocked with blocking buffer (PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3) for 2 h at room temperature, and washed with washing buffer (0.05% Tween-20 in PBS). Samples (100 μl) and standards were incubated for 2 h at room temperature, washed, and incubated with 20 ng/ml polyclonal biotinylated Abs to MIP-1α (R&D Systems), MIP-1β (R&D Systems), IL-8 (R&D Systems), or MCP-1 (PharMingen) for 2 h at room temperature. After washing, 0.1 ng/ml streptavidin-HRP (Zymed, South San Francisco, CA) was added for 20 min at room temperature, washed, and developed with tetramthylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 20 min. The peroxidase reaction was stopped by adding 0.5 volumes of 1 M phosphoric acid. The plate was read at 450 nm in an ELISA plate reader (Anthos, Durham, NC).

Phosphorylation of ERK-1/ERK-2

HMC-1 cells (1×10^6/sample) were stimulated with fMLP (1 μM) or C3a (10 nM) in HEPES-buffered saline, and the reaction was stopped at different time periods by the addition of a 3-fold excess of ice-cold PBS containing 1 mM sodium orthovanadate. Cells were harvested by centrifugation and resuspended in 50 μl of lysis buffer of the following composition: 150 mM NaCl, 50 mM Tris (pH 8.0), 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 100 μg/ml 1-chloro-3-tosylamido-4-phenyl-2-butanone, 50 μg/ml 1-chloro-3-tosylamido-7-amino-2-heptanone, and 10 μg/ml PMSF. The samples were centrifuged (10,000 × *g*, 10 min) to remove insoluble debris, and the supernatant was mixed with an equal volume of 2× SDS sample buffer and heated to 90°C for 10 min. Samples were electrophoresed in 10% SDS-polyacrylamide gels and transferred onto a nitrocellulose filter. The filter was treated with 3% nonfat milk in PBS and incubated with an Ab specific for phosphorylated ERK-1/ERK-2. The reaction was detected by enhanced chemiluminescence. The membrane was stripped and reprobed with an Ab that reacts with unphosphorylated ERK-1 or ERK-2.

Construction of HA-C3aR and transient transfection in HMC-1 cells

A nucleotide sequence encoding the nine-amino acid HA peptide (YPY DVPDYA) was inserted between the N-terminal initiator methionine and the second amino acid of C3aR by PCR. The 5′ oligonucleotide, in a 5′-3′ order, contained six miscellaneous bases, six bases encoding *Bam*HI, three bases encoding methionine, 27 bases encoding the HA tag, and 27 bases complementary to the cDNA of the C3aR encoding aa 2–10 of the receptor. The 3′-oligonucleotide was complementary to the C-terminal seven amino acids and a stop codon with a 5′ *Hin*dIII and ligated into a mammalian expression vector, pRK5 (13). Transient transfection of HMC-1 cells with empty vector (mock) or vector containing cDNA encoding HA-C3aR was performed as described above for FR, but the cells were used 16–18 h after transfection. Mock or C3aR-transfected cells were incubated with 12CA5 Ab followed by FITC-labeled goat anti-mouse IgG. The cells were washed, and receptor expression was analyzed by flow cytometry.

Nuclear extracts and EMSA

Nuclear extracts were prepared according to the method of Shreiber et al. (15) with some modification. HMC-1 cells (1×10^7) were treated with or without fMLP (1 μM) or C5a (100 nM) for 1 h at 37°C. Cells were washed in PBS and resuspended in 10 ml of ice-cold buffer A (10 mM HEPES (pH 7.6), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermidine, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin) and incubated for 15 min on ice. Nonidet P-40 was added from a 10% stock solution to a final concentration of 0.6%, and the sample

was vortexed for 10 s. Samples were centrifuged at 3,000 rpm for 10 min at 4°C, and the nuclei were resuspended in 10 ml of buffer C (20 mM HEPES (pH 7.6), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinin, and 2 μg/ml pepstatin). Suspensions were agitated for 30 min at 4°C, and nuclear extracts were separated from debris by centrifugation at 15,000 $\times$ *g* for 15 min at 4°C. Nuclear extracts were frozen at −70°C in aliquots containing 20% glycerol.

NFATc and AP-1 consensus and mutant double-stranded oligonucleotides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Top-strand sequences are NFATc consensus site 5′-CGCCAAAGAGGA AAATTTGTTTCATA, NFATc mutant site 5′CGCCCAAAGCTTAAA ATTTGTTTCATA, and AP-1 consensus site 5′-CGCTTGATGACTAC AGCCGGAA. Annealed binding sites were radiolabeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Radiolabeled oligonucleotides were purified by electrophoresis through an 8% polyacrylamide gel, overnight elution from gel slices at 37°C, concentration using an Elutip-d (Schleicher & Schuell, Keene, NH), and ethanol precipitation. EMSAs were performed as described previously (16) with some modifications. Nuclear extracts (3 μg) were incubated with 1 μg of poly(dI-dC) carrier in a 25-μl reaction mix containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol for 30 min either at room temperature (to test Ab inhibition) or at 4°C (to test oligonucleotide competition). For Ab inhibition experiments, 0.2 μg of goat affinity-purified anti-NFATc (SC-1149X, Santa Cruz Biotechnology) or goat control IgG was included in the incubation. For oligonucleotide competition experiments, unlabeled oligonucleotides (250 fmol) were included in the incubation. Labeled binding site probes (5 fmol, ~5–6 $\times$ 10^4 cpm) were then added for an additional 20 min of incubation at 4°C. Samples were electrophoresed through a 4% polyacrylamide gel containing 22.5 mM Tris-borate and 0.5 mM EDTA at 4°C.

Results

Characterization of expressed HA-FR in HMC-1 cells

HMC-1 cells, which endogenously express receptors for C3a and C5a at low levels, were stably transfected with cDNA encoding HA-FR. The transfectants were analyzed for their ability to bind [^{3}H]fMLP and to mobilize Ca^{2+} in response to fMLP. Saturation binding studies revealed the presence of 54,354 ± 3,478 binding sites/cell, which compares to 55,000–120,000 binding sites for natively expressed receptors in human neutrophils (17, 18). As shown in Fig. 1*A*, fMLP stimulated a dose-dependent increase in peak Ca^{2+} mobilization in transfected HMC-1 cells with an EC_{50} value of 80 nM, which is identical with the EC_{50} value reported for fMLP-stimulated degranulation in rat basophilic leukemia (RBL-2H3) cells expressing HA-FR (13). C3a and C5a also stimulated dose-dependent increases in Ca^{2+} mobilization, but with EC_{50} values of 0.1 and 3.3 nM, respectively (Fig. 1*A*). Although the peak intracellular Ca^{2+} mobilizations to all three ligands were similar in magnitude, there were remarkable differences in their ability to activate a sustained Ca^{2+} mobilization. Responses to all three stimulants reached a peak within 5 s after stimulation. However, C3a and C5a-induced Ca^{2+} mobilizations returned to basal levels within 2–3 min after stimulation. In contrast, fMLP-induced Ca^{2+} mobilization remained elevated for the duration of the experiment (up to 15 min). Ptx (100 ng/ml for 16 h) blocked Ca^{2+} mobilization to C3a, C5a, and both the initial Ca^{2+} spike as well as the sustained response to fMLP (data not shown).

Expression of chemokine mRNA and protein by HMC-1 cells

To determine the effects of signaling through chemoattractant receptors on cytokine expression, transfected HMC-1 cells were stimulated for different time periods with a concentration of chemoattractant that resulted in maximal Ca^{2+} mobilization; C3a (10 nM), C5a (100 nM), or fMLP (1 μM) and cytokine mRNAs were quantified by RPA. Resting HMC-1 cells expressed no mRNAs encoding cytokines IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-10, IL-13, IL-14, IFN-γ, oncostatin M, TGF-β2, TGF-β3, G-CSF, GM-CSF, or stem cell factor as determined by RPA, and stimulation with C3a, C5a, or fMLP did not induce their expression (not shown). HMC-1 cells constitutively expressed low levels of mRNAs encoding the cytokines IL-6, IL-15, leukocyte inhibitory factor, lymphotoxin β, M-CSF, and TGF-β1, but their expression was not changed by stimulation with C3a, C5a, or fMLP. For chemokines, neither unstimulated nor chemoattractant-stimulated HMC-1 cells expressed mRNAs for lymphotactin, I-309, INF-γ-inducible protein of 10 kDa, or RANTES (Fig. 2*A*). HMC-1 cells did express low levels of MIP-1β, MCP-1, and IL-8 transcripts. Stimulation with fMLP, but not C3a or C5a, for 3 h caused an up-regulation of MIP-1β and MCP-1 mRNAs (31 ± 17-fold for MIP-1β and 10 ± 2-fold for MCP-1; n = 4; Fig. 2, *A–C*). This effect of fMLP was due to the activation of its receptor, because fMLP did not stimulate this response in untransfected cells (Fig. 2*A*). The effect of fMLP on MIP-1α mRNA expression in this assay was not clear, but C3a or C5a did not cause up-regulation of mRNA for MIP-1α (Fig. 2*A*).

The ability of fMLP to up-regulate MIP-1β and MCP-1 mRNA reached a maximum by 3 h after stimulation and returned to baseline levels by l4 h (Fig. 2, *A* and *B*). To determine whether the up-regulation of mRNA by fMLP resulted in protein production,

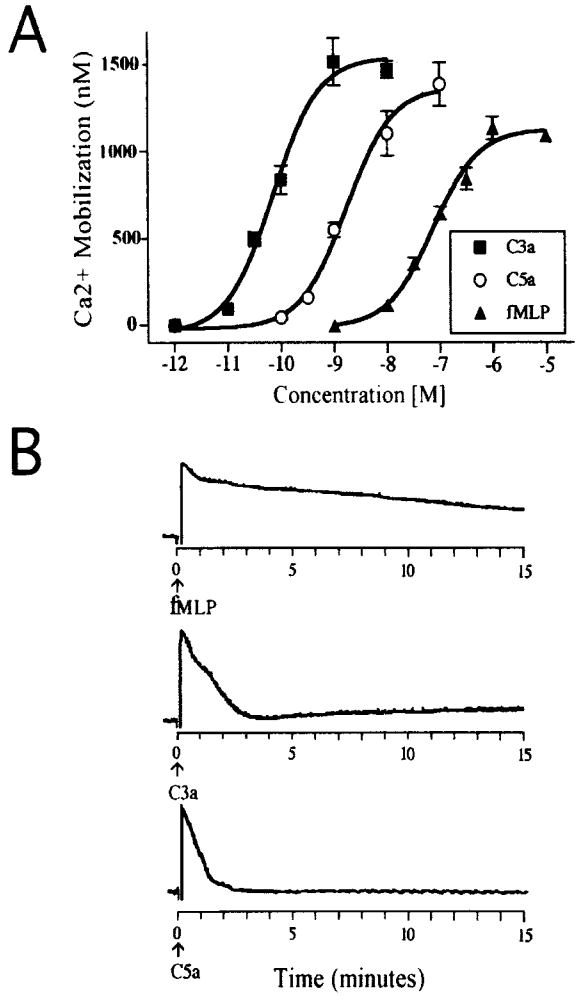

FIGURE 1. Calcium mobilization by fMLP, C3a, and C5a in HMC-1 cells. HMC-1 cells expressing FR (3 $\times$ 10^6/sample), were loaded with indo-1 and stimulated with different concentrations of C3a, C5a, or fMLP, and peak Ca^{2+} mobilization was determined (*A*). The basal response in the absence of chemoattractant was 199 ± 8 nM (n = 36). Points are the mean ± SE of three experiments. *B*, Tracing for Ca^{2+} in response to optimal concentrations of fMLP (1 μM), C3a (10 nM), and C5a (100 nM). Data are representative of three experiments.

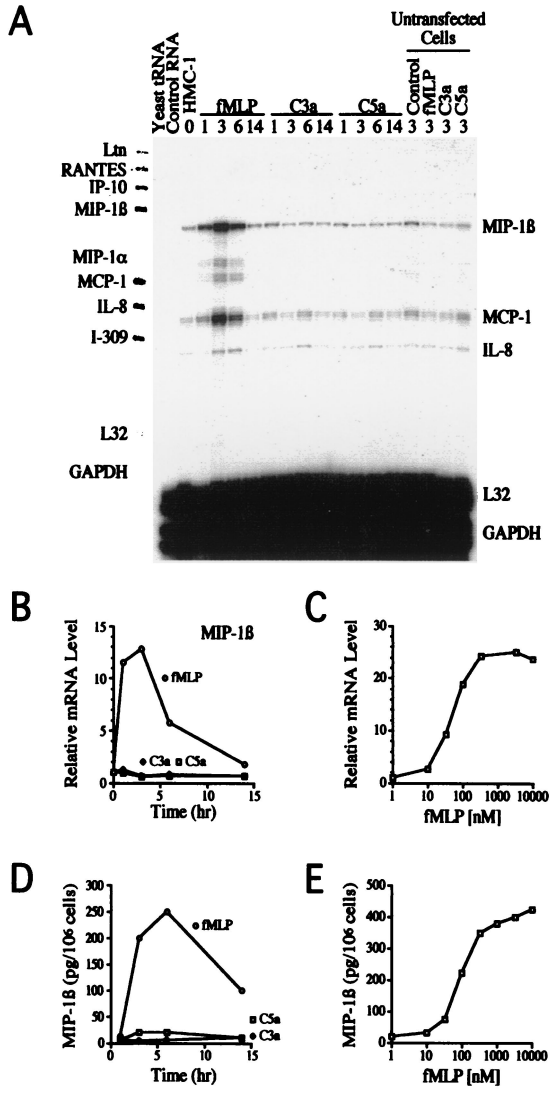

FIGURE 2. GPCR-mediated chemokine production in HMC-1 cells as determined by RPA and ELISA. *A*, For RPA, HMC-1 cells expressing FR (8×10^6 in 3 ml of medium) were stimulated with fMLP (1 μM), C3a (10 nM), or C5a (100 nM) for 0, 1, 3, 6, and 14 h. For a control, untransfected cells were also stimulated with fMLP, C3a, and C5a for 3 h. Total cellular RNA was isolated and hybridized with ^{32}P-labeled riboprobes. Following RNase digestion, the samples were resolved by SDS-PAGE, and the gels were exposed in a phosphorimager. *B*, Time course of fMLP-induced MIP-1β mRNA up-regulation in HMC-1 cells. Quantitation of mRNA for MIP1-β was performed with ImageQuant software and is expressed as a percentage of GAPDH. *C*, Dose-response curve of MIP-1β mRNA expression at 3 h in response to varying doses of fMLP. Data are expressed as a percentage of GAPDH. For the assay of chemokine protein production, HMC-1 cells (1×10^6 cells/1 ml medium) were incubated with fMLP (1 μM), C3a (10 nM), or C5a (100 nM) for different time periods (*D*) or with different concentrations of FMLP for 6 h (*E*). The samples were centrifuged, and supernatants were used to determine MIP-1β by ELISA. The data shown are representative of three experiments.

supernatants from fMLP-, C3a-, and C5a-stimulated HMC-1 cells were tested for MIP-1β and MCP-1 proteins by ELISA. Unstimulated cells secreted little or no MIP-1β. However, stimulation of HMC-1 cells with fMLP resulted in a time- and dose-dependent increase in MIP-1β protein production (Fig. 2, *D* and *E*). The production of MIP-1β was maximal at 6 h after stimulation and declined thereafter (Fig. 2*D*). As for Ca^{2+} mobilization, the EC_{50} for both fMLP-induced MIP-1β mRNA up-regulation (Fig. 2*C*) and protein release (Fig. 2*E*) was about 80 nM. Neither C3a (10 nM) nor C5a (100 nM) stimulated MIP-1β production in HMC-1 cells either at the mRNA or protein level (Fig. 2, *A*, *B*, and *D*). Neither C3a nor C5a, up to a concentration of 1 μM, stimulated MIP-1β production (data not shown). In human lung mast cells and HMC-1 cells, MCP-1 is released into the supernatant in a constitutive manner (19). We also found that incubation of HMC-1 cells with medium alone for 6 h resulted in production of MCP-1 to a level of 195 ± 26 pg/10^6 cells. Stimulation of cells with fMLP for 6 h resulted in a net increase of 730 ± 143 pg/10^6 cells. Under this condition, neither fMLP, C3a, nor C5a stimulated secretion of MIP-1α, RANTES, or IL-8 proteins in HMC-1 cells (data not shown).

Roles of G protein, protein kinase C, and ERK phosphorylation in fMLP-induced MIP1-β production in HMC-1 cells

FR couples to G_i-like G proteins in leukocytes (13, 18, 20). To test the role of signaling through $G_i\alpha$ proteins in chemokine gene expression, we incubated HMC-1 cells with ptx (100 ng/ml, overnight), which inhibits signaling through $G_i\alpha$, and stimulated cells with fMLP (1 μM) for 2 h (mRNA) and 6 h (chemokine release). Ptx had no effect on basal responses, but almost completely abolished both fMLP-stimulated mRNA induction and protein release (Fig. 3). To determine which signal transduction pathways may be involved in the generation of chemokines by GPCR activation, we tested the effects of inhibitors of protein kinase C (bisindolylmaleimide), PI3 kinase (wortmannin), mitogen-activated protein kinase kinase (MEK) (PD98059), and a membrane-permeable cAMP analogue, cpt-cAMP, an activator of PKA. Bisindolylmaleimide had no effect on fMLP-induced chemokine production at either the mRNA or protein level (Fig. 3). Stimulation of cells with PMA and the Ca^{2+} ionophore A23187 resulted in a much greater level of MIP-1β production than that in fMLP-stimulated cells (net increase of $\geq$2000 pg/10^6 cells compared with $\sim$300 pg/10^6 cells). Bisindolylmaleimide, which had no effect on fMLP-induced chemokine production, inhibited the response to PMA/A23187 by $>$95% (not shown). Cpt-cAMP (1 mM) or PD98059 (100 μM) caused substantial inhibition of fMLP-induced MCP-1 and MIP-1β mRNA up-regulation as well as MIP-1β protein release (Fig. 3). Wortmannin partially blocked fMLP-induced responses. These data suggest that the ability of fMLP to stimulate the expression of MCP-1 and MIP-1β genes in HMC-1 cells requires GPCR-mediated activation of MAPK, but not protein kinase C.

To further test the role of MAPK activation on chemokine production, the effect of fMLP on ERK phosphorylation was determined. HMC-1 cells were stimulated with fMLP (1 μM) or C3a (10 nM) for different time periods, and ERK phosphorylation was determined by Western blotting using an Ab that specifically recognizes the phosphorylated ERK-1 and ERK-2. As shown in Fig. 4, fMLP caused phosphorylation of both ERK-1 and ERK-2. The response reached a peak within 1 min after stimulation and was sustained for up to 15 min. Although C3a also caused an increase in ERK phosphorylation within 1 min, this response was transient and returned to basal within 5 min after stimulation (Fig. 4). The effects of the MEK-1 inhibitor PD98059 on fMLP-stimulated MIP-1β production, phosphorylation of both ERK and its downstream effector Elk-1 were tested. As shown in Fig. 5, PD98059 inhibited fMLP-induced MIP-1β production, ERK and ELK-1 phosphorylation in a dose-dependent manner. Ptx had no effect on basal ERK-2 phosphorylation, but substantially inhibited the response to fMLP (Fig. 6). Bisindolylmaleimide had no effect on fMLP-induced ERK phosphorylation (Fig. 6) or chemokine production (Fig. 3). Surprisingly, neither wortmannin nor cpt-cAMP inhibited fMLP-induced ERK-2 phosphorylation (Fig. 6) despite

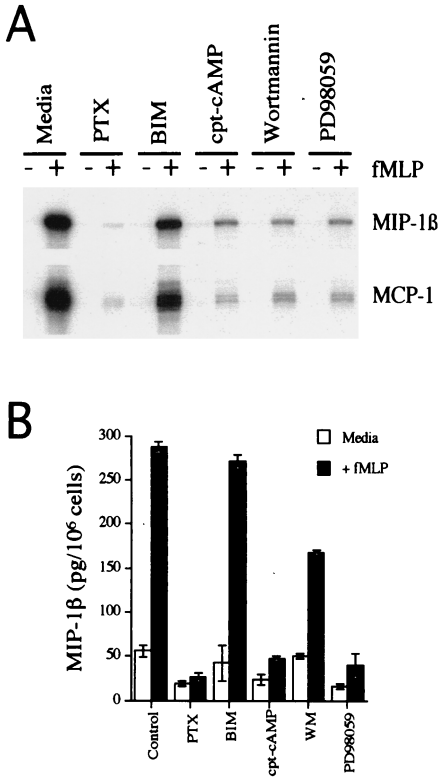

FIGURE 3. Effects of inhibitors on fMLP-stimulated chemokine production. HMC-1 cells expressing FR (8×10^6 in 3 ml medium for RPA (*A*) or 1×10^6 cells/1 ml medium for ELISA (*B*)) were incubated with medium, ptx (100 ng/ml, overnight), bisindolylmaleimide (BIM; 10 μM, 10 min), cpt-cAMP (1 mM, 10 min), wortmannin (100 nM, 30 min), or PD98059 (100 μM, 30 min) and stimulated WM; fMLP. *A*, The reaction was continued for 3 h after stimulation. Total cellular RNA was isolated and hybridized with ^{32}P-labeled riboprobes. Following RNase digestion, the samples were resolved by SDS-PAGE, and the gels were exposed in a phosphorimager. *B*, The reaction was stopped 6 h after stimulation. The samples were centrifuged, and supernatants were used to determine MIP-1β production by ELISA. The data shown are representative of three experiments.

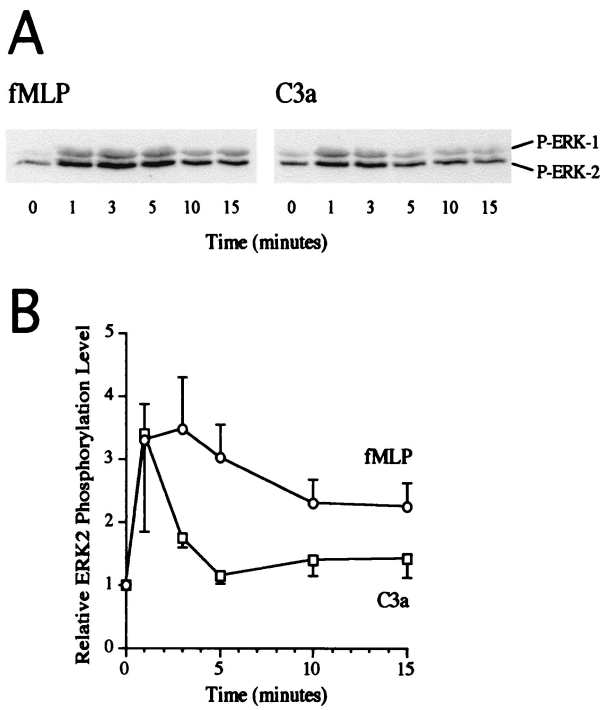

FIGURE 4. Kinetics of fMLP- and C3a-induced ERK phosphorylation. *A* HMC-1 cells were stimulated with fMLP (1 μM) or C3a (10 nM), and the reactions were stopped at different times by the addition of ice-cold PBS. Cell pellets were lysed, proteins separated on a 10% SDS-polyacrylamide gel, and Western blotting was performed with an Ab that specifically recognizes the phosphorylated forms of ERK-1 and ERK-2. Equal amounts of protein loading were confirmed by stripping the membrane and performing a Western blot with ERK-2 Ab (not shown). *B*, The extent of ERK-2 phosphorylation was expressed as the fold increase over the basal unstimulated response. The data shown are the mean ± SEM of four experiments.

the fact that they blocked the chemokine response to fMLP (Fig. 3). These findings suggest that fMLP-induced chemokine production involves synergistic interaction of MAPK with another signaling pathway.

Role of extracellular Ca^{2+} on GPCR-induced MIP-1β production in HMC-1 cells

EGTA binds to extracellular Ca^{2+} and blocks Ca^{2+} influx in response to receptor stimulation. To test the role of extracellular Ca^{2+} on fMLP-induced responses, HMC-1 cells were exposed to EGTA (5 mM) before stimulation with the ligand. As shown in Fig. 7, *A* and *B*, EGTA had no effect on the initial Ca^{2+} spike in response to fMLP, but it blocked the sustained Ca^{2+} mobilization. EGTA also completely inhibited fMLP-stimulated MIP-1β production (Fig. 7*C*). Calmodulin is a signaling molecule that mediates the effects of Ca^{2+} mobilization in many systems (21). To test the role of Ca^{2+}-mediated signaling on MIP-1β production, HMC-1 cells were pretreated with a calmodulin inhibitor fluphenazine before stimulation with fMLP. As shown in Fig. 7*C*, fluophenazine almost completely blocked the response to fMLP.

Unlike FR, which are expressed on the surface of leukocytes at high levels (50,000–120,000 receptors/cell), C3aR and C5aR are less abundant (3,000–15,000 receptors/cell) (22). To test whether increasing the level of C3aR expression could result in C3a-induced chemokine production, HMC-1 cells were transiently transfected with vector alone (mock) or with cDNA encoding HA-tagged C3aR. FACS analysis with 12CA5 Ab, which recognizes the HA tag on the transfected receptor but does not bind to the native receptor, revealed that ~40% of the cells expressed HA-tagged C3aR (Fig. 8*A*). As expected, C3a stimulated a transient Ca^{2+} mobilization in mock-transfected cells, but this response was more sustained in cells transiently expressing C3aR (Fig. 8*B*). This sustained Ca^{2+} response was associated with C3a-induced MIP-1β production (Fig. 8*C*). Similar data were obtained with HMC-1 cells transiently expressing C5aR (not shown). These findings suggest that the signaling pathway that synergizes with MAPK activation for GPCR-induced chemokine production involves sustained Ca^{2+} mobilization.

Role of NFAT activation on fMLP-induced chemokine production in HMC-1 cells

Given that elevation of intracellular calcium was a prominent feature of the response to fMLP, we hypothesized that Ca^{2+}-dependent activation of calcineurin and, subsequently, NFAT may also be involved in GPCR-induced chemokine gene expression. Certainly, Ag stimulation of T cells and mast cells causes calcineurin activation (23). We first tested the effect of cyclosporin A (CsA), a potent inhibitor of calcineurin, on fMLP-induced MIP-1β production. CsA (100 nM) inhibited fMLP-induced MIP-1β production by 80 ± 3% (Fig. 9*A*). To test whether fMLP stimulates

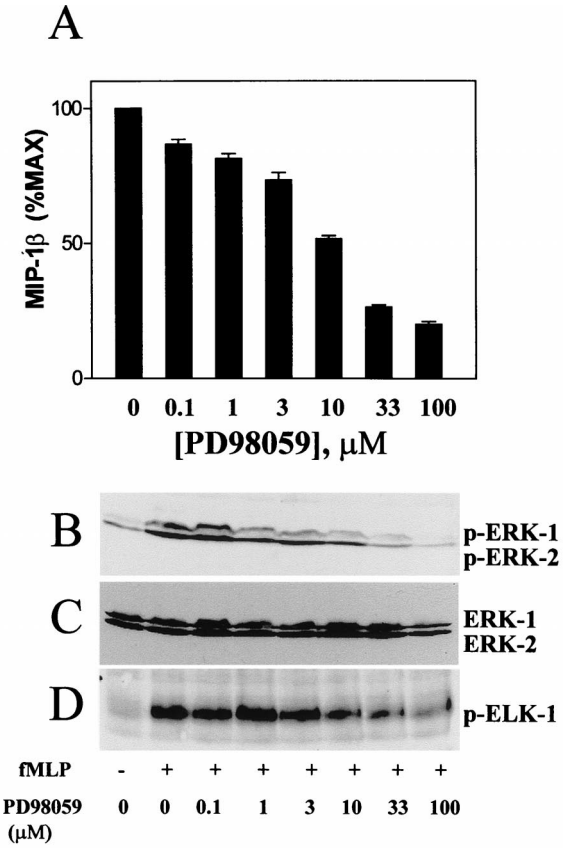

FIGURE 5. Effect of PD98059 on fMLP-induced MIP-1β production, ERK and ELK-1 phosphorylation. *A*, HMC-1 cells were incubated with different concentrations of PD98059 for 30 min and were stimulated with fMLP (1 μM) for 6 h. MIP-1β production was determined by ELISA. The data are expressed as the percentage of the fMLP-stimulated response (350 $\pm$ 23 pg/10^6 cells) after the basal level (50 $\pm$ 4 pg/10^6 cells) was subtracted. PD98059 had no significant effect on the basal response. *B*, Cells were incubated in the absence or the presence of different concentrations of PD98059 and were exposed to buffer (−) or fMLP (+) for 3 min. Western blotting was performed with phospho-ERK Ab as described. *C*, Stripping the membrane and performing Western blotting with ERK-2 Ab confirmed equal amounts of protein loading. *D*, The same membrane was stripped again, and Western blotting was performed with a phospho-ELK-1 Ab. Data shown are representative of three similar experiments.

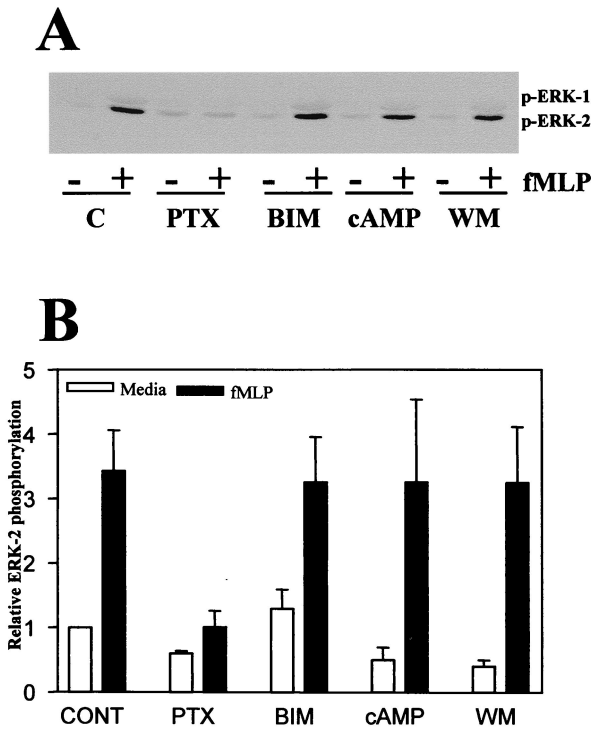

FIGURE 6. Regulation of fMLP-induced ERK phosphorylation in HMC-1 cells. Cells (1×10^6/ml) were incubated with medium (C/CONT), ptx (100 ng/ml, overnight), bisindolylmaleimide (BIM; 10 μM, 10 min), cpt-cAMP (cAMP; 1 mM, 10 min), and wortmannin (WM; 100 nM, 30 min) and then exposed to buffer (−) or fMLP (1 μM) and incubated for 3 min. The reactions were stopped by the addition of ice-cold PBS. Cell pellets were lysed, proteins were separated on a 10% SDS-polyacrylamide gel, and Western blotting was performed with phospho-ERK Ab (*A*). *B*, The extent of ERK-2 phosphorylation was expressed as the fold increase over the basal unstimulated response. The data shown are the mean $\pm$ SEM of four experiments.

NFAT activity, nuclear extracts from untreated and fMLP-treated HMC-1 cells were used to perform EMSA. As shown in Fig. 9*B*, fMLP caused the up-regulation of a protein-DNA complex consistent with NFAT activation. This complex formed with a wild-type NFAT binding site (compare *lanes 2* and *6*), but not with a mutant binding site (compare *lanes 1* and *5*). Moreover, unlabeled wild-type competitor blocked the fMLP-induced complex formation, whereas the mutant competitor had no effect (Fig. 9*B*, *lanes 7* and *8*). To further test for specificity, the EMSA reaction was performed in the presence of anti-NFAT or control Ab. Anti-NFAT Ab inhibited formation of the fMLP-induced complex, whereas the control Ab did not (Fig. 9*C*). Ag-stimulated cytokine gene expression involves activation of both NFAT and AP-1. Therefore, we determined whether fMLP also causes activation of AP-1 in HMC-1 cells. For these experiments EMSA was first performed with nuclear extract from unstimulated HMC-1 cells. As shown in Fig. 9*D* (*lane 1*), AP-1 binding activity was detected in HMC-1 cell extracts. This activity represents AP-1, as incubation with excess AP-1 oligonucleotide blocked formation of the protein-DNA complex, but a nonspecific control oligonucleotide had no effect. Unlike its effect on NFAT, fMLP had no effect on the AP-1 response (Fig. 9*D*). To test whether C5a stimulated transcription factor activation, nuclear extracts from fMLP- and C5a-stimulated HMC-1 cells were compared for their ability to stimulate NFAT and AP-1 activation. FMLP caused a 4.2 $\pm$ 0.02-fold increase in NFAT activity, whereas C5a had no effect.

Discussion

In this study we have used HMC-1, which endogenously expresses receptors for C3a and C5a at low levels and generated stable transfectants expressing FR. We used this system as a model to study the role of GPCR-mediated cytokine production in leukocytes. Using RPA, we initially screened for the ability of fMLP, C3a, and C5a to induce mRNA accumulation of 33 cytokines. We found that of these cytokines, mRNAs for MIP-1β and MCP-1 were up-regulated in response to fMLP, but not to C3a or C5a. This mRNA up-regulation was associated with a time- and dose-dependent release of chemokines as measured by ELISA.

It is important to note that in leukocytes, FR are expressed at much higher levels than C3aR or C5aR. For example, 50,000–120,000 FR are present on neutrophils (17, 18). In contrast, C3aR expression in monocytes, neutrophils, eosinophils, and basophils ranges from 3,000 to 10,000 copies/cell (22). Also, basophils, eosinophils, and HMC-1 cells express ~15,000 C5aR/cell. Although most leukocytes express FR, C3aR, and C5aR, the ability of these

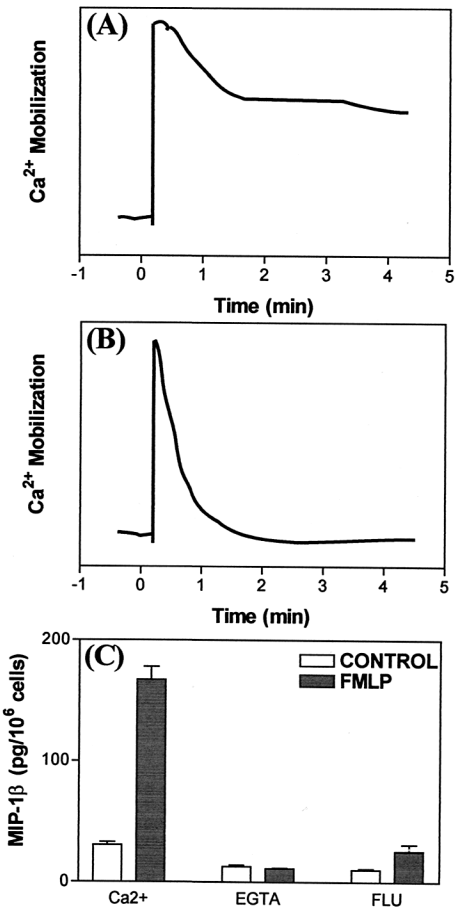

FIGURE 7. Effect of extracellular Ca^{2+} on fMLP-induced Ca^{2+} mobilization and MIP-1β production. HMC-1 cells were loaded with indo-1, washed, and resuspended in HEPES buffer containing 1 mM Ca^{2+} (*A*) or in the same buffer that was supplemented with EGTA (5 mM; *B*). FMLP (1 μM) was added (time zero), and intracellular Ca^{2+} levels were measured for 4–5 min. *C*, Cells were incubated with medium containing Ca^{2+} alone or medium supplemented with EGTA (5 mM) or fluphenazine (FLU; 50 μM) and stimulated with fMLP (1 μM) for 6 h, and MIP-1β release was determined by ELISA.

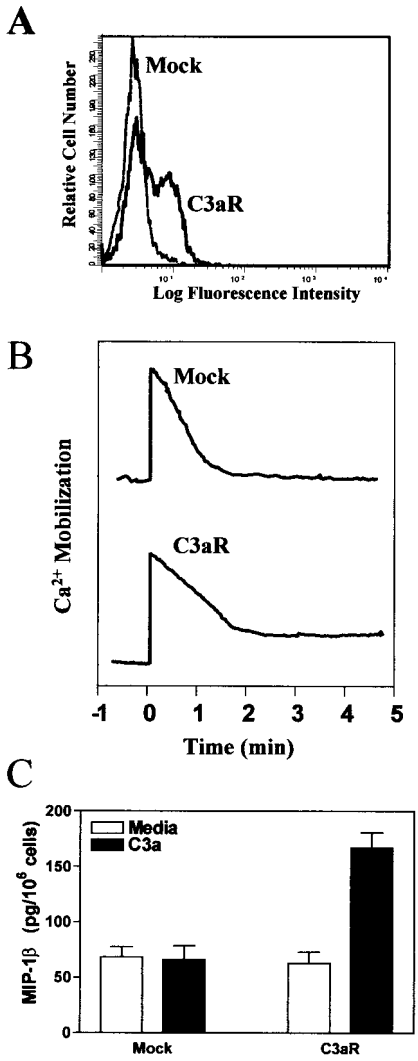

FIGURE 8. Effect of transient C3aR expression on C3a-mediated Ca^{2+} mobilization and MIP-1β production in HMC-1 cells. Cells were transiently transfected with empty vector (mock) or with cDNA encoding HA-tagged C3aR (C3aR). *A*, FACS analysis was performed with 12CA5 Ab. *B*, Indo-1-loaded mock-or C3aR-transfected cells were stimulated with C3a (10 nM; at time zero) and intracellular Ca^{2+} mobilization was determined for 4–5 min. *C*, HMC-1 cells were incubated with C3a (10 nM) for 6 h, and MIP-1β production was determined by ELISA. Data shown are representative of three similar experiments.

receptors to stimulate biological responses has been well documented only in human basophils. The differences in the biochemical and functional properties of the GPCRs described herein for HMC-1 cells, which express 54, 354 ± 3,478 FR/cell, are very similar to those reported for the same receptors in basophils. For example, as in basophils (1), fMLP stimulated a sustained Ca^{2+} mobilization, whereas the response to C5a was transient (Fig. 1). Furthermore, fMLP causes leukotriene production in basophils, whereas C3a or C5a has no effect (2, 24, 25). The findings in the present study that C3a and C5a stimulate transient Ca^{2+} mobilization in HMC-1 cells and that overexpression of C3aR or C5aR leads to both a sustained Ca^{2+} mobilization and MIP-1β production demonstrate that the ability of GPCR to stimulate chemokine production depends on the level of receptor expression.

An interesting aspect of the present work was that although FR, C3aR, and C5aR all couple to the same ptx-sensitive G protein, the differences in their ability to stimulate chemokine production reflected the length of their signaling time. Thus, fMLP-stimulated chemokine production was associated with both sustained Ca^{2+} mobilization and ERK phosphorylation. Furthermore, C3a and C5a, which induced both transient Ca^{2+} mobilization and ERK phosphorylation, did not stimulate chemokine production. Evidence that fMLP-induced ERK phosphorylation was necessary for chemokine production was provided by the demonstration that ptx and PD98059, which respectively block G protein and MAPK activation, inhibited fMLP-induced ERK phosphorylation and chemokine production. Furthermore, fMLP stimulated the phosphorylation ELK-1, which is a transcription factor that is activated by ERK. In addition, PD980589 blocked fMLP-induced ELK-1 phosphorylation in a dose-dependent manner. These findings suggest that fMLP-induced chemokine production in HMC-1 cells is mediated via signaling pathways that include sustained Ca^{2+} mobilization and ERK phosphorylation.

The mechanism by which fMLP stimulates ERK phosphorylation in HMC-1 cells is not known. It is generally accepted that G_i-coupled receptors use the $\beta\gamma$ subunits of G protein to activate MAPK via a mechanism involving the following pathway: G$\beta\gamma\rightarrow$Src$\rightarrow$Shc/Grb/SOS$\rightarrow$Ras$\rightarrow$Raf$\rightarrow$MEK$\rightarrow$ERK (26). In

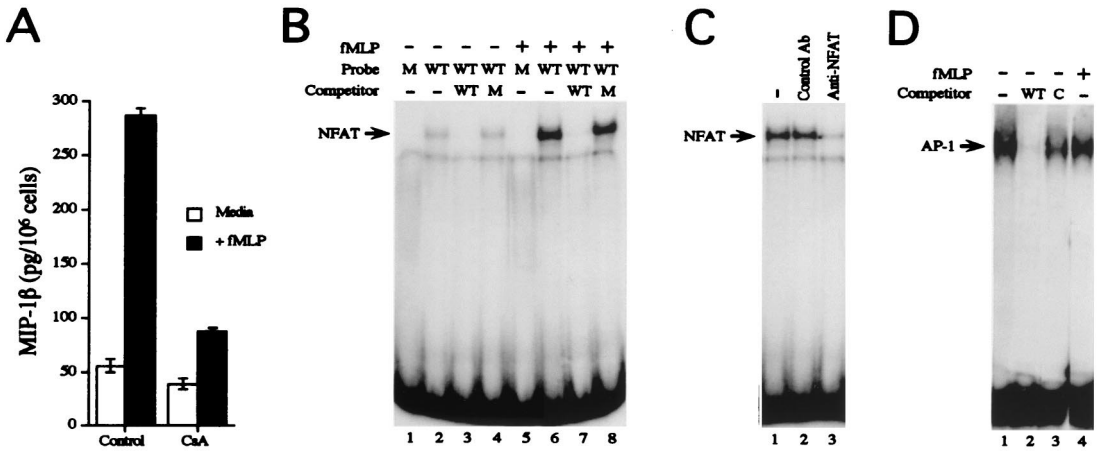

FIGURE 9. Role of NFAT in fMLP-stimulated chemokine production in HMC-1 cells. *A*, Cells were preincubated in the absence or the presence of CsA (100 nM, 30 min) and exposed to medium (−) or fMLP (+) for 6 h. MIP-1β was determined by ELISA. *B*, Radiolabeled wild-type (WT) or mutant (M) NFAT double-stranded oligonucleotides were incubated with nuclear extracts from control or fMLP-stimulated HMC-1 cells in the absence of competitor or in the presence of a 50-fold molar excess of wild-type (WT) or mutant (M) competitors. DNA protein complex was resolved by electrophoresis. The NFAT-containing complex is marked. *C*, Radiolabeled NFAT double-stranded oligonucleotides were incubated with nuclear extracts from fMLP-treated HMC-1 cells in the presence of anti-NFAT or control polyclonal Abs. The NFAT-containing complex is marked. *D*, Radiolabeled AP-1 double-stranded oligonucleotides were incubated as described in *A* to detect AP-1 binding. Competition was performed with a wild-type (WT) or nonspecific control (C) oligonucleotide. The AP-1-containing complex is marked.

neutrophils, PI3 kinase is involved in fMLP-induced ERK phosphorylation, possibly via its direct effect on MEK (G$\beta\gamma\rightarrow$PI3 kinase$\rightarrow$MEK$\rightarrow$ERK) (8). Thus, cpt-cAMP and wortmannin, which inhibit Raf-1 and PI3 kinase, respectively, block fMLP-stimulated ERK phosphorylation in human neutrophils (8, 27). The finding in the present study that neither wortmannin nor cAMP blocked fMLP-induced ERK phosphorylation in HMC-1 cells is inconsistent with a role for Raf or PI3 kinase in MAPK activation in this cell line. This type of inconsistency has recently been reported for other receptors. For example, in CHO cells expressing CXCR1 and CXCR2, IL-8-induced MAPK activation is mediated independently of Raf or PI3 kinase (28). In Swiss-3T3 and COS-7 cells, epidermal growth factor and lysophosphatidic acid-induced MAPK activities are mediated in a Raf-1-independent and a cAMP-insensitive manner (29). In PC12 cells, Ras-dependent MAPK activation also does not require PI3 kinase or Raf activation (30). These findings suggest the existence of a MEK kinase different from classic Raf kinases that might be involved in ERK phosphorylation in HMC-1 cells and other cell types. The identity of this kinase and the mechanism by which fMLP activates ERK phosphorylation remain to be determined.

An important and unexpected finding of the present study was that although cpt-cAMP and wortmannin had no effect on fMLP-stimulated ERK phosphorylation, they blocked chemokine production in response to fMLP. This raises the intriguing possibility that fMLP-stimulated chemokine production involves synergistic interaction of ERK phosphorylation with another signaling pathway. NFAT is a cytosolic transcription factor that regulates the activation of cytokine genes in Ag-stimulated T cells and mast cells (10, 11). Ag stimulation of these cells results in a Ca^{2+}-dependent activation of the phosphatase calcineurin, which dephosphorylates NFAT. This dephosphorylation allows NFAT to translocate to the nucleus, where it combines with the AP-1 complex to regulate the transcription of early cytokine genes (10, 23). The immunosuppressive drug CsA inhibits cytokine gene expression by blocking calcineurin. Although recent studies have shown that GPCR could activate NFAT in lymphoid (31) and microglial cells (32), its biological significance is not known. The demonstration herein that CsA blocked fMLP-induced MIP-1β production indicated that the signal needed to synergize with ERK phosphorylation to induce chemokine production might be the activation of NFAT (Fig. 7). This possibility was supported by the finding that fMLP, but not C5a, stimulated NFAT activation in nuclear extracts of HMC-1 cells. Although NFAT regulates cytokine gene expression in Ag-stimulated mast cells and T cells, the present study demonstrates a previously unrecognized role of this transcription factor in GPCR-induced chemokine production.

In summary, we have used a human mast cell line that natively expresses C3aR and C5aR at low levels and generated stable transfectants expressing physiologic levels of FR as a model for GPCR activation. Using this system we have shown that bacterial products such as fMLP can induce cells of innate immunity to produce the proinflammatory chemokines MIP-1β and MCP-1 that may, in turn, recruit other leukocytes to sites of infection. Furthermore, we have shown that the selectivity of fMLP vs C3a or C5a to induce

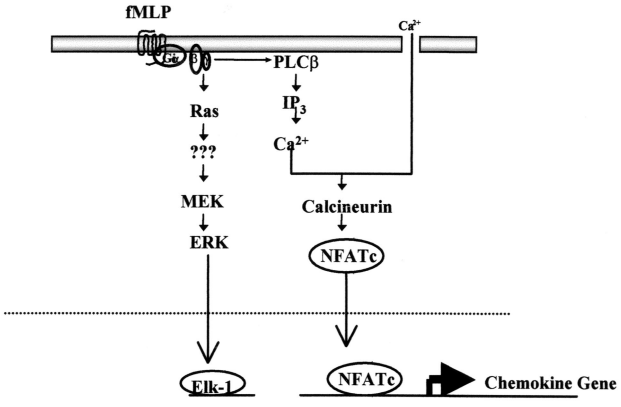

FIGURE 10. Model for fMLP-induced chemokine gene expression in HMC-1 cells. FMLP activates MAPK via a pathway that does not appear to involve Raf. This response synergizes with NFAT activation for the induction of chemokine gene expression.

chemokine production is due to the level of receptor expression, the length of their signaling time, and the synergistic interaction of ERK phosphorylation, sustained Ca^{2+} mobilization, and NFAT activation (see model in Fig. 10).

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