Ca\(^{2+}\)-independent Protein Kinase Cs Mediate Heterologous Desensitization of Leukocyte Chemokine Receptors by Opioid Receptors*

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Heterologous desensitization of chemokine receptors by opioids has been considered to contribute to their immunosuppressive effects. Previous studies show that Met-enkephalin, an endogenous opioid, down-regulates chemotaxis of selected chemokine receptors via phosphorylation. In the present study, we further investigated the molecular mechanism of such cross-regulation. Our data showed that preincubation with Met-enkephalin inhibited both MIP-1\(\alpha\)-mediated chemotaxis and Ca\(^{2+}\) flux of monocytes in a dose-dependent manner. The inhibitory effects were maximal using nanomolar concentrations of activating chemokines, a concentration found in physiological conditions. A decrease both in chemokine receptor affinity and in coupling efficiency between receptors and G protein were observed, which directly contributed to the desensitization effects. However, comparing with chemokines such as MIP-1\(\alpha\) and MCP-1, opioids did not elicit a calcium flux, failed to induce MIP-1\(\alpha\) receptors internalization, and mediated a less potent heterologous desensitization. We hypothesized that these differences might originate from the involvement of different protein kinase C (PKC) isotypes. In our studies, opioid-mediated down-regulation of MIP-1\(\alpha\) receptors could be blocked by the general PKC inhibitor calphostin C, but not by the calcium-dependent classic PKC inhibitor Go6976. Western blotting analysis and immunofluorescent staining further showed that only calcium-independent PKCs were activated upon opioid stimulation. Thus, opioids achieve desensitization of chemokine receptors via a unique pathway, involving only calcium-independent PKC isotypes.

Long term opioid usage induces immunosuppression by modulating a spectrum of immune activities, such as inhibition of lymphocyte proliferation, decreased production of interferon \(\gamma\), interleukin 2, and interleukin 4 by activated lymphocytes, enhanced synthesis of tumor necrosis factor \(\alpha\) and interleukin 1\(\beta\) in activated macrophage, enhanced production of MCP-1, RANTES (regulated on activation normal T-cell expressed and secreted), and IP-10 in peripheral blood mononuclear cells, and reduction in antibody production (1–5). The fact that accelerated human immunodeficiency virus pathogenesis occurred in patients who abuse heroin is consistent with the immunosuppressive activity of the opioids (6). Although previous studies indicate that opioids may regulate immune responses through their action on central nervous system or sympathetic nervous system, the discovery of opioid receptors on peripheral blood mononuclear cells suggested that these opioid receptors could directly modify the response of proinflammatory receptors (2–4, 7). Data from knock-out mice and \textit{in vitro} studies suggest that such suppression is mediated largely by \(\mu\), \(\delta\), and \(\kappa\) opioid receptors. Opioid receptors, members of the seven-transmembrane receptor family, perform their function by coupling to heterotrimeric G\(_{\alpha}\) proteins. Their activation leads to inhibition of adenyl cyclase by G\(_{\alpha}\) inhibition of voltage-dependent calcium channels, and activation of G protein-coupled inwardly rectifying K\(^{+}\) channels by G\(_{\beta\gamma}\).

In cells expressing multiple G protein-coupled receptors (GPCRs),\(^1\) prolonged activation of one receptor has been shown to result in the down-regulation of other GPCR through a process called heterologous desensitization. Accumulating data have demonstrated that heterologous down-regulation of GPCR is mediated by protein kinase A and protein kinase C (PKC) (8, 9, 11). Calcium flux, a potent activator of PKC, has been considered essential for heterologous desensitization of chemokine receptors in peripheral blood mononuclear cells (8–10). Interaction of chemokine receptors with their ligands activates G\(_{\alpha}\) proteins, induces the production of DAG, and releases Ca\(^{2+}\), followed by activation and translocation of PKC to the plasma membrane. This process is associated with desensitization of other GPCRs in the same cell by phosphorylation of their consensus sites (10, 13). The family of PKC consists of more than 12 isozymes, and each of them exhibits a unique pattern of tissue distribution, subcellular translocation, and function (14). For instance, PKC\(\gamma\) is essential in mediating neutrophil chemotaxis and in regulating the polarity of astrocytes during wound healing process (15, 16). PKC\(\theta\) is predominantly expressed in lymphocytes and is recruited to the membrane during antigen presentation to T-cells (17). The 12 PKC isozymes can be divided into three subfamilies: classical PKCs (cPKCs), such as \(\alpha\), \(\beta\), \(\beta\)II, and \(\gamma\), require both Ca\(^{2+}\) and DAG for activation; novel PKCs (nPKCs), such as \(\delta\), \(\epsilon\), \(\theta\), and \(\eta\), are DAG-dependent but Ca\(^{2+}\)-independent; and atypical PKCs, such as \(\zeta\) and \(\lambda\), require

\(^{1}\) The abbreviations used are: GPCR, G protein-coupled receptor; PKC, protein kinase C; DAG, diacylglycerol; cPKC, classical PKC; nPKC, novel PKC; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; GTP\(\gamma\)S, guanosine 5’-3-O-(thio)triphosphate; CTOP, Cys\(^2\), Tyr\(^3\), Orn\(^9\), Pen\(^7\) amide; eGFP, green fluorescent protein.
neither Ca²⁺ or DAG. Eight PKC isozymes, α, β1, β2, δ, ε, η, μ, and ζ, have been identified in human blood monocytes (18). However, their contribution to heterologous desensitization of chemokine receptors has not been defined.

Preincubation with µ- or δ-opioid agonists has been shown to inhibit MIP-1α-mediated chemotaxis of monocytes and neutrophils (7, 21). Such inhibition can be blocked by the nonselective opioid antagonist naloxone or the µ- or δ-selective antagonists CTOP and naltrindole (7). Preincubation with opioids has been shown to enhance phosphorylation of CXCXR1, correlating with modest impairment of chemotaxis. Compared with other chemokines, opioids are less capable of mediating heterologous desensitization. Heterologous desensitization of chemokine receptors seems to follow a hierarchy; certain receptors are more potent in desensitizing than others (19, 20). The capacity of a receptor to cross-desensitize GPCRs has been proposed to correlate with its ability to induce a greater phosphoinositide hydrolysis (8). We set out to investigate the possibility that the hierarchy may be related to the PKC isotypes.

In this study, we first show that, in monocytes, opioid-mediated heterologous desensitization inhibits not only chemokine-induced chemotaxis but also Ca²⁺ mobilization in a dose-dependent manner. Such inhibitory effects result from a decrease in chemokine receptor affinity and their coupling efficiency to G protein. Furthermore, we find that, unlike chemokine receptors, opioid receptors fail to elicit a calcium flux and only activate novel and atypical PKC, resulting in modest suppression of chemokine receptor function. Our data suggest that the relative potency of G protein-coupled receptors in heterologous desensitization is dependent on which subfamily of PKC is activated. A robust heterologous desensitization requires both calcium-dependent and -independent kinase C. Opioids are unique and only utilize a calcium-independent PKC pathway to phosphorylate and inactivate heterologous receptors.

**MATERIALS AND METHODS**

**Chemicals and Materials**—MCP-1 and MIP-1α were obtained from PeproTech (Rocky Hill, NJ); I²[125I]-MIP-1α, 3H-DAMGO, and Tog polysac-24e were from PerkinElmer Life Sciences; Met-encephalin was from Peninsula Laboratories, Inc. (San Carlos, CA); DAMGO was from Sigma; calphostin C and G60976 were from Alexa Biochemicals (San Diego, CA); chemotaxis chamber and membrane were from Neuroprobe (Gaithersburg, MD); polyclonal anti-PKCα (sc-216), anti-PKCα, β, γI (c-20), and anti-PKCδ (c-17) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and ECL reagents were from Pierce. All other reagents were reagent grade and were obtained from standard suppliers.

**Cells and Cell Culture**—Human peripheral monocytes were obtained from healthy donor blood packs and isolated from Buffy coats (Trans-24usion Medicine Department, National Institutes of Health Clinical Center, Bethesda, MD) by iso-osmotic Percoll gradient. The monocytes were >90% pure by nonspecific esterase staining or by morphological characterizations. In brief, fresh monocytes were stored in ice-cold PBS for 90 min and used for experiments on the same day. HEK293, vector/HEK293, and µ-opioid receptor/HEK293 cells were grown in DMEM (Biowhit-24aker, Walkersville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1 mM glutamine (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). CCR1/HEK293, µ-receptor/CCR1/HEK293, and vector/CCR1/HEK293 cells were grown in the same buffer with 400 µg/ml Gentamicin (Invitrogen). Macrophages were generated in vitro by incubating freshly isolated monocytes 1 × 10⁶/ml in RPMI 1640 medium and 10% fetal bovine serum in the presence of recombinant human macrophage colony-stimulating factor (50 ng/ml) at 37°C in a humidified CO₂ (5%) incubator for 7 days with the addition of fresh recombinant human macrophage colony-stimulating factor-containing medium every 2–3 days (22).

**Plasmid and Stable Cell Line Constructions**—PCR fragments of full-length CCR1 were inserted into pcDNA 3.1 to make pcP1. pcCCR1 was linearized with ScaI and electroporated into HEK293 cells. After selection with 800 µg/ml Gentamicin, the CCR1 level was screened by Western blotting, and a single colony with high expression of CCR1 was selected to generate CCR1/HEK293. Full-length µ-opioid receptor was inserted into pLZRS-IRE-EGFP retroviral vector to generate µ-opioid receptor. The empty vector was used as a control. Phoenix-Amphotropic Retroviral Packaging cells were plated at 1.5–2 million cells/60-mm plate in DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% glutamine, 18–24 h prior to transfection (23). Approximately 2 µg of each plasmid containing the desired inserts (pLZRS-IRE-EGFP) were prepared for transfection into cells by using FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer’s instructions (23, 24). The LZRS vector replicates episomally via use of the EBNA-1 protein and also contains a puromycin resistance gene. Finally, the inclusion of the IRES-EGFP expression cassette allows for sorting of infected target cells by flow cytometry. After transfection, the Phoenix-Ampho cells were selected with 5 µg of puromycin per ml and were incubated at 7 days, at which time the population consisted of virtually 100% EGFP-expressing cells. Infectious virus-containing supernatants were propagated by growing parent cell lines infected with recombinant retroviral vectors as described above, in T75 flasks to ~80% confluency and then overlaying this culture with 10 ml of complete medium to allow cells to produce virus overnight. At the same time, target cell populations were prepared by plating cells into suitable tissue culture vessels and overlaying the target culture with 0.22 µm of filtered, recombinant retrovirus-infected supernatants the following morning. Infectious supernatants were supplemented with 5 µg/ml polyelectrolyte to assist in virus attachment. 3–5 days post-infection, the target cells were sorted by flow cytometry and analyzed as described under “Results.”

**Chemotaxis**—Chemotaxis was performed as described by the manufacturer (Neuroprobe). In brief, the monocytes were pretreated with PBS, MIP-1α, MCP-1, Met-encephalin, or morphine for 30 min at 37°C. The cells were then washed twice with binding medium (RPMI from Biowhitaker, 1% bovine serum albumin, and 20 mM HEPES) and loaded into the upper chemotaxis chambers. Chemokines, diluted to various concentrations were loaded into the lower chambers. The chambers were incubated at 37°C, 100% humidity, and 5% CO₂ for 1 h. The 5-µm filter between the chambers was then washed, fixed, and stained. The cells that migrating through the filter were counted by microscopy. The chemotaxis index was the ratio of chemotactic cell numbers in a chemotactic gradient versus the cell numbers in a medium control. For HEK293 cells, the polycarbonate filter was pretreated with 50 µg/ml of collagen in binding medium at 4°C overnight. Chemotaxis of HEK293 cells was assayed after 5 h. The statistical analysis of chemotactic responses was performed by PRISM3.0.

**Ligand-induced Calcium Mobilization**—Calcium flux was measured as described by Badolato et al. (25). In brief, the cells were incubated at 10⁷/ml for 30 min at room temperature in DMEM containing 1 µM Fura-2. The cells were then washed with DMEM once, washed with Hanks’ balanced salt solution twice, and diluted into 2 × 10⁶/ml. The cells were then loaded into a 2-ml cuvette at 37°C, and the relative ratio of fluorescent emission at 510 nm when excited by 340 nm and 380 nm was recorded as a PerkinElmer Life Sciences luminescence spectrometer. For heterologous desensitization experiments, the cells were first incubated with Met-encephalin or chemokines at 37°C for 30 min before adding Fura-2.

**Ligand Binding Analysis**—The ligand binding assays were carried out as described by Grimm et al. (7) with modifications. The cells were preincubated with MIP-1α, MCP-1, or Met-encephalin for 30 min at 37°C, washed extensively, and resuspended in binding medium at 10⁷/ml. The binding assays were carried out on ice using 0.5 nM I²[125I]-MIP-1α in the presence of increasing concentration of competing unlabelled-MIP-1α. The cells were incubated at 4°C for 30 min, and the ligand/antiligand bindings were separated by a low temperature gradient. The level of binding was determined in a γ-counter. Nonlinear regression analysis of data was performed by a PRISM3.0 program by fitting the following equation.

\[ \text{Total Binding} = B_o + \text{Satellite} \]

**Non-Nonspecific Binding**

\[ \text{B}_o = \frac{\text{Nonspecific Binding}}{\text{Eq. 1}} \]

**PKC Translocation Assay**—Assay of PKC translocation in monocytes were performed as described by Laudanna et al. (15) with minor modifications. In brief, fresh monocytes were stored in ice-cold PBS for 90 min to decrease the membrane-bound PKC before stimulation. Cock-
tails of protease inhibitors were added. The cells were then stimulated at 37 °C, stopped by ice-cold PBS, homogenized by sonication, and underwent ultra-centrifugation at 100,000 g for 1 h. The supernatant was kept as the cytosolic fraction. The precipitates were sonicated in one half volume of PBS with 1% Triton X-100 and centrifuged at 100,000 g again for 30 min. The solubilized precipitates were kept as the membrane fraction. The samples were then loaded on 10% SDS-PAGE, followed by Western blotting analysis. Confocal microscopic analysis of immunofluorescent staining of PKC isotypes was carried out as described by Etienne-Manneville (16). The cells were pretreated with 100 ng/ml MCP-1 or 10^{-7} M Met-enkephalin for 10 min, then fixed, permeabilized, and stained by rabbit antibody to PKC isotypes followed by fluorescein isothiocyanate-labeled goat anti-rabbit antibody. The cells were visualized using a Zeiss inverted fluorescent confocal microscope.

RESULTS

Opioids Induced Monocyte Chemotaxis, but Not Calcium Flux—First, we compared the degree of Met-enkephalin-mediated chemotaxis of freshly isolated monocytes to those induced by conventional chemokines, MIP-1α and MCP-1. As shown in Fig. 1A, MCP-1, presumably by activating chemokine receptor, CCR2, induced a robust chemotaxis response, which peaked at 3 \times 10^{-10} M. MIP-1α, the endogenous ligand for receptor CCR1 and 5, was also a potent chemoatractant of monocytes. However, the chemotactic response of Met-enkephalin on monocytes, although significant, was less potent, with an index ranging from 2 to 4.5. Morphine was also a weaker stimulus of monocyte chemotaxis (data not shown). The lower chemotactic activity of opioids may be due to the lower receptor expression on cell surface or perhaps due to inefficient coupling between opioid receptors and G_{i} proteins. Furthermore, although MCP-1 and MIP-1α induced monocyte chemokinesis, the background level of monocyte motility was unchanged when treated with opiates over a wide concentration ranging from 10^{-11} to 10^{-8} M (data not shown).

Because opioid receptors couple to G_{i} proteins and induce chemotaxis, we determined to assess their capacity to elicit a concomitant Ca^{2+} response. As shown in Fig. 1B, both MCP-1 and MIP-1α rapidly induced a potent Ca^{2+} flux in monocytes, confirming that the inositol 1,4,5-trisphosphate-induced Ca^{2+} flux is functional (26). However, Met-enkephalin, at concentrations from 10^{-10} to 10^{-4} M, failed to initiate a detectable Ca^{2+} response in monocytes (Fig. 1B and data not shown). Morphine also did not induce any measurable Ca^{2+} mobilization (data not shown). The deficiency of opioid-induced Ca^{2+} mobilization is likely due to insufficient production of inositol 1,4,5-trisphosphate by activated G_{i}-coupled PLCβ. These data further suggest that the µ- and/or δ-opioid receptors are less potent in inducing chemotactic G_{i} signaling in monocytes than chemokine receptors.

Pretreatment with Opioids Inhibited Chemokine-mediated Chemotaxis and Calcium Response in Monocytes—To evaluate the potency of opioid-induced desensitization, the inhibitory effects of Met-enkephalin on MIP-1α-induced chemotaxis were compared with the desensitizing effects of MIP-1α and MCP-1 on chemokine receptors. Freshly isolated monocytes pretreated with 100 ng/ml MIP-1α exhibited a reduction of more than 85% of the chemotactic response to MIP-1α, because of homologous desensitization (Fig. 2A). This homologous desensitization was dose-dependent (Fig. 2B). Monocytes pretreated with 100 ng/ml MCP-1 for 30 min also showed a marked decrease in their chemotactic response to MIP-1α, because of heterologous desensitization of chemokine receptors (Fig. 2, A and B). Pretreatment with 10^{-6} M of Met-enkephalin significantly impaired MIP-1α-mediated chemotaxis but to a lesser degree (Fig. 2A). Met-enkephalin exhibited dose-dependent heterologous desensitization effects with optimal inhibitory activity of 50% at 10^{-6} M (Fig. 2B).

To determine the effect of opioid mediated heterologous desensitization on Ca^{2+} flux, we compared the dose dependence of
the MIP-1α-elicited Ca\(^{2+}\) response of monocytes, which were pretreated cells with 1000 ng/ml of MIP-1α, MCP-1, or 10\(^{-7}\) M Met-enkephalin. MIP-1α, at 0, 5, 15, 50, 150, and 500 ng/ml, induced Ca\(^{2+}\) flux in monocytes in a dose-dependent manner (Fig. 2C and data not shown). To directly compare homologous and heterologous desensitization, the maximal value of each Ca\(^{2+}\) flux was used to plot the dose response of the stimulus (Fig. 2D). Monocytes pretreated with 1000 ng/ml MIP-1α for 30 min followed by three washes failed to exhibit a detectable response to 5 and 15 ng/ml MIP-1α (Fig. 2D). Homologous desensitization also decreased the response to MIP-1α at 150 and 500 ng/ml. MCP-1 pretreatment severely reduced the MIP-1α-induced Ca\(^{2+}\) response, but the inhibitory effects decreased as the concentration of stimulus was increased and became undetectable at 150 ng/ml of MIP-1α (Fig. 2D). Pretreatment with Met-enkephalin resulted in moderate inhibition of Ca\(^{2+}\) flux (Fig. 2D). Met-enkephalin-mediated inhibitory effects were maximal when chemokine concentration was low and decreased as the chemokine concentration was increased (Fig. 2D). Both chemotaxis and calcium flux data show that the heterologous desensitization of the MIP-1α response by Met-enkephalin was significant but less potent than either homologous or heterologous desensitization of chemokine receptors by chemokines.

We further examined the effect on MIP-1α-mediated chemotaxis in \textit{in vitro} macrophage colony-stimulating factor-stimulated macrophages. As shown in Fig. 3, although opioids also did not induce calcium flux in activated macrophage, they...
induced similar heterologous desensitization of chemokine receptors in activated macrophages.

Met-enkephalin Pretreatment Reduced Binding Affinity of MIP-1α Receptors and Coupling Efficiency between MIP-1α Receptors and G Protein—Desensitization of seven-transmembrane receptors may involve internalization of receptors, decrease in ligand binding affinity, or impaired interaction with G proteins (11–13, 27–31). I²53-MIP-1α was used to monitor the effect of Met-enkephalin on the total numbers of MIP-1α-binding sites and binding affinity on monocytes. Homologous competitive binding analyses showed a 3-fold decrease in MIP-1α binding affinity after Met-enkephalin pretreatment, whereas the total number of binding sites was unchanged (Fig. 4, A and B). In contrast, preincubation with MIP-1α reduced the number of surface binding sites by over 70%, presumably because of receptor internalization during homologous desensitization (Fig. 4C). Activation of MCP-1 receptors also decreased the number of MIP-1α receptors by more than 35% (Fig. 4C). In contrast with our results, previously reported binding analysis, carried out at room temperature, did not reveal any detectable affinity change (7). Our binding assays were performed at 4 °C to prevent MIP-1α-induced homologous desensitization.

Upon ligand binding, chemokine receptors activate downstream heterotrimeric G proteins by enhancing the exchange of bound GDP to GTP. A desensitized GPCR shows a decrease in its capability to induce the binding of [³²S]GTPγS to membrane G proteins. Pretreatment with Met-enkephalin for 30 min resulted in a 34% loss of MIP-1α-stimulated [³²S]GTPγS binding on the membrane, indicating an impairment of the coupling efficiency between chemokine receptors and downstream G protein (Fig. 4D). In contrast, the capability of MIP-1α receptors to enhance membrane [³²S]GTPγS binding was severely inhibited (up to 62–70%) after pretreatment with MCP-1 or MIP-1α (Fig. 4D). The decrease in both receptor affinity and coupling efficiency to G protein may directly contribute to the desensitization of MIP-1α-mediated chemotaxis, calcium flux, and other signals.

Overexpression of Opioid Receptors Did Not Rescue Ca²⁺ Flux Defects—The expression level of opioid receptors on monocytes and macrophages is very low relative to that of chemokine receptors. Less than 10⁵ binding sites were detected using [³⁵S]GTPγS to membrane in monocytes pretreated with MIP-1α, MCP-1, or Met-enkephalin. D, comparison of MIP-1α-stimulated binding of [³⁵S]GTPγS to membrane in monocytes pretreated with MIP-1α, MCP-1, or Met-enkephalin. (A and B, one set of representative data from two independent experiments. In each experiment, each data point is the average of duplicated binding assays. C and D, Each data point is the average of triplicate binding assays. The data were repeated twice.)
provide further evidence that long term activation of opioid receptors leads to a moderate level of down-regulation of chemokine receptor function through Ca\(^{2+}\)-independent heterologous desensitization.

**Met-enkephalin-induced Chemokine Receptor Desensitization**

Could Be Blocked by Calphostin C but Not Go6976—Second messenger-stimulated kinases, protein kinase A and PKC, are involved in heterologous desensitization of G protein-coupled receptors via feedback regulation (8, 9, 11). Previous studies have repeatedly shown that, upon chemokine binding, receptors elicit a Ca\(^{2+}\) flux response, which in turn activates PKC, resulting in heterologous desensitization of other types of chemokine receptors. However, because Met-enkephalin did not induce a detectable Ca\(^{2+}\) response, it therefore presumably used another mechanism. We used calphostin C, an inhibitor of nPKCs and cPKCs, to evaluate whether PKCs play any role in opioid receptor-mediated inhibition. When monocytes were pre-

**FIG. 5.** Effects of Met-enkephalin on CCR1/HER293 cells infected to overexpress \(\mu\)-opioid receptors. A, effects of Met-enkephalin on Ca\(^{2+}\) flux in \(\mu\)-receptor overexpression cells. B, effect of Met-enkephalin on CCR1 internalization in \(\mu\)-receptor overexpression cells. C, effect of Met-enkephalin on heterologous desensitization by \(\mu\)-receptor overexpression cells. (A–C, a set of representative data from at least two independent experiments. B, each data point is an average of triplicate assays; \(p < 0.026\). C, each data point is an average of quadruplicate assays; \(p < 0.041\).)

**FIG. 6.** Calphostin C, not Go6976, blocks Met-enkephalin-mediated heterologous desensitization. A, effects of calphostin C on MCP-1 and Met-enkephalin desensitization of chemotactic response (\(p < 0.047\)). B, effects of Go6976 on desensitization of chemotactic response to MIP-1\(\alpha\) by MCP-1 and Met-enkephalin (\(p < 0.05\)). (Each data point is an average of quadruplicate data.)

**Opioids Down-regulate Chemotaxis**

with 200 nm calphostin C, the inhibition of MIP-1\(\alpha\) chemotaxis by opioids was largely blocked, indicating the involvement of PKCs in heterologous desensitization (Fig. 6A). MCP-1-mediated heterologous desensitization was also blocked by calphostin C treatment (Fig. 6A). However, calphostin C did not interfere with MIP-1\(\alpha\)-mediated chemotaxis but only with heterologous desensitization. To determine which subfamily of PKCs were involved, we used Go6976, a specific inhibitor for cPKC but not nPKC isozymes. This drug only partially impaired the inhibition by MCP-1 pretreatment and had no effect on the inhibition induced by opioids (Fig. 6B). These data suggest that only Ca\(^{2+}\)-independent PKC isotypes are involved in opioid-mediated heterologous desensitization, consistent with a lack of Ca\(^{2+}\) flux upon opioid treatment.

**Met-enkephalin Elicited Activation of Calcium-independent PKC, not cPKC**—The translocation of PKCs from cytosol to different membrane compartments is a hallmark of PKC activation (14). The activation of each isozyme can be monitored by its redistribution to the membrane. To confirm the observations obtained with PKC inhibitors, we investigated the translocation of different PKC isozymes in monocytes upon MCP-1 and Met-enkephalin stimulation. As shown in Western blotting analysis, in freshly isolated monocytes, PKCs distributed in the cytosol (Fig. 7A). When stimulated with 1000 ng/ml MCP-1,
monocytes responded with a membrane enrichment of both Ca\(^{2+}\)-dependent and -independent PKCs, such as \(\alpha, \beta I, \beta II, \delta,\) and \(\zeta\) isozymes (Fig. 7A). The translocation occurred in as little as 30 s (data not shown). Similar activation patterns were also found in MIP-1\(\alpha\)-activated cells (data not shown). However, only Ca\(^{2+}\)-independent PKCs, such as \(\delta\) and \(\zeta\), were activated by 10\(^{-7}\) M Met-enkephalin. We further confirmed the Western blotting data by immunofluorescent staining of PKC isotypes upon MCP-1 or Met-enkephalin stimulation.

Our data suggest that calcium-dependent PKCs are also critical for robust heterologous desensitization. MCP-1 elicited a marked calcium response, which is necessary for the internalization of GPCR-binding sites and a marked decrease in the coupling efficiency between receptors and G protein. Such inhibitory effects could be blocked by the PKC inhibitor calphostin C. In contrast, Met-enkephalin failed to activate classic PKCs and consequently had moderate inhibitory effects and no receptor internalization. Thus, we speculate that the hierarchy of heterologous desensitization partially relies on the capacity of receptors to activate different isotypes of PKC; opioid receptors that fail to activate cPKC can only mediate moderate heterologous desensitization.

Chemotaxis requires less signaling input from activated receptors than Ca\(^{2+}\) flux. Most chemokines induce both chemotaxis and a Ca\(^{2+}\) flux response in a G-protein-dependent fashion. It was even suggested that chemotaxis requires a Ca\(^{2+}\) signal. Our data clearly show that chemotaxis by opioids can occur in the absence of Ca\(^{2+}\) mobilization. Furthermore, our data suggest that chemotaxis is more sensitive to ligand stimulation. We speculate that Met-enkephalin receptors induce a low level of phospholipid metabolism, enough for chemotaxis, but generate insufficient DAG to elicit a detectable Ca\(^{2+}\) flux. Indeed, other weak chemotactants, such as certain autoantigens, fail to elicit Ca\(^{2+}\) responses.

Opioid receptors may serve as a link between immune and nervous systems by modulating PKC activity of leukocytes. In neuronal systems, opioids elicit activation of G protein-coupled inwardly rectifying K\(^{+}\) channels, inhibition of Ca\(^{2+}\)-channel, and adenylyl cyclase activity, eventually resulting in analgesia (33). By coupling to G\(\alpha\), opioid receptors on leukocytes mediate chemotaxis, suggesting that they may share similar downstream signaling pathways with chemokine receptors (7). Three mechanisms have been proposed to explain opioid-mediated immunomodulation. First, continuous activation of opioid receptors in the central nervous system may elevate the level of circulating corticosterone, which in turn suppresses the im-
mune system (34, 35). Second, opioids may activate the sympathetic nervous system to increase the level of epinephrine or norepinephrine (36, 37). The discovery of opioid receptors in peripheral blood mononuclear cells added a third mechanism based on data showing that these opiate receptors can directly modify the response of proinflammatory receptors (2–4, 7). Our data suggest that long term usage of opioids directly interferes with chemokine receptor function and provide a molecular mechanism for such heterologous desensitization. The dose-response curve of Met-enkaphalin-mediated inhibition showed that Met-enkephalin inhibited chemotaxis at concentrations as low as 10<sup>−9</sup> M, comparable with the blood level of opiates in patients or addicts (12). In addition, prolonged exposure to drugs has been shown to increase the expression of opioid receptors, which may lead to greater inhibitory effects. Opioid-induced heterologous desensitization suppresses chemokine responses in leukocytes when the concentration of chemokine is in the nanomolar range. For instance, dosage curves of Ca<sup>2+</sup> mobilization showed that opioid inhibition was most effective when the concentration of MIP-1α was between 1–50 ng/ml, equivalent to its level in vivo. This leads us to suggest that such cross-desensitization in leukocytes may directly contribute to well documented opioid-mediated immunosuppression (1–3). Such down-regulation of chemokine receptor function is not limited to monocytes in the resting state. The fact that Met-enkephalin pretreatment also impaired the chemotaxis response of monocyte-derived macrophages suggests a potential inhibitory role of opioids during inflammation. Nevertheless, the inhibitory effects of heterologous desensitization appear to be limited and constitute only one part of the complex immunomodulatory effects of opioids.

Overall, our studies have identified Ca<sup>2+</sup>-independent PKCs as participants in opioid desensitization of chemokine receptors and provided additional insights of the molecular mechanism of opioid-mediated immunosuppression. This is a first report on the different roles of nPKCs and cPKCs in heterologous desensitization. Based on our results, specific inhibitors for nPKC isozymes can be used to develop strategies to prevent opiate-mediated immunosuppressive effects. Inhibitors that do not block cPKC presumably will have fewer undesirable negative effects on endogenous heterologous desensitization of chemokine receptors.

Acknowledgments—We thank Dr. Ji Ming Wang, Dr. O. M. Zack Howard, and Dr. Deborah Schechtmann for inspiring discussion. We thank for Edward Cho for technical assistance.

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